

Critical Review

Yeast Programmed Cell Death: An Intricate Puzzle

P. Ludovico¹, F. Madeo² and M. T. Silva³

¹*Life and Health Research Institute, Health Sciences School, Minho University, Campus de Gualtar, Braga, Portugal*

²*IMB, Karl-Franzens University, Graz, Austria*

³*Fish Immunology and Vaccinology, Institute for Molecular and Cell Biology (IBMC), Porto, Portugal*

Summary

Yeasts as eukaryotic microorganisms with simple, well known and tractable genetics, have long been powerful model systems for studying complex biological phenomena such as the cell cycle or vesicle fusion. Until recently, yeast has been assumed as a cellular ‘clean room’ to study the interactions and the mechanisms of action of mammalian apoptotic regulators. However, the finding of an endogenous programmed cell death (PCD) process in yeast with an apoptotic phenotype has turned yeast into an ‘unclean’ but even more powerful model for apoptosis research. Yeast cells appear to possess an endogenous apoptotic machinery including its own regulators and pathway(s). Such machinery may not exactly recapitulate that of mammalian systems but it represents a simple and valuable model which will assist in the future understanding of the complex connections between apoptotic and non-apoptotic mammalian PCD pathways. Following this line of thought and in order to validate and make the most of this promising cell death model, researchers must undoubtedly address the following issues: what are the crucial yeast PCD regulators? How do they play together? What are the cell death pathways shared by yeast and mammalian PCD? Solving these questions is currently the most pressing challenge for yeast cell death researchers.

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INTRODUCTION

Programmed cell death (PCD) refers to an active process characterized by a course of endogenous molecular events, representing a form of ‘cell suicide’ (1). PCD is a highly complex, regulated process requiring the coordinated activa-

tion and execution of multiple sub-programmes that include both caspase-dependent or –independent mechanisms. Genetically defined PCD programs dictate different phenotypes ranging from apoptotic to necrotic to autophagic, with intermediate and overlapping forms.

Unlike apoptosis, the non-apoptotic modes of PCD including programmed necrosis, autophagic cell death and mitotic catastrophe are still difficult to distinguish unambiguously and remain poorly characterized (2–4). For several years, apoptosis was wrongly understood as synonymous with PCD and was assumed to be confined to multicellular organisms, with *Caenorhabditis elegans* and *Drosophila melanogaster* being chosen as core models for cell death research.

The completion of the *Saccharomyces cerevisiae* genome sequence in 1996 and the initial failures to detect orthologues of apoptosis-related proteins encoded by the yeast genome, made yeast a ‘clean room’ system for the study of heterologous expressed mammalian apoptotic regulators such as proteins from the Bcl-2 family. Unexpectedly, the expression of the pro-apoptotic protein Bax in yeast resulted in cell death (5) with an apoptotic nature (6), which could be prevented by the co-expression of the antiapoptotic Bcl-2 protein suggesting some specificity of the killing effect (5). It is seven years since the yeast *S. cerevisiae* appeared as the simplest eukaryotic organism displaying PCD with an apoptotic phenotype (7). Since then the number of reports of this phenomenon increases almost daily, leading to the emergence of a new, fruitful and promising research field that profits from the recognized advantages of yeasts for the study of biological processes.

In our opinion, it is now time to look carefully into the phenomenon and to find answers to some urgent questions: what are the yeast apoptotic regulators? How do they play together? What are the cell death pathways shared by yeasts and mammals? Finally, are we in danger of over interpreting the data concerning apoptosis and disregarding the possible occurrence of non-apoptotic PCD processes as has happened

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Address correspondence to: Paula Ludovico, Life and Health Research Institute, Health Sciences School, Minho University, Campus de Gualtar, 4710-057 Braga, Portugal.

Tel: 00 351 253604800. Fax: 00 351 253604809.

E-mail: pludovico@ecsaude.uminho.pt

in the last decades with the study of cell death in mammalian systems? These are the current major challenges for researchers in the yeast apoptosis field that will be discussed in the following sections.

