REVIEW ARTICLE

Fungal apoptosis: function, genes and gene function

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Received 3 December 2008; revised 10 February 2009; accepted 12 March 2009. Final version published online 20 April 2009.

DOI:10.1111/j.1574-6976.2009.00180.x

Editor: Martin Kupiec

Keywords
apoptosis; PCD; fungi; cell death.

Abstract

Cells of all living organisms are programmed to self-destruct under certain conditions. The most well known form of programmed cell death is apoptosis, which is essential for proper development in higher eukaryotes. In fungi, apoptotic-like cell death occurs naturally during aging and reproduction, and can be induced by environmental stresses and exposure to toxic metabolites. The core apoptotic machinery in fungi is similar to that in mammals, but the apoptotic network is less complex and of more ancient origin. Only some of the mammalian apoptosis-regulating proteins have fungal homologs, and the number of protein families is drastically reduced. Expression in fungi of animal proteins that do not have fungal homologs often affects apoptosis, suggesting functional conservation of these components despite the absence of protein-sequence similarity. Functional analysis of *Saccharomyces cerevisiae* apoptotic genes, and more recently of those in some filamentous species, has revealed partial conservation, along with substantial differences in function and mode of action between fungal and human proteins. It has been suggested that apoptotic proteins might be suitable targets for novel antifungal treatments. However, implementation of this approach requires a better understanding of fungal apoptotic networks and identification of the key proteins regulating apoptotic-like cell death in fungi.

Introduction

The term apoptosis is used to describe a morphologically distinct form of programmed cell death (PCD) (Collins *et al*., 1992). Apoptotic cell death is a vital process in multicellular organisms: it normally occurs during development and is associated with maintenance of cell homeostasis, elimination of damaged cells, response to infectious agents, aging and differentiation, as well as in the adaptive responses of cells to biotic and abiotic stresses (Danial & Korsmeyer, 2004; Green, 2005). Apoptosis networks are composed of hundreds of proteins that are tightly regulated by complex signaling cascades at multiple points. Inappropriate apoptosis due to interference with this regulatory network can have multiple effects and is associated with many human malignancies, including neurodegenerative diseases, autoimmune disorders and cancer (Elmore, 2007). Many of the key apoptotic proteins have been identified. However, in most cases, the molecular mechanisms of these proteins are only partially understood and much of the research continues to focus on the elucidation of apoptosis-signaling networks and analysis of the proteins' mechanisms of action. Because of its central role in development and disease, apoptosis has long been recognized for its immense therapeutic potential and much effort is being invested in discovering and designing novel apoptosis-based drugs (Zhang, 2002; Andersen *et al*., 2005; Fesik, 2005; Nguyen *et al*., 2007).

Although apoptosis is mainly recognized for its developmental roles in higher eukaryotes, it is not restricted to metazoans: it appears to occur in most living systems, including plants, fungi, and bacteria (Lewis, 2000; Golstein *et al*., 2003; Lu, 2006; Ramsdale, 2006; Robson, 2006). Apoptotic-like cell death was first described in *Saccharomyces cerevisiae* over 10 years ago, but yeast apoptosis remained controversial, mainly due to its questionable physiological relevance and a lack of molecular and genomic data (Fröhlich & Madeo, 2000; Fabrizio & Longo, 2008). Later studies, including the identification and analysis of homologs of apoptotic genes, confirmed the existence of apoptotic-like cell death in fungi. These studies also showed the connection between apoptotic-like cell death and

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important biological processes such as development, aging, stress responses and pathogenesis. The emerging role of apoptosis as a key regulator of fungal development suggests that it might be possible to develop new means of controlling fungal infections through manipulation of apoptosis. However, apoptosis has been described and studied in only a few fungal species, and although homologs of apoptotic genes can be identified in all fungal genomes, to date only a handful of genes have been functionally analyzed. Further research is needed to identify the molecular components and cellular mechanisms controlling apoptosis in fungi.

Recognition of the importance of apoptosis for fungal development has led to increased interest and more intense research in recent years. Several excellent reviews have been recently published, which provide information on various aspects related to fungal apoptosis (Hamann *et al.*, 2008; Ramsdale, 2008; Sharon & Finkelshtein, 2008). Here, we provide a general overview of the current knowledge on the processes of fungal apoptosis and then focus on recent molecular and genomic studies of fungal apoptotic networks and genes. For convenience, we start with a brief overview of apoptosis in mammals, which we will use later as a reference when discussing fungal apoptosis.

Hallmarks of apoptosis

Apoptosis is one of several types of energy-dependent PCD processes in which dying cells undergo controlled decomposition and their components are recycled. This is in contrast to necrotic cell death, which is energy-independent and is associated with cell perturbation and inflammatory response. Apoptosis can follow two major routes, known as the extrinsic (or death receptor) and intrinsic (or mitochondrial) pathways (Elmore, 2007). The extrinsic pathway is initiated by extracellular ligands, such as Fas or tumor necrosis factor, toxins, or other external signals that bind and activate death receptors on the cell membrane. The intrinsic pathway can be activated by cell damage or during specific developmental stages. It involves a diverse array of stimuli that activate intracellular targets and mitochondria-initiated events (Box 1). The extrinsic and intrinsic pathways converge on the same execution pathway, which is initiated by the cleavage of caspase-3.

To date, evidence only exists for components of an intrinsic-like pathway in fungi: it is unclear whether the extrinsic pathway is altogether missing or regulated by as-yet unidentified proteins.

Apoptosis is defined by a sequence of unique morphological changes. The first visible process is cell shrinkage and chromatin condensation. Next, extensive plasma membrane blebbing occurs followed by nuclear fragmentation and formation of apoptotic bodies, which are subsequently engulfed by phagosomes. The process terminates with decomposition of the apoptotic bodies within the phagosomes and complete recycling of the components. It should be noted that although the mechanisms and morphologies of apoptosis and necrosis differ, there is considerable overlap between the two, and it is not always possible to distinguish apoptosis from necrosis using conventional microscopy. Therefore, determination of apoptosis cannot rely solely on cell morphological markers and must also be supported by the presence of additional apoptosis-specific markers. Among the well-known biochemical and cytological responses of apoptotic cells are accumulation of reactive oxygen species (ROS), activation of caspases, DNA cleavage by specific endonucleases and externalization of the inward-facing phosphatidylserine in the cell's lipid bilayer (Elmore, 2007). These changes can be monitored by a variety of direct and indirect methods, which are used to determine apoptosis. However, not all methods are appropriate to all situations, and the choice of method must take into consideration its relevant advantages and disadvantages.

Box 1. Intrinsic pathway in vertebrates

Activation of the intrinsic apoptotic response is mediated by the mitochondria. Apoptotic signals cause changes in the inner mitochondrial membrane that result in opening of the mitochondrial permeability transition (MPT) pore and release of certain mitochondrial proteins, which associate with and activate downstream components of the apoptotic machinery. These proteins can be classified into two groups according to their role and mode of action. The first group of proteins activates the caspase-dependent mitochondrial pathway and consists of cytochrome *c*, Smac/DIABLO and the serine protease Htr2/Omi. Cytochrome *c* binds and activates apoptosis-inducing factor (Apaf-1), and this complex recruits procaspase-9, forming the apoptosome complex (Jiang & Wang, 2004). Activated caspase 9 then cleaves and activates downstream caspases including caspases 3. Smac/DIABLO and HtrA2/Omi antagonize the anti-apoptotic activity of IAPs (Verhagen *et al.*, 2001). The second group of proteins, AIF, endonuclease G and CAD, are released from the mitochondria later during apoptosis, after the cell has committed to die. Following release from mitochondria, these nucleases are translocated into the nucleus and cleave nuclear DNA. AIF and endonuclease G both function in a caspase-independent manner (Susin *et al.*, 1999; Daugas *et al.*, 2000; Li *et al.*, 2001). These mitochondrial events are regulated by pro- and anti-apoptotic proteins, members of the Bcl-2 family. These proteins are important regulators of the mitochondrial apoptotic pathway and can determine if the cell commits to apoptosis or aborts the process.

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The following methods are widely used to determine apoptotic-like cell death in fungi:

- Accumulation of ROS can be detected by various oxidation-sensitive chemicals, which change their absorbance spectrum or emit fluorescence in the presence of ROS.
- Cleavage of nuclear DNA by Ca^{2+}- and Mg^{2+}-dependent endonucleases during apoptosis results in specific DNA fragmentation that can be visualized by gel electrophoresis. When separated on an agarose gel, the fragments form a 'DNA ladder,' which is considered a hallmark of apoptosis.
- DNA cleavage can also be detected by the terminal dUTP nick end-labeling (TUNEL) method, in which a terminal transferase is used to add flourescein-labeled UTP to the 3′-end of the DNA fragments, which can then be detected by fluorescence microscopy.
- Annexin V is a recombinant protein that interacts specifically and strongly with phosphatidylserine residues. Fluorescently labeled Annexin V is used to detect the externalization of phosphatidylserine on the outer leaflet of the plasma membrane during apoptosis. Membranes of necrotic cells are also labeled by Annexin V. To differentiate between necrotic and apoptotic cells, the Annexin V-positive cells are costained with membrane-impermeable nucleic acid dyes such as propidium iodide (PI), which are excluded from cells with intact membranes and therefore stain only necrotic cells.
- Changes in caspase activity can be detected in a number of ways. Modified caspase substrates are used, which, when cleaved by caspases, release a fluorescent product that can be detected and quantified.

Hallmarks of fungal apoptosis

Fungal apoptosis was overlooked for many years. Budding yeasts (and other fungi) were considered apoptotic-null systems for several reasons: lack of genetic evidence of homologs of known apoptotic genes, the assumption that PCD cannot exist in unicellular organisms and limited research interest in PCD in systems other than metazoans. The first indication of PCD in these organisms involved a temperature-sensitive mutant of *S. cerevisiae*, *cdc48*: under nonpermissive temperature, cells of this mutant exhibited various apoptotic markers, including chromatin condensation and nuclear fragmentation, and positive staining with Annexin V and TUNEL (Madeo *et al*., 1997). This discovery forced the rethinking of PCD in budding yeast and other systems, and signaled a new phase in apoptosis research in fungi as well as in other lower eukaryotes. Apoptotic-like PCD has since been demonstrated in a variety of organisms, including plants, fungi and protists (Maercker *et al*., 1999; Vardi *et al*., 1999; Al-Olayan *et al*., 2002; Jin & Reed, 2002). Certain types of PCD have also been demonstrated in bacteria (Ameisen, 1996; Engelberg-Kulka & Glaser, 1999; Lewis, 2000; Sat *et al*., 2001). It is now accepted that PCD is part of almost every form of life; however, the apoptotic apparatus varies considerably in its molecular components, regulation and the role it plays in different living systems. Some arguments remain as to whether PCD in fungi is indeed true apoptosis, and many researchers prefer the term apoptotic-like cell death. We agree with this terminology, mainly because the roles that PCD seems to play in fungi are different from those in mammals. For simplicity, in this review, we generally use the term apoptosis to refer to apoptotic-like fungal cell death.

