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Programmed Cell Death in Unicellular Versus Multicellular Organisms

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Abstract

Programmed cell death (self-induced) is intrinsic to all cellular life forms, including unicellular organisms. However, cell death research has focused on animal models to understand cancer, degenerative disorders, and developmental processes. Recently delineated suicidal death mechanisms in bacteria and fungi have revealed ancient origins of animal cell death that are intertwined with immune mechanisms, allaying earlier doubts that self-inflicted cell death pathways exist in microorganisms. Approximately 20 mammalian death pathways have been partially characterized over the last 35 years. By contrast, more than 100 death mechanisms have been identified in bacteria and a few fungi in recent years. However, cell death is nearly unstudied in most human pathogenic microbes that cause major public health burdens. Here, we consider how the current understanding of programmed cell death arose through animal studies and how recently uncovered microbial cell death mechanisms in fungi and bacteria resemble and differ from mechanisms of mammalian cell death.

1. INTRODUCTION

Cell death research has entered an exciting new era due to the emergence of elegantly defined suicidal death pathways encoded by bacteria and fungi, settling the long debate about whether programmed cell death/regulated cell death (PCD/RCD) arose before multicellular animals. This crucial aspect of biology has been long neglected because the evolutionary selection of suicidal death in unicellular species seemed improbable. Very recently, empirical evidence has persuasively demonstrated that altruistic cell death is critical for the survival of microbial populations in the laboratory. These mechanisms are best characterized in bacteria and a few fungi but are currently uncharacterized in parasites such as *Toxoplasma* and *Plasmodium* that infect many people worldwide. The diversity of microorganism cell death mechanisms may far exceed those identified thus far (**Figure 1**).

2. CONCEPTUALIZING PROGRAMMED CELL DEATH: A MULTISTEP JOURNEY

The concept of PCD/RCD arose from observations of disappearing cells during metamorphosis, adult tissue remodeling, and phagocytosed microorganisms (21). The systematic disappearance (presumed death) of cells observed in animals was considered to be purposeful, but the dying cell was considered a passive participant, dying from a lack of essential resources, malfunction, or other challenges rather than from active self-killing mechanisms (27, 59). The concept that cells could contribute to their own death was solidified with the discovery of lysosomes in 1955, which could potentially rupture inside cells and release degradative enzymes into the cytoplasm, a cell death process now known as lysosome membrane permeabilization (LMP), though the mechanisms remain unclear (90, 114). However, at the time it was not yet conceptualized that evolutionarily selected suicidal cell death machinery is encoded by nearly every animal cell.

In the mid-nineteenth century, a distinctive cell morphology characterized by chromatin condensation and degradation, known as chromatolysis, was commonly observed within dying cells during insect morphogenesis and mammalian tissue remodeling (21), and, 130 years later, chromatolysis became known as apoptosis (72). The term apoptosis was coined to describe the morphology of dying cells (including chromatolysis) that occurred at delayed times after the initial wave of necrotic cell death in damaged tissues and therapeutically regressing tumors, implying an internally directed death of cells responding to pathological conditions (72, 90, 91).

However, the concept that nearly every animal cell is capable of suicidal death did not become indelibly sealed and widely accepted until studies in *Caenorhabditis elegans* provided definitive evidence of the first prodeath gene, *ced-3* (154). Normal *C. elegans* development is accompanied by the death of 131 cells, producing an adult worm composed of 959 somatic cells. Mutations in *C. elegans ced-3* rescued all 131 cells observed to die during normal development (154). The powerful genetic tools for *C. elegans* and *Drosophila* uncovered many conserved cell death regulators and were instrumental in shaping the understanding of apoptosis, the best-understood form of cell death. Apoptosis is mediated by a subset of animal caspases (e.g., *C. elegans* CED-3, *Drosophila* DRICE, and mammalian caspase-3) (5, 25, 42, 79, 144, 154). It is now well accepted that specific cell populations are required to die for normal animal development and adult homeostasis. Well-known examples include the death of ~50% of neurons born during brain development; the interdigital cells that remain only in waterfowl, amphibians, and a few mammals with webbed feet; and the >95% of thymocytes that die during thymic selection. In total, billions of cells are estimated to die daily in human adults.

The first antiapoptotic human gene, *BCL2*, was identified as a candidate oncogene driving tumorigenesis of follicular lymphomas (hence the name, B-cell lymphoma/leukemia 2) but was

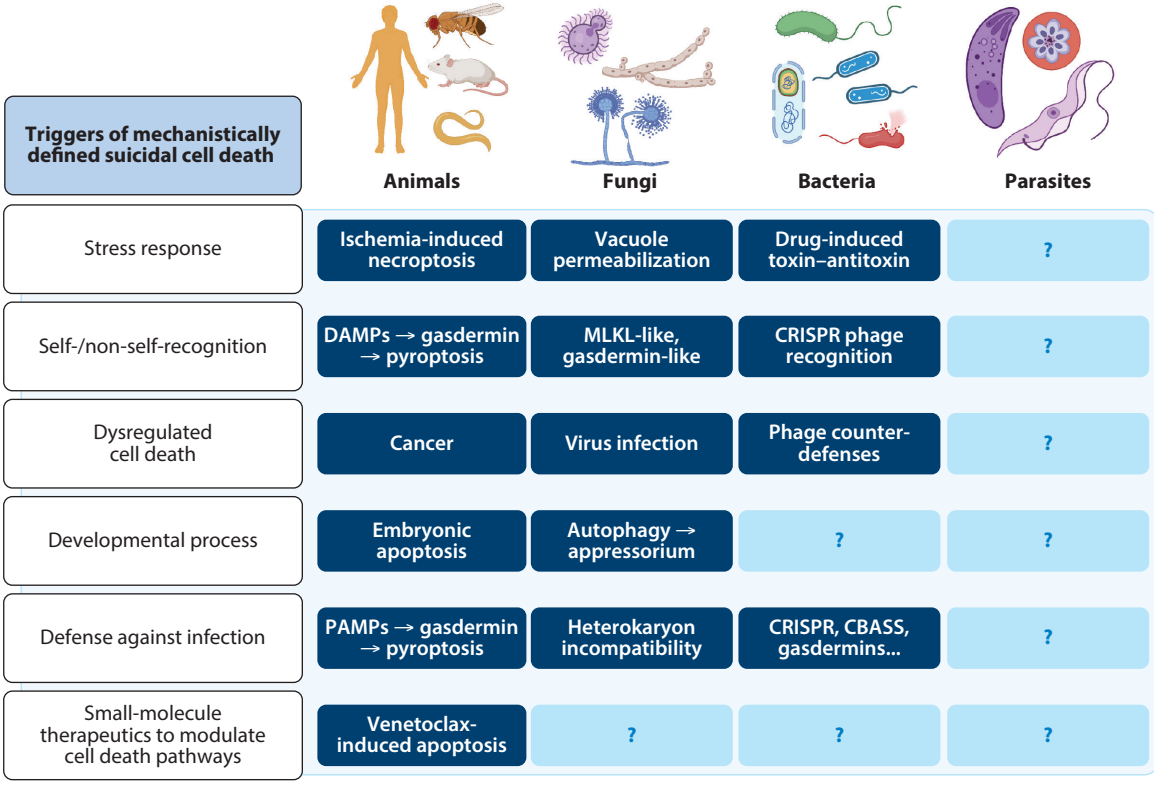

Figure 1

Defined cell death mechanisms occur in diverse species in response to diverse stimuli. While all species are expected to encode some form of self-inflicted cell death orchestrated by specific cell death effector molecules or by self-sabotage, molecularly and genetically regulated cell death pathways have been delineated only in relatively few species thus far. Dark boxes indicate that one or more species in this group of organisms has at least one genetically or molecularly defined cell death effector pathway reported; only salient examples are listed. A myriad of stressors lead to physiological, pathological, and drug-induced cellular responses that commonly lead to activation of death pathways when normal repair mechanisms falter. Similarly, detection of foreign material or cell damage can also lead to death, and in some cases exaggerated innate immune responses (e.g., inflammation) are detrimental and contribute to cell death. Normal triggers of cell death that are not fully executed due to defects in the activation (or inhibition) of cell death also compromise cell health. Thus far, the evidence that fungal cell death is a protective response to virus infection is far less well supported than for bacteria and animals, in part because fungal viruses remain cell associated and are transmitted vertically to offspring, reducing the options for studying suicidal cell death. Abbreviations: CBASS, cyclic oligonucleotide–based antiphage signaling system; DAMP, damage-associated molecular pattern; MLKL, mixed lineage kinase domain–like; PAMP, pathogen-associated molecular pattern. Figure adapted from images created with BioRender.com.

