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Review

Non-apoptotic cell death in animal development

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Programmed cell death (PCD) is an important process in the development of multicellular organisms. Apoptosis, a form of PCD characterized morphologically by chromatin condensation, membrane blebbing, and cytoplasm compaction, and molecularly by the activation of caspase proteases, has been extensively investigated. Studies in *Caenorhabditis elegans*, *Drosophila*, mice, and the developing chick have revealed, however, that developmental PCD also occurs through other mechanisms, morphologically and molecularly distinct from apoptosis. Some non-apoptotic PCD pathways, including those regulating germ cell death in *Drosophila*, still appear to employ caspases. However, another prominent cell death program, linker cell-type death (LCD), is morphologically conserved, and independent of the key genes that drive apoptosis, functioning, at least in part, through the ubiquitin proteasome system. These non-apoptotic processes may serve as backup programs when caspases are inactivated or unavailable, or, more likely, as freestanding cell culling programs. Non-apoptotic PCD has been documented extensively in the developing nervous system, and during the formation of germline and somatic gonadal structures, suggesting that preservation of these mechanisms is likely under strong selective pressure. Here, we discuss our current understanding of non-apoptotic PCD in animal development, and explore possible roles for LCD and other non-apoptotic developmental pathways in vertebrates. We raise the possibility that during vertebrate development, apoptosis may not be the major PCD mechanism.
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Facts

- Linker cell-type death (LCD) is the first cell death program found to proceed independently of caspases, and as a normal part of animal development.
- Germ cell death in *Drosophila* requires the serine protease HtrA2/Omi and involves mitochondria and lysosomes.
- Disruption of apoptotic genes in the mouse does not block most developmental cell death.

Open Questions

- Why is caspase-independent cell death often associated with germline and gonadal development?
- Is vertebrate LCD a freestanding developmental cell death program, or a backup to apoptosis?
- How prevalent is LCD in vertebrate development, and how conserved are the molecular players?
- How can non-apoptotic developmental cell death in vertebrates be tracked?

The term programmed cell death (PCD) was first coined to describe cell elimination that occurs at precise locations and times during animal development.[1] This process is key for sculpting tissues and organs, for removing excess or unnecessary cells, and for tissue homeostasis. The reproducible and consistent patterns of cell death in developing animals led to the idea that specific genes drive the phenomenon. Indeed, genes promoting apoptosis, a form of PCD characterized by chromatin condensation, membrane blebbing, and cytoplasm compaction[2] (Figure 1a), were initially isolated in *Caenorhabditis elegans.*[3] The discovery that the *C. elegans* caspase gene *ced-3* is required for developmental apoptosis, and the subsequent realization that caspase homologs in *Drosophila* and in vertebrates also promote apoptosis, demonstrated that underlying the stereotypical morphological signature is a conserved molecular program.[4,5] In species as diverged as *C. elegans* and the mouse, apoptosis is mediated by caspase proteases, activated by a conserved scaffolding protein called CED-4 in *C. elegans* and Apaf-1 in the mouse. Bcl-2 family proteins act upstream of CED-4/Apaf-1 to control its activation. This occurs by direct binding in *C. elegans,*[6] or through release of mitochondrial cytochrome *C*, which binds Apaf-1 in mammals.[7] For a comprehensive review see Fuchs and Steller.[8]

Although initial reports of mice harboring knockout mutations in caspase genes, in *Apaf-1*, or in both *Bax* and *Bak* suggested that these genes play important roles in vertebrate developmental cell death,[9–14] breeding mutants onto different genetic backgrounds revealed that homozygous knockout mice were not only born, but could survive to adulthood, often exhibiting only minor defects.[14–17] For example, while initial reports suggested that *Apaf-1* mutant mice exhibit inappropriate webbing between the pentadactyl-limb digits, later

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analysis revealed only a delay in the process, with complete culling within 2 days.[12,13] Mutations in *Caspase-3* or *Caspase-9* do not affect this process.[11,18] Furthermore, while persistence of small webs is observed in *Bax; Bak* double mutants, this surviving tissue is a small fraction of what survives in *Bmp* mutants, where cell death is entirely blocked.[19,20] Similarly, early studies of *Caspase-3* mutants revealed mice with apparent skull fractures and protruding brain tissue, suggesting a vast excess of neurons, consistent with the observation that in some brain regions up to 80% of cells that are originally produced undergo PCD.[10,21] Subsequent examination, however, revealed no increase in neuronal cell number.[22] Rather, defects in the sutures that bind skull bones together appear to be the cause of skull disruption,[23] and oozing of brain tissue is likely a consequence of the release of intracranial pressure to which the brain is normally subjected.

Although the absence of massive cell survival in *Caspase-3* or *Caspase-9* mutants can be explained by redundant activities of these enzymes, as the mouse harbors 13 caspase genes,[8] only a single *Apaf-1* gene exists in the murine genome.[12] Furthermore, studies of *Bax; Bak* double mutants suggest that developmental apoptosis is nearly entirely abrogated, yet some animals still develop normally.[14] Thus, an alternative explanation may be the existence of caspase-independent non-apoptotic processes. Cells dying with non-apoptotic features during development have been extensively described,[24] yet little is known about the underlying molecular effectors of these alternative death programs, or their *in vivo* relevance.

The past decade has seen somewhat of a renaissance in studying non-apoptotic cell death programs, and gene pathways controlling these processes in developing animals are emerging. While most developmental cell death in *Drosophila* and *C. elegans* proceeds via apoptosis, these organisms also provide highly amenable settings to discover non-apoptotic pathways. Here, we describe our current understanding of molecularly characterized non-apoptotic cell death programs that operate during development. These include germ cell death, nurse cell death and salivary gland cell death in *Drosophila*, and linker cell death in *C. elegans.* We investigate possible conservation in vertebrates, and discuss ultrastructural studies of developing vertebrate embryos that support an important role for non-apoptotic cell death. We suggest, specifically, that the linker cell-type death (LCD) caspase-independent program acts as a primary cell death mechanism, or as a backup when caspase-dependent processes fail in vertebrates.

