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Role of apoptotic regulators in the protective effect of acetic acid against ethanol - induced cell death in *Saccharomyces cerevisiae*

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Mestrado em Bioquímica Aplicada

Trabalho efectuado sob a orientação de
Professora Doutora Manuela Côrte-Real

Trabalho efectuado sob a co-orientação de
Professora Doutora Maria João Sousa

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DECLARAÇÃO

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(Natália Cristina Alves Rocha)

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Abstract

Ethanol is a well-know end product of alcoholic fermentation carried by *Saccharomyces cerevisiae*. At relatively low concentrations it is an inhibitor of yeast growth, while, high ethanol concentrations reduces cell viability (Birch and Walker, 2000). During fermentation, acetic acid produced by yeast metabolism may accumulate in growth medium and enhance ethanol toxicity (Gibson *et al.,* 2007). By contrast, studies conducted in our laboratory have shown that *S. cerevisiae* cells exposed simultaneously to toxic concentrations of ethanol and low concentrations of acetic acid displayed a higher survival than cells treated only with ethanol (Vieira *et al.*, unpublished results). Later, Trindade showed that Hog1p and Fps1p, involved in acetic acid resistance, do not have a role in the protective effect of acetic acid seen by Vieira (Trindade, 2009). Subsequently, it was also shown that the osmolyte trehalose, the heat shock protein Hsp12p, the MAP kinase Slt2/MPK1 and functional mitochondria play a role in the protection by the undissociated form of acetic acid against ethanol induced cytotoxicity (Afonso, 2011).

With the present master thesis we intended to further understand how acetic acid protects against ethanol-induced cytotoxicity, determining the role of known apoptotic regulators in this protection. We also intended to characterize the death of cells exposed to toxic concentrations of ethanol since this knowledge is important to dissect the role of acetic acid in its inhibition.

This study showed that the deletion in *CYC3* or *ATP2, ATP10* and *NUC1* abolish and decrease, respectively, the protection of acetic acid against loss of cell viability, loss of plasma membrane integrity and the accumulation of superoxide anion, induced by ethanol. Death induced by ethanol was associated with typical markers of apoptosis but also of necrosis. In particular caspase activation and exposure of phosphatidylserine was detected mainly in cells with compromised plasma membrane, but also HMGB1/Nhp6A translocates from the nucleus to the cytosol in response to ethanol. Moreover, the absence of known apoptotic regulatory proteins abrogate (*atp2Δ, atp10Δ, cyc3*Δ, *por1*Δ, *cpr3*Δ and *yca1*Δ mutants), exacerbate (*aif1Δ, nuc1Δ,* and *cyc1Δcyc7Δ* mutants) or have no effect (*pep4Δ* mutant) in ethanol induced cell death. Altogether the results obtained with the mutants phenotypes and the characterization of cell death markers indicate that ethanol triggers a regulated cell death process that shares features typical of both apoptosis and necrosis. Whether

the necrotic features reflect a programmed necrosis or a secondary necrosis of apoptotic cells committed to death in response to ethanol, requires further studies.

Resumo

O etanol é um produto final bem conhecido da fermentação alcoólica conduzida por *Saccharomyces cerevisiae*. Em concentrações relativamente baixas é um inibidor do crescimento da levedura, enquanto em concentrações elevadas induz perda da viabilidade celular (Birch e Walker, 2000). Durante a fermentação, o ácido acético produzido pelo metabolismo da levedura pode acumular-se no meio extracelular e aumentar a toxicidade de etanol (Gibson *et al.,* 2007). Por outro lado, estudos realizados no nosso laboratório mostraram que células de *S. cerevisiae* simultaneamente expostas a concentrações tóxicas de etanol e baixas concentrações de ácido acético exibiam uma maior sobrevivência do que aquelas tratadas apenas com etanol. (Vieira *et. al.,* resultados não publicados). Mais tarde, Trindade mostrou que as proteínas Hog1p e Fps1p, envolvidas na resistência ao ácido acético, não apresentam um papel no efeito protetor do ácido acético observado por Vieira (Trindade, 2009). Posteriormente, mostrou-se que o osmólito trealose, a proteína de choque térmico Hsp12p, a MAP quinase Slt2/MPK1 e mitocôndrias funcionais têm um papel na proteção da forma não dissociada do ácido acético contra citotoxicidade induzida por etanol (Afonso, 2011).

Com a presente dissertação de mestrado pretendeu-se compreender melhor como o ácido acético protege contra a citotoxicidade induzida por etanol, determinando o papel de reguladores conhecidos da apoptose nesta proteção. Também se pretendeu caracterizar a morte de células expostas a concentrações tóxicas de etanol uma vez que este conhecimento é importante para dissecar o papel do ácido acético, na sua inibição.

Este estudo mostrou que a ausência dos genes *CYC3* ou *ATP2, ATP10* e *NUC1* elimina e diminui, respetivamente, a proteção do ácido acético contra a perda induzida pelo etanol da viabilidade celular, da integridade da membrana plasmática e da acumulação de anião superóxido. A morte induzida pelo etanol foi acompanhada de marcadores celulares típicos de apoptose, mas também de necrose. Em particular foi detetada ativação de caspases e exposição de fosfatidilserina principalmente em células com a membrana plasmática comprometida, mas também a translocação do HMGB1/Nhp6A do núcleo para o citosol em resposta ao etanol. Adicionalmente, a ausência de reguladores apoptóticos reduziram (nos mutantes *atp2Δ, atp10Δ, cyc3*Δ, *por1*Δ, *cpr3*Δ e *yca1*Δ), potenciaram (nos mutantes *aif1Δ, nuc1Δ,* e *cyc1Δcyc7Δ*) ou não afetaram a

sobrevivência celular (no mutante *pep4Δ*) em resposta ao etanol. Os resultados respeitantes aos fenótipos dos mutantes estudados e à caracterização dos marcadores de morte celular indicam que o etanol induz uma morte regulada que partilha características típicas de apoptose e necrose. Se as características de necrose refletem uma necrose programada ou uma necrose secundária de células que desencadearam um processo de morte apoptótico, requer mais estudos no futuro.

Index

Abbreviations

AA – Acetic Acid
ABC – ATP – Binding Casset
ADP – Adenosine Diphosphate
AIF – Apoptosis Inducing Factor
AK – Adenylate Kinase
AnnV – Annexin V
ANT – Adenine nucleotide transcolator
ATP – Adenosine Triphosphate
CCHL – Cytochrome C the Heme Lyase
CFU – Colony Forming Units
CS – Citrate Synthase
Cyt c – Cytochrome c
DAPI – Diamino – 2 – phenyl – indole dihydrochloride
DHE – Dihydroethidium
$DIOC_6$ – 3,3' – Dihexyloxacarbocyanine Iodide
DNA – Deoxyribonucleic Acid
Endo G – Endonuclease G
Et – Ethanol
FITC – Fluoroisothiocyanate
gTME – global Transcription Machinery
HEPES - 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HMGB1 – High Mobility Group Box 1
HPSs – Heat Shock Proteins
HSFs – Heat Shock factors
H_2O_2 – Hydrogen Peroxide
IAP – Inhibitors of Apoptosis Proteins
MAP Kinases – Mitogen – Activated Protein Kinases
MES – N – Moroholino Ethanesulfonic Acid
min – minutes
MMP – Mitochondrial Membrane Permeabilization
MOMP – Mitochondrial Outer membrane Permeabilization
MPTP – Mitochondrial permeability transition pore
N.s. – no significant
NAD^+ - Nicotinamide Adenine Dinucleotide
NCCD – Nomenclature Committee on Cell Death
Omi/HtrA2 – High Temperature Requirement Proteins A2
OMM – Outer Mitochondrial Membrane
PARP1 – Poly ADP – ribose Polymerase
PBS - Phosphate Buffered Saline
PCD – Programmed Cell Death
pH – Potential oh Hydrogen
PHD – Plant Homeo Domain
PI – Propidium Iodide
PS – Phosphatidylserine
RIP1 – Receptor-interacting serine Protein 1
RNA – Ribonucleic Acid
ROS – Reactive Oxygen Species
rpm – rotations per minute
SC URA – Synthetic Complete Uracil
SD – Standard Deviation
STRE – Transcriptional factor interact with stress response element
TCA – Trichloroacetic
TNFR1 – Tumor Necrosis Factor Receptor 1
TUNEL – Terminal dUTP Nick – End Labeling
UFAs – Unsaturated Fatty Acids
VDAC1 – Voltage Dependent Anion – selective Channel
YPD – Yeast Peptone Dextrose
YPDA - Yeast Peptone Dextrose Agar

INTRODUCTION

Yeast: biotechnology relevance and applications in fundamental and applied research

Yeasts are eukaryotic unicellular fungi with similar characteristics to higher eukaryotic cells. These microorganisms nowadays are relevant in both biotechnology, with important economic impact in several fields, and in fundamental research (Sousa *et al.,* 2011). Its simplicity, receptivity to genetic manipulation and traceability make it a powerful tool to elucidate the mechanisms underlying the fundamental cellular processes of eukaryotes and their modes of regulation (Walker, 1998).

Saccharomyces cerevisiae, the most commercially exploited yeast species, has been traditionally used in the brewing, baking and wine-making industries (Muthaiyan and Ricke, 2009). With the advances in the biotechnological field it has been gaining importance in different industries, including the chemical and food industries, as well as in the health and biological, biomedical and environmental fields (Walker, 1998). This yeast species is widely used for the production of macromolecular cellular components such as lipids, proteins including enzymes, and vitamins (Bigelis, 1985; Stewart and Russell, 1985). With a long history of industrial applications, *S. cerevisiae* has been the subject of various studies of microbiology (Anderson, 1992). *S.cerevisiae* has also been used as a model for physiological studies on the effects of osmotic and oxidative stress induced by ethanol and weak acids among other compounds (Braun *et al.,* 2006). The completion of the DNA sequence of the yeast *S. cerevisiae* genome has made possible research approaches that allow analysis of cellular processes at a whole-genome scale. Many of the proteins encoded by yeast bear a striking primary sequence and functional homology to corresponding proteins found in higher eukaryotic organisms. This similarity accelerated the description of the function of numerous mammalian gene products and contributed to the deciphering of the basic physiological processes of eukaryotic cells. Yeast cells, due to their ability to express functionally proteins of therapeutic interest, have also become an important tool in the drug discovery process (Pausch *et al.,* 2001).

The presence of an apoptotic regulatory network in yeast that encompasses many of the crucial events that occur in mammalian cells stimulated several studies in disease related proteins that have no homologues in this organism and created a new research field with the so-called humanized yeast systems, providing cell-based assays to discover novel medicinal compounds (Mager and Winderickx, 2005). More recently it was recognized that yeast, like mammalian cells, can also dye by programmed necrosis, which enlarge the possibilities to exploit this cellular model to

understand different forms of regulated cell death in higher eukaryotes. Moreover, the knowledge of cell death processes in yeast and of the mechanisms underlying their regulation allows to improve the performance of the fermentative yeast with relevant impact on biotechnological applications.

Stress in yeast

Yeasts require specific internal conditions for optimal growth and functioning, thus fluctuations in available nutrients, temperature, osmolarity and acidity of their environment and the presence of noxious agents such as radiation and toxic chemicals which represent a stress to yeasts (Gasch *et al.,* 2000). Stress can also be imposed by the host defense mechanism on the case of pathogenic yeasts (Moye-Rowley, 2003).

In order to minimize the effects of stress on the survival and proliferation of cells different molecular responses can be induced. An adaptive response can result from exposure to low levels of a stress and may result in transient resistance when the levels of the same stress increase. This adaptation to stress can also lead to increased resistance (or cross-protection) to other types of stress (Jamieson 1992; Lee *et al.,* 1995; Moradas-Ferreira and Costa, 2000). The adaptive response requires new protein synthesis, indicating that changes in gene expression are fundamental. The phenomenon of cross-protection suggests either that different stress conditions can activate similar defense mechanisms or that there is a general stress response that can confer a basic level of protection (Chen *et al.,* 2003).

Studies at the whole genome scale in the yeast *S. cerevisiae* have revealed that 10% to 14% of all genes are induced or repressed in response to a wide range of stresses. Induced genes are involved in various processes, including carbohydrate metabolism, detoxification of reactive oxygen species (ROS), protein folding and degradation, vacuolar and mitochondrial functions, autophagy, and metabolite transport. Repressed genes are generally involved in energy consuming and growth-related processes, including RNA processing, transcription and translation, and biosynthesis of ribosomes and nucleotides (Causton *et al.,* 2001; Gasch *et al.*, 2000).

The major yeast stress-response mechanisms that protect cells against various types of stress are: accumulation of trehalose, synthesis of molecular chaperones, antioxidant proteins and hydrophylins, accumulation of compatible solutes, and changes in the composition of the plasma

membrane. A specific mechanism to protect cells from different stresses have also been described, for instance, expression of aquaporins has been described as a protection mechanism against freeze stress (Tanghe *et al.*, 2002).

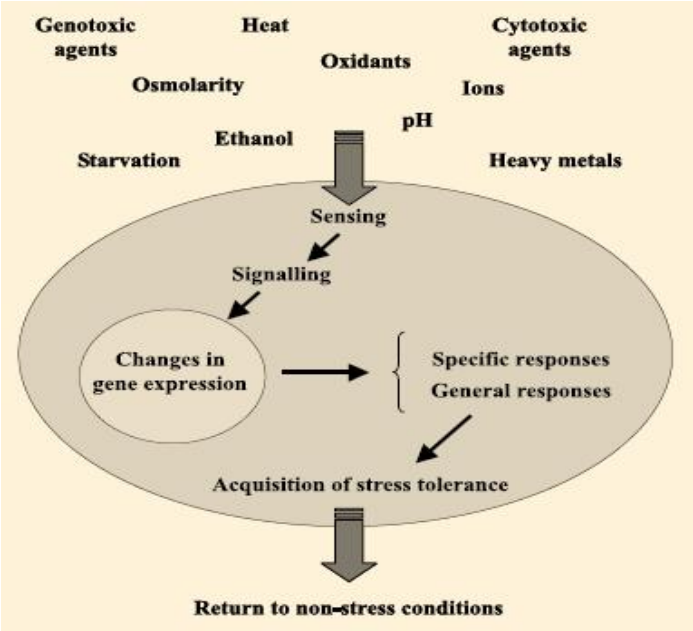

Figure 1 - Schematic representation of cell events triggered by environmental changes and leading to adaptation to stress (adapted from Salvador *et al.,* 2009)

Ethanol stress

One of the most common stresses that yeast cells encounter during fermentation is the exposure to increasing concentration of ethanol in the medium (Ding *et al.,* 2009). At relatively low concentrations ethanol is an inhibitor of yeast growth, inhibiting cell division, decreasing cell volume and specific growth rate, while high ethanol concentrations reduce cell vitality and increase cell death (Birch and Walker, 2000). Ethanol also influences cell metabolism and macromolecular biosynthesis by inducing the production of heat shock-like proteins, lowering the rate of RNA and protein accumulation, enhancing the frequency of petite mutations, altering metabolism, denaturing intracellular proteins and glycolytic enzymes and reducing their activity (Hu *et al.*, 2007). The main sites for ethanol effects in yeast are the cellular membranes (Mishra and Prasad, 1989), hydrophobic and hydrophilic proteins and the endoplasmic reticulum (Walker, 1998).

In order to circumvent the damage caused by the increasing concentration of ethanol, the yeast cells have created mechanisms of defense (Ding *et al.,* 2009). Thus, the yeast cells can change the plasma membrane composition for antagonizing fluidization and to improve its stability, specifically it has been show that the levels of unsaturated fatty acids (UFAs) (You *et al.,* 2003) and ergosterol (Daum *et al.*, 1998; Swan and Watson, 1998) increase in response to the high concentration of ethanol. The addition of some types of amino acids (Hu *et al.,* 2005; Takagi *et al.,* 2005) and inositol (Kelley *et al.,* 1998) has also been described as a way to improve tolerance to ethanol, probably increasing the stability of the membrane.

Accumulation of trehalose and induction of heat shock proteins (HSPs), factors that stabilize and/or repair denatured proteins in yeast cells, appear to have a role in the cells ability to tolerate alcohol (Swan and Watson, 1998; Vianna *et al.,* 2008). Some candidate proteins involved in the expression of stress related genes like the zinc finger proteins (MacPherson *et al.,* 2006), and the recently reported alcohol sensitive ring/PHD finger 1 protein (Asr1p) (Betz *et al.,* 2004) also play a role in ethanol tolerance in *S. cerevisiae.* The global transcription machinery engineering (gTME) technology has also been used to reprogram gene transcription and then improve glucose/ethanol tolerance of yeast cells (Alper *et al.,* 2006).

Table 1 – Factors related with ethanol tolerance of *S. cerevisiae* and their function during ethanol stress (adapted from Ding *et al.,* 2009).

Factors involved in ethanol tolerance	Function	References
Composition of yeast plasma membrane		You *et al.* (2003); Xiao *et al.* (2008); Ingram (1990) and Weber and de Bont (1996). Inoue *et al.* (2000) and Swan and Watson (1998). Hu *et al.* (2005) and Takagi *et al.* (2005). Cartwright *et al.* (1987); Furukawa *et al.* (2004); and Fernandes and Sa-Correira (2003).
Unsaturated fatty acids (UFAs) Ergosterol Amino acids Inositol and H^+-ATPase	Increase membrane stability and antagonize the fluidity caused by ethanol	
Factors that stabilize or repair denatured proteins		Gomes *et al.* (2002); Ogawa *et al.* (2000); Barry and Gawrisch (1995); and Lucero *et al.* (1997). Craig *et al.* (1993); Seymour and Pipper (1999); Quan *et al.* (2004); and Pipper *et al.* (1994, 1997).
Trehalose Heat shock proteins (HSPs)	Stabilize or repair denatured proteins caused by ethanol	
Transcriptional factor interact with stress response element (STRE)		Schuller *et al.* (1994); Marchler *et al.* (1993); Moskvina *et al.* (1998); Watanabe *et al.* (2007); Gorner *et al.* (1998, 2002) and Hirata *et al.* (2003). Hahn *et al.* (2004); Wu (1995); Takemori *et al.* (2006); Sorger and Pelham (1988); Guo *et al.* (2008); and Liu *et al.* (2008). Daulny *et al.* (2008); Betz *et al.* (2004); Izawa *et al.* (2006); Aravind *et al* (2003); Capili e*t al.* (2001); Kubota *et al.* (2004); and van Voorst *et al.* (2006).
Heat shock factors (HSFs) Alcohol sensitive ring/PHD finger 1 gene (Asr1) Some others genes		

Acetic acid stress

Acetic acid is a normal by-product of the alcoholic fermentation carried out by *S. cerevisiae*. It can also be produced by contaminating lactic and acetic acid bacteria (Du Toit and 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). This weak acid can have negative effects in industrial fermentation processes such as wine production (Garay-Arroyo *et al.,* 2004; Vilela-Moura *et al.,* 2010), or lignocellulosic fermentations for bioethanol production (Klinke *et al.,* 2004; Liu and Blaschek, 2010; Mira *et al.,* 2010b) underpinning its biotechnological relevance.

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 (Casal *et al.,* 1996). 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 and Arneborg, 1998; Leão and van Uden, 1986; Pampulha and 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). Thus, the undissociated form of this acid causes toxic effects that result in the inhibition of growth and fermentation rates (Pampulha and Loureiro, 1989; Phowchinda *et al.*, 1995). *S. cerevisiae* cells are normally able to grow on acetic acid medium (pH 5.5), which is used as the sole carbon and energy source. Under this condition the weak acid is partially found in the dissociated form and acetate is transported across the plasma membrane through an electroneutral proton symport transporter. Two genes have been associated with acetate transporter *ADY2* (Casal *et al.,* 1996; Paiva *et al.,* 2004) and *JEN1,* encoding a general monocarboxylate transporter (Casal *et al.,* 1999). Acetate taken up by cells is converted to acetyl-CoA by one of either peroxisomal or cytosolic acetyl-CoA synthetases. Acetyl-CoA is then consumed in the glyoxylate shunt or oxidized in mitochondria through the tricarboxylic acid cycle (Lee *et al.,* 2011; Vilela-Moura *et al.,* 2008). Under certain conditions, acetic acid is also responsible for inducing two types of cell death, high- and low-enthalpy (Pinto *et al.,* 1989). Exponential-phase cultures of *S. cerevisiae* treated with high concentrations of acetic acid (120-200 mM), exhibited a necrotic

phenotype, while cultures treated with low doses (20-80 mM) evidenced an apoptotic programmed cell death (PCD) process (Ludovico *et al.*, 2001).

Most common mechanisms of yeast adaptation to stress involve plasma membrane transporters and the proton-translocating ATPase. The plasma membrane transporter Pdr12p, a member of ATP-binding cassette (ABC) transporter family, is strongly induced by weak acids, but this response seems not to be very important for acetic acid resistance (Piper *et al.,* 1998). In order to confer resistance to stress, Pdr12p may bind acid anions incorporated in the inner leaflet of the plasma membrane and transport them to the opposite side of the membrane in order promote their transport out of the cell (Weber and de Bont, 1996). In the absence of stress by weak acids, the carrier is not active (Holyoak *et al.,* 2000).

The plasma membrane H^+-ATPase, an ATP-driven proton efflux pump has also been shown to be important in the stress response caused by weak acids (Serrano, 1991). Holyoak and collaborators have observed that the low expression of *PMA1* gene, encoding the H^+-ATPase, increases the cell sensitivity to weak acids (Holyoak *et al.,* 1996).

