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Targeting intrinsic cell death pathways to control fungal pathogens

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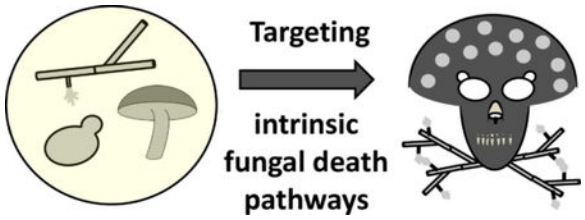
Abstract

Fungal pathogens pose an increasing threat to public health. Limited clinical drug regimens and emerging drug-resistant isolates challenge infection control. The global burden of human fungal pathogens is estimated at 1 billion infections and 1.5 million deaths annually. In addition, plant fungal pathogens increasingly threaten global food resources. Novel strategies are needed to combat emerging fungal diseases and pan-resistant fungi. An untapped mechanistically novel approach is to pharmacologically activate the intrinsic cell death pathways encoded by pathogenic fungi. This strategy is analogous to new anti-cancer therapeutics now entering the clinic. Here we summarize the best understood examples of cell death mechanisms encoded by pathogenic fungi, contrast these to mammalian cell death pathways, and highlight the gaps in knowledge towards identifying potential death effectors as druggable targets.

Graphical Abstract



Fungal species appear to undergo forms of programmed cell death though the detailed mechanisms are not yet known.

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Keywords

fungi; cell death; pathogen; yeast; necrosis; apoptosis

1. Introduction

An estimated 3.8 million fungal species make up a significant portion of global biodiversity [1]. Of these, the small fraction that is pathogenic has significant impact on global health and mortality. Annual global incidence of acute invasive fungal diseases was recently estimated at 1.9 million, primarily attributed to cryptococcal meningitis, invasive aspergillosis, pneumocystis pneumonia, and invasive candidiasis [2]. Another three million live with chronic pulmonary aspergillosis, while over 10% of the world population has non-invasive fungal infections [2, 3]. Despite the importance to public health, fungal infections are more neglected than other major killers. Diagnostics for fungal infections have greatly improved but remain limited or unavailable. Treatment strategies for invasive species are challenged by the small arsenal of anti-fungal agents, the susceptibility of immunocompromised populations, the rise of drug resistance across major fungal pathogens including *Candida*, *Cryptococcus*, *Aspergillus* and new emerging strains [2, 4–6]. In addition, fungal infections of unknown proportions affect terrestrial, avian and marine wildlife and cause significant agricultural losses [7–11].

The rise in outbreaks of fungal pathogens has been attributed to a number of factors including contaminated medical devices, organ transplants, and patient immune status [7, 12, 13]. Rising global temperatures are predicted to select for fungal thermal tolerance, which may facilitate breaching mammalian defenses, though direct evidence is limited to date [8, 9, 14]. Beyond human pathogens, plant fungal pathogens such as *Magnaporthe oryzae* (rice blast) threaten global food security by infecting economically significant cereal crops, typically claiming 10–30% of rice harvests in parts of the Americas, Asia and Africa [15–17]. Epidemics of rice blast can devastate entire fields, potentially impacting approximately half the world's population dependent on rice as a primary staple, compounded by the high costs of anti-fungals for treating crops [15].

In light of these challenges, new out-of-the box strategies are needed to combat fungal pathogens. One possibility on the horizon is pharmacologic manipulation of intrinsic cell death mechanisms encoded by fungi. Precedence for this concept is provided by the cancer field. A new class of drugs emerged from the discovery of a deep binding cleft on human anti-apoptotic proteins BCL2 and BCLxL where their natural inhibitors bind, and where small molecule mimics of these inhibitors also bind [18]. In 2016, three decades after the discovery of BCL2 [19–23], a BCL2 antagonist (Venetoclax/ABT-199) was approved for clinical use in a subset of cancer patients [24–27], and many related compounds are currently in clinical trails [28] – an exciting new era. While similar approaches are being explored for the BCL2 homologs in viruses [29–31], fungi lack BCL2 homologs and therefore are not amenable to this approach. Nevertheless, there is interest in this general direction [32], and feasibility is suggested by growing evidence indicating that molecular death mechanisms exist in multicellular and filamentous fungal pathogens (e.g.

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Magnaporthe and *Neurospora crassa*) and likely also exist in single-cell yeast pathogens (e.g. *Cryptococcus* and *Candida*).