Germ Cell Death in *Drosophila*

Pre-meiotic male germ cells undergo stochastic PCD in the adult fly testes, and dying cells display some apoptotic features, including cytoplasmic compaction and chromatin condensation[25] (Figure 2a). Dying germ cells are also TUNEL (terminal deoxynucleotidyl nuclear transferase dUTP nick end-labeling)-positive, suggesting chromosomal DNA fragmentation. Surprisingly, these wild-type cells are not stained with an antibody against cleaved Caspase-3, which binds the executioner caspases Drice and Dcp-1.[25] Furthermore, cells still die when these caspases are knocked down, suggesting that germ cell death is non-apoptotic. Correspondingly, unlike apoptotic cells, mitochondria of dying wild-type germ cells appear deformed and swollen.[25]

Although germ cell death does not employ executioner caspases, it does require an Apaf-1-independent function of the initiator caspase Dronc.[25] Mutations in *Apaf-1/ark* increase cell death, perhaps because more Dronc is available to induce non-apoptotic death.[25,26] Mutations in *dronc* result in a 40–60% decrease in death. This defect is specific, as lesions in the initiator caspases *strica* and *dredd* have no effect. The mechanism of Dronc action is not fully understood. However, lysosomal biogenesis proteins and the lysosomal protease cathepsinD are required for efficient demise, and an increase in reactive oxygen species (ROS) and cytoplasm acidification occurs.[25] ROS and Dronc may promote lysosome membrane permeabilization, allowing release of cathepsinD and DNaseII into the cytosol[25] (Figure 2b). The release of DNaseII may explain TUNEL staining in the absence of cleaved Caspase-3.

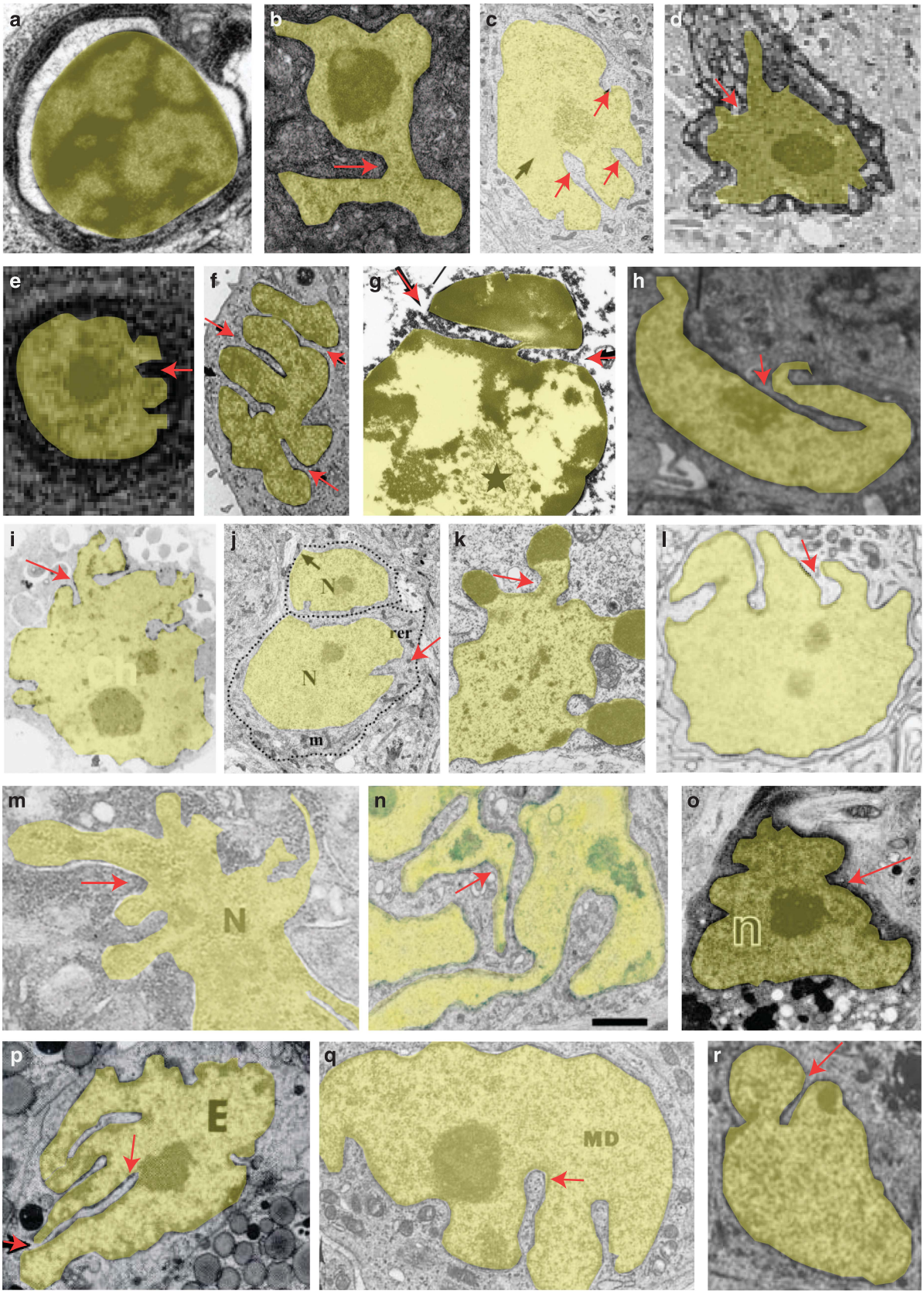
Yacobi-Sharon *et al*.[25] discovered that the mitochondrial serine protease HtrA2/Omi plays an important role in germ cell death. *Drosophila* carrying deletions of either one or both *htrA2/omi* copies are viable, but exhibit male sterility associated with a decrease in germ cell death. Remarkably, *htrA2/omi* lesions in humans are associated with Parkinson's disease,[27] and mutations in the Parkinson's disease- and mitochondrial-associated gene *pink1* also cause a decrease in *Drosophila* germ cell death. Overexpression of a cytosolic version of HtrA2/Omi promotes caspase-independent cell death in mammalian cells,[28] accompanied by morphological changes similar to *Drosophila* germ cell death, although nuclear changes are not evident.[25,28] Roles for mitochondria in *Drosophila* germ cell death are also supported by the findings that the Bcl-2 family proteins Debcl and Buffy, and the mitochondrial nuclease EndoG, promote death.[25]

In rodents, male germ cells undergo stochastic cell death that may be caspase independent.[29,30] Mutations in the pro-apoptotic gene *Bax* do not block these deaths, suggesting possible involvement of non-apoptotic programs.[31,32] Furthermore, mutations in the testes-specific serine protease inhibitor *spink2* result in increased germ cell death in male mice.[30] These observations provide circumstantial evidence that conserved pathways may control stochastic male germ cell death across species.

