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REVIEW

Alternative cell death mechanisms in development and beyond

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A canonical regulatory pathway involving the members of the Bcl-2 and caspase families has been established to regulate developmental apoptosis in nematodes and flies. However, mutant mice that have major deficiencies in this apoptosis pathway show only relatively minor developmental defects. Recent revelations indicate that multiple mechanisms are involved in regulating cell death during mammalian development, tissue homeostasis, and pathological cell loss. Here, we critically evaluate the evidence demonstrating the existence of alternative cell death mechanisms, including apoptosis of lower organisms in the absence of canonical apoptosis mediators, autophagic cell death, necroptosis, elimination by shedding, keratinocyte death by cornification, and cell–cell cannibalism by entosis. The physiological relevance of alternative cell death mechanisms as primary and backup mechanisms is discussed.

Apoptosis is a regulated form of cellular suicide that is tightly controlled by a genetic program (Yuan and Horvitz 2004). In the early days of apoptosis research, many research papers were introduced with statements such as “Apoptosis, or programmed cell death, is essential for development and tissue homeostasis.” Since apoptosis was discovered as a major sculpting force in the development of *Caenorhabditis elegans* (Yuan and Horvitz 2004), this cell death modality was expected to be important for the development of mammals as well. Thus, it was highly surprising that mice lacking the key elements of the core machinery of apoptosis (including multiple caspase mutants, *apaf-1*^{-/-}, or *bax*^{-/-}; *bak*^{-/-} double-knockout mice) can survive embryonic development and develop into largely normal adulthood (Chautan et al. 1999; Lindsten and Thompson 2006). Although redundancy of different caspases may explain the survival of caspase-deficient mice, major apoptotic defects are well demonstrated to affect tissues and cells from *apaf-1*^{-/-} and *bax*^{-/-}; *bak*^{-/-} mice.

How can a major defect in apoptosis be conciliated with a near-to-normal physiology in mammals? There are only

two ways out of the dilemma. Either cell death is not important for development and adult tissue homeostasis—a hypothesis that is refuted by multiple lines of evidence—or alternative cell death mechanisms may operate to eliminate cells in the absence of the apoptotic machinery. Thus, it is our firm belief that a complete description of the nonapoptotic pathways that cause cellular demise is crucial for the comprehension of normal physiology as well as pathological cell losses. Here, we discuss the major alternative routes to developmental cell death.

Suppressing apoptotic and nonapoptotic cell death during development

Interdigital cell death during embryonic development has been used as a quintessential example to underscore the cardinal importance of apoptotic cell death as a sculpting force during morphogenesis. Surprisingly, however, none of the apoptosis-deficient mutant mice manifested severe syndactyly, the fusion among fingers or toes that results from the persistence of interdigital membranes. Among different genetic models of apoptosis deficiency, *bax*^{-/-}; *bak*^{-/-} double-knockout mice provide the best example of syndactyly (Lindsten and Thompson 2006). However, compared with that of mutants in the Wnt signaling pathway (MacDonald et al. 2004; Johnson et al. 2005; Morello et al. 2008), the syndactyly phenotype exhibited by *bax*^{-/-}; *bak*^{-/-} mice can only be considered as a partial defect. Although it is possible that the defects in morphogenesis (not just cell death) also contribute to the severe phenotype of syndactyly that is observed in certain mutants of the Wnt pathway, we should consider the possibility that the severe syndactyly phenotype involves the suppression of both apoptosis and backup cell death mechanisms. Consistent with this possibility, a detailed analysis of the interdigital cell death in *apaf-1* mutant mice, which lack the key adaptor protein for mediating caspase-9 activation, led to the discovery that some cell deaths exhibit features of necrosis (Chautan et al. 1999; Cande et al. 2002). This result suggests the activation of alternative, nonapoptotic cell death mechanisms when *apaf-1*-dependent caspase activation is blocked. Indeed, interdigital cell death is only slightly

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delayed in *apaf-1*-deficient mice, suggesting that nonapoptotic mechanisms come into action as a backup mechanism when apoptosis is inhibited.

On the other hand, constitutive activation of the FGF (fibroblast growth factor) pathway due to loss-of-function mutations of its negative regulator (BMP [bone morphogenetic protein]) (Guha et al. 2002) or gain-of-function mutations of FGF receptors promotes syndactyly in humans (Macias et al. 1996). One possible signaling pathway by which these mutations cause syndactyly may be through suppression of the expression of *Dickkopf1* (*Dkk1*), an inhibitor of the Wnt pathway that is highly expressed in regressing interdigital tissues. Mice deficient in *Dkk1* exhibit severe syndactyly (MacDonald et al. 2004). The role of Wnt in regulating interdigital cell death is further strengthened by the syndactyly phenotype of loss-of-function mutants of other WNT inhibitors: *Sfrp2* (secreted frizzled-related protein-2) (Morello et al. 2008) and *Megf7/LRP4*, a member of the low-density lipoprotein (LDL) receptor-related proteins (LRPs) that antagonize LRP6-mediated activation of canonical Wnt signaling (Johnson et al. 2005). Since the activation of Wnt is sufficient to induce syndactyly, Wnt signaling is probably involved in regulating both apoptotic and nonapoptotic cell death mechanisms in the interdigital tissue. This also raises the possibility for a role of nonapoptotic cell death mechanisms in tumorigenesis, because constitutive activation of the Wnt pathway, often due to loss-of-function mutations in its negative regulator, *APC* (*adenomatous polyposis coli*), is common in familial and sporadic colorectal carcinomas (Gregorieff and Clevers 2005).

An intriguing—yet unproven—working hypothesis would predict that prolonged clonogenic survival, in either interdigital tissue or cancer cells, would rely on the simultaneous blockade of both apoptotic and nonapoptotic cell death mechanisms.