soon shown to maintain cell survival independently of cell proliferation, an important new concept in cancer biology (137). Another important concept enforced by this study was that cell death is not simply the loss of life. How does BCL-2 keep cells alive in the absence of growth factors—by actively sustaining survival or by actively suppressing cell death? The answer is likely both. Soon, BCL-2 was demonstrated to actively repress cell death machinery, building on evidence that *C. elegans* CED-9, homolog of BCL-2, inhibits cell death induced by the CED-3 caspase during worm development (61). Thus, BCL-2 and CED-9 became known as the first antiapoptotic proteins. These and other revolutionary discoveries planted the seed that drove the new field of apoptosis—the first defined cell death pathway. Later, BCL-2 family proteins were found to have

additional prosurvival functions independent of their roles in apoptosis, for example, by adjusting mitochondrial energetic efficiencies (18). These nonapoptotic functions are less well understood, but likely contribute importantly to cell death resistance.

The vast expanse of knowledge about apoptosis and BCL-2 family proteins enabled the development of a first-in-class, US Food and Drug Administration (FDA)–approved, BCL-2-specific inhibitor used to effectively treat several cancers by inducing cell death (119). Could this success be recapitulated for microorganisms? By analogy, growing knowledge of microorganism cell death is expected to reveal mechanisms for activating microbial death pathways using small-molecule therapeutics. Thus far, several groups are developing therapeutic bacteriophages capable of triggering bacterial cell suicide to treat infections (47, 76).

3. MAMMALIAN CELL DEATH WITH AND WITHOUT PARALLELS IN MICROBES

Aside from apoptosis in mammals, three nonapoptotic cell death pathways are at the forefront: pyroptosis, necroptosis, and ferroptosis, which are forms of regulated necrosis resulting in cell lysis. New mammalian cell death pathways, such as copper-induced death termed cuproptosis (132), and new components of established pathways, such as the pore-forming protein Ninj1 in pyroptosis, continue to emerge (71). Although mammalian cells activate cell death pathways in response to invasion by microorganisms, whether any mammalian-like cell death pathways also occur within microorganisms has been long debated. The answer has become evident with the recent discovery of many newly defined suicidal death pathways in microbes. Here, we consider how some of these are related to mammalian cell death.

3.1. Apoptosis: A Broadly Used Term with Evolving Definitions

The word apoptosis is widely used by many scientists, educators, students, and the lay public to mean PCD/RCD. According to definitions derived from several online authorities (e.g., the National Human Genome Research Institute and Merriam-Webster), apoptosis is a form of genetically programmed, self-inflicted cell death to eliminate damaged or exhausted cells that occurs both during fetal development and in adults. Most online audio pronunciations verbalize the second "p" in apoptosis. To distinguish apoptosis from several other forms of cell death, researchers studying mammalian/animal cell death restrict the definition of apoptosis (often pronounced with the second "p" silent) to refer to suicidal cell death mediated by apoptotic caspases, especially caspase-3, resulting in typical apoptotic cell morphologies. Apoptotic cell morphology includes cell shrinkage, chromatin condensation, nuclear fragmentation, plasma membrane blebbing without loss of membrane integrity, and subsequent engulfment by neighboring cells in vivo.

However, definitions evolve, which is evident from the series of guidelines published by the Nomenclature Committee on Cell Death (NCCD) in 2005, 2009, 2012, 2015, 2018, and 2023. In the first NCCD guidelines published in 2005 (77, p. 1465), strict adherence to the morphological definition was enforced, although it was said that apoptosis could occur with or "without evidence of caspase activation." However, by 2009 (78, p. 5), it was acknowledged that "caspase activation may be necessary for. . .apoptotic morphology," and that permeabilization of the outer mitochondrial membrane is an important commitment point during apoptosis. Remaining uncertainty about the requirement for caspases stemmed from the observation that caspase inhibitors blocked apoptotic morphologies but did not ultimately block cell death. However, we now know that apoptotic cells treated with caspase inhibitors can die by necroptosis and that nonapoptotic caspases (e.g., caspase-1 and caspase-11) can activate pyroptosis, although caspase-1 and caspase-11

have other key functions, especially in releasing the inflammatory cytokine interleukin-1β (IL-1β) (discussed in Section 4). By the NCCD's 2012 version (51), caspase activation was still not a strict requirement for apoptotic death, but by 2015 (49), the term caspase-independent cell death referred only to regulated necrosis pathways, and apoptosis was defined morphologically and biochemically as caspase-dependent (falling short of caspase-mediated). However, by 2018 (50), apoptotic caspase-3 was unexpectedly found to cleave gasdermin E (GSDME) to switch cells from apoptosis to pyroptosis. Thus, a more nuanced distinction is made between caspase-3-dependent death by pyroptosis (dependent on caspase-3 cleavage of GSDMD) and caspase-3-mediated apoptosis.

The main ambiguity that arose in the past 20 years regarding the word apoptosis was the assumption that mammalian apoptosis assays, when applied to yeast, parasites, and other eukaryotic microbes, reflected mammalian-like molecular dying processes orchestrated by yeast and parasite caspase-like proteases (2). Currently, these assumptions lack generally accepted evidence, and microorganisms lack several key morphological and biochemical features of mammalian apoptosis (discussed below). However, microbial proteases and nucleases that are detected by mammalian apoptosis assays could ultimately be found to participate in yeast/parasite cell death.

The latest version of the NCCD guidelines (139) recognizes 13 nonapoptotic forms of RCD [mitochondrial permeability transition (MPT)-driven necrosis, necroptosis, pyroptosis, ferroptosis, autosis, parthanatos, entotic cell death, NETotic cell death, lysosome-dependent cell death (related to lysosome membrane permeabilization), autophagy-dependent cell death, alkaliptosis, cuproptosis, and PANoptosis (the simultaneous activation of pyroptosis, apoptosis, and necroptosis)]. Nine other cell death names recognized in earlier NCCD guidelines but not mentioned in 2023 include autophagy (autophagy-mediated cell death), necrosis/oncosis, mitotic catastrophe, mitotic death, excitotoxicity, Wallerian degeneration, cornification, paraptosis, and pyronecrosis. However, autophagy-dependent (potentially autophagy-mediated) death occurs during the life cycle of fungal plant pathogens (e.g., *Magnaporthe oryzae*), and mechanisms analogous to Wallerian degeneration have an interesting overlap with recently identified cell death and immunity in bacteria and plants (discussed in Sections 4.5 and 5.1, respectively). Cell death nomenclature has helped drive the field despite continuous revisions.

3.2. Nomenclature for Microorganism Cell Death

Currently, there is no word or term that is generally accepted to refer to programmed and/or regulated microorganism cell death. Thus, most keyword searches to identify publications on this topic instead retrieve papers describing cell death of mammalian host cells induced by invading microbes. The recommendation from a group of scientists studying mammalian and microbial cell death at a Banbury meeting in late 2022 is to avoid terms ending in "-tosis" to prevent automatic misconceptions about potential similarities to mammalian/animal cell death pathways. They also recommend that new terms be applied to molecularly defined pathways, for example, Thoeris, a recently defined altruistic bacterial cell suicide pathway activated by phage infection (discussed in Section 5.1). However, searching PubMed or Google Scholar with linked terms such as "bacterial-cell-death" or "fungal-cell-death," with or without "programmed," retrieves several related articles but few of the studies cited here. Nevertheless, continued use of these and similar search terms will help newcomers seeking information.

3.3. Programmed Versus Regulated Cell Death

In recent years, the NCCD has recommended reserving the widely known term PCD for developmental and physiological cell death (referring to animals), reasoning that the term was coined

to describe cell death observed in the maturing silk moth (91). The NCCD offers a newer term unfamiliar to most microbiologists, RCD, which includes PCD and any additional forms of regulated/druggable cell death, especially pathological states where the mechanisms are not known to have normal physiological roles. While this raises further questions about the evolutionary origins of genetically regulated pathological cell death, it alleviates the need to address physiological roles.