YEAST APOPTOSIS TRIGGERS

The first observations of yeast PCD were made on a *S. cerevisiae* strain harbouring a mutation in the *CDC48* gene which encodes a protein required for vesicle trafficking (7). Surprisingly, dying cells of this mutant displayed an apoptotic phenotype characterized by several hallmark mammalian apoptotic markers such as phosphatidylserine exposure, condensation and margination of chromatin and DNA fragmentation. Soon after the discovery that the *CDC48* mutant could undergo a form of PCD, researchers showed that exogenous toxic agents such as hydrogen peroxide (8) or acetic acid (9) trigger PCD with an apoptotic phenotype in *S. cerevisiae*. From hereon, several reports have implicated the impairment of different cellular functions caused by different exogenous stress stimuli or deletion of certain genes in the induction of an apoptotic phenotype in *S. cerevisiae* (see reviews 10, 11). Conditions triggering a yeast apoptotic phenotype include sugar (12) and salt (13) stresses; starvation for carbon source on expired medium (14) or of essential amino acids like lysine and histidine in auxotrophic cells (15); impairment of exocytosis by deletion of the *SRO7/SOP1*-encoded tumor suppressor homologue (16), DNA damage (see review 11) and truncation or deletion of genes encoding proteins involved in mRNA stability (17) and cell-cell communication (18).

Yeast apoptotic cell death processes have also been linked to other physiological traits such as aging. Curiously, mutations leading to an increase in actin dynamics promote yeast longevity, whereas decreased actin dynamics causes an enhanced metacaspase activity and mitochondrial membrane depolarization leading to an augment in reactive oxygen species (ROS) and cell death (19). The described role of actin cytoskeleton dynamics in the regulation of yeast apoptosis (19) indicates that this pathway may likely be part of the natural aging process in yeasts and potentially in mammalian cells as well, reinforcing the use of yeasts as model organisms for research on cell aging and death. In fact, yeast cells dying after chronological aging display stereotypical apoptotic markers indicating that long-term cultivation can induce altruistic mortality of an apoptotic nature. The elimination of old cells, that are less robust and may become deleterious to the well being of the population, confers a selective and adaptative advantage for the healthier members of the community (14). Recently, yeasts were shown to undergo 'programmed aging' involving a cell death process mediated partially by superoxide (20). These authors propose that early death and high mutation frequency have evolved to promote early adaptation. Mutations increasing longevity

and antioxidant protection or the overexpression of superoxide dismutases and catalase or deletion of the yeast metacaspase were proven to affect adaptative regrowth, raising the possibility that natural selection has prevented alleles that confer longevity extension from becoming fixed (20).

The number of reports implicating and relating yeast apoptotic PCD with diverse cellular functions increases daily but the gaps in our knowledge regarding yeast cell death pathways and regulators presses us even further towards the necessity for a greater understanding of yeast PCD processes.

ROS, METACASPASE, CYTOCHROME C, APOPTOSIS INDUCING FACTOR AND HTRA2/OMI: HOW DO THEY PLAY TOGETHER?

Given the evidence in favour of yeast PCD it is pertinent to ask how is the yeast cell death program regulated when most of the crucial regulator genes known in mammalian systems are missing? From the beginning, yeast PCD with an apoptotic phenotype was associated with ROS production leading to the assumption that these radicals were the only regulators in yeast apoptotic cell death. However, the precise role of ROS in PCD is still matter of controversy even in mammalian systems. Indeed, the intracellular redox state has been shown to modulate the permeability transition pore opening (21), cell cycle checkpoints (22), p53 transactivation (23) and caspase-3 activity (24). Alternatively, ROS might only be secondary apoptotic messengers resulting from the upstream activation of the PCD machinery. In *S. cerevisiae*, ROS accumulation is apparent in almost every apoptotic scenario and probably plays a causal role. This may point to an ancestral mechanism of apoptotic regulation, although one should not discard the possibility that ROS have a dual character in yeast PCD, acting as secondary apoptotic messengers and/or regulators of the process.

The description of a yeast metacaspase (*YCA1*), a caspase-related protease with caspase activity (25), has altered how science is looking into the phenomenon of apoptosis in yeasts because for the first time a specific molecule can be implicated as an executor of a wide range of apoptotic stimuli. The discovery of a yeast molecule homologous to human caspases, that when disrupted rescues yeasts from a variety of different lethal stimuli, reinforces the concept of an ancestral PCD machinery in yeast cells. In the mammalian apoptotic process the activation of the 'executioner' caspases and subsequent cleavage of their substrates within the cell can be linked directly to the characteristic morphological and biochemical features of the apoptotic phenotype. Although the phenotypical alterations observed in yeast cells dying by PCD resemble the apoptotic phenotype observed in mammalian cells, there are no known yeast metacaspase substrates; therefore the role of the caspase-like protein Yca1p in yeasts is still poorly understood.

