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Ubiquitin-mediated Regulation of Cell Death, Inflammation and Defense of Homeostasis

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Abstract

Cell death and inflammation are ancient processes of fundamental biological importance in both normal physiology and human disease pathologies. The recent observation that apoptosis regulatory components have dual roles in cell death and inflammation suggests that these proteins function, not primarily to kill, but to coordinate tissue repair and remodeling. This perspective unifies cell death components as positive regulators of tissue repair that replaces malfunctioning or damaged tissues and enhances the resilience of epithelia to insult. It is now recognized that cells that die by apoptosis do not do so silently, but release a variety of paracrine signals to communicate with their cellular environment to ensure tissue regeneration, and wound healing. Moreover, inflammatory signalling pathways, such as those emanating from the TNF-receptor or Toll-related receptors, take part in cell competition to eliminate developmentally aberrant clones. Ubiquitylation has emerged as crucial mediator of signal transduction in cell death and inflammation. Here we focus on recent advances on ubiquitin-mediated regulation of cell death and inflammation, and how this is used to regulate the defense of homeostasis.

Keywords

caspases; Ubiquitin; IAPs; RIPK1; homeostasis

Introduction

The remarkable capacity to correct for tissue stress and malfunction is one of the most fascinating hallmarks of multicellular organisms. While we marvel at the high level of tissue plasticity during animal development, we struggle with the incredible ability of cancer cells to escape growth inhibitory signals or develop resistance to treatments. For the maintenance of tissue homeostasis and tissue fitness it is essential that damaged or malfunctioning cells are detected, eliminated and replaced (Jacobson, Weil, & Raff, 1997). The decision as to whether a cell lives or dies relies not just on the regulation of the intrinsic apoptotic

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programme, but critically depends on paracrine interactions between damaged cells and cells of the surrounding tissue (Neves, Demaria, Campisi, & Jasper, 2015). It is now clear that cellular malfunction, and this includes activation of oncogenes or loss of tumour suppressor proteins, results in the production of cytokines, chemokines and mitogens, which stimulate an adaptive response for the defense of homeostasis (Medzhitov, 2008). Activation of the cell death programme is, thereby, critically important to eliminate and replace malfunctioning or damaged cells (Thompson, 1995).

Studies in flies show that tissue homeostasis in epithelia is governed by 'collective' decision mechanisms that determine cell death and proliferation across tissues (Vincent, Fletcher, & Baena-Lopez, 2013). These mechanisms include cell competition and apoptosis-induced compensatory proliferation (Fig. 1). Cell competition is a process in which fast-growing fitter cells (winners) kill neighboring slow growing, 'less fit' (losers) cells, even when the weaker cells are fully viable in a non-mosaic tissue. While cell competition and compensatory proliferation have been studied extensively in flies, recent studies reveal the existence and importance of similar processes in mammals (Bondar & Medzhitov, 2010; Oertel, Menthena, Dabeva, & Shafritz, 2006; Oliver, Saunders, Tarle, & Glaser, 2004). For example, cell competition selects the fittest stem cell in the epiblast that gives rise to the entire organism (Claveria, Giovinazzo, Sierra, & Torres, 2013), while in young individuals, cell competition is used to ensure tissue fitness (Amoyel & Bach, 2014). Due to chronic engagement of the tissue repair programme in older individuals, age-related decline of tissue homeostasis can occur, resulting in degeneration, metabolic dysfunction, and cancer (Martins et al., 2014; Neves et al., 2015). Consistently, loss of homeostasis is one of the hallmarks of aging (Chovatiya & Medzhitov, 2014).

Ubiquitin as mediator of signalling events

While superficially tissue stress, inflammation and cell death appear separate phenomena, recent advances in our understanding of these stress responses strongly indicate that the underlying signalling events are in fact linked. Ubiquitylation, thereby, has emerged as crucial mediator and regulator of signal transduction in cell death and inflammation (Dikic, Wakatsuki, & Walters, 2009) (Fig. 1).

The covalent attachment of ubiquitin (Ub) to target proteins can alter the protein's conformation or binding properties and thus influences protein activities, localization or stability. Ub is a small protein modifier that is covalently attached to proteins in a step-wise process that involves Ub activating enzymes (E1), Ub conjugating enzymes (E2) and Ub protein ligases (E3) (Hershko, Ciechanover, & Varshavsky, 2000). E3s confer substrate specificity by bringing Ub-loaded E2 to targets substrates and promoting the formation of an isopeptide linkage between the carboxyl-terminus of Ub (glycine (G)76) and the amino group of a lysine (K) residue of the substrate.

Ub can be conjugated either as a single moiety or as chains of variable length (Komander, 2009) (Fig. 1). Different linkage types provide further complexity, as Ub moieties can be conjugated to one another via each of the seven K residues within Ub, or via ubiquitin's N terminal methionine. This allows the formation of homotypic chains linked via ubiquitin's

K6, K11, K27, K29, K33, K48, K63 or M1 (Kirisako et al., 2006). In addition to homotypic chains that are sequentially linked through the same successive linkage type, mixed-linkage chain types also exist in which several distinct K residues are used to connect consecutive Ub moieties (Meyer & Rape, 2014). The complexity and versatility of Ub-dependent modifications are further increased through the generation of heterologous chain types where Ub is connected with other Ub-like modifiers, such as SUMO (Tatham et al., 2008).

The eight different types of homotypic Ub chains exert distinct effects on cellular processes (Bhoj & Chen, 2009). This is because the differently linked poly-Ub chains adopt distinct structures. For instance, K48-linked poly-Ub chains take up a kinked topology while K63- and M1-linked chains adopt an open configuration that resembles ‘beads-on-a-string’ (Komander, 2009). While it is well established that K48-linked modifications can promote degradation through recognition by the 26S proteasome, recent evidence indicate that other linkage types, such as M1, K29, K33 and K63, can regulate biological processes in a degradation-independent manner (Komander & Rape, 2012). Whether ubiquitylation targets proteins for degradation or mediates non-degradative signalling depends on protein-interactions between the ubiquitylated protein and Ub-binding proteins - often termed Ub "receptors" (Hoeller, Hecker, & Dikic, 2006). Ub-receptors (UBRs) carry small Ub-binding domains (UBDs) that bind to the Ub modification via low-affinity, non-covalent interactions. Currently more than 20 different types of UBDs are known that detect overlapping as well as distinct Ub modifications. UBRs that selectively recognize K48-linked poly-Ub chains, such as the proteasome subunit Rpn13 (Lundgren, Masson, Realini, & Young, 2003), recruit modified proteins to the proteasome for degradation. In contrast, UBRs that bind to mono-Ub, K63-linkages or linear (M1) Ub allow Ub-dependent association with signalling molecules (Hoeller et al., 2006). In particular, K63-linked Ub chains, and their respective UBRs, are critical for tumour necrosis factor (TNF)-mediated NFkB activation and cell survival (Stickle et al., 2004).

Ub as arbiter of life and death – TNF signalling as paradigm

The role of Ub as crucial mediator and regulator of signal transduction in the defense of homeostasis is best illustrated by the signalling pathways emanating from the TNF receptor (Fig. 2). TNF is a major inflammatory cytokine that was first identified for its ability to induce rapid hemorrhagic necrosis of experimental cancers (Carswell et al., 1975). Now it is clear that TNF functions as a master regulator of the cytokine network that coordinates defense of homeostasis via controlling inflammation, cell proliferation, differentiation, survival and death (Balkwill, 2009).

In mammals, binding of TNF to its extracellular receptor TNFR1 triggers either prosurvival/inflammatory or pro-death signaling pathways in a strictly Ub-dependent manner (Walczak, 2013) (Fig. 2). TNF can regulate tissue homeostasis in at least three different ways: through 1) activation of NF-kB-dependent and MAPK/JNK-dependent transcriptional programmes, 2) induction of caspase-8-dependent apoptosis or 3) stimulation of Receptor interacting protein kinase (RIPK)-mediated necrosis (necroptosis) (Declercq, Vanden Berghe, & Vandenabeele, 2009).

