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PRIMER

Cell death in animal development

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ABSTRACT

Cell death is an important facet of animal development. In some developing tissues, death is the ultimate fate of over 80% of generated cells. Although recent studies have delineated a bewildering number of cell death mechanisms, most have only been observed in pathological contexts, and only a small number drive normal development. This Primer outlines the important roles, different types and molecular players regulating developmental cell death, and discusses recent findings with which the field currently grapples. We also clarify terminology, to distinguish between developmental cell death mechanisms, for which there is evidence for evolutionary selection, and cell death that follows genetic, chemical or physical injury. Finally, we suggest how advances in understanding developmental cell death may provide insights into the molecular basis of developmental abnormalities and pathological cell death in disease.

KEY WORDS: Cell death, Apoptosis, Caspase, Non-apoptotic cell death, Linker cell-type death, LCD, Pathological cell death, Cell compartment elimination

Introduction

Cell death is prevalent in and crucial for animal development (Fuchs and Steller, 2011). The notion that cell death is regulated during animal growth first emerged from studies of motoneuron development in the chick embryo. These studies led to the discovery of nerve growth factor (NGF), the first described regulator of cell survival (Hamburger and Levi-Montalcini, 1949). Research on amphibian and insect metamorphosis revealed that developmental cell death is commonplace and predictable (Glücksmann, 1965; Kerr et al., 1972; Lockshin and Williams, 1965); the term programmed cell death (PCD) was coined to acknowledge this reproducibility. Cell death plays many roles in development, from tissue sculpting, to controlling cell numbers, to quality control (Box 1). It is not surprising, therefore, that blocking cell death has severe consequences. PCD-defective mutants of the nematode *Caenorhabditis elegans* develop to adulthood, but produce fewer progeny, grow more slowly, and exhibit nervous-system and behavior defects (Avery and Horvitz, 1987; Ellis et al., 1991; White et al., 1991). In *Drosophila melanogaster* and vertebrates, PCD appears to be important for viability (White et al., 1994), and vertebrate PCD defects result in developmental abnormalities and pathologies including cancer and neurodegeneration (Fuchs and Steller, 2011).

Although developmental cell death was originally imagined as a passive withering process, studies in *C. elegans* identified conserved cell-autonomous machinery driving cell elimination (Conradt and Horvitz, 1998; Ellis and Horvitz, 1986; Hengartner and Horvitz, 1994b; Yuan and Horvitz, 1992; Yuan et al., 1993). Thus, in development, cells fated to die activate an evolutionarily-selected self-culling cascade to cause their own demise. To date, most studies of developmental cell death use *C. elegans* and *D. melanogaster* as model systems.

In this Primer, we summarize mechanisms underlying developmental cell death and describe their control, focusing on apoptotic and non-apoptotic pathways. Next, we discuss the different ways cell death is initiated in the context of embryonic development. We also contrast developmental cell death with pathological cell death (Box 2, Table 1) and discuss repurposing of cell death pathways for eliminating subcellular compartments. We do not review dying-cell phagocytosis (efferocytosis), although rapid and efficient clearance is essential for cell elimination, and readers are directed to excellent reviews on the subject, e.g. Arandjelovic and Ravichandran (2015). Finally, although cell death has been studied for nearly a century, we highlight fascinating remaining problems that fuel current excitement.

Apoptosis

Apoptosis (Greek: 'falling off') is a type of PCD with a distinct ultrastructure, characterized by cytoplasm compaction, condensed chromatin and, occasionally, plasma membrane blebbing. Intracellular organelles remain morphologically intact until late in the dying process (Clarke, 1990; Kerr et al., 1972). Apoptotic cell death is prevalent during development: in *C. elegans* hermaphrodites, 131 of 1090 of somatic cells generated, and half of germ-line cells, die apoptotically (Sulston et al., 1983). In *Drosophila*, apoptosis begins at 7 h of embryogenesis (Abrams et al., 1993), and in vertebrates apoptosis is evident in early developing tissues (Bedzhov and Zernicka-Goetz, 2015). Apoptosis is regulated by a conserved caspase-dependent molecular program (Fuchs and Steller, 2011; Yuan et al., 1993).

The apoptotic machinery

Caspases

Insights into the roles of caspases in the apoptotic program came initially from *C. elegans* (Horvitz, 2003). Cloning of the cell-death gene *ced-3*, and recognition that it encodes a protein similar to mammalian IL-1β converting enzyme (caspase 1), catapulted the caspase family to recognition as apoptotic executioners (Yuan et al., 1993). Caspases (cysteine-aspartic acid proteases), a group of aspartate-directed cysteine proteases, are activated following cleavage of an inactive precursor at specific aspartates (Thornberry et al., 1992). Such activation can be either self-catalytic or via other caspases (Slee et al., 1999; Thornberry et al., 1992). In *C. elegans*, the caspase CED-3 promotes apoptosis (Yuan et al., 1993). Three additional caspases, CSP-1, -2 and -3, are encoded by the genome (Shaham, 1998). CSP-1 may have pro-apoptotic functions, whereas CSP-2 and CSP-3 curtail CED-3 auto-activation. Unlike loss of CED-3, mutations in these caspases only weakly influence apoptosis progression (Geng et al., 2009).

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Box 1. Important roles for cell death in development

Morphogenesis

Cell death drives morphogenesis and tissue sculpting, as during removal of inter-digital webbing [see figure (i)] (Lindsten et al., 2000), or tube hollowing, as in pro-amniotic cavity (Coucouvanis and Martin, 1995), neural tube and lens formation (Glücksmann, 1951).

Deleting structures

Vestigial or transient structures are removed by cell death. During mammalian embryogenesis, pronephric tubules (Baehrecke, 2002) and subplate neurons (Jacobson et al., 1997) die. The tadpole tail and insect larval tissues are removed during metamorphosis (Baehrecke, 2002). In mammals, Müllerian [see figure (ii)] and Wolffian ducts degrade sex-specifically in males and females, respectively (Jacobson et al., 1997).