2. Distinctions between cell death programs in animals versus fungi

The systematic disappearance/death of cells observed in developing animals was once assumed to result from depletion of essential resources for those cells. In this case, cell death could simply be the absence of life without any active participation by the cell destined to die. However this philosophy changed with delineation of the first molecularly defined cell death pathway, a cell autonomous caspase-dependent process genetically mapped in *C. elegans* and mammals [33–36]. This apoptotic death pathway is inhibited by the CED9/BCL2 proteins and is required to eliminate many cells during embryonic development [23]. Apoptosis can be induced in mammalian cells by a variety of stimuli from within the cell (e.g. DNA damage) and by extracellular ligand-induced signaling pathways that converge to activate caspase 3, the primary effector molecule of apoptosis (Fig 1). The morphological features of apoptotic mammalian cells are attributed to actions of caspase 3 that prepare apoptotic cell corpses for engulfment and degradation by neighboring cells. Caspases are also widely studied for their roles in non-death related cellular processes including differentiation, proliferation, and neuronal function [37–41]. However, biochemical mechanisms analogous to mammalian caspase-dependent apoptosis have not been identified in fungi (see nomenclature conflict, section 4).

Most mammalian caspases do not promote apoptosis but several caspases can activate pyroptosis (programmed necrosis) by cleaving and activating the pore-forming protein gasdermin D that lyses mammalian cells from the inside of the plasma membrane [42, 43] (Fig 1). Cell death pathways can become intertwined, for example when apoptotic caspase 3 promotes pyroptosis by cleaving and activating a different gasdermin protein, gasdermin E [44]. The human gasdermin E/DNFA5 C-terminal domain is conserved in some species of yeast (Cdc23/Mcm10), but these yeast proteins lack the relevant death-inducing pore-forming domain and are not known to promote yeast cell death [45]. Other widely studied non-apoptotic (necrotic) mammalian death pathways are necroptosis and more recently ferroptosis, which play important roles in disease pathogenesis including neurodegeneration and infection [46–51]. Fungi may have semblances of mammalian necrosis pathways (see section 5).

Unlike mammalian cells, fungi have cell walls, live in distinctly different environments, and are genetically diverse. Thus, it seems likely that fungal mechanisms of cell death would have been selected under equally diverse driving forces. Thus, fungi can be expected to have diverse biochemical death processes to build morphologically distinct structures or suppress the spread of fungal viruses, and therefore may share more dissimilarities than similarities with mammals. By extension, applying mammalian apoptosis assays and reporters (e.g. TUNEL assays, zVAD caspase inhibitors/reporters, and Annexin V to detect exposed phosphatidylserine) to study fungi may not answer the important mechanistic details of fungal cell death [52–54]. Mammalian apoptosis assays have been applied in studies of *Saccharomyces cerevisiae*, *Cryptococcus neoformans, Candida* species, and *Aspergillus*

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fumigatus among other pathogenic fungi but without identifying the precise causes of reporter activity or the direct effectors of cell death [54–58].

3. Rationale for the existence of cell death in multi- and unicellular fungi

Definitive molecular evidence for a deliberate program of self-inflicted injury in fungi has not yet been achieved, at least not in a manner analogous to the salient discoveries that finally squelched remaining skepticism about the existence of programmed cell death in animals [20, 59, 60]. The existence of cell death mechanisms in multi-cellular filamentous fungi (e.g. *Aspergillus*) is more easily rationalized because it was originally assumed that programmed cell death machinery arose during evolution with multicellular species. Among multi-cellular fungal species, there are clear examples where fungal cell death appears to be deliberately orchestrated and even required for the fungal life cycle, for example in the plant pathogen *Magnaporthe oryzae* [61] and in *Podospora anserina* and related filamentous fungi [62]. Some of the molecular mechanisms have been partially delineated, providing potential pharmacologic targets (see section 5).

Even the communities of single cell yeast species such as the human pathogens *Candida albicans* and *Cryptococcus neoformans* as well as the laboratory workhorse *Saccharomyces cerevisiae* exhibit characteristics of multi-cellularity [63]. Like multicellular organisms that undergo distinct pathways of differentiation to fulfill specific roles required for organismal viability, unicellular fungi also encode differentiation processes to form multicellular communities. Cell morphological changes between smooth, wrinkled and pseudohyphae have long been observed to correlate with phenotypic/functional changes including virulence in *Cryptococcus neoformans* and *Candida albicans* [64, 65]. Different gene expression profiles in *Saccharomyces cerevisiae* cells are associated with different cell behaviors in aggregates, hyphae and biofilms [63, 66–68]. The transition from colonies to biofilms by *Saccharomyces cerevisiae* is orchestrated in part by the opposing actions of two transcription factors Tup1, which promotes biofilm formation, and Cyc8/Ssn6, which inhibits Flo11 expression and represses biofilm formation [69]. Long non-coding RNAs (lncRNAs) are differentially expressed in *Saccharomyces cerevisiae* colonies versus biofilms [70]. Although a role for cell death in prokaryote biofilm formation is not understood, dying bacterial cells have been observed within biofilms and proposed to be beneficial by preventing extensive disruption of colony architecture [71, 72].

