Review

The Great Debate: Regulated Cell Death in Fungi and the Role of Metacaspases

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Review

The Great Debate: Regulated Cell Death in Fungi and the Role of Metacaspases

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Abstract

Caspases orchestrate metazoan apoptosis, regulating processes such as embryogenesis, the death of old and infected cells and immune tolerance. Structural orthologs of caspases have been identified in bacteria, plants, protists and fungi and regulated cell death has been demonstrated in these organisms. This led some researchers to conclude that fungal metacaspases might perform a similar function to caspases. This review discusses regulated cell death, beginning with an account of RCD and the central role of caspases in mammalian RCD. It goes on to give examples of RCD in fungi, compares the structure and activity of caspase orthologs and outlines examples of metacaspase-dependent and metacaspase-independent cell death in fungi, focusing on *S. cerevisiae*. Finally, it addresses the question "are metacaspases caspases?", identifies alternative cell death proteases and recommends future research objectives.

Keywords: apoptosis; PCD; RCD; caspase; metacaspase; fungi

1. Introduction

Apoptosis, a form of regulated cell death (RCD), is vital for mammalian development, positive and negative selection of immune cells and turnover of old and worn-out cells [1–15]. Apoptosis of superfluous cells sculpts precise shapes, such as fingers and toes, organs and tissues. B- and T-lymphocytes that do not produce operational antigen receptors are removed via apoptosis. Weak interactions of T-cells with the major histocompatibility complex and strong reactions to self-antigens trigger apoptosis. B-cells are also subject to negative selection. Exactly 131 *Caenorhabditis elegans* somatic cells undergo apoptosis during development. Balanced mitosis and apoptosis maintain cell numbers and dysfunctional apoptosis can lead to cancer or inflammation.

The discovery of structural caspase orthologs [16] and apoptosis-like cell death in fungi, protists, plants etc. suggested that apoptosis is conserved throughout the tree of life [17–21]. Caspase orthologs were also identified in cyanobacteria, brown algae, proteobacteria, archaea, actinobacteria and viruses [22–27]. RCD may be apoptotic, autophagic or necrotic (reviewed by [28]).

This review describes mammalian apoptosis, regulation by caspases and techniques for detecting RCD characteristics. It also discusses RCD and caspase orthologs in other organisms, focusing mainly on fungi. Methods for detecting fungal RCD are considered, as is the great debate: are metacaspases caspases?

2. Characterization of Mammalian Apoptosis

2.1. Physiological Changes During Apoptosis

During apoptosis, mammalian cells (Figure 1) undergo blebbing of the plasma membrane (PM), flipping of phosphatidylserine (PS) to the outer PM layer, reduced mitochondrial membrane potential, cell shrinkage, chromatin condensation, DNA fragmentation and disintegration of the cell into apoptotic bodies [29–32] that are cleared by phagocytes so that cell contents do not damage

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surrounding tissues or trigger inflammatory responses [33–35]. Apoptosis is ordered cell death, regulated by genetically encoded proteins and conserved pathways, and necrosis (except programmed necrosis) is accidental cell death [36,37]. Necrosis involves swelling of cells and organelles, PM disruption and no DNA fragmentation.

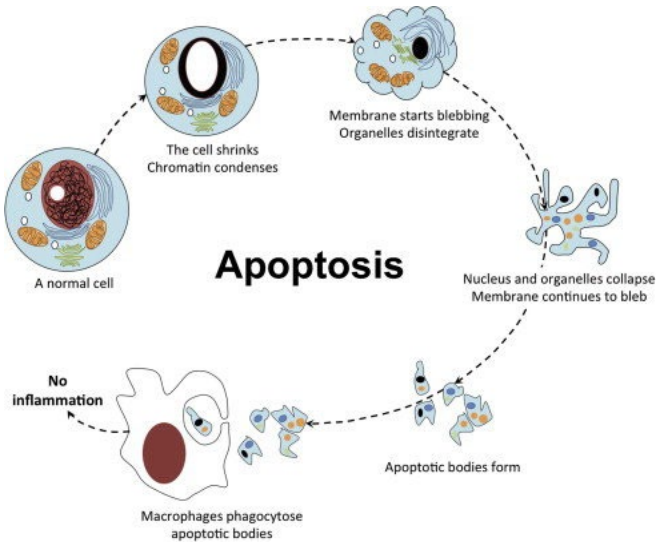

Figure 1. Apoptosis. Cells shrink, chromatin condenses, the PM blebs, organelles break down, and apoptotic bodies form that are removed by phagocytes. Reproduced from [32]. © 2015 Abou-Ghali and Stiban. CC BY-NC-ND License (http://creativecommons.org/licenses/by-nc-nd/4.0/).

2.2. Detecting Apoptosis

Cell shrinkage, membrane blebbing, chromatin condensation and the formation of apoptotic bodies may be observed using light or electron microscopy and chromatin condensation by fluorescence microscopy and DNA staining with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI) or Hoechst stain [38–42]. Prior staining with eosin-methylene blue helps to distinguish the cytoplasm and nucleus and DNA fragmentation may be studied using flow cytometry or gel electrophoresis. Flipping of PS to the outer PM leaflet is visualized using annexin V, which binds strongly to PS. DNA strand breaks expose 3′ end hydroxyl groups that can be visualized via dUTP Nick End Labeling (TUNEL) staining [43]. Terminal deoxynucleotidyl transferase (TdT) attaches 5-bromo-2'-deoxyuridine 5'-triphosphate (Br-dUTP) to 3′ end hydroxyl groups. The labelled antibody Ab-FITC binds to Br-dU (Figure 2) and the fluorescein isothiocyanate (FITC) detected using fluorescent microscopy.

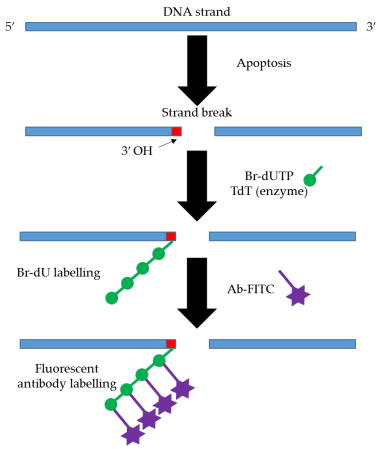

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Figure 2. TUNEL staining. During apoptosis, DNA strand breaks expose 3′ end hydroxyl (-OH) groups. TdT labels 3′ -OH with Br-dUTP and Ab-FITC binds to Br-dU chains. TdT — terminal deoxynucleotidyl transferase; Br-dUTP — *5-Bromo-2′-Deoxyuridine 5′-Triphosphate; Ab-FITC — anti-Br-dU antibody bound to* fluorescein isothiocyanate. Adapted from [43].

2.3. Mammalian Caspases

Apoptosis is orchestrated by caspases, i.e. **C**ysteine-dependent, **ASP**artyl prote**ASE**s, which cleave proteins after aspartate and have a conserved cysteine/histidine catalytic dyad [44,45]. Proteolysis is activated via cleavage and association of the large and small subunits. Some caspases regulate inflammation and others regulate apoptosis. The latter include initiator and executioner caspases (Figure 3). Initiator caspases are activated by intrinsic or extrinsic signaling and activate executioner caspases, which cleave downstream proteins, leading to apoptosis. Caspase-8 associates with adapter proteins (Figure 4), bringing two proteins closer and activating proteolysis [46]. Two large subunits and two small subunits form the active caspase-8 heterotetramer. Caspase-3 forms dimers with one monomer inverted with respect to the other. Activated initiator caspases activate caspase-3 via cleavage between the large and small subunits.

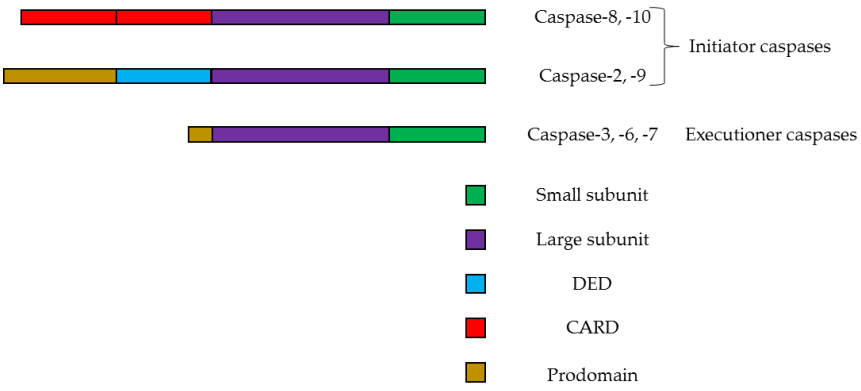

Figure 3. Human initiator and executioner caspases. Caspases-2, -8, -9 and -10 are initiator caspases while caspases-3, -6 and -7 are executioner caspases. Caspases-8 and-10 include caspase recruitment domains (CARDs) and caspases-2 and -9 include death effector domains (DED) which mediate protein interaction. Adapted from [44].

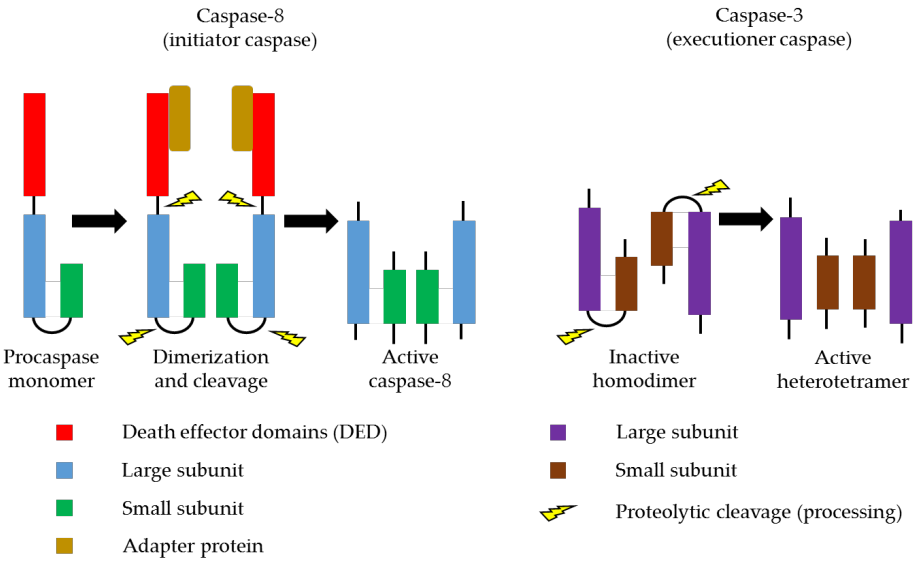

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Figure 4. Activation of initiator and executioner caspases. Procaspase-8 is cleaved between the large and small subunit and between the large subunit and pro-domain, forming active caspase-8. Caspase-3 forms dimers and cleavage between the large and small subunits forms active caspases. Adapted from [46].

2.4. Apoptosis Pathways

Apoptosis is triggered via different pathways [15,47]. The intrinsic (mitochondrial) pathway occurs in response to e.g. lack of oxygen (Figure 5) via altered mitochondrial physiology and cytochrome c release. Cytochrome c, caspase-9 and Apaf-1, form the apoptosome and activate caspase-9. Extracellular signaling activates the extrinsic (death receptor) pathway via binding of ligands (e.g. tumor necrosis factor alpha — TNFα) to surface receptors. Adapter proteins and caspase-8, form the death-inducing signaling complex (DISC) and activate caspase-8. The perforin/granzyme pathway involves permeabilization of the PM by perforin, secreted by cytotoxic T cells. This activates caspase-independent apoptosis via granzyme A, DNA cleavage by the SET complex and granzyme B activation, triggering caspase-3activation, directly or via caspase-10.