Nurse Cell Death in *Drosophila*

In the *Drosophila* ovary, 15 nurse cells provide a developing oocyte with proteins, mRNA, and organelles[33] (Figure 2c). After extruding their cytoplasmic contents into the oocyte, nurse-cell remnants die. Dying wild-type cells exhibit chromatin condensation, but also autophagosomes, ruptured lysosomes and large vacuoles.[34,35] Dying cells stain with acidification markers, autophagic markers and TUNEL,[34–37] raising the possibility that more than one cell death program is involved.

Whether caspases promote nurse cell death is debated. Some studies report no activated caspases in dying cells,[38,39] whereas others report immunoreactivity using an activated Caspase-3 antibody.[36,37] Mutants in *Drosophila* inducers of apoptosis do not exhibit nurse cell survival, suggesting a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caspase-independent process,[40,41] and some studies suggest that overexpression of the caspase inhibitors *diap1* or *p35* also does not perturb death.[37,38] However, other studies suggest that such overexpression weakly blocks death.[42]

Genetic studies demonstrate that nurse cell death still proceeds in *dronc, dredd*, *strica, dcp-1* or *drice* caspase single mutants.[42–44] However, *dredd;strica* or *dcp-1;drice* double mutants exhibit weak survival, suggesting that caspases may be relevant, but functionally redundant.[42] Congruently, mutants in *Apaf-1/ark*, which functions upstream of initiator caspases, also exhibit weak nurse cell death defects.[41]

In developing nurse cells *in vitro*, the Caspase-3-inhibitor Z-DEVD-FMK blocks DNA fragmentation but not chromatin condensation.[45] While mutations in inhibitor of caspase-activated DNase (ICAD) cause a decrease in cleaved DNA, nurse cells still clear their DNA efficiently.[46] In another study, however, mutants in *dICAD* displayed weak nuclear persistence, similar to *Apaf-1*/*ark* mutants.[41] Together, these data suggest that caspases may play some role in nurse cell death.

The role of autophagy in nurse cell death is also unclear. Despite the presence of autophagosomes, autophagy-related protein levels are not increased in dying cells.[35,38] However, one study reported that nurse cell nuclei persist in mutants of the autophagy genes *atg1* and *atg13.*[36] This defect, however, is not fully penetrant, suggesting other processes are probably involved.[36] *atg7;dcp-1* double mutants exhibit similar defects to *atg7* single mutants,[47] indicating that these other pathways may be caspase independent.

Lysosomes may play important roles in nurse cell death and removal (Figure 2d). Mutants in *DNaseII* and the lysosomal trafficking protein *deep orange/Vps18* exhibit persisting nurse cell nuclei,[35,47] as do mutants in the lysosomal fusion protein *Spinster* and the protease *CathepsinD.*[35] While Deep Orange is required in the engulfing follicle cells, DNaseII and Spinster appear to function cell autonomously.[35] Thus, lysosomes may have multiple activities in nurse cell death, although these results are complicated by the fact that some of these mutants perturb cytoplasm transfer, which may delay or impede death.[34]

Recent research raises the possibility that the primary nurse cell death mechanism may be phagoptosis, such that nurse cell death is non-autonomously promoted by surrounding follicle cells.[48] Consistently, mutants in the engulfment genes *draper* and *ced-12* display nurse cell survival, and Draper is required for nurse cell acidification.[48] Furthermore, ablation of surrounding follicle cells strongly inhibits death, although cytoplasm transfer is also blocked.[48] Thus, it is possible that nurse cell death may be an assisted suicide.

Salivary Gland Cell Death in *Drosophila*

Drosophila salivary glands are larva-specific structures that are rapidly degraded after puparium formation[49,50] (Figure 2e). Dying wild-type cells exhibit apoptotic features, but also an abundance of autophagosomes and nuclear separation from the cytoplasm.[51,52] Both autophagy and caspase genes are induced during salivary gland cell death,[53,54] although the precise contribution of each is not fully understood. Caspases may be dispensable for death. Loss-of-function mutants in *dronc* or *drice* caspase genes or in *Apaf-1*/*ark* do not strongly perturb salivary gland death and degradation.[50,55] While expression of the caspase inhibitor p35 halts DNA fragmentation and nuclear lamin cleavage,[51,52] it does not result in intact cells.[54] Instead, fragments appear to persist inappropriately,[50] suggesting a cell degradation role for caspases.

Autophagy is induced prior to salivary gland cell death, and in the absence of the autophagy-related gene *atg18*, salivary glands are not properly degraded, exhibiting vacuolated cell fragments[50] (Figure 2f). These findings provide *in vivo* evidence for a role of autophagy in degrading dying cells.[50] Combining autophagy and caspase inhibition blocks gland

