1. Phylogeny  
   MLKL (Mixed Lineage Kinase Domain‐Like protein) is a conserved vertebrate pseudokinase that has been identified in a broad range of species including human, mouse, rat, horse, pig, chicken, stickleback fish, frog, and tuatara. Its occurrence in organisms spanning mammals to lower vertebrates illustrates its deep evolutionary conservation as part of a distinct subgroup within the protein kinase superfamily that lacks catalytic activity. Although MLKL shares structural similarities with active kinases, phylogenetic analyses position it firmly within the pseudokinase branch, and studies have revealed that its evolution involved divergence from canonical kinases as its role shifted from catalysis to serving as a regulated effector of cell death. Comparative investigations of MLKL orthologues have further demonstrated species‐specific differences in domain architecture and activation mechanisms that underscore its adaptive evolution across vertebrates (davies2020distinctpseudokinasedomain pages 1-2, tanzer2016evolutionarydivergenceof pages 1-2).
2. Reaction Catalyzed  
   Unlike conventional protein kinases that catalyze the transfer of a phosphate group from ATP to target proteins, MLKL does not perform any phosphoryl transfer reaction. Although it contains a kinase‐like domain that structurally resembles a catalytically competent active site, MLKL lacks the residue motifs necessary for enzymatic activity, and no reaction such as ATP + substrate → ADP + phospho–substrate + H⁺ has been detected. Instead, MLKL functions solely as a phosphorylation substrate for RIPK3 and, upon phosphorylation, undergoes a conformational switch that mediates downstream necroptosis, rather than catalyzing the chemical reaction itself (murphy2013thepseudokinasemlkl pages 9-9, liao2017mixedlineagekinase pages 10-10).
3. Cofactor Requirements  
   In catalytically active kinases, cofactors such as Mg²⁺ (or Mn²⁺) are indispensable for coordinating ATP binding and facilitating phosphoryl transfer. However, given that MLKL lacks intrinsic kinase activity and does not catalyze ATP-dependent reactions, it does not require such cofactors for catalytic function. Although MLKL is capable of binding ATP under certain conditions, its nucleotide binding is atypical and occurs in a manner that is independent of the divalent cations normally essential in active kinases. Thus, no cofactor such as Mg²⁺ is necessary for MLKL’s membrane-targeting and oligomerization functions that occur downstream of its phosphorylation by RIPK3 (murphy2013thepseudokinasemlkl pages 3-4, osinski2022biochemicalandstructural pages 18-24).
4. Substrate Specificity  
   Because MLKL is catalytically inactive, it does not display the substrate specificity characteristic of enzyme-active serine/threonine kinases that recognize and phosphorylate target motifs. Instead, MLKL itself serves as a substrate for the active upstream kinase RIPK3, which phosphorylates specific serine/threonine residues found within its activation loop. No consensus recognition sequence or substrate motif is defined for MLKL because it does not perform enzymatic functions; its primary sequence and conformational features are instead adapted to enable it to undergo conformational changes and oligomerize following phosphorylation. In this role, MLKL is part of a signaling cascade in which substrate specificity is determined by RIPK3 rather than by MLKL itself (murphy2013thepseudokinasemlkl pages 2-3, liao2017mixedlineagekinase pages 10-10).
5. Structure  
   MLKL exhibits a bipartite domain organization consisting of an N‐terminal four-helix bundle (4HB) domain and a C‐terminal pseudokinase domain. The N-terminal 4HB domain functions as the executioner module, which when liberated, mediates membrane permeabilization and consequent necroptotic cell death. The C-terminal pseudokinase domain, although sharing the canonical bilobal architecture (with a smaller N-lobe comprised primarily of β-strands and a larger C-lobe predominated by α-helices) seen in active kinases, lacks key catalytic motifs (such as the HRD and DFG motifs) and the requisite residues for magnesium coordination. Detailed crystallographic studies in species such as rat and horse have revealed that the MLKL pseudokinase domain can adopt distinct conformations–with conformational differences (for example, an additional helix in the β3-αC loop in horse MLKL) that impact its interaction with RIPK3. In many MLKL orthologues, the activation loop is sequestered within the pseudoactive site, and phosphorylation by RIPK3 prompts a conformational change that destabilizes this arrangement, allowing for MLKL oligomerization and translocation to the plasma membrane. The overall three-dimensional organization of MLKL, as supported by both crystallographic and AlphaFold modeling studies, reflects its dual role as both a structural switch and a mediator of membrane disruption, even though its pseudokinase domain is catalytically inert (davies2020distinctpseudokinasedomain pages 4-5, murphy2013thepseudokinasemlkl pages 2-3, liao2017mixedlineagekinase pages 10-10).
