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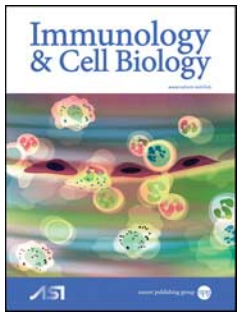

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Insane in the membrane: a structural perspective of MLKL function in necroptosis

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Abstract

Necroptosis (or "programmed necrosis") is a caspase-independent cell death pathway that operates downstream of death receptors, including Tumour Necrosis Factor Receptor-1 (TNFR1), and the Toll-like receptors, TLR3 and TLR4. Owing to its immunogenicity, necroptosis has been attributed roles in the pathogenesis of several diseases, including inflammatory bowel disease and the tissue damage arising from ischemic-reperfusion injuries. Only over the past 7 years has the core machinery of this pathway, the receptor-interacting protein kinase-3 (RIPK3) and the pseudokinase, Mixed Lineage Kinase domain-Like (MLKL), been defined. Our current understanding of the pathway is that RIPK3-mediated phosphorylation activates cytoplasmic MLKL, which is the most terminal known effector in the pathway, leading to MLKL's oligomerisation, translocation to, and permeabilisation of, the plasma membrane. Here, we discuss the insights gleaned from structural and biophysical studies of MLKL and highlight the known unknowns surrounding MLKL's mechanism of action and activation.

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Introduction

Necroptosis is distinct from the better-understood apoptosis pathway at both the mechanistic and phenotypic levels. In contrast to apoptosis, necroptosis occurs independently of proteolytic caspase activity. Instead, the core pathway is controlled by the conventional protein kinases, receptor-interacting protein kinase (RIPK)-1 [1, 2] and RIPK3 [3-5], and the terminal effector pseudokinase, Mixed Lineage Kinase-domain Like (MLKL) [6-9]. Phenotypically, the hallmark of a cell undergoing necroptosis is cell swelling ("oncosis"), believed to arise from compromise of plasma membrane integrity. The resulting leakage of cellular contents, including damage-associated molecular patterns (DAMPs), into the surrounding milieu is believed to underlie the immunogenicity of necroptosis. Consequently, necroptotic cell death has been linked to innate immune responses (Oberst, this edition of ICB), viral defence (Kaiser, this edition of ICB), and various inflammatory diseases. In contrast, apoptosis is generally considered to be immunologically inert, which is reflected in roles for apoptosis in development. Rather than undergoing cell swelling, apoptotic cell membranes remain intact, bleb and shed 'apoptotic bodies', which are cleared by macrophages.

Because of the implication of necroptosis in inflammatory responses and pathologies, there is unquestionable therapeutic potential in the development of molecules that can modulate necroptosis. To this end, the cell death field is invested in understanding the protein interactions and precise choreography of the necroptotic pathway. More specifically, it remains of enormous interest to better understand how instigation of necroptotic death leads to permeabilisation of the plasma membrane. Keeping in mind the many possible open questions and approaches to study necroptosis, this review will focus on the mechanistic insights achieved through structural biology and biophysical techniques.

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The necroptotic pathway

Extrinsic factors known to initiate necroptosis include the Toll-like receptors, TLR3 and TLR4, by their ligands, viral double stranded DNA (or its laboratory surrogate, polyI:C) and lipopolysaccharide (LPS), respectively [10], and death receptors, including Fas/CD95 upon engagement of Fas ligand [2]. However, it is tumour necrosis factor (TNF) engagement of the TNF receptor that is the best-characterised necroptosis initiator *in vitro* [11].

The precise biological mechanism(s) regulating the trifurcation between TNF-induced pro-inflammatory cytokine production via the NF-κB pathway and death via apoptosis or necroptosis remain to be established, and have been considered elsewhere [11]. Simplistically, TNF is believed to signal via the NF-κB pathway as a default setting. When RIPK1 ubiquitylation is compromised, such as by inhibition or depletion of the E3 ubiquitin ligases, the inhibitor of apoptosis proteins (IAPs; reviewed by Wong et al. in this ICB issue), RIPK1 can participate in death signalling. In cases where caspase-3 and caspase-8 activity operates, RIPK1 [12], CYLD[13] and possibly RIPK3 [14] are cleaved and apoptosis prevails. However, compromise of caspase activity allows necroptosis to proceed, with RIPK3-mediated phosphorylation of MLKL believed to be the kiss of death. The mechanism of RIPK3 activation remains the subject of ongoing interest. Conventionally, RIPK3 was thought to be activated by RIPK1 kinase activity in a simple signalling cascade, although this has not been formally shown. Instead, it appears that RIPK3 oligomerisation, an event reliant on RIPK3 abundance [15] and mediated by the ~19 amino acid C-terminal RIP Homotypic Interaction Motif (RHIM) [16], is the key step in progressing necroptosis [17-19]. RIPK1 and RIPK3 have been shown biophysically to assemble to into the "necrosome" via the organisation of their RHIMs

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into amyloid fibrils [20], although whether RIPK1 promotes or hinders RIPK3 activation appears to depend on cellular context[21-23], including as-yet possibly unidentified factors.

