Conformational interconversion of MLKL and disengagement from RIPK3 precede cell death by necroptosis

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Phosphorylation of the MLKL pseudokinase by the RIPK3 kinase leads to MLKL oligomerization, translocation to, and permeabilization of, the plasma membrane to induce necroptotic cell death. The precise choreography of MLKL activation remains incompletely understood. Here, we report Monobodies, synthetic binding proteins, that bind the pseudokinase domain of MLKL within human cells and their crystal structures in complex with the human MLKL pseudokinase domain. While Monobody-32 constitutively binds the MLKL hinge region, Monobody-27 binds MLKL via an epitope that overlaps the RIPK3 binding site and is only exposed after phosphorylated MLKL disengages from RIPK3 following necroptotic stimulation. The crystal structures identified two distinct conformations of the MLKL pseudokinase domain, supporting the idea that a conformational transition accompanies MLKL disengagement from RIPK3. These studies provide further evidence that MLKL undergoes a large conformational change upon activation, and identify MLKL disengagement from RIPK3 as a key regulatory step in the necroptosis pathway.
Necroptosis is a lytic form of programmed cell death that is dysregulated in multiple human pathologies, including inflammatory diseases, inflammatory bowel disease, and ischemia-reperfusion injury. The necrotic pathway is considered to have originated as a host defense mechanism against invading pathogens. The "ubiquitin ligase family, and the apoptotic proteolytic enzyme, threonine protein kinase (RIPK)-1 is unable to be ubiquitylated well-studied. In scenarios where receptor-interacting serine/threonine protein kinase (RIPK)-1 is activated by autophosphorylation, the high molecular weight complex termed the "necosome" is formed. Within the necosome, RIPK1 and RIPK3 form a heterooligomeric complex, and RIPK3 is activated by autophosphorylation. The precise molecular details of what follows RIPK3 activation are currently not well understood, but it is proposed that RIPK3 recruits and phosphorylates the terminal effector protein, mixed lineage kinase domain-containing protein (MLKL).

Recent studies indicate there are important mechanistic differences between MLKL activation between species and MLKL comprises an N-terminal four-helix bundle (4HB) domain, which is responsible for membrane disruption and cell death, a two-helix connector termed the brace helices, and a regulatory C-terminal pseudokinase domain. Phosphorylation of the MLKL pseudokinase domain by RIPK3 prompts an oligomerization event prior to plasma membrane translocation, where it enacts lytic permeabilization in a manner that is still currently debated. The precise molecular mechanisms underlying MLKL activation between species are not well understood, although the extent of mechanistic diversity remains unclear. Considering MLKL orthologs are present in most chordate lineages. The MLKL-RIPK3 cognate pair appear to have evolved as a signaling cassette, which can be ubiquitylated by the cellular inhibitors of apoptosis proteins (cIAPs) E3 ubiquitin ligase family, and the apoptotic proteolytic enzyme. Caspase-8 is inhibited, a high molecular weight complex termed the "necosome" is formed. Within the necosome, RIPK1 and RIPK3 form a heterooligomeric complex, and RIPK3 is activated by autophosphorylation. The precise molecular details of what follows RIPK3 activation are currently not well understood, but it is proposed that RIPK3 recruits and phosphorylates the terminal effector protein, mixed lineage kinase domain-containing protein (MLKL).

We sought to dissect the nature and precise choreography of the human RIPK3:MLKL interaction during necroptosis signaling by developing synthetic protein ligands, termed Monobodies, that recognize distinct conformers of the MLKL pseudokinase domain. Monobodies are derived from a human fibronectin type-III domain scaffold, and bind to their target proteins with high affinity and high specificity. The lack of disulfide bonds enables Monobodies to be expressed as genetically-encoded ligands in the reducing environment of the cytosol, where binding to their protein targets can be used to dissect signaling events. We recently reported Monobodies that bind the human MLKL 4HB domain to inhibit necroptosis by blocking recruitment of essential adapters for MLKL membrane translocation. Here, we present Monobodies that recognize distinct human MLKL pseudokinase domain conformers, thus allowing the signaling events underlying RIPK3-mediated activation of MLKL to be mapped. We show that endogenous human MLKL and RIPK3 are pre-associated under basal conditions and, upon necrotic stimulation and accompanying phosphorylation, MLKL disengages from RIPK3. Crystal structures of human MLKL pseudokinase domain in complex with Monobodies identified both open and closed forms in which the activation loop and αC helix adopts distinct conformations. Because monobody (Mb27) recognition of MLKL is only observed following MLKL phosphorylation by, and release from, RIPK3, in cells, our structure identifies the closed form of MLKL containing an unstructured activation loop as the activated form of MLKL. Together, these studies reveal human MLKL dissociation from RIPK3 as an additional checkpoint in the necroptosis signaling pathway.

Results
Mb27 binding requires MLKL dissociation from RIPK3. We developed monobodies that target the C-terminal pseudokinase domain of human MLKL: Mb(MLKL_26), Mb(MLKL_27), and Mb(MLKL_32) (referred to as Mb26, Mb27, and Mb32, respectively). Mb26 bound the human MLKL pseudokinase domain with a $K_D$ value of 176 ± 13 nM; Mb27 with $K_D$ value of 75 ± 14 nM (Supplementary Fig. 1a–c); Mb32 bound with a $K_D$ of 37.1 ± 2.4 nM, as previously reported. To dissect protein interactions during necroptosis, we stably introduced monobodies under a doxycycline (dox)-inducible promoter into the human colon cancer cell line, HT29 (Supplementary Fig. 2a). These monobody constructs bear an N-terminal FLAG tag for detection of expression and immunoprecipitation, and a C-terminally fused GFP. Following dox-induced expression of these Monobodies, HT29 cells were exposed to a necroptosis stimulus (TSI: TNF (T); the Smac mimetic IAP antagonist, Compound A (S); and the pan-Caspase inhibitor, IDN-6556 (I)). In contrast to previously-reported monobodies directed toward the 4HB domain of human MLKL, Mb26, Mb27, and Mb32, which bind the pseudokinase domain, did not inhibit necroptosis (Supplementary Fig. 2b–d).