The transcription factor Haa1p is required for a rapid yeast adaptation to acetic acid. The protective effect exerted by Haa1ptowards weak acids decreased steeply with the increase of the liposolubility of the weak acid, being maximal for acetic acid (Fernandes *et al.*, 2005). This transcription factor, directly or indirectly regulate most of the acetic acid induced gene expression, being the main player in reprogramming yeast genomic expression in the adaptation to growth in the presence of acetic acid stress (Mira *et al.,* 2011). The transcription of nine Haa1p-target genes, seem to have an important role in the adaptation of yeast to stress caused by weak acids. Those having more prominent effect were *TPO2, TPO3, SAP30*, *HRK1* (Mira *et al.,* 2011) and *YGP1* (Fernandes *et al.,* 2005).

Other transcription factors such as, Msn2p/Msn4p, War1p, Rim101p and Pdr1p/Pdr3p are known as mediators in response to stress caused by weak acids (Mira *et al.,* 2010a).

The stress caused by acetic acid, at low pH leads to activation of two mitogen- activated protein (MAP) kinases, Hog1p and Slt2p. Hog1p-dependent phosphorylation of aquaglyceroporin Fps1p results in its ubiquitination, endocytosis, and final degradation in the vacuole (Mollapour *et al.,* 2009). The Hog1p-directed destabilization of Fps1p decreases acetic acid intracellular accumulation, leading to resistance to acetic acid (Piper, 2011; Zhang *et al.,* 2011).

Protection against acetic acid-induced stress, a condition known to induce a severe amino acid-starvation response, was also associated with reduced levels of the translation factors eIF4A, eEF1A, eEF2 and eEF3A (Almeida *et al.,* 2009).

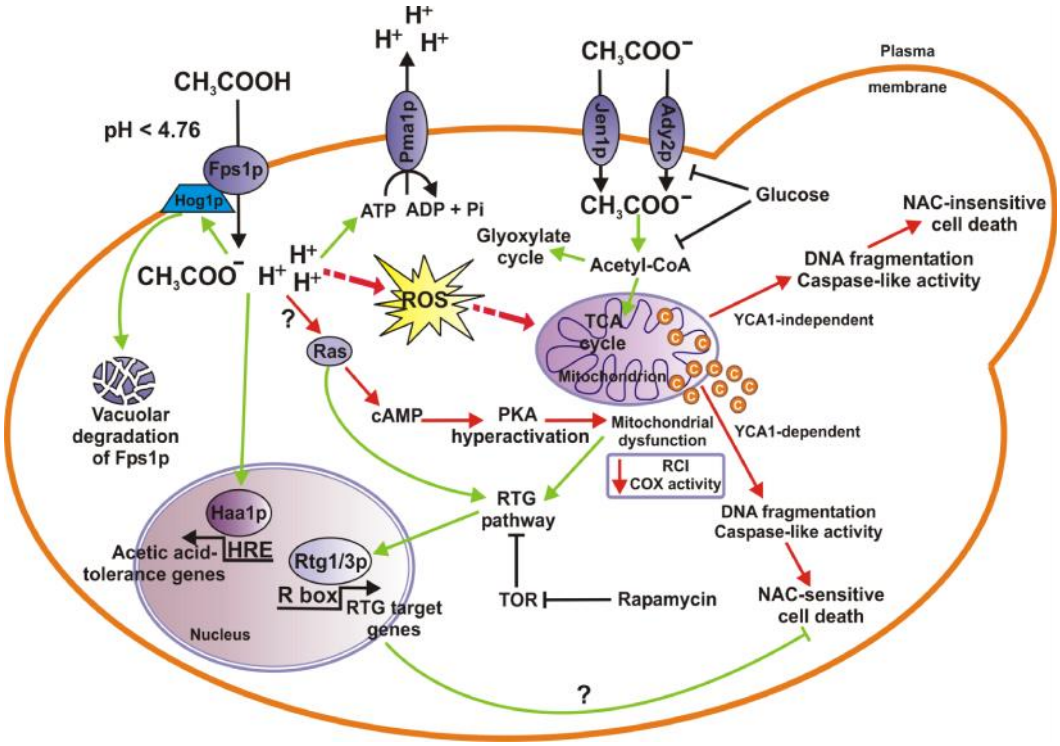

Figure 2 - Mechanisms of acetic acid stress response in *S. cerevisiae cells*. When yeast cells utilize acetic acid as the sole carbon source, acetate anion enters cells through a monocarboxylate proton symporter (potentially by Jen1p or Ady2p monocarboxylate transporter) where it is converted into acetyl-CoA, which enters the TCA or the glyoxylate cycle. Both acetate transport and metabolism are inhibited by glucose. At low pH (p*Ka*= 4.76), in the presence of glucose, acetic acid enters cells in its undissociated form by facilitated diffusion or through the Fps1p aquaglyceroporin channel. Once inside the cell the more neutral cytosolic pH causes its dissociation into anions and protons. Concomitant cytoplasmic acidification by protons induces the activation of the Pma1p, a plasma membrane ATPase that pumps protons out of the cell. Stress induce by acetic acid may activate Hog1p, a MAP-kinase involved in phosphorylation and subsequent ubiquitination, endocytosis, and final vacuolar degradation of Fps1p, and transcription factor Haa1p enabling cells to adapt to acetic acid (adapted from Giannattasio *et al.,* 2013).

Cell Death

Cell death has many forms and shapes. Cell death research encompasses not only the study of apoptosis, autophagic cell death, necrosis and other modes of cellular demise, but also the role these phenomena play in physiological and pathological processes including development, aging, and disease (Fulda *et al.,* 2009). In the last decades, cell death attracted growing interest of the scientific community, mostly because of its crucial role in tissue homeostasis and development of multicellular organisms (Baehrecke, 2002). Since the first descriptions of programmed cell death mechanisms, several attempts have been made to classify cell death subroutines based on morphological and biochemical characteristics (Galluzzi *et al.,* 2012).

In 2009, the Nomenclature Committee on Cell Death (NCCD) proposed a set of recommendations for the definition of distinct cell death morphologies and for the appropriate use of cell death-related terminology, including 'apoptosis', 'necrosis' and 'mitotic catastrophe' (Kroemer *et al.,* 2009). Recently, NCCD proposed a functional classification of cell death subroutines that applies to both *in vitro* and *in vivo* settings and includes extrinsic apoptosis, caspase-dependent or -independent intrinsic apoptosis, regulated necrosis, autophagic cell death and mitotic catastrophe (Galluzzi *et al.,* 2012).

Apoptosis

Apoptosis is a programmed cell death process that is important for many biological functions. This process occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. It also occurs as a defense mechanism in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001). Apoptosis is a type of cell death with its own characteristics, such as exposure of phosphatidylserine from the inner leaflet to the external leaflet of the plasma membrane, chromatin condensation, internucleosomal DNA fragmentation, cell volume decrease and finally formation of apoptotic bodies which are subsequently removed by phagocytes without causing an inflammatory response (Lawen, 2003).

The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events. It is known that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Igney and Krammer, 2002). These pathways converge on the same terminal or execution pathway and are initiated by the cleavage of caspase-3 (Martinvalet *et al.,* 2005). Briefly, the apoptotic process consists of 3 consecutive phases: trigger by extracellular or intracellular stimuli, execution by activation of intracellular proteases and elimination of dead cells by engulfment of cell debris by neighboring cells or macrophages (Saikumar *et al.*, 1999).

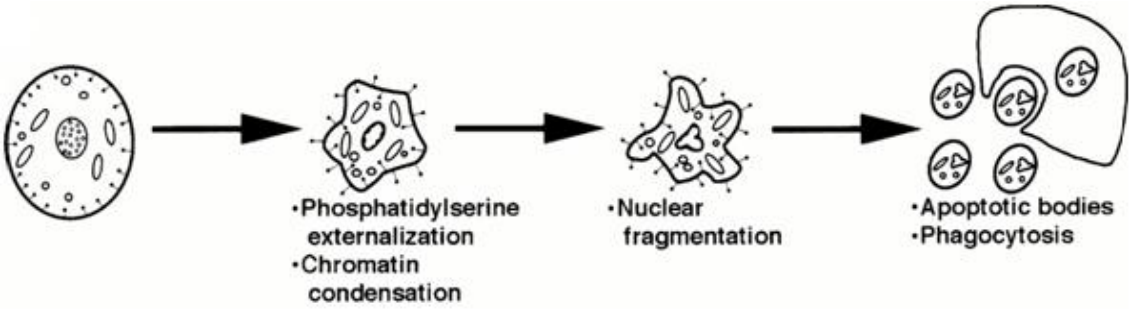

Figure 3 – Schematic representation of the morphological features of apoptosis (adapted by Jones, 1997).

It has become clear that, among other cellular processes, the apoptotic core machinery is conserved in yeast, which brings great advantages for the discovery of basic mechanisms that may also be extended to human apoptosis (Gutiérrez *et al.,* 2010). In 1997 yeast apoptosis was firstly observed (Madeo *et al.,* 1997) and since then multiple yeast orthologues of crucial mammalian apoptotic proteins have been identified, such as caspases (Madeo *et al.,* 2002), AIF (Wissing *et al.,* 2004), Omi/HtrA2 (Fahrenkrog *et al.*, 2004), IAP (Walter *et al.*, 2006) and Endo G (Büttner *et al.*, 2007).

Over the years, different agents capable of inducing apoptosis in yeast were identified, such as acetic acid, ethanol, hydrogen peroxide, sorbitol or glucose, sodium chloride, among others (Pereira *et al.,* 2008). In order to detect apoptosis in yeast, various tests have been developed; they include determination of viability [by colony forming units], ROS accumulation [by dihydroethidium

(DHE) staining], DNA fragmentation [using TUNEL (Terminal dUTP nick-end labeling) assay], exposure of phosphatidylserine [by Annexin V (AnnV) staining], chromatin condensation [by DAPI (4,6-diamino-2-phenyl-indole dihydrochloride) staining] and cell integrity [by Propidium iodide (PI) staining] (Fig. 4) (Gutiérrez *et al.,* 2010).

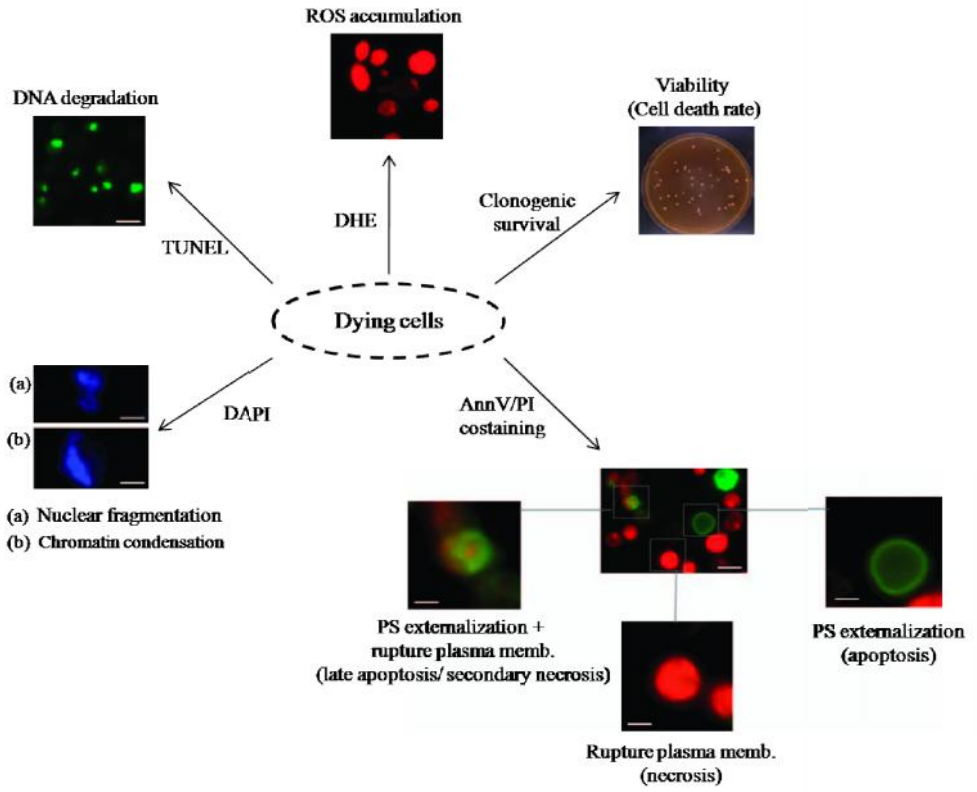

Figure 4 - Assays routinely used in the field of yeast PCD. Co-staining of annexinV (AnnV) and propidium iodide (PI) allows discrimination between early apoptotic cells exhibiting phosphatidylserine (PS) externalization (Ann^+, PI), cells showing rupture plasma membrane indicate of primary necrosis (Ann^-, PI^+) and late apoptotic/secondary necrotic cells, which show both PS exposition and membrane permeability (Ann^+/PI^+). DNA fragmentation is measured using the TUNEL test accumulation of ROS is usually measured using dihydroethidium (DHE). Nuclear fragmentation and chromatin condensation can be observed upon DAPI staining (adapted from Gutiérrez *et al.,* 2010).

Necrosis

Necrosis occurs generally in response to physico-chemical stress, including hypoxia, ischemia, hypoglycemia, extreme temperature changes and nutrient deprivation (Nicotera *et al.,* 1999) and is morphologically characterized by a gain in cell volume (oncosis), swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents (Kroemer *et al.,* 2009). It is known that this process triggers various cellular phenomena, such as uncoupling of the respiratory chain, production of ROS, nitroxidative stress by nitric oxide or similar compounds, and mitochondrial membrane permeabilization (MMP) often controlled by cyclophilin D, lysosomal changes, ROS production by Fenton reactions, lysosomal membrane permeabilization, nuclear changes (hyperactivation of PARP-1 and concomitant hydrolysis of NAD^+), lipid degradation (following the activation of phospholipases, lipoxygenases and sphingomyelinases), increase in the cytosolic concentration of calcium (Ca^{2+}) that result in mitochondrial overload and activation of noncaspase proteases (e.g., calpains and cathepsins) (Nicotera *et al.,* 1999).

The necrotic death was long considered an unregulated process. However, it is increasingly clear that this process does not occur by accident and that there are multiple signaling pathways implied in it (Vanlangenakker *et al.,* 2008). Thus, the term programmed necrosis has emerged to identify a cell death process characterized by rapid loss of plasma membrane integrity prior to the exposure of a phagocytic signal. Studies on this issue have shown that there are several stimuli capable of triggering regulated necrosis, including alkylating DNA damage, excitotoxins and the ligation of death receptors (He *et al.,* 2009; Zhang *et al.,* 2009), for example, TNFR1, and Toll-like receptors (Vanlangenakker *et al.,* 2008).

This new concept of necrosis, as a regulated entity, represents a paradigm shift in biology and medicine, may shed new light on disease pathogenesis and provide opportunities for therapeutics not thought possible in the recent past (Kung *et al.,* 2011).

Table 2 – Features of necrosis (adapted from Kung *et al.,* 2011).

Necrosis	
Morphology	
Cell	Swelling
Mitochondria	Marked swelling
Chromatin condensation	Usually not prominent
Cell fragmentation	Cell rupture
Membrane blebbing	Not characteristic
Membrane integrity	Defective at early stages
Tissue inflammation	Severe
Functions	
Cellular ATP levels	Markedly depleted
Production of ATP	Markedly decreased
Consumption of ATP	Continued
MPTP opening	An early defining event in the mitochondrial necrosis pathway
Loss of $\Delta\Psi_m$	An early defining event in the mitochondrial necrosis pathway
Release of apoptogenic factors	Not classic, but may be present because of OMM rupture following MPTP opening
Caspase activation	Not classically present but may occur with OMM rupture
Activation of other proteases	Calpains, cathepsins, and other lysosomal proteases sometimes activated and which contribute to cell death

Necrosis in yeast

Although apoptosis is the most discussed type of cell death and is recognized as the primary cause of cell death, necrosis has also been described in yeast. This kind of death does not occur only as a result of brutal chemical or physical insults, but can occur in physiological conditions, as reasonable concentrations of a compound may induce it (Eisenberg *et al.,* 2010). Acetic acid and hydrogen peroxide are examples of agents capable of inducing not only apoptosis, but also necrosis in yeast. The type of death is dependent on the dose, i.e., apoptosis is triggered by low doses and higher doses of the same compound may lead to necrosis. This applies to acetic acid (Ludovico *et al.*, 2001), to copper and manganese (Liang *et al.*, 2007), to the antifungal agent amphotericin B (Phillips *et al.,* 2003) and possibly also to pheromones (Zhang *et al.,* 2006). Merely increasing the dosage of typical apoptotic triggers may, in most cases, stimulate non-regulated type of necrotic

death, likely resulting from radical damage of cellular structures and integrity. In the case of acetic acid, however, it is also feasible that increasing concentrations interfere with intracellular pH homeostasis in a regulated way that might resemble the detrimental effects of vacuolar dysfunction (Hauptmann *et al.,* 2006). Tunicamycin-induce cell death in *S. cerevisiae* is accompanied by an excessive production of ROS, by a large loss of plasma membrane integrity, while at the same time lacking apoptotic DNA fragmentation. The sensitive to this agent is increased in calcineurin-deficient mutants or by the calcineurin inhibitor FK506. This allowed to conclude that tunicamycin induces a non-apoptotic, possibly necrotic death in yeast (Dugeon *et al.,* 2008). Hsp90p, cytosolic chaperone for many kinases including RIP1 which serves a pro-necrotic signaling function in mammalian cells (Lewis *et al.,* 2000), has been functionally associated with the regulation of necrosis as well (Dugeon *et al.,* 2008). Disruption of Hsp90p function with the inhibitor radicicol, prevent the massive cell death described above, thus it is plausible that calcineurin and Hsp90p act with opposing functions in the regulation of controlled necrotic cell death in yeast (Dugeon *et al.,* 2008).

Since in yeast there is no clear homolog of RIP1, exact molecular function of Hsp90p during yeast necrosis is unknown. Other types of yeast serine/threonine kinases such as Slt2p (Mpk1p), have been suspected to be targets of Hsp90p and might bear a comparable role to RIP1 (Truman *et al.,* 2007; Zhao *et al.,* 2007).

It is known that mitochondria play a role in apoptosis in both yeast and mammals, as well as in necrosis of mammalian cells (Kroemer *et al.,* 2007; Eisenberg *et al.,* 2007 and Pozniakovsky *et al.,* 2005). The production of ROS has been linked to apoptosis under diverse conditions, although it has also been described that ROS accumulation increases in necrosis in mammalian cells. To which extent ROS may also play an executor role during necrotic cell death in yeast remains unknown (Festjens *et al.,* 2006).

Some studies, demonstrated that yeast Endo G encompasses a vital (anti-necrotic) as well as a lethal (pro-apoptotic) function (Büttner *et al.,* 2007). Dual-vital and lethal-functions are well described for several other crucial regulators of apoptosis, including caspases, cytochrome *c* or the apoptosis inducing factor AIF (Launay *et al.,* 2005).

Disruption of normal vacuolar/lysosomal function derives in necrotic cell death, at least in part due to failure of intracellular pH control. Intriguingly, lysosomal proteins (i.e. cathepsins, which need a rather acidic pH to exhibit their proteolytic function) together with non-lysosomal proteases

(i.e. calpains) have been directly linked to necrotic cell death execution. This raises the possibility that dysfunction of vacuoles/lysosomes (and thus disruption of homeostatic pH control) may trigger necrosis by release of pro-necrotic proteases, which would find an optimal pH for their enzymatic activity in the acidified cytosol (Nakayama *et al.,* 2002; Yamashima *et al.,* 1998).

Several studies have addressed the possible link between peroxisomal function and necrosis in yeast. Deletion of *S.cerevisiae PEX6*, encoding a protein crucial for peroxisomal protein import, increased the sensitivity towards both acetic acid stress and stress upon entry into early stationary phase. This was accompanied by excessive ROS production and markers of necrosis (Jungwirth *et al.,* 2008).

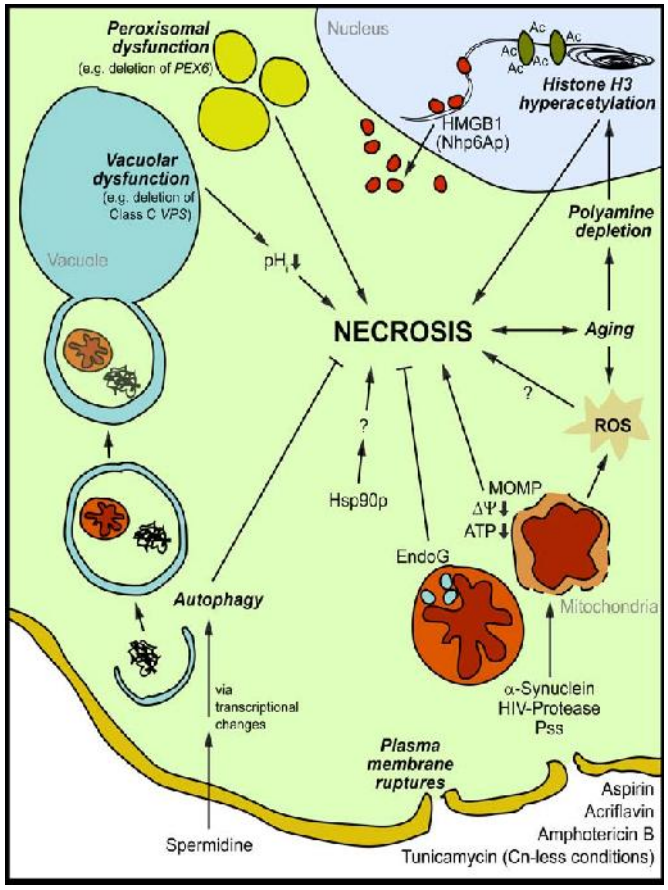

Figure 5 - Schematic view of stimuli and cellular processes that interfere with yeast necrosis. Autophagy as well as proper vacuolar and peroxisomal functions are homeostatic processes that counteract necrosis in yeast. Dysfunction of these processes induces defined cellular events (e.g. cytosolic acidification) that can culminate into increased necrotic death accompanied by typical morphological and cell biological changes. These include plasma membrane rupture, mitochondrial outer membrane permeabilization (MOMP), dissipation of mitochondrial potential ($\Delta\Psi m\downarrow$), ATP depletion (ATP↓), overproduction of ROS and nuclear release of high mobility group box-1 (HMGB1) protein. Besides external stimuli (e.g. Acriflavin or Amphotericin B), endogenous triggers of yeast necrosis exist including Endo G depletion, heterologous expression of α- synuclein, HIV protease and proteinaceouselicitor harpin (Pss). In addition, chronological aging represents a physiological scenario closely interrelated to necrotic cell death, which is regulated by a network of epigenetic histone modifications and intracellular levels of polyamines (adapted from Eisenberg *et al.,* 2010).

