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STATE-OF-THE-ART REVIEW

Surviving death: emerging concepts of RIPK3 and MLKL ubiquitination in the regulation of necroptosis

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Lytic forms of programmed cell death, like necroptosis, are characterised by cell rupture and the release of cellular contents, often provoking inflammatory responses. In the recent years, necroptosis has been shown to play important roles in human diseases like cancer, infections and ischaemia/reperfusion injury. Coordinated interactions between RIPK1, RIPK3 and MLKL lead to the formation of a dedicated death complex called the necrosome that triggers MLKL-mediated membrane rupture and necroptotic cell death. Necroptotic cell death is tightly controlled by post-translational modifications, among which especially phosphorylation has been characterised in great detail. Although selective ubiquitination is relatively well-explored in the early initiation stages of necroptosis, the mechanisms and functional consequences of RIPK3 and MLKL ubiquitination for necrosome function and necroptosis are only starting to emerge. This review provides an overview on how site-specific ubiquitination of RIPK3 and MLKL regulates, fine-tunes and reverses the execution of necroptotic cell death.

Introduction

Mammalian cells are able to actively decide between survival and cell death and have the option to undergo a rich palette of different forms of programmed cell death (PCD). An adequate balancing of the fundamental decisions about life and death underlies organismal development and tissue homeostasis, in which some cells are instructed to die, while others are prompted to divide and proliferate [1–3]. Disturbances in activating specific PCD pathways underlie a wide variety of pathophysiological conditions, ranging from tumour formation to inflammation, infection and acute kidney injury and organ damage caused by ischaemia-reperfusion [4–6].

At present, around 15 distinct modes of PCD have been identified, some of which are based on strictly dedicated signalling pathways, while others use shared signalling networks [7,8]. Necroptosis, a prototypic form of lytic PCD, occurs upon membrane permeabilization that depends on the pseudokinase Mixed Lineage Kinase Domain-Like Protein (MLKL) [8]. Necroptotic cell death decisions rely on the type of stimulus and cellular context and are controlled by numerous checkpoints, fail-break mechanisms and regulatory systems [8]. Among these are proteolytic and non-proteolytic forms of ubiquitination, in which proteins are post-translationally modified with ubiquitin

Abbreviations
4HB, four-helix bundle; BR, brace region; DD, death domain; DUB, deubiquitinating enzyme; I, isoleucine; IAP, inhibitor of apoptosis protein; K, lysine; KD, kinase domain; MLKL, mixed lineage kinase domain-like protein; NF-κB, nuclear factor-κB; PCD, programmed cell death; PS, phosphatidylserine; PsKD, pseudokinase domain; RHIM, RIP homotypic interaction motif; RIPK, Receptor-interacting serine/threonine-protein kinase; TNFR1, TNFα receptor 1; TNFα, tumour necrosis factor α; Ub, ubiquitin; UBD, Ub-binding domain; UbL, Ub-like; USP, Ub-specific proteases; zVAD.fmk, n-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethylketone.

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(Ub). Although the role of ubiquitination is reasonably well-understood in the regulation of upstream necroptosis signalling, for example, RIPK1 ubiquitination, it still remains unclear how end-point modifications of necroptosis executioner proteins, such as RIPK3 and MLKL, control or counteract necroptosis. Therefore, this review provides an overview on recent advances in site-specific ubiquitination and deubiquitination in the control of the final execution of necroptosis.

Ubiquitination and regulation by deubiquitinating enzymes

Ubiquitination refers to the covalent modification of substrate proteins with the small and conserved Ub protein [9]. Ub is highly conserved and widely expressed; it modifies the vast majority of cellular proteins during their lifetime in eukaryotes and is therefore an important factor in many biological processes. Failure to adequately balance ubiquitination is a major cause of a wide variety of human diseases, like genetic disorders, neurodegenerative diseases, infections and inflammation [10].

Substrates can be conjugated with one or multiple single Ub molecules, or with polymeric chains of different lengths. Ub chains are generated through linkages via the internal lysine (K) residues (K6, K11, K27, K29, K33, K48 and K63) or via the N-terminal methionine-1 (linear, head-to-tail or M1) [9,11,12] of Ub. Apart from homotypic chain types, consisting of a single type of linkage, also heterotypic, branched and hybrid chains, for example, K11/K48 and M1/K63, have been identified [13–15]. Ubiquitination critically controls the stability and abundance of substrates by mediating their recognition and degradation by the 26S proteasome [9]. Apart from substrate degradation, Ub also serves non-degradative functions, including DNA damage responses and signal transduction [12]. In general, mammalian ubiquitination occurs through the concerted activation of Ub-activating (E1s), -conjugating (E2s) and -ligating (E3s) enzymes that use adenosine triphosphate (ATP) for Ub activation and modification of substrates [16–18].

Besides ubiquitination, around 10 different Ub-like protein (UbL) families have been discovered, like interferon (IFN)-stimulated gene 15 (ISG15), small Ub-related modifier (SUMO) and the neural precursor cell expressed, developmentally downregulated 8 (NEDD8), each equipped with dedicated E1-E2-E3-like conjugation machineries and specific biological functions [19]. Extensive cross-modifications of Ub, UbL and additional post-translational modifications have been reported [20], for example, the Really Interesting New Gene (RING)-type E3 Ub ligase RNF4 mediates polyubiquitination of SUMO chains [21] and hybrid Ub-NEDD8 chains protect against proteotoxicity [22].

Ub signals are recognised by proteins that contain Ub-binding domains (UBDs) [23], such as Ub-associated (UBA), Ub-interacting motifs (UIM), zinc finger (ZnF) and the UBAN [Ub-binding domain in A20-binding inhibitor of Nuclear Factor-κB (NF-κB) activation (ABIN and NF-κB essential modulator (NEMO)]. Up till now, around 25 subfamilies have been identified that display specificity and selectivity in Ub-binding targets [20,23].

