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REVIEW ARTICLE

The regulation of necroptosis by post-translational modifications

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Abstract

Necroptosis is a caspase-independent, lytic form of programmed cell death whose errant activation has been widely implicated in many pathologies. The pathway relies on the assembly of the apical protein kinases, RIPK1 and RIPK3, into a high molecular weight cytoplasmic complex, termed the necrosome, downstream of death receptor or pathogen detector ligation. The necrosome serves as a platform for RIPK3-mediated phosphorylation of the terminal effector, the MLKL pseudokinase, which induces its oligomerization, translocation to, and perturbation of, the plasma membrane to cause cell death. Over the past 10 years, knowledge of the post-translational modifications that govern RIPK1, RIPK3 and MLKL conformation, activity, interactions, stability and localization has rapidly expanded. Here, we review current knowledge of the functions of phosphorylation, ubiquitylation, GlcNAcylation, proteolytic cleavage, and disulfide bonding in regulating necroptotic signaling. Post-translational modifications serve a broad array of functions in modulating RIPK1 engagement in, or exclusion from, cell death signaling, whereas the bulk of identified RIPK3 and MLKL modifications promote their necroptotic functions. An enhanced understanding of the modifying enzymes that tune RIPK1, RIPK3, and MLKL necroptotic functions will prove valuable in efforts to therapeutically modulate necroptosis.

Facts

- Post-translational modifications of the RIPK1 and RIPK3 kinases and MLKL pseudokinase regulate their localization, activity, conformation, oligomerization, and protein interactions
- To date, principally activating modifications have been reported for RIPK3 and MLKL
- Most of the reported post-translational modifications in the necroptosis pathway affect the apical kinase, RIPK1
- RIPK1 post-translational modifications govern whether it participates in NF-κB, apoptotic or necroptotic signaling

Open questions

- Are RIPK3 and MLKL disarmed by enzymes that remove activating post-translational marks?
- What is the function of MLKL ubiquitylation?
- Do kinases other than RIPK3 phosphorylate MLKL to promote necroptotic death, and what are their identities?
- How do RIPK3 and MLKL modifications control their protein interactions and prompt cell death?

Introduction

Necroptosis is a caspase-independent, pro-inflammatory programmed cell death pathway that was first described towards the end of the 20th century [1–5]. The term 'necroptosis' was subsequently coined [6] to connote the better-understood programmed cell death pathway, apoptosis, to convey that necroptosis is similarly triggered by specific signals and is tightly regulated. Morphologically, apoptotic cells are marked by cell shrinkage, chromatin condensation, and cellular fragmentation [7] and, as a mechanism of normal cell development and homeostasis, it is considered immunologically silent [8]. On the contrary, necroptosis is characterized by organelle swelling, plasma membrane rupture and, more importantly, leakage of

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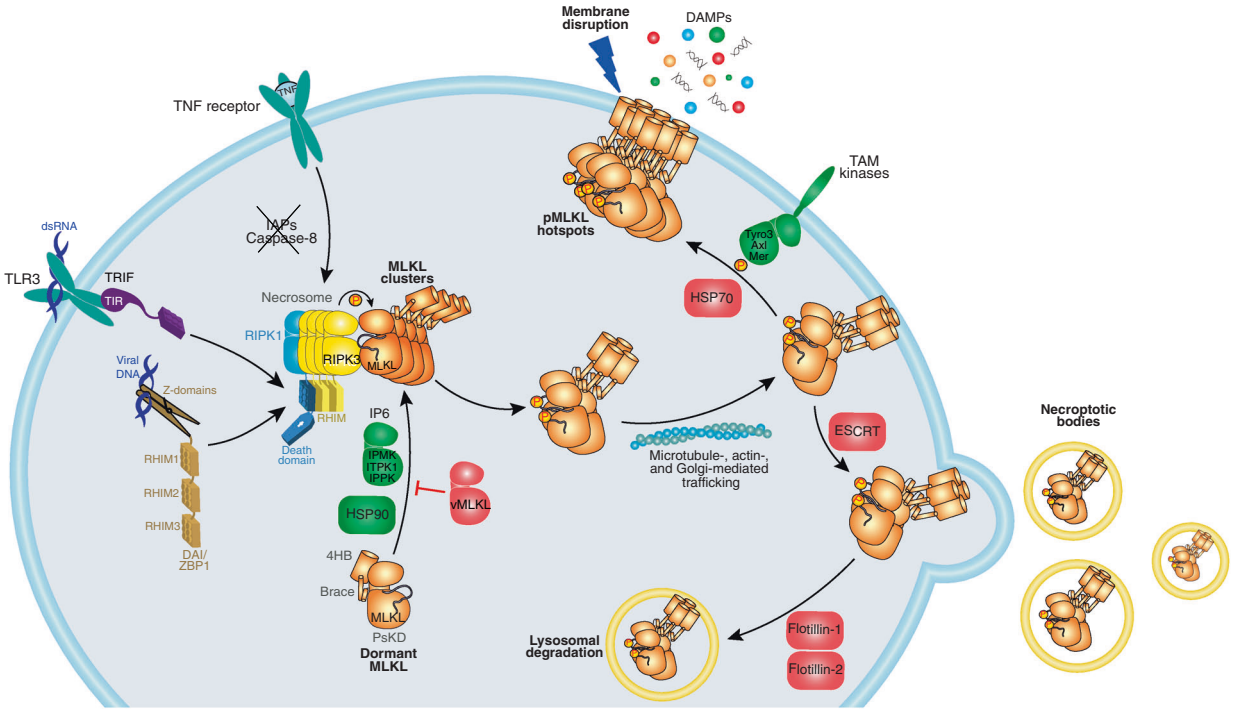

Fig. 1 Overview of the necroptosis pathway. Necroptosis can be induced by various receptors (TNF-receptor, Toll-like receptors (TLRs), Z-DNA-binding protein 1 (ZBP1)/DAI) upon binding to their respective ligands. TNF-induced necroptosis requires the absence of cIAP and Casp8 activity. Under such conditions, RIPK1 and RIPK3 oligomerize as a RHIM-mediated amyloid fibril, resulting in RIPK3 activation by autophosphorylation, the recruitment of MLKL and its activation via RIPK3-mediated phosphorylation. Phosphorylated MLKL is thought to be activated by undergoing a conformational change to expose the killer 4HB domain [30, 43, 178, 186] and assemble into oligomers as necrosomal clusters before being trafficked to the plasma membrane via microtubule-, actin-, and Golgi-dependent mechanisms [51]. Activated MLKL accumulates into hotspots at the plasma membrane and, when a threshold is surpassed, disrupts the plasma membrane to cause death and the release of DAMPs [51]. This pathway is tightly regulated by various accessory components (positive regulators in green; negative regulators in red). Among these, certain inositol phosphates [196, 197], the chaperone HSP90 [198–200], and TAM kinases [187] promote necroptosis by positively regulating MLKL activation, translocation, and clustering at plasma membrane respectively. Conversely, necroptosis has been shown to be negatively regulated by blocking MLKL recruitment to RIPK3 (viral MLKL homolog [19]), preventing MLKL accumulation at plasma membrane (HSP70 [201]), and the removal of membrane bound MLKL (ESCRT [50, 202], flotillins [203]).

intracellular components known as damage-associated molecular patterns (DAMPs), which propagate secondary inflammatory responses [9, 10]. In keeping with its pro-inflammatory nature, necroptosis has likely evolved as an innate immunity mechanism to complement apoptosis as a means of eliminating pathogens, such as in scenarios where pathogen-encoded proteins block apoptosis [11, 12]. The idea that necroptosis arose as an ancestral host defense mechanism is supported by the discovery of viral and bacterial proteins that block necroptosis at various steps of the signaling pathway in animals [13–23], and the recent discovery of convergently-evolved pathway effectors that contribute to plant host defense [24].

Necroptosis is effected by receptor-interacting serine/threonine protein kinase (RIPK)-3 [25–27] and the terminal effector, the mixed-lineage kinase domain-like (MLKL) pseudokinase [28–30] (Fig. 1). Ligand binding to a variety of death receptors, such as tumor necrosis factor (TNF) receptor 1 (TNFR1), can induce necroptosis by prompting association and activation of their respective cytoplasmic adaptor proteins. In cellular contexts where the cellular inhibitors of apoptosis E3 ligase family (cIAPs) [31–33] or its downstream protein kinase TAK1 [34] and the extrinsic apoptosis effector, Caspase-8 [35–37], are disarmed or depleted, death receptor ligation promotes autophosphorylation of RIPK1 kinase and assembly into high molecular weight complexes with RIPK3. Such an assembly depends on the formation of functional amyloid via their RIP homotypic interaction motifs (RHIM) [38, 39]. Subsequently, RIPK3-mediated phosphorylation provokes the killing activity of MLKL [28, 30, 40] by prompting translocation to, and disruption of, the plasma membrane [40–44]. While the precise mechanisms underlying MLKL activation, translocation, and membrane compromise are still emerging (reviewed in ref. [45, 46]), the phosphorylation of MLKL on its pseudokinase domain activation loop (S345 in mouse; S358 in human MLKL) is considered the hallmark of its activation and has been widely used as a

biomarker for necroptotic death [28, 40, 47, 48]. However, necroptosis also depends on multiple downstream checkpoints that can be blocked by necrosulfonamide (NSA) [40, 49], ESCRT-III [50], Golgi/microtubule/actin trafficking pathway inhibitors [51], and MLKL killer/four-helix bundle (4HB) domain binding monobodies [52], indicating that additional steps beyond MLKL phosphorylation itself are required to induce cell death.

