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Stress and Cell Death in Yeast Induced by Acetic Acid

M. J. Sousa[1], P. Ludovico[2,3], F. Rodrigues[2,3], C. Leão[2,3] and M. Côrte-Real[1]
[1]*Molecular and Environmental Research Centre (CBMA)/Department of Biology, University of Minho, Braga*
[2]*Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga*
[3]*ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães*
Portugal

1. Introduction

Yeasts are nowadays relevant microorganisms in both biotechnology, with important economic impact in several fields, and fundamental research where *Saccharomyces cerevisiae* appears as one of the most used and versatile eukaryotic cell models. In industrial fermentations, yeasts are subjected to different stress conditions, such as those imposed by low water activity and by the presence of cytotoxic compounds. Yeast cells react to adverse conditions by triggering a stress response, enabling them to adapt to the new environment. However, upon a severe cell cue the elicited stress responses may be insufficient to guarantee cell survival and cell death may occur. The simplicity of yeast and its amenability to manipulation and genetic tractability make this unicellular eukaryotic microorganism a powerful tool in deciphering the mechanisms of eukaryotic cellular processes and their modes of regulation. Despite the differences in signalling pathways between yeast and higher eukaryotes current knowledge on cellular stress responses and programmed cell death confirms that several steps are phylogenetically conserved and therefore yeasts are ideal model systems to study the molecular pathways underlying these processes.

In this chapter we focus on the molecular mechanisms associated with stress response and cell death in yeast triggered by acetic acid. We start with a general introduction devoted to the physiological responses to acetic acid, and to the high resistance of the food spoilage yeast *Zygosaccharomyces bailii* to this acid in comparison with *S. cerevisiae* and other yeast species. Basic aspects of programmed cell death are also covered. The subsequent sections are dedicated to an overview of ours and other authors' studies highlighting the kinetics, components and pathways already identified in acetic acid-induced cell death.

1.1 Acetic acid physiological responses

Acetic acid is a normal by-product of the alcoholic fermentation carried out by *S. cerevisiae* and of contaminating lactic and acetic acid bacteria (Du Toit & Lambrechts, 2002; Pinto et al., 1989; Vilela-Moura et al., 2011) or it can be originated from acid-catalyzed hydrolysis of

lignocelluloses (Lee et al., 1999; Maiorella et al., 1983). Above certain concentrations accepted as normal (0.2 to 0.6 g/l), acetic acid has a negative impact on the organoleptic qualities of wine and may affect the course of fermentation, leading to sluggish or arrested fermentations (Alexandre & Charpentier, 1998; Bely et al., 2003; Santos et al., 2008). In bioethanol production from lignocellulosic acid hydrolysates, acetic acid may also be associated with the inhibition of alcoholic fermentation, limiting the productivity of the process (Lee et al., 1999; Maiorella et al., 1983; Palmqvist & Hahn-Hägerdal, 2000). Therefore, acetic acid has a negative impact on yeast performance, restraining the production efficiency of wine, bioethanol or of products obtained by heterologous expression with engineered yeast cells under fermentative conditions. On the other hand, the cytotoxic effect of acetic acid is exploited in food industry, where it is used as a preservative. Some non-*Saccharomyces* species such as *Z. bailli* are highly resistant to acetic acid. Understanding the molecular determinants underlying such acid resistance phenotype is relevant for the design of strategies aiming at the genetic improvement of industrial *S. cerevisiae* strains, and the prevention of food and beverage spoilage by resistant species.

In most strains of *S. cerevisiae*, acetic acid is not metabolized by glucose-repressed yeast cells and enters the cell in the non-dissociated form by simple diffusion. Inside the cell, the acid dissociates and, if the extracellular pH is lower than the intracellular pH, this will lead to an intracellular acidification and to the accumulation of its dissociated form (which depends on the pH gradient), affecting cellular metabolism at various levels (Casal et al., 1996; Guldfeldt & Arneborg, 1998; Leão & van Uden, 1986; Pampulha & Loureiro, 1989;). Intracellular acidification caused by acetic acid leads to trafficking defects, hampering vesicle exit from the endosome to the vacuole (Brett et al., 2005). Though acetic acid induces plasma membrane ATPase activation (50 mM, pH 3.5), this enhanced activity is not enough to counteract cytosolic and vacuolar acidification (Carmelo et al., 1997). The toxic effects of the undissociated form of the acid also translate into an exponential inhibition of growth and fermentation rates (Pampulha & Loureiro, 1989; Phowchinda et al., 1995). Studies on glucose transport and enzymatic activities showed that the sugar uptake is not inhibited and that enolase is the glycolitic enzyme most affected by acetic acid, presumably resulting in a limitation of glycolytic flux (Pampulha & Loureiro-Dias, 1990). As revealed by the proteomic analysis of acetic acid-treated cells, carbohydrate metabolism is strongly affected, in agreement with a decreased glycolytic rate. Levels of the glycolytic proteins phosphofructokinase (Pfk2p) and fructose 1,6-bisphosphate aldolase (Fba1p) were decreased whereas the pyruvate decarboxylase isoenzyme (Pdc1p) suffered several post-translational modifications (Almeida et al., 2009). Growth in batch cultures following cellular adaptation to acetic acid is associated not only with a decrease in the maximum specific growth rate and in the ATP yield, but also with a recovery in intracellular pH and an increase in the specific glucose consumption rate, indicating that metabolic energy was diverted from metabolism (Pampulha & Loureiro-Dias, 2000). Using anaerobic chemostat cultures, it was shown that higher trehalose contents induced by lower growth rates or by the presence of ethanol are related to higher tolerance of *S. cerevisiae* to acetic acid (Arneborg et al., 1995, 1997). However, internal acidification caused by the acid can lead to the activation of trehalase (Valle et al., 1986). Hypersensitivity to acetic acid was observed in auxotrophic mutants with requirements for aromatic amino acids. Consistently, prototrophic *S. cerevisiae* strains are more resistant to acetic acid treatment (Gomes et al., 2007). Though there is no direct evidence, these phenotypes are probably explained by an

inhibition of the amino acid uptake, since sensitivity is suppressed by supplementing the medium with high levels of tryptophan (Bauer et al., 2003). Accordingly, it was recently shown that acetic acid causes severe intracellular amino-acid starvation (Almeida et al., 2009), as referred below (section 4.4.). In another study, it was found that deletion of *FPS1*, coding for an aquaglyceroporin channel, abolishes acetic acid accumulation at low pH (Mollapour & Piper, 2007). This observation was explored to improve acetic acid resistance and fermentation performance of an ethanologenic industrial strain of *S. cerevisiae* through the disruption of *FPS1* (Zhang et al., 2011). The acetic acid-tolerance phenotype of the disrupted mutant was mainly explained by the preservation of plasma membrane integrity, higher *in vivo* activity of the H^+-ATPase, and lower oxidative damage after acetic acid treatment.

1.2 The high resistance of *Zygosaccharomyces bailii* to acetic acid

Acetic acid, due to its toxic effects, is used in food industry as a preservative against microbial spoilage. As a weak monocarboxylic acid with a pK_a of 4.76, its toxicity is strongly dependent on the pH of the medium, exerting an antimicrobial effect mainly at low pH values (below pK), where the protonated form predominates. However, there are some yeast species that are able to spoil foods and beverages due to their capacity to survive and grow under these stress conditions where other microorganisms are not competitive. *Z. bailli* is one of the most widely represented spoilage yeast species, particularly resistant to organic acids in acidic media with sugar (Thomas & Davenport, 1985). Another interesting feature of *Z. bailii* is its ability to grow under strictly anaerobic conditions (with trace amounts of oxygen) in complex medium, whereas in synthetic medium under strictly anaerobic conditions *Z. bailii* displays an extremely slow and linear growth compatible with oxygen-limitation (Rodrigues et al., 2001). These differential requirements for anaerobic growth, different from those associated with Tween 80 and ergosterol, are still a matter of debate (Rodrigues et al., 2005). This species is much more tolerant to acetic acid than *S. cerevisiae* and is able to grow in medium with acetic acid concentrations well above those tolerated by the later yeast, a phenotype that seems to be related to the metabolism of the acid. Glucose respiration and fermentation in *Z. bailii* and *S. cerevisiae* express different sensitivity patterns to ethanol and acetic acid. Inhibition of fermentation is much less pronounced in *Z. bailii* than in *S. cerevisiae*, and the inhibitory effects of acetic acid on *Z. bailii* are not significantly potentiated by ethanol (Fernandes et al., 1997).

