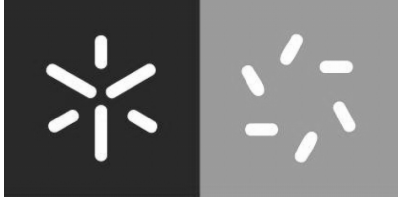

Universidade do Minho
Escola de Ciências

António Miguel Araújo Rego

Characterization of the role of sphingolipids in the modulation of acetic acid-induced apoptosis

Tese de Mestrado em Genética Molecular

Trabalho efectuado sob a orientação da
Professora Doutora Manuela Côrte-Real
e co-orientação do
Professor Doutor Vítor Costa

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1. **É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE, APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.**

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António Miguel Araújo Rego

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Characterization of the role of sphingolipids in the modulation of acetic acid-induced apoptosis

Abstract

The yeast *Saccharomyces cerevisiae* can undergo programmed cell death in response to different stimuli. Exposure of yeast cells to acetic acid has been shown to trigger a mitochondrial pathway displaying, as in mammalian cells, typical apoptotic markers such as externalization of phosphatidylserine, DNA fragmentation, chromatin condensation, mitochondrial dysfunction with cytochrome c release and production of reactive oxygen species (ROS).

Sphingolipids are lipid second messengers generated in response to different physiological signals and stress stimuli. They affect multiple aspects of cellular function, including apoptosis. Changes in sphingolipid metabolism have been linked to apoptosis and oxidative stress in both yeast and mammalian cells. The increase of ceramide and sphingosine levels leads to cell growth arrest and apoptosis whereas the increase of sphingosine-1-phosphate levels promotes proliferation and inhibits apoptosis. Moreover, ceramides have been detected in mitochondria and accumulate upon stress treatments, increasing the permeability of the mitochondria to cytochrome *c* and leading to the generation of ROS.

Our working hypothesis was that acetic acid may elicit ceramide production and, therefore, may trigger apoptosis by a signal transduction pathway modulated by ceramide. For that reason, we aimed to characterize the relative contribution of biosynthesis *versus* catabolism of ceramides to the apoptotic cell death induced by acetic acid in yeast. For our studies, yeast cells lacking Lag1p, Lac1p (unable to generate ceramide by *de novo* synthesis), Ydc1p and Ypc1p (unable to breakdown ceramide) and Isc1p (unable to generate ceramide by degradation of inositolphosphosphingolipids), were generated by homologous recombination.

Our results showed that *lag1*Δ and *isc1*Δ mutant cells exhibited a higher resistance to acetic acid that was correlated with lower levels of ROS production and reduced mitochondrial alterations. In comparison with the wild-type strain, *lag1*Δ and *isc1*Δ mutant cells display, under acetic acid stress, lower levels of mitochondrial fragmentation and degradation, and reduced alterations of the mitochondrial membrane potential. Associated with these events, there was also less translocation of cytochrome *c* to the cytosol in response to acetic acid than in the wild-type strain.

In conclusion, our results suggest that ceramide production contributes to cell death induced by acetic acid, especially through the hydrolysis of complex lipids catalyzed by Isc1p and through *de novo* synthesis catalyzed by Lag1p.

Caracterização do papel dos esfingolípidos na modulação da apoptose induzida por ácido acético

Resumo

A levedura *Saccharomyces cerevisiae* pode sofrer morte celular programada em resposta a diferentes estímulos. O tratamento de células de levedura com ácido acético tem sido descrito como capaz de ativar a via mitocondrial apresentando, exposição de marcadores celulares típicos de apoptose de mamíferos tais como externalização de fosfatidilserina, fragmentação de DNA, condensação da cromatina, disfunção mitocondrial, incluindo a libertação de citocromo *c*, e produção de espécies reativas de oxigénio (ROS).

Os esfingolípidos são lipídos mensageiros gerados em resposta a diferentes sinais fisiológicos e estímulos de stress. Alterações no metabolismo de esfingolípidos têm sido associadas a apoptose e stress oxidativo tanto em leveduras como em células de mamíferos. O aumento dos níveis de ceramidas e esfingosina promove a paragem do crescimento celular e a apoptose, enquanto o aumento dos níveis de esfingosina-1-fosfato promove a proliferação e inibe a apoptose. Adicionalmente, tem-se verificado a deteção e acumulação de ceramidas nas mitocôndrias após tratamentos de stress, aumentando a permeabilidade da mitocôndria ao citocromo *c* e levando à produção de ROS.

A hipótese de trabalho deste projeto consistiu na suposição de que o ácido acético pode levar à produção de ceramidas e portanto, desencadear a apoptose por via de transdução de sinal modulado por ceramida. Por essa razão, foi nosso objetivo caracterizar a contribuição relativa da biossíntese e do catabolismo de ceramidas para a morte celular por apoptose induzida por ácido acético na levedura. Mutantes de levedura deficientes nas enzimas Lag1p e Lac1p (incapazes de gerar ceramidas pela síntese *de novo*), Ydc1p e Ypc1p (incapazes de catabolizar ceramidas) e Isc1p (incapaz de gerar ceramidas pela degradação da inositolfosfoesfingolípidos), foram gerados por recombinação homóloga.

Os resultados mostraram que os mutantes *lag1*Δ e *isc1*Δ exibem uma maior resistência ao ácido acético que se correlaciona com níveis baixos de produção de ROS e alterações mitocondriais menos intensas. Em comparação com a estirpe selvagem, *lag1*Δ e *isc1*Δ exibem, sob stress de ácido acético, menores níveis de fragmentação e degradação mitocondrial e reduzidas alterações do potencial de membrana mitocondrial. Associados a estes eventos, observou-se igualmente uma menor translocação de citocromo *c* para o citosol do que na estirpe selvagem.

Em conclusão, os resultados sugerem que a produção de ceramidas contribui para a morte celular induzida por ácido acético, especialmente através da hidrólise de lípidos complexos catalisada por Isc1p e através de síntese *de novo* catalisada por Lag1p.

Index

Abbreviations

AIF - Apoptosis-inducing Factor
ANT - Adenine Nucleotide Translocator
Apaf-1 - Apoptotic Protease Activating Factor-1
ATP - Adenosine Triphosphate
BSA - Bovine Serum Albumin
c.f.u. - Colony forming units
CAPK - Ceramide-activated Protein Kinase
CAPPs - Ceramide-activated Protein Phosphatase
CARD - Caspase Recruitment Domain
c-FLIP - Cellular-FLICE (FADD-like IL-1β-converting enzyme)-inhibitory Protein
CK - Creatine Kinase
Cyp D - Cyclophilin D
DAPI - 4,6-Diamino-2-phenyl-indole dihydrochlorid
DD - Death Domain
DED - Death Effector Domain
DHE - Dihydroethidium
DHS - Dihydrosphingosine
$DiOC_6$ - 3,3′-Dihexyloxacarbocyanine iodide
DISC - Death Inducing Signaling Complex
DNA - Deoxyribonucleic Acid
DNP - Dinitrophenyl
DNPH - 2,4-Dinitrophenylhydrazine
DR - Death Receptors
DTNB - 5,5′-Dithiobis (2-nitrobenzoic acid)
DTT - Dithiothreitol
EDTA - Ethylenediaminetetraacetic acid
Endo G - Endonuclease G
ER - Endoplasmatic Reticulum
FADD - Fas-Associated Death Domain
GFP - Green Fluorescent Protein
H_2O_2 – Hydrogen Peroxide
HtrA2/Omi - High Temperature Requirement Protein A2
IAPs - Inhibitors of Apoptosis Proteins
IPC - Inositolphosphorylceramide
KSR - Kinase Suppressor of Ras
LCB – Long Chain Base
$M(IP)_2C$ - Mannosyldiinositolphosphorylceramide
MAC - Mitochondrial Apoptosis-induced Channel
MAPK - Mitogen-activated Protein Kinases
MIPC – Mannosylinositolphosphorylceramide
MOMP - Mitochondrial Outer Membrane Permeabilization
NADH - Nicotinamide Adenine Dinucleotide
NADPH - Nicotinamide Adenine Dinucleotide Phosphate
NO - Nitric Oxide
PARP-1 – polyADP-ribose Polymerase
PBR - Peripheral Benzodiazepine Receptor
PCD – Programmed Cell Death
PCR - Polymerase Chain Reaction
PHS - Phytosphingosine
PI - Propidium Iodide
PKC - Protein Kinase C
PTP - Permeability Transient Pore
Rb - Retinoblastoma Product Gene
ROS - Reactive Oxygen Species
SAPK - Stress-activated Protein Kinase
SC Gal - Synthetic Complete Galactose medium
SDK1 - Sphingosine-dependent Protein Kinase
SDS - Sodium Dodecyl Sulfate
SK1 - Sphingosine Kinase
Smac/Diablo - Second Mitochondria-derived Activator of Caspases/Direct Inhibitor of Apoptosis Protein (IAP)-Binding Protein With Low Pi
TNF-R - Tumor-Necrosis Factor Receptor
TRADD - TNF-R-Associated Death Domain
TRAIL-R - TNF-related Apoptosis-Inducing Ligand Receptor
TUNEL - Terminal dUTP Nick-end Labeling
VDAC - Voltage-Dependent Anion Channel

1. INTRODUCTION

1.1. Cell death

In the last decades, the understanding of cell death has attracted a lot of attention, mostly because of its crucial role in tissue homeostasis and development of multicellular organisms (Baehrecke, 2002). Cell death may occur through different processes, some of which may have a physiological role. Classification of the types of cell death and associated terminology has evolved since the 19th century. Initially, isolated studies recognized that cell death occurs during metamorphosis and embryogenesis, but studies on dying cells led Lockshin to introduce the concept of programmed cell death (PCD) in 1965. PCD was categorized as a type of cell death that was not accidental (Necrosis), but a genetically controlled sequence of steps that lead to morphological and biochemical changes (Lockshin and Zakeri, 2001). Later, in 1972, Kerr and coworkers coined the term apoptosis to define a new pattern of cell death genetically controlled, with specific morphological features (Kerr *et al*., 1972). Apoptosis was later considered synonym of PCD and cell death classified into apoptosis and necrosis. However, since it was later found that necrosis may also be highly regulated (Ellis and Horvitz, 1986) and that exist other forms of cell death, including autophagic cell death, this classification was abandoned (Debnath *et al*., 2005).