Even within simple colonies of *Saccharomyces cerevisiae*, transcriptional profiling has revealed some of the inner workings of yeast colony life and the changes that occur during aging [73, 74]. Simple yeast colonies of *S. cerevisiae* are stratified in cell layers with distinguishable morphological and metabolic characteristics [75]. In general, yeast cells in upper colony layers are larger and undergo autophagy, while cells in lower layers are smaller, rely more on respiration and are more sensitive to stress [75]. By occupying specific niches, cells in yeast communities can perform unique roles such as adhesion, invasion, and nutrient recycling that can reasonably be expected to facilitate community function and survival [67, 69]. Yeast colony subdomains that are prone to cell death also could potentially benefit the surviving population [76].

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Beneficial yeast cell death has been suggested to occur during failed mating attempts, stress responses to adverse environmental conditions, and cell death could conceivably have a role in self-recognition that also limits the spread of viral pathogens [77–79]. Altruistic cell death of aged yeast cells has also been reported [80, 81]. In search of gene-dependent yeast cell death programs, we and others have identified candidate genes in genome-wide screens of the yeast *Saccharomyces* knockout collection in response to heat stress, heat shock, ER stress or acetate treatment [82–86], but the precise death mechanisms and death effectors are not yet known. Thus, the possibility of unicellular programmed cell death remains a philosophical question until compelling molecular pathways are defined [87].

4. Evidence for and against Apoptosis-Like Cell Death in Fungi

A conspicuous nomenclature problem stems from the application of the term “apoptosis” to yeast and other fungi. The original definition and rationale for coining the word apoptosis in 1972 was to infer a deliberate cell death process occurring in mammalian tissues, long before any molecular mechanisms were known [88]. The original evidence for such was limited to morphological/pathological changes, some of which resemble dying yeast cells [55]. Thus, apoptosis began to be defined morphologically. The nomenclature conflict arises because yeast biologists/geneticists continue to apply the term apoptosis as originally defined or by morphological features and mammalian apoptosis reporters. Meanwhile, the definition of apoptosis in the larger human/animal cell death field further evolved over the decades beginning with the identification of pro-apoptotic caspases in the 1990’s (see section 2). Thus, those who were taught that apoptosis is caspase-dependent might assume incorrectly that the prevalent use of the term ‘yeast apoptosis’ in the literature is supported by a body of evidence demonstrating caspase-dependent death. However, this appears unlikely, as fungi do not encode orthologs of mammalian apoptosis mediators (e.g. true caspases or BCL2 family proteins). Thus, it is more likely that fungi die by alternative necrotic processes [52, 89, 90].

Fungi encode metacaspases, which are related to mammalian caspases but differ in substrate specificity and mechanisms of activation [91]. *Saccharomyces cerevisiae* has a single metacaspase Mca1 (renamed Yca1), while *Aspergillus fumigatus* has two, CasA and CasB [92]. While *Saccharomyces* Yca1 has been implicated in cell death under some circumstances [93], *Aspergillus* CasA and CasB appear not to promote death and are even protective [92]. Given that caspases have both death-jobs and day-jobs in mammalian cells (see section 2), *Saccharomyces* metacaspase Mca1/Yca1 may also have a day-job, such as clearing protein aggregates [94]. A few metacaspase substrates have been reported, for example GAPDH, which is also cleaved in mammalian cells by caspases [95, 96]. How these substrates contribute to yeast cell death is unclear. An alternative interpretation is that the enhanced cell survival by *MCA1/YCA1* deletion strains are an indirect or compensatory effect of *MCA1/YCA1* deficiency [52]. Several other yeast homologs of mammalian cell death regulators have been reported to have some involvement in yeast cell death processes, including orthologs of EndoG, AIF, cytochrome *c* and others, but the detailed death mechanisms are not clarified [97–101] (note that some of these references use apoptosis to mean programmed/regulated cell death).

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5. Programmed Cell Death in Growth and Development of Fungi

In our view, the best examples of fungal cell death occurring in natural settings, and for which some of the molecular death machinery is known, are the economically critical plant pathogen *Magnaporthe oryzae* and the model organism *Podospora anserina*.

5.1 Autophagy-dependent cell death in fungal development

Plant pathogen *Magnaporthe oryzae* (also known as *Pyricularia oryzae*) is a filamentous ascomycete fungus that infects the world's most valuable cereal crops, including rice, barley, wheat, millet, oat, and ryegrass [15, 102]. A three-celled spore (conidium) produces an infectious structure, the appressorium, which has sufficient turgor to facilitate entry through the outer cuticle of the plant cell wall to reach the inner plasma membrane and invade [102, 103]. The fungus then develops invasive hyphae that spread within the plant and release new spores through lesions formed in the host [102, 104]. The fungus induces necrotic plant cell death and can spread across fields within days. Estimated annual losses are sufficient to feed millions of people, underscoring the need for quick acting fungicidal compounds [61, 105, 106].