Fungal apoptotic cells exhibit many of the morphological and biochemical changes seen in mammalian apoptosis, and therefore, the assays used to monitor fungal apoptosis are similar to those used in mammals. However, not all apoptotic markers are readily detected in fungi, and most methods require some modifications due to structural differences, particularly the presence of a cell wall. Differences also exist among fungi, especially between yeasts and filamentous species, due to their different morphologies. Morphological and cytological markers are very useful for monitoring apoptosis in yeast cells, because differences between cell populations can be quantified by comparing the number of apoptotic cells in each population (Narasimhan *et al*., 2001; del Carratore *et al*., 2002; Huh *et al*., 2002; Granot *et al*., 2003). The use of such markers is less obvious in filamentous species for a number of reasons: firstly, filamentous fungi are multicellular, making it difficult to quantify these changes. Secondly, there is a lack of uniformity within the mycelium: parts of the same colony might be of different age or at different developmental stages, and hyphae may have different morphologies. Furthermore, many fungi are multinucleated, and the nuclei are not always of uniform size, which makes nuclear shrinkage a less obvious measure in some cases. For example, in the multinucleate gray mold fungus *Botrytis cinerea*, nuclei range in size and exhibit high natural size and shape variability when stained with either 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst. Nuclear shrinkage and fragmentation is, therefore, a less reliable marker in this fungus and should be used with caution, in combination with other assays. To circumvent these limitations, the use of single-cell structures such as spores or protoplasts might be considered. These structures are more uniform and provide a means of obtaining quantitative data (Cheng *et al*., 2003; Mousavi & Robson, 2004; Leiter *et al*., 2005; Semighini *et al*., 2006a, 2008).

Biochemical markers of apoptosis, including ROS accumulation, DNA cleavage and externalization of phosphatidylserine, have also been used to detect apoptosis in fungi. An oxidative burst, which is represented by an accumulation of ROS, is an immediate and common response in fungal PCD. ROS levels can be estimated via reaction with a variety of chemicals. Commonly used compounds include

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dihydrorhodamine 123, dichlorodihydrofluorescein diacetate and dihydroethidium. The amount of ROS can be estimated by fluorescent microscopy, or quantified by fluorescence-activated cell sorting (FACS – in single cells) or by measuring relative fluorescence (in mycelia) (Madeo *et al.*, 1999; del Carratore *et al.*, 2002; Chen & Dickman, 2005; Kitagaki *et al.*, 2007).

A positive TUNEL assay result is taken as strong evidence for apoptosis in both yeasts and fungi (Madeo *et al.*, 1999; Cheng *et al.*, 2003; Mousavi & Robson, 2003; Chen & Dickman, 2005). DNA degradation can also be visualized by gel electrophoresis, but it usually results in a smear without distinct bands rather than the classical DNA ladder (Roze & Linz, 1998; Madeo *et al.*, 1999; Granot *et al.*, 2003; Mousavi & Robson, 2004; Kitagaki *et al.*, 2007). The lack of a clear DNA ladder in *S. cerevisiae* might be due to little or no linker DNA between the nucleosomes (Lowary & Widom, 1989). Apoptosis without the occurrence of a DNA ladder has also been described for several types of metazoan cells (Oberhammer *et al.*, 1993). Therefore, the lack of a classical DNA ladder in fungi should not hinder the use of DNA cleavage as a marker of fungal apoptosis. Apoptosis-associated DNA damage also involves specific cleavage of several RNA species (Degen *et al.*, 2000). Pulsed-field gel electrophoresis was recently used to show specific degradation of rRNA in apoptotic yeast cells (Mroczek & Kufel, 2008).

Annexin V staining of phosphatidylserine on the external plasma membrane has been widely used to show apoptosis in fungi (Madeo *et al.*, 1997; Mousavi & Robson, 2003; Phillips *et al.*, 2003; Leiter *et al.*, 2005; Semighini *et al.*, 2006a). Fungal cells cannot be directly stained with Annexin V because of their cell wall and must therefore be first treated with cell-wall-degrading enzymes to release protoplasts. This is a harsh process, which in itself can cause significant cell death. It also imposes many restrictions on the experimental system, and therefore, Annexin V staining is not always a viable option. One advantage of using protoplasts is that the cells can be counted by FACS, thus providing a quantitative measure of apoptosis (Baek *et al.*, 2004; Li *et al.*, 2006). Co-staining with PI must always be performed in order to discriminate between necrotic and apoptotic cells. Additional parameters that have been used to determine apoptosis in fungi include measurement of caspase activity (Huh *et al.*, 2002; Madeo *et al.*, 2002b; Mousavi & Robson, 2003; Silva *et al.*, 2005; Ito *et al.*, 2007; Kitagaki *et al.*, 2007) and changes in mitochondrial transmembrane potential (Ito *et al.*, 2007).

It should be remembered that determination of apoptosis is mostly circumstantial and that the changes detected by each of these assays may also reflect processes other than apoptosis, or may be an artifact of the system. For example, DNA fragmentation can also be associated with certain forms of necrosis (Collins *et al.*, 1992); some cell morphologies can be shared between apoptosis and other types of PCD, and detection of caspase activity *in situ* may result in artifacts (Vachova & Palkova, 2007). It is therefore important to use multiple assays, which detect different parameters of apoptotic cell death, in order to properly distinguish apoptosis from other types of fungal cell death. In addition, other types of cell death might have somewhat different phenotypes, particularly autophagy, which represents a unique type of PCD.

Autophagy

Fungal autophagy is a specific type of PCD (also known as type II PCD) that differs from apoptotic cell death in several features and has different roles. Recent studies have shown an association between autophagy and several processes in fungi, including development and pathogenesis. Here, we briefly describe fungal autophagy. For a comprehensive review of this subject, the reader is referred to a recent review by Pollack *et al.* (2009).

Autophagy is a ubiquitous, nonselective degradation process in eukaryotic cells. Autophagic cell death is characterized by the degradation of cytoplasmic components before nuclear collapse, unlike apoptosis in which the order of these events is reversed (Clarke, 1990). One of the hallmarks of autophagy is the dynamic rearrangement of membranes, which leads to the formation of autophagosomes (Baba *et al.*, 1994). Autophagy genes act in a conjugation cascade controlling the process's initiation and execution: these molecular processes have been found to be highly conserved from yeasts to humans (Reggiori & Klionsky, 2002). In *S. cerevisiae*, autophagy is associated with differentiation, development (e.g. reproduction and spore germination) and stress responses (reviewed in Levine & Klionsky, 2004). Yeast autophagy is connected with nitrogen starvation and pseudohyphal growth: both pseudohyphal growth and autophagy are induced under nitrogen depletion, whereas inhibition of autophagy results in increased pseudohyphal growth (Gimeno *et al.*, 1992). Furthermore, autophagy-deficient yeast strains induce pseudohyphal growth at higher nitrogen concentrations. These results suggest a model in which autophagy mitigates nutrient stress and delays the onset of pseudohyphal growth (Ma *et al.*, 2007).

Autophagy in filamentous fungi is typically induced by carbon and nitrogen starvation (Dementhon *et al.*, 2003; Pinan-Lucarré *et al.*, 2005). Nutrient recycling through autophagy is the first type of starvation-induced PCD before induction of more detrimental cell-death processes such as cellular degradation, autolysis and apoptotic cell death (Levine & Klionsky, 2004; Emri *et al.*, 2005; Yorimitsu & Klionsky, 2005; Richie *et al.*, 2007a). Autophagy is also associated with growth, morphogenesis and development, and it has been suggested to play a role in protection from cell death (Pollack *et al.*, 2009). Fungal autophagy is typically

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accompanied by rapid hypervacuolization and vacuole enlargement (Pollack *et al.*, 2008). Similar to other systems, fungal autophagy is characterized by the presence of autophagosomes and is controlled by target of rapamycin kinase.

Two authophagy genes have been studied in fungi, orthologs of yeast *ATG1* and *ATG8*. Defects in these autophagy genes influence morphogenesis and development. For example, *Magnaporthe oryzae mgatg1* or *mgatg8* deletion mutants show reduced aerial hyphae, disrupted conidiation and delayed germination (Liu *et al.*, 2007). Mutants in these autophagy genes, or their deletion, also exhibit reduced formation of sexual reproductive organs, such as protoperithecia in *Podospora anserina* and perithecia in *M. oryzae* (Pinan-Lucarré *et al.*, 2005; Liu *et al.*, 2007). In *M. oryzae*, deletion of the *MgATG8* gene prevented the spore-cell death that is necessary for completion of the early stages of plant infection (Veneault-Fourrey *et al.*, 2006). Although appressoria were produced, they could not penetrate the plant cuticle and the *mgatg8* mutant strains were completely nonpathogenic. *Colletotrichum lindemuthianum clk1* (homolog of *ATG1*) deletion mutants ware also defective in cuticle penetration (Dufresne *et al.*, 1998). Thus, autophagy-mediated cell death might be necessary for plant pathogenesis in these species. In *P. anserina*, autophagy is induced early on in the incompatibility (cell death) reaction. Knockout of the *ATG1* ortholog, which is essential for autophagosome formation in *P. anserina*, prevented autophagy; however, neither cell death nor vacuolization were affected, suggesting that in this species, these processes are autophagy-independent (Pinan-Lucarré *et al.*, 2005).

Developmentally regulated fungal apoptosis

Reproduction

Suicidal cell death has very different roles in different biological systems. The main role of apoptosis in mammals is regulation of normal development, although it is also associated with various other processes, including adaptive responses of cells to stress and elimination of pathogens. In fungi, apoptotic cell death is more tightly connected to stress adaptation, while involvement in development is less general and is mainly associated with various aspects of fungal reproduction and aging. We will first review the evidence connecting apoptosis with fungal development, particularly during reproduction and aging.