Figure 1 Crenellated nuclei (pseudocolored yellow, red arrows) are prevalent in disease models and in normal development. (**a**) In apoptosis, chromatin is highly condensed and darkly staining, but the nuclear envelope remains round (*C. elegans* cell). (**b**) In linker cell death, the nuclear envelope is crenellated, with indentations apparent even using Nomarski optics. Reprinted from Blum *et al.*[63] (**c**) In a mouse model of Huntington's disease, crenellated nuclei are prominent in almost all the cells of the striatum. Black arrow, neuronal nuclear inclusion. Reprinted from Davies *et al.*[70] with permission from Elsevier. (**d**) They are also prevalent in dark neurons of a mouse model of Huntington's disease.[71] Copyright 2000 National Academy of Sciences. (**e**) In brain autopsy samples of a Huntington's disease patient, crenellated nuclei with uncondensed chromatin are present. Reprinted from Maat-Schieman *et al.*[77] with permission from Oxford University Press. (**f**) In a cell line overexpressing a pathogenic Ataxin-3, crenellated nuclei were apparent prior to non-apoptotic cell death. Reprinted from Evert *et al.*[74] with permission from Oxford University Press. (**g**) In the CAG repeat disease dentatorubral-pallidoluysian atrophy, autopsy samples from patients have nuclear membrane indentations in some granule cells in the cerebellar cortex. Star, intranuclear inclusion. Reprinted from Takahashi *et al.*[75] with permission from Elsevier. (**h**) Dying cells from the developing palatal shelf display crenellated nuclei. Reprinted from Schweichel and Merker[83] with permission from John Wiley and Sons (Publisher). (**i**) While *Caspase-9* null embryonic stem cells rarely die after UV treatment, those that do display LCD features, including open chromatin, swollen organelles, and crenellated nuclear envelope. Ch, chromatin. Reprinted from Hakem *et al.*[9] with permission from Elsevier. (**j**) Degenerating motoneurons in *Bak* knockout mice have crenellated nuclei, and are small and atrophied. N, nucleus. m, mitochondria. rer, rough endoplasmic reticulum. Black arrow, synapse. Dotted line, soma. Republished with permission from Society for Neuroscience, from Sun *et al.*;[88] permission conveyed through Copyright Clearance Center, Inc. (**k**) Degenerating motoneurons in a wild-type stage 24 chick spinal cord have indented nuclei.[91] Adapted from ©1974 O'Connor and Wyttenbach. *Journal of Cell Biology* **60**: 448–459. DOI: 10.1083/jcb.60.2.448. (**l**) Non-apoptotic dying retinal ganglion cells in the same organism have crenellated nuclei after axotomy. Reprinted from Borsello *et al.*[93] with permission from John Wiley and Sons (Publisher). (**m**) Peripherally deprived ciliary ganglion neurons in the embryonic chick display highly indented nuclei.[94] N, nucleus. Adapted from ©1976 Pilar and Landmesser. *Journal of Cell Biology* **68**: 339–356. DOI: 10.1083/jcb.68.2.339. (**n**) Avian motoneurons in culture adopt either an apoptotic morphology or an LCD morphology as shown here, with highly crenellated nuclei, and swollen organelles at later timepoints (not shown). Scale bar = 1 μm. Reprinted from Brunet *et al.*[95] with permission from John Wiley and Sons (Publisher). (**o**) In the presence of caspase inhibitors, motoneurons in the chick acquire non-apoptotic morphology. n, nucleus. Reprinted from Yaginuma *et al.*[96] with permission from Elsevier. (**p**) Mouse uterine epithelial cells die shortly after implantation, and these cells display irregularly shaped nuclei. Reprinted from Parr *et al.*[101] with permission from Oxford University Press. (**q**) Regressing Müllerian duct cells in wild-type mice have open chromatin, crenellated nuclei, and swollen organelles. Reprinted from Dyche[108] with permission from John Wiley and Sons (Publisher). MD, Müllerian duct epithelial cell. (**r**) Explant cultures of degenerating Müllerian duct cells from rats share the same features. Reprinted from Price *et al.*[111] with permission from John Wiley and Sons (Publisher)

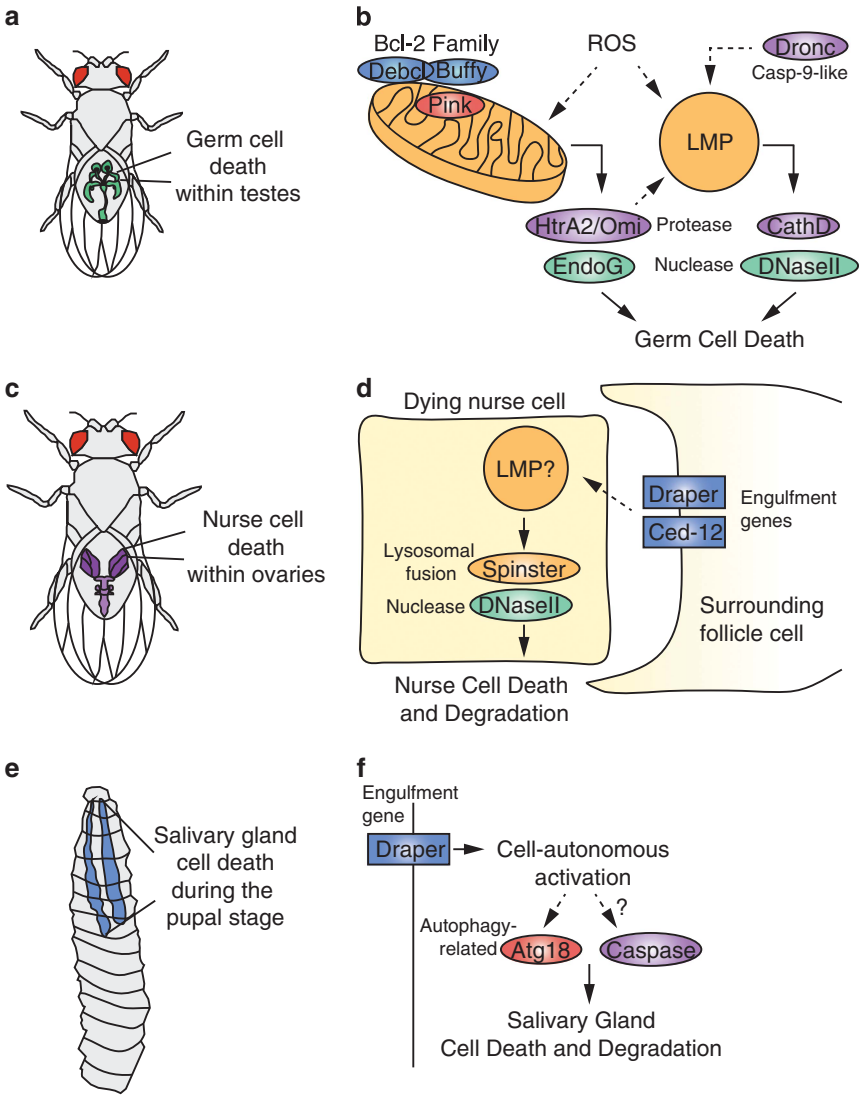

Figure 2 Non-apoptotic cell death in *Drosophila*. (**a**) In adult males, germ cell death occurs within the testes (green). (**b**) Dronc, a Caspase-9-like protease, functions in an apoptosome-independent fashion to perhaps trigger lysosome membrane permeabilization (LMP), allowing the release of the protease CathepsinD and the nuclease DNaseII. At the mitochondria, Bcl-2 family members Debcl and Buffy function on the outer membrane, and the Parkinson-related protein Pink1 functions within. ROS may trigger release of the serine protease HtrA2/Omi and the nuclease EndoG.[25] (**c**) In the female adult fly, nurse cell death occurs within the ovaries (purple). (**d**) The surrounding follicle cells may induce nurse cell death, using the engulfment receptors Draper and Ced-12 via phagoptosis. Downstream events may lead to lysosome membrane permeabilization (LMP) and the release of the lysosomal fusion protein Spinster and the nuclease DNaseII. Caspases and autophagy may be minimally involved. (**e**) After puparium formation, large larval salivary glands (blue) die and degrade before the adult fly hatches. (**f**) The engulfment gene Draper is required cell-autonomously within the salivary glands to drive cell death, and may lead to downstream activation of autophagy genes like Atg18. Caspases may work in parallel

degradation further, but not cell death, suggesting these pathways function in parallel.[50] Cell death can be inhibited by overexpression of the PI3K active subunit Dp110 and p35, suggesting involvement of a PI3K target.[50]