Ubiquitination is counterbalanced and Ub signals are dynamically removed from substrates and hydrolysed by the action of deubiquitinating enzymes (DUBs) [24,25]. Around 100 DUBs have so far been identified that, based on their domain structure, can be classified as Ub-specific proteases (USP), ovarian tumour (OTU), Machado-Josephin domain (MJD), Ub C-terminal hydrolase (UCH), motif interacting with Ub-containing novel DUB family (MINDY), the ZnF and UFSP domain protein (ZUFSP)-related DUBs and the metalloproteases belonging to the Jab1/Mov34/Mpr1 Pad1 N-terminal+ (MPN+) (JAMM) domain family [24,26–30]. Deubiquitination is mediated through at least one Ub-binding site (S1 site) at the surface of DUBs that recognise residues within the C-terminus and the I36 and I44 hydrophobic patches on the surface of Ub [25,31]. DUBs may be non-specific, substrate-specific, either alone or incorporated in macromolecular complexes, such as the proteasome [32] or the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex [33,34], or may act specifically for certain chain-types, like specific peptidase 30 (USP30) and cylindromatosis (CYLD), that allow direct recognition of Ub chains and targeted degradation of selective Ub chain types [25]. Additional determinants that define DUB activity are distal end processing (exo-DUB) or in-chain processing (endo-DUB), Ub chain length as well as the removal of mono-Ub modifications or complete chains *en bloc* [25].

Apart from removing Ub modifications from substrates, DUBs also serve important additional roles in maintaining cellular Ub homeostasis. Ub is expressed either as inactive precursors, in which single Ub molecules are fused to the ribosomal proteins L40 and S27A (UBA52 and UBA80, respectively), or as extended Ub polymers (UBB and UBC). Importantly, DUBs are also required to cleave these precursors and to control the pool of free Ub that can subsequently be used for conjugation by the E1-E2-E3 machinery [25,35–37].

Besides enzymes that remove Ub modification, UbL modifications are also dynamically regulated by specialised DUB-like enzymes, such as the NEDD8-specific

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proteases [38] or the SUMO-specific proteases [39] that catalyse the removal of NEDDylation or SUMOylation, respectively. DUBs distinguish Ub and UbL modifications with high accuracy due to the recognition of unique hydrophobic surfaces on Ub and selective binding of residues flanking inter-Ub bonds [9,40].

DUB function is strictly controlled by modulating cellular localisation, abundance and enzymatic activity. For example, phosphorylation, as well as ubiquitination itself, regulates the cellular localisation of specific DUBs [38,39,41–43], while the abundance of other DUBs is induced by transcriptional regulation, as is the case for A20 and CYLD by NF-κB activation [44–46]. DUBs with higher abundance often serve housekeeping functions, like proteasome- and Ub-mediated protein degradation [24,25], while others are associated with specific organelles or cellular structures, such as the nucleus, nucleolus or endoplasmic reticulum (ER) and mitochondria or microtubuli [25]

DUB activity is also controlled by phosphorylation, such as inhibitor of nuclear factor kappa B kinase subunit beta (IKKβ)-mediated phosphorylation of the Ub carboxyl-terminal hydrolase CYLD that positively regulates its activity [47]. Furthermore, additional modifications, like SUMOylation and ubiquitination, are also regulating DUB activity and function [48,49]. Post-translational modifications of Ub itself may also add additional layers of regulation to DUB function, as is the case for USP30 that exhibits lesser activity towards phosphorylated K6-linked poly-Ub compared with unmodified Ub [50,51].

The extensive regulation of DUB functions ensures important roles of DUBs in organising DNA damage responses [52,53], cell cycle [34], inflammation [54,55] and cell death pathways [40,56]. Failure to regulate the function of DUBs often leads to dramatic physiological consequences and underlies many diseases, such as several neurological disorders [57–59]. In addition, several DUBs are well-known oncogenes or tumour suppressors and are intimately linked to tumour formation and progression [60–63].

The role of ubiquitination in PCD signalling

Cell death occurs in many ways and E3- and DUB-mediated ubiquitination is an important regulator in the control of cellular signalling and the final execution of several PCD pathways. In contrast to passive cell death (necrosis), which is mostly a consequence of exposure to toxic substances, temperature or pressure, active or programmed forms of cell death rely on genetically encoded signal transduction pathways. Some forms of PCD are essential for organismal development and tissue homeostasis, while others play clearly defined roles in human diseases and are often dysregulated in inflammation and oncogenesis [64]. For example, loss-of-function mutations in key PCD effector proteins contribute to chemotherapy resistance, tumour formation and inflammatory responses in the tumour microenvironment (TME) [64–67].

At present, around 15 different types of PCD have been identified, ranging from apoptosis to necroptosis and ferroptosis [7]. Many modes of PCD are characterised by shared and overlapping signalling pathways, specific morphological features and different functional effects on the cellular microenvironment, for example, lytic or non-lytic forms with direct implications for local inflammation (necro-inflammation) [4,64]. While some forms of PCD are initiated by strictly dedicated and highly specialised signalling networks, others share similar signalling pathways. Although biochemically, morphologically and functionally relatively distinct, necroptosis largely shares NF-κB signalling with (extrinsic) apoptosis. As described in further detail later, ubiquitination and DUBs serve as important molecular switches and checkpoints in the control of Tumour Necrosis Factor α (TNFα)-mediated activation of TNF Receptor 1 (TNFR1)-induced NF-κB signalling and the balance between apoptotic and necroptotic cell death. Apart from TNFα-mediated activation, NF-κB can also be initiated via activation of TNFR2, Interleukin (IL)-1β, nucleotide-binding oligomerisation domain-containing protein 2 (NOD2) or toll-like receptors (TLRs), with important functions in the control of PCD [68–70]. In addition, ubiquitination and deubiquitination tightly control downstream necroptotic events as well and are critically involved in the timed initiation and fine-tuning of necroptosis execution [71].