Fungi are capable of sexual (meiotic) and asexual (mitotic) reproduction. While most species can reproduce sexually, some seem to have lost the ability to mate and are considered asexual (traditionally classified as Deuteromycetes). When mating is possible, it can be of two basic types: homothallic species (capable of self-fertilization) and heterothallic species (non-self-fertilizing). Reproduction in heterothallic species is initiated by pheromones, which trigger the fusion of hyphae from sexually compatible strains. In *S. cerevisiae*, there are two mating types termed ‘a’ and ‘α’. Cells of each mating type produce and secrete either ‘a’ or ‘α’ factors (pheromones), which trigger mating in cells of the opposite mating type. Severin & Hyman (2002) showed that in the absence of an appropriate mating partner, exposure of cells to pheromones of the opposite mating type leads to ROS accumulation, DNA degradation and cell death. As might be expected, deletion of STE20 kinase (a key enzyme in the pheromone-induced MAP kinase signal cascade) prevented this pheromone-induced cell death. The authors further demonstrated ROS-associated cell death in cell populations, which was in opposite correlation to mating success. These findings were taken as evidence for the possible occurrence of apoptotic cell death during unsuccessful mating in natural yeast populations. It should be noted, however, that pheromone-induced cell death was observed at pheromone concentrations that were 10-fold higher than physiological concentrations; no cell death was induced by 10-fold lower (physiological) concentrations, which were sufficient for shmoo induction.

In filamentous fungi, vegetative hyphae commonly fuse. These hyphal fusions occur during colony formation as well as between hyphae of different strains as part of parasexual reproduction (Saupe, 2000; Glass & Kaneko, 2003; Glass & Dementhon, 2006). The fusion between hyphae from different strains forms a heterokaryon, a situation in which cells contain nuclei of different genetic backgrounds. Fungi have evolved specific heterokaryon-incompatibility (HI) loci, which determine hyphal-fusion compatibility (Leslie & Zeller, 1996; Glass *et al.*, 2000). Hyphae must be vegetatively compatible with each other in order to sustain the heterokaryon. When they are incompatible, the HI genes activate a rapid, localized cell-death response, which specifically kills the fusion cell (Glass & Kaneko, 2003). In many ways, HI resembles the well-known hypersensitive response (HR) in plants, during which localized apoptotic-like cell death prevents pathogen spreading (Lam *et al.*, 2001). Both HI and HR are accompanied by classical apoptotic markers and have been widely studied (del Pozo & Lam, 1998; Jacobson *et al.*, 1998; Glass *et al.*, 2000; Saupe, 2000; Marek *et al.*, 2003; Glass & Dementhon, 2006; Paoletti & Clave, 2007; Williams & Dickman, 2008). During HI, the fusion hyphae undergo a series of apoptosis-associated morphological changes, including cytoplasm condensation, vacuolization and shrinkage of the plasma membrane (Glass & Kaneko, 2003; Marek *et al.*, 2003; Glass & Dementhon, 2006). Nuclear fragmentation and positive TUNEL staining have also been reported. The widespread occurrence and high number of HI loci in filamentous fungi argues for their importance. Unlike the case of yeast pheromones, apoptosis is a general phenomenon of HI and occurs naturally.

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Therefore, HI demonstrates an important process in which apoptosis plays a major role.

Apoptotic-like cell death has been associated with spore formation, both sexual and asexual (conidia). In some homothallic species, the immature asci contain eight ascospores, but four of them die during maturation, resulting in four-spore asci. Studies in *Coniochaete tetrasperma* showed that PCD occurs during spore maturation, eliminating four of the eight ascospores (Raju & Perkins, 2000). Cytological markers of apoptosis were observed in meiotic mutants of *Coprinopsis cinereus* (syn. *Coprinus cinereus*). In these mutants, the nuclei in immature basidiospores arrested after meiotic metaphase I and then underwent apoptotic-like cell death (Lu *et al.*, 2003). Unlike in *C. tetrasperma*, in which apoptosis occurs during normal development of the wild-type spores, here this process served to eliminate mutant spores. In *P. anserina*, deletion of the two metacaspase genes caused defects in ascospore formation (Hamann *et al.*, 2007). Overexpression of the anti-apoptotic Bcl-2 gene in *Colletotrichum gloeosporioides* caused a sharp increase in conidial production (Barhoom & Sharon, 2007). These last two examples, although circumstantial, suggest that changes in the regulation of apoptosis can affect sexual and asexual sporulation. In *Aspergillus nidulans*, apoptotic markers and induction of caspase activity were observed during sporulation (Thrane *et al.*, 2004). Caspase activity was shown by hydrolysis of substrates specific for caspase-3- and -8-like activities. These activities were repressed by the caspase-3- and -8-specific irreversible peptide inhibitors, but were not affected by the nonspecific inhibitor E-64. *Aspergillus nidulans* extract contained two proteins that revealed caspase-like activity: one of them degraded both caspase-3- and -8-specific substrates, whereas the other only degraded the caspase-8 substrate. Two metacaspases and a single poly-ADP ribose polymerase (PARP)-like protein were identified in *A. nidulans*. The PARP-like protein was detected in mycelia until the start of conidial formation, and it was then degraded along with increased caspase activity (Thrane *et al.*, 2004).

These examples show the involvement of apoptosis in fungal reproduction. It can occur during both meiotic and mitotic spore formation, during normal development and in cases of abnormalities, in which apoptosis might serve as a control mechanism by eliminating the genetically damaged spores. In these cases, apoptosis appears to be closely associated with the cell cycle and might be triggered by improper regulation of cell-cycle progression (Madeo *et al.*, 1997; Lu *et al.*, 2003).

Aging

Aging is a process of progressive decline in the ability to withstand stress, damage and disease. In multicellular organisms, apoptosis has been documented as an anti-aging mechanism. Apoptosis eliminates damaged cells by the coordinated activity of gene products that regulate cell death and induce cell proliferation, so that the old cells are replaced. ROS-induced cell damage is a major process in aging and there is a correlation between extended life span and increased oxidative resistance (Lorin *et al.*, 2006). *Saccharomyces cerevisiae* and *P. anserina* are two model organisms that have been used to study aging. In *S. cerevisiae*, cells bud a limited number of times, producing a daughter cell each time. The measure of yeast's life span is thus the number of divisions of a mother cell before it dies (Mortimer & Johnston, 1959). In this replicative aging, life span is not correlated with chronological time and it is not directly affected by nutrient availability. Another type of aging in budding yeasts is chronological aging, in which cells that are still reproductively young undergo a process of senescence due to lack of nutrients in the stationary phase. The chronological life span of *S. cerevisiae* can be measured by monitoring the mean and maximum survival times of populations of postmitotic cells, which is analogous to the way life span is monitored in metazoan organisms (Longo *et al.*, 1996; Steinkraus *et al.*, 2008). Appearance of apoptotic markers and elevated caspase activity were detected in *S. cerevisiae* during both types of aging (Laun *et al.*, 2001; Buttner *et al.*, 2006). *Yca1* is the only metacaspase gene known in *S. cerevisiae*; however, enhanced caspase activity has been reported in aged *yca1*Δ cultures, suggesting the involvement of additional caspase-related proteases in chronological aging (Herker *et al.*, 2004). The old cells release substances into the medium that promote colony growth and survival. Apoptotic cell death was observed in the center of a colony in which chronologically mature cells died while releasing materials such as ammonia that enabled young cells at the periphery of the colony to escape death (Herker *et al.*, 2004; Vachova & Palkova, 2005). Increased cell densities and changes in culture conditions also lead to apoptosis and might cause cell death in aged cultures. However, genetic evidence from mutant and transgenic strains shows that aging-related apoptosis occurs independently of environmental conditions; blocking of apoptotic cell death by knockout of pro-apoptotic genes or by overexpression of anti-apoptotic genes often leads to extended chronological as well as replicative life span in yeast (Mortimer & Johnston, 1959; Madeo *et al.*, 2002b; Fabrizio *et al.*, 2004; Herker *et al.*, 2004; Li *et al.*, 2006). Together, these studies confirm the association of apoptosis with yeast aging and suggest that it is an essential component of an 'aging program' that blocks cell protection and accelerates death, thereby regulating life span in budding yeasts (Fabrizio *et al.*, 2004; Herker *et al.*, 2004; Fabrizio & Longo, 2008). The existence of such a program may seem unlikely in a unicellular organism. Nevertheless, there are several possible

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explanations for such a phenomenon: (1) aging yeasts accumulate mutations and therefore must be removed from the culture. Indeed, DNA mutations can lead to accumulation of ROS and other apoptotic markers (Madeo *et al*., 2002a, b; Huang *et al*., 2003; Fabrizio *et al*., 2004; Herker *et al*., 2004; Li *et al*., 2006); (2) in the context of a large population, the 'suicide' response represents a survival strategy for the group in which dying cells are recycled and provide nutrients to the remaining population. After most of the population is dead, growth can resume (Fabrizio *et al*., 2004).

In *P. anserina*, senescence is characterized by an age-related decrease in mycelium growth rate, reduction in aerial hypha formation, increased pigmentation and eventual death of peripheral hyphae (Albert & Sellem, 2002). At the microscopic level, the peripheral hyphae show abnormal branching and swelling. There is a correlation between the aging process and accumulation of mutated mtDNA leading to mitochondrial genome instability (Osiewacz & Borghouts, 2000; Albert & Sellem, 2002). Strains selected for increased life span were found to be deficient in cytochrome *c* oxidase (COX) activity as they carried deletions of the first exon of the *COX1* gene. Deletion of the *COX5* gene (encoding subunit V of COX) resulted in a severe decrease in growth rate but a 30-fold increase in life span, decreased ROS production and a drastic reduction in the rearrangement of mtDNA (Dufour *et al*., 2000). Mutants with deletions in genes encoding the other COX subunits had similar phenotypes (Lorin *et al*., 2006). In these mutants, respiration was carried out via alternative oxidase-dependent pathways, producing approximately one-third of the energy generated by the cytochrome pathway. Genetic manipulation that restored ROS production to wild-type levels also restored the mutated mtDNA and led to decreased life span, indicating that ROS damaged mtDNA directly or via oxidation of cell-cycle-related proteins. Deletion of a gene encoding mitochondrial fission factor (*PaDNM1*) increased fungal life span and resistance to the apoptosis-inducing compound etoposide, further demonstrating the central role of mitochondrion-mediated apoptosis in the aging of this fungus (Scheckhuber *et al*., 2007). Collectively, these results indicate that increased ROS levels during aging trigger mitochondrion-dependent PCD in senescent cultures of *P. anserina*.