One of the peculiar traits of *Z. bailii* is the mechanism underlying the transport of acetic acid into the cell and its regulation, the first step of acid metabolism. Either glucose or acetic acid grown cells display activity of mediated transport systems for acetic acid (Sousa et al., 1996). This is in contrast with what has been described so far in other yeast species, namely *S. cerevisiae, Candida utilis,* and *Torulaspora delbrueckii* where active transport of acetate by a H^+-symport is inducible and subject to glucose repression (Casal & Leão, 1995; Cássio et al., 1987, 1993; Leão & van Uden, 1986). Additionally, in the presence of glucose, *Z. bailii* displays a reduced passive permeability to the acid when compared with *S. cerevisiae* (Sousa et al., 1996). Unlike most strains of *S. cerevisiae*, which are unable to metabolize acetic acid in the presence of glucose, *Z. bailii* is able to simultaneous use the two substrates due to the high activity of the enzyme acetyl-CoA synthetase (Sousa et al., 1998). Thus, it appears that in *Z. bailii* both membrane transport and acetyl-CoA synthetase could assume particular

physiological relevance in regards to the high resistance of this yeast species to environments containing mixtures of sugars and acetic acid, such as those often present during wine fermentation. Under these conditions, both the membrane transport flux and the intracellular metabolic flux of the acid seem to be regulated in such a way that cell can cope with the cytotoxic effects of the acid. These physiological traits have been related to the high resistance of *Z. bailii* to acidic media containing ethanol since this alcohol inhibits the mediated transport of the acid (Sousa et al., 1998).

1.3 A brief overview of programmed cell death: From multicellular organisms to yeast

The designation "Programmed Cell Death" (PCD) was first introduced by Lockshin (Gewies, 2003). Though PCD was initially related to the physiological cell death during organism's development, it has been generalised to alternative suicide processes that cells activate in response to various environmental aggressions. The term active cell death means that the process is genetically regulated in opposition to passive or accidental death, which is an uncontrolled death that occurs after exposure to an excessive dose of the lethal agent. These processes play an important role in the normal development, homeostasis mechanisms and disease control of multicellular organisms. Among the different forms of PCD (Kromer et al., 2009), namely apoptosis, autophagic cell death and programmed necrosis, apoptosis is the most common morphological expression of PCD. The main morphological features of an apoptotic cell, since the initial description by Kerr, Wyllie and Currie (1972), are the reduction of cellular volume (pyknosis), chromatin condensation and nuclear fragmentation (karyorrhexis) and engulfment by resident phagocytes (*in vivo*). All these changes take place in cells which display little or no ultrastructural modifications of cytoplasmic organelles and maintain plasma membrane integrity until the final stages of the process. Exposure of posphatidylserine on the outer leaflet of the plasma membrane of apoptosing cells, which promote phagocytosis by scavenging macrophages, is often an early event of apoptosis. Though not exclusive of apoptosis, other biochemical and functional changes such as oligonucleosomal DNA fragmentation, and the presence of proteolytically active caspases (cysteine-dependent aspartate-specific proteases) or of cleavage products of their substrates, may accompany the dismantling of the apoptotic cell. While in some settings apoptosis occurs independently of caspases, in others these proteases are key regulators of the death process and responsible for morphological and biochemical alterations typical of apoptosis (e.g., cellular blebbing and shrinkage, DNA fragmentation, and plasma membrane changes), as well as for the rapid clearance of the dying cell (Hengartner, 2000).

At least two major apoptotic pathways have been described in mammalian cells. One requiring the participation of mitochondria, called "intrinsic pathway," and another one in which mitochondria are bypassed and caspases are activated directly, called "extrinsic pathway" (Hengartner, 2000; Matsuyama et al., 2000). Regarding the mitochondrial pathway, two main events have been proposed as integral control elements in the cell's decision to die, namely, the permeabilization of the mitochondrial membrane and the release of several apoptogenic factors like cytochrome *c* (cyt *c*), apoptosis inducing factor (AIF), endonuclease G (Endo G), HtrA2/OMI and Smac/DIABLO (Hengartner, 2000; Matsuyama et al., 2000). Release of cyt *c* to the cytosol drives the assembly of a high-molecular-weight complex, the apoptosome, that activates caspases (Adrian & Martin,

2001). Translocation of cyt *c* to the cytosol is, therefore, a pivotal event in apoptosis. Cyt *c* is a soluble protein loosely bound to the outer face of the inner mitochondrial membrane, and its release is associated with an interruption of the normal electron flow at the complex III site, that could divert electron transfer to the generation of superoxide (Cai & Jones, 1998).

Beside caspases, members of the Bcl-2 protein family are key regulators of apoptosis, playing a crucial role in the regulation of the mitochondrial apoptosis pathway in vertebrates (Roset et al., 2007). The Bcl-2 family members have been identified and classified accordingly to their structure and function. At first, this family was usually divided in anti- and pro-apoptotic members. Currently, with new results obtained for a sub-group of this family, the BH-3 only proteins, they are divided into four categories (Chipuk et al., 2010). The anti-apoptotic Bcl-2 proteins (A1, Bcl-2, Bcl-w, Bcl-xL and Mcl-1), Bcl-2 effector proteins, (Bak and Bax), direct activator BH3-only proteins (Bid, Bim and Puma) and sensitizer/de-repressor proteins (Bad, Bik, Bmf, Hrk and Noxa). Complex interactions between members of this family control the integrity of the mitochondrial outer membrane (Green et al., 2002). The pro-apoptotic members of this family (Bax and Bak) are critical for mitochondrial membrane permeabilization, since deletion of both proteins impairs this event (Wei et al., 2001). Multicellular organisms have developed different regulatory complex mechanisms that coordinate cell death and cell proliferation and guarantee tissue homeostasis and normal development. Dysfunction of apoptosis is associated with severe human pathologies such as cancer and neurodegenerative diseases. Therefore, the identification of components of the different apoptotic pathways and the understanding of mechanisms underlying their regulation is critical for the development of new strategies for prevention and treatment against those diseases.

For several years, *Caenorhabditis elegans* and *Drosophila melanogaster* have been chosen as core models for cell death research, and until a decade ago it was not conceivable that unicellular organisms including yeast could possess a PCD process. This assumption was supported by the absence of key regulators of mammalian PCD in yeast, as indicated by plain homology searches and by the difficulty to explain the sense of cell suicide and its evolutionary advantage in a unicellular organism. However, in the late 1990s early 2000s, evidence indicating the presence of some basic features characteristic of an apoptotic phenotype in *S. cerevisiae* was reported (Madeo et al., 1997). This study showed that the expression of a point-mutated *CDC*48 gene (cdc48^{S565G}), essential in the endoplasmic reticulum (ER)-associated protein degradation pathway, leads to a characteristic apoptotic phenotype. Later on it was shown in *S. cerevisiae* that depletion of glutathione or exposure to low external doses of H_2O_2 triggers the cell into apoptosis, whereas depletion of reactive oxygen species (ROS) or hypoxia prevents apoptosis (Madeo et al., 1999). In addition, an intracellular accumulation of ROS was detected in the cell cycle mutant cdc48^{S565G} of *S. cerevisiae* and in yeast cells expressing mammalian Bax (Ligr et al., 1998). These results allowed the identification of ROS production as a key cellular event common to the known scenarios of apoptosis in yeast and animal cells (Madeo et al., 1999). Subsequent studies revealed that acetic induces apoptosis in *S. cerevisiae* through the involvement of mitochondria, indicating the conservation in yeast of an intrinsic death pathway (Ludovico et al., 2001, 2002). These former studies led to the emergence of a new research field that profited from the recognized advantages of yeast for the study of biological processes. Currently, there is

increasing evidence that apoptotic-like cell death pathways exist in unicellular organisms such as yeast and that this ability confers selective advantage in adapting to adverse environmental conditions and thus ensuring survival of the clone (Herker et al., 2004). Therefore, it is consensual that yeast can undergo cell death with typical markers of mammalian apoptosis in response to different stimuli and possess orthologs of mammalian apoptosis regulators, supporting the existence of a primordial apoptotic machinery similar to that present in higher eukaryotic cells (for a revision see Carmona-Gutierrez et al., 2010; Pereira et al., 2008).

