Conformational switching of the pseudokinase domain promotes human MLKL tetramerization and cell death by necroptosis

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Necroptotic cell death is mediated by the most terminal known effector of the pathway, MLKL. Precisely how phosphorylation of the MLKL pseudokinase domain activation loop by the upstream kinase, RIPK3, induces unmasking of the N-terminal executioner four-helix bundle (4HB) domain of MLKL, higher-order assemblies, and permeabilization of plasma membranes remains poorly understood. Here, we reveal the existence of a basal monomeric MLKL conformer present in human cells prior to exposure to a necroptotic stimulus. Following activation, toggling within the MLKL pseudokinase domain promotes 4HB domain disengagement from the pseudokinase domain αC helix and pseudocatalytic loop, to enable formation of a necroptosis-inducing tetramer. In contrast to mouse MLKL, substitution of RIPK3 substrate sites in the human MLKL pseudokinase domain completely abrogated necroptotic signaling. Therefore, while the pseudokinase domains of mouse and human MLKL function as molecular switches to control MLKL activation, the underlying mechanism differs between species.
Necroptosis is a regulated cell death mechanism in which the plasma membrane is compromised, allowing escape of the cell's contents and instigation of an inflammatory response. In contrast to apoptosis, necroptosis is a caspase-independent cell death pathway, which is executed by the terminal protein, mixed lineage kinase domain-like (MLKL), following activation by the conventional protein kinase, receptor interacting protein kinase (RIPK)-3. RIPK3 hetero-oligomerizes with another protein kinase, RIPK1, to form the "necrosome": a high molecular weight signaling platform that has been variously reported to activate MLKL via stable recruitment or by a transient enzyme–substrate interaction with RIPK3. RIPK3-mediated phosphorylation of the activation loop residues, mouse S345,43 or human T357/S358, in the MLKL pseudokinase domain (PsKD) is widely thought to be the trigger for MLKL activation. MLKL phosphorylation promotes oligomerization, translocation to the inner plasma membrane, and ensuing necrotic cell death by membrane permeabilization, although the precise molecular details of this event are the subject of ongoing debate.

MLKL comprises an N-terminal four-helix bundle (4HB) domain connected to a C-terminal PsKD via a two-helix linker, which we termed the brace helices. The N-terminal 4HB domain was shown by us and others using cellular and biochemical assays to be responsible for lipid engagement and membrane permeabilization. The PsKD is so-named because it has a conserved kinase-like fold, although it lacks the residues that are necessary for catalytic activity in canonical protein kinases. As a result, we hypothesized that the PsKD functions as a molecular switch, where phosphorylation of the MLKL PsKD activation loop by RIPK3 leads to a conformation change and relief of an inhibitory protein–protein interaction with the executioner 4HB domain. Despite lacking catalytic activity, MLKL has retained the ability to bind ATP, although the role of nucleotide binding in modulating the molecular switch and regulating MLKL's necrototic function are currently unclear.

How the PsKD might suppress the executioner function of the 4HB domain, and the nature of the conformational change, if any, that occurs upon MLKL activation, remain unknown. Much of our understanding of mechanism extends from the structure of full-length mouse MLKL, where the 4HB domain is solvent exposed and would thus be available to participate in necrotic killing. In contrast, no structure of full-length human MLKL has been reported to date, and while recombinant mouse MLKL forms trimers in solution, the stoichiometry of human MLKL oligomers has been a matter of debate. Intriguingly, in contrast to mouse MLKL, human MLKL 4HB domain expression does not induce cell death in mouse or human cells in the absence of forced oligomerization. Additionally, in contrast to recombinant mouse MLKL, human MLKL 4HB+brace and full-length human MLKL, human MLKL 4HB+brace exhibited modest activity in liposome permeabilization assays in vitro, implying a crucial role for the PsKD in a necrotic human MLKL assembly.

Using biophysics, mass spectrometry (MS), and cellular assays, we reveal a role for the PsKD in directing the transition of human MLKL from a basal monomeric state to a pro-necroptotic tetramer. Wild-type hMLKL assembled into tetramers in vitro, robustly permeabilized liposomes, and reconstituted necrotic signaling in MLKL U937 cells. In contrast, hMLKL PsKD mutants, including some identified in cancers, stabilized a monomeric state, leading to deficiencies in membrane permeabilization in liposome dye release assays and in cell death induction. These data support the idea that mutations or binding of ligands, such as ATP, within the PsKD favor a monomeric form of hMLKL that exists basally in the cytoplasm prior to the cell receiving a necrototic stimulus. Using crosslinking MS (XL-MS) to direct molecular modeling, we found the 4HB domain was bound in cis by the suppressor PsKD, which thereby restricted its executioner activity. The transition to the hMLKL tetramer was modeled from small-angle X-ray scattering (SAXS) data and molecular dynamics (MD), with intermolecular cross-links as constraints, yielding a model in which the second brace helix serves an important role in higher-order assembly: an assertion further supported by hydrogen–deuterium exchange MS (HDX-MS).

Previous studies have reported that hMLKL oligomerization, and thus activation, can be promoted by introduction of negatively charged residues to mimic hRIPK3-mediated phosphorylation within the PsKD activation loop, T357 and S358. However, reconstitution of MLKL with U937 and HT29 cells with hMLKL constructs encoding phosphomimetic (Glu or Asp) mutations of T357 and S358 neither induced constitutive cell death nor reconstituted cell death upon treatment with necrotic stimuli. These data indicate that, contrary to its mouse counterpart, activation of human MLKL depends on additional steps beyond modification by RIPK3 for necrotic signaling to ensue. Overall, this work provides insights into how residues within the MLKL PsKD promote MLKL structural change and the self-association essential for necroptosis.

Results

hMLKL exists in three conformational states. In cells, cytosolic MLKL is monomeric prior to phosphorylation within the pseudokinase site by RIPK3. This led us to hypothesize that rearrangement within the PsKD, induced by phosphorylation of the activation loop, drives oligomer formation in vivo. Recombinant full-length hMLKL has been reported to exist in an oligomer:monomer equilibrium, suggesting a dynamic interconversion in solution. E351 is part of the GFE motif present in MLKL orthologs, which is the equivalent of the Mg2+-binding DFG motif in active kinases. E351 resides adjacent to activation loop RIPK3 substrate residues, T357 and S358, proximal to other features common to kinases: the VAIK motif and catalytic loop HRD motif counterpart (HG330K331) (Fig. 1a). We previously observed that introduction of an E351K mutation, which was first identified in a lung carcinoma, into the recombinant PsKD led to enhanced ATP binding. To determine whether enhanced ATP binding within the PsKD might perturb the oligomer:monomer equilibrium, we compared full-length E351K and wild-type hMLKL. While wild-type hMLKL eluted as distinct peaks from size exclusion chromatography (SEC), as expected, E351K eluted as a single species at a retention time equivalent to the 55 kDa wild-type hMLKL monomer (Supplementary Fig. 1a, b). We analyzed the self-association of full-length wild-type and E351K hMLKL at 1, 0.5, and 0.25 mg/mL using sedimentation velocity (SV) analytical ultracentrifugation (AUC). At each concentration, two species were observed for wild-type hMLKL with sedimentation velocity (SV) analytical ultracentrifugation (AUC). At each concentration, two species were observed for wild-type hMLKL with sedimentation coefficients of 3.5 S and 8.2 S, while only one 3.5 S species was observed for E351K (Supplementary Fig. 1c, d). While the addition of 300 μM ATP did not alter the positions of either protein at 1 mg/mL, ATP skewed the equilibrium of wild-type hMLKL toward the smaller 3.5 S species (Fig. 1b). Under these conditions, E351K remained monomeric (Fig. 1c).

