CELL DEATH AND AUTOPHAGY

Live to die another way: modes of programmed cell death and the signals emanating from dying cells

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Abstract | All life ends in death, but perhaps one of life's grander ironies is that it also depends on death. Cell-intrinsic suicide pathways, termed programmed cell death (PCD), are crucial for animal development, tissue homeostasis and pathogenesis. Originally, PCD was almost synonymous with apoptosis; recently, however, alternative mechanisms of PCD have been reported. Here, we provide an overview of several distinct PCD mechanisms, namely apoptosis, autophagy and necroptosis. In addition, we discuss the complex signals that emanate from dying cells, which can either trigger regeneration or instruct additional killing. Further advances in understanding the physiological roles of the various mechanisms of cell death and their associated signals will be important to selectively manipulate PCD for therapeutic purposes.

Life and death are one thread, the same line viewed from different sides. Lao Tzu (ancient Chinese philosopher)

The concept of naturally occurring cell death dates back to 1842, when Karl Vogt showed that the notochord of the midwife toad is removed by cell death during metamorphosis, thereby facilitating the formation of vertebrae[1]. However, for many years, cell death was assumed to be no more than an inevitable and passive finale of life. This notion was challenged in the mid-1960s by the observation that developmental cell death depends on the expression of endogenous genes, thus giving rise to the term programmed cell death (PCD)[2,3]. Another important contribution came from electron microscopy studies carried out by Kerr, Wyllie and Currie[4] in the 1970s that defined the ultra-structural hallmarks of different types of cell death. Although various types of cell death had already been described in the nineteenth century, electron microscopy enabled a clear distinction to be made between these different modes of death. Cells that die during normal development and tissue homeostasis have morphological hallmarks, which include cytoplasmic shrinkage, nuclear condensation and the retention of membrane and organelle integrity — a process termed apoptosis[4]. By contrast, cells that swell and rupture as a result of overwhelming trauma die in a mode of cell death named necrosis.

Historically, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus* have been instrumental in developing our understanding of PCD and its role in animal development (BOX 1). Pioneering work in *C. elegans* by Horvitz and colleagues[5,6] defined the core apoptotic pathway and showed the conserved role of caspases in apoptosis (FIG. 1a). Since then, additional mechanisms of cell death have been reported, indicating that apoptosis is not the only mode of PCD. Here, we provide an overview of several important PCD mechanisms and discuss the biological significance of these pathways *in vivo*. Additional details of cell-based and biochemical studies for individual types of PCD are summarized in several excellent recent reviews[7–15]. Another rapidly expanding area of research discussed in this Review is signalling by apoptotic cells. Traditionally, dying cells were thought to have limited signalling capacity, as they are rapidly cleared by phagocytes. However, it is now clear that apoptotic cells release many signals that profoundly affect their cellular environment. These signals include mitogens, which promote proliferation and tissue repair, and death factors, which stimulate coordinated cell killing. This extraordinary complexity in the regulation and execution of cell death poses substantial experimental challenges but also presents exciting new opportunities for clinical translation.

Type I cell death: apoptosis

Caspases: the cellular executioners. Apoptosis is the most prominent and best-studied mode of PCD during development[9,16]. This conserved process, which can

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doi:10.1038/nrm3999
Published online 20 May 2015

Box 1 | **Programmed cell death in model organisms**

The *Caenorhabditis elegans*, *Drosophila melanogaster* and mouse (*Mus musculus*) model systems have shaped our understanding of how cells undergo programmed cell death (PCD). *C. elegans* provides unique opportunities for experimentation owing to its defined and invariant cell lineage. In ontogeny of the hermaphrodite worm, 131 of 1,090 somatic cells are eliminated by PCD, which results in adult organisms comprised of 959 cells[172]. In loss-of-function mutants for the pro-apoptotic genes *egl-1*, *ced-3* and *ced-4*, cell death is blocked, which results in survival of these 131 cells; however, the lifespan and behaviour of the worm are not affected.

D. melanogaster development is considerably more complex: cell fate and the number of cells are not predetermined but depend on extracellular signals and environmental factors. Therefore, *D. melanogaster* offers unique opportunities for studying PCD in the context of developmental plasticity and tissue homeostasis. The most common form of developmental PCD in the fly is apoptosis, and inhibition of this process causes severe developmental defects, malformations and organismal lethality[40–42,173]. However, inhibition of apoptosis does not affect the elimination of specific cells such as nurse cells, which indicates that apoptosis is not the only mechanism of PCD in flies[174].

Consistent with increased organismal complexity, the apoptotic machinery in vertebrates is even more intricate than that in flies and is involved in the regulation of crucial events throughout the lifespan of the organism. Therefore, it was surprising that mice in which genes encoding key components of the apoptotic machinery were deleted only have minor developmental defects and can reach adulthood[11]. The simplest explanation for the lack of overt phenotypes is that there may be functional redundancy between apoptotic proteins[22]. However, another possibility is that cells are eliminated by alternative PCD mechanisms when apoptosis is blocked[11]. Nevertheless, in many situations, the inhibition of apoptosis causes embryonic lethality, developmental abnormalities and various pathologies (TABLE 1).

These developmental studies have been complemented by various models to explain why cells need to die during development, indicating that the reasons include sculpting and deletion of structures, supply of nutrients, regulation of cell number and elimination of abnormal cells[8,175].

be triggered both intrinsically (for example, by DNA damage) and extrinsically (for example, by growth factor withdrawal, steroid hormones or ligation of death receptors), culminates in the activation of caspases, which are a class of cysteine proteases that are expressed as inactive zymogens in almost all cells[17,18] (FIG. 1). Interestingly, whereas *C. elegans* has 4 caspases, flies and mice contain 7 and 13 caspases, respectively, which suggests that higher organismal complexity is matched with a greater number of caspases. Although many caspases have crucial roles in apoptosis, these proteins also have non-apoptotic functions in various processes, including immunity, cellular remodelling, learning, memory and differentiation[8,9,19].

Traditionally, caspases have been subdivided into two categories: initiator caspases, which include Death-related ced-3/Nedd2-like caspase (Dredd) and Death regulator Nedd2-like caspase (Dronc) in *D. melanogaster*, and caspases 1, 2, 4, 5, 8, 9, 10, 11 and 12 in mammals; and executioner caspases, which include Ice (also known as Drice) and Death caspase 1 (Dcp1) in *D. melanogaster*, and caspases 3, 6, 7 and 14 in mammals. Some members of the caspase family can be compensated for, whereas others have non-redundant essential functions *in vivo*[10,11,13] (TABLE 1). Initiator caspases have long amino-terminal pro-domains, which facilitate the formation of protein platforms that regulate caspase activation. An example is the interaction of the caspase 9 pro-domain with apoptotic protease-activating factor 1 (APAF1) and cytochrome *c*, which produces the apoptosome that is thought to initiate apoptosis[20]. Alternatively, it has been suggested that this protein platform is responsible for signal amplification rather than for initiating the apoptotic cascade, which could explain how executioner caspases can be activated in mice (and flies) that have mutations in the genes encoding APAF1 (Ark (also known as Dark) in *D. melanogaster*) and caspase 9 (Dronc in *D. melanogaster*)[21–23]. This also provides a potential mechanism for how the executioner caspases 3 and 7 can regulate upstream events such as mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome *c*[24,25]. According to this model, executioner caspases may actually function upstream of initiator caspases, in at least some scenarios.

Apoptosome formation. MOMP, which results in the release of cytochrome *c* from the mitochondrion, is a decisive point that determines whether the cell commits to apoptosis. MOMP is regulated by members of the BCL-2 family, which contains both anti-apoptotic and pro-apoptotic members[10] (FIG. 1). The interplay between BCL-2 proteins determines whether a cell undergoes apoptosis. The assembly of pro-apoptotic BAX (BCL-2-associated X protein) and BAK (BCL-2 antagonist/killer) oligomers in the mitochondrial outer membrane promotes MOMP and the release of intermembrane proteins, such as cytochrome *c*, to the cytosol. This in turn promotes apoptosome formation and the activation of executioner caspases, thus propagating a proteolytic cascade that leads to the cleavage of many crucial cellular target proteins, including the inactivating cleavage of a DNase inhibitor, which causes nuclear DNA fragmentation[17,22,26].

The activation of caspases can also occur downstream of death receptor ligation, which results in binding of the initiator caspase 8 to its adaptor FAS-associated death domain protein (FADD) and assembly of the death-inducing signalling complex (DISC), as discussed in the necroptosis section below.

Inhibitor of apoptosis proteins. Another important layer of PCD regulation involves the inhibition of caspases by the inhibitor of apoptosis protein (IAP) family[8] (FIG. 1). IAPs were originally discovered in insect viruses and contain at least one baculovirus IAP repeat domain (BIR domain)[27,28]. Many cellular IAPs (cIAPs) have been described in insects and vertebrates and have been grouped into a 'family', but it is important to recognize that these are functionally diverse proteins, many of which have no direct role in apoptosis. Compelling evidence for a crucial role for some IAPs in the regulation of apoptosis came from analyses of loss-of-function and gain-of-function mutations in *D. melanogaster* Death-associated IAP 1 (*Diap1*)[29–31]. Inactivation of *Diap1* causes large-scale caspase activation and apoptosis, whereas gain-of-function mutations inhibit cell death, indicating that Diap1 has an essential role in the regulation of caspases *in vivo*. IAPs can bind to and inhibit caspases *in vitro* through their BIR domains[28]. Several IAPs also have a RING

Initiator caspases
Caspases that cleave inactive forms of executioner caspases.

Executioner caspases
Caspases that cleave various cellular proteins, often leading to apoptosis.

Apoptosome
A protein platform that is formed during apoptosis. This platform comprises cytochrome *c* that has translocated from the mitochondria to the cytoplasm and apoptotic protease-activating factor 1 (APAF1).

Mitochondrial outer membrane permeabilization
(MOMP). An event regulated by the BCL-2 protein family that is considered to be the 'point of no return' at which the cell commits to apoptosis.

