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EDITORIAL

MLKL ubiquitylation: more than a makeover

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For proteins after biosynthesis, the post-translational modifications are like the "makeovers." These secondary changes have wide-ranging effects on protein functions. For example, MLKL, the executor for necroptosis, can only perform plasma membrane pore-forming and trigger damage repair until phosphorylated by kinase RIPK3 on T357/S358 (human) or S345 (murine) [1–3]. This phosphorylation also promotes endosomal trafficking, either for releasing extracellular vehicles (EVs) or lysosomal fusion [4, 5]. Instead of RIPK3, in response to serum and amino acid deprivation, calcium/calmodulin-dependent protein kinase II (CAMK2/CaMKII) phosphorylates MLKL on the same sites to facilitate autophagic flux [6].

Other phosphorylation sites of MLKL include Y376 (human) or Y363 (murine) by T.A.M. kinases. The tyrosine phosphorylation also facilitates MLKL oligomerization at the plasma membrane [7]. In addition, murine MLKL phosphorylation on S441 played a role in provoking MLKL-directed myelin breakdown, rather than cell death, in Schwann cells [8]. This phosphorylation is distinct from the necroptosis-inducing phosphorylation mediated by RIPK3, and the kinase responsible for S441 phosphorylation is still unknown. MLKL phosphorylation may also show inhibitory effects. The phosphorylation on S83 (human) or S82 (murine) blocks MLKL mediated cell death downstream of RIPK3 [9]. The responsible kinase is also unclear. The S83 is in the N-terminal helix bundle domain of MLKL. Covalent targeting of C86 (human) in this domain by necrosulfonamide (NSA) also blocks necroptosis [3], suggesting the importance of covalent modification within this region.

Recent studies reveal the MLKL ubiquitylation during necroptosis [10–12], including the one from Yoon et al., published on this issue [12]. Both the mono-ubiquitylation and poly-ubiquitylation, at multiple sites, in human or murine systems were observed in these reports. Based on the approach with the deubiquitylating enzyme (DUB) USP21, which can cleave the covalent bond between a protein substrate and the first added ubiquitin, it is shown that MLKL can be mono-ubiquitylated at multiple sites, such as K50 (human), K51 (human + murine), K77 (murine), K172 (murine), and K219 (murine). Meanwhile, Yoon et al. demonstrated the conjugation of K63-linked polyubiquitin chains to K50/K51 (human) and K51 (murine) residues [12]. The K63-linked Ub chain could be removed by K63-specific DUBs CYLD or AMSH, but not K48-specific DUB Otubain1. They also identified that E3 ligase ITCH is the ubiquitin ligase for the K63-linked MLKL ubiquitylation.

Multiple mutagenesis approaches were adapted to dissect the relationship of MLKL phosphorylation, oligomerization, pore-forming activity, and ubiquitylation. MLKL undergoes ubiquitylation after phosphorylation by RIPK3, supported by the evidence that the ubiquitylation is correlated with S345 phosphorylation, and RIPK3 phosphorylation site mutations abolish the ubiquitylation. Moreover, MLKL oligomerization is required for its ubiquitylation, as MLKL R105A/D106A mutations in the four-helix bundle (4HB) prevent the oligomerization and ubiquitylation. Note that RIPK3 association and MLKL phosphorylation are not required for ubiquitylation. It appears that as long as MLKL can oligomerize, such as by Q343A (murine) mutation [13], it can be ubiquitylated even in RIPK3 deficient cells, bypassing S345 phosphorylation. MLKL ubiquitination is not a consequence of necroptosis. The N-terminus flag tagged MLKL or E109A/E110A MLKL mutant, unable to induce necroptosis yet can still be phosphorylated and then ubiquitylated.

MLKL is predominantly ubiquitylated in the cytosolic compartment before translocation to other membrane structures, such as plasma membranes or endosomes. Using in situ proximity ligation assay (PLA) and subcellular fractionation approaches, Garcia et al. showed that the necroptosis associated prominent MLKL-ubiquitin PLA speckles, first localized to the cytosol before membrane translocation. The subcellular fractionation assay further confirmed that as cell death progressed, more ubiquitinated MLKL accumulated in the membrane-enriched fractions [10].

Ubiquitination of MLKL has profound biological consequences. MLKL K219 ubiquitylation contributes to its cytotoxic activity. K219 forms a hydrogen bond with Q343 in the crystal structure [13], which contributes to keeping MLKL in a non-cytolytic conformation. K219 ubiquitylation may disrupt this interaction, therefore, contribute to MLKL killing activity. MLKL K219R mutation, deficient in ubiquitination and predicted to maintain the hydrogen bond with Q343, did not trigger cell death in the absence of necroptotic stimuli but could partially block necroptosis when stimulated from the receptors or by MLKL S345D mutation. In contrast, MLKL K219M mutation disrupted the hydrogen bond with Q343 and ubiquitination, was cytotoxic, bypassing extracellular stimuli. Thus, ubiquitylation of K219 contributes to the cytotoxic potential of MLKL, possibly by breaking the interactions with Q343 and mimicking the K219M mutation. Physiologically, *Mlkl*$^{K219R/K219R}$ knock-in can protect cells from TNF and MCMV-driven necroptosis and necroptosis-induced tissue injury in a skin ulceration and inflammation animal model [10]. K219 of mouse MLKL is conserved in human and corresponds to K230. However, human K230 ubiquitylation was only found from a whole ubiquitinome-wide analysis [14] but not in necroptotic HT-29 cells [12]. Different from K219, murine MLKL K51, and K77 ubiquitylation seems not to affect MLKL's killing potentials.