Binding of TNF to TNFR1 results in the assembly of a protein complex at the receptor's cytoplasmic tail. This complex, which is frequently referred to as complex-I (Micheau & Tschopp, 2003), consists of TNFR1, the adaptors TRADD, TRAF2, the kinase RIPK1 and the E3 Ub-ligases cellular Inhibitor of Apoptosis cIAP1 and cIAP2. Within this complex, RIPK1 is rapidly conjugated with poly-Ub chains by cIAPs. cIAP-mediated conjugation of Ub to RIPK1 allows subsequent recruitment of the Linear Ubiquitin chain Assembly Complex (LUBAC, composed of HOIL/HOIP/Sharpin), the kinase complexes TAK1/TAB2/TAB3, and IKK (composed of NEMO/IKKa/IKKb) (Silke, 2011). Ub-dependent recruitment of LUBAC, TAK1/TAB2/TAB3, and IKKs is mediated by UBDs present in TAB2, NEMO and HOIP. Once recruited, LUBAC then modifies NEMO and RIPK1 with M1-linked Ub chains, resulting in increased stability of the TNF signalling complex. Additionally, the binding of NEMO to M1-linked Ub chains causes a conformational change of the IKK complex that is thought to facilitate its activation (Rahighi et al., 2009). Complex-I then signals inflammation and cell survival through TAK1 and IkB kinase (IKK)-dependent activation of NFkB. cIAPs are also required for JNK signaling (Gardam et al., 2011; Matsuzawa et al., 2008). This has been most clearly demonstrated for signaling that emanates from CD40, a TNF-super family receptor, but similar concepts likely hold true for TNFR1 signaling too. Ultimately this drives expression of a transcriptional programme that restores homeostasis and lowers the apoptotic threshold through the induction of anti-apoptotic molecules (Fig. 2).

As its name suggest, TNF can also potently induce cell death. This is mediated by a RIPK1-based secondary complex that is frequently referred to as complex-II or necrosome (Pasparakis & Vandenabeele, 2015; L. Wang, Du, & Wang, 2008). This RIPK1-based complex can either kill via caspase-8 leading to apoptosis, or through RIPK3 and MLKL, which results in necroptosis. Under normal conditions, a small fraction of RIPK1 dissociates from complex-I within 30min to three hours, and together with TRADD associates with the adaptor protein FADD and procaspase-8 to form complex-II (Micheau & Tschopp, 2003) or necrosome (Pasparakis & Vandenabeele, 2015). Whether lethal levels of complex-II forms critically depends on the ubiquitylation status of RIPK1 in complex-I, RIPK1 deubiquitylation by deubiquitylating enzymes such as CYLD, or NF-kB-dependent expression of anti-apoptotic genes, such as cFLIP or cIAP2. The formation and activity of complex-II is tightly regulated by cIAPs, LUBAC, NEMO and TAK1. cIAPs, NEMO and TAK1 not only suppress formation complex-II via activation of NF-kB but also thwart transition of complex-I to complex-II through mechanisms that are NF-kB-independent. It is believed that the Ub chains conjugated to RIPK1 by cIAP1/2 and LUBAC in complex-I constitute the decisive factor preventing RIPK1 from forming complex-II, and limiting its killing potential. The ability of IAPs to ubiquitylate RIPK1 and suppress its killing potential is antagonist by the deubiquitylating enzyme CYLD, which can cleave M1, and K63-linked Ub chains on RIPK1 (Komander et al., 2008). It is currently thought that deubiquitylation of RIPK1 drives complex-II formation and favors RIPK1-dependent apoptosis. However, when the levels of RIPK3 and MLKL are sufficiently high and caspase-8 activity is reduced, blocked or absent, complex-II can recruit and activate RIPK3, which in turn can drive MLKL-dependent necroptosis (Pasparakis & Vandenabeele, 2015). Caspase-8 together with $cFLIP_L$ reportedly inhibits necroptosis through cleaving RIPK1 and RIPK3 (Feng et al.,

2007; Lin, Devin, Rodriguez, & Liu, 1999). In addition, caspase-8 can also cleave CYLD (O'Donnell et al., 2011), which removes Ub chains from RIPK1 and contributes to necroptosis *in vitro* and *in vivo*.

Ub-dependent regulation of TNF-induced cell death is evolutionary conserved, and is critically important for the maintenance of tissue fitness and the elimination of developmentally aberrant cells in *Drosophila* (Igaki & Miura, 2014). The *Drosophila* genome encodes a single member of the TNF family, named Eiger (Igaki et al., 2002; Moreno, Yan, & Basler, 2002) (Fig. 2). Eiger is required for inducing the death of cells mutant for apico-basal polarity genes, such as *scribbled* (*scrib*), *discs large* (*dlg*), and *lethal giant larvae* (*lgl*). In the absence of Eiger, such mutant clones grow aggressively and develop into neoplastic tumours. This suggests that in *Drosophila* Eiger functions as part of a surveillance programme that actively eliminates oncogenic polarity-deficient cells from the tissue (Igaki & Miura, 2014). Intriguingly, the Eiger-dependent elimination system is the result of cell competition that only operates when mutant cells are confronted with wild-type cells in a mosaic tissue. While *Drosophila* imaginal epithelium entirely mutant for *scribbled* or *discs large* results in tumorous overgrowth, such polarity-deficient oncogenic mutant cells, in an otherwise wild-type tissue, do not overgrow but are instead eliminated from the tissue by cell death.

Eiger-dependent cell elimination critically depends on Ub-dependent activation of TAK1 and JNK signalling, which in turn drives a cell death programme that relies on inputs from *Drosophila* initiator caspase DRONC and the metabolic state of a cell (Igaki & Miura, 2014). Eiger-mediated cell death requires the E3 ligase TRAF2 and the K63-selective E2-heteromeric complex Bendless (Ubc13)/dUev1a (Ma et al., 2014; Ma et al., 2013). In addition, the E3 ligase NOPO (no poles), which is the *Drosophila* orthologue of mammalian TRAF-interacting protein TRIP, also contributes to Eiger-induced cell death (Ma et al., 2012). Most likely, TRAF2 in conjunction with Bendless/dUev1a, promotes the conjugation of K63-linked Ub chains that in turn allows recruitment and activation of TAK1 via its Ub-receptor TAB2. Activation of TAK1 subsequently results in activation of Hemipterous (also known as JNKK or MKK7) and the *Drosophila* JNK orthologue Basket (Igaki & Miura, 2014). Through a process that is molecularly ill defined, Eiger-mediated activation of Basket results in the induction of cell death signalling.

Intriguingly, Eiger-mediated cell death is strictly dependent on deubiquitylation. In the absence of the deubiquitylating enzyme CYLD, Eiger-mediated cell death, and elimination of neoplastic tumours is blocked (Xue et al., 2007). Given that mammalian CYLD is an M1 and K63-selective deubiquitylating enzyme (Komander et al., 2008), and that the catalytic domains of *Drosophila* and mammalian CYLD are 53% identical, it is highly likely that the removal of non-degradative, K63-linked Ub chains is critical for the execution of cell death. The current literature suggests that CYLD promotes Eiger-induced cell death via the removal of degradative K48-poly-Ub chains on dTRAF2, thereby stabilizing TRAF2 and allowing efficient TRAF2-dependent activation of TAK1, Hep and JNK (Xue et al., 2007). Such a model is, however, inconsistent with the notion that TRAF2 and Bendless/Uev1a exclusively promotes the formation of K63-linked non-degradative Ub chains, and that

CYLD, at least in mammals, lacks affinity for K48 di-Ub chains and instead preferentially cleaves K63- and M1-Ub linkages (Komander, Clague, & Urbe, 2009).

Taken together, in both *Drosophila* and mammals, ligation of TNF and Eiger to their cognate receptors results in the transient formation of Ub-dependent signalling hubs that allow recruitment and activation of kinases via specialized adaptor molecules with Ub-binding domains. While assembly of these Ub-dependent signalling centers can mediate various cellular phenotypes ranging from cytokine production, proliferation, canalization, pain sensitization to host defense, TNF/Eiger-dependent cell death appears to be achieved through specific deubiquitylation events that tip the balance of TNF/Eiger-signalling in favor of death.

Ub-dependent regulation of RIPK1 and the ripoptosome

In mammals, RIPK1-dependent cell death not only occurs in response to TNF, but also operates downstream of many other cytokine receptors, damage associated molecular pattern receptors, pathogen associated molecular pattern receptors or in response to genotoxic stress (Pasparakis & Vandenabeele, 2015) (Fig. 2). This suggests that RIPK1 functions as a more generic stress sentinel. Under these conditions, RIPK1 assembles a protein complex containing the core components RIPK1, FADD, and caspase-8 (Feoktistova et al., 2011; Tenev et al., 2011a). While these are the same components as the ones of complex-II (Micheau & Tschopp, 2003; L. Wang et al., 2008), the fact that this complex forms independently of TNFR1 indicates that it cannot constitute complex-II, which, per definition, originates from complex-I (Micheau & Tschopp, 2003; L. Wang et al., 2008). To uncouple it from the TNF-dependent complex-II, TNFR1-independent assembly of the RIPK1/FADD/caspase-8 complex is, therefore, better referred to as ‘ripoptosome’ (Feoktistova et al., 2011; Tenev et al., 2011a). Although the core of this complex consists of RIPK1, FADD and caspase-8, the ripoptosome can also include additional proteins such as FLIP and RIPK3, depending on cell type and stimulus (Green, Oberst, Dillon, Weinlich, & Salvesen, 2011).