RIPK3 oligomerisation is believed to induce transphosphorylation of a loop at the foot of the RIPK3 C-lobe (T231/S232 in mouse; S227 in human RIPK3), rather than activation loop phosphorylation, to stimulate RIPK3 catalytic activity [7, 24]. In turn, RIPK3 proceeds to phosphorylate the pseudokinase domain of MLKL [6, 7, 9, 25], although it is currently unclear whether this occurs subsequent to stable recruitment of MLKL to the necrosome or whether the RIPK3:MLKL encounter is transient, and indeed whether the stability of this interaction depends on the cellular context or necroptotic stimulus. Regardless of the precise mechanism, it is believed that RIPK3-mediated phosphorylation induces a conformational change in MLKL [6, 26], which leads to exposure of the N-terminal four-helix bundle (4HB) domain [27], MLKL oligomerisation and translocation to membranes, membrane permeabilisation and cell death [27-32].

We note that while it is clear that MLKL phosphorylation, oligomerisation and membrane translocation are required for membrane permeabilisation, the exact mechanism of membrane disruption is still a matter of contention (Figure 1). Here, we will describe in detail the field's accumulated knowledge of MLKL's structure, mechanism of activation and interactions with lipids and other proteins.

How do the domains of MLKL contribute to its function?

MLKL is the most terminal known protein in the kinase cascade that leads to necroptosis. The protein has two functional domains, an N-terminal four-helix bundle (4HB) and a C-terminal

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pseudokinase domain (PsKD), which are tethered by a two-helix linker (the "brace" helices). The structural contribution of each domain to the function of MLKL will be discussed.

The NTD of MLKL kills like no other

The N-terminal 4HB domain (residues 1-125 in mouse; 1-124 in humans) is the 'killer' domain. In HT-29 cells, an isoform of MLKL was identified in which the PsKD is deleted due to alternative mRNA splicing [33]. Although the physiological context during when this isoform is synthesized by cells is currently unknown, its expression in cultured cells resulted in constitutive necroptosis. Similarly, inducible expression of mouse 4HB domain (residues 1-125) leads to cell death in the absence of stimuli [27].

The 4HB domain does not share significant sequence or structural homology to other protein families. This presents both intrigue and frustration, as it is not possible to infer how the 4HB domain permeabilises membranes based on structure/function studies of homologous proteins. Using Hidden Markov Models, Daskalov *et al.* proposed homology to the HeLo-like domain of the fungal protein HELLP [34]. Interestingly, the HELLP protein contains an amyloid-forming region that is reminiscent of the fibril-fostering RHIM domains of the RIPK1/3 proteins [20]. However, any functional parallels, especially regarding a possible role of the MLKL pseudokinase domain as an oligomerisation or protein interaction domain, require further investigation.

The N-terminal domain (NTD) is made up of the 4HB domain and two brace helices. The structure of full-length mouse MLKL was solved by X-ray crystallography [6] and the human NTD (residues 1-154) by Nuclear Magnetic Resonance (NMR) spectroscopy [29] subsequently (Figures 2 and 3). Both structures contain a common 4HB domain core, with helices 2 and 4

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packed against the N-terminal portion of the first of the two brace helices (Figure 2A,B). Notably, there were few inter-residue nuclear Overhauser effect resonances (NOEs; signals between NMR-susceptible nuclei proximal in 3D space, rather than those connected by chemical bonds) observed between helix-2 and the first brace helix, suggesting that the 4HB domain:brace interaction may be dynamic, although how this relates to MLKL function has yet to be fully explored. An additional helix was present in the human structure in the loop connecting helices 3 and 4, which capped one end of the bundle (Figure 2B). Density for this helix was absent from the mouse crystal structure [6], consistent with the dynamic nature of this helix in the human MLKL NMR structure.

The brace helices: More than just a tether.

The brace helices that tether the 4HB domain to the PsKD contribute more to MLKL function than simply connecting the two domains. As will be discussed, the brace helices are bi-functional, transmitting conformational changes induced by phosphorylation of the PsKD to the 4HB domain, as well as providing an interface for MLKL oligomerisation. It is worth noting the brace region of mouse and human MLKL differ in length by 9 amino acids owing to an insertion in the first brace helix of human MLKL. When the NMR structure of the human NTD (residues 2-154) are overlayed with the full length mouse MLKL structure, steric clashes between the helix in the loop connecting helices 3 and 4 and the mouse PsKD were observed [29], suggesting the differences between the positions of the helix insertion might occur between species or conformers, or that the α3-α4 loop helix does not universally adopt a helix in all orthologous MLKL structures.

The PsKD - an actively dead kinase.