NF-κB signalling typically relies on a well-orchestrated interplay of degradative and non-proteolytic forms of ubiquitination and DUB action. NF-κB comprises a family of transcription factors, like RelA/p65, that control the expression of numerous genes involved in pro-inflammatory responses, proliferation, migration, invasion and angiogenesis [68–70,72]. NF-κB is essential for cell fate control and normal cellular homeostasis and controls cellular responses on exposure to many different stimuli, like cytokines, toxic substances or pathogenic microorganisms [68]. Dysregulation of NF-κB underlies cancer, inflammation and autoimmune diseases as well as a defective development of immune responses [73]. For example, constitutive NF-κB activation is a common feature in a wide variety of tumours, including haematological

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malignancies like leukaemia, myelomas and B-cell lymphomas [74,75].

Activation of TNFR1 initiates the recruitment of the adaptor protein TNFR-associated death domain protein (TRADD) and receptor-interacting serine/threonine-protein kinase 1 (RIPK1) to the TNFR1 death domain (DD), through homotypic DD interactions [76]. TRADD serves as platform that further recruits the E3 ligase TNF-associated factor 2 (TRAF2) and the cellular Inhibitor of Apoptosis (IAP) protein 1 and 2 (cIAP1/2), creating the pro-survival TRADD- and RIPK1-dependent complex I [77–79]. cIAP1/2 modifies several targets in complex I with K11-, K48- and K63-linked poly-Ub [80–84], that further recruits the linear Ub-specific E3 ligase chain assembly complex (LUBAC). LUBAC is composed of the central catalytic subunit Ran-binding protein 2 (RanBP2)-type and C3H4-type ZnF containing 1 (HOIP), RING finger protein 31 (RNF31, HOIL) and SH3 and multiple ankyrin repeat domains protein 3 (SHANK)-associated RH domain interactor (SHARPIN) [85–88]. LUBAC modifies several targets, including K63-linked poly-Ub, TNFR1, RIPK1 and TRADD in complex I [13,89–94] and together with cIAP1/2-mediated ubiquitination recruits and activates several kinase complexes, like inhibitor of NF-κB kinase subunits α and β (NEMO/IKKα/IKKβ), transforming growth factor-β-activated kinase 1 and MAP3K7-binding protein 1/2 (TAB1/2)/TGF-β-activated kinase 1 (TAK1) and TAK1-binding proteins 1 and 2 (TAB2/3) [68,69,95]. TAK1 phosphorylates IKKβ, leading to IKKβ-mediated phosphorylation and K48-dependent ubiquitination and proteasomal degradation of NF-κB inhibitor α (IκBα) [68–70,95]. IκBα normally retains NF-κB transcription factors within the cytosol and IKKα/β-mediated degradation of IκBα allows the nuclear translocation of NF-κB and activation of pro-survival gene expression [68,95].

In addition, cIAP1/2 negatively regulates non-canonical NF-κB signalling by controlling the constitutive ubiquitination and proteasomal degradation of NF-κB-inducing kinase (NIK) [96–102]. Importantly, upon chemical inhibition or loss of cIAP1/2 expression, NIK is stabilised and induces the processing of p100 to p52 and translocation of the NF-κB p52-RelB dimers to the nucleus to activate non-canonical NF-κB signalling [98,101,102].

Apoptosis

Apoptosis plays important roles in cellular homeostasis and is involved in the clearance of damaged and transformed cells, T-cell development and embryogenesis [1,103]. Central effectors in apoptosis are cysteine-aspartic proteases, or caspases, that are activated by autocatalytic cleavage from inactive precursors (pro-caspases) and mediate substrate cleavage and cell death [104–106]. Apoptosis is characterised by caspase-mediated condensation of chromatin, nuclear DNA fragmentation, cell shrinkage, membrane blebbing, loss of adhesion and exposure of phosphatidylserine (PS) [1,103].

Generally, two distinct modes of apoptosis can be distinguished. Intrinsic apoptosis is triggered by internal cell stress and involves pro- and anti-apoptotic B-cell lymphoma 2 (BCL-2) proteins that mediate mitochondrial outer membrane permeabilisation (MOMP), release of cytochrome c and second mitochondria-derived activator of caspases (Smac/DIABLO) [107], leading to the formation of the caspase-9-containing apoptosome, that activates caspase-3 and -7 [108,109] (Fig. 1).

In contrast, extrinsic apoptosis is mediated by extracellular cytokines, like CD95 ligand (TNFR superfamily member 6; Fas), TNFR superfamily member 10C (TRAIL) and TNFα [76]. Activation of these receptors induce the assembly of the death-inducing signalling complex (DISC) [110–112] that activates caspase-8, leading to caspase-3 and -7 activation and cleavage of the BH3-interacting domain death agonist (BID), that promotes cytochrome c release and apoptotic cell death [113,114]. Upon TNFα-mediated TNFR1 activation, the cytoplasmic TRADD-dependent complex IIa, composed of TRADD, Fas-associated death domain protein (FADD), FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (FLIP) and caspase-8 or a RIPK1-dependent complex IIb, consisting of RIPK1, FADD, caspase-8, FLIP and RIPK3 (ripoptosome) are formed [115–117]. CYLD is an important DUB that mediates RIPK1 deubiquitination to facilitate formation of the TRADD-dependent complex IIa that induces caspase-8 activation and apoptosis [118]. Loss of the cIAP1/2 E3 ligases, for example, by SMAC mimetic-induced K48-linked autoubiquitination followed by proteasomal degradation, leads to the formation of the RIPK1-dependent complex IIb, or ripoptosome, that triggers RIPK1-dependent apoptosis [119]. Finally, when caspase-8 is inactivated or inhibited by broad-range caspase inhibitors, like *n*-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethylketone (zVAD.fmk), quinoline-Val-Asp-difluorophenoxymethyl ketone (Q-VD-OPh) and Emricasan (IDN-6556), RIPK1 and RIPK3 cooperate to form the necrosome complex that allows MLKL activation and necroptosis induction (discussed later) [120,121] (Fig. 1).