To accurately determine the molecular weight of the higher-order species of MLKL, we performed native MS analysis on the wild-type hMLKL oligomer SEC fraction. The mass spectrum revealed a charge envelope consistent with a tetrameric protein complex (216,190 Da) (Fig. 1d). In contrast, the most abundant wild-type species in the E351K mass spectrum corresponds to a monomer (55,350 Da) in addition to a very small population of dimer
Fig. 1 Tetrameric hMLKL(2−471) is destabilized by ATP or PsK domain mutation to a monomeric intermediate. **a** Homology model of hMLKL. The αC helix is highlighted green, the VAIK maroon, HGK blue, GFE orange, and the RIPK3 phosphorylation site, Thr357/Ser358, in purple. **b, c** Standardized continuous sedimentation coefficient [$c(S_{20,w})$] distribution of wild-type and E351K hMLKL(2−471) in the presence (red) and absence (blue) of 300 μM ATP at 1 mg/mL. Residuals for the best fit of the raw radial absorbance sedimentation velocity data to a continuous sedimentation coefficient [$c(S)$] distribution model are shown as an inset for corresponding protein at 1 mg/mL in the absence of ATP (top) and presence of 300 μM ATP (bottom). **d, e** Mass spectra of wild-type and E351K hMLKL(2−471) under native conditions. **f** Liposomes containing self-quenching dye 5(6)-carboxyfluorescein were added to 1 μM of wild-type hMLKL (dark blue), wild-type hMLKL + ATP (red), E351K hMLKL (orange), E351K hMLKL + ATP (light blue), and hMLKL(2−154) (green), and dye release was monitored by spectrophotometry at 485 nm over 30 min. Data represent mean ± SEM of three independent assays.
These results confirm that hMLKL forms a tetrameric complex, consistent with the larger species observed by SV-AUC, which is disrupted by the introduction of the E351K mutation in the PsKD.

Bilayer permeabilization driven by wild-type and E351K hMLKL was measured using a liposome dye release assay. E351K and the N-terminal domain (hNTD; comprising residues 2–154) induced modest dye release (~15% of maximum) (Fig. 1f), while wild-type hMLKL caused rapid 5(6)-carboxyfluorescein release to ~80% of maximum dye release. Addition of ATP to the system enhanced E351K and lowered wild-type hMLKL activity to converge at ~40% maximum dye release (Fig. 1f). Taken together with the AUC and native MS results, the activity of these proteins in the liposome dye release assays indicates that recombinant hMLKL forms a tetrameric structure in solution that is destabilized by conformational changes induced by ATP binding or mutations in the vicinity of the PsKD activation loop. Moreover, the liposome permeabilization activity of E351K hMLKL suggests that MLKL can transition between three distinct conformational states: a basal monomer, a nucleotide-loaded transition-state monomer with intermediate liposome permeabilization activity, and the necroptotic tetramer.
The 4HB domain packs against the PsKD αC helix in monomeric hMLKL. We hypothesized E351K hMLKL reflects the basal monomeric conformation of cytosolic hMLKL prior to necrototic stimuli and sought to model the structure using a chemical XL-MS approach. Using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) coupling, “zero-length” crosslinks between proximal aspartic/glutamic acid and lysine side chains were identified by MS (Supplementary Data 1, Supplementary Fig. 2a). From an initial open model of hMLKL,21(1a), generated using the hMLKL N-terminal domain nuclear magnetic resonance structure and PsKD crystal structure by homology with the crystal structure of full-length mouse MLKL, the lowest-energy large-amplitude normal mode that brought the 4HB and PsKDs into contact with one another was traced. This unbiased domain reorientation yielded a structure in good agreement with the chemical crosslinking results. High-scoring links corresponded to Ca–Ca separations less than the upper-bound limit of 30 Å (Supplementary Data 1). Thus the resulting model reveals a compact structure in which the 4HB domain is packed against the αC helix and pseudocatalytic loop of the PsKD (Fig. 2a).

Addition of ATP to E351K hMLKL resulted in a transition to a conformer with increased ability to disrupt liposomes (Fig. 1). To analyze changes in secondary structure (via hydrogen bonding) and dynamics of this transition from a basal to a pro-necrotic state, we used HDX-MS in the presence or absence of ATP. ATP binding to E351K hMLKL resulted in a reduction of deuterium exchange in the regions surrounding the ATP-binding pocket in the PsKD (Fig. 2b–d; Supplementary Fig. 3) and increased in deuterium exchange in the PsKD αC helix (Arg-244 to Leu-263) and in the α3 and α4 helices from the 4HB domain that abut the αC helix in our model (Fig. 2b–d). This suggests that, upon ATP binding, the 4HB and αC helices became disengaged leading to an increase in secondary structure dynamics and a reduction in stability of the α-helices in each domain as the protein transitions to a pro-necrotic conformation.

hMLKL tetramer assembles into a daisy chain configuration. In previous mutational studies of the human MLKL PsKD, we observed that introduction of a K331N substitution in the pseudocatalytic loop decreased ATP affinity.14 Here, in the context of full-length hMLKL, K331N substitution markedly favored tetramer formation over monomer by SV-AUC sedimentation experiments (Supplementary Fig. 4a), thus identifying K331N hMLKL(2–471) as an ideal candidate for biophysical characterization of the hMLKL tetramer. Recombinant K331N permeabilized liposomes equivalently to wild-type hMLKL in the absence of ATP, with only modest perturbation by the addition of ATP (Fig. 3a), indicating that this mutation promotes a hMLKL conformation that is predisposed to forming higher-order assemblies. To define the in-solution structure of the hMLKL tetramer, we performed SAXS analysis on recombinant K331N hMLKL eluted by inline SEC into the path of the X-ray beam (statistics shown in Supplementary Table 1). Scatter profiles corresponding to the apex of the tetramer SEC peak were averaged and background subtracted, with Guinier analysis indicating that the data were monodisperse (Fig. 3b) with a maximum particle dimension (Dmax) of 170 Å (Fig. 3c). We fitted a tetrameric model of hMLKL to these scattering data by rigid-body modeling of the reported hMLKL model21 using maximum 35 Å distance restraints between K157–K157, K173–K173 and K305–K305 pairs in neighboring protomers (Fig. 3d). These contacts were experimentally derived from crosslinks identified in hMLKL tetramers by MS following treatment with the primary amine crosslinkers, disuccinimidyl suberate (DSS) and Bis(sulfosuccinimidyl) suberate (BS5) (Supplementary Fig. 2b, c; Supplementary Data 1). These restraints are few, because only the like-to-like crosslinks mediated by DSS and BS5 could be unambiguously assigned as intersubunit linkages within the hMLKL tetramer. Molecular dynamics refinement and simulated annealing minimization were used to further refine the tetramer model. Theoretical scatter calculated for our model was consistent with the experimental data (χ = 0.542 using CRYSOIL; Fig. 3b). The tetramer model (Fig. 3d) revealed an arrangement of protomers in which residues previously reported to be involved in lipid engagement17 were surface exposed (Fig. 3e, f) and all 4HB domains presented in a plane that would allow simultaneous lipid engagement (Fig. 3e, f). The daisy chain-like tetramer arrangement of hMLKL formed through heterologous interfaces, rather than a dimer of dimers with isologous interfaces, is consistent with the AUC and native MS analyses, which suggest that the protein exists in monomer and tetramer states but not dimers and trimers.