We next examined whether Mb26, Mb27, and Mb32 could bind MLKL within HT29 cells by immunoprecipitating these monobodies via their N-terminal FLAG tags and examining the pulled down proteins by immunoblot. Consistent with our earlier report, Mb32 bound human MLKL following treatment with the necroptotic stimulus, TSI, and additionally immunoprecipitated MLKL under basal conditions in the absence of stimulation (Fig. 1a). In contrast, Mb27 and Mb26 did not immunoprecipitate MLKL under basal conditions, and only recognized MLKL upon phosphorylation by RIPK3 following necroptotic stimulation with TSI (Fig. 1a; Supplementary Fig. 2e). We also examined whether MLKL was immunoprecipitated in complex with RIPK3. While Mb32-immunoprecipitated MLKL was bound to RIPK3 in both unstimulated cells and cells exposed to a necroptotic stimulus, Mb27-bound MLKL did not co-precipitate RIPK3 following treatment with the TSI necroptotic stimulus (Fig. 1a). Based on these data, we hypothesized that under basal conditions, MLKL is bound to RIPK3, both in the steady-state and following recruitment to the necosome, and that RIPK3 binding to MLKL occludes the binding sites for Mb26 and Mb27. Consistent with this idea, we observed that Mb27 and Mb26 immunoprecipitated MLKL in the absence and presence of a necroptotic stimulus in RIPK3−/− HT29 cells (Fig. 1b; Supplementary Fig. 2f, g). Notably, Mb27 and Mb26 bound MLKL in RIPK3−/− HT29 cells even though MLKL was not phosphorylated (Fig. 1b), which was
expected because RIPK3 is the only kinase identified thus far to phosphorylate the MLKL activation loop\textsuperscript{43}. These data indicate that RIPK3 binding to MLKL under basal conditions occludes both Mb27 and Mb26 binding to MLKL, and that necroptotic stimuli induce MLKL disengagement from RIPK3 to present the Mb27 and Mb26 binding sites. We further investigated whether Mb27 or Mb26 and RIPK3 compete for an overlapping binding site in MLKL using recombinant proteins. Here, we co-expressed and purified the heterocomplex of human RIPK3 kinase domain and the MLKL pseudokinase domain from insect cells and examined whether the purified MLKL:RIPK3 complex could be bound by His\textsubscript{6}-tagged Mb26, Mb27, Mb32 and a
Previously reported control monobody, Mb33, which recognizes the human MLKL 4HB domain\(^{31}\) that is absent from MLKL in these constructs (Supplementary Fig. 3a–e). Strikingly, Mb26 and Mb27 could only bind the MLKL pseudokinase domain (Supplementary Fig. 3c, d), leading to dissociation of the MLKL:RIPK3 complex. In contrast, Mb32 bound MLKL alone and in complex with RIPK3 (Supplementary Fig. 3a, b), while, as expected, the control 4HB domain ligand, Mb33, did not recognize any form of the MLKL pseudokinase domain (Supplementary Fig. 3e). These data are concordant with our observations in human cells, and support the idea that RIPK3 engagement occludes the Mb27 and Mb26 binding site on the MLKL pseudokinase domain.

Recognition of MLKL by Mb27 mapped with the kinetics of RIPK3-mediated MLKL phosphorylation following treatment with the necroptotic stimulus, TSI. MLKL was bound by Mb27 two hours post-TSI treatment, following the emergence of MLKL phosphorylation 1.5 h post-TSI stimulation (Fig. 1c). Similarly, MLKL binding by Mb27 was observed at 7.5 h post-stimulation (Fig. 1d), which corresponds to the typical onset of HT29 cell death following treatment with the TSI necroptotic stimulus\(^{32}\). At 7.5 h post-TSI, we observed that phosphorylated RIPK3 (pRIPK3; upper band; antibody also recognizes unmodified RIPK3, which is the lower band\(^{44}\)) was present in complexes immunoprecipitated by Mb27. We attribute this phenomenon to the assembly of MLKL oligomers at the necrosome into clusters prior to cell death, as we recently described\(^{32,44}\), where phosphorylated MLKL (pMLKL) protomers are retained at the necrosome within an MLKL oligomer, but have disengaged from RIPK3 to expose the Mb27 binding site.

As these results indicate that phosphorylation of MLKL by RIPK3 leads to release of MLKL from the necrosome and exposure of the epitope for Mb27, we further examined the determinants of MLKL release. We reconstituted RIPK3\(^{−/−}\) HT29 cells with FLAG-tagged human RIPK3 and immunoprecipitated RIPK3 complexes by FLAG pulldown. MLKL was observed in complex with RIPK3 under basal conditions, consistent with a previous overexpression study in HeLa and HT29 cells\(^{4}\), as well as after 3 h of necroptotic stimulation (Fig. 1e). Notably, no phosphorylated MLKL was detected in complex with RIPK3, supporting the idea that phosphorylation destabilizes the MLKL:RIPK3 interaction. To further investigate this possibility, we examined the role of phosphorylation and binding of Mg\(^{2+}\), ATP and the non-hydrolyzable ATP mimetic, adenylyl-imidodiphosphate (AMPPNP), on stability of the human RIPK3 kinase domain and His\(_{6}\)-tagged MLKL pseudokinase domain heterocomplex co-expressed and purified from insect cells (Fig. 1f). While ATP or Mg\(^{2+}\) alone, or AMPPNP in the presence of Mg\(^{2+}\), did not impact stability of the heterocomplex, incubation of this complex with ATP and Mg\(^{2+}\) led to the disengagement of MLKL and RIPK3. These data implicate the catalytic activity of RIPK3, which relies on the presence of both ATP and Mg\(^{2+}\), rather than solely binding to ATP, AMPPNP or Mg\(^{2+}\) alone, as driving the dissolution of the RIPK3 and MLKL heterocomplex. These findings provide compelling support for the idea that RIPK3-mediated MLKL phosphorylation serves as the cue for MLKL dissociation from the necrosome.

We further validated our observations from immunoblot studies that Mb32 binds MLKL constitutively, while Mb27 binds MLKL only post-necroptotic stimulation, by examining the Mb27 and Mb32 interactomes by mass spectrometry. Consistent with our immunoblot results, Mb32 bound MLKL and RIPK3 both under basal conditions and following treatment with the TSI necroptotic stimulus, whilst Mb27 displayed MLKL binding only after necroptotic stimulation and did not measurably bind RIPK3 (Supplementary Fig. 4a, b). Importantly, Mb32 immunoprecipitation of RIPK3 did not arise from non-specific binding; Mb32 did not capture RIPK3 from MLKL\(^{−/−}\) HT29 cells (Supplementary Fig. 2h, i), confirming that Mb32-immunoprecipitated RIPK3 in complex with MLKL from wild-type HT29 cell lysates.

**Mb27 binding is centered on the human MLKL ATP-binding cleft.** To define the mechanism of action for Mb27, which binds human MLKL only after necroptotic stimulation, we crystallized the complex of Mb27 and the human MLKL pseudokinase domain (residues 190–471) and determined its crystal structure at 2.6 Å resolution (Fig. 2a–c, Table 1). This crystal structure revealed that Mb27 binds human MLKL via an epitope centered on the ATP-binding cleft of the pseudokinase domain (Fig. 2a, b).