Recently, the Nomenclature Committee on Cell Death proposed a unified criterion for the definition of cell death, according to different morphological characteristics. Cell death is now classified into three major pathways: apoptotic, necrotic and autophagic *(*Kroemer *et al*., 2009).

1.2. Apoptosis

The term "apoptosis" derived from an ancient Greek word meaning "falling petals of flowers" or "fall of leaves in autumn". It was the term chosen by John Kerr and his coworkers Andrew Wyllie and Alastair Currie in 1972 to define a new type of death (Kerr *et al*., 1972). Apoptosis is a type of cell death with morphological characteristics distinct from those found in necrosis and autophagy (Figure 1).

In necrosis, the cell content is released in an uncontrolled manner, resulting in damage to neighboring cells and a strong inflammatory response in the corresponding tissue (Proskuryakov *et al*., 2003). In autophagy, the cells recycle their own damaged intracellular components via the lysosome when the nutrients are scarce (Codogno and Meijer, 2005). In apoptosis, cells undergo a series of morphological changes 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 (Saraste and Pulkki, 2000; Lawen, 2003).

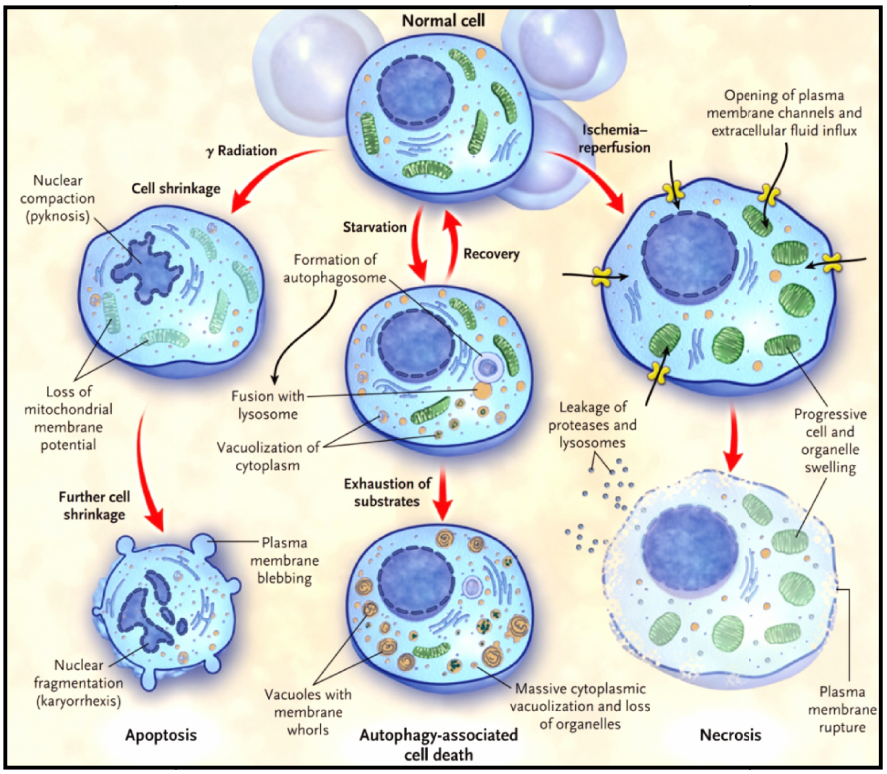

Figure 1. Molecular and morphological events associated with the different types of cell death: apoptosis, autophagy and necrosis (Hotchkiss *et al.*, 2009)

1.2.1. Caspases

Most of the alterations observed during apoptosis are caused by proteases called caspases (Cysteine-dependent aspartate-specific proteases). Conserved through evolution, caspases can be found in humans, insects, nematodes and hydra. Caspases contain a cysteine residue in the active site that is critical for their proteolytic activity and exhibit a high affinity for aspartate (Asp), cleaving their substrates after these residues (Cohen, 1997). Close to one hundred caspase substrates have already been identified, ranging from complex macromolecular complexes (e.g. actin network) to single enzymes [e.g. polyADP-ribose polymerase (PARP-1)] (Fischer *et al.*, 2003).

Caspases are synthesized as inactive zymogens, named procaspases. Procaspases can be proteolytically cleaved between the large and small subunit by an upstream caspase, resulting in the separation of these subunits and activation of

procaspases, now known only as caspases (Figure 2). Caspases can also be activated by induced proximity, where the low intrinsic protease activity of procaspases is sufficient to allow them to mutually cleave and activate each other (Chowdhury *et al*., 2008).

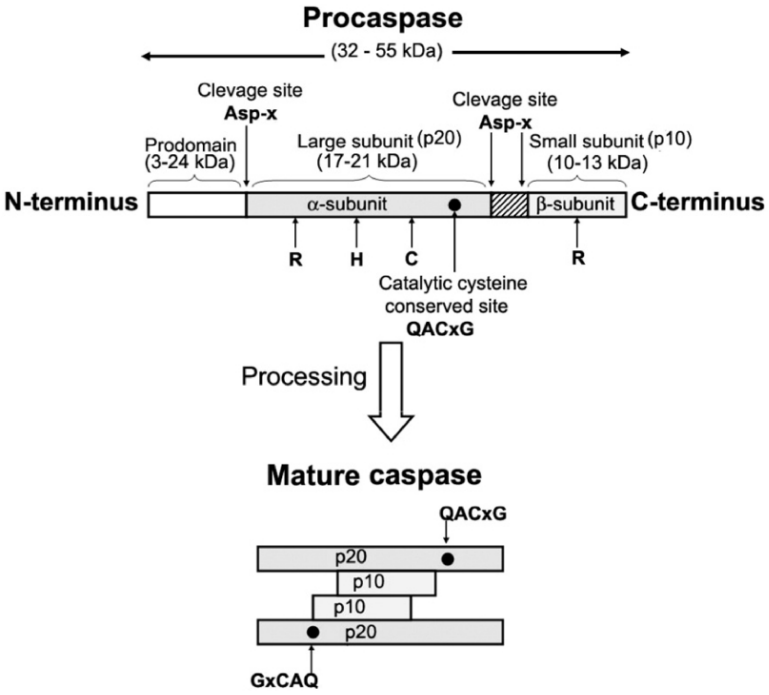

Figure 2. Schematic representation of caspase structure and processing. The active site residues are represented by R, H and C (Chowdhury *et al*., 2008).

Caspases can be divided into initiators (caspases-2, -8, -9 and -10) and executioners (caspases-3, -6, and -7). Executioner caspases have a small pro-domain, while initiator caspases have a long pro-domain, and a Death Effector Domain (DED), in the case of caspases -8 and -10, or a Caspase Recruitment Domain (CARD), in the case of caspases -2 and -9 (Budihardjo *et al*., 1999).

1.2.2. The Bcl-2 family members

The gene encoding the protein Bcl-2 (B-cell lymphoma 2) was the first proto-oncogene to be related to the regulation of cell cycle progression. However, its oncogenic characteristic stems from its ability to prevent apoptosis rather than promoting proliferation. Since then, several homologues of Bcl-2 have been identified that can be defined by the presence of conserved domains (BH1-BH4) necessary for their anti- or pro-apoptotic functions (Tsujimoto, 1998). Based on structural and

functional similarities, the Bcl-2 family of proteins can be divided into four sub-families: anti-apoptotic (A1, Bcl-2, Bcl-w, Bcl-xL and Mcl-1), effector proteins (Bax and Bak), direct activator BH3-only proteins (Bid and Bim) and de-repressor/sensitizer BH3-only proteins (Bad, Bik, Bmf, Hrk, Noxa and Puma) (Chipuk *et al*., 2010) (Figure 3). In viable cells, pro-apoptotic proteins are antagonists of anti-apoptotic proteins, and the levels of pro- and anti-apoptotic proteins determine susceptibility to apoptosis (Korsmeyer, 1995).

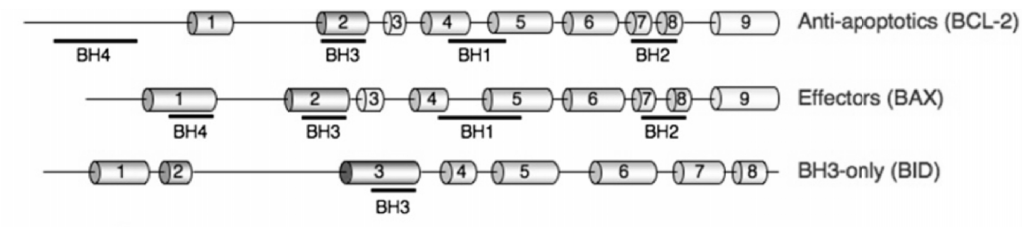

Figure 3. Schematic representation of a member of each Bcl-2 sub-family. The conserved domains are underlined. Cylinders represent α helices (Chipuk *et al*., 2010).