In the regulation of cell fate, RIPK1 acts as central post-translational hub and can be modified with poly-

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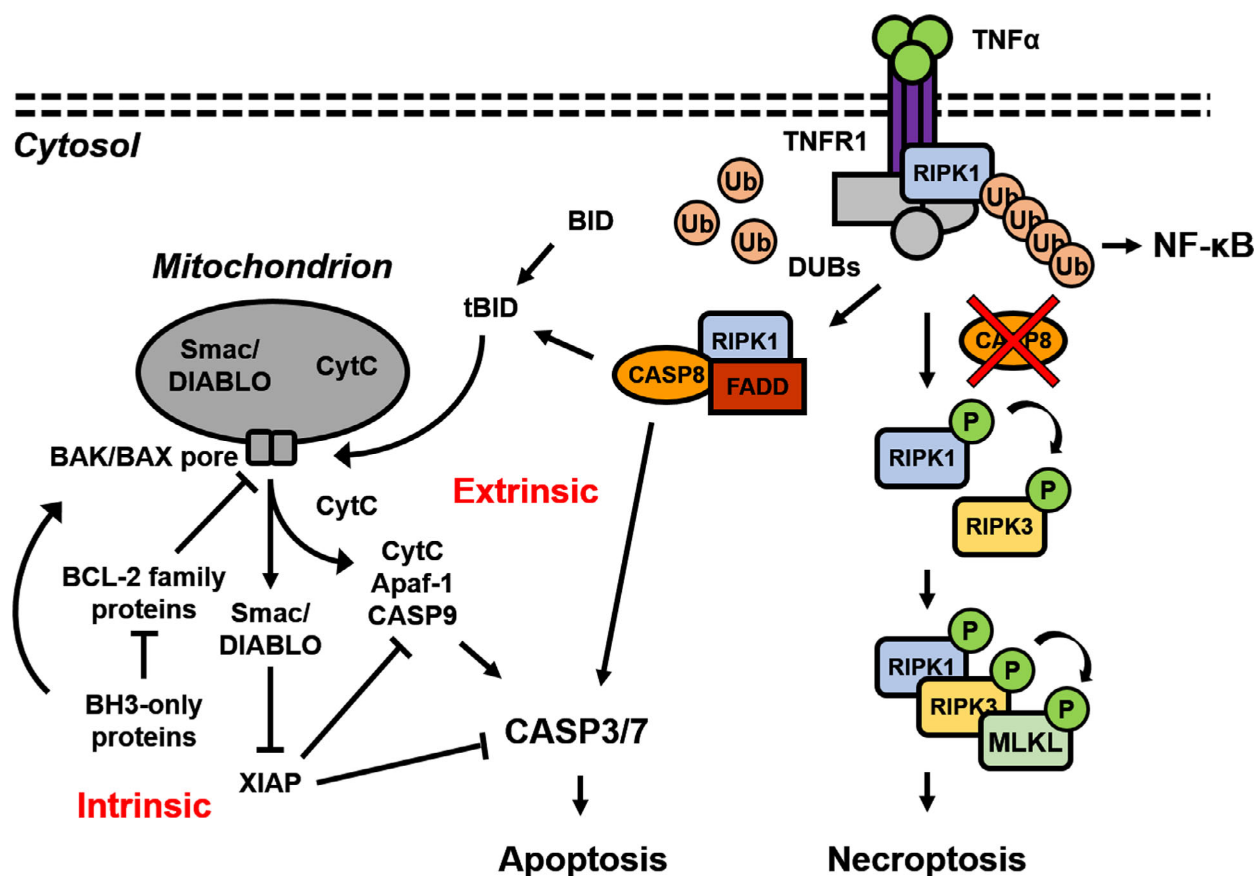

Fig. 1. Shared and unique features of apoptosis and necroptosis. Activation of death receptors (DRs), like TNFR1 by TNFα, triggers pro-survival NF-κB signalling or, depending on RIPK1 ubiquitination and the caspase-8 (CASP8) status, either RIPK3- and MLKL-induced necroptosis, or extrinsic apoptosis. CASP8 activates caspase-3 and -7 (CASP3/7) and cleaves BID into truncated BID (tBID) that induces loss of mitochondrial membrane potential via BAK/BAX oligomerisation and the formation of pro-apoptotic BAK/BAX pores. These pores enable the release of mitochondrial proteins, like cytochrome c (CytC), that together with Apaf-1 and caspase-9 (CASP9) forms the apoptosome complex. The apoptosome activates the effector caspases CASP3/7 to induce apoptosis. In addition, Smac/DIABLO is also released from mitochondria and inhibits X-linked IAP (XIAP) to further activate CASP9 and CASP3/7. In contrast, the intrinsic apoptosis pathway is regulated by the balance between apoptosis-initiating BH3-only proteins and pro-survival BCL-2 proteins, controlled by cellular damage, oxidative stress, oncogenes and growth factor deprivation. Ub, ubiquitin, P, phosphorylation.

mono Ub as well as M1-, K11-, K48- and K63-linked Ub chains [80,82,84,86,93,122–124], and perhaps additional linkages as well [125], often in a complex interplay with phosphorylation [126]. Several DUBs, like CYLD, A20 and potentially OTULIN, have been reported to be involved in the removal of Ub signals from RIPK1 and to determine cell fate decisions. However, many details of RIPK1 deubiquitination and implications for cell survival still remain unclear.