Death-inducing signalling complex
(DISC). A protein platform formed by death receptors that can drive apoptosis.

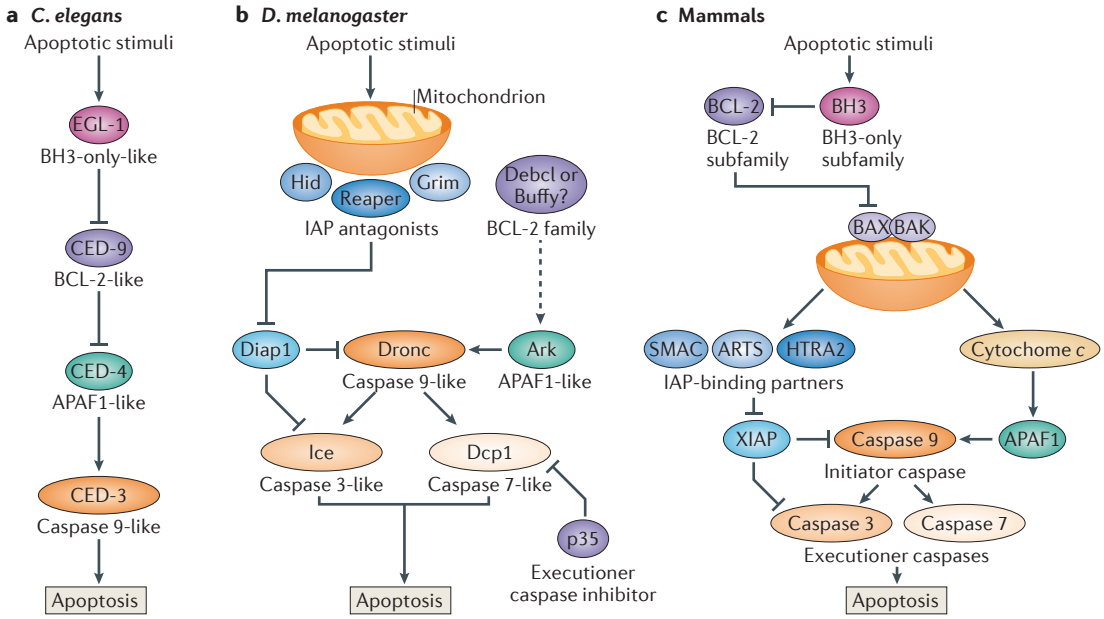


Figure 1 | **The core of the apoptotic machinery is conserved.** Functional homologues of apoptotic proteins in *Caenorhabditis elegans*, *Drosophila melanogaster* and mammals show the evolutionary conservation of the apoptotic pathway. **a** | In *C. elegans*, EGL-1 (which is a BH3-only protein homologue) binds to and inhibits CED-9 (a BCL-2-family homologue), resulting in the release of CED-4 (a homologue of apoptotic protease-activating factor 1 (APAF1)) from the CED-9–CED-4 complex. This enables cells to be eliminated by CED-3 (which is a caspase). **b** | In *D. melanogaster*, inhibitor of apoptosis protein (IAP) antagonists Reaper, Head involution defective (Hid) and Grim (which are known as the RHG proteins) mediate the degradation of Death-associated IAP 1 (Diap1), thereby liberating the executioner caspases Ice and Death caspase 1 (Dcp1). In addition, this enables Death regulator Nedd2-like caspase (Dronc), which is a caspase 9 homologue, to interact with Ark (an APAF1 homologue) and form the apoptosome that efficiently activates the executioner caspases. The protein p35 is a specific inhibitor of executioner caspases, and the activation of the apoptosome may be regulated by the BCL-2 family members Debcl and Buffy (hence depicted by a dashed line and question mark). **c** | In mammals, the crucial decision as to whether a cell commits to apoptosis is regulated by an interplay between the anti-apoptotic BCL-2 subfamily of proteins and the pro-apoptotic BH3-only subfamily. During apoptosis, BH3-only proteins facilitate a BAX (BCL-2-associated X protein)- and BAK (BCL-2 antagonist/killer)-dependent release of cytochrome *c* from mitochondria; cytochrome *c* binds to APAF1 and gives rise to the apoptosome. In parallel, IAP antagonists such as second mitochondria-derived activator of caspase (SMAC), HTRA2 and apoptosis-related protein in the TGF-β signalling pathway (ARTS) translocate from mitochondria and release caspases from negative regulation by IAPs. In particular, caspase 9 is liberated from X-linked IAP (XIAP) and activated by the apoptosome, which stimulates the executioner caspases 3 and 7. Homologous proteins are depicted in the same colours in the three panels.

motif, which enables them to function as E3 ubiquitin ligases and to promote the ubiquitylation of key cell death proteins[28,32,33]. In addition, some IAPs contain a ubiquitin-associated domain, which facilitates their interaction with ubiquitylated proteins, and a caspase-recruitment domain that mediates homophilic interaction with caspases[32,33]. Diap1 has two important functions that involve its RING domain: first, it ubiquitylates caspases, thus suppressing apoptosis; second, it auto-ubiquitylates and targets itself for degradation once the cell has committed to apoptosis[30,34–36]. Interestingly, Diap1-mediated ubiquitylation involves both degradative ubiquitylation and non-degradative polyubiquitylation that can regulate caspase activity[37,38].

The mammalian orthologue of Diap1 is X-linked IAP (XIAP), which also functions as an E3 ligase and is considered to be the most potent caspase inhibitor *in vitro*[39]. XIAP-deficient mice are viable and, for many years, they were thought to have only 'mild' phenotypes[32]. However, it has recently become clear that XIAP has a crucial role in stem cell apoptosis (see below).

Baculovirus IAP repeat domain
(BIR domain). A domain present in inhibitor of apoptosis proteins (IAPs) that can bind to caspases as well as to pro-apoptotic factors such as IAP antagonists.

Inhibitor of apoptosis protein antagonists. For a cell to commit to apoptosis, IAPs must be inactivated by endogenous antagonists (FIG. 1). A family of naturally occurring IAP antagonists was originally discovered by screening for cell death-defective mutants in *D. melanogaster*[40]. This revealed the essential role of three closely linked genes — *reaper* (*rpr*), *head involution defective* (*hid*) and *grim*, which encode the RHG proteins — in the induction of apoptosis[40–42]. RHG proteins have a common N-terminal IAP-binding motif (IBM) that is structurally conserved between flies and mammals and that is essential for IAP binding, IAP inactivation and cell killing[43].

Deletion of the genes encoding RHG proteins blocks apoptosis, and the ectopic expression of these genes is sufficient for the killing of many cell types[40–42,44]. In addition, mutations in *Diap1* that result in reduced binding of Diap1 to RHG proteins protect against apoptosis, indicating that Diap1 is a direct and functionally relevant target for the pro-apoptotic activity of RHG proteins (FIG. 1b). Furthermore, RHG proteins physically interact with each other to form heteromeric complexes at the mitochondrial

Table 1 | **Physiological functions of key cell death genes**

Cell death genes	Functional classification	Loss-of-function mutant phenotype	Physiological function from mutant and RNAi studies
Mice			
Bcl-2	BCL-2 subfamily	Abnormal death of renal epithelial progenitors, leading to fatal kidney disease	Inhibits apoptotic induction
Puma, *Bid* and *Bim*	BH3 subfamily	Similar to *Bax* and *Bak* mutants	Essential for initiation of apoptosis at the mitochondria
Bax and *Bak1*	BAX subfamily	• Most mice die during embryogenesis • Neonates show partial syndactyly, imperforated vaginas and an increase in haematopoietic cell number	Essential for apoptosis induction
Smac	IAP antagonist	Mice look normal	Physiological function found with caspase 3 deletion (see below)
Htra2	IAP antagonist	Lethal postnatal neurodegenerative disease	Phenotype does not seem to be related to apoptosis
Sept4	IAP antagonist	Increased numbers of apoptotic-resistant stem cells, improved regeneration and increased malignancies	Important for stem cell apoptosis, tumorigenesis and regeneration
Apaf1	Caspase 9 adaptor	• Similar to *Casp9* loss-of-function mutant phenotype • Some mice survive to adulthood	Essential for activation of caspase 9
Xiap	IAP	Mice have impaired wound healing, and hair follicle stem cells have increased susceptibility to apoptosis	Important for stem cell apoptosis and skin regeneration
Casp3	Executioner caspase	Phenotype depends on genetic background; perinatally lethal in FVB129 mice and no phenotype in C57BL/6 mice, probably owing to differences in caspase 7 expression levels	Essential for neuronal cell death and may have a redundant role in other tissues
Casp7	Executioner caspase	Mice look normal	• No apparent apoptotic defects in mutants • Seems to compensate in the absence of caspase 3
Casp3 and *Casp7*	Executioner caspases	• Perinatal lethality • Defects in cardiac development • MEFs show apoptotic resistance	Caspases 3 and 7 are required for apoptosis
Casp3 and *Smac*	Executioner caspase and IAP antagonist, respectively	• Perinatal lethality • Similar to *Casp3*$^{-/-}$ and *Casp7*$^{-/-}$ mice	Sensitizing deletion of caspase 3 shows that SMAC modulates caspase 7 through IAPs
Casp8	Initiator caspase	• Embryonic lethality • Death receptor-mediated apoptosis is completely impaired	Essential for extrinsic apoptosis mediated by the TNF family and for inhibition of necroptosis
Fadd	Caspase 8 adaptor	Similar to *Casp8*$^{-/-}$ mice	Essential for activation of caspase 8
Casp8 and *Ripk3*	Initiator caspase and necroptosis factor, respectively	Mice develop normally but have lymphadenopathy by 4 months of age	Caspase 8 and RIPK3 together seem to be dispensable for mammalian development
Casp9	Initiator caspase	• Perinatal lethality, but some animals survive to adulthood • Defects in the CNS	Required for efficient apoptosis in some cell types

outer membrane that are crucial for cell killing *in vivo*[45]. Importantly, RHG genes are transcriptionally regulated by embryonic patterning pathways and by many different pro-apoptotic signals[14]. This indicates that the convergence of different death-inducing signals occurs, at least in part, through a transcriptional mechanism. Finally, the pro-apoptotic activity of Hid is blocked upon phosphorylation by the MAPK Rolled, and Hid is targeted by the Epidermal growth factor receptor–Ras signalling pathway, resulting in cell survival[46,47]. Collectively, these studies established the important physiological role of IAPs and their antagonists and showed that caspases are controlled by both activating and inhibitory pathways[48,49].