1.2.3. Apoptotic pathways

Similarly to other signaling pathways, the events responsible for the apoptotic death process are mediated by two classical pathways: the extrinsic and intrinsic pathways, which are activated by the binding of ligands to death receptors and by stress triggered by oncogenes, irradiation, reactive oxygen species (ROS) and exposure to several drugs, respectively (Figure 4).

1.2.3.1. Extrinsic pathway

The extrinsic pathway is one of the best characterized apoptotic signaling pathways. This pathway involves binding of specific extracellular ligands to their cognate cell surface death receptors (DR) such as Tumor-Necrosis Factor Receptor (TNF-R1), CD95 (also called as Apo-1 or Fas), TNF-related Apoptosis-Inducing Ligand Receptor (TRAIL-R1/2) and DR3/6 (Sartorius *et al*., 2001). Subsequent signaling is mediated by the cytoplasmic domain of the DR, named Death Domain (DD). Adapter proteins such as Fas-Associated Death Domain (FADD) or TNF-R-Associated Death Domain (TRADD) have their own DD and are recruited to the DD of the activated death

receptors. These adapter proteins also have a death-effector domain (DED), with which the DED of procaspase-8 can interact with to form the Death Inducing Signaling Complex (DISC). Concentration of several pro-caspase-8 molecules within DISC leads to autocatalytic cleavage, activation and release of active caspase-8, which initiates a cascade of caspases by processing caspases -3, -6 and -7, which then cleave certain substrates. This eventually leads to the morphological and biochemical features of apoptosis. DISC signaling can be inhibited by expression of cellular-FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP), a caspase-8 inhibitor, leading to inactivation of DISC (Hengartner, 2000; Lawen, 2003).

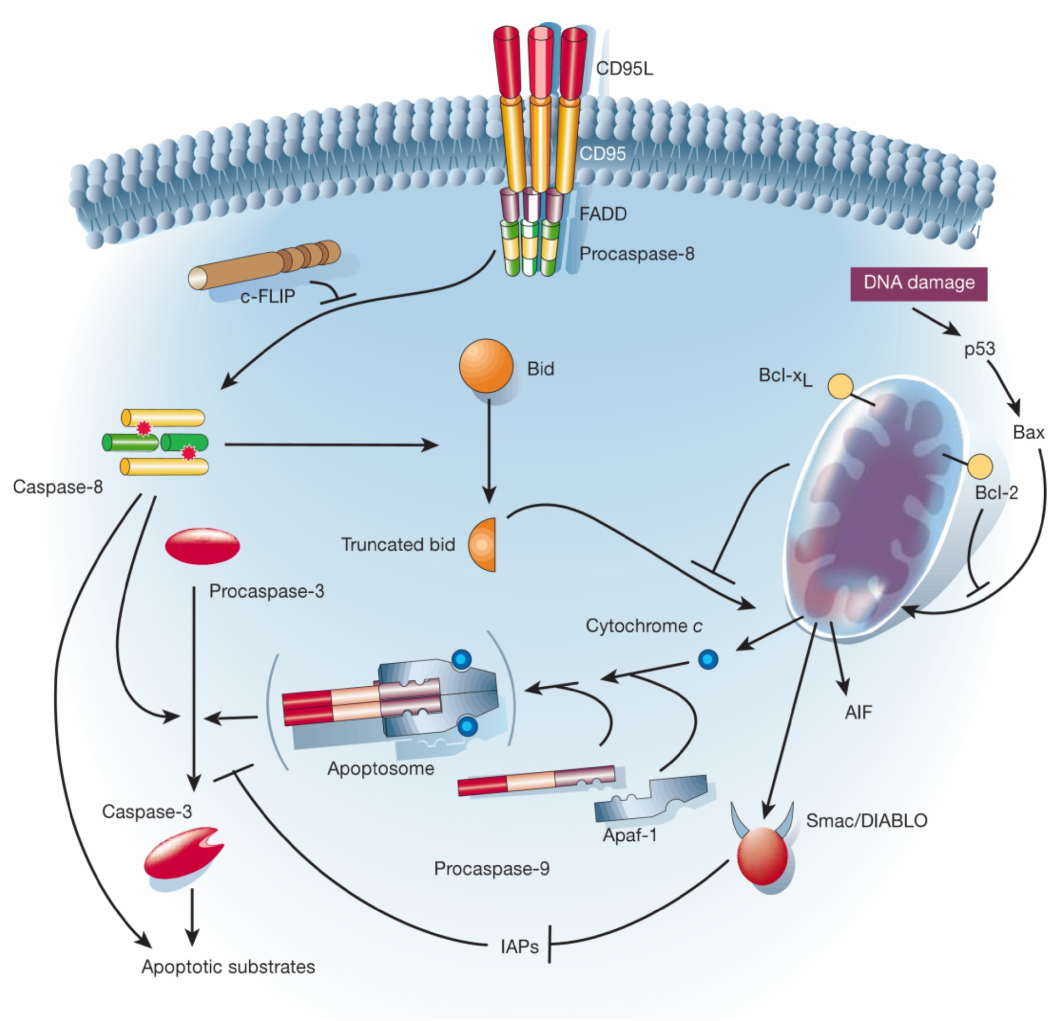

Figure 4. Schematic representation of the two major apoptotic pathways in mammalian cells: the extrinsic and intrinsic pathways (Hengartner, 2000).

1.2.3.2. Intrinsic pathway

In the intrinsic pathway, mitochondria have a central role in the induction of apoptosis. Mitochondria are able to propagate the death signals generated in the cells and/or amplify the apoptotic signal from the extrinsic pathway through mitochondrial outer membrane permeabilization (MOMP) and release of pro-apoptotic proteins. The connection between the extrinsic and intrinsic pathways and amplification of death signal is mediated by Bid, a pro-apoptotic Bcl-2 family member. Bid is cleaved by caspase-8 and when the truncated form (tBid) is translocated into the mitochondria it acts to induce MOMP and release of pro-apoptotic proteins (Luo *et al*., 1998).

MOMP occurs at the early stages of the intrinsic pathway, though the precise mechanism has not been determined (Figure 5). According to one model, MOMP is mediated by members of the pro-apoptotic Bcl-2 family, Bax and Bak, which interact and form the mitochondrial apoptosis-induced channel (MAC), a pore that allows the release of pro-apoptotic proteins. In fact, Mcdonnell proposed that cleavage of Bid changes the conformation of the protein, leading to exposure of hydrophobic residues that allows the insertion of tBid into the membrane and binding of its BH3 domain to Bax and Bak to form pores (Mcdonnell *et al*., 1999). Moreover, it was observed that Bcl-2 and Bcl-xL can directly induce changes in conformation of the proteins Bax and Bak, preventing their activation and polymerization, and blocking the release of pro-apoptotic mitochondrial factors that lead to apoptosis (Letai *et al*., 2002). Another model suggests a permeability transition pore (PTP) is formed, which allows the passage of solutes and water into the mitochondrial matrix, causing mitochondrial depolarization, uncoupling of oxidative phosphorylation and osmotic swelling. This leads to the rupture of the outer membrane and subsequently to the release of pro-apoptotic proteins that circulate freely in the intermembrane space. The precise localization and composition of PTP has not been fully determined, but it appears to be localized at the site of contact between the inner and outer mitochondrial membranes and to contain as main components the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), hexokinase, creatine kinase (CK), the peripheral benzodiazepine receptor (PBR), and the mitochondrial matrix cyclophilin D (Cyp D) (Garrido *et al*., 2006).

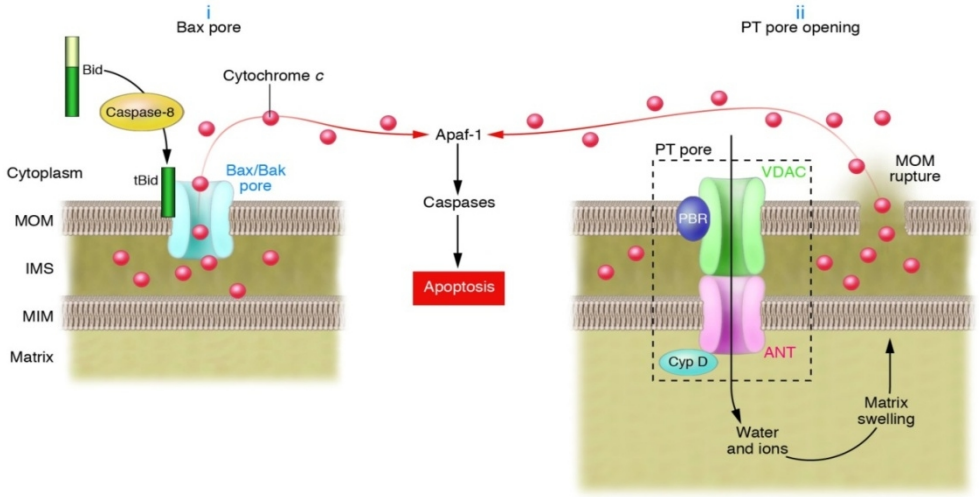

Figure 5. Molecular mechanisms of MOMP: (i) Bax/Bak pore formation and (ii) PTP opening (Bouchier-Hayes *et al*., 2005).