Therapeutic targeting of necroptosis in human disease

As an innate immune mechanism, necroptosis plays a key role in protection against pathogens in humans [13–17, 19, 21–23, 53, 54]. On the other hand, necroptosis has attracted much attention due to its dysregulation in a cornucopia of human inflammatory diseases. Hypo- and hyperactivation of necroptosis, as well as dysregulation of the balance between apoptosis and necroptosis, can contribute to disease pathologies (reviewed in ref. [55, 56]). Consequently, errant necroptosis has been implicated in the pathology of inflammatory syndromes [57], atherosclerosis [58], cardiac [59] and kidney [60] ischemia/reperfusion injury, sepsis [61–63], inflammatory bowel disease [64], neurodegenerative diseases [65], and cancer [66], although some of these attributions remain the matter of debate [62, 67–72]. In addition, damage caused by some bacterial infections have been proposed to be attributable to excessive necroptosis and inflammation [73, 74]. Taken together, ample evidence suggests that restricting necroptosis will serve as a useful therapeutic strategy for the treatment of various infectious and non-infectious inflammatory diseases.

Targeting the post-translational modifications that promote necroptotic cell death is an attractive avenue for clinical intervention in many diseases. To date, most effort in targeting the pathway has been focused on RIPK1 kinase —the apical, instigating kinase in necroptosis signaling— with several inhibitors progressing into early phase clinical trials (as reviewed in ref. [75]). The rationale for targeting RIPK1 is clear; patient variants in RIPK1 that prevent cleavage and thus negative regulation by Casp8 were reported to underlie an inflammatory disease termed CRIA (cleavage-resistant RIPK1-induced autoinflammatory syndrome) [37, 76, 77]. Furthermore, colitis patients harboring inactivating substitutions in NEMO exhibit elevated RIPK1 kinase activity and cell death [78], providing further support that targeting RIPK1 and the modifying enzymes that promote its necroptotic activity could be therapeutically advantageous. There has been some debate about whether targeting the downstream kinase, RIPK3, could be a viable therapeutic strategy, because in some contexts RIPK3 inhibitors and kinase-inactivating mutations appear to favor a kinase domain conformation that promotes constitutive apoptosis [79, 80], rather than simply inhibiting the catalytic activity of RIPK3 to prevent the phosphorylation and activation of the terminal effector, MLKL [81]. It is foreseeable that targeting MLKL could be advantageous because of MLKL's specific role in executing necroptosis, although the only selective agent reported to date, NSA, is a covalent modifier of MLKL and is thus not suitable for clinical development [28]. Recent studies suggest that small molecules that target RIPK1, RIPK3, and MLKL simultaneously may prove an effective strategy in targeting necroptotic diseases pharmacologically [63].

Activation of necroptosis signaling downstream of receptors

A number of cell surface receptors can trigger necroptosis upon binding to their respective ligands, including TNFR1, interferon receptors (IFNRs), Toll-like receptors (TLRs), and DAI/ZBP1 (Fig. 1). While the adaptor proteins involved in each receptor complex vary depending on the type of receptor, they all signal through the necrosome to cause necroptotic death. The exact composition of the necrosome may also vary, but the core components comprise RIPK1, RIPK3, and MLKL. Necrosome assembly and MLKL activation occurs downstream of various stimuli, of which the TNFR1 pathway is the most well-studied. It is presumed that other death receptors function analogously, although like TLRs [82], it is possible distinct adaptor proteins connect the receptor signals to the necrosome. Activated IFNRs signal via JAK kinases and their cognate STAT proteins, leading to a transcriptional program that results in synthesis of necrosome modulator proteins [83]. An understanding of the connection between IFNR signaling and necroptosis is still in its infancy, but was reported to involve PKR kinase and the DAI/ZBP1 sensor protein [83, 84]. DAI/ZBP1 is best understood as a multi-RHIM-containing DNA sensing protein that detects, and is activated by, viral dsDNA. DAI/ZBP1 was proposed to activate RIPK3 directly via a RHIM–RHIM interaction [22] to promote MLKL phosphorylation and necroptotic cell death, which can be attenuated by RIPK1 sequestration of DAI/ZBP1 [85, 86].

Recent studies have revealed the existence of multiple signaling checkpoints that control necroptosis [19, 28, 30, 40, 42, 46, 51, 52, 87–89]. At the heart of these regulatory checkpoints lie many post-translational modifications that can promote (Fig. 2) or attenuate (Fig. 3) signaling flux via the necroptosis pathway. Here, we review our current understanding of the modifications that influence the activity, localization, and stability of the RIPK1,

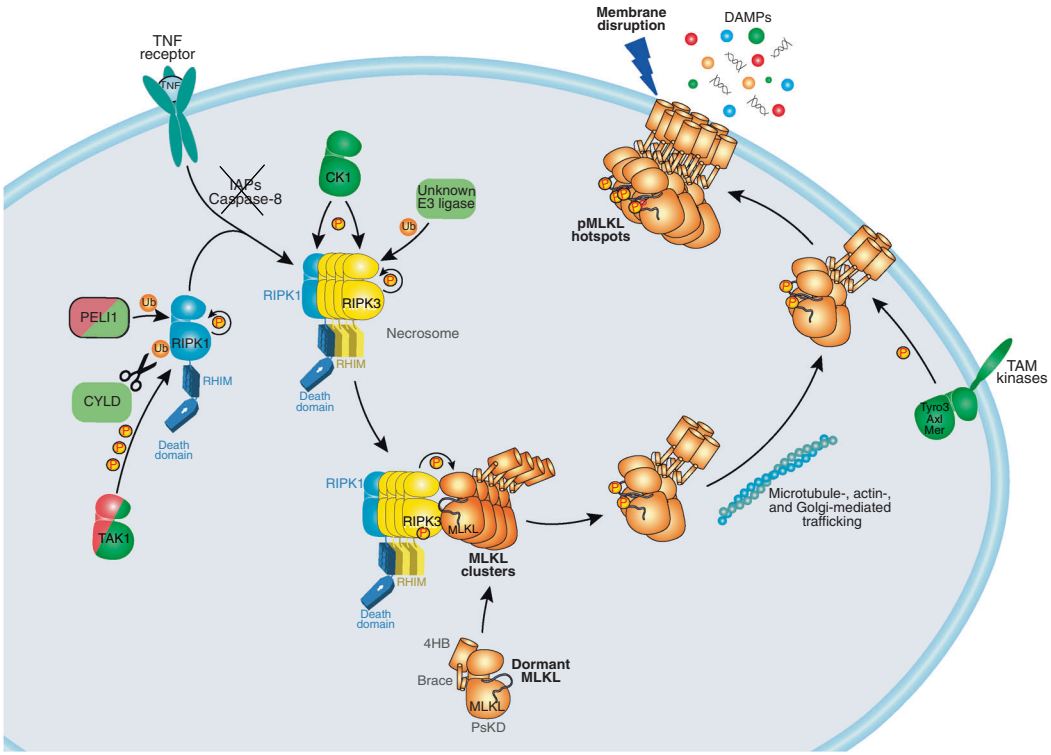

Fig. 2 Post-translational modifications positively regulate necroptosis. RIPK1 is positively regulated by K63-linked ubiquitination (PELI1), removal of M1-linked ubiquitin chains (CYLD), and autophosphorylation. RIPK3 autophosphorylation is required for its interaction with, and phosphorylation of, MLKL. This is promoted by RIPK3 ubiquitination at K5, as well as CK1-mediated phosphorylation in some contexts. TAM kinase-mediated phosphorylation was proposed to promote MLKL clustering at plasma membrane into hotspots.

RIPK3, and MLKL: the core proteins of the necroptosis machinery.

Overview of the necroptosis signaling pathway

As a hub for cell death signaling, RIPK1 is involved in the regulation of both pro-survival NF-κB activation, apoptosis, and necroptosis (reviewed in ref. [90]). Upon TNF ligation, RIPK1 along with other adaptor proteins and other signaling components are recruited to the cytosolic death domain of TNFR1 to form the TNF-receptor-associated complex I, which serves as a platform for signaling via the pro-survival NF- κB pathway [91, 92]. RIPK1 is heavily ubiquitylated in complex I, which serves to confine RIPK1 to this death receptor-nucleated complex to suppress cell death and promote inflammatory gene expression. When one of the checkpoints in complex I fails or is negated, it can lead to RIPK1-dependent apoptosis in certain scenarios [93], but apoptosis can also be promoted via RIPK1 kinase-independent mechanisms [94]. The role of RIPK1 in necroptosis, however, is complicated because, depending on cellular context, both positive (necroptosis-promoting) and negative (necroptosis-attenuating) regulatory functions for RIPK1 have been reported. Murine cells expressing kinase-inactive RIPK1 are protected from TNF-induced necroptosis, implicating its kinase activity in driving necroptosis via the TNF pathway [95]. Because RIPK1 knock-out mice die perinatally [96], yet mice bearing either of two distinct kinase-inactive mutant RIPK1 knock-in alleles (K45A or D138N) are viable and fertile [80, 95], it can be concluded that RIPK1's scaffolding function is important in restricting necroptosis. This idea is supported by the observation that cells lacking RIPK1 undergo increased spontaneous RIPK3-dependent death [95, 97–99], indicating that depending on cellular context, RIPK1 can either promote or negate RIPK3 activation and therefore cell death.