The human MLKL pseudokinase domain adopts a conventional, bilobal kinase fold, where the N-lobe comprises principally β-strands and the larger C-lobe mainly contains α-helices, with the ATP-binding cleft situated between these lobes. As expected, the MLKL pseudokinase domain structure closely resembles the previously determined high-resolution crystal structure of the apo human MLKL pseudokinase (Fig. 3a, b; PDB accession 4MWI, RMSD = 0.568 Å over 188 Ca atoms)\(^{45}\). The greatest deviation is in the N-lobe, which is shifted away from the C-lobe by 7.8° and opens a larger ATP-binding cleft between the lobes. Since Mb27 binds within this ATP-binding cleft, this modest structural change likely reflects a conformer stabilized by Mb27 binding. This does not change the overall topology, conformation, or preclude assembly of an intact regulatory (R)-spine (M254, I265, H329, F350) and the conserved salt-bridge between K230 and E250 that are characteristic of a closed, “active-like” kinase conformation (Fig. 3b; a representative electron density map is shown in Supplementary Fig. 5a).
Mb27 primarily interacts with C-lobe residues, forms an interface area of 777 Å² and is estimated to release solvation free energy of 8.3 kcal/mol, as predicted by PDBePISA. The interface area between other alternative pairs of monobody and MLKL in the crystal lattice is minimal, and a complexation significance score (CSS) of 1.0 at this interface is consistent with this being the biological assembly in solution. Mb27 originated from the “loop only” monobody library designed to form a convex binding surface (Supplementary Fig. 1a), and accordingly primarily penetrates into the ATP-binding cleft of MLKL via its BC, DE, and FG loops (Fig. 2a, b), which fills in a large proportion of the space within the ATP-binding cleft. The primary interface is centered around the Mb27 BC loop, which binds the ATP-binding cleft side of the C-lobe, involving the αF-αG and β7–β8 loops, the C-terminus of the activation loop, as well as the αD helix. The Mb27 FG loop also forms a lesser interface with the loop preceding the β1 strand of the pseudokinase domain N-lobe. E213 and R333 of human MLKL appear to mediate the key electrostatic interactions by satisfying multiple hydrogen bonds and salt bridges with D36, R39, Y81, Y83, and W87 of Mb27 (Fig. 2c). At the centre of the C-lobe interface, W34 and Y83 of Mb27 interact with the sidechains of A375, Y376, I410, and P411 via hydrophobic interactions. To our knowledge, this is the first example of a monobody that binds to the respective active or pseudoactive site of a kinase or pseudokinase.

Mb32 captures human MLKL in an open conformation. To define the human MLKL binding mode of Mb32, which can pulldown MLKL and RIPK3 from cell lysates both under basal conditions and following post-necroptotic stimulation (Fig. 1a), we crystallized the human MLKL pseudokinase domain in complex with Mb32 and determined its crystal structure to 2.4 Å resolution (Fig. 2d–f, Table 1). Mb32 binds to an epitope centered on the human MLKL C-lobe αE and αI helices and engages the β7–β8 loop at the hinge and the loop preceding the N-lobe β1 strand. This epitope is on the opposite face of the pseudokinase domain to the pseudoactive site to which Mb27 binds (Fig. 2a–c). Consistent with Mb32 originating from the “side and loop” monobody library designed to form a concave binding surface (Supplementary Fig. 1a), the elongated FG loop, C- and D-strands, which are the principal sites of variation within this library, feature prominently in human MLKL binding (Fig. 2d–e). Accordingly, a series of FG loop residues (Y80, F82, M83, Y84, Y87, Y89) sit at the center of the epitope and bind V308, L311, Y318, L473, L466 of human MLKL via hydrophobic interactions (Fig. 2f). Mb32 binding buries a surface area of 1058 Å² and is estimated to release solvation free energy of 8.3 kcal/mol on average (between two copies of the interface in an asymmetric unit), as predicted by PDBePISA. The interface area between other alternative pairs of monobody and MLKL in the crystal lattice is minimal, and a complexation significance score (CSS) of 1.0 at this interface supports this arrangement as the biological assembly in solution. The capacity of Mb32 to bind human MLKL both under basal conditions and following necroptotic stimulation indicates its epitope is accessible under both conditions. Whether Mb32 selectively recognizes the open conformer of MLKL is currently unclear, however. It is possible that Mb32 could bind via the hinge region of both the open and closed forms of MLKL, and that the conformational changes that occur during necroptotic signalling have minimal effect on Mb32 recognition of the hinge region. On the other hand, superimposing the open conformer of human MLKL (crystallized in complex with Mb32) on the human MLKL:Mb27 complex revealed extensive clashes, primarily with the activation loop, that would preclude Mb27 recognition of the open MLKL conformer (Supplementary Fig. 5b). Owing to these steric clashes, we, therefore, conclude...
that Mb27 likely specifically recognizes the closed, active-like conformer, and not the open, activation loop helix form, of human MLKL.

Surprisingly, the pseudokinase domain in complex with Mb32 adopts a conformation distinct from the previously determined crystal structures of human MLKL pseudokinase alone (exemplified by PDB accession 4MW145). RMSD = 0.476 Å over 174 Ca atoms) or in complex with Mb27 (Fig. 3a–c, g). In all previous human MLKL structures25,45,47,48, the pseudokinase adopts a characteristic closed kinase conformation, including an intact R-spine and a conserved salt bridge between K230 and E250. An analogous closed, active-like conformation was also conserved in rat and horse MLKL pseudokinase domain structures35 (Fig. 3d, e). Unexpectedly, the Mb32-bound human MLKL structure adopted an open conformation that resembles that of the mouse MLKL pseudokinase domain6 (Fig. 3f). In this form, the R-spine and conserved β3 strand lysine C helix glutamate (K230:E250) salt-bridge are perturbed by the activation loop adopting a helical conformation, which displaces the αC helix. Accordingly, in the Mb32-bound structure, human MLKL adopts an open, inactive kinase-like conformation where the angle between N- and C-lobes is increased by 20.2° and the R-spine residues (M254, I265, H329, F350) are no longer aligned. Analogous to the mouse MLKL structure (PDB 4BTF20), where this conformation is stabilized by a hydrogen bond between K219 in the ATP-binding β3 strand and Q343 in the activation loop helix, a similar interaction is present within human MLKL between K230 and Q356 (Fig. 3c; representative electron density shown in Supplementary Fig. 5c). The similarity between the two MLKL structures strongly suggests that the human MLKL conformation captured in the Mb32:MLKL complex represents a low energy state within the conformational equilibrium of MLKL, rather than a high-energy or distorted state that is somehow induced by Mb32 binding. The low likelihood of capturing such high-energy structures with Monobodies or other crystallization chaperones has been detailed previously49. In mouse MLKL, disruption of the K219-Q343 bond triggers stimulus-independent MLKL activation leading to cell death by necroptosis6. By contrast, disruption of the analogous interaction in human MLKL upon introduction of the K230M or Q356A (Supplementary Fig. 2j) did not trigger constitutive activation, consistent with the reported differences between mouse and human MLKL activation25,27,29,35,36,45. Similarly, introduction of the E250A substitution in human MLKL did not influence necroptotic signaling when expressed in MLKL−/− HT29 cells (Supplementary Fig. 2k). E250 resides in the αC helix and forms a salt-bridge with the β3 strand lysine, K230, that is synonymous with the closed, active-like conformation of protein kinases. These data indicate that additional interactions likely govern MLKL conformation, which can compensate for loss of Q356 or E250 in cellular contexts.

The Mb32-bound complex enabled us to model the complete structure of the human MLKL pseudokinase domain from residues 191 to 471, including the activation loop in which residues R353–S360 formed a short α-helix antiparallel to the αC helix within the N-lobe (electron density exemplified in Supplementary Fig. 5c). In previously-reported human MLKL structures25,45,48, in addition to the Mb27-bound complex reported here, much of the activation loop, where the T357/S358 RIPK3 substrates reside, was not modeled due to lack of observed electron density. While the C-lobe of Mb32-bound MLKL is almost identical to that of the apo human MLKL pseudokinase domain (residues 286–471; RMSD = 0.296 Å, over 141 Ca atoms; Fig. 3g), the activation loop helix displaces the αC helix, resulting in a twist in the N-lobe by 20.2° that opens the ATP-binding cleft. The subsequent portion of the activation loop (residues L361–K372) runs adjacent to the pseudoactive site alongside the β1–β2 loop in the N-lobe and proximal to the αD helix in the C-lobe, which contributes D296 to a salt bridge with R370 in the activation loop.