Mammals also have IAP-binding proteins, most notably second mitochondria-derived activator of caspase (SMAC; also known as DIABLO) and the mitochondrial serine protease HTRA2 (also known as OMI or PARK13)[50–52]. Many more mitochondrial proteins that can interact with IAPs through their N-terminal IBMs have been identified but remain to be functionally characterized[53]. SMAC and HTRA2 are localized to the mitochondrial intermembrane space and translocate to the cytoplasm following MOMP (FIG. 1c). These proteins are thought to promote cell death following cytoplasmic release by binding to IAPs, which promotes caspase activation. However, rigorous genetic evidence to support this

Table 1 (cont.) | **Physiological function of key cell death genes**

Cell death genes	Functional classification	Loss-of-function mutant phenotype	Physiological function from mutant and RNAi studies
Caenorhabditis elegans			
egl-1	BH3-only-like	Animals are viable and have an additional 131 cells	Essential for inhibiting CED-9
ced-9	BCL-2-like	Developmental lethality is due to massive cell death	Inhibits cell death
ced-4	APAF1-like	Animals are viable and have an additional 131 cells	Essential for activating CED-3
ced-3	Caspase 9-like	Animals are viable and have an additional 131 cells	Essential for cell death
Drosophila melanogaster			
hid	IAP antagonist	Mutants die as embryos owing to extensive defects in apoptosis	Essential for most apoptosis
rpr, *hid* and *grim*	IAP antagonists	Mutants die as embryos owing to extensive defects in apoptosis	Essential for most apoptosis
Ark	APAF1-like adaptor	• Pupal lethality • Defects in developmental cell death	Essential for most apoptosis
Diap1	IAP	Embryonic lethality is due to excessive apoptosis	Inhibits Dronc, Ice and Dcp1
Dronc	Caspase 9-like	• Pupal lethality • Defects in developmental cell death	Essential for most apoptosis
Dcp1	Executioner caspase	Mutants are viable	Can be compensated for by Ice, similarly to mammalian caspases
Ice and *Dcp1*	Executioner caspases	• Prepupal lethality • Phenotype is similar to *Dronc* mutants	Essential for apoptosis

APAF1, apoptotic protease-activating factor 1; *Bak1*, BCL-2 antagonist/killer 1; *Bax*, BCL-2-associated X protein; *Bid*, BH3-interacting domain death agonist; *Casp*, caspase; CNS, central nervous system; *Dcp1*, Death caspase 1; *Diap1*, Death-associated inhibitor of apoptosis 1; *Dronc*, Death regulator Nedd2-like caspase; *Fadd*, FAS-associated death domain protein; *hid*, head involution defective; IAP, inhibitor of apoptosis; MEF, mouse embryonic fibroblast; *Ripk3*, receptor-interacting serine/threonine protein kinase 3; *rpr*, *reaper*; *Smac*, second mitochondria-derived activator of caspase; TNF, tumour necrosis factor, *Xiap*, X-linked inhibitor of apoptosis.

model *in vivo* is still lacking. *Htra2*$^{-/-}$ animals and cells do not have reduced cell death rates and actually lose a population of neurons in the striatum, which results in a lethal postnatal neurodegenerative disorder with features of Parkinson disease. This phenotype seems to be due to loss of the protease function of HTRA2 rather than to loss of its IBM[54].

Similarly, *Smac*$^{-/-}$ mice have no overt phenotype, and *Smac*$^{-/-}$ mouse embryonic fibroblasts (MEFs), lymphocytes and hepatocytes respond normally to apoptotic stimuli[55]. Although a redundancy between SMAC and HTRA2 has been suggested, mice that are deficient in both of these proteins have similar phenotypes to their parental single-knockout strains[54]. An alternative explanation for the physical association of SMAC and HTRA2 with XIAP is that binding promotes the ubiquitylation and proteasome-mediated degradation of SMAC and HTRA2 following release into the cytoplasm (a type of clean-up mechanism)[53]. Small-molecule IAP antagonists that are based on the conserved IBM of RHG proteins and SMAC (which are often referred to as SMAC mimetics) have been developed and are currently under clinical investigation as potential pro-apoptotic cancer therapeutics[56]. These compounds, which were initially designed to inhibit XIAP, target primarily cIAP1 and cIAP2 and sensitize cancer cells to tumour necrosis factor (TNF)-related apoptosis[57,58].

Another mammalian IAP antagonist is apoptosis-related protein in the transforming growth factor-β (TGFβ) signalling pathway (ARTS), which is encoded by *SEPT4*. ARTS does not contain an IBM but, similarly to the RHG proteins, it is localized to the mitochondrial outer membrane[59,60]. ARTS functions upstream of MOMP and mitochondrial protein release[25]. *Sept4*$^{-/-}$ mice are defective in caspase-mediated elimination of bulk cytoplasm during spermiogenesis, have increased numbers of haematopoietic stem and progenitor cells and have increased susceptibility to developing malignancies[61,62]. XIAP is the direct biochemical and physiological target of ARTS, and both of these proteins are crucial for the regulation of cell death in hair follicle stem cells (HFSCs)[60,63]. Loss of ARTS protects HFSCs against apoptosis, increases the number of HFSCs and leads to marked improvement in wound healing[63]. Furthermore, *Sept4*$^{-/-}$ mice have improved regeneration of hair follicles from the wound bed and develop significantly smaller scars[63]. Conversely, inactivation of XIAP abrogates these phenotypes and impairs wound healing, which indicates an *in vivo* role for XIAP and shows the importance of apoptosis in the regulation of HFSC number[63].

In summary, opposing pathways simultaneously regulate caspase activity during apoptosis through either caspase activation (by APAF1) or caspase inhibition (by IAPs); these pathways are subjected to many layers of coordinated upstream regulation. The cell is killed by the apoptotic machinery only after several checkpoints have been cleared, to ensure that effector caspase activity is sufficiently widespread and at a high enough level to cause extensive proteolysis of crucial cellular proteins. Finally, even at a late stage in apoptosis, cells can sometimes recover through a phenomenon termed 'anastasis' (Greek for 'rising to life')[64]. This could be a mechanism that the body uses to preserve cells that are difficult to replace.

Hair follicle stem cells (HFSCs). Adult stem cells that are normally situated in a niche called the bulge and that function to replenish the hair follicle.

Box 2 | **Linker cell death**

The linker cell in *Caenorhabditis elegans* migrates along a predetermined path and completes its migration when positioned between the gonad and cloacal tube, which functions as the exit channel for the sperm. Death of the linker cell before completion of its migration leads to severe defects in gonadal elongation and to impaired male fertility[65]. The microRNA let-7 and its downstream target *lin-29* are crucial components in the regulation of linker cell death. In let-7- and LIN-29-mutant animals, the linker cell survives and the connection between gonadal and cloacal tubes fails to form, resulting in sperm accumulation[65]. However, LIN-29 is also expressed in other cells that are not destined to die, indicating that LIN-29 is necessary but not sufficient for linker cell death. Recently, it was elegantly shown that linker cell death also requires SEK-1, TIR-1 and the polyglutamine-repeat protein PQN-41 (REF. 176). However, ongoing work continues to elucidate the downstream targets that are responsible for the demolition phase of the linker cell, as well as other key death-inducing genes that control linker cell death. Of particular interest, the *Drosophila melanogaster* and mouse orthologues of TIR-1 (Ect4 and SARM1, respectively) have a role in Wallerian degeneration. Following injury, loss of Ect4 or SARM1 suppresses axonal degeneration, which indicates that axons can actively promote their own demise and that TIR-1, Ect4 and SARM1 are implicated in non-apoptotic mechanisms of programmed cell death[177].

Dying linker cells have non-apoptotic characteristics, such as nuclear envelope crenellation, lack of chromatin condensation, organelle swelling and an increased number of cytoplasmic membrane-bound structures[65,176]. These morphological features resemble cell death during mammalian development, as in the case of chick ciliary ganglion cells and chick spinal cord motor neurons, suggesting that linker cell death is a morphologically conserved form of cell death. Furthermore, nuclear envelope crenellation is implicated in several polyglutamine expansion diseases, raising the possibility that neurodegeneration is promoted by inappropriate activation of a mechanism similar to that involved in linker cell death. Future work will shed light on the evolutionary conservation of this intriguing cell death programme and its role in development and disease.

Non-apoptotic programmed cell death

Although apoptosis is the most common form of PCD during development, emerging evidence indicates that it is not the only mechanism by which PCD takes place[11]. A recent report describes an alternative PCD pathway in *D. melanogaster*, which constantly eliminates 20% of pre-meiotic germ cells in the adult testis[178]. This pathway, termed germ cell death (GCD), is independent of the main effector caspases and involves an apoptosome-independent function of the initiator caspase Dronc. In addition, two main pathways, involving the lysosomes and the mitochondria, were found to mediate GCD, the latter of which requires the catalytic activity but not the IAP-antagonizing activity of HtrA2. In *C. elegans*, almost all PCD occurs by apoptosis, except in the case of the linker cell, which dies in a caspase-independent manner[5,65]. Dying linker cells have unique morphological features, which are also seen in certain neurons that die during spinal cord development in vertebrates, suggesting that this mechanism of PCD is conserved throughout evolution (BOX 2). In addition, as detailed in the following sections, autophagy and necroptosis have been intensively studied in the context of PCD.

Linker cell
A migratory cell of the *Caenorhabditis elegans* male gonad that dies in a non-apoptotic, caspase-independent manner.