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The pseudokinase domain (PsKD) functions as a molecular switch that toggles between the activated and inactive conformations of MLKL. While pseudokinase domains topologically resemble protein kinase domains, they lack critical residues that are necessary for phosphoryl-transfer rendering them catalytically-defective or dead [35-37]. Therefore, even at high concentrations it does not detectably auto-phosphorylate or phosphorylate other proteins, such as bovine serum albumin [6, 7]. The typical structure of a kinase is bi-lobal, in which the N-terminal lobe contains five antiparallel β-strands and one α-helix (called the αC helix), which packs against the larger C-terminal lobe almost entirely comprising α helices. In a conventional (active) protein kinase, three essential motifs are conserved. The first Val-Ala-Ile-Lys (VAIK) is important for ATP positioning during catalysis, in which the side-chain of the Lys residue positions the α- and β- phosphates during phosphotransfer. The second motif, His-Arg-Asp (HRD) resides within the activation loop and the side-chain of the Asp contributes to catalytic activity in conjunction with Asp of the third motif (Asp-Phe-Gly) to bind to a Mg^{2+} ion which in turn co-ordinates the β- and γ-phosphates of the ATP molecule [38]. In most species, MLKL retains the VAIK motif, however the HRD motif is replaced by divergent sequences and the DFG has been modified in most species to Gly-Phe-Glu (GFE). Intriguingly, MLKL has retained the ability to bind ATP, ADP and ATP analog, AMP-PNP, in the absence of Mg^{2+} and Mn^{2+}, despite an absence of hydrolytic activity [6, 26], however it remains of outstanding interest how nucleotide binding might relate to MLKL regulation or biological function.

The ‘activation loop’ of mouse MLKL (Ser340-Ile346) adopts an α-helical structure, synonymous with inactive protein kinase structures (Figure 3A), although the extended nature of the helix is rather unusual. Moreover, the activation loop helix was packed against the αC helix in the N-lobe in the full length mouse MLKL structure, precluding the typical kinase

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fold in which the αC helix Glu (E239) would ion pair with the catalytic Lys of the VAIK motif (K219 in mouse MLKL) (Figure 3D). Instead K219 interacts with Q343 in the activation loop helix, a residue largely conserved amongst MLKL orthologs, whilst the shunned E239 of the αC helix is directed towards solvent. The functional importance of the K219:Q343 interaction to mouse MLKL was verified by mutating either residue individually to Ala, which triggered necroptosis in the absence of stimuli [6]. Q343 is proximal to S345, S347 and T349 in mMLKL, which are sites subject to RIPK3 phosphorylation [6], of which S345 phosphorylation is thought to be the key trigger for MLKL activation [6, 25, 39]. These findings indicate that the K219:Q343 interaction may have evolved as an important regulator of the conformational changes that switch MLKL from inactive to active forms [6].

The mouse MLKL PsKD has also been solved in a 1:1 complex with the kinase domain of mouse RIPK3 (Figure 3B) [40]. This structure indicates that the MLKL pseudokinase domain does not undergo gross conformational rearrangement when engaged with RIPK3 [40]. The domains pack against each other in a head-to-head fashion through an interface that is dominated by van der Waals interactions, H-bonds and π-stacking of N-lobe phenylalanine rings from both RIPK3 (F27) and MLKL (F234). Mass spectrometry confirmed both RIPK3 kinase and MLKL pseudokinase domains were phosphorylated in this complex, however density was present only for the RIPK3 phospho-T231/S232 sites, the latter of which is positioned at the interface with MLKL's C-lobe (Figure 3B). Curiously, within the RIPK3:MLKL complex, both the RIPK3 kinase domain and the MLKL PsKD adopt inactive-like conformations with disrupted hydrophobic spines. This raises the interesting possibility that MLKL engagement by RIPK3 in this mode locks the molecular switch to prevent MLKL activation, or conversely that MLKL's PsKD suppresses RIPK3. The former possibility is supported by elevated necroptotic death by MLKL harbouring mutations to phosphosites that

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reside in the RIPK3:MLKL interface when expressed in fibroblasts lacking endogenous RIPK3 [39].

The uncomplexed ("*apo*") structure the human MLKL PsKD has been solved as an independent entity [26, 40]. Overlay of this crystal structure with the mouse counterpart reveals differences in the activation loop, where electron density for the human activation loop (residues 357-368) is absent (Figure 3D). This suggests the region is dynamic, unlike the unconventional helix observed in the mouse PsKD. Additionally, the human PsKD adopts a closed conformation synonymous with active protein kinase structures. Whether these two conformations are representative of two phases of a molecular switch remains a subject of enormous interest.