The salivary glands are large structures, and their phagocytosis by neighboring cells is not well understood. The engulfment gene *draper* is required to induce autophagy cell-autonomously in the salivary gland cells, and not for engulfment,[52,56] suggesting an unexpected and intimate connection between engulfment and degradation genes. Such a connection had been described for nurse cell death (see above[48]). In *C. elegans*, engulfment genes also appear important in some contexts for promoting nuclease activation for cell degradation.[57]

Linker Cell Death in *C. elegans*

The *C. elegans* male-specific linker cell leads gonad elongation. During the L4 larva-to-adult transition, the cell dies, allowing gonad–cloaca fusion for sperm release and male fertility (Figure 3a). The dying linker cell is engulfed by its neighboring U.l/rp cells.[58] Morphologically, wild-type linker cell death is non-apoptotic: chromatin is not condensed, the nuclear envelope becomes crenellated, and mitochondria and

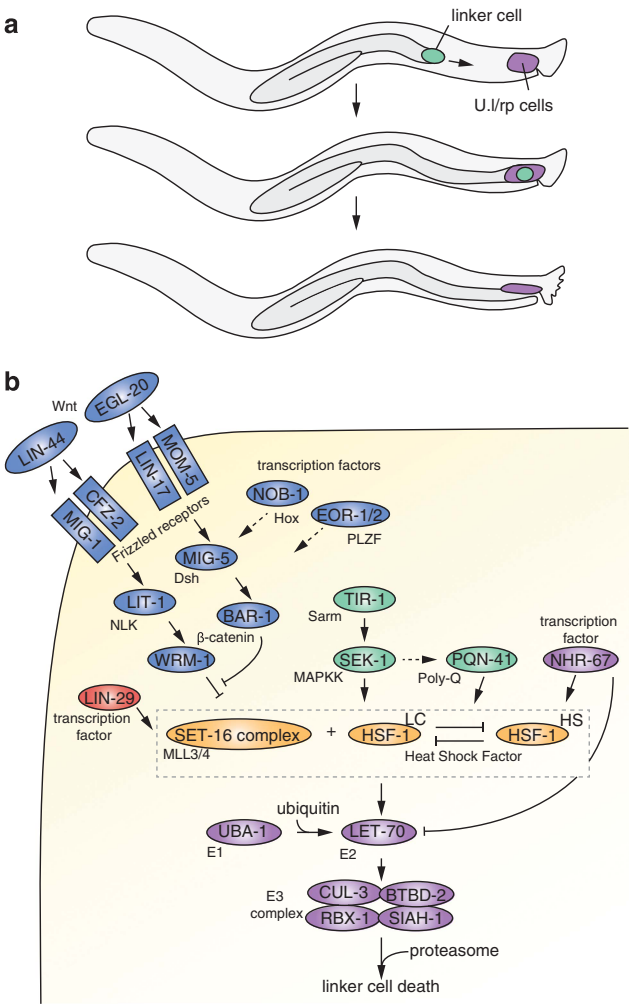

Figure 3 The linker cell dies independent of caspases. (**a**) The linker cell (green) is a male-specific cell that leads the elongation of the developing gonad. Once it reaches its destination, it dies, and is engulfed by the U.l/rp cells (purple), allowing fusion of the gonad with the cloaca. (**b**) Multiple regulatory pathways are required to ensure the linker cell dies at the proper place and time. These pathways include two antagonistic Wnt pathways (blue), the developmental timing protein LIN-29 (red), Sarm/TIR-1, the MAPKK SEK-1, and the polyglutamine protein PQN-41 (green), and the nuclear hormone receptor NHR-67 (purple). These pathways converge on the heat-shock factor HSF-1, which acts in parallel with the SET-16 histone methyltransferase complex (orange). The pro-death function of HSF-1 competes with its pro-survival function. HSF-1 may transcriptionally upregulate the E2 ubiquitin-conjugating enzyme LET-70 (purple). The transfer of ubiquitin from the E1 UBA-1, through LET-70, to an E3 complex may confer substrate specificity, leading perhaps to degradation of key proteins by the proteasome, resulting in linker cell death

endoplasmic reticulum swell[58] (Figure 1b). Death proceeds even when all four caspase genes are inactivated, and mutations in other core apoptotic genes also fail to prevent death.[58,59] Thus, linker cell death represents the first example of a non-apoptotic caspase-independent cell death process required for development. The morphology of dying linker cells is frequently seen in vertebrate development, and we have termed this appearance LCD. Importantly, although invaginated membranes are often artifacts of EM fixation, this is not the case for linker cell nuclear crenellation, as this feature is detected in the living animal under light microscopy. In vertebrate settings, LCD ultrastructural features are distinguished from EM artifacts or natural variability as they are seen only at specific developmental times, and accompany only populations of dying cells and not their neighbors (see below).

