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Exploring advanced *Drosophila* cell death techniques and cancer-related studies

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Cell death is an essential physiological process for the survival of multicellular organisms. Our understanding of programmed cell death in development, immune function maintenance, and adult tissue repair has significantly advanced over the past decade. However, there are still gaps in our knowledge about the induction, regulation, and checkpoints of this process due to the diverse forms of cellular suicide and the rapid nature of the process. Molecular advancements such as specific cell death sensors, RNA-seq, single-cell RNA-seq, and proteomics have allowed for identifying new factors and a better understanding of the molecular networks and pathways that regulate these processes. Programmed cell death also plays a role in cancer, both limiting and facilitating aspects of the malignant process, making its analysis and inhibition challenging. This review discusses the field's advancements using the model organism *Drosophila melanogaster*, the types of cell death in development and adult tissues, the techniques for studying it, and its role in cancer.

KEYWORDS

programmed cell death, sensors, methods, -omics, development, cancer, *Drosophila*

Introduction

The ability of an organism to induce its own cells to die is an evolutionarily advantageous process that safeguards the organism and ensures only the fittest cells survive. Early studies characterizing the distinct morphologies associated with cell death processes revealed the inherently regulated and intentional nature of programmed cell death (PCD) (Kerr et al., 1972). PCD occurs under many normal physiological conditions and through many different means, playing an essential role in the removal of unfitted or surplus cells (Fuchs and Steller, 2011). The pioneering genetic and molecular studies on the regulation of PCD were conducted using *C. elegans* (Ellis and Horvitz, 1986). In *C. elegans*, PCD is essential for germ-cell death during oogenesis (Lettre and Hengartner, 2006) and protection against pathogen attack (Aballay and Ausubel, 2001). Examples of PCD during development are the hormonal signalling pathways selectively eliminating Müllerian ducts in males and Wolffian ducts in females (Arya and White, 2015), the developing of digits in higher vertebrates (Fuchs and Steller, 2011) or the regular lattice of the retina of insects (Cagan and Ready, 1989). PCD also acts in adult organisms to eliminate surplus progenitor cells in the mammalian immune system (John Cohen, 1991) and the excess enteroblast cells during the repair and homeostasis of the adult intestine of fruit fly (Reiff et al., 2019). PCD is also a critical process in cancer as evading cell death is one of the biological processes that allow cancer cells to thrive, survive, and facilitate invasion and migration of metastatic cells (Koren and Fuchs, 2021).

The fruit fly *D. melanogaster* is an excellent model for investigating the complex interplay between cell death, cell competition, and tumorigenesis (Adrados et al., 2024; Mirzoyan et al., 2019; Parvy et al., 2018; Pinal et al., 2019; Sollazzo et al., 2023). For instance, cell competition, initially discovered in *D. melanogaster*, plays a crucial role in both fly and human cancer development as an intrinsic tumour suppression mechanism (Morata and Ripoll, 1975; Simpson and Morata, 1981). Studies in the fruit fly of this evolutionarily conserved process provide valuable insights into clonal evolution and tumour heterogeneity in human cancers, potentially leading to novel therapeutic approaches (Cong and Cagan, 2024). The genetic tools available for this animal model allow for precise manipulation of genes involved in cell death and the cancer-related processes, making it an ideal system for studying the molecular pathways that regulate these phenomena (Hay et al., 2004; Munnik et al., 2022). Additionally, the conservation of signalling pathways between *D. melanogaster* and humans has contributed significantly to understanding cancer hallmarks such as genomic instability, resistance to cell death, altered metabolism, inflammation, and immune evasion (Baonza et al., 2022; García-López et al., 2021; Jiang et al., 2022; Shan and Mollereau, 2024; Stefanatos and Vidal, 2011).

This review aims to critically analyse the most recent advancements for investigating cell death and cancer-related cell death in the *D. melanogaster* model. By integrating and synthesizing the latest developments in techniques and methodologies, we aim to provide researchers with a comprehensive interpretation of the cutting-edge tools available to uncover the fundamental biological processes that underlie PCD. Here we summarize and exemplify the roles of PCD to equip researchers with the knowledge and insights necessary to advance the field and make significant contributions to understanding cell death and cancer in *D. melanogaster*.

The multifaceted roles of PCD in *D. melanogaster*

Cell death during development

Studies on cell death in *D. melanogaster* have offered valuable insights into the complex mechanisms that regulate this fundamental process in development and biological homeostasis [reviewed in Yalonetskaya et al. (2018)]. From embryonic stages to metamorphosis, *D. melanogaster* has proven to be an exceptional model for unravelling the mysteries of PCD in diverse physiological contexts orchestrating essential processes for maintaining cellular equilibrium. The *D. melanogaster* life cycle comprises four distinct stages: embryo, larva (first, second and third instar), pupa (prepupal and pupal stage) and adult. Notably, cell death manifests early in embryogenesis, with apoptotic cells appearing approximately 7 hours after egg deposition and subsequently becoming more widespread throughout the embryo (Abrams et al., 1993). During the larval stages, PCD occurs in several tissues, with a pronounced presence in the peripheral and central nervous system (CNS). Crucially, specific signalling pathways tightly regulate cell death, playing a vital role in the selective elimination of cells during tissue morphogenesis (Rusconi et al., 2000). Examples of that are the waves of PCD among neurons, initiated during mid-to-late embryogenesis, moulding the CNS development (Abrams et al., 1993). The first wave of neurogenesis in the embryonic stages establishes the larval nervous system, while a second wave, which develops during the larval and pupal stages, shapes the remaining components of the CNS that will function in the adulthood (Rogulja-Ortmann et al., 2007). This process, however, is not static, as numerous larval neurons meet their fate in cell death during metamorphosis, underlining the dynamics and plasticity of these events (Truman and Bate, 1988). In addition to the death of neuroblasts, neurons and glia also die throughout development, both to establish appropriate cell numbers and to remove cells that are no longer required in later stages (Pinto-Teixeira et al., 2016).