Autophagic cell death
A reported mode of programmed cell death that is associated with the presence of autophagosomes and that depends on autophagy-related proteins.

Type II cell death: autophagy

Macroautophagy (here referred to as autophagy) is a catabolic process that involves the degradation of cytoplasmic components, protein aggregates and organelles through the formation of autophagosomes, which are degraded by fusion with lysosomes[66]. This process has been extensively studied in the response to starvation of *Saccharomyces cerevisiae*, in which it protects cells from death by recycling cell contents. Autophagy depends on a large group of evolutionarily conserved autophagy-related (ATG) genes[66]. In accordance with the protective, pro-survival function of autophagy, silencing and deletion of ATG genes resulted in accelerated cell death[12,67]. However, in certain scenarios (and depending on the organism), it has been suggested that autophagy can lead to or contribute to cell death.

Historically, type II cell death was termed autophagic cell death, which perhaps gave the misleading impression that cell death is achieved by the autophagic machinery[12]. However, this name simply indicates that cell death is accompanied by the presence of large numbers of autophagosomes, thus raising the question of whether autophagy is an unsuccessful attempt at survival rather than a bona fide cell death mechanism.

In *D. melanogaster*, dying cells of the salivary glands contain autophagic vacuoles and express ATG genes[7,68]. Moreover, deletion of ATG genes attenuates both autophagy and PCD[69]. Interestingly, the engulfment receptor Draper is required for autophagy during the death of salivary gland cells[70]. Furthermore, compelling evidence indicates that caspase activity is necessary for the degradation of these cells, suggesting the involvement of an apoptotic mechanism. First, anti-apoptotic *Diap1* is repressed, and pro-apoptotic genes (*rpr* and *hid*) are activated before the death of salivary gland cells[71]. Second, dying cells are positive for apoptotic markers[66] (which can be detected using acridine orange and TUNEL assays). Third, deletion of *Ark* (the *D. melanogaster* orthologue of *Apaf1*) or caspase inhibition rescues these cells from death[68,72–76]. Thus, autophagy alone is not sufficient for salivary gland cell death.

Autophagic cell death is also associated with the destruction of *D. melanogaster* mid-gut cells. Death of these cells has been characterized by both caspase activation and autophagy; however, recent experiments showed that mutations in the genes encoding caspases or their regulators do not rescue these cells from destruction[77]. By contrast, the inhibition of Atg1, Atg2 and Atg18 expression delays mid-gut degradation without a decrease in caspase activity, which indicates that the death of mid-gut cells involves a caspase-independent form of PCD that requires autophagy[77]. Intriguingly, this work also suggests that the activation of caspases occurs by an unknown mechanism in these cells, as Death executioner caspase related to Apopain/Yama (Decay) was activated in the absence of the activation of initiator caspase Dronc.

In mice, it has also been suggested that autophagy is an alternative killing mechanism in situations in which apoptosis is not possible. MEFs that are deficient in both BAX and BAK are resistant to various inducers of apoptosis but can undergo cell death in what seems to be an autophagy-mediated manner[78,79]. Knockdown of the mammalian ATG gene beclin 1 (*Becn1*; *ATG6* (also known as *VPS30*) in yeast) or of *Atg5* reduced cell death in response to etoposide or staurosporin in $Bax^{-/-}$ $Bak^{-/-}$ MEFs[73]. Intriguingly, autophagy was not induced when downstream apoptotic processes were inhibited in

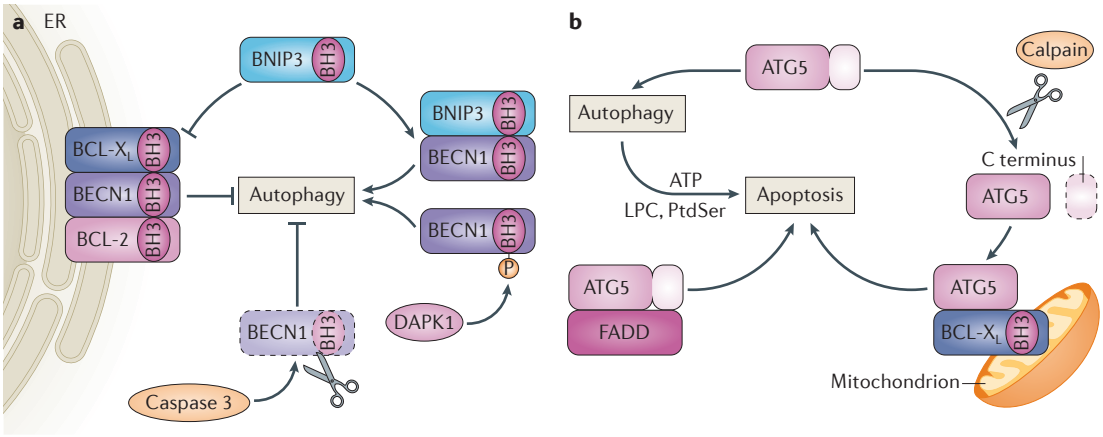


Figure 2 | **Crosstalk between autophagy-related and apoptosis-related proteins.** **a** | The BH3 domain of the autophagy-related (ATG) protein beclin 1 (BECN1) enables the formation of a BCL-2– BECN1–BCL-X_L complex that localizes to the endoplasmic reticulum and inhibits autophagy. Competitive interaction by the BH3-only protein BCL-2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and phosphorylation (P) by death-associated protein kinase 1 (DAPK1) regulate the dissociation of this complex and drive autophagy. BECN1 has also been found to be a substrate of caspase 3. **b** | ATG5 can undergo calpain-dependent cleavage, which switches its function from pro-survival to pro-death. This carboxy-terminal cleavage gives rise to a pro-apoptotic product that translocates to the mitochondrion, binds to BCL-X_L and induces apoptosis. ATG5 can also induce apoptosis by binding to FAS-associated death domain protein (FADD), and apoptotic features such as phosphatidylserine (PtdSer) exposure, release of lysophosphatidylcholine (LPC) and membrane blebbing seem to depend on autophagy for the provision of energy in the form of ATP. ER, endoplasmic reticulum.

Casp9$^{-/-}$ or *Apaf1*$^{-/-}$ MEFs or in response to the addition of the pan-caspase inhibitor Z-VAD-FMK, which indicates that BAX and BAK might have specific roles in regulating autophagy. In this regard, it is intriguing that BECN1 has a BH3 domain, through which it interacts with pro-survival BCL-2 family members (BCL-2, BCL-X_L and myeloid cell leukaemia sequence 1 (MCL1))[12,80]. This interaction is conserved and is also seen in *C. elegans*, in which BEC-1 (the orthologue of mammalian BECN1) binds to CED-9 (a BCL-2-like protein)[81]. The direct interaction of BCL-2, BCL-X_L and BECN1 inhibits the pro-autophagy function of BECN1 and is dependent on subcellular localization[80] (FIG. 2a). The autophagy-inhibiting BCL-2–BCL-X_L–BECN1 complexes localize to the endoplasmic reticulum, indicating that autophagy is regulated in an organelle-specific manner[82] (FIG. 2a). Interestingly, the BH3 domain of BECN1 also regulates dissociation of this complex. Death-associated protein kinase 1 (DAPK1) can phosphorylate the BH3 domain of BECN1, resulting in reduced affinity for BCL-X_L and dissociation of the complex, which leads to the induction of autophagy[83]. In addition, by competitive interaction, BH3-only BCL-2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) can also cause dissociatiozn of the BCL-2–BCL-X_L–BECN1 complex[84]. Finally, BECN1 has been found to be a substrate of caspase 3, and caspase-mediated cleavage of BECN1 abrogated its interaction with BCL-2 (REF. 85) (FIG. 2a). Taken together, these results indicate that the BH3 domain of BECN1 can regulate the association–dissociation kinetics of the BCL-2–BCL-X_L–BECN1 complex and thus can shift the balance between autophagy and apoptosis[85].

ATG5 may also have a dual role in the regulation of both autophagy and apoptosis. ATG5 can undergo calpain-dependent cleavage, which switches its function from a pro-survival autophagy protein to a death -induction protein. This cleavage removes the carboxy-terminal domain of ATG5, giving rise to a pro-apoptotic product that translocates to the mitochondria, where it induces the intrinsic apoptotic programme[86] (FIG. 2b). Interestingly, ATG5 can also bind to FADD through its C-terminal domain and thereby stimulate caspase-dependent apoptotic cell death[87].

There is some indication that autophagy contributes to the classic hallmarks of apoptosis such as phosphatidylserine exposure and membrane blebbing. These processes depend on ATP, and autophagy may help in this regard by generating the metabolic substrates that are required for the bioenergetic needs of the dying cell. An elegant study showed that ATP produced by the autophagic machinery is responsible for the generation of the 'find-me' and 'eat-me' signals for phagocytes (lysophosphatidylcholine and phosphatidylserine, respectively) in mouse embryoid bodies[88] (FIG. 2b). Embryoid bodies that were derived from *Becn1*$^{-/-}$ or *Atg5*$^{-/-}$ embryonic stem cells were able to undergo the initial stages of apoptosis but were unsuccessful in producing the signals required for phagocytic clearance[88]. In addition, autophagy might supply the ATP that is required for membrane blebbing during apoptosis[89].

An important unresolved question is to what extent autophagy actually mediates cell death rather than simply occurring at the same time. It was recently shown, using a systematic approach to examine the existence of autophagic cell death in mammalian cells, that none of the 1,400 death-inducing compounds that were screened eliminated cells through the induction of autophagy[90]. Silencing of key autophagy genes was not only ineffective in preventing cell death but actually accelerated it[90]. As these are negative results, they do not exclude the

possibility that cell death can be mediated by autophagy, but they do indicate that autophagy does not have a substantial role in promoting cell death.