Both mouse and human MLKL PsKDs bind ATP, ADP or AMP-PNP with low micromolar affinity, but only in the absence of cations [6, 26, 36]. Evolutionary divergence of pseudoactive site residues that mediate nucleotide binding was evident from comparison of the mouse and human PsKD structures[26, 41]. Most strikingly, the VAIK motif Lys residue, K219 in mouse MLKL, was essential for nucleotide binding, whilst mutation of the human counterpart (K230) compromised, but did not abrogate, nucleotide binding. The involvement of the VAIK Lys in ATP binding was unexpected, because this is considered a catalytic, not nucleotide binding, residue in conventional protein kinases [42]. In addition, the human MLKL catalytic loop has evolved an HGK motif, of which K331 contributes to ATP binding whilst no obvious counterpart is apparent in mouse MLKL [26]. These observations suggest that the MLKL pseudoactive site has taken advantage of the lack of selective pressure to maintain active site geometry in the absence of a catalytic function, allowing elements of the pseudoactive site to evolve nucleotide-binding functions. Whether ATP binding contributes

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to, or is a hallmark of, MLKL's molecular switch mechanism or whether it is an evolutionary remnant of an ancestral MLKL function remains unclear. Such detailed insights into the molecular switch mechanism and the means by which conformational changes in the PsKD are transmitted to the 4HB domain via the brace helices await future structural studies.

MLKL PsKD as a protein interaction domain

The PsKD has a central role in controlling the dynamics of MLKL to release the 4HB "killer" domain. It also serves as a protein interaction domain (or "adaptor"). In addition to the described association with the RIPK3 kinase domain, three recent publications identified MLKL as a client of Hsp90 and the protein kinase co-chaperone CDC37 [43-45]. Our data suggests this association is via the PsKD and not the NTD, because Hsp90 inhibition blocked death of fibroblasts expressing an activated mutant form of full-length mouse MLKL, but did not impact MLKL NTD-mediated killing [43]. Additionally, inhibition of Hsp90 activity imposed a blockade on MLKL oligomerisation and membrane translocation [43].

A role for the Hsp90:CDC37 system in necroptosis has previously been implicated through association with RIPK1 [46-51] and RIPK3 [3, 52, 53], consistent with better-understood chaperone functions of Hsp90 for conventional protein kinases. The mechanistic details of this interaction remains unclear, although our data suggests the Hsp90:CDC37:MLKL PsKD complex is transient [43]. Whether other effectors may be recruited to MLKL via the PsKD and if these associations can be therapeutically targeted to modulate the necroptotic pathway remain to be established.

The role of MLKL post-translational modification in formation of the necrosome.

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In the necroptosis pathway, both ubiquitination and phosphorylation are important events that promote the recruitment of binding partners, control protein levels and localisation, and regulate activation of necroptotic effectors (reviewed in ref.[54]). Although ubiquitination of RIPK1, RIPK3 and MLKL have been reported, it is their phosphorylation that is better understood as essential to the propagation of the necroptotic signalling cascade. The phosphorylation states of RIPK1 and RIPK3 cement the formation of the necrosome, while phosphorylation of MLKL is the cue for oligomerisation and translocation to the plasma membrane [7, 40]. RIPK1 is thought to autophosphorylate its activation loop [1], and can also be phosphorylated by RIPK3 [3]. In contrast, formal evidence that RIPK1 phosphorylates RIPK3 is yet to be reported, although it was noted that levels of phosphorylated RIPK3 decreased in cells treated with the RIPK1 inhibitor necrostatin-1 [3]. Consequently, the RIPK1>RIPK3>MLKL activation sequence is markedly more complicated than a simple hierarchical phosphorylation cascade and suggests that the kinase domains of RIPK1 and RIPK3 can augment the protein interaction functions of their RHIMs.

Experiments with kinase-dead RIPK3 revealed autophosphorylation of the kinase domain is required for association with MLKL [24]. Both human and mouse MLKL have been shown to be phosphorylated by RIPK3 at Ser/Thr residues (T357/S358 in human and S345, S347, T349 and S352 in mouse)[7, 40]. Alanine ablation of the T357/S358 phospho-sites in human MLKL blocked necroptosis, but did not preclude binding to RIPK3, establishing that phosphorylation of MLKL is essential to MLKL activation and secondary to RIPK3 engagement [7].

Additional phosphorylation sites, S158, S228 and S248, have been identified in mouse MLKL using Mass Spectrometry [39]. S228 and S248 were identified following *in vitro* kinase assays using recombinant mRIPK3 and mMLKL, while S158 was identified in MLKL affinity-

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purified from whole cell lysates. Mutation of S158 or S248 to alanine (phospho-ablating), but not aspartate or glutamate (phospho-mimetic), led to an increase in necroptosis in the absence of stimuli in wild-type, *Mlkl*$^{-/-}$, and *Mlkl*$^{-/-}$*/Ripk3*$^{-/-}$ fibroblasts, inferring phosphorylation may negatively regulate MLKL activity [39]. In contrast, mutation of S248 to Glu or Ala promoted MLKL-mediated killing, most profoundly in fibroblasts lacking endogenous RIPK3 and MLKL. Taken together, the impact of MLKL phosphorylation appears to be site and scenario dependent and can both positively and negatively tune necroptotic signalling. The protein kinase responsible for mMLKL S158 phosphorylation is yet to be identified, but signifies more broadly that kinases in addition to RIPK3 are likely to contribute to MLKL activation. Consistent with this idea, MLKL was recently shown to induce necroptosis independently of RIPK3 in an inflammation-dependent hepatitis model, and using recombinant proteins, RIPK1 was excluded as the candidate kinase mediating MLKL phosphorylation [55].