Mechanisms of programmed cell death in the nematode *C. elegans*

The seminal work by Horvitz and colleagues (Yuan and Horvitz 2004) in the late 1980s and early 1990s provided first insights into the molecular mechanism that mediates developmental cell death in *C. elegans*. The gene products of *ced-3* (a caspase), *ced-4* (a caspase activator homologous to Apaf-1), *ced-9* (a multidomain protein of the Bcl-2 family), and *egl-1* (a proapoptotic BH3-only protein) constitute the prototypic apoptotic machinery that regulates the programmed death of 131 cells during development. Curiously, the development and adult lives of *ced-3* and *ced-4* mutant animals, which carry a significant number of extra cells that normally would have died in wild-type animals, are largely normal (Ellis and Horvitz 1991). This could be explained in a number of ways. First of all, it is possible that, in a primitive organism such as *C. elegans*, this cell death mechanism does not serve a major function from an organismal point of view—at least in the rather protective laboratory environment. It has been proposed that *ced-3* and *ced-4* are indispensable for the host defense against pathogenic bacteria (Aballay and Ausubel

2001); thus, the advantage of carrying intact *ced-3* and *ced-4* genes might become manifest only when the nematodes are kept in their natural habitat: soil. Alternatively, it is conceivable, yet remains to be investigated in detail, that at least a portion of “undead” cells may eventually be eliminated from *ced-3* and *ced-4* mutant nematodes through alternative nonapoptotic mechanisms.

There is compelling evidence for the existence of nonapoptotic developmental cell death in *C. elegans*. One clear example is the death of linker cells in male *C. elegans* that occurs normally in *ced-3* or *ced-4* mutant males (Abraham et al. 2007). The disappearance of linker cells facilitates the connection of the male reproductive system to the exterior. Although the linker cell death was initially believed to be a “murder” by engulfment (Sulston et al. 1980), subsequent detailed analysis demonstrated that engulfment is not required for linker cell death. The death of linker cells is controlled by developmental timing because, in *lin-29* mutants, which exhibit retarded development, linker cells persist beyond the point at which they would have died in wild-type animals. Dying linker cells do not manifest any signs of nuclear condensation, the morphological hallmark of apoptosis, yet extensively accumulate cytoplasmic vesicles with single membranes. These cytoplasmic vesicles are believed to be distinct from autophagosomes, as knockout of major autophagy genes—including *bec-1* and *unc-51*, the *C. elegans* orthologs of *Beclin 1* and *Atg1/Unc1*, respectively—have no effect on linker cell death (Abraham et al. 2007). While the molecular mechanism that mediates the death of linker cells is still not clear, the morphology of dying linker cells shares some resemblance with nonapoptotic cell deaths in vertebrates. One such example is the “dark” cell death of spinal motor neurons in developing *caspase-3*^{-/-} or *caspase-9*^{-/-} mouse embryos that is characterized by mitochondrial swelling and electron-translucent cytoplasmic vacuoles (Oppenheim et al. 2001) similar to that of dying linker cells. While it is clear that the nonapoptotic cell death modality that operates in male linker cells is not a “backup” mechanism that becomes manifest only when apoptosis is suppressed, it remains to be seen whether the “dark” cell death may be activated in the absence of the normal programmed cell death machinery, such as in *ced-3* and *ced-4* mutants.

In *C. elegans* expressing a mutant MEC-4 protein (which is an amiloride-sensitive Na⁺ channel from the degenerin family), mechanosensory neurons undergo necrosis (Driscoll and Chalfie 1991). The dominant gain-of-function mutant MEC-4 protein mediates an increased ion flow across the plasma membrane, eventually leading to the activation of aspartyl and calpain proteases and lysosome-mediated necrosis (Syntichaki et al. 2002). Importantly, knockdown of several autophagy genes coding for the nematode orthologs of *Beclin 1*, *Atg8/LC3*, and *Atg18* prevented necrosis induced by the mutant MEC-4 protein (Samara et al. 2008), underscoring the importance of the lysosomal cell death pathway for necrotic cell death. The integrity of lysosomes may be regulated by serpins in *C. elegans*, as *srp-6* mutant animals show enhanced sensitivity toward cellular stresses such as hypostomic shock, heat shock,

oxidative stress, and hypoxia (Luke et al. 2007). However, it is not clear to what extent this lysosomal pathway is regulatable and whether it contributes to cell death during *C. elegans* development and tissue homeostasis.

'Apoptosis' without the canonical regulators of apoptosis

Caspases are phylogenetically restricted to animals only. Although the genomes of plants and single-cell eukaryotes do not encode canonical caspases, they nevertheless undergo regulated cell death with morphological features similar to that of apoptosis. For example, yeast cells can undergo cell death associated with chromatin condensation, DNA fragmentation, and phosphatidylserine exposure; when yeast is made to express a CDC48 mutant (Madeo et al. 1997); when yeast is treated with external stressors, including hydrogen peroxide or acetic acid; or when yeast undergoes chronological aging (Madeo et al. 2002). This has given rise to the notion that yeast can also undergo apoptosis, which is at odds with the idea that apoptosis must be mediated by canonical regulators including proapoptotic multidomain Bcl-2 family proteins and caspases. Many investigators in the cell death field argue that, since yeast does not encode any canonical caspase or (known) Bax/Bak analogs, yeast cell death should not be termed "apoptosis." However, it is intriguing that dying yeast cells can acquire the morphological features of apoptosis (Carmona-Gutierrez et al. 2010), suggesting that several hallmarks of apoptosis—including nuclear pyknosis, chromatin condensation, and phosphatidylserine exposure—are not truly specific biomarkers for caspase activation and thus must be interpreted with caution. For example, cell death with morphological features of apoptosis during the development of vertebrates has traditionally been regarded as evidence of the activation of one uniform, caspase-driven biochemical cell death pathway. However, we must consider the possibility that at least some of those cell deaths with morphological features of apoptosis that occur during the development of vertebrates arise without the obligatory contribution of the canonical apoptosis pathway.