The identification of the necrotic cell death occurs due to the absence of apoptotic markers studied extensively and by the occurrence of early plasma membrane permeabilization (Eisenberg *et al.,* 2009). Figure 4 summarizes in more detail the available methodologies and outlines a strategy to determine the different modes of cell death in yeast.

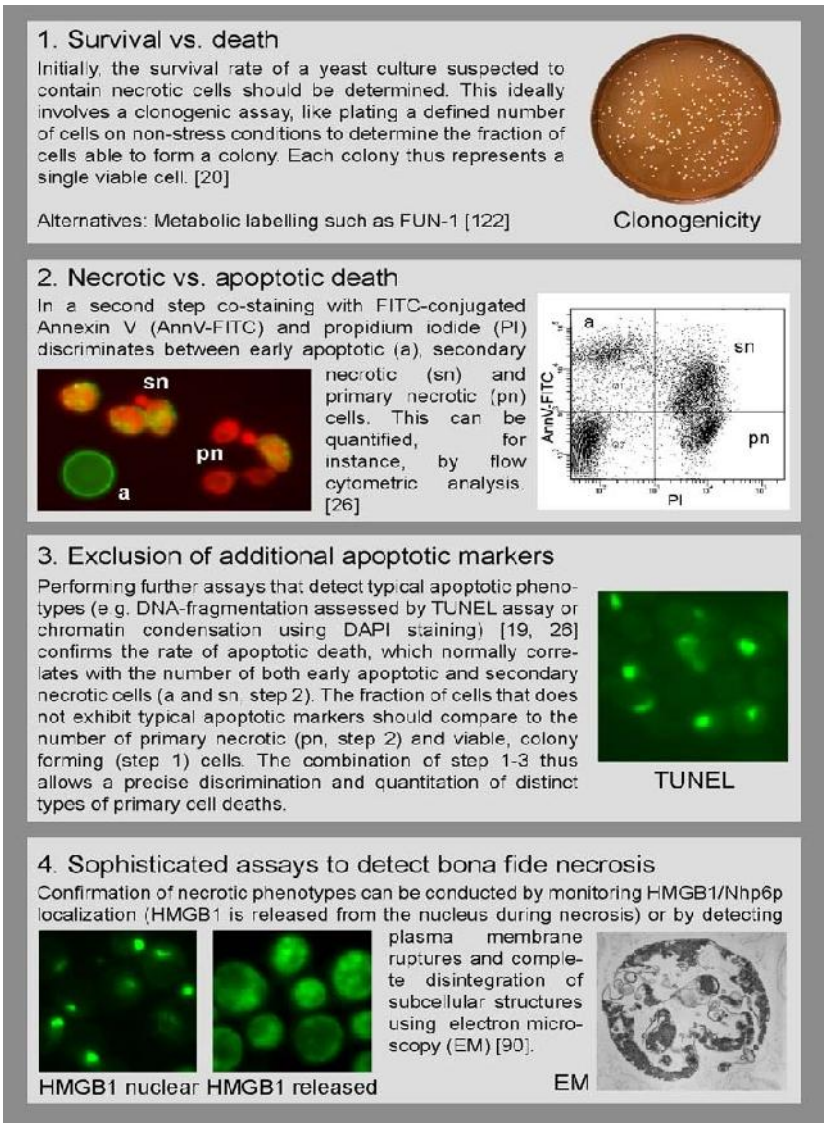

Figure 6 - A strategy to determine primary necrotic cell death in yeast (adapted from Eisenberg *et al.,* 2010).


AIM

Previous data obtained in our lab showed that *S. cerevisiae* cells exposed simultaneously to toxic concentrations of ethanol and low concentrations of acetic acid displayed a higher survival (measured either by CFU or by propidium iodide staining) than cells treated only with ethanol. These results indicated that acetic acid induces a cellular response that provides protection against the cytotoxic effect of ethanol (Vieira *et al.,* unpublished results). Evidence of the involvement of the aquaglyceroporin Fps1p and of the MAP kinase Hog1p in acetic acid stress (Mollapour and Piper, 2006) triggered new studies. To address the possible involvement of the aquaglyceroporin Fps1p and of the MAP kinase Hog1p in the protector effect of acetic acid against ethanol stress (Mollapour and Piper, 2006), the loss of cell viability and plasma membrane integrity induced by ethanol in absence and presence of ethanol were determined. We found that the deletion of these genes did not abrogate the protection effect suggesting that it does not depend on Hog1p and Fps1p (Trindade, 2009).

Later Afonso showed that the osmolyte trehalose, the heat shock protein Hsp12p, the MAP kinase Slt2/MPK1 and functional mitochondria play a role in the protection by the undissociated form of acetic acid against ethanol induced cytotoxicity (Afonso, 2011).

With the present master thesis we intended to further understand how acetic acid protects against ethanol-induced cytotoxicity, determining the role of mitochondria in this protection. We also intended to characterize the death of cells exposed to toxic concentrations of ethanol since this knowledge would be important to dissect the role of acetic acid in its inhibition.To achieve these goals, cell viability and cytometric assays were performed on wild type cells and in knockout mutant cells, lacking known apoptototic regulators that have been involved in mitochondrial mediated death, during exposure to 13% (v/v) ethanol and to 13% (v/v) ethanol and 0.1% (v/v) of acetic acid.

MATERIALS AND METHODS

Yeast Strains

Saccharomyces cerevisiae strain BY4741 (*MATa his3*Δ*1 leu2*Δ0 met15Δ0 ura3Δ0) and the respective knockouts mutant strains in *ATP2, ATP10, POR1, YCA1, AIF1, PEP4, CPR3, CYC3, NUC1, AAC1-3* and *CYC1CYC7* genes, were used in this work and were obtained from EUROSCARF collection, except the *Δcyc1Δcyc7* strain that was kindly provided Dr. Bjorn Johansson (CBMA, UM).

Media and Growth Conditions

For cell viability experiments, yeast cells were grown on YPDA plates (1% yeast extract, 2% bacto-peptone, 2% glucose and 2% agar) at 30 °C for 2-3 days. After growth on YPDA, cells were then inoculated (OD_{640nm}=0. 5-0.6) in Erlenmeyers 50ml with 10 ml of YPD medium (ratio 1:5), at 26°C overnight with shaking (120 rpm).

For fluorescence microscopy observations, yeast cells were grown under the same conditions with the exception of culture medium. For this purpose a defined minimal medium (SC medium) containing 2% glucose, 0.17 % yeast nitrogen base without amino acids, 5 % ammonium sulphate, 0.2% Drop-out mixture lacking histidine, lysine, metionine, leucine and uracil, 0.008% histidine, 0.008% lysine, 0.04% leucine and 0.008% methionine.

Cell viability assays

For cell viability assays, 1.3 ml of $1x10^7$cells/ml (OD_{640} = 0.5 – 0.6) were transferred to microcentrifuge tube with a small hole in the lid to allow some transfer of oxygen. The cells were centrifuged and resuspended in 1.3 ml of YPD medium at pH 3.5. Cell viability assays were done using 13% (v/v) ethanol or 13% (v/v) ethanol and 0.1% (v/v) of acetic acid. Ethanol and acetic acid used were obtained from stock solutions of absolute ethanol (> 99%, v/v) and of 1M acetic acid, at pH 3.5. Cells were incubated at 26 °C with shaking (120 rpm), during 3 hours. Samples of 50 µl were collected at different times (60, 120, 180 min). After 3 dilutions (one dilution at 1:10 and two dilutions at 1:20), 7 drops of 40 µl of the last dilution were plated on YPDA. Cell viability was analyzed by counting colony forming units (c.f.u) after 2-3 days of growth at 30°C. The percentage of

survival for the different times was calculated by the formula: number of colonies in time X min (TX)/ number of colonies in time 0 (T0) x100.

Flow cytometric assays

In all the flow cytometric assays the samples were analyzed in an Epics® XL™ (Beckman Coulter) flow cytometer, and data were analyzed with version 2.5.0 of the flowing software. At least 20,000 cells from each sample were analyzed.

Assessment of plasma membrane integrity

Integrity of the cell plasma membrane was analyzed by flow cytometry using the impermeable dye propidium iodide (PI) (Molecular Probes, SIGMA). 100 µl of samples were collected at specific intervals (60, 120 and 180 min). Cells were harvested by centrifugation, ressuspended in 500 µL PBS (1x) to which 1 µl of a PI stock solution (1mg/mL) was added and incubated for 10 minutes at room temperature in the dark.

Assessment of superoxide anion intracellular accumulation

Intracellular accumulation of superoxide anion was detected by flow cytometry using Dihydroethidium (DHE) (Molecular Probes, SIGMA).100 µl of samples were collected at specific intervals (60, 120 and 180 min). DHE was added to yeast cell suspensions ($1x10^6$ cells /ml) to a final concentration of 5 µg /ml, and incubated for 40 minutes in the dark at room temperature.

Assessment of mitochondrial membrane potential

Mitochondrial membrane potential was measured by flow cytometry, using cells stained with 3,3'-Dihexyloxacarbocyanine iodide ($DiOC_6$). Cells were collected and suspended in suspension buffer [10 mM 2-(N-morpholino)ethanesulfonic acid (MES), 0.1 mM $Mgcl_2$ and 2% glucose, pH 6.0 (set with $Ca(OH)_2$]. After addition of 1nM $DiOC_6$, cells were incubated at 30°C for 30 minutes in the dark. For

double staining, cells were subsequently incubated with 1 µl of a PI stock solution (1mg/mL) for 10 minutes at room temperature.

Detection of caspase activation

Detection of caspase activation was performed using "caspACE, FITC-VAD- fmk In Situ Marker" (Promega). Briefly, for the caspACE kit $1x10^6$ cells were washed in PBS, suspended in 100 µl staining solution containing 50 µM of FITC-VAD-fmk and incubated for 20 minutes at 30°C in the dark. After incubation cells were washed once and suspended in PBS. For double staining with PI, cells were subsequently incubated with 1 µl of a PI stock solution (1mg/mL) for 10 minutes at room temperature prior to analysis by flow cytometry.

Assessment of exposure of phosphatidylserine in the outer surface of the plasma membrane

Phosphatidylserine exposure was detected by a FITC-coupled Annexin V reaction with the ApoAlertAnnexin V Apoptosis Kit (BD Biosciences). For that purpose cells were harvested (0.3 OD) and washed in sorbitol buffer (1.2 M sorbitol; 0.5 mM MgCl; 35mM K_2HPO_4, pH 6.8). To facilitate the probe diffusion through cell wall, an incubation step with glusulase (3%, v/v) and 7 U/ml of lyticase at 28 °C was performed. Phase-contrast microscopy was used to monitor cell wall digestion step, controlling in this way damage to the unfixed spheroplasts. Cells were subsequently centrifuged (10 min at 1500 rpm) and ressuspended in 200 µl of binding buffer (1.2 M sorbitol; 10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$). 2 µl Annexin V (1 µg/ml) and 1 µl PI (4 µg/ml) were added to 40 µl of the cell suspension and the mixture incubated for 20 min at room temperature in the dark. Finally, extra 400 µl of binding buffer were added to the mixture just prior to analysis by flow cytometry.

Assessment of the translocation of Nhp6Ap from the nucleus into the cytosol by fluorescence microscopy

BY4741 strain and the respective knockouts mutant strains in *ATP2, ATP10, CYC3, CPR3 and POR1* genes were transformed with the plasmids pUG35 and pUG35-NHP6A-EGFP, kindly provided by Dr. Frank Madeo (University of Gratz, Austria) by standard protocol. Yeast cells were grown in defined minimal medium (SC medium), as described above. 100 µl of samples were collected at specific intervals (60, 120 and 180 min). Cells were harvested by centrifugation, ressuspended in 10 µl of water. Cells were visualized in a Leica Microsystems DM5000B epifluorescence microscope using appropriate filter settings with a 100x oil immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

Assessment of the translocation of Nhp6Ap from the nucleus into the cytosol by flow cytometry

Cells expressing GFP-tagged Nhp6Ap were harvested by centrifugation, ressuspended in 500 µL PBS (1x) to which 1 µl of a PI stock solution (1mg/mL) was added and incubated for 10 minutes at room temperature in the dark prior to analysis by flow cytometry. Samples of 100 µl were collected at specific intervals (60, 120 and 180 min).

Reproducibility and statistical analysis of the results

The results obtained are represented by the mean and standard deviation (SD) values of at least three independent experiments. Statistical analyses were carried out using GraphPad Prism Software v 6.00. P-values lower than 0.05 were assumed to represent a significant difference.

RESULTS

Acetic acid protects cells from ethanol-induced cell death in *Saccharomyces cerevisiae*

As referred above ethanol is the main end product of alcoholic fermentation carried out by *S.cerevisiae* and above a certain concentration exerts cytotoxic effects through perturbation of multiple processes responsible of cellular homeostasis. During fermentation, some weak acids such as acetic, butyric and pyruvic acids, produced by yeast metabolism, may accumulate in the growth medium and enhance ethanol toxicity, which results for instance in a higher inhibition of yeast growth and fermentation (Gibson *et al.,* 2007). However, previous data obtained in our laboratory showed that *S. cerevisiae* cells exposed simultaneously to toxic concentrations of ethanol and low concentrations of acetic acid displayed higher survival than cells treated only with ethanol. These results indicated that acetic acid induces a cellular response that provides protection against the cytotoxic effect of ethanol (Vieira *et al.,* unpublished results; Andreia *et al.*, 2011).

In this thesis, as referred above, one of our goals was to further understand how acetic acid protects cells from ethanol-induced death. In a first phase we evaluated cell viability by CFUs counts and plasma membrane integrity by PI staining of *S. cerevisiae* BY4741 cells exposed to 13% (v/v) ethanol either without or with 0.1% (v/v) acetic acid, pH 3.5, for 3 hours. The presence of acetic acid led to an increase of the percentage of CFUs associated with a decrease in the percentage of cells stained with PI when compared with cells only treated with ethanol, though only reaching statistical significance for the loss of cell viability (Fig. 7A, B). Subsequently, we evaluated the intracellular levels of superoxide anion with DHE, as an indicator of oxidative stress (Fig.7C) and found that acetic acid lead to a decreased accumulation of superoxide though this protective effect was only statistically different after 60 min.

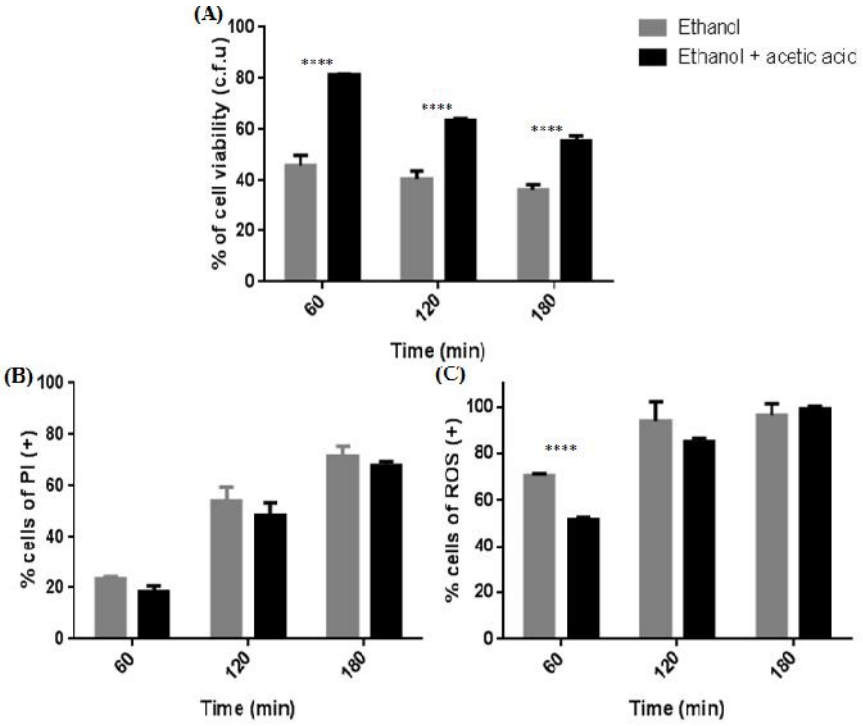

Figure 7 - Acetic acid protects *S. cerevisiae* BY4741 cells from ethanol cytotoxicity. (A) – Cell viability (%) assessed by CFUs counts. 100% corresponds to the number of CFUs at time 0 min. The differences between the two cultures are statistically significant for time 60, 120 and 180 min. ($P<0.0001$). (B) – Cells with loss of plasma membrane integrity expressed as a percentage of PI positive stained cells. The differences between different cultures are not statistically significant. (C) - Cells displaying accumulation of superoxide expressed as a percentage of DHE positive stained cells. The differences between the two cultures are statistically significant for time 60 min. ($P<0.0001$). All experiments were performed in YPD medium at pH 3.5. Cells were incubated with 13% (v/v) of ethanol (Et) or co-incubated with 13% (v/v) ethanol and 0.1% (v/v) of acetic acid (Et + AA), for 3 hours. All the values represent the average of 3 independent experiments and the respective standard deviations.

Protective effect of acetic acid against ethanol stress: the role of known yeast apoptotic regulators

Data obtained in our laboratory (Afonso, 2011), showed that cell cultures without functional mitochondrial did not displayed protection by acetic acid against ethanol-induced cell death when assessed by CFUs counts. However, under this condition a significant protection against loss of plasma membrane integrity was still observed. Thus, mitochondrial functions, namely respiration, appear important for acetic acid to increase cell viability of cells exposed to ethanol but is dispensable for the preservation of plasma membrane integrity. In order to try to understand the exact role of mitochondria in the protection by acetic acid, we selected a group of *S. cerevisiae* knockout mutants in the BY4741 genetic background lacking known regulators of yeast apoptosis, some of which are involved in mitochondrial mediated apoptotic death, and evaluated the loss of cell viability, plasma membrane integrity and the levels of intracellular accumulation of superoxide anion as described above (Fig. 8, 9, 10 and 11). The experiments were performed using 13% (v/v) of ethanol in the absence or presence of 0.1% (v/v) of acetic acid, pH3.5. The mutant strains selected lack oxidative phosphorylation, cytochrome *c*, orthologues of putative components of mammalian permeability transition pore (PTP), and the yeast orthologues of the mammalian ENDO G, AIF, Cathepsin D and of the yeast metacaspase Yca1p.

Involvement of the ATP synthase subunits Atp2p and Atp10p

Synthesis of ATP from ADP and phosphate, catalyzed by F_0F_1-ATP synthases, is the most abundant physiological reaction in almost any cell. F_0F_1-ATP synthases are membrane-bound enzymes that use the energy derived from an electrochemical proton gradient for ATP formation (Diez *et al.,* 2004). *ATP2* encodes the beta subunit of F_1 sector (Saltzgaber-Muller *et al.,* 1983), and *ATP10* is a mitochondrial inner membrane protein component that acts as Atp6p-specific chaperone and is required for the biogenesis of the hydrophobic F_0 sector of the mitochondrial ATPsynthase complex (Paul *et al.,* 2000). Although Atp2p is essential for the function of ATP synthase, it is not essential for life in yeast. Deletion of this gene, like deletions in many other genes necessary for the function or maintenance of mitochondria, lead to a "petite" phenotype that is associated with a slow-growing in glucose and inability to growth on non-fermentable carbon sources (Saltzgaber-Muller *et al.,* 1983).

The protective effect of 0.1% (v/v) of acetic acid on 13% (v/v) of ethanol-induced death verified in the wild-type strain was not abolished by deletion of *ATP2* or *ATP10* suggesting that these proteins are not involved in the protection by the acid (Fig. 8A). However, when the mutant cells *atp2*Δ and *atp10*Δ treated with 13% (v/v) of ethanol were compared with cells treated with 13% (v/v) of ethanol and 0.1% (v/v) of acetic acid, there was no protection against the loss of plasma membrane integrity by acetic acid in the case of the *atp2*Δ mutant, and with the *atp10*Δ mutant there was even an increase in the percentage of cells with compromised plasma membrane (Fig. 8B). However, and consistent with these data, while 0.1% (v/v) acetic acid decreased the accumulation of superoxide anion induced by 13% (v/v) ethanol in the *atp2*Δ mutant for 60 and 120 min., it had the opposite effect in the *atp10*Δ mutant (Fig. 8C).