Regulating cell number

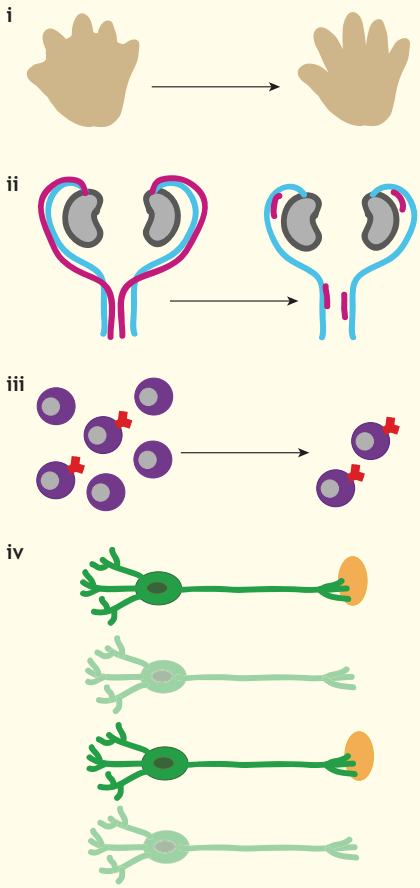
Cell numbers are maintained through a balance between cell division and death. In developing tissues, cells are often overproduced and then removed. More than 50% of generated mammalian CNS neurons die [see figure (iii)] (Oppenheim, 1991), and 80% of oocytes in human females succumb before birth (Reynaud and Driancourt, 2000).

Quality control

Cell death also exerts protective roles, removing damaged or dangerous cells. In the immune system, auto-reactive B- and T-lymphocytes recognizing self or lacking functional receptors are eliminated [see figure (iv)] (Opferman and Korsmeyer, 2003).

Evolutionary origins

In prokaryotes, cell death may have arisen in single-cell aggregates (Shub, 1994; Yu and Snyder, 1994). In the slime mold *Dictyostelium*, stalk cells die during starvation-induced differentiation, to facilitate dissemination (Cornillon et al., 1994; Loomis and Smith, 1995). Cell death also occurs in plant development, driving xylem, ovule and flower formation (Greenberg, 1996). Somatic cell death early in development promotes reproduction of the green algae *Volvox*.



Drosophila also has several caspase genes, with three of the seven having key cell-death roles (Cooper et al., 2009). The caspase Dronc cleaves, and functions upstream of caspases Drice and Dcp-1 (Hawkins et al., 2000; Xu et al., 2006). This hierarchy, which also appears in vertebrates (Inoue et al., 2009; Slee et al., 1999), suggests subdivision of caspases to initiators, such as Dronc, or effectors, such as Drice. The assignment relies, in part, on presence or absence of a large N-terminal prodomain found in initiators but absent in effectors (Sakamaki and Satou, 2009). Nonetheless, this classification is almost certainly an oversimplification, because initiator caspases such as Dronc can promote cell death even in the absence of effector caspases (Gorelick-Ashkenazi et al., 2018; Xu et al., 2006).

The caspase family expands further in vertebrates. The human genome, for example, encodes 14 caspases (Pop and Salvesen, 2009), with initiators (caspase 2, 8, 9 and 10) and effectors (caspase 3, 6 and 7) controlling apoptosis. The remaining seven caspases do not direct cell death, having specialized functions in inflammation (Labbé and Saleh, 2008) and differentiation (Hoste et al., 2013).

Activation of initiator caspases, and of CED-3 in *C. elegans*, is mediated by binding to adapter proteins, forming a super-structure called the apoptosome. The apoptosome is thought to bring pro-caspases into proximity for cross activation (Zou et al., 1999). The *C. elegans* apoptosome contains eight CED-4 adapter moieties, as determined by X-ray diffraction, which bind only two CED-3 caspase molecules (Qi et al., 2010) (Fig. 1A). Cryo-electron-microscope structures of the *Drosophila* apoptosome suggest eight copies of CED-4-like Dark (also known as dApaf-1 or HAC-1) and of Dronc (Yuan et al., 2011) (Fig. 1B). In humans, CED-4-like APAF1 assembles as a heptamer, binding three or four pro-caspase 9 proteins (Acehan et al., 2002) (Fig. 1C). Why stoichiometry of these complexes differs is not understood, nor are consequences of these differences.

Regulation of the apoptosome

The apoptosome is regulated by mitochondrial outer membrane proteins of the BCL2 family. In *C. elegans*, loss-of-function mutations in the *Bcl2*-related gene *ced-9* activate apoptosis in developing cells that normally live (Hengartner et al., 1992). In these cells, CED-9 protein normally binds CED-4, preventing apoptosome activation (Chinnaiyan et al., 1997; Spector et al., 1997; Wu et al., 1997) (Fig. 1A). Nonetheless, CED-9 may also have pro-apoptotic functions (Hengartner and Horvitz, 1994a; Shaham and Horvitz, 1996), and these could, in part, be a consequence of its effects on mitochondrial morphology (Jagasia et al., 2005). In vertebrates, pro/anti-apoptotic roles are divided among BCL2 family proteins. Some BCL2 family proteins, including BCL2, the first pro-survival protein to be discovered in any species (Vaux et al., 1988), inhibit apoptosis, whereas others, such as Bax, Bak (Bak1) and Bok, promote it (Youle and Strasser, 2008) (Fig. 1C). Binding of BCL2 family members to Apaf1 is likely not an important regulatory mechanism in vertebrates. Instead, these proteins govern the release of mitochondrial Apaf1-binding factors, chief among them, cytochrome C, which activates Apaf1 (Zou et al., 1997) (Fig. 1C). *Drosophila* BCL2-related proteins appear not to have apoptotic developmental functions, but influence DNA-damage induced apoptosis (Brachmann et al., 2000; Igaki et al., 2000; Monserrate et al., 2012). Although a role for cytochrome C in *Drosophila* apoptosis is not known, testes-specific cytochrome C activates caspases during spermatogenesis (Arama et al., 2003), suggesting conserved roles. BCL2 family protein activities are controlled through binding of small BCL2 homology domain 3 (BH3)-only proteins (Conradt and Horvitz, 1998; Youle and Strasser, 2008) (Fig. 1B).

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Box 2. Pathological cell death

Many cell death forms occur under non-physiological conditions, including necroptosis, ferroptosis and others. In these, an essential cellular function is disrupted, leading to cell loss. The term 'regulated cell death' is used to group these cell death forms with apoptosis (Galluzzi et al., 2014; Tang et al., 2019). However, this is misleading, as it implies that, like apoptosis, these death forms have been evolutionarily selected for their cell-lethal functions, a claim that is unsupported. Although apoptosis can also occur in pathology, and apoptotic proteins can have non-cell-death roles, mutations in apoptotic components clearly disrupt developmental cell death. Proteins implicated in non-physiological cell death forms generally have key functions entirely unrelated to cell death, and loss of these regulators has no effect on physiological cell death. A more apt term for these cell death events is, therefore, pathological cell death, which also emphasizes their possible relevance in clinical settings.