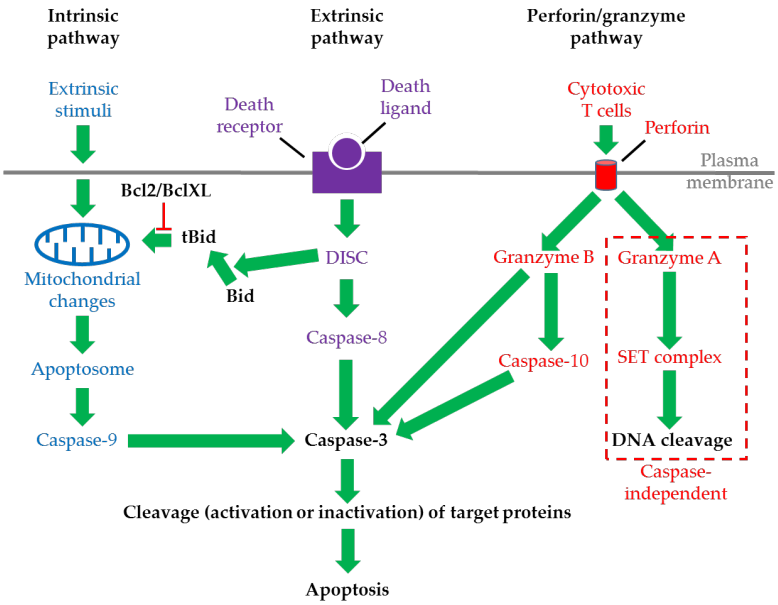

Figure 5. Intrinsic, extrinsic and perforin/granzyme pathways. Intrinsic and extrinsic apoptosis and one branch of the perforin/granzyme pathway converge on caspase-3, which cleaves target proteins, activating some and inactivating others, triggering apoptosis. Adapted from [15,47].

2.5. Extrinsic Apoptosis and the DISC

Death ligands induce apoptosis by attaching to death receptors [47–49]. For example, tumor necrosis factor-α (TNF-α) binds to tumor necrosis factor receptor 1 (TNFR1) Decoy receptor 3 (DcR3) competitively binds FasL and TL1A. When FasL binds to Fas (Figure 6), the receptors cluster, promoting interaction with the Fas-associated protein with death domain (FADD). The latter recruits procaspase-8 via death effector domains (DED), forming the Death-induced signaling complex (DISC) (Figure 6). This promotes cleavage of the procaspase and formation of a heterotetramer (active caspase-8), which activates caspase-3. An inhibitor of caspase-8, c-FLIP (cellular FLICE inhibitory protein) competes with caspase-8 for binding to the DED of FADD. When the ratio of caspase-8 to c-FLIP is high, caspase-8 binds FADD and is cleaved and activated. However, when the ratio is low, there are two possible mechanisms of inhibition. The long isoform, c-FLIP$_L$ stalls caspase processing after formation of the p43 product and before further processing to the p18 and p10 fragments. The short isoform, c-FLIP$_S$ blocks all processing of procaspase-8, though the zymogen binds to FADD.

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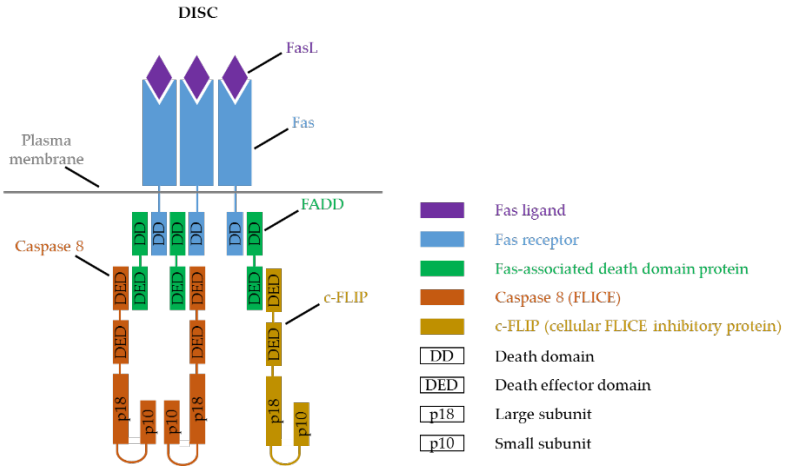

Figure 6. Death-inducing signaling complex. The DISC mediates extrinsic apoptosis and is formed when death ligands bind to death receptors at the cell surface, causing the death receptors to cluster together. Death receptors associate with the adapter protein FADD via their death domains and FADD with caspase-8 (or caspase-10) via death effector domains. Clustering of death receptors brings two caspase proteins close together and they form an active heterotetramer. cFLIP competes with caspase-8 for binding to FADD. Adapted from [47].

2.6. Intrinsic Apoptosis and the Apoptosome

Stimuli, such as changes in metabolism or the cell cycle, DNA damage, growth factor withdrawal, some drugs, lack of oxygen, heat shock, radiations and viruses activate the intrinsic apoptosis pathway (Figure 7) by inducing changes in mitochondria that lead to mitochondrial outer membrane permeability (MOMP) and the release of cytochrome c and s*econd mitochondria-derived activator of caspases* (SMAC) from the mitochondria [50].

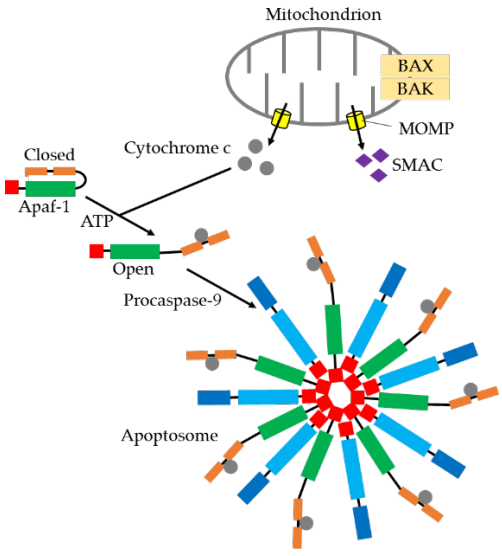

Figure 7. Apoptosome. Once pro-apoptotic factors trigger the permeabilization of the mitochondrial membrane, cytochrome c is released from the mitochondria, and in the presence of ATP, associates with Apaf-1 and procaspase-9 to form the apoptosome, which promotes apoptosis via proximity-dependent activation of caspase-9. Orange — WD40 motif; dark blue — small subunit; light blue — large subunit; red — caspase recruitment domain (CARD); MOMP — mitochondrial outer membrane permeabilization; SMAC — second mitochondria-derived activator of caspases; Apaf-1 — apoptotic protease-activating factor 1. Adapted from [51].

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3. Regulated Cell Death in Fungi

3.1. Why should Fungi Undergo Regulated Cell Death?

It seems strange that programmed cell death should have evolved in single-celled organisms since loss of the whole organisms would prevent the inheritance of pro-cell death genes to offspring unless the gains were indirect, benefitting closely related individuals [52]. Apoptosis-like cell death was detected in the slime mold, *D. discoideum* and parasitic *Trypanosoma* and *Leishmania* species and it was suggested that RCD of some *D. discoideum* cells to produce the stalk of the fruiting body enhances the dispersal of closely related spores while apoptosis-like RCD among parasitic protists is a way of avoiding a pro-inflammatory reaction that would threaten the survival (and therefore reproduction) of closely-related individuals in the host body (reviewed by [53]. It has been suggested that the RCD machinery was acquired by the ancestors of modern eukaryotes from the endosymbiotic proteobacterial and cyanobacterial ancestors of modern mitochondria and chloroplasts respectively and that modern RCD programs and complex apoptotic machinery arose from an arms race between eukaryote and endosymbiont. The two participants had developed an "addictive" relationship and targeting the endosymbionts/mitochondria with pore-forming proteins became a means of self-destruction (apoptosis). The endosymbiont may have evolved the means to kill the host cell when conditions deteriorated (e.g. Bax/Bak), and the host evolved ways to inhibit this destruction (e.g. Bcl-2/Bcl-X_L). There is also some evidence to suggest that most cell death proteins also have cell survival roles and did not evolve specifically for the purpose of mediating apoptosis. Büttner et al. [54] suggested that there are several good reasons why a single celled organism should evolve RCD, including the ability to kill unrelated individuals and thus benefit kin, the ability to remove sexually incompatible individuals from the population and the ability of older, less fit cells to undergo cell death, sparing environmental nutrients, and releasing their own cell contents, to help power growth of fitter kin.

3.2. Early Examples of RCD in Fungi

Unbalanced Growth

In the mid-20th century it was shown in several fungal species, including *Ophiostoma multiannulatum, Neurospora crassa* and *Aspergillus nidulans*, (Table 1) that when some mutant strains were deprived of a particular nutrient, they underwent regulated cell death (RCD) due to an imbalance in metabolism, while others did not [55–58]. Deleting another gene, which affected a different branch of metabolism, appeared to restore balance and rescue the mutant strain from RCD.

Table 1. Identification of RCD in fungi.

Species	RCD trigger	References
Ophiostoma multiannulatum	Unbalanced growth	[55]
Neurospora crassa	Unbalanced growth	[56,57]
Aspergillus nidulans	Unbalanced growth	[58]
Podospora anserina	Heterokaryon incompatibility	[59–61]
Cochliobolus heterostrophus	Heterokaryon incompatibility	[62–65]
Cryphonectria parasitica	Heterokaryon incompatibility	[66]
Neurospora crassa	Heterokaryon incompatibility	[67–71]
Podospora anserina	Ascospore abortion	[72–74]
Neurospora spp.	Ascospore abortion	[74–80]
Schizosaccharomyces pombe	Ascospore abortion	[74,81,82]

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Venturia inaequalis	Ascospore abortion	[83]
Fusarium verticillioides	Ascospore abortion	[84–87]
Bipolaris maydis	Ascospore abortion	[74,88,89]
Coniochaeta tetraspora	Ascospore abortion	[90]
Coprinopsis spp.	Fruiting body development	[91,92]
Agaricus bisporus	Fruiting body development	[93–95]
Psilocybe spp., *Panaeolus* spp, Stropharia rugosoannulata, Coprinellus domesticus, Candolleomyces candolleanus, Tremella mesenterica, Otidea onotica, Peziza ostracoderma	Fruiting body development	[94]
Saccharomyces cerevisiae	Yeast killer toxin	[96–100]
Saccharomyces cerevisiae	Sugar	[101–106]
Saccharomyces cerevisiae	$cdc48^{S565G}$ mutant	[107]
Saccharomyces cerevisiae	Bax expression in yeast	[17,108–111]
Saccharomyces cerevisiae	Oxygen stress	[112]
Saccharomyces cerevisiae	Acetic acid	[113–117]
Saccharomyces cerevisiae	Plant defense compound osmotin	[118]
Saccharomyces cerevisiae	Aging	[119–122]
Saccharomyces cerevisiae	Pheromone	[19]
Saccharomyces cerevisiae	Sodium chloride	[123]
Saccharomyces cerevisiae	Defects in mRNA decapping	[124]
Saccharomyces cerevisiae	Aspirin	[125]
Saccharomyces cerevisiae	Hypochlorous acid (HOCl)	[126]
Saccharomyces cerevisiae	Defects in DNA replication initiation	[127]
Saccharomyces cerevisiae	Hyperosmotic stress	[128]
Saccharomyces cerevisiae	Reduced sister chromatid cohesion	[129]
Saccharomyces cerevisiae	N-glycosylation defect-induced ER stress	[130]
Saccharomyces cerevisiae	Copper or manganese	[131]
Saccharomyces cerevisiae	Formic acid	[132]
Saccharomyces cerevisiae	Palmitate-induced ER stress	[133]
Saccharomyces cerevisiae	Accumulation of Ras2p in mitochondria due to WHI2, HXK2 or SNF1 deletion	[134–136]
Saccharomyces cerevisiae	Lack of potassium	[137]
Saccharomyces cerevisiae	Lack of H2B K123 ubiquitination	[138]
Saccharomyces cerevisiae	Lead	[139]
Saccharomyces cerevisiae	Gefitinib (EGFR inhibitor)	[140]
Saccharomyces cerevisiae	Cisplatin	[141]
Saccharomyces cerevisiae	Heat shock (45 °C)	[142]
Saccharomyces cerevisiae	Anacardic acid	[143]
Saccharomyces cerevisiae	Cold plasma	[144,145]
Saccharomyces cerevisiae	Nickel oxide nanoparticles	[146]
Saccharomyces cerevisiae	Citral and geraniol	[147]
Saccharomyces cerevisiae	Expression of caspase-1 in yeast	[148]