Proper regulation of RIPK1, and the ripoptosome, is critically important for normal development and physiology (Bonnet et al., 2011; Dannappel et al., 2014; Declercq et al., 2009; Dondelinger et al., 2013; Duprez et al., 2011; Ermolaeva et al., 2008; Green et al., 2011; O'Donnell et al., 2011; Oberst et al., 2011; Pasparakis & Vandenabeele, 2015; Polykratis et al., 2014; Vandenabeele, Galluzzi, Vanden Berghe, & Kroemer, 2010; Welz et al., 2011). Consistently, genetic deletion of RIPK1 in mouse models results in lethality due to systemic inflammation. Although cell death is frequently considered to be the result of inflammation, recent evidence suggest that cell death may in fact precede, trigger or amplify the inflammatory response. Indeed, neither apoptosis nor necroptosis are ‘silent’ processes. Although caspase-mediated apoptosis is widely thought to be immunologically inert or even tolerogenic, it is now clear that activation of caspases not only contributes to apoptosis but also leads to the generation of paracrine signals that ensure tissue homeostasis and coordinate tissue repair (Fuchs & Steller, 2011; Martin, Henry, & Cullen, 2012). Further, a complex relationship exists between caspase activation, apoptosis and necroptosis (Green et al., 2011). Accordingly, necroptosis typically occurs under circumstances in which caspase

activation is absent or blocked. Because necroptotic death results in the spillage of cytosolic components and alarmins, this form of death triggers secondary inflammation. The important physiological role of necroptosis was highlighted by a number of genetic studies showing that caspase-8 or FADD deficiency cause embryonic lethality and trigger inflammation *in vivo* by sensitizing cells to RIPK3-mediated necroptosis (Bonnet et al., 2011; Dannappel et al., 2014; Declercq et al., 2009; Dondelinger et al., 2013; Duprez et al., 2011; Ermolaeva et al., 2008; Green et al., 2011; O'Donnell et al., 2011; Oberst et al., 2011; Pasparakis & Vandenabeele, 2015; Polykratis et al., 2014; Vandenabeele et al., 2010; Welz et al., 2011). The activity of RIPK3 is subject to modulation by RIPK1. This is achieved through the RIP homotypic interaction motif (RHIM) present in both RIPK1 and RIPK3 that allows the formation of RIPK1:RIPK3 protein complexes. The recruitment of RIPK1 to RIPK3 not only allows activation of RIPK3, but also permits RIPK1-dependent negative regulation of RIPK3, most likely via recruitment of caspase-8/FLIP heterodimers, which cleave and inactivate RIPK3 (Feng et al., 2007).

Formation and activity of the ripoptosome is also subject to tight regulation by multiple members of the IAP protein family. Of the IAPs, cIAP1 and cIAP2 are the most critical regulators of ripoptosome assembly (Feoktistova et al., 2011; Geserick et al., 2009; Tenev et al., 2011a). Nevertheless, XIAP also contributes to the regulation of this RIPK1-based platform because in the absence of XIAP, depletion of cIAPs results in increased assembly of this complex (Tenev et al., 2011a). The extent to which individual IAPs contribute to the inhibition of ripoptosome assembly most likely depends upon cell type and stimulus. IAP-mediated inactivation of RIPK1 and/or ripoptosome occurs in an Ub-dependent manner, most likely by targeting RIPK1, and other components of the ripoptosome, for proteasomal degradation. Caspase-8-mediated cleavage of cFLIP generates cFLIP(p43), permitting its binding to TRAF2 and the formation of cFLIP(p43)-caspase-8-TRAF2 tertiary complex (Kataoka & Tschopp, 2004; Micheau et al., 2002). TRAF2 then recruits cIAPs which target cFLIP(p43) and caspase-8 for ubiquitylation (Tenev et al., 2011b). This indicates that cIAP1 and cIAP2 target ‘active’ cFLIP-caspase-8 complexes for ubiquitylation and inactivation. The importance of IAP-mediated regulation of RIPK1-based death complexes is illustrated by the notion that *Xiap*$^{-/-}$*Ciap1*$^{-/-}$ and *Ciap1*$^{-/-}$ *Ciap2*$^{-/-}$ animals are embryonic lethal, and that this lethality is rescued by crossing the *Xiap*$^{-/-}$*Ciap1*$^{-/-}$ and *Ciap1*$^{-/-}$*Ciap2*$^{-/-}$ mice to *Ripk1*$^{-/-}$ and *Ripk3*$^{-/-}$ mice (Moulin et al., 2012). This demonstrates that multiple IAPs function together to regulate an embryonic decision point involving RIP kinase activity.

IAP-mediated regulation of caspases

In both *Drosophila* and mammals, members of the IAP protein family are the most prominent E3 ligases that modulate caspases and apoptosis. Apoptosis regulatory IAPs carry either two or three NH_2-terminal BIR domains and a C-terminal RING finger that provides them with Ub E3-ligase activity (Silke & Meier, 2013). The BIR domain mediates protein interactions, and in most cases, binds to IAP-binding motifs (IBM) present in active caspases and IAP-antagonists such as mammalian Smac/DIABlO and Omi/HtrA2 or *Drosophila* REAPER, GRIM and Head Involution Defective (HID) (Shi, 2002a). The main feature of an IBM is the presence of an NH_2-terminal alanine (Fig. 3). However, in some cases IBMs can also harbor a serine at the first position (Verhagen et al., 2007). The NH_2-

terminal alanine or serine, which must be exposed and unblocked (devoid of NH_2-terminal acetylation), inserts into the extensive hydrophobic cleft on the surface of BIRs and forms hydrogen bonds with neighboring residues, thereby anchoring the IBM-carrying protein to IAPs (Wu et al., 2000). Subtle changes in the peptide-binding groove of BIR domains alter their preference for particular client proteins with IBMs. Therefore, proteins with IBMs display differential and selective binding to specific BIR domains. Apoptosis regulatory IAPs such as XIAP, cIAP1, cIAP2 and *Drosophila* IAP1 (DIAP1) and DIAP2 carry two BIR domains capable of binding IBMs. The tandem arrangement (i) increases the repertoire of proteins with which they can interact, and (ii) potentially enhances the binding-affinity to particular IBM-containing target proteins, particularly when they are dimeric or oligomeric in nature.

In *Drosophila*, DIAP1, encoded by the *thread* (*th*) gene, was the first BIR-containing IAP identified. DIAP1-mediated inhibition of caspases is essential for cell survival as loss of DIAP1 function instigates spontaneous caspase-mediated cell death (Goyal, McCall, Agapite, Hartwieg, & Steller, 2000; Lisi, Mazzon, & White, 2000; S. L. Wang, Hawkins, Yoo, Muller, & Hay, 1999). Conversely, gain-of-function mutations significantly suppress cell death, and lead to over-growth phenotypes due to supernumerary cells (Goyal et al., 2000; Lisi et al., 2000; S. L. Wang et al., 1999).

The BIR domain, in combination with its flanking regions, functions as a protein interaction module that, for DIAP1, mediates binding to both initiator (DRONC) and effector caspases (drICE and DCP-1) (Fig. 3). Importantly, different caspases bind to distinct BIRs: while the BIR1 region of DIAP1 is essential for binding to the effector caspases drICE and DCP-1 (Hawkins, Wang, & Hay, 1999; Kaiser, Vucic, & Miller, 1998; Zachariou et al., 2003), the BIR2 region directly associates with the initiator caspase DRONC (Meier, Silke, Leevers, & Evan, 2000). As a consequence of this differential binding, one molecule of DIAP1 can bind simultaneously to DRONC and drICE or DCP-1 (Zachariou et al., 2003). Physical association between DIAP1 and effector caspases is essential for cell survival. Embryos homozygous for *diap1* loss-of-function mutations that completely abrogate binding to effector or initiator caspases, die during embryogenesis due to inappropriate cell death (Goyal et al., 2000; Lisi et al., 2000; Rodriguez, Chen, Oliver, & Abrams, 2002; S. L. Wang et al., 1999; Zachariou et al., 2003). On the other hand, mutations that enhance DIAP1's ability to associate with activated effector caspases result in a gain-of-function phenotype (Goyal et al., 2000; Zachariou et al., 2003). Therefore, DIAP1:caspase association represents a pivotal step in the regulation of the apoptotic caspase cascade.