The differentiation of the adult eye from imaginal tissue during pupal development is another clear example of PCD, which involves the precise patterning of the interommatidial cells surrounding the photoreceptor clusters. Here, apoptosis serves as the mechanism for eliminating superfluous cells, highlighting its role in the formation of the mature organ (Cagan and Ready, 1989; Wolff and Ready, 1991). The larval-to-pupal transition represents a crucial stage characterised by dramatic cell death, devised primarily by the steroid hormone ecdysone (Jiang et al., 1997). This process engages the elimination of many larval tissues, a phenomenon tightly controlled by nuclear hormone receptors and transcription factors that provide spatial and temporal regulation (Garelli et al., 2012). This massive cell death during the larval-to-pupal transition appears to involve mechanisms beyond canonical apoptosis, underscoring the complexity of the regulatory landscape. During the third instar larva stage of development, autophagy has been shown to function as a key cell death mechanism. Removing the obsolete larval midgut and fat body is a prime example of autophagy-dependent cell death through regulation of the PI3K pathway (Berry and Baehrecke, 2007; Rusten et al., 2004). In some cases, this process requires autophagy but occurs independently of apoptosis (Denton et al., 2009), but in others, like salivary gland degradation, it also involves both autophagy and apoptosis working in concert (Martin et al., 2007). This process, triggered by the steroid hormone ecdysone, involves the activation of autophagy-related genes as well as apoptotic machinery.

In the context of oogenesis, the *D. melanogaster* ovary offers a fascinating terrain for studying PCD. This process is also notable for its complexity, with hundreds of ovarian chambers progressing through defined stages of development. Here, nurse cells, essential for oogenesis, undergo PCD after transferring their cytoplasmic contents to oocytes, and are subsequently eliminated by a subset of follicular epithelial cells. This meticulous process reveals the interconnection between cell death and reproductive development (Timmons et al., 2016).

The *D. melanogaster* cell death machinery is primarily represented by apoptosis, a caspase-dependent cell death pathway highly conserved among metazoans. Caspases, cysteine proteases, play a central role in apoptosis, where the initiator caspases Dronc respond to apoptotic stimuli and the effector caspases Drice and Dcp-1 cleave substrates to induce cell death (Kumar and Cakouros, 2004). In *D. melanogaster*, several types of PCD have been identified, contributing to various developmental processes (Figure 1). Briefly, prominent forms of cell death and related processes include:

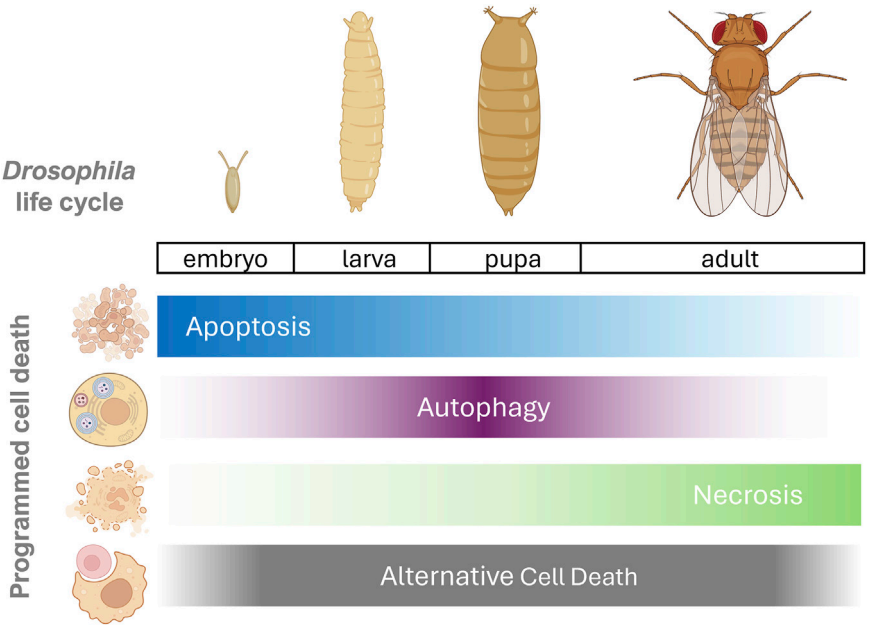

FIGURE 1
D. melanogaster developmental cell death. An illustration of main programmed cell death programs activated during the *D. melanogaster* life cycle. Apoptosis is initiated in early embryogenesis and continues during neurodevelopmental stages. Despite most *Drosophila* tissues dying via apoptosis, in the larval and pupal stages, autophagic-dependent cell death is activated in response to ecdysone. During adulthood, male spermatogonia cyst cells and female nurse cells die by necrotic process. Alternative cell death processes occur during all stages of *D. melanogaster* development, i.e., parthanatos at embryogenesis, ferroptosis during wing disc development and phagoptosis (also called phagocyte-driven cell death) during spermatogenesis. Created with BioRender.com.

Apoptosis

This is a well-characterized and extensively studied form of PCD in *D. melanogaster*. It plays a crucial role in development, tissue homeostasis, and the removal of unwanted or damaged cells (White et al., 1994). The proapoptotic genes *grim*, *reaper*, *hid*, and *sickle*, collectively known as RHG, initiate apoptosis in response to specific developmental cues (Bergmann et al., 1998; Goyal et al., 2000; Hay et al., 1995; White et al., 1996). Other routes besides the RHG motif could activate apoptosis. For instance, mitochondrial cytochrome c (Cyt c) that regulates apoptosis in the developing eye (Mendes et al., 2006) and is involved in caspase activation for spermatid individualization (Arama et al., 2006) and salivary gland degradation (Long et al., 2024).