Although RIPK1 S161 autophosphorylation appears to precede necrosome formation [86, 100, 101] and drives RIPK1-RIPK3 interaction, it remains unclear how interactions among RHIM proteins are regulated [38, 39]. Studies using RIPK1 and RIPK3 fused to dimerization domains indicate that RIPK1-RIPK3 interaction alone is insufficient to cause necroptosis in mouse cells, while RIPK3-RIPK3 homo-interaction is both necessary and sufficient to induce necroptosis [94, 102, 103]. More recently, attention has turned to the possibility that, in addition to the RHIM, the RIPK3 kinase domain contributes to dimerization (Fig. 4b), as would be expected considering the importance of RIPK3

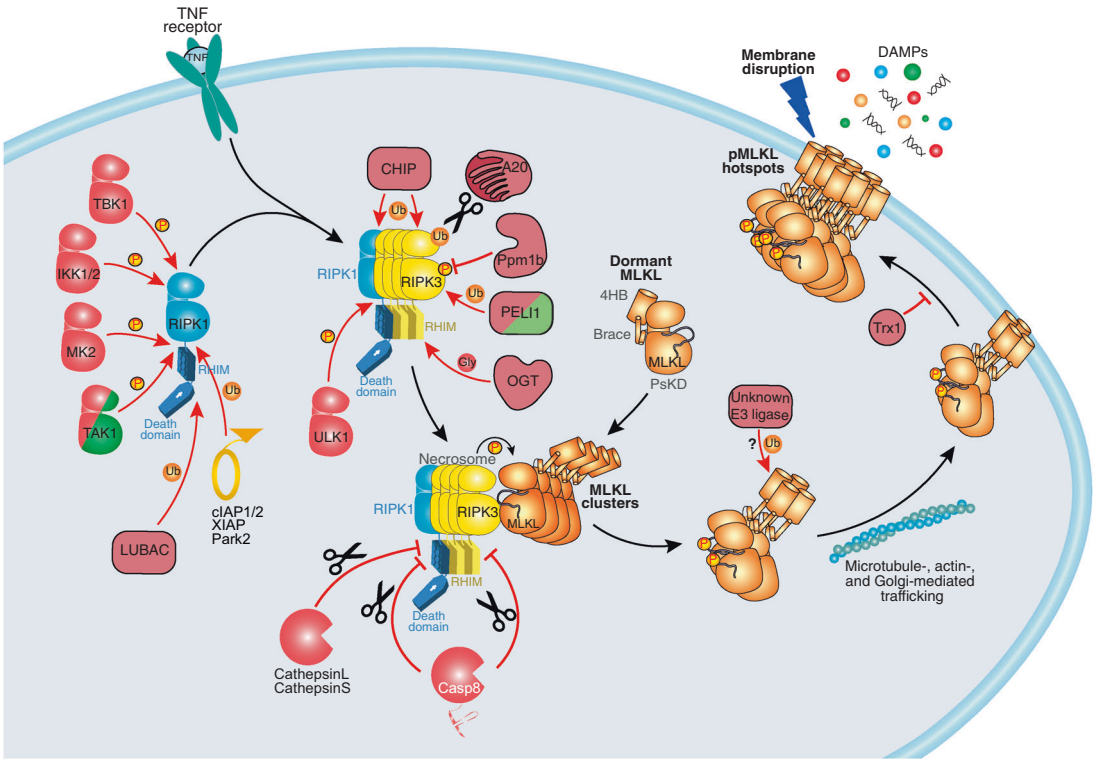

Fig. 3 Post-translational modifications negatively regulate necroptosis. Many components of TNFR1 complex I that contribute to pro-survival signaling negatively regulate necroptosis via RIPK1. These include inhibitory phosphorylation by kinases TBK1, IKK1/2, MK2, TAK1, and ULK1; as well as ubiquitylation by E3 ligases LUBAC, cIAP1/2, XIAP, and Park2. CHIP- and A20-mediated ubiquitylation targets RIPK1 and RIPK3 for degradation. The Ppm1b phosphatase subtly attenuates necroptosis by removing RIPK3 autophosphorylation. PELI1-mediated ubiquitination is proposed to target RIPK3 towards proteasomal degradation. Cleavage of RIPK1 by cathepsins, RIPK1, and RIPK3 by Casp8 inhibits necroptosis. MLKL is ubiquitylated by unknown E3 ligases. Reduction of disulfide bonds by Trx1 was proposed to prevent MLKL from assembling into higher-order species.

phosphorylation to induction of necroptosis [81]. These studies point towards a model where RIPK1 seeds the amyloid fibril by RIPK1-RIPK3 interaction, which is insufficient to propagate necroptotic signals. When more RIPK3 molecules are recruited to the necrosome, RIPK3-RIPK3 homo-interaction presumably enables RIPK3 autophosphorylation, which is a known prerequisite for RIPK3 engagement and activation of MLKL by phosphorylation [28, 104, 105]. RIPK3-MLKL interaction is mediated by their respective kinase and pseudokinase domains, as demonstrated by the crystal structure of murine RIPK3-MLKL complex [105], but curiously this interaction can only be detected biophysically when mixing recombinant proteins of the human, but not the murine, system [19, 30, 89]. Nevertheless, RIPK3-MLKL interaction is clearly necessary for necroptotic signaling; mutations to RIPK3 [28, 104] or MLKL [89] at the interaction interface, or binding of viral MLKL orthologs [19] to RIPK3, can block necroptotic signaling in both human and murine cell lines. Furthermore, overexpressed human RIPK3 and MLKL have been observed pre-associate under basal conditions in the cytoplasm [28], before higher order necrosomes assemble in the perinuclear space, and recruit RIPK3-MLKL subcomplexes [51, 106]. Taken together, although RIPK3 autophosphorylation, oligomerization, and interaction with MLKL are critical for necroptosis signaling, the interplay among these events remains unclear.

MLKL consists of an N-terminal four-helix bundle domain (4HB), followed by a two-helix brace region and the C-terminal pseudokinase domain (Fig. 4), designated based on the topology observed in the full-length mouse MLKL crystal structure [30]. The MLKL 4HB is a lipid-binding domain [40, 44, 89, 107], which executes necroptosis by permeabilizing the plasma membrane. The underlying lytic mechanism, such as whether MLKL oligomers assemble into a transmembrane pore or merely perturb the lipid bilayer, remains heavily debated in the absence of structural studies of the 4HB embedded in a lipid environment (reviewed in ref. [88]). While the 4HB appears to be the direct executioner of plasma membrane rupture, the C-terminal pseudokinase domain acts to suppress the lytic function of the 4HB domain [43]. The pseudokinase domain is regulated by interaction with RIPK3 and RIPK3-mediated phosphorylation, which has led to the proposal that this domain acts as a molecular switch that can be toggled by phosphorylation by RIPK3 [30, 89, 108] and possibly other

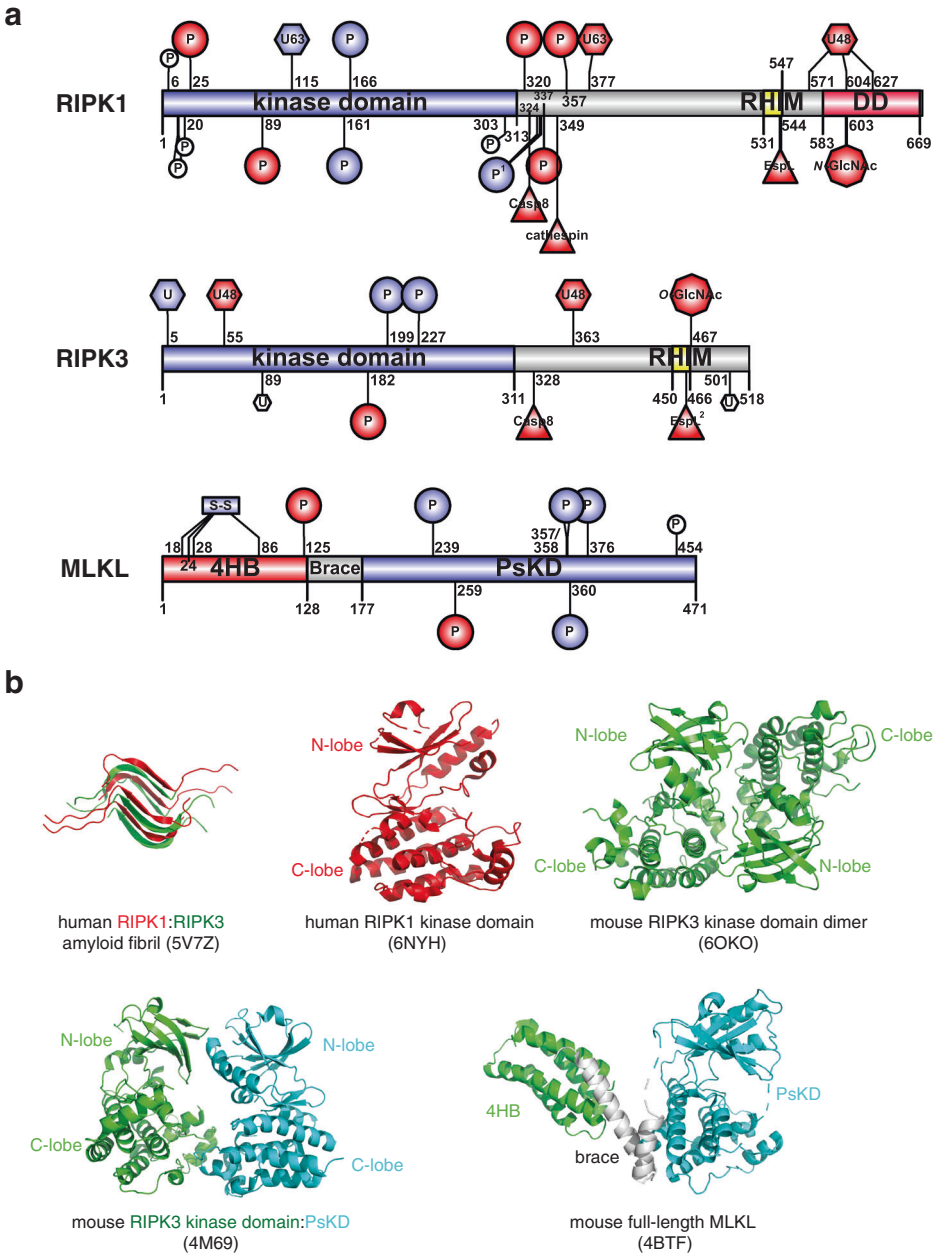

Fig. 4 Domain architecture and known post-translational modification sites of the necrosome components. a Residues are numbered based on human ortholog sequence. Modifications are colored based on proposed effect to necroptotic cell death. Necroptosis-promoting (positive regulatory) events are colored blue; negative events are colored red. Modifications that were observed but have no known function in necroptosis are in white. Sequence diagrams were generated using IBS [204]. 4HB four-helix bundle domain, PsKD pseudokinase domain, RHIM RIP homotypic interaction motif, DD death domain. **b** Representative structures of RIPK1 and RIPK3 component domains and full-length MLKL (PDB accession numbers: 5V7Z [38]; 6NYH [71]; 6OZO [205]; 4M69 [105]; 4BTF [30]).

kinases [47]. Following activation, MLKL assembles into oligomers and subsequently into higher order assemblies, which are trafficked to the plasma membrane via Golgi-microtubule-actin-dependent mechanisms [43, 51, 89, 108]. It has been proposed that when the accumulation of MLKL at the plasma membrane surpasses a critical threshold level at focal "hotspots", membrane integrity is compromised and cell death ensues [51]. The activities of each of the core elements of the necroptosis machinery, RIPK1, RIPK3, and MLKL, can be tuned by post-translational modifications. Below we describe these events and evaluate evidence for their roles in promoting or negating necroptosis signaling.