Inspection of crystal packing did not implicate crystal contacts as the likely basis of this open conformation in *crystallo*. Molecular dynamics simulations indicated that within pMLKL, pS358 formed stable interactions with T246 on the αC helix (Fig. 3h), while pT357 was highly dynamic with a fully solvated side chain. Additionally, the activation loop helix is stable throughout these simulations and was not perturbed by phosphorylation of T357/S358 on the microsecond timescale.
Using enhanced sampling techniques, further MD simulations indicated that MLKL activation loop phosphorylation favors the transition of the MLKL pseudokinase domain from an open conformation with an activation loop helix to the closed form containing an unstructured activation loop (Fig. 3i). Free energy surfaces for MLKL and pMLKL were calculated as a function of the K230–E250 salt bridge distance using the weighted histogram analysis method (WHAM)

\[ \Delta G = -4.02 \text{ kcal/mol} \] indicates the change in \( \Delta G \) upon phosphorylation. A negative value suggests that phosphorylation stabilizes the closed, active-like conformation.
Overall, the change in ΔG upon phosphorylation (ΔΔG = −4.02 kcal/mol) suggests that activation loop phosphorylation greatly increases the likelihood of MLKL adopting a closed, active-like conformation over the open conformation. It is important to note that, to date, only the phosphomimetic T357E/S358E MLKL, which exhibited the closed conformation, but no pMLKL, pseudokinase domain structure has been reported, and thus formal validation of these models awaits further experimentally-determined structures. Together, these data support the idea that the open conformation with a helical activation loop observed in the Mb32-bound human MLKL complex structure reflects an alternative state of human MLKL present in solution.

Mb27 and RIPK3 bind overlapping sites on MLKL. The underlying regulation of the interaction between MLKL and RIPK3 is of enormous interest. Because Mb27 selectively recognizes human MLKL only in the absence of RIPK3 or following necrotic stimulation, Mb27 can be used as a tool to dissect the regulation of an MLKL-RIPK3 interaction. To date, the only reported structure of an MLKL pseudokinase domain-RIPK3 kinase domain heterocomplex is that from mouse (PDB 4M69), with no structures reported for the human MLKL-RIPK3 complex nor human RIPK3 kinase domain alone. The pseudokinase domains of mouse and human MLKL exhibit only 64.3% amino acid identity. The sequence of and around the activation loop harbors the most divergence, which includes the interface between mouse MB27 and RIPK3 C-lobes that was observed in the complex structure (Fig. 4a, b). We superimposed the human MLKL pseudokinase domain-Mb27 complex structure upon the mouse MLKL-RIPK3 complex (PDB accession 4M69; RMSD = 0.735 Å over 183 Ca atoms) to examine whether the Mb27 and RIPK3 binding epitopes overlap (Fig. 4c–d). This analysis revealed that the Mb27 and RIPK3 binding sites on human MLKL pseudokinase domain are located adjacent to one another, which partially overlap at the C-terminus of the activation loop (V371, K372, S373) and the N-terminus of the αG helix (S417). Therefore, we expect RIPK3 binding to MLKL will sterically-occlude Mb27 binding if the human MLKL-RIPK3 complex were analogous to the mouse MLKL-RIPK3 heterocomplex structure.

We identified 19 human MLKL residues within the Gly-rich loop and β2-β3 loop in the N-lobe, at the end of the catalytic and activation loops, on the αD helix and preceding the αG helix in the C-lobe as proximal to Mb27 in the crystal structure (Fig. 4c). To probe whether these residues might contribute to the RIPK3 binding site, we individually substituted each of these residues in full-length human MLKL with alanine and stably-introduced each mutant into MLKL−/− HT29 cells (Supplementary Fig. 6a). In parallel, we also introduced wild-type full-length human MLKL and the T357E/S358E mutant in which the RIPK3 substrate residues in the human MLKL activation loop are substituted with Glu to mimic phosphorylation. Following doxycycline treatment to induce expression, we examined the capacity of wild-type and mutant human MLKL constructs to reconstitute the necrotic signaling pathway by quantifying SYTOX Green uptake as a measure of cell death using IncuCyte live cell imaging. Ala substitutions in the Gly-rich loop (R210A, E211A, N212A, and E213A), β2-β3 loop (Q236A, S239A), the catalytic (R333A, S334A, S335A) and activation (D369A, R370A, K372A, S373A, T374A) loops, within the αD helix (E293A, D296A) and preceding the αG helix (Q413A, N416A) did not measurably impact the necrotic cell death after 20 h of necrotic (TSI) stimulation (Fig. 4e). These mutants exhibited similar cell death kinetics to wild-type human MLKL (Supplementary Fig. 6b–u), indicating that, individually, each residue is dispensable for RIPK3 binding and activation. Because the death invoked by these mutants mirrored the activity of wild-type MLKL, we conclude that these mutants can bind and be phosphorylated by RIPK3 to a comparable degree to wild-type MLKL. Intriguingly, only the αD helix mutant, R292A human MLKL, exhibited deficits in necrotic cell death signaling (Fig. 4e Supplementary Fig. 6p). While these deficits are likely attributable to the disruption of key structural interactions (Fig. 4f), the extent of phosphorylated MLKL after necrotic (TSI) stimulation was comparable that of wild-type MLKL (Fig. 4g). These data indicate that RIPK3 interaction was not perturbed, but instead downstream signaling by R292A MLKL is compromised. It is notable that R292A human MLKL was expressed at lower levels than most mutants, consistent with mutation compromising R292A stability by disrupting important structural interactions (Supplementary Fig. 6a). However, because the expression level was comparable to R379A human MLKL, which could reconstitute cell death signaling in MLKL−/− HT29 cells, deficits in R292A MLKL signaling are not likely attributable solely to a lower level of expression. Furthermore, R292A MLKL induced comparable phosphatidylserine (PS) exposure to wild-type MLKL and other mutants when Annexin V-binding was monitored by IncuCyte imaging. PS-exposure is known to precede necroptosis, and because this was comparable between wild-type MLKL and all mutants, indicates MLKL expression is not likely to be a limiting factor (Supplementary Fig. 7a). This raises the possibility that although R292A MLKL can reach the plasma membrane, there may be deficits in assembly into the higher order hotspots responsible for membrane permeabilization and cell death. Consistent with previous studies, phosphomimetic Glu substitution of the MLKL activation loop residues, T357/S358, which are located adjacent to the Mb27 binding site (Fig. 4d), compromised reconstitution of the necrotic pathway in MLKL−/− HT29 cells (Fig. 4e). While alanine substitution of T374 in human MLKL did not impact necrotic signaling, introduction of the phosphomimetic T374D mutation abrogated necrotic signaling (Fig. 4e Supplementary Fig. 6o) by impeding MLKL binding to, and phosphorylation by, RIPK3 (Fig. 4g). Human MLKL T374 was identified to be phosphorylated in a cell cycle-dependent manner, although at present the kinase responsible for this modification is unknown. Our data indicate that T374, which resides within the Mb27-binding epitope on MLKL, contributes to RIPK3 engagement and necrotic signaling, consistent with RIPK3 binding being centered on the adjacent MLKL activation loop residues, T357 and S358, and neighboring F386 in the αE-αF loop. To determine if the mutants, R292A and T374D, could still bind to Mb27, we stably expressed both Mb27 and MLKL mutants in MLKL−/− HT29 and performed Mb27 immunoprecipitations (Supplementary Fig. 7b). We detected no binding between Mb27 and R292A or T374D, validating the epitope identified in the Mb27:MLKL structure and further supporting the conclusion that the Mb27 and RIPK3 binding sites on MLKL partially overlap.

