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# Model systems in cell death-grand challenge

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## Introduction

In 1929, the Nobel Prize winning physiologist August Krogh observed that “*For a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied.*” (Krogh, 1929). Known as the Krogh Principal, this appreciation of a “model systems” approach has been foundational for many aspects of basic biology, from the use of the squid giant axon to define the ionic basis of the action potential to the use of the fruit fly to unlock the molecular basis of biological clocks. While the ultimate goal for many researchers may be to gain a better understanding of human development and/or pathogenesis, the complexity of mammalian systems often makes direct analyses challenging. Invertebrates and other “simpler” model systems often display adaptations that exaggerate normal cellular processes that make them attractive vehicles for the analysis of specific traits.

This approach has also proven to be foundational for the study of cell death. The term “programmed cell death” (PCD) (now commonly referred to as “regulated cell death” to distinguish it from “accidental cell death” (Galluzzi et al., 2018)) was coined by Lockshin and Williams in 1965 to describe the precisely timed loss of the intersegmental muscles of Lepidoptera at the end of metamorphosis (Lockshin and Williams, 1965). These giant cells (each of which is ~5 mm long and up to 1 mm in diameter depending on the species) initiate PCD coincident with the emergence of the adult moth from the overlying pupal cuticle. Few other naturally occurring examples of PCD are so exquisitely timed or offer such prodigious amounts of clean cellular material for molecular and biochemical analyses (e.g., Tsuji et al., 2020). However, it was another invertebrate model, the nematode *Caenorhabditis elegans*, that propelled the field of cell death from a small cottage industry with a few dozen investigators in the 1970s and 1980s into a massive research enterprise that has produced more than 560,000 publications during the past 30 years. One of the unique features of *C. elegans* that make it such an attractive model is that it displays “cell consistency”, meaning that every individual has the same number of somatic cells. By performing detailed lineage analyses, the identity and fate of every single cell was described by Sulston and Horvitz (Sulston and Horvitz, 1977). For about 20% of the cells, their fate is to die, primarily *via* apoptosis. At the time this work was conducted it was not well understood if PCD during development reflected the simple wasting away of surplus/unnecessary cells, active murder by neighboring cells, or cell-autonomous suicide. Using a clever genetic trick that prevented dying cells from being phagocytosed and thus rapidly removed, the Horvitz lab demonstrated that the ability of cells to die required the activity of specific genes that acted in a cell autonomous manner, and thus represented

cellular suicide (Ellis and Horvitz, 1986; Yuan and Horvitz, 1990). This model allowed them to identify both Ced-3 (caspase) and Ced-4 (Apaf1) as essential components of the killing machinery (Yuan and Horvitz, 1992; Yuan et al., 1993). Subsequent studies demonstrated that Ced-9 encodes a potent and essential survival protein that protects most cells from death during development (Hengartner et al., 1992). Apoptosis is such an ancient and evolutionarily conserved program that the human anti-apoptosis protein Bcl-2 can functionally substitute for Ced-9 in transgenic worms, despite a having last shared a common ancestor some 600 million years ago (Vaux et al., 1992).

While *C. elegans* has been invaluable for defining the core machinery of apoptosis, it has more limited utility for some other lines of investigation. For example, early in the history of the field, three primary mechanisms of cell death were recognized—apoptosis (type I degeneration), necrosis, and type II degeneration (autophagy dependent cell death (ADCD) (Schweichel and Merker, 1973; Clarke, 1990). Even though *C. elegans* has been foundational for the study of apoptosis, this organism does not appear to significantly employ ADCD to mediate cell loss during development, meaning that other organisms have proven to be more useful for exploring this mechanism (Takacs-Vellai et al., 2005). (This highlights the reality that while a given model system may represent an exceptional choice for one line of investigation, it may be more limited utility for another).