Recognizing this issue, an attempt to link entosis, pathological death of healthy tumor cells through engulfment by neighboring tumor cells, to physiological LCD in *C. elegans*, revealed similarities in cell adhesion and actin localization (Lee et al., 2019). Yet, other results argue strongly against a link. Adhesion and cytoskeletal proteins are not unique markers of entosis, and mutants lacking these proteins were never shown to exhibit linker cell survival. Many LCD genes act within the linker cell and not in the engulfing cell (Abraham et al., 2007; Blum et al., 2012; Kinet et al., 2016; Malin et al., 2016), contradicting the definition of entosis as death by engulfment (Overholtzer et al., 2007). Nuclear crenellations, an early sign of cell death, precede linker cell engulfment (Keil et al., 2017), also contradicting the definition of entosis. In fact, the linker cell can die in the complete absence of engulfment (Abraham et al., 2007; Keil et al., 2017). Furthermore, linker cell engulfment is mediated by RAB-35 and ARF-6 GTPases (Kutscher et al., 2018), not implicated in entosis. Nonetheless, such efforts to link pathological cell death types to developmental processes are important, as they may uncover novel modes of developmental cell death.

Although BCL2-related proteins play more prominent roles in *C. elegans* development than in *Drosophila*, the opposite is true for Inhibitor-of-Apoptosis (IAP) proteins. *Drosophila* DIAP1 and mammalian XIAP are E3 ubiquitin ligases that target caspases for inhibition and/or degradation (Ryoo et al., 2002; Schile et al., 2008; Vaux and Silke, 2005). *Drosophila* proteins Reaper, Hid and Grim (White et al., 1994), and mammalian mitochondrial proteins Smac (Diablo), ARTS (Septin4) and HtrA2 (Omi; a mitochondrial serine protease) (Larisch et al., 2000; Suzuki et al., 2001; Verhagen et al., 2000) regulate IAPs and cell death induction (Fig. 1B,C).

Alternative mechanisms of caspase activation

In addition to the intrinsic, mitochondrial-release pathway, mammals also activate apoptosis through extrinsic pathways, involving cell-surface receptor engagement by ligands, such as tumor necrosis factor (TNF). This promotes formation of death-induced signaling complexes (DISCs), which contain receptors, linking proteins and an initiator caspase, caspase 8. Like apoptosomes, DISCs facilitate self-cross-activation of pro-caspase 8 moieties, which in turn activate effector caspases (Mace and Riedl, 2010; Yu and Shi, 2008) (Fig. 1C).

Targets of the apoptotic machinery

Although apoptotic roles of caspases were discovered three decades ago, we only have limited understanding of how caspases bring about cell death. Whether caspases cleave a few crucial substrates to effect cell demise or whether wholesale protein degradation is required remains unknown. Hundreds of proteins can be cleaved by caspases (Julien and Wells, 2017); however, functional roles for cleavage are only documented in a few cases.

In vertebrates, for example, DNA degradation accompanies apoptosis and is initiated by caspase-activated DNAse (CAD; DFFB) (Halenbeck et al., 1998). CAD associates with an inhibitor, ICAD (DFFA), which is cleaved during apoptosis, releasing CAD and allowing it to generate blunt-end double-strand DNA breaks (Sakahira et al., 1998). CAD-dependent DNA cleavage is not required for apoptosis, but DNA fragmentation/elimination is delayed in CAD mutants (Kawane et al., 2003).

Cleavage substrates also promote dying-cell phagocytosis. In *C. elegans* and the mouse, caspases cleave Xk family proteins, which regulate plasma-membrane lipid asymmetry (Stanfield and Horvitz, 2000; Suzuki et al., 2013). Xk protein processing leads to phosphatidyl-serine exposure on plasma membrane outer leaflets, attracting phagocytes that engulf the cell. As with CAD, neither *C. elegans* CED-8 nor mouse Xkr8 Xk proteins are required for apoptosis, but their loss alters elimination kinetics.

Non-apoptotic cell death

Murine *Casp3*, *Casp9* or *Apaf1* gene knockouts cause perinatal lethality (Kuida et al., 1998, 1996; Yoshida et al., 1998). These mutants have cranial disruption, suggesting excess neuron survival, and persistence of inter-digital webbing, which initially pointed to key developmental roles for apoptosis. Nonetheless, subsequent studies have questioned these interpretations. For one, cell death during early embryo cavitation proceeds unabated in these mutants (Coucouvanis and Martin, 1995). Furthermore, although perinatal lethality is common, even triple-mutant mice lacking Bax, Bak and Bok, in which no apoptosis occurs, can develop to adulthood (Ke et al., 2018). Furthermore, although inter-digital webbing persists for a while, it is eventually eliminated (Chautan et al., 1999). Thus, other developmental cell death forms must exist. Linker cell-type death (LCD) has emerged as a leading candidate, along with other, less well characterized, pathways.

Linker cell-type death

Studies of the *C. elegans* linker cell provided the first direct evidence that caspase-independent non-apoptotic cell death operates during animal development (Fig. 2). The linker cell, a male-specific leader cell that guides gonad elongation, dies to facilitate vas deferens and cloacal fusion (Kimble and Hirsh, 1979). Mutants in which linker cell death does not occur retain sperm and are likely infertile (Abraham et al., 2007). Importantly, linker cell death still occurs in animals lacking all four *C. elegans* caspase-related genes (Abraham et al., 2007; Denning et al., 2013). Similarly, other apoptosis genes are not required, nor are genes implicated in autophagy or necrosis. Consistent with these observations, dying linker cell ultra-structure differs from apoptotic morphology (Fig. 2A,B) and is characterized by lack of chromatin condensation, a crenellated nucleus and organelle swelling (Abraham et al., 2007). Thus, LCD represents a novel cell death program.

A central LCD regulator in *C. elegans* is HSF-1, a conserved transcription factor, which adopts death-promoting roles that are distinct from its well-described protective functions in heat-shock response. *let-70* (encoding a conserved E2 ubiquitin-conjugating enzyme) is a key HSF-1 target. LET-70 (E2), ubiquitin and proteasome component expression increases preceding LCD. CUL-3 (cullin-3), RBX-1, BTBD-2 and SIAH-1 E3 ligase components function with LET-70 for *C. elegans* LCD (Kinet et al., 2016) (Fig. 2C).