To understand the dynamic changes that occur to MLKL upon tetramer formation, we performed HDX-MS on the E351K (monomer) and wild-type (tetramer) hMLKL proteins (Fig. 3g, h; Supplementary Fig. 2d–f). We observed markedly reduced deuterium exchange in wild-type relative to E351K hMLKL in the brace region (residues 151 and 194) (Fig. 3h) and increased deuterium exchange in the PsKD αC helix and in the 4HB domain α4 helix within the tetramer (Fig. 3g), consistent with 4HB domain reorientation away from the PsKD within the tetramer.

PsKD switch mutations compromise liposome permeabilization. To understand how changes in the PsKD of MLKL control the transition between monomer and tetramer, we introduced a point mutation corresponding to E351K in the human MLKL PsKD to evaluate the effect of this mutation on the tetramerization of hMLKL. As shown in Supplementary Fig. 7a, mutation of E351K to an alanine residue dramatically reduced the ability of hMLKL to form tetramers or to interact with liposomes, as measured by SV-AUC and native MS. However, the daisy chain-like tetramer arrangement of a E351K hMLKL model indicates that it remains structurally similar to wild-type hMLKL tetramer (Fig. 3d), thus suggesting that the tetramerization event itself is preserved, but that the mutation disrupts the ability of hMLKL to effectively interact with lipid bilayers

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**Fig. 2** 4HB packs against the αC helix in the hMLKL PsKD and is displaced upon ATP binding. a E351K hMLKL(2–471) structure modeled using molecular dynamics based on distance restraints defined by DMTMM crosslinking experiments (Supplementary Fig. 2a, Supplementary Data 1). The αC helix is highlighted in green, while α3 and α4 of the 4HB domain (yellow) pack against the αC helix. The RIPK3 substrates, Thr357 and Ser358, are shown as sticks. b Mirror plot demonstrating deuteration (percentage of theoretical maximum) of E351K hMLKL peptides ±ATP over the indicated time points. Error bars represent SEM of triplicate experiments. Each tick on the x axis represents an individual peptide. Overlapping or adjacent peptides were identified for all regions of the protein except for a small section of the N-terminal 4HB. A line break in this region has been introduced to illustrate the gap in sequence coverage (see Supplementary Fig. 3a for peptide sequence coverage). Overlapping regions of peptide coverage are illustrated using tiled lines below the x axis. All SEM values did not exceed ±5.96 from n = 3 independent time course experiments; error bars omitted for clarity. c Differential deuteration uptake of E351K hMLKL peptides ±ATP over the indicated time points (calculated as ATP deuterated minus -ATP deuterated). As in b, each tick represents an individual peptide with a line break introduced to illustrate a gap in sequence coverage. Peptides corresponding to overlapping sequences are shown as tiles below the plot. The corresponding regions of hMLKL the peptides map to are shown schematically between c and d, with sequence elements that undergo differential exchange±ATP highlighted in blue. d Differential hydrogen–deuterium exchange (E351K hMLKL±ATP, 60 min time point) for each analyzed peptide was averaged across overlapping sequences per amino acid residue (excluding the first 2 residues of each peptide due to high rates of back exchange) and mapped onto the E351K hMLKL model (see Supplementary Fig. 3b). Dark gray coloring represents regions of the protein excluded from analysis owing to insufficient sequence coverage.
series of mutations into full-length hMLKL guided by XL-MS, HDX-MS, and residues mutated in human cancer specimens (Fig. 4a, Table 1). Mutations in hMLKL PsKD annotated in human tumors are of interest because defective necroptosis may underlie persistence of tumors. To benchmark activities in liposome permeabilization assays, we compared recombinant wild-type or mutant full-length hMLKL to a previously reported control mutant, K16A/R17A hMLKL, which exhibits compromised liposome permeabilization17 (Fig. 4b).

The activation loop of the hMLKL PsKD contains E351 of the GFE (“DFG”) motif, K354/T355, and the RIPK3 substrates, T357 and S358 (Fig. 4a). In our model, E351 is surrounded by other residues of interest: K230 of the VAIK motif, which positions ATP for catalysis in active protein kinases and was previously...
**Fig. 3** hMLKL(2–471) tetramerizes in a daisy-chain arrangement. a Liposome dye release assay of K331N hMLKL(2–471) at 1 μM in the absence (blue) and presence (red) of 500 μM ATP. Dye release was monitored by spectrophotometry at 485 nm over 30 min. Data represent mean ± SEM of three independent assays. b Small angle scattering profile of averaged and background subtracted data from the apex of inline size exclusion peak (black circles). c Interatomic distance distribution plot, $P(r)$, profile calculated from scattering data with GNOM. Maximum particle dimension, $D_{max}$, was estimated as 170 Å. d Arrangement of subunits within the hMLKL tetramer. e Electrostatic surface of one subunit in the tetramer. Charge distribution is consistent with available presentation of residues with positive charge in the 4HB domain to engage lipid (lipid-binding residues defined in ref.19) and mapped onto the crosslink-refined E351K hMLKL monomer model (g) or the hMLKL tetramer model shown with 4HB down (left) or PsK down (right; h). Dark gray coloring represents regions of the protein excluded from analysis owing to insufficient sequence coverage.
implicated in hMLKL PsKD ATP binding\(^{14}\); G330 of the HGK motif (equivalent to the catalytic "HRD" motif in active kinases); and the αC helix residue, E258. Mutation of each of these residues led to deficits in liposome permeabilization (Fig. 4), which could not be overcome by composite mutations, such as E258K/E351K. E258 formed DMTMM crosslinks with K351 within the E351K hMLKL monomeric mutant (Supplementary Fig. 2a, Supplementary Data 1), indicative of a salt bridge present in these mutants that locks the protein in a suppressed conformation.