Apart from M1, K11, K48 and K63 Ub chains, additional chain linkages are also involved in survival and cell death signalling. For example, the E3 ligase tripartite motif-containing protein 13 (TRIM13) promotes TRAF6 modification with K29-linked Ub chains to regulate NF-κB signalling upon activation of TLR2 [127]. In addition, TRAF6 and TRAF7 mediate K29 ubiquitination and degradation of the anti-apoptotic protein cFLIP$_L$ [128]. Furthermore, the NF-κB adaptor NEMO becomes modified with K27 Ub chains that inhibit NF-κB in dendritic cells, and TRIM23-mediated K27 ubiquitination of NEMO mediates antiviral inflammatory responses as well [129].

Finally, ubiquitination controls not only regulatory signalling towards apoptosis, but also the abundance and function of apoptotic effector proteins (for an overview see [130]). Generally, apoptotic cells and cellular debris associated with apoptosis are taken up by neighbouring cells, among them macrophages, thereby often suppressing local immunological responses [64–67].

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Necroptosis

In contrast to apoptosis, necroptosis, or programmed necrosis, is morphologically characterised by cell swelling and membrane rupture. By doing so, this lytic form of PCD generally releases damage-associated molecular patterns (DAMPs), like high mobility group box 1 (HMGB1), free DNA, RNA and cytokines, such as TNFα and IFNs [64–67], that further provoke inflammatory responses in the cellular microenvironment (necro-inflammation) [131,132].

Necroptosis can be triggered via activation of TNFRs, TRAILRs, CD95, TLRs, NOD2-like receptors (NLRs), as well as via recognition of double-stranded RNA or receptor activation by IFNs [2–4,112,119,133]. Necroptosis plays important roles in a wide variety of human diseases, including inflammatory bowel diseases, ischaemia-reperfusion injury, cancer and infections [132]. A common pathway of necroptosis activation relies on activation of the NF-κB signalling cascade but, in contrast to extrinsic apoptosis, requires inhibition, or loss, of the cIAP1/2 E3 ligase activity to prevent RIPK1 ubiquitination and NF-κB-mediated pro-survival signalling and loss of caspase-8 activity [133] (Fig. 2).

RIPK1 contains an N-terminal kinase domain (KD), a RIP homotypic interaction motif (RHIM) and a C-terminal DD [134,135]. RIPK1 provides a molecular scaffold that promotes pro-survival functions through DD-mediated interactions of RIPK1 with TNFR1, TRADD and FADD [78,136]. In contrast, the catalytic activity of RIPK1 mediates apoptotic and necroptotic functions [137] and is further fine-tuned by post-translational modifications and RHIM-based interactions with other RHIM-domain containing proteins, like Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), Z-DNA Binding Protein 1 (ZBP1) and RIPK3 [138–140]. RIPK3 contains an N-terminal kinase domain and a RHIM motif, flanked by unstructured C-terminal regions [4,133,141–144]. Activated RIPK1, together with RIPK3, initiates auto- and trans-phosphorylation steps [145–147] that induce the formation of a heteroamyloid complex, the necrosome [148], of which especially RIPK3 oligomerisation is the driving force in necroptosis [148–154]. In humans, phosphorylation of RIPK3 at S227 recruits MLKL into the necrosome, which becomes phosphorylated by RIPK3 at residues T357 and S358 [142,151,155], although RIPK3 can also pre-assemble with MLKL [142,156]. In mice, phosphorylation of MLKL S345 is essential for necroptosis progression, illustrating important species-specific differences in the regulation of necroptosis at the level of RIPK3 and MLKL activation [157–159]. MLKL contains a four-helix bundle (4HB), an auto-inhibitory brace region (BR) and a C-terminal pseudokinase domain (PsKD). RIPK3-mediated MLKL phosphorylation inducing conformational changes in MLKL that expose the MLKL 4HB domain [156,159,160] thereby inducing the formation of necroptosis-proficient MLKL oligomers which are recruited to biological membranes, including the plasma membrane [157,159–162], the ER [163], mitochondria [163] and autophagic compartments, like lysosomes [163–166]. Although activated MLKL is located on several cellular membranes, MLKL accumulation at the plasma membrane mediates the release of cellular contents [132,133,161,163] and most likely leads to necroptotic cell death [133,151,155,157,163,167] (Fig. 2). How and in what kind of higher-order structures MLKL mediates membrane permeabilisation still remains unclear and MLKL trimers [160,168] and tetramers [159], but also hexamers [163], octamers [169] and higher-order polymers [150,170,171] have been described. In addition, the exact mechanisms of how MLKL mediates membrane disruption remain unclear. Positively charged amino acids in the human MLKL 4HB interact with phosphatidylinositol phosphates (PIPs) and regulate MLKL binding to biological membranes [162,167,172]. In addition, murine MLKL residues in the α3 and α4 helix as well as residues in the first brace helix are important for necroptosis [173]. Moreover, highly phosphorylated forms of inositol phosphate 6 (IP6), generated by the IP kinases inositol polyphosphate multikinase (IPMK), inositol-tetrakisphosphate 1-kinase (ITPK1) [174] and inositol pentakisphosphate 2-kinase (IPPK) [175] bind to the MLKL 4HB and displace the BR to regulate necroptosis. The appearance of MLKL hotspots at the plasma membrane correlates with necroptotic membrane damage [170], increased Ca^{2+} influx and accumulation of PS at the outer leaflet of the plasma membrane [170,176,177].