However, applying the terms PCD and RCD to microorganism cell death is not uniformly straightforward. For example, Thoeris, a genetically encoded altruistic bacterial suicide pathway, was shown to become activated in the first bacterial cell in the population to become infected by a phage, thereby blocking the production of new virus and saving the entire bacterial culture from annihilation (111) (discussed in Section 5.1). The genome structure and mechanics of Thoeris and other bacterial death pathways strongly indicate that they arose through evolution to cause suicidal cell death. Thus, Thoeris can be considered PCD on this basis but can also be considered RCD, given the lack of evidence thus far that Thoeris is activated in the normal bacterial life cycle. Currently, the choice of PCD versus RCD nomenclature is left to the investigator's discretion.

3.4. Apoptosis by Its Restricted Definition Applies to Animals, Not Microbes

Decades of research have exquisitely delineated many aspects of apoptosis, defined by characteristic cell morphologies, and molecularly defined as caspase-mediated (e.g., caspase-3-mediated) suicidal cell death. BCL-2 family proteins are important regulators of apoptosis upstream of caspase-3. Apoptosis is required for normal embryonic development and successful cancer therapy. Various technologies over the last 15 years have expanded the human BCL-2 protein family to 14, and these proteins are generally grouped as anti- or proapoptotic, though only half have been extensively studied (4). The short BH3 sequence motif (one of four BCL-2 homology/BH motifs) of the proapoptotic BCL-2 homologs BAX and BAK binds to a deep cleft on the antiapoptotic proteins BCL-2, BCL-xL, BCL-W, and MCL-1, and the resulting heterodimers are considered mutually inhibitory. BCL-2 homologs are found widely in metazoans, including simple animals lacking organs, and in several tumor-associated viruses (3). Deletion of one or more BCL-2 family members in cultured cells demonstrates that these proteins are not essential, but genetic knockouts of one or more of these family members in mice typically have severe consequences, testifying to their roles in development as well as normal physiology. BCL-2, BCL-xL, and MCL-1 are particularly well known to be elevated in many cancers. Mammalian BCL-2 family proteins also influence the outcome of infection, and subversion of virus-induced cell death by BCL-2 attenuates Sindbis virus virulence and preserves neuronal survival despite long-term virus persistence in the brain (85–87).

In contrast to mammalian and *C. elegans* BCL-2 family proteins, the functions of *Drosophila* BCL-2 orthologs Debcl and Buffy have been difficult to pin down, though recent advances support their roles in apoptosis (65). BCL-2 homologs are not known to exist in plants, fungi, bacteria, or other single-cell species, although low sequence conservation of BCL-2 across diverse species does not rule out structurally similar proteins not yet identified.

Intense interest has focused on a collection of diverse BH3-only proteins, one of which can be classified as a BCL-2 family member based on structure (BID), and possibly others in the BID clade. As the name implies, sequence conservation between BH3-only proteins and the BCL-2 homologs is limited to a single short, linear BH3 motif (4). BH3-only proteins are suggested to be sensors of cellular status, and, in turn, their BH3-containing α-helix engages anti- and/or proapoptotic BCL-2 homologs to modulate cell death susceptibility. Of particular interest are BH3-only proteins (e.g., BIM and BID) that trigger BAX and BAK to oligomerize and form pores in the mitochondrial membrane, leading to mitochondrial outer membrane permeabilization (MOMP),

considered a commitment point in apoptosis with a few exceptions (11, 81). Small molecule mimics of BH3 motifs inhibit antiapoptotic BCL-2 family proteins and are actively pursued as anticancer therapeutics, leading to the FDA-approved compound venetoclax (trade name Venclexta®).

While the binding specificities of mammalian BH3 motifs, especially BH3-containing peptides, have been extensively characterized, much less is known about the functions of full-length BH3-only proteins, which can have different partner selections compared to their peptides (13). Aside from the eight canonical BH3-only proteins, a long list of other, unrelated mammalian proteins are reported to contain a functional BH3 that binds one or more BCL-2 family members (4). Perhaps the best-studied noncanonical BH3-only protein is mammalian Beclin 1 (*Saccharomyces cerevisiae* yeast Atg6), which binds and restrains the lipid kinase activity of the autophagy-inducing VPS34 complex, until Beclin 1 becomes phosphorylated and dissociates, along with BCL-2, from the VPS34 complex (112, 151). An analogous mechanism was not found for yeast Atg6. However, a putative yeast BH3-only protein Ybh3/Bxi1 (YNL305C) was reported to coprecipitate with BCL-xL (98). This raises questions about the definition of a BH3-only protein without yeast BCL-2 family proteins to interact with. Additionally, *S. cerevisiae* is not an intracellular pathogen that might encounter host BCL-2 proteins. This does not rule out the potential importance of Ybh3/Bxi1 calcium channel activity in regulating cell survival and death. A mammalian ortholog of Ybh3/Bxi1, RECS1/TMBIM1, was recently found to induce cell death by LMP, potentially by its calcium channel activity (114). However, the first *Drosophila* BH3-only protein was uncovered only very recently (65), a reminder that many questions remain.

Proapoptotic BCL-2 homologs BAX and BAK permeabilize the mitochondrial outer membrane to induce MOMP, releasing several mitochondrial factors into the cytoplasm, including cytochrome *c* (which transfers electrons in the mitochondrial electron transport chain). Although cytochrome *c* can be released from mitochondria during cell death in yeast and some nonmammalian animals, the importance to cell death is unclear because these species lack Apaf1 apoptosomes, which are the targets that are activated by mammalian cytochrome *c*. The death function of cytochrome *c* is also not conserved in *C. elegans* and *Drosophila* despite having Apaf1 counterparts. Another open question is the nature of the BAX/BAK pore that releases cytochrome *c* from mitochondria during apoptosis, which is suggested to be a lipidic pore rather than a protein-lined channel (131, 143). The Apaf1 apoptosome recruits and activates caspase-9, which in turn cleaves and activates caspase-3, a major effector of apoptosis. Caspase-3 is also activated by caspase-8 via death receptor signaling (e.g., TNFR1) from the cell surface (10, 83). Caspase-3 cleaves after selected aspartate residues in several hundred cellular substrates to activate or inactivate their functions in a manner that promotes cell death. Cleavage of caspase substrates is believed to cause the classical morphological appearance and biochemical hallmarks of apoptotic cells that were first recognized as chromatolysis (28, 64).

Salient examples of caspase-3 substrates believed to contribute to apoptosis include the inhibitor of caspase-activated DNase (iCAD), which is the inhibitory chaperone of DNA-laddering endonuclease CAD. CAD cleaves nuclear DNA between nucleosomes, resulting in DNA ladders on gels (not observed in most species). This finding inspired the development of the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) cell death assay in the early 1990s. However, a TUNEL assay that does not include evidence of DNA ladders on gels is not specific to apoptosis, as other regulated mammalian necrosis pathways generate DNA breaks labeled by the TUNEL assay. Drawing conclusions about yeast cell death pathways based on the TUNEL assay poses additional challenges. A study in yeast reported DNA laddering induced by the yeast endonuclease Nuc1 (YJL208C), a homolog of mammalian EndoG, in a model of nutrient deprivation (low carbon) during sporulation, when a portion of spores die (40). However, spore death proceeds normally without Nuc1. Thus, Nuc1 lacks a role in cell death and instead is important

postdeath for the degradation of DNA that is exposed to Nuc1 nuclease activity because it failed to become encased in the nucleus of a new spore.

Another classic example is caspase-3 cleavage of scramblases (activating XKR8) and flippases (inactivating ATP11A/ATP11C P4-type ATPases that bind/require CDC50A), which promotes externalization of phosphatidylserine (PS) on the outer leaflet of the plasma membrane of animal cells. The exposed PS is then available for detection by Annexin V staining of intact, nonpermeable cells. PS exposure in mammals, *Drosophila*, and *C. elegans* is a signal for engulfment and degradation of dead and dying cells by neighboring cells in tissues (105). Genetic screens in model organisms that defined the early apoptosis pathway also identified many conserved engulfment genes.

Thus, apoptosis is more than a cell death program; it also disposes of cell corpses, thereby reducing inflammatory potential. For example, inflammation is avoided during massive cell death occurring in germinal centers during B cell development and maturation when >95% of B cells die. The resulting apoptotic cell bodies are rapidly engulfed by resident tangible body macrophages, named for the prominent remnants of chromatolytic apoptotic bodies derived from dying B cells that are readily observed within their phagocytic vacuoles (58).