Cytochrome c (cyt c) is another molecule implicated in yeast apoptosis. Resembling mammalian systems, yeast cyt c is translocated from mitochondria to the cytosol under apoptotic conditions such as Bax expression (26), deletion of the histone chaperone *ASF1/CIA1* (27), acetic acid treatment (28) and PCD induction by mating pheromone (18). Moreover, cells without mature cyt c were shown to be more resistant to death induced by acetic acid (28). However, the interaction of cyt c with other cytoplasmic proteins has so far not been revealed and the elucidation of the specificity of its release from mitochondria is yet to be demonstrated.

Two basic key players of mammalian apoptosis, HtrA2/Omi and Apoptotic Inducing Factor (AIF), were shown to be present in the yeast genome (29, 30). Fahrenkrog and coworkers (29) identified and characterized Nma111p, an HtrA-like protein of *S. cerevisiae*, that under certain stress conditions, aggregates inside the nucleus mediating yeast apoptotic PCD. In addition, we have proven that Aif1p a yeast homologue of mammalian Aifp is also a cell death mediator that is translocated from mitochondria to the nucleus in response to apoptotic stimuli (30). Recently, yeast homologues of mitochondrial fission factors such as *FIS1* and *DNM1* were reported to also regulate yeast PCD, suggesting one more cellular process involved in yeast and mammalian PCD processes (31).

Although these advances have highlighted the existence of an ancestral apoptotic machinery in *S. cerevisiae* sharing amazing homologies with that of mammalian systems, all of them constitute pieces of a puzzle that is far from being solved. Besides the observation that the effect of Aif1p overexpression is attenuated by disruption of *YCA1* (30), no other relationships have been established between the currently identified yeast apoptotic players. The clarification of the hierarchy of these yeast cell death players as well as their interactions during apoptosis will allow the drawing of the yeast PCD pathway(s), further contributing to the elucidation of the roots of apoptotic PCD in higher eukaryotes.

YEAST APOPTOTIC PATHWAY(S)?

As previously discussed, it is still not possible to outline the putative death pathway(s) playing in yeast apoptotic PCD due to the lack of knowledge concerning the interactions between the few identified regulators. Once again, it is necessary to stress that at this point yeast cell death researchers must focus on this issue in order to further validate and take advantage of this promising cell death model. Results described in literature suggest that similar to mammalian cells, yeast cells would also possess different apoptotic pathways. Some of those results clearly show a crucial involvement of mitochondria in a number of apoptotic PCD pathways triggered by different stimuli or deletion of some genes. Manon and coworkers (26) pointed out that Bax-induced growth arrest of yeast cells is related to defects in the respiratory chain such as a decrease in

the amount of cytochrome c oxidase (COX) complex and the release of cyt c to the cytosol.

On the other hand, the *S. cerevisiae* strain lacking the histone chaperone *ASF1/CIA1* also displayed mitochondrial dysfunctions associated to typical apoptotic markers including a reduction of the mitochondrial membrane-potential, dysfunction of the mitochondrial proton pump, and release of cyt c to cytoplasm (27). Our previous results showed that acetic acid-induced apoptosis in *S. cerevisiae* is also mediated by a mitochondria-dependent apoptotic pathway (28): release of cyt c and ROS production were associated with a reduction in oxygen consumption and a decline of COX activity coupled with a specific decrease in the amounts of COX II subunit. An apoptotic phenotype related to mitochondria was also observed in yeast cells subjected to DNA damage due to a mutation in the *CDC13* gene which encodes a yeast telomere binding protein with an essential role in protecting yeast telomeres (32). In fact, mitochondrial proteins have been identified as multicopy suppressors of cell death in the *CDC13* mutant strain. Transformation with a human HeLa cDNA library produced one clone that was identified as the mitochondrial protein MTOC3 (subunit 3 of human COX) while transformation with a yeast genomic library produced two clones identified as fragments of YDR333C and antisense of CYC8. YDR333C was found to interact with the mitochondrial ribosomal protein Mrp51p (involved in the regulation of the expression of subunits 2 and 3 of yeast COX) and CYC8 encodes a protein that regulates expression of COX3 and COX6 of COX. Interestingly, these suppressors are directly or indirectly related to mitochondrial COX whose inhibition is known to trigger apoptosis in mammalian cells (33, 34). Somehow, data available concerning yeast cell death induced by Bax expression, acetic acid treatment or deletion of either *ASF1/CIA1* or *CDC13* genes, clearly point out that COX complex is playing a role in yeast apoptosis resembling mammalian systems. Mitochondria involvement in yeast apoptosis was also supported by recent results indicating that mitochondrial fragmentation is also regulating yeast PCD (31). Moreover, mitochondria have been implicated at a general level in yeast PCD by the results obtained with the rho0 mutant strain (lacking mitochondrial DNA), revealing that the apoptotic signals induced by Bax expression, acetic acid treatment or deletion of *CDC13* were suppressed in a rho0 mutant (26, 28, 32). Curiously, the rho0 strain is able to undergo hydrogen peroxide- (our unpublished results) or essential amino acid starvation-induced apoptosis (15) suggesting a different role for mitochondria in these apoptotic processes. Supporting these results, yeast metacaspase, the described executor of PCD induced by a wide range of apoptotic stimuli, has a significantly lower activity in cells dying by acetic acid treatment when compared with the activity seen in cells dying by hydrogen peroxide exposure (25). These results suggest that acetic acid-induced PCD