Discussion

The implication of necrotic cell death in the pathophysiology of a range of human diseases, including inflammatory diseases and those involving ischemic-reperfusion injury, has focused attention on therapeutically targeting the pathway. Because of its recent discovery, however, our current understanding of the pathway, including the molecular mechanism by which the terminal effector, MLKL, is activated by the upstream kinase, RIPK3, is incomplete. Here, we report the development of Monobodies that bind the human MLKL pseudokinase domain and serve as powerful tools for dissecting events in the chronology of necroptosis signaling. One monobody, Mb32, bound MLKL constitutively, and via Mb32 immunoprecipitation enabled us to validate the pre-association of endogenous MLKL and RIPK3 under basal conditions. Two further Monobodies, Mb26

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and Mb27, bound MLKL only after cells were exposed to a necroptotic stimulus, and their binding corresponded with MLKL phosphorylation and dissociation from the necrosome. As such, Mb26 and Mb27 can be used as molecular probes to monitor MLKL activation in human cells.

Our findings define two upstream events in the necroptosis signaling pathway that had been proposed, but lacked formal supporting evidence: (1) association of endogenous MLKL with RIPK3 under basal conditions; and (2) that MLKL phosphorylation leads to disengagement from RIPK3 following exposure to a necroptotic stimulus (Fig. 5). Here, we provide formal validation that endogenous RIPK3 and MLKL form a heterocomplex under basal conditions in human cells: an observation mirrored by the stoichiometric complex formed by recombinant MLKL and RIPK3 proteins when recombinantly expressed and purified from insect cells. The existence of an MLKL:RIPK3 heterocomplex under basal conditions was inferred from previous studies in which overexpressed RIPK3 and MLKL coimmunoprecipitated from HeLa and HT29 lysates; and from the observation that MLKL-like proteins encoded by some poxviruses bound the RIPK3 kinase domain and occluded cellular MLKL binding to disrupt necroptotic signaling.

Recent studies indicate that after MLKL disengagement from necrosomal RIPK3, higher-order phosphorylated MLKL (pMLKL) complexes are trafficked to the plasma membrane in vesicles via the Golgi-, actin- and microtubule-trafficking machinery. The identities of the chaperones that

| H. sapiens | 1 | 90 | QE–QI KEI KKEQLSGSPW I LLRENEVSTLYKGEYHRAPVAI KVFKKLQA
| M. musculus | 179 | Q D L Q I K | E PKEHL – GPPW T K L
| H. sapiens | 238 | G SI A I V R Q F N K E I K T M K F E S P N I L R I F G I C I D E TV P P Q F S I V ME Y C 288
| M. musculus | 227 | E B V G I V R F T N D E I K T M K F D S P N I L R I F G I C I D Q T V K P E F S I V ME Y C 275
| H. sapiens | 290 | E L GT L R E L D R E K D L T G K R M V L G A A R G L Y R L H S E A P L E E 335
| M. musculus | 279 | E L G T L R E L D R E K D L T M S V R L L V L A A R G L Y R L H S E A P L E E 322
| H. sapiens | 334 | E D C P S E L R E I D E C R A H O P S V R P S V D E I L K K L S T F S K 471
| M. musculus | 323 | S F L V A G G Y Q V K L A – R K L V A V K R Q Q E P L 433
| H. sapiens | 385 | V Y Q V K S E I Y S F G I V L W E I A T Q D I P F Q G C N S E K I R K L V A V K 493
| M. musculus | 374 | V Y Q V K S E I Y S F G I V L W E I A T Q D I P F Q G C D S K K I R E L V A D K K Q E P 443
| H. sapiens | 434 | G E D C P S E L R E I D E C R A H O P S V R P S V D E I L K K L S T F S K 471
| M. musculus | 421 | G E D C P S E L R E I D E C R A H O P S V R P S V D E I L K K L S T F S K 464

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Our findings define two upstream events in the necroptosis signaling pathway that had been proposed, but lacked formal supporting evidence: (1) association of endogenous MLKL with RIPK3 under basal conditions; and (2) that MLKL phosphorylation leads to disengagement from RIPK3 following exposure to a necroptotic stimulus (Fig. 5). Here, we provide formal validation that endogenous RIPK3 and MLKL form a heterocomplex under basal conditions in human cells: an observation mirrored by the stoichiometric complex formed by recombinant MLKL and RIPK3 proteins when recombinantly expressed and purified from insect cells. The existence of an MLKL:RIPK3 heterocomplex under basal conditions was inferred from previous studies in which overexpressed RIPK3 and MLKL communoprecipitated from HeLa and HT29 lysates; and from the observation that MLKL-like proteins encoded by some poxviruses bound the RIPK3 kinase domain and occluded cellular MLKL binding to disrupt necroptotic signaling. Recent studies indicate that after MLKL disengagement from necrosomal RIPK3, higher-order phosphorylated MLKL (pMLKL) complexes are trafficked to the plasma membrane in vesicles via the Golgi-, actin- and microtubule-trafficking machinery. The identities of the chaperones that

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Fig. 4 Mutation of MLKL R292 and T374 in the Mb27 binding epitope inhibits necroptosis. a Sequence alignment of human and mouse MLKL pseudokinase domains. Residues of mouse MLKL that interact with RIPK3 in the MLKL:RIPK3 complex structure (PDB accession 4M69) as predicted by PDBePISA in red text; key structural elements in teal; activation loop residues phosphorylated by RIPK3 in green. b The mouse MLKL pseudokinase domain (cyan):RIPK3 kinase domain (purple) co-crystal structure (PDB accession 4M69) with non-conserved residues in human MLKL and RIPK3 in red and yellow, respectively. c, d The human MLKL:Mb27 structure was superimposed on the mouse MLKL:RIPK3 complex structure (PDB 4M69), with Mb27-proximal residues identified for characterization in c as shown as sticks (c). d The Mb27-binding interface on the human MLKL pseudokinase (cyan surface), residues within 4.5 Å of the superimposed mouse RIPK3 kinase domain (PDB 4M69) (purple surface) and overlapped residues in the Mb27- and mouse RIPK3-epitopes, V371, K372, S373, and S417 (orange surface) are shown. The αC helix (yellow), and previously implicated RIPK3-interacting regions of human MLKL, the activation loop (green) and F386 (blue sticks)17,25, and T374 implicated herein, are highlighted. e Evaluation of necroptotic signaling by wild-type and Mb27 binding epitope mutants of full-length human MLKL in MLKL−/− HT29 cells. Wild-type or mutant human MLKL expression was induced with doxycycline (Dox) and cell death was measured by SYTOX Green uptake (1/mm²) quantified using IncuCyte S3 live cell imaging in the presence or absence of a necrototic stimulus (TNF, Smac mimetic, IDN-6556, TSI) for 20 h. Two independent cell lines were generated for WT, S239A, Q236A, and one for other MLKL mutants; WT lines were assayed in n = 4 independent experiments; other mutants in n = 3 independent experiments. Data are plotted as mean ± SEM. f Alanine substitution of MLKL R292 likely disrupts key interactions to neighboring residues. g Wild-type and R292A, but not T374D, human MLKL expressed in MLKL−/− HT29 cells were phosphorylated in response to 3 h TSI treatment. Data are representative of duplicate independent experiments.