Intriguingly, accumulation of activated MLKL at the plasma membrane is not the endpoint of necroptotic cell death and several cell-type specific biological mechanisms have been identified that can resuscitate necroptotic near-death experiences (Fig. 2). For example, membrane accumulation and necroptotic cell death downstream of activated MLKL can be inhibited by monobodies that specifically bind the 4HB of human MLKL [170]. Phosphorylated MLKL associates with the lipid raft-resided proteins flotillin-1 and -2 at the plasma membrane and becomes endocytosed and degraded by lysosomes in a cell-line specific manner [164]. Interestingly, plasma membrane-accumulated MLKL is also controlled by endosomal sorting

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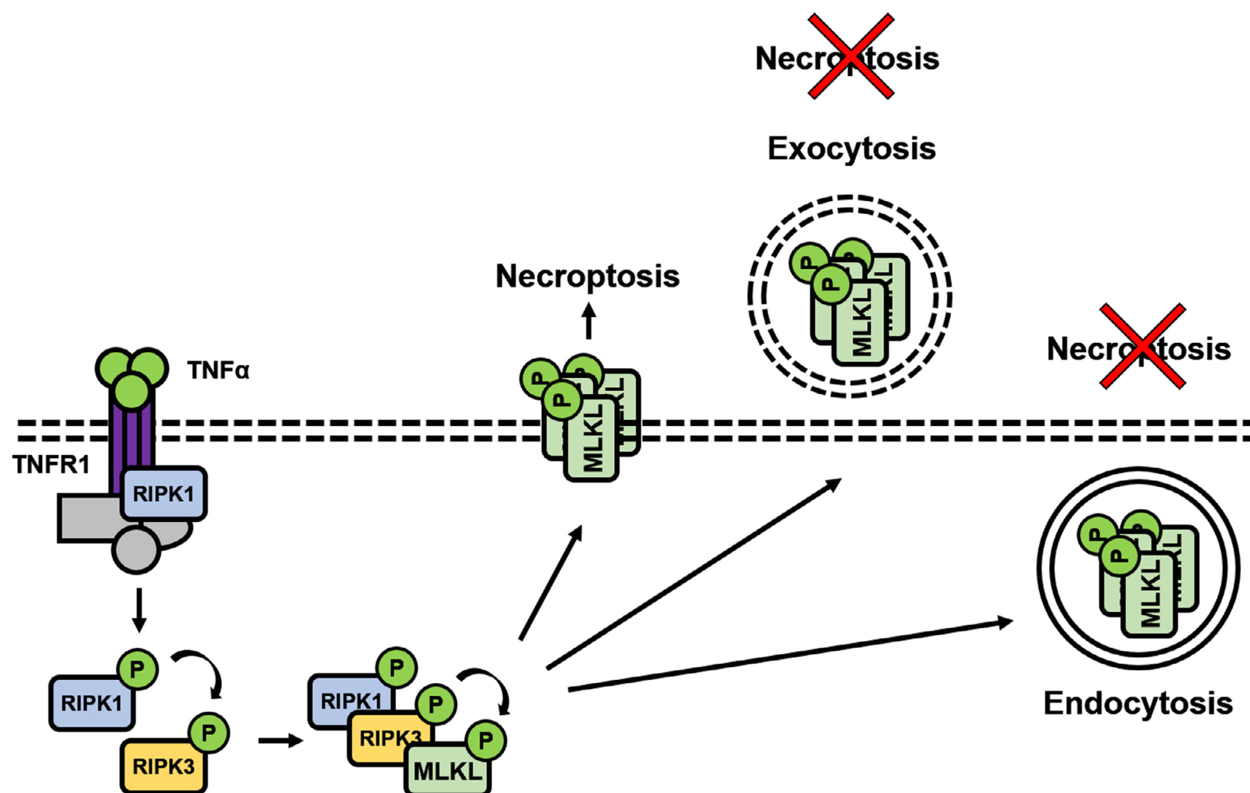

Fig. 2. Regulation of necroptosis execution. Necroptosis typically occurs upon activation of RIPK1, that involves a complex interplay between phosphorylation and ubiquitination. Activated RIPK1 interacts with RIPK3 through the RHIM domain, triggering auto- and transphosphorylation, leading to necrosome formation. Activated RIPK3 recruits and phosphorylates MLKL after which MLKL translocates to cellular membranes to mediate necroptosis. The initiation of necroptosis can be counteracted by exocytosis and endocytosis of activated MLKL in a context- and cell type-specific manner. A potential role of site-specific ubiquitination in these events remains unknown. P, phosphorylation.

complexes required for transport (ESCRT-), ALIX-, syntenin- and Rab27-mediated exocytosis that promote the shedding of MLKL in extracellular necroptotic vesicles, or exosomes, and balance membrane repair [164,177–180]. By doing so, both endocytic and exocytic measures balance the execution of necroptosis at the later stages of MLKL activation (Fig. 2).

Regulation of necroptosis by selective (de)ubiquitination of RIPK3 and MLKL

Not only the initiation, but also the execution of necroptosis, as well as the cellular sensitivity towards necroptotic cell death, is controlled in a fine-tuned manner by selective ubiquitination. Apart from Ub modification of RIPK1, site-specific ubiquitination of RIPK3 and MLKL is emerging as regulatory mechanism of necroptosis execution.

RIPK3 is extensively modified with different types of poly-Ub linkages on multiple residues scattered throughout the protein (Fig. 3A,B). Site-selective RIPK3 ubiquitination affects the RIPK1-RIPK3 interaction, the kinase activity of RIPK3 and necrosome formation. RIPK3 ubiquitination occurs in species-, cell type- and stimulus-specific manners, with different functional consequences related to degradative and non-degradative forms of ubiquitination as well as positive and negative effects on necroptosis (Fig. 3A,B). For example, the E3 ligase Pellino 1 (PELI1) mediates K48-based ubiquitination, proteasomal degradation and necroptosis inhibition of human RIPK3 via residue K363, in a manner dependent on the kinase activity [181]. Of note, RIPK3 K363 is not present in murine RIPK3. Interestingly, PELI1 also modifies RIPK1 on K115, a residue that is conserved between mouse and human, with K63-linked poly-Ub, depending on the RIPK1 kinase activity and promotes the RIPK1-RIPK3 interaction to facilitate necroptosis [182] (Fig. 3A,B).