Autophagy

This process involves the degradation and recycling of cellular components within lysosomes. Autophagy is essential for maintaining cellular homeostasis, and its dysregulation has been implicated in various diseases including cancer (Debnath et al., 2023). In *D. melanogaster*, autophagy plays a role in various developmental stages and responses to nutrient availability (Berry and Baehrecke, 2007; Denton et al., 2009). While autophagy is a well-studied mechanism of cell death, it can also contribute to the pro-survival mechanism, modulating necrotic cell death in *Drosophila* neurons and potentially acting as a protective mechanism against stress-induced necrosis (Lei et al., 2017). Furthermore, autophagy has been found to regulate necrosis in specific contexts, while necrosis signalling can influence autophagic activity in others (Park et al., 2020).

Necrosis

While apoptosis is the primary mode of PCD in *D. melanogaster*, instances of necrosis have also been observed, particularly in response to specific stress conditions (Park et al., 2020). Necrotic cell death involves rapid cellular swelling and membrane rupture, leading to inflammation (Yacobi-Sharon et al., 2013).

Efferocytosis

A process closely related to cell death, important for development and homeostasis, and responsible for removing apoptotic cells by phagocytes. This process is critical for maintaining tissue integrity and preventing inflammation. In *D. melanogaster*, efferocytosis occurs in nearly all tissues, including the CNS, where phagocytic glia and haemocytes play essential roles in clearing apoptotic cells (Davidson and Wood, 2020; Zheng et al., 2017; 2021).

Cancer cell death mechanisms

Cell death is an essential area of study for understanding the fundamental biological processes underlying cancer development and progression. *D. melanogaster* is a valuable model organism for studying these mechanisms due to its genetic similarity to humans and its well-characterized genetics and developmental biology (Jennings, 2011). Until now, cancer-related research has focused on understanding cell death evasion mechanisms and developing promising anticancer strategies that inhibit them. In this context, several forms of PCD have been identified and shown to play crucial

roles in modulating the tumour microenvironment (TME), making their study attractive in cancer research (Parvy et al., 2018).

Studies carried out in *D. melanogaster* have made it possible to identify PCD associated with tumour initiation and progression processes. An example of this is the phenomenon called "cellular competition," which plays a role in eliminating oncogenic cells or selecting fitter cells. This effect has been demonstrated in the fruit fly intestinal tumours, where oncogenic cells hack the system, competing with surrounding cells and inducing their elimination, creating a permissive environment for tumour growth (Suijkerbuijk et al., 2016). Furthermore, in different *D. melanogaster* cancer models, it has been shown that epithelial tumours exhibit a high level of cell death when grown under competitive stress. This pressure allows excessive growth of the cancerous mass, mainly dependent on the activation of caspases since their inhibition is sufficient to reduce the size of the tumours (Sollazzo et al., 2023).

In addition, autophagy can also play an important role in cancer cell death. In *D. melanogaster*, the induction of autophagy in the TME is mediated by ROS accumulation and activation of the JNK signalling pathway in tumour cells. Notably, the active transport of nutrients from cells surrounding the tumour sustains tumour growth, indicating that tumour cells proliferate and grow at the expense of their neighbouring normal cells through non-cell autonomous autophagy (Zhao et al., 2021). JNK signalling also contains tumour growth through necrosis, an alternative PCD activated when Egr/JNK-mediated apoptosis fails to inhibit the oncogenic growth of *scrib* mutant cells. This activation of necrosis is mediated by the initiator caspase Dronc (Li et al., 2019).

Despite the significant advances in the study of cell death in the context of cancer, there is still much to understand about this process and its dual role in suppressing and promoting tumours. Past and present studies highlight the power of *D. melanogaster* model to genetically dissect the complex interplay between the cell death pathways and oncogenic signalling. Inhibiting apoptosis can lead context and stage-dependent effects, sometimes promoting senescence and tumour growth while restraining malignant traits in other cases.

Techniques used to study cell death in *D. melanogaster*

The precise characterization of different types of cell death does not rely on a single technical method. Instead, it uses a multifaceted approach that combines various markers and techniques (Napoletano et al., 2019). Distinguishing between different cell death processes is achieved through the integration of both classical and modern methods. This comprehensive strategy includes the use of electron microscopy to observe ultrastructural changes, analysis of specific biochemical markers, real-time imaging techniques to track the progression of cell death, and advanced molecular methods such as single-cell RNA sequencing. Additionally, functional assays and genetic manipulations are employed to validate the signalling pathways involved. This combination of approaches allows for a more robust and reliable characterization of cell death mechanisms, overcoming the limitations of any individual method and providing a more complete understanding of these complex biological processes.

Classical approaches

In situ approaches have been utilized to study PCD during development of *D. melanogaster*, allowing for the visualization and quantification of cell death processes at the single-cell level (Denton et al., 2008; Denton and Kumar, 2015). These methods enable the detection of morphological and biochemical changes associated with PCD, such as DNA fragmentation, chromosome condensation, and nuclear deformation, providing insights into the dynamics of cell death during development (Richardson and Kumar, 2002).