2. Stress response pathways and key components

Under unfavourable environmental conditions, the yeast cell induces a common set of functional changes as a broad response to stress. These changes include, on one hand, reduction in activities linked with cell proliferation and protein synthesis, anabolic pathways and other processes associated with high energy expense, and, on the other, increase in activities related to protection and repair of damage of different molecules (DNA, proteins and lipids) and cellular structures. Gasch et al. (2000) reported that there are changes in a common set of about 900 genes (termed "environmental stress response" - ESR) in response to 12 different adverse environmental transitions, which mainly depend on the transcription factors Yap1, Msn2 and Msn4. From the 900 genes of the ESR, ≈600 are repressed and ≈ 300 are induced. The last set incorporates approximately 50 genes previously described as part of general stress response and which bear the stress response element (STRE) promoter sequence recognized by Msn2p and Msn4p. The different environmental conditions not only produce a set of common changes, probably accounting for the cross-resistances to different unrelated stress, but also generate specific responses reflecting the particular cell targets for each stress. Genome-wide functional analyses using the yeast disruptome, as well as gene expression profiling, have been exploited to identify key components of stress response induced by different weak carboxylic acids, namely sorbic, citric, benzoic, propionate, lactic and acetic acids (Abbot et al., 2008; Kawahata et al., 2006; Mira et al., 2009, 2010; Mollapour et al., 2004; Schuller et al., 2004). The first studies combining the two approaches were performed with sorbic acid. They were used to identify key players in biological response to the acid and to differentiate the essential genes from those displaying expression changes but which were not critical, or even relevant, for the ability of the cell to cope with a particular stress (Schuller et al., 2004). In this line, it was observed that although most of the genes induced by sorbic acid stress were dependent on the Msn2/4 transcription factors, the double knockout mutant was not more sensitive to sorbate stress. Resistance to sorbic acid, on the other hand, is predominantly associated with the activities of the previously described efflux pump Pdr12 (Piper et al., 1998) and of its dedicated transcription factor War1p. Oxidative stress-sensitive mutants, as well as mutants defective in mitochondrial function, vacuolar acidification and protein sorting (vps), ergosterol biosynthesis (erg mutants) and in actin and microtubule organization were also identified as sorbate-sensitive by genome-wide screening (Mollapour et al., 2004). Sorbate resistance increased with deletion of 34 genes categorize in several different functions, including *TPK2*, coding for one of the protein kinase A (PKA) isoforms, and the genes coding for the Yap5 transcription factor, two B-type cyclins (Clb3p, Clb5p), and a plasma membrane calcium channel activated by endoplasmic reticulum stress (Cch1p/Mid1p).

Lawrence et al. (2004) combined genome-wide phenotypic studies, expression profiling and proteome analysis to investigate citric acid stress. These authors described for the first time the involvement of mitogen-activated protein kinase (MAPK) high-osmolarity glycerol (HOG) pathway in the regulation of stress induced by a weak acid. Sixty nine mutants displaying sensitivity to 400 mM citric acid (pH 3.5) were detected in the screening, but no resistant strains were found. Citric acid up-regulated many stress response genes. However, in accordance with the results from the sorbic acid study, little correlation is observed between gene deletions associated with the citric acid-sensitive phenotype and those with measurable changes in the levels of transcript or protein expressed, although they belong to the same gene ontology families. Also, as found for sorbic acid, vacuolar acidification seems to be crucial for adaptation to citric acid. Transcription factors mediating glucose derepression, enzymes involved in amino acid biosynthesis and a plasma membrane calcium channel seem essential for adaptation to citric acid as well.

Applying genome-wide functional analysis and gene expression profiling to the study of acidic stress caused by lactic and acetic acid revealed a connection between Aft1p-regulated intracellular metal metabolism and resistance (Kawahata et al., 2006). As for sorbic and citric acids, vacuolar acidification and the Hog1p pathway seem to be important for resistance to lactic and acetic acid at low pH. In accordance, a sub-lethal growth inhibitory concentration of acetic acid was shown to promote the phosphorylation of Hog1p and Slt2p, two MAP kinases in *Saccharomyces cerevisiae* (Mollapour & Piper, 2006). However, from the 101 viable kinase mutants of the Euroscarf collection, only *hog1*Δ, *pbs2*Δ, *ssk1*Δ and *ctk2*Δ exhibited deficient growth in the presence of acetic acid. Activation of Hog1p by acetic acid was shown to depend on the presence of *SSK1* and *PBS2*, but not of *SHO1* or *STE11.* In the same screening, loss of the cell integrity MAP kinase (Slt2p/Mpk1p) was found to slightly increase acetate resistance. In what concerns the known plasma membrane sensors of MAPK pathways, acetate-induced Hog1p activation appears to involve the Sln1p, as also found for citric acid (Lawrence et al., 2004), whereas Slt2p activation was dependent on Wsc1p (Mollapour et al., 2009). It was also shown that the activation of Hog1p by acetic acid causes the removal of protein-channel Fps1p from the plasma membrane and limits the accumulation of the acid (Mollapour & Piper, 2007). The transcription factor Haa1p was also associated with resistance to acetic acid in glucose medium, where the knockout mutant displayed an increased lag phase (A. R. Fernandes et al., 2005). This effect was mainly attributed to the downregulation of genes coding for the plasma membrane multidrug transporters, *TPO2* and *TPO3,* and for the cell wall glycoprotein, *YGP1*. Genome-wide screening of the *S. cerevisiae* Euroscarf mutant collection identified 650 determinants of acetic acid tolerance, clustering essentially in the functional categories of carbohydrate metabolism, transcription, intracellular trafficking, ion transport, biogenesis of mitochondria, ribosome and vacuole, and nutrient sensing and response to external stimulus (Mira et al., 2010). Accordingly, a proteomic analysis of *S. cerevisiae* cells treated with acetic acid revealed that proteins from amino-acid biosynthesis, transcription/translation machinery, carbohydrate metabolism, nucleotide biosynthesis, stress response, protein turnover and cell cycle are affected (Almeida et al., 2009). Twenty eight transcription factors were identified as required for acetic acid resistance, from which Msn2p, Skn7p and Stb5p were found to have the highest percentage of targets among the genes required for acetic acid tolerance. The transcription factor Rim101p, previously described to counteract propionic acid-induced toxicity (Mira et al., 2009), was also found to

be necessary for acetic acid resistance. Differential transcriptome profiling in response to acetic acid revealed changes in the expression of 227 genes (Li & Yuan, 2010). The downregulated genes are associated with mitochondrial ribosomal proteins and with carbohydrate metabolism and regulation, whereas those related to arginine, histidine, and tryptophan metabolism were upregulated. Data indicated that acetic acid disturbs mitochondrial functions at translation, electron transport chain and ATP production levels, interrupts reserve metabolism (glycogen and trehalose metabolism and glucan synthesis), and regulates the central carbon metabolism and amino acid biosynthesis in yeast.