The permeabilization of mitochondria results in the release of pro-apoptotic proteins into the cytoplasm, such as cytochrome *c*, Apoptosis-inducing factor (AIF), Endonuclease G (Endo G), Second Mitochondria-derived Activator of Caspases/Direct Inhibitor of Apoptosis Protein (IAP)-Binding Protein With Low Pi (Smac/Diablo) and High Temperature Requirement protein A2 (HtrA2/Omi) (Shimizu *et al*., 2001; Gulbins *et al*., 2003).

The cytochrome *c* released from the intermembrane space of mitochondria contributes to the formation of the apoptosome, together with Apoptotic Protease Activating Factor-1 (Apaf-1) and deoxyadenosine triphosphate (dATP). The apoptosome activates caspase-9, which further mediates the activation of the caspase cascade and execution of apoptosis (Acehan *et al*., 2002). Smac/Diablo inhibits the Inhibitors of Apoptosis Proteins (IAPs), which inhibit the activity of executing caspases. When translocated into the nucleus, AIF induces deoxyribonucleic acid (DNA) fragmentation and chromatin condensation, whereas Endo G induces internucleosomal DNA fragmentation (van Loo *et al*., 2001). In addition to the mitochondrial factors released, dissipation of the membrane potential also causes the loss of cell homeostasis via generation of ROS that quickly saturate the antioxidant systems and, consequently, led to cessation of ATP synthesis, Ca^{2+} release, oxidation of redox molecules such as nicotinamide adenine dinucleotide reduced form (NADH), nicotinamide adenine dinucleotide phosphate reduced form (NADPH) and glutathione, and activation of stress response genes (Kroemer *et al*., 2007).

1.3. Yeast apoptosis

It has become clear that the apoptotic program is not restricted to multicellular organisms, but also occurs in unicellular organisms such as the budding yeast *S. cerevisiae*. The physiological role of apoptosis in single-cell organisms was initially questioned because there was no obvious reason for a unicellular organism to commit suicide. However, yeast tends to cluster and form communities. It was hypothesized that apoptosis may occur in yeast during chronological and replicative aging, unsuccessful mating processes, and to remove virus-infected and damaged cells from colonies. This altruistic cell death spares nutrients for younger cells and releases nutrients that can be metabolized by younger cells, contributing to the viability and reproductive success of healthier members of the community (Figure 6) (Büttner *et al*., 2006).

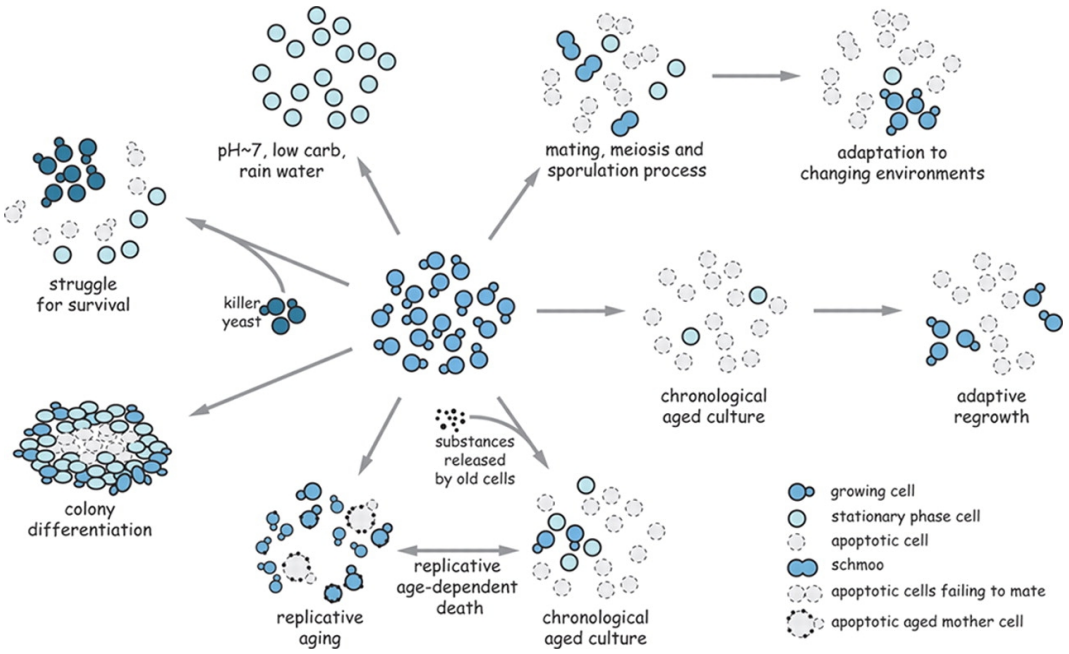

Figure 6. Scenarios of yeast apoptosis (Büttner *et al*., 2006).

Several studies have shown the occurrence of cell death in yeast can display with some characteristics of apoptosis similar to that of mammalian cells. The first observation of apoptosis in yeast was made in a temperature-sensitive mutant of *S. cerevisiae* (mutant Cdc48^{S565G}). *CDC48* is an essential gene that encodes an AAA-ATPase localized in the endoplasmic reticulum and necessary for vesicle trafficking/ translocation of ubiquitinated proteins from the endoplasmic reticulum to the

proteasome for degradation. Surprisingly, when incubated above the restrictive temperature, $Cdc48^{S565G}$ cells showed an apoptotic phenotype characterized by phosphatidylserine exposure, DNA damage, chromatin condensation and fragmentation, release of cytochrome *c* and ROS production (Madeo *et al*., 1997; Braun *et al*., 2006). Since then, several studies also identified yeast orthologs of several members of the mammalian apoptotic machinery, including 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). In addition, apoptosis has often been associated with scenarios involving apoptotic mitochondrial fragmentation (Fannjiang *et al*., 2004) and cytochrome *c* release (Ludovico *et al*., 2002). Regulators such as Apaf-1 and of most members of the Bcl-2 family proteins seem to be absent in yeast. Until now, only a yeast BH3-only protein was identified. Ybh3p translocates to the mitochondria and is capable of mediating the mitochondrial pathway of apoptosis (Büttner *et al*., 2011). However, heterologous expression of Bax in yeast leads to apoptotic cell death that can be prevented by heterologous expression of anti-apoptotic Bcl-2 and Bcl-xL, suggesting the function of Bcl-2 family proteins is potentially conserved in yeast (Hanada *et al*., 1995).

Several assays for apoptosis detection are routinely used in yeast. They include determination of viability, ROS accumulation, DNA fragmentation [TUNEL (Terminal dUTP nick-end labeling) assay], exposure of phosphatidylserine (Annexin-V staining), chromatin condensation [DAPI (4,6-diamino-2-phenyl-indole dihydrochloride) staining] and cell integrity [Propidium iodide (PI) staining] (Carmona-Gutierrez *et al*., 2010). Using these assays, it was found that exposure of yeast cells to a variety of stimuli such as acetic acid, sodium chloride, ethanol, hypochlorite, amiodarone, gallium arsenide, pheromones, valproic acid, edelfosine, jasplakinolide, glucose or sorbitol, or high concentrations of glucose in the absence of other nutrients can trigger the apoptotic process (for a review see Pereira *et al*., 2008).

1.4. Acetic acid as an inducer of apoptosis

Acetic acid is a weak acid that can be formed as an end sub-product of alcoholic fermentation by *S. cerevisiae*. This compound is mainly produced by yeast strains in order to equilibrate the intracellular redox balance. In response to hyperosmotic stress caused by high sugar concentrations, yeasts increase glycerol production, oxidizing NADH to NAD^+. In order to regenerate reducing equivalents, yeasts increase the oxidation of ethanol to acetate, thus increasing the production of acetic acid (Nissen *et al*., 2000).

As all weak carboxylic acids, acetic acid is partially ionized in solution. It has a pKa value of 4.75, and the extracellular pH determines the proportion between the undissociated and anionic form (acetate) and the main mechanism of acetic acid cellular uptake. Acetate can be transported by two different carriers: an acetate-propionate-formate permease (Paiva *et al*., 1999) or an acetate-proton symport enconded by *JEN1* (Casal *et al*., 1999). In glucose-repressed yeast cells, acetic acid enters the cell in the undissociated form by simple diffusion (Casal *et al*., 1996), but also potentially by a Fps1p channel. Mollapour and Piper demonstrated that deletion of *FPS1*, a gene that encodes an aquaglyceroporin channel, abolishes the accumulation of undissociated acetic acid in the cell. Moreover, they correlated loss of Fps1p with resistance to acetic acid and found that Fps1p is regulated by Hog1p signaling. Hog1p directly phosphorylates Fps1p, targeting the channel for endocytosis and degradation in the vacuole (Mollapour and Piper, 2007). After acetic acid entry, it dissociates (when the intracellular pH is higher than the extracellular pH), compromising cell viability (Pinto *et al*., 1989), leading to the intracellular acidification (Casal *et al*., 1996) and induction of apoptosis (Ludovico *et al*., 2001).