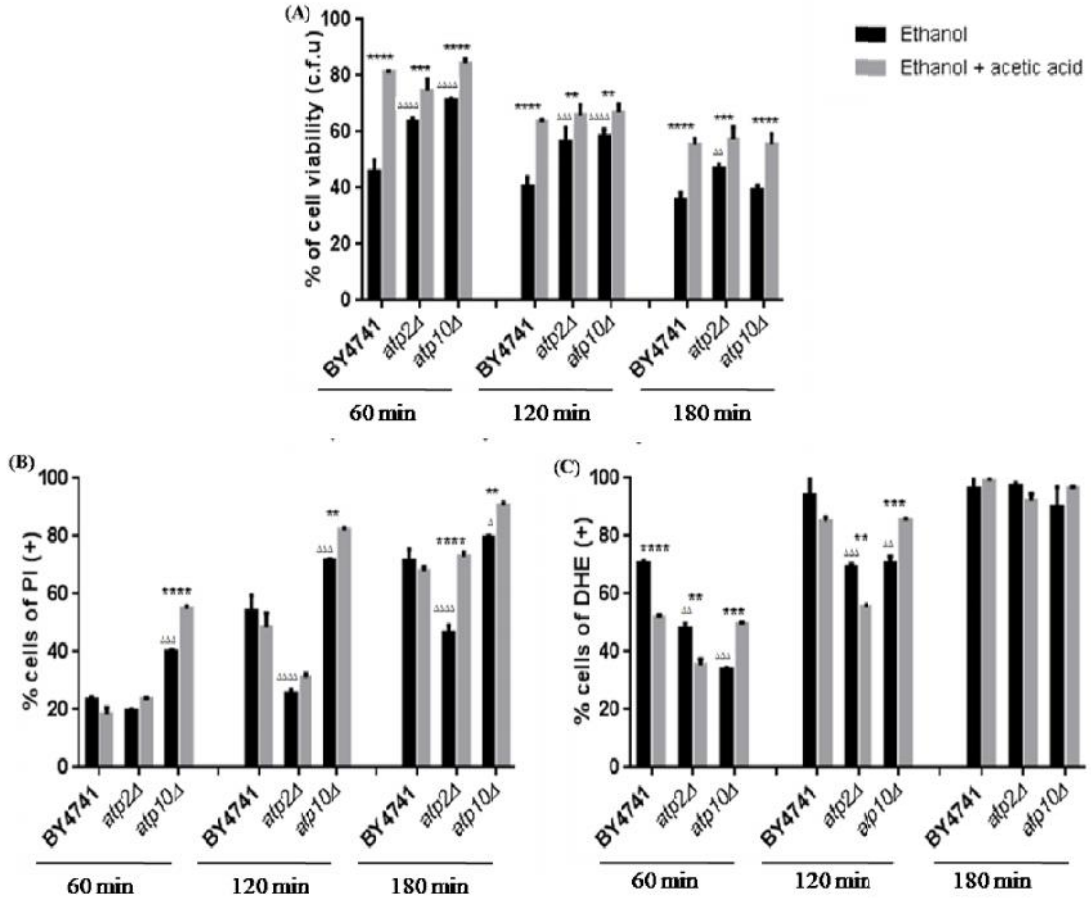

Figure 8 - Acetic acid protects *S. cerevisiae* BY4741, *atp2Δ* and *atp10Δ* cells from ethanol cytotoxicity. (A) – Cell viability (%) assessed by CFUs counts. 100% corresponds to the number of CFUs at time 0 min. (B) – Cells with loss of plasma membrane integrity expressed as a percentage of PI positive stained cells. (C) - Cells

displaying accumulation of superoxide expressed as a percentage of DHE positive stained cells. All experiments were performed in YPD medium at pH 3.5. Cells were incubated with 13% (v/v) of ethanol (Et) or co-incubated with 13% (v/v) of ethanol and 0.1% (v/v) of acetic acid (Et + AA), for 3 hours. All the values represent the average of 3 independent experiments and the respective standard deviations. * Represent statistical analysis of cells treated only with ethanol and Δ represent statistical analyses of cells treated with ethanol and acetic acid. ****/ΔΔΔΔ: P<0.0001; ***/ΔΔΔ: P<0.001; **/ΔΔ: 0.01and */Δ: P<0.05.

Involvement of the heme lyase Cyc3p and of the cytochrome *c* isoforms Cyc1p and Cyc7p

CYC3 gene encodes cytochrome *c* heme lyase (CCHL), essential for the covalent binding of the heme group to isoforms 1 and 2 of the apocytochrome *c* and thus to the formation of the mature cytochrome *c*.

The presence of 0.1% (v/v) of acetic acid did not cause a significant increase in cell survival in the *cyc3Δ* mutant at any time of treatment (Fig. 9A). Similarly, the protective effect of acetic acid against loss of plasma membrane integrity and accumulation of intracellular superoxide was not significant at any time of treatment suggesting that Cyp3p has a role in the protective effect of the acid against ethanol (Fig. 9A, B).

CYC1 and *CYC7* encode the iso-1-cytochrome *c* and iso-2-cytochrome *c*, respectively.

Cells lacking both cytochrome *c* isoforms exhibited increase cell survival in response to 13% (v/v) of ethanol plus 0.1 % (v/v) of acetic acid after 60 and 120 min., but lost the protective effect of acetic acid after 180 min (Fig. 9A). Though there was no protection against the loss of plasma membrane integrity at any time, 0.1% (v/v) of acetic acid decreased significantly the level of superoxide anion after 180 min. (Fig. 9B, C).

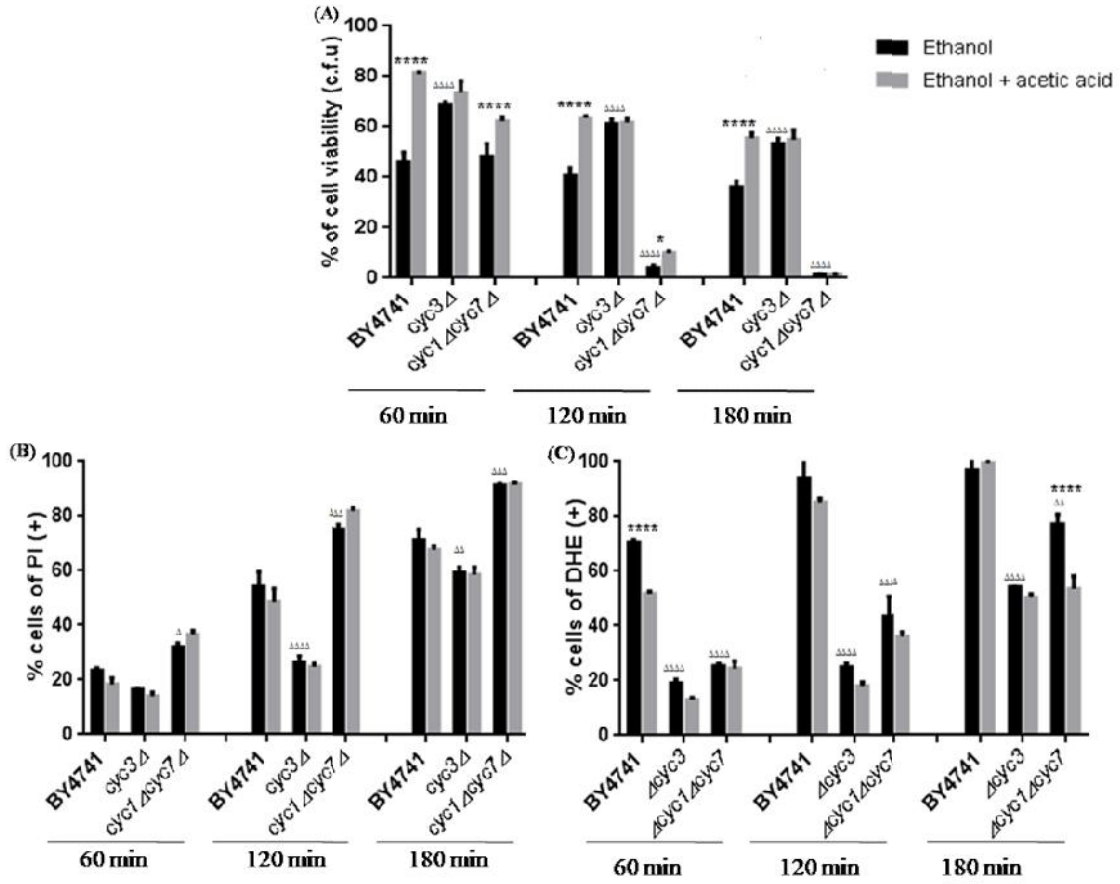

Figure 9 - Acetic acid protects *S. cerevisiae* BY4741, *cyc3Δ* and *cyc1Δcyc7Δ* cells from ethanol cytotoxicity. (A) – Cell viability (%) assessed by CFUs counts. 100% corresponds to the number of CFUs at time 0 min. (B) – Cells with loss of plasma membrane integrity expressed as a percentage of PI positive stained cells. (C) - Cells displaying accumulation of superoxide expressed as a percentage of DHE positive stained cells. All experiments were performed in YPD medium at pH 3.5. Cells were incubated with 13% (v/v) of ethanol (Et) or co-incubated with 13% (v/v) of ethanol and 0.1% (v/v) of acetic acid (Et + AA), for 3 hours. All the values represent the average of 3 independent experiments and the respective standard deviations. * Represent statistical analysis of cells treated only with ethanol and Δ represent statistical analyses of cells treated with ethanol and acetic acid. ****/ΔΔΔΔ: P<0.0001; ***/ΔΔΔ: P<0.001; **/ΔΔ: 0.01and */Δ: P<0.05.

Involvement of the yeast voltage dependent anion channel Por1p, ADP/ATP carrier proteins Aac1-3p and of cyclophilin Cpr3p

POR1 gene, encodes the yeast orthologue of the well-characterized voltage dependent anion-selective channel (YVDAC1) of the mitochondrial outer membrane, responsible for the permeability to most metabolites. Since YVDAC1 provides the primary pathway for the movement of metabolites across the outer mitochondrial membrane, cells in which the *POR1* gene has been deleted (*por1*Δ cells) are deficient for respiration (Dyson *et al.,* 1997).

A significant protective effect of 0.1% (v/v) of acetic acid on 13% (v/v) of ethanol-induced death was observed in the absence of Por1p after 120 and 180 min. of treatment, suggesting that protective effect of acetic acid is independent of this protein (Fig. 10A). Acetic acid (0.1%, v/v), had also a significant protective effect against the loss of plasma membrane integrity induced by 13% (v/v) of ethanol, but not against accumulation of superoxide anion, which oppositely increased the accumulation of this ROS at 180 min., in comparison with ethanol alone (Fig. 10B, C).

AAC1, AAC2, and *AAC3* encode the ADP/ATP carrier in *S. cerevisiae*. While the *AAC1* and *AAC2* genes are expressed preferentially under derepression conditions in the presence of oxygen, the *AAC3* gene is expressed only under anaerobic conditions. Strains lacking the three *AAC* genes (*AAC1, AAC2* and *AAC3*) are defective in oxidative phosphorylation, have reduced respiration rates, and ATP levels do not allow growth on non-fermentable carbon sources. The absence of these genes leads to a decrease in apoptosis induced by acetic acid associated with inhibition of MOMP and cyt *c* release, suggesting a crucial role of *AAC* in yeast apoptosis (Pereira *et al.,* 2007).

Acetic acid (0.1%, v/v) does not protect *aac1-3*Δ mutant cells against 13% (v/v) ethanol induced cell death, suggesting a crucial role of these carrier proteins in the protection (Fig. 10A). However, the absence of this protein did not abolish the protective effect of 0.1% (v/v) of acetic acid against loss of plasma membrane integrity and accumulation of superoxide anion (Fig. 10B, C).

Cyclophilins are a family of ubiquitous proteins that are the intracellular target of the immunosuppressant drug cyclosporin A. These proteins catalyze peptidylprolyl cis-trans isomerization *in vitro* and accelerate the refolding of a fusion protein synthesized in a reticulocyte lysate and

imported into the matrix of isolated yeast mitochondria. *S. cerevisiae* contains at least five cyclophilins, one of these, Cpr3p, is found in the mitochondrial matrix (Liang & Zhou, 2007). Strains lacking mitochondrial cyclophilin are respiratory competent (Matouschek *et al.,* 1995).

In the absence of Cpr3p the significant increase in cell survival caused by 0.1% (v/v) of acetic acid at 60 min. was lost at 120 min., and at 180 min. the acid enhanced the decrease in cell survival in response to 13% (v/v) of ethanol (Fig. 10A). The loss of plasma membrane integrity and the increase in the accumulation of superoxide anion induced by 13% (v/v) of ethanol was higher in the presence of 0.1% (v/v) of acetic acid (Fig. 10B, C).

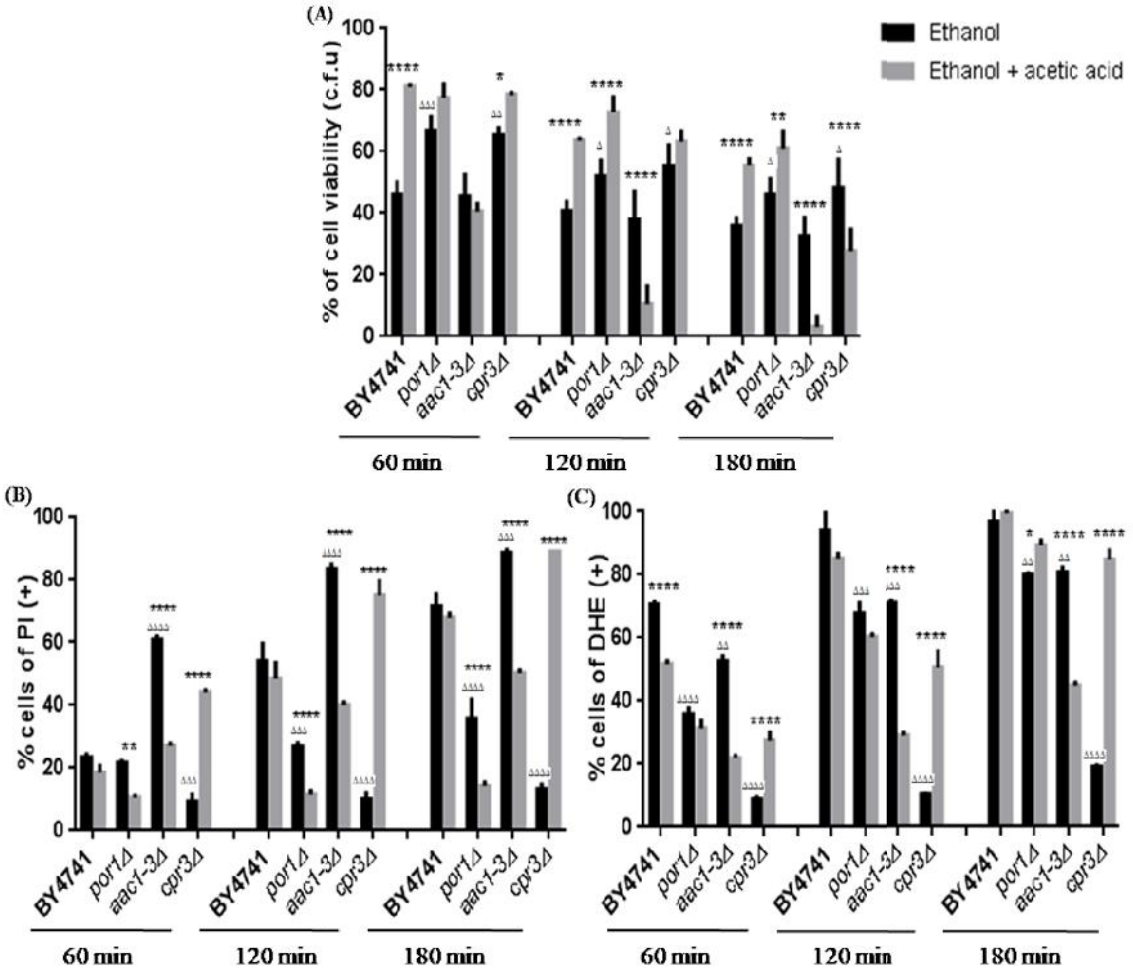

Figure 10 - Acetic acid protects *S. cerevisiae* BY4741, *por1Δ*, *aac1-3Δ and cpr3Δ* cells from ethanol cytotoxicity. (A) – Cell viability (%) assessed by CFUs counts. 100% corresponds to the number of CFUs at time 0 min. (B) – Cells with loss of plasma membrane integrity expressed as a percentage of PI positive stained cells. (C) - Cells displaying accumulation of superoxide expressed as a percentage of DHE positive stained

cells. All experiments were performed in YPD medium at pH 3.5. Cells were incubated with 13% (v/v) of ethanol (Et) or co-incubated with 13% (v/v) of ethanol and 0.1% (v/v) of acetic acid (Et + AA), for 3 hours. All the values represent the average of 3 independent experiments and the respective standard deviations. * Represent statistical analysis of cells treated only with ethanol and Δ represent statistical analyses of cells treated with ethanol and acetic acid. ****/ΔΔΔΔ: P<0.0001; ***/ΔΔΔ: P<0.001; **/ΔΔ: 0.01and */Δ: P<0.05.

Involvement of the yeast ENDO G Nuc1p, Apoptosis Inducing factor Aif1p, metacaspase Yca1p, and the vacuolar protease Pep4p

NUC1 gene encodes a major mitochondrial nuclease found in *S. cerevisiae,* and is the yeast homolog of the metazoan endonuclease G (EndoG), a mitochondrial protein with DNase/RNase activity involved in apoptotic DNA degradation. Overexpression of Nuc1p promotes yeast PCD. When mitochondrial respiration is increased *NUC1* deletion inhibits apoptotic death, whereas under respiration repression conditions, *NUC1* deletion sensitizes yeast cells to non-apoptotic death indicating a dual, pro-life and pro-death role for *NUC1* (Büttner *et al.,* 2007).

*nuc1*Δ cells only display a significant higher cell survival in response to ethanol when 0.1% (v/v) of acetic acid is present at 60 min. At 180 min the presence of the acid rather than protecting enhanced the toxic effect of ethanol (Fig. 11A). This observation suggests that Nuc1p plays an important role in protection by this weak acid. Though the protective effect of 0.1% (v/v) of acetic acid on cell survival at 60 min. was consistently associated with a significant decrease on the accumulation of superoxide anion, it was connected to a significant increase in the loss of plasma membrane integrity (Fig. 11B, C).

The yeast caspase-1 protein (Yca1p), the only metacaspase in *S. cerevisiae*, was recently identified as a Ca^{2+}-activated cysteine protease that may cleave specific substrates during stress response in yeast, seems to play an important role in the apoptotic process (Wong *et al.,* 2012).

The protective effect of 0.1% (v/v) of acetic acid remained in the absence of Yca1p, except for 180 min. where no significant differences of cell survival in response to 13% (v/v) of ethanol in the absence or presence of the acid were observed (Fig. 11A). No significant protection of acetic acid

against 13% (v/v) of ethanol induced loss of plasma membrane integrity and accumulation of superoxide anion was observed at any time (Fig. 11B, C).

PEP4 gene encodes a protease (proteinase A) that is required for the processing of vacuolar protein precursors to their mature forms. Overexpression of Pep4p promotes cell survival by specifically reducing necrosis; deletion exacerbates both apoptosis and necrosis. Suppression of apoptosis by Pep4p relies on its proteolytic activity, whereas the repression of necrosis is mediated by the proteolytically inactive Pep4p propeptide, which promotes survival via a novel pathway, the stimulation of spermidine biosynthesis (Gutiérrez *et al.,* 2011).

In cells lacking Pep4p the protective effect of 0.1% (v/v) of acetic acid against 13% (v/v) of ethanol-induced death was only statistically significant at 60 min. (Fig. 11A). However, the presence of 0.1% (v/v) of acetic acid led to a protection against loss of plasma membrane integrity, though not associated with decreased intracellular levels of superoxide anion suggesting that it may act downstream of superoxide anion accumulation to protect against plasma membrane damage (Fig. 11B, C).

Aif1p, similar to mammalian Apoptosis-Inducing Factor (*AIF)*, is located in mitochondria and translocates to the nucleus of yeast cells in response to apoptotic stimuli. Therefore, Aif1p is required for efficient apoptotic cell death in budding yeast. Overexpression of Aif1p can sensitize yeast to apoptotic stimuli (Wissing *et al.,* 2004).

In the absence of this protein 0.1 % (v/v) of acetic acid does not protect against cell death induced by 13% (v/v) of ethanol and even significantly potentiated ethanol cytotoxicity, suggesting that Aif1p plays a crucial role in the protection (Fig. 11A). Curiously, this negative effect of the acid was associated with a higher loss of plasma membrane integrity and increase in accumulation of superoxide anion. Instead, it was observed a significant decrease in the loss of plasma membrane integrity, at 60 and 120 min., and in the accumulation of superoxide anion at 120 min., while for the other time points no differences were detected (Fig. 11B, C).