Work in our lab revealed multiple control pathways promoting linker cell death. This death must be tightly regulated, as premature demise blocks gonad elongation, resulting in sterility.[60] Two opposing Wnt pathways control death onset (Figure 3b). One pathway, consisting of the Wnt ligand LIN-44, secreted by some tail cells, the Frizzled receptors MIG-1 and CFZ-2, the Nemo-like kinase LIT-1 and the β-catenin WRM-1, all acting in the linker cell, appears to block linker cell death, and may prevent premature death.[61] The LIN-44/Wnt pathway is inhibited by a second Wnt pathway, consisting of EGL-20/Wnt, secreted by the engulfing cells, and LIN-17/Frizzled and MOM-5/Frizzled, acting within the linker cell with MIG-5/Disheveled and BAR-1/β-catenin, which promotes death.[61] Two transcription factors, NOB-1/Hox and the EOR-1/-2/PLZF complex, likely control the EGL-20/Wnt pathway.[62]

In parallel, TIR-1, the *C. elegans* ortholog of mammalian Sarm, activates the MAPKK protein SEK-1 to promote death.[63] SEK-1 may promote expression of the polyglutamine-repeat protein PQN-41. PQN-41 expression is induced in the linker cell at the onset of death, and a GFP-tagged version forms puncta within the linker cell.[63]

The zinc-finger protein LIN-29, a component of a developmental timing program,[64] also promotes death, as does the nuclear hormone receptor NHR-67.[62] These genes appear to act independently of and in parallel to the Wnt and MAPKK pathways.[62]

Linker cell death can be restored in mutants carrying lesions in the upstream control pathways by a gain-of-function mutation in HSF-1, a highly conserved transcriptional regulator of the heat shock and other stress responses. HSF-1 loss-of-function blocks death. Thus, HSF-1 likely functions downstream of or in parallel to the regulators described above.[61] A pro-death role for HSF-1 is surprising, as HSF-1 is typically responsible for promoting survival in response to stress. In animals exposed to a heat-shock 4 h before death onset, the linker cell survives inappropriately, suggesting that the pro-death and pro-survival functions of HSF-1 may compete.[61] Genetically, HSF-1 functions at the same step as the SET-16/MLL3/4 H3K4 histone methyltransferase complex.[62]

Genetic, expression and functional studies suggest that HSF-1 may work by transcriptionally activating components of the ubiquitin proteasome system.[61] The single *C. elegans* E1 enzyme, UBA-1, is required through activity of the E2 ubiquitin-conjugating enzyme LET-70, homologous to mammalian UBE2D2. LET-70/UBE2D2 appears to act through an E3 ubiquitin ligase complex comprising the cullin CUL-3, the ring box protein RBX-1, the BTB domain-containing protein BTBD-2 and perhaps SIAH-1. Substrates of this E3 complex are unknown, but may provide critical clues to how this caspase-independent cell death is executed. It is possible, for example, that degradation of a critical substrate is the key to the lethal effects of the pathway. Correspondingly, proteasome 19 S regulatory domain components are required for linker cell death.[61]

Molecular components related to those promoting linker cell death have been implicated in degeneration and death in other settings. Wallerian degeneration is a process by which the distal process of an injured axon degenerates, leaving the cell body intact. Wallerian degeneration slow (Wld^S) mice have persistent axotomized axons[65] caused by an abnormal protein fusion between an NAD^+ synthesis protein, NMNAT, and the ubiquitin factor E4B (Ube4b).[66] Recent work has revealed that mutations in *dSarm*, the *Drosophila* homolog of *C. elegans tir-1* required for linker cell death, also suppress Wallerian degeneration in the fly.[67] Likewise, murine *Sarm* promotes Wallerian degeneration *in vivo*.[67] Overexpression of the SAM-TIR domains of murine Sarm in cell culture promotes death that appears non-apoptotic, as it proceeds in the presence of caspase inhibitors.[68]

In the linker cell, TIR-1/Sarm1 regulates expression of the polyQ-repeat protein, PQN-41.[63] While PQN-41 function is not well understood, its sequence raises the hypothesis that polyQ-expansion neurodegenerative diseases, such as Huntington's disease, may be instances of a developmental cell death program gone awry.[69] Supporting this, in a mouse Huntington's disease model, dying cells exhibit swollen mitochondria and Golgi, and crenellated nuclei, strikingly reminiscent of linker cell death[70] (Figures 1c and d). TUNEL staining is not detected in dying neurons.[71] Crenellated nuclei are also found in dying cells of other polyQ disease models and in human patients[72–77] (Figures 1e–g).

Intersegmental muscles (ISMs) of the moth, *Manduca sexta,* may also prove fertile ground for exploring similarities with linker cell death. Wild-type ISM cells that die during metamorphosis exhibit non-apoptotic features: chromatin condensation is not seen, nor are DNA fragmentation, or membrane blebbing.[78] However, ubiquitin expression is induced, similar to linker cell death.[79] Furthermore, an E1 ubiquitin-activating enzyme, several E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases are induced.[80] Molecular characterization of these proteins and their targets may reveal similarities to linker cell death.