Nonetheless, yeast possess some elements that resemble those involved in the canonical pathway of animal cell apoptosis. Mitochondria are involved in mediating yeast cell death (Cheng et al. 2008), and the yeast genomes do encode homologs of mammalian cell death effectors, including a metacaspase termed YCA1 (Madeo et al. 2002), apoptosis-inducing factor (Aifp) (Wissing et al. 2004), and HtrA2/Omi (Nma111p) (Fahrenkrog et al. 2004). Since yeast Aifp is a mitochondrial DNase that translocates to the nucleus upon lethal stress with H₂O₂ or acetic acid (Wissing et al. 2004), while Nma111p is a serine protease that is normally localized in the nucleus (Fahrenkrog et al. 2004), activation of Aifp and Nma111p might contribute to the acquisition of the apoptotic phenotype. Moreover, YCA1 overexpression can induce yeast cell death with morphological features of apoptosis, and its deficiency reduces cell death induced by oxidative stress, acetate, or chronological aging (Madeo et al. 2002).

Intriguingly, plant metacaspases, which are distant phylogenetic relatives of animal caspases, as is YCA1, can cleave some nuclear substrates that are also cleaved by animal caspases (Sundstrom et al. 2009). Thus, although metacaspases have a different cleavage specificity than caspases—they hydrolyze proteins after arginine or lysine (basic residues), not after aspartate (an acidic residue)—they can share substrates over large evolutionary distances. Nonetheless, alternative interpretations are possible, and YCA1 might regulate autophagy in an indirect fashion. For instance, YCA1 has been shown to be a stress response protein associated with chaperones. Loss of YCA1 in *Δyca1* cells induces an increase in stress response protein levels and the formation of autophagosomes (Lee et al. 2010). It remains to be seen if the increased resistance of *Δyca1* yeast cells to H₂O₂ or acetic acid is due exclusively to the lack of proteolytic execution, or whether it might involve a constitutively activated stress response that mediates an elevated level of cytoprotection.

The fact that yeast apparently lacks a canonical apoptosis pathway, but nonetheless can acquire an "apoptotic" morphology in response to lethal stimuli, points to the possibility that mammalian cells may similarly succumb to an "apoptotic" cell death without the canonical apoptosis effectors coming into action. For example, the caspase-independent, phylogenetically conserved death effector termed AIF has been shown to mediate chromatin condensation and induce phosphatidylserine exposure as a backup mechanism in both human cells (Susin et al. 2000) and *C. elegans* (Wang et al. 2007) when caspase activation is inhibited. In some paradigms of yeast cell death (Wissing et al. 2004) as well as in mammalian neurons (Cregan et al. 2004), AIF is important for cell death induction, suggesting that caspase-independent apoptosis may be mediated, at least in some instances, by AIF. However, systematic studies are needed to explore the question regarding in which cases cells dying with an apoptotic morphology succumb to caspase-independent programs.

Autophagic cell death—elimination of cells in toto

Morphological studies of developing embryos have led to the detection of certain dying cells with extensive accumulation of vesicles, a phenomenon that has been called "autophagic cell death" (Schweichel and Merker 1973). However, the activation of autophagy serves in most conditions to promote survival (Levine and Yuan 2005); the existence of autophagic cell death and the exact contribution of the canonic autophagic pathway to "autophagic cell death" are not yet established.

Insect metamorphosis provides a convenient and highly synchronized model of developmental cell death, as larval organs are destroyed in toto during a short period of time. Metamorphic cell death of the salivary glands from developing *Drosophila melanogaster* larvae is initiated by a rise in ecdysone, which promotes the up-regulation of several caspases (*dronc*, *drice*, and *strica*) and *ark*, along with several *Atg* genes (Berry and Baehrecke 2007). However, inhibition of caspases is not sufficient to block the degeneration of the salivary gland, suggesting the involvement of

additional mechanisms. When combined with caspase inhibition, mutations in *Atg5* or loss-of-function mutations in *Atg2*, *Atg3*, or *Atg18* lead to the persistence of salivary glands for ~24 h beyond the time when they normally would have died in control larvae. Thus, autophagy, or at least certain autophagic genes, are involved in the death of salivary gland cells during metamorphosis. However, it is also possible that only a selected few among all of the genes involved in mediating canonical autophagy are involved in mediating “autophagic cell death.” This could explain why deficiencies in some autophagy genes (e.g., *atg1*, *atg2*, and *atg18*) have stronger effects on metamorphic cell death than those in others (e.g., *atg7*).

Interestingly, *drpr*, the fly homolog of the *C. elegans* engulfment gene *ced-1*, as well as several factors involved in the engulfment of dying cells from programmed cell death—such as *six microns under (simu)*, *croquemort (crq)*, *ced-6*, *src oncogene at 42 A (Src42A)*, *ced-12*, *crk*, and *myoblast city (mbc)*—were found recently to be involved in the activation of autophagy during metamorphosis of salivary gland. Furthermore, Drpr was found to be required for induction of both autophagy and cell death in metamorphic salivary glands (McPhee et al. 2010). Thus, the mechanism of engulfment may provide a direct link to connect cell death to autophagy.