Post-translational modifications of RIPK1

Phosphorylation

RIPK1 is heavily regulated with post-translational modifications. A number of phosphosites that influence RIPK1's catalytic activity and thus engagement in death signaling have been reported (Fig. 4a, Table 1). These include autophosphorylation sites, which mark its activation towards necroptosis, as well as several phosphosites by accessory kinases, which were proposed to inhibit RIPK1's kinase activity.

Table 1 Post-translational modifications of RIPK1.

Domain	Residue[a]		PTM	Modifying enzyme	Effect on necroptosis	Proposed mechanism	Ortholog studied	Cell model[b]	Mouse model	Recombinant protein	Reference
	Human	Mouse									
Kinase	S6	**S6**	Phosphorylation	IKK1/2	No effect	–	Mouse	MEF, MDF	No	No	[121]
Kinase	**S14/15**	S14/15	Phosphorylation	Auto	No effect	–	Human	293T	No	No	[109]
Kinase	**S20**	–	Phosphorylation	Auto	No effect	–	Human	293T	No	No	[109]
Kinase	S25	**S25**	Phosphorylation	IKK1/2	↓ Inhibit RIPK1 S166 autophosphorylation and cell death	Inhibit kinase activity	Mouse	MEF, MDF, BMDM	Yes	Yes	[121]
Kinase	**S89**	S89	Phosphorylation	Unknown	↓ Attenuates kinase activity	Unknown	Human	Jurkat	No	No	[87]
Kinase	**S161**	**S161**	Phosphorylation	Auto	no effect	–	Human	Jurkat	No	No	[87]
					↑ Drives RIPK1-RIPK3 interaction	Conformational change	Mouse	L929	No	No	[100]
					No effect	–	Mouse	BMDM, MDF, MEF	No	No	[101]
Kinase	S166	**S166**	Phosphorylation	Auto	↑ Drives RIPK1-RIPK3 interaction	Conformational change	Mouse	BMDM, MDF, MLF, MEF	Yes	No	[86, 101]
Intermediate	**S320**	**S321**	Phosphorylation	TAK1	↓ Attenuates complex II formation, RIPK1 S166 autophosphorylation, and RIPK1-dependent cell death	Inhibit kinase activity	Mouse	L929, MEF	No	No	[124]
				MK2	↓ Attenuates complex II formation and RIPK1-dependent cell death	Inhibit kinase activity	Human, mouse	MEF, BMDM, BT549, HT29	Yes	Yes	[122]
					No effect	–	Mouse	MEF, BMDM	Yes	Yes	[123]
Intermediate	S331	**S332**	Phosphorylation	TAK1	↑ Hyperphosphorylation promotes RIPK3 interaction and necroptosis	Unknown	Mouse	L929, MEF	No	No	[124]
Intermediate	S333	**S334**	Phosphorylation								
Intermediate	S335/ T337	**S336**	Phosphorylation	MK2	↓ Attenuates complex II formation and RIPK1-dependent cell death	Inhibit kinase activity	Mouse	MEF, BMDM	Yes	Yes	[123]
Intermediate	**S357**	**S356**	Phosphorylation	ULK1	↓ Attenuates complex II formation and RIPK1-dependent cell death	Unknown	Mouse, human	L929, MEF, 293T	No	Yes	[125]
Intermediate	**K377**	**K376**	K63-linked ubiquitylation	cIAP1/2	↓ Inhibits complex II formation and cell death	Prevent RIPK1 dissociation from complex I; recruit LUBAC, TAK1, IKK1/2, and NF-κB activation	Mouse	MEF	Yes	No	[127, 129–131]
	K377	**K376**		Park2	↓ Delayed necroptosis	Recruit LUBAC, TAK1, IKK1/2 and NF-κB and MAPK activation	Human, mouse	MEF, 293T, 661W	No	No	[132]
	K377	**K376**	Deubiquitylation (K63-linked)	A20	↓ Inhibits complex II formation and cell death	Targets RIPK1 for proteasomal degradation	Mouse, human	MEF, K562, A549, mouse T cells	Yes	No	[147, 148, 151–154, 184]
–	**Unknown**	**Unknown**	K48-linked ubiquitylation								

Table 1 (continued)

Domain	Residue[a]		PTM	Modifying enzyme	Effect on necroptosis	Proposed mechanism	Ortholog studied	Cell model[b]	Mouse model	Recombinant protein	Reference
	Human	Mouse									
–	Unknown	**Unknown**	M1-linked ubiquitylation	LUBAC (HOIL, HOIP, SHARPIN)	↓ Inhibits TNF-induced cell death	Recruit TAK1, IKK1/2, A20, CYLD, and NF-κB activation	Mouse	MEF	Yes	No	[114, 134–141]
–	**Unknown**	**Unknown**	Deubiquitylate K63- and M1-linked Ub	CYLD	↑ Promote necrosome formation and necroptosis	Promote RIPK1 kinase activity	Mouse, human	MEF, HT29, L929, BMDM	Yes	No	[142, 143, 149, 150]
Kinase	**K115**	**K115**	K63-linked ubiquitylation	PELI1	↑ Required for necrosome formation	Promote RIPK1 kinase activity	Mouse, human	MEF, HT29	Yes	No	[162, 163]
Intermediate	**K571**	–	K48-linked ubiquitylation	CHIP	↓ Inhibits TNF-induced necroptosis	Targets RIPK1 for lysosomal degradation	Mouse, human	MEF, L929, HT29	Yes	No	[156–158]
Death	**K604**	K589									
Death	**K627**	K612									
–	**Unknown**	Unknown	K11-linked ubiquitylation	UbcH5, cIAP1	Unknown	Promote K11-linked polyubiquitination which recruits NEMO	Human	293T, HeLa S3, HT1080, HT29	No	Yes	[133]
Intermediate	**D324**	**D325**	Cleavage	Casp8	↓ Inhibits necroptosis	Protease cleavage	Human, mouse	HT29, MEF, L929, BMDM	Yes	Yes	[35–37, 76, 171]
Intermediate	L349	**L348**	Cleavage	Cathepsin	↓ Attenuates necroptosis	Protease cleavage	Mouse	BMDM	No	Yes	[175]
RHIM	Y544	**H533**	Cleavage	EspL	↓ (Pathogen) inhibits necroptosis	Protease cleavage	Mouse	MDF, BMDM	Yes	Yes	[17]
Death	R603	R588	*N*-GlcNAc	NleB	↓ (Pathogen) inhibits TNF-induced cell death	Inactivates death domain-mediated interactions	Mouse, human	293T, HeLa, MEF, HT29	Yes	Yes	[20, 21]

[a]Equivalent residues in human and mouse orthologs are inferred by sequence homology. Bold indicates the ortholog residue with direct evidence of such post-translational modification.

[b]Cell line abbreviations: *MEF* mouse embryonic fibroblasts, *MDF* mouse dermal fibroblasts, *293T* human embryonic kidney cell line HEK293T, *BMDM* bone marrow-derived macrophages, *Jurkat* human T lymphocyte Jurkat cells, *L929* mouse fibrosarcoma cells, *MLF* mouse lung fibroblasts, *BT549* human breast cancer cells, *HT29* human adenocarcinoma cells, *661W* mouse cone photoreceptor cells, *K562* human chronic myelogenous leukemia cells, *A549* human adenocarcinomic alveolar basal epithelial cells, *HeLa (S3)* human adenocarcinoma cells, *HT1080* human fibrosarcoma cells.

Autophosphorylation upregulates RIPK1 activity

The primary function of RIPK1's kinase domain is generally accepted to be autophosphorylation [100, 101, 109]. RIPK1 only exhibits modest kinase activity, and RIPK1-mediated phosphorylation of RIPK3 or other substrates has not been convincingly shown experimentally. Therefore, RIPK1 does not promote necroptosis via a classical phosphorylation cascade, but instead, relies on RIPK1 autophosphorylation for pathway initiation. The autophosphorylation sites S161 and S166 have emerged as biomarkers for RIPK1 activation, but the biological functions of these phosphorylation events remain the subject of debate [86, 100, 101, 109]. A recent study failed to detect S161 phosphorylation in endogenous RIPK1, suggesting that the contribution of this phosphorylation event to RIPK1 regulation might depend on the cellular and stimulus context [101]. Studies of phosphoablating mutations have yielded conflicting results. While S161A reconstituted necroptosis similarly to wild-type RIPK1 [87], subsequent work [100] argued that S161A is not a faithful mimetic of unmodified S161 because it disrupts the S161-D156 hydrogen bond observed in the structure of RIPK1 [110], and allows the activation loop to adopt an activated kinase conformation. On the contrary, studies of S161N RIPK1 support a functional role for S161 phosphorylation, because this mutant both failed to reconstitute necroptosis and prevented its co-localization with RIPK3 [100].