The mechanism of caspase-binding differs greatly depending on the caspase involved (Fig. 3). While zymogenic DRONC readily binds to the BIR2 region of DIAP1, DIAP1 only associates with proteolytically cleavage forms of drICE/DCP-1. As proteolytic cleavage and removal of the pro-domain of drICE or DCP-1 results in activation of thee effector caspases, this indicates that DIAP1 only regulates active versions of drICE and DCP-1. The mechanism behind this selectivity resides in the exposure of an IBM at the neo-NH2 terminus of drICE and DCP-1, which is uncovered following cleavage and activation of these effector caspases (Tenev, Zachariou, Wilson, Ditzel, & Meier, 2005). Although IAP:caspase association is the decisive step in the regulation of apoptosis in *Drosophila*,

physical interaction between DIAP1 and caspases alone is insufficient to regulate caspases. This is evident because DIAP1-bound effector caspases remain catalytically active under *in vitro* conditions (Tenev et al., 2005). Moreover, DIAP1 mutants with a dysfunctional RING finger completely fail to suppress caspase-mediated cell death, even though these proteins bind to caspases with the same affinity as their wild-type counterparts. Ultimately, suppression of caspases and apoptosis results from DIAP1-mediated ubiquitylation of the zymogenic form of DRONC and active drICE or DCP-1 (Chai et al., 2003; Ditzel et al., 2008; Lisi et al., 2000; Wilson et al., 2002).

The mechanism by which ubiquitylation of DRONC causes its inactivation appears to be context-dependent, involving degradative as well as non-degradative ubiquitylation. Outside of the apoptosome, DIAP1-mediated ubiquitylation of DRONC neutralizes it through an unknown mechanism that operates independent of the proteasome (Lee et al., 2011) (Fig. 3). However, when part of the apoptosome, DIAP1 conjugates K48-linked poly-Ub chains to DRONC, targeting it for proteasomal destruction (Shapiro, Hsu, Jung, Robbins, & Ryoo, 2008). Hence, only apoptosome-associated DRONC (as well as DARK itself), but not free monomeric DRONC, is targeted for proteasomal degradation. Interestingly, DRONC-mediated cleavage of DARK is required for proteasomal degradation of the DRONC/DARK complex. This suggests that the cleavage event recruits the E3 ligase (Shapiro et al., 2008).

DIAP1-mediated regulation of effector caspases is also dependent on the conjugation of Ub. Attachment of non-degradative (K63-linked) poly-Ub chains to the effector caspase drICE (homologue of caspase-3/-7) directly reduces its proteolytic potency, affecting kinetic parameters of the enzyme (Ditzel et al., 2008). Computational modelling of an ubiquitylated effector caspase suggests that the Ub chains sterically occlude the catalytic pocket of the caspase and would interfere with substrate entry.

In addition to Ub, DIAP1 can also inactivate effector caspases via the covalent attachment of the Ub-like modifier NEDD8. NEDD8-mediated suppression of drICE occurs via a mechanism that relies on non-competitive inhibition, most likely through a NEDD8-induced conformational change of the caspase. Disruption of drICE ubiquitylation or NEDDylation, either by loss of DIAP1's E3 activity or generation of a non-modifyable form of drICE, renders this effector caspase resistant to DIAP1-mediated inactivation (Broemer et al., 2010; Ditzel et al., 2008).

Surprisingly, DIAP1 in its full-length form is incapable of binding and regulating caspases, or acting as an E3 ligase. To activate its anti-apoptotic potential, it requires proteolytic cleavage. Removal of the first 20 amino acid residues of DIAP1 radically changes its properties: the cleaved form interacts with caspases far better than full-length, non-cleaved DIAP1. It seems that full-length DIAP1 resides in an inactive, ‘closed’ configuration that precludes caspase binding (Yan, Wu, Chai, Li, & Shi, 2004). Only when it is cleaved can it bind tightly to DRONC or effector caspases, and function as an E3 (Ditzel et al., 2008). In this respect, caspases activate their own inhibition in a regulatory feedback loop.

Cleavage of DIAP1 not only removes the presumptive inhibitory NH_2-terminal portion of the protein, it also exposes a new docking site for UBR-containing E3 ligases of the NH_2-

end rule pathway (Ditzel et al., 2003; Herman-Bachinsky, Ryoo, Ciechanover, & Gonen, 2007) (Fig. 3). DIAP1's two ubiquitylation-associated activities - UBR-E3 recruitment and DIAP1's own RING finger – are both required for its full anti-apoptotic activity. The presence of a functional RING alone seems not to be sufficient, since a DIAP1 mutant that retains a functional RING but fails to bind UBRs also fails to protect from cell death induced by ectopic expression of proapoptotic proteins. Likewise, DIAP1 that has a defective RING but is otherwise fully competent in recruiting UBR-E3s also fails to regulate apoptosis properly. Among the different UBRs, UBR3 appears to be particularly important for the regulation of caspases (Q. Huang et al., 2014), as RNAi-mediated knockdown of *ubr3* leads to caspase activation and cell death. Intriguingly, in the absence of *ubr3*, over-expression of DIAP1 fails to suppress caspases; corroborating the notion that DIAP1 relies on recruitment of the NH_2-end rule system to suppress caspase activity. Therefore, UBR-recruitment and DIAP1's RING are both required for DIAP1's full anti-apoptotic activity (Ditzel et al., 2008; Ditzel et al., 2003; Muro, Means, & Clem, 2005).

drICE, but not DRONC or DCP-1, is also regulated by the second *Drosophila* IAP, DIAP2. Intriguingly, DIAP2 functions as a mechanism-based regulator of drICE, acting as a pseudo-substrate, which, following cleavage, traps the active caspase via a covalent linkage between DIAP2 and the catalytic machinery of drICE (Ribeiro et al., 2007) (Fig. 3). Despite direct inhibition of drICE's catalytic cysteine, DIAP2's E3 ligase activity also contributes to proper drICE inhibition (Ribeiro et al., 2007). Despite the ability of DIAP2 to regulate drICE, this function seems not to be essential for cell viability as *diap2* mutant animals are fertile and fully viable. A cell death phenotype in *diap2*-mutant animals is only revealed when flies are subjected to ionizing radiation (Ribeiro et al., 2007).

While DIAP2-mediated regulation of caspases is less important for the regulation of cell death, recent evidence indicate that the ability of DIAP2 to regulate caspases is essential for proper regulation of the *Drosophila* innate immune response to infection with Gram-negative bacteria. DIAP2-mediated conjugation of K63-linked Ub chains on DREDD, the *Drosophila* orthologue of caspase-8, thereby allows Ub-mediated aggregation and activation of DREDD (Meinander et al., 2012) (Fig. 4). Active DREDD subsequently cleaves IMD (*immune deficiency*) (Paquette et al., 2010). Upon cleavage, IMD exposes an IBM at its neo-NH_2 terminus, which binds to the BIR2/3 of DIAP2. This provides DIAP2 with an additional docking site, reinforcing complex stability and allowing DIAP2-mediated ubiquitylation of IMD, and quite possibly other components of the signaling complex (Paquette et al., 2010). The Ub chains on IMD and DREDD appear to serve as scaffolds for the recruitment of dTAK1, IKK and the precursor form of the NF-kB transcription factor RELISH (Ferrandon, Imler, Hetru, & Hoffmann, 2007; Kanayama et al., 2004; Kleino et al., 2005; Lu, Wu, & Anderson, 2001; Rutschmann et al., 2000; Rutschmann, Kilinc, & Ferrandon, 2002; Silverman et al., 2003; Silverman et al., 2000; Vidal et al., 2001; Zhuang et al., 2006). This brings RELISH into close proximity of ubiquitylated and active DREDD, allowing DREDD-mediated proteolysis of Relish. The proximity to the signaling complex also allows phospho-mediated activation of RELISH (Erturk-Hasdemir et al., 2009). Subsequently, cleaved and phosphoryated RELISH translocates to the nucleus where it drives expression of anti-microbial peptide genes.

While ubiquitylation of DREDD and IMD are essential for activation of RELISH, several deubiquitylating enzymes have been identified that negatively regulate the ability of IMD and DREDD to drive induction of RELISH-dependent target genes (Engel et al., 2014; Thevenon et al., 2009). Among these are USP36, USP2 and USP34, which prevent inappropriate activation of IMD-dependent immune signalling in unchallenged conditions.