Fig. 5 Summary of regulatory checkpoints in human MLKL activation. Necroptosis is initiated by ligand binding to cell surface death receptors, such as TNF receptor 1, when the IAP E3 ubiquitin ligases and Caspase-8 are depleted or inhibited. Immunoprecipitation using Mb32 identified MLKL to exist in complex with RIPK3 prior to initiation of necroptosis. The RIPK3:MLKL complex is recruited to a RIPK1-nucleated platform (the necrosome) following induction of necroptosis, where RIPK3 is autophosphorylated and MLKL phosphorylated by RIPK3. Subsequently, oligomeric phosphorylated MLKL (pMLKL) disengages from RIPK3, which permits its recognition by Mb27. Phospho-MLKL oligomers are subsequently trafficked to the plasma membrane via the Golgi-/actin-/microtubule-trafficking machinery32. pMLKL accumulates at the plasma membrane into higher order hotspots, and when a threshold amount of MLKL is surpassed, MLKL releases the plasma membrane to induce cell death and release of pro-inflammatory DAMPs32. Monobody-27 and Monobody-32 are depicted in light and dark purple colors, respectively; dashed lines represent the interactions observed for Mb27 and Mb32 in HT29 cells. The skull and crossbones image (Mycomorphbox_Deadly.png; artist, Sven Manguard) was sourced under a Creative Commons Attribution-Share Alike 4.0 license.

Traffic MLKL to the plasma membrane are yet to be discovered, although we pinpointed their site of interaction as the α4 helix of the N-terminal executioner four-helix bundle domain of MLKL using the inhibitory monobodies, Mb33 and Mb37, in our previous study31. Once pMLKL reaches the plasma membrane, a critical threshold amount of MLKL must be reached via assembly of pMLKL membrane "hotspots" to achieve membrane disruption and consequent lytic cell death32. Several proteins and post-translational modifications have been reported to modulate MLKL activity to date (reviewed in ref. 29,43), and it is likely that further auxiliary proteins contribute to tuning of MLKL’s killer function34,35. With the availability of monobodies that can detect pMLKL release from the necrosome (Mb27 and Mb26, reported here) and block pMLKL membrane translocation (Mb33, Mb37), we expect future studies will be enabled to dissect which regulated steps in the necroptosis pathway, which we term checkpoints, are influenced by modulatory proteins and post-translational modifications.

By solving the structures of human MLKL pseudokinase domain in complex with Mb27 and Mb32, we identified two distinct MLKL conformers. Mb32 enabled crystallization of a human MLKL conformer in which the activation loop formed an atypical helix that buttresses against, and displaces, the αC helix, leading to an open conformation. This conformation is reminiscent of the mouse MLKL pseudokinase domain5, which to date has only been observed in this form. Now, with the illustration that human MLKL can adopt a similar conformation, additional support is offered to the ideas that MLKL behaves as a biphasic switch55 and, more broadly, that pseudokinases can function as dynamic modulators in signal transduction56–58. Molecular dynamics simulations indicate this conformation to be stable on the microsecond timescale, while enhanced sampling methods
indicate phosphorylation serves to lower the energy barrier for the transition of MLKL from the open form with an activation loop helix to the closed, active-like conformation. Accordingly, we reasoned that the open conformation of human MLKL pseudokinase domain likely reflects the inactive, RIPK3 binding conformation for several reasons. Firstly, mouse MLKL adopts a similar conformation in its complex with the mouse RIPK3 kinase domain57, raising the possibility that human MLKL interacts with human RIPK3 via an analogous mode. Secondly, Mb27, which binds the pro-necrotic form of MLKL in cells, bound the closed MLKL conformer in which the site of RIPK3-mediated phosphorylation—the activation loop—was unstructured. Notably, Mb27 bound human MLKL via an epitope that overlaps the activation loop and, owing to the mutual exclusivity of Mb27 and RIPK3 binding, allowed us to pinpoint a role for the MLKL activation loop in RIPK3 engagement. In addition, previous studies have highlighted the activation loop as a dynamic element in MLKL vertebrate orthologs, with phosphorylation proposed to trigger conformational interconversion to a pro-necrotic form and/or pro-necrotic RIPK3 dissociation35. Our structural studies indicate that Mb27 is a specific probe for detecting the closed, active-like conformer of MLKL, which emerges following necroptotic stimulation, RIPK3-mediated phosphorylation of MLKL, and MLKL disengagement from the necrosome. The precise molecular basis underlying RIPK3 recognition of MLKL and how RIPK3 maintains MLKL in an inactive form awaits high resolution structural analyses.

This study is a second example of monobody-assisted studies of pseudokinases in which monobodies developed under the same experimental conditions captured two distinct conformations of pseudokinases. In a previous study of Aurora A kinase, Monobodies targeted to its allosteric effector-binding site captured active and inhibited forms of the kinase domain59. In solution, these conformational states of Aurora A kinase are populated at substantial levels in the absence of a bound monobody50. The fact that Mb27 and Mb32 were developed from the same monobody screen under identical conditions strongly suggests that these two conformations represent major conformers of MLKL in solution and points to the broad utility of monobodies in identifying and capturing distinct conformers of an inherently dynamic proteins, such as signaling kinases49,61. Collectively, our findings offer formal support for the idea that RIPK3 binds and maintains human MLKL in an inactive form under basal conditions, and that necroptotic stimulation triggers MLKL phosphorylation, conformational interconversion, and dissociation from RIPK3. By mapping the early events in MLKL activation that precede MLKL membrane translocation and plasma membrane disruption, our studies have unveiled additional signaling checkpoints that could be targeted pharmacologically to inhibit pathological necroptotic cell death.

Methods

Generation of monobodies. For monobody screening, bait protein was generated from a bacmid prepared in DH10MUltiBac E. coli (ATCC Biosynthetic) from a pPSfBac H1b (Thermo) vector encoding a TEV (tobacco etch virus) protease-cleavable His fusion N-terminal to the human MLKL pseudokinase domain (residues 190–471), which was C-terminally fused to a non-cleavable flexible-penta-Ser linker and AviTag (ASSSSGLNDIFEAQKIEWHE) using the Bac-to-Bac system in S2 insect cells, as reported for untagged counterparts25,26,45,49. The AviTag of human MLKL pseudokinase domain was biotinylated using recombinant HIV-1 Tat N-terminal to the NPLκ domain, including a biotinylated glutamine (residue 191–470) and subsequently ligated into pSuper2 S200 100 300 GL size-exclusion chromatography column in 0.2 M NaCl, 20 mM HEPES pH 7.5, 5% v/v glycerol. The bait was immobilized by binding to Streptavidin MagneSphere Paramagnetic Particles (Promega) and subjected to four rounds of screening of monobody phage-displayed libraries, before enriched sequences were subcloned into a yeast display library and subjected to two further rounds of yeast displayed screening, using established procedures31,39,42.