The primary mechanism of autophagy, macro-autophagy, involves the sequestration of damaged organelles and bulk cytoplasm into double membrane vesicles that ultimately fuse with lysosomes to form autophagosomes, where the contents are degraded and recycled (Gómez-Virgilio et al., 2022). In many cases this process allows cells to survive starvation and other insults and can even serve to suppress apoptosis in some situations (Bata and Cosford, 2021). Consequently, it was unclear if autophagy during cell death reflects an aborted effort for survival or an authentic cell killing mechanism (Schwartz, 2021). In fact, it was suggested that the term “autophagic cell death” may be a misnomer and that “cell death with autophagy” might be a more accurate descriptor of the process (Kroemer and Levine, 2008). In this regard, the fruit fly *Drosophila* has proven to be perhaps the best model for addressing this question. (While lacking the cell consistency of *C. elegans*, *Drosophila* does support an exceptional array of genetic tools that allow researchers to analyze and/or manipulate essentially any gene, at any stage of development, in almost any cell (e.g., Heigwer et al., 2018; Zirin et al., 2022). The ability to perform genome-wide RNAi and CRISPR screens has proven to be an invaluable approach for identifying key components of genetic pathways and modifiers that regulate them.

Genetic studies targeting the autophagy pathway in *Drosophila* have demonstrated unambiguously that autophagy is required for the loss of specific cells during metamorphosis (reviewed in Zhang and Baehrecke, 2015; Yalonetskaya et al.,

2018). During metamorphosis, larval-specific tissues are degraded or remodeled while adult-specific structures form to meet the animal's new lifestyle. Among the tissues that experience extensive cell death are the salivary gland and portions of the gut (Restifo and White, 1992). The Baehrecke lab employed gene inactivation or knockdown to target components of the apoptosis and autophagy pathways in order to investigate the molecular mechanisms that mediate the loss of these cells. They found that salivary gland death requires both apoptosis and autophagy, while the demise of the midgut depended exclusively on autophagy (Martin and Baehrecke, 2004; Berry and Baehrecke 2007; Denton et al., 2009, 2012). These studies were foundational for demonstrating unambiguously that autophagy can mediate the death of some cells.

Independent studies with the slime mold *Dictyostelium* further supported a functional role for autophagy in cell death during development. These social amoebae live most of their lives as solitary cells consuming bacteria and other organic substrates. However, when faced with starvation, individual cells aggregate and form a multicellular fruiting body with ~100,000 members. The stalk cells die as a normal component of development, which facilitates the dissemination of the spores. Genetic and pharmacological studies suggest that the loss of these cells is dependent on the autophagy pathway although some questions remain (Mesquita et al., 2017).

These examples highlight some of ways in which model systems have proven to be invaluable tools for examining foundational aspects of cell death. Obviously, many other key insights have not been included in this brief summary. That said, there are still major areas of the field where our understanding is far from complete. Some examples of where model systems could provide invaluable insights to advance the field are described below.

## Novel cell death pathways and their evolution

For many researchers, the terms “programmed cell death” and “apoptosis” are used interchangeably. This is clearly an oversimplification that ignores the fact that more than a dozen distinct cell death programs have been described, each with its own underlying biochemical mechanisms and roles in development, homeostasis, and/or pathogenesis. In addition to the classic programs of necrosis, apoptosis, and ADCD described above, other distinct pathways include: ferroptosis, parthanatos, entosis, necroptosis, pyroptosis, NETotic cell death, mitochondrial permeability transition, linker cell death, and mitotic catastrophe (Abraham et al., 2007; Galluzzi et al., 2018; Tang et al., 2019; Nirmala and Lopus, 2020). This year alone, copper-dependent cell death, cuproptosis, and erebosis, a cell death process that involves the loss of the cytoskeleton and

the accumulation of Angiotensin-Converting Enzyme, were described for the first time as novel cell death pathways (Ciesielski et al., 2022; Tsvetkov et al., 2022). It should be noted that erebosis was identified as a potentially distinct mode of cell death that occurs during gut cell turnover during fruit fly metamorphosis (Ciesielski et al., 2022).