Like DIAP2, mammalian XIAP similarly functions both as mechanism-based caspase inhibitor and potent regulator of Ub-dependent activation of NF-kB (Fig. 5). XIAP-mediated inactivation of caspase-3, -7 and -9 does not require a functional RING finger under *in vitro* conditions or when over-expressed (Shi, 2002b). Residues located immediately upstream of XIAP's BIR2 domain directly bind to the active site pocket of caspase-3 and –7 and obstruct substrate entry (Chai et al., 2001; Y. Huang et al., 2001; Riedl et al., 2001; Silke et al., 2001; Suzuki, Nakabayashi, Nakata, Reed, & Takahashi, 2001). XIAP-mediated inactivation of caspase-9 occurs differently, namely by keeping caspase-9 in a monomeric, inactive state (Shiozaki et al., 2003).

Even though the RING domain of XIAP is not required for caspase inhibition *in vitro*, it contributes to XIAP's function *in vivo*. A recent report now finds that XIAP's RING indeed is critical for its anti-apoptotic function (Schile, Garcia-Fernandez, & Steller, 2008). Using gene targeting of endogenous XIAP, the authors show that deletion of the RING finger sensitized fibroblasts to TNFα-induced cell death and led to increased rates of apoptosis in an Eμ-Myc mouse lymphoma model. In both these systems, XIAPΔ^{RING} mutant cells responded in the same way as XIAP null cells, even though XIAPΔ^{RING} protein was abundantly expressed. Moreover, following apoptosis induction caspase activity was significantly higher in XIAPΔ^{RING} cells compared to wild-type controls. This indicates that the BIR domains are not sufficient on their own to block caspase activity *in vivo*. Consistent with a requirement of the RING finger, caspase-3 poly-Ubiquitylation was reduced in mutant cells. Remarkably, no increased levels of caspase-3 were observed, suggesting a non-degradative mode of caspase ubiquitylation, a finding that contradicts an earlier study (Suzuki, Nakabayashi, & Takahashi, 2001). In addition, XIAP reportedly also ubiquitylates caspase-9 (Morizane, Honda, Fukami, & Yasuda, 2005), though the functional outcome of this yet to be determined.

As indicated above, XIAP not only regulates cellular processes by controlling caspases, but also by mediating activation of NF-kB. In particular, XIAP is indispensable for innate immune signalling triggered by NOD1 and NOD2 (Bauler, Duckett, & O'Riordan, 2008; Bertrand et al., 2009; Krieg et al., 2009). NODs belong to the nucleotide-binding oligomerisation domain family that contain an NH_2-terminal caspase recruitment domain (CARD), a centrally located nucleotide-binding and oligomerisation domain (NBD), and 10 tandem leucine-rich repeats (LRRs) in their C-terminus through which they detect bacterial peptidoglycans shed during bacterial growth (Chen, Shaw, Kim, & Nunez, 2009).

The pathways activated by NODs are highly reminiscent to the Ub-dependent signal transduction cascade downstream of TNF-R1 (Fig. 5). Like TNF-R1, NODs stimulate Ub-mediated activation of NF-kB and the MAP kinases p38 and JNK. Stimulation of NOD1 and NOD2 receptors triggers the formation of a NOD-dependent multi-protein complex that

recruits RIPK2, a close homologue of RIPK1 (Inohara et al., 2000). XIAP in conjunction with LUBAC then conjugate RIPK2 with M1- and K63-linked Ub chains (Bertrand et al., 2009; Hasegawa et al., 2008; Yang et al., 2007), which subsequently result in TAK1/TAB2/3-mediated activation of MAPK and NF-kB pathways leading to production of cytokines, chemokines and antimicrobial peptides that help to defend against invading microbes and ensures the defense of homeostasis (Bertrand et al., 2009; Hasegawa et al., 2008; Park et al., 2007; Yang et al., 2007).

Although XIAP is involved in regulating NOD signalling, XIAP deficiency manifests in a wide range of clinical immune phenotypes, including EBV-associated hemophagocytic lymphohistiocytosis, Crohn-like bowel disease, severe infectious mononucleosis, splenomegaly, uveitis, periodic fever, fistulating skin abscesses, and severe Giardia enteritis (Speckmann et al., 2013). These clinical phenotypes are not shared by mutations in other components of the NOD-dependent multi-protein complex, and thus suggest other immune-related roles of XIAP.

IAPs other than cIAPs and XIAP also play important roles in regulating apoptosis as well as non-apoptotic signalling events in an Ub-dependent fashion. BRUCE/Apollon is a membrane-associated IAP that carries only one BIR domain. Additionally, it also contains an Ub-conjugating (UBC) motif that can function as an Ub-E2, transferring Ub to substrates. In addition to contributing to cytokinesis, BRUCE/Apollon also safe guards cell viability by targeting caspase-9, and the IAP-antagonist protein SMAC/Diablo, for Ub-mediated proteasomal degradation (Bartke, Pohl, Pyrowolakis, & Jentsch, 2004; Hao et al., 2004). In *Drosophila,* the activity of dBRUCE is indispensable for controlled activation of caspases required for spermatide individualization (Arama, Agapite, & Steller, 2003). Further, dBRUCE also targets the IAP-antogonist Reaper and Grim for proteasomal degradation, thereby contributing to the apoptotic threshold (Domingues & Ryoo, 2012; Vernooy et al., 2002).

Although IAPs are the most prominent class of E3 ligases that target caspases for ubiquitylation, IAPs are not the only E3s that control caspases. Components of a testis specific Cullin-3 based E3 Ub-ligase complex is particularly important for caspase activation during sperm differentiation (Arama, Bader, Rieckhof, & Steller, 2007; Bader, Arama, & Steller, 2010; Kaplan, Gibbs-Bar, Kalifa, Feinstein-Rotkopf, & Arama, 2010). Defects in any of the Cullin-3 components decreases caspase activity and causes male sterility due to individualization defects. Similarly caspases are activated during mammalian spermatogenesis and disrupting the cell death machinery results in infertile males due to defects during late spermatid maturation. In *Drosophila*, spermatid individualization is a process in which 64 interconnected spermatids separate from one another and eliminate the majority of their cytoplasmic contents (Feinstein-Rotkopf & Arama, 2009). This process requires the activity of Cullin-3, the substrate binding BTB protein KLHL10 and the pseudosubstrate inhibitor Soti (Arama et al., 2007; Bader et al., 2010; Kaplan et al., 2010). Cullin-3/KLHL10 allows sub lethal activation of caspases by targeting the caspase inhibitor dBRUCE for ubiquitylation and proteasomal degradation. Interestingly, it was recently reported that mutations in the human form of KLHL10 are associated with male infertility

and low sperm count (Yatsenko et al., 2006), indicating that ubiquitylation may be used in a similar fashion for cell sculpting in mammals.

Concluding remarks

The maintenance of homeostasis in multicellular organisms depends on a continuous, coordinated response to external and internal insults that challenge cellular and tissue integrity throughout life. Loss of homeostasis is a hallmark of aging, resulting in pathologies often caused by defective or deregulated tissue damage responses. Inflammation and cell death are essential defense responses that are induced by infection or injury. However, inflammation and cell death are also induced by tissue stress and malfunction to maintain tissue homeostasis under a variety of noxious conditions. A number of dedicated sensors have evolved to detect different stressors and induce appropriate adaptive responses. Common to these pathways is the conjugation of non-degradative Ub chains that produce robust signalling networks, which coordinate tissue remodeling and adaptation to tissue stress via transcriptional programmes, the induction of apoptosis or necroptosis. Most, if not all, cellular stress responses, in addition to cell–autonomous adaptive changes, produce secreted factors that affect other cells in the tissue. This coordinates tissue remodeling that replaces malfunctioning or damaged tissues (Grivennikov, Greten, & Karin, 2010).

Whatever the cause of the inflammatory response, its ‘purpose’ is to remove the source of the disturbance, to allow the host to adapt to the abnormal conditions, and, ultimately, to restore functionality and homeostasis to the tissue. Much more needs to be learnt about how the Ub-signalling system impacts on inflammation and adaptation to tissue stress. Further, unraveling how the Ub-signal is conjugated, recognized and disassembled will be critically important to gain a better understanding of the regulatory processes that control cell death, inflammation and stress responses, and might contribute to disease pathologies such as cancer.