Expression constructs. Monobody sequences were amplified by PCR from library clone templates and ligated into a derivative of the doxycycline-inducible, puromycin-selectable vector, pTRE3G PGK puro, encoding an N-terminal FLAG and C-terminal GFP sequence (synthesized by ATUM, CA). For immunoprecipitation of mutant MLKL proteins, monobody sequences were introduced into the doxycycline-inducible, hygromycin-selectable vector, pTRE3G PGK hygro, encoding an N-terminal FLAG and C-terminal GFP sequence. Mutations were introduced into a human MLKL DNA template (from DNA2.0, CA) using oligonucleotide-directed overlap PCR or were synthesized by ATUM (CA) and subcloned into pTRE3G PGK puro as BamHI-EcoRI fragments. A gene encoding N-terminally FLAG-tagged human RIPK3 was synthesized by ATUM (CA) and ligated into pTRE3G PGK puro. Insert sequences were verified by Sanger sequencing (Micromon DNA Sequencing Facility, VIC, Australia). Oligonucleotide sequences are listed in Supplementary Table 1. Vector DNA was co-transfected into HEK293T cells with pVsVg and pCMV AR8.2 helper plasmids to generate lentiviral particles, which were transduced into HT29 or U937 and selected for genomic integration using puromycin (2.5 μg/mL; StemCell Technologies) using established procedures25,26.

Reagents and antibodies. Primary antibodies used in this study were: rat anti-MLKL (clone 3H1, produced in-house; 1:1000 dilution; available as MABC064, EMD Millipore, Billerica, MA, USA); rat anti-human MLKL pseudokinase domain (clone 7G2, produced in-house; 1:2000 dilution)52; rabbit anti-human MLKL phospho-S258 (AB67901, Abcam; 1:3000); mouse anti-Actin (A-1978, Sigma-Aldrich, St Louis, MO, USA); P-P38; mouse anti-MLKL (P393, produced in-house; 1:1000)17, rabbit anti-human phospho-S227 (D6W2ST, CST; 1:1000), mouse anti-FLAG M2 (Sigma-Aldrich, F1804, 1:3000). Recombinant hTNF-Fc, produced in-house, and the Smac mimetic, Compound A, have been previously described34,46. The pan-caspase inhibitor, IDN-6556/emricasan, was provided by TetraLogic Pharmaceuticals.

Generation of RIPK3 /− HT29 cells. HT29 cells expressing humanised S. pyogenes casp25 were transduced with a lentivirus encoding a doxycycline-inducible sgRNA sequence specifically targeting the second exon of RIPK3 (GAATTCTGGTCGCGCGCTAGA) via a lentiviral vector, pCMV ΔR8.2, in which the viral integrase gene was mutated by oligonucleotide-directed PCR to introduce the ΔR8 mutation to ablate reverse transcription. This vector was packaged with a pVsVg helper plasmid in HEK293T cells to generate lentiviral particles. RIPK3 editing was initiated using an established procedure25; sgRNA expression was induced using doxycycline (1 μg/mL); cells were sorted as pools by flow cytometry for high expression of sgRNA vector-encoded GFP after two days, and after further 6 weeks, cells were single-cell sorted based on absence of GFP to confirm absence of integration. Effective knockout of RIPK3 was confirmed by culturing in TSI to ensure non-responsiveness to necroptotic stimuli, using anti-RIPK3 western blot and next-generation sequencing (Illumina).

Cell culture. The human colorectal adenocarcinoma HT29 (a gift from Mark Hampton; and their MLKL /− /− and /− /− [this work] counterparts) were cultured in human DMEM (Gibco) media and human monocytic lymphoma MLKL /− /− U937 cells were cultured in human RPMI media. DMEM and RPMI media were supplemented with 8% vol/vol fetal calf serum (FCS; Sigma), with puromycin (2.5 μg/mL; StemCell Technologies) added for lines stably transduced with inducible MLKL or monobody constructs. Routine PCR testing confirmed cell lines to be mycoplasma-negative.

Flow cytometry cell death assay. HT29 cells were plated 3.75 × 104 cells/well in 24-well plate, allowed to settle 16−24 h and then treated with doxycycline (20 ng/mL) for 3 h to induce expression of the relevant monobody construct prior to stimulation. U937 cells were plated at 5 × 104 cells/well into 96-well plates and treated with doxycycline, as above, overnight to induce expression of the hTNF-Fc construct. MLKL /− /− U937 and HT29 cells were then treated with doxycycline (20 ng/mL) in combination with TFN (100 ng/mL), the Smac-mimetic compound A (500 nm) and the pan-caspase inhibitor IDN-6556 (5 μM) to induce necroptosis. Cell death was quantified by propidium iodide (PI; 1 μg/mL; Sigma #P4170) uptake using Flow cytometry 24 h post stimulation. Data were collected in duplicate, using 5 replicate experiments and presented as mean ± SEM. Data were collected using a BD FACSCalibur instrument and analysis was performed using FlowJo (v10.17) using the gating strategy depicted in Supplementary Fig. 2f, i, m.

Incucyte cell death assay. HT29 cells were seeded into 48-well plates at 3 × 103 cells/well and left to settle for 16−24 h prior to treatment with doxycycline (20 ng/mL) overnight or right before induction of MLKL construct. Cells were then treated with TFN (100 ng/mL), the Smac-mimetic compound A (500 nm) and the pan-caspase inhibitor IDN-6556 (5 μM) to induce necroptosis in phenol red-free media supplemented with 1% FCS, 1 μM Na pyruvate (ThermoFisher Scientific #11360070), 1 mM L-GlutMAX and SYTOX Green (ThermoFisher Scientific #S7020) or AlexaFluor488-conjugated Annexin V (ThermoFisher Scientific #A13201) as before32. Cells were moved into the Incucyte S3 System (Essen Bioscience) and imaged using default bright-field and green
channel settings on 10x objective. Scans were obtained every hour for 20 or 24 h, and number of SYTOX Green- or Annexin V- positive cells per mm² over time was quantified using IncuCyte S3 v2018A software (Essen Bioscience).

**Western blot**. HT29 cells were seeded into 24-well plates at 7 × 10⁴ cells/well and induced overnight with 20 ng/mL doxycycline for Mb or MLKL expression, or 2.5 mg/mL TNF for RIPK3 expression. Cells were harvested in 2× SDS Laemmli lysis buffer, sonicated, boiled at 100 °C for 3–5 min, then resolved by 4–15% Tris-Glycine gel (Bio-Rad). Proteins were transferred to nitrocellulose or PVDF membrane and probed with antibodies as indicated.