Phosphorylation of the activation loop residues, T357 and T358, by RIPK3 or replacement with phosphomimetic Asp or Glu residues was reported to promote oligomer formation of hMLKL\(^{12,13}\). We introduced phosphomimetic Glu residues into both wild-type and E351K hMLKL. T357E/S358E hMLKL behaved similarly to wild-type hMLKL except that its ability to permeabilize liposomes was not diminished in the presence of ATP. Alanine substitution of the adjacent K354/T355 led to a lack of attenuation upon ATP. The effects of activation loop substitution are consistent with the idea that phosphomimetic mutations, and perhaps phosphorylation itself, stabilize MLKL tetramers and ATP does not induce dissociation of this stabilized form (Fig. 4h). Somewhat surprisingly, T357E/S358E/E351K hMLKL was unable to disrupt liposomes and was unresponsive to ATP, indicating a block in the molecular switch-driven transition between the three conformational states of hMLKL (Fig. 4i).

In both the wild-type and E351K hMLKL monomers, a pair of lysines adjacent to the aforementioned E258 in the αC helix, K255 and K256 (Fig. 4a), were observed to form zero-length (DMTMM) crosslinks with E111 in the 4HB domain (Supplementary Data 1). These crosslinks were not detected in the wild-type hMLKL tetramer, raising the possibility that K255/K256 contribute to maintaining the monomeric conformation. The K255A/K256A and D107A/E111A hMLKL mutants exhibited liposome permeabilization comparable to wild-type hMLKL (Fig. 4j, k). Liposome permeabilization by these mutants was only modestly impacted by ATP and, to comparable extents, consistent with reciprocal interaction of these sites within the 4HB domain-restrained monomeric conformation (Fig. 4j, k). Deficits in liposome permeabilization observed among PsKD mutants were not attributable to compromised lipid binding, because E258K and G330E hMLKL bound membrane lipid arrays with a preference for phosphatidylserine, phosphatidylinositol-4-phosphate, and cardiolipin, like wild-type and D107A/E111A hMLKL (Supplementary Fig. 5), and consistent with similar reports for wild-type hMLKL\(^{10,12}\).

Our recent studies have implicated the brace helices in communicating activating signals from the PsKD to the executioner 4HB domain and as an important contributor to MLKL oligomerization\(^{22}\). Mutation of brace residue, K157, which formed DSS/BS\(^3\) crosslinks to K157 of another protomer within the wild-type hMLKL tetramer (Supplementary Fig. 2a, b), induced deficits in liposome permeabilization (~40% dye egress vs 80% for wild-type hMLKL; Fig. 4l). PsKD mutations restrict molecular switch toggling by ATP. The observation that ATP binding destabilized full-length wild-type hMLKL tetramer formation and attenuated liposome permeabilization activity led us to examine whether ATP binding might restrict MLKL activation. Using a thermal stability shift assay\(^{16,23}\), we measured the affinity of full-length wild-type and mutant hMLKL for ATP (Table 1; Supplementary Fig. 4). While wild-type hMLKL bound ATP with a \(K_D\) of 36 μM, curiously, the liposome permeabilizing activities of T357E/S358E, E258K, and G330E hMLKL were unaffected by ATP (Fig. 4) despite comparable affinities, suggesting that these mutations perturb (or override) any conformational change that may impact MLKL activation upon ATP binding. In contrast, E351K hMLKL bound ATP with a \(K_D\) of 26 μM, and ATP binding induced elevated liposome permeabilization activity (Fig. 1f), consistent with a molecular switch that can be toggled by ATP in E351K hMLKL to relieve the repression of hMLKL activation. Unsurprisingly, K230M, K331N, K354A/T355A, and T357E/S358E/E351K hMLKL did not markedly respond to ATP in the liposome dye release assays, consistent with their ATP-binding deficits (Supplementary Fig. 4). Overall, residues within the PsKD play key roles in governing hMLKL activation, and their mutation can severely impact (or override) the sensitivity of hMLKL to ATP-binding-induced conformational changes. We expected mutations outside the PsKD to have little impact on ATP binding.

### Table 1 Summary of wild-type and mutant hMLKL properties

| hMLKL mutation | Location | Liposome permeabilization | ATP-binding \(K_D\) (±SEM; μM) | Necroptotic killing function | Tumor association |
|----------------|----------|---------------------------|-------------------------------|-------------------------------|-------------------|
| Nil (wild type) |          | +/+/+/+                   | 36 ± 4                        | +                             | –                 |
| K230M          | PsK β3 strand | ++/+                       | –                            | Delayed                       | K230Q in colon carcinoma |
| K255A/K256A    | PsK αC helix | ++/+/+                     | ND                           | +                             | –                 |
| E258K          | PsK αC helix | +/+/+                     | 24 ± 2                       | Delayed                       | E258K in colon and endometrial carcinomas\(^{19}\) |
| G330E          | PsK catalytic loop | +/+                        | 28 ± 6                       | Delayed                       | G330E melanoma; G330R endometrial carcinoma\(^{19}\) |
| K331N          | PsK catalytic loop | +/+/+/+                    | –                            | +                             | –                 |
| E351K          | PsK catalytic loop | +/+/+/+/+                  | 26 ± 6                       | Delayed                       | E351K lung carcinoma\(^{19}\); E351Q prostate adenocarcinoma |
| K354A/T355A    | PsK activation loop | ++++/++++                 | –                            | ND                            | –                 |
| T357E/S358E    | PsK activation loop | ++++/+++++                | 56 ± 8                       | –                             | –                 |
| K16A/R17A      | 4HB α1 helix  | ++/+                      | ND                           | Delayed                       | R17W in endometrial cancer\(^{19}\) |
| D107A/E111A    | 4HB α4 helix  | ++++/++                   | 23 ± 2                       | –                             | –                 |
| K157A          | Brace helices | +/+/+                     | 164 ± 31                     | Delayed                       | –                 |

ND not determined; –, not detected
Although true of D107A/E111A hMLKL, surprisingly, K157A hMLKL bound ATP with the relatively weak $K_d$ of ATP of 164 μM, which led to minimal impact of ATP on K157A hMLKL liposome permeabilization.

Monomer promoting PsKD mutations delay necroptosis. Having identified residues in the hMLKL PsKD as important determinants of activation in in vitro assays, we sought to establish their contribution to cellular necroptosis signaling. To this end, we generated MLKL$^{−/−}$ U937 cells by CRISPR editing using a non-integrating lentivirus and reconstituted the necroptosis pathway by expressing wild-type or mutant hMLKL for 16 h via a doxycycline-inducible lentiviral vector before sensitivity to death stimuli was assessed. MLKL knockout was verified by western blot (Fig. 5a) and next-generation sequencing, and this end, we generated

Western blot analysis (Supplementary Fig. 1), indicating that any deficits in necroptotic signaling are not a consequence of reduced protein expression. Cell lines showed comparable sensitivity to the apoptotic stimulus, T5, in the presence and absence of doxycycline-induced protein expression. As expected, doxycycline was essential for responsiveness to the necroptotic stimulus, TSI, in reconstituted MLKL$^{−/−}$ U937 lines (Supplementary Fig. 7).