Although the published data (McPhee et al. 2010) is consistent with a cell-autonomous mode of action for Drpr to mediate autophagy activation during degeneration of salivary gland, an alternative, yet-to-be-excluded possibility is that Drpr may activate autophagy in cells other than the dying ones, and that this distant autophagy may be required for the coordination of the cell death process that culminates in the attrition of an entire tissue. In this regard, it might be interesting to examine if dying cells in metamorphic salivary glands also express Pretaporter, a ligand that binds to the extracellular region of Drpr and participates in the clearance of apoptotic cells (Kuraishi et al. 2009), and whether Pretaporter exposure is regulated by *atg* genes.

During the developmental programmed cell death in *C. elegans*, two pathways control the engulfment of dying cell corpses on the side of engulfing cells. The first pathway—which includes *ced-1*, *ced-6* (GULP), *ced-7* (ABC transporter), and *dyn-1* (Dynamain, a large GTPase)—functions to recognize dying cells and transduce engulfment signals. The second pathway—including *ced-2* (CrkII), *ced-5* (Dock 180), *ced-10* (Rac guanosine triphosphatase), *ced-12* (ELMO), and *psr-1* (phosphatidylserine receptor)—activates cytoskeleton rearrangement in the engulfing cells (Reddien and Horvitz 2004). During phagocytosis, the receptor CED-1 clusters in the phagocytic cup before quickly encircling the cell corpse. Mutations in engulfment genes, such as *ced-1*, can enhance the persistence of undead cells caused by partial loss-of-function mutations in killer genes, such as *ced-3* (Reddien et al. 2001). Thus, the engulfment process may have a role in ensuring the completion of the cell death process. On the other hand, degradation of apoptotic cells in the engulfing cells involves the sequential enrichment of CED-1, DYN-1, phosphatidylinositol 3-phosphate (PI3P), and the small GTPase RAB-7 on the phagocytic

membranes of the engulfing cells, followed by the fusion of endosomes and lysosomes to phagosomes (Yu et al. 2008). Thus, an alternative possibility is that Drpr performs a similar function in dying cells as that in the engulfing cells by inducing the formation of (auto)phagosomes during the metamorphic death of salivary gland cells.

Degeneration of midgut during metamorphosis of *Drosophila* provides another example of autophagic cell death. Like in salivary glands, programmed cell deaths triggered by steroids lead to the destruction of larval midgut structures. Despite high levels of caspase activity in metamorphic midgut in wild-type *Drosophila*, caspases are not required for midgut degradation. Dying midgut cells manifest the accumulation of pGFP-Atg8a in cytoplasmic puncta, which is a sign of autophagy. Furthermore, knockdown of several *Atg* genes that are transcriptionally upregulated at the time of midgut cell death severely delays midgut degradation (Denton et al. 2009). These data indicate again an important role for *Atg* genes in midgut destruction, yet do not clarify whether these genes act in a cell-autonomous fashion.

Autophagic cell death has also been noted in *Dictyostelium discoideum*, a simple eukaryote that lives in the soil and feeds on bacteria (Giusti et al. 2009b). In the vegetative state, when food is abundant, *Dictyostelium* cells divide by binary fission; however, when food is exhausted, starvation triggers the formation of multicellular aggregates in which coordinated morphogenesis and cellular differentiation facilitates the formation of a fungus-like structure that carries spore-forming cells on the top of a stalk composed of cells that have undergone programmed death and lignification. The *Dictyostelium* genome does not encode caspases or Bcl-2 family proteins, yet does contain a set of *atg* genes similar to those found in yeast and mammals. One particular *Dictyostelium* cell line can be driven into programmed death by starvation and simultaneous addition of the differentiation factor DIF. This programmed death is autophagic in that it is accompanied by the accumulation of vacuoles, which are suppressed by mutations in *atg1*. However, *atg1* mutant *Dictyostelium* cells still die in response to starvation plus DIF, while manifesting a necrotic phenotype characterized by mitochondrial uncoupling, the clustering of organelles, lysosomal permeabilization, and eventual disruption of the cells into debris (Giusti et al. 2009a). Although Atg1 is clearly required for the vacuolization of *Dictyostelium*, the manner in which Atg1 is involved in cell death is still debatable. It is possible that Atg1 is indeed required for cell death of *Dictyostelium* induced under starvation condition with the presence of DIF. In the absence of Atg1, however, a “backup” cell death mechanism would be activated to mediate cell death. Alternatively, Atg1 may be required only for vacuolization, which would constitute an epiphenomenon that accompanies cell death but is not required for the lethal process. In the Atg1 mutant, the cell death exhibits an alternative morphology, but the fundamental mechanism that mediates cell death may not have changed (Kosta et al. 2004).

Transgene-enforced overexpression of *Atg1* in *Dictyostelium* is sufficient to induce ectopic autophagy and cell

death in several *Drosophila* tissues, including the imaginal discs, fat body, and salivary glands (Scott et al. 2007). Cell death induced by overexpression of *Atg1* shows typical signs of apoptosis, including caspase activation and DNA fragmentation, and is inhibited by the caspase inhibitor p35. Thus, in contrast to metamorphic cell death of the salivary gland, where caspase activation occurs upstream of or in parallel with autophagy activation, high levels of autophagy induced by *Atg1* overexpression may lead to cell destruction in a caspase-dependent manner. Since *Atg1* clearly has functions other than mediating autophagy—e.g., regulating axonal transport (Toda et al. 2008)—it remains to be seen whether *Atg1*-induced caspase activation is truly due to excessive autophagy.