In addition, Carboxyl terminus of Hsp70-interacting protein (CHIP)-mediated K48-linked ubiquitination of

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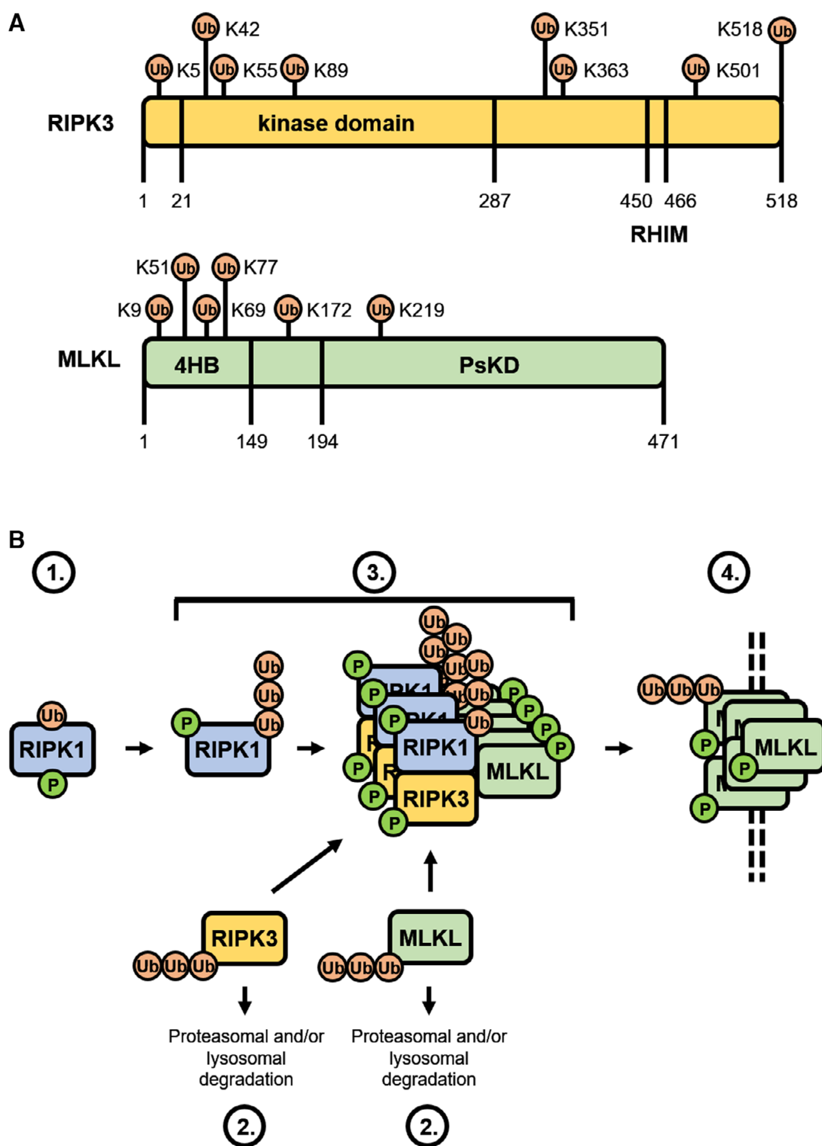

Fig. 3. The role of site-specific ubiquitination of RIPK3 and MLKL in necroptosis. (A) Ub modifications on RIPK3 and MLKL. Schematic representation of the domain structures of human RIPK3 (Uniprot ID: Q9Y572) and MLKL (Uniprot ID: Q8NB16), with identified Ub acceptor sites. Ub: ubiquitin, RHIM: RIP homotypic interaction motif, 4HB: four-helix bundle, PsKD, pseudokinase domain. Representations are not drawn on scale. (B) (Potential) Roles of ubiquitination in the control of necroptosis. Ubiquitination and deubiquitination of RIPK1 are important determinants for initiating necroptotic signalling (1). Ubiquitination also controls RIPK3 and MLKL stability and proteasomal/lysosomal degradation of un-complexed RIPK3 and MLKL, thereby regulating their availability for necroptosis (2). In addition, ubiquitination regulates early and late stage RIPK1 and RIPK3 functions as well as the formation of the necrosome (3). Finally, site-specific ubiquitination of MLKL influences MLKL oligomerisation, membrane localisation and membrane rupture, either by directly influencing MLKL function or affecting additional, yet unidentified, UBD-containing effector proteins (4). Of note, species- and cell type-specific differences in necroptotic signalling and spatio-temporal control of necroptosis might be influenced in different ways by ubiquitination as well. Ub, ubiquitin, P, phosphorylation.

human RIPK3 at K55, K89, K363 and K501 has been described, of which modification of the conserved K55 and K363 are the main effector lysine residues that control RIPK3 degradation by the lysosomal pathway [183]. Loss of CHIP expression stabilises RIPK3 and sensitises cells towards necroptotic cell death. The regulatory roles of proteasomal degradation in the control of RIPK3 expression and necroptosis have been further confirmed by the observation that the proteasome inhibitors MG132 and bortezomib induce accumulation of K48-linked poly-Ub chains at the conserved K264 in murine RIPK3 [184]. Functionally, blockage of proteasome-mediated RIPK3 degradation triggered the increased appearance of RIPK3 oligomerisation, MLKL activation and necroptosis independently of caspase-8 [184].