Induced fungal apoptosis

Physical and chemical stress

Several lines of evidence connect apoptosis with stress responses in fungi. Although triggered by different stimuli, stress responses are connected with the genetic program for aging and both pathways seem to share common components, which eventually lead to apoptotic cell death. Similar to aging, stress responses are usually initiated by an oxidative burst, which, depending on the conditions, can lead to either increased stress resistance or initiation of apoptotic cell death. Conditions that induce fungal apoptosis include various types of stress, such UV or oxidative stress, treatment with broad-spectrum elements such as salts and acids, or challenge with specific compounds, including known antifungal agents. While induced apoptosis has been described in several species, most of the available information still comes from studies in *S. cerevisiae*. Examples of conditions and chemicals that induce fungal and yeast apoptosis are summarized in Table 1.

Induction of apoptotic cell death by oxidative conditions has been reported in several fungi, including *S. cerevisiae*, *Candida albicans* and *Aspergillus fumigatus* (Madeo *et al*., 1999; Phillips *et al*., 2003; Mousavi & Robson, 2004). Treated cells of these fungi exhibited DNA fragmentation, as reflected by TUNEL staining and DNA gel electrophoresis (*S. cerevisiae* and *A. fumigatus*). Staining-treated *S. cerevisiae* cells with DAPI showed nuclear condensation. Chromatin condensation and nuclear degradation were detected in *S. cerevisiae* and *C. albicans* by transmission electron microscopy (TEM) following H_2O_2 treatment. Positive Annexin V and negative PI staining were observed in *A. fumigatus* and *C. albicans*. Pretreatment of *S. cerevisiae* and *A. fumigatus* with cycloheximide blocked H_2O_2-induced apoptotic phenotypes. Above a certain threshold concentration of H_2O_2, the amount of apoptotic cells decreased along with an increase in the number of necrotic cells. Taken together, these analyses show an orderly execution of apoptotic-like cell death in these fungi, following oxidative stress. Importantly, however, there was no increase in caspase activity during H_2O_2-induced cell death in *A. fumigatus*, and pretreatment with the broad-spectrum caspase inhibitor Z-VAD-FMK did not affect the amount of TUNEL-positive cells. Thus, in *A. fumigatus*, H_2O_2-induced apoptosis is probably caspase-independent.

UV irradiation induces apoptosis in mammalian cells (Gottlieb *et al*., 1996; Zhai *et al*., 1996) and has a similar effect in budding yeasts. Exposing *S. cerevisiae* to UV irradiation resulted in cell death with apoptotic characteristics (del Carratore *et al*., 2002). TUNEL-positive cells were detected after UV treatment and the amount of TUNEL-positive cells increased in a dose-dependent manner, peaking at $120\,J\,m^{-2}$. Although higher doses resulted in greater loss of viability, the amount of TUNEL-positive cells declined, suggesting a shift from apoptosis to necrosis. FACS analyses revealed an increased number of cells with sub-G1 DNA content, in correlation with the TUNEL results. TEM investigation of UV-irradiated sub-G1 cells revealed that most of them contained condensed chromatin and vacuolization, which increased over time. UV-induced mortality in *C. gloeosporioides* was reduced in Bcl-2-expressing strains, which are also protected from other stresses (Barhoom & Sharon, 2007).

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Table 1. Conditions and compounds that induce fungal apoptosis

Treatment	Species	Cas*	Description	References
Stresses				
Oxidative	*S. cerevisiae*	NR	H_2O_2 and depletion of glutathione-induced apoptosis	Madeo *et al*. (1999)
	C. albicans	NR	H_2O_2-induced apoptotic cell death in both species	Phillips *et al*. (2003, 2006)
	A. fumigatus	Ind		Mousavi & Robson (2004)
UV	*S. cerevisiae*	NR	Exposure to UV resulted in apoptotic-like cell death	del Carratore *et al*. (2002)
Heat	*S. cerevisiae*	NR	Heat stress caused apoptotic cell death. The effect was mediated by RAS1 and RAS2	Shama *et al*. (1998)
Osmotic	*S. cerevisiae*	Dpn	High glucose or sorbitol concentrations induced apoptotic cell death	Silva *et al*. (2005)
Nutrients				
Amino acids	*S. cerevisiae*	NR	Starvation for lysine or histidine led to apoptotic cell death	Eisler *et al*. (2004)
Carbon	*A. nidulans*	NR	Carbon starvation caused apoptosis in both fungi	Emri *et al*. (2005)
	N. crassa	NR		Jacobson et al. (1998)
Glucose	*S. cerevisiae*	NR	Glucose induces cell death in the absence of nutrients to support growth	Granot *et al*. (2003)
Ethanol	*S. cerevisiae*	Dpn	Treatment with up to 23% ethanol reduced survival along with apoptotic markers	Kitagaki *et al*. (2007)
Acids/salt/ions				
Acetic acid	*S. cerevisiae*	NR	Treatment with low concentrations of acetic acid induced	Ludovico *et al*. (2001)
	C. albicans	NR	apoptosis both species	Phillips *et al*. (2003, 2006)
Valpuric acid	*S. cerevisiae*	Dpn	Low concentration of valpuric acid induced cell death with apoptotic markers	Mitsui *et al*. (2005)
Formic acid	*S. cerevisiae*	Ind	Formic acid induced Yca1p-independent apoptosis-like cell death	Du *et al*. (2008)
NaCl	*S. cerevisiae*	NR	Exposure to 1.5 M NaCl inhibited growth and promoted	Huh *et al*. (2002)
		Dpn	apoptotic cell death	Wadskog *et al*. (2004)
Copper	*S. cerevisiae*	Ind	Cu^{2+} ions caused a time- and concentration-dependent apoptotic cell death. The response was ROS-dependent	Liang & Zhou (2007)
Manganese	*S. cerevisiae*	Dpn	In Mn^{2+}, ions the response was ROS-independent	Liang & Zhou (2007)
Cadmium	*S. cerevisiae*	Dpn	Low cadmium concentrations induced a glucose-dependent apoptosis	Nargund *et al*. (2008)
Arsenic	*S. cerevisiae*	Dpn	Arsenic caused apoptosis, which was abolished in *yca1*Δ strain.	Du *et al*. (2007)
Antifungal				
AmB	*A. fumigatus*	Ind	AmB induced a dose-dependent apoptotic cell death in *A. fumigatus*	Mousavi & Robson (2004)
	C. albicans	NR	AmB induced apoptotic cell death in *C. albicans*	Phillips *et al*. (2003)
Osmotin	*S. cerevisiae*	NR	The tobacco antifungal protein osmotin induced an ROS-dependent apoptotic cell death	Narasimhan *et al*. (2001)
α-Tomatin	*F. oxysporum*	Dpn	α-Tomatin induced an ROS-dependent apoptotic cell death	Ito *et al*. (2007)
PHS/DHS	*N. crassa*	NR	PHS induced apoptosis-like cell death	Castro *et al*. (2008)
	A. nidulans	Ind	PHS and DHS induced ROS-independent apoptosis	Cheng *et al*. (2003)
Lovastatin	*M. racemosus*	NR	Lovastatin induces apoptotic cell death in both species	Roze & Linz (1998)
	C. gloeosporioides	NR		Barhoom & Sharon (2007)
Farnesol	*S. cerevisiae*	NR	Farnesol-treated cells exhibited reduced growth rate and apoptotic markers	Machida *et al*. (1998), Machida & Tanaka (1999)
	A. nidulans	NR	Farnesol induced growth arrest and ROS-mediated apoptosis in	Semighini *et al*. (2006a)
	F. graminearum	NR	both species	Semighini *et al*. (2008)
PAF	*A. nidulans*	NR	PAF induces ROS-mediated apoptotic cell death	Leiter *et al*. (2005)
Killer toxin	*S. cerevisiae*	Dpn	Killer toxin caused apoptosis in susceptible *S. cerevisiae* cells	Reiter *et al*. (2005), Mazzoni & Falcone (2008)
Developmental				
Pheromone	*S. cerevisiae*	NR	α-Factor pheromone induced apoptotic cell death in population of a-type cells	Severin & Hyman (2002)
Stationary	*A. fumigatus*	Dpn	Apoptotic cell death appeared in hyphae during stationary phase	Mousavi & Robson (2003)

*Involvement of caspase: Dpn, caspase-dependent; Ind, caspase-independent; NR, not reported.
UV, ultraviolet; PSH, phytosphingosine; DHS, dihydrosphingosine; PAF, an antifungal protein from *Penicillium chrysogenum*.

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Stationary cultures of *A. fumigatus* developed apoptotic markers along with increased activities of caspase-1 and -8, followed by loss of membrane integrity and cell viability (Mousavi & Robson, 2003). Addition of cycloheximide or the caspase inhibitor Z-VAD-FMK to cultures before transition to the stationary phase blocked apoptotic cell death, suggesting that this process is caspase-dependent and requires *de novo* protein synthesis. Cell death induced by physical damage was not affected by cycloheximide or Z-VAD-FMK, further supporting the induction of apoptosis upon entry into the stationary phase. Various other stimuli that induce fungal apoptosis have been reported (Hamann *et al.*, 2008; Ramsdale, 2008). In *S. cerevisiae*, hyperosmotic stress caused by high glucose or sorbitol was associated with increased caspase activity. Sensitivity to hyperosmotic conditions was reduced in *yca1*Δ cells, suggesting activation of a caspase-dependent pathway (Silva *et al.*, 2005). Similar effects were observed by exposure of cells to high salt (1.5 M NaCl) concentrations (Huh *et al.*, 2002), which can be attributed to either the osmotic stress or toxic effects induced by the salt. Enhanced caspase activity was monitored in NaCl-treated cells, and *yca1*Δ cells exhibited reduced sensitivity to high salt concentrations, consistent with a caspase-mediated response (Wadskog *et al.*, 2004). Low concentrations of acetic acid and other weak acids can also induce apoptosis in fungi (Ludovico *et al.*, 2001; Phillips *et al.*, 2003). Yeast cells incubated with a low concentration of valpuric acid showed various apoptotic markers, including exposure of phosphatidylserine and ROS accumulation (Mitsui *et al.*, 2005). This response appeared to be caspase-dependent because *yca1*Δ cells were less sensitive to the treatment and showed reduced TUNEL and Annexin V staining. The *yca1*Δ mutant cells stained positive with dihydroethidium, indicating that ROS generation was an early response, upstream of Yca1p. Induction of yeast apoptosis by Cu^{+2} and Mn^{+2} ions was reported recently. Interestingly, the response to copper ions was ROS-dependent but caspase-independent, while the response to manganese ions did not involve ROS but was caspase-dependent (Liang & Zhou, 2007). As can be seen from these examples, most of the stimuli that induce cell death in *S. cerevisiae* are caspase-dependent.