Growing evidence indicates that programmed cell death of *Magnaporthe oryzae* conidia is required for successful invasion of plants, and the mechanism of cell death involves autophagy (Fig 1). Upon adhesion of a conidial spore to a leaf surface, a germ tube emerges from the conidium and its distal end subsequently differentiates into the infective appressorium. To generate the appressorium, the conidium undergoes one round of mitosis allowing one daughter nucleus to migrate through the germ tube to the maturing appressorium. Within hours, the conidium undergoes nuclear collapse, which is accompanied by the formation of autophagosomes and vacuoles [61, 105]. Interestingly, conidia lacking the autophagy factor *Mgatg8* (homolog of mammalian LC3 and hallmark of autophagy), which exhibit phenotypes indicative of defective autophagy as expected, fail to die and also fail to form the appressorium required for infectivity [61]. Thus, conidial cell death appears to be essential for the life cycle of *Magnaporthe oryzae.* Based on sequence homology, other components of the autophagy pathway were identified in *Magnaporthe oryzae*. Remarkably, individual deletion of 16 non-selective macroautophagy genes resulted in continued survival of conidia and failure to form the appressorium or to infect plants. [107]. Thus, autophagy is required for the fungus to develop its appressorium structure needed to traverse the plant cell wall [108, 109]. In contrast, deletion of genes predicted to be involved in pexophagy or mitophagy (forms of selective autophagy) did not affect development of the appressorium or subsequent infection [170].

How is autophagy related to conidial cell death? The differentiation process is stimulated by nutrient deprivation, as conidia incubated in high nutrients do not proceed to form an appressorium, but instead form undifferentiated hyphae and continue to undergo mitosis [108, 109]. Nutrient depletion in fungi and mammals is known to downregulate cell growth by inhibiting the Tor1 kinase complex (TORC1) to derepress autophagy [110]. We recently identified *Saccharomyces cerevisiae* Whi2 as a novel inhibitor of TORC1 activity specifically in low amino acid conditions [111, 112]. Because TORC1 suppresses autophagy, sustained TORC1 activity in yeast lacking Whi2 results in failure to induce

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autophagy [113]. These functions of yeast Whi2 appear to be conserved in the human KCTD proteins, and children with bi-allelic mutations in *KCTD7* have a severe neurodevelopmental disorder, potentially due to defective autophagy required to recycle nutrients and sustain neuronal functional and viability [111, 113]. Conversely in the case of *Magnaporthe* conidia, autophagy genes are critical for conidial cell death, likely because the nutrients derived from conidia are donated to build the appended appressorium, resulting in conidial cell sacrifice. However, it is not known if the Whi2 homolog in *Magnaporthe oryzae* is required for developmental cell death and appressorium formation. If so, Whi2 inhibition is a potential strategy to control *Magnaporthe oryzae*. *Saccharomyces cerevisiae* lacking Whi2 have reduced fitness and are super sensitive to many death stimuli, further supporting this approach [83, 114]. Other pro-survival proteins, such as the conserved BIR1 (baculovirus inhibitor of apoptosis repeat) proteins of human pathogen *Aspergillus fumigatus*, may also be viable therapeutic targets based on a recent study [58]. Mammalian IAP family proteins are also being targeting in pre-clinical trials as therapeutics [115–117], potentially paving the way for fungal inhibitors.

Definitive demonstration that autophagy is a direct versus indirect mediator of *Magnaporthe oryzae* conidial cell death is challenging. There is a distinction between autophagy-dependent and autophagy-mediated cell death. In the latter case, cells die as a direct consequence of overzealous autophagy, while in the former case, autophagy could be indirectly involved, for example by degrading a key pro-survival factor required to sustain conidial cell viability. An analogous situation occurs during *Drosophila* development. The essential *Drosophila* inhibitor of apoptosis (IAP) protein DIAP1 constitutively inhibits the *Drosophila* caspase Dronc [118–121]. Degradation of DIAP1 by the proteasome and possibly by autophagy leads to rapid activation of Dronc and cell death [118, 121, 122]. Therefore, the caspase Dronc, and not the proteasome/autophagosome, is the direct mediator of death. Furthermore, evidence for the existence of autophagic cell death in mammalian cells is limited and has been reclassified as autophagy-dependent cell death [123, 124]. Despite efforts, no alternative death effectors have been identified thus far in *Magnaporthe*, and the death of conidia concomitant with building the appressorium under nutrient deprivation conditions, makes a case for autophagic cell death. In either case, it can surely be agreed that conidial cell death is dependent on autophagy and that conidial cell death constitutes a deliberate developmental form of programmed cell death in the life cycle of *Magnaporthe*.