The study of DNA fragmentation as a marker of apoptosis in *D. melanogaster* has been facilitated by several *in situ* techniques. The TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling) assay is widely used to detect apoptosis by identifying DNA breaks (Figure 2A), labelling their 3′-hydroxyl termini and allowing visualization of apoptotic cells in *D. melanogaster* tissues like imaginal discs (Gavrieli et al., 1992). The vital dye acridine orange (AO) is another method that has been used for decades to detect apoptosis in *D. melanogaster* tissues and cells (Figure 2B; Abrams et al., 1993; Wolff and Ready, 1991), as AO is a fluorescent dye that intercalates with DNA and can identify cells undergoing apoptosis-associated DNA fragmentation (Spreij, 1970). These well-established assays have made it possible to catalogue the pattern of cell death during *D. melanogaster* embryogenesis and metamorphosis over the years (Abrams et al., 1993; Baechrecke, 2000; Pazdera et al., 1998; Peterson et al., 2002). These methods continue to be employed to confirm and differentiate apoptosis from other types of cell death, such as necrosis. Their enduring relevance is further enhanced by refinements in detection techniques, improving sensitivity and specificity (Chimata et al., 2022). In addition, immunohistochemistry assays have been used to study apoptosis, detecting proteins such as p53, annexin V, and caspases. These *in situ* techniques enable the detection and quantification of apoptotic cells undergoing DNA fragmentation in *D. melanogaster* tissues during development and in response to various stimuli (Dichtel-Danjoy et al., 2012; Robin et al., 2019; Sarkissian et al., 2014; Shklyar et al., 2013).

The most commonly used *in situ* approaches to study PCD in *D. melanogaster* include *in situ* hybridization and *in situ* optical imaging. *In situ* hybridization is used to determine gene expression patterns by detecting RNA transcripts within cells or tissues (Van De Corput et al., 1998). However, this method has been optimized to target specifically cell death genes in *D. melanogaster* during development (Lécuyer et al., 2008; Tan et al., 2011). On the other hand, *in situ* optical imaging techniques, such as fluorescence, confocal, or multiphoton microscopy, offer a powerful approach to studying the metabolic dynamics of lipids and proteins during ageing, allowing for the visualization of cellular processes associated with PCD at a high resolution. These methods are particularly useful for studying PCD during *D. melanogaster* oogenesis, which is regulated by mechanisms different from those that control cell death in other tissues (Foley and Cooley, 1998; Nezis et al., 2000). New approaches have emerged combining traditional ones with advanced imaging systems such as direct image of lipid metabolic changes by Raman spectroscopy (DO-SRS) or mitochondrial dynamics by fluorescence wide-field microscopy (LaJeunesse et al., 2004; Li et al., 2022). This emerging technology has allowed the identification of unsaturated lipids

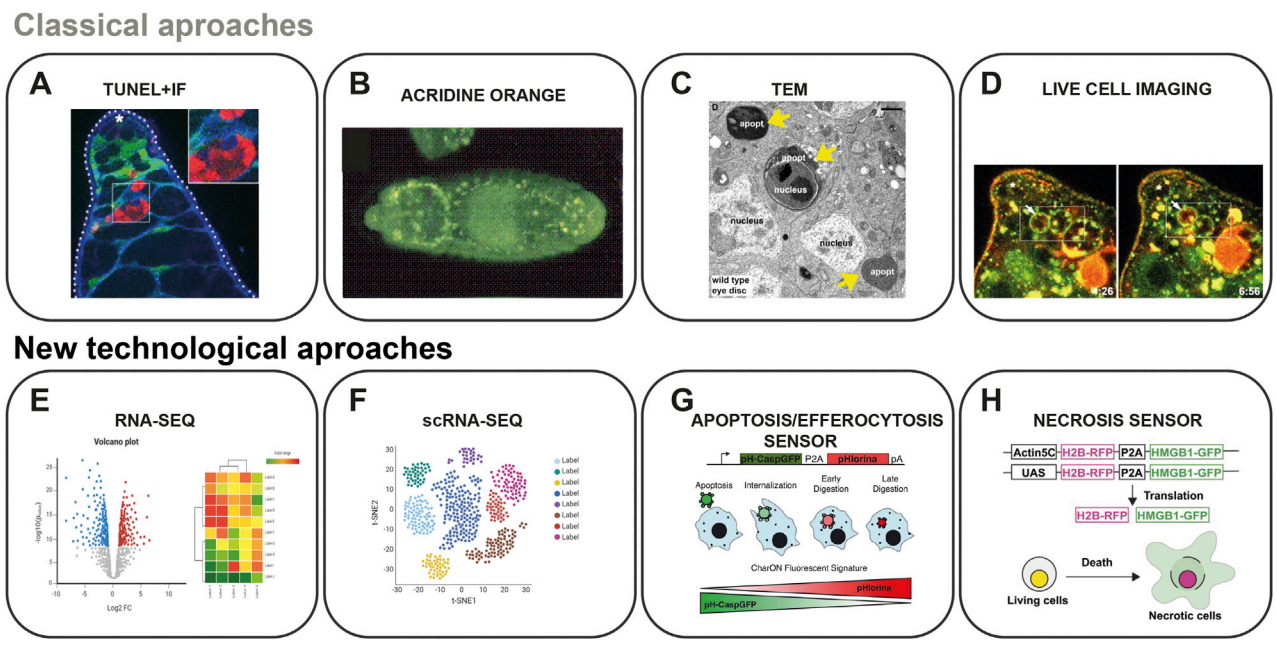

FIGURE 2
Approaches to study programmed cell death in *D. melanogaster*. This figure showcases the array of techniques available for studying PCD in *D. melanogaster*. Example results for classical approaches **(A–D)** and schematic representations for new technology approaches **(E–H)**. **(A)** The apical tip of a testis showing the expression of cytGFP in cyst cells (green), the immunofluorescence (IF) of Drpr (blue) and the TUNEL staining (red, dying germ cells). Asterisk marks the hub.**(B)** Dorsal view of a stage 14 embryo stained with Acridine Orange. **(C)** TEM photography revealing apoptotic bodies (yellow arrows) induced by clonal overexpression of *Hid* in eye imaginal disc cells. Apoptotic cells appear darker than healthy cells. **(D)** Live-imaged testis from Rab7-YFP (green) marked with LysoTracker (red). Boxed region, highlight late endosomes (white arrow) surrounding live germ cells that are gradually filled with LysoTracker. **(E)** Common graphical tools for interpretation of the RNA-seq data. **(F)** Schematic representation for visualization of scRNA-seq spatial transcriptomic data. **(G)** Example diagram of the genetic sensor CharON. The construct design is shown on the top, and at the bottom is a representation of the mechanism of an apoptotic CharON-expressing cell (green or red) engulfed by a macrophage (blue). **(H)** Diagram of the genetic sensor Necrosensor 2. The necrosensor (HMGB1-GFP) is connected with H2B-RFP via P2A. HMGB1-GFP is released into the extracellular space upon necrotic stimuli (green), whereas H2B-RFP remains in the nucleus (red). Panels A and D adapted from Zohar-Fux et al. (2022), C from Nagy et al. (2015), G from Raymond et al. (2022) and H from Nishida et al. (2024) under Creative Commons CC-BY licenses. Panel B adapted with permission from Developmental Journal (Abrams et al., 1993). Panels E and F were created with BioRender.com.