Type III cell death: regulated necrosis

For many years, necrosis was regarded as an unregulated mode of cell death that was caused by overwhelming trauma. However, many recent studies indicate the existence of several modes of regulated necrosis[15]. Necrosis is characterized by swelling of organelles and cells, rupture of the plasma membrane and release of the intracellular contents[15]. Different modes of regulated necrosis share common morphological features[15]. Below, we focus on the best-studied form of regulated necrosis, termed necroptosis, which is a type of necrotic cell death that depends on receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and/or RIPK3 (REFS 13–15). Additional terms have been proposed to describe necrosis that is induced by different stimuli, but it remains to be shown that these actually involve different mechanisms for programmed necrosis[15].

Necroptosis. The term 'necroptosis' was coined to describe a non-apoptotic mode of cell death that is elicited by ligation of TNF receptor 1 (TNFR1; also known as TNFRSF1A) when caspases are inhibited[91]. This form of PCD can be inhibited by necrostatin 1 (REF. 91). Although necroptosis is only one type of regulated necrosis, the terms are often regarded as being synonymous[91,92].

Necroptosis can be induced by the ligation of various death receptors, which include FAS (also known as TNFRSF6 or CD95), TNFR1 and TNFR2, various Toll-like receptors and intracellular sensors such as DNA-dependent activator of interferon-regulatory factors (DAI; also known as ZBP1) and protein kinase R (PKR; also known as EIF2AK2)[93]. The TNFR signalling pathway best exemplifies how the necroptotic programme can be induced. Following binding of TNF, TNFR1 recruits RIPK1, TNFR1-associated death domain (TRADD), cIAP1 and cIAP2, and TNFR-associated factor 2 (TRAF2) and TRAF5, which form complex I[94] (FIG. 3a). cIAPs mediate Lys63-linked ubiquitylation of RIPK1, which enables the docking of TGFβ-activated kinase 1 (TAK1; also known as MAP3K7), TAK1-binding protein 2 (TAB2) and TAB3 and results in the activation of the inhibitor of nuclear factor-κB (NF-κB) kinase (IKK) complex. This complex, in turn, targets inhibitor of NF-κB-α (IκBα) for degradation, which liberates NF-κB. NF-κB translocates to the nucleus, where it drives transcription of pro-survival genes as well as genes encoding negative-feedback proteins such as IκBα and interleukin-17 (IL-17) receptor D (also known as SEF)[95–98]. Two such pro-survival NF-κB target genes are those encoding zinc-finger protein A20 (also known as TNFAIP3) and FLICE-like inhibitory protein (FLIP; also known as CFLAR). A20 polyubiquitylates RIPK1 with Lys48-linked chains, thereby marking it for proteasomal degradation (FIG. 3a), whereas the deubiquitylating enzyme cylindromatosis (CYLD) eliminates Lys63-linked ubiquitin chains from RIPK1, leading to the dissociation of RIPK1 from complex I[98,99]. This deubiquitylation event changes the function of RIPK1 from promoting survival to promoting death through formation of the DISC (also known as complex IIa), which comprises RIPK1, RIPK3, TRADD, FADD, caspase 8 and FLIP[15,94]. The DISC has a dual role: it facilitates the cleavage and degradation of CYLD, RIPK1 and RIPK3 to promote survival; and it enables the homodimerization and catalytic activation of caspase 8 to stimulate apoptosis (FIG. 3a). In mice, deletion of the genes encoding the complex IIa components caspase 8 and FLIP results in embryonic lethality[100–102]. However, these mice are rescued when *Ripk3* is inactivated (TABLE 1), suggesting that a key function of complex IIa is to regulate necroptosis (see below)[103–105]. Furthermore, deletion of *Ripk1* results in postnatal lethality that can be prevented by the ablation of *Ripk3* together with either *Casp8* or *Fadd*[106,107] (TABLE 1).

When caspase 8 is inactivated, RIPK1 associates with RIPK3, resulting in autophosphorylation, transphosphorylation and formation of the necrosome[108–110] (FIG. 3a). Accordingly, targeting the kinase domain of RIPK1 with necrostatin 1 inhibits the interaction between RIPK1 and RIPK3 but does not affect the pro-survival NF-κB pathway downstream of RIPK1 (REFS 91,111,112). Phosphorylation of RIPK3 (on Ser227 in humans or Ser232 in mice) results in the recruitment of mixed-lineage kinase domain-like protein (MLKL), which initiates necroptosis[113,114]. MLKL is phosphorylated by RIPK3, which leads to the formation of MLKL oligomers that translocate to the plasma membrane, bind to phosphatidylinositol phosphates and form membrane-disrupting pores[115,116]. The resulting perturbation of membrane integrity seems to induce influx of both Na^+ and Ca^{2+}, leading to a rise in osmotic pressure that causes membrane rupture[116–118] (FIG. 3a).

Ripk3$^{-/-}$ or *Mlkl*$^{-/-}$ mice do not have developmental or homeostatic defects[106,119]. However, as many of the downstream signalling components of necroptosis are unknown, it is too early to conclude whether necroptosis has a role in developmental or homeostatic processes. By contrast, there is evidence that necroptosis is activated in response to bacterial and viral infection[104]. When *Ripk3*$^{-/-}$ mice are exposed to vaccinia virus, they have increased viral titres and succumb to infection more rapidly than wild-type mice[109]. This is a result of impaired virus-induced tissue necrosis, inflammation and control of viral replication. Hence, necroptosis has two distinct functions: on the one hand, it functions as a back-up mechanism to eliminate infected cells when apoptosis is inhibited; on the other hand, it promotes the release of damage-associated molecular patterns (DAMPs) to induce the immune response. Importantly, viral genes encode specific inhibitors, such as viral inhibitor of caspase activation, viral FLIPs and viral inhibitor of RIPK1 (REFS 120,121), that can affect necroptosis. Interestingly, some viruses (such as murine cytomegalovirus) can block both apoptosis and necroptosis because their genomes encode both a caspase 8 inhibitor and a RIPK3 inhibitor[122,123].

The induction of a pro-inflammatory response following necroptosis has been observed in various organs such as the skin and intestine. Deletion of *Fadd* and

Necrostatin 1
A potent and selective inhibitor of necroptosis that was originally reported as a selective allosteric inhibitor of the death domain receptor-associated adaptor kinase RIP1 in the necroptosis pathway.

Damage-associated molecular patterns
(DAMPs; also known as alarmins). Molecules that are released by stressed cells and that function as endogenous danger signals to initiate and propagate the inflammatory response.

Casp8 in epidermal cells results in skin inflammation, which can be reversed by *Ripk3* deletion[124,125]. Similarly, mice in which *Casp8* is deleted in intestinal epithelial cells have ileitis as a result of increased necroptosis within the stem cell crypts[126]. These data, together with the ability of caspase 8 to cleave RIPK1 and RIPK3, identify caspase 8 as a key regulator of inflammation that is situated at the crossroads of apoptosis and necroptosis.