Magnaporthe oryzae also causes the death of its host plant cell upon egress. Inhibition of a single fungal kinase Pmk1 results in trapping of the fungus in the host cell and blocking its migration to infect a second cell, thereby protecting the plant and rendering the fungus non-infective [104]. Interestingly, pharmacologic inhibition of Pmk1 also blocked appressorium formation. This raises the exciting possibility for targeted inhibition of Pmk1 and potentially other inhibitors of appressorium formation as potential control strategies for agriculture.

5.2 Heterokaryon Incompatibility may resemble mammalian necroptosis

The second example of apparent fungal programmed cell death that occurs in some filamentous fungi is known as vegetative or heterokaryon incompatibility (HI). Hyphal cells

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can fuse to form multinucleated cells termed heterokaryons, a process allowing cytoplasmic exchange and the formation of a syncytial network that facilitates the flow of resources and biochemical signals [125, 126]. Cell fusion and formation of the heterokaryon involves mechanisms to avoid fusion between incompatible non-self cells. The ability to distinguish between self and non-self is determined by genetic differences at *het* loci [125, 127, 128]. Fusion between cells with identical/compatible *het* loci will form stable heterokaryons. In contrast, fusion between cells with distinct *het* loci are incompatible and will trigger a type of programmed cell death characterized by rapid compartmentalization, septal plugging and eventual death of the original two cells [125, 127, 129, 130]. The compartmentalization and septal plugging during HI has been reported to restrict horizontal transfer of mycoviruses, suggesting one possible benefit for the evolution of programmed cell death mechanisms [131–133].

Work done particularly in *Podospora anserine, Neurospora crassa* and *Cryphonectria parasitica* (chestnut blight) [125, 126, 130, 134–139] has uncovered some of the mechanisms of cell death due to incompatibility [140]. These fungi encode multiple HI systems that have been identified molecularly or genetically [125]. A prominent morphological feature of HI cell death is vacuolization, raising the possibility that vacuolar leakage or rupture contributes to cell death. This would be akin to cell death by lysosomal membrane permeability (LMP) in mammals, such as occurs during mammary gland involution [141], and has been implicated in fungal cell death in different scenarios and species [84, 97, 137]. However, biochemical explanations for the morphological changes during HI death are uncertain, but appear not to require metacaspases [134]. Transcriptional profiling implicates increased reactive oxygen species production along with calcium and phosphatidylinositol signaling pathways [134]. Autophagy is also induced during HI [139], but is not required for cell death [138]. In *N. crassa*, transcription factors CZT-1 [135] and VIB1 [136] are required for activation of the HI cell death program, and the *P. anserina* transcription factor *idi-4,* has been implicated in cell death induction [140]. However, the link between these transcription factors and the cell death effector proteins directly responsible for cytological-morphological features of HI are only partially delineated, predominantly in *P. anserina*, but are potential drug targets in pathogenic strains.

One candidate mechanism of cell death during HI in *Podospora anserina* may share features reminiscent of mammalian necroptosis (Fig 1) (see section 2). Different subpopulations of *P. anserina* can be distinguished by two allelic variants at the *het-s* locus, encoding two highly similar proteins HET-S and HET-s. Cell-cell fusion of populations differing at this locus results in cell death due to HI [142, 143]. The *het-s* allelic variant encodes a soluble cytoplasmic protein that can spontaneously fold into an amyloid-like prion state via a C-terminal domain [142, 144, 145]. Despite being 96% identical to *het-s*, the *het-S* allelic variant product does not spontaneously adopt the prion-like state [143, 146]. However, HET-S proteins can be induced to form prion-like structures upon physical interaction with the prion-like state of HET-s as a consequence of heterokaryon formation. This leads to adaptation of HET-S to a prion-like fold and subsequent partial unfolding of the HET-S N-terminal HeLo protein domain [143, 145, 147]. This HeLo domain unfolding leads to conversion of HET-S into an integral membrane protein that appears to oligomerize into a pore-forming complex mediating cell death during HI [147] (Fig 2). Interestingly, the HeLo

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domain has predicted structural similarity to the N-terminal domain of the mammalian pseudokinase MLKL (e.g. Phyre2). Mammalian MLKL is thought to oligomerize and form pores from the cytosolic side of the plasma membrane resulting in cell lysis, and thus acts as the effector protein of necroptosis through its pore-forming properties [62, 148–150] (Fig 1). HeLo-like domains termed HELLP present in other fungal species are suggested to have some sequence similarity to the pore-forming domain of MLKL, even though true homology may not be present [62]. Pore-forming proteins in fungal programmed cell death represents a potentially conserved mechanism across many fungal species and regulation of such may serve as a platform for the development of antifungal drugs.