3. Cell death induced by acetic acid and its dependence on the temperature

Temperature profiles are an expression of the temperature dependence of growth and death in batch culture. Metabolites that accumulate in the medium and added drugs of industrial, medical or general scientific interest may profoundly change the temperature profile of yeast. Analysis of such modified profiles may shed light on the nature and localization of the targeted sites. Moreover, this analysis allows for predictions of the temperature-dependence of yeast performance in industrial fermentations and the effects of the temperature on the cytoxicity of preservatives on yeast in food, wine and other beverages (van Uden, 1984). It was shown that in *S. cerevisiae*, under certain conditions, acetic acid compromises cell viability and ultimately results in two types of cell death, high (HED) and low enthalpy (LED) cell death (Pinto et al., 1989). At concentrations similar to those that may occur during vinification and other alcoholic yeast fermentations, acetic acid and other weak acids enhance thermal death, causing a shift of the lethal temperatures of glucose-grown cell populations of *S. cerevisiae* to lower values. This type of cell death (HED) represents a thermal death enhanced exponentially by the acid which predominates at lower acetic acid concentrations (<0.5%, w/v) and higher temperatures. The knowledge acquired by the study of HED is of practical importance since the HED contributes to the so called "heat-sticking" of alcoholic yeast fermentations, particularly of red wine and fuel ethanol fermentations in warm countries in the absence of efficient temperature control. The second type of death (LED) induced by acetic acid occurred at intermediate and lower temperatures at which thermal death is not detectable, and could be considered a consequence of the cytoplasm acidification. Ethanol and other alkanols also induced these types of cell death, but acetic acid is over 30-times more toxic than ethanol. Cell death induced by acetic acid alone or with ethanol is strongly dependent not only on growth phase and pre-culture conditions of the cells before exposure to acetic acid, but also on the experimental conditions. Culture parameters such as temperature, pH, oxygen and nutrient availability, and, in particular, glucose concentration which determines the proportion between fermentative and respiratory metabolism, influence the percentage of dead cells in response to a given dose of acetic acid.

It was also observed that acetic acid and other weak acids enhanced death in glucose-grown cells of the spoilage yeast *Z. bailii*, but the effects were much lower than those described for *S. cerevisiae*, and only detectable at higher acid concentrations (Fernandes et al., 1999). *Z. bailii* is more resistant than *S. cerevisiae* to short-term intracellular pH changes caused by acetic acid (Arneborg et al., 2000). Furthermore, while in *S. cerevisiae* the enhancement of death by weak acids at intermediate temperatures could be considered a consequence of the acidification of the cytoplasm, in *Z. bailii* the intracellular acidification induced by weak

acids is less pronounced and appears not to have a significant role in death at such temperature range. This reinforces the idea that in *Z. bailii*, as opposed to *S. cerevisiae*, weak acids in general and acetic acid in particular only enhance thermal death and not LED, which may occur at lower temperatures. Furthermore, significant HED at these lower temperatures requires rather high concentrations of the toxic compounds, which, at least in the case of acetic acid, are much less realistic for alcoholic fermentations than the ones that induced significant death of this type in *S. cerevisiae*. As a consequence, it could be postulated that cell viability of *Z. bailii* will not be significantly affected, even at the end of the normal alcoholic fermentation processes, where the concentration of ethanol is high. Specifically in wine, this property of *Z. bailii* may be associated with its presence at the end of the process where the environmental conditions are too severe to allow survival of *S. cerevisiae*. As referred above the ability of *Z. bailii* to use acetic acid simultaneously with glucose, even at low pH values such as 3.5, in contrast to *S. cerevisiae*, which is often unable to metabolize the acid under these conditions, probably contributes to those different patterns of behavior between the two species (Sousa et al., 1998). The responses of the yeast to stress conditions could be considered using both non- and adapted cells. Adaption of cells to acetic acid in the growth medium modifies the cell death sensitivity pattern to acid environments. In *Z. bailii*, the negative effects induced by acetic acid in cell viability were only slightly lower in adapted than in non-adapted cells, which is consistent with the fact that in *Z. bailii* transport and intracellular acetic acid metabolism operate independently of the presence of glucose in the growth medium.

4. Acetic acid as an inducer of programmed cell death

In previous sections we focused on the cytotoxic effects of acetic acid and on the cellular responses triggered by the acid. This section encompasses the characterization of the cell death process induced by acetic acid, and covers the main molecular components/pathways involved and their regulation.

4.1 Molecular components and pathways

The first studies regarding the assessment of cell structural and functional changes associated with acetic acid-induced cell death in populations of *S. cerevisiae* were performed by flow cytometry multiparametric analysis combining different viability dyes (Prudêncio et al., 1998). Kinetic changes in esterase activity, intracellular dye processing, and membrane integrity were monitored, and to detect those changes three assays involving fluorescein diacetate hydrolysis, FUN-1 processing, and propidium iodide exclusion, were used, respectively. This approach allowed establishing the temporal order of appearance of the cell changes that pointed to the decrease in the ability to process FUN-1, which preceded the decrease in esterase activity, and was followed by the loss of cell membrane integrity after incubation with acetic acid. Together, these results suggested an intracellular localization of the acetic acid cellular target(s) in an early phase of cell death, rather than on the cellular membrane which occurred much later. Nevertheless, the flow cytometric analysis of mitochondrial membrane potential, $\Delta\Psi m$, (determined by rhodamine 123 staining) and plasma membrane integrity (determined by PI staining) showed that in *S. cerevisiae* acetic acid treatment (1.0% and 1.8% v/v, pH 3.0 for 130 min.) affects the proliferative capacity that is followed by the loss of plasma membrane integrity, and later by the loss of ability

of mitochondria to specifically stain with Rh123. In contrast, acetic acid treatment (1.8% and 3% v/v, pH 3.0 for 130 min.) of *Z. bailii* cells affects much less the ability of mitochondria to specifically stain with Rh123, and the loss of plasma membrane integrity observed for higher acetic acid concentrations is correlated with the loss of proliferative capacity (Ludovico 1999). Altogether these results clearly indicate that plasma membrane and mitochondria are targeted by acetic acid in *S. cerevisiae* at lower concentrations than in *Z. bailii* cells in accordance with the higher resistance phenotypes of the latter species. As mentioned above, acetic acid induces a PCD process in *S. cerevisiae* which shares common features with an apoptotic phenotype (Ludovico et al., 2001). It was found that acetic acid in concentrations between 20 and 120 mM induces a cycloheximide-inhibitable PCD process in exponentially growing *S. cerevisiae* cells that displays the most common apoptotic hallmarks, such as: (i) chromatin condensation along the nuclear envelope verified by transmission electron microscopy and DAPI staining; (ii) exposure of phosphatidylserine on the surface of the cytoplasmic membrane revealed by the FITC–annexin V reaction; and (iii) occurrence of internucleosomal DNA fragmentation demonstrated by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Exposure of cells to a higher acetic acid concentration (200 mM) resulted in cell death that was not inhibited by cycloheximide and was accompanied by ultrastructural alterations typical of necrosis. Pulsed field gel electrophoresis of chromosomal DNA from stationary phase cells dying by apoptosis after exposure to acetic acid (175 mM) revealed DNA breakdown into fragments of several hundred kilobases, consistent with the highly order chromatin degradation preceding DNA laddering in apoptotic mammalian cells (Ribeiro et al., 2006).