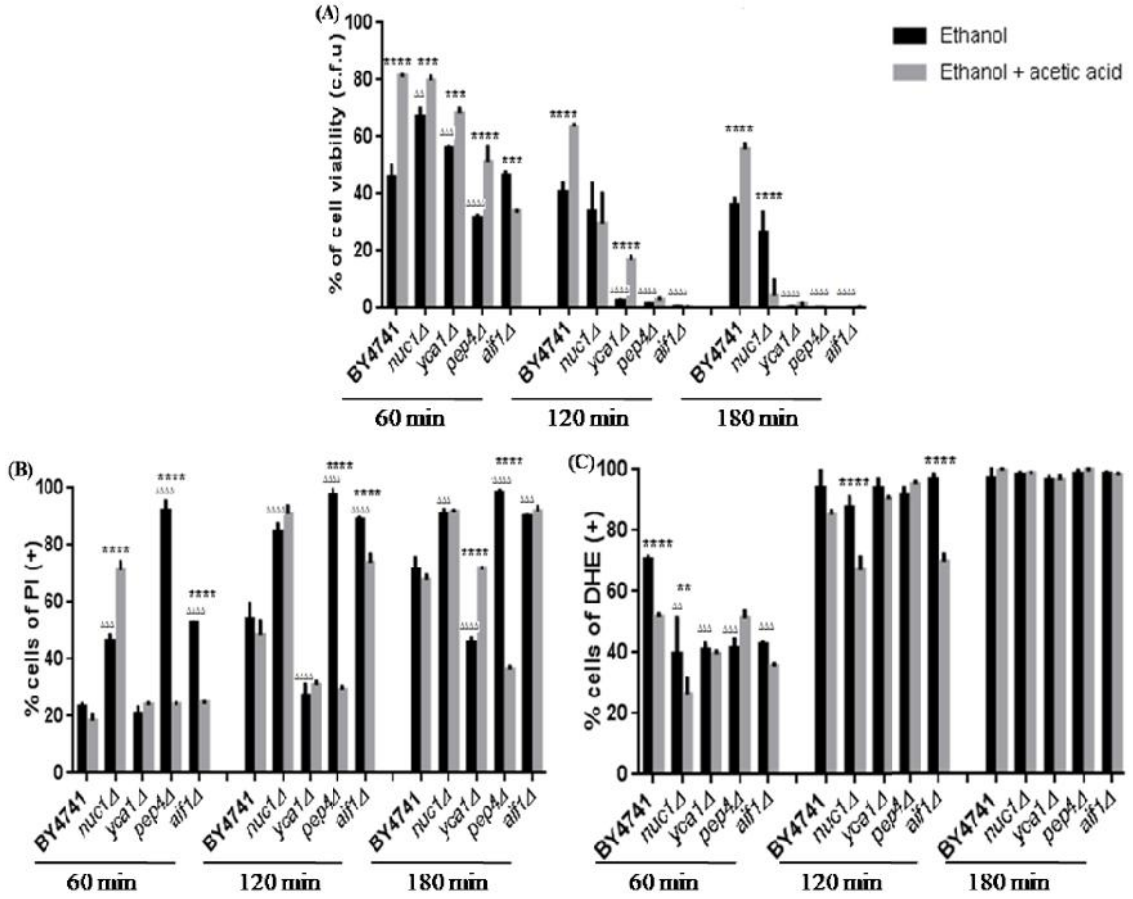

Figure 11 - Acetic acid protects *S. cerevisiae* BY4741, *nuc1Δ*, *yca1Δ, pep4Δ* and *pep4Δ* cells from ethanol cytotoxicity. (A) – Cell viability (%) assessed by CFUs counts. 100% corresponds to the number of CFUs at time 0 min. (B) – Cells with loss of plasma membrane integrity expressed as a percentage of PI positive stained cells. (C) - Cells displaying accumulation of superoxide expressed as a percentage of DHE positive stained cells. All experiments were performed in YPD medium at pH 3.5. Cells were incubated with 13% (v/v) of ethanol (Et) or co-incubated with 13% (v/v) of ethanol and 0.1% (v/v) of acetic acid (Et + AA), for 3 hours. All the values represent the average of 3 independent experiments and the respective standard deviations. * Represent statistical analysis of cells treated only with ethanol and Δ represent statistical analyses of cells treated with ethanol and acetic acid. ****/ΔΔΔΔ: P<0.0001; ***/ΔΔΔ: P<0.001; **/ΔΔ: 0.01and */Δ: P<0.05.

Does ethanol induce a programmed necrosis in *Saccharomyces cerevisiae*?

Previous studies provided evidence that ethanol induces a cell death process which displays common features with apoptosis, namely chromatin condensation and fragmentation, internucleosomal DNA degradation, and dependence on *de novo* protein synthesis (Kitagaki *et al.,* 2007). Moreover, ethanol-induced cell death was associated with an accumulation of ROS and a Fis1p-mediated mitochondrial fragmentation. These authors also assessed the role of some of the known apoptotic regulators including, Yca1p, Aif1p, Cyc3p, and Nuc1p, in cell death induced by 21% of ethanol. They found that with exception of Nuc1p, which exhibited a significant lower survival as compared to the wild-type, none of the other mutations had a significant effect on sensitivity to ethanol.

From our study on the involvement of known apoptotic regulators in the protection by acetic acid described above, we could also conclude on their role in ethanol-induced cell death. Cells of *atp2Δ and atp10Δ* mutants, exposed to 13 % (v/v) of ethanol, displayed an increased survival at all treatment times (Fig. 8A). While the increase in cell viability of the *atp2*Δ mutant was accompanied by a decrease in the percentage of cells with loss of plasma membrane integrity and in the accumulation of superoxide anion, the *atp10*Δ mutant showed a decrease in the percentage of cells with superoxide accumulation but surprisingly an increase in the percentage of cells with loss of plasma membrane integrity. The higher ethanol resistance of *atp2*Δ and *atp10*Δ mutants was associated with a decrease in intracellular levels of superoxide anion (Fig. 8B, C).

Cells lacking Cyc3p also showed a significant increase in cell survival when treated with 13% (v/v) of ethanol as compared with wild-type cells (Fig. 9A), and this was associated with a significant decrease in the loss plasma of membrane integrity and in the accumulation of intracellular levels of superoxide anion (Fig. 9B, C). Together these results suggest that as observed in cells committed into apoptosis in response to acetic acid and hyperosmtic stress (Ludovico *et al.,* 2002; Silva *et al.,* 2005) the mature form of cytochrome *c* is required for cell death induced by ethanol.

Cells lacking both cytochrome *c* isoforms (*cyc1*Δ*cyc7*Δ), when exposed to 13% (v/v) ethanol showed a decrease in cell viability after time 120 and 180 min., but not after 60 min. in comparison to the wild-type (Fig. 9A). The absence of these genes led to an increase in the loss of plasma

membrane integrity but, in contrast, to a decrease in the accumulation of superoxide anion induced by ethanol (Fig. 9B, C).

In the cultures of *por1Δ* exposed of 13% (v/v) of ethanol, we observed an increase in cell viability (Fig. 10A); this increased was accompanied by a decrease in the loss of plasma membrane integrity and accumulation of superoxide anion (Fig. 10B, C).

The absence the Aac1-3p led to a decreased in cell viability, except at 60 min. of treatment where the difference between this mutant and wild-type was not significant (Fig. 10A). The decreased in cell viability was accompanied by increase in the loss of plasma membrane integrity, on the other hand, the accumulation of superoxide anion decreased (Fig. 10B, C).

Cells lacking Cpr3p exhibit a higher resistance to 13% (v/v) of ethanol, compared to the wild-type (Fig. 10A). In accordance with the increase in cell survival the deletion of *CPR3* led to a reduction in the loss of plasma membrane integrity and in accumulation of superoxide anion (Fig. 10 B, C).

Cells lacking *NUC1* showed a higher resistance at 60 min. treatment with 13% (v/v) of ethanol, but at 120 and 180 min. a decreased in cell viability was observed (Fig. 11A). The absence of this gene led to an increase in the loss of plasma membrane integrity (Fig. 11B). Regarding the accumulation of superoxide anion, it was decreased at 60 min., but for 120 and 180 min. of treatment it did not display any change (Fig. 11C).

YCA1 deletion led to a significant increase of cell viability for time 60 min. of treatment, but at 120 and 180 min. of treatment we observed a marked decrease in cell viability (Fig. 11A). The deletion of this protein led to a decrease in the loss of plasma membrane integrity, the same was observed after 60 min. for the accumulation of superoxide anion, but no significant differences were observed between this mutant and wild-type for the remaining treatment time (Fig. 11B, C).

Finally, cells lacking *PEP4* or *AIF1 (pep4Δ or aif1Δ*), showed a drastic decrease of cell viability at 120 and 180 min. of treatment (Fig. 11A). For both mutants, was observed an increased in loss of plasma membrane integrity, such as in accumulation of superoxide anion (Fig.11 B, C).

Mitochondrial membrane potential

Mitochondrial membrane potential is an important aspect of mitochondrial function. It is responsible for the production of ATP and is generated by the electron transport chain. Loss of mitochondrial membrane potential has been described as an early defining event in the mitochondrial necrosis pathway (Kung *et al.,* 2011). To ascertain the occurrence of this mitochondrial dysfunction during ethanol induced cell death we determined changes in mitochondrial membrane potential in the wild-type and in the mutant strains, which exhibited a resistant phenotype in response to 13% (v/v) ethanol, pH 3.5. Variations in mitochondrial membrane potential were analyzed by flow cytometry using the membrane potential-sensitive probe $DiOC_6$. This fluorescent probe accumulates in the mitochondria as a function of its membrane potential. The changes in relative fluorescence reflecting alterations in mitochondrial membrane potential were expressed in relative values (Log FL-1/Log FS), comparatively with time zero. This ratio allows making the fluorescence measurements independent of the cell size and hence reflecting only changes in mitochondrial membrane potential. The cells stained with $DiOC_6$ were co-stained with PI in order to allow monitoring changes in mitochondrial membrane potential both in the PI positive and negative stained cell sub-populations.

The mutant strains *atp2Δ, atp10Δ* and *cyc3Δ* showed a lower hyperpolarization than the wild-type strain, in accordance with their higher resistance to ethanol-induced cell death (Fig. 12A). Curiously the hyperpolarization appears to be less pronounced in the cell subpopulations with compromised plasma membrane integrity for all strains, as well as the hyperpolarization displayed by the mutant strains as compared to the wild-type strain (Fig. 12B).

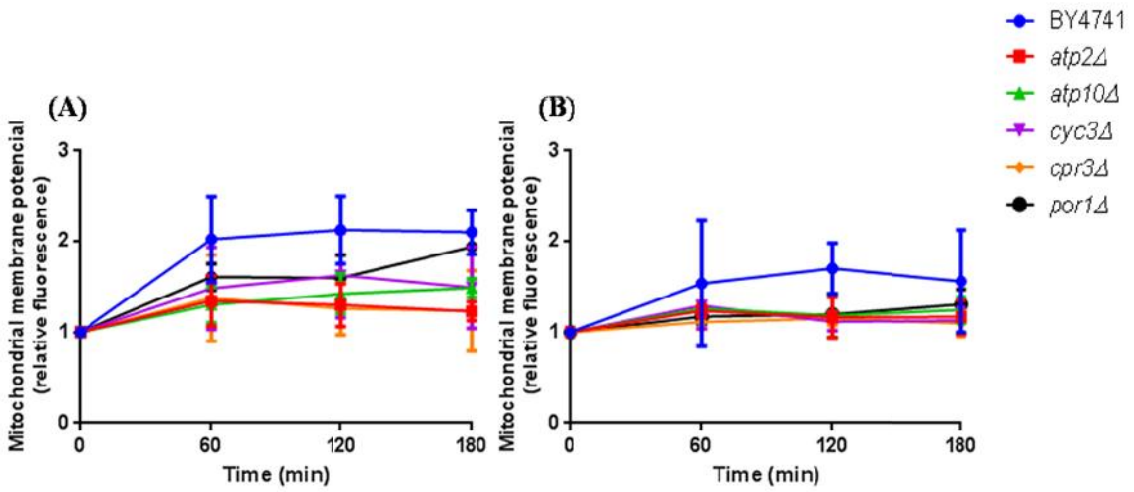

Figure 12 - Mitochondrial membrane potential in BY4741, *atp10Δ*, *atp2Δ, cyc3Δ ,cpr3Δ* and *por1Δ* cells after exposure to 13% (v/v) of ethanol, pH 3.5, for 3 hours. (A) - PI negative cells. Values significantly different for *cpr3Δ* and *atp2Δ*: P<0.0001; *atp10Δ*: P<0.001 and *cyc3Δ*: P<0.01. (B) – PI positive cells. Values significantly different for *atp2Δ, cyc3Δ and cpr3Δ*: P<0.01; *atp10Δ* and *por1Δ*: P<0.05. All experiments were performed in YPD medium. Values are mean ± SD of at least 3 independent experiments.

Detection of caspase activation

Most of the alterations observed during apoptosis are caused by proteases called caspases. The activation of these proteases leads to an apoptotic phenotype (Bursch, 2001). To further characterize death induced by ethanol, caspase activation was assessed by flow cytometry using the "CaspACE, FITCVAD-fmk In Situ Marker" together with PI staining (Fig. 13).

The results obtained showed an increase of the green fluorescence along treatment with 13% (v/v) of ethanol. However, in particular for 120 and 180 min, this was associated with a simultaneous increase of the red fluorescence indicating a simultaneous loss of plasma membrane integrity. Unspecific staining with FITC-VAD-fmk has been shown to occur in cells with compromised plasma membrane (Wysocki *et al.,* 2004). However, the detection of cells with increased levels of green fluorescence but which preserved plasma membrane integrity at 60 min. indicates that caspase activation precedes loss of plasma membrane integrity, and the increased percentage of cells displaying double staining at 120 and 180 min correspond to late apoptotic cells that are dying by secondary necrosis. The activation of caspases in response to lethal concentrations of ethanol is

in accordance with the *yca1*Δ significant higher survival and lower accumulation of intracellular superoxide anion at 60 min, and to the delayed decrease of plasma membrane integrity at 120 and 180 min., as compared o the wild-type.

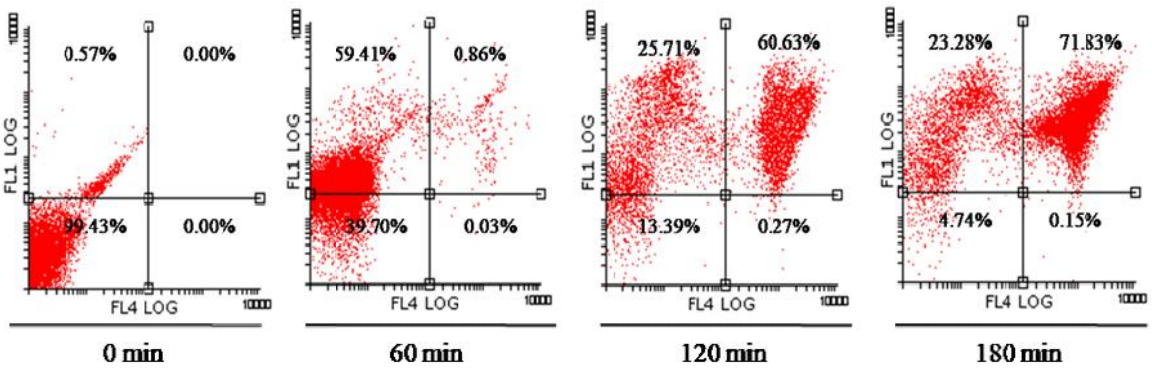

Figure 13 – Assessment of caspase activation during ethanol-induced cell death. Biparametric histograms of FITC and PI fluorescences of wild-type (BY4741) exposed to 13% (v/v) of ethanol, pH 3.5, for 3 hours. All experiments were performed in YPD medium. Data represent one of 3 independent experiments.

Exposure of phosphatidylserine

Phosphatidylserine has an asymmetric distribution in the lipid bilayer of the cytoplasmic membrane. The exposure of phosphatidylserine (PS) at the outer surface of the cytoplasmic membrane occurs at the early stages of apoptosis (Martin *et al.,* 1995). We checked this through the FITC-coupled Annexin V reaction followed by flow cytometry analysis of cells co-stained with Annexin V and PI. This experiment was performed with the wild type strain (BY4741), exposed to 13% (v/v) ethanol, pH 3.5, for 2 hours. Cells treated with 3 mM of hydrogen peroxide for 120 min. were used as a positive control of exposure of PS. Though the percentage of cells with Annexin V positive staining was low it was possible to discriminate the two sub-popupulations (Annexin V+, PI-) and (Annexin V+, PI+) from the Annexin negative staining population (Annexin V-, PI+) (Fig. 14B).

Cells exposed to 13% (v/v) ethanol displayed an increase in the green fluorescence (22.93%) indicative of Annexin positive staining (Fig. 14A). However, since most of these cells were PI positive it would be necessary to assess whether Annexin V staining precedes loss of plasma membrane

integrity at shorter times of treatment with ethanol in order to conclude that PS exposure is an early event of ethanol-induced cell death.

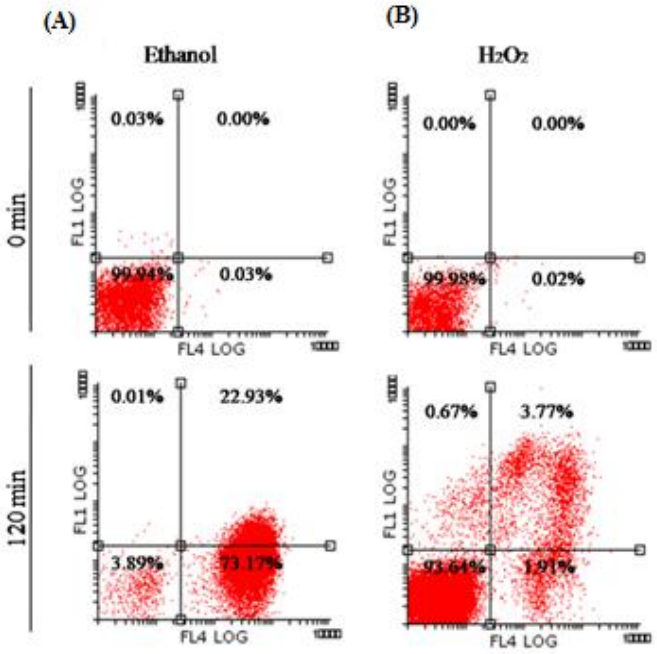

Figure 14 – Ethanol does not induce exposure of phosphatidylserine. (A) - Biparametric histograms of Annexin V and PI stained cells of the wild-type (BY4741) exposed to 13% (v/v) of ethanol, pH 3.5, for 2 hours. (B) - Biparametric histograms of Annexin V and PI stained cells of the wild-type (BY4741) exposed to 3mM of hydrogen peroxide, pH 3.5, for 2 hours. All experiments were performed in YPD medium. Data represent one of 2 independent experiments.

As discussed in the introduction it has been proposed that the rapid loss of plasma membrane integrity, characteristic of cell death by necrosis (He *et al.,* 2009; Zhang *et al.,* 2009) may be also associated with a regulate process of cellular demise. Since all the strains under study when exposed to 13% (v/v) of ethanol showed a high percentage of cells with loss of plasma membrane integrity associated with loss of cell survival, we addressed whether this could be the case. In order to test this hypothesis, we monitored nucleus-cytosolic translocation of Nhp6Ap as discussed below.

Nucleus-cytosolic translocation of Nhp6Ap

Necrosis can be monitored by the nucleus - cytosolic translocation of the human chromatin bound non-histone protein HMGB1 (high mobility group Box1). The nuclear release of Nhp6Ap, the yeast homologue of HMGB1, has also been observed in dying yeast cells and has therefore been considered a marker of necrosis in this organism (Eisenberg *et al.,* 2009). To ascertain whether ethanol induces a regulated form of necrosis, mutant strains, which revealed a resistance phenotype in the survival assays with ethanol, were transformed with an Nhp6A-EGFP expression plasmid. The transformants were then treated with 13% (v/v) of ethanol, pH 3.5, for 3 hours and observed by fluorescence microscopy. Cells treated with a dose of hydrogen peroxide (3 mM) that triggers an apoptotic death in *S. cerevisiae* were used as a negative control.

All the transformants, including the wild-type (BY4741) and mutant strains, exhibited a bright nuclear fluorescence at time zero. After 60 and 120 min. exposure to ethanol some fluorescence was observed in cytosol whereas after 180 min., a diffuse fluorescence was observed throughout the cell indicating the translocation of Nhp6Ap from the nucleus to the cytosol. In cells exposed to hydrogen peroxide there was no relocalization of the fluorescence into the cytosol after 3 hours of treatment indicating that, upon induction of apoptosis, Nhp6Ap is not translocated to the cytosol (Fig. 15). The translocation of Nhp6Ap from the nucleus to the cytosol was delayed in the mutants *atp10Δ, atp2Δ* and *cyc3Δ.* However, while we detected a diffuse cellular fluorescence for *atp10Δ* strain, in the latter two mutants the fluorescence was still preferentially localized in the nucleus. These observations indicate that in contrast to *atp10Δ* mutant, but in accordance with the higher resistance phenotypes of *atp2Δ* and *cyc3Δ,* Nhp6Ap was only partially translocated to the cytosol in these two mutants. *cpr3Δ* and *por1Δ* exhibit a behavior similar to the wild-type strain. The release of Nhp6Ap began after the first hour of treatment, and was completed after 3 hours of treatment, which allows us to think that these cells commit into a necrotic regulated death.