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Saccharomyces cerevisiae	Mito/autophagy defects due to deletion of PIL1	[149]
Saccharomyces cerevisiae	Synthetic antimicrobial peptides	[150]
Saccharomyces cerevisiae	Nano plastic-induced oxidative stress	[151]
Saccharomyces cerevisiae	Silver nanoparticles	[152]
Saccharomyces cerevisiae	Cohesin dysfunction	[153]
Saccharomyces cerevisiae	Deletion of AP-3 components or downstream kinase	[154]
Saccharomyces cerevisiae	Enhanced mitochondrial DNA damage due to HAP4 deletion	[155]
Schizosaccharomyces pombe	Bax/Bak expression	[156–158]
Schizosaccharomyces pombe	Deficiency in diacylglycerols	[159]
Schizosaccharomyces pombe	Replication stress	[160]
Schizosaccharomyces pombe	Entry of DAG-deficient mutants into stationary phase	[161]
Schizosaccharomyces pombe	Inositol deprivation-induced ER stress	[162]
Schizosaccharomyces pombe	Chronological aging	Reviewed by [161]
Candida albicans	Hydrogen peroxide, acetic acid and amphotericin B	[163,164]
Candida albicans	Caspofungin	[165]
Candida albicans biofilm	Amphotericin B	[166]
Candida albicans	Farnesol	[167]
Candida albicans	Aureobasidin A	[168]
Aspergillus fumigatus	Stationary phase	[169]
Aspergillus nidulans	Sporulation	[170,171]
Aspergillus nidulans Aspergillus fumigatus	Farnesol	[167,172]
Aspergillus flavus	Essential oils	[173]
Aspergillus fumigatus	UPR/antifungal drugs	[174]
Aspergillus flavus	Perillaldehyde	[175]
Aspergillus flavus	Hexanal	[176]
Cryptococcus neoformans	Radiation	[177]
Histoplasma capsulatum	Radiation	[177]
Colletotrichum trifolii	Ras mutant under starvation UV light Hydrogen peroxide Heat shock Sodium chloride	[178]
Colletotrichum gloeosporioides	Bcl-2	[179]
Fusarium oxysporum, Colletotrichum graminicola.	Killer toxin	[100,180]

Heterokaryon Incompatibility

In 1952 Professor George Rizet reported that crossing strains of *Podospora anserina* (one with an S allele, the other with an s allele) yielded S and S^s but no s offspring, that the S^s individuals were compatible with either parent and that the S^s strain sometimes reverted to the s genotype, which then

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grew to dominate the culture [59]. We now know this phenomenon as heterokaryon incompatibility (HI — a form of non-self-recognition), which is governed by the *het-S*/*het-s* locus [60,61]. When incompatible mycelia fuse, they form a heterokaryon and the resulting fusion cell and surrounding cells die via vacuolar lysis. The *het-S*/*het-s* gene product exists in three forms — HET-S, HET-s and HET-s* and the HET-s form is a prion-forming protein while the HET-s* form is soluble. Crossing the *het-s** strain with *het-s* produces offspring, harboring the HET-s prion (stacks of blue squares, Figure 8A). The *het-s** and *het-S* strains are compatible and produce healthy offspring (Figure 8B). However, when *het-s* is crossed with *het-S*, the prion alters the conformation of the HET-S protein, exposing a transmembrane domain. HET-S forms pores in the membrane, triggering cell death (Figure 8C). HI has also been demonstrated in *Cochliobolus heterostrophus* [62–65], *Cryphonectria parasitica [66]*, *Neurospora* spp. [67–70]. HI-induced cell death involves DNA damage, shrinking of the cytoplasm and the formation of apoptotic bodies, which are hallmarks of apoptosis [71]. Numerous vacuoles appear, which may be autophagosomes — typical markers of autophagy and autophagic cell death. Some morphological changes (e.g. chromatin condensation and nuclear fragmentation) have been observed during both apoptotic and autophagic cell death.

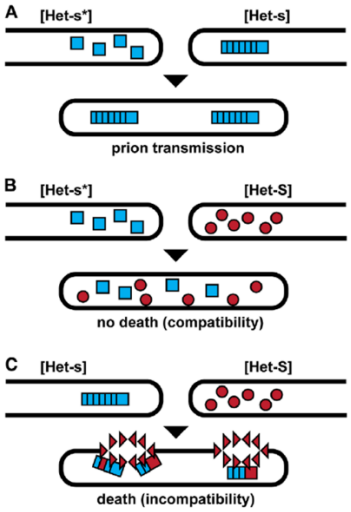

Figure 8. Heterokaryon incompatibility in *P. anserina*. Two mycelia fuse to form a heterokaryon but a cross between incompatible mycelia results in death of the heterokaryon and surrounding cells. Het-s*: strain produces soluble s protein (separate blue squares); Het-s: strain produces s prion (stacked blue squares); Het-S: produces S protein (red circles). Het-s prions proliferate in offspring of Het-s* x Het-s cross (A). Het-s* x Het-S: no prion involved so compatible (B). Cross of incompatible Het-s and Het-S: Het-s prions alter conformation of Het-S protein (red squares), exposing transmembrane domain and leading to pore formation (triangles) and death of heterokaryon (C). Reproduced from [61]. Creative Commons Attribution License.

Ascospore Abortion

Ascospore abortion has been documented in many different species, including *Podospora anserina* [72–74]. In *P. anserina* and *P. comata* ascospore abortion is driven by the interaction of two genes: *het-S* and *het-s*, the latter of which encodes a protein product that can form a prion, triggering the death of *het-S* spore. *Neurospora* spp. undergo 2 rounds of meiosis, followed by a round of mitosis to form eight spores and, in a cross between spore-killer (*Sk*) and *Sk*-sensitive cells, half of the spores die [74–80]. Once the spore nuclei divide, sensitive cells lose their typical vacuolated appearance, the cytoplasm becomes disordered and the nuclei begin to break down [76]. Possession of *rsk* (resistance to spore killer) genes determines whether a spore will survive but *rsk* genes are specific to one or more *Sk* genes[80]. These two early observations of fungal regulated cell death (RCD) are examples of meiotic drive elements, which are overrepresented among offspring of a mating, at the expense of other alleles (Table 1). *N. crassa* possesses two different killer genes, *Sk-2*^{K} and *Sk-3*^{K} and both the killer genes and resistance genes are only common in a few sites in Indonesia and Papua New Guinea. *N.*

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tetrasperma normally forms 4 ascospores. When the two *N. crassa* killer genes were transferred to *N. tetrasperma*, the *Sk-2*^{K} x *Sk-3*^{K} cross produced 4 heterokaryotic spores. However, when the 8-spore gene, *E* was also transferred to *N. tetrasperma*, the *Sk-2*^{K} x *Sk-3*^{K} cross yielded 4 live spores and 4 dead ones. During the second meiotic division, the nuclei from a- and α-mating type parents migrate to opposite ends of the ascus in *N. crassa* and each of the 8 nuclei contains either a Mat-A or a Mat-a mating type nucleus (Figure 9). On the other hand, in *N. tetrasperma*, Mat-A- and Mat-a nuclei divide, parallel to one-another and only 4 spores are formed, so each spore contains one Mat-A and one Mat-a nucleus. This strategy of producing four spores with one nucleus from each parent is termed pseudohomothallism and occurs in *Gelasinospora tetraspora* and *Agaricus bisporus*. Sometimes, one of the expected Mat-A/Mat-a (A+a) spores is replaced by one Mat-A (A+A) and one Mat-a (a+a) spore, after the nuclei segregate in unexpected ways.

Two types of spore killing mechanisms have been described [74] — the "killer + target" and the "poison + antidote" systems. The killer + target mechanism involves a killer gene and a target gene, and only spores with the target gene are killed (Figure 10A). The poison + antidote mechanism involves a gene that encodes both a poison and its antidote (Figure 10B) and spores that possess the gene are protected from the poison by the antidote. The Sk system in *Neurospora* is an example of the killer + target mechanism while the Spok genes of *P. anserina* and *P. comata* and the *wtf* genes of *S. pombe* are examples of the poison + antidote mechanism. The Spok protein product acts as both poison and antidote while the wtf locus encodes overlapping genes, encoding a Wtf^{poison} and a Wtf^{antidote} protein [81,82]. There are many different wtf genes and the antidote, encoded in a particular wtf locus is specific to the poison, encoded at the same locus.

Other species that have been shown to undergo ascospore abortion include *Schizosaccharomyces pombe* [74,81,82], *Venturia inaequalis* [83], *Fusarium verticillioides* [84–87], *Bipolaris maydis* [88,89], *Coniochaeta tetraspora* [90].

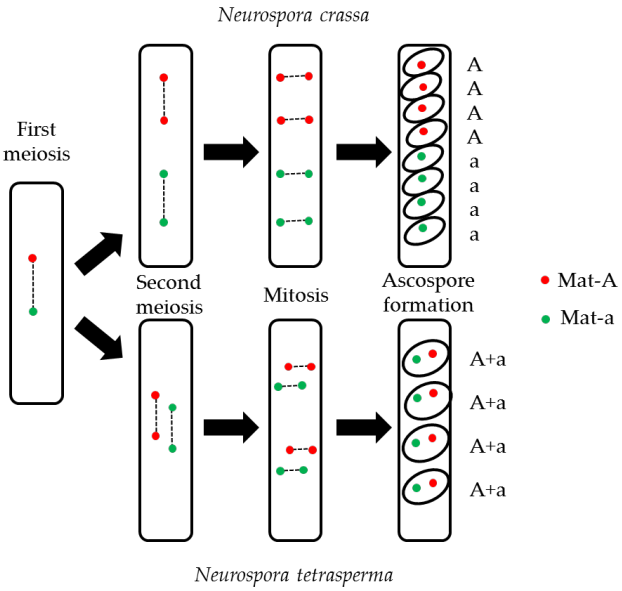

Figure 9. Spore development in *Neurospora* species. Following fusion of Mat-A and Mat-a gametes to form diploid zygotes, both *N. crassa* and *N. tetrasperma* undergo two rounds of meiosis and one of mitosis prior to ascospore formation. Mat-A and Mat-a nuclei segregate to opposite ends of the ascus during meiosis II in *N. crassa* but in *N. tetrasperma* the spindles overlap and nuclei of opposite mating types remain close together. Also, *N. crassa* produces eight haploid ascospores while *N. tetrasperma* produces four diploid ascospores. *N. crassa* therefore produces ascospores with nuclei from only one parent while *N. tetrasperma* produces spores with nuclei from both parents. Adapted from [181].

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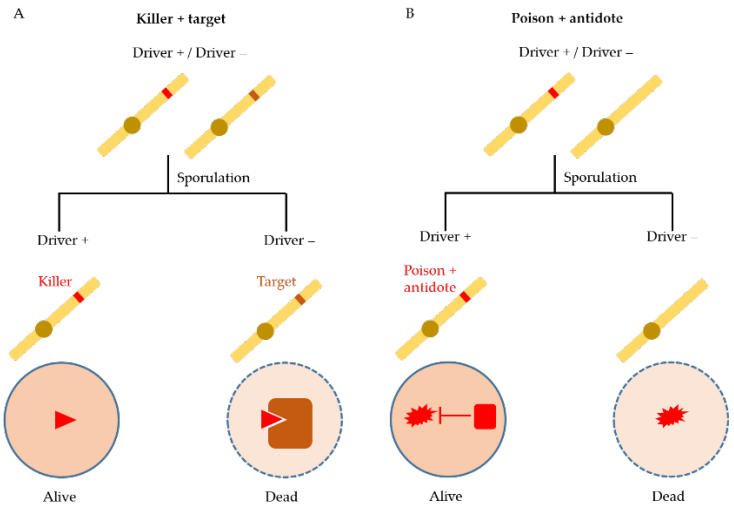

Figure 10. Spore killing mechanisms. In the killer + target mechanisms (A) one gene encodes the killer protein (red triangle) and another the target protein (brown square). If one or more spores inherit a killer gene (the driver), all spores are exposed to it but only spores that inherit the target gene die. In the poison + antidote mechanism (B) the poison (explosion symbol) and antidote (red square) proteins are encoded in the same gene (or overlapping genes). If one or more spores inherit this gene/these genes (the driver), all spores are exposed to the poison but only spores that inherited the driver benefit from the antidote and spores without the driver die. Adapted from [74].