Exposure of *S. cerevisiae* to low doses to acetic acid at pH 3.0 results in cell death with features of mammalian apoptosis. Cells exposed to low doses of acetic acid exhibit chromatin condensation, exposure of phosphatidylserine and DNA strand breaks (Ludovico *et al*., 2001). Like in mammalian cells, yeast apoptosis induced by acetic acid was linked to mitochondria. It was shown that acetic acid can lead to the release of cytochrome *c*, ROS production, transient hyperpolarization of mitochondria followed by depolarization, decrease of mitochondrial respiration associated with decrease in cytochrome oxidase activity (Ludovico *et al*., 2002) and mitochondrial ultrastructural changes, namely decrease of cristae number, formation of myelinic bodies, and swelling (Ludovico *et al*., 2003). Acetic acid has been extensively used as an inducer of apoptosis in namely in the study of the involvement of the yeast AIF1 (Wissing *et al*., 2004), mitochondrial fragmentation (Fannjiang *et al*., 2004), modulation of mammalian protein kinase C (PKC) (Saraiva *et al*., 2006), involvement of metacaspase YCA1 (Guaragnella *et al*., 2006), MOMP (Pereira *et al*., 2007) and Pep4p involvement (Pereira *et al*., 2010).

1.5. Sphingolipids

Sphingolipids were considered for a long time simply structural molecules residing in membranes. They are now known to be important in cell stress responses and act as messengers in a variety of signaling pathways such as senescence,

differentiation, apoptosis, cell-cycle arrest, proliferation, mitogenesis, inflammation, migration, and angiogenesis (Hannun and Obeid, 2008), and are associated with several human diseases such as sphingolipidoses, cancer, neurodegenerative diseases, and cardiovascular pathologies (Ozbayraktar and Ulgen, 2009). Consequently, understanding how sphingolipid metabolism regulates these signaling pathways and the mechanisms underlying these diseases is of utmost importance.

Sphingolipids were first named by Johann Thudichum in 1884 to describe the enigmatic nature and properties of complex lipids present in brain tissue. Several species of sphingolipids have been identified since then, and nowadays sphingolipids are one of the major classes of membrane lipids with a structural role in the eukaryotic lipid bilayer (Futerman and Hannun, 2004). As all membrane lipids, sphingolipids are amphipathic molecules, i.e. molecules with both hydrophilic and hydrophobic properties. The hydrophobic region is constituted by a sphingoid long-chain base (LCB), which forms the backbone of sphingolipids, to which a fatty acid is attached by an amide bond. The hydrophilic region is constituted by a polar head group (Figure 7). The type of LCB, fatty acid, and polar head group determine the type of sphingolipid (Dickson, 1998; Ozbayraktar and Ulgen, 2009).

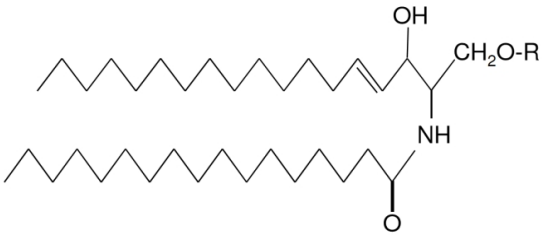

Figure 7. General structure of sphingolipids. The LCB is linked to a fatty acid by an amide bound and a polar head group, R (Fuller, 2010).

1.5.1. Yeast sphingolipid metabolism

The study of sphingolipids is a recent field. Simple model organisms such as the yeast *S. cerevisiae* have been used to uncover the pathways involved in sphingolipid metabolism and function. All the genes that encode the enzymes involved in yeast sphingolipid metabolism are known, the steps involved take place in the same organelles as in mammals and most of the enzymes have orthologs in mammalian cells. *S. cerevisiae* sphingolipid metabolism has ceramides as central molecules and comprises a *de novo* biosynthesis pathway as well as sphingolipid turnover (Figure 8).

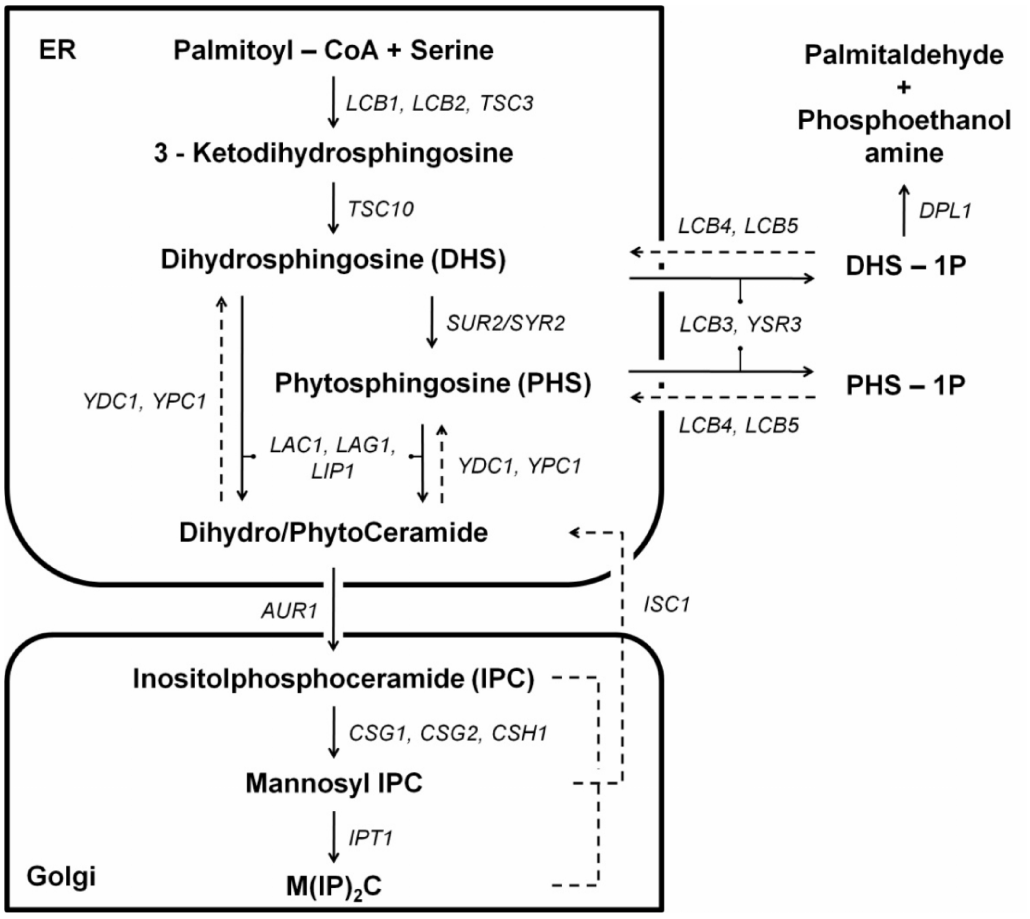

Figure 8. Schematic overview of yeast sphingolipid metabolism displaying the metabolic intermediates, genes involved and cellular locations of the enzymatic reactions.

1.5.2. *De novo* synthesis

Phytoceramide and dihydroceramide are assumed to be the yeast counterparts of mammalian ceramides, and were found to mediate regulation of cell growth and stress responses. As in all organisms, sphingolipid synthesis in yeast begins in the endoplasmatic reticulum (ER) with the condensation of serine and palmitoyl-CoA by serine palmitoyltransferase to yield 3–ketodihydrosphingosine and release of carbon dioxide and Coenzime A (CoA) (Dickson and Lester, 1999). This membrane-bound enzyme is composed of two homologous subunits encoded by *LCB1* and *LCB2*, required for its activity (Nagiec *et al*., 1994), and a third small subunit, encoded by *TSC3* (temperature-sensitive suppressor of calcium sensitivity), a post-translational activator that is essential at high temperatures (Gable *et al*., 2000).

In the next step of the sphingolipid metabolism, 3–ketodihydrosphingosine is reduced and converted to dihydrosphingosine (DHS) by the NADPH-dependent 3–ketoreductase encoded by *TSC10*. Deletion of the *TSC10* gene confers an unviable

phenotype that can be rescued only when the medium is supplemented with DHS or phytosphingosine (PHS) (Beeler, 1998). DHS can be hydroxylated at C-4 by Sur2p/Syr2p hydroxylase to form PHS and both long chain bases, DHS and PHS, can suffer phosphorylation and N-acylation. The Sur2p/Syr2p hydroxylase is not essential for cell growth; however, deletion results in a mutant cell that has only DHS, whereas wild-type cells have mostly PHS (Haak *et al*., 1997).

DHS and PHS can be phosphorylated by two LCB kinases, encoded by *LCB4* and *LCB5*, forming DHS-1-phosphate and PHS-1-phosphate, respectively. Finally, these phosphorylated products can either be dephosphorylated back to DHS and PHS by the phosphatases Lcb3p/Ysr2p and Ysr3p or catabolized by dihydrosphingosine-1-phosphate lyase (Dpl1p) to release palmitaldehyde and phosphoethanolamine (Sims *et al*., 2004).

In the N-acylation step, C_{26} fatty acyl-CoA is added via an amide bond to DHS and PHS to yield dihydroceramide and phytoceramide, respectively. N-acylation of both long chain bases requires two ceramide synthases, encoded by *LAG1* (longevity assurance gene 1) and *LAC1* (longevity assurance gene 1 cognate) (Guillas *et al*., 2001). *LAG1* was first identified by D'mello in 1994 as a gene involved in cell aging whose expression is decreased in aged yeast cells and its deletion results in an increased lifespan (D'mello *et al*., 1994). *LAC1* was later identified as a homologue of *LAG1* (Jiang *et al*., 1998), and since then both genes have been implicated in acyl-CoA-dependent ceramide synthesis (Schorling *et al*., 2001) and shown to play a role in the transport of glycosylphosphatidylinositol-anchored proteins from the ER to the Golgi (Barz and Walter, 1999). In addition, Lip1p forms a heteromeric complex with Lac1p and Lag1p and is essential for ceramide synthase activity *in vivo* and *in vitro* (Vallée and Riezman, 2005).