Specific compounds

Most compounds with antifungal activity impair essential processes, mainly cell wall and cell membrane integrity. The mode of action of these compounds has been postulated from their primary target site, which in many cases is associated with inhibition of ergosterol biosynthesis. For example, polyenes are assumed to kill fungal cells by forming pores in the plasma membrane due to their high affinity for fungal ergosterol. Other common drugs inhibit processes that are essential to cell wall biosynthesis. For example, echinocandins inhibit β-1,3-glucan synthase, which participates in the synthesis of cell wall glucans. The recognition of apoptosis as a viable and important process in fungi has led to a re-evaluation of the mode of action of leading antifungal compounds. Recent studies have shown that some compounds actually induce apoptotic cell death in the treated pathogen, raising the possibility that these compounds' antifungal activity might be mediated by induced apoptosis. A range of additional compounds, including antifungal proteins, have been shown to induce apoptosis in fungi. Here, we describe examples of compounds of different origin and chemical nature that have been found to induce apoptotic cell death. However, the number of compounds known to induce PCD in fungi is much larger. For a more comprehensive list, the reader is referred to a recent review on the topic (Ramsdale, 2008).

The polyene amphotericin B (AmB) has been used to treat fungal infections in humans for over 30 years (Bratjburg *et al.*, 1990). Similar to other polyene antibiotics, AmB has high affinity to sterols, particularly ergosterol. It has been accepted that AmB antifungal activity is due to interaction with ergosterol in the plasma membrane, which causes pore formation and distortion of cell integrity (Liao *et al.*, 1999). More recently, it was shown that AmB induces apoptotic-like cell death in fungi. *Aspergillus fumigatus* treated with AmB showed dose-dependent TUNEL and Annexin V staining (Mousavi & Robson, 2004). Notably, above 1 $\mu g\, mL^{-1}$ AmB, cell death shifted from apoptotic to necrotic, as determined by an increase in PI-positive and decrease in TUNEL-positive cells. The appearance of apoptotic markers could not be blocked or reduced by caspase inhibitors, nor were any changes recorded in caspase activity, suggesting a caspase-independent cell-death process. Induction of apoptotic-like cell death by AmB has also been reported in *C. albicans* (Phillips *et al.*, 2003). Markers used to determine apoptosis in this fungus included ROS accumulation and nuclear fragmentation. Possible involvement of caspases was not determined in that study. Additional antifungal drugs of different chemical groups and primary targets have been reported to induce apoptotic cell death in several fungi, suggesting that induced PCD might be a common mode of action (Ramsdale, 2008).

The induction of apoptosis by AmB might be due to the release of membrane components, particularly sphingolipids. Sphingolipid metabolism is associated with a wide range of cellular activities, including stress response, apoptosis, inflammation, cell-cycle regulation and cancer development (Dickson, 1998; Kolesnick & Kronke, 1998; Hannun & Luberto, 2000; Hannun *et al.*, 2001). Two major sphingoid bases of fungi – dihydrosphingosine and phosphingosine – induced ROS accumulation and cell death with typical markers of apoptosis in *A. nidulans* (Cheng *et al.*, 2003).

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Pretreatment of protoplasts with ROS scavengers prevented ROS accumulation but had no effect on the appearance of other apoptotic markers, suggesting an ROS-independent response. Induction of apoptosis by dihydrosphingosine was also metacaspase-independent, because there was no difference in apoptotic cell death of a *casA*Δ metacaspase-deleted mutant. However, this conclusion requires further validation because *A. nidulans* has another metacaspase gene (*CasB*), which might also be involved. Interestingly, dihydrosphingosine-induced cell death has been shown to mediate phytotoxicity of the fungal-produced AAL toxin. The AAL toxin, produced by *Alternaria alternata*, belongs to a class of host-selective fungal mycotoxins that are structurally related to sphinganine, a precursor in plant sphingolipid biosynthesis. AAL toxin kills the cells of sensitive host plants by inducing apoptotic cell death (Brandwagt *et al*., 2000). Administration of AAL toxin to sensitive tissues blocks sphingolipid biosynthesis and leads to accumulation of dihydrosphingosine. AAL-insensitive plants contain the *ASC-1* resistance gene, a homolog of the yeast longevity assurance gene (*LAC1*). Asc1p modifies sphingolipid metabolism in AAL-treated cells, thereby preventing accumulation of dihydrosphingosine and induction of apoptosis (Brandwagt *et al*., 2000; Spassieva *et al*., 2002).

Manipulation of plant apoptosis is a strategy used by pathogens to weaken and accelerate cell death in the host plant (Sharon & Finkelshtein, 2008). New evidence supports the possibility that plants might exploit fungal apoptosis in order to block pathogen invasion by secreting apoptosis-inducing antifungal compounds. The tobacco pathogenesis-related protein osmotin induces apoptotic cell death in *S. cerevisiae* (Narasimhan *et al*., 2001). This induced cell-death activity is ROS-dependent, because blocking ROS production rescued cells from osmotin-induced death. Although, so far, osmotin induction of apoptosis has only been demonstrated in *S. cerevisiae*, additional antifungal peptides are known from other organisms that can induce apoptotic cell death in different fungi (Ramsdale, 2008). The plant saponin α-tomatin is an antifungal sesquiterpene glycoside produced by tomatoes. α-Tomatin was thought to promote fungal death by disruption of membrane integrity (Friedman, 2002). However, it was recently shown that it induces apoptotic cell death in the plant pathogen *Fusarium oxysporum* (Ito *et al*., 2007). These authors also showed that apoptosis was necessary for the antifungal activity of this compound: blocking *de novo* protein synthesis using cycloheximide reduced apoptotic cell death in a dose-dependent manner. In addition, the fungicidal action of α-tomatin was suppressed by the mitochondrial electron transport inhibitor oligomycin, suggesting a role for mitochondria in the process. ROS scavengers (ascorbic acid and dimethylthiourea) and inhibition of caspases (using Z-VAD-FMK) reduced cell death in a dose-dependent manner, suggesting that α-tomatin-induced cell death in *F. oxysporum* is ROS- and caspase-dependent.

A relatively large number of fungally produced compounds, both secondary metabolites and peptides, can cause apoptotic cell death in fungi. Lovastatin is a secondary metabolite produced by several filamentous fungi. It is known for its inhibitory effect on 3-hydroxy-3-methylglutaryl-CoA reductase and is widely used as a cholesterol-lowering drug. In addition, lovastatin triggers apoptosis in human cell lines by disturbing prenylation-dependent signal-transduction pathways (Wu *et al*., 2004; Shellman *et al*., 2005). In one of the earliest reports on fungal apoptosis, Roze & Linz (1998) showed induction of apoptotic cell death by lovastatin in the mold *Mucor racemosus*. Later studies showed similar effects in *C. gloeosporioides* (Barhoom & Sharon, 2007) and *B. cinerea* (I. Hatam & A. Sharon, unpublished data). Lovastatin-induced apoptosis in *C. gloeosporioides* was reduced in transgenic strains expressing the anti-apoptotic Bcl-2 gene, supporting an apoptotic-based cell death. The isoprenoid farnesol is a quorum-sensing molecule secreted by *C. albicans*. It inhibits the yeast-to-hypha transition in dense cultures without restricting growth of the fungus (Hornby *et al*., 2001). In other fungi, farnesol induces apoptosis. Yeast cells treated with farnesol exhibited reduced growth rate, ROS accumulation and hyperpolarization of mitochondrial membrane potential (Machida *et al*., 1998; Machida & Tanaka, 1999). In *A. nidulans* and *Fusarium graminearum*, farnesol caused growth arrest and the appearance of apoptotic markers, including ROS accumulation, phosphatidylserine externalization and rapid DNA condensation and fragmentation (Semighini *et al*., 2006a, 2008). PAF is a cysteine-rich antifungal protein secreted by *Penicillium chrysogenum*. The protein is actively internalized by sensitive fungi and is inhibitory to various plant and zoopathogenic fungi (Kaiserer *et al*., 2003). In *A. nidulans*, PAF induced ROS-mediated apoptotic cell death accompanied by hyperpolarization of the plasma membrane, phosphatidylserine externalization and accumulation of DNA-strand breaks (Leiter *et al*., 2005). As can be seen from these examples, most compound-induced apoptotic cell death in fungi is caspase-dependent.

While until recently, even the mere existence of apoptosis in fungi was questionable, the above examples clearly show that it is a general process in all studied species, leaving little doubt as to the significance of apoptotic cell death in fungi. These studies substantiate apoptosis as an essential process that is associated with fungal development, defense and stress adaptation. The abundance of fungal-produced compounds that induce apoptosis in other fungi indicates that it might be a general strategy used by fungi to protect themselves from pathogenic and competing species. The recognition of apoptotic cell death as a central regulator of

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fungal lifestyle makes it an attractive target for antifungal therapies and provides novel pathways to manipulate fungi, for example for improved longevity and biomass production. However, molecular information on the regulation of apoptosis in fungi is limited and further research is needed to discover and analyze the regulatory genes and proteins involved. In the following sections, we review the genomic and molecular information available on apoptotic fungal genes.