MLKL ubiquitylation may also antagonize necroptosis. The experimental evidence is primarily based on the MLKL-USP21 fusions. Given USP21 can remove all ubiquitin from MLKL in vitro, fusing the catalytic domain of USP21 to MLKL could serve as a total "loss-of-function" approach for MLKL ubiquitination study. Employing this approach, Liu et al. showed that MLKL-USP21 fusions could reconstitute the capacity of *Mlkl*$^{-/-}$ cells to undergo necroptosis as WT MLKL molecules [11]. However, different from WT MLKL, expression of MLKL-USP21 fusion can cause cell death independent of RIPK3. This indicates that the MLKL ubiquitylation, at least on some of the amino acid residues, can antagonize MLKL killing capacity and restrain the basal levels of MLKL activation.

In this issue, Yoon et al. reported the MLKL K50/K51 ubiquitylation, which may explain the results from MLKL-USP21

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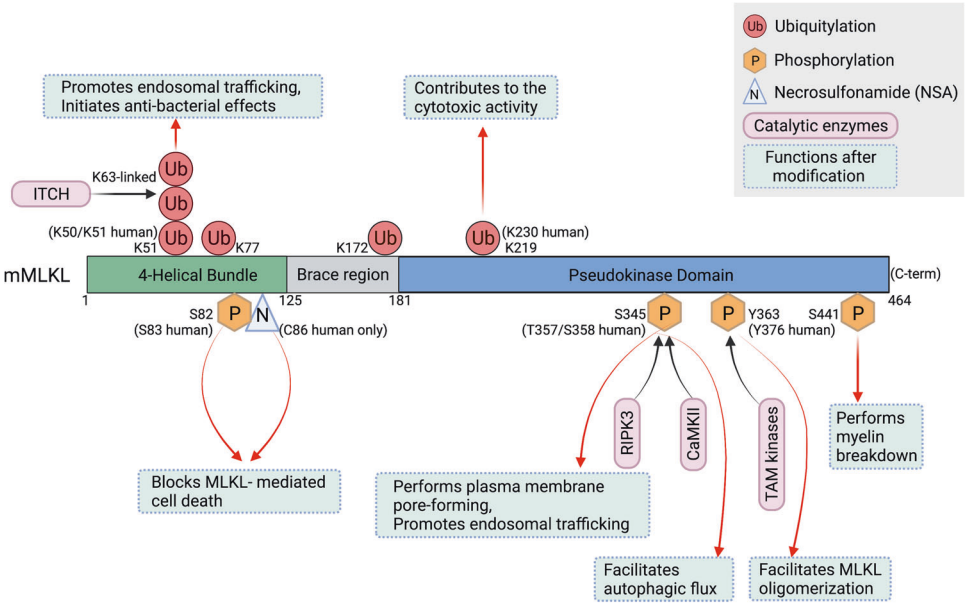

Fig. 1 Post-translational modifications of MLKL and their functions. This figure summarizes the covalent post-translational modifications on MLKL, including modification types, sites, responsible enzymes and biological consequences.

fusion [12]. They showed ITCH-mediated K63-linked poly-ubiquitylation on K50/K51 dictated the endosomal association of the phosphorylated MLKL. While a portion of WT MLKL translocated to endosome upon phosphorylation and oligomerization, MLKL K50R mutation did not display these associations with the Rab5$^+$ or Rab7$^+$ endosomes. Since the association of ubiquitylated MLKL with endosomes enhanced the release of activated MLKL via extracellular vesicles or traffic for lysosome degradation, MLKL K50R/K51R mutations or ITCH knockout augmented necroptosis. This mechanism may serve as one possible explanation of the necroptosis antagonizing role of MLKL ubiquitylation.

Further, MLKL, once ubiquitinated on K50/K51 (human and murine), acquires the ability to initiate anti-bacterial effects. K50/K51 ubiquitinated MLKL can target the bacteria, such as *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Escherichia coli*, to endosomal-lysosome trafficking system. K50R (human) or the K50R/K51R (murine) MLKL mutants, or silencing of ITCH, abolished the anti-pathogen effects. This anti-pathogen effect is distinct from the MLKL cytotoxic effect, as MLKL L58G/I76G mutant, which can be phosphorylated, oligomerized, and probably ubiquitinated, yet fail to mediate plasma membrane pore-forming, still suppresses *L. monocytogenes* growth. Thus, MLKL ubiquitination also serves as an immune defense mechanism.

Altogether, MLKL, the initially identified effector molecule in necroptotic death [15], has multiple non-deadly functions. Molecular determinants, such as the various covalent modifications discussed above (Fig. 1), define the roles that MLKL takes in different situations. These modifications are not just--"makeovers."

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST
The authors declare no competing interests.

ADDITIONAL INFORMATION
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