Necroptosis—receptor-interacting protein 1 (RIP1) kinase activity-dependent necrotic cell death

Necrosis had traditionally been considered an accidental cell death that is not subject to cellular regulations. Necrotic cell death is morphologically characterized by an increase in cell volume, swelling of organelles, and plasma membrane rupture, followed by leakage of intracellular contents. However, it has now been established that at least a part of necrotic cell death may be executed through a mechanism termed “necroptosis” (Degterev et al. 2005). Recent advances have begun to elucidate the molecular mechanisms that ignite and execute this necrotic cell death program. Necroptosis may be activated upon stimulation by tumor necrosis factor α (TNF α), FasL, and TRAIL, the very same ligands that can activate apoptosis in the death receptor pathway. Thus, cell death induced by the activation of the death receptor may be executed through alternative cell death pathways, apoptosis, or necroptosis.

The kinase activity of RIP1 is specifically required as an upstream regulator of necroptosis

RIP1, a death domain-containing kinase, plays a critical role in necroptosis. Its serine/threonine kinase activity is essential for the necroptotic death pathway, but is dispensable for both NF- κ B activation and apoptosis, which rely on the intermediate and death domains of the RIP1 protein (Holler et al. 2000). Importantly, a small molecule inhibitor of necroptosis, necrostatin-1 (Nec-1), is a potent inhibitor of RIP1 kinase activity and necroptosis (Degterev et al. 2008).

Under apoptosis-competent conditions, TNF α stimulation sequentially induces the formation of two protein complexes, complex I and complex IIa, which stimulate NF- κ B activation and apoptosis, respectively (Fig. 1; Micheau and Tschopp 2003; Declercq et al. 2009). TNF α binding to TNF receptor 1 (TNFR1) induces the recruitment of RIP1 and other proteins to the receptor to form complex I. Subsequently, these components dissociate from TNFR1, and RIP1 can be found in the cytosol within complex IIa, which includes RIP1, caspase-8, and Fas-associated death domain (FADD). The signal that stimulates the transition from complex I to complex IIa is currently unclear. An alternative multiprotein aggregate, called complex IIb, may form in apoptosis-deficient con-

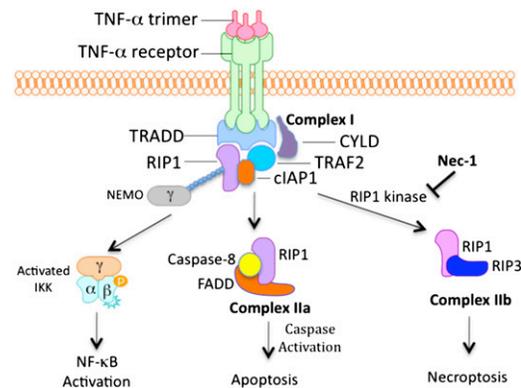


Figure 1. The signaling complexes are induced by TNF α to mediate NF- κ B activation, apoptosis, and necroptosis. Stimulation of TNFR1 by TNF α leads to the formation of an intracellular complex at the cytoplasmic membrane (complex I) that includes TRADD, TRAF2, RIP1, and cIAP1. Ubiquitination of RIP1 at K377 by cIAP1 leads to the recruitment of NEMO, a regulatory subunit of the IKK complex that in turn activates the NF- κ B pathway. RIP1 is also involved in the formation of complex IIa, including FADD and caspase-8, to activate a caspase cascade to mediate apoptosis. Under apoptosis-deficient conditions, or when cells are infected by certain viruses, RIP1 interacts with RIP3 to form complex IIb, which is involved in mediating necroptosis. The formation complex IIb requires the kinase activity of RIP1 that is inhibited by Nec-1. (Reproduced from Christofferson and Yuan [2009] with permission from Elsevier [© 2009].)

ditions; in particular, in the presence of caspase-8 inhibitors. Complex IIb contains at least one additional protein component: RIP3. Treatment with the RIP1 kinase inhibitor Nec-1 prevents the recruitment of both RIP1 and RIP3 to complex IIb, suggesting that RIP1 kinase activity is important for this complex formation in necroptotic cells (Cho et al. 2009; He et al. 2009). The interaction of RIP1 and RIP3 via their RIP homotypic interaction motif (RHIM) domains plays a critical role in mediating downstream necroptotic event (Sun et al. 2002).

CYLD is an important regulator of necroptosis

In a genome-wide siRNA screen, Hitomi et al. (2008) identified CYLD to be vital for mediating both apoptosis and necroptosis in response to TNF α stimulation. CYLD is a deubiquitinase that functions as a tumor suppressor. Loss-of-function mutations in CYLD give rise to familial cylindromatosis, a rare skin condition in which patients develop benign tumors in hair follicles and sweat glands of the head and neck (Bignell et al. 2000). CYLD is recruited to the TNFR1 complex upon stimulation by TNF α . Since RIP1 kinase is ubiquitinated within the TNFR1 complex, its deubiquitination by CYLD may be important for the signal transduction of both apoptosis and necroptosis. A number of E3 ligases have been implicated in RIP1 ubiquitination, including cIAP1/2, TRAF2, TRAF6, and A20 (Park et al. 2004; Wertz et al. 2004; Festjens et al. 2007). Thus, it is possible that CYLD regulates necroptosis by directly modulating RIP1 ubiquitination. However, it has been reported recently that the loss of CYLD

enhances Wnt/ β -catenin signaling through an increased K63-linked ubiquitination of Dishevelled (Dvl) (Tauriello et al. 2010). Since activating mutations in the Wnt pathway cause syndactyly, likely due to the suppression of apoptotic and nonapoptotic cell death pathways (see above), it is possible that inhibition of CYLD contributes to the suppression of necroptosis by up-regulating Wnt signaling. This intriguing possibility remains to be explored.