While S166 is widely used as a biomarker for RIPK1 activation [109, 111], phosphorylation of this site appears to be insufficient for necroptosis. It was proposed that S166 may not be a necroptosis-specific phosphorylation event, because it was detected in both TNF and TZ (TNF plus pan-caspase inhibitor Z-VAD-FMK) treatments, and this autophosphorylation did not require RIPK1 ubiquitination [86]. Recent studies of mice bearing a S166A RIPK1 knock-in allele indicates that S166 phosphorylation promotes and is coincident with, but insufficient to cause, cell death [101]. These studies raise the possibility that autophosphorylation of additional sites may be promoted by S166 phosphorylation for RIPK1 to reach its full kinase activity.

IKK1/2, TAK1, MK2, and ULK1-mediated phosphorylation downregulates RIPK1

RIPK1 was reported to be phosphorylated by IKK1/2, TAK1, and MK2 to regulate its kinase activity. These phosphorylation sites are located either on the N-lobe of the kinase domain, or in the intermediate region located between the kinase domain and RHIM (Fig. 4a, b), each resulting in inhibition of its kinase activity. TAK1 and IKK1/2 protein kinases are recruited to the TNFR1 complex I via the ubiquitin chains added by cIAP proteins and linear ubiquitin chain assembly complex (LUBAC, consisting of HOIL/HOIP/SHARPIN) [112] to RIPK1 [92, 113–117], where they are best known for stimulation of the NF-κB pathway. IKK1/2-mediated phosphorylation of RIPK1 within TNFR1 complex I was reported to protect cells from RIPK1-dependent apoptosis and necroptosis independent of NF-κB signaling [118]. RIPK1-dependent apoptosis is mediated by the cytosolic complex II (Casp8, FADD, cFLIP, and RIPK1), which forms after RIPK1 dissociates from complex I and drives Casp8 activation [119, 120]. Additionally, IKK1/2 enzymatic activity was reported to prevent RIPK1 integration into complex II via phosphorylation of S25 in the kinase domain of RIPK1 [121]. Introduction of the phosphomimetic S25D mutation into RIPK1 reduced kinase activity and S166 phosphorylation, thereby accounting for the protective role served by the introduction of this mutation, and S25 phosphorylation, in cells and in mice [121]. The nature of the S25 substitution appears to play an important role, however, because S25E mouse RIPK1 could reconstitute signaling in *Ripk1*$^{-/-}$ L929 cells [100]. These findings raise the possibility that some substitutions at this site may compromise RIPK1 kinase domain structure and/or protein interactions, rather than simply emulating phosphorylation. Another N-lobe inhibitory phosphosite, S89, was identified similarly by mutational analyses, where S89A increased human RIPK1 kinase activity while S89D attenuated it [87], though the identity of the kinase that modifies this site remains of outstanding interest. In concert, S6 in RIPK1 was also reported to be phosphorylated by IKK1/2, but no functional role was attributed to this phosphorylation event [121].

Both TAK1 and MK2 were proposed to phosphorylate S321 of mouse RIPK1 and the equivalent residue in human RIPK1, S320, to attenuate RIPK1 kinase activity and, accordingly, cell death [122–124]. Because TAK1 and MK2 function within the same signaling cascade, the relative contribution of each kinase to this modification is currently debated [122–124]. These studies concordantly found that S321/S320 phosphorylation in complex I attenuated complex II formation and cell death in a cIAP1/2-dependent manner downstream of TNF signaling. In addition to phosphorylation of S321, each of S332, S334 and S336 were identified as phospho-sites in RIPK1, with S336 an additional substrate of MK2 [123, 124], suggesting a role for multiple phosphorylation events within the intermediate region in promoting necroptosis. The observation that S336A mutation, but not S321A, sensitized mouse embryonic fibroblasts (MEFs) to cell death led to the proposal that phosphorylation of S336 has a more prominent role in restricting RIPK1-dependent cell death [123].

Taken together, IKK1/2, TAK1, and MK2 phosphorylation of RIPK1 kinase represent multiple mechanisms by which necroptosis can be suppressed during pro-survival

NF-κB signaling. In addition to these, the autophagy-initiating kinase ULK1 was recently proposed to add another layer of regulation by phosphorylating an additional site, S357, C-terminal to the kinase domain [125]. However, ULK1 activity only modestly impacted necroptosis, suggesting a non-obligate function of S357 phosphorylation in tuning necroptosis.

Ubiquitylation

RIPK1 is heavily ubiquitylated in the TNFR1 complex I, which serves as a signaling platform for the regulation of cell survival, apoptotic and necroptotic death. Several types of ubiquitylation have been observed on RIPK1, including Met1-, K11-, K48-, and K63-linked ubiquitylation (reviewed in ref. [126]) (Table 1). A number of E3 ligases and deubiquitinases have been proposed to mediate ubiquitin modifications. The best characterized examples are of the complex I components, cIAP1/2, LUBAC, A20, and CYLD. However, few ubiquitylation sites have been characterized, and evidence connecting ubiquitylation sites and ubiquitin-modifying enzymes is scarce. Recently, several ubiquitin-modifying enzymes have been implicated as positive and negative regulators of RIPK1-dependent cell death.

Downregulation of RIPK1-mediated cell death by ubiquitylation

cIAP-mediated K63-linked ubiquitylation of RIPK1 is well-established to suppress RIPK1-mediated cell death [127] (and reviewed in ref. [128]). These ubiquitylation events enable recruitment of complex I components, including LUBAC, TAB1/2, TAK1, NEMO, and IKK1/2, to activate the pro-survival NF-κB pathway and inhibit cell death. Recently, K63-linked ubiquitylation on K376 of mouse RIPK1 (equivalent to K377 of human RIPK1) was identified as an important mechanism to suppress cell death [129–131]. The RIPK1 K376R mutation caused embryonic lethality in knock-in mice, which was attributed to increased kinase activity and elevated sensitivity to RIPK1-dependent cell death. These studies support a role of K376 ubiquitylation in suppressing RIPK1 kinase activity, although formal evidence that K376 is the direct substrate of cIAP1/2 is still lacking. While the substrate site(s) in RIPK1 remain unknown, another E3 ligase, Parkin (Park2), was reported to bind RIPK1 in complex I and perform similar functions to cIAP1/2 in some contexts [132]. Loss of Park2 had only a modest impact on necroptosis, which suggests that Park2 performs an auxiliary role for cell death, the details of which await further investigation. Additionally, cIAP1 facilitates K11-linked polyubiquitylation of RIPK1 during TNF-induced NF-κB signaling. This action is dependent on the UbcH5 family of E2 enzymes [133]. Although K11-linked polyubiquitylation is more commonly known to be a degradation signal, the observation of NEMO binding to this K11-linked polyubiquitin chain led to the proposal of a possible non-degradative signaling role [133], whose biological effect on necroptotic cell death awaits further characterization.

LUBAC negatively regulates RIPK1-mediated cell death, but the precise mechanisms and ubiquitylation sites remain unknown. Downstream of cIAP1/2, LUBAC is recruited to complex I where it catalyzes M1-linked (linear) ubiquitylation of TNFR1, TRADD, and RIPK1. Such LUBAC-mediated ubiquitylation reinforces complex I, enables recruitment of the NEMO/IKK1/IKK2 complex to drive NF-κB activation, and negatively regulates RIPK1-mediated cell death [114, 134–141]. Much like deubiquitylation of the cIAP-mediated K63-linked ubiquitin chain from RIPK1, removal of M1-linked ubiquitin chains by CYLD is required for RIPK1-dependent cell death to proceed [142, 143]. Consistent with these in vitro observations, mice with impaired LUBAC components display severe inflammation or embryonic lethality that could be rescued by co-deletion of Casp8 with RIPK3 or MLKL in some cases [141, 144–146]. Paradoxically, LUBAC is required in complex I to recruit A20 and CYLD, each of which exhibit opposing effects on necroptosis [142, 143, 147–150]. They further modify the ubiquitylation of RIPK1 to exert pro-death effects that are countered by M1-linked ubiquitylation [143].

A20 is a bifunctional ubiquitin-modifying enzyme that restricts NF-κB activation and apoptosis following TNF- and TLR-stimulation [147, 148]. The N-terminal domain of A20 is a deubiquitinase that removes K63-linked ubiquitin chains from RIPK1, while the C-terminal zinc finger (ZnF7) domain functions as a ubiquitin ligase that polyubiquitylates RIPK1 with K48-linked ubiquitin chains to target it for proteasomal degradation. Accordingly, perinatal lethality and widespread tissue inflammation phenotype were observed in A20-deficient mice [151]. A20 is recruited to LUBAC-mediated M1-linked ubiquitylation sites via its ZnF7 domain, and A20 binding also protects M1-linked ubiquitin chains from deubiquitylation or degradation [152, 153]. On the other hand, contrary to the anti-inflammatory roles proposed by the above studies, A20 was proposed to bind cytosolic complex II via a ZnF7-dependent mechanism and potentiate apoptotic cell death [154]. However, why this promotes cell death instead of inhibiting it and whether this recruitment to the necrosome is dependent on re-ubiquitylation of necrosomal RIPK1 by HOIP remains to be established [155].

Carboxyl-terminus of Hsp70-interacting protein (CHIP) is an E3 ligase that introduces K48-linked ubiquitin chains on K571, K604, and K627 of RIPK1, leading to its

lysosomal degradation [156–158]. CHIP-deficiency led to increased levels of RIPK1 and increased sensitivity to TNF-induced necroptosis in cell lines and provoked an inflammatory postnatal lethal phenotype in mice. These data support a role for CHIP as a negative regulator of necroptosis.

Optineurin (OPTN) is a ubiquitin-binding protein that has been implicated in regulating necroptosis by regulating turnover of RIPK1 [65]. OPTN-depletion increased expression levels of RIPK1, RIPK3 and MLKL, and sensitized cultured cells to necroptosis. Consequently, OPTN was proposed as a negative regulator of necroptosis; however, since OPTN is not a E3 ligase itself, the mechanism by which OPTN promotes RIPK1 modification and regulation is unclear.