and Cyt c protein accumulated simultaneously in egg chambers from old flies that could induce cell death, as demonstrated during salivary gland degradation and neuronal apoptotic cell death (Hung et al., 2021; Long et al., 2024).

PCD is also observed in patches of cells during the larval stages, but during metamorphosis most larval tissues undergo this process mainly regulated by the steroid hormone 20-hydroxyecdysone (ecdysone). This process plays a crucial role in eliminating obsolete larval tissues and organs, allowing for the formation of the adult body structure (Jiang et al., 1997). In addition to apoptosis, autophagy contributes to this tissue remodelling during a developmental programmed 5-day starvation period. It involves the degradation of cellular components through the formation of autophagosomes, which can have survival and death functions depending on the context (Berry and Baehrecke, 2007; Denton et al., 2009; Rusten et al., 2004). Analysis of autophagy in *D. melanogaster* encompasses various techniques to monitor and analyse the PCD at different stages. Fluorescent markers, such as GFP-tagged Atg8a, allow visualization of autophagosome formation through fluorescence microscopy (Juhasz and Neufeld, 2008). Transmission electron microscopy (TEM; Figure 2C) provides high-resolution ultrastructural analysis of autophagic vesicles (Eskelinen et al., 2011). Autophagic flux assays, using lysosomal inhibitors like chloroquine or bafilomycin A1, measure the dynamic process of autophagy by blocking autophagosome-lysosome fusion (Nagy et al., 2015).

Necrosis, traditionally viewed as a response to severe damage and stress, can also occur as a physiological event in the absence of external insult in organisms. *Drosophila* spermatogenesis provided the first evidence of physiologically programmed necrosis controlled by p53, a crucial mechanism in tumour suppression. This discovery illustrates a p53-dependent mechanism that is evolutionarily preserved in mammals (Napoletano et al., 2017; Yacobi-Sharon et al., 2013). Necrosis also plays a crucial role in regulating female nurse cell death during the developmental processes of oogenesis (Bass et al., 2009). The main method for studying necrosis in these models has been the propidium iodide (PI) staining or PI/TUNEL double labelling combined with TEM and immunohistochemistry (Bass et al., 2009; Napoletano et al., 2017).

Alternative PCD forms have also been studied in *D. melanogaster,* which are activated during early embryogenesis. One of these processes is parthanatos, triggered by the overexpression or activation of the enzyme poly (ADP-ribose) polymerase-1 (PARP-1) after DNA damage caused by genotoxic stress or excitotoxicity (Wang and Ge, 2020). In *D. melanogaster,* parthanatos-like cell death is activated in 30% of primordial germ

cells, usually eliminated during embryogenesis and is detected using a Top I-mediated ligation assay, allowing the visualization of DNase II-induced DNA cleavage (Tarayrah-Ibraheim et al., 2021). Phagoptosis, another form of PCD, is also activated in *D. melanogaster* germ cells (Kanaan et al., 2023; Zohar-Fux et al., 2022). In this process, phagocytes engulf and degrade viable cells in response to an "eat-me" signal or the loss of "don't-eat-me" signals (Brown and Neher, 2012). Ferroptosis also emerged as a new PCD that has been studied in the fruit fly, a nonapoptotic form of cell death that results from iron accumulation and lipid peroxidation in cells (Saini and Owusu-Ansah, 2023). This alternative form has been demonstrated during the development of wing disc cells through morphological changes in the mitochondria and ROS accumulation (Mumbauer et al., 2019). Ferroptosis can be detected through direct measurement of lipid peroxidation by assessing the absorbance of samples at 532 nm, as well as measuring Fe levels in haemolymph using a colourimetric assay (Gomes et al., 2023). On the other hand, erebosis, a unique type of cell death, is found in the gut cells of adult fruit flies. Unlike apoptosis or necrosis, it involves a gradual loss of cell components and structure without triggering typical stress or immune responses, helping maintain gut health by replacing old cells with new ones (Bergmann, 2022; Ciesielski et al., 2022). A new proposed form of autophagy-associated cell death is karyoptosis, identified in a *D. melanogaster* model of neurodegenerative disease (Baron et al., 2017). Karyoptosis is triggered by chronic inhibition of autophagy and can be identified by detecting Lamin B1 in the cytoplasm colocalizing with autophagic markers, such as LC3 or p62 (Napoletano et al., 2019). The ancestral origin of mammalian pyroptosis, termed proto-pyroptosis, has also been described in *D melanogaster*. This inflammatory form of cell death is characterized by the recruitment of crystal cells to sites of injury and was first observed through live imaging of wounded *Drosophila* larvae (Dziedziech and Theopold, 2022). All these alternative forms of cellular death have been studied using TEM, *in vivo* live imaging and immunofluorescence techniques with fluorescent dyes such as lysotracker and Hoechst (Figure 2D).