A question of size – the oligomeric state of MLKL

MLKL oligomers have been observed at the plasma membrane of necroptotic cells in a number of studies. The arrangement of these oligomers is controversial and reports include trimers [27, 28], tetramers [30] and even hexamers [56]. The interface through which MLKL self-associates remains unknown, however it is unlikely to be dominated by the PsKD which shows no evidence of multimerisation in solution in Size Exclusion Chromatography or small angle X-ray scattering (SAXS; a low resolution in solution structural technique) experiments [6, 26], nor in crystal structures [26].

MLKL has been reported to migrate as a high molecular weight complex on non-reducing SDS-PAGE, suggesting the formation of intermolecular disulfide bonds [28, 30, 31, 56]. However,

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currently, it is unclear whether these disulfide bonds are formed naturally within a cell or post-cell lysis [28].

The two-helix brace connecting the 4HB domain and PsKD appears to be involved in oligomerisation [27, 29, 57]. Recombinant mouse MLKL (1-169), which contains the brace region, was shown by analytical ultracentrifugation (AUC; a method to examine protein molecular weights and oligomerisation states in solution) to exist in a monomer-trimer equilibrium, with a K_d for self-association of 7.2 μM [27]. More recently, Quarato *et al.* performed complementary AUC analyses of recombinant human counterparts. The construct that contained the brace region (1-182) was shown to exist in an equilibrium of monomer (13 %), dimer (21 %) and Trimer/Tetramer (66 %) at 0.87 mg/ml [57]. This equilibrium was skewed by necrosulfonamide, a necroptosis inhibitor that covalently modifies Cys86 in the human MLKL 4HB domain [7], with no monomeric species observed [57]. A truncated construct (1-140) lacking the brace region was found to be monomeric, consistent with the earlier NMR study of human MLKL (2-154), where no evidence of oligomerisation was reported [29]. Further support for the brace region mediating higher order assembly was obtained by expressing the brace region (residues 140-182) as a Cerulean fusion in 293T cells and crosslinking with DSS[57]. These studies implicate the two-helix brace in oligomerisation, although how this relates to MLKL's activation mechanism in cells remains unclear. Considering that expression of the mouse MLKL 4HB domain (residues 1-125), which lacked the brace helices, was sufficient to kill mouse fibroblasts [27, 32], it is possible that brace mediated-oligomerisation serves to augment, or contribute to, rather than govern MLKL's killing activity.

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Phosphorylation of human MLKL at T357/S358 by RIPK3 appears to serve as a switch that predisposes MLKL to oligomerisation. Unphosphorylated recombinant full length human MLKL exists in a monomer/oligomer equilibrium as assessed by Size Exclusion Chromatography (SEC) [56], with the monomeric state favoured by introduction of phospho-ablating alanine mutations. In contrast, introduction of Glu/Asp (phospho-mimetic) mutations push the equilibrium toward oligomerisation [56]. These observations using recombinant proteins are well supported by cellular data for both mouse and human MLKL.

Taken together a multi-step model for oligomerisation emerges where RIPK3 phosphorylation of the MLKL PsKD induces a conformational change to release the 4HB domain and expose the brace helices. The brace helices can then interact with the brace region of another activated MLKL molecule to assemble into higher order species. The exact intermolecular interactions that facilitate this interaction, and the precise stoichiometry, the packing of MLKL oligomers and the role of the 4HB domain and PsKD in mediating higher order assembly are yet to be determined.

Is there a role for mitochondria in necroptosis ?

Early reports favoured the idea that, like apoptosis, mitochondria were crucial to execution of the necroptotic cell death program, with the mitochondrial proteins, PGAM5 and Drp1, initially implicated as effectors downstream of MLKL [58]. The model that MLKL might target mitochondria for permeabilisation was supported by the observations that (a) recombinant MLKL binds the mitochondrial phospholipid, cardiolipin, and can permeabilise cardiolipin-containing liposomes *in vitro*; and (b) MLKL was found to be localised to membranes throughout the cell, including mitochondrial membranes [56].

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However, subsequent studies indicate that PGAM5 or Drp1, the mitochondrial localisation of MLKL, and even the necessity of mitochondria themselves, are dispensable for necroptotic death [59-63]. Recent *in vitro* studies with recombinant MLKL revealed that cardiolipin was dispensable for liposome permeabilisation, and in fact MLKL preferentially permeabilised liposomes composed of a more plasma membrane-like phospholipid composition [32]. These data suggest that should mitochondria be involved in necroptosis, the mechanism may involve a parallel pathway, or as a secondary event subsequent to instigation of necroptotic death, or simply via a mechanism not universal to all model cell lines.

What is MLKL doing at the plasma membrane?