Upregulation of RIPK1-mediated necroptosis by ubiquitin-modifying enzymes

CYLD is another deubiquitinase that is recruited to complex I downstream of LUBAC [143], but unlike A20, CYLD is recruited to LUBAC via the adaptor SPATA2 [159]. CYLD removes K63- and M1-linked ubiquitin chains from RIPK1 and TRAFs, thereby promoting formation of cytosolic RIPK1-nucleated signaling platforms, including the necrosome, which directs necroptosis [142, 143, 149, 150]. Whether CYLD catalyzes deubiquitylation in complex I or the necrosome is a matter of debate [143, 149], but since LUBAC was shown to ubiquitylate RIPK1 in both complex I and the necrosome, it is possible CYLD can be recruited to both complexes. Furthermore, cleavage of CYLD by Casp8 was proposed as a mechanism to hold aberrant necroptosis in check downstream of death receptor stimulation [160]. However, knock-in mice containing the CYLD D215A allele, a form of CYLD that cannot be cleaved by Casp8, were viable [37], suggesting this mechanism may be dispensable during homeostasis. Taken together, several lines of evidence support a role for CYLD as a positive regulator of necroptosis, although it is not essential in all cell types and therefore likely performs an auxiliary role in tuning necroptotic signaling [161].

Several additional ubiquitin-modifying enzymes have been proposed to function as regulators of necroptosis. PELI1, an E3 ligase known to be involved in mediating TLR3/4 signaling, was shown to K63-linked ubiquitylate RIPK1 on K115 during TNF-induced necroptosis in a RIPK1 kinase-dependent manner [162, 163]. PELI1 deficiency was reported to abrogate the RIPK1-RIPK3 interaction and necroptosis, suggesting PELI1 functions as an essential positive regulator of necroptosis. Paradoxically, PELI1 was reported to exhibit the opposite effect in the context of RIPK3 ubiquitylation by targeting RIPK3 for degradation [164]. Therefore, the net effect of PELI1 on necroptotic signaling remains unclear. In a subsequent study, RIPK1 K115R knock-in mice were found to be viable with only modestly elevated responsiveness to TNF-induced death [129], suggesting a less prominent role for this ubiquitination site during steady state. Lastly, APC11, an E3 ligase of the APC/C complex that targets its substrates for degradation via K11- and K48-linked polyubiquitination, was identified in an siRNA knockdown screen as a RIPK1, and therefore necroptosis, activator [93]. Further biochemical and in vivo data are required to confirm the mechanism and physiological relevance by which APC11 regulates necroptosis.

Other post-translational modifications

It is well-established that necroptosis is suppressed by Casp8, whose cellular inhibitors and substrates also regulate necroptosis by extension. Activated Casp8 in cytosolic complex II cleaves human RIPK1 at D324 [35, 37, 76, 77, 165] (mouse RIPK1 D325) and possibly RIPK3 at D328 [36] (mouse RIPK3 D333), thereby severing their kinase domains from the RHIM oligomerization modules to limit the capacity of RIPK1 and RIPK3 to assemble into the necrosome and execute necroptosis [166]. Inhibition or deficiency of Casp8 or its binding partner, the FADD adaptor protein, enables necroptosis to ensue [167, 168]. The catalytic activity of Casp8 is tightly controlled by two cFLIP isoforms [169, 170]. $cFLIP_L$ is a pseudoprotease that forms a heterodimer with Casp8 and inhibits apoptosis. Formation of this heterodimer prevents the Casp8 autoprocessing that is required for apoptosis to proceed, while retaining cleavage activity towards RIPK1 and potentially RIPK3 to block necroptosis [171]. $cFLIP_S$ on the other hand inhibits Casp8-mediated cleavage of RIPK1 and RIPK3 in addition to inhibition of Casp8 autoprocessing, and thus functions as a pro-necroptotic factor [32]. XIAP is another direct inhibitor of apoptotic caspases. Similar to cIAP1/2, XIAP is an E3 ligase of the inhibitor of apoptotic protein (IAP) family, but its mechanism is more enigmatic. Loss of XIAP confers some sensitivity to TNF- and TLR-induced cell death in macrophages and dendritic cells [172, 173], but in neutrophils this requires co-deletion with cIAP1/cIAP2 [174]. Furthermore, an aforementioned positive regulator of necroptosis, CYLD, has also been shown to be a Casp8 substrate whose cleavage was proposed to limit TNF-induced necroptosis [160].

It appears likely that proteolytic post-translational modifications more broadly function to regulate necroptotic flux. Cathepsin B and S were reported to cleave RIPK1 during TNF- or TLR-induced necroptosis in macrophages [175]. Knocking down the expression of cathepsins resulted in decreased RIPK1 cleavage and slightly increased sensitivity to necroptosis. Further in vivo data will be useful to confirm

the physiological relevance of cathepsins as necroptosis regulators. Enteropathogenic *Escherichia coli* (EPEC) also encode a cysteine protease, EspL, that cleaves RHIM-containing proteins (RIPK1, RIPK3, TRIF, and ZBP1/DAI) [17], as well as an arginine glycosylation enzyme, NleB, which inhibits the interactions among the same proteins via N-acetylglucosamine modifications [20, 21]. These represent mechanisms by which post-translational modifications are utilized by pathogens to restrict necroptosis and evade the associated host immune response. Collectively, these studies highlight that, while among the necroptotic machinery, our knowledge of the post-translational modifications that regulate RIPK1 is the most advanced, it is likely that additional modifications that tune RIPK1's activity, localization, protein interactions, and stability remain still to be discovered.

Post-translational modifications of RIPK3

Phosphorylation

To date, all known phosphorylation events in RIPK3 have been reported to promote necroptotic signaling (Fig. 2, Table 2). RIPK3 autophosphorylation is a crucial event for induction of cell death by necroptosis, and is thought to arise following the self-association of RIPK3 protomers within the necrosome [25, 26]. Although inhibition of RIPK1 kinase activity also inhibits RIPK3 phosphorylation [25, 80, 111, 163], no phosphorylation of RIPK3 by RIPK1 was observed in in vitro kinase assays [25]. These data support the idea that RIPK1 activates RIPK3 via a scaffolding function [25], which seeds the formation of higher-order RIPK3 complexes. This is further supported by multiple reports of overexpression of RIPK3 driving autophosphorylation and necroptosis [27, 94, 176] because a surfeit of RIPK3 will obviate the need for RIPK1 to nucleate higher-order RIPK3 assemblies of increased local concentration for autophosphorylation. In keeping with this idea, activation of RIPK3 can also mediated by the RHIM-containing proteins TRIF [82] and ZBP1 [22, 84], even though neither is a kinase, supporting the notion that the activation of RIPK3 does not require direct phosphorylation by RIPK1.

Three autophosphorylation sites have been discovered within human RIPK3, which are proposed to contribute to RIPK3's function in necroptotic signaling. S227 in human RIPK3 and the counterpart in mouse RIPK3, T231/S232, are located in the kinase C-lobe and are the best characterized RIPK3 phosphosites (Fig. 4a, b), with verified functions in both human and mouse cells [28, 87, 104]. S227 phosphorylation is dispensable for human RIPK3 kinase activity, but is critical for recruitment of MLKL and subsequent induction of necroptosis. Introduction of the phosphoablating mutation, S227A, into human RIPK3 completely abrogated necroptotic cell death [28, 104]. In contrast to human RIPK3, mouse RIPK3 has an additional, adjacent phosphosite, T231, which was reported to contribute to mouse RIPK3's necroptotic activity [104]. The additional requirement for T231 phosphorylation likely contributes to the inability of mouse and human RIPK3 to respectively bind and activate mouse and human MLKL [104, 177, 178]. Nevertheless, human RIPK3 S227 and mouse RIPK3 T231/S232 phosphorylation are widely used as biomarkers for RIPK3 activation and the instigation of necroptosis. Although a role for Casein Kinase (CK)1α in regulating necroptosis was previously suggested by its reported binding and phosphorylation of RIPK1 [179], recently CK1γ2 and γ3 [180], CK1α, CK1δ, and CK1ε [181] were additionally proposed to phosphorylate S227 of human RIPK3 within the necrosome. In contrast, CK1γ2 was proposed to serve an entirely opposite function by binding and negatively-regulating RIPK3 activity, and therefore necroptosis, in the testes [182]. The relative contribution to RIPK1 and RIPK3 regulation by CK1 proteins, the contexts under which regulatory function occurs, whether different CK1 isoforms serve different functions, and whether they are positive or negative regulatory events, remain of outstanding interest. Dephosphorylation of the counterpart of human RIPK3 S227 in mouse RIPK3, T231/S232, was reported to be catalyzed by the Ppm1b phosphatase to restrict necroptosis, with Ppm1b deletion leading to a modest increase in RIPK3 phosphorylation, death of cultured cells, and tissue damage in the TNF-induced sterile sepsis mouse model [183]. This raises the possibility that other phosphatases may antagonize phosphorylation of the necrosome and act as additional negative regulators of necroptosis.

Beyond S227 and T231/S232 in the C-lobes of human and mouse RIPK3, respectively, the phosphorylation of additional sites has been reported to promote necroptosis. Phosphorylation of human RIPK3 S199 was also proposed to be required for necroptotic cell death in HT29 cells [26], although subsequent studies indicate S199 phosphorylation is dispensable [104]. Alanine substitution of the mouse RIPK3 counterpart of S199, S204, on the other hand compromised mouse RIPK3 kinase activity and necroptosis when introduced in *Ripk3*$^{-/-}$ fibroblasts [87]. While substitution of mouse RIPK3 S204 with the phosphomimetic residue, Asp, did not lead to constitutive killing, this mutant could reconstitute necroptotic signaling in *Ripk3*$^{-/-}$ fibroblasts to a comparable extent to wild-type RIPK3. In contrast to wild-type mouse RIPK3, treatment of RIPK1 with small-molecule inhibitor, Nec-1, did not block S204D mouse RIPK3-mediated death following necroptotic stimulation [87]. These findings support the idea that mouse

Table 2 Post-translational modifications of RIPK3.