Fruiting Body Development

Lu [91] reported that, during the development of fruiting bodies in *Coprinopsis cinereus*, the nascent gills begin as ridges, then cells between ridges break down, leaving pieces of cells and membranes, hydrolytic enzymes and multivesicular bodies. However, others have suggested that these markers of RCD were artifacts, resulting from the preparation of tissue for electron microscopy [92]. However, during fruiting body development, a kind of autolytic cell death removes gills that might be an obstacle to spore release, splits gills to allow opening of the cap or facilitates the release of copious amounts of extracellular matrix, which drives differentiation [93–95]. RCD is important in fruiting body development in many other species, including *Agaricus bisporus, Psilocybe* spp., *Panaeolus* spp., *Stropharia rugosoannulata*, *Coprinellus domesticus*, *Candolleomyces candolleanus, Candolleomyces candolleanus, Tremella mesenterica*, *Otidea onotica* and *Peziza ostracoderma* [93–95].

3.3. RCD in Saccharomyces cerevisiae

Yeast Killer Toxin

At low pH, some strains of *S. cerevisiae* secrete a substance that kills susceptible strains of yeast or closely related species [96–100] but are immune to the effects of this substance themselves. Following filtration, the medium in which killer yeast were grown retains the capacity to kill susceptible strains [96]. Treatment of a non-susceptible, killer strain with cyclohexamide converts it into a susceptible, non-killer strain [97], suggesting that a protein product of a yeast gene is required for production of the killer toxin. The killer yeast strain produces a 32 kDa protein, M-P1 that includes the killer toxin [98] and a non-killer, non-susceptible strain was shown to produce a similarly sized protein, implying that this strain has a defect in the dsRNA, encoding the protein, or in a host gene that is necessary for expression of the killer toxin. Killer yeast contain long (L) and medium (M) dsRNA, encoding the viral capsule and encapsulation machinery and the toxin and resistance proteins respectively [99]. It is now known [100] that the M dsRNA is produced by a killer virus (ScV-M1, ScV-M2 and ScV-M28) and encodes both toxin and resistance factor (Figure 11) while the L-A dsRNA is produced by a helper virus ScV-L-A and encodes the Gag (capsule protein) and Gag-Pol (polymerase necessary for viral maintenance/encapsulation etc.) In the case of K28 toxin, a high dose

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leads to cell death via necrosis (i.e. membrane disruption) while a low dose triggers cell death with hallmarks of apoptosis (membrane blebbing, PS flipping etc.) The ca. 20 kDa K28 toxin enters the nucleus and disrupts the cell cycle by interfering with certain proteins.

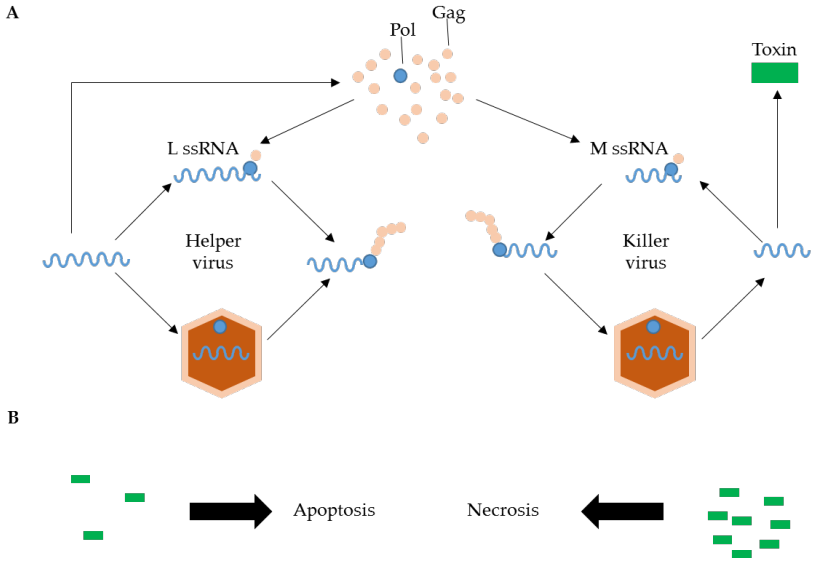

Figure 11. Yeast killer toxin. **A.** Killer strains produce medium (M) RNA that encodes both the toxin and antidote (green square). A helper virus produces long (L) RNA, encoding both the Gag protein that makes up the viral capsule and Pol (polymerase). The killer and helper viruses both use Pol and Gag to build capsules and to replicate and maintain RNA. **B.** Low concentrations of toxin induce cell death via apoptosis while and high concentrations via necrosis. Adapted from [100].

Sugar

Stationary phase *S. cerevisiae* cells lose viability when cultured in media containing hexose sugars (such as glucose or fructose) but lacking other nutrients, whereas there is no loss of viability in water or in media containing all nutrients except sugar [101–104]. "Sugar-induced cell death" requires phosphorylation of glucose (or fructose) but non-fermentable carbon sources, such as ethanol or acetate, generate phosphorylated sugars via glycolysis and these can then trigger cell death.

When exponentially growing cells were cultured in the presence of glucose (but not other nutrients) S-phase cells underwent programmed necrosis [105,106], as demonstrated by the permeability of the cell membrane to PI and FITC-labelled dextran (FD). This loss of membrane integrity was not secondary to apoptosis, since cycloheximide did not counteract this effect. Furthermore, annexin V and DAPI staining showed that exponential cells undergoing SICD did not resemble apoptotic (hydrogen peroxide-treated) cells with regards to PS flipping and chromatin fragmentation respectively.

cdc48^{S565G} Mutation

In 1997, Frank Madeo and coworkers [107] reported on a *Saccharomyces cerevisiae* strain with a mutation in the cell cycle gene *CDC48* — serine 565 was mutated to a glycine residue in the temperature-sensitive mutant. The mutation led to cell cycle arrest and the group found that arrested cells bore hallmarks of apoptosis (Figures 12, 13 and 14). DAPI staining of mutant cells from both 2 day and 5-day-old cultures revealed chromatin fragmentation (Figure 12), which was much less prevalent in wild type cells from stationary culture (Figure 12a) and highly pronounced in the *cdc48*^{S565G} mutant, incubated at 37 °C for 3 hours (Figure 12b), and in *cdc48*^{S565G} mutant cells from stationary cultures (Figures 12c, n, p and r). They used TUNEL staining to reveal extensive DNA strand breaks in the mutant strain (Figure 13a) compared to the wild type control (Figure 13b). They also used annexin V staining to show that phosphatidylserine had flipped to the outer layer of the

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cell membrane in the mutant (Figure 14a) but not in the wild type (Figure 14c) and propidium iodide (PI) staining to show that some cells of the mutant strain had suffered membrane disruption (Figure 14b). Furthermore, electron microscopy revealed extensive chromatin condensation and fragmentation of nuclei in the *cdc48*^{S565G} mutant strain, compared to the wild type.

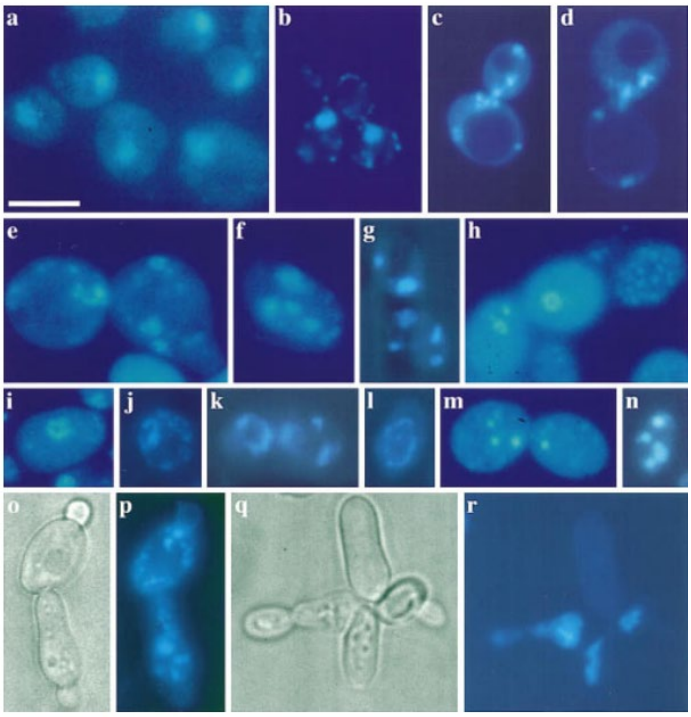

Figure 12. Chromatin fragmentation. DAPI-stained (a-n and p and r) and phase-contrast (o and q) images. All panels show strain KFY437, possessing a mutated /*cdc48*^{S565G} gene, except negative control, which shows strain KFY417, with wild type *CDC48* (a) and positive control, which shows strain rE24-15, with a temperature-sensitive *cdc48- 3*^{ts} gene (b). Cells, grown on YEPD medium and harvested during log phase (i-k) or in stationary phase after 2 days (a, c-h, l-n) or after 5 days (o,p,q,r). Log phase rE24-15 cells were incubated at 37°C for 3 hours to arrest cell cycle (b). Bar — 10 μ. Reproduced from [107] with permission from Rockefeller University Press.

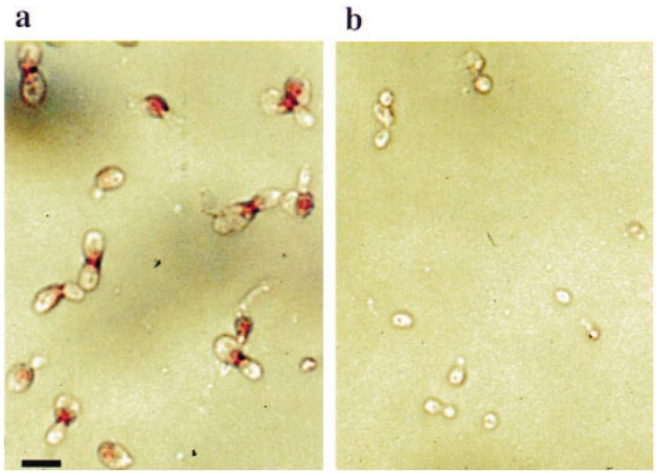

Figure 13. DNA strand breaks. Strain KFY437, with *cdc48- 3*^{ts} mutant gene (a) and negative control strain KFY417, with wild type CDC48 gene (b) were grown on YEPD for 36 h (to end of log phase) then TUNEL staining was used to identify DNA strand breaks. Bar — 10 μ. Reproduced from [107] with permission from Rockefeller University Press.

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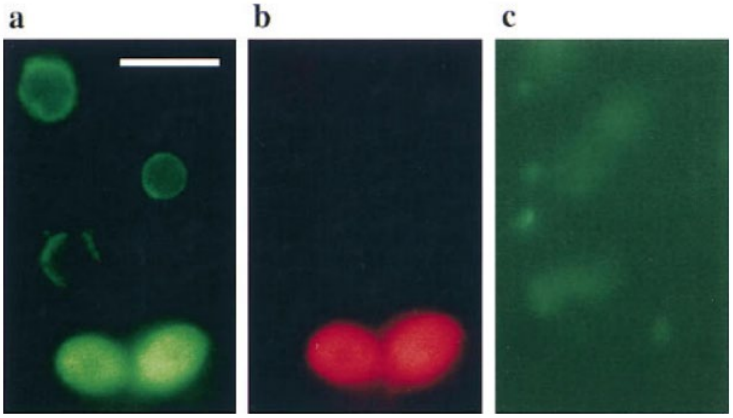

Figure 14. Flipping of phosphatidylserine. Strain KFY437, with *cdc48- 3*^{ts} mutant gene (a and b) and negative control strain KFY417, with wild type CDC48 gene (c) were grown on YEPD for 12 h then stained with FITC-labelled annexin V to identify flipping of phosphatidylserine from the inner to the outer layer of the plasma membrane (a and c) and with propidium iodide to identify cells with plasma membrane damage (b). Bar — 10 μ. Reproduced from [107] with permission from Rockefeller University Press.