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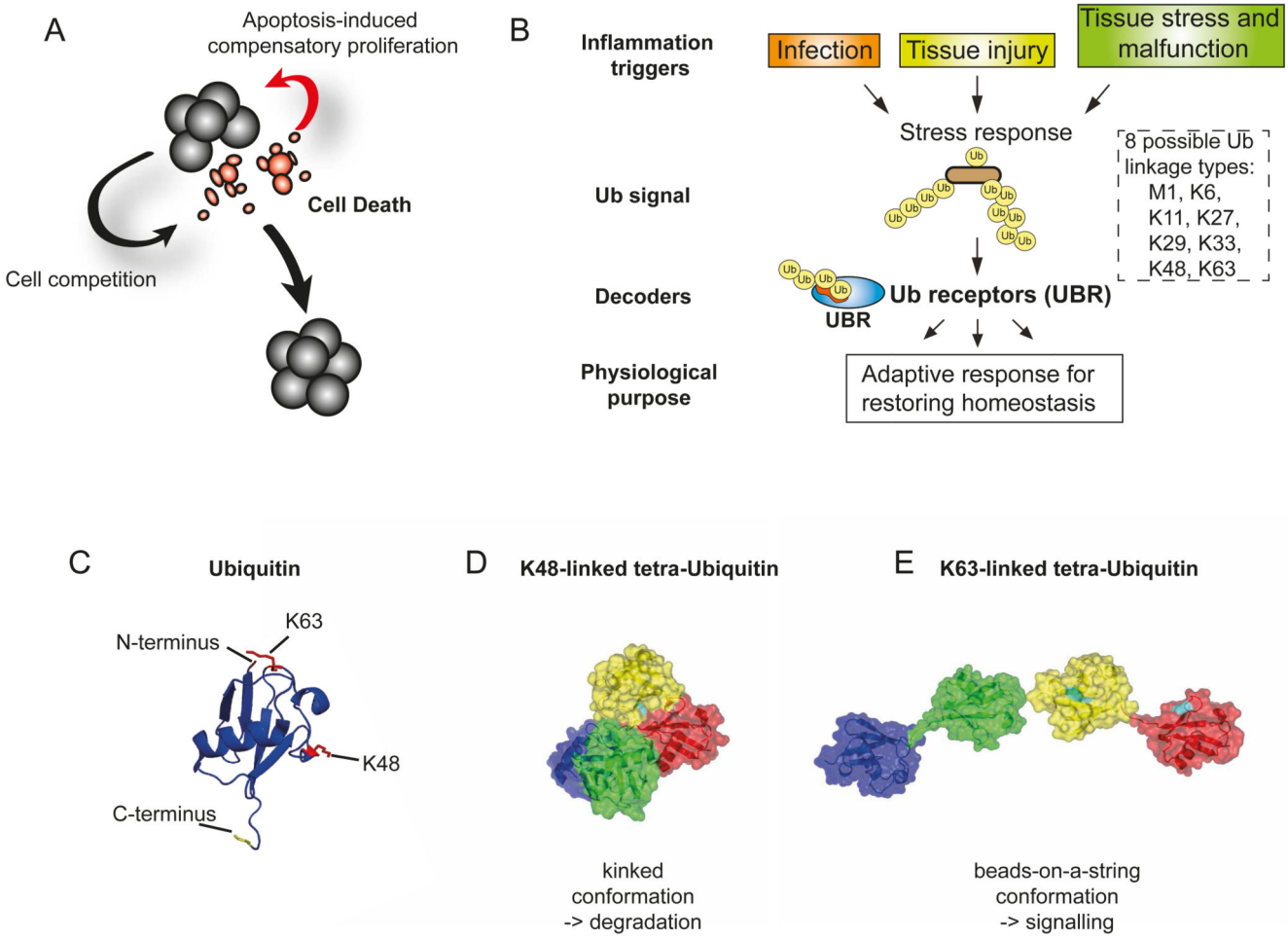

Figure 1. Ubiquitylation as mediator and regulator of signal transduction in cell death, inflammation, and defense of homeostasis.

(A) Tissue malfunction results in a secretery programme and inflammatory response whose purpose it is to restore homeostasis. Tissue homeostasis is regulated by a collective decision mechanism that influences cell death and proliferation across tissues. These include cell competition and apoptosis-induced compensatory proliferation. **(B)** Tissue stress response and inflammation underlie a common principle in which the conjugation of typical Ub-chains produces robust networks that are decoded by Ub-receptors whose actions serve to coordinate adaptation to tissue stress. **(C)** Ribbon Structure of Ub. Lysines at position 48 and 63 of Ub are highlighted. **(D, E)**Topology of K48- (C) and K63-linked (D) tetra Ub chains.

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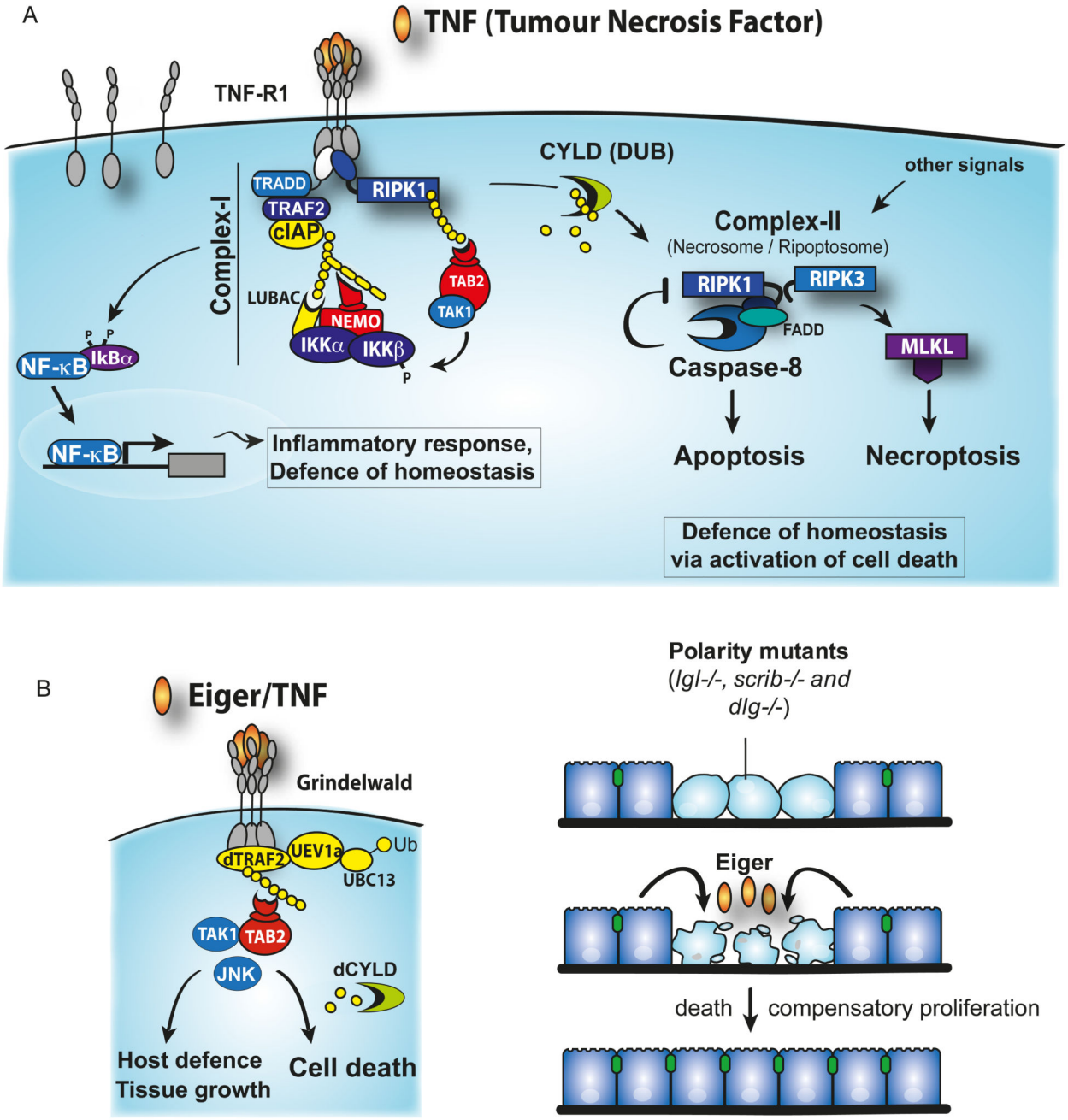

Figure 2. Evolutionary conservation of TNF-induced cell death and the defense of homeostasis.