This list includes several forms of programmed necrosis, including: ferroptosis, necroptosis, pyroptosis, and NETosis (Galluzzi et al., 2018). These deaths result in cellular swelling and ultimately lysis, leading to the release of highly inflammatory cellular constituents termed DAMPs (damage-associated molecular patterns) (Nakano et al., 2022). While these pathways were initially described in mammals, they appear to represent ancient programs. For example, ferroptosis has been observed in such diverse organisms as insects (Mumbauer et al., 2019) and plants (Distéfano et al., 2021). Pyroptosis, a process whose best described role is cytokine activation, has been observed in corals (Jiang et al., 2020). While largely presumed to function as a defense against pathogens, necrotic cell deaths may serve other roles as well. For instance in flies, a p53-dependent necrotic program is activated during spermatogenesis, unrelated to infection (Napoletano et al., 2017). How did these ancient necrotic programs evolve and how are they controlled?

Non-mammalian models, with their diverse developmental strategies and powerful genetic tools, represent promising vehicles for discovering yet new cell death programs. In addition, employing a phylogenetic approach should provide fundamental insights into the breadth and evolution of cell death mechanisms.

## Interplay between different cell death programs

Not surprisingly, the regulation of cell death is a complex and carefully orchestrated set of regulatory processes and there are numerous examples of interplay between different cell death programs. The activation of autophagy following nutrient deprivation can serve to enhance survival and prevent the activation of apoptosis (reviewed in Ploumi et al., 2022). While there are many molecular mechanisms for this cross-talk, perhaps the best characterized example is Beclin-1, which sits at the intersection of autophagy and apoptosis (He and Levine, 2010). Beclin-1 binds to class III phosphatidylinositol-3 kinase, a key mediator of autophagosome formation (Kihara et al., 2001). As a BH3-only protein, it can also bind the anti-apoptotic protein Bcl-2, which functions to inhibit apoptosis. Other BH3-only proteins can displace the Bcl-2 from Beclin-1, leading to the initiation of apoptosis (Elgendy et al., 2011). Beclin-1 is also a caspase-3 substrate, and its cleavage produces a C-terminal fragment that can directly trigger mitochondrial permeability and the subsequent release of pro-

apoptotic factors (Wirawan et al., 2010; Zhu et al., 2010). Thus, there are numerous cross-regulatory interactions between the proteins that mediate autophagy and apoptosis.

The pro-inflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) can drive both apoptosis and necroptosis by binding to membrane-associated TNF Receptors (TNFR). This leads to the formation of the death-inducing signaling complex (DISC) and a cascade of events that results in the autocatalytic activation of pro-caspase-8. This in turn drives the extrinsic apoptosis pathway *via* caspase-3 activation, and the intrinsic pathway by converting BID into the mitochondrially targeted tBid (Li et al., 1998). Separately, TNFR activation can also recruit RIPK1 and other proteins to form the necrosome, a signal transduction cascade that triggers necroptosis (Li et al., 2021). There is significant crosstalk between these two cell death pathways. For example, caspase-8 can cleave RIPK1, and thus simultaneously drive apoptosis and inhibit necroptosis. Paradoxically, in addition to its well-defined role in necroptosis, RIPK1 can also trigger pro-caspase activation and apoptosis (Zhang et al., 2019). Thus, like autophagy and apoptosis, there is a complex interplay between necroptosis and apoptosis.

As the old proverb states, “the devil is in the details”. If all of these different cell death programs arose independently during evolution, when and how did they develop the capacity to regulate one another? Are they all regulated in the same manner within the same tissue or within the same organism? Did the capacity to cross regulate different cell death programs provide organisms with fundamentally new developmental or homeostatic capabilities? Defining these signal transduction pathways will be essential to understanding fundamental aspects of development, immune responses, and pathogenesis.

## Non-apoptotic roles for caspases

Caspases are best known for driving apoptosis. Initiator caspases, like caspases-8 and -9, activate executioner pro-caspase zymogens (eg. caspases-3, -6, -7, and/or -10), which in turn cleave a myriad of cellular substrates resulting in the well-orchestrated process of apoptosis (Green, 2022). Caspase-1 (also known as interleukin-1 beta-converting enzyme) was the first caspase described and it plays essential roles in inflammation by cleaving pro-inflammatory cytokines like interleukins-1 $\beta$  and -18 (Kostura et al., 1989). These cytokines then drive a range of potent inflammatory responses including pyroptosis, a form of programmed necrosis that facilitates the clearance of pathogens (reviewed in Kesavardhana et al., 2020).