Ultra-structural similarities to *C. elegans* LCD abound in developing vertebrates. For example, murine embryonic stem cells lacking caspase 9 undergo normal culling, but with LCD morphology (Hakem et al., 1998). Spinal cord motoneuron death

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Table 1. Pathological cell death

Type of death	Key triggers	Features/characteristics	Molecular events	References
Necrosis	Accidental, passive, cell death. Can be triggered by oxidative stress, calcium overload, trauma or ischemia.	Swelling and rupture of the cell and its organelles; leakage of cellular contents; possibly triggering inflammation.	Can be driven by sudden loss of mitochondrial potential.	Allard et al., 2000; Izzo et al., 2016; Kerr et al., 1974; Orvis et al., 2008; Roberts et al., 2002; Vanden Berghe et al., 2014
Necroptosis	Combination of death-receptor activation and loss of caspase 8.	Morphologically necrotic.	Initiated by death or pathogen-recognition receptors. Depends on RIPK3 kinase activation by RIPK1, leading to MLKL pseudokinase phosphorylation and activation. MLKL oligomers can translocate to the plasma membrane leading to permeabilization and death.	Galluzzi and Kroemer, 2008; Linkermann and Green, 2014; Murphy et al., 2013
Ferroptosis	ROS and iron-dependent intracellular lipid peroxidation.	Morphologically necrotic.	A number of molecular players have been identified. The best characterized is the reduced glutathione (GSH)-dependent enzyme glutathione peroxidase 4 (GPX4), which has a pro-survival function and inhibits ferroptosis.	Dixon, 2017; Xie et al., 2016; Yang and Stockwell, 2016
Pyroptosis	Perturbations of extracellular or intracellular homeostasis associated with innate immunity. Has pro-inflammatory effects.	Unusual chromatin condensation; plasma membrane permeabilization.	Although morphologically non-apoptotic, pyroptosis is caspase-dependent. Death occurs through proteolytic cleavage of gasdermin D (GSDMD) by inflammatory caspases.	Aachoui et al., 2013; Jorgensen and Miao, 2015; Wang et al., 2017
Parthanatos	DNA damage.	Membrane rupture without swelling; DNA fragmentation; chromatin condensation.	Requires polyADP-Ribose polymerase 1 (PARP-1), a chromatin-associated nuclear protein important for DNA repair. PARP1 hyper-activation leads to ATP depletion and accumulation of poly (ADP-ribose) polymers. Parthanatos is caspase-independent but requires mitochondria-associated apoptosis inducing factor (AIF) to which poly (ADP-ribose) polymers bind. AIF translocates to the nucleus and mediates DNA fragmentation and chromatin condensation. Cytosolic AIF promotes translocation of macrophage migration inhibitory factor (MIF) into the nucleus, where it cleaves DNA.	David et al., 2009; Fatokun et al., 2014; Virág et al., 2013; Wang et al., 2011; Yu et al., 2006, 2002
Entosis	Epithelial tumor state.	Invasion and internalization of a healthy living cell by a neighboring cell, forming cell-in-cell structures. Cell death is one possible fate of the internalized cell.	Involves cell adhesion and formation of adherens junctions by E cadherin (Cdh1). Cytoskeletal rearrangement requiring the actomycin complex RHOA (ras homolog family member), and ROCK (rho associated coiled coil protein). The host cell digests the internalized cell through LC3-associated phagocytosis (LAP) and the lysosomal degradation pathway.	Coucouvanis and Martin, 1995; Krishna and Overholtzer, 2016; Overholtzer et al., 2007

occurs unabated in caspase or *Apaf-1* mutants (Kuida et al., 1998, 1996; Yoshida et al., 1998); and although some extra motoneurons accumulate in *Bax* mutants, these fail to make synapses, have short axons and exhibit crenellated nuclei (Sun et al., 2003). LCD morphology is also observed in normally dying chick ciliary ganglion neurons (Chu-Wang and Oppenheim, 1978; O'Connor and Wyttenbach, 1974). In the reproductive system, dying Müllerian or Wolffian duct cells also exhibit LCD hallmarks

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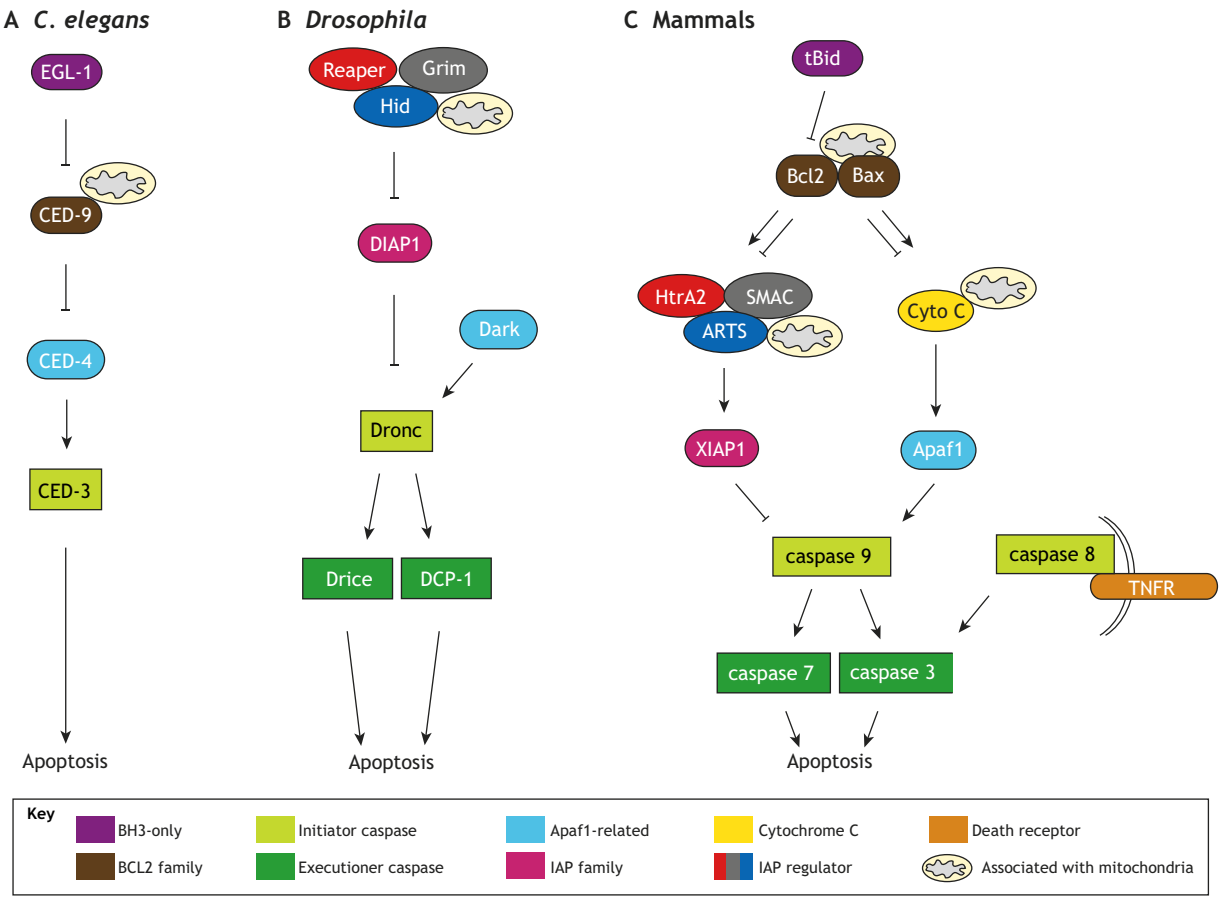