After generation of ceramides, they are transported to the Golgi for incorporation into complex sphingolipids. Ceramides are first converted into inositol phosphorylceramide (IPC) by transferring of a phosphorylinositol group from phosphatidylinositol to ceramide with release of diacylglycerol. This step is catalyzed by the IPC synthase encoded by *AUR1*, a essential gene whose deletion is lethal (Nagiec *et al*., 1997). More recently, Kei1p was identified as a novel component of IPC synthase. It was observed that Kei1p interacts with Aur1p and is essential for its enzymatic activity and localization (Sato *et al.*, 2009). The complex sphingolipid IPC can further be mannosylated to form mannosylinositolphosphorylceramide (MIPC) via three enzymes, encoded by *CSG1*, *CSG2* and *CSH1*. The enzymes can form two complexes, composed of Csg1p-Csg2p and Csh1p-Csg2p, that function as two different IPC mannosyltransferases, which transfer the mannose from the nucleotide sugar GDP-mannose to the inositol group in IPC (Uemura *et al*., 2003). The Ca^{2+}-

binding protein Csg2p functions as a regulatory subunit in the complex because it regulates the transport and protein levels of the Csg1p and Csh1p (Uemura *et al*., 2007).

The final step in sphingolipid synthesis is the synthesis of the most abundant complex lipid in yeast, mannosyldiinositolphosphorylceramide ($M(IP)_2C$). $M(IP)_2C$ is synthesized by the addition of another inositol phosphate group to MIPC by inositolphosphotransferase (Ipt1p) (Dickson *et al*., 1997).

1.5.3. Sphingolipid turnover

Yeast ceramides can be catabolized by two homologous alkaline ceramidases. Dihydroceramidase (Ydc1p) and phytoceramidase (Ypc1p) are associated with the deacylation of dihydroceramide and phytoceramide, respectively (Mao *et al*., 2000a; Mao *et al*., 2000b).

Ceramides can also be produced through the turnover of complex sphingolipids. This reaction is performed by inositol phosphosphingolipid phospholipase C (Isc1p), which has phospholipase-C type activity and hydrolyses the polar head groups from complex sphingolipids, releasing dihydroceramide and phytoceramide. Isc1p is activated by phosphatidylserine, phosphatidylglycerol, and cardiolipin, and is dependent on the presence of Mg^{2+} and inhibited by Mn^{2+}. Isc1p overexpression results in an increase of ceramide levels, whereas Isc1p knockout results in an accumulation of complex lipids (Sawai *et al*., 2000). In the pre-diauxic phase, i.e. fermentation phase, in which the cells preferentially metabolize the sugar, Isc1p is located in the ER whereas in the post-diauxic phase, i.e. respiration phase, in which cells utilize the ethanol produced during the fermentative phase, Isc1p is located in the mitochondria (Vaena de Avalos *et al*., 2004). *ISC1* deleted strains grow very slowly in media with nonfermentable carbon sources such as glycerol, lactate, ethanol, or acetate (Vaena de Avalos *et al*., 2005), and have an altered mitochondrial lipid profile (lower content of α-hydroxylated phytoceramide) (Kitagaki *et al*., 2007). This suggests Isc1p has a critical role in mitochondrial function and/or in the regulation of pre/post-diauxic shift, because respiration and utilization of nonfermentable carbon sources require intact mitochondrial function. In addition, *ISC1* deletion has been associated with premature aging and decreased cellular resistance to hydrogen peroxide (H_2O_2) (Almeida *et al*., 2008), ethidium bromide (Kitagaki *et al*., 2007), genotoxic agents (methyl methanesulfonate and hydroxyurea) (Matmati *et al*., 2009), and increased cellular resistance to high concentrations of NaCl and LiCl (Betz *et al*., 2002).

1.6. Sphingolipids and cell fate

Over the past years, sphingolipids have generated considerable interest, not only due to their structural role but also as secondary signal effector molecules that can control vital biological functions.

Ceramide, a central molecule in the sphingolipid pathway, can be generated by *de novo* synthesis and by catabolism of complex lipids and has a number of metabolic fates, including catabolism to sphingosine and sphingosine-1-phosphate. Cells maintain a dynamic equilibrium of the levels of ceramide, sphingosine and sphingosine-1-phosphate. The relative amounts of these different sphingolipids and lipid–protein interactions determine cell fate. In mammalian cells, the increase of ceramide and sphingosine levels leads to cell growth arrest and apoptosis, whereas the increase of sphingosine-1-phosphate levels promotes proliferation and inhibits apoptosis (Futerman and Hannun, 2004).

1.6.1. Ceramides

Cellular ceramide levels increase in response to a variety of stimuli either by *de novo* synthesis, breakdown of complex sphingolipids, or inhibition of ceramidases. The established idea that ceramide has a role as a bioactive lipid in apoptosis is based on the identification of putative and direct targets for ceramide action (Pettus *et al*., 2002). The effects of ceramide appear to be mediated by activation of protein kinases and phosphatases, such as ceramide-activated protein kinase (CAPK), PKC, mitogen-activated protein kinases (MAPK) and ceramide-activated protein phosphatases (CAPPs), or through the interaction with caspases and mitochondria (Mathias *et al*., 1998).

Kinase suppressor of Ras (KSR) is a direct target of ceramide. It was first identified as a CAPK and required for both inflammatory responses and ceramide-inducing stress (Zhang *et al*., 1997). Through phosphorylation, KSR activates Raf-1, which phosphorylates MEK, activating MAPK (Yan and Polk, 2001) and promoting apoptosis in cells expressing small amounts of the pro-apoptotic protein BAD (Basu *et al*., 1998). Ceramide has also been directly associated with PKC ζ. Cells treated with ceramide exhibit a high activation of PKC ζ, which promotes the activation of the stress-activated protein kinase (SAPK) pathway and suppression of cell growth (Bourbon *et al*., 2000). Moreover, activation of PKC ζ by ceramide seems to be essential in the formation of a pro-apoptotic complex in differentiating stem cells (Wang *et al*., 2005).

Another target of ceramide is the CAPP: PP2A and PP1. Ceramide-activated PP2A is able to mediate the apoptotic process by dephosphorylation and consequent inactivation of pro-growth kinases such as PKC α (Lee *et al*., 1996) and Akt (Schubert *et al*., 2000), and anti-apoptotic proteins such as Bcl-2 (Ruvolo *et al*., 1999) or activation of pro-apoptotic proteins such as Bad (Xin and Deng, 2006). On the other hand, PP1 was shown to be involved in ceramide-induced dephosphorylation of the retinoblastoma product gene (Rb), interfering with cell cycle regulation (Kishikawa *et al*., 1999).

Cathepsin D (endosomal acidic aspartate protease) was identified by Heinrich in 1999 as a novel ceramide-binding protein. This interaction induces the autocatalytic proteolysis of the pro-enzyme to the active form of cathepsin D (Heinrich *et al*., 1999). Since then, cathepsin D has been implicated in apoptosis due its role in cleavage and activation of Bid, Bax activation and translocation to the mitochondria, destabilization of mitochondria, cytochrome *c* release and caspase activation (Guicciardi *et al*., 2004).

Several studies have demonstrated that ceramide has a role in mitochondria-involving apoptosis. First, cellular ceramide levels increase prior to the activation of the mitochondrial pathway of apoptosis (Rodriguez-Lafrasse *et al*., 2001). Second, ceramide has been shown to interact with and inhibit components of the mitochondrial respiratory chain in isolated mitochondria (Gudz *et al*., 1997), induce ROS production in cells (France-Lanord *et al*., 1997) and isolated mitochondria (García-Ruiz *et al*., 1997), mitochondrial depolarization and dysfunction (Hearps *et al*., 2002), and release of pro-apoptotic proteins, such as cytochrome *c* and AIF (Di Paola *et al*., 2004; Zhang *et al*., 2008). Third, the discovery that mitochondria contain the enzymes involved in ceramide synthesis and the observation that several agents such as TNF, UV radiation and Fas increase the levels of ceramide in isolated mitochondria confirmed that apoptosis occurs via an increase in mitochondrial ceramide levels (Siskind, 2005).

Siskind and Colombini showed that ceramide can form large and stable channels with an estimated diameter of about 10 nm (Samanta *et al*., 2011) (Figure 9) that allows the release of proteins in the intermembrane space with a molecular weight up to 60 kDa (Siskind *et al*., 2002) such as cytochrome *c* (12 kDa), AIF (57 kDa), Endo G (28 kDa) and Smac/DIABLO (42 kDa). In addition, ceramide channels can be disassembled by binding of anti-apoptotic proteins, such as Bcl-xL (Ganesan and Colombini, 2010), and that Bax seems to be responsible for enlargement of the ceramide channels, improving mitochondrial membrane permeability (Ganesan *et al*., 2010). Furthermore, ceramide channels are specifically formed in mitochondrial membranes at physiologically concentrations of ceramide. Indeed, at concentrations 20 times higher than those required for channel formation in mitochondrial, ceramide

channel formation does not occur in the plasma membrane of erythrocytes (Siskind *et al*., 2006).