(A) TNF signaling and the transition of complex-I to complex-II. Upon TNF-binding, cIAPs are recruited to the TNF-R1 signaling complex (Complex-I) via TRADD/TRAF2. cIAPs ubiquitylate several molecules within the complex. RIPK1 ubiquitylation is the most readily observed. Ubiquitylation of components of complex-I, such as RIPK1, drives the recruitment of HOIL-1/HOIP/Sharpin that together form the Linear Ubiquitin Assembly Complex (LUBAC). LUBAC generates linear Ub chains on NEMO and RIPK1 that in turn recruits more NEMO molecules via its linear Ub binding UBAN domain. NEMO is

probably constitutively associated with IKKa/IKKb and IKKb is phosphorylated and activated by TAK1 that is independently recruited to ubiquitylated complex-I via its Ub receptors TAB2 and TAB3 that bind only to K63-linked Ub chains. Phosphorylated and activated IKKb in turn phosphorylates IκBα, which leads to recruitment of a HECT E3 ligase. This E3 ligase promotes K48-linked ubiquitylation and proteasomal degradation of IκBα, allowing translocation of NFkB subunits p50/p65 to drive production of cytokines. p50/p65 also promote expression of *IκBα*, to cause feedback inhibition, as well as genes such as *cFLIP* that are required to protect cells from complex-II-induced cell death. The numbered arrows provide a tentative indication of temporal sequence. Complex-II is most likely generated from complex-I, in an as yet undefined manner, and comprises RIPK1, FADD and caspase-8. Deubiquitylation by CYLD is thereby a decisive step in the transition of complex-I to complex-II. Caspase-8 limits Complex-II formation by cleaving and inactivating RIPK1. Consequently, loss of IAPs, LUBAC or caspase-8 activity results in formation of Complex-II that is able to drive necroptosis. Formation of complex-II, Necrosome or ripoptosome can also occur following stimulation of Pattern Recognition receptors or genotoxic stress. **(B)** Eiger-mediated signalling that regulates a variety of cellular and tissue processes, including the elimination of polarity mutant cells. Eiger mediates its effect through binding to its cognate receptor Grindelwald. This results in activation of JNK in a DTRAF2/Bendless/dUev1A dependent manner. The *Drosophila* homologue of TAB2/3 (dTAB2) links TAK1 to the presumptive Ub chains conjugated by DTRAF2. dCYLD influences the decision as to whether JNK drives cell death or non-cell death processes (see text for further details).

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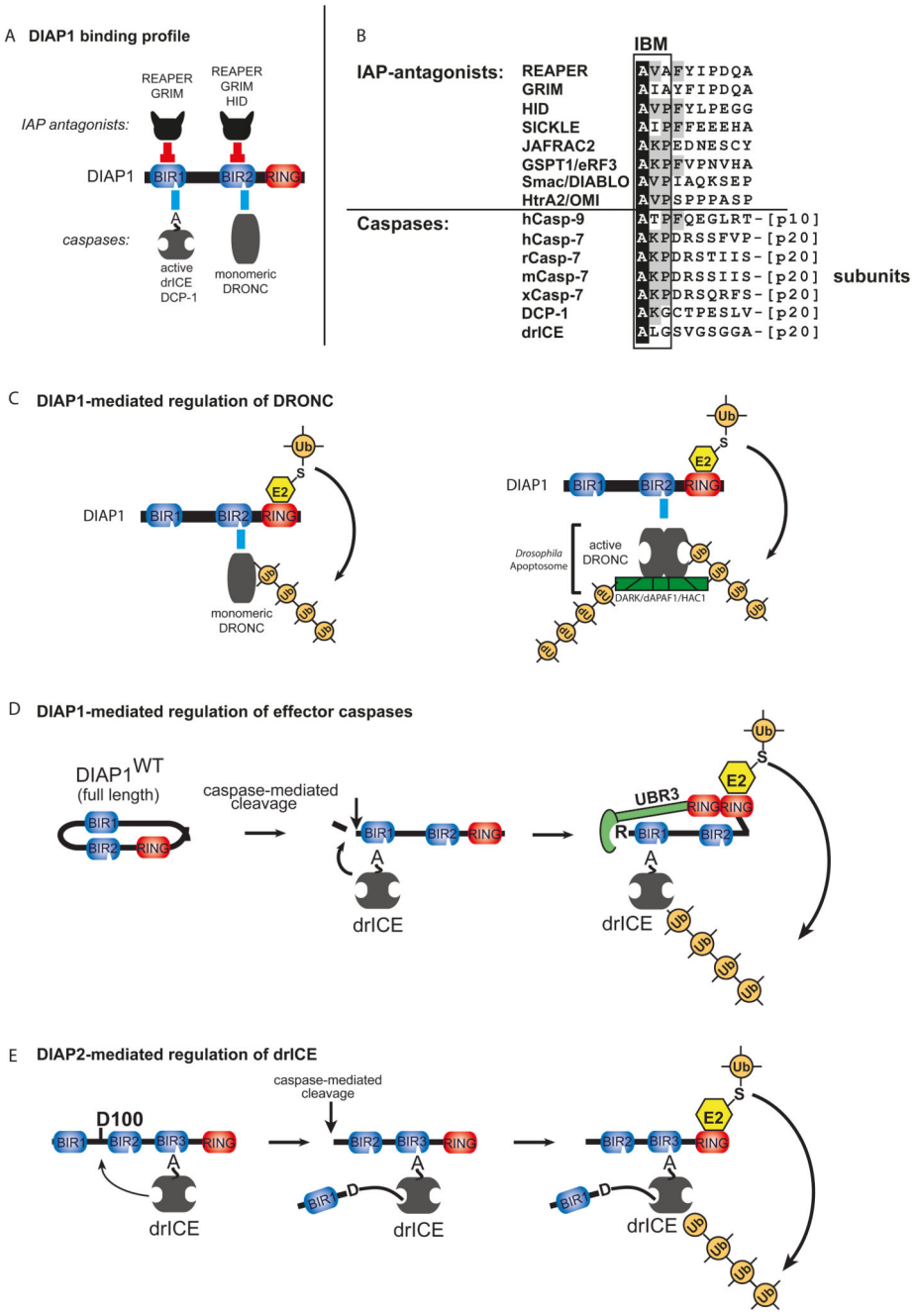

Figure 3. IAP-mediated regulation of caspases in *Drosophila*.

(A) Binding profile of DIAP1 with caspases and IAP antagonists. Direct physical interaction with the effector caspases drICE or DCP-1 and the initiator caspase DRONC is mediated through DIAP1's BIR1 and BIR2 domains, respectively. Following their activation, drICE and DCP-1 expose an NH_2-terminal IBM (depicted as A), which allows their binding to BIR1. **(B)** Sequence alignment of IBM-bearing proteins. Identical residues are highlighted in black. Residues conserved in four or more IBM-proteins are indicated in grey. **(C)** DIAP1's BIR2-DRONC association is essential for DIAP1 to neutralise DRONC. Following binding,

DIAP1's RING-finger promotes Ub conjugation of DRONC, leading to its inactivation through non-degradative ubiquitylation of monomeric DRONC (left panel), and by targeting apoptosome-associated active DRONC for degradation (right panel). **(D-E)** Mechanism of effector caspase (drICE) inactivation by DIAP1 (D) and DIAP2 (E). **(D)** Full-length wild-type DIAP1 is held in an inactive conformation, and requires caspase-mediated proteolytic cleavage at residue 20 for its activation. After cleavage, BIR-mediated caspase-binding occurs more efficiently. Cleavage also facilitates recruitment of N-end rule UBR E3 ligases, which together with DIAP1's RING domain, promote ubiquitylation and inactivation of drICE and DCP-1. **(E)** drICE is also subject to regulation by DIAP2. drICE binds to the BIR3 of DIAP2 in an IBM-dependent manner, and following binding cleaves DIAP2 at D100. DIAP2 cleavage results in a covalent adduct between D100 and the catalytic machinery of drICE, trapping the caspase. Full inactivation of drICE is achieved through RING-mediated ubiquitylation.