Current models favour the plasma membrane as the primary site of MLKL action, with MLKL's translocation to the membrane known to correlate with its phosphorylation [39]. However, a lag between MLKL membrane translocation and cell death of ~6 hours was observed in U937 cells [39], and begs the question: what needs to happen at the membrane before MLKL can induce death? A number of competing models have emerged for how MLKL might compromise plasma membrane integrity to induce cell death (Figure 1), although the precise mechanism remains a matter of ongoing debate. Current models include multi-step, partial insertion mediated by specific side-chain interactions between positive residues in the 4HB domain and negatively-charged phospholipids to disrupt membranes [27, 31] and the whole embedment of oligomers to form pores [56]. Other models include auxiliary interactions with ion channels including TRPM7 to mediate osmolysis [24, 28] or that MLKL itself could form a cation channel [64]. Current structure/function studies are focused on understanding the affinity of MLKL interaction with the membrane, the depth of penetration, the specific side chains involved whether conformational changes occur within the 4HB

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domain to accommodate membrane engagement. Progress to answering these questions will be reviewed below.

Which residues within the 4HB domain mediate membrane engagement?

MLKL has been shown to preferentially bind to a number of phosphatidylinositol (PI) species, in addition to cardiolipin, but not other potential components of the bilayer including diacylglycerol, cholesterol and sphingomyelin [31, 56]. The preference for PIs has guided structural studies looking to identify side chains of the 4HB that initially engage the membrane. Dondelinger *et al.* demonstrated mutation of the 9 residues with positive charges on the surface of human 4HB domain to either alanine or glutamate were inactive in liposome assays and lipid arrays using phosphoinositides immobilised on membrane "PIP strips" [31]. Introduction of these mutations into full length human MLKL did not thwart expression in cells, suggesting no significant structural defects, however neither mutant oligomerised or translocated to the plasma membrane. Of note, N-terminal FLAG-tagged MLKL can still oligomerise and translocate to the plasma membrane but cannot lyse cells [27]. Additionally, recombinant mouse, chicken and frog 4HB domains can disrupt liposomes and expression of these domains kills mouse fibroblasts, despite poor conservation of the 9 positive residues in human [27, 32]. Therefore the deficiency of these mutants may relate to lost oligomerisation, which precludes translocation to the membrane, rather than an inability to engage.

How far into the membrane does the 4HB penetrate?

The 4HB domain has been shown to interact with free lipids and liposomes to cause disruption [29, 31, 32, 56, 57]. Two independent studies have shown while the 4HB domain permeablises liposomes, it cannot interact with nanodiscs of the same lipid composition [7, 56]. Nanodiscs are lipid bilayers contained by scaffolding protein belts, which are commonly used

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as model systems for *in vitro* studies of membrane binding. Su *et al.* attributed this finding to the inability of nanodiscs to accommodate embedment of the 4HB domain, which would cause displacement of lipids that is not possible in such a highly constrained system [29], but equally suboptimal presentation of lipid headgroups would also preclude association with nanodiscs. Su *et al.* concluded, through NMR titrations and monitoring fluorescence changes upon lipid binding, that the four core helices of the 4HB domain and the α3-α4 loop helix enter the liposome, but that the first brace helix does not [29]. Notably, it was reported that the inclusion of the first brace helix to the construct reduced liposome leakage, suggesting a regulatory role for the brace helices as via a "plug" mechanism [29].

The model of MLKL N-terminal region:lipid engagement was further extended in a recent paper by Quarato *et al*. [57], who proposed a two-step binding mode. An array of inositol phosphates (IP), the polar head group of PIPs, were titrated against ^{15}N hMLKL(2-156) by NMR spectroscopy to determine any affinity preferences or structural changes. Each of the IPs tested exhibited weak millimolar binding affinity, with IP_4(1,3,4,5) binding with the highest affinity (K_d ~1.4 mM). Based on the NMR assignments of the human NTD [29], two potential phospholipid binding hotspots were identified: one centred on basic residues at the C-terminus of helix-1 and the N-terminus of helix-2 with a K_d ~5-10 mM; and another higher affinity site (K_d ~1-2 mM) centred on the α3-α4 loop helix and preceding residues in α3 (Figure 2B). The reassignment of resonances in the presence of PI(4,5)P_2 suggested an opening of the first brace helix to facilitate binding, consistent the model earlier proposed by Su *et al.* [29, 57].

In the mouse 4HB domain, two clusters were also reported to be functionally significant based on mutational analyses (Figure 2A) [27]. One cluster (yellow side chains, Figure 2A)

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centred on the α3 and α4 helices appeared to block cell death by impacting oligomerisation when expressed in mouse dermal fibroblasts. Mutations in the second cluster, centred on the α1 and α2 helices (green side chains, Figure 2A), blocked cell death, despite oligomerisation and translocation to the plasma membrane. The latter finding was unexpected, but suggests the second cluster may underpin protein interactions with either other MLKL protomers within a higher order complex or with other effectors to drive necroptosis [27].

Conclusions and future directions.