Fig. 1. Conserved apoptotic pathways. (A-C) Apoptotic cascades are initiated at the mitochondrion in *C. elegans* (A) and *Drosophila* (B) and also at the cell surface in mammals (C), resulting in caspase activation. Based on Fuchs and Steller (2011).

(Djehiche et al., 1994; Dyche, 1979; Price et al., 1977). Vertebrate homologs of some *C. elegans* LCD genes have been implicated in cell degenerative processes (see below). LCD, therefore, appears to be a prevalent non-apoptotic program operating across animals (Kutscher and Shaham, 2017).

Autophagy-associated cell death

Autophagy, bulk degradation of cytoplasm and organelles by the lysosome, accompanies cell death in some developmental contexts (Allen and Baehrecke, 2020). Whether autophagy drives cell death, or is a protective cellular response, remains hotly debated. Nonetheless, the occurrence of autophagy morphologically distinguishes autophagic death from standard apoptosis. Autophagic death has been studied extensively in *Drosophila* salivary glands, larval structures degraded after pupa formation (Berry and Baehrecke, 2007; Jiang et al., 1997). Although expression of caspase genes is induced in this structure (Lee et al., 2003), mutations in caspase genes, or in *Dark*, do not block salivary gland elimination (Berry and Baehrecke, 2007; Muro et al., 2006). Instead, cell fragments persist inappropriately. Autophagy genes are also induced early during salivary gland elimination, and in *atg18a* mutants glands are not fully degraded. Combined autophagy and caspase inhibition blocks degradation further, but not cell death initiation. Cell death is inhibited by overexpression of the PI3K active subunit Dp110 (PI3K92E), suggesting that a PI3K target drives death (Berry and Baehrecke, 2007). Cell death in the *Drosophila* midgut is also autophagic, with similar characteristics to salivary gland death (Denton et al., 2009).

Cell death by extrusion

Dying epithelial cells lose contact with neighbors and are shed in a process termed anoikis (Frisch and Francis, 1994). Although caspase activation accompanies anoikis, cell elimination by shedding is still observed in caspase mutants. Thus, contact loss may be an independent cell-elimination program. In *C. elegans* lacking CED-3 caspase, a few cells slated to die are extruded into the embryonic fluid. Shedding requires the PIG-1 AMP-activated serine/threonine kinase (Denning et al., 2012), which also promotes apoptotic death of neuroblast daughter cells (Cordes et al., 2006). PIG-1 may prevent expression of cell adhesion molecules of cells destined to die. A complex containing PAR-4, the homolog of the mammalian tumor-suppressor kinase LKB1 (STK11), may target PIG-1 to facilitate cell extrusion. In *Drosophila*, wing imaginal disc cells harboring homozygous mutations in the cell-growth gene *apterous* are removed by apoptosis and basal extrusion. When apoptosis is blocked, extrusion eliminates all dying cells (Klipa and Hamaratoglu, 2019). Shedding also occurs in vertebrate epithelia: in zebrafish, dying cells express the cell-surface lipid sphingosine 1-phosphate, allowing binding to neighboring cells. These initiate intercellular actomyosin ring contraction that ejects cells from the epithelium (Gu et al., 2011). In the mouse intestine, physiological enterocyte shedding involves redistribution of the tight-junction protein ZO-1 (TJP1; Guan et al., 2011).

Cell death with non-canonical lysosome or mitochondria involvement

Perhaps the strongest evidence that lysosomes promote developmental cell death comes from studies of *Drosophila* germ cells. Here, the