Like the TUNEL labeling assay, the Annexin V staining assay, which is used to detect surface exposure of PS, has also been applied to detect dying fungi and parasites. Although both subunits of the homologous yeast flippase (P4-type ATPase Drs2/YAL026C) and required binding partner Cdc50/YCR094W were identified in our yeast cell death screen (130), the relevance of PS exposure beneath the yeast cell wall is uncertain, and the engulfment of a dying yeast cell by a neighboring yeast cell has not been described. While the Annexin V assay may serve to detect dying and dead microbes, the significance is uncertain because the mechanisms involved have not been demonstrated to be causal for microbial cell death.

A major debate has transpired over whether yeast/fungal and plant metacaspases, which have limited sequence similarity to animal caspases, participate in yeast/fungal or plant cell death (134). Many studies have proposed that metacaspases facilitate apoptosis-like death in yeast and plants. Although several metacaspase substrates have been identified in several species, their role in promoting cell death is uncertain, and distinguishing the potential cell death functions of metacaspases from the other roles of metacaspases in healthy cells is challenging. That said, very few mammalian caspase substrates have been validated in knock-in mice expressing uncleavable caspase substrates with functional consequences (17, 110). To address the debate about the relationship between metacaspases and their distant caspase relatives, which have distinct cleavage site preferences and other differences, researchers in the field have worked together to address the issues by providing metacaspase nomenclature (104). Interestingly, ancestral members of the caspase superfamily appear to be involved in pyroptosis-like bacterial cell death.

4. PYROPTOSIS DEFINED BY GASDERMINS IN MAMMALS, FUNGI, AND BACTERIA

Except for apoptosis, most other forms of self-inflicted mammalian cell death cause cell lysis by disruption of the plasma membrane and thus are regarded as forms of regulated necrosis. The best studied are pyroptosis and necroptosis, which have known pore-forming proteins as terminal death effectors: gasdermins (GSDMs) and MLKL (mixed lineage kinase domain–like) psuedokinase, respectively. GSDMs and MLKL assemble inside cells and insert into the cytoplasmic side of the plasma membrane to permeabilize mammalian cells. Recently, GSDM-like proteins were identified in bacteria, fungi, archaea, and other microbes (68) (**Figure 1**). Remarkably, microbial GSDMs exhibit many genetic, biochemical, and biological characteristics of mammalian GSDMs, including the ability to cause suicidal cell death, which Johnson & Kranzusch (67) refer to as

bacterial pyroptosis. These impactful discoveries reveal compelling evidence of microbial PCD/RCD and imply ancient evolutionary origins of mammalian cell death and innate immunity.

There are also some distinctions from bacteria, as mammalian GSDMs fulfill additional roles in signaling inflammatory innate immune responses (102, 116, 149). At the heart of the mammalian pyroptosis pathway is the production of proinflammatory cytokine IL-1β. Caspase-1 is activated by inflammasomes and by caspase-11 (70, 99, 141). Caspase-1 has a critical role in releasing IL-1β from living mammalian cells. Caspase-1 and other proteases can cleave GSDMD, which triggers cytoplasmic GSDMD to form plasma membrane pores required to release IL-1β from cells (15). Although not definitively proven in vivo, but consistent with evidence from coronavirus disease 2019 (COVID-19) patients (69), the accumulation of caspase-1-cleaved GSDMD appears to promote mammalian cell death when GSDM pores are inadequately repaired.

4.1. Mammalian Pyroptosis: An Inflammatory Cell Death Pathway

Infected mammalian (and microbial) cells are replication factories for intracellular pathogens. Several cell death pathways serve as host defenses to eliminate infected cells and induce innate immune signaling and inflammation in mammals. Inflammatory signaling also recruits cleanup and repair crews. Mammalian pyroptosis is a form of inflammatory self-induced necrotic cell death activated by cell damage and invading pathogens (26, 93, 95).

The millions to billions of microbial spores inhaled daily, indoors and outdoors, require management by host innate immune responses. Conserved components of incoming pathogens such as DNA, RNA, and lipopolysaccharide (LPS), known collectively as pathogen-associated molecular patterns (PAMPs), as well as components of damaged host tissues, known as self-damage-associated molecular patterns (DAMPs), are detected by a wide range of different mammalian pattern recognition receptors (PRRs). PRRs include the membrane-associated Toll-like receptors (TLRs) and cytoplasmic PRRs, including the inflammasomes (e.g., NLRP3), NOD-like/nucleotide-binding leucine-repeat receptors (NLRs), RIG-I-like receptors (RLRs), and Z-DNA-binding protein 1 (ZBP1). PRRs induce immune defense signaling, and activation of some PRRs leads to cell death, such as inflammasomes, which induce pyroptosis, and ZBP1, which induces necroptosis. For immune signaling, the Toll/interleukin-1 receptor (TIR) domain of the TLR4 protein interacts with the TIR domain in its adaptor protein MyD88 [which also contains a death domain (DD), similar to adaptors for death receptor–dependent apoptosis]. MyD88 together with other factors further signals proliferative as well as protective inflammatory defense mechanisms, such as inflammasome NLRP3 expression needed to control infections, but if not controlled can also contribute to disease pathogenesis (140). TLR4 signaling through the adapter protein TIR domain–containing adapter-inducing interferon-β (TRIF) is reported to directly activate necroptosis (57). Interestingly, the TIR domains in the mammalian/animal adaptor protein SARM1 involved in Wallerian degeneration (i.e., the death of severed axons), as well as in some plant NLRs (involved in hypersensitivity reactions including cell death) and bacterial cell death systems (e.g., Thoeris), were found to have additional enzymatic activities linked to cell death (43, 148) (discussed in Section 5.1).

Multiple different inflammasome proteins recognize and bind to different PAMPs and DAMPs, triggering inflammasome complex assembly. Inflammasome complexes are platforms for activating nonapoptotic caspases, which have intertwined roles in immunity and cell death. Caspase-1 [also known as IL-1β-converting enzyme (ICE)] was originally identified as the protease required to cleave and convert proinflammatory cytokine pro-IL-1β into its active form. Active IL-1β is released from cells when caspase-1 cleaves and activates GSDMD to form pores in the plasma membrane, eventually causing cell death (89, 149, 155). Noncanonical inflammasomes activate

mouse caspase-11 (human caspase-4 and caspase-5), which also cleave GSDMD to form pores required to release cytokines.

Like other cell death factors, caspases have additional functions. In addition to cleaving IL-1β and GSDMs, caspase-1 cleaves and activates caspase-7, which is historically regarded as a weaker version of apoptotic caspase-3. In contrast, caspase-7 was recently found to counteract GSDMD-mediated pyroptosis in hepatocytes by enabling plasma membrane repair. Caspase-7 released from cells through GSDMD pores cleaves and activates acid sphingomyelinase to produce ceremide to rapidly repair membranes damaged by GSDMD and perforin pores (108). However, this function of caspase-7 does not ultimately prevent cell death. Instead, caspase-7 appears to only delay cell lysis via GSDMD, thereby allowing time for the infected cell to exist in an apoptotic state that is critical for clearance of important intracellular pathogens *Chromobacterium* and *Listeria*. In contrast to hepatocytes, this mechanism is not needed to clear these infections from macrophages (108). Interestingly, the death-delaying function of caspase-7 has another role, as caspase-7 knockout mice are defective for extrusion of cells from the intestinal epithelium into the gut lumen, a mechanism of eliminating old cells in normal physiology, which presumably avoids inflammation (109).

Pathogenic consequences of excessive host inflammatory responses have long been known (56) but have risen to the forefront, inspired by the COVID-19 era as patients succumb to excessive immune reactions after severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus titers decline. The role of pyroptosis in contributing to inflammatory diseases is continually unfolding. Antibody-mediated SARS-CoV-2 uptake via Fcγ receptors on monocytes and macrophages was recently found to trigger NLRP3 and AIM2 inflammasome activation and pyroptosis (69). While pyroptotic cell death is beneficial in that it aborts SARS-CoV-2 virus production, pyroptosis may also contribute to systemic inflammation that causes severe illness. Chronic inflammation is currently suggested to underlie a myriad of disorders, including Alzheimer's disease and neuroinflammatory syndromes following fungal infections (35, 103, 122).