In addition to pore formation, the amyloid-prion assembly phenomenon to induce fungal cell death may have additional conceptual counterparts in the fungal kingdom (Fig 1). Like other types of intracellular signaling platforms, cell death pathways in mammals, including apoptosis, have an amplification step that is mediated by large dedicated protein complexes (apoptosome, inflammasome, necrosome) that recruit and activate death effectors such as caspases or other. To build these large death-osomes, their protein components undergo oligomerization. The mammalian necrosome turns out to be an amyloid with a function. Unstructured regions of necrosome components RIPK1 and RIPK3 preferentially assemble into a stable hetero-amyloid that is required for necroptotic cell death [150, 151]. The prion protein HET-s of filamentous fungi also folds into an amyloid fibril to promote fungal cell death by heterokaryon incompatibility [152, 153]. Recently, an new HI mechanism in *Neurospora crassa* was reported that involves a compatibility locus encoding two interacting proteins, the SNARE protein SEC-9 and a NOD-like receptor (NLR) protein PLP-1 with features similar to NLRs encoded by plants, mammals and other fungi [154]. Fusion of germinated asexual spores with incompatible SEC-9– PLP-1 proteins induces PLP-1 oligomerization and cell death. In this way, fungal incompatibility mechanisms resemble mammalian innate responses where NLRs recognize foreign molecular signatures and pathogens leading to NLR oligomerization to signal cell death by pyroptosis [155, 156].

The mammalian pyroptosis pathway adaptor protein ASC can assemble into filaments through the cooperative interactions of its PYD domain, which binds to the PYD domain of NOD-like inflammasome receptor protein (NLRP3) to facilitate caspase 1 recruitment and activation [153]. One study found that swapping the PYD domains of both NLRP3 and its adaptor ASC with the corresponding region of a NOD-like fungal protein and the prion domain of fungal HET-s, respectively, could induce inflammasome assembly [157]. The yeast prion protein SUP35 found in *Saccharomyces cerevisiae* and other fungi could also functionally replace a region of mammalian inflammatory response protein MAVS [157]. Although many details remain unaddressed, it is possible that prion-like amplifications are a cell death theme across species. This concept has been capitalized by a number of investigators using yeast *Saccharomyces cerevisiae* to study mechanisms of neuronal toxicity induced by human disease-associated amyloids, made popular by Susan Lindquist [158].

Ferroptosis is an emerging and exciting new research area in part because of its relevance to disease states involving neuronal or renal damage [50]. Ferroptosis is thought to result from loss of cell integrity due to lipid peroxidation that damages cell membranes, but the exact

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molecular effectors and mediators of mammalian cell ferroptosis are just beginning to be delineated. The working definition of ferroptosis is cell death inhibited by iron chelators [48], a phenomenon observed to occur in diverse cell types including fungi [159] (Fig 1).

6. Conclusions

Fungi represent a significant public health burden and require a reinforcement of current therapeutics. One attractive therapeutics approach is pharmacological manipulation of intrinsic cell death pathways to induce the death of fungi. Targeting intrinsic fungal cell death programs may allow for the development of fast acting and pathogen-specific anti-fungal agents. The existence for programmed cell death mechanisms in multi- and unicellular fungal species is currently challenged for the lack of molecularly defined death mechanisms equivalent to what is available for animals. However, death programs can be reasonably assumed to exist based on observations of fungal cell death that occurs in normal development, for example during host cell invasion by the economically important plant pathogen *Magnaporthe oryzae*, as well as in germlings and mycelial growth of several filamentous fungi that undergo incompatibility loci-induced cell death. Furthermore, new evidence reveals the complex order within colonies of unicellular fungi that potentially require cell death as a mechanism to maintain long-term population dynamics and pathogen evasion. However, with these exceptions, little is known about fungal cell death effectors in either multi- or unicellular species and further work is needed to address the gaps in knowledge about cell death modalities across the diverse kingdom of fungi. New nomenclature may assist in addressing the nuances of fungal cell death that differ from mammalian pathways and to avoid confusion when applying mammalian assays to fungi [53]. With stringency in the assays applied to fungi, the field will move to uncover the effectors of cell death in fungi. Delineating these pathways can lead to a better understanding of novel targets for controlling pathogenic fungi.