Caspases (cysteine aspartic proteases), key components of the mammalian apoptotic machinery, have a crucial role in cell dismantling. The metacaspase Yca1p, the only yeast ortholog of mammalian caspases identified so far, is activated in cells undergoing acetic acid-induced apoptosis in a manner strongly dependent on the cell growth phase (Pereira et al., 2007). Kex1p has been characterized as a serine carboxypeptidase B-like protease responsible for processing of prepro-factor (mating pheromone) as well as K1 and K2 killer toxin precursors (Bussey 1988; Fuller et al., 1988) while traversing the secretory pathway. This protease, besides being involved in PCD caused by defective N-glycosylation (Hauptmann et al., 2006), also contributes to the active cell death program induced by acetic acid stress. In fact, during cell death induced by acetic acid, the deletion of *KEX1* led to increased survival of cells and also a reduced production of ROS (Hauptmann et al., 2008). Though, as referred above, Yca1p was shown to be involved in acetic acid induced cell death, a caspase-independent route was also postulated (Guaragnella et al., 2006). Kex1p may be engaged in and contribute to this pathway, albeit its precise function in the cascade remains to be determined. The role of the proteasome in PCD is rather controversial. While proteosome inhibition has been found to induce PCD in certain mammal and plant cell contexts (Shinohara et al., 1996; Kim et al., 2003), transient proteasome activation is necessary for protein degradation during acetic acid-induced apoptosis in yeast (Valenti et al., 2008).

Similar to *S. cerevisiae*, acetic acid also induces in *Z. bailii* either an apoptotic or a necrotic death process, depending on the acid concentration. However, in *Z. bailii* the PCD process was found to occur at higher acetic acid concentrations (320-800 mM), described to be necrotic for *S. cerevisiae*. This is consistent with the higher resistance of *Z. bailii* compared to

that of *S. cerevisiae* as discussed above. The observation that acetic acid-induced PCD can occur not only in *S. cerevisiae* but also in *Z. bailii* (Ludovico et al., 2003) and *Candida albicans* (Phillips et al., 2003) reinforces the concept of a physiological role of the PCD in the normal yeast life cycle and raises the possibility that this mode of cell death is generalized in yeast.

4.2 The involvement of mitochondria

Like in mammalian cells, the PCD process triggered by acetic acid in yeast can be mediated by mitochondria (Ludovico et al., 2002). Biochemical and molecular evidence provided by such studies included the accumulation of mitochondrial reactive oxygen species (ROS), transient hyperpolarization followed by depolarization, decrease in cytochrome oxidase activity (COX) linked to a specific decrease in the amounts of COX II subunit, affecting mitochondrial respiration, and release of lethal factors like cytochrome *c* (cyt *c*). Though in apoptosis induced by hyperosmotic shock (Silva et al., 2005) a causal relationship between cyt *c* release and caspase activation was established, this has not been shown for most of the apoptotic scenarios in yeast. Moreover, components downstream release of cyt *c* have not yet been identified in yeast. Therefore, the formation of a mammalian apoptosome-like structure in yeast and the precise role of cyt *c* release in cells undergoing apoptosis need further research. The acetic acid-induced PCD process was found to be independent of oxidative phosphorylation because it was not inhibited by oligomycin treatment. The inability of *S. cerevisiae* mutant strains (lacking mitochondrial DNA, heme lyase, or ATPase) to undergo acetic acid-induced PCD and the absence of cyt *c* release in the ATPase mutant (knockout in *ATP10*) provides further evidence that the process is mediated by a mitochondria-dependent apoptotic pathway (Ludovico et al., 2002). Accordingly, disruption of the genes *CYC1* and *CYC7* rescued cells against acetic acid-induced PCD (Guaragnella et al., 2010a). ROS, in particular hydrogen peroxide, are mediators rather than by-products in *S. cerevisiae* cells committed to apoptosis triggered by acetic acid (Guaragnella et al., 2007). This interpretation was further confirmed by the protection against acetic acid-induced PCD afforded by the overexpression of cytosolic catalase (Guaragnella et al., 2008). As aforementioned, mitochondrial outer membrane permeabilization (MOMP) is a crucial step in the apoptotic pathway. This triggers the release of proteins from the mitochondrial intermembrane space into the cytosol, where they ensure propagation of the apoptotic cascade and execution of cell death. Opening of a mitochondrial pore called the permeability transition pore complex (PTPC), which leads to the swelling of mitochondria and rupture of the mitochondrial outer membrane, has been put forward as one of the mechanisms underlying mammalian MOMP. Although the molecular composition of the pore is not completely defined, it has been proposed that its major components are the adenine nucleotide transporter (ANT), the voltage dependent anion channel (VDAC) and cyclophilin D (for a review see Kinnally et al., 2011). Yeast genetic approaches revealed that while deletion of *POR1* (yeast VDAC) enhances apoptosis triggered by acetic acid, absence of ADP/ATP carrier (AAC) proteins (yeast orthologs of ANT) protects cells exposed to acetic acid (Pereira et al., 2007). Absence of AAC proteins and the consequent impairment of cyt *c* release do not completely prevent acetic acid-induced apoptosis, suggesting that alternative cyt *c*-independent pathways are involved. One such pathway may be the translocation of Aif1p, the yeast apoptosis inducing factor, from the mitochondria to the nucleus in response to acetic acid (Wissing et al., 2004). Mammalian AIF is a bifunctional NADH oxidase which has a pro-survival role when localized in the mitochondrial

intermembrane space through its involvement in mitochondrial respiration, and a lethal function upon translocation to the nucleus through a caspase-independent apoptotic process (Vahsen et al., 2004 ; Susin et al., 1999). Yeast Aif1p shares the same localization and death executing pathways as mammalian AIF, and dependence on cyclophilin A (CypA) but is partially dependent on caspase action (Wissing et al., 2004). It was recently shown that mammalian AIF mediates a programmed necrosis pathway independent of apoptosis and involving other molecules such as poly(ADP-ribose) polymerase PARP-1, calpains, Bax, Bcl-2, histone H2AX, and cyclophilin A (Delavallée et al., 2011). The yeast Yca1p is required for cyt *c* release (Guaragnella et al., 2010b) and the antioxidant N-Acetyl-L-cysteine (NAC) prevents acetic acid induced cell death by scavenging hydrogen peroxide, and inhibiting cyt *c* release and caspase activation (Guaragnella et al., 2010a). This supports the occurrence of a ROS-dependent acetic acid induced PCD. Because NAC does not prevent cells lacking Yca1p and cyt *c* to undergo PCD induced by acetic acid, it was proposed that a ROS-independent PCD can also be induced by the acid.

It was observed that during a variety of apoptotic scenarios in mammalian cells the interconnected mitochondrial network converts into punctiform morphology at early times, a process known as thread–grain transition (see reviews Parone et al., 2006; Scorrano et al.; 2005; Youle et al., 2005). This apoptotic fragmentation was proposed to be due to the activation of the physiological fission machinery, which proved also to influence the path of apoptosis (Frank et al., 2001). Inhibition of the mitochondrial fission machinery in mammals, including Drp1, ortholog of yeast Dnm1p, and Fis1p, impairs not only apoptotic fragmentation of the mitochondrial network but also cyt *c* release and the process of death itself (Frank et al., 2001; James et al., 2003). In yeast apoptosis induced by different compounds (Fannjiang et al., 2004; Kitagaki et al., 2007; Pozniakovsky et al., 2005) or upon heterologous Bax expression (Kissova et al., 2006) mitochondria also undergo extensive fragmentation suggesting it to be a general feature of yeast PCD. The mitochondrial fission protein Dnm1p and its two interactors, Mdv1p and Fis1p, have been implicated in the execution of the yeast apoptotic program induced by acetic acid (Fannjiang et al., 2004). Deletion of *DNM1* or *MDV1*/*NET2* inhibits cell death induced by acetic acid. Interestingly, Dnm1p-deficiency protected cells from death more efficiently than from mitochondrial fragmentation. This suggests that the absence of Dnm1p in yeast might confer protection against cell death also by mechanisms other than those related to fission of the mitochondria. In *S. cerevisiae*, Fis1p is evenly distributed along the mitochondrial surface, where it functions as a receptor to recruit Dnm1p from the cytosol to mitochondria (Modzi et al., 2000). The fact that the double mutant *dnm1*Δ*fis1*Δ behaved like *dnm1*Δ led Fannjiang et al. (2004) to propose that Fis1p inhibits the action of Dnm1p, thus promoting cell survival. The pro-survival role of Fis1p in yeast was a surprise since in mammalian cells overexpression of *FIS1* not only triggers mitochondrial fission but also cyt *c* release and apoptosis (James et al., 2003). Nevertheless, a recent study revealed that deletion of *FIS1* essentially selects for a secondary mutation in the stress-response gene *WHI2* that confers sensitivity to cell death (Cheng et al., 2006).