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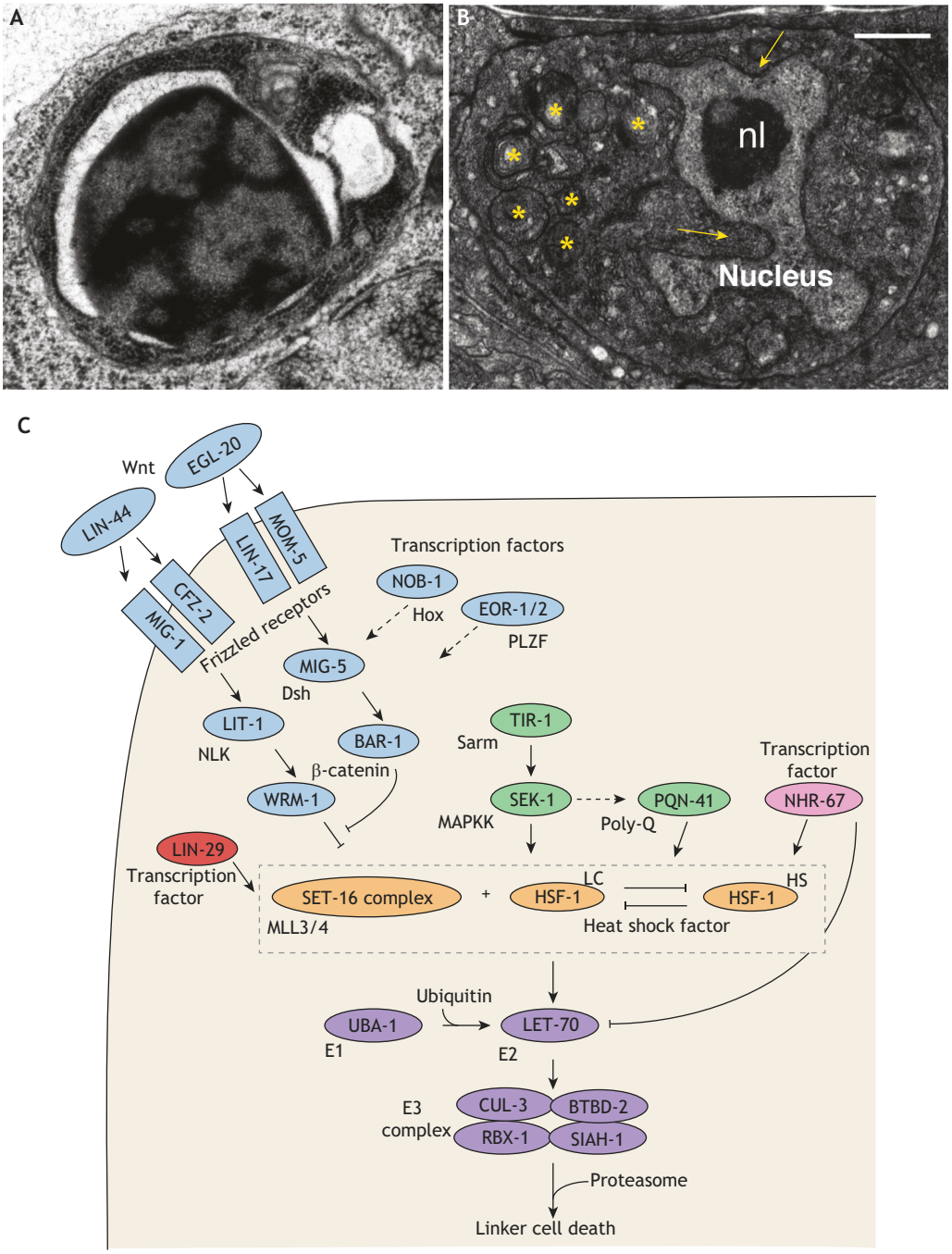

Fig. 2. Regulation of linker cell-type death in *C. elegans*. (A) Electron micrograph of a *C. elegans* apoptotic cell. (B) Electron micrograph of a *C. elegans* linker cell dying by LCD. nl, nucleolus. Arrows, nuclear invaginations/crenellations. Asterisks, swollen organelles. (C) Pathway for LCD in *C. elegans*. LCD in worms is subject to both cell-autonomous transcriptional control and non-autonomous control through multiple control pathways. Death onset is controlled by two opposing Wnt pathways (blue). The Wnt ligand LIN-44 acts non-autonomously in conjunction with the Frizzled receptors MIG-1 and CFZ-2, the Nemo-like kinase LIT-1 and the β-catenin WRM-1 in the linker cell to possibly prevent premature death. A second Wnt pathway inhibits the LIN-44/Wnt pathway. Here, EGL-20/Wnt acts non-autonomously, and LIN-17/Frizzled and MOM-5/Frizzled act in the linker cell with MIG-5/Disheveled and BAR-1/β-catenin, promoting death. This second pathway is likely controlled by two additional transcription factors, NOB-1/Hox and the EOR-1/-2/PLZF complex. In a parallel pathway (green), death is promoted by TIR-1, the *C. elegans* ortholog of mammalian Sarm, which activates the MAPKK protein SEK-1, which in turn may promote expression of the polyglutamine-repeat protein PQN-41 in the linker cell. The zinc-finger protein LIN-29 (red), which regulates developmental timing, and the nuclear hormone receptor NHR-67 (pink) appear to act independently of and in parallel with the Wnt and MAPKK pathways to promote death. In addition, HSF-1 may transcriptionally activate components of the ubiquitin proteasome system (purple).The pro-death function of HSF-1 (LC) competes with the pro-survival function (HS). Adapted from Kutscher and Shaham (2017). Scale bars: 0.5 µm.

initiator caspase Dronc is required, independently of Dark or effector caspases, to promote lysosome membrane permeabilization. Germ-cell death is reduced in lysosome biogenesis mutants and in mutants of the lysosomal protease-encoding gene *Cathepsin D* (Yacobi-Sharon et al., 2013). Lysosomes also promote the death of nurse cells, which provide developing *Drosophila* oocytes with mRNA, proteins and organelles (Jenkins et al., 2013). Here, loss of lysosomal DNaseII Deep orange (Vps18; a lysosomal trafficking protein), Spinster (a lysosomal fusion protein) or Cathepsin D results in nurse cell nuclei persistence. Deep orange functions in the engulfing follicle cells, whereas DNaseII and Spinster act cell autonomously (Bass et al., 2009; Peterson and McCall, 2013).

Although release of mitochondrial proteins can lead to caspase activation and apoptosis, mitochondria may also direct alternative cell dismantling. Mitochondrial HtrA2 can promote germ-cell death in *Drosophila* independently of caspases (Yacobi-Sharon et al., 2013). In mammalian cells, HtrA2 overexpression also promotes caspase-independent cell death, accompanied by morphological changes resembling dying *Drosophila* germ cells (Suzuki et al., 2001).

Developmental regulation of cell death

Transcription

Cell death is activated in developing tissues in a myriad of ways. Apoptosis in *C. elegans* is usually induced by transcriptional activation of *egl-1*/BH3-only (Malin and Shaham, 2015) (Fig. 1A). For example, the sex-determination protein TRA-1A (TRA-1) represses *egl-1* transcription in HSNs of hermaphrodites, but not

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males, driving sexually-dimorphic survival of this cell (Conradt and Horvitz, 1999). The Hox gene *lin-39* suppresses *egl-1* transcription in VCs, preventing apoptosis (Potts et al., 2009). Transcription of *ced-3* caspase also controls apoptosis onset (Malin and Shaham, 2015). In the tail-spike cell, which dies independently of EGL-1, apoptosis initiation follows *ced-3* caspase gene transcription by PAL-1, a caudal-type homeodomain protein (Maurer et al., 2007). In *Drosophila*, the Hox genes *Deformed* and *Abd-B* promote apoptosis at intersegmental boundaries by regulating *reaper* expression (Aachoui et al., 2013; Lohmann et al., 2002; Miguel-Aliaga and Thor, 2004; Suska et al., 2011).