Other alternative cell death mechanisms include entosis, a form of non-apoptotic cell death that occurs when one cell actively invades and becomes engulfed by a neighbouring cell (Overholtzer et al., 2007). Entosis has been linked to earlier studies on cell competition in *D. melanogaster*, but this mechanism requires apoptotic programs. This phenomenon is typically investigated using clonal analysis and tissue mosaics (Morata, 2021), and it has generated considerable attention in cancer research, where it is thought to play a critical role in tumour progression and the regulation of cell populations (Cong and Cagan, 2024). Alternative cell context-dependent PCD, such as excitotoxicity, have also been studied in the fruit fly. The overstimulation of neurons triggers excitotoxicity, leading to cell death through excessive excitatory neurotransmitters, especially glutamate (Sattler and Tymianski, 2001). In *D. melanogaster*, this process has been studied using calcium and glutamate live imaging combined with locomotion experiments to detect behavioural patterns after neuronal death (Peng et al., 2019; Xu and Xu, 2018).

There are also key determinants that support PCD during *D. melanogaster* development, such as efferocytosis. During embryogenesis, efferocytosis is essential for the removal of apoptotic cells from the nervous system, allowing for the proper formation and function of neurons (Kurant et al., 2008). Similarly, in the germline cells, efferocytosis helps to remove apoptotic cells and maintain tissue homeostasis during *D. melanogaster* development (Etchegaray et al., 2012; Timmons et al., 2016). The process of efferocytosis in the fruit fly has traditionally been assessed using a combination of methods, including TEM, AO staining, TUNEL assay, and immunohistochemistry.

Molecular techniques

New molecular technologies have allowed the advancement of our understanding of cell death in the fly model (Figures 2E, F). For instance, gene expression comparisons using the Gene Chip *D. melanogaster* Genome 2.0 arrays in young (two-day-old) and old (45-day-old) flies have revealed upregulation of genes promoting cell death in older flies, including the caspase genes *Damm*, *Strica* and *Decay*, as well as changes in apoptosis regulation in ageing tissues, suggesting that tissue-specific changes occur in the regulation of apoptosis as the organism ages, rather than a generalised increase in programmed cell death across all tissues (Bordet et al., 2021).

Additionally, RNA-sequencing (RNA-seq) analysis performed on the eyes of fruit fly pupae at two developmental stages, 21 and 40 h after pupa formation has provided insights into the regulation of cell death during development (DeAngelis et al., 2021). Comparing the temporal and spatial gene expression in apoptotic and non-apoptotic tissues during metamorphosis enabled the identification of a reduction in the ecdysone-induced gene *E93*, a critical regulator of cell death, in non-apoptotic tissues despite caspase activation (Ojha and Tapadia, 2020). These studies compared larval and pupal salivary glands, which undergo cell death during metamorphosis, with Malpighian tubules, which avoid apoptosis, utilising the Affymetrix *D. melanogaster* Genome 2.0 microarray chip.

Omics technologies have also addressed PCD in cancer and other pathological states. A major gene linking PCD and cancer is *TP53*, whose mutations are highly prevalent in human tumours. (Olivier et al., 2010). The p53 transcription factor coordinates various cellular responses to stress, including the initiation of apoptosis (Vousden and Prives, 2009). Using RNA-seq combined with chromatin immunoprecipitation sequencing (ChIP-Seq), the function of p53 was interrogated in postmitotic and embryonic *D. melanogaster* tissues (Kurtz et al., 2019). In the developing embryo, p53 robustly activates key apoptotic genes in response to radiation-induced DNA damage. The p53 enhancer near the cell death gene *reaper* forms chromatin contacts, facilitating the activation of p53 targets over long genomic distances. Interestingly, this typical p53 apoptotic response is absent in adult heads, a postmitotic tissue, and this lack of response is not associated with changes in chromatin contacts.

PCD in other diseases has also been investigated employing omics techniques. One example is the autosomal dominant retinitis pigmentosa (ADRP), an age-related degenerative retinal disease (Sung et al., 1991), in which the chronic perturbation of the endoplasmic reticulum induces apoptosis. To understand the pathways that mediate apoptosis related to ER stress, Park and collaborators used an ADRP *D. melanogaster* model, and found that Wg/Wnt1 signalling mediates this process. Subsequent analysis by

RNA-seq of eye imaginal discs showed that the ER stress-associated serine protease (Erasp) is a downstream target of Wg/Wnt1 during ER perturbation (Park et al., 2023).

RNA-seq analysis can be performed on whole *D. melanogaster*, dissected tissues, body parts, or at the single-cell level to examine healthy and disease cells and the PCD process. Using single-cell RNA sequencing (scRNA-seq), the impact of an *Rbf* mutation during *D. melanogaster* eye development was investigated (Ariss et al., 2018). The *Rbf* gene encodes the retinoblastoma protein (pRB) *D. melanogaster* orthologue, a tumour suppressor that blocks cell-cycle progression and is inactivated in human cancers (Dick et al., 2018). Analysis of the transcriptome profiles of wild-type and *Rbf* mutant eye imaginal disc cells revealed a mutant-specific cell population exhibiting intracellular acidification due to increased glycolytic activity. These metabolic changes, confined to this *Rbf* mutant population, sensitise cells to apoptosis and define the pattern of cell death in the *Rbf* mutant (Ariss et al., 2018).

The proteomic tools have also been instrumental in elucidating the molecular mechanisms underlying steroid-triggered autophagic cell death of the dying *D. melanogaster* salivary glands. These studies have confirmed the caspase-dependent autophagic transcriptional cascade, and additionally uncovered novel regulators, such as the cell cycle protein Warts, which participate in caspase-independent degradation pathways (Martin et al., 2007). By comparing the proteomes of salivary glands undergoing developmental versus stress-induced autophagic cell death, researchers have identified additional factors required for proper cell degradation (McPhee et al., 2012), highlighting the power of integrating genomic and proteomic approaches to obtain a comprehensive understanding of the complex cell death programs.