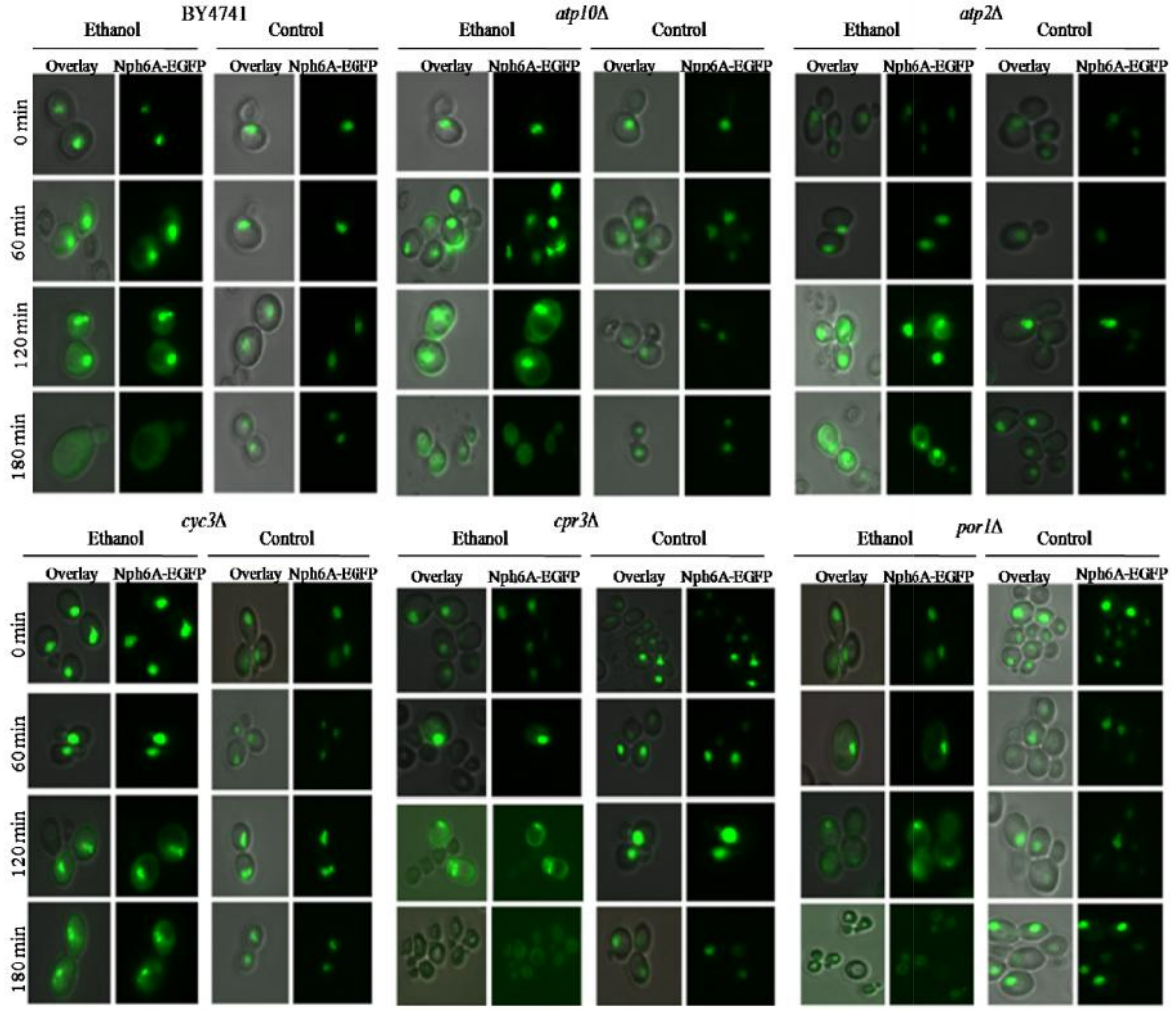

Figure 15 - Fluorescence microscopy of BY4741, *atp10Δ*, *atp2Δ, cyc3Δ, cpr3Δ* and *por1Δ* cells expressing Nhp6A-EGFP treated with 13% (v/v) of ethanol or hydrogen peroxide (3mM) (0, 60, 120 and 180 min.). All experiments were performed in SC URA- medium at pH 3.5, for 3 hours. Data represent one of 3 independent experiments. Bar, 10 μm.

In order to quantify the decrease in green fluorescence upon translocation of Nhp6Ap, we stained the cells with PI and analysed byflow cytometry, both the PI positive and negative stained cell sub-populations. The changes of green fluorescence were expressed in relative values comparatively with time zero (Fig. 16). The results obtained for each mutant are in accordance with those obtained by fluorescence miscroscopy (Fig. 15). Indeed there was a marked decrease in the green

fluorescence in the wild-type reflecting the release of Nhp6Ap, and the mutants *atp2Δ, atp10Δ* and *cyc3Δ* showed a delay in the decrease in green fluorescence, which is consistent with their resistance phenotype, as referred above (Fig. 16).

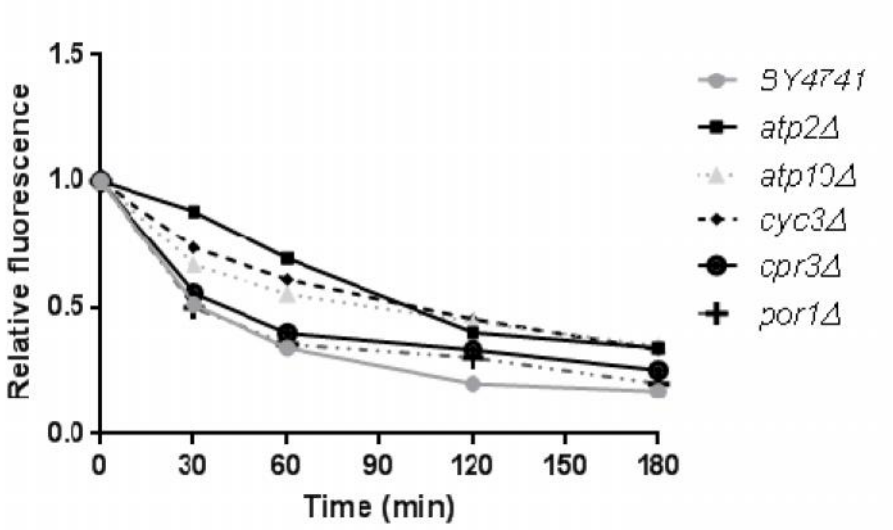

Figure 16 - Reduction of the green fluorescence (relative fluorescence) determined by flow cytometry in BY4741, *atp10Δ*, *atp2Δ, cyc3Δ, cpr3Δ* and *por1Δ* expressing Nhp6A – EGFP, exposed of 13% (v/v) of ethanol, pH 3.5, for 3 hours. All experiments were performed in SC URA- medium. The values represent the average of 2 independent experiments.

DISCUSSION AND FUTURE PERPECTIVES

From a biotechnology point of view, ethanol is the most important microbial fermentation product. It is associated with many aspects of human life, including consumption of alcoholic beverages and use of ethanol-based biofuels. Production of industrial ethanol has been gaining increased relevance as an alternative energy source due to the reduced availability of petroleum derived fuels. However, efficient ethanol production faces a bottleneck regarding ethanol yield since the yeast *S. cerevisiae,* commonly used for fermentation, dies when ethanol levels exceed a certain concentration. For these reasons, elucidation of ethanol-induced cell death in yeast is likely to have a significant impact on the fermentation industry.

Previous studies reported that cell death induced by ethanol shows characteristics of an apoptotic process associated with Fis1p mediated mitochondrial fragmentation and accumulation of ROS (Kitagaki *et al.,* 2007). However, while Fis1p and Nuc1p were found to protect from ethanol-induced cell death, no other protein conferring resistance to ethanol-induced cell death was identified. During fermentation, some weak carboxylic acids such as acetic, produced by yeast metabolism, accumulate in the growth medium. This accumulation enhances ethanol cytotoxicity and can result in growth inhibition or arrest of fermenting cells, and decreases the efficiency of grape must fermentation or the production of industrial ethanol (Gibson *et al*., 2007). However, previous data obtained showed that *S. cerevisiae* cells exposed simultaneously to toxic concentrations of ethanol and low concentrations of acetic acid displayed higher survival (measured either by CFUs or by PI staining) than cells treated only with ethanol. These results indicated that acetic acid induces a cellular response that provides protection against the cytotoxic effect of ethanol (Vieira *et al.,* unpublished results). Data obtained later in our laboratory (Afonso, 2011), showed that cell cultures without functional mitochondrial did not display protection by acetic acid against ethanol-induced cell death. Since superoxide anion is mainly produced at the mitochondrial compartment and the protection by the acid depends on the mitochondrial function, namely on respiration, it appears that respiring mitochondria are required for acetic acid to increase cell viability in response to ethanol.

Aiming to understand how acetic acid inhibits ethanol induced cell death we sought to analyze the role of proteins that reside in mitochondria, as well as of the yeast metacaspase Yca1p and the vacuolar protease Pep4p in the acid protection effect. This analysis also allowed further characterization of molecular components/regulators involved in ethanol-induced cell death.

Table 3 shows how the absence of known apoptototic regulatory proteins abrogate, exacerbate or have no effect on death induced by ethanol. The mutant phenotypes can be essentially associated to one of the four following types: R) resistant phenotype associated with a higher survival, lower loss of plasma membrane integrity and lower accumulation of superoxide anion; r) resistant phenotype associated with a higher survival, higher loss of plasma membrane integrity and lower accumulation of superoxide anion; IS) sensitivity similar to wild-type regarding cell survival but with higher loss of plasma membrane integrity and lower accumulation of superoxide anion; and S) sensitive phenotype associated with a lower cell survival, higher loss of plasma membrane integrity and lower accumulation of superoxide anion.

Table 3 – Role of known apoptotic regulators on ethanol-induced cell death in *S. cerevisiae*. Mutant phenotypes were assessed by CFUs counts, PI staining and accumulation of intracellular superoxide in response to 13% (v/v) ethanol. N.s refers to results not statistically different as compared with the wild-type. In case these differences are significant for only one or two time points, the time points are specified.

Ethanol- induced cell death			
Mutants	**Viable cells (%)**	**Cells with plasma membrane integrity (%)**	**Cells with superoxide accumulation (%)**
atp2Δ	Higher	Lower (120 and 180 min)	Lower (60 and 120 min)
atp10Δ	Higher (60 and 120 min)	Higher	Lower (60 and 120 min)
cyc3Δ	Higher (60 and 120 min)	Lower (120 and 180 min)	Lower
cyc1Δcyc7Δ	Lower (120 and 180 min)	Higher	Lower
por1Δ	Higher	Lower (120 and 180 min)	Lower
aac1-3Δ	N.s.	Higher	Lower
cpr3Δ	Higher	Lower	Lower
nuc1Δ	Higher (60 min)	Higher	Lower (60 min)
yca1Δ	Higher (60 min) and lower (180 min)	Lower (120 and 180 min)	Lower (60 min)
pep4Δ	Lower	Higher	Lower (60 min)
aif1Δ	Lower (120 and 180 min)	Higher	Lower (60 min)

The R mutant phenotype includes the mutant strains *atp2Δ, cyc3*Δ, *por1*Δ, *cpr3*Δ and *yca1*Δ indicating that mitochondria is involved in ethanol-induced cell death and requires the ATPsynthase complex, mature cytochrome *c*, two yeast orthologues of putative components of the

mammalian PTP, and depends on yeast metacaspase Yca1p. The effect of the oxidative phosphorylation inhibitor oligomycin on ethanol-induced cell death will allow assessing whether inhibition of cell death is due to ATP deficiency or to the absence of a functional ATPsynthase. Recent studies propose that PTP is formed by dimers of the ATPsynthase independently of OMM components, which share common regulators with PTP (such as Ca^{2+}, Mg^{2+}, adenine nucleotides and Pi) (Bonora *et al.,* 2013; Giorgio *et al.,* 2013). The relevance of ATP synthase or of the ATP pool is further supported by the higher resistance of the *atp10*Δ mutant, which lacks an assembly factor of the ATP synthase. The r phenotype of *atp10*Δ is associated with a lower superoxide anion accumulation but unlike the *atp2*Δ. The discrepancy between cell survival and loss of plasma membrane integrity may be explained by the differences in the methods used. Indeed, while cell survival is assessed after serial dilutions and after plating in a rich medium allowing some cells with compromised plasma membrane to recover, PI staining is performed immediately after ethanol treatment, which may lead to a percentage of cell survival lower than the percentage of cells with loss of plasma membrane integrity. Curiously, cells lacking Atp2p or Atp10p are more sensitive and more resistant to acetic acid-induced cell death, respectively (Pereira *et al.,* 2007; Ludovico *et al.,* 2002). One possible explanation for the opposite phenotypes of *atp2Δ* rely on a distinct dependence of the two cell death processes on ATP derived from oxidative phosphorylation. Indeed, it was shown that acetic acid-induced apoptosis is not inhibited by oligomycin, and it is possible that ethanol-induced cell death is sensitive to this inhibitor. However, this hypothesis is not consistent with the unaltered cell survival displayed by the *aac1-3*Δ, as discussed below, and which is defective in oxidative phosphorylation.

Ethanol metabolism in yeast involves its oxidation to acetaldehyde and then to acetate. It could be therefore expected that both ethanol and acetic acid could share the same death pathway. However, ethanol oxidation is subject to glucose repression and thus is not occurring under the death inducing conditions used in the assays. This may determine some differences in the cell death pathways triggered by these two lethal stimuli.

The resistance phenotype of *atp10*Δ to acetic acid-induced apoptosis was attributed to the non release of cytochrome *c*. The observation that this mutant displays a similar phenotype regarding ethanol-induced cell death supports the notion that a fully assembled F0 F1-ATPase is required for the death process and that ATP synthase complex would also be involved in the mechanism of cyt *c*

release from mitochondria. Though the events downstream cyt *c* release have not been completely clarified in yeast, since no apoptosome-like structure has been identified, it was found a causal relationship between metcaspase activation and cyt *c* during apoptosis induced by hyperosmotic stress (Silva *et al.,* 2005) as well as abrogation of hyperosmotic- and acetic acid-induced apoptosis in the absence of mature cyt *c* (Silva *et al.,* 2005; Ludovico *et al.,* 2002). A similar behavior was observed for ethanol-induced cell death pointing to a role of cyt *c* in the death process. Since ethanol-induced cell death also appears to depend on Yca1p, as absence of this caspase leads to increased cell survival, as observed in different yeast apoptotic scenarios (Madeo *et al.,* 2002), the assessment of caspase activation in the *cyc3*Δ mutant would allow confirming if, like in hyperosmotic-induced apoptosis, caspase activation depends on cyt *c*.

Deletion of *CPR3*, encoding the yeast orthologue of mammalian cyclophilin D, resulted in increased resistance to ethanol-induced cell death compared to the wild-type suggesting that it is involved this cell death process. Cyclophilin D is a prolylisomerase located within the mitochondrial matrix, which together with the adenine nucleotide translocator (ANT), and the voltage dependent anion channel (VDAC), have been proposed as putative components of the mitochondrial permeability transition pore (mPTP). Openning of mPTP is associated with mitochondrial swelling, outer membrane rupture, and the release of apoptotic mediators (Halestrap, 2005). Mammalian cyclophilin D has been implicated in both necrosis and apoptosis programs (Halestrap, 2005; Schneider, 2005) but other authors claimed that it is involved in necrotic, but not apoptotic, cell death (Baines *et al.,* 2005; Nakagawa *et al.,* 2005). Accordingly, it was found that mitochondrial cyclophilin (Cpr3p) does not play a role in acetic acid-induced apoptosis. This conclusion was supported by the lack of effect of cyclosporin A, known to block mPTP opening by binding to cyclophilin D in mammalian cells, as well as by the observation that *CPR3* deletion does not affect loss of cell viability, and the emergence of apoptotic markers or cyt *c* release. However a genetic screen identified Cpr3p as mediating the Cu-induced apoptotic program (Liang & Zhou, 2007) like we found for ethanol-induced cell death. The study of the effect of cyclosporin A, in ethanol-induced cell death will allow to further support the involvement of Cpr3p in cell demise in response to ethanol. In line with these results we also found that *por1*Δ cells exhibited a significant increase in cell survival in response to ethanol, suggesting a key role of mPTP played in ethanol-induced cell death. However, in contrast to acetic-acid induced apoptosis, cell survival was not affected by the absence of Aac1-3p

proteins the yeast orthologues of the mammalian ANT. The lower accumulation of superoxide anion can be explained by the reduced respiration rates. Indeed most of the superoxide anion generated by mitochondria is thought originate from electron leakage at the components of the mitochondrial electron transport chain, and reduced electron transport rates associated with low respiration rates will generate less superoxide anion.

The S mutant phenotype includes the mutant strains: *aif1Δ, pep4Δ* and *cyc1Δcyc7Δ.* Aif1p similar to mammalian AIF is required for efficient apoptotic cell death in yeast. Aif1p has been shown to be involved in apoptosis induced by H_2O_2 and chronological aging. Overexpression of Aif1p might sensitize yeast to apoptotic stimuli (Wissing *et al.,* 2004). *PEP4* gene encodes a protease (proteinase A) that is required for the processing of vacuolar protein precursors to their mature forms, thus the *pep4* mutants of *S. cerevisiae* accumulate inactive precursors of vacuolar hydrolases and deletion exacerbates both apoptosis and necrosis (Gutiérrez *et al.,* 2011). Pep4p has a role in mitochondrial degradation in yeast cells undergoing AA-PCD and confers protection against these apoptotic stimuli (Pereira *et al.,* 2010). *CYC1* and *CYC7* encode the iso-1-cytochrome *c* and iso-2-cytochrome *c*, respectively. In hyperosmotic stress the absence of holocytochrome *c* in *cyc1Δcyc7Δ* strain enhances cell survival (Silva *et al.,* 2005). The absence of any of these three proteins led to a decrease in cell viability, accompanied by an increase in loss of plasma membrane integrity and suggests a role for cyt *c*, Aif1p and Pep4p in the resistance to ethanol. The high rate of loss of plasma membrane integrity, as described above, suggests that death is occurring through necrosis. The lower accumulation of superoxide anion in the case of *aif1Δ* and *cyc1Δcyc7Δ* may be explained by a reduced or absence of respiration in these mutants. Though the results obtained are in agreement with the expected for the *pep4Δ* mutant, as a reduction in cell viability is observed for both apoptosis and necrosis (Carmona-Gutierrez *et al.,* 2004), it disagrees with the previously described apoptosis resistance of cells lacking Aif1p and cyt *c*.

The yeast Aif1p like its mammalian orthologue is a mitochondrial flavoprotein that translocates to the nucleus and takes part in DNA degradation in cells undergoing apoptosis (Wissing *et al.,* 2004). Mammalian AIF acts as an NAD(P)H oxidase (Miramar *et al*, 2001), and the AIF knockdown or knock-out compromises the utilization of NAD(P)H and the maintenance of GSH in oxidant-stressed cells (Cande *et al,* 2004b). It was shown that human or mouse cells lacking AIF exhibit a compromised oxidative phosphorylation. This dysfunction is associated with a high lactate

production and enhanced dependency on glycolytic ATP generation, due to severe reduction of respiratory chain complex I activity. Accordingly, the absence of AIF also compromised OXPHOS in yeast (which does not possess a full complex I), indicating a general, pleiotropic role of AIF in the assembly and/or maintenance of respiratory complexes (Vahsen *et al.,* 2004).

Altogether, the sensitive phenotype of *aif1*Δ and *cyc1Δcyc7*Δ mutants point to a crucial role of respiration in the protection against ethanol-induced death. However, whether and how oxidative phosphorylation influences the fate of the cell is less clear and requires further studies. It appears that oxidative phosphorylation, even at a reduced level due to glucose repression, has a protective role in the absence of accumulation of ROS, based on the *aif1*Δ sensitive phenotype, but a deleterious role taking into account the resistance phenotype of *atp2*Δ and *atp10*Δ mutants. However, it cannot be discarded that Aif1p and ATP synthase may have interfere with the cell response to ethanol in a way that does not depend on oxidative phosphorylation.

Nuc1p is a yeast orthologue of mammalian endonuclease G (Endo G). As mammalian Endo G (Büttner *et al.,* 2007), Nuc1p is located in mitochondria and translocates to the nucleus upon apoptosis induction. This mutant when exposed to ethanol presented resistance at 60 min, with a decrease in viability in the other treatment times. An increase in the loss of membrane integrity but not in the accumulation of superoxide anion was also observed. Disruption of *NUC1* causes an inhibition of apoptotic cell death when mitochondrial respiration is high, but enhances necrotic death when oxidative phosphorylation is repressed (Büttner *et al.,* 2007). Since cell death induced by ethanol was carried in rich medium with glucose, which is associated with reduced levels of oxidative phosphorylation, the observed decrease in cell survival of *nuc1*Δ mutant accompanied by an in increase in the loss of plasma membrane integrity suggests the occurrence of a necrotic cell death, like observed in previous studies. This is also consistent with a lower accumulation of ROS in *nuc1*Δ mutant as compared to the wild-type strain.

In order to further characterize the death process induced by ethanol other cell death markers such as alteration of the mitochondrial membrane potential, caspase activation and exposure of phosphatidylserine on the outer surface of the plasma membrane and the translocation of Nhp6Ap from the nucleus to the cytosol, were monitored in the wild-type and in the mutants *atp10Δ*, *atp2Δ*, *cyc3Δ*, *por1Δ* and *cpr3Δ* that showed an increased survival in response to ethanol 13% (v/v), compared to the wild-type strain.

Loss of membrane potential is an early defining event in the mitochondrial necrosis pathway (Kung *et al.,* 2011). Our results show that cells with compromised plasma membrane integrity exhibit an increase of the mitochondrial membrane potential though lower than the hyperpolarization observed in cells that preserved the plasma membrane integrity. Positive and negative PI stained cell sub-populations of the ethanol resistant mutants exhibited no changes and slight increase in mitochondrial membrane potential in comparison to the wild-type, respectively.

Though apoptosis can occur through a caspase independent pathway the activation of these proteases leads, normally, to an apoptotic phenotype (Bursch, 2001). Also exposure of phosphatidylserine at the outer surface of the cytoplasmic membrane occurs at early stages of apoptosis (Martin *et al.,* 1995) and is a characteristic marker of this type of regulated death. Cell death induced by ethanol was associated with caspase activation at 60 min. mainly in cells, which lost their integrity of the plasma membrane. This result suggests caspases are activated and is consistent with the phenotype obtained for the *yca1*Δ mutant. Externalization of phosphatidylserine was also observed after 120 min. but most cells had their plasma membrane compromised. These observations are typical of late apoptotic cells, which undergo a secondary necrosis in the final step of the apoptotic process.

Though most studies in the field of yeast PCD have addressed the process of apoptosis, it is increasingly recognized that necrosis as a primary cause of cell death also exists in yeast. Importantly, evidence is accumulating that necrotic cell death does not only follow cell exposure to chemical or physical aggressions but also occurs under normal physiological conditions and reasonable concentrations of cell death inducing substances (Eisenberg *et al.,* 2010). Several studies have shown that necrotic cell death is morphologically characterized by rupture of the plasma membrane and subsequent loss of intracellular contents, which does not occur in death by apoptosis (Kroemer *et al.,* 2009). The high percentage of cells unable to exclude PI after exposure to ethanol indicative of loss of plasma membrane integrity pointed to a necrotic death. The *atp10Δ* strain reached approximately 80% of PI positive cells while the other resistant mutants did not showed such high levels, with a percentage of PI positive cells ranging between 20% and 40%.