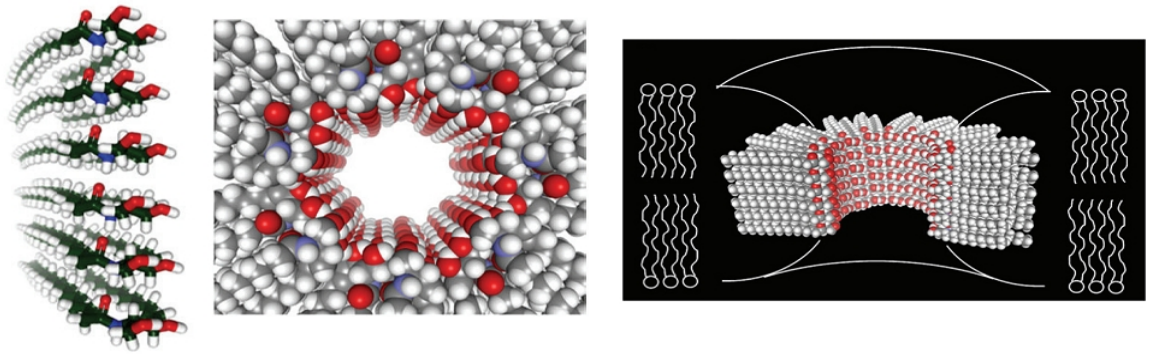

Figure 9. Structure of ceramide pores. They are composed of a ring of a variable number of columns each consisting of six ceramides interconnected by hydrogen bonds between the amide linkage (Siskind and Colombini, 2000).

1.6.2. Ceramides and its metabolites: Sphingosine and Sphingosine-1-phosphate

Sphingosine and sphingosine-1-phosphate are sphingolipids derived from ceramides that can also function as signaling molecules. Sphingosine has been shown to play a role in the induction of apoptosis in several types of cells, such as HL-60 cells (Sakakura *et al*., 1996), human neutrophils (Ohta *et al*., 1994), cardiac myocytes (Krown *et al*., 1996), neurons and astrocytes (Kanno and Nishizaki, 2011), among others. Sphingosine interacts with several signaling pathways, including activation of JNK, p38 MAPK, sphingosine-dependent protein kinase (SDK1) and caspases, stimulation of PARP-1 cleavage, induction of BID and BAX truncation and cytochrome *c* release, inhibition of PKCα, Akt kinase, ERK1/ERK2 kinases, 14-3-3 chaperone protein, β1 integrin and Ca^{2+}/calmodulin-dependent protein kinase and reduction of the expression of Bcl-2 and Bcl-xL (Figure 10). In addition, sphingosine is able to stimulate the activation of Rb and promotes cell cycle arrest (Cuvillier, 2002, Taha *et al*., 2006). On the other hand, sphingosine-1-phosphate functions as an antagonist of ceramide and sphingosine and plays a crucial role in the promotion of survival, proliferation and inhibition of apoptosis. Moreover, the fact that sphingolipids are interconvertible led to the proposal of the so-called “sphingolipid rheostat” model, which postulates that the relative levels of these lipids determine the cell fate (Spiegel and Milstien, 2003). Indeed, it has been proposed that the sphingolipid signaling pathway is a target of interest for cancer therapy (Cuvillier *et al*., 2010). Many external stimuli, namely growth factors, cytokines and mitogens, were shown to activate the sphingosine kinase (SK1),

leading to increased sphingosine-1-phosphate levels and decreased ceramide levels (Alvarez *et al*., 2007). Once generated, sphingosine-1-phosphate can act either extracellularly, by binding to G-protein-coupled receptors present on the cells, initiating downstream G-protein-mediated signaling pathways, or intracellularly, by regulating the calcium levels, activating pro-survival mediators like nitric oxide (NO), ERK, Akt, or by inhibiting the mitochondrial pathway by blocking JNK or activating several transcriptional factors such as AP-1 and NF-κB (Spiegel and Milstien, 2003; Pitson, 2011).

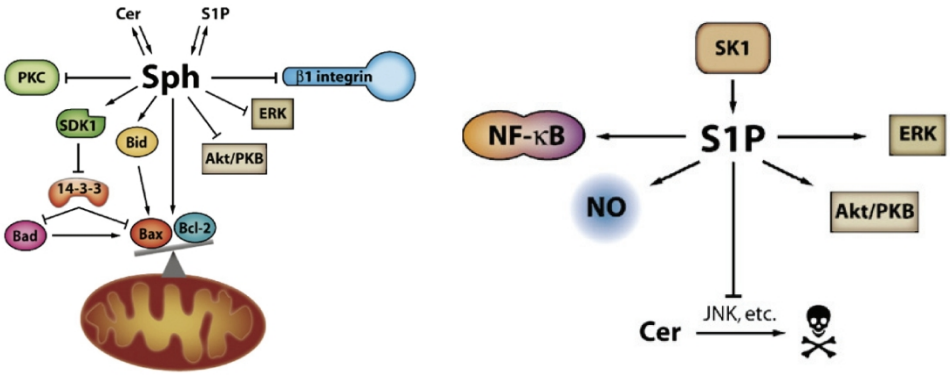

Figure 10. Sphingosine (Sph) and sphingosine-1-phosphate (S1P) targets (Taha *et al*., 2006).

1.7. Sphingolipids and yeast apoptosis

Until now, few studies were performed in yeast to address the involvement of sphingolipids in apoptosis. Siskind observed that expression of either recombinant Bcl-xL or CED-9, homologues of Bcl-2 proteins, disassemble ceramide channels in isolated mitochondria of yeast cells (Siskind *et al*., 2008). In another study, overexpression of Ydc1p ceramidase triggered vacuolar and mitochondrial fragmentation and dysfunction, shortened chronological lifespan and increased apoptosis (Aerts *et al*., 2008). Moreover, Isc1p deletion is associated with up-regulation of the iron regulon and leads to an overload of iron, which catalyzes the production of the highly reactive hydroxyl radicals via the Fenton reaction, and increases apoptotic cell death caused by exposure to hydrogen peroxide (Almeida *et al*., 2008). Recently, it was described that Isc1p is an upstream regulator of Sit4p, the catalytic subunit of PP2A in yeast. Deletion of the *SIT4* gene in *isc1*Δ abolishes the premature ageing and oxidative stress sensitivity of this strain by reversing the mitochondrial dysfunction of *isc1*Δ cells (Barbosa *et al*., 2011).

2. OBJECTIVES

Nowadays, it is consensual that the yeast *S. cerevisiae* undergoes apoptosis in a manner similar to that of mammalian cells. Several members of the mammalian apoptotic machinery have already been identified, and implicated in scenarios of yeast apoptosis. However, there are still numerous apoptotic regulators that remain to be discovered, and others whose function and hierarchy in apoptotic cell death has not yet been determined.

This work aimed to understand the role of ceramides in the mitochondrial apoptotic pathway induced by acetic acid, by taking advantage of bakers´s yeast as a powerful genetic system, and using the following approaches:

1. Construction of several mutants involved in sphingolipid metabolism;
2. Identification of the enzymes associated with sphingolipid metabolism that involved in acetic acid-induced apoptosis;
3. Characterization of the involvement of these enzymes in oxidative stress and mitochondrial apoptotic markers induced by acetic acid.

3. MATERIALS AND METHODS

3.1. Yeast strains

All *Saccharomyces cerevisiae* strains used in this study are listed in Table I. *S. cerevisiae* strain CG379 was used as the wild-type. The *lac1*Δ, *lag1*Δ, *ydc1*Δ, *ypc1*Δ and *isc1*Δ mutants were constructed in CG379 by homologous recombination with disruption cassettes (*KanMX4*) amplified by Polymerase Chain Reaction (PCR), using the oligonucleotides listed in Table II (numbers 1-10) and genomic DNA isolated (as described below in 3.3) from the respective mutants of the yeast strain BY4741 (Euroscarf collection, Germany).

CG379 cells were transformed by electroporation (as described below in 3.4) and transformants selected on rich medium [YPD; 1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose] containing 200 μg/mL geneticin. The correct integration of the disruption cassettes was confirmed by PCR using oligonucleotides (numbers 11-20) that bind upstream and downstream of the insertion, plus an additional oligonucleotide (number 21) binding within the kanamycin gene (Figure 11). In addition, *lag1*Δ and *isc1*Δ were transformed by electroporation with pYES2-*LAG1* and pYES2-*ISC1* vectors, respectively. For mitochondrial studies, wild-type, *lag1*Δ and *isc1*Δ strains were transformed with pYES2-*mtGFP*.

Table I. List of *S. cerevisiae* strains used in this study.

Strain	Genotype	Reference/Source
CG379	Matα, ade5, his2, leu2-112, trp1-289, ura3-52	Yeast Genetic Stock Center, University of California, USA
CG379 pYES2	CG379 harboring pYES2	This study
CG379 pYES2-*mtGFP*	CG379 harboring pYES2-*mtGFP*	This study
*lac1*Δ	CG379 *lac1*Δ :: *KanMX4*	This study
*lag1*Δ	CG379 *lag1*Δ :: *KanMX4*	This study
*lag1*Δ pYES2	*lag1*Δ harboring pYES2	This study
*lag1*Δ pYES2-*LAG1*	*lag1*Δ harboring pYES2-*LAG1*	This study
*lag1*Δ pYES2-as*LAC1*	*lag1*Δ harboring pYES2-as*LAC1*	This study
*lag1*Δ pYES2-*mtGFP*	*lag1*Δ harboring pYES2-*mtGFP*	This study
*ydc1*Δ	CG379 *ydc1*Δ :: *KanMX4*	This study
*ypc1*Δ	CG379 *ypc1*Δ :: *KanMX4*	This study
*ypc1*Δ pYES2	*ypc1*Δ harboring pYES2	This study
*ypc1*Δ pYES2-as*YDC1*	*ypc1*Δ harboring pYES2-as*YDC1*	This study
*isc1*Δ	CG379 *isc1*Δ :: *KanMX4*	This study
*isc1*Δ pYES2	*isc1*Δ harboring pYES2	This study
*isc1*Δ pYES2-*ISC1*	*isc1*Δ harboring pYES2-*ISC1*	This study
*isc1*Δ pYES2-*mtGFP*	*isc1*Δ harboring pYES2-*mtGFP*	This study

Table II. List of oligonucleotides used in this study for the construction of yeast mutants. Restriction sites are marked in bold in respective oligonucleotide sequence.