4.2. Bacterial Gasdermins: The Original Pyroptosis

Pathogen recognition receptors and inflammasomes leading to mammalian cell pyroptosis have some corollaries in bacteria and fungi, but the most obvious conservation across species is that of the GSDMs, the effectors of pyroptotic cell death (**Figure 2**). Predecessors of the mammalian GSDMs are represented in microbial genomes. Informatics have identified GSDM-like proteins in fungal species (Ascomycota), diverse bacteria, archaea, and several hundred other prokaryotes from metagenomics, despite low sequence conservation (31, 68). Microbial GSDM-like proteins correspond to the N-terminal pore-forming death effector domain of mammalian GSDMs involved in pyroptosis. Prokaryotic and eukaryotic microbial GSDMs initially appeared to lack the regulatory C-terminal domain of mammalian GSDMs. However, crystal structures were solved for two GSDM-like proteins from soil bacteria, *Bradyrhizobium* and the relatively new genus *Vitiosangium* (68), revealing a different schema. Instead of the large C-terminal inhibitory domain present in mammal GSDMs, the bacterial GSDMs have a short C terminus that enwraps the characteristic bundled core domain to stabilize the inactive bacterial GSDMs. Thus, the short tail serves a similar role as the longer mammalian C terminus. Importantly, microbial GSDMs, analogous to their mammalian counterparts, are activated by proteolytic cleavage of this short inhibitory C terminus to induce oligomerization and facilitate membrane pore formation and bacterial cell death.

The proteases responsible for activating bacterial and fungal GSDMs are often encoded by adjacent genes. Predicted proteases can be identified in close proximity to GSDM genes in the vast majority of microbial species (31, 68). Biochemical studies further support proteolytic activation upon cleaving the short inhibitory C terminus and reveal some species specificity between GSDMs

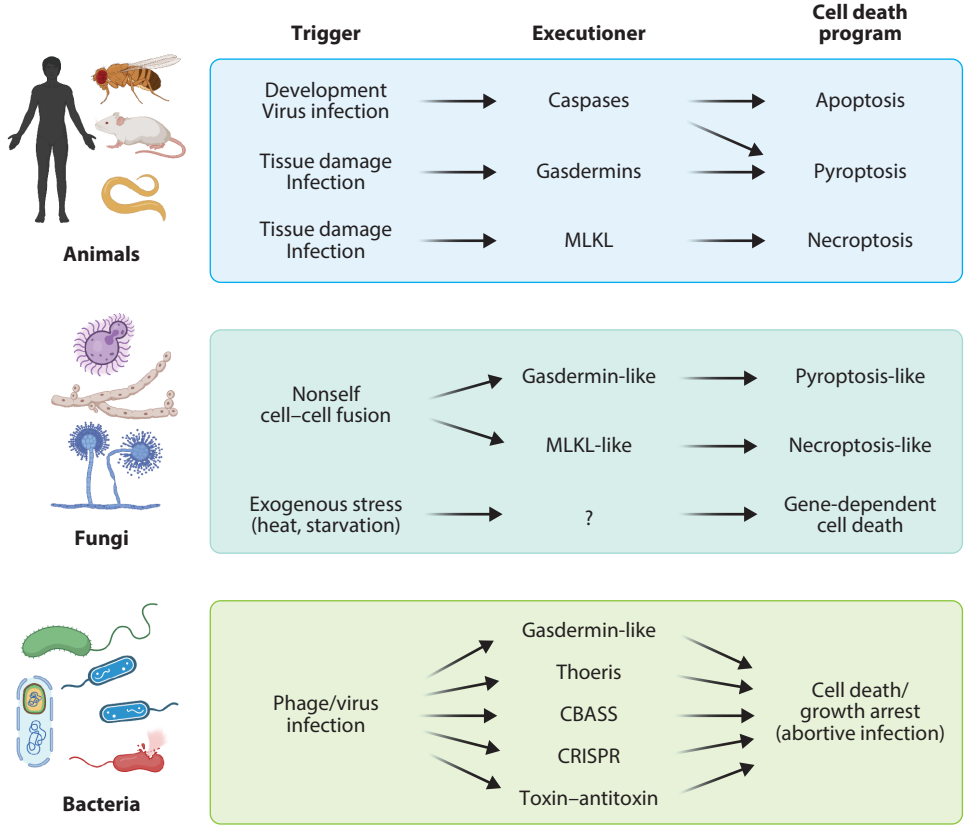

Figure 2

Partially conserved molecular cell death effector mechanisms across species. Except for CRISPR and possibly toxin–antitoxins, these cell death mechanisms share obvious resemblances to elements present in human innate immunity and cell death pathways, including bacterial and fungal proteases with caspase-like domains that cleave and activate fungal (Rcd-1 and Het-Q1) and bacterial gasdermin-like proteins and, thus, are regulated similarly to mammalian gasdermins. Thoeris signals suicidal death using TIR domains found in human TLRs and other immune regulators, and there are resemblances to cGAS-STING in bacterial CBASS. Fungal amyloid-like MLKL-like proteins mediate death in heterokaryon incompatibility, as do fungal gasdermin-like proteins, except via distinct molecular mechanisms. It is expected that the full repertoire of death pathways may far exceed those identified to date, some potentially unique to a species. Abbreviations: CBASS, cyclic oligonucleotide–based antiphage signaling system; cGAS-STING, cyclic GMP-AMP synthase–stimulator of interferon genes; MLKL, mixed lineage kinase domain–like; Rcd-1, regulated cell death 1; TIR, Toll/interleukin-1 receptor; TLR, Toll-like receptor. Figure adapted from images created with BioRender.com.

and their adjacent proteases. Intriguingly, the bacterial proteases and other adjacent genes contain additional protein domains that are found in mammalian/animal immune signaling and apoptosis machinery. These include nucleotide-binding/ATPase (NACHT) domains as well as tetratricopeptide repeat (TPR), leucine-rich repeat (LRR), or WD40 repeat domains, which define mammalian NLRs that sense invading pathogens and tissue damage and that signal in the pyroptosis pathway (discussed above in Section 4). Thus, intrinsic inflammatory signaling pathways upstream of mammalian pyroptosis pathways may also have potential corollaries in bacteria.

Involvement of bacterial GSDMs and their activating proteases in altruistic bacterial cell death was tested in a phage infection model by expressing the four-gene operon encoding the bacterial

GSDM, two trypsin-like proteins, and an ATPase from *Lysobacter* in a heterologous bacterium, *Escherichia coli* (68). Cultures of *E. coli* carrying the *Lysobacter* antiphage defense system are resistant to several coliphages (T4, T5, and T6) attributed to altruistic suicidal death of the infected bacterial subpopulation, thereby blocking phage propagation. Importantly, this altruistic death was abolished by mutation of the protease active site in the second trypsin-like gene. Similarly, mutation of the active site (H796 and C804) in the caspase-like protease encoded adjacent to the GSDM gene in the bacterium *Runella* also blocked bacterial cell death, resulting in collapse of the bacterial population following phage infection. In addition, mutation of the caspase cleavage site (P1 and P1′ residues) in *Runella* GSDM abolished cell death. These critical results surpass current evidence available in mammals that GSDM cleavage is an important mediator of pyroptotic cell death in vivo. The interpretation is that during the initial wave of phage infection at low multiplicity of infection, the production of progeny phages is thwarted by the bacterial GSDM-containing cassette because these bacteria underwent GSDM-dependent cell suicide, allowing the remaining uninfected bacterial population to regrow (68). Dozens of defense islands have been identified in bacterial genomes, although the phages they defend against are not yet known.