Bax Expression

During a yeast two-hybrid screen to identify interactions among human Bax and other Bcl-2 family members, it was found that a fusion protein containing Bax killed yeast and that another fusion protein containing Bcl-2 counteracted Bax-dependent killing [108]. Greenhalf and co-workers [109] showed that Bax arrests cell growth but that this only results in cell death when functional mitochondria are present. These findings are important because they indicate that the pro-cell death role of Bax and the anti-cell death roles of the Bcl proteins might be conserved in yeast and that apoptosis in yeast might resemble metazoan intrinsic apoptosis. Another group [17] showed that expressing Bax in yeast under the control of the *GAL10* promoter results in reduced levels of cytochrome c oxidase in mitochondria and release of cytochrome c but this effect is counteracted if Bcl-x_L (the longer splice variant of Bcl-x and a member of the Bcl-2 family) is also expressed. Overexpressing mammalian Bax in yeast produces changes in cell morphology that are typical of apoptosis — flipping of PS, chromatin condensation, membrane blebbing and DNA strand breaks [110]. Vacuolation of the cell during Bax-induced yeast cell death resembles that observed when Bax is overexpressed in human cells while caspase activity is inhibited [111]. This suggests that Bax-induced yeast cell death may resemble RCD in a common ancestor of yeast and humans, before the evolution of caspases. Xu and co-workers proposed using *S. cerevisiae* or S. *pombe* as models of Bax-induced PCD and published detailed protocols for expressing Bax in the fungal species and for detecting cell death [111].

Oxygen Stress

Exposing yeast to oxygen stress via treatment with low concentrations of hydrogen peroxide (H_2O_2) or by deleting the glutathione (antioxidant) gene, led to enhanced apoptosis, as confirmed by TUNEL and DAPI staining and by electron microscopy [112]. Higher concentrations of H_2O_2 did not induce apoptosis but triggered loss of membrane integrity, as confirmed by PI staining and treating cells with cycloheximide abolished the pro-apoptotic effect of low-dose H_2O_2, indicating that new protein synthesis was required for this process. Furthermore, apoptosis in the temperature-sensitive *cdc48* mutant (above) and apoptosis in cells, expressing bax, was accompanied by the accumulation of ROS, and the addition of ROS scavengers (or growth under anaerobic conditions) reduced the incidence of apoptosis.

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Acetic Acid

It was reported as early as 1989 [113] that acetic acid induces two kinds of cell death: a low enthalpy death at high acetic acid concentration and low temperature and a high enthalpy death at high temperature and low acetic acid concentration. Prudêncio and coworkers [114] monitored Fun1p processing and PI staining to show that high concentrations of acetic acid reduce vacuolar processing and plasma membrane integrity. Ludovico and co-workers [115] showed that low-dose acetic acid induced apoptosis in yeast while a higher dose led to necrosis, as confirmed using annexin V, TUNEL and PI staining and electron microscopy. Low-dose, apoptotic cell death (measured by counting CFUs) was blocked by cycloheximide but high-dose, necrotic cell death was not. For technical reasons, it was not possible to study the effect of cycloheximide on PS flipping or DNA strand break formation. It was further shown [116] that acetic acid-induced cell death in yeast involved ROS accumulation, activation of the proteasome then loss of cytochrome c from the mitochondria. Two pathways of acetic acid-induced cell death were described — one was inhibited by N-acetyl cysteine (a scavenger of ROS) while the other was not (reviewed by [117]).

Osmotin

The tobacco defense compound osmotin was shown to induce RCD in *S. cerevisiae* in a ROS- and Ras2-dependent manner [118]. The authors demonstrated that osmotin induced ROS accumulation, DNA strand breaks and nuclear fragmentation as well as loss of viability, shrinking of the cytosol, vacuolation, membrane blebbing and formation of apoptotic bodies. Many of these are typical markers of apoptosis.

Aging

Deletion of antioxidant genes or increased oxygenation leads to reduced replicative lifespan (RLS) while the antioxidant glutathione increases RLS [119]. Laun and coworkers [119] showed that replicatively old cells accumulate mitochondrial ROS and exhibit signs of apoptosis, such as DNA strand breaks and PS flipping. Chronologically old cells accumulate ROS, lose viability and exhibit DNA strand breaks, PS flipping and chromatin condensation [120,121]. Furthermore, adding dried secretions from aged cultures to 7-day-old stationary cultures (but not log-phase cultures) led to an 8-fold increase in cell survival, while secretions from log-phase cultures had a much lower effect. This suggests that aging cells release substances during cell death that promote the survival of more viable cells. In aging yeast colonies on solid agar [122] the production of ammonia by colony cells triggers RCD of central cells, releasing nutrients for use by outer cells. Central cells display elevated levels of ROS accumulation, DNA strand breaks, DNA fragmentation and cell shrinkage than outer cells, taken from the same colonies. In colonies of a *sok2* knockout mutant, unable to produce ammonia, outer cells were subject to significant levels of RCD too, implying that the ammonia-induced death of central cells released nutrients that were used by outer cells.

Pheromone

A low dose of alpha-factor pheromone (produced by yeast cells of mating type α) induced shmoo formation and mating in cells of mating type a [19]. A dose, ten times higher, induced RCD in unmated a-type cells, involving an increase in ROS, DNA strand breaks, DNA fragmentation and membrane disruption. Blocking protein biosynthesis or opening of the permeability transition pore (PTP) or knocking out the MAP kinase component gene, *STE20*, counteracted this effect while deleting genes, encoding components of the calcineurin/calmodulin system, rendered yeast even more sensitive to pheromone-induced cell death.

Other Stimuli that Trigger Yeast RCD

Other factors that induce RCD in yeast (Table 1) include sodium chloride [123], defects in mRNA decapping [124], aspirin [125], hypochlorous acid [126], defects in DNA replication [127],

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hyperosmotic stress [128], reduced sister chromatid cohesion via *PDS5* mutation [129], defects in N-glycosylation due to *OST2* or *WBP1* deletion or in temperature-sensitive mutants of either gene at 37 °C [130], copper or manganese [131], formic acid [132], palmitate-induced ER stress [133], accumulation of Ras2 in mitochondria due to deletion of *WHI2*, HXK2 or *SNF1* ([134–136], low availability of potassium [137], a lack of H2B K123 ubiquitination [138], exposure to lead [139], the epidermal growth factor receptor (EGFR) antagonist geftinib [140], cisplatin [141], heat shock [142], anacardic acid [143], cold plasma [144,145], nickel oxide nanoparticles [146], citral or geraniol [147], expression of human caspase-1 in yeast [148], defects in mitophagy or autophagy due to deletion of *PIL1* [149], synthetic antimicrobial peptides [150], oxidative stress induced by polyethylene terephthalate Nano plastic [151], silver nanoparticles [152], cohesion dysfunction [153], Deletion of AP-3 components or downstream kinase [154], and enhanced mitochondrial DNA damage due to *HAP4* deletion [155].

3.4. RCD in Schizosaccharomyces pombe

Expressing Bax or Bak in *S. pombe* led to RCD, which was characterized by vacuolarization of the cytoplasm, DNA condensation and disintegration of the nuclear envelope and RCD was mediated via cell cycle arrest and was abrogated by Bcl-2 or Bcl-X_L [156–158]. Deleting either of two genes, involved in the last step of diacylglycerol biosynthesis causes *S. pombe* cells to undergo RCD upon the onset of stationary phase [159]. TUNEL staining showed increased DNA strand breaks, DAPI staining of the nucleus confirmed increased DNA fragmentation, annexin V staining revealed flipping of PS in the plasma membrane and PI staining showed an increased incidence of cells with membrane damage in the mutant strains, compared with the wild type. Mutations in DNA synthesis initiation genes leads to the accumulation of ROS and cell death and delaying replication fork progress via treatment with hydroxyurea increases ROS accumulation and cell death [160]. Mutants that lack the genes for Plh1p and Dga1p, which catalyze the final esterification step of diacylglycerol production, display characteristic signs of RCD upon entry into stationary phase [161]. Inositol starvation induces cell death in fission yeast that is dependent on the chaperone calnexin ([162]. Chronological aging also causes ROS accumulation and cell death in S. pombe and deleting *SCH9* or *PCA1* partially counteracts this effect while dysfunctional antioxidant defenses exacerbate the effect [161].

3.5. RCD in Human Fungal Pathogens

Candida albicans

Low doses of acetic acid, hydrogen peroxide and amphotericin B elicit RCD in *C. albicans* with hallmarks of apoptosis, including PS flipping, DNA strand breaks, chromatin condensation, nuclear fragmentation and ROS accumulation [163]. Higher doses elicited necrotic cell death. Cell death was later shown to be mediated by Ras-cAMP-PKA signaling and blocking this signaling pathway delayed cell death while increasing Ras-cAMP-PKA activity accelerated RCD [164]. Another antifungal drug, caspofungin induces cell death in *C. albicans* [165]. At low doses, about 25% of cells showed hallmarks of apoptosis (DNA strand breaks, ROS accumulation, loss of mitochondrial membrane potential, chromatin condensation and blebbing of the nucleus) while a smaller percentage stained with PI, implying necrosis. The fraction of necrotic cells increased at higher concentrations of the drug. Amphotericin B was shown to induce RCD in *C. albicans* even when cells were embedded in the extracellular matrix of biofilms [166] and there was some evidence that histone acetylation might promote AmB-induced RCD. *C. albicans* secretes the quorum sensing messenger farnesol, which inhibits the switch to hyphal morphology at physiological concentrations but induced RCD in *C. albicans* at high concentrations (100 μM) [167]. It was shown that cells upregulated heat shock and antioxidant protein expression while downregulating metabolic enzyme expression. Treated cells died with hallmarks of apoptosis (DNA strand breaks, ROS accumulation and mitochondrial fragmentation etc.). The plant metabolite, aureobasidin A promotes *C. albicans* RCD

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by inhibiting inositol phosphorylceramide synthase, which leads to altered membrane fluidity, reduced membrane potential and increased ROS accumulation [168].

Aspergillus species

Farnesol induces RCD in other organisms, including *A. nidulans* [172]. Treating *A. nidulans* with farnesol, blocked hyphal formation and growth and induced condensation of the nucleus. It also induced DNA strand break formation, flipping of PS and accumulation of ROS, implying an apoptosis-like RCD. Clove and rosemary oil both induced RCD with hallmarks of apoptosis in the plant, animal and human pathogen, *A. flavus* [173]. Inhibition of the unfolded protein response enhanced antifungal drug susceptibility and drug-induced cell death in *A. fumigatus* [174]. The plant essential oil, perillaldehyde induced RCD in *A. flavus* by altering metabolism, leading to a scarcity of reducing equivalents and therefore increased accumulation of ROS [175]. Similarly, carvacrol, a constituent of plant essential oils, induced *A. niger* RCD via increased hydrogen peroxide accumulation, downregulated expression of NADPH oxidase, oxidative stress and peroxidation of lipids [182]. Furthermore, hexanal triggered RCD in *A. flavus* via loss of mitochondrial membrane potential, oxidative stress and DNA damage [176].

Cryptococcus neoformans and *Histoplasma capsulatum*

Dadachova et al. [177] set out to test the possibility of treating fungal infections with radiation but fungal pathogens may be thousands of times more resistant to gamma rays than human cells. They attached radioactive isotopes to antibodies that targeted *C. neoformans* or *H. capsulatum* and showed that the former is 1,000 times and the latter 100 times more sensitive to radioisotopes emitting alpha or beta radiation than gamma rays. Low dose alpha or beta radioimmunotherapy elicited apoptosis-like RCD in most cells of both species while high dose gamma rays produced similar changes in only 30% of cells.

3.6. RCD in Plant Fungal Pathogens

Colletotrichum trifolii expressing constitutively active Ras underwent RCD via ROS accumulation, PS flipping, DNA fragmentation etc. when grown on minimal medium *[178]* but proline addition prevented RCD, possibly via ROS scavenging. Sodium chloride, heat shock, UV radiation and hydrogen peroxide also triggered RCD in *C. trifolii*. Barhoom and Sharon *[179]* found that expression of Bax in *Colletotrichum gleosporioides* was lethal so they co-expressed Bax with inducible Bcl-2. Bcl-2 suppressed Bax lethality, but when Bcl-2 expression was switched off, fungal cells underwent RCD. Zygocin, the yeast killer toxin produced by *Zygosaccharomyces bailii* was shown to inhibit aerial mycelia growth of the plant pathogens, *Colletotrichum graminicola* and *Fusarium oxysporum [100,180]*, as well as several human fungal pathogens. Further investigation, using *S. cerevisiae*, showed that zygocin induced apoptosis-like RCD.