The fungal apoptotic network

As mentioned already, apoptotic pathways in fungi seem to be mitochondrion-dependent, and can be either caspase-dependent or independent. Similar to the situation in metazoans, ROS accumulation is a common, although not obligatory response, as apoptosis induced by some stimuli is ROS-independent. Thus, the general characteristics of fungal apoptosis resemble the intrinsic mammalian pathway (see Box 1). However, because the lifestyle of fungi is fundamentally different from that of mammals, apoptosis in these organisms has evolved to fulfill different roles and is expected to be regulated in different ways. Thus, while the core apoptotic machinery in fungi and higher organisms might be similar, other components are likely to be different. Indeed, database searches of the available fungal genomes reveal putative homologs of apoptotic genes in yeasts and fungi. Identified genes are homologs of mitochondrion-associated regulators of apoptosis, such as the mitochondrion-secreted proteins cytochrome *c* and Omi/HtrA2, and downstream components such as metacaspases and inhibitors of apoptosis [inhibitor of apoptosis proteins (IAPs)] (Fig. 1). Significantly, a caspase-independent pathway appears to be highly conserved in fungi, and known elements of this pathway have been identified in both yeast and fungal genomes (Halestrap, 2005). More than 50 putative human and mouse PCD-associated genes have been described in *Aspergillus* (Fedorova *et al*., 2005). In addition to conserved core components of the metazoan apoptotic machinery, this list includes many fungus-specific genes, such as *het* loci, and species-specific protein families. Interestingly, some of these proteins, such as Apaf1 (involved in apoptosome formation) and PARP, are not represented in *S. cerevisiae*.

Close examination of putative fungal apoptotic genes reveals that in most cases, they are not true homologs of their mammalian counterparts. Homology between fungal and mammalian apoptotic proteins usually centers on specific domains within the protein, but otherwise there is high sequence divergence between fungal genes and their mammalian orthologs (Fig. 2). Mammalian apoptotic proteins may contain several different domains, whereas only a single type of apoptotic domain is often present in corresponding fungal orthologs (Reed *et al*., 2004). Furthermore, in most cases, the fungal domain is similar, but not identical, to the domain present in mammals. For example, human caspases are synthesized as inactive zymogens that consist of an N-terminal prodomain, followed by a large subunit of about 20 kDa and a small subunit of about 10 kDa. Two types of caspases are distinguished: initiator caspases, which are activated by apoptotic stimuli, and effector caspases, which upon activation by initiator caspases cleave the so-called death substrates. The initiator caspases are characterized by a long prodomain comprising a caspase-recruitment domain (CARD) or death-effector domain (DED), which are necessary for their interaction with adaptor proteins that mediate caspase heterodimerization (Earnshaw *et al*., 1999; Salvesen, 2002; Fuentes-Prior & Salvesen, 2004). Caspases specifically cleave after an aspartate residue located in the P1

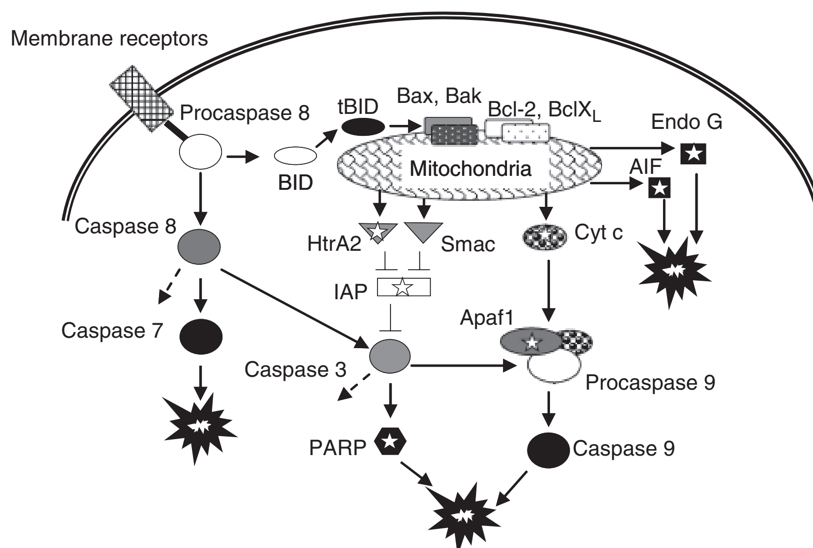

Fig. 1. Schematic presentation of apoptotic pathways. Only major regulators are shown. When fungal homologs exist, proteins are marked with a star. No fungal homologs have been identified for proteins consisting the death receptor pathway (indicated on the left side). Caspase-independent apoptosis is mediated by the nucleases EndoG and AIF (indicated on the right side). ✸ – Apoptosis.

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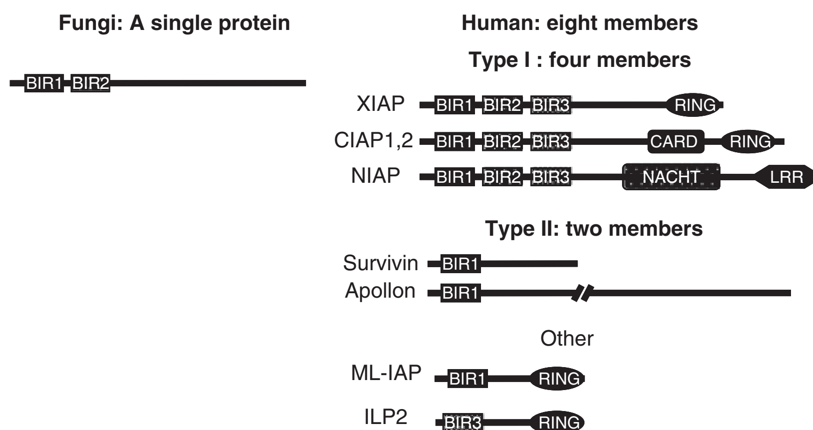

Fig. 2. Differences between human and fungal BIR-containing proteins. Human IAPs include eight members, six of them are subdivided into IAP (four members) and IAP-like (two members) proteins (Reed *et al.*, 2004). The IAP-like protein members usually contain a single BIR domain, while domains such as RING, CARD and LRR seem to be absent. BIR domains in IAP-like proteins have a longer primary sequence than those in IAPs, which is *c.* 100 amino acids long, vs. *c.* 70 amino acids in IAPs. When this distinction is made, the IAPs and IAP-like BIR domains are named BIR type I and BIR type II, respectively (Verhagen *et al.*, 2001). Most fungi have only a single, BIR type II-containing protein, which is most closely related to survivin. Some species, however, do not have any BIR-containing proteins.

position of the tetrapeptide recognition motif X-Glu-X-Asp, and have a functional cysteine at the base of their active site (Lavrik *et al.*, 2005). Caspases have not been identified in fungi. Instead, fungi (and plants) have a class of related proteases called metacaspases, which are considered to represent an ancient form of caspases (Uren *et al.*, 2000). Overall sequence similarity between caspases and metacaspases is very low and homology is only revealed through iterative domain search. Metacaspases do not contain CARD or DED, and homology to caspases is restricted to the active site. Furthermore, there are structural differences between the active sites of metacaspases and caspases, which imply possible differences in substrate specificity (reviewed in Vachova & Palkova, 2007; Vercammen *et al.*, 2007).

Another example of architectural differences between fungal and mammalian proteins is presented by the IAPs, which prevent apoptosis by blocking caspases under normal conditions. IAPs are typically characterized by the presence of three baculovirus IAP repeat (BIR) domains that mediate their interaction with caspases (Verhagen *et al.*, 2001; Dohi *et al.*, 2004; Reed *et al.*, 2004). IAP proteins may also contain a C-terminal RING-finger domain with E3 ubiquitin ligase activity and a CARD that may mediate the interaction with initiator caspases (Holcik & Korneluk, 2001; Verhagen *et al.*, 2001). IAP homologs have not been found in fungi. Instead, a related BIR-containing protein can be identified in most (but not all) of the available fungal genomes (our unpublished data). The BIR domain of these IAP-like proteins is slightly larger than that found in IAP proteins, and is therefore called type II BIR (Verhagen *et al.*, 2001). The fungal IAP-like proteins contain a single or two BIR domains, compared with three BIR domains in IAPs; they do not contain any of the other domains found in IAPs, and except for homology at the BIR domains, they are highly variable in size and structure (Fig. 2).

These two examples suggest that components of fungal apoptotic networks can be structurally very different from their mammalian orthologs, retaining only the basic core elements that are associated with their apoptotic role. This raises the possibility that fungal apoptotic networks are less complex, with fewer interactions and a reduced ability to fine-tune their response. This view is also supported at the genome level. Regulators of apoptosis in mammals are often members of large protein families. Although the different protein members may exhibit functional overlap, they are also involved in different aspects of apoptosis and respond to different stimuli. In contrast, in most fungi, only a single or two genes are present. For example, there are 14 different caspases in humans, which are divided into initiator and effector caspases. Some of them, such as caspase-8, are specific to the death-receptor (extrinsic) pathway; others, such as caspase-9, are specific to the mitochondrial (intrinsic) pathway; and some, such as caspase-3, are common to both pathways. Similarly, there are eight IAP members in humans (Fig. 2). Although not fully characterized, it is known that different IAP members regulate and interact with different caspases (Reed *et al.*, 2004). In contrast, there is only a single metacaspase (Yca1p) and a single BIR-containing protein (Bir1p) in *S. cerevisiae* (Uren *et al.*, 1999). In filamentous fungi, two metacaspases and a single BIR-containing protein are usually found. However, some fungi do not have any BIR-containing proteins, suggesting that either the functions of this protein might be executed by different proteins or the protein might not be essential in these organisms. In this respect, it should be noted that some important regulators of apoptosis are completely

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missing in fungi, particularly the upstream regulatory components that mediate the apoptotic signals. For example, there are no representatives of the Bcl-2 family of proteins in fungal genomes. This important family of pro- and anti-apoptotic proteins includes over 25 members in humans. The Bcl-2 superfamily is divided into subgroups, according to the existence in the protein of up to four different domains called BH (Bcl-2 homology) domains 1–4. The largest single subgroup includes BH-3-only proteins, all of which are pro-apoptotic. An important part of their function is interaction with anti-apoptotic Bcl-2 members via binding of the BH-3 domain in the Bcl-2 hydrophobic groove. Surprisingly, however, heterologous expression of Bcl-2 proteins either promotes (pro-apoptotic members) or suppresses (anti-apoptotic members) apoptotic cell death in yeast (Longo *et al*., 1997; Fröhlich & Madeo, 2000; Polcic & Forte, 2003). Similarly, expression of the anti-apoptotic Bcl-2 protein in the plant pathogen *C. gloeosporioides* blocked lovastatin- and Bax-induced PCD and conferred longevity and stress resistance to the transgenic strains (Barhoom & Sharon, 2007). A positive interaction was found between Bcl-2 and a *C. gloeosporioides* homolog of the human translationally controlled tumor protein (TCTP) (Barhoom & Sharon, 2007). Mutations in the Bcl-2 hydrophobic groove blocked the interaction with TCTP and abolished the anti-apoptotic activity of Bcl-2. Although TCTP may not be associated with the phenotypes of Bcl-2, this result demonstrates the functional importance of the Bcl-2 hydrophobic groove, and suggests that similar to humans, it might be essential for the interaction of Bcl-2 with fungal proteins (I. Agmon *et al*., unpublished data). Thus, although structural homologs of Bcl-2 have not been identified in fungal genomes, part of their function might be executed by nonhomologous fungal proteins. This example may also hold true for additional elements, such as the apoptosis regulator P53 or the mitochondrial protein SMAC/Diablo. The existence of a pathway that mediates extrinsic signals that include death receptors together with downstream components should also not be excluded, despite an absence of recognized components.