The use of structure/function studies and biophysical techniques have greatly enhanced our understanding of the mechanism by which MLKL engages and permeabilises the plasma membrane. These studies provide a starting point for a more detailed examination of MLKL's mechanism of action at the membrane using contemporary membrane protein biophysical techniques, such as lipid cubic phase crystallization and cryo-electron microscopy. Coupling these studies with cellular assays will be crucial in resolving the debate about the precise mechanism of MLKL action and, furthermore, in identifying the other cellular factors that promote or negate MLKL-mediated necroptosis.

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Figure Legends

Figure 1: Proposed models of MLKL action at the plasma membrane.

A. Partial insertion mediated by two-step binding mode of the 4HB domain[57].

B. MLKL association with auxiliary transporters, such as TRPM7, or transmembrane ion channels to facilitate osmolysis[28, 30].

C. Rearrangement of the MLKL 4HB domain to become a selective cation channel[64].

D. Oligomerisation of MLKL mediated by the brace region to disrupt the plasma membrane and allow the release of cellular contents[27, 32].

Figure 2: Structural comparison of the 4HB domains of mouse and human NTD.

A. Structure of mouse MLKL N-terminal region (1-182) from the full length crystal structure (2-456) (PDB:4BTF)[6]. Mutagenesis studies revealed Cluster 1 (*yellow*) alanine substitution mutants cannot form oligomers and consequently do not translocate to the plasma membrane and cause cell death. Cluster 2 (*green*) mutations block cell death, but not the ability to form oligomers and localise to the plasma membrane[27].

B. Structure of human MLKL (1-154) solved by NMR (PDB:2MSV)[29]. Two regions were mapped to the structure by Quarato *et al*. showing K_d ~5-10 mM binding affinity for inositol phosphates (orange) and K_d ~1-2 mM (pink)[57].

C. Overlay of the mouse (*purple*) and human (*cyan*) NTD structures illustrates the overall similarity between their folds.

Figure 3. Structural insights into the mouse and human MLKL pseudokinase domains

A. The full length structure of mouse MLKL solved by X-ray crystallography (PDB:4BTF)[6], featuring the 4HB domain (left) and pseudokinase domain (right) joined by the brace helices.

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B. Co-complex structure (PDB:4MB9) of mouse MLKL PsKD (residues 182-459) bound to mouse RIPK3 kinase domain (residues 12-312)[40]. The two RIPK3 phosphosites, S231 and T232, observed in the structure at the MLKL binding interface are shown as orange sticks.

C. The human MLKL PsKD (PDB:4MWI)[26] adopts an active protein-kinase like structure, with a more conventional helix αC position without the activation loop helix seen in the mouse MLKL PsKD structure.

D. Magnification of the human (cyan) PKD overlayed with mouse (purple). Human MLKL has retained the canonical kinase interaction between K230:E250 (blue), while mouse has diverged to an unconventional K219/Q343 hydrogen bond.

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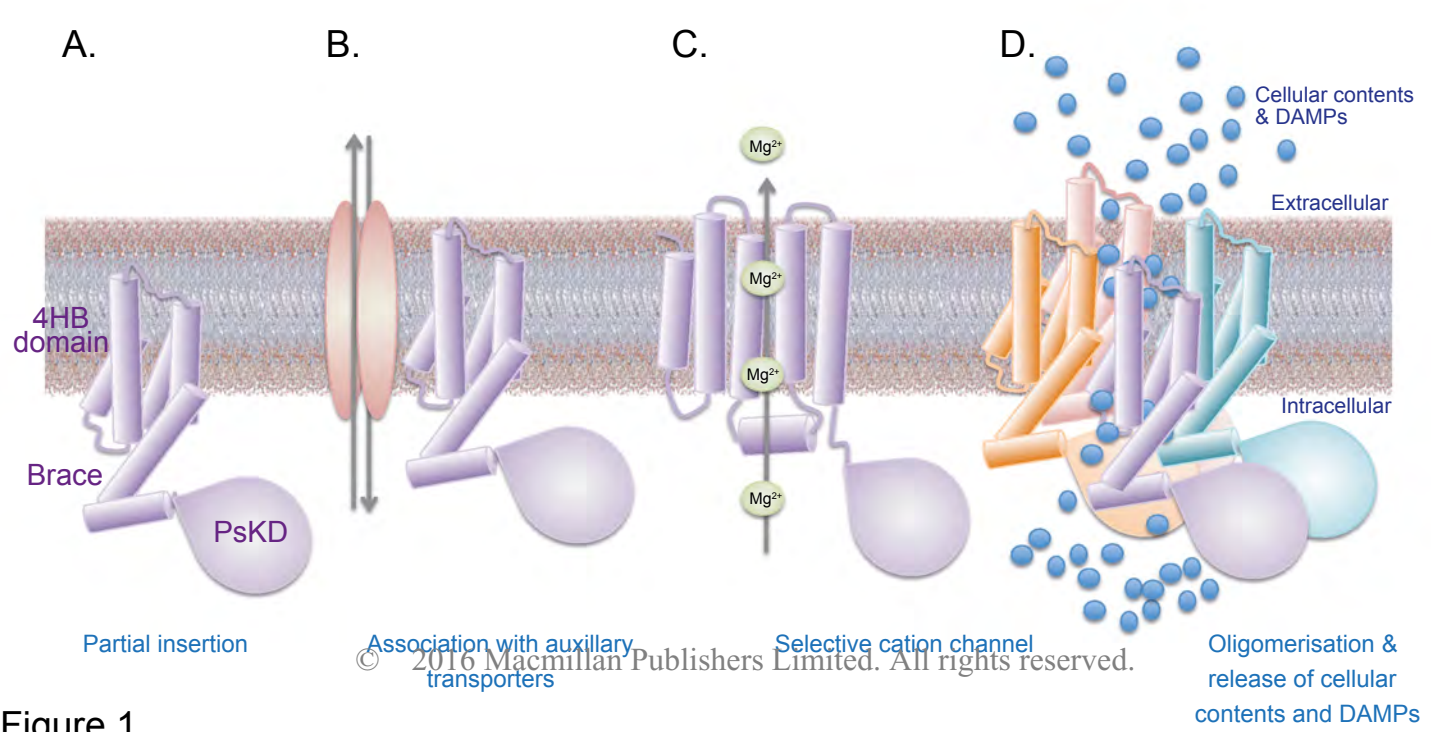