4. Caspase Orthologs and Other Death Protein Orthologs

Orthologs of proteins with roles in metazoan RCD have been discovered in yeast (Figure 15), including orthologs of caspases (metacaspase Mca1p), cytochrome c, AIF/AMID (Aif1p and Ndi1p), IAP (Bir1p) and Omi/HtrA2 (Nma111p) [54,183].

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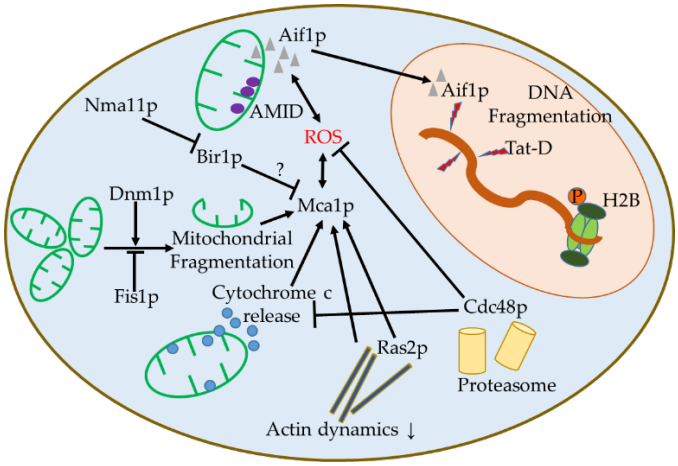

Figure 15. Yeast RCD mediators. Mca1p, ROS and cytochrome c release were shown to be involved in yeast RCD. The yeast inhibitor of apoptosis (IAP), Bir1p may inhibit Mca1p. The ortholog of Omi/HtrA2, Nma11p cleaves Bir1p. Cytochrome c release, mitochondrial fragmentation and reduced actin dynamics (via Ras2p) activate Mca1p. Mca1p activation, dysfunctional protein aggregate degradation and release of Aif1p promote ROS accumulation. Cdc48p, and the proteasome drive the degradation of protein aggregates, counteracting ROS accumulation and cytochrome c release. Aif1p promotes DNA strand breakage by the endonuclease, Tat-D. Phosphorylation of histone H2B has also been shown to promote chromatin condensation and DNA fragmentation. Adapted from [54].

It has been suggested that the eukaryotic RCD machinery, including caspases and caspase orthologs, may have been inherited from the bacterial symbiont that evolved into mitochondria [53] and that this development may have arisen from a mutual addiction relationship. Bacterial/mitochondrial genes have mostly been relocated to the host cell nucleus and are transcribed and translated by the host before migration to the mitochondria. Therefore, mitochondria cannot survive independently of the host cell and eukaryotes rely on mitochondria for respiration. RCD effectors such as the pro-apoptotic Bcl-2 may have evolved from bacterial proteins that allowed the symbiont to kill the host cell when conditions became unfavourable.

4.1. Discovery of Metacaspases, Paracaspases and Orthocaspases

Multiple rounds of searches using the basic local alignment search tool (BLAST) at the EMBL/EBI protein database [184], using a consensus sequence generated via multiple alignment of known caspases, identified distantly related proteins with significantly similar sequences, including conserved active site residues [18]. Further research was carried out, using the sequence of this new caspase ortholog and a further consensus sequence, generated by another round of alignments. The newly discovered caspase orthologs included proteins from *Caenorhabditis elegans*, *Dictyostelium discoideum*, *Streptomyces coelicolor*, and a *Rhizobium* species. Based on these findings, further rounds of iterative BLAST searches were carried out and identified three new families of caspase orthologs [16]. These families were paracaspases (in metazoa and *D. discoideum*), metacaspases (in plants, algae, fungi, and protists) and distant caspase orthologs in bacteria, such as gingipain R in *Porphyromonas gingivalis*. More recently, various groups have identified proteases, termed "prokaryotic metacaspase-like proteases" or "orthocaspases" [25].

4.2. Structure of Caspase Orthologs

The structures of caspases, metacaspases and paracaspases differ (Figure 16). Paracaspases (found in animals and slime molds) include immunoglobulin-like domains and death domains [185]. Type I metacaspases (found in fungi, protists and brown algae etc.) have an N-terminal, proline-rich region and may, or may not, have a zinc finger motif [25,186,187]. Type II metacapases (found in plants and green algae) have a long linker region between the large and small subunits. Type III

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metacaspases (found in phytoplankton) [185]. It has been proposed that the presence of type I metacaspases in fungi, protists and brown algae etc. but type I and type II metacaspases in plants may have originated via horizontal gene transfer of primitive metacaspases from the ancestors of modern mitochondria and chloroplasts respectively [188].

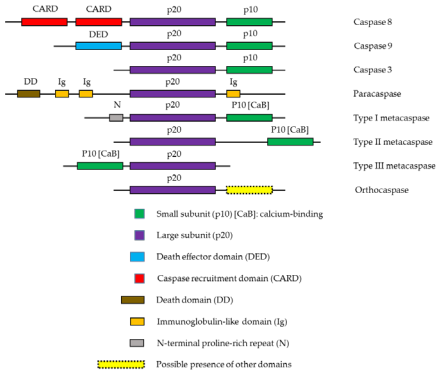

Figure 16. Structures of caspase orthologs — paracaspases and type I, II and III metacaspases differ in the presence of various domains. The small subunit is absent from paracaspases and may be absent from orthocaspases (metacaspase-like proteases). The positions of the small and large subunits are reversed in type III metacaspases. The structures of caspases-3, -8 and -9 are shown for comparison. The small subunits of metacaspases have calcium binding (CaB) domains while those of caspases do not. Adapted from [185] and [44].

4.3. Mechanism of Metacaspase Activity

Like caspases, metacaspases have a large and a small subunit, with the catalytic Cys/His dyad in the hemoglobinase fold within the large subunit [189]. *Arabidopsis thaliana* metacaspases AtMC4 and AtMC9 were shown to cleave substrates with Arg or Lys at the P1 position rather than aspartate [186]. Also, it was shown that the glycine and arginine residues that form the basic S1 pocket of caspases (into which the acidic aspartate residue fits) are replaced by aspartate or glutamate residues in metacaspases, forming an acidic pocket into which the basic arginine fits [186]. Furthermore, the initial cleavage target lysine between the large and small subunits of AtMC4 is masked by N-terminal and C-terminal loops and calcium ions activate cleavage at this and other basic amino acid residues, separating the subunits and activating proteolytic activity [190]. Treatment of *Trypanosoma brucei* with calcium induced metacaspase MCA2 activation, which was blocked by the addition of the calcium chelation agent EGTA [191]. Similarly, activation of *A. thaliana* metacaspases AtMCP2a and AtMCP2b, as well as the yeast metacaspase Mca1p, requires relatively high concentrations of calcium, the concentration of which is increased during certain types of stress [192].

4.4. Metacaspase-Dependent RCD in Fungi

Many groups have demonstrated the activation of caspase-like proteases during RCD using fluorescent versions of pan-caspase inhibitors, e.g. FITC-VAD-FMK (fluoroisothiocyanate-labelled valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) [127]. Chronological aging and killer toxin promote cell death via Mca1p [19,120,122,193], as does hyperosmotic stress, due to high glucose, sorbitol or sodium chloride concentration [128,194]. Valproic acid-, arsenic-, caffeine- and metal ion-induced cell death are also Mca1p-dependent [131,195–197].

Aging, Mca1p and Cell Death

Age-induced RCD in yeast is mediated by yeast metacaspase Mca1p [20,120]. It was shown that Mca1p was cleaved to remove a small subunit and thus activate the large subunit in a similar manner to mammalian caspases but mutation of catalytic cysteine 297 abolished cleavage. They further showed that a fluorescent initiator caspase substrate, Ac-IETD-AMC (acetyl-isoleucyl-glutamyl-threonyl-aspartyl-amino-4-methylcoumarin) was robustly cleaved by Mca1p while an executioner

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caspase substrate, Ac-DEVD-AMC (acetyl-aspartyl-glutamyl-valyl-aspartyl-amino-4-methylcoumarin) was not. In strain *mca1*^{C296A}, in which the catalytic cysteine residue was mutated to alanine, caspase-like activity was not as high as in the wild type but was higher than in the empty vector control, which now seems questionable as the mutation of the cysteine should have abolished "caspase" activity (though not aspartyl proteases activity). The authors used TUNEL and DAPI (*4′,6-diamidino-2-phenylindole*) staining to show that hydrogen peroxide-induced cell death was accompanied by markers of apoptosis — breakage of DNA strands and condensation of chromatin respectively and that these were abrogated by treatment with cell-permeable pan-caspase inhibitor zVAD-fmk (karbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) or by mutation of the catalytic cysteine to alanine. This indicated that Mca1p did play a role in linking apoptogenic stimuli with the downstream effects of RCD and with the increase in caspase activity, but cleavage of caspase substrates by Mca1p may be non-specific and the metacaspsase may act upstream of other activated aspartyl proteases.

Herker and coworkers [120] measured "caspase activation" during chronological aging using FITC-VAD-fmk (a fluorescent analog of a pan-caspase inhibitor) and flow cytometry. FITC-VAD-fmk fluorescence was higher at 3 days and 5 days than in 4 hour-old cultures and deleting *MCA1* abolished most of this activity in 3-day-old cultures but had a much smaller effect on 5-day-old cultures. One possibility is that Mca1p plays a role in aspartyl protease activation during early RCD or when stress levels are below a particular threshold but is not as important once cells have committed to the cell death program.

Salt and Osmotic Stress

Deleting *MCA1* alleviates symptoms of RCD, induced by sodium chloride in a mutant strain, deleted for tumor suppressor homolog *SRO7* [194]. "Caspase activity during apoptosis" was detected using FITC-VAD-fmk. RCD in response to sorbitol- or glucose-induced hyperosmotic stress was shown to be dependent on the presence of intact cytochrome c and Mca1p but independent of the apoptosis-inducing factor ortholog Aif1p [128]. Apoptosis-like cell death was confirmed using electron microscopy and TUNEL, DAPI and PI staining, as well as dihydroethidium (DE) or 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) to measure reactive oxygen species (ROS). The authors also used a double staining technique involving FITC-VAD-fmk and PI to detect "caspase activity". The fact that deletion of *MCA1* abrogates "yeast apoptosis" and that "yeast apoptosis" involves "caspase" activity did not necessarily show that Mca1p was responsible for the "caspase" activity detected.

Farnesol

Shirtliff et al. [167] found that during farnesol-induced RCD in *C. albicans*, *MCA1* expression was upregulated, that cells died with hallmarks of apoptosis (DNA strand breaks, ROS accumulation and mitochondrial fragmentation etc.). They also found that farnesol induced caspase-like proteolytic activity in a dose-dependent manner. At the time, caspase activity was seen by many as a defining characteristic of apoptosis, and this may have influenced the enthusiasm with which some researchers sought to prove that RCD was accompanied by increased caspase activity. Unfortunately, the group did not report whether the FLICA kit they used was for detecting pan-caspase or specific caspase-type activity.

Antifungal Drugs and Fungicides

Hao and coworkers [165] used the multi-caspase substrate (aspartyl)$_2$-Rhodamine 110 (D2R), cleavage of which releases fluorescent rhodamine, to show that caspofungin-induced RCD was accompanied by "caspase activity" in *C. albicans*. We now know that fungi do not possess *bona fide* caspases. Al-Dhaheri and Douglas [166] demonstrated caspase-like activity during amphotericin B-induced RCD in *C. albicans* biofilms, using both SR-FLICA (a poly-caspase substrate) and D$_2$R. They

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also showed that caspase inhibitors, particularly those that blocked caspases-1, 2, 3 and 5, enhanced the survival of AmB-treated biofilm cells. The caspase inhibitor was Z-X-FMK, where X is a synthetic caspase substrate (e.g. YVAD) and is attached to a fluromethylketone (FMK) group, which covalently (and irreversibly) binds to the catalytic cysteine via its sulfur atoms, blocking enzymatic activity. There is no data on how the use of caspase inhibitors affected the level of aspartic protease activity during RCD. The agricultural fungicide, mancozeb kills yeast via upregulated ROS production and increased mitochondrial membrane potential, eliciting alterations in yeast cells that are typical of apoptosis and is dependent on Mca1p [198].