Nuc1p, the yeast ortholog of the mammalian endonuclease G, also mediates apoptosis induced by acetic acid (Buttner et al., 2007). This protein shares with other mitochondrially located yeast cell-death regulators, like cyt *c* and Aif1p, and with their mammalian counterparts, a role in cell proliferation and in cell death. Furthermore, it was found that Nuc1p-mediated death is independent of Yca1p and Aif1p. Instead, the Aac2p,

karyopherin Kap123p, and histone H2B interact with Nuc1p and are required for cell death upon Nuc1p overexpression, suggesting a pathway in which mitochondrial pore opening, nuclear import, and chromatin association are successively involved in EndoG-mediated death (Buttner et al., 2007). Ysp2p is another mitochondrial protein with a direct function in mitochondria-mediated PCD, since its absence hinders mitochondrial thread-to-grain transition and confers resistance to acetic acid-induced PCD (Sokolov et al., 2006).

4.3 The involvement of the vacuole

In the last decade, it has been demonstrated that organelles other than mitochondria are also engaged in the regulation of mammalian cell death processes (for a revision see Boya et al., 2008; Johansson et al., 2010; Repnik et al., 2010). Mammalian cells in response to different death stimuli may entail mitochondrial membrane permeabilization associated with the release of pro-apoptotic factors into the cytosol, as well as lysosomal membrane permeabilization (LMP) coupled with the release of cathepsins. Cathepsin D (CatD) has emerged as a central player in the apoptotic response. It was found that, following LMP, CatD is released into the cytosol and triggers a mitochondrial apoptotic cascade. However, CatD can have anti-apoptotic effects in some cellular types and specific contexts. Indeed, it is generally accepted that CatD is overexpressed and plays an important role in cancer cells (Masson et al., 2010). Pro-CatD outside the cells induces proliferation, angiogenesis, invasion and metastasis (Benes et al., 2008). Additionally, it was demonstrated that inhibition of CatD with pepstatin A induces caspase-dependent apoptosis in neuroblastoma cell lines (Kirkegaard & Jäättelä, 2008) and that overexpression of intracellular CatD in mouse xenografs using rat derived cell lines inhibits apoptosis (Masson et al., 2010). It was also reported that CatD downregulation sensitizes neuroblastoma cells to doxorubicin-induced apoptosis, while the opposite effect is observed for CatD overexpression (Sagulenko et al., 2008). In contrast, CatD mediates cyt *c* release and caspase activation in staurosporin-induced apoptosis in human fibroblasts (Johansson et al., 2003). It is therefore apparent that CatD can have opposite roles in apoptosis and that the lysosome is intrinsically connected to apoptosis through LMP.

In yeast, the vacuole seems to play a similar role to lysosomes in the regulation of apoptosis. The first study on the involvement of the vacuole in yeast apoptosis, concerns the translocation of the vacuolar protease Pep4p, the ortholog of the human CatD, into the cytosol during H_2O_2-induced apoptosis (Mason et al., 2005). It was found that, in an early phase of cell death, ROS levels and nuclear permeability increase while cell viability drops. In a later phase, the vacuolar membrane becomes permeable and provides access of the protease to nucleoporin substrates. Similar to the partial lysosomal membrane permeabilization observed during mammalian apoptosis, the release of Pep4p-EGFP from the vacuole is not linked to a rupture of the vacuolar membrane, as evidenced by a vacuolar lumen morphologically distinct from the cytosol. However, *PEP4* deleted cells are not protected from H_2O_2-induced cell death. This may be explained by the fact that migration of Pep4p out of vacuoles and nucleoporin degradation occurs only after the cells are nonviable. Release of Pep4p from the vacuolar compartment is also observed in an End3p deficient mutant displaying actin cytoskeleton stabilization-induced apoptosis (Gourlay et al., 2006). However, a role for this protease in actin-stabilized dying cells was not ascertained by the authors. Another study also documented the involvement of the vacuole in yeast apoptosis.

Deletion of class C vacuolar protein sorting genes results in drastically enhanced sensitivity of yeast to treatment with acetic acid and leads to a necrotic death, whereas death is mainly apoptotic in the wild type strain. These data indicate that a functional vacuole is required for a regulated cell death process through apoptosis (Schauer et al., 2009).

The occurrence of mitochondrial degradation following apoptosis induction is a common feature of mammalian cells and is often considered critical for its progression (reviewed in Tolkovsky et al., 2002). This event is generally mediated by lysosomes and usually occurs through an autophagic process that shows selectivity for mitochondria, termed mitophagy (Lemasters, 2005). Recent evidence supports the view that the PTP could be the trigger for mitochondrial degradation (Kim et al., 2007; Rodriguez-Enriquez et al., 2006). However, removal of mitochondria is not always dependent on the autophagic machinery (Matsui et al., 2006). In yeast cells undergoing apoptosis, mitochondrial degradation has been reported (Fannjiang et al., 2004). Heterologous expression of Bax (Kissova et al., 2007), mitochondrial dysfunction (Priault et al., 2005), osmotic swelling (Nowikovsky et al., 2007) and homeostasis of stationary phase cells (Tal et al., 2007) were also associated to selective removal of mitochondria. It was recently found that autophagy is not active during acetic acid-induced apoptosis (Pereira et al., 2010). Indeed, no increase in the amount of Atg8p, an essential autophagosome component, or in the activity of a truncated form of alkaline phosphatase, which activation is dependent on the induction of autophagy, is detected during acetic acid-induced apoptosis. Accordingly, deletion of *ATG5*, another component of the yeast autophagic machinery, did not affect cell survival. Alternatively to autophagy, the vacuolar protease Pep4p, ortholog of the human Cat D, was translocated to the cytosol and played an important role in mitochondrial degradation. Transmission electron microscopy analysis of the dying cells showed that vacuolar membrane integrity was preserved and plasma membrane integrity was maintained. Hence, Pep4p release seems to involve partial permeabilization of the vacuolar membrane rather than an extensive permeabilization typical of necrotic death. Taken together, these results suggested that Pep4p could have a role in apoptotic cell death similar to that of mammalian CatD. Instead, deletion of Pep4p confers higher susceptibility to acetic acid (Pereira et al., 2010) and leads to combined apoptotic and necrotic cell death during chronological aging (Carmona-Gutierrez et al., 2011) pointing to a function in cell protection rather than in the execution of cell death. Sustaining this hypothesis, cells overexpressing Pep4p displayed a higher resistance to acetic acid (Pereira et al., 2010) and an extension of chronological aging particularly through the anti-necrotic function of this protease rather than through its anti-apoptotic role (Carmona-Gutierrez et al., 2011). Pep4p deficient cells, like the wild type strain, exhibit mitochondrial dysfunction but are delayed in mitochondrial degradation during acid-induced apoptosis. On the other hand, Pep4p overexpression slightly enhanced mitochondrial degradation under the same conditions. Therefore, the process of removing damaged mitochondria apparently has a protective role in acetic acid-treated cells, although it is likely not the only factor affecting cell viability. Though the involvement of the vacuole and Pep4p in mitochondrial degradation is autophagy-independent, the precise mechanism is unknown. It is however apparent that this process also involves non-vacuolar proteins. Indeed, AAC-deficient cells show a decrease in mitochondrial degradation in response to acetic acid as well, and are not defective in Pep4p release. Therefore, AAC proteins seem to affect mitochondrial degradation at a step downstream to Pep4p release, possibly triggering degradation through their involvement in mitochondrial permeabilization. Accordingly, the

sensitization of cells to acetic acid by deletion of *PEP4* was dependent on AAC proteins, again suggesting these proteins act downstream of Pep4p in the apoptotic cascade (unpublished results). Moreover, it was proposed that the AAC proteins relay a signal of mitochondrial dysfunction, targeting their destruction. Taken together, the aforementioned observations suggest that vacuole and mitochondria destabilization, as measured by Pep4p and cyt *c* release, respectively, are events in the cell death cascade. Even though CatD, the mammalian ortholog of Pep4p, was shown to have a role in cell death by triggering mitochondrial dysfunction and subsequent release of mitochondrial proteins, some studies have shown an inhibitory role for CatD in apoptosis. Since autophagy is not active in cells undergoing acetic acid-induced apoptosis, vacuolar membrane permeabilization associated with the release of Pep4p may act as an alternative mitochondrial degradation process. The cytosolic acidification induced by acetic acid, associated with inhibition of autophagy may favor the activity of Pep4p after its release from the vacuole. These results unveil a complex regulation and interplay between mitochondria and the vacuole in yeast PCD.