New therapeutic approaches are justified as there are only four major groups of antifungal drugs currently available, azoles (e.g. fluconazole affecting ergosterol biosynthesis), polyenes (e.g. amphotericin B and nystatin affecting membrane ergosterol), flucytosine (anti-metabolite) and echinocandins (interfere with cell wall biosynthesis), which have been used for decades, have off target effects and have contributed to resistant infections [160]. Pan-resistant fungi such as the transmissible human pathogen *Candida auris* and *Lomentospora prolificans* are emerging currently [161–163]. Fungal infections can be difficult to clear. A small portion of fungal cell populations are not killed by anti-fungals either because they are drug-resistant (e.g. enhanced efflux mechanisms) or because they are persisters in a metabolic state that renders them drug insensitive. Persisters are not drug-resistant if they reenter the growing state, but can be highly drug insensitive in their more dormant persister state [164]. Thus, persisters are insensitive to drugs directed only to active metabolic pathways. Approaches that target viability per se may be more effective against persisters, which likely contribute to clinically reticent infections. Counter intuitively, slow growing fungal persister cells with decreased ergosterol or cell wall synthesis can be more tolerant to metabolic drug pressures [164]. Persistence of *Candida albicans* and *Candida glabrata* has been attributed to defects in ribosome synthesis and mutations in the target of rapamycin (TOR) pathway that promotes cell growth and protein translation [160].

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Development of combination anti-fungal therapies are needed to effectively kill both growing and persistent fungal pathogens, analogous to anti-bacterials for tuberculosis [163]. The 9–12 month treatment for *Mycobacteria tuberculosis* was reduced to 6 months by including pyrazinamide, which kills bacterial persisters, in combination with rifampin and isoniazid, which kill growing mycobacteria [165].

An important next step is to identify and characterize the key molecular players in microbial cell death. Similar to the current situation for fungi and other microbes, the early mammalian/animal cell death field was viewed with skepticism until the molecular effectors of animal cell death were identified, making the existence of programmed cell death irrefutable. Thus, the current fungal cell death field is in a similar position as the mammalian and animal cell death field about 30 years ago, but faster progress can be expected since the paths to the major concepts and potentially to some of the molecular similarities have been paved by the animal cell death field.

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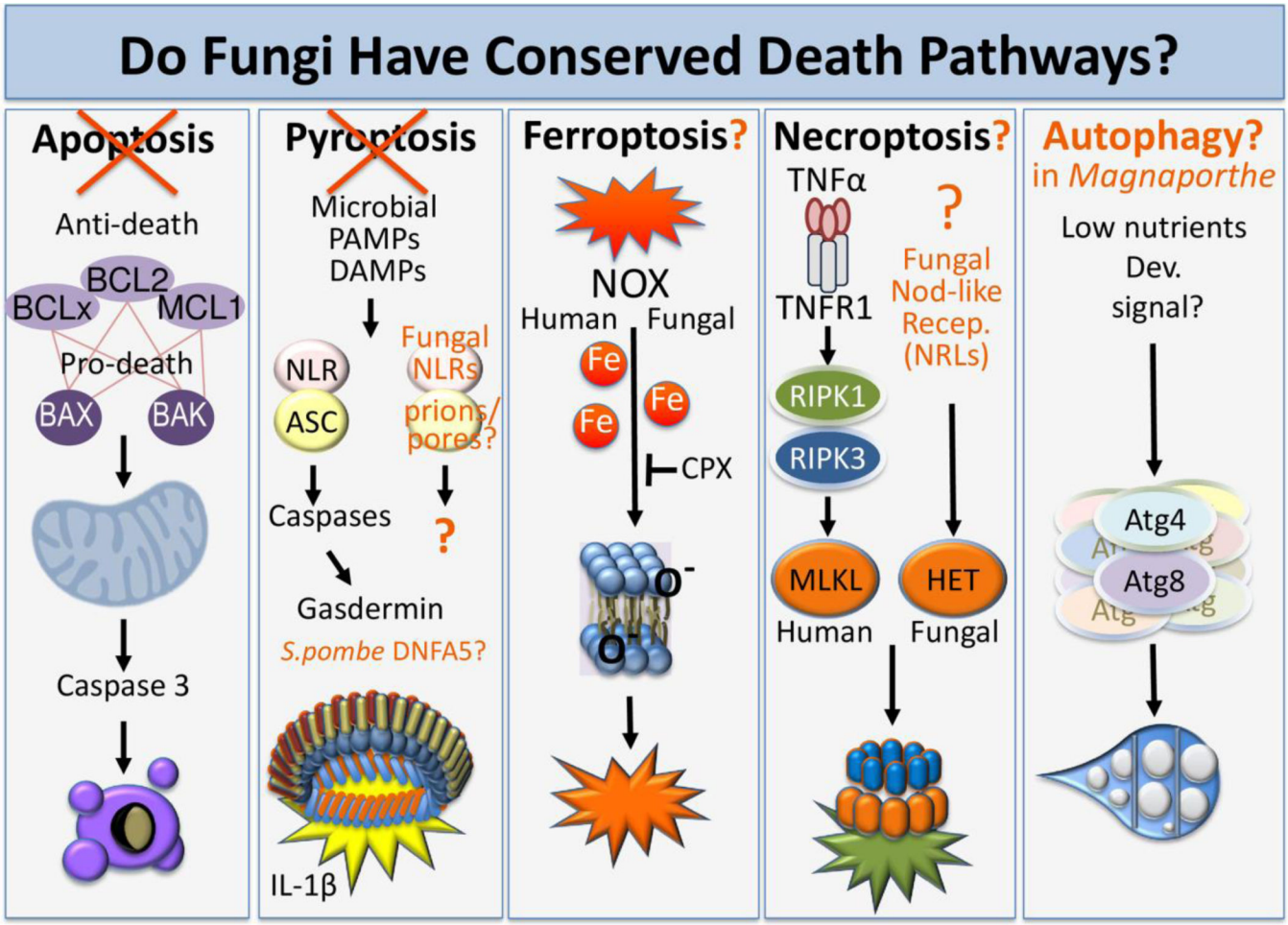