RNA inhibition

Post-transcriptional mechanisms also control apoptosis. In the *C. elegans* germ-line, for example, *ced-3* caspase mRNA is repressed by four conserved RNA-binding proteins (Subasic et al., 2016). Likewise, the *Drosophila* microRNA *bantam* is a potent inhibitor of apoptotic cell death during development (Brennecke et al., 2003). *C. elegans bantam*-related microRNAs *mir-35* and *mir-58* also inhibit apoptosis by inhibiting *egl-1* mRNA accumulation (Sherrard et al., 2017).

Signaling

Cell-cell signaling pathways in *Drosophila* and vertebrates often initiate apoptosis. In *Drosophila*, Notch signaling promotes apoptosis of neuronal hemilineages in the post-embryonic ventral nerve cord (Truman et al., 2010). The Hippo signaling pathway also regulates cell death through the transcriptional co-activator Yorkie, which inhibits *hid*, leading to DIAP1 activation and cell survival (Huang et al., 2005). In the murine skin and nervous system, loss of Ras pathway components promotes apoptosis (Satoh et al., 2011; Scholl et al., 2007), suggesting this developmental pathway normally blocks death.

External signals also regulate LCD (Fig. 2C). In *C. elegans*, linker cell LCD is controlled by the EGL-30 pro-death and LIN-44 pro-survival Wnt signals that together function redundantly with developmental timing (LIN-29, Zn-finger) and SEK-1/MAPKK pathways. These pathways control non-canonical HSF-1 activity (Kinet et al., 2016). In vertebrates, Müllerian duct degeneration, which appears to proceed by LCD, is initiated by a TGF-β-related anti-Müllerian hormone (Cate et al., 1986) and by Wnts (Allard et al., 2000; Orvis et al., 2008; Roberts et al., 2002).

Engulfment assistance

Signaling from engulfing cells has emerged as an important mechanism for guaranteeing apoptosis fidelity. For example, neighboring engulfing cells non-autonomously assist killing of *C. elegans* B.al/rapaav cells (Johnsen and Horvitz, 2016), and engulfment gene mutations enhance cell survival in animals homozygous for weak *ced-3* caspase mutations (Hoeppner et al., 2001; Reddien et al., 2001). Engulfing cells may promote polarized CED-3 caspase distribution in precursor cells, resulting in death of one daughter cell and survival of the other (Chakraborty et al., 2015). Similar non-autonomous requirements for engulfment genes regulate *Drosophila* nurse cell death. Loss of the engulfment receptor Draper, homologous to *C. elegans* CED-1 and vertebrate MEGF10, from surrounding follicle cells blocks nurse cell genome fragmentation and death (Timmons et al., 2017).

Cell compartment elimination

Subcellular compartments are often selectively eliminated during development, a process that has been termed ‘pruning’. In the nervous system, axon or dendrite fragmentation removes exuberant connections to refine and sculpt activity (Box 1; Fig. 3A). Such remodeling occurs in *Drosophila* mushroom body gamma neurons (Technau and Heisenberg, 1982; Watts et al., 2003) and dendritic arborization (da) neurons (Williams and Truman, 2005), as well as in murine L5 cortical neurons (Bagri et al., 2003). Neuronal pruning can also be achieved by process retraction, or ‘dying back’, without fragmentation (Fig. 3B), as in mammalian hippocampal infrapyramidal neurons. Receptors for axon guidance, TGF-β and so-called death receptors initiate pruning (Bagri et al., 2003; Low et al., 2008; Nikolaev et al., 2009; Yan et al., 2005; Yu and Schuldiner, 2014; Zheng et al., 2003). Downstream players include ubiquitin proteasome system components, calcium-activated calpains, kinases and cytoskeletal regulators (Chen et al., 2012; Ghosh et al., 2011; Watts et al., 2003; Williams and Truman, 2005; Zhai et al., 2003).

Caspases are important for developmental pruning. In *Drosophila*, Dronc, Drice and DCP-1 are essential for da neuron dendrite culling (Kuo et al., 2006; Schoenmann et al., 2010; Williams et al., 2006). In mammals, caspase 3 and caspase 6 promote developmental pruning of retinocollicular axons (Simon et al., 2012). Caspase-dependent axon degeneration can also occur following NGF deprivation. Here, a retrograde signal from the axon induces a transcriptional response that feeds back into the axon to effect its destruction. Thus, pruning can be a cell-wide phenomenon, and requires communication between cell compartments (Simon et al., 2016).

Caspase-dependent cell compartment-specific elimination is also found during *Drosophila* spermatogenesis. Here, before individualization, 64 spermatids remain interconnected by cytoplasmic bridges. An actin-based complex traverses the length of the spermatid axonemes away from the nuclei, resolves the cytoplasmic bridges and extrudes the cytoplasm between the spermatid tails, leaving behind individualized spermatids. Caspase mutants retain cytoplasm-filled cystic bulges (Tokuyasu et al., 1972; Fabrizio et al., 1998; Arama et al., 2003).

Even when an entire morphologically complex cell is eliminated in development, different parts of the cell can die by different mechanisms, as with the *C. elegans* tail-spike cell, a morphologically complex cell with a long microtubule-rich process that shapes and extends to the tip of the tail. The tail-

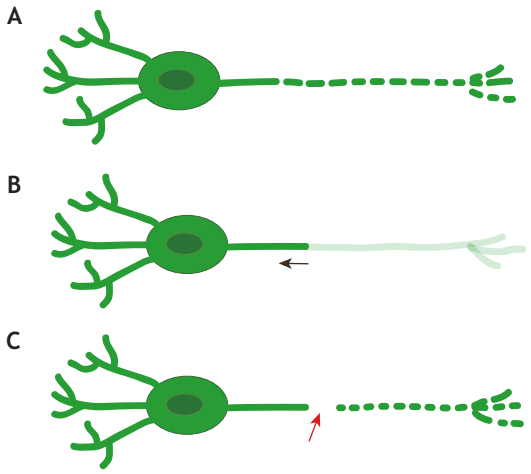

Fig. 3. Neurite-specific elimination. (A) Cell process fragmentation. (B) Cell process retraction, or ‘dying back’. (C) Wallerian degeneration: following axon severing (red arrow) the cell body remains intact while the axon fragments.