Metals

Nickel oxide nanoparticle-induced RCD in yeast was found to be dependent on Yca1p and Aif1p [146]. Manganese-, but not copper-induced RCD is dependent on Mca1p and the latter involves accumulation of ROS while the former does not [131].

4.4. Metacaspase-Independent RCD

Sphingolipids

Dihydrosphingosine and phytosphingosine induced RCD in *A. nidulans* [199]. This involved ROS accumulation, but ROS scavenging did not abrogate RCD, suggesting that RCD was not dependent on ROS production. This sphingolipid-induced RCD resulted in increased PS flipping and DNA strand breakage and the latter required active protein synthesis, implying that cell death was programmed. The RCD process was not affected by *MCA1* deletion but required functional mitochondria.

Altruistic Death of Yeast Cells in Colonies

The survival of outer cells in yeast colonies [122] at the expense of central cells (which undergo RCD) is independent of Mca1p and Aif1p. Ammonia is used as a messenger to regulate coordinated cell death among central cells. Colony cells, deleted for *MCA1* stain with D_2R, indicating the presence of an active aspartyl protease. This could explain the many reports of aspartyl protease activity during yeast RCD, even after *MCA1* deletion or inhibition with z-VAD-fmk.

Antimicrobial Peptides

A truncated (but active) version of the tree frog antimicrobial peptide, dermaseptin S3 was found to kill yeast, with increased ROS production, flipping of PS and DNA strand breakage . Dermaseptin-induced cell death was dependent on Aif1p and on two proteins (Stm1p and Izh2p) that had been implicated in other forms of yeast cell death, but not on Mca1p.

Acetic Acid

Mca1p was not essential for acetic acid-induced RCD [116] since acetic acid induced two forms of RCD — an Mca1p-dependent and an Mca1p-independent pathway [117]. Deletion of *MCA1* did not abolish acetic acid-induced RCD but reduced the rate of cell death. Furthermore, inhibition of Mca1p with z-VAD-fmk did not rescue WT or deletant cells from RCD, implying that neither form of cell death was dependent on the catalytic function of Mca1p.

Metals

Copper-induced yeast RCD was also shown to be independent of Mca1p, involved production of copious ROS and was alleviated by the addition of antioxidants (unlike manganese-induced RCD) [131] Anacardic acid was shown to induce RCD in *S. cerevisiae* with hallmarks of apoptosis [143]. RCD was not suppressed by knocking out metacaspase gene *MCA1* or by the presence of caspase inhibitor Z-VAD-fmk, implying that anacardic acid-induced cell death in yeast is independent of Mca1p.

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Formic Acid

Low doses of formic acid induce RCD with hallmarks of apoptosis [132] including DNA strand breaks, chromatin fragmentation, nuclear condensation, flipping of PS, reduced potential in the mitochondrial membrane and accumulation of ROS. Deletion of *MCA1* resulted in an earlier and stronger burst of ROS production but *MCA1* deletion did not abrogate RCD.

Caspofungin

The antifungal drug caspofungin blocks β-1,3-glucan synthesis, which is important for cell wall integrity, and elicits RCD in *S. cerevisiae* and *C. albicans* with ample ROS accumulation and DNA strand breakage [200]. Caspofungin-induced RCD was abrogated by *AIF1* deletion but not by *MCA1* deletion.

4.5. Non-Cell Death Roles of Yeast Metacaspase

Clearance of Protein Aggregates

Mca1p has a non-RCD role, helping to clear protein aggregates [201]. The metacaspase was shown to localise to protein aggregates and interact with heat shock proteins in a way that was dependent on its polyglutamine-rich domain. Deletion of *MCA1* or altering the gene to express catalytically inactive Mca1p resulted in an increased buildup of aggregated proteins.

Cell Cycle Control

Mca1p also appeared to play a role in regulation of the cell cycle . Deletion of *MCA1*, or mutation to abolish Mca1p catalytic activity, extended the G1/S transition of the cell cycle [202]. Furthermore, cells with reduced Mca1p catalytic activity did not arrest when treated with nocodazole (a microtubule inhibitor). This implied that Mca1p was also involved in the G2/M checkpoint. This phenomenon was not dependent on ROS production. Interestingly, metacaspases have also been implicated in growth and cell cycle regulation of the protist human parasites, *Trypanosoma brucei* and *Leishmania* major [203].

5. The Debate

Fungi undergo RCD in response to various stimuli, sometimes with characteristics of metazoan apoptosis [54,204]. The discovery of caspase orthologs, as well as orthologs of other metazoan cell death proteins, in fungi suggested that fungi might possess similar cell death pathways [16]. Frank Madeo has been, perhaps the greatest proponent of the theory that metacaspases are caspases, and named the yeast metacaspase gene "Yeast Caspase 1" (*YCA1*) after demonstrating its involvement in yeast RCD [20]. Some forms of yeast RCD are dependent on Mca1p and some are not [205,206], Mca1p has non-cell death roles [201,202], and some parasitic protists have metacspases that regulate growth, the cell cycle and differentiation [203]. In line with caspases, and other caspase orthologs, metacaspases are clan CD, family C14 proteases (https://www.ebi.ac.uk/merops) with a characteristic active site (the hemoglobinase fold) and (with some exceptions) a catalytic cysteine/histidine dyad [186]. Metacaspases cleave peptides after arginine or lysine, whereas caspases cleave after aspartate [1]. Furthermore, metacaspases require calcium for activation [190].

5.1. Are Metacaspases Caspases?

Vercammen et al. [188] wrote an editorial entitled "Are metacaspases caspases?" Referring to the finding of Madeo's team [20] that Mca1p mediates cell death, they remarked that upregulated stress following overexpression of a stress-inducing protease was no surprise. They further queried the Madeo et al. [20] report of RCD-related caspase activity as it was based on the use of unreliable synthetic caspase substrates, and Mca1p from cells undergoing hydrogen peroxide-induced cell

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death had been shown to cleave after Arg or Lys, not Asp [192]. Vercammen et al. [188] went on to question the involvement of some metacaspases in RCD, particularly those with roles in development and growth.

5.2. Metacaspases are Caspases, Doubt No More

In their editorial, (title above), Carmona-Gutierrez et al. [207] (Madeo's team) acknowledged that caspases and metacaspases cleave at different residues but theorized that they would cleave similar substrates as part of a conserved cell death process. They went on to comment on the discovery that the Norway spruce (*Picea abies*) metacaspase, MCII-Pa cleaved Tudor staphylococcal nuclease (TSN) during RCD, induced by stress or related to development [208] and that human TSN was cleaved by caspase-3 during apoptosis. Human TSN is involved in splicing and transcription and cleavage of TSN led to reduced expression of some pro-survival mRNAs and this resulted in cell death. Madeo and his team [207] argued that this was evidence that caspase substrates, and therefore the cell death regime, was conserved in yeast.

5.3. Metacaspases are Not Caspases - Always Doubt

In response, Enoksson and Salvesen [209] wrote an editorial with the title shown above. They begin by agreeing that the naming of caspase orthologs as metacaspases and paracaspases was confusing and then stated that it was unscientific to suggest there was no doubt about the relationship between caspases and metacaspases since nothing can be proved in science, only disproved. They went on to say that apoptosis arose only in multicellular organisms and that MCII-Pa cleaves PaTSN in 4 different places instead of one specific place, thus resembling protein degradation rather than cell death-related cleavage. Enoksson and Salvesen [209] argued that the definition of caspases (cysteine dependent aspartyl proteases) specifically stipulates cleavage after aspartate and therefore excludes metacaspases.

6. Alternative Cell Death Proteases in Yeast

As discussed above, RCD resulting from various stresses is sometimes dependent on Mca1p and sometimes not. Also, deletion of *MCA1* may or may not affect the level of "caspase" activity, detected in dying cells. The involvement of metacaspase-independent protease activity in RCD has been documented in several examples of RCD in *S. cerevisiae* [210]. It is possible that other proteases are responsible for the aspartyl protease activity that was detected during cell death in response to certain stimuli. In some experiments (above) a caspase inhibitor blocks cell death and/or aspartyl protease activity. Since there are no caspases in fungi, this may best be explained by a protease, with a catalytic cysteine, acting up- or downstream of an aspartyl protease.

6.1. Esp1p

For example, the yeast separin Esp1p is a clan CD protease with a catalytic cysteine and cleaves after glutamate and occasionally aspartate residues (MEROPS database, https://www.ebi.ac.uk/merops/ accessed 29.03.2026). *ESP1* is an essential gene with roles in mitosis and meiosis and cannot be deleted but overexpression leads to increased RCD (Saccharomyces Genome Database [SGD] https://frontend.qa.yeastgenome.org/, accessed 29.03.2026). During hydrogen peroxide-induced RCD, Esp1p is released from its inhibitor Pds1p and cleaves the cohesin complex component, Mcd1p, allowing the Mcd1p C-terminal fragment to migrate to the mitochondria and trigger cytochrome c-dependent RCD via loss of mitochondrial membrane potential [211]. It should be mentioned that Esp1p and Mcd1p are two of a number of proteins with roles in both cell death and the cell cycle [212]. During the cell cycle, Esp1p elicits the separation of Mcd1p from chromatin, allowing Mcd1p to promote the separation of sister chromatids (Figure 17).

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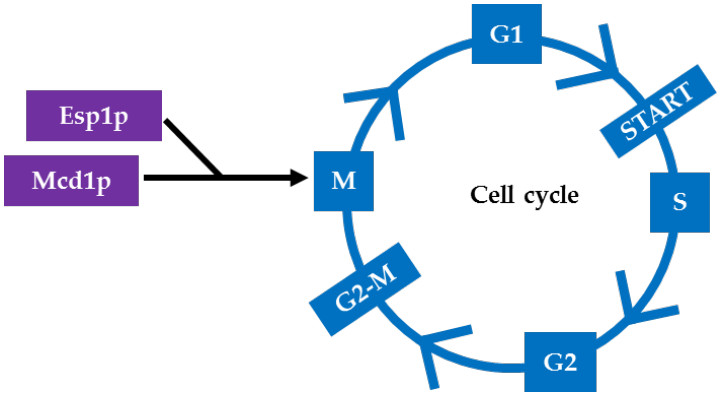

Figure 17. Involvement of cell death proteins, Esp1p and Mcd1p in the cell cycle. As well as triggering mitochondrial-dependent cell death after cleavage by Esp1p, Mcd1p promotes sister chromatid separation during the cell cycle after being freed from chromatin by Esp1p. Adapted from [212].

6.2. Nma111p

Nma111p is a clan PA, S1 (chymotrypsin) family serine endopeptidase (MEROPS database, https://www.ebi.ac.uk/merops/ accessed 29.03.2026). Deletion of *NMA111* leads to loss of or reduced RCD while overexpression increases RCD (SGD https://frontend.qa.yeastgenome.org/, accessed 29.03.2026). Nma111p, with roles in chromosome segregation and cytokinesis, is a homolog of the metazoan apoptosis-inducing mitochondrial protease Omi/HtrA2, and cleaves the inhibitor of apoptosis (IAP) protein, Bir1p (Figure 15) leading to RCD [213,214].

6.3. Kex1p

Defective N-glycosylation of proteins in a temperature-sensitive mutant was shown to induce cell death with typical apoptosis characteristics (DNA strand breaks, fragmentation of chromatin, PS flipping and ROS accumulation), accompanied by an increase in "caspase" activity (measured using a FITC-labeled pan-caspase substrate) and RCD was abrogated by the application of a pan-caspase inhibitor [130]. The use of different specific caspase substrates showed that N-glycolysation defect-dependent caspase activity was mainly directed towards substrates containing the caspase-6 target sequence VEID, followed by the caspase-8/10 target sequence, IETD, though cross-reactivity could not be ruled out. The serine carboxypeptidase, Kex1p mediated RCD, triggered by defective N-glycosylation, acetic acid and chronological aging and deletion of *KEX1* reduced markers of RCD and "caspase" activity in cells with defective N-glycosylation [215]. It should be noted that when tested in a cell-free experiment, Kex1p did not cleave the synthetic substrate containing the caspase-6 target sequence VEID, suggesting that the N-glycosylation defect-dependent caspase activity, mentioned above, might not be directly attributable to Kex1p.