The proteome of *D. melanogaster* ovary, a robust system for investigating physiological cell death related to cell migration and other critical cell behaviours using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis uncovered critical regulator factors (Velentzas et al., 2015). This study identified signalling pathways previously analysed in mammals and showed a more comprehensive network of known factors such as p53, IGF, and PI3K. The study also contributed to linking distinct cell death sub-routines involved in the *D. melanogaster* ovary, indicating the co-expression and probably synergistic effects of cell death programs in the egg chamber compartments, during development and under stress conditions.

Autophagy plays a crucial role in cell survival and death, and its progression and resolution depend on lysosome function. A study by Xu *et al.*, used a label-free LC-MS/MS approach to identify a group of proteins involved in the autophagy-dependent cell death program during degradation of *D. melanogaster* larval midguts (Xu et al., 2021). The study clarified how the lysosome contributes to this process through the essential function of cathepsins in the regulation of autophagic flux by maintaining a degradative environment inside the lysosome. Similarly, an optimised method for isolating autophagic structures from adult flies, with subsequent lipidomic analysis using MS/MS-based method, has contributed to pointing out the critical lipid transport function of the Atg2 protein in the *de novo* synthesis of early autophagic organelles (Laczkó-Dobos et al., 2021).

In a 2020 study, researchers used hydrophilic interaction LC-MS method to investigate controlled overexpression of Atg1 in specific tissues of *D. melanogaster* larvae. The study found that this overexpression increases mild autophagy and extends the lifespan of the flies. Although these flies were more sensitive to starvation, they also have an increased mitochondrial metabolism, which could be related to their longevity (Bjedov et al., 2020).

Furthermore, a study by Gao and collaborators combined RNA-seq and co-immunoprecipitation coupled LC-MS/MS of S2 cells to demonstrate that Wunen2 (Wun2) protein is required for efferocytosis both *in vitro* and *in vivo* (Gao et al., 2022). The study also revealed that Wun2 has a role in preventing the lysosomal degradation and transport of βν integrin from recycling endosomes to the plasma membrane to promote apoptotic cell clearance in *D. melanogaster*.

Genetically tractable systems

Genetically encoded sensors are powerful tools for studying PCD in the *D. melanogaster* model system (Figures 2G, H). Given the evolutionary conservation of core PCD mechanisms, insights gained from *D. melanogaster* PCD sensors are highly relevant for understanding cell death processes in human diseases like cancer.

There are multiple genetically encoded molecular sensors based on fluorescence for monitoring cell death in the fruit fly (Baena-Lopez et al., 2018; Nishida et al., 2024; Raymond et al., 2022; Schott et al., 2017) even though dying cells could be labelled *in situ* with vital dyes (Gavrieli et al., 1992). The sensors include the possibility of detecting cell death and associated processes in real-time (Raymond et al., 2022; Schott et al., 2017; To et al., 2015), overcoming the limitation of antibody staining. The fluorescence-based probes enable high-fidelity recording of processes with single-cell and subcellular spatial resolution (Greenwald et al., 2018). The genomic encoding of the sensors allows consistency and reliability in the detection of programmed cell death, making it easier to achieve accurate conclusions about this process (Table 1).

Many genetically encoded sensors engineered for studying cell death in *D. melanogaster* rely on caspase activity. These sensors become fluorescent upon the activation of caspases (Baena-Lopez et al., 2018; Lee et al., 2018; Raymond et al., 2022; Schott et al., 2017; To et al., 2015). For instance, the Apoliner sensor consists of two fused fluorescent proteins, mRFP and eGFP, separated upon caspase activity. When caspases are activated, the sensor is cleaved, causing eGFP to move to the nucleus while mRFP remains in the membranes (Bardet et al., 2008). Similarly, the infrared fluorescent executioner-caspase reporter iCasper becomes infrared fluorescent when apoptotic mechanisms start in cells. The iCasper apoptotic sensor has been used to measure apoptosis throughout developmental stages, demonstrating a spatiotemporal correlation between apoptosis and embryonic morphogenesis. Furthermore, it has been employed to investigate the dynamics of apoptosis during *D. melanogaster* brain tumour formation (To et al., 2015).

Another example of the development of sensors based on caspase activity and fluorescent proteins is the GFP-based variant of caspase 3-like protease activity indicator (GC3Ai). The expression of GC3Ai produces a non-fluorescent GFP that contains a caspase-1 recognizing sequence. After cleavage by active caspases, GFP becomes fluorescent and allows the visualization of apoptotic cells (Schott et al., 2017). Transgenic UAS-GC3Ai flies are

TABLE 1 Available genetic sensors to study programmed cell death mechanisms across different *D. melanogaster* tissues.

Biosensor	Cell death mechanism	Tissue	References
Apoliner	Apoptosis	Whole embryo	Bardet et al. (2008)
iCasper	Apoptosis	Embryo and larval CNS	To et al. (2015)
GC3Ai	Apoptosis	Eye-antennal discs; pupal leg disc	Schott et al. (2017)
DBS-S	Apoptosis	Wing imaginal disc	Baena-Lopez et al. (2018)
DBS-S-QF	Apoptosis	Larval wing imaginal disc; adult eyes and posterior midgut	Baena-Lopez et al. (2018)
CharON	Apoptosis, efferocytosis	Embryo CNS and hemocoel	Raymond et al. (2022)
CasExpress	Anastasis	Embryo; larval oenocytes, eye-antennal, leg and wing imaginal discs, CNS, gut; adult gut, brain, VNC, visceral and body wall muscles, oviduct, ovary, antenna and eye	Ding et al. (2016)
CaspaseTracker	Apoptosis, anastasis	Adult egg chambers, ovary, CNS, gut, Malpighian tubules	Tang et al. (2015)
Necrosensor	Necrosis	Embryo; larval wing imaginal disc, fat body and gut; adult testis	Nishida et al. (2024)
Casor	Apoptosis	Larval neurons	Lee et al. (2018)
mCherry-DmAtg8a	Autophagy	Adult egg chambers	Nezis et al. (2009)
GFP-mCherry-DmAtg8a	Autophagy	Adult nurse cells and egg chambers	Nezis et al. (2010)

available, as well as other alternative transgenic lines with different fluorescent proteins, including Cerulean and Venus, that are also appropriate for apoptosis detection in both live and fixed tissues. The use of the GC3Ai sensor permitted the description of the first apoptosis-inducing BH3-only protein (sayonara) in *D. melanogaster* (Ikegawa et al., 2023).