While a prevailing assumption in the field is that caspase activation represents an irrevocable “go/no go” event in apoptosis, focal caspase activation has been shown to serve important developmental roles in several model systems. Indeed, fruit flies expressing the fluorescent caspase reporter

CasExpress reveal that caspases are active in a range of tissues during development without the subsequent demise of the cell (Ding et al., 2016). A number of studies have demonstrated that transient caspase activity is required for normal development and homeostasis in animals (reviewed in Nakajima and Kuranaga, 2017). For example, activation of the initiator caspase Dronc in fruit flies (a caspase-9 ortholog) has been shown to mediate elasticity in the salivary gland by cleaving actin without triggering concurrent cell death (Kang et al., 2017). Focal caspase activation can also drive mitosis by releasing mitogens in an apoptosis-induced regeneration process in the wing disc (Huh et al., 2004; Amcheslavsky et al., 2018; Sun et al., 2020). As well, during neurodevelopment, focal caspase activation prunes dendrites in the nervous system, thus facilitating the removal of surplus or inappropriate synaptic connections without overt damage to the cell (Williams et al., 2006; Ertürk et al., 2014).

Is the transient activation of caspases a more generalized process during development? If so, what regulatory processes restrict caspase activity so that it doesn't snowball into apoptosis? Some mechanisms that focally restrain caspase activity include restricting caspases to specific subcellular locations and/or the judicious expression of inhibitor of apoptosis proteins (Vasudevan and Ryoo, 2015). Are there others? Understanding how caspases can be exploited to help refine developmental processes is both a fundamental and largely unexplored topic. It is likely that the diversity of cellular and developmental specializations displayed by different invertebrates may represent useful models for exploring these questions.

## Programmed cell death in plants

Like animals, plants employ PCD at multiple stages of development (reviewed in Sueldo and van der Hoorn, 2017). During the formation of the xylem, a structure that facilitates the transport of water and nutrients from the roots to the rest of the plant, tracheary cells undergo an autolytic cell death in order to form the hollow vessel. The death of tapetum cells in the anthers of flowering plants is required for proper pollen formation. During seed development the suspensor cells transport nutrients from parental tissues to the early embryo and then undergo cell death as embryogenesis progresses.

Plants can also reactively trigger the hypersensitive response (HR), a rapid onset focal cell death process that restricts the spread of pathogens (Balint-Kurti, 2019). In most cases, HR death is mediated by a family of nucleotide-binding site and leucine-rich repeat domains (NLR) resistance genes (Chakraborty et al., 2018). HR-mediated death displays some aspects of apoptosis (cellular shrinkage, genomic DNA degradation, TUNEL staining, mitochondrial depolarization)

and certain features of autophagy (vesicles with cellular constituents) but does not cleanly fall into any of the cell death programs described for animal cells (Mur et al., 2008). Indeed, it has been assumed that apoptosis does not exist in plants (Locato and De Gara, 2018). This raises several questions about the evolution of cell death programs in plants and how they may have evolved from a common eukaryotic ancestor. For example, while plants do not encode caspases, they do utilize cysteine metacaspases in some cell death processes (Minina et al., 2017). Unlike canonical caspases that cleave after aspartates, the metacaspases cleave after lysine and arginine residues (Fagundes et al., 2015). Another possible mechanism for HR-mediated death is the NLR protein ZAR1. It has been shown to associate with other binding partners to form a membrane associated pentameric channel known as the resistosome (Wang et al., 2019). It is possible that this triggers a necrotic type of cell death that nevertheless displays some apoptotic features.

Far less is known about the mechanisms of cell death in plants relative to animals. This is disappointing since plants make up the majority of the biomass on the planet and they serve essential ecological roles in terms of creating diverse habitats, food, CO<sub>2</sub> sequestration, and even atmospheric oxygen. Consequently, a deeper analysis of plant cell death would not only enhance our understanding of important basic biological processes, it may also empower us to better help plants survive in a rapidly changing environment.