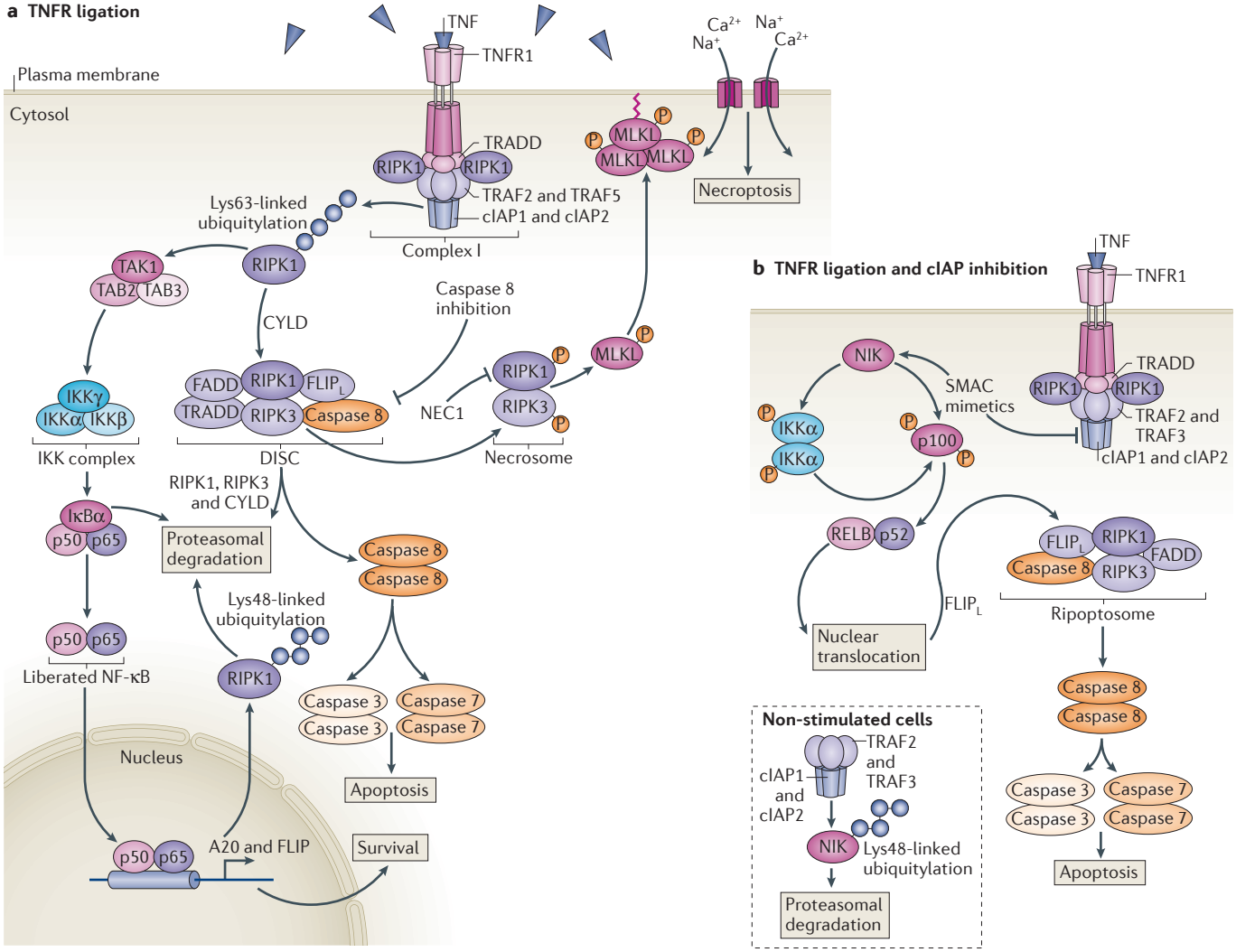


Figure 3 | **Tumour necrosis factor-mediated survival, apoptosis and necroptosis.** **a** | Ligation of tumour necrosis factor (TNF) receptor 1 (TNFR1) results in the recruitment of receptor-interacting serine/threonine protein kinase 1 (RIPK1), TNFR1-associated death domain (TRADD), cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2, and TNFR-associated factor 2 (TRAF2) and TRAF5 to TNFR1 to form complex I. cIAP1 and cIAP2 mediate Lys63-linked ubiquitylation of RIPK1, which facilitates docking of transforming growth factor-β (TGFβ)-activated kinase 1 (TAK1) and its binding partners TAK1-binding protein 2 (TAB2) and TAB3. The signal is then propagated to the inhibitor of nuclear factor-κB (NF-κB) kinase (IKK) complex, which promotes the degradation of IκBα, the cytoplasmic inhibitor of canonical NF-κB. Once liberated from IκBα, NF-κB translocates to the nucleus, where it drives the transcription of pro-survival genes as well as feedback antagonists. These pro-survival NF-κB-target genes include those encoding A20 and FLICE-like inhibitory protein (FLIP), which promote the association of RIPK1 with cytoplasmic RIPK3, TRADD, FAS-associated death domain protein (FADD), caspase 8 and the NF-κB target FLIP to form the death-inducing signalling complex (DISC). The long isoform of FLIP ($FLIP_L$) can heterodimerize with caspase 8 and facilitate the cleavage and degradation of cylindromatosis (CYLD), RIPK1 and RIPK3. However, the DISC also causes homodimerization and catalytic activation of caspase 8, which activates caspase 3 and caspase 7 to induce apoptosis. When caspase 8 is deleted or inhibited, RIPK1 interacts with RIPK3, leading to the formation of the necrosome; this interaction can be inhibited by necrostatin 1 (NEC1). RIPK3 recruits and phosphorylates (P) mixed-lineage kinase domain-like protein (MLKL), leading to the formation of MLKL oligomers that translocate to the plasma membrane. Once at the plasma membrane, MLKL forms membrane-disrupting pores, which regulate influx of both Na^+ and Ca^{2+}, resulting in membrane rupture. **b** | cIAPs also function as negative regulators of the non-canonical NF-κB pathway. In resting, non-stimulated cells, a complex comprising cIAP1, cIAP2, TRAF2 and TRAF3 targets and degrades NF-κB-inducing kinase (NIK). Following ligation of TNFR1, the TRAF2–TRAF3–cIAP1–cIAP2 complex is recruited to the receptor, changing the substrate specificity of the complex from NIK to the components of the complex itself. This results in increased NIK levels, phosphorylation of IKKα and p100, proteolytic processing of p100 to p52 and translocation of p52 to the nucleus. When cIAPs are depleted, the canonical NF-κB pathway is inhibited, whereas the non-canonical NF-κB pathway is not. Under these conditions, the large (~2 MDa) ripoptosome is assembled and, similarly to the DISC, can stimulate caspase 8-mediated apoptosis. SMAC, second mitochondria-derived activator of caspase.

Programmed cell death through the ripoptosome

In addition to their function as positive regulators of canonical NF-κB signalling, cIAPs have a key role in the regulation of the non-canonical NF-κB pathway[57,127,128]. In resting cells, non-canonical NF-κB signalling is repressed by a complex consisting of cIAP1, cIAP2, TRAF2 and TRAF3 (REF. 129). This E3 ligase complex binds to NF-κB-inducing kinase (NIK; also known as MAP3K14), conjugates Lys48-linked ubiquitin chains and targets NIK for proteasomal degradation. Following ligation of a subset of TNFR-superfamily members (by CD40L, TWEAK or BAFF), the TRAF2–TRAF3–cIAP complex is recruited to the receptor, which changes the substrate specificity of the complex from NIK to any of the components of the complex in a manner that depends on the cellular context and the receptor type. As a result, NIK levels are increased, leading to phosphorylation of IKKα and of the NF-κB precursor p100 (FIG. 3b). Sequentially, homodimers of IKKα further phosphorylate p100 to promote its proteolytic processing to p52, which can bind to other NF-κB subunits and translocate to the nucleus. In accordance, when cIAPs are depleted (owing to chemotherapeutic drugs, cytokine stimulation or SMAC mimetics), the canonical NF-κB pathway is inhibited, whereas the non-canonical NF-κB pathway is activated. Under these conditions, TNF is produced and another death-inducing platform (termed complex IIb or the ripoptosome) is assembled[130,131]. The ripoptosome forms in the cytoplasm independently of TNF, FASL, TRAIL (also known as TNFSF10), death receptors and mitochondrial pathways. This large (~2 MDa) complex comprises RIPK1, FADD and caspase 8, and is negatively regulated by cIAP1, cIAP2 and XIAP. Similar to the DISC, the ripoptosome can stimulate caspase 8-mediated apoptosis as well as necroptosis in a $FLIP_L$- and caspase 8-dependent manner ($FLIP_L$ refers to the long isoform of FLIP) (FIG. 3b).

Signalling from dying cells

Apoptotic cells are rarely visible *in situ* owing to efficient clearance mechanisms. When clearance is impaired, uncleared corpses become necrotic and induce inflammation and autoimmunity[132]. To ensure proper cell clearance, apoptotic cells secrete 'find-me' signals that instruct phagocyte attraction. These signals include lysophosphatidylcholine, sphingosine-1-phosphate and the nucleotides ATP and UTP[132,133]. In addition, apoptotic cells secrete 'eat-me' signals, of which phosphatidylserine is the best known. In viable cells, phosphatidylserine is exclusively retained on the inner leaflet of the lipid bilayer, but it is exposed on the extracellular layer during apoptosis[133]. Recent work has elegantly shown that, in response to apoptotic stimuli, caspase 3 cleaves XK-related protein 8, which leads to phosphatidylserine exposure[134]. Interestingly, CED-8, which is the only *C. elegans* XK-family homologue, also promotes phosphatidylserine exposure and the engulfment of cell corpses in a caspase-mediated manner[135]. Although the exposure of phosphatidylserine during apoptosis is conserved in evolution, the exact mechanism by which it facilitates cell clearance remains controversial[132].

As apoptotic cells are rapidly cleared, it was long thought that they had limited signalling capacity other than secreting 'find-me' and 'eat-me' signals. However, recent advances have shown that apoptotic cells are a rich source of signals that profoundly affect their cellular environment, including mitogens that instruct proliferation and regeneration, and death factors that trigger additional apoptosis.

Apoptosis-induced proliferation. Regeneration is a process of regrowth or repair that enables organisms to survive overwhelming trauma[136]. Research in diverse model systems has now shown that apoptotic cells can secrete mitogens to directly stimulate cell proliferation. Apoptosis-induced proliferation can aid regeneration and explain how developing tissues can compensate for massive cell loss in response to stress or injury.

The original observation that apoptotic cells can secrete mitogens to stimulate the proliferation of adjacent precursor cells came from studies in *D. melanogaster*[36,137,138] (FIG. 4a). A technical problem in these studies was the rapid clearance of dying cells. The key to overcoming this hurdle was to block the completion of apoptosis by expression of p35, which specifically inhibits executioner caspases. The resulting 'undead' cells remain in a prolonged apoptotic state and stimulate tissue overgrowth by releasing mitogenic signals[139,140] (FIG. 4a). Candidates for these mitogens include Wingless (Wg; a WNT orthologue) and Decapentaplegic (Dpp; the orthologue of mammalian TGFβ and bone morphogenetic proteins), as these proteins are activated under the relevant conditions[36,138] (FIG. 4a). Furthermore, Wg signalling is required for cell proliferation in this system[36]. Finally, the Jun N-terminal kinase (Jnk) pathway also has a key role in promoting apoptosis-induced proliferation and wound healing in *D. melanogaster*[36,141].

Initially, it was not clear whether Jnk functions independently of the apoptotic programme, whether it is a downstream target of Dronc or whether it is an inducer of pro-apoptotic genes[36,142–144]. However, a recent study reconciled these different models by identifying a positive-feedback loop in which Jnk and p53 function both upstream and downstream of Dronc and RHG proteins to amplify the apoptotic signal[145] (FIG. 4a). Interestingly, disruption of the *D. melanogaster* Cdc42–Par6–atypical protein kinase C epithelial polarity complex stimulates Jnk-dependent apoptosis and apoptosis-induced proliferation[146].

These observations were further extended in p35-independent systems, which also showed the requirement of Wg and Jnk for proper regeneration[147,148] (FIG. 4b). Notably, in the differentiating *D. melanogaster* retina, apoptosis-induced proliferation involves Hedgehog as the primary mitogenic signal[149]. Hedgehog secretion is induced in apoptotic photoreceptor neurons in a manner that depends not only on the initiator caspase Dronc but also on the executioner caspases Ice and Dcp1 (FIG. 4c). Therefore, p35 blocks apoptosis-induced proliferation in this tissue (but not in the wing disc), which indicates that distinct mechanisms of apoptosis-induced proliferation might operate in different cell types and tissues.