For its part, the Drice-based sensor (DBS) is another useful PCD sensor. Drice is a critical effector of apoptotic caspases, and after a two-step enzymatic process involving Dronc-mediated cleavage, forms two subunits—large and small—that associate to create the active caspase (Lannan et al., 2007). Baena-Lopez and collaborators developed a genetically encoded reporter system to detect early cell death stages, termed CD8-DriceC211A-short-Histone-GFP (DBS-S). Without caspase activation, DBS-S remains outside the nucleus. Upon cell death induction, the Drice subunit excision allows Histone-GFP to translocate to the nucleus, correlating with cleaved caspase-3 immunoreactivity (Baena-Lopez et al., 2018). The sensor allows studying temporal dynamics of cell proliferation and apoptosis after DNA damage (Ruiz-Losada et al., 2022), as well as investigating the involvement of different caspases in the cell death process (Aggarwal et al., 2022). To further increase the uses of the DBS sensor, Histone-GFP was replaced with the transcriptional activator QF to generate the DBS-S-QF, enabling genetic manipulation of caspase-activating cells using the QUAS-Gal4/UAS system, and lineage tracing of cells activating by Dronc (Baena-Lopez et al., 2018; Reiff et al., 2019).

In certain circumstances, some cells survive PCD despite caspase activation, a phenomenon known as anastasis. The CasExpress sensor facilitates the identification of these cells by driving the expression of fluorescent proteins, transiently or permanently, in cells that persist after caspase activation (Ding et al., 2016). The use of this anastasis biosensor led to comprehend that cell survival after caspase activation is a physiological tissue repair mechanism that can be disrupted in an oncogene-driven overgrowth context (Sun et al., 2020). Another system to detect anastasis in *D. melanogaster* tissues is CaspaseTracker, a biosensor based on a caspase-activatable Gal4 and the G-TRACE fluorescent protein system (Tang et al., 2015).

PCD can also occur independently of caspase activity, as it occurs in autophagy or necrosis. For autophagy, the mCherry-DmAtg8a reporter was engineered to detect autophagosomes and autolysosomes (Nezis et al., 2009). A step forward is the double-tagged GFP-mCherry-DmAtg8a sensor, that is effective for detecting and discriminating the autophagosomes in yellow (red and green merged) and the autolysosomes in red fluorescence due to the acidic environment of the latter (Nezis et al., 2010).

In contrast, no genetic biosensor has been available to detect necrosis *in vivo* in any organism until 2024 when Necrosensor (Figure 2H) was developed (Nishida et al., 2024). This necrosis sensor employs the nuclear protein HMGB1 (high-mobility group box 1) as a marker, because HMGB1 is released during necrosis in tissue cultures (Scaffidi et al., 2002). By fusing HMGB1 with GFP, it enables the detection of necrosis *in vivo* without the need for live staining (Nishida et al., 2024).

As mentioned before, PCD is followed by the engulfment and degradation of dead cells by phagocytes through efferocytosis (Davidson and Wood, 2020; Zheng et al., 2017). One of the major limitations of fluorescent-based sensors in studying cell death is the high pH sensitivity of fluorescent proteins and their weak resistance to photo-quenching in the acidic conditions of lysosomes during phagocytosis (Shinoda et al., 2018). Mutating the CG3Ai sensor led to the creation of ph-CaspGFP, a GFP-based apoptosis sensor designed to resist photo-quenching. The red fluorescent pHlorina sensor tracks apoptotic corpses during phagosome acidification, increasing fluorescence as pH decreases. The combination of both generates the CharON sensor (Figure 2G),

which shows GFP-positive cells undergoing apoptosis and increased fluorescence in phagocytes during efferocytosis in real-time (Raymond et al., 2022).

Conclusion and future perspective

The field of programmed cell death (PCD) has made significant strides, expanding beyond the classical apoptosis model to recognize a diverse array of pathways. This comprehensive understanding demands the refinement and adaptation of traditional techniques used to study PCD. The fruit fly has emerged as an invaluable model organism in this endeavour, providing insights into the intricate mechanisms of PCD in development, tissue homeostasis, and cancer biology. Its genetic malleability and decades of accumulated knowledge have facilitated the development of new methodologies for studying PCD.

Throughout the *D. melanogaster* life cycle, from embryonic stages to metamorphosis and adult tissues, PCD plays multifaceted roles. Researchers have identified and characterized various forms of cell death, including apoptosis, autophagy, necrosis, and emerging types such as parthanatos and phagoptosis. These findings highlight the complex regulation of PCD in diverse physiological contexts. Moreover, *D. melanogaster* has provided crucial insights into cancer-related cell death mechanisms, unveiling cellular competition, tumour microenvironment modulation, and the dual role of PCD in suppressing and promoting tumours (Diwanji and Bergmann, 2019). The progression of techniques used to study PCD, from traditional approaches to advanced genetic sensors, has equipped researchers with a powerful toolkit to investigate cell death processes at an unprecedented resolution. As new technologies emerge and our understanding deepens, the *D. melanogaster* model continues to offer immense potential for unravelling the complexities of PCD and its implications in development and disease, promising future breakthroughs in cell death research.

Author contributions

DT-L: Writing–original draft, Writing–review and editing. MD: Writing–review and editing. MU: Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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