The downstream mechanisms of necroptosis

We still know very little about the downstream mechanism mediating the execution of necroptosis. Reactive oxygen species (ROS) production has been proposed to be an executioner of necroptosis in certain cell types. L929 fibrosarcoma cells and mouse embryonic fibroblasts (MEFs) produce ROS in response to TNF α stimulation (Goossens et al. 1999; Lin et al. 2004). Treatment with an antioxidant such as butylated hydroxyanisole (BHA) quenches ROS and prevents cell death in some cell types (Lin et al. 2004). However, antioxidant treatment is unable to rescue all cell lines from necroptosis (Degterev et al. 2005), suggesting the cell type-dependent involvement of ROS in the execution of this cell death modality.

RIP3 has been reported to interact with several metabolic enzymes, including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL), and glutamate dehydrogenase 1 (GLUD1) (Zhang et al. 2009). These enzymes increase the abundance of substrates for use in oxidative phosphorylation, which is a major source of ROS in the cell. RIP3-deficient cells have reduced ROS production downstream from TNF α signaling (Cho et al. 2009).

The mitochondrion is well known for its function in apoptotic cell death, and has also been implicated downstream from RIP1 (Kroemer et al. 2007). A role for adenine nucleotide translocase (ANT) and cyclophilin D (CypD) in the mitochondrial permeability transition has been proposed in necroptosis. In a cardiac ischemia and reperfusion injury, CypD-deficient mice had decreased infarct size and reduced levels of necrotic cell death compared with control animals (Nakagawa et al. 2005). A clinical trial confirmed that treatment with cyclosporine A, an inhibitor of CypD, can reduce tissue damage when administered at the time of percutaneous coronary intervention (PCI) after acute myocardial infarction (Piot et al. 2008). CypD is involved in necrotic cell death, although its role in necroptosis has not been specifically tested. TNF α and zVAD-fmk treatment results in a mitochondrial defect in the ADP/ATP exchange through ANT, which is dependent on RIP1 (Temkin et al. 2006). However, there has been no detailed analysis of mitochondrial functions in necroptosis.

Autophagic vesicles are commonly observed in necroptotic cells, leading some investigators to propose that autophagy is an execution mechanism for necroptosis. In L929 cells, treatment with autophagy inhibitors 3-MA and wortmannin are able to partially inhibit zVAD-fmk-induced cell death. Knockdown of autophagy-related genes such as *Beclin 1* and *Atg7* also inhibits necroptosis in L929 cells (Yu et al. 2004). However, in other cell types, this finding has not been confirmed. Jurkat cells, MEFs, and

Balb c/3T3 cells were tested extensively for the cytoprotective effect of autophagy inhibitors or knockdown of *Beclin 1* and *Atg5*. None of these inhibitors of autophagy were able to reduce cell death (Degterev et al. 2005). Necroptosis signaling may, in fact, activate autophagy, perhaps as a clean-up mechanism for cell death. Experiments using proliferating T cells have shown that loss of FADD or caspase-8 increases cell death with features of autophagy, while it limits cell proliferation (Bell et al. 2008; Ch'en et al. 2008; Osborn et al. 2010). Addition of Nec-1 subverts this phenotype, suggesting that the cells undergo RIP1 kinase-dependent necroptosis (Bell et al. 2008). Thus, in this case, it seems that what was initially thought to be autophagic cell death is actually necroptosis. It remains to be determined if autophagy is actually a response to necroptotic cell death.

Necroptosis has been implicated mainly in mediating cell death under pathological conditions. Nec-1 protects neurons against excitotoxins. This applies to glutamate-induced death of hippocampal HT-22 cells (Xu et al. 2007) and NMDA-induced death of rat cortical neurons (Li et al. 2008). Excitotoxicity is involved in the pathologies associated with chronic neurodegeneration such as Alzheimer's disease and Parkinson's disease, and also in acute neuronal loss resulting from stroke (Moskowitz et al. 2010). Consistently, Nec-1 significantly decreases infarct size upon middle cerebral artery occlusion in a mouse model of stroke (Degterev et al. 2005). There is also accumulating evidence suggesting that necroptosis can occur in the immune system. A systematic screening effort designed to identify genes involved in necroptosis led to the identification of multiple genes that are expressed more in immune cells (and neuronal tissues) than in other cell types (Hitomi et al. 2008). Accordingly, the activation of innate immune signaling through Toll-like receptor 3 (TLR3) in conjunction with IFN γ treatment can induce necroptosis. Importantly, necroptosis may act as a second line of defense against viral infections. Many viruses encode Bcl-2 homologs or caspase inhibitors to block apoptosis (Galluzzi et al. 2008), and a viral inhibitor of RIP1, the cytomegalovirus protein M45, has been discovered (Mack et al. 2008), suggesting that regulation of necroptosis may be involved in the virus-host interaction. Tissues from *RIP3*^{-/-} are protected against necrotic cell death after vaccinia virus infection. However, *RIP3*^{-/-} mice succumb to vaccinia virus infection in conditions in which wild-type mice readily survive (Cho et al. 2009), suggesting that some degree of necroptosis is indispensable for alerting antiviral immune responses.

Shedding—physical elimination of cells

The intestinal epithelium is a self-renewing monolayer of epithelial cells arising from stem cells localized at the base of the crypts. In the mouse, it takes 2–3 d for the epithelial cells to migrate from the crypts to the tips of villi, where they are shed at a rate of 1400 cells per villus per day. Thus, the intestinal epithelium has one of the highest turnover rates in the body (Watson and Pritchard 2000). Shedding of cells leads to their separation from the

extracellular matrix and hence is expected to lead to anoikis, a specific form of apoptosis that is induced by detachment. However, apoptosis-deficient (e.g., *Casp-3*^{-/-}, *Apaf-1*^{-/-}, and *Bax*^{-/-}; *Bak*^{-/-}) mice fail to exhibit a defect in the intestinal epithelium. Although *Bcl-2*^{-/-} mice might have supernumerary apoptotic cells in the intestine, no defect in the villus dimension of mice overexpressing *Bcl-2* in the intestine, as well as in *Bax*^{-/-}; *Bak*^{-/-} mice, has been reported, suggesting that the turnover of intestinal epithelial cells may involve a nonapoptotic cell death mechanism.