Monitoring HMGB1/ Nhp6Ap release from the nucleus to the cytosol during cell death has been used as a bona fide marker of necrosis (Eisenberg *et al.,* 2010) and could give strength to our hypothesis. H_2O_2 is an exogenous trigger commonly used to induce apoptosis in yeast (Madeo *et al.*,

1999; Ludovico *et al.*, 2001), and thus has been used as a control of apoptosis. Under these conditions, there was no decrease of green fluorescence, which discards the occurrence of death by necrosis and validates the translocation of Nhp6Ap to the cytosol to monitor necrosis.

Cell death induced by ethanol was associated with the translocation of Nhp6Ap from the nucleus to the cytosol, typical of necrosis. The *atp10Δ*, *atp2Δ* and *cyc3Δ* mutants, showed a delay in the re-localization of the nuclear green fluorescence to the cytosol, in agreement with their resistant phenotype cited above.

Altogether the results obtained with the mutants phenotypes and the characterization of cell death markers suggest that ethanol triggers a regulated cell death process that shares feature typical of both apoptosis and necrosis. Whether the necrotic features reflect a programmed necrosis or are a secondary necrosis of apoptotic cells committed to death in response to ethanol requires further studies. Regarding the protective effect of acetic acid against loss of cell viability induced by ethanol we show that all the mutants displayed a decrease in the protection by acetic acid excepting *cyc3*Δ and *pep4*Δ mutant, in which protection was abolished and maintained, respectively, as compared to the wild-type strain (Table 4). While in *cyc3*Δ mutant the protection was also abrogated in what regards the preservation of plasma membrane integrity and superoxide accumulation the same was not apparent in the other mutants strains, or was only observed for some time point. Moreover, in some deletion mutants the influence on the protection had opposite effects on cell survival, loss of plasma membrane integrity and accumulation of superoxide anion. This indicates that the proteins studied have different roles in cell viability, plasma membrane integrity and oxidative stress, making the interpretation of the results quite difficult and pointing to the need of further studies. However, the mutants *atp2*Δ, *atp10*Δ and *nuc1*Δ display a decrease of protection by acetic acid against loss of cell viability, plasma membrane integrity and accumulation of superoxide, which indicates that this proteins play a relevant role in the protection effect.

Table 4 – Role of known apoptotic regulators on the protective effect of acetic acid against ethanol-induced cell death in *S. cerevisiae*. Cellular phenotypes were assessed by CFUs counts, PI and DHE staining of cells in response to 13% (v/v) ethanol in the absence and presence of 0.1% (v/v) acetic acid. In the table "Decreased" refers to a protective effect significantly inhibited; "Increased" to a protective effect significantly enhanced; "Maintained" refers to maintenance of the significant protective effect, and "Absence" to the absence a

significant a protective effect. When the differences were significant for only one or two time points, the time points are specified.

Protective effect of acetic acid assessed by:			
Mutants	**CFUs (%)**	**Cells with plasma membrane integrity (%)**	**Cells with superoxide accumulation (%)**
atp2Δ	Decreased	Decreased (180 min)	Decreased (60 min) and increased (120 min)
atp10Δ	Decreased (60 min)	Decreased	Decreased (60 min) and increased (120 min)
cyc3Δ	Absent	Absent	Absent
cyc1Δcyc7Δ	Decreased (120 min)	Absent	Maintained (180 min)
por1Δ	Decreased (180 min)	Increased	Decreased (180 min)
aac1-3Δ	Decreased (120 and 180 min)	Increased	Increased
cpr3Δ	Decreased (60 and 180 min)	Increased	Decreased
nuc1Δ	Decreased (60 and 180 min)	Decreased (60 min)	Decreased (60 min)
yca1Δ	Decreased (60 min)	Decreased (180 min)	Absent
pep4Δ	Maintained (60 min)	Increased	Absent
aif1Δ	Decreased (60 min)	Increased (60 and 120 min)	Increased (120 min)

Our study complements the existing information in the literature addressing ethanol induced cell death process. We show the role of regulators of apoptosis in yeast in the protection exerted by acetic acid in ethanol-induced death. In the characterization of this cell death, we used different tests, including the translocation of HMGB1, activation of caspases and externalization of phosphatidylserine, as well as broaden the study to other mutants.

In the future, in order to better understand the protective effect of acetic acid on cell death by ethanol, it would be important to carry further studies. To better understand the role of mitochondria other structural and functions alterations of this compartment should be assessed namely mitochondrial fragmentation and degradation, permeabilization of the outer and inner mitochondrial membrane, by monitoring the mitochondrial contents of citrate synthase (CS) and adenylate kinase (AK), located in mitochondrial matrix and the mitochondrial intermembrane space, respectively. Since it is described in literature that during necrosis there is an increase in the cytosolic

concentration of calcium (Ca^{2+}) and cellular ATP levels are markedly depleted (Kung *et al.,* 2011), the levels of Ca^{2+} and ATP during death induced by ethanol could complete the characterization of the different functional changes involved in the death process. Besides cyclophilin, other molecular components have been involved in necrotic death scenarios such as calpains and cathepsins and their involvement in ethanol-induced death should be determined.

BIBLIOGRAPHY

Afonso, A. F. (2011). "Protective effect of acetic acid against ethanol-induced cell death in *Saccharomyces cerevisiae".* Departamento de Biologia, Universidade do Minho, Portugal.

Almeida, B., Ohlmeier, S., Almeida, A.J., Madeo, F., Leao, C., *et al.* (2009). "Yeast protein expression profile during acetic acid-induced apoptosis indicates causal involvement of the TOR pathway". *Proteomics,* **9:** 720–732.

Alper, H., Moxley, J., Nevoigt, E., Fink, G.R. and Stephanopoulos, G. (2006). "Engineering yeast transcription machinery for improved ethanol tolerance and production".*Science,* **314**: 1565–1568.

Anderson, A. (1992). "Yeast genome project: 300,000 and counting". *Science,* 256-462.

Aravind, L., Iyer, L.M. and Koonin, E.V. (2003). "Scores of RINGS but no PHDs in ubiquitin signaling". *Cell Cycle,* **2:**123–126.

Baehrecke, E.H. (2002). "How death shapes life during development". *Nat Rev Mol Cell Biol*, **3:** 779–787.

Baines, C.P., Kaiser, R.A., Purcell, N.H., Blair, N.S., Osinska, H., Hambleton, M.A., *et al.* (2005) "Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death." *Nature*, **434:** 658–662.

Barry, J.A. and Gawrisch, K. (1995). "Effects of ethanol on lipid bilayers containing cholesterol, gangliosides, and sphingomyelin".*Biochemistry*, **34:**8852–8860.

Betz, C., Schlenstedt, G. and Bailer, S.M. (2004). "Asr1p, a novel yeast ring / PHD finger protein, signals alcohol stress to the nucleus".*J Biol Chem,* **279**: 28174–28181.

Bigelis, R. (1985)." Primary metabolism and industrial fermentations.In Gene Manipulations in Fungi". J.W. Bennet and L.L. Lasure (ed.) *Academic Press*, New York, NY, p. 357.

Birch, R.M. and Walker, G.M. (2000)."Influence of magnesium ions on heat shock and ethanol stress responses of *Saccharomyces cerevisiae".Enzyme Microb Technol,* **26:** 678–687.

Braun R.J., Zischka H., Madeo F., Eisenberg T., Wissing S., Büttner S., Engelhart S.M., Büringer D., and Ueffing, M. (2006). "Crucial Mitochondrial Impairment upon *CDC48* mutation inapoptotic yeast". *J.Biol. Chem.*, **281:** 25757-25767.

Brett, C.L., Tukaye, D.N., Mukherjee, S. and Rao, R. (2005). "The yeast endosomal Na+(K+)/H+ exchanger Nhx1 regulates cellular pH to control vesicle trafficking". *Mol Biol Cell*, **16:** 1396–1405.

Bursch, W. (2001). "The autophagosomal-lysosomal compartment in programmed cell death". *Cell.Death. Differ*, **8:** 569-81.

Büttner, S., Carmona-Gutierrez, D., Vitale, I., *et al.,* (2007)."Depletion of endonuclease G selectively kills polyploid cells". *Cell Cycle*, **6:** 1072–1076.

Cande, C., Vahsen, N., Metivier, D., Tourriere, H., Garrido, C., Tazi, J., Kroemer, G. (2004b) "Regulation of cytoplasmic stress granules by apoptosis-inducing factor." *J Cell Sci,* **117:** 4461–4468.

Capili, A.D., Schultz, D.C., Rauscher, I.F. and Borden, K.L. (2001). "Solution structure of the PHD domain from the KAP-1 corepressor: structural determinants for PHD, RING, and LIM zinc-binding domains". *EMBO J.* **20:**165–177.

Cartwright, C.P., Veazey, F.J. and Rose, A.H. (1987). "Effect of ethanol on activity of the plasma membrane ATPase in, and accumulation of glycine by *Saccharomyces cerevisiae".J Gen Microbiol,* **133:**857–865.

Carmona – Gutiérrez, D., Bauer, M.A., Ring, J., Knauer, H.,*et al.,* (2004). "The propeptide of yeast cathepsin D inhibits programmed necrosis". Cell Death and Disease, 2, e161; doi: 10.1038/cddis.2011.43.

Casal, M., Cardoso, H. and Leão, C. (1996)."Mechanisms regulatingthe transport of acetic acid in *Saccharomyces cerevisiae".Microbiology,* **142**: 1385-1390.

Casal, M., Paiva, S., Andrade, R. P., Gancedo, C., and Leão, C. (1999). "The lactate-proton symport of *Saccharomyces cerevisiae* is encoded by JEN1". *J. Bacteriol.,***181:** 2620–2623.

Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G., Lee, T.I., True, H.L., Lander, E.S., and Young, R.A. (2001)." Remodeling of yeast genome expression in response to environmental changes". *Mol. Biol. Cell,* **12:** 323–337.

Chen, D., Toone W. M., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N., and Bahler, J. (2003). "Global transcriptional responses of fission yeast to environmental stress". *Mol. Biol. Cell,* **14:** 241-229.

contamination in bioethanol fermentors". *Bioresour Technol,* **101:** 5033-42.

Craig, E.A., Gambill, B.D. and Nelson, R.J. (1993). "Heat shock proteins: molecular chaperones of protein biogenesis". *Microbiol Rev*, **57:**402–414.

Daulny, A., Geng, F., Muratani, M., Geisinger, J.M., Salghetti, S.E. and Tansey, W.P. (2008)."Modulation of RNA polymerase II subunit composition by ubiquitylation". *Proc Natl Acad Sci U S A*, **105:** 19649–19654.

Daum, G., Lees, N.D., Bard, M. and Dickson, R. (1998). "Biochemistry, cell biology, and molecular biology of lipids of *Saccharomyces cerevisiae*". *Yeast* **14:**1471–1510.

Diez, M., Zimmermann, B., Borsch, M., *et al.,* (2004). "Proton-powered subunit rotation in single membrane bound F_oF_1-ATP synthase". *Nature structural & Molecular Biology,* **11:** 135-141.

Ding, J., Huang, X., Zhao, N., Yang, D. and Zhang, K. (2009)." Tolerance and stress response to ethanol in the yeast *Saccharomyces cerevisiae*".*Appl Microbiol Biotechnol,* **85:** 253-263.

Du Toit, W.J. and Lambrechts, M.G. (2002). "The enumeration and identification of acetic acid bacteria from south african red wine fermentations". *Int J Food Microbiol*, **74:** 57-64.

Dudgeon, D.D., Zhang, N., Ositelu, O.O., Kim, H. and Cunningham, K.W. (2008). " Nonapoptotic death of *Saccharomyces cerevisiae* cells that is stimulated by Hsp90 and inhibited by calcineurin and *Cmk2* in response to endoplasmic reticulum stresses. *Eukaryot Cell,* **7:** 2037–2051.

Dyson, E. B., Song, J., Wolfgang, W. J., Colombini, M. and Forte, M. (1997). " Multicopy suppressors of phenotypes resulting from the absence of yeast VDAC encode a VDAC-like protein." *Molecular and cellular biology*, **17**: 5227-5738.

Eisenberg, T., Knauer, H., Schauer, A., *et al.,* (2009). "Induction of autophagy by spermidine promotes longevity". *Nat Cell Biol*, **11:** 1305–1314.

Eisenberg, T., Büttner, S., Kroemer, G. and Madeo, F. (2007). "The mitochondrial pathway in yeast apoptosis. *Apoptosis*, **12:** 1011–1023.

Eisenberg, T., Gutierrez, C. D., Büttner, S., Tavernarakis, N. and Madeo, F. (2010). "Necrosis in yeast". *Springer science + Business media*, **15:** 257-268.

Fahrenkiog, B., Sauder, U., Aebi, U. (2004). "The *S. cerevisiae* HtrA-like protein Nma111p is a nuclear serine protease that mediates yeast apoptosis". *Journal of Cell Science*, **117:** 115 – 126.

Fernandes, A. R., Mira, N. P., Vargas, R. C., Canelhas, I., and Sa-Correia, I. (2005). "*Saccharomyces cerevisiae* adaptation to weak acids involves the transcription factor Haa1p and Haa1p-regulated genes". *Biochem.Biophys. Res. Commun.,***337:** 95–103.

Fernandes, A.R. and Sa-Correia, I. (2003). "Transcription patterns of PMA1 and PMA2 genes and activity of plasma membrane H+-ATPase in *Saccharomyces cerevisiae* during diauxic growth and stationary phase". *Yeast,* **20:** 207–219.

Festjens, N., Vanden Berghe, T. and Vandenabeele, P. (2006). "Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response". *Biochim Biophys Acta,* **1757:** 1371–1387.

Fulda S., Gorman MA., Hori O. and Samali A. (2009). "Cellular stress responses: cell survival and cell death". *International Journal of Cell Biology.* Article ID 214074, 23 pages. doi: 10.1155/2010/214074.

Furukawa, K., Kitano, H., Mizoguchi, H. and Hara, S. (2004). "Effect of cellular inositol content on ethanol tolerance of *Saccharomyces cerevisiae* in sake brewing".*J Biosci Bioeng*, **98:** 107– 113.

Galluzzi, L. (2012). "Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012". *Cell Death Differ*, **19:** 107-120.

Garay-Arroyo, A., Covarrubias, A. A., Clark, I., Nino, I., Gosset, G., andMartinez, A. (2004). "Response to different environmental stress conditions of industrial and laboratory *Saccharomyces cerevisiae* strains". *Appl. Microbiol. Biotechnol,* **63:** 734–741.

Gasch, A. P., Spellman, P.T., Kao, C.M., Harel, O.C., Eisen, M.B., Storz, G., Botstein, D., and Brown, P.O. (2000). "Genomic expression programs in the response of yeast cells to environmental changes". *Mol. Biol. Cell,* **11:** 4241-4257

Giannattasio, S., Guaragnella, N., Ždralevi´c, M., Marra, E. (2013). "Molecular mechanisms of *Saccharomyces cerevisiae* stress adaptation and programmed cell death in response to acetic acid". *Microbiology*, **4:** 1-7.

Gibson, B. R., Lawrence, S. J., Leclaire, J. P., Powell, C. D. and Smart, K. A. (2007). "Yeast responses to stresses associated with industrial brewery handling". *FEMS Microbiol Rev,* **31:**535-69.

Gomes, F.C., Pataro, C., Guerra, J.B., Neves, M.J., Correa, S.R., *et al.,* (2002). "Physiological diversity and trehalose accumulation in *Schizosaccharomyces pombe* strains isolated from spontaneous fermentations during the production of the artisanal Brazilian cachaca". *Can J Microbiol*, **48:**399–406.

Gorner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., *et al.*,(1998). "Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity". *Genes Dev,* **12:**586–597.

Gorner, W., Durchschlag, E., Wolf, J., Brown, E.L., Ammerer, G., *et al.,* (2002). "Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor". *EMBO J*, **21:**135–144.

Guldfeldt, L.U. and Arneborg, N. (1998). "Measurement of the effects of acetic acid and extracellular pH on intracellular pH of nonfermenting individual *Saccharomycescerevisiae*cells by fluorescence microscopy". *Appl Environ Microbiol*, **64:** 530–534.

Guo, L., Chen, S., Liu, K., Liu, Y., Ni, L., *et al.,* (2008). "Isolation of heat shock factor HsfA1a-binding sites in vivo revealed variations of heat shock elements in Arabidopsis thaliana". *Plant Cell Physiol,* **49:**1306–1315.

Guttiérrez, D.C., Bauer, M.A., Ring, J., *et al.,* (2011). "The propeptide of yeast cathepsin D inhibits programmed necrosis". *Cell death and disease*, doi: 10.1038/cddis.2011.43.

Gutierrez, D.C., Eisenberg, T., Büttner, S., Meisinger, G. and Madeo, F. (2010). "Apoptosis in yeast triggers pathways, subroutines". Cell Death and Differentation", **17:** 763 – 773.

Hahn, J.S., Hu, Z., Thiele, D.J. and Iyer, V.R. (2004). "Genome-wide analysis of the biology of stress responses through heat shock transcription factor". *Mol Cell Biol*, **24:**5249–5256.

Halestrap, A. (2005). "Biochemistry: a pore way to die." *Nature,* **434:** 578–579.

Hauptmann, P., Riel, C., Kunz-Schughart, L.A., Frohlich, K.U.,Madeo, F. and Lehle, L. (2006). "Defects in N-glycosylation induceapoptosis in yeast". *Mol Microbiol,* **59:** 765–778.

He, S., Wang, L., Miao, L., Wang, T., Du, F., Zhao, L. and Wang, X. (2009). "Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha". *Cell*, **137:** 1100- 1111.

Hirata, Y., Andoh, T., Asahara, T. and Kikuchi, A. (2003). "Yeast glycogen synthase kinase-3 activates Msn2p-dependent transcription of stress responsive genes". *Mol Biol Cell*, **14:**302–312.

Holyoak, C. D., Stratford, M., McMullin, Z., Cole, M. B., Crimmins, K., Brown, A. J. & Coote, P. J. (1996). "Activity of the plasma membrane H(+)-ATPase and optimal glycolytic flux are required for rapid adaptation and growth of *Saccharomyces cerevisiae* in the presence of the weak-acid preservative sorbic acid". *ApplEnviron Microbiol,* **62:** 3158 - 3164.

Holyoak, C. D., Thompson, S., Ortiz Calderon, C., Hatzixanthis, K., Bauer, B., et al., (2000). "Loss of Cmk1 ca^{2+}/ calmodulin-dependent protein kinase in yeast results in constitutive weak organic acid resistance, associated with a posttranscriptional activation of the Pdr12 ATP-binding cassette transporter". *Mol Microbiol,* **37:** 595 - 605.

Hu, C.K., Bai, F.W. and An, L.J. (2005). "Protein amino acid composition of plasma membranes affects membrane fluidity and thereby ethanol tolerance in a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae"*. *Sheng Wu Gong Cheng Xue Bao,* **21**:809–813.

Hu, X.H., Wang, M.H., Tan, T., Li, J.R., Yang, H., Leach, L., Zhang, R.M. and Luo, Z.W. (2007). "Genetic dissection of ethanol tolerance in the budding yeast *Saccharomyces cerevisiae".Genetics,* **175**: 1479–1487.

Igney, F.H., Krammer, P.H. (2002) "Death and anti-death: tumour resistance to apoptosis". *Nat Rev Cancer,* **2:** 277–288.

Ingram, L.O. (1990). "Ethanol tolerance in bacteria".*Crit Rev Biotechnol,* **9:**305–319.

Inoue, T., Iefuji, H., Fujii, T., Soga, H. and Satoh, K. (2000)."Cloning and characterization of a gene complementing the mutation of an ethanol-sensitive mutant of sake yeast".*Biosci Biotechnol Biochem,* **64:**229–236.

Izawa, S., Ikeda, K., Kita, T. and Inoue, Y. (2006). " Asr1, an alcohol-responsive factor of *Saccharomyces cerevisiae*, is dispensable for alcoholic fermentation". *Appl Microbiol Biotechnol*, **72:**560–565.

Jamieson, D.J. (1992)." *Saccharomyces cerevisiae* has distinct adaptive responses to both hydrogen peroxide and menadione". *J. Bacteriol*, **174:** 6678–6681.

Jones, B.A., Gores, G.J. (1997). "Physiology and pathophysiology of apoptosis in epithelial cells of the liver, pâncreas, and intestine".*Journal of Physiology*, **273:** 1174 – 1188.

Jungwirth, H., Ring, J., Mayer, T., *et al.,* (2008). "Loss of peroxisome function triggers necrosis". *FEBS Lett,* **582:** 2882–2886.

Kelley, M.J., Bailis, A.M., Henry, S.A. and Carman, G.M. (1998). "Regulation of phospholipid biosynthesis in *Saccharomyces cerevisiae* by inositol. Inositol is an inhibitor of phosphatidylserine synthase activity". *J Biol Chem*, **263:**18078–18085.

Kitagaki, H., Araki, Y., Funato, K., Shimoi, H. (2007) "Ethanol-induced death in yeast exhibits features of apoptosis mediated by mitochondrial fission pathway" *FEBS letters*, **581:** 2935-2942.

Klinke, H. B., Thomsen, A. B., and Ahring, B. K. (2004). "Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass". *Appl. Microbiol. Biotechnol,* **66:** 10– 26.

Kroemer, G., Galluzzi, L. and Brenner, C. (2007). " Mitochondrial membrane permeabilization in cell death".*Physiol Rev,***87:** 99–163.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E.S., Baehrecke, E.H. *et al.*, (2009). "Classification of cell death: recommendations of the Nomenclature Committee on CellDeath 2009". *Cell Death Differ*, **16:** 3–11.

Kubota, S., Takeo, I., Kume, K., Kanai, M., Shitamukai, A., *et al.,* (2004), "Effect of ethanol on cell growth of budding yeast: genes that are important for cell growth in the presence of ethanol". *Biosci Biotechnol Biochem*, **68**:968–972.