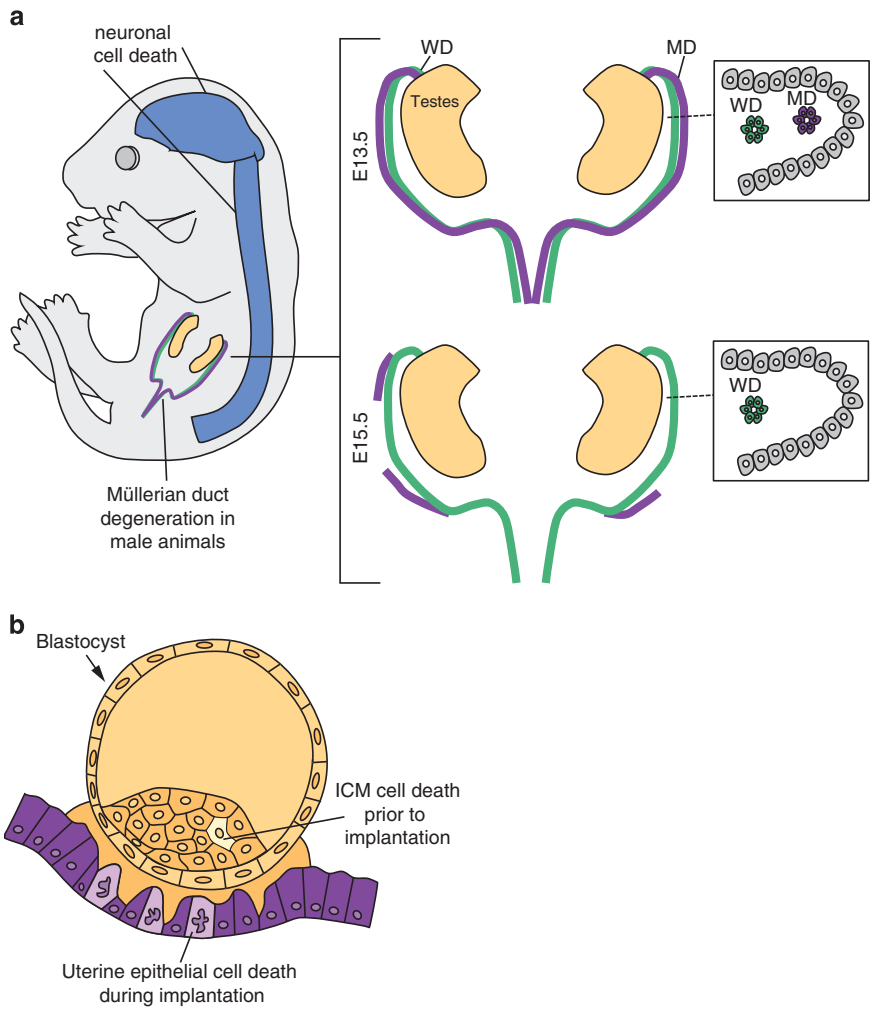

Figure 4 Non-apoptotic cell death occurs in vertebrate development. (**a**) In the developing mouse embryo, neurons within the brain and spinal cord (blue) die. In male mice at E13.5, the Müllerian duct (MD, purple) and the Wolffian duct (WD, green) are both present in the immature reproductive system. Signal from the testes cause the Müllerian duct to degenerate by E15.5. (**b**) Cell death occurs in the inner cell mass of the blastocyst (yellow) prior to implantation. Uterine epithelial cells (purple) die upon implantation of the blastocyst and may be entosed by the trophoblast cells

Non-apoptotic Cell Death in Vertebrate Development

Although non-apoptotic cell death pathways like necroptosis have been described in vertebrate cells, none appear to be required for normal development, as elimination of key genes regulating these processes does not affect development or developmental cell death.[81,82] Nonetheless, evidence that non-apoptotic cell death has important roles in vertebrate development abounds, including but not limited to the formation of the mammalian palate[83] (Figure 1h), cornification of epidermal keratinocytes,[84] and lysosome-mediated mammary gland involution post-lactation.[85] In the following subsections, we highlight four salient examples that raise the possibility that non-apoptotic cell death programs may indeed be the norm during vertebrate development. Whether such programs back up, act in parallel, or function independently of apoptosis is not easy to distinguish, as a key test for their non-apoptotic nature is whether they proceed without apoptotic genes- inherently unnatural settings. We have therefore focused on processes that are genetically non-apoptotic, and which also have obvious non-apoptotic features in the developing wild-type animal.

Cell death in the vertebrate nervous system. Initial studies of mice harboring lesions in core apoptotic components, including *Apaf-1*, the initiator *Caspase-9*, and the executioner *Caspase-3*, suggested important roles for these genes in nervous system development. *Apaf-1*$^{-/-}$, *Caspase-3*$^{-/-}$, and *Caspase-9*$^{-/-}$ animals exhibited exencephaly associated with neural tube defects, and most animals died perinatally,[9–13] and presented with mitotically active immature neurons.[86] Later studies revealed that genetic background was a strong contributor to these defects, and that mice carrying the same lesions in other genetic contexts could develop normally to adulthood. For example, *Caspase-3*$^{-/-}$ mice in a C57BL/6J genetic background survive to adulthood, but the same mutation in 129X1/SvJ mice induces perinatal lethality.[15] How then does cell death still occur? A number of studies suggest that alternative cell death pathways are likely to be at least as important as apoptosis in these mutants.

While embryonic stem cells lacking *Caspase-9* exhibit resistance to pro-apoptotic agents,[9] some mutant cells die and exhibit non-apoptotic LCD-like morphology, including a crenellated nuclear envelope, uncondensed chromatin, and swollen organelles (Figure 1i), suggesting that a process akin to linker cell death may be functioning. Supporting this, death of post-mitotic spinal cord and brain stem motoneurons (Figure 4a) appears to proceed normally in *Caspase-3*$^{-/-}$ and *Caspase-9*$^{-/-}$ animals even though TUNEL staining decreases,[86] suggesting that caspase-activated DNase (CAD), one of the enzymes responsible for cleaving chromosomal DNA during apoptosis,[87] is not induced. Thus, cells appear to be dying in the absence of caspase activation. Indeed, dying cells lacking *Caspase-3* or *Caspase-9* have different morphologies than wild-type cells, with reduced chromatin condensation.[86] Similar studies in *Bax*$^{-/-}$ animals reveal an increase in cell number, but the extra cells are small and atrophied, are not synaptically connected, and exhibit crenellated nuclei[88] (Figure 1j). In *Apaf-1*$^{-/-}$ mutants, spinal and cranial motoneurons, and dorsal root ganglion sensory cell death numbers are normal compared with wild type, again suggesting that caspase-independent cell death mechanisms are important in the developing nervous system.[89]

Similar to mice, motoneurons are normally produced in excess during chick spinal cord development, and many subsequently die.[90] Ultrastructurally, these dying wild-type cells possess either apoptotic or non-apoptotic morphologies, the latter exhibiting dilated organelles, open chromatin and an irregular nucleus[91,92] (Figure 1k). Axotomized retinal ganglion cells or peripherally deprived ciliary ganglion cells have the same indented nuclear structure[93,94] (Figures 1l and m). Interestingly, wild-type chick motoneurons in culture can adopt similar morphologies (Figure 1n), and while these dying cells can be engulfed, they lack cleaved Caspase-3 staining or TUNEL staining.[95] *In vivo*, these motoneurons still die in the presence of the Caspase-3 inhibitor Ac-DEVD-CHO or the stronger pan-caspase inhibitor Boc-D-FMK.[96] Intriguingly, dying cells treated with caspase inhibitors have a crenellated nucleus, open chromatin, and swollen organelles,[96] all features of LCD (Figure 1o).

Cell death in the inner cell mass. Cell death is an early occurrence in vertebrate development, and is evident in the inner cell mass (ICM) prior to implantation[97] (Figure 4b). These cell deaths in a wild-type mouse blastocyst are characterized by condensed chromatin and loss of nuclear membrane integrity, but also swelling of the endoplasmic reticulum.[98] Single mutants of apoptotic factors do not seem to block blastocyst development, suggesting that cell death likely proceeds normally.[97] Supporting this notion, when hamster or mouse embryos are cultured from the 8-cell stage to the blastocyst stage *in vitro* in the presence of the Caspase-3 inhibitor Ac-DEVD-CHO, no noticeable effects on development are observed.[99] Dying cells in the ICM of wild-type bovine blastocysts exhibit a mix of apoptotic and non-apoptotic features.[100] Some dying cells have condensed chromatin but weak TUNEL staining, while others have moderate TUNEL staining.[100] Furthermore, only low transcript levels of key apoptotic factors, including *Caspase-3* and *Caspase-9*, are detected.[100] Thus, caspases, and perhaps other apoptotic proteins, may not be required for ICM cell death.