Phosphomimetic PsKD mutants do not induce necroptosis. In contrast to mouse MLKL$^{1,11}$, introduction of point mutations in the hMLKL PsKD did not induce stimulus-independent death but rather led to defective necroptotic activity (Fig. 5c, d). To further probe the differences in activation mechanisms between mouse and human MLKL, we introduced phosphomimetic (acidic) or phosphoablating (alanine) residues at T357 and S358 in the hMLKL PsK activation loop to, respectively, emulate or block the key activation step, phosphorylation by RIPK3. Introduction of the phosphomimetic substitution, S345D, into mouse MLKL to emulate RIPK3-mediated phosphorylation leads to robust stimulus-independent death in mouse cells$^{8,25}$. By contrast, not only did phosphomimetic T357E/S358E and T357E/S358D or phosphoablating T357A/S358A hMLKL mutants fail to induce constitutive cell death, each could not reconstitute necroptosis signaling in MLKL$^{−/−}$ U937 (and HT29) cells and no cell death was observed 24 (and 48) hours post-stimulation with TSI (Fig. 5e).

In our MLKL monomer model arising from XL-MS, the αC helix residues, K255 and K256, are adjacent to D107 and E111, which led to minimal impact of ATP on K157A hMLKL liposome
Notably, similar results were obtained using MLKL$^{-/-}$ HT29 cells, where inducibly expressed wild-type, but not T357E/S358E or T357E/S358D, hMLKL constructs could reconstitute the necroptosis signaling pathway (Fig. 5f). Furthermore, recombinant wild-type, but not T357E/S358E, hMLKL robustly bound immobilized Streptavidin-binding peptide (SBP)-tagged hRIPK3 kinase domain in surface plasmon resonance (SPR) experiments (Fig. 5g). These data suggest deficits in necrosome
recruitment likely underlie defective necrototic signaling by T357E/S358E hMLKL (Fig. 6). Defective T357E/S358E hMLKL function is not attributable to the mutation compromising MLKL folding or structure, because the crystal structure of T357E/S358E hMLKL PsKD (residues 190–471) solved to 2.8 Å resolution was comparable to that of wild-type hMLKL PsK (RMSD 0.78 Å) (Supplementary Fig. 4b, c; Supplementary Table 2). Additionally, introduction of T357E/S358E substitutions failed to overcome deficits in necrototic signaling in E351K hMLKL, with negligible cell death observed 24 h post-stimulation with TSI. Introduction of single phosphomimetic and phosphoablating mutations at T357 or S358, on the other hand, did not preclude reconstitution of single phosphomimetic and phosphoablating mutations at hMLKL to necroptotic death assessed at 6, 12, and 24 h post-TSI treatment by PI uptake and with doxycycline-inducible wild-type or mutant T357E/S358E, T357E/S358D, T357A/S358A, T357E/S358E/E351K, T357A, T357D, S358A, and S358E hMLKL (analyte applied over 0–24 h) and GAPDH (cytoplasmic). All blots are representative of three independent experiments in the absence of an upstream trigger. Here we reveal the existence of a basal, monomeric conformer in which the executioner 4HB domain interacts with the PsKD via an interface centered around the aC helix to attenuate its killing activity. Using XL-MS and HDX, coupled with in vitro liposome permeabilization assays and biophysical and cellular studies, we implicated a core of pseudoactive site residues centered around the activation loop and aC helix of the human MLKL PsKD in controlling assembly of hMLKL into the tetramers that mediate necroptotic cell death.

The stoichiometry of the human MLKL oligomer has been a matter of debate in the literature, with trimers, tetramers, hexamers, and octamers variously proposed, with few studies using high-resolution approaches. Here we used native MS to define the hMLKL oligomer as a tetramer. Further evidence for hMLKL-forming tetramers was obtained from SALS data, which in combination with XL-MS and MD, enabled low-resolution modeling of the hMLKL tetramer. These data are consistent with a daisy chain assembly, in which the 4HB domain and brace helices abut the foot of the PsKD C-lobe in the neighboring hMLKL protomer. However, detailed knowledge of the precise intersubunit interactions that govern tetramer assembly awaits high-resolution tetramer structure.

Discussion
MLKL is ubiquitously expressed1,26 and, as such, mechanisms must exist to prevent MLKL activation and necroptotic cell death in the absence of an upstream trigger. Here we reveal the existence of a basal, monomeric conformer in which the executioner 4HB domain interacts with the PsKD via an interface centered around the aC helix to attenuate its killing activity. Using XL-MS and HDX, coupled with in vitro liposome permeabilization assays and biophysical and cellular studies, we implicated a core of pseudoactive site residues centered around the activation loop and aC helix of the human MLKL PsKD in controlling assembly of hMLKL into the tetramers that mediate necroptotic cell death.

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within our tetramer model, the four 4HB domains (and component residues implicated in lipid binding) are largely surface exposed and are orientated at the outer torus surface, which would allow simultaneous membrane docking. Although not observed in our studies, we cannot exclude the possibility that higher-order oligomers, such as the octamers reported to arise from tetramers via an interface surrounding the 4HB domain α4 loop residue, C862^7, may arise from hMLKL tetramers in cells.

Our findings indicate that in solution hMLKL populates either tetramer or monomer forms and suggest an intrinsic difference from mouse MLKL, whose 4HB brace was found to form homotrimers in our earlier AUC^11 and SAXS^22 studies. Inter-species differences in oligomeric status are not surprising considering the evolutionary divergence in oligomeric status of other effectors in other death pathways, including the apoptosis scaffold APAF1^23. In the case of human MLKL, our observations that ATP binding destabilized the hMLKL tetramer and modulated liposome permeabilization indicate that hMLKL can occupy three distinct states: a monomeric form with attenuated activity, which we propose corresponds to the deactivation form a tetramer, which corresponds to the form that mediates necroptosis; and an intermediate form, in which the PsKD molecular switch mechanism is locked, preventing communication of changes from the PsKD to the executioner 4HB domain.

Consistent with the importance of the “pseudoactive” site in hMLKL to activation, mutations to K230, E258, G330, and E351 have been identified in tumor genomes. Not only did K230M, E258K, G330E, and E351K mutations compromise hMLKL activity in in vitro liposome permeabilization assays but also necroptotic signaling in reconstituted MLKL^−/− U937 cells. Importantly, it is the network of residues centered on the activation loop and αC helix (Fig. 4a) that underpins hMLKL activation, rather than simply ATP-binding propensity. This parallels the situation in mouse MLKL where mutation of the key ATP-binding residue, K219 (the mouse counterpart of human K230), and a partner residue in the activation loop not involved in ATP binding, Q343, can toggle the molecular switch to induce constitutive activation of mMLKL^1. In hMLKL, mutation of pseudoactive site residues induce the opposite outcome, giving rise to deficits in hMLKL activation. Together, these studies indicate that ATP-binding propensity is a hallmark of a PsKD that can behave as a conformational switch but that ATP binding itself is not necessarily a physiological modulator of MLKL activation. Rather, mutations that favor maintenance of monomeric forms of human MLKL lead to deficits in cell death as a consequence of delayed engagement and phosphorylation by necroosomal hRIPK3. Because E258K hMLKL can still undergo RIPK3-mediated phosphorylation, this supports the idea that monomeric hMLKL mutants have retained the capacity to engage RIPK3. However, the delayed kinetics of oligomerization and RIPK3-mediated phosphorylation of these mutants are consistent with the notion that they are locked in a pre-necroptotic monomeric conformation in the cytosol, which slows their recruitment to RIPK3 and subsequent activation in cells.