4.3. Fungal Gasdermins for Pyroptosis-Like Death

Similar to bacteria, fungi were thought to lack known corollaries to inflammatory signaling that originally defined mammalian pyroptosis, until the identification of fungal GSDM-like pyroptosis death effector proteins. Filamentous fungi grow by extending hyphae that can branch and fuse to form mycelial networks and can also fuse with hyphae from neighboring fungi of the same species. However, some strains of *Neurospora crassa* cannot survive fusion with one another. The fusion of two cells encoding incompatible allorecognition proteins results in septation to isolate the two recently fused cells from neighboring cells, followed by suicidal cell death. Several genetic loci responsible for this phenomenon, known as heterokaryon incompatibility (HI), have been mapped to specific genetic loci (31). Diverse types of allorecognition mechanisms have been identified, and a few of these are characterized in greater detail. The general theme is that each type consists of two variants of the same gene, and coexpression of an incompatible pair in the same cell upon fusion results in rapid cell death. Thus, HI loci serve as self- versus non-self-recognition modules and are thought to protect from invading genetic elements and mycoviruses. The cell death machinery encoded by one of these allorecognition genes in the model organism *N. crassa* is the regulated cell death 1 (RCD-1) protein. RCD-1 shares a predicted structure and limited sequence similarity with the N-terminal pore-forming domain of mammalian GSDMs (31, 34). The fusion of two cells carrying antagonistic alleles, *rcd-1-1* and *rcd-1-2*, induces fungal cell death. Co-expression of *rcd-1-1* and *rcd-1-2* in mammalian HEK293T cells will also kill mammalian cells. Purified RCD-1-1 plus RCD-1-2 proteins together, but not separately, permeabilize liposomes containing PS and cardiolipin, similar to the lipid composition preferences of mammalian GSDMs (30, 31). Thus, the two proteins may induce conformational changes that stimulate oligomerization and pore formation, conceptually analogous to RIPK3–MLKL interactions in mammalian necroptosis.

A more common type of GSDM-like genetic locus is the allorecognition system resulting in cell death via HI in *Podospora anserina*, another nonpathogenic ascomycete model organism (22, 31). In this case, the two alternate alleles *het-Q1* and *het-Q2* encode unrelated proteins (idiomorphic genes). Similar to human GSDMD and the *Lysobacter* GSDM discussed in Section 4.2, the *P. anserina* GSDM-like HET-Q1 is proteolytically activated. HET-Q1 becomes cytotoxic when cleaved by the subtilisin-like serine protease HET-Q2 (22). Exogenous coexpression of HET-Q1 and HET-Q2 in yeast and mammalian cells also causes cell death (22). A search for HET-Q1 homologs identified more than 1,800 additional GSDM-like sequences in ∼400 fungal genomes

(predominantly Ascomycota). However, unlike the *P. anserina* idiomorphic arrangement but more similar to the bacterial GSDM cassettes, the majority of *het-Q1*-like genes were found in a two-gene cluster together in the same locus with a *het-Q2* protease-like domain–containing protein (22, 31). Thus, additional mechanisms must regulate the initiation of cell death, such as different signals required to activate different HET-Q2 proteases or dictate their cleavage specificities for heterologous HET-Q1 proteins. Similarly, different mammalian GSDMs are cleaved by cysteine proteases (e.g., caspase-1 and caspase-3) or serine proteases (granzymes) (155). The majority of HET-Q2-like fungal proteases identified thus far are subtilisin-like serine proteases and a few CHAT domain–cysteine proteinases belonging to the CHAT peptidase domain family pfam12770 that includes caspases. Thus, the winding cell death trail across species is now coming into view.

Interestingly, and similar to bacteria, fungal HET-Q2-like proteases typically contain additional protein domains. Some HET-Q2-like proteins contain NLR-like domains, including a P-loop between the protease and a WD40 repeat, analogous to mammalian NLRs that sense PAMPs derived from invading pathogens and DAMPs derived from host tissue damage to activate mammalian inflammation and pyroptosis (discussed in Section 4.1). Thus, cell death as well as immunity pathways present in mammals appear to have ancient origins in bacteria, fungi, and plants (6, 101).

4.4. Mammalian Necroptosis and Fungal Corollaries Beyond MLKL Death Effectors

Mammalian necroptosis is a form of regulated necrosis activated by tumor necrosis factor (TNF) signaling through the TNF receptor 1 (TNFR1) pathway leading to activation of the kinase RIPK1 (150). RIPK1 can signal to activate apoptosis or necroptosis. For necroptosis, RIPK1 phosphorylates and activates RIPK3, promoting formation of the necrosome, which leads to phosphorylation of MLKL, the necroptosis pore-forming death effector. Apoptotic mammalian caspase-8, which is also activated by the intracellular complex induced by TNFR1 activation, can cleave and inhibit RIPK1, shifting the balance toward apoptosis. Higher-order multimerization of amyloid-like RIPK1–RIPK3 complexes can adopt different shapes, which appear to be important determinants for activating the pore-forming activity of MLKL (20, 115). Like other cell death effectors, MLKL has other functions. MLKL can be found in the nucleus and in association with cytoplasmic (membrane repair) ESCRT proteins and endosomes independently of its necroptotic function following phosphorylation by RIPK3 (153). Extracellular vesicles were also found to contain phosphorylated MLKL and proinflammatory cytokine IL-1β, suggesting complex regulation of necrotic cell death and nondeath functions of MLKL (54, 152, 153). RIPK1 activation leads to GSDM pore formation but can also lead to initiation of apoptosis.

MLKL-like proteins have been identified in fungi and plants (8). Fungal MLKL-like proteins appear to have the mammalian necroptotic cell death effector function. Fungal MLKL-like gene clusters are often found in the same fungal genomes with HET-Q/GSDM gene clusters and represent another type of cell death via HI involving self- and non-self-discrimination. In *P. anserina*, self- versus non-self-allorecognition is also determined by the genetic differences between two *het* loci encoding HET-S or HET-s (33) (**Figure 1**). Fusion of hyphal cells with compatible *het* loci forms stable heterokaryons, while fusion of two cells encoding incompatible *het* loci (HET-S and HET-s) triggers rapid compartmentalization, septation, vacuolization, and death of the two recently fused cells (33). Cell death occurs upon fusion of HET-S and HET-s strains, resulting in prion-like structures that facilitate unfolding of the HET-S N-terminal HeLo domain and subsequent conversion to a pore-forming integral membrane protein leading to cell death (121). The HeLo domain has predicted structural similarity to the four-helix-bundle

N-terminal domain of the mammalian necroptosis executioner protein MLKL, which also contains a helical connector (brace) region and a C-terminal pseudokinase domain. Like mammalian MLKL, activation of the HeLo domain of HET-S in the fungal cytoplasm forms pores in the plasma membrane, resulting in cell lysis (32). Plants also encode NLRs and MLKL-/HeLo-like proteins that can cause cell death following infection with fungal pathogens (95, 97).

4.5. Autophagy-Dependent Cell Death in Fungal Development and Virulence

Autophagy (also known as macroautophagy) is critical for survival, and its role in cell death remains a debated topic in mammalian cell biology and has been reviewed by others (106). *M. oryzae* is a filamentous ascomycetes fungus that infects valuable cereal crops, including rice, barley, wheat, millet, oat, and ryegrass (100), causing estimated annual losses of crops that would have been sufficient to feed millions of people (136). Infection of a plant leaf is initiated when the three-celled asexual fungal spore (conidium) produces an appressorium that penetrates through the plant cell wall (100, 138). Invasive hyphae then develop, spread throughout the plant, and release new spores through lesions that cause necrotic cell death of the host plant.

PCD is part of the natural life cycle of *M. oryzae* and is required for virulence (73, 138). Formation of the infectious fungal appressorium is initiated when the apical cell of the three-cell conidium (asexual spore) undergoes mitosis and one daughter nucleus migrates through a germ tube to the maturing appressorium structure (100). Within hours, the three conidial nuclei collapse and the conidial cells undergo vacuolization and death, leaving an intact infectious appressorium (138). Interestingly, autophagy is required for conidial cell death to occur. Conidia lacking the autophagy factor *MgATG8* (homolog of mammalian LC3 and hallmark of autophagy) exhibit defective autophagy, fail to undergo nuclear degradation and cell death, and fail to form the appressorium required for infectivity (138). In fact, deletion of any 1 of 16 autophagy genes resulted in the extended survival of conidia, failure to form the appressorium, and inhibition of the infection cycle (73) (**Figure 1**). Definitive demonstration of autophagy as a direct (autophagy-mediated) versus indirect (autophagy-dependent) mediator of cell death is challenging in this case. For example, conidial cell death could potentially occur following autophagic degradation of an unidentified cell death inhibitor, analogous to degradation of *Drosophila* DIAP1. Direct cell death effector proteins have not yet been identified in *Magnaporthe*, although recent studies have suggested ferroptosis in conidial cell death.