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spike cell dies after guiding tail formation in the late-stage embryo through a non-canonical apoptotic pathway that depends on CED-3 caspase and CED-4/Apaf1, but not EGL-1/BH3-only (Maurer et al., 2007). Initiation of cell death depends on transcriptional activation of *ced-3* by PAL-1, a homolog of mammalian Cdx homeodomain protein. The tail-spike cell is relatively long-lived, dying after differentiation, lending itself to a more detailed examination than most cells fated to die in the nematode. The tail-spike cell dies through three compartment-specific programs, in which the proximal process fragments and clears first, the cell body then rounds, and the distal process retracts (Ghose et al., 2018) (Fig. 4). This multi-faceted degeneration program, which we have termed compartmentalized cell elimination (CCE), also promotes removal of *C. elegans* CEM neurons, which die sex-specifically in the hermaphrodite embryo (Ghose et al., 2018). This suggests existence of a conserved mode of complex cell elimination. Nonetheless, our understanding of pruning mechanisms is rudimentary, and elucidation of compartment-specific dismantling mechanisms remains an important goal for the field.

Cell death and disease

The 2002 Nobel Prize was awarded, in part, for discovering genes controlling cell death in animal development. These studies, initiated when *C. elegans* was still an obscure model system, provided a blueprint for identifying cell death pathways in other animals, revealing broad conservation of the apoptotic program. Apoptosis research quickly gained traction once roles in human disease became apparent, with the discovery of mutations in regulators such as BCL2 and the death receptor Fas in lymphoproliferative disease and in cancer. Nonetheless, rather than the end of the story, the Nobel recognition coincided with the realization that much more needs to be investigated before cell death is declared understood. The identification of non-apoptotic programs in development reflects this current momentum, and association of these programs with human diseases (Box 3) once again drives wide interest.

Studies of cell compartment elimination in development may also shed light on related human pathologies. Perhaps the most well-studied example of pathological neurite degeneration is Wallerian degeneration (Fig. 3C; Coleman and Freeman, 2010). Here, distal axons, severed from the cell body, fragment. In Wallerian degeneration slow (Wld^s) mice, expressing an abnormal protein fusion between a NAD^+ synthesis protein, NMNAT, and the ubiquitin factor E4B (Ube4b), axons persist after severing, indicating that axon degeneration is not passive (Mack et al., 2001). That the gene *Sarm* is required for Wallerian degeneration in mammals and *Drosophila* (Hoopfer et al., 2006; Osterloh et al., 2012; Xiong et al., 2012), and for developmental LCD in *C. elegans* (Blum et al., 2012), suggests that developmental cell death components may underlie pathological cell or process elimination.

Box 3. Linking developmental cell death to human disease

A number of developmental cell death genes have been associated with disease states. Homologs of several *C. elegans* LCD pathway components, for example, promote cell-degenerative processes or tumorigenesis in vertebrates. *C. elegans pqn-41*, which encodes a self-aggregating glutamine-rich protein, is reminiscent of polyQ proteins that cause neurodegeneration (Blum et al., 2012), and polyQ-associated death is characterized by nuclear crenellations like those seen in LCD (Davies et al., 1997). *tir1*/Sarm, which functions with PQN-41/Q-rich, promotes distal axon degeneration following axotomy in mice and *Drosophila* (Osterloh et al., 2012). The LCD regulators *let-7*/microRNA and HSF-1 are often altered in tumors (Jiang et al., 2015; Nguyen and Zhu, 2015). Homologs of the transcriptional regulators SET-16/MLL, NHR-67/TLX, and EOR-1/PLZF, which also regulate *C. elegans* LCD, are altered in – and causal for – some tumors (Chen et al., 1994; Jackson et al., 1998; Ruault et al., 2002). HTRA2 lesions in humans are associated with Parkinson's disease, and Pink1, a Parkinson's disease and mitochondria-associated protein, promotes *Drosophila* germ-cell death (Strauss et al., 2005).

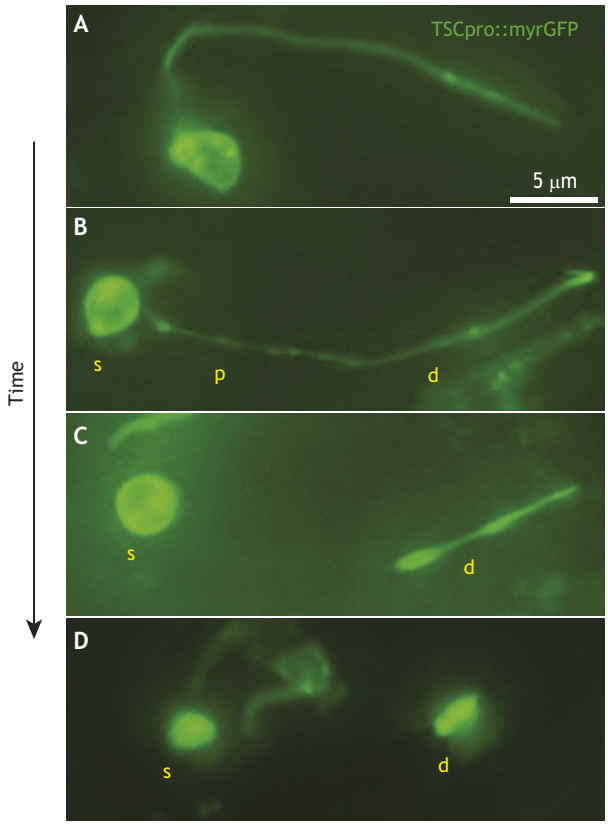

Fig. 4. Compartmentalized cell elimination. (A-D) Images of a dying *C. elegans* tail-spike cell at different stages of death. (A) An intact tail-spike cell with a soma and long process. The proximal process segment (p) undergoes localized beading and fragmentation (B) and is eliminated before the soma (s) and distal process (d) (C). The soma rounds (B,C). The distal segment undergoes bidirectional retraction into a compact structure (C,D) and is eventually engulfed and removed. The entire cell is eliminated in ∼150 min. The caspase CED-3 acts independently in each compartment. Adapted from Ghose et al. (2018). TSCpro, tail-spike cell promoter; myrGFP, myristoylated green fluorescent protein (GFP).

Future perspectives

Many questions about the basic mechanisms of cell death in development remain. Perhaps most important is an understanding of what it means for a cell to die. Are specific cellular pathways targeted, disruption of which is the point of no return? Do all cell death pathways converge on these same targets? Is the end of metabolism the end of life for a cell, or is cell death only truly effected through phagocytic degradation? Touching on the realm of philosophy, it is possible that delving deeper into cell death mechanisms will reveal meaningful responses to these metaphysical inquiries.

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