**Immunoprecipitation for western blot**. HT29 cells seeded into 10 cm plates at 2.5 × 10⁵ cells/plate or 6-well plates at 3.5 × 10⁵ cells/well were induced overnight with 20 ng/mL doxycycline and treated with TNF (100 ng/mL), Smac-mimetic (Compound A; 500 nM) and pan-caspase inhibitor IDN-6565 (5 μM) (TSI) for 3 or 7.5 h. Cells were harvested in lysis buffer (50 mM Tris-HCl pH 7.4, 1% (v/v) Triton X-100, 1 mM PMSE, 1 mM EDTA, 150 mM NaCl, 2 mM sodium Vanadate, 10 mM NaF, Complete protease inhibitor tablet (Roche)) and supernatants were mixed with Anti-FLAG M2 Affinity Gel (Millipore). Beads were washed in lysis buffer and proteins harvested using 2× SDS lysis buffer or eluted with 1× SDS FLAG peptide. FLAG elution samples were mixed with 5× SDS lysis buffer and pre-run on SDS-PAGE, then used for western blotting with antibodies as indicated.

**Mass spectrometry**. HT29 cells seeded into 15 cm plates at 10⁶ cells/plate were induced overnight with 20 ng/mL doxycycline and treated with TNF (100 ng/mL), Smac-mimetic (Compound A; 500 nM) and pan-caspase inhibitor IDN-6565 (5 μM). Cells were harvested in lysis buffer (50 mM Tris-HCl pH 7.4, 1 % (v/v) Triton X-100, 150 mM NaCl, 1 mM EDTA, 2 mM sodium Vanadate, 10 mM NaF, 1 mM PMSE, Complete protease inhibitor tablet (Roche)) and supernatants were mixed with Anti-FLAG M2 Affinity Gel (Millipore). Beads were washed in lysis buffer and proteins eluted using 2× SDS lysis buffer or eluted with 1× SDS FLAG peptide. FLAG elution samples were mixed with 5× SDS lysis buffer and pre-run on SDS-PAGE, then used for western blotting with antibodies as indicated.

**Protein crystallisation and structure determination.** Recombinant human MLKL pseudokinase domain (residues 190–471) and either Mb27 or Mb32 were coeluted by Superdex 75 10/300 GL size-exclusion chromatography (Cytiva) and concentrated to 5 mg/mL using a 10 kDa MWCO spin column. Crystallization trials were conducted at the Crystallisation Facility (Pap Hille, UQ) with 150 mL protein solution mixed with 150 mL reservoir volume in sitting drops, with 50 μL reservoir volume. Mb27:MLKL pseudokinase domain crystals grown in 0.2 M magnesium chloride, 20% w/v PEG 3350 at 8 °C, and Mb32:MLKL pseudokinase domain crystals grown in 0.1 M Bis-Tris chloride (pH 6.5), 20% w/v PEG monomethyl ether at 8 °C were soaked in high chloride reservoir (8 M NaCl) with 25% w/v ethylene glycol and flash-cooled in liquid nitrogen. X-ray diffraction data were collected at the Australian Synchrotron MX2 beamline using the EGER X 16M detector using the inhouse MX beamline control software. Diffraction data were indexed and integrated in XDS66, and merged and scaled in AIMLESS67. Phases were solved by molecular replacement using the published human MLKL pseudokinase domain structure (PDB accession 4M67)47, Mb33 structure31 (PDB accession 6UX8, for Mb27), and another monobody structure68 (PDB accession 6UX8, for Mb27), and another monobody structure68 (PDB accession 4ME7Y,69, Mb33 structure31 (PDB accession 6UX8, for Mb27), and another monobody structure68 (PDB accession 5N7E, for Mb32) as search models in PHASER70. COOT and phenix.refine were used for iterative real-space and reciprocal-space refinement by manual model building and phase refinement71. After initial molecular replacement, part of the structural model deviated significantly from the observed electron density in the N-lobe of MLKL in complex with Mb32, as it adopts a different conformation to the search model. This part of the model was deleted and manually rebuilt in COOT through iterative real-space and reciprocal-space refinement by manual model building and phase refinement71. Structure models were validated using MolProbity72. All structural figures were generated using PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC.).

**Molecular dynamics.** The MLKL pseudokinase domain coordinates from the Mb32 complex were used as the starting conformation for molecular dynamics simulations. Post-translational modifications were introduced using PyMts73. Unbiased all-atom molecular dynamics (MD) simulations were performed using GROMACS 2016.474. Structures were parameterized using the CHARMM3375 force field and solvated with the TIP3P water model. Random solvents molecules were replaced with sodium or chloride ions to neutralize the charge of the system. The system was contained in a dodecahedron at least 1 nm larger than the protein from all sides with periodic boundary conditions. Long-range interactions were
calculated with particle mesh Ewald. Neighbor lists were maintained using the Verlet cutoff scheme. The system underwent steepest descent minimization until the maximum force was <100 kJ/mol. Canonical ensemble was used to heat the system from 0 to 310 K in 100 ps. Isothermal-isobaric ensemble was applied for 100 ps. Positional restraints were applied during equilibration. Production run used 2 fs time steps. Enhanced sampling methods were implemented using PLUMED-patched GROMACS. Structures of dephosphorylated and phosphorylated MLKL were minimized and equilibrated as previously described for unbiased simulations. Steered MD simulations were used to estimate intermediate conformations between the human MLKL open, inactive (MLKL-Mb32) and closed, active state (PDB ID: 4MWI). Moving restraints for steering included a combination of dihedral, center-of-mass distance, and RMSD restraints which were manually optimized to accurately steer the system to the active state. All moving restraints were implemented as a harmonic potential with a center moving linearly over time which were added to the system Hamiltonian. Umbrella samples were used to calculate free energy surfaces with the Weighted Histogram Analysis Method (WHAM) as implemented by WHAM v2.0.18 (Alan Grossfield). Free energy surfaces were calculated as a function of the K230-E250 salt bridge distance which was defined as the distance between two atoms: K230-NZ and E250-CD. We simulated umbrella samples from 110 intermediate states each dephosphorylated and phosphorylated MLKL spanning a range of K230-E250 salt bridge distances from ~3 to 18 Å. Umbrella sampling was implemented as a harmonic potential with a static center defined by the initial KE salt bridge distance and a spring constant parameter of 60. These potentials were added to the Hamiltonian of each umbrella sample. Umbrella samples were simulated for 80 ns each for a total of (110 × 80 ns) of simulation time for each dephosphorylated and phosphorylated MLKL.

**Data availability**

Data, including expression construct sequences, are available from the corresponding authors upon request. The atomic coordinates for the human MLKL pseudokinase domain complexes with Mb27 and Mb32 have been deposited in the Protein Data Bank (www.rcsb.org) and a spring constant parameter of 60. These potentials were added to the system Hamiltonian. Umbrella samples were used to calculate free energy surfaces with the Weighted Histogram Analysis Method (WHAM) as implemented by WHAM v2.0.18 (Alan Grossfield). Free energy surfaces were calculated as a function of the K230-E250 salt bridge distance which was defined as the distance between two atoms: K230-NZ and E250-CD. We simulated umbrella samples from 110 intermediate states each dephosphorylated and phosphorylated MLKL spanning a range of K230-E250 salt bridge distances from ~3 to 18 Å. Umbrella sampling was implemented as a harmonic potential with a static center defined by the initial KE salt bridge distance and a spring constant parameter of 60. These potentials were added to the Hamiltonian of each umbrella sample. Umbrella samples were simulated for 80 ns each for a total of (110 × 80 ns) of simulation time for each dephosphorylated and phosphorylated MLKL.

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