4.6. Ferroptosis: Mammals, Fungi, and a Parasite

Ferroptosis is suggested to be an important contributor to disease pathology, especially in the brain. Ferroptosis differs from the other three well-studied mammalian cell death pathways as it lacks a known death effector protein. Instead of pore-forming proteins as in apoptosis (BAX/BAK), pyroptosis (GSDMs and NINJ1), and necroptosis (MLKL) (46, 71), cells die by ferroptosis following membrane damage due to lipid peroxidation of polyunsaturated fatty acids (PUFAs). Mammalian ferroptosis is also defined as an iron-dependent process inhibitable by iron chelators. There are two prominent antiferroptosis factors in mammalian cells: the enzyme glutathione peroxidase 4 (GPX4), a selenocysteine protein with phospholipid hydroperoxidase activity, and the cystine–glutamate antiporter system x_c^-, which maintains intracellular cysteine and glutathione levels to support GPX4 function and avoid accumulation of lethal levels of lipid hydroperoxides (37, 145). While the plasma membrane of mammalian cells becomes compromised during ferroptosis, whether it is the first target of lipid peroxidation at the initiation of ferroptosis is an open debate. The term ferroptosis was coined to describe cell death induced by the cancer cell–selective small-molecule erastin, which inhibits system x_c^-, resulting in depletion of cellular

cysteine and glutathione and leading to inhibition of GPX4 (37). Iron chelators and lipophilic antioxidants suppress ferroptosis.

Genetic regulation of mammalian ferroptosis is likely more complex than delineated thus far. Integrating information from two dozen published screens suggests that lipid peroxidation is downstream of many distinct stimuli and is particularly sensitive to cell context–dependent effects (96). In particular, ACSL4 involved in PUFA synthesis is important for ferroptosis when GPX4 is inhibited but not for ferroptosis induced by cystine depletion. By contrast, alkylglycerone phosphate synthase involved in the synthesis of PUFA-containing ether lipids was not important for ferroptosis induced by GPX4 inhibition. Another recent interrogation of the ferroptosis pathway by screening compounds identified a subset of mechanistic target of rapamycin (mTOR) inhibitors [adenosine triphosphate (ATP)-dependent] that suppress ferroptosis, perhaps by depleting cells of specific amino acids such as cysteine via sustained protein synthesis driven by mTOR (24).

It is conceivable that ferroptosis-like death might be an ancient form of RCD. Iron chelation was reported to prevent the developmental cell death of conidial cells required for completion of the fungal life cycle of the rice blast fungus *M. oryzae*, an important plant pathogen (29, 88, 123). Thus, both iron accumulation and autophagy appear to be important for conidial cell death, which is required to produce the infectious appressorium, a high-turgor structure required to infect plants. However, it appears that ferric ions promote death independently of the Fenton reaction, as argued for mammals (117).

The extracellular single-celled parasite *Trypanosoma brucei*, the causative agent of sleeping sickness/trypanosomiasis in people and livestock, was reported to undergo ferroptosis. Trypanosome death is elevated in mutants lacking a distant ortholog of human GPX4, tryparedoxin hydroperoxidase, and cell death can be suppressed by iron chelators and lipophilic antioxidants, analogous to mammals (12). Heat stress–induced ferroptosis has also been described in a plant and in cyanobacteria (1, 36). However, *S. cerevisiae* yeast are highly resistant to lipid peroxidation and can tolerate high levels of PUFAs, apparently due to endogenous coenzyme Q biosynthesis (124), suggesting that ferroptosis may not play a major role in some cell death paradigms.

5. MULTIPLE ANTIPHAGE DEFENSE SYSTEMS BY ALTRUISTIC CELL SUICIDE–ABORTIVE INFECTION

5.1. Role of TIR Domains in Mammalian and Microbial Cell Death

As discussed above, mammalian PRRs recognize the conserved elements (e.g., DNA, RNA, and lipids) of incoming pathogens or damaged host cells and signal to activate immune defenses and/or cell death. TLRs are a type of PRR that contain a TIR domain. The TIR domains in mammalian TLRs are important for transmitting immune signals and engaging cell death via the TIR-containing adapter TRIF, but they lack enzymatic activity (113). By contrast, the TIR domains in three diverse systems—mammalian SARM1, which promotes death of neuronal axons through Wallerian degeneration; some plant NLRs involved in pathogen-induced cell death; and the TIR domain–containing bacterial antiphage protein ThsB of the Thoeris phage defense system—all exhibit NADase enzymatic activity, cleaving NAD^+ to generate adenosine diphosphate (ADP)-ribose (ADPR) and nicotinamide (111). Plant TIR domain proteins and bacterial ThsB have an additional cyclic nucleotide synthase activity that produces the cellular messenger cyclic ADPR (cADPR) (43). Variants of cADPR are produced by other bacterial TIR proteins. The cADPR product of ThsB activates the SIRT2 domain protein ThsA, which consumes NAD^+ to cause bacterial cell death or growth arrest, while plant TIRs signal to regulate calcium signaling. By contrast, mammalian SARM1 is thought to consume NAD^+ to cause neuronal death. Thus, all three diverse organisms appear to act differently, though the full extent of their overlapping

functions is not yet known. ATP depletion by conversion to ITP (inosine triphosphate) was reported to serve as another antiphage defense mechanism called RADAR, in which the adenosine deaminase RdrB and the AAA ATPase RdrA form a large complex that depletes the cell ATP (39).

Thoeris is responsible for an abortive infection, also known as Abi, and promotes an altruistic cell death mechanism (92, 142). Expression of the two-gene Thoeris system from *Bacillus cereus* transplanted into *Bacillus subtilis* cells induced rapid suicidal cell death prior to completion of the phage replication cycle. This suicidal death prevents progeny virus production and spread to nearby bacterial cells, thereby protecting the population from a lytic phage infection and total collapse of the bacterial population (111). Phages also counteract bacterial antiviral defense mechanisms. For example, the bacteriophage protein Thoeris antidefense 1 (Tad1) acts like a sink to sequester the glycocyclic nucleotides that bacterial Thoeris TIR domains produce to signal bacterial immune responses. Thus, viral Tad1, which is found in many phage families, thwarts bacterial antiviral defenses by decoupling bacterial phage sensing from effector functions (82). The defenses and counterdefenses between bacteria and phage are often referred to as an arms race.

TIR domains are also found in metazoan and bacterial immunity pathways known as cyclic GMP-AMP synthase (cGAS)–stimulator of interferon genes (STING) in mammals and cyclic oligonucleotide–based antiphage signaling system (CBASS) in bacteria. In mammals, some inflammasomes and TLRs, as well as the nucleic acid–sensing protein cGAS, sense viral nucleic acids. cGAS catalyzes the synthesis of unconventional cyclic dinucleotides, which bind and activate the STING protein. Activated STING transits from the endoplasmic reticulum to the Golgi apparatus and to vesicles in mammalian cells, while TBK1 kinase signaling and subsequent activation of the transcription factor IRF3 activate innate immune and inflammatory signaling (62).

Bacterial cGAS-like nucleotidyltransferase (CD-NTase) is activated by phage infection to synthesize versions of cyclic dinucleotides as part of the CBASS. In addition to the CD-NTase, CBASS operons also encode CD-NTase-associated proteins (Cap) that are activated by nucleotide binding and induce bacterial cell death by different mechanisms, including membrane permeabilization, nucleic acid degradation, and metabolite depletion by TIR-STING (Cap12) (43). Thus, CBASS is an antiviral defense mechanism in which a dicyclic nucleotide binds and activates a receptor that facilitates cell death prior to successful phage replication (23, 38). Another system known as Pycsar (pyrimidine cyclase system for antiphage resistance) plays a role in phage defense through the production of cyclic pyrimidines 3′,5′-cyclic cytidine monophosphate (cCMP) and 3′,5′-cyclic uridine monophosphate (cUMP) that signal activation of bacterial effector proteins that mediate cell death (128).