In contrast to our earlier studies of mouse MLKL^11,11, none of the mutations we introduced into the human MLKL PsKD in the present work led to stimulus-independent cell death. Fundamental differences between the mouse and human necroptosis pathways are not altogether surprising considering 71.5%, 60.5%, and 61% amino acid identity between mouse and human RIPK1, RIPK3, and MLKL, respectively, and the inability of human MLKL to reconstitute the necroptosis pathway in mouse Mlkl^−/− fibroblasts^7. However, the inability of hMLKL PsKD mutants to induce cell death in our study was surprising in light of previous reports^12,18,20–22. The basis for these differences are unclear, although it is possible that the choice of cell line, expression vector, and mode, and whether endogenous MLKL is present, are contributing factors. Moreover, not only did the T357E/S358E or T357E/S358D hMLKL double mutants fail to induce constitutive cell death when expressed in MLKL^−/− U937 and HT29 cells in our hands but it also completely abrogated sensitivity to necroptotic stimuli. Our earlier studies hinted at this possibility: expression of T357E/S358E hMLKL in parental U937’s did not induce constitutive necroptosis but instead led to a dominant-negative effect when cells were treated with necroptotic stimuli^24, presumably owing to hetero-oligomerization with endogenous hMLKL poisoning assembly of active oligomers. While reconstitution of MLKL^−/− U937 and HT29 cells with T357E/S358E hMLKL led to abrogation of necroptotic killing, individual substitution of S358 with Glu or Ala and T357 with Ala led to defective, but not abrogated, sensitivity to necroptotic stimuli in U937 cells. In contrast to mouse MLKL, where a transient “hit-and-run” interaction with RIPK3 to phosphorylate MLKL is sufficient for its activation and can be emulated by the constitutive killer S345D mutation^14,15, our findings suggest a model in which RIPK3 recruits hMLKL to the necrosome via a stable interaction. This assembly precedes RIPK3-mediated phosphorylation to facilitate hMLKL tetramer assembly, reorganization, or disengagement from the necrosome, to allow translocation to the plasma membrane where permeabilization ensues (Fig. 6a)

Defective necroptosis arose upon mutation of the hMLKL Pskdosequative site or RIPK3 substrate sites (Fig. 6b), which could be attributed to deficits in RIPK3-mediated recruitment of hMLKL to the necrosome. In this study, we have unveiled crucial, here-to-fore unrealized, mechanistic differences in activation between mouse and human MLKL. An appreciation of species-dependent differences underlying the PsKD molecular switch mechanism is essential to understanding how MLKL drives necroptosis and will prove invaluable to structure-guided design of modulatory small molecules.

**Methods**

**Protein expression and purification.** Full-length human MLKL and mutants were expressed in Sf21 insect cells as previously described^7. Briefly, DNA encoding wild-type (synthesized by DNA2.0) or mutant hMLKL (residues 2–471) were cloned into a pFastBac-derived vector as in-frame fusions with a TEV protease-cleavable N-terminal GST tag. Bacmids generated using the Bac-to-Bac system (Invitrogen) following transformation of DH10Multbac E. coli cells were used to transfect Sf21 insect cells to generate P1 baculovirus according to the Bac-to-Bac manual (Invitrogen). Following a second viral amplification step, P2 virus (10% v/v) was used to infect 0.5 L Sf21 cultures proteins in 2.8 L Fernbach flasks shaking at 90 rpm, 27 °C for 48 h. Human MLKL N-terminal domain (2–154) was expressed via a pGEX-derived vector encoding an in-frame fusion with an N-terminal, TEV-protease-cleavable GST tag in E. coli BL21 Codon Plus, as previously described^7. Cell pellets were resuspended in lysis buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 10% glycerol, 0.5 mM TCEP) and cells lysed via sonication. Cell debris was separated from soluble protein via centrifugation at 45,000 x g and lystate filtered with a 0.45 μm filter prior to incubation with pre-equilibrated glutathione agarose (UibPbo) at 4 °C for 1 h with gentle agitation. Beads were then collected via centrifugation and washed exhaustively with lysis buffer and mixed with 200 μg of TEV for 2 h at 20 °C or overnight at 4 °C. The supernatant was filtered through a 0.45-μm filter, concentrated via centrifugal ultrafiltration (30 kDa molecular weight cutoff; Millipore) and loaded onto a Superdex S200 gel filtration column (GE Healthcare) equilibrated in gel filtration buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 5% glycerol). For AUC experiments, glycerol was omitted from the buffer. Proteins eluted from SEC column were assessed for purity by reducing sodium dodecyl sulfate (SDS)-PAGE with Sim-Stain visualization (ThermoFisher). Except where indicated, the protein eluted as oligomer and monomer peaks, the two peaks were pooled. Proteins were concentrated to approximately 5 mg/mL and snap frozen for storage at −80 °C. N-terminally SPB-tagged hRIPK3 (2–356; DNA synthesized by ATUM) was expressed in S21 cells via a bacmid generated from pFastBac Htb (Invitrogen), which encodes a TEV protease-cleavable N-terminal His6 fusion, using methods analogous to those described for mouse RIPK3^33.

**Liposome dye release assays.** Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and resuspended in chloroform at 20 mg/mL to emulsify a plasma membrane composition (20% POPE, 40% POPC, 10%
phosphoinositol, 20% DOPS, 10% POPG), as previously reported7. Dried lipids were resuspended in 1 mL 50 mM (S)-carboxyfluorescein solutions, vortexed, and sonicated. Liposomes were rehydrated through an extended process. Prior to use, liposomes were purified from excess (S)-carboxyfluorescein using PD-10 column (GE Healthcare) equilibrated with 10 mM HEPES pH 7.5, 135 mM KCl. Samples in the presence of ATP were preincubated with 500 μM ATP on ice for 30 min. For dye release assays, 8 μM of liposomes were mixed with 1 μM protein in Falcon 96-well plates and immediately measured every 60 s (excitation 485 nm, emission 535 nm; HiDex Plate reader) at 20°C for 30 min. 100% dye release was determined by the incubation of liposomes with 1% Triton X-100 and data were represented as a percentage of maximum release. All assays were performed in triplicate; data are presented as a mean ± SD of three independent assays.

Native mass spectrometry. Recombinant protein was buffer exchanged into 250 mM ammonium acetate using a MicroBio-Spin 6 column (Bio-Rad, USA). MS spectra were acquired on a Bruker Maxis II ETD using an off-line nano electro-spray ionization source (Bruker, Bremen, Germany). Samples were introduced into the ion source via a nanoflow capillary emitter tip prepared in-house with spray current introduced via a titanium wire in the rear of the capillary. The optimized instrument parameters were used as follows: Spectra rate 0.5 Hz, Capillary voltage 1000 V, Dry Temp 60 °C, Funnel 1 RF 300Vpp, Transfer Time 120 μs, Pre Pulse Voltage 20 μs, Gas Flow Rate 20%. Spectra were analyzed using the Unicore software.