would be strictly dependent on mitochondria activating an intrinsic-like pathway, while the one triggered by hydrogen peroxide would be mitochondria-independent using mitochondria only as an amplification signal in an extrinsic-like pathway. Moreover, even $\Delta YCA1$ cells show rapid death in presence of high concentrations of the described apoptotic inducing agents, indicating the presence of alternative mechanisms of cell death. Altogether, these data can be used to suggest in all probability the existence of more than one cell death pathway in yeasts.

Apoptotic proteins such as BI-1 or IAP have counterparts in yeast cells (35, 36), but they have not yet been implicated in yeast PCD. The role of Bir1p (a yeast IAP homologue) on yeast PCD and its putative role on the regulation of metacaspase has not been studied so far. However, as suggested by Fahrenkrog and coworkers (29), Yca1p, Nma111p and Bir1p could be engaged in the regulation of yeast PCD in a similar way to their mammalian homologues. Furthermore, because of Nma111p and Bir1p nuclear localization, the nucleus appears to play a significant role in yeast apoptosis (29). Future studies regarding these putative orthologue proteins might contribute to a better understanding of yeast PCD as the genetic tractability of these microorganisms may lead to the implication of other proteins in yeast associated PCD pathway(s). Nevertheless, the evolutionary roots of PCD processes still remain a mystery and the elucidation of yeast PCD pathway(s) appears as one of the greatest challenges for yeast cell death researchers.

YEAST AS AN 'UNCLEAN' MODEL FOR APOPTOSIS RESEARCH

As stated earlier, yeast has proven to be a powerful model system for studying complex biological phenomena and was considered to be a 'clean room' system for examining the effect of heterologous expression of pro- and anti-apoptotic mammalian proteins as well as the identification of novel apoptotic activators or inhibitors (37–39). In fact, genetic screens allowed the identification of mitochondrial subunits (e.g., from mitochondrial F₀F₁-ATPase proton pump) as critical for Bax-mediated killing in *S. cerevisiae* (37) and novel Bax inhibitors such as Bax inhibitor-1 (BI-1) (38) or Ku70 (39). The lethal effect of the expression of several mammalian pro-apoptotic regulators such as Bax (5) or caspases (40) although fruitful for the study of those regulators, was always intriguing for scientists. For several years the rescue of cell lethality, caused by expression of Bax or Bak by co-expression of anti-apoptotic proteins such as Bcl-2 or Bcl-x_l, was not considered evidence in favour of the existence of an endogenous PCD process. Several authors argued that in most cases this suppression would be due to direct binding of the mammalian anti-apoptotic proteins to their mammalian apoptotic-promoting counterparts. None-

theless, the occurrence of a yeast endogenous PCD process is no longer a subject for speculation as an ancestral PCD machinery is undoubtedly present in this unicellular eukaryote. Ascertaining whether or not the rescue by anti-apoptotic proteins of lethality induced by expression of Bax or Bak is due to direct binding of the mammalian anti-apoptotic proteins to their mammalian apoptosis-promoting counterparts or to the inactivation of the yeast endogenous PCD program is still a matter of debate. It is clearly important to recognize that yeasts must be viewed as an 'unclean system' to study pro- and anti-apoptotic mammalian proteins. The employment of yeasts as models for apoptosis research to investigate the functions and interactions of heterologously expressed mammal proteins should not ignore the existence of an endogenous yeast PCD pathway. In fact, one must consider the possibility that the heterologously expressed apoptotic regulator proteins could somehow interact with the native machinery that promotes or inhibits the endogenous yeast PCD. Therefore, one should take into consideration that even though yeasts are an 'unclean system' they are still a promising model for apoptosis research, provided that all the results are analysed and interpreted in light of the existence of an endogenous yeast PCD pathway(s).