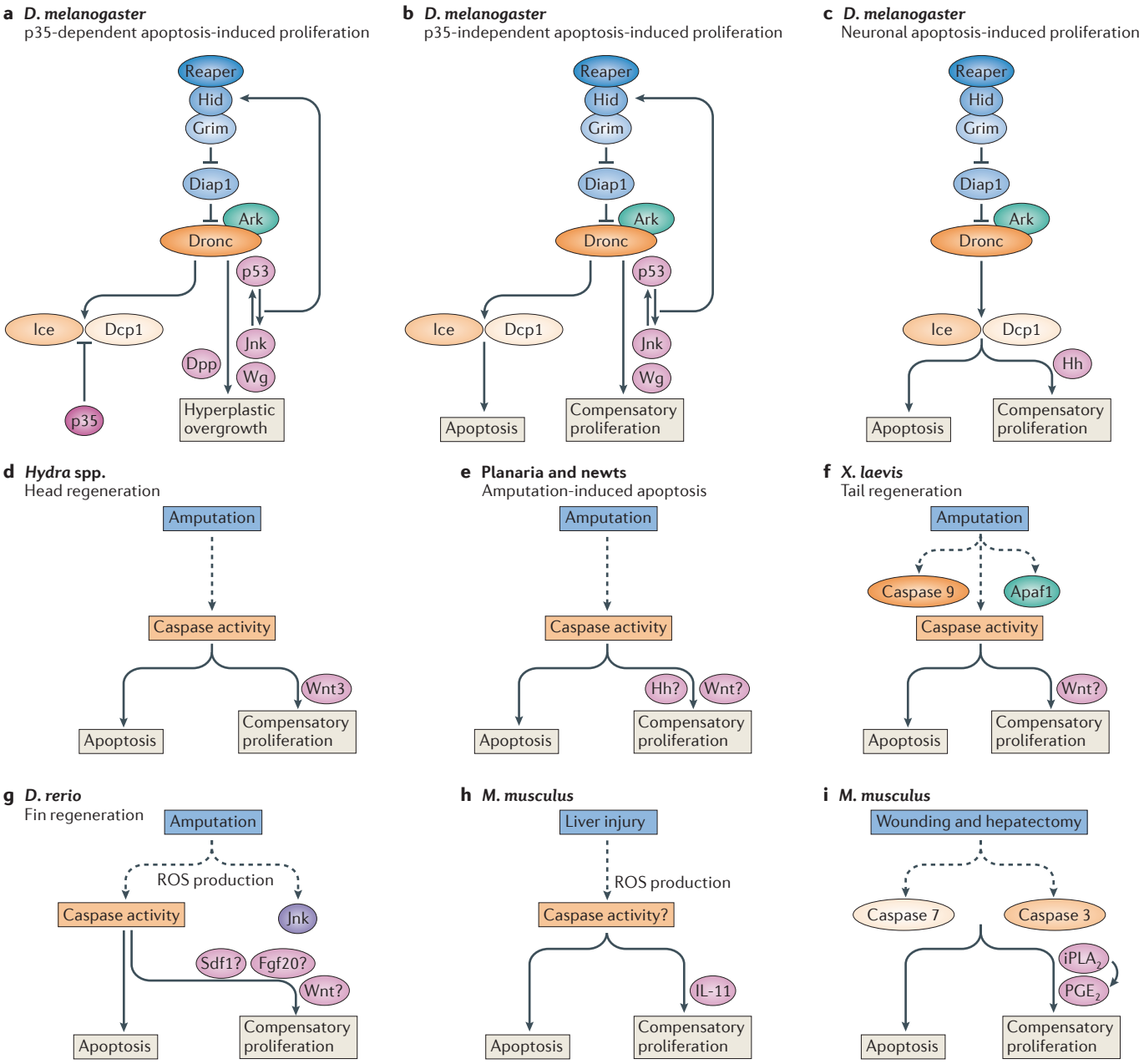


Figure 4 | **Apoptosis-induced proliferation.** Apoptotic cells can secrete mitogens, which stimulate growth and regeneration. Caspase targets and mitogenic factors are depicted in pink, and question marks indicate uncertainty. Dashed arrows indicate cases in which it is not clear how injury results in caspase activation in the different model systems. **a** | In *Drosophila melanogaster*, p35 inhibits executioner caspases (Ice and Death caspase 1 (Dcp1)) and retains the cells in an 'undead' state. Under these conditions, p53 and Jun amino-terminal kinase (Jnk) are activated and promote the release of Wingless (Wg; a homologue of WNT) and Decapentaplegic (Dpp; a homologue of transforming growth factor-β (TGFβ) and bone morphogenetic proteins), thereby promoting hyperplastic overgrowth through apoptosis-induced proliferation. **b** | In *D. melanogaster*, in a naturally occurring p35-independent system, apoptosis induces tissue regeneration by secretion of Wg. **c** | In *D. melanogaster*, differentiated neurons induce apoptosis-induced compensatory proliferation through Hedgehog (Hh), which stimulates non-neuronal cell proliferation. In this scenario, the signal is downstream of Ice and Dcp1 and not of Death regulator Nedd2-like caspase (Dronc). **d** | In *Hydra* spp., head regeneration is a caspase-dependent process: apoptotic cells secrete Wnt3, leading to apoptosis-induced compensatory proliferation. **e** | In Planaria and newts, apoptotic cells and caspase activity are seen at the amputation site; however, it remains to be seen whether the apoptotic cells are responsible for the secretion of Wnt and Hh. **f** | In *Xenopus laevis*, tail amputation leads to caspase activity, and inhibition of caspase 3 and caspase 9 inhibits proliferation and regeneration. It is unclear whether this form of compensatory proliferation is mediated by Wnt. **g** | In *Danio rerio*, fin amputation results in of the production of reactive oxygen species (ROS), which induce caspase and Jnk activation. It remains to be established whether fibroblast growth factor 20 (Fgf20), Wnt and stromal cell-derived factor 1 (Sdf1) are secreted from apoptotic cells to regulate apoptosis-induced compensatory proliferation. **h** | In *Mus musculus*, liver injury results in ROS production and the secretion of interleukin-11 (IL-11) from hepatocytes, which facilitates apoptosis-induced compensatory proliferation. **i** | In *M. musculus*, wound repair and liver regeneration depend on caspase 3 and caspase 7. Caspase 3 mediates the proteolysis of calcium-independent phospholipase A2 ($iPLA_2$) to produce prostaglandin E_2 (PGE_2), which is known to stimulate stem cell proliferation, wound repair and regeneration. Apaf1, apoptotic protease-activating factor 1; Diap1, Death-associated inhibitor of apoptosis 1.

Subsequently, apoptosis-induced proliferation was also observed in other organisms, including *Hydra* spp., Planaria, newts, *Xenopus laevis*, zebrafish and mice. In *Hydra* spp., apoptosis is both required and sufficient to induce Wnt3 production for head regeneration after mid-gastric bisection[150]. The Mapk–cyclic AMP-responsive element-binding protein pathway triggers apoptosis in *Hydra* spp. and, reminiscent of the *D. melanogaster* apoptosis-induced proliferation mechanism, apoptotic cells release Wnt3 in a caspase-dependent manner to promote cell proliferation and regeneration[150,151] (FIG. 4d). Planaria and newts also have large numbers of apoptotic cells at the site of amputation, but whether these cells drive regeneration through apoptosis-induced proliferation is still unknown[152–156] (FIG. 4e). In the *X. laevis* tadpole, many apoptotic cells can be detected 12 hours after tail amputation, and caspase activity is necessary for tail regeneration[157]. However, it remains to be seen whether Wnt, which is known to have a key role in *X. laevis* regeneration, is secreted from apoptotic cells (FIG. 4f). In zebrafish, reactive oxygen species (ROS) are produced in response to adult fin amputation. ROS stimulate caspases and Jnk in parallel, both of which are required for apoptosis-induced proliferation. Furthermore, the data suggest that fibroblast growth factor 20, stromal cell-derived factor 1 (also known as Cxcl12a) and Wnt proteins might be secreted from dying cells in this scenario[158] (FIG. 4g). Similarly, it was recently shown in mice that IL-11, which is a member of the IL-6 pro-inflammatory family of cytokines, is produced by hepatocytes in a ROS-dependent manner. IL-11 is released from apoptotic hepatocytes and induces phosphorylation of signal transducer and activator of transcription 3 (STAT3) and apoptosis-induced proliferation[159] (FIG. 4h). Interestingly, production of ROS can also result in hepatocyte necrosis that releases IL-1, which in turn stimulates and induces apoptosis-induced proliferation[160]. In addition, dying MEFs can stimulate the proliferation of stem and progenitor cells in a caspase-dependent manner[161]. Mice that are deficient in caspase 3 and caspase 7 have impaired wound healing and liver regeneration. Interestingly, the downstream target of these caspases is prostaglandin E_2, a promoter of stem or progenitor cell proliferation and tissue regeneration[161] (FIG. 4i).