Apart from K48 Ub chains, the hybrid E3 ligase and DUB A20 affect necroptosis by removing K63-linked poly-Ub chains from murine RIPK3 at the conserved K5 and thereby preventing the RIPK1-RIPK3 interaction and necroptosis [56]. In addition, the E3 ligase Parkin suppresses necrosome formation by promoting K33-linked poly-ubiquitination of human RIPK3 at residues K197, K302 and K364, which are not conserved in murine RIPK3 [185].

The tripartite motif E3 ligase TRIM25 interacts also with RIPK3, but not with RIPK1 or MLKL, and mediates ubiquitination of human RIPK3 at K501 [186]. Although loss of TRIM25 expression sensitised human and murine cell lines towards necroptosis, due to TRIM25- and K48-linked poly-Ub-mediated degradation of RIPK3 by the 26S proteasome, RIPK3

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K501 is not conserved in mice and the main RIPK3 acceptor lysine in mice remains unknown. Interestingly, TRIM25 has been demonstrated to mediate K63 poly-Ub of RIG-I and IFIH1 [187,188], while NLRP12 inhibits TRIM25 ubiquitination [189], suggesting potential roles of the regulation of RIPK3 expression in viral responses and innate immunity. In line with this, a viral inducer of RIPK3 degradation (vIRD) was identified in orthopoxviruses that bridge RIPK3 to the SKP1-Cullin1-F-box E3 ligase complex to mediate RIPK3 degradation and subsequently inhibition of necroptosis [190]. Infection of mice with vIRD-deficient viruses reduced inflammation, viral replication and mortality, linking RIPK3 stability and necroptosis induction to inflammation and virus-host evolution.

The RIPK3 ubiquitination status is regulated not only by E3 ligases, but also by DUBs. Recently, USP22, a DUB which is part of the ATXN7L3, ATXN7 and ENY2 DUB module of the SAGA complex [34,191,192] has been reported to regulate RIPK3 ubiquitination and necroptotic cell death [193]. Three novel, USP22- and TBZ-regulated human RIPK3 ubiquitination sites, K42, K351 and K518, have been identified, of which only K351 is not conserved in mice. The RIPK3 lysine-to-arginine (K-to-R) mutations K518R and K42R/K351R/K518R increased necroptotic cell death altering the RIPK3 ubiquitination status, necrosome formation and MLKL phosphorylation [193].

In contrast to the widespread ubiquitination of RIPK1 and RIPK3, Ub modification of MLKL and its relevance for necroptotic cell death remains largely unexplored. MLKL is modified with Ub during necroptosis [194–196] and the molecular mechanisms and cellular consequences of MLKL ubiquitination are starting to emerge (Fig. 3A,B). A proteome-scale yeast two-hybrid screen to identify protein-protein interactions in the human liver identified an interaction of MLKL with the E3 ligase RAD18 [197]. Interestingly, a modification of MLKL with mono-Ub has very recently been discovered that likely occurs after MLKL oligomerisation at membranes and might target MLKL for proteasomal and lysosomal degradation [198]. Four Ub sites have been identified in murine MLKL (K9, K51, K69 and K77), located in the 4HB, and although mutation of these lysine residues did not affect necroptosis, genetic fusion of the pan-DUB USP21 with subsequent loss of MLKL ubiquitination sensitised cells towards necroptosis, even in the absence of necroptotic stimuli [198]. Although the E3 ligase responsible for MLKL mono-ubiquitination remains to be identified, RAD18 is well-known to modify PCNA with mono-Ub at stalled replication forks in DNA damage tolerance [199,200]. The potential involvement of RAD18 in MLKL ubiquitination remains unclear.

In addition, MLKL has been found to be modified by K63-linked poly-Ub prior to membrane accumulation in a manner requiring RIPK3-induced phosphorylation. Four endogenously ubiquitinated MLKL lysine residues (K51, K77, K172 and K219) have been identified in necroptotic murine dermal fibroblasts (MDFs) with K219 boosting necroptosis. Human MLKL K230, that corresponds with murine MLKL residue K219, has been reported as ubiquitination site as well [201], whereas the MLKL K230Q mutation has also been identified in colon carcinoma patients [159]. MLKL$^{K219R/K219R}$ bone-marrow derived macrophages (BMDMs) and MDFs are resistant to necroptosis induced by various stimuli, while MLKL$^{K219R/K219R}$ mice are protected against necroptotic tissue injury induced by the combination of the IAP antagonist ASTX660 and the caspase inhibitor Emricasan [196].

Concluding remarks

The first glimpses of selective RIPK3 and MLKL ubiquitination already suggest intriguing and crucial regulatory roles for necroptosis execution. Multiple forms of degradative and non-degradative Ub modifications have been detected in RIPK3 and MLKL, with different effects on necroptosis. However, many fundamental questions remain unanswered. For example, why are so many different residues on RIPK3 and MLKL modified with so many different types of Ub modifications? Can multiple Ub modifications occur on the same RIPK3 or MLKL molecules or are there specific RIPK3 and MLKL sub-populations that are selectively modified? How is RIPK3 and MLKL ubiquitination regulated in time and cellular space? Which E3 ligases and DUBs are involved in modifying RIPK3 and MLKL with ubiquitination? What kind of signalling functions do non-degradative chains on RIPK3 and MLKL have and is there any interplay with other UbL systems? Do Ub chains attract UBD proteins and are these connected to existing or novel cellular functions of RIPK3 and MLKL, perhaps even unrelated to cell death? Unravelling necroptosis signalling in greater detail will likely answer these questions and provide a deeper understanding of selective ubiquitination in necroptosis and its role in human diseases.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

RK and SJLvW both wrote and edited the manuscript.

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