Acetic acid has also been shown to induce apoptosis in a mammalian cell model. It has been demonstrated that the short chain fatty acids (SCFA) acetic and propionic acids produced by dietary propionibacteria in the human intestine induce cell death in colorectal carcinoma cell lines (CRC) by a mitochondrial-dependent apoptotic pathway (Jan et al., 2002; Lan et al., 2007) as described for yeast cells (Ludovico et al., 2001, 2002). SCFA induced nuclei shrinkage, chromatin condensation, nuclei fragmentation into apoptotic bodies and activation of pro-caspase 3. Moreover, it was shown that the mitochondrial dysfunctions induced by SCFA in CRC cells are similar to those observed in yeast, and can also be partially inhibited by expression of anti-apoptotic members of the Bcl-2 protein family (Jan et al., 2002; Saraiva et al., 2006). Jan and co-workers determined that the adenine nucleotide transporter (ANT), a putative component of the mammalian PTPC, was a potential SCFA target. Likewise, AAC proteins, the yeast orthologs of ANT, are targets in the acetic acid-induced apoptosis pathway (Pereira et al., 2007). The observation that acetic acid triggers a mitochondrial apoptotic pathway in both yeast and CRC cells further supports the use of the yeast model system to provide insights towards enhanced understanding of the function of mitochondria in cell death. It would be interesting to assess whether acetic acid induces LMP and release of CatD in CRC cell lines. This may provide valuable insights into the enhanced understanding of the function of lysosomes in cell death and their crosstalk with mitochondria.

The peroxisome is another organelle that is involved in cell death in yeast (Jungwirth et al., 2008). Indeed, cells lacking *PEX6*, encoding a peroxisomal membrane protein involved in a key step of peroxisomal protein import, display an increased accumulation of reactive oxygen species and an enhanced loss of viability upon acetic acid treatment associated with markers of necrosis. Nevertheless, it remains to be elucidated whether this necrotic death is a regulated process.

4.4 Apoptotic signaling pathways

To date, few reports on the apoptotic-related signal transduction pathways have been published in yeast. Indeed, most studies regarding yeast apoptosis have encompassed mainly the identification of different apoptotic triggers and the components/regulators of apoptotic death. Several functional genetic analysis and pharmacological-based approaches

allowed identifying components of yeast signalling cascades that, similarly to their mammalian counterparts, are engaged in conveying the information to the apoptotic apparatus. Not surprisingly, cell death signalling pathways in high eukaryotes are conserved and involved in the modulation of apoptosis in yeast.

The first evidence linking RAS/cAMP/cAMP-dependent protein kinase (PKA) pathway signalling to acetic acid-induced apoptosis in yeast came from the study by Phillips et al. (2006) with *Candida albicans*. Mutations that block Ras–cAMP–PKA signalling (*ras1*Δ, *cdc35*Δ, *tpk1*Δ, and *tpk*2Δ) suppress or delay the apoptotic response, whereas mutations that stimulate signalling (*RAS1*^{val13} and *pde*2Δ) accelerate the rate of entry into apoptosis. Consistently, pharmacological inhibition or stimulation of Ras signalling delay or promote apoptosis. Similar to the *C. albicans ras1* mutant, *RAS2* deletion is able to decrease cell death induced by acetic acid in *S. cerevisiae* (Ramsdale, 2006; Burtner et al., 2009). Transient mitochondrial hyperpolarization and ROS production (Ludovico et al., 2002) together with an increase in intracellular Ca^{2+} concentration (Pereira, C., Sousa, M.J. & Côrte-Real M., unpublished data) was found in response to acetic acid, pheromone and amiodarone. This indicates that these three stimuli converge into common death pathways. Moreover, since both pheromone- and acetic acid-induced apoptosis are inhibited by cycloheximide, it presumes transcriptional activation of target genes by upstream signalling cascades. Indeed, pheromone activates a calmodulin/calcineurin-controlled MAPK pathway (Severin et al., 2002) and the Ras pathway signals yeast apoptosis induced by harsh environmental stress, such as acetic acid (Phillips et al., 2003). Intracellular Ca^{2+} increase and ROS production are common features in these three scenarios, whereas intracellular acidification was only reported when death is induced by weak carboxylic acids, including acetic acid (Cardoso & Leão, 1992; Sokolov et al., 2006). Notably, death during chronological aging, measured as the loss of cell viability over time of stationary phase cells, is associated with acidification of the medium, and its neutralization by pH buffering compounds abolishes death. Sod2p and signalling through Ras–cAMP–PKA, including the transcription factors Msn2 and Msn4, were also shown to play a crucial role in the regulation of yeast chronological aging and death program (Fabrizio et al., 2004). Recently, a model for acetic acid as a cause of chronological aging has been proposed based on the observation that many modifiers of chronological life span also modulate acetic acid resistance. Most relevant, and besides Ras2p referred above, deletion of Sch9p kinase increases both CLS and acetic acid resistance in a Rim15p and Gis1p dependent manner (Burtner et al., 2009).

More recently, the combination of a proteomic approach using 2-DE and MS for the analysis of total cellular extracts, together with functional studies of *S. cerevisiae* cells treated with acetic acid, indicated that acetic acid causes severe intracellular amino-acid starvation, involving the general amino-acid control system as well as the TOR pathway (Almeida et al., 2009). Indeed, cells lacking Gcn4p/Gcn2p and Tor1p displayed a higher resistance to acetic acid, which in the latter case was associated with a TUNEL negative phenotype and lower ROS levels. In addition, cells lacking downstream mediators of the TOR pathway revealed that apoptotic signaling involves the phosphatases Pph21p and Pph22p but not Sit4p.

5. Conclusion

Acetic acid was early recognised as a common toxic agent present in different biotechnological processes associated with negative effects on the fermentative yeast. As

mentioned above, knowledge on yeast stress mechanisms in response to acetic acid has already had an impact on the construction of industrial strains with improved performance in biotechnological processes (Zhang et al., 2011). However, most studies on stress response, including the latest on the evaluation of resistance to acetic acid at a genome-wide scale, have only been focused on the ability of yeast to divide and grow in the presence of toxic agents. Studying the effect of gene deletions on cell growth, however, does not provide the full picture of the determinants for stress resistance and survival under stress. In the last decade, acetic acid has been identified as an inducer of a programmed cell death (PCD) process. The recognition that different lethal stimuli, such as acetic acid, trigger a regulated death in yeast provides a new basis for future breeding strategies of industrial strains with improved cell survival. Thus far, the occurrence in yeast of a regulated cell death has not been exploited to control yeast performance in industrial processes. Moreover, no genome-wide studies have been performed until now regarding the elucidation of the mechanisms underlying acetic acid-induced PCD and of its regulatory pathways. Therefore, a complete picture of the main executors and regulators involved in acetic acid-induced PCD in yeast is still missing. A new genome-wide analysis could allow identifying genes involved in the execution of acetic acid induced-PCD and also of those involved in its regulation. This high-throughput approach will likely provide information on new putative targets for the control of acetic acid-induced PCD and ultimately will allow improving the performance of industrial yeast strains, and to design new strategies for food preservation by inhibiting or activating the PCD process, respectively. An overview of the current knowledge on targets and pathways underlying PCD induced by acetic acid is shown in Fig. 1.