Kung, G., Konstantinidis, K. and Kitsis R.N. (2011) "Programmed necrosis, not apoptosis, in the heart." *Circulation Research*, **108:** 1017–1036.

Launay, S., Hermine, O., Fontenay, M., Kroemer, G., Solary, E. and Garrido, C. (2005)."Vital functions for lethal caspases".*Oncogene,* **24:** 5137–5148.

Lawen, A. (2003). "Apoptosis-an introduction".*BioEssays,***25:** 888 – 896.

Leão, C. and van Uden, N. (1986). "Transport of lactate and other monocarboxylates in the yeast *Candida utilis".* *Appl Microbiol Biotechnol*, **23:** 389-393.

Lee, J., Dawes, I.W., and Roe, J.H. (1995). "Adaptive response of *Schizosaccharomyces pombe* to hydrogen peroxide and menadione".*Microbiology* ,**141:** 3127–3132.

Lee, R. E., Brunette, S., Puente, L. G. and Megeney L. A. (2010). "Metacaspase Yca1 is required for clearance ofinsoluble protein aggregates". *PNAS*, **107:** 13348-13353.

Lee, Y.Y., Iyer, P. & Torget, R.W. (1999)."Dilute-acid hydrolysis of lignocellulosic biomass".*Adv Biochem Eng Biotechnol*, **65:** 93–115.

Lewis, J., Devin, A., Miller, A., *et al.,* (2000). "Disruption of hsp90 function results in degradation of the death domain kinase, receptor-interacting protein (RIP), and blockage of tumor necrosis factor-induced nuclear factor-kappaB activation. *J Biol Chem,***275:** 10519–10526.

Liang, Q. and Zhou, B. (2007) "Copper and manganese induce yeast apoptosis via different pathways". *Mol Biol Cell,* **18:** 4741–4749.

Liu, K.H., Zhang, L.M., Ding, X.W., Deng, B.W. and Chen, W.Q. (2008). "Analysis of in vivo binding of yeast heat shock factor to promoter DNA".*AJB*, **7:**1069–1071.

Liu, Z. L., and Blaschek, H. P. (2010). "Biomass conversion inhibitors and in situ detoxification," in *Biomass to Biofuels: Strategies for Global Industries*,eds A. Vertes, N. Qureshi, H. Yukawa, and H. Blaschek. (Oxford: John Wiley & Sons, Ltd.), 233–259.

Lucero, P., Penalver, E., Moreno, E. and Lagunas, R. (1997). "Moderate concentrations of ethanol inhibit endocytosis of the yeast maltose transporter". *Appl Environ Microbiol*, **63:**3831–3836.

Ludovico, P., Rodrigues, F., Almeida, A., *et al.,* (2002). "Cytochrome c realease and mitochondria involvement in programmed cell death induced by acetic acid in *Saccharomyces cerevisiae*". *Mol. Biol Cell*, **13:** 2598-2606.

Ludovico, P., Sousa, M.J., Silva M.T., Leão, C. and Côrte-Real, M. (2001). "*Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid". *Microbiology,* **147:** 2409-2415.

MacPherson, S., Larochelle, M. and Turcotte, B. (2006). "A fungal family of transcriptional regulators: the zinc cluster proteins". *Microbiol Mol Biol*, **70**:583–604.

Madeo, F., Frohlich, E., Frohlich, K.U. (1997)." A yeast mutant showing diagnostic markers of early and late apoptosis".*J Cell Biol*, **139:** 729–734.

Madeo, F., Frohlich, E., Ligr, M., *et al.,* (1999). "Oxygen stress: a regulator of apoptosis in yeast". *J Cell Biol,***145:** 757–767.

Madeo, F., Herker, E., Maldener, C., Wissing, S., *et al.,* (2002)." A caspase-related protease regulates apoptosis in yeast", *Molecular Cell,* **9:** 911 – 917.

Mager, W. H. and Winderickx, J. (2005). "Yeast as a model for medical and medicinal research". *Trends Pharmacol Sci,***26:** 265-73.

Maiorella, B., Blanch, H.W. & Wilke, C.R. (1983)."By-product inhibition effects on ethanolic fermentation by *Saccharomyces cerevisiae".Biotechnol Bioeng*, **25:** 103–121.

Marchler, G., Schuller, C., Adam, G. and Ruis, H. (1993). "A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions". *EMBO J*, **12:**1997–2003.

Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M. and Green, D.R. (1995) "Early redistribution of plasma membrane phosphatidylserine is a generalfeature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl."*J Exp Med,* **182:** 1545-56.

Martinvalet, D., Zhu, P., Lieberman, J. (2005). "Granzyme A induces caspase- independent mitochondrial damage, a required first step for apoptosis". *Immunity,* **22**:355–370.

Matouschek, A., Rospert, S., Schmid, K. and Glick, B.S. (1995). "Cyclophilin catalyzes protein folding in yeast mitochondria" *Biochem,* **92:** 6319-6323.

Mira, N. P., Henriques, S. F., Keller, G., Teixeira, M. C., Matos, R. G., Arraiano, C. M., *et al.,* (2011). "Identification of a DNA-binding site for the transcription factor Haa1, required for *Saccharomyces cerevisiae* response to acetic acid stress". *Nucleic Acids Res,* **39:** 6896–6907.

Mira, N. P., Palma, M., Guerreiro, J. F., and Sa-Correia, I. (2010b). "Genomewide identification of *Saccharomycescerevisiae* genes required for tolerance to acetic acid". *Microb. Cell Fact,* **9:** 79.

Mira, N. P., Teixeira, M. C., and Sa-Correia, I. (2010a). "Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: a genome-wide view". *OMICS,* **14:** 525–540.

Miramar, M.D., Costantini, P., Ravagnan, L., Saraiva, L.M. et al., (2001). "NADH-oxidase activity of mitochondrial apoptosis inducing factor (AIF)". *J Biol Chem,* **276:** 16391–16398.

Mishra, P. and Prasad, R. (1989). "Relationship between ethanol tolerance and fatty acyl composition of *Saccharomyces cerevisiae*" .*Appl Microbiol Biotechnol,* **30**: 294–298

Mollapour, M. and Piper, P.W. (2006)." Hog1p mitogen-activated protein kinase determines acetic acid resistance in *Saccharomyces cerevisiae". FEMS Yeast Res*, **6:** 1274-1280.

Mollapour, M., Shepherd, A., and Piper, P. W. (2009). " Presence of the Fps1p aquaglyceroporin channel is essential for Hog1p activation, but suppresses Slt2 (Mpk1)p activation, with acetic acid stress of yeast". *Microbiology* **155:** 3304–3311.

Moradas-Ferreira, P., and Costa, V. (2000)."Adaptive response of the yeast *Saccharomyces cerevisiae* to reactive oxygen species: defenses, damage and death". *Redox*, *5:* 277–285.

Moskvina, E., Schuller, C., Maurer, C.T., Mager, W.H. and Ruis, H. (1998). "A search in the genome of *Saccharomyces cerevisiae* for genes regulated via stress response elements". *Yeast*, **14:**1041–1050.

Moye-Rowley, W. S. (2003). "Regulation of the transcriptional response to oxidative stress in fungi: similarities and differences". *Eukaryot Cell,* **2:** 381-389.

Muthaiyan, A. and Ricke, S. C. (2009). "Current perspectives on detection of microbial contamination in bioethanol fermentors".*Bioresour Technol,* **101**: 5033-42.

Nakagawa, T., Shimizu, S., Watanabe, T., Yamaguchi, O., Otsu, K., Yamagata, H., *et al.* (2005) "Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death." *Nature* **434:** 652–658.

Nicotera P. and Lipton S. A. (1999). "Excitotoxins in neuronal apoptosis and necrosis." *Journal of Cerebral Blood Flow andMetabolism*, **19:** 583–591.

Norbury, C.J., Hickson, I.D. (2001). "Cellular responses to DNA damage". *Annu Rev Pharmacol Toxicol,* **41:** 367–401.

Ogawa, Y., Nitta, A., Uchiyama, H., Imamura, T., Shimoi, H. and Ito, K. (2000). "Tolerance mechanism of the ethanol-tolerant mutant of sake yeast". *J Biosci Bioeng,* **90:**313–320.

Paiva, S., Devaux, F., Barbosa, S., Jacq, C., and Casal, M. (2004). "Ady2p is essential for the acetate permease activity in the yeast *Saccharomycescerevisiae". Yeast,* **21:** 201–210.

Pampulha, M. E. & Loureiro, V. (1989).**"**Interaction of the effects of acetic acid and ethanol on inhibition of fermentation in *Saccharomycescerevisiae".Biotechnol Lett,* **11**: 269-274.

Paul, M.F., Barrientos, A. and Tzagoloff, A. (2000). "A single amino acid changes in subunit 6 of the yrast mitochondrial ATPases suppresses a null mutations in ATP10". Biol. Chem., 275: 29238-29243.

Pausch, H.M., Kirsch, R.D. and Silverman, J.S. (2001) "Saccharomyces cerevisiae: Applications". *Encyclopedia of life sciences.Nature pulishing group.*

Pereira, C., Camougrand, N., Manon, S., Sousa, M.J. and Côrte-Real, M. (2007). "ADP/ATP carrier is requeried for mitochondrial outer membrane permeabilization and cytochrome c release in yeast apoptosis". *Molecular Microbiology,* **66:** 571-582.

Pereira, C., Chaves, S., Alves, S., Salin, B., Camougrand, N., Manon, S., Sousa, M.J. and Côrte-Real, M. (2010) "Mitochondrial degradation in acetic acid-induced yeast apoptosis: the role of Pep4 and the ADP/ATP carrier." *Mol. Microbiol.* **76**, 1398–1410.

Pereira, C., Silva, R.D., Saraiva, L., Johansson, B., Sousa, M.J., Côrte-Real, M. (2008). "Mitochondrial – dependent apoptosis in yeast".*Biochimica et Biophysica Acta*, **1783:** 1286 – 1302.

Phillips, A.J., Sudbery, I. and Ramsdale, M. (2003) "Apoptosis induced by environmental stresses and amphotericin B in Candida albicans". *Proc Natl Acad Sci USA,* **100:**14327–14332.

Phowchinda, O., Délia-Dupuy, M.L. and Strehaiano, P. (1995)." Effects of acetic acid on growth and fermentative activity of *Saccharomyces cerevisiae".Biotechnol Lett*, **17:** 237–242.

Pinto, I., Cardoso, H. and Leão, C. (1989).**"**High enthalpy and lowenthalpy death in *Saccharomyces cerevisiae* induced by aceticacid".*Biotechnol Bioeng,* **33**: 1350-1352.

Piper, P., Mahé, Y., Thompson, S., *et al.,* (1998). "The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast". *EMBO J,* **17:** 4257- 4265.

Piper, P. W. (2011). "Resistance of yeasts to wek organic acid food preservatives," in *Advances in Applied Microbiology*, eds A. Laskin, G. Gadd, and S. Sariaslani (Burlington: AcademicPress), 97–113.

Piper, P.W., Ortiz-Calderon, C., Holyoak, C., Coote, P. and Cole, M. (1997). "Hsp30, the integral plasma membrane heat shock protein of *Saccharomyces cerevisiae*, is a stress-inducible regulator of plasma membrane H(+)-ATPase". *Cell Stress Chaperones,* **2:**12–24.

Piper, P.W., Talreja, K., Panaretou, B., Moradas-Ferreira, P., Byrne, K. *et al.*, (1994). "Induction of major heat shock proteins of Saccharomyces cerevisiae, including plasma membrane Hsp30, by ethanol levels above a critical threshold". *Microbiology,* **140**: 3031–3038.

Pozniakovsky, A.I., Knorre, D.A., Markova, O.V., Hyman, A.A., Skulachev, V.P. and Severin, F.F. (2005). "Role of mitochondria in the pheromone- and amiodarone-induced programmed death ofYeast". *J Cell Biol,* **168:** 257– 269.

Quan, X., Rassadi, R., Rabie, B., Matusiewicz, N. and Stochaj, U. (2004). "Regulated nuclear accumulation of the yeast hsp70 Ssa4p in ethanol-stressed cells is mediated by the N-terminal domain, requires the nuclear carrier Nmd5p and protein kinase C". *FASEB J,* **18:** 899–901.

Saikumar, P., Dong, Z., Mikhailov, V., Denton, M., Weinberg, J.M., Venkatachalam, M.A. (1999). "Apoptosis: Definition, Mechanisms and relevance to disease". *The American Journal of Medicine,* **107:** 489-506.

Saltzgaber-Muller, J., *et al.* (1983). **"**Nuclear genes coding the yeast mitochondrial adenosine triphosphatase complex. Isolation of ATP2 coding the F1-ATPase beta subunit". *J Biol Chem,* **19:**11465-11470.

Salvador, Vanessa Alexandra Guerreiro (2009). "Avaliação de apoptose e necrose em *saccharomyces cerevisiae* em fermentos vinários". Dissertação (Mestrado em Engenharia Alimentar). Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Lisboa.

Schuller, C., Brewster, J.L., Alexander, M.R., Gustin, M.C. and Ruis, H. (1994). "The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae* CTT1 gene". *EMBO J,* **13:** 4382–4389.

Schneider, M. D. (2005). "Cyclophilin D: knocking on death's door. *Sci STKE*, pe26.

Serrano, R. (1991). Transport across yeast vacuolar and plasma membranes. In *The Molecular Biology of the Yeast Saccharomyces*, pp. 523±586. Edited by J. R. Broach, J. Pringle & E. W. Jones. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Seymour, I.J. and Piper, P.W. (1999). "Stress induction of HSP30, the plasma membrane heat shock protein gene of *Saccharomyces cerevisiae*, appears not to use known stress-regulated transcription factors". *Microbiology,* **145:**231–239.

Silva, R.D., Sotoca, R., Johansson, B., *et al.,* (2005). "Hyperosmotic stress induced metacaspase and mitochondria- dependent apoptosis in *Saccharomyces cerevisiae*". *Mol Mocrobiology*, **58:** 824-834.

Sorger, P.K. and Pelham, H.R. (1988). "Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation". *Cell*, **54:**855–864.

Sousa, M.J., Ludovico, P., Rodrigues, F., Leão, C. and Côrte-Real, M. (2011). "Stress and cell death in yeast induced by acetic acid", *www. Intechopen.com*, **4:** 73-100.

Stewart, G.C. and Russell, I. (1985). "The biology of *Saccharomyces Cerevisiae".*In A.L. Demain and N.A. Solomon, (eds.),Biology of industrial organisms.*Benjamin Cummins Publishers*, Menlo Park, p. 511-536.

Swan, T.M. and Watson, K. (1998). "Stress tolerance in a yeast sterol auxotroph: role of ergosterol, heat shock proteins and trehalose". *FEMS Microbiol Lett,* **169**:191–197.

Takagi, H., Takaoka, M., Kawaguchi, A. and Kubo, Y. (2005). "Effect of L-proline on sake brewing and ethanol stress in *Saccharomyces cerevisiae*".*Appl Environ Microbiol,* **71**:8656–8662.

Takemori, Y., Sakaguchi, A., Matsuda, S., Mizukami, Y. and Sakurai, H. (2006). "Stress-induced transcription of the endoplasmic reticulum oxidoreductin gene ERO1 in the yeast *Saccharomyces cerevisiae*".*Mol Genet Genomics,* **275:**89–96.

Tanghe, A., Van Dijck, P., Dumortier, F., Teunissen, A., Hohmann, S. and Thevelein, J. M. (2002)." Aquaporin expression correlates with freeze tolerance in baker's yeast, and overexpression improves freeze tolerance in industrial strains".*ApplEnviron Microbiol,* **68:** 5981-5989.

Trindade, D. A. M. (2009). "Papel da Aquagliceroporina Fps1 na Sensibilidade ao Etanol de *Saccharomyces cerevisiae*, e o Efeito do Tratamento Cruzado com Ácido Acético" *Departamento de Biologia*, Universidade do Minho.

Truman, A.W., Millson, S.H., Nuttall, J.M., Mollapour, M., Prodromou, C. and Piper, P.W. (2007). "In the yeast heat shock response, Hsf1directed induction of Hsp90 facilitates the activation of the Slt2 (Mpk1) mitogen-activated protein kinase required for cell integrity. *Eukaryot Cell,* **6:** 744–752.

Vahsen, N., Candé, C., Brière, J.J., Bénit, P., Joza, N., Larochette, N., Mastroberardino, P.G., Pequignot, M.O., Casares, N., Lazar, V., Feraud, O., Debili, N., Wissing, S., Engelhardt, S., Madeo, F., Piacentini, M., Penninger, J.M., Schägger, H., Rustin, P., Kroemer, G. (2004). "AIF deficiency compromises oxidative phosphorylation". *EMBO J.* **23:** 4679 -89.

van Voorst, F., Houghton-Larsen, J., Jonson, L., Kielland-Brandt, M.C. and Brandt, A. (2006). "Genome-wide identification of genes required for growth of *Saccharomyces cerevisiae* under ethanol stress". *Yeast*, **23:**351–359.

Vanlangenakker,N., Berghe,T.V., Krysko, D.V., Festjens, N. and Vandenabeele,P. (2008). "Molecular mechanisms and pathophysiology of necrotic cell death," *Current MolecularMedicine*, **8:** 207–220.

Vianna, C.R., Silva, C.L., Neves, M.J. and Rosa, C.A. (2008). "*Saccharomyces cerevisiae* strains from traditional fermentations of Brazilian cachaca: trehalose metabolism, heat and ethanol resistance". *Antonie Van Leeuwenhoek,* **93:** 205–217.

Vilela-Moura, A., Schuller, D., Mendes-Faia, A., and Corte-Real, M. (2008)."Reduction of volatile acidity of wines by selected yeast strains". *Appl. Microbiol. Biotechnol.,* **80:** 881–890.

Vilela-Moura, A., Schuller, D., Mendes-Faia, A., Silva, R. D., Chaves, S.R., Sousa, M. J., *et al.*(2010). "The impact of acetate metabolism on yeast fermentative performance and wine quality: reduction of volatile acidity of grape musts and wines". *Appl. Microbiol. Biotechnol,* **89:** 271–280.

Vilela-Moura, A., Schuller, D., Mendes-Faia, A., Silva, R.D., Chaves, S.R., Sousa, M.J. and Côrte Real, M. (2011). "The impact of acetate metabolism on yeast fermentativeperformance and wine quality: reduction of volatile acidity of grape musts andwines". *Appl Microbiol Biotechnol*, **89:** 271–280.

Walker, G.M. (1998). "Yeast Physiology and Biotechnnology". West Sussex, England: John Wiley and sons Ltd.

Walter, D., Wissing, S., Madeo, F, Fahrenkrog, B. (2006). "The inhibitor – of – apoptosis protein Bir1p protects against apoptosis in *S. cerevisiae* and is a substrate for yeast homologue of Omi/HtrA2". *Journal of Cell Science*, **199:** 1843 – 1851.

Watanabe, M., Tamura, K., Magbanua, J.P., Takano, K., Kitamoto, K., *et al.,* (2007).“Elevated expression of genes under the control of stress response element (STRE) and Msn2p in an ethanol tolerance sake yeast”.*J Biosci Bioeng,* **104:**163–170.

Weber, F.J. and de Bont J.A. (1996). “Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes”.*Biochim Biophys Acta,* **1286**: 225–245.

Wissing, S., Ludovico, P., Herker, E., *et al.,* (2004). “An AIF orthologue regulates apoptosis in yeast”. *The journal of cell biology*, **16:** 964-974.

Wong, A., Yan, C. and Shi, Y. (2012). “Crystal structure of the yeast metacaspase Yca1”.*Journal of biological chemistry*, **287:** 29251-29259.

Wu, C. (1995). “Heat shock transcription factors: structure and regulation”. *Annu Rev Cell Dev Biol,* **11:**441–469.

Wysocki R, Kron S.J. (2004). “Yeast cell death during DNA damage arrest is independent of caspase or reactive oxygen species” J. Cell Biol. 2: 166 – 311.

Xiao, D., Wu, S., Zhu, X., Chen, Y. and Guo, X. (2008). “Effects of Soya Fatty Acids on Cassava Ethanol Fermentation”.*Appl Biochem Biotechnol*, doi:10.1007/s12010-008-8344-7.

Yamashima, T., Kohda, Y., Tsuchiya, K., *et al.,* (1998). “Inhibition of ischaemic hippocampal neuronal death in primates with cathepsin B inhibitor CA-074: a novel strategy for neuroprotection based on ‘calpain-cathepsin hypothesis’”. *Eur J Neurosci*, **10:** 1723–1733.

You, K.M., Rosenfield, C.L. and Knipple, D.C. (2003). “Ethanol tolerance in the *yeast Saccharomyces cerevisiae* is dependent on cellular oleic acid content”. *Appl Environ Microbiol,* **69**:1499–1503.

Zhang, D.W., Shao, J., Lin, J., Zhang, N., Lu, B.J., Lin, S.C., Dong, M.Q. and Han, J. (2009). “RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis”.*Science*. **325:** 332–336.

Zhang, J.G., Liu, X.Y., He, X.P., Guo, X.N., Lu, Y. and Zhang, B.R. (2011). “Improvement ofacetic acid tolerance and fermentation performance of *Saccharomyces cerevisiae* by disruption of the *FPS1* aquaglyceroporin gene”.*Biotechnol Lett*, **33:** 277-284.

Zhang, N.N., Dudgeon, D.D., Paliwal, S., Levchenko, A., Grote, E. and Cunningham, K.W. (2006) “Multiple signaling pathways regulate yeast cell death during the response to mating pheromones”.*MolBiol Cell,* **17:** 3409–3422.

Zhao, R. and Houry, W.A. (2007). “Molecular interaction network of the Hsp90 chaperone system”.*Adv Exp Med Biol,* **594:** 27–36.