Domain	Residue[a]		PTM	Modifying enzyme	Effect on necroptosis	Proposed mechanism	Ortholog studied	Cell model[b]	Mouse model	Recombinant protein	Reference
	Human	Mouse									
Kinase	**T182**	T187	Phosphorylation	Auto	Both ↑ and ↓, net effect unclear	Promotes RIPK3 kinase activity; but also recruits PELI1 which targets RIPK3 for proteasomal degradation	Human	HT29, 293T	No	Yes	[164]
Kinase	**S199**	**S204**	Phosphorylation	Auto	↑ Promotes necroptosis	Promotes RIPK3 kinase activity	Human, mouse	MEF, Jurkat, HT29	No	Yes	[87]
Kinase	**S227**	**T231/S232**	Phosphorylation	Auto	↑ Promotes necroptosis	Promotes interaction with MLKL	Mouse, human	MEF, Jurkat, HT29	No	Yes	[28, 87]
	S227	T231/S232		CK1α, CK1δ, and CK1ε			Human	HeLa	No	Yes	[181]
	S227	**T231/S232**	Dephosphorylation	Ppm1b	↓ Restrict necroptosis	Antagonize essential phosphorylation	Human, mouse	293T, L929, HeLa	Yes	Yes	[183]
Kinase	K5	**K5**	Deubiquitination	A20	↓ Inhibits complex II formation and cell death	A20 deubiquitinate K63-linked ubiquitin chain of RIPK3 at K5, which is required for necrosome formation	Mouse	T cell, MEF	No	No	[184]
Kinase	**K55**	K56	K48-linked ubiquitination	CHIP	↓ Attenuates necroptosis	Targets RIPK3 for lysosomal degradation	Mouse, human	MEF, L929, HT29	Yes	No	[156]
Kinase	**K89**	–	K48-linked ubiquitination	CHIP	–	–	–	–	–	–	[156]
Intermediate	**K363**	–	K48-linked ubiquitination	CHIP	↓ Attenuates necroptosis	Targets RIPK3 for lysosomal degradation	Mouse, human	MEF, L929, HT29	Yes	No	[156]
		–	K48-linked ubiquitination	PELI1	↓ Attenuates necroptosis	Targets RIPK3 for proteasomal degradation	Mouse, human	MEF, HT29	No	No	[164]
Intermediate	**K501**	–	K48-linked ubiquitination	CHIP	–	–	–	–	–	–	[156]
Intermediate	**D328**	**D333**	Cleavage	Casp8	↓ Inhibits necroptosis	Protease cleavage	Human	HeLa, 293T	No	Yes	[36, 171]
RHIM	N462	**Y453**	Cleavage	EspL	↓ (Pathogen) inhibits necroptosis	Protease cleavage	Mouse	MDF, BMDM	Yes	Yes	[17]
RHIM	**T467**	Unknown	*O*-GlcNAc	OGT	↓ Attenuates necroptosis	Steric hinderance prevent RHIM-mediated RIPK1-RIPK3 interaction	Human, mouse	293T, L929, THP-1, BMDM	Yes	No	[185]

[a]Equivalent residues in human and mouse orthologs are inferred by sequence homology. Bold indicates the ortholog residue with direct evidence of such post-translational modification.

[b]Cell line abbreviations: *HT29* human adenocarcinoma cells, *293T* human embryonic kidney cell line HEK293T, *MEF* mouse embryonic fibroblasts, *Jurkat* human T lymphocyte Jurkat cells, *HeLa* human adenocarcinoma cells, *L292* mouse fibrosarcoma cells, *BMDM* bone marrow-derived macrophages, *THP-1* human monocyte-like cells.

RIPK3 S204 phosphorylation is necessary for necroptosis, and that mimicking phosphorylation alleviates the need for RIPK1 activity to promote RIPK3 activity. Precisely why RIPK1 activity is required for mouse RIPK3 to become phosphorylated on S204 is unclear, because RIPK1 is not known to phosphorylate RIPK3. It remains of interest to deduce whether Nec-1 binding to RIPK1 leads to an unproductive conformation or if it is the blockade of RIPK1 activity and autophosphorylation that thwarts RIPK3 interaction and activation.

It was proposed that phosphorylation of a site at the C-terminal end of the human RIPK3 activation loop, T182, was required to prime S227 phosphorylation and therefore promote RIPK3 necroptotic activity [164]. Phosphorylation of T182 was reported to be dependent on RIPK3 kinase activity, so it is presumed to be an autophosphorylation site. While the cellular contexts under which T182 phosphorylation are required for RIPK3 activation are not fully understood, it was proposed that this modification is required for interaction with PELI1, an E3 ubiquitin ligase that catalyzes K48-linked polyubiquitination to target RIPK3 for proteasomal degradation [164]. Hence, this is a possible mechanism by which phosphorylated, and therefore activated, RIPK3 could be selectively targeted for degradation to restrict necroptosis.

Ubiquitylation

RIPK3 is regulated by E3 ubiquitin ligases, including A20, CHIP, and PELI1, which are also known to ubiquitylate RIPK1 and negatively regulate necroptosis. Because RIPK3 ubiquitylation at K5 by an unknown E3 ligase under basal conditions is required to support the formation of the necrosome and promote necroptosis, the removal of K63-linked ubiquitin chains at this site by A20 restricts necroptosis [184]. It is notable that although A20 restricts RIPK1 and RIPK3 necroptotic activities, the underlying mechanisms appear to differ. A20 ubiquitylation of RIPK1 was reported to target RIPK1 for proteasomal degradation, however A20 ubiquitylation of RIPK3 has not been observed and, instead, RIPK3 negative regulation appears to rely on K5 deubiquitylation.

The cellular levels of RIPK3 appear to be regulated by K48-linked ubiquitylation by the E3 ubiquitin ligases, CHIP and PELI1, which induces RIPK3 degradation to suppress necroptosis [156, 164]. CHIP was reported to ubiquitylate RIPK3 to regulate protein levels via lysosomal degradation [156], similar to CHIP's function as a regulator of RIPK1. While human RIPK3 is ubiquitylated at K55, K89, K363, and K501 by CHIP, it appears that K55 and K363 are the critical sites for RIPK3 regulation. Although CHIP-mediated ubiquitylation of RIPK3 led to lysosomal targeting, PELI1-mediated K48-ubiquitylation of K363, a site also modified by CHIP, targeted RIPK3 to the proteasome for degradation [164]. CHIP- or PELI1-depletion in human and mouse cells increased RIPK3 levels and correspondingly increased sensitivity to TNF-induced necroptosis, supporting their functions as negative regulators of RIPK3 and therefore necroptosis. However, the net effect of PELI1 on necroptosis is unclear, because PELI1 is also reported to regulate RIPK1 to promote RIPK1-RIPK3 interaction and necroptosis [162, 163].

Other post-translational modifications

Similar to RIPK1, RIPK3 cleavage by Casp8 during apoptotic conditions is proposed to serve as a mechanism to restrict necroptosis to favor the non-death outcomes of TNF signaling [36, 171]. Casp8 was proposed to cleave human RIPK3 at D328 in the intermediate region between the kinase and RHIM domains, which is a similar position to RIPK1 cleavage site (Fig. 4). While this cleavage was not observed in human cells overexpressing the D328A mutant RIPK3 [36], the precise physiological significance of RIPK3 cleavage by Casp8 is unclear.

O-GlcNAc transferase (OGT) of the hexosamine biosynthetic pathway was reported to mediate *O*-linked β-N-GlcNAcylation of mouse RIPK3 on T467, adjacent to the RHIM motif [185]. This modification was proposed to inhibit the interaction between RIPK1 and RIPK3, possibly due to steric hindrance of interactions between RHIM domains. The universality of this regulatory mechanism between species, tissues and necroptotic stimulation remains unclear at present. Like the reported ubiquitylation sites in RIPK3, the OGT substrate residue in mouse RIPK3, T467, is poorly conserved amongst RIPK3 orthologs. Therefore, it remains to be established whether adjacent sites could be targeted by OGT and other glycosylation enzymes in other species to regulate RIPK3's necroptotic function.

Post-translational modifications of MLKL

Phosphorylation

Phosphorylation of the activation loop of pseudokinase domain of MLKL by RIPK3 is considered a hallmark of MLKL activation [28, 30, 40, 47, 48] (Figs. 2 and 4, Table 3). Phosphorylation of the activation loop of the pseudokinase domain (S345 of murine MLKL [30]; T357/S358 of human MLKL [28]) by RIPK3 precedes necroptotic death in both human and murine cell lines. Phosphorylation is proposed to trigger a conformational change in the MLKL pseudokinase domain [30, 178, 186], which is communicated via a two-helix connector termed the brace

Table 3 Post-translational modifications of MLKL.