Electron microscopic studies revealed a fascinating diversity of cell death morphologies during the shedding of mammalian small intestine cells (Fig. 2; Mayhew et al. 1999). A large percentage of cell deaths occurs upon the formation of a tight junction between the cell that is going to be extruded and neighboring epithelial cells, which may “push” the cell that is destined to die toward the lumen. In this case, live cells are extruded into the lumen and the shed cells acquire an apoptotic morphology only when they have lost contact with the epithelium. However, some shed cells with an anuclear phenotype may die before being shed. Alternatively, certain epithelial cells die in situ and are engulfed by macrophages. Finally, certain epithelial cells die with a morphological resemblance to necrosis, including cell swelling, a decrease in cell electron density, and complete degradation of organelles and membranes. The mechanism that regulates necrosis in the mammalian intestine is unknown. Nonetheless, these morphological studies suggest that multiple mechanisms may be involved in the turnover of the small intestine, consistent with the normal intestinal phenotype of apoptosis-deficient mice.

LKB1, a serine–threonine kinase, is a tumor suppressor that is inactivated in Peutz-Jeghers syndrome, an inherited condition characterized by the development of multiple benign hamartomatous polyps in the gastrointestinal tract. A reduction of apoptosis (determined by the TUNEL method) was found in small intestines of patients with Peutz-Jeghers syndrome, and the expression of LKB1 can stimulate p53-dependent apoptosis (Karuman et al. 2001). Thus, the loss of LKB1 may be sufficient to reduce the rate of intestinal epithelial turnover. How this is achieved is not entirely clear. LKB1 catalyzes the activating phosphorylation of kinases of the AMPK family, which regulates cell cycle and cell growth. Moreover, LKB1 can activate SIK1 (salt-inducible kinase 1), a key modulator of anoikis (Cheng et al. 2009). However, since the turnover of intestine epithelial cells may involve multiple cell death mechanisms beyond anoikis, it appears plausible that LKB1 must affect other mechanisms of cell loss in order to promote the formation of hamartomatous polyps.

Cornification—the specialized death of keratinocytes in skin

Our skin is covered by dead cells, so-called corneocytes, which perform an essential function as a physical and chemical barrier. Keratinocytes, the major cell type in the epidermis, undergo a specialized form of programmed cell death, termed cornification, that is different from canon-

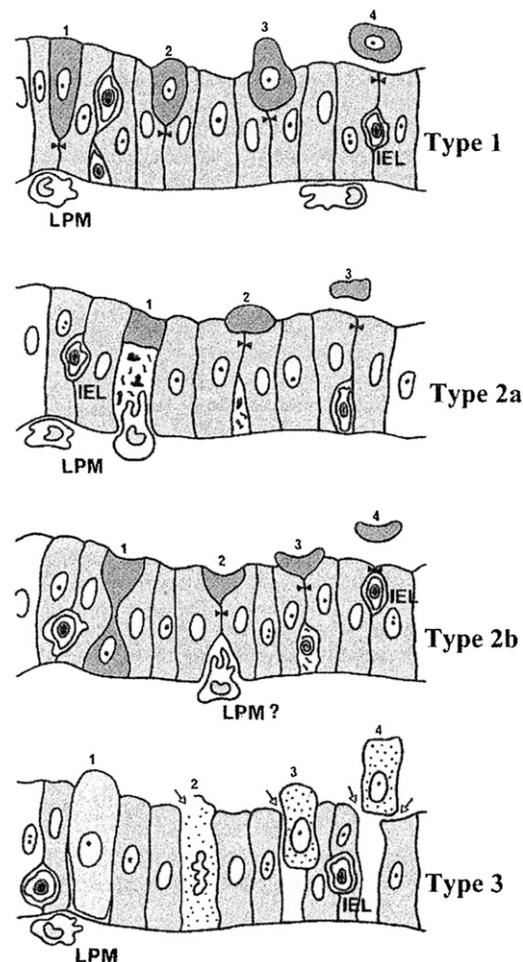


Figure 2. Types of cell death seen in small intestine. (Reproduced with permission from Mayhew et al. [1999].) In type 1 cell death, a sequence of changes (1–4) leads to complete cells being extruded into the lumen. In type 2 cell death, only anucleate apical cell fragments are extruded. Two variants are distinguishable by the fate of the nucleated basal cell fragments. Type 2a cell death (sequence 1–3) creates large intercellular spaces extending from the apical fragment to the basal lamina and containing debris for phagocytosis. Type 2b cell death involves cell shrinkage and in situ degeneration of nucleated fragments in narrow intercellular spaces between adjacent enterocytes (sequence 1–4). The type 1 and type 2 cell deaths exhibit features of apoptosis. Type 3 cell death is reminiscent of necrosis. Cell swelling (1) and degradation (2–4) lead to breaks in epithelial continuity (open arrows), and end in spillage of degraded cell remnants into the lumen (2) or subtotal degradation and extrusion of a complete cell remnant (3–4). (LPM) Lamina propria macrophages; (IEL) intraepithelium lymphocytes.

ical apoptosis. During cornification, the keratinocytes lose their organelles, including the nucleus, to become dead corneocytes. In the final stage, corneocytes are shed from the skin by a process termed desquamation (Lippens et al. 2009). Cornification does not require the canonical apoptosis mediators, as none of the apoptosis-deficient mouse models manifests a defect in cornification. Skin development does require caspase-14, a nonapoptotic caspase expressed exclusively in the cornifying epithelial

cells and activated during cornification (Denecker et al. 2007). Caspase-14^{-/-} mice show defects in stratum corneum formation and reduced skin barrier. In caspase-14^{-/-} mice, profilaggrin, a caspase-14 substrate, is not properly processed into mature filaggrin, which is required for the proper moisturizing and formation of skin barrier (Denecker et al. 2007). However, it is clear that the cornification and desquamation process is largely normal in caspase-14^{-/-} mice; thus, the mechanism that leads to the death of keratinocytes during cornification that removes all of the organelles is still not clear.