5.2. Other Bacterial Defense Systems

Toxin–antitoxin (TA) modules are widespread in bacteria encoded on plasmids or chromosomal DNA and are composed of a toxic protein and a counteracting antitoxin, which is either a protein or a noncoding RNA (133). Five major classes of TA modules are described, and type II are the best studied. Type II systems reside on the same operon and code for small proteins, and inhibition of the toxin is carried out through protein–protein interactions. The toxin is a stable protein, while the antitoxin is unstable due to rapid degradation by bacterial proteases, usually Lon or ClpP (120). Pharmacologically activating TA systems has been proposed as a potential pathogen control strategy. Further studies are needed to identify druggable targets and to potentially uncover novel connections between cell death resistance and mechanisms of bacterial persistence (133). The mazEF TA module in *E. coli* prevents the spread of phage (60). Many bacteria acquire resistance to phage infection by integrating DNA from phage genomes in CRISPR and utilize CRISPR-associated Cas proteins to initiate an antiviral response (9, 14). A plethora of distinct new

CRISPR defense mechanisms (e.g., the type III CRISPR system) involving inhibition of bacterial cell growth or cell death continue to be identified (7).

6. WHY UNICELLULAR FUNGI NEED CELL DEATH

Fungi occupy diverse environments and therefore are likely to encode diverse mechanisms of cell death. However, detailed molecular cell death pathways are known only for filamentous fungi that are considered to exhibit multicellular behaviors (discussed in Section 4). Nevertheless, important unicellular fungal pathogens, including yeast such as *Candida albicans*, *Candida auris*, and *Cryptococcus neoformans*, can also be expected to undergo PCD. For example, yeast naturally live in colonies that exhibit characteristics of multicellularity (135). Like multicellular animals that undergo differentiation to fulfill specific roles required for organismal viability, unicellular fungi also encode differentiation programs to form multicellular communities observed inside both simple and structured yeast colonies. Moreover, morphological switches by colonies between smooth, wrinkled, mucoid, and pseudohyphae have correlated with phenotypic behaviors, including virulence of *C. neoformans* and *C. albicans* (48, 125).

The application of the term apoptosis to yeast cell death has helped and hindered progress, as yeast lack core apoptosis factors, most notably BAX/BAK and caspase-3 (59) (various definitions of apoptosis are discussed in Section 3.1). The utility and interpretation of any mammalian apoptosis assays applied to yeast are unclear; these include fluorogenic caspase reporters that cleave after aspartate residues (e.g., zVAD-fmk), PS exposure detected by Annexin V, and TUNEL labeling of DNA fragments. All of these assays are thought to detect the direct consequences of apoptotic caspases in mammals, worms, and flies. Fungi encode metacaspases, which are related to mammalian caspases but differ in substrate specificity and mechanism of activation, but are unlikely to directly activate these apoptosis reporters (discussed in Section 3.4). Other yeast homologs of mammalian apoptotic cell death factors have been reported to have some involvement during the dying process in yeast cells, including orthologs of EndoG, AIF, cytochrome *c*, and others, but the mechanisms are unclear or do not support a causal role in death (41, 94, 147) (discussed in Section 3.4).

The widely used laboratory model *S. cerevisiae* is highly tractable for genetic manipulation of cell death mechanisms (19, 129). Studies of cell death induced in knockout yeast strains by exposure to heat-ramp, hydrogen peroxide, acetic acid, and endoplasmic reticulum stressors indicate that cell death is genetically regulated (53, 129, 130). For example, disruption of the yeast AP-3 vesicle trafficking pathway, which delivers membrane-associated proteins such as the Yck3 kinase to the vacuole membrane, protects yeast cells from loss of vacuolar membrane integrity and cell death (75, 127). Vacuole membrane permeabilization occurs prior to plasma membrane permeabilization, suggesting that a commitment point to death in yeast may occur at the vacuole (74, 75, 127). The evidence further suggests that yeast proteins trafficked by AP-3 to vacuole may cause membrane permeabilization and cell death. Thus, yeast appear to have a death pathway conceptually similar to mammalian LMP death by lysosome permeabilization. However, a cell death effector protein that directly forms pores in the vacuole/lysosome membrane to cause cell death has not been identified in yeast or mammals. The release of vacuolar proteases into the yeast cytoplasm has been suggested to contribute to yeast cell death, though the evidence is mixed. A more attractive possibility is that, like the late steps of apoptosis (discussed in Section 3.4), digestion of yeast cell milieu by vacuolar proteases after the cell has already committed to death serves instead to provide ready nutrient sources for surviving neighbor cells (127).

Evidence that starvation conditions may have driven the selection for cell death mechanisms is found in yeast. *S. cerevisiae* yeast colonies exhibit features of multicellularity and are comprised of metabolically distinguishable upper and lower cells that may facilitate community survival (16, 135, 146). Changes in gene expression profiles in *S. cerevisiae* cells inhabiting aggregates and

biofilms have been associated with changes in behaviors (107). In response to starvation, *S. cerevisiae* undergoes mating, meiosis and sporulation to gain resilience and conserve energy. Each mating gives rise to four spore progeny (i.e., a tetrad). Under extreme nutrient limitation conditions, one or more of the four spores in each tetrad dies, accompanied by vacuole permeabilization and nuclear degradation of the dead spores as well as the mother cell that underwent sporulation, potentially liberating nutrients to support spore development of the survivors (40) (mechanism discussed in Section 3.4).

Cell death may limit the spread of viral pathogens throughout the yeast population (via vertical transmission) (66). Altruistic death of aged yeast appears to support long-term survival of the population, where death-prone yeast cultures survive longer than cultures of death-resistant yeast (44). Involvement of mitochondrial fission/fusion integrity in altruistic cell death is suggested by the protective effect of Δ*dnm1* deletion against killer virus–infected cultures (45, 66). The identification of naturally pathogenic mycoviruses may reveal ancient immune mechanisms encoded by fungi to combat infection by inducing fungal cell death, analogous to bacterial models of cell death.

7. EXPLOITING CELL DEATH THERAPEUTICALLY

Precedence for successful targeting of mammalian apoptosis therapeutically has been established. Small-molecule BH3 mimetics bind into the cleft of antiapoptotic BCL-2 proteins and displace their proapoptotic binding partners to activate apoptosis. Many companies have generated BH3 mimetics and mimetics of other molecules in pursuit of cancer therapeutics. Only one of these has been FDA approved thus far; Venclexta® (ABT-199, venetoclax) is a selective inhibitor of BCL-2 but does not appreciably bind its closest homolog BCL-xL and is a frontline therapeutic for treating chronic lymphocytic leukemia (126).

The prospect of clinical intervention of regulated necrosis pathways, such as disulfiram to inhibit GSDM pore formation (63), NLRP3 inflammasome inhibitors (55), and RIPK1 inhibitors (103), has also been investigated for the therapeutic treatment of inflammatory and degenerative diseases by intervening in necroptotic signaling, for use against inflammatory bowel disease, amyotrophic lateral sclerosis, and Alzheimer's disease (35, 103, 122).

The search for new antibiotics has nearly ground to a halt, and novel control measures are needed, driving the development of phage therapies for patients and kiwi plants (47, 76). Although this search was first pursued by Félix d'Hérelle in the early twentieth century to combat dysentery in French troops, success required considerably more detailed information, and recent advancements on this front have led to new start-ups, including BiomX, which seeks approaches for chronic inflammatory disorders, and the biotech company EcoPhage, which is developing phage for protecting crops.

Although knowledge of PCD pathways in fungi is in its infancy, clear examples of deliberate fungal cell death in self-recognition, fungal life cycle, and virulence present opportunities for the development of interventions (32, 53, 118, 138). The burden of fungal infections is underreported, and the arsenal of antifungal agents is remarkably small. Bioinformatics-based approaches are being applied to discover new cell death genes in diverse microbes (52, 148). Thus, new control measures could involve fungal cell death manipulations. A recent summit held by the Canadian Institute for Advanced Research and the Burroughs Wellcome Fund convened a workshop to consider the challenges and threats of fungal infections in which several recommendations for future needs and directions were made, though fungal cell death research was not yet ready for such discussions.

Although the prospect of targeting cell death pathways for control of microbes remains understudied, there is interest in this general direction (80, 84), and feasibility is suggested by growing

evidence indicating that molecular death mechanisms exist in multicellular and filamentous fungal pathogens (e.g., *M. oryzae* and *N. crassa*) (53) and also likely exist in single-cell yeast pathogens (e.g., *Cryptococcus* and *Candida*) and appear to involve lysosome/vacuole membrane permeabilization (127). The identification of microbial cell death pathways with druggable targets represents a novel platform for future antifungal drug development (31, 80). A renaissance in cell death research has occurred through the study of regulated cell death in microbes.

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