YEAST AND NON-APOPTOTIC PCD

The mechanisms that regulate non-apoptotic cell death remain even more dubious than those that regulate apoptosis. Yeast contributions have considerably promoted our understanding of several aspects of non-apoptotic PCD and are expected to increase in the near future.

Necrotic PCD, an active controlled cell death process with a phenotype reminiscent of passive accidental necrosis, has been found to be implicated in several instances of neurodegeneration, inflammatory diseases, infection and ischemia (2–4). In this form of PCD, apoptotic markers are lacking; instead, the cell membrane is damaged early, organelles are disorganized and the cell swells and eventually lyses. Instances of programmed necrosis occur after triggering of TNF or Fas death receptors when apoptosis is chemically or genetically blocked (reviewed in 41). In these situations PARP (poly ADP-ribose polymerase) is not cleaved by caspases and leads to energy depletion resulting in death. Programmed necrosis can be triggered in more 'natural' situations, namely in viral infections where participation of RIP or the serine protease activity of HtrA2/Omi have been implicated (41). In many instances of programmed necrosis, pieces of the apoptotic death machinery are shared by the necrotic death program. However, the final stages of the necrotic process require the utilization of specific genes including those encoding for enzymes used in the terminal dismantlement of the dying cell, like calpains and cathepsins (42).

Even though mechanisms of programmed necrosis are conserved from worms and flies to humans (42), programmed necrosis has not been specifically studied in yeasts. However, it is important to bear in mind that contrary to apoptosis in multicellular organisms, where apoptotically dying cells are removed and eliminated by scavenger cells before being lysed by a terminal necrotic process (post-apoptotic secondary necrosis), apoptosis in yeast inevitably ends with the lytic disintegration of the doomed cells. This has been a source of confusion in the characterization of yeast apoptotic processes, because necrosis indicators (like cell membrane damage) can occur in an otherwise typical apoptotic process. The same problem arises in studies concerning apoptosis using *in vitro* cultured mammalian cell lines. Primary necrosis, but not programmed necrotic cell death, was observed when *S. cerevisiae* (9), *Zygosaccharomyces bailii* (43) or *Candida albicans* (44) were exposed to high concentrations of acetic acid.

Autophagic cell death (ACD) refers to a separate form of PCD where autophagy is excessive and lysosomal activity exaggerated, leading to active destruction of the cytoplasm before nuclear changes become apparent (45). ACD has been observed in several physiological situations in embryogenesis and in adulthood and has been associated with the pathogenesis of some neurodegenerative disorders and cancer. Studies on yeast autophagy have been instrumental in the advance of the understanding of autophagy and ACD, with most autophagy-related genes identified and characterized by genetic analysis in yeasts (46). Genome sequence analysis have revealed that similar genes are implicated in autophagy in worms, flies and humans, and links between yeast autophagy genes and human cell death have also been made (47). Yeast cells undergo autophagic death when arrested at any stage of the cell cycle (48, 49) or following activation of protein phosphatase 2A (50). The borderline between ACD and apoptosis can be unclear and links between both cell death pathways have been disclosed (51), indicating the possibility of some overlapping or interdependence between them (3).

Mitotic catastrophe represents a less debated form of caspase-independent PCD caused in mammalian cells and yeasts by aberrant mitosis. It was initially described in *Schizosaccharomyces pombe* (52), although its PCD character was not recognized at that time. The regulatory mechanisms of this peculiar form of cell demise remain to be elucidated (4).

So far, few biological models have been used to investigate PCD mechanisms. A large extent of the knowledge in the PCD field, which as reviewed here is still very scarce, resulted from the use of two invertebrate (*C. elegans* and *D. melanogaster*) and two vertebrate (human and mouse) animal models. Despite the contribution of these models to our current understanding of cell death, further progress in the resolution of a number of questions resulted from the parallel use of classical and alternative biological models (53). The lessons learned from yeast PCD, besides yielding new insights into the

evolution of the apoptotic pathways in eukaryotic cells, will produce knowledge relevant to human health. One should keep in mind that the easy genetic manipulation of yeast cells may not always be beneficial and could reveal a 'dark side'. The genetic plasticity of yeast cells allows them to readapt to new conditions when genes are deleted giving rise to pleiotropic resistances. Consequently, 'innocent bystander' genes could be misinterpreted as relevant genes, leading to the necessity of a multifaceted approach to properly implicate their product on yeast PCD. Although yeasts have turned out to be important models in cell death research, they are unlikely to reveal all the aspects of complex cell death mechanisms in multicellular organisms.

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