Crosslinking mass spectrometry. Wild-type or E351K hMLKL (2.5 μg) were crosslinked in the presence and absence of 500 μM ATP. DSS and BS2 were used at 0.1, 0.25, 0.5, 1.0, and 2.0 mM while DMTMM was used at 100, 30, 15, and 7.5 mM. All reactions were performed at 20°C for 30 min and stopped by addition of reducing SDS loading dye, before boiling and resolution by 4–20% Tris-Glycine SDS-PAGE (BioRad, CA). Protein bands were visualized with SimplyBlue SafeStain (ThermoFisher) and manually excised for in-gel reduction with 10 mM dithiothreitol (DTT, Sigma) for 30 min, alkylated for 30 min with 50 mM iodoacetamide (Sigma), and digested with 35 ng trypsin gold (Promega) for 16 h at 37 °C. The extracted peptide solutions were then acidified (0.1% formic acid) and concentrated to 10 μL by centrifugal lyophilization using a SpeedVac AES 1010 (Savant). Extracted peptides were injected and separated by reversed-phase liquid chromatography (LC) on a 60 cm × 7.8 mm i.d. UHPLC system (Waters, USA) using a 250 mm × 75 μm coloLiner packed emitter tip (Ion Opticks, Australia) with a linear 90-min gradient at a flow rate of 400 nL/min from 98% solvent A (0.1% Formic acid in Milli-Q water) to 35% solvent B (0.1% Formic acid, 99.9% acetonitrile). The nano-UPLC was coupled on-line to a Q-Exactive Orbitrap mass spectrometer equipped with a nano-electron spray ionization source (Thermo Fisher Scientific, Bremen, Germany) or an Impact II UHPLC-TripleQuad (Bruker, Bremen, Germany). High-mass accuracy MS data were obtained in a data-dependent acquisition mode. Raw files were analyzed using MaxQuant (version 1.5.5.1). The database search was performed using the Uniprot Homo sapiens database plus common contaminants with strict trypsin specificity allowing up to two missed cleavages. MaxQuant was converted to MGF files using the APL to MGF converter software (https://www.wehi.edu.au/people/andrew-webb/1298/apl-mgf-converter). Crosslinked peptides were identified from the MGF files using the StavroX software (version 3.6.0.1). Lysines, protein N-termini, serines, threonines, and tyrosines were set as reaction sites of the crosslinker NHS esters (DSS and BS2) or Lysines or protein N-termini, serine, threonine, and tyrosine residues (DMTMM). Trypsin was set as the enzyme allowing for three missed cleavages at lysines and two at arginines. Precursor precision was set at 10 ppm with fragment ion precision set at 20 ppm. Deuterium output was performed using in-house scripts written in R (version 3.3.1). Deuterium exchange per residue was calculated by summing and averaging deuterium exchange across overlapping peptide regions for each residue. The first two residues of each peptide were not included in this calculation due to known issues with rapid back exchange 36.

Modeling of the monomeric and tetrameric conformers. An initial monomeric structural model was generated using MODELLER using the PDB entries, 4BTF, 2M5V, and 4MVL as templates against the hMLKL sequence. Conformational change in this model was predicted using normal mode analysis (NMA) employing the ProDy software37. Since large amplitude motions are not harmonic, an eigenvector tracking approach was used to model domain motion and reorientation. Using the X-ray crystal structure, the eigenvector corresponding to the lowest-frequency normal mode was determined using the ProDy NMA application. Additional coordinates were distance for this eigenvector were minimized using the YASARA software. NMA was performed upon the new geometry, and atomic coordinates again displaced in the direction of the eigenvector with the largest overlap with the original (X-ray structure) lowest-energy eigenvector. This procedure was repeated until the two domains were in contact. To the final structure obtained following NMA tracking, 30 ns of MD simulation was applied using the YASARA software.

The tetramer model generated from SAXS data by SASREF rigid-body fit was subjected to MD refinement using the YASARA software (www.yasara.org). Initial simulated annealing minimization was proceeded by 1 ns of MD using the YASARA knowledge-based force field with a target temperature of 300 K and minimization. The N2 atoms of lysine residues 157, 173, and 305 were restrained to the same residue in each of the neighboring monomers with a weak harmonic restraint of 10/Nm with data of 30 Å. All bonds to hydrogen and angles involving hydrogen were fixed, the simulation temperature was set at 298 K, and the time step used was 2 fs.

Antibodies and reagents. Primary antibodies used in this study were: rat anti-MLKL (clone 3H1, produced in-house1; available as MABC604, EMD Millipore, Billerica, MA, USA; 1:1000), rabbit anti-MLKL phospho-S358 (AB187091, Abcam; 1:4000), anti-GAPDH (2118, Cell Signaling Technology, Danvers, MA, USA; 1:2000), anti-Axin-2 (A-1987, Sigma-Aldrich, St Louis, MO; 1:3000), and anti-VAD/CIAP (AB10527, EMD Millipore; 1:5000). Recombinant hTNF-α, produced in-house, and the Smac mimetic, Compound A, have been previously described38,39. The pan-caspase inhibitor, IDN-6556/enemicas, was provided by Tetrologic Pharmaceuticals.

Generation of MLKL ΔN cell lines and expression constructs. Third-generation lentiviral vectors (pVSvG and pCMVΔR8.2) were used to generate lentiviral particles in HEK293T cells to stably infect U937 with humanized Streptococcus pyogenes cas9 using FUCas9Cherry40, which was a gift from Marco Herold. FhH1UTG, also from Dr Herold, with an inducible sgRNA sequence specifically targeting the first exon of MLKL (TCCCGGATCTCCGTGTTACTTC) was then introduced using an integrase-deficient lentivector, pFPAK2-D64V, which was a gift from David Rawlings and Andrew Scharenberg (Addgene plasmid #63586)41. For this second infection, virus particles were generated in HEK293T cells seeded into 10 cm plates, co-transfected with these plasmids and a pVSvG helper plasmid. Viral supernatant was harvested at 48 and 72 h postinfection, pooled, and passed through 0.45 μm pore filters, then centrifuged at 20,000 × g for 4 h on a sucrose cushion (20% w/v sucrose in 50 mM Tris- HCl, pH 7.4, 100 mM NaCl, 0.5 mM EDTA42). Pellets were resuspended in 500 μL RPMI medium (8% fetal calf serum (FCS)), with 4 μg/mL polybrene, and used to infect 5 × 105 cells. Cells were spun at 200 × g for 32 °C for 1 h, then incubated overnight at 37 °C. The following day, 1 mL additional media was added, and sgRNA expression was induced using doxycycline (1 μg/mL). Two days after induction, cells were fluorescence-activated cell sorted into pools based on high expression of green fluorescent protein (GFP) to confirm the presence of the guide vector, and after a further week, cells were single-cell sorted based on the absence of GFP to confirm absence of integration. Clones were expanded and treated with TNF (100 ng/mL) and the Smac-mimetic Compound A (500 nM) and the pan-caspase inhibitor IDN-6556 (10 μM) to determine clones no longer responsive to necroptotic stimuli. Effective knockout of MLKL was confirmed using anti-MLKL western blot and Next-Generation sequencing (Illumina).