In summary, the above examples demonstrate that the core apoptotic network in fungi consists of proteins that have retained the basic regulatory domains found in related mammalian apoptotic proteins (Fig. 1). The domains found in fungal genes are not always identical to those in their mammalian orthologs and they seem to represent a more ancient form of the apoptotic domains. Overall, the fungal apoptotic network appears to be less complex than in mammals, and some of the known apoptotic proteins are not present in fungi. These proteins are either not essential for fungal apoptosis, or their activity may be executed by nonhomologous proteins that share sufficient functional conservation despite lack of sequence similarity. Conversely, the role of putative orthologs as regulators of apoptosis requires functional verification, as they may play roles that are very different from those of their mammalian orthologs.

Functional analysis of fungal apoptotic genes

Over 50 fungal genomes are currently available through public domains, enabling extensive gene search and comparative analyses of fungal traits (Xu *et al*., 2006a). As already mentioned, putative orthologs of mammalian apoptotic genes can be found in all fungal genomes. However, to date, only a few of these genes have been analyzed. This situation is expected to change soon as apoptotic genes are being analyzed in several different filamentous species, but until then, studies in *S. cerevisiae* remain the main source of information on the role of fungal apoptotic genes. Analyses of *S. cerevisiae* apoptotic genes have been recently reviewed (Fröhlich *et al*., 2007). Here, we briefly describe the main yeast apoptotic genes with a focus on results obtained in other species.

The discovery that Bcl-2 proteins can induce or prevent apoptosis in *S. cerevisiae* (Longo *et al*., 1997) led to the development of yeasts as screening systems for the identification of novel apoptotic genes (Jin & Reed, 2002). In a screen for human suppressors of apoptosis, a protein called BI-1 (for Bax inhibitor 1) was isolated, which blocked Bax-induced apoptotic cell death in *S. cerevisiae* (Xu & Reed, 1998). BI-1 is a membrane protein with six transmembrane helices, and it has been suggested that similar to Bcl-2 proteins, it can form pores in membranes. However, unlike proteins of the Bcl-2 family, which are absent in fungi, putative BI-1 orthologs with a moderate level of homology to human BI-1 can be identified in fungal genomes. To date, only the *S. cerevisiae* BI-1 has been analyzed. Yeast cells overexpressing the yeast *BI-1* had higher resistance to heat or oxidative stress and were protected from Bax-induced cell death (Chae *et al*., 2003). Similar to mammalian BI-1, deletions of the cytosolic domain of yeast BI-1 abolished its anti-apoptotic function. There is no information on possible targets of BI-1 or on this protein's mode of action and additional studies, including those in species other than *S. cerevisiae*, are needed to determine the precise role of these proteins. The question of whether fungal BI-1 might provide some of the functions of the 'missing' Bcl-2 proteins is of special interest.

In mammals, Bcl-2 proteins regulate mitochondrial permeability and thus secretion of certain pro-apoptotic proteins, including cytochrome *c*, HtrA2/Omi and SMAC/Diablo, and the endonucleases EndoG and apoptosis-inducing factor (AIF). Except for SMAC/Diablo, putative homologs of all other mentioned mitochondrion-residing proteins have been identified in fungi (Fig. 1). In *S. cerevisiae*,

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cytochrome *c* is secreted from mitochondria following apoptosis-inducing stimuli (Roucou *et al.*, 2000). However, the possible role of cytochrome *c* in yeast apoptosis is unclear. A cytochrome *c*-encoding gene has also been analyzed in *P. anserina*. Mutants in the *CYC1* gene displayed increased longevity, reduced growth rate and reduced ROS production, along with stabilization of mtDNA (Sellem *et al.*, 2007). These phenotypic defects, common to complex III and IV loss-of-function mutants, probably result from reduced ATP production. Similar to *S. cerevisiae*, the role of cytochrome *c* in *P. anserina* apoptosis remains unclear. In mammals, cytochrome *c* facilitates formation of apoptosomes in an ATP-dependent manner. There is no evidence for the existence of apoptosomes in fungi. Therefore, cytochrome *c* is either not directly involved in apoptosis or its mode of action is very different from that in mammals. In mammals, AIF and EndoG are two DNA nucleases that promote DNA degradation in a caspase-independent manner. Upon stimulation of apoptosis, they are translocated from the mitochondria to the nucleus and cleave chromosomal DNA. AIF also exhibits NADH dehydrogenase activity, and as such it is required for efficient respiration (Vahsen *et al.*, 2004), but this activity is unrelated to apoptosis induction (Cheung *et al.*, 2006). The mode of action of the *S. cerevisiae* homolog Aif1p closely resembles that of mammalian AIF. Aif1p is a mitochondrial protein, it is translocated to the nucleus under apoptosis-inducing conditions or during aging and it is involved in nuclear DNA fragmentation and chromatin condensation (Ye *et al.*, 2002; Parrish & Xue, 2003). Consistent with a pro-apoptotic role, *aif1Δ* strains show reduced stress- and age-induced apoptosis, while *AIF1*-overexpressing strains exhibit increased sensitivity to apoptosis-inducing conditions (Weinberger *et al.*, 2003; Wissing *et al.*, 2004; Fröhlich *et al.*, 2007). *Podospora anserina* contains putative homologs of AIFs [i.e. AIFs and 'AIF-homologous mitochondrion-associated inducers of death' (AMIDs)]. PaAmid1 deletion strains have a moderately extended life span (59–78%). In humans, AIF is a member of a small protein family that includes AMID and AIFl (AIF-like). AMID is an avoprotein with NAD(P)H oxidase activity. It induces caspase- and p53-independent apoptosis, but unlike AIF, it lacks a mitochondrial localization signal and is associated with the outer membrane of the mitochondria (Wu *et al.*, 2002). In *S. cerevisiae*, there are two AMID homologs, *NDE1* and *NDI1*. Overexpression of *NDI1*, but not *NDE1*, caused enhanced aging along with activation of apoptotic markers in yeast cells when grown on glucose-containing media, while deletion of *NDI1* decreased ROS production and prolonged the yeast's chronological life span (Li *et al.*, 2006). Deletion of an AIF homolog in *Neurospora crassa* resulted in strains that were more resistant to the drug phytosphingosine and to H_2O_2 (Castro *et al.*, 2008). In contrast, a strain containing a deletion in a gene encoding an AMID-like polypeptide was more sensitive to both treatments. Similarly, in *A. nidulans*, Aif was found to be involved in protection from apoptosis as Δ*aifA* mutants were more sensitive to farnesol- and H_2O_2-induced cell death (Savoldi *et al.*, 2008). The *A. nidulans* AifA protein did not migrate to nuclei following apoptotic stimulus, suggesting a mode of action different from that of human Aif. AIF and AMID homologs were also identified in *P. anserina*. Δ*paamid1* mutants had an extended life span, although the effect was somewhat smaller compared with a metacaspase deletion mutant (Hamann *et al.*, 2007). These studies show the high degree of functional conservation between fungal and human AIF and AMID proteins. The effect of AMID on longevity and cell death is indirect and likely due to its role in respiration and ROS production, whereas AIF seems more directly associated with regulation of apoptosis, probably through DNA degradation.

The second mitochondrion-secreted nuclease, EndoG, also translocates into nuclei upon exposure to apoptosis-inducing stimuli; however, its role as a regulator of apoptosis is more controversial. The yeast homolog Nuc1p is localized in the mitochondria and translocates to nuclei following H_2O_2 treatment. Overexpression of *NUC1* led to increased apoptosis upon treatment with either acetic acid or H_2O_2, whereas deletion of this gene protected cells from apoptosis (Burhans & Weinberger, 2007). Nuc1p-mediated death is independent of Yca1p and Aif1p (Buttner *et al.*, 2007). A role for EndoG in other fungi has not been reported. In *B. cinerea*, the EndoG homolog *BcNUC1* was found to localize in mitochondria, but its overexpression had no obvious phenotype (I. Hatam & A. Sharon, unpublished data).

The human serine protease HtrA2/Omi promotes apoptosis by antagonizing the apoptosis-inhibiting function of IAPs. HtrA2/Omi binds and degrades certain IAP proteins, thereby allowing caspase activation. The yeast homolog Nma111p is also pro-apoptotic; however, unlike HtrA2/Omi, Nma111p is a nuclear protein (Fahrenkrog *et al.*, 2004). Δ*nma111* strains survive elevated temperatures or H_2O_2 better than wild-type cells and show no apoptotic hallmarks, whereas overexpression of *NMA111* enhances apoptotic-like cell death. Although homologs of *NMA111* have been identified in additional fungal species, functional analyses have not been reported. Interestingly, homologs in *Aspergillus* are more closely related to the human than yeast protein (Fedorova *et al.*, 2005). We have recently cloned the *NMA111* homolog from *B. cinerea*. A Δ*bcnma* strain exhibited delayed germination, enhanced longevity but no change in oxidative-stress resistance (A. Finkelshtein & A. Sharon, unpublished data). In fresh, healthy-looking hyphae, the BcNma protein was distributed in the cell within small vesicles and possibly also in mitochondria. Computational analysis revealed that BcNma has an IAP-binding domain, which is also present in the human but not in the

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S. cerevisiae protein. This domain seems necessary for interaction of HrtA2/Omi with XIAP (Walter *et al.*, 2006). Thus, although the BcNma protein exhibits higher sequence homology to Nma111p of *S. cerevisiae*, it is functionally more similar to the human HrtA2/Omi protein.