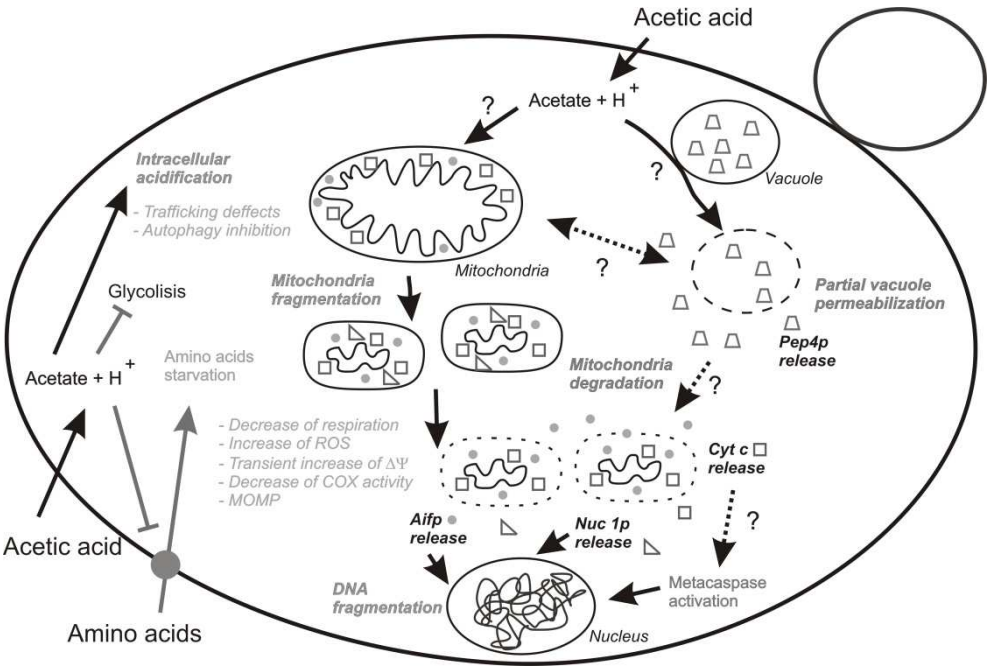

Fig. 1. Current knowledge on targets and pathways underlying PCD induced by acetic acid in *Saccharomyces cerevisiae*.

A major challenge in the future regarding stress and PCD in response to acetic acid will be to get a clearer picture on the different activated signalling pathways and of the crosstalk between them, and to comprehend how multiple cell targets and different types of damage lead the yeast cell to commit to a regulated form of suicide. As discussed above, different signalling pathways share several components, allowing the cell to convey the signal through a complex communication network. Regarding PCD induced by acetic acid, it was already shown that it targets mitochondria and the vacuole and probably other organelles, and induces different types of damage. It would be important to clarify how these organelles communicate and the different types of damage interact to ultimately trigger cell death, and whether they act in an independent, additive or synergistic manner. Understanding how the distinct stress signalling pathways communicate and how different types of death-associated damage contribute to cellular dysfunction in yeast is likely to prove informative about mammalian stress responses and PCD induced by acetic acid. Although acetic acid has been shown to target mitochondria and induce several damages in CRC cells, so far no stress signalling pathways have been identified.

The non-dissociated form of the acid enters glucose repressed cells by simple diffusion. Once inside the cell acetic acid dissociates and if the extracellular pH is lower than the intracellular pH this will lead to the accumulation of acetate and to the acidification of the intracellular environment. The effects promoted by these events are diverse but include inhibition of amino acid uptake and carbohydrate metabolism. Acetic acid also affects mitochondria function and dynamics triggering the release to the cytosol of mitochondria resident apoptogenic molecules such as cyt *c* and Aifp, the latter leading to DNA fragmentation. Partial permeabilization of the vacuolar membrane and release of Pep4p also occur during acetic acid-induced PCD. The link between vacuolar release of Pep4p and mitochondria degradation is still elusive. Once released, Pep4p could be directly or indirectly involved in the proteolytic degradation of mitochondria. However, mitochondria could be the first organelle affected by acetic acid, promoting a subsequent vacuole permeabilization followed by Pep4p release. Finally, acetic acid could act directly and simultaneously at the vacuole and mitochondria, which may further amplify the signal by a positive feedback mechanism. Broken arrows represent hypothetical pathways.

S. cerevisiae, the common baker's yeast, is a unicellular fungus of the ascomycete family. This unicellular eukaryote is one of the best established experimental model organisms and has been extensively used to unveil the mechanisms of eukaryotic cellular processes and their modes of regulation, including cell cycle control (McInerny, 2011), ageing, stress responses (Gasch & Werner-Washburne, 2002) and programmed cell death (Carmona-Gutierrez et al., 2010). More recently, it has been used as a valuable tool to understand the development of some human diseases such as degenerative disorders, including Huntington's and Parkinson's disease (Petranovic et al., 2008). The features that make this simple eukaryote a model system of choice are numerous: i) its cultivation is simple, fast and inexpensive; ii) it is a haplodiplont enabling sexual crossing and clonal division; iii) genetic manipulations, such as gene disruption, insertion or mutation are easy to perform, owing to the presence of a very efficient homologous recombination pathway; iv) heterologous expression of genes either from an episomal plasmid or from chromosomal integration can be achieved; v) the genome sequence and a collection of single deletion mutants for diploid cells and for non-essential genes of haploid cells is available. Moreover, *S. cerevisiae* is the organism with the most comprehensive datasets gathered from high-throughput data collected by functional

genomic tools including transcriptome, proteome, metablome, interactome, locasome and flux analysis (see Petranovic et al., 2008). Moreover, there is a high degree of conservation between yeast and higher eukaryotes, with respect to numerous basic biological processes, and approximately half of the genes involved in human hereditary diseases are predicted to have yeast homologues (Hartwell, 2004). Nonetheless, it has to be mentioned that yeast also harbours some relevant differences comparatively with other organisms. For instance, yeast is highly specialized to grow on glucose medium and, unlike higher eukaryote cells, it easily forms respiratory deficient mutants which have little or none mitochondrial respiration. This ability to survive under conditions in which mitochondrial respiration is impaired or absent represents an ideal model, specifically with regard to the study of the involvement of mitochondrial proteins in PCD. Despite the vast differences in complexity between yeast and humans, the study of stress responses and PCD in yeast has provided insights into pathways that modulate response to stress and PCD in mammals. Thus, advances in the elucidation of cell death in yeast not only assume relevance in terms of biotechnological processes, but also in biomedicine. The studies that many yeast biologists have performed contributed to establish *S. cerevisiae* as an accepted model and will allow it to continue at the forefront of model organisms used to answer to many relevant biological questions.

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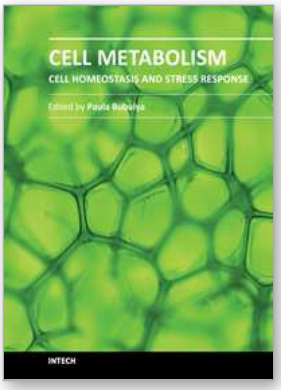

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A global research community of scientists is teasing out the biochemical mechanisms that regulate normal cellular physiology in a variety of organisms. Much of current research aims to understand the network of molecular reactions that regulate cellular homeostasis, and to learn what allows cells to sense stress and activate appropriate biochemical responses. Advanced molecular tools and state-of-the-art imaging techniques discussed in this book continue to provide novel insights into how environmental changes impact organisms, as well as to develop therapeutic interventions for correcting aberrant pathways in human disease.

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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821