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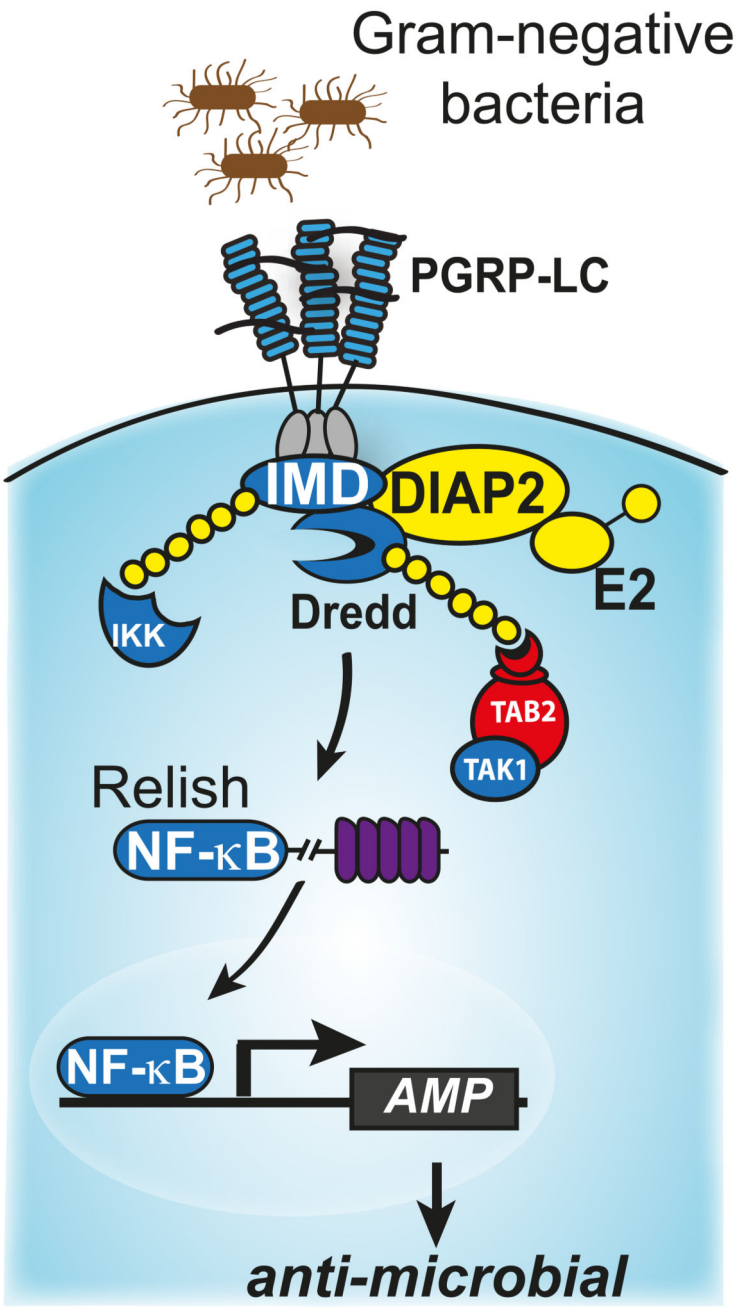

Figure 4. Model depicting Ub-dependent activation of NF-κB during *Imd* signalling.

Ligand binding induces recruitment of Imd and dFADD to PGRP-LC. This allows binding of DREDD to dFADD, which leads to dimerisation-induced activation of DREDD. Active DREDD subsequently cleaves IMD, which leads to the exposure of an IBM at the neo-amino terminus of cleaved IMD. This in turn allows recruitment of DIAP2 into the signalling complex, followed by DIAP2-mediated ubiquitylation of various components of this complex, such as IMD and DREDD. Ubiquitylation of DREDD is key for DREDD-mediated cleavage of RELISH. The Ub chains of DREDD may serve as a scaffold for the

recruitment of IKK. Since IKK binds to RELISH, as evidenced by its ability to phosphorylate RELISH, IKK might bring RELISH into close proximity of DREDD for proteolysis. Ubiquitylation of components of the IMD pathway also leads to activation of TAK1 and IKK, which in turn phosphorylates RELISH. **(6.)** Phosphorylated and cleaved RELISH subsequently translocates to the nucleus where it drives expression of RELISH target genes.

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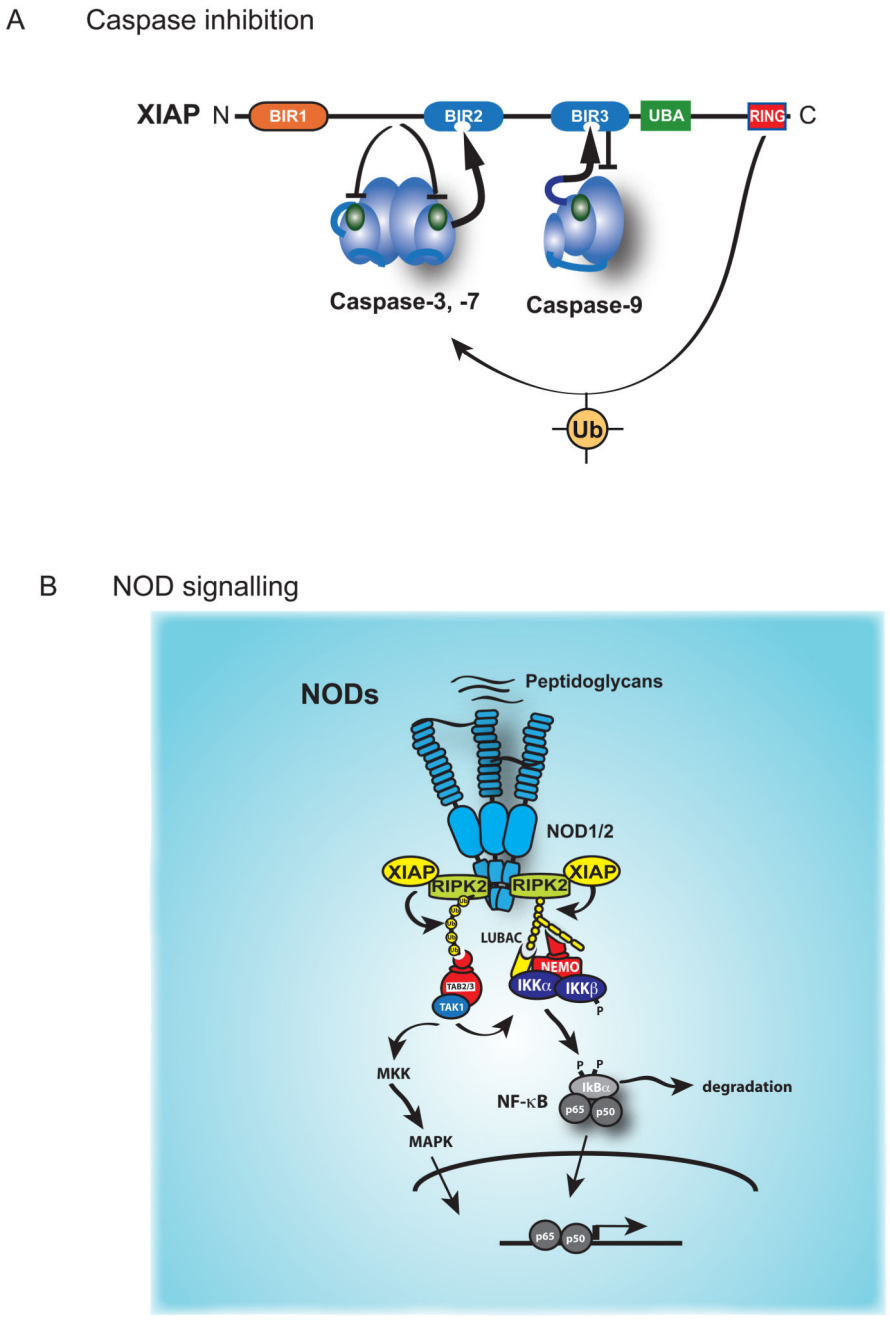

Figure 5. XIAP-mediated regulation of caspases and NF-κB.

(A) XIAP directly inhibits the effector caspase-3 and caspase-7, and the initiator caspase-9. The sequence preceding the BIR2 domain of XIAP occupies the catalytic pocket of caspase-3 or caspase-7, thereby blocking substrate entry. In addition, the BIR2 domain interacts with the IBM of caspase-3 or caspase-7 that is exposed following their proteolytic activation (shown as an arrow). XIAP-mediated inhibition of caspase-9 requires proteolytic cleavage of caspase-9, which exposes an IBM that binds to the BIR3 of XIAP. Caspase-9 activity is blocked because XIAP prevents caspase 9 dimerization, a prerequisite for initiator

caspasenactivity. The RING domain of XIAP also contributes to caspase inhibition. (**B**) NOD-mediated activation of NF-kB and MAPK signalling. Detection of bacterial peptidoglycans by NOD1 and NOD2 results in the formation of an oligomeric signalling complex that recruits RIPK2, and XIAP. XIAP mediated ubiquitylation of RIPK2 allows the recruitment of TAB2/TAB3/TAK1 and IKKs, thereby triggering NF-kB and MAPK signalling.

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