Death of uterine epithelial cells during implantation. At the site of implantation, uterine epithelial cells die and are internalized by embryonic trophoblast cells[101] (Figure 4b). These dying epithelial cells in normal development exhibit compacted chromatin, but also swollen organelles and irregular nuclear envelopes[101,102] (Figure 1p). One study reported activated Caspase-3 in TUNEL-positive dying cells in wild-type hamster and mouse uterine epithelial cells at the site of implantation.[99] Additionally, inhibiting Caspase-3 with Ac-DEVD-CHO in the uterus stopped implantation in both species, suggesting that Caspase-3 may be necessary for cell death.[99] However, *Caspase-3*$^{-/-}$ or *Apaf-1*$^{-/-}$ adult female mice are fully fertile.[15,16] Furthermore, a subsequent detailed examination of the process revealed that neither cleaved Caspase-3 nor TUNEL are detected in dying wild-type epithelial cells immediately adjacent to trophoblast cells.[103] Entosis, engulfment of a living cell followed by

degradation, has been suggested as a mechanism, as the kinase ROCK, which controls entosis in cell culture,[104] appears to be required.[103] The ultrastructure of these dying cells was not reported, and could be used to distinguish among different alternative cell death mechanisms.

Cell death in the Müllerian duct. In developing mammals, sex specification occurs early in embryogenesis. Initially, both sexes form a Wolffian duct (WD), which develops into male urogenital structures, and a Müllerian duct (MD), which gives rise to female reproductive structures. In females, the WD degenerates, whereas the MD regresses in males[105] (Figure 4a). MD regression is initiated by the secreted TGF-β protein AMH (anti-Müllerian hormone).[106] In the absence of AMH, the MD persists, and results in female organs alongside the male urogenital system. This leads to infertility, as the ectopic organs physically block sperm release.[107]

The morphology of dying wild-type MD epithelial cells in rats, mice and rabbits, is non-apoptotic: chromatin in degenerating cells is uncondensed, and the nuclear envelope is crenellated[108–110] (Figure 1q). These features are reminiscent of linker cell death, suggesting that MD degeneration proceeds similarly. Degenerating female rabbit WD cells also have crenellated nuclei.[110] MD and WD degeneration can be reproduced in an organ culture system, and dying cells here exhibit open chromatin and nuclear crenellation[111] (Figure 1r).

Regressing wild-type MD cells are TUNEL positive,[112] and cleaved Caspase-3 is detected in some dying cells.[107] However, neither *Caspase-3* nor other apoptosis mutants are reported to harbor defects in urogenital tract development. There are conflicting reports regarding the sterility of *Apaf-1*$^{-/-}$ mutants males; however, not all males are infertile.[16,17] *Caspase-3*$^{-/-}$ male mice in the C57BL/6J genetic background are fully fertile.[15] Thus, it is possible that LCD is the main cell death mechanism during MD and WD degeneration, with *Apaf-1* and *Caspase-3* either playing minor roles, or specifically promoting corpse degradation (see, e.g., ref. 96).

While the genes directly driving MD cell death are unknown, some of the upstream signaling events have been explored. Wnt7a signaling from MD epithelial cells allows surrounding mesenchymal cells to respond to AMH,[113] likely by regulating expression and/or activation of receptors in the mesenchyme.[114–116] Receptor engagement leads to activation of SMAD proteins, which then induce expression of Wnt4, and possibly other Wnts.[116] Possible autocrine signaling leads to the activation of β-catenin, which promotes regression in the epithelial cells.[112] β-Catenin accumulates in the mesenchymal cytoplasm, and is present near the plasma membrane of MD epithelial cells.[112] Mesenchyme-specific knockdown of β-catenin prevents MD degeneration.[107] While both MD regression and linker cell death use β-catenin signaling to initiate cell death, β-catenin appears to function cell non-autonomously in the MD.

The matrix metalloproteinase MMP2 may be involved in signaling to initiate regression.[117] *Mmp2* is expressed at higher levels in the male mesenchyme, and this increase is abolished in the absence of AMH.[117] In organ culture, blocking MMP2 prevents MD degeneration, while activating MMP2 causes degeneration.[117] However, MMP2 mutant mice are fertile, suggesting other genes are involved.[118] Roles for MMPs in linker cell death have not been described, but the adjacent gonad is known to secrete these proteins.[119]

Together, the examples described make a compelling case for the prevalence of caspase-independent non-apoptotic cell death during vertebrate development, with LCD playing, perhaps, an important role.

Looking forward

The studies reviewed here demonstrate that developmental cell death is a far more complex and poorly understood process than has been generally acknowledged, and that the often-interchangeable usage of the terms apoptosis and programmed cell death is highly misleading. Using single assays, such as TUNEL or activated caspase staining, appears insufficient to assign a mode of cell death as apoptotic or non-apoptotic, and may give a misleading impression of the relative importance of each. In germ cell death in *Drosophila*, for example, TUNEL-positive cells do not overlap with cleaved Caspase-3 staining, suggesting non-apoptotic events.[25] Conversely, lack of TUNEL staining or caspase reactivity does not mean cell death is not occurring. For example, dying spinal motoneurons with LCD features are TUNEL negative.[95,96] We therefore believe that assigning a death type requires multiple corroborating assays, among which ultrastructural characterization of the dying cells is likely to provide the most information, and can be easily used, for example, to distinguish LCD and apoptosis.[95]

Non-apoptotic cell death is an exciting and largely unexplored facet of development. Because some of these cell deaths may affect animal fertility and nervous system development, the genes involved could be highly conserved and of major clinical relevance. Model systems such as *C. elegans* and *Drosophila* provide unique gene-discovery arenas that can propel our molecular understanding of non-apoptotic processes in vertebrates in much the same way these animals were used to reveal the underpinnings of apoptosis.[120]

Conflict of Interest

The authors declare no conflict of interest.

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