6. Regulation  
   The primary regulatory mechanism for MLKL activation is phosphorylation by RIPK3, an upstream serine/threonine kinase that specifically targets residues within MLKL’s activation loop. In several studies, phosphorylation events (notably at serine and threonine residues such as S345 in mouse MLKL and T357/S358 in human MLKL) have been shown to trigger a conformational change in the pseudokinase domain. This phosphorylation-induced change alleviates the inhibitory interactions between the pseudokinase domain and the N-terminal 4HB domain, permitting MLKL to oligomerize and translocate to the plasma membrane where it executes necroptotic cell death. Experimental evidence also shows that mutations mimicking phosphorylation in the absence of RIPK3 can induce constitutive necroptosis, thereby underscoring the importance of the phosphorylation event in regulating MLKL’s conformational switch. Post-translational modifications other than phosphorylation have not been documented as significant for MLKL regulation, and the enzyme machinery that unidirectionally activates MLKL is limited to RIPK3. Thus, the regulation of MLKL is achieved primarily through phosphorylation-dependent allosteric modulation that governs its oligomerization and membrane-targeting properties (murphy2013thepseudokinasemlkl pages 7-8, davies2020distinctpseudokinasedomain pages 7-8, liao2017mixedlineagekinase pages 7-10).
7. Function  
   MLKL serves as the terminal effector in the programmed necroptosis pathway, a form of regulated necrotic cell death predominantly initiated by death receptor signaling such as that induced by tumor necrosis factor (TNF). Once phosphorylated by RIPK3, MLKL undergoes an irreversible conformational change that drives its oligomerization. Activated MLKL oligomers translocate from the cytosol to cellular membranes—most notably the plasma membrane—where the N-terminal 4HB domain disrupts lipid bilayer integrity. This membrane permeabilization results in calcium influx and ultimately cell lysis. In addition to its role in TNF-induced necroptosis, emerging evidence indicates that MLKL may also contribute to nuclear necroptosis in response to viral infection via activation by ZBP1 and nuclear RIPK3. The downstream effects of MLKL activation include the release of damage-associated molecular patterns (DAMPs), which can further amplify inflammatory responses. In this manner, MLKL is positioned as both an executioner of cell death and a mediator of inflammation, integrating signals from upstream kinases and contributing to pathophysiological outcomes in contexts such as ischemia–reperfusion injury and virus-induced inflammation (liao2017mixedlineagekinase pages 10-10, wood2023pseudomonasaeruginosacytotoxins pages 22-23, chan2015programmednecrosisin pages 4-4).
8. Other Comments  
   Given its central role as the executioner in necroptosis, MLKL is a subject of intense research both for understanding cell death mechanisms and for potential therapeutic intervention in inflammatory and degenerative diseases. Inhibitory strategies targeting MLKL aim to modulate its oligomerization and membrane translocation rather than its non-existent kinase activity. In addition, while several small molecules have been developed to inhibit other key players in necroptosis (such as RIPK1 and RIPK3), specific inhibitors that directly target MLKL’s conformational switch have also shown promise in modulating necroptosis. Moreover, the clinical relevance of MLKL is underlined by its association with tissue injury in conditions including neurodegeneration, renal ischemia, and inflammatory bowel disease. MLKL’s status as a pseudokinase further distinguishes it within the kinome and has led to the recognition of distinct classes of pseudokinases as nonenzymatic signaling regulators in cell death and inflammatory pathways. Additional evolutionary insights are provided by studies in plants where MLKL\_NTD domains are found in kinase fusion proteins with functional divergence relative to their animal counterparts, highlighting both the evolutionary adaptability and the specialized signaling function of MLKL (he2024akinasefusion pages 4-6, tanzer2016evolutionarydivergenceof pages 1-2).
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