6.4. Pep4p

The vacuolar aspartic protease, Pep4p was shown to migrate to the mitochondria during acetic acid-induced RCD, where it mediated direct or indirect autophagy-independent degradation of damaged mitochondria, resulting in a reduction in cell death markers and enhancing viability *[216]*. Deleting *PEP4* was shown to stave off RCD while overexpression enhanced RCD. The role of Pep4p in mitochondrial degradation induced by apoptogenic levels of acetic acid was later shown to be dependent on its catalytic activity and on the ADP/ATP carrier Aac2p but not on the voltage-dependent channel Por1p *[217]*. Pep4p may be directly or indirectly responsible for at least some of the cell death-related "caspase" activity, detected by various groups but Pep4p plays a pro-survival, rather than a pro-death, role in this example.

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6.5. Proteasome

Using 3 fluorogenic artificial substrates, [218] demonstrated a 45%, 60% and 30% increase in proteasomal trypsin, chymotrypsin and peptidyl-glutamyl peptide bond hydrolysing (PDPH) activity respectively during acetic acid-induced RCD in yeast. It was shown that that proteasome inhibition blocked acetic acid-induced cell death and that the increase in proteolytic activity was not due to an increase in proteasome component abundance. The authors speculated that activation/maturation/increased efficiency led to increased proteolysis.

6.6. Other Candidate Death-Inducing Proteases

Fluorogenic caspase-1, caspase-6 and caspase-8 substrates were used to screen a library of yeast strains, deleted for protease genes [210] and they were further screened for susceptibility to acetic acid-induced cell death. Six strains were found to have reduced caspase-1/6/8 activity and increased resistance to apoptogenic doses of acetic acid.

Yps7p

Deleting *YPS7* reduced caspase-6 and caspase-8 activity and increased survival during acetic acid-induced cell death. Yps7p (Yapsin 7 protein) is a putative GPI-anchored aspartic protease with a role in cell wall integrity (CWI) (SGD https://frontend.qa.yeastgenome.org/, accessed 29.03.2026). Deletion of *YPS7* might be expected to reduce resistance to acetic acid due to loss of CWI. However, it has been shown in *N. glabratus* that yapsins play a role in maintaining vacuolar pH [219] leading one to theorize that loss of Yps7 in *S. cerevisiae* might mediate RCD via the release of Pep4p from the vacuole.

Aap1p

Deleting *AAP1* reduced caspase-6 activity and enhanced cell survival following treatment with apoptogenic doses of acetic acid. Aap1p is an alanine/arginine metalloaminopeptidase, involved in promoting glycogen accumulation during the diauxic shift, as well as positively regulating the heat shock response, both of which are pro-survival roles (SGD https://frontend.qa.yeastgenome.org/, accessed 29.03.2026).

Pim1p

Deleting *PIM1* also reduced caspase-6 activity and enhanced survival of acetic acid-treated cells. Pim1p is an ATP-dependent Lon protease with roles in mitochondrial maintenance and respiration (SGD https://frontend.qa.yeastgenome.org/, accessed 29.03.2026). Interestingly, Pim1p is required for biosynthesis of mitochondrial intronic gene products, including cytochrome c oxidase subunit I (CoxIp). This suggests a possible link with cytochrome c, release of which drives some forms of RCD, and raises the possibility that loss of mitochondria and respiration (which promote ROS accumulation and therefore RCD) might be beneficial. In fact, expressing human α-synuclein in yeast leads to mitochondrially mediated cell death and yeast counteract this phenomenon by becoming rho$^-$ petites, which lack mitochondria and respiration (Akintade and Chaudhuri, 2020).

Lap2p

The *LAP2* deletion mutant was defective in caspase-6 activity and was better able to survive acetic acid cell death conditions. Lap2p (leukotriene A4 hydrolase) is a leucyl aminopeptidase that also hydrolyses epoxide (SGD https://frontend.qa.yeastgenome.org/, accessed 29.03.2026). The protein is involved in both protein catabolism and lipid metabolism and heat sensitivity is reduced in the deletant strain. Little is known about its involvement (if any) in cell death but if the protease mediates sensitivity to heat, it may promote sensitivity to other stresses and could be a pro-death

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protease under RCD-inducing conditions. Alternatively, it could be another pro-survival protein with indirect effects on aspartyl protease activity.

Map1p

MAP1 deletion reduces acetic acid-induced cell death under apoptogenic conditions and has a deficiency in caspase-3 activity. Map1p is a methionine aminopeptidase that deletes N-terminal methionine residues, of newly synthesized proteins and inhibits gene expression (SGD https://frontend.qa.yeastgenome.org/, accessed 29.03.2026). Deletion of *MAP1* has many detrimental effects including reduced RLS, cell size and survival under various stress conditions. Its localization includes stress granules, and it has been shown that the yeast metacaspase Mca1p has a role in clearance of protein aggregates, thus counteracting RCD by restoring proteostasis [201].

Rbd2p

In the *RBD2* deletion mutant, caspase-3 and caspase-8 activity are depressed, relative to the wild type and the mutant strain is more resistant to acetic acid-induced RCD (SGD https://frontend.qa.yeastgenome.org/, accessed 29.03.2026). Rbd2p is a putative clan ST rhomboid protease, and predicted membrane protein, that localizes to the COPI-coated vesicles, Golgi and nuclear periphery (SGD https://frontend.qa.yeastgenome.org/, accessed 29.03.2026; (MEROPS database, https://www.ebi.ac.uk/merops/ accessed 29.03.2026). In *S. pombe*, hypoxic conditions are believed to promote cleavage of the sterol regulatory element binding protein (SREBP) Sre1p, leading to migration of the N-terminal fragment (a transcription factor) to the nucleus, where it activates hypoxia response genes [220]. It was shown that Sre1p cleavage (and therefore growth under hypoxic conditions) was dependent on Rbd2p. Similar SREBP regulation of hypoxia genes also exists in *Aspergillus fumigatus* and *Cryptococcus neoformans*. If *S. cerevisiae* Rbd2p has a similar function, it is another pro-survival protein and its exact role in promoting acetic acid-induced RCD remains to be established.

7. Defining and Testing for Regulated Cell Death

7.1. Defining Different Forms of Fungal Cell Death

The use of defunct terms such as apoptosis and apoptosis-like cell death to describe fungal RCD is frowned upon because the modern definition of apoptosis includes the involvement of caspase-3, which does not exist in fungi [221]. A group of fungal cell death researchers issued guidelines for the definition of different forms of cell death in yeast [222] and suggesting the use of specific terms. They made recommendations on how to distinguish between apoptosis, regulated necrosis, autophagy-dependent cell death (ADCD), accidental necrosis and "cytoprotective" autophagy. They also stressed that PCD is a specialized form of RCD, related to development (e.g. apoptosis of cells to sculpt fingers and toes). Apoptosis, regulated necrosis and ADCD are all forms of RCD while accidental necrosis (AN) is "unplanned" cell death, resulting from cellular insult, such as antifungal drugs, starvation, toxins and physical damage. General autophagy is a mechanism for recycling cellular material via the vacuole.

7.2. Testing for Different Forms of Fungal Cell Death

The group also suggested techniques for distinguishing among the different cellular mechanisms (reviewed by [222]). Cell death may be assessed using PI staining and identifying the percentage of cells that are "PI-positive" while viability can be measured using counts of colony-forming units (CFUs) or growth rate assays. A positive PI (or trypan blue) result could relate to primary or secondary necrosis, but annexin V staining (for PS flipping) can help to distinguish between the two. Annexin V staining also highlights apoptosis, but it should be noted that yeast cells need to be spheroplasted prior to use (possibly altering cell behavior) and that PS flipping also occurs

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during some forms of necrosis. Measuring CFUs fails to distinguish between dead and senescent cells. A reduced growth rate could indicate a change in the cell cycle rather than dying of cells. Yeast vitality may be assessed by observing the conversion of methylene blue to a colorless form, the export of red phloxine B or specific enzyme activities. Of course, some activities occur in dying cells and others are not necessary for life. The production of reactive oxygen species (ROS) is another characteristic of cell death and may be measured using e.g. dihydroethidium, 2,7-dichlorodihydrofluorescein diacetate, but different stains detect a different range of ROS.

Other techniques, the authors recommend for confirming apoptosis, include DAPI staining and electron microscopy to visualize chromatin condensation, TUNEL staining to identify DNA strand breaks, western blot or immunofluorescence microscopy etc. to detect loss of certain proteins from the mitochondrial intermembrane space and fluorescent probes that highlight a loss of mitochondrial membrane potential via changes in fluorescence or localization [222]. However, all these techniques detect changes that are not unique to apoptosis but may also occur during necrosis. The authors suggest using a combination of annexin V and PI staining to reveal the status of different cells. When staining occurs with PI but not annexin V, cells are undergoing primary necrosis. Staining with annexin V but not PI indicates probable apoptosis. Staining with both annexin V and PI is a sign of secondary necrosis — necrosis that follows apoptosis as the cells lose membrane integrity. Finally, staining with neither PI nor annexin V would suggest that the cells are healthy.

Wloch-Salamon and Bem [223] reviewed 3 types of cell death in *S. cerevisiae* (apoptotic, autophagic and necrotic) and methods for identifying them in yeast cells. They also pointed out advantages and disadvantages of each method. For example, they state that the preparation technique can lead to false positives during TUNEL testing. Also, annexin V staining relies upon spheroplasting of cells before preparation and this can lead to artifactual PS exposure. Tests for caspase activity (or rather, aspartase activity with specific P1-P4 preferences) may be complicated by unspecific substrate proteolysis. One way to distinguish between RCD and non-regulated necrosis is to determine whether new protein biosynthesis is necessary for the phenomena to occur (i.e. whether it is abolished in the presence of cycloheximide) (reviewed by [142].

7.3. Testing for Protease Activity

Synthetic caspase substrates and inhibitors are unsuitable for assessing metacaspase activity in yeast as they produce a significant number of false positives [224–226]. Caspases cleave peptides after aspartate at the P1 position but metacaspases cut after arginine or lysine . Based on the autoprocessing and substrate cleavage preferences of various metacaspases, it was suggested that better fluorogenic substrates be used, such as z-VRPR-AMC and corresponding inhibitors such as z-VRPR-FMK [227–229]. A new generation of non-peptide metacaspase inhibitors has also been developed, particularly those that inhibit parasitic protist metacaspases with pro-growth/development roles [229,230].

8. Conclusions and Recommendations

At least some metacaspases appear to have roles in RCD but they are not caspases. They have different P1 substrate specificities and P1 specificity is defined in the word “caspase”. Most metacaspases are activated by calcium whereas caspases are activated via proximity of caspase proteins and interaction between them. Metacaspases appear to be less specialized than caspases as cell death regulators and have pro-survival roles, including cell cycle regulation, growth, differentiation and proteostasis. Having said that, metacaspases are certainly structurally related to caspases and at least one substrate is conserved between humans and yeast. The substrate is cleaved at one specific residue (an aspartate) by caspase-3 but at 4 different residues (arginine or lysine) by MCII-Pa. However, it is believed that the ancestors of caspases and caspase orthologs resembled a metacaspase. It is logical to expect that metazoa, with more complex cell death programs should have evolved caspases with much more specific cleavage targets.

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The most important future aims are to use metacaspase-specific substrates and inhibitors to study fungal cell death and associated protease activity and to identify the degradomes of fungal metacaspases and to elucidate the regulatory pathways in fungal RCD.

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Abbreviations

The following abbreviations are used in this manuscript:

PCD	Programmed cell death
RCD	Regulated cell death
DAPI	4,6-diamidino-2-phenylindole-dihydrochloride
TUNEL	d**U**TP **N**ick **E**nd **L**abeling
TdT	Terminal deoxynucleotidyl transferase
Br-dUTP	*5-bromo-2'-deoxyuridine 5'-triphosphate*
FITC	Fluorescein isothiocyanate
Caspase	Cysteine-dependent aspartyl protease
DED	Death effector domain
CARD	Caspase recruitment domain
DD	Death domain
Apaf-1	Apoptotic protease-activating factor 1
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1
FasL	Fas ligand
TL1A	Tumor necrosis factor-like cytokine 1A
DR3	Death receptor 4
DR4	Death receptor 4
DR5	Death receptor 5
DcR3	Decoy receptor 3
FADD	Fas-associated protein with death domain
FLICE	Fas-associated death domain protein interleukin-1β-converting enzyme
c-FLIP	Cellular FLICE inhibitory protein
DISC	Death-inducing signaling complex

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