Mutations were introduced into the hMLKL template (from DNA2O, CA) using oligonucleotide-directed overlap PCR. Wild-type and MLKL mutant DNA sequences were introduced into the doxycycline-inducible, pyruvycin-selectable vector, pTREG3 PGK puromycin (kindly supplied by Dr Toru Okamoto) as BamHI-EcoRI fragments, as previously described12,13, and inserted were verified by Sanger sequencing (Micromon DNA Sequencing Facility, VIC, Australia). Vector DNA was co-transfected into HEK293T cells with pVSvG and pCMVΔR8.2 helper plasmids to generate lentiviral particles as above.

Cell death assays. The histiocytic lymphoma U937 (sourced from ATCC) and colorectal adenocarcinoma HT29 (a kind gift from Professor Mark Hampton and Dr Andreas Konigstorfer, University of Otago, New Zealand) cell lines were cultured in

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human tonicity RPMI medium and Dulbecco’s Modified Eagle’s medium, respectively, supplemented with 8% v/v FCS, with puromycin (5 μg/mL) added for stably expressing MLKL constructs. Routinely, all plasmids were transfected into 293T cells (Biozare IBA, Göttingen, Germany) using Lipofectamine 3000 (ThermoFisher Scientific, Waltham, MA). Cells were harvested after 48 h post-transfection.

Western blot and Blue Native PAGE. Expression analyses were done in parallel with cell death assays, with cells seeded into 96-well plates at 5 × 10^4 cells/well, and induced overnight with doxcycline, as indicated above, or untreated. The cell death assay reached 6 and 12 h time points post-TS/TSI treatment, cells for expression analysis were harvested using a 2× SD Laemmli lysis buffer, sonicated, boiled at 100 °C for 5 min, and resolved by 4–20% Tris-Glycine gel (Biorad). After transfer onto polyvinylidene difluoride (PVDF), membranes were blocked with 5% skim milk and then probed with antibodies as indicated. For BN-PAGE, wild-type U937 and U937 MLKLΔ1–Δ160 cells expressing mutant MLKL variants were seeded into 12-well plates at 5 × 10^4 cells/well 16 h before treatment. Expression of mutant MLKL constructs was induced with 20 ng/mL doxycycline for 4 h, then treated with TSI as described above for time course indicated in Fig. 6 and Supplementary Fig. 8, or left untreated, as indicated. Wild-type U937 were treated with TSI for time course indicated in Fig. 6 or left untreated. For all cell lines, untreated cells were harvested at the longest time point assessed in that experiment. Cells were fractionated into cytoplasmic and membrane fractions as previously described1,7–9,15,16 using a Corbett Real Time PCR machine with proteins diluted in 150 μM ATP were performed on 1 mg/mL hMLKL samples using an XL-I analytical ultracentrifuge (Beckman Coulter) equipped with a UV/Vis scanning optics, as before.23 Buffer reference (20 mM HEPES pH 7.5, 200 mM NaCl) and 380 μL of hMLKL sample solutions at 0.25, 0.5, or 1 mg/mL were loaded into 12 mm double-sector cells with quartz windows and the cells were mounted in an An-60Ti 4-hole rotor or An-50Ti 8-hole rotor. Experiments in the presence or absence of 300 μM ATP were performed on 1 mg/mL hMLKL samples and buffer reference containing 300 μM ATP was used. All experiments were conducted at 50,000 rpm (201,600 × g) and 20 °C, and radial absorbance data were collected at 280 nm in continuous mode. Data were fitted to a continuous sedimentation coefficient distribution function $c(s)$ model and converted to standardized sedimentation coefficient $c(s)_{st}$ distributions using SEFDRT4. The protein partial-specific volumes, buffer density, and buffer viscosity were calculated using SEDNTHER48.

Thermal shift assays. Thermal shift assays were performed as described previously using a Corbett Real Time PCR machine with proteins diluted in 150 mM NaCl, 20 mM Tris pH 8.0, 1 mM DTT to 0.5 μg in a total reaction volume of 25 μL. SYPRO Orange (Molecular Probes, CA) was used as a probe with fluorescent detection at 530 nm. Thermal stability was examined in the presence and absence of 0.2 mM ATP, with no divalent cations added. A positive transition $\Delta T_m$ value indicated that ATP binding protected the protein from denaturation. The $\Delta T_m$ for ATP binding was determined by titrating ATP concentrations 0–400 μM. Two transitions were observed for some variants and the $K_w$ was calculated based on the $\Delta T_m$ of the first transition state. Two independent assays were performed for wild-type and mutant hMLKL proteins; averaged data ± SD are shown for each in Supplementary Fig. 4 and Kd estimates in Table 1.

Surface plasmon resonance. SPPB-tagged hRIPK3 kinase domain (residues 2–356) was immobilized on a dextran chip with preimobilized streptavidin (Biacore SA chip). Briefly, the surface was activated with 50 mM NaOH + 1 M NaCl solution and SPPB-hRIPK3 injected at a concentration of 0.04 mg/mL in 20 mM HEPES buffer (pH 8.0) containing 200 mM NaCl. Data were collected using a Spectroflow detector (GE Healthcare, Uppsala, Sweden). As a control, the first flow cell was used as reference surface to correct for bulk refractive index, matrix effects, and nonspecific binding. The second flow cell was immobilized with SPPB-hRIPK3. Solutions of full-length wild-type hMLKL and T357E/S358E hMLKL were flowed over the chip at 1, 0.5, 0.25, 0.125, 0.0625, and 0 μM in triplicates, at 30 μL/min, and the amount of bound material as a function of time was recorded as sensograms. The dissociation phase was monitored in 20 mM HEPES (pH 8.0) containing 200 mM NaCl at the same flow rate. This was followed by one injection of 50 mM NaOH + 1 M NaCl to regenerate a fully active capturing surface. All experiments were performed at 25 °C.

Data availability. Atomic coordinates for the T357E/S358E hMLKL (190–471) have been deposited in the Protein Data Bank under the accession number 6BWK. The data that support the findings of this study are available from the corresponding author upon request.

Received: 9 January 2018 Accepted: 17 May 2018

Published online: 21 June 2018

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Title:
Conformational switching of the pseudokinase domain promotes human MLKL tetramerization and cell death by necroptosis

Date:
2018-06-21

Citation:
Petrie, E. J., Sandowl, J. J., Jacobsen, A., Smith, B. J., Griffin, M. D. W., Lucet, I. S., Dai, W., Young, S. N., Tanzer, M. C., Wardak, A., Liang, L.-Y., Cowan, A. D., Hildebrand, J. M., Kersten, W. J. A., Lessene, G., Silke, J., Czabotar, P. E., Webb, A. & Murphy, J. M. (2018). Conformational switching of the pseudokinase domain promotes human MLKL tetramerization and cell death by necroptosis. NATURE COMMUNICATIONS, 9 (1), https://doi.org/10.1038/s41467-018-04714-7.

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