Number	Name	Oligonucleotide Sequence
1	Lac1Fw	5´- GGAGGGAGAAAGTATTGGAATCT – 3´
2	Lac1Rv	5´- GAAAGCACTAACATCAACATGGA – 3´
3	Lag1Fw	5´- CGTCATCTTCCATTTGAAATCC – 3´
4	Lag1Rv	5´- TCTTACTAGGAGTCTTGGCGAGA – 3´
5	Ydc1Fw	5´- TGTCCGATAGCGTACGCCA – 3´
6	Ydc1Rv	5´- GCCGGTTTTCCAAGCAG – 3´
7	Ypc1Fw	5´- CGCGAGACATCGGAAAATA – 3´
8	Ypc1Rv	5´- CATGTCCCGAATTAGCTAACAA – 3´
9	Isc1Fw	5´- AGGTCGACTGCCGTCTAGAT – 3´
10	Isc1Rv	5´- GCGGACTTCATTTTACTCCAGAC – 3´
11	Lac1KanFw	5´- TGGGCATTGTACCTGATCATG – 3´
12	Lac1KanRv	5´- GGCCTACTATGACAACGATAGCT – 3´
13	Lag1KanFw	5´- CCAGTCCGTCAAGACTAATATCG – 3´
14	Lag1KanRv	5´- CGATGATTCATTGAGATCTGTCA – 3´
15	Ydc1KanFw	5´- AAATCCCTCGTTCCCGG – 3´
16	Ydc1KanRv	5´- TATGTGCCGCCGACATG – 3´
17	Ypc1KanFw	5´- GGACGGATTATCACGCAAGT – 3´
18	Ypc1KanRv	5´- CAGAAGCCAAAATAGCATTCAA – 3´
19	Isc1KanFw	5´- TTGCAGCAGCGAGTCCA – 3´
20	Isc1KanRv	5´- CGAACGAGGCAGTAGTCATGTT – 3´
21	KanRv	5´- AATCGAATGCAACCGGC – 3´
22	LAG1_HindIII_Fw	5´- ACGAC**AAGCTT**AACATGACATCAGCTACGGACAAAT - 3´
23	LAG1_XhoI_Rv	5´- AGATA**CTCGAG**CGTTTATTCACACTTTTCCTTAGAT - 3´
24	asLAC1Fw	5´- TAA**AAGCTT**GCTTCATCGACAATAAGCCAAG - 3´
25	asLAC1Rv	5´- CAC**CTCGAG**CCTATGAATATCCTTTTTCGTTGGAGTA - 3´
26	asYDC1Fw	5´- GAAA**AGCTTC**AATTACTGTTCAGCTGGCCTTATCCA - 3´
27	asYDC1Rv	5´- CAA**CTCGAG**TCCATGGTTATTCTTTTTTGTTTCATCATC - 3´

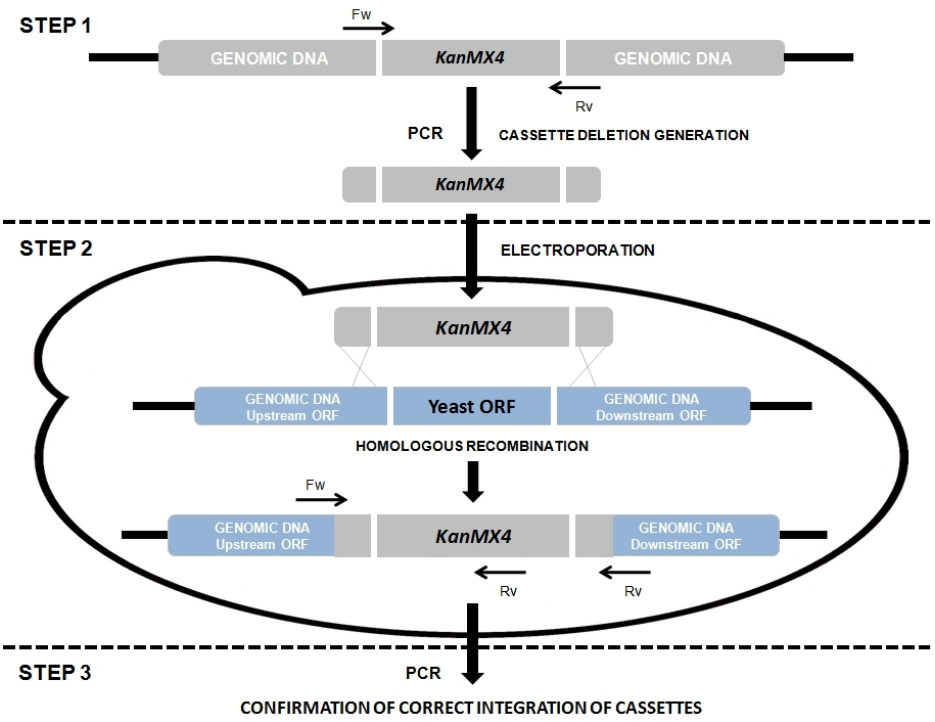

Figure 11. General scheme of the strategy used for construction of yeast mutants. Step 1 represents the procedure used for generation of *KanMX4* cassettes from the respective mutants in strain BY4741, step 2 the homologous recombination mechanism, and step 3 the confirmation of the correct integration of disruption cassettes in proper position of genome of CG379 strain with the primers represented in step 2.

The construction of double mutants was performed by silencing the *YDC1* gene in *ypc1*Δ mutants and the *LAC1* gene in *lag1*Δ mutants using an antisense gene expression vector. The *ypc1*Δ and *lag1*Δ mutants were transformed by electroporation with pYES2-as*YDC1* and pYES2-as*LAC1*, respectively (Figure 12).

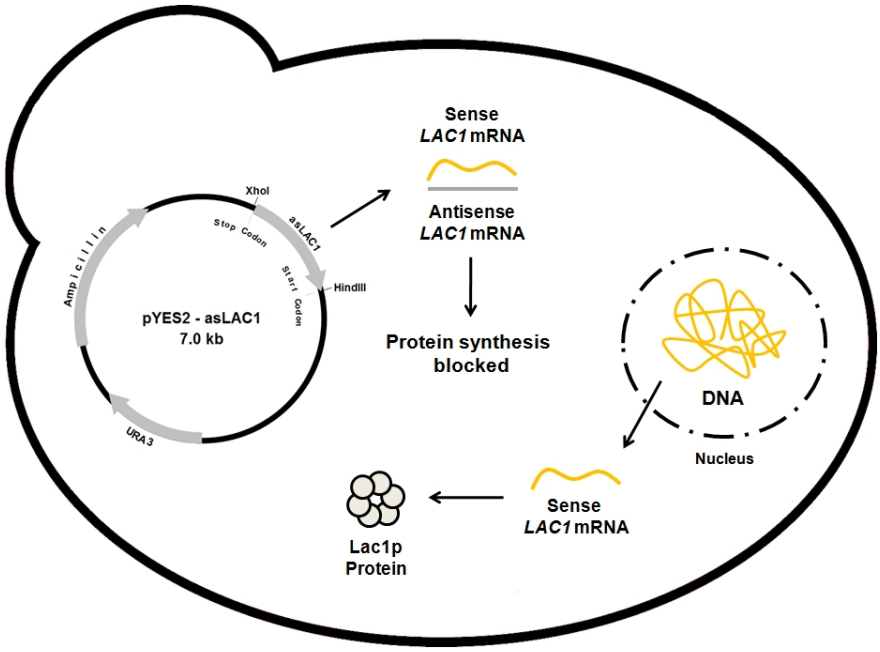

Figure 12. General scheme of the strategy used for construction of double mutants. When antisense mRNA from the plasmid is expressed in yeast cells, it hybridizes with the sense mRNA from the nucleus, blocking synthesis of the protein.

3.2. Plasmids

All the plasmids used in this study are listed in Table III. For expression of *LAG1*, pYES2-*LAG1* was constructed. The *LAG1* gene was amplified by PCR from genomic DNA isolated from the CG379 strain using the oligonucleotides LAG1_HindIII_Fw (number 22) and LAG1_XhoI_Rv (number 23), which introduce *Hin*dIII and *Xho*I restriction sites in the flanks, and cloned into pYES2 using these enzymes (Figure 13).

Table III. List of plasmids used in this study.

Plasmid	Description	Reference/Source
pYES2	*URA3*; *AmpR*	Invitrogen
pYES2-*LAG1*	*LAG1* inserted in pYES2	This study
pYES2-*ISC1*	*ISC1* inserted in pYES2	Y. Hannun, Medical University of South Carolina, Charleston, USA
pYES2-as*LAC1*	Antisense - *LAC1* inserted in pYES2	This study
pYES2-as*YDC1*	Antisense - *YDC1* inserted in pYES2	This study
pYES2-*mtGFP*	*mtGFP* inserted in pYES2	Westermann and Neupert, 2000