Figure 1. Are there conserved molecular death pathways in mammals and fungi?

Features of the best characterized mammalian cell death pathways and potentially analogous mechanisms present in fungal species. Fungi lack the mammalian apoptosis pathway in which caspase 3 activation is regulated by BCL2 family proteins, and also lack the caspases 1, 4, 5 and 11, and pore-forming gasdermins (unlike related fungal proteins) that mediate programmed necrosis by pyroptosis, although fungal NLR-like receptors can trigger cell death upon cell-cell fusion of highly related but incompatible fungal cells. Iron-dependent cell death via ferroptosis due to lipid peroxidation may be generalizable across a wide range of species. The fungal pore-forming domain of HET-S thought to mediate incompatibility cell death has predicted structural similarity to the mammalian pore-forming domain of MLKL, mediator of necroptosis.

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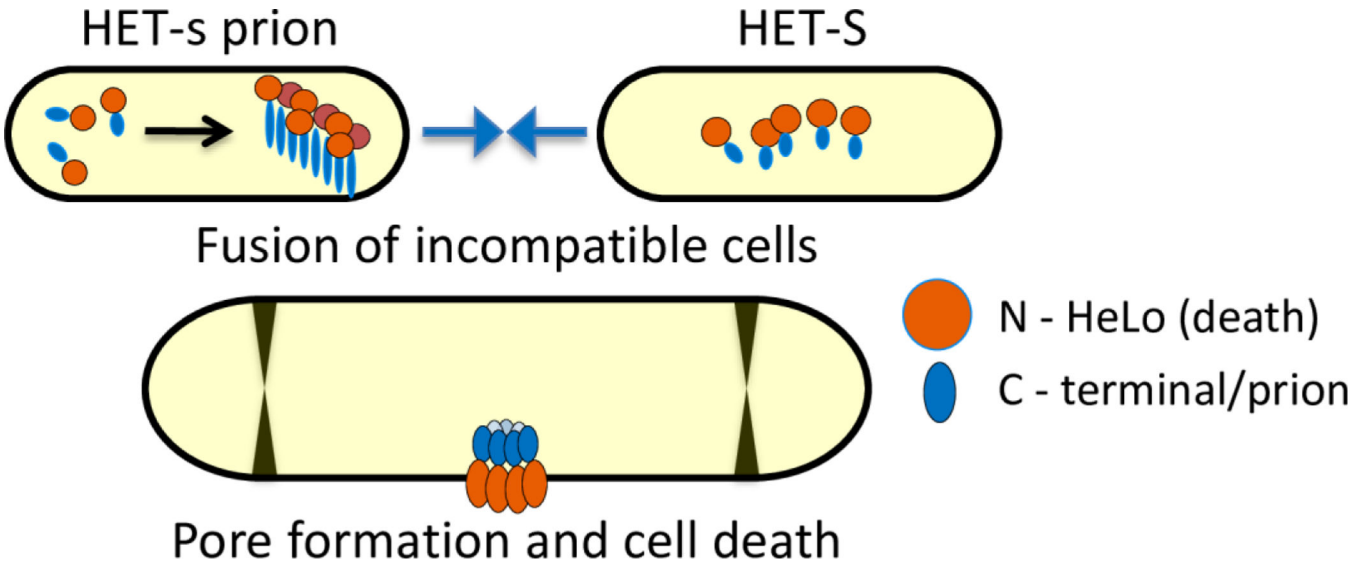

Figure 2. Fungal cell death regulated by HET proteins during heterokaryon incompatibility.

The C-terminal prion-like domain is thought to trigger conformational changes and membrane association by the N-terminal HeLo domain to induce pore formation following cell-cell fusion between incompatible fungal cells with non-self *het* loci.