## Programmed cell death in prokaryotes

PCD serves a myriad of roles in eukaryotes, including: sculpting the body (e.g., loss of interdigital cells during limb formation), removing deleterious cells (e.g., self-reactive thymocytes during negative selection), matching populations of interacting cells (e.g., motor neurons and muscles), etc. (reviewed in Milligan and Schwartz, 1997; Fuchs and Steller, 2011). However, at first glance, it is counter intuitive that PCD might serve some beneficial role for prokaryotes, unicellular organisms that lack tissues. However, there are many circumstances where bacteria can aggregate to form multicellular communities (Lyons and Kolter, 2015). Within this context, the ability of some cells to undergo an altruistic death can confer significant benefits to the larger community, and thus ultimately benefit the clonally-related condemned cell. These deaths can aid in sporulation, biofilm maintenance, mycelium formation, the uptake of extracellular DNA *via* transformation, antibiotic production, etc. (reviewed in Allocati et al., 2015). For example, biofilms, which can contribute to pathogenesis in several human disorders including cystic fibrosis, urinary tract infections and endocarditis, display cell death at specific stages of

development. These autolytic deaths provide nutrients for the colony and thus facilitate survival.

Three primary cell death pathways have been described in bacteria: apoptosis-like death (ALD), thymineless death (TLD), and toxin-antitoxin (TA) mediated death (Lee and Lee, 2019). ALD displays many features of apoptosis, including DNA fragmentation and phosphatidylserine externalization. TLD-mediated death occurs following thymine restriction and results in substantial single-stranded DNA breakage and chromosome segregation failure during replication. Separately, some bacteria produce toxins that can induce the death of the bacterial cell but are held in check by the co-expression of an anti-toxin protein. During times of stress, the destruction of the antitoxin functionally de-represses toxin activity, resulting in the demise of the cell.

The range of cell death mechanisms available to prokaryotes has likely not been fully described. In addition, it is not clear if and how these more ancient prokaryotic cell death programs may have contributed to the evolution of eukaryotic cell death mechanisms like apoptosis. Indeed, the death of prokaryotes has been referred to as “proapoptosis” (Hochman, 1997). There is compelling evidence that mitochondria represent ancient bacterial symbionts (Margulis, 1975). Did these prokaryotic cells bring their own cell death mechanisms to eukaryotes (Koonin and Aravind, 2002)? Mitochondria play essential roles in driving the intrinsic apoptosis pathway and are central to other cell death programs as well, like autophagy (Abate et al., 2020). While caspases are not present in the bacterial genome, some bacteria like *Helicobacter pylori*, display DNA fragmentation and electron dense DNA when they die (Cellini et al., 2001). Is this process driven by the larger metacaspase family of proteases or some other enzymatic system yet to be characterized (Basmaciyan and Casanova 2019)?

Understanding prokaryotic cell death will not only enhance our fundamental understanding of the origin and diversification of cell death programs, they may also provide tools for the prevention or remediation of some of the more devastating plant and animal bacterial pathogens. Early work on the use of PCD as a target in antibiotic treatment (Tanouchi et al., 2013) provides just one example of some of the potential uses of prokaryotic PCD mechanisms to improve both agricultural productivity and human and animal health.

## Final thoughts

While there has been amazing progress in the field of cell death research over the past 30 years, many aspects remain to be discovered. The PCD community has moved away from the rather simplistic view that all cell deaths can be binned into one of three programs (apoptosis, necrosis and ADCD) and now recognize that there are perhaps a dozen or more distinct

pathways. How many still await description? How did these programs evolve and how are they used to help engineer embryogenesis and maintain homeostasis? How does misregulation of cell death contribute to pathogenesis and can we use these insights to develop better therapeutics? The use of non-mammalian model systems can help advance these goals. For example, a variety of genetic engineering approaches can be used to create humanized animal models in worms, flies and fish can be exploited for high throughput drug screens to identify new therapeutics (Rajan et al., 2020; Savitsky et al., 2020; Zhu et al., 2020). One of the great promises of the field is that we will be able to activate or inhibit cell death in a lineage-specific manner to block disease or enhance regeneration. For us to achieve that potential will require substantially more basic research to define the molecular mechanisms and their interactions that drive cell death. *Model systems in cell death (MSCD)* looks forward to publishing papers that help meet these challenges.

## Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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