Entosis—cell eating cell

Apoptotic cells are commonly engulfed by professional phagocytes (such as macrophages) or nonprofessional neighboring cells. However, live cells may also be engulfed by another cell, a process termed entosis, providing an interesting case of “cannibalism” in cell biology (Fig. 3; Overholtzer et al. 2007). Entosis was first discovered in human tumors, and may be activated when cells lose their attachment to the extracellular matrix. It is still unclear what might be the “eat-me” signal for entosis, as the internalized live cells do not expose phosphatidylserine residues on the outer leaflet of their plasma membranes, and hence lack (one of) the most important engulfment signals of apoptotic cells. The entotic cell–cell interaction requires cadherin to establish cell contact. The entotic internalization process then requires actin polymerization and myosin II, Rho, and Rho kinase ROCK activity in the internalized cells. Once internalized, the engulfing cell appears to use the lysosome to mediate the degradation of the intruded cell without the involvement of caspases (Overholtzer et al. 2007). Although entosis has been described to occur only in tumors, it is conceivable that this mechanism may also contribute to cell death during development and tissue homeostasis.

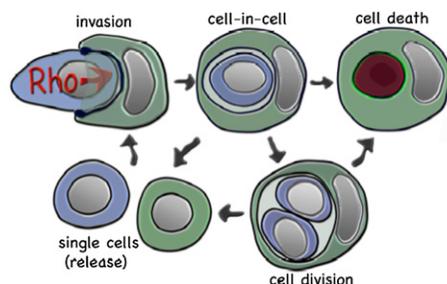


Figure 3. An illustration of entosis. A matrix-detached target cell (top left, blue) invades (arrow) into a neighboring host cell (turquoise) by a Rho-dependent contractile actomyosin force-based mechanism, requiring E-cadherin and adherens junction formation (blue junction at the cell–cell interface). The target cell is then internalized by the host. An internalized cell can be degraded by lysosomal enzymes (the red cell), can undergo cell division inside the host, or can be released from the host. (Kindly contributed by Michael Overholtzer and Joan S. Brugge.)

Physiological significance of alternative cell death mechanisms

Although apoptosis has been initially viewed as the primary regulated mechanism of cell death in higher animals, the importance of alternative cell death mechanisms is underscored by the largely normal development of mutant mice with mutations in the canonical apoptosis mediators. Morphological observations suggest that additional nonapoptotic cell death pathways may operate in the normal development of mammalian embryos (Schweichel and Merker 1973). Although nonapoptotic mechanisms are most clearly illustrated under apoptotic-deficient conditions, it is also possible that nonapoptotic cell death mechanisms might function at the front line as the primary cell death mechanism in a cell type-dependent and stimulus-dependent manner. For example, although necroptosis may be used largely as a backup mechanism under physiological conditions when stimulation of death receptors fails to activate apoptosis, necroptosis may also operate as one of the primary mechanisms that mediates pathological cell death. Moreover, the mechanisms that mediate the turnover of epithelial cells, such as intestinal epithelial cells and keratinocytes, appear primarily nonapoptotic. Finally, since yeast cells, which lack a canonical apoptosis pathway, can acquire a typical apoptotic morphology, we must consider the possibility that some of the “apoptotic” cell death that occurs during development may be executed through noncanonical pathways.

How may cells choose to die through apoptotic versus nonapoptotic pathways? Apoptotic cell death is highly regulatable, and thus may operate in individual cells to lead to selective elimination. In contrast, autophagic cell death such as that which occurs during metamorphosis may function to eliminate a tissue in toto. For epithelial cells that are exteriorly exposed, physical removal of dying or dead cells through shedding may be the most efficient mechanism of elimination.

Apoptosis is executed through a highly stereotyped series of biochemical events that guarantee a swift and noninflammatory removal of corpses. Although the translocation of phosphatidylserine to the outer leaflet has been known to be a caspase-dependent event in apoptosis, the exposure of phosphatidylserine can also occur in nonapoptotic cell death (Hirt et al. 2000) as a result of cathepsin B activation (Foghsgaard et al. 2001), perturbed Ca²⁺ homeostasis, or activation of PKC. Thus, the same mechanism that is employed to engulf apoptotic cells may also be used to clean up nonapoptotic cell death.

While apoptosis has been studied extensively for more than two decades, nonapoptotic cell death mechanisms are only beginning to be elucidated. In spite of the initial consensus that apoptosis would be the mechanism of programmed cell death, it turned out that apoptosis-deficient mice have largely normal phenotypes, indicating that nonapoptotic cell death mechanisms must play a major role in development and tissue homeostasis. Given that the vast plant kingdom and primitive eukaryotes such as yeast and *Dictyostelium* lack the major player(s) of the canonical, caspase-driven apoptotic pathways,

yet effectively control and execute their own death, nonapoptotic cell death pathways may be evolutionarily more ancient than that of the apoptotic pathway. The mechanistic elucidation of these alternative cell death mechanisms is likely to lead to exciting new insights into the regulation of programmed and accidental cell death, with major implications for the fields of biology and medicine.

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