Domain	Residue[a]		Modification	Modifying enzyme	Effect on necroptosis	Proposed mechanism	Ortholog studied	Cell model[b]	Mouse model	Recombinant protein	Reference
	Human	Mouse									
4HB	**S125**	**S124**	Phosphorylation	Unknown	—Inconclusive	–	Human, mouse	293T, MDF	No	No	[47, 188, 189]
Brace	–	**S158**	Phosphorylation	Unknown	↓ Inhibits necroptosis	Unknown	Mouse	MDF	No	No	[47]
PsKD[c]	S239	**S228**	Phosphorylation	RIPK3	↑ Modestly enhances necroptosis	Unknown	Mouse	MDF	No	No	[47]
PsKD	S259	**S248**	Phosphorylation	RIPK3	↓ Inhibits necroptosis	Unknown	Mouse	MDF	No	No	[47]
PsKD	**T357/ S358**	**S345**	Phosphorylation	RIPK3	↑Promotes necroptosis	Promotes MLKL oligomerization and membrane translocation	Human, mouse	HeLa, NIH 3T3, HT29	No	Yes	[28, 30, 40, 47, 48]
PsKD	S360	**S347**	Phosphorylation	RIPK3	↑Promotes necroptosis slightly	Unknown	Mouse	MEF	No	No	[30, 48]
PsKD	–	**T349**	Phosphorylation	RIPK3	No effect	–	Mouse	MEF	No	No	[30, 48]
PsKD	**T374**	S360	Phosphorylation	Unknown	Unknown	–	Human	HeLa	No	No	[188, 189]
PsKD	**Y376**	Y363	Phosphorylation	TAM kinases	↑Promotes necroptosis	Promotes MLKL oligomerization at the plasma membrane	Human, mouse	HT29, MEF, Jurkat, L929	Yes	No	[187]
PsKD	S454	**S441**	Phosphorylation	Unknown	No effect	Mediates myelin breakdown independent from necroptosis pathway	Mouse	Schwann cells	Yes	No	[190]
4HB[c]	**C18**	C18	Disulfide bond	Unknown	↑Promotes necroptosis	Mediates polymer formation	Human, mouse	HT29, L929	No	Yes	[193, 195]
4HB	**C24**	C24	Disulfide bond	Unknown	↑Promotes necroptosis	Mediates polymer formation	Human, mouse	HT29, L929	No	Yes	[193, 195]
4HB	**C28**	C28	Disulfide bond	Unknown	↑Promotes necroptosis	Mediates polymer formation	Human, mouse	HT29, L929	No	Yes	[193, 195]
4HB	C18/ C24/ C28	**C18/ C24/ C28**	Disulfide bond	Unknown	↑Promotes necroptosis	Possible structural function	Mouse	MDF	No	No	[43]
4HB	**C86**	–	Disulfide bond	Unknown	↑Promotes necroptosis	Mediates polymer formation	Human, mouse	HT29, L929	No	Yes	[193]
		–	Disulfide bond reduction	Thioredoxin-1	↓ Suppress necroptosis	Prevents polymer formation	Human	HeLa, HT29, 293T	No	Yes	[194]

[a]Equivalent residues in human and mouse orthologs are inferred by sequence homology. Bold indicates the ortholog residue with direct evidence of such post-translational modification.

[b]Cell line abbreviations: *MDF* mouse dermal fibroblasts, *HeLa* human adenocarcinoma cells, *NIH-3T3* immortalized mouse embryonic fibroblasts, *HT29* human adenocarcinoma cells, *MEF* mouse embryonic fibroblasts, *Jurkat* human T lymphocyte Jurkat cells, *L929* mouse fibrosarcoma cells, *293T* human embryonic kidney cell line HEK293T.

[c]*4HB* four-helix bundle domain, *PsKD* pseudokinase domain.

region to the N-terminal 4HB domain [43, 177]. These steps are presumed to induce exposure of the 4HB domain, which enables MLKL translocation to the plasma membrane where the 4HB domain permeabilizes the membrane to cause cell death. Broadly, these principles are thought to be common to MLKL activation across species, although the underlying mechanisms appear to have diverged between MLKL orthologs [30, 89, 108, 178]. Principally, the precise function of activation loop phosphorylation appears to differ between species. In mouse cells, mutations to mimic phosphorylation of the MLKL activation loop at S345 suffice to induce cell death, whereas those in rat MLKL lead to a partial activation, and those in human and horse MLKL do not [178]. These findings have led to the proposal that MLKL activation loop phosphorylation may serve a range of functions in promoting MLKL activation, from triggering the conformational interconversion of mouse MLKL to the active form with unleashed 4HB domain to inducing dissociation from RIPK3 to enable membrane translocation and disruption in the case of human MLKL. The underlying molecular basis is that endogenous mouse RIPK3 and MLKL do not appear to stably interact [30], leading to the model that mouse RIPK3 can transiently interact with and phosphorylate mouse MLKL [46, 88]. In human MLKL, on the other hand, phosphomimetic substitutions of T357/S358 failed to reconstitute necroptosis in human cell lines and recombinant human MLKL harboring these substitutions exhibited deficits in human RIPK3 binding [89], raising the question whether human MLKL requires RIPK3 interaction or phosphorylation of other sites to execute necroptosis.

Beyond the activation loop, additional phosphorylation sites in mouse MLKL were identified as mouse RIPK3 substrates by mass spectrometry [30, 47, 48]. Further residues adjacent to S345 in the mouse MLKL activation loop residues, S347 and T349, are also phosphorylated by mouse RIPK3 [30, 48]. S347 phosphorylation appears to serve an auxiliary role in activating MLKL, while T349 phosphorylation does not measurably contribute [48]. Non-activation loop residues of mouse MLKL were also found to be phosphorylated, including S228 and S248 in the MLKL N-lobe by RIPK3, as well as S158 in the brace region by an unknown kinase [47]. Mouse MLKL bearing phospho-ablating and phosphomimetic mutations at these sites could reconstitute necroptotic signaling in *Mlkl*$^{-/-}$ fibroblasts, indicating that phosphorylation of these sites is not essential to MLKL activation, but instead likely tunes the necroptotic signal. S228A, S228E, and S158A mouse MLKL mutants induced stimulus-independent cell death, which was aggravated by deletion of RIPK3, leading to the proposal that RIPK3 binding may also act as a negative regulator of necroptosis [47]. These phosphosites are conserved in human MLKL, except S158, which is replaced with Asn, and it remains of interest whether the orthologous residues (and other phosphosites) perform similar functions in the regulation of human MLKL killing function.

Members of the TAM (Tyro3, Axl, and Mer) receptor tyrosine kinases are known to activate multiple pro-survival and proliferation signaling cascades, such as PI3K, MAPK, and Rac pathways, but curiously, they were recently reported to mediate necroptosis by phosphorylating Y376 of human MLKL [187]. Phosphorylation of Y376 was proposed to serve as a secondary cue to induce MLKL oligomerization at the plasma membrane, although the precise chronology remains an open question after MLKL was observed to form oligomers prior to membrane translocation [51, 52]. While a human MLKL Y376F mutant failed to reconstitute necroptosis in HeLa cells overexpressing RIPK3 [187], it remains to be established under what circumstances Y376 is solvent exposed. In human MLKL structures, Y376 is located in the short helix at the C-terminal end of the activation loop and performs a structural role within the C-lobe of the pseudokinase domain. Thus, it is of interest to understand the conditions that lead to its exposure for phosphorylation and the ubiquity of this phenomenon.

Additional phosphosites in MLKL have been identified by mass spectrometry, although these have not yet been attributed functions in necroptotic signaling and nor have the responsible kinases been identified. S125 and T374 in human MLKL were phosphorylated in a cell cycle dependent manner [188, 189], while mouse MLKL phosphorylation on S441 was attributed a role in provoking MLKL-directed myelin breakdown in Schwann cells [190]. The occurrence of these events raises the possibility that additional, as yet unidentified sites are likely to be phosphorylated within MLKL in different cellular contexts upon exposure to different stimuli. It is also noteworthy that some species, including chickens, express MLKL, but not RIPK3 [19, 191, 192], raising the possibility that, in addition to RIPK3, other kinases may phosphorylate MLKL to provoke its necroptotic activity. Currently, however, the existence and identities of these kinases, and the stimuli and cellular contexts that might provoke their activity toward MLKL, are unknown.

Ubiquitylation

Ubiquitylation of MLKL has been observed following necroptotic stimulation in bone marrow-derived macrophages in a TRIF-dependent manner [172]. However, the function, underlying mechanisms, ubiquitylation sites, and the responsible E3 ligases remain unclear to date.

Other post-translational modifications

MLKL has been reported to form a disulfide-dependent amyloid-like polymer during necroptosis in cells [193], with thioredoxin-1 (Trx-1) proposed to suppress necroptosis by reducing MLKL 4HB domain cysteines to prevent oligomer formation [194]. However, the precise function and the ubiquity of MLKL amyloid forms remain unclear. It is notable that disulfide-bonded MLKL tetramers have become a widely used diagnostic for MLKL activation by non-reducing SDS-PAGE, although formation of disulfide bonds between MLKL protomers in cells prior to lysis has not been clearly demonstrated. Accordingly, these oligomers, which represent a small proportion of cellular MLKL in most studies, could well represent disulfide linkages that arise as a consequence of cell lysis owing to the proximity of MLKL protomers to one another within the necrosome. Therefore, the physiological function of MLKL oligomers formed from intersubunit disulfide bonds requires further investigation.

Conclusions and open questions

Among the post-translational modifications reported to regulate the core necroptotic proteins—RIPK1, RIPK3, and MLKL—the preponderance is reported to target the apical effector, RIPK1. In addition to RIPK1 having been studied for many years more than RIPK3 and MLKL, RIPK1 participates in pro-inflammatory TNF signaling, and cell death by apoptosis and necroptosis. Consequently, many post-translational modifications serve to divert RIPK1's involvement between these different signaling conduits, whereas those in RIPK3 and MLKL direct their activation in cell death signaling. To date, the bulk of post-translational modifications in RIPK3 and MLKL are reported to activate their necroptotic functions, and consequently it remains to be discovered whether negative regulatory modifications exist and whether the activating post-translational modifications can be defused with other modifying enzymes, such as deubiquitinases and phosphatases. Thus far, only A20 has been identified as a deubiquitinase and Ppm1b a phosphatase for RIPK3, while none have been reported for MLKL. As our understanding of differences in signaling events between species, cell lines and necroptotic stimuli grows, we expect that future studies will reveal further post-translational modifications within RIPK3 and MLKL and unearth negative regulators to remove post-translational marks and halt the progression of necroptotic death. Such studies will reveal further checkpoints in the signaling pathway, which could be targeted therapeutically to counter necroptotic pathologies.

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Compliance with ethical standards

Conflict of interest PEC and JMM contribute to a project developing necroptosis inhibitors in collaboration with Anaxis Pharma. The other authors declare no conflict of interest.

Ethics statement Not applicable.

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