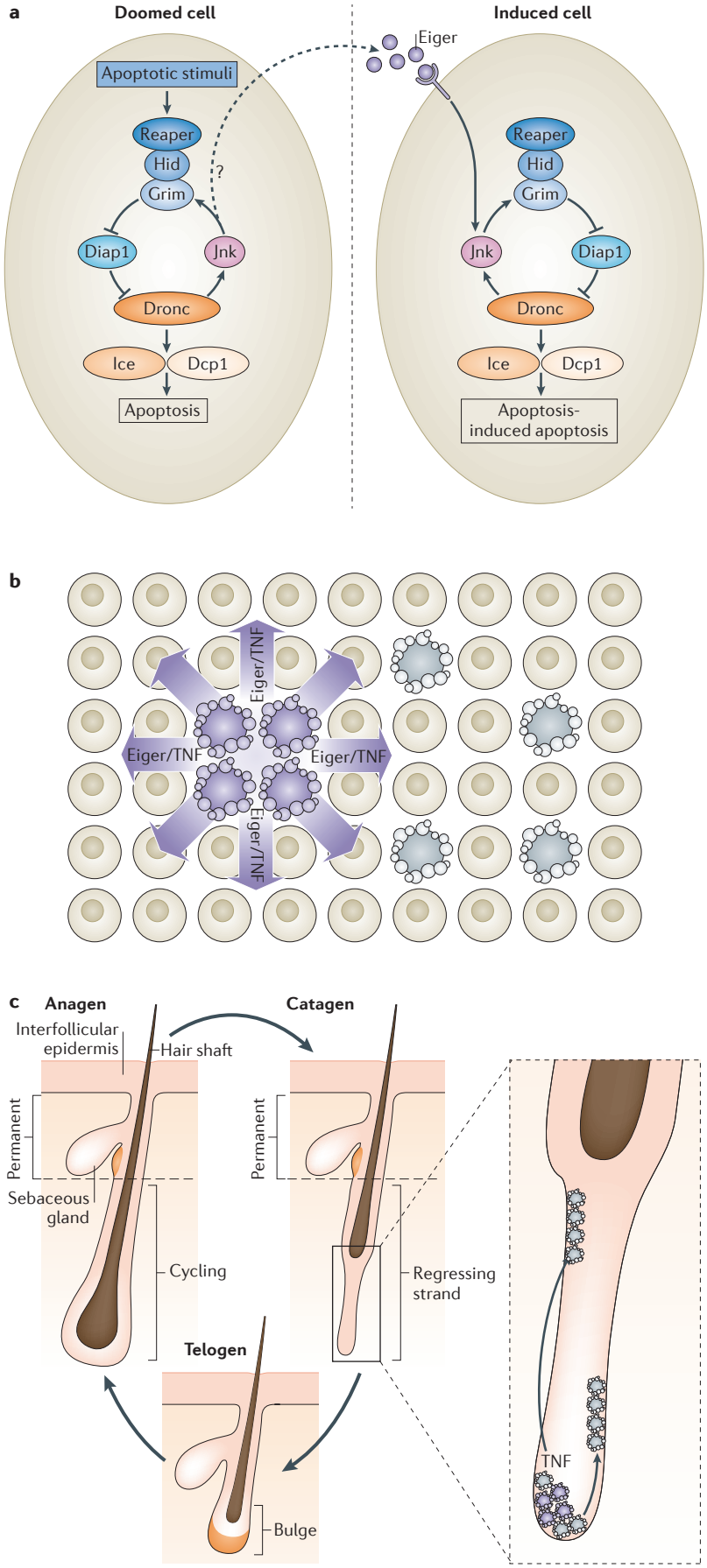
Taken together, there is now a considerable body of evidence indicating that a key function of caspases in dying cells is to facilitate the release of mitogens that stimulate stem or progenitor cell proliferation and thereby mediate regeneration. Given the strong connections between stem cells, regeneration and tumour growth, apoptosis-induced proliferation may also contribute to cancer. Collectively, these findings challenge the simplistic view of apoptosis as a tumour-suppressive or tumour-preventive mechanism. On the one hand, apoptosis suppresses tumour formation by removing damaged and unwanted cells; on the other hand, apoptosis may stimulate tumour growth through apoptosis-induced proliferation. Additional support for this idea comes from the finding that prostaglandin E_2 is released by bladder cancer cells and stimulates the proliferation of cancer stem cells, thereby leading to tumour repopulation[162]. Furthermore, deficiency in caspase 3 causes sensitivity towards radiotherapy, suggesting the existence of a cell death-induced tumour repopulation mechanism[163].

In the future, it will be interesting to examine apoptosis-induced proliferation in different human cancers and to explore dual-agent therapies designed to kill cancer cells while simultaneously blocking mitogens that might induce proliferation.

Apoptosis-induced apoptosis. Developmental apoptosis often involves the removal of large groups of cells in a cohort[8,16]. Examples of such 'communal suicide' include various morphogenic events in *D. melanogaster*, the elimination of the *X. laevis* tadpole tail and removal of the interdigital webbing in vertebrates. However, how this collective death occurs remains largely undefined. New insights have come from the recent finding that apoptotic cells can instruct additional death in their neighbourhood in a phenomenon termed apoptosis-induced apoptosis[164]. In the *D. melanogaster* wing disc, which comprises an anterior and a posterior compartment, induction of large-scale apoptosis in the posterior compartment surprisingly resulted in the apoptosis of cells that were located at a considerable distance in the anterior compartment[164].

Apoptosis-induced apoptosis in the wing disc uses Eiger (an orthologue of mammalian TNF) to instruct death from a distance. Eiger is produced by dying cells of the posterior compartment and activates Jnk in anterior compartment cells to initiate apoptosis[164] (FIG. 5a,b). Apoptosis-induced apoptosis also takes place in vertebrates and has an important physiological function in the coordinated elimination of hair follicle cells. The hair follicle undergoes cycles of rest (telogen), growth (anagen)

Figure 5 | **Apoptosis-induced apoptosis.** **a** | Expression of the inhibitor of apoptosis antagonists Hid or Reaper in 'doomed' cells (left panel) in *Drosophila melanogaster* can cause apoptosis in distant cells (right panel). This is very likely to be true for Grim as well, but this has not yet been demonstrated experimentally. The apoptotic signal is propagated by Eiger (a homologue of mammalian tumour necrosis factor (TNF)), which can cross compartmental borders (marked by a dashed line) to activate Jun amino-terminal kinase (Jnk) and instruct apoptosis in distant cells. The production of Eiger is thought to depend on Jnk (depicted by a question mark). **b** | Apoptosis-induced apoptosis is represented schematically. Purple cells represent the initial apoptotic focal point. These cells emit Eiger in *D. melanogaster* and TNF in mice, resulting in secondary apoptosis (grey cells). **c** | Hair follicles cycle through phases of growth (anagen), destruction (catagen) and rest (telogen). During catagen, apoptosis eliminates the lower two-thirds of the hair follicle. Post-catagen, the hair follicle enters telogen, which is followed by a new cycle of hair growth (anagen). The inset illustrates how apoptosis-induced apoptosis regulates the hair follicle cycle. During catagen, a primary apoptotic event (purple cells) induces a wave of secondary apoptosis (grey cells) that is required for the cohort elimination of transient hair follicle cells. Dcp1, Death caspase 1; Diap1, Death-associated inhibitor of apoptosis 1; Dronc, Death regulator Nedd2-like caspase.



and degeneration (catagen; in which cells that are located in the lower portion of the hair follicle are eliminated by apoptosis)[165]. During catagen, apoptotic cells produce TNF, which is required for cell death and for progression of the hair follicle cycle[164] (FIG. 5c). Similarly, in situations in which DNA damage is too great to repair, TNF is expressed by apoptotic cells. In this scenario, ataxia telangiectasia mutated (ATM) activates NF-κB, which induces expression of TNF, thus creating a feedforward loop that promotes apoptosis and secretion of IL-8 (REF. 166). Interestingly, in contrast to these findings, Tnf that is produced by dying retinal neurons in zebrafish drives glial cell proliferation during retinal regeneration[167].

In Planaria, amputation induces two successive waves of apoptosis: an immediate, localized response (which peaks after 1–4 hours) and a second, systemic response (which peaks 3 days post-amputation) that can be induced in uninjured organs, presumably to ensure proper remodelling[168].

Inter-organ communication can also be seen in *D. melanogaster* in response to tissue damage. Wounding the cuticle results in apoptosis in the mid-gut, an organ that is distant from the wound site. ROS can mediate the induction of apoptosis in mid-gut cells, which activates a tissue stem cell regeneration pathway. Intriguingly, blocking mid-gut cell death post-wounding leads to mortality in flies, suggesting that this mechanism dampens the dangerous systemic wound reaction[169].

The observation that apoptotic cells can facilitate additional cell death potentially has many important implications. Apoptosis-induced apoptosis may function in sculpting and deletion of tissues in which the complete elimination of entire cell groups is required. In addition, this process might be an efficient means to inhibit viral spreading and might occur in pathologies that are associated with extensive cell death, such as ischaemic stroke, liver failure and neuronal degeneration. Furthermore, apoptosis-induced apoptosis may provide an explanation for the 'bystander effect', whereby non-irradiated cells respond in the same manner as radiation-exposed cells[170,171].

In summary, apoptotic cells have the unexpected capacity to stimulate either cell proliferation or further cell death. At this time, it is not clear how the decision between these opposing outcomes is made, and we still have a very incomplete understanding of the role and regulation of cell death *in vivo*. Further work in this area is likely to yield many surprises and to facilitate the design of rational therapies that will target cell death pathways.

Looking ahead

Since the original proposition of a cellular mechanism of PCD, tremendous progress has been made towards elucidating the biochemical mechanism of apoptosis. In retrospect, it is remarkable how the discovery of the apoptotic cell death machinery in *C. elegans* paved the way for unravelling the more intricate systems seen in *D. melanogaster* and mammals. However, we are only now beginning to understand the *in vivo* role of key apoptotic components in mammals, which holds much promise for the treatment of various human disorders.

The synonymy of PCD with apoptosis is now outdated, as alternative cell death mechanisms have been described. One prominent process that is often associated with apoptosis is autophagy. However, additional work is needed to clarify whether autophagy is a bona fide mechanism for cell killing *in vivo*.

Another cell death process that is being intensely studied is necroptosis. Perhaps the main obstacle that limits our understanding of this process is the challenge of identifying necroptotic cells *in vivo* and distinguishing whether a cell dies from necroptosis or from necrosis generated in another manner. In addition, it remains to be clarified whether necroptosis drives inflammation or is driven by it.

At this time, we do not understand why there are so many different ways for a cell to die, how cells are selected *in vivo* to activate any of these mechanisms of PCD or how survival and death signals are integrated to direct a specific cell fate. Finally, an exciting field of research concerns signals emanating from dying cells. Much remains to be discovered about the complex signals that dying cells use to instruct their neighbours and the role of these signals under physiological conditions. Given the importance of cell death during development, homeostasis and disease, deciphering the intriguing 'last words' of dying cells promises exciting new discoveries that may have considerable therapeutic value.

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Acknowledgements
The authors thank Y. Fox for help with the figures and F. Kol for ongoing support. H.S. is an investigator with the Howard Hughes Medical Institute and is supported by the US National Institutes of Health grant RO1GM60124. Y.F. is supported by the Deloro Career Advancement Chair and the Alon Fellowship.

Competing interests statement
The authors declare no competing interests.