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Figure 1

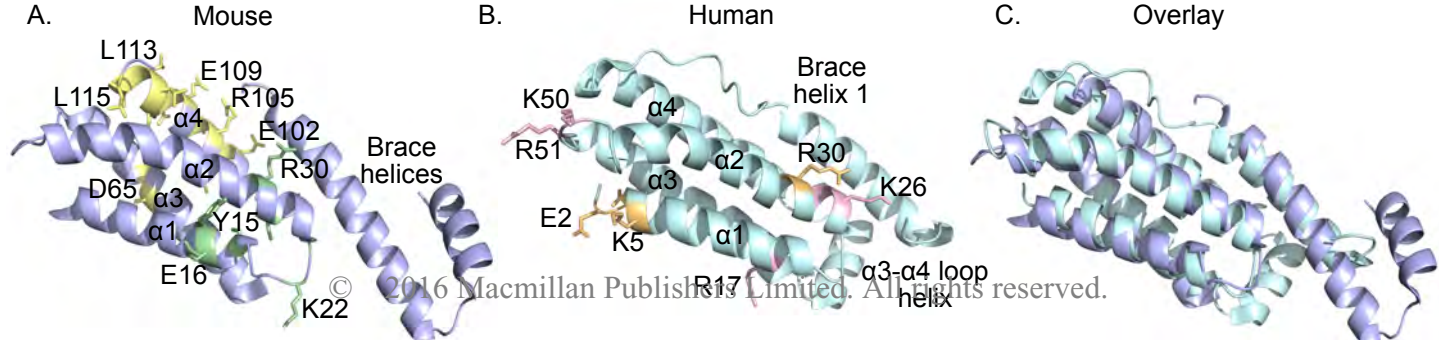

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Figure 2

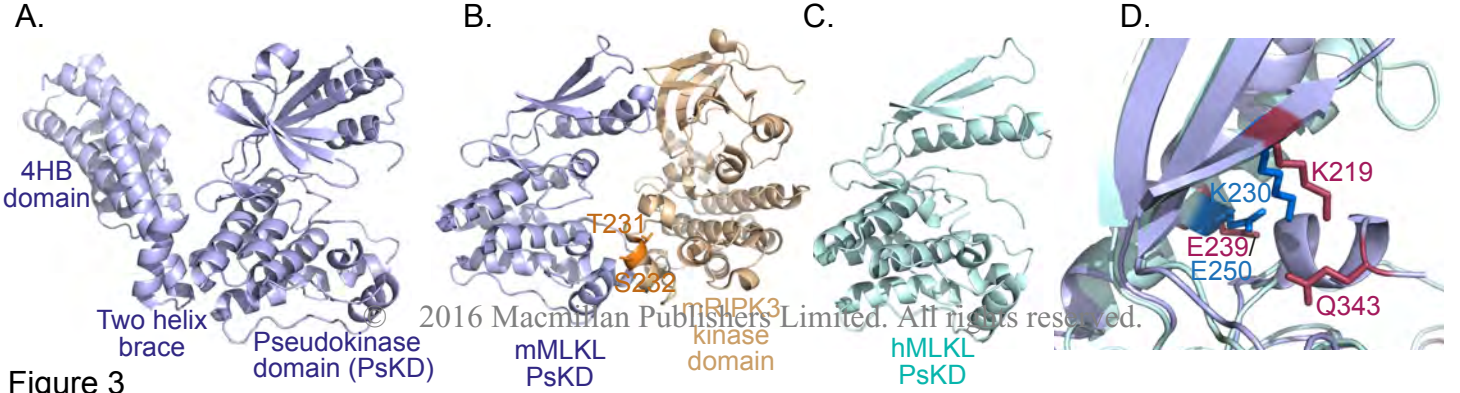

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Figure 3