The *S. cerevisiae* IAP-like protein Bir1p has anti-apoptotic activity, which is antagonized by Nma111p. Like its mammalian homolog survivin, Bir1p is also involved in chromosome segregation (Rajagopalan & Balasubramanian, 2002). The two BIR domains in the N-terminal part of the protein are necessary for anti-apoptotic activity, while the C-terminal part is necessary for the cell-cycle control properties (Widlund *et al.*, 2006). IAP proteins in mammals bind to caspases through the CARD that is present in both proteins. Bir1p lacks a CARD and it does not interact with Yca1p, suggesting that similar to the human survivin, the anti-apoptotic activity of Bir1p might occur via binding and stabilization of other cellular proteins rather than by direct inhibition of caspases (Walter *et al.*, 2006). We have recently isolated the BIR-containing protein from *B. cinerea*. The gene was found to be essential, as homokaryon knockout mutants could not be generated (Shlezinger *et al.*, 2008). Overexpression of *BcBIR1* resulted in prolongation of the growth phase and reduced ROS accumulation in batch cultures, as well as reduced sensitivity to H_2O_2 and salt stresses (Shlezinger *et al.*, 2009). Preliminary data indicate that BcBir1 might be degraded in a BcNma-dependent manner (our unpublished data). These results highlight the similarities and differences in the roles and modes of action of these proteins in *S. cerevisiae* and *B. cinerea*. Most apoptosis-related proteins play a role in processes other than cell death, which in many cases may be their primary role. Bir1p, for example, is involved in cell division. The structural and functional differences between Bir1p and BcBir1 are probably associated with the different lifestyles of the single cell, budding yeast and multicellular filamentous species.

Caspases are at the end of the apoptotic chain. In most cases, a single or two metacaspase genes can be identified in fungal genomes. Metacaspases have now been analyzed in several fungi and in all cases they have been associated with apoptotic cell death, although some PCD scenarios do not involve metacaspases (summarized in Table 1). The single yeast metacaspase Yca1p exhibits high proteolytic activity toward the mammalian caspase substrates VEID-AMC and IETD-AMC. This activity is completely inhibited by the pan-caspase inhibitor Z-VAD-FMK (Madeo *et al.*, 2002b). Disruption of *YCA1* reduced cell death and formation of apoptotic markers in aged cultures. The Δ*yca1* strain accumulated less ROS and exhibited enhanced resistance to oxygen stress, whereas overexpression of *YCA1* had the opposite effect and colonies were hypersensitive to apoptotic stimuli (Madeo *et al.*, 2002b). Two metacaspases, *CasA* and *CasB*, have been identified and cloned in *A. fumigatus* (Richie *et al.*, 2007b). Phosphatidylserine exposure was prevented in a Δ*casA*/Ä*casB* double mutant but cell viability of the mutant was not altered. The Δ*casA*/Δ*casB* double mutant also retained wild-type virulence and showed no difference in sensitivity to various apoptosis-inducing stimuli. Thus, although required for the loss of membrane phospholipid asymmetry in the stationary phase, other apoptotic phenotypes and PCD induced by external conditions, such as oxidative stress and antifungal compounds, are independent of these metacaspases. However, the Δ*casA*/Δ*casB* double mutant retained some caspase activity in cells entering the stationary phase, suggesting the existence of additional, as-yet uncharacterized caspase-like proteases (Richie *et al.*, 2007b). These results are in contrast to yeast, in which Yca1p is necessary for cell death induced by various external stimuli (Eisler *et al.*, 2004; Silva *et al.*, 2005; Vachova & Palkova, 2007). Caspase activity was also found in aging cultures of *P. anserina*. Two metacaspases, *PaMCA1* and *PaMCA2*, were isolated and knockout mutants were generated and characterized. Knockout of either *PaMCA1* or *PaMCA2* led to an 80% or 148% increase in life span, respectively (Hamann *et al.*, 2007).

Only a few caspase target proteins have been identified in fungi, one of which is PARP. In metazoans, PARP plays an important role in modulating the cellular response to stress and apoptosis (reviewed by Koh *et al.*, 2005). There is no homolog of PARP in *S. cerevisiae*, but homologs can be found in filamentous species (Fedorova *et al.*, 2005). The *A. nidulans* PrpA is an 81-kDa protein that does not contain the classical caspase-3 or -8 cleavage sites, but a KVVDK site is recognized, which results in a 60-kDa product when incubated in the presence of fungal extracts with high caspase activity (Thrane *et al.*, 2004). A Δ*prpA* mutant showed reduced apoptosis after farnesol treatment (Semighini *et al.*, 2006b). This observation implicates PrpA in at least one fungal cell-death pathway. A second protein target of caspases, DAP-3, is a mitochondrial mediator of apoptosis, which may act in mammals both up- and downstream of caspase-8. Disruption of Ygl129c, the yeast *DAP-3* homolog, completely prevented induction of apoptosis induced by overexpression of *YCA1*, placing it downstream of caspase activation. The human nuclear protein Rad21 is cleaved by caspases after exposure to apoptotic stimuli and the C-terminal product is translocated to the cytoplasm, where it acts as a nuclear signal for apoptosis (Chen *et al.*, 2002; Pati *et al.*, 2002). Mcd1p, the *S. cerevisiae* Rad21 homolog, is required for progression from the S phase through mitosis. Cleavage of Mcd1 by the nuclear caspase-like protease Esp1p at the metaphase-to-anaphase transition promotes loss of cohesin, followed by dissociation of Mcd1p from the chromatids (Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Uhlmann *et al.*, 1999). Recently, it was reported that Mcd1p is cleaved

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upon H_2O_2-induction of apoptosis in *S. cerevisiae*. The C-terminal fragment is translocated to the mitochondria, where it leads to a decrease in mitochondrial membrane potential and amplification of cell death in a cytochrome *c*-dependent manner (Yang *et al.*, 2008). Furthermore, cleavage of Mcd1 during H_2O_2-induced apoptosis in yeast was promoted by Esp1, and could be blocked by caspase-1 inhibitor and the broad-spectrum caspase inhibitor Z-VAD-FMK. These new results might help explain the residual caspase activity that is often observed during apoptosis in Δ*yca1* cultures.

Overall, these analyses show that fungal homologs of known apoptotic proteins may be involved in regulation of apoptotic cell death; however, their function does not always correspond to their predicted activity. In *S. cerevisiae*, most of the analyzed genes either protected or promoted apoptosis in a conserved, caspase-dependent manner, but in filamentous species, results were mixed: some proteins had effects similar to those observed in budding yeasts, while others had either no effect or one that was opposite to that expected. It is also apparent that caspase-independent cell death is more common in filamentous species than in budding yeasts. However, this might be partly due to the higher number of metacaspases in filamentous species, an issue that requires further verification. Although the number of fungal genes that have been analyzed is still small, these findings suggest that the function of specific apoptosis-related proteins is not always conserved between fungi and mammals, or even between *S. cerevisiae* and filamentous species. While it is highly possible that PCD plays different roles in filamentous species, the contribution of apoptotic cell death to unicellular organisms is less intuitive. Herker *et al.* (2004) showed that deletion of *YCA1*, although conferring stress-resistance and longevity in specific yeast cells, was disadvantageous in a yeast population. Deletion of YCA1 increased the survival rate of chronologically aged cells; however, after preculturing for 35 days, only 1% of Δ*yca1* cells developed colonies in a plating assay compared with 7% of the wild-type cells. This work demonstrates the importance of PCD for long-range survival of a population, despite the increased longevity in single *yca1* cells. It should also be noted that caspase-like activity was reported during chronological aging in Δ*yca1* cultures (Herker *et al.*, 2004; Vachova & Palkova, 2005), suggesting the possible existence of additional caspase-related proteins in *S. cerevisiae*.

Concluding remarks

Apoptotic-like fungal cell death has gained recognition in recent years with the discovery of apoptotic genes and the partial elucidation of the biological function of apoptosis in fungi. It is now generally accepted that fungi undergo PCD, which is highly similar to mammalian apoptosis and is part of the normal development and stress adaptation, in both unicellular (budding) and multicellular (filamentous) species. Molecular and genomic data confirm the existence of apoptotic machinery that is conserved between fungi and higher eukaryotes. Fungal apoptotic networks consist of protein homologs of known mammalian proteins as well as more unique components that regulate apoptosis in fungal-specific processes. Still, research into fungal apoptosis is relatively new and many questions remain open, such as: (1) Are there fungal paralogs to foreign apoptotic genes that, although not found in fungal genomes, can nevertheless induce or prevent apoptosis in fungi? Moreover, in some cases it has been shown that the function of such heterologous proteins in fungi is highly similar to their mode of action in the original host. Identification of the fungal paralogs might reveal novel regulators of fungal development and help elucidate the connection between apoptosis and development in fungi. (2) What is the function of the putative homologs of known apoptotic proteins that can be identified in the available fungal genomes? Recent analyses of a few homologs in filamentous species have revealed significant differences from previously reported results in *S. cerevisiae*, which might be related to the differences in cellular organization, life cycle, and lifestyle between budding yeasts and filamentous species. Further analyses of such homologs in a wider range of species with different lifestyles will help determine the level of functional conservation of fungal apoptotic proteins as well as highlight fungus-unique functions and differences between species, particularly pathogens vs. saprophytes. (3) What are the roles of fungal-specific apoptotic genes? Previous bioinformatics searches have revealed the existence of fungal-specific apoptotic genes such as *het* loci and have also indicated changes in copy number and domain architecture. The availability of genome sequences enables more robust bioinformatics analyses, which can be used to delineate the complete fungal apoptotic inventory, identify cryptic apoptotic genes and shed light on the evolutionary origin of apoptosis in fungi compared with other systems. (4) What are the signal cascades that regulate apoptosis in fungi? Several signaling elements have been associated with fungal apoptosis, particularly cAMP, Ras and MAP kinases, but information is still very limited and the signal cascades that regulate apoptosis are not well understood. Signaling cascades in fungi have been extensively studied in recent years and major signal cascades have been elucidated in great detail in a number of species. This knowledge should be useful in elucidating the signals and signal pathways that activate and regulate apoptosis. Finally, the use of apoptosis-triggering compounds to develop novel antifungal treatments has been proposed and holds great potential. This possibility is supported by recent studies, showing that induced apoptosis

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might be necessary for the antifungal activity of many compounds, including known antifungal drugs. Further research, particularly a more precise determination of the role of apoptosis in fungi and a better characterization of putative apoptotic proteins, is needed in order to exploit the full potential of this approach.

Acknowledgements

This work was supported by the Israel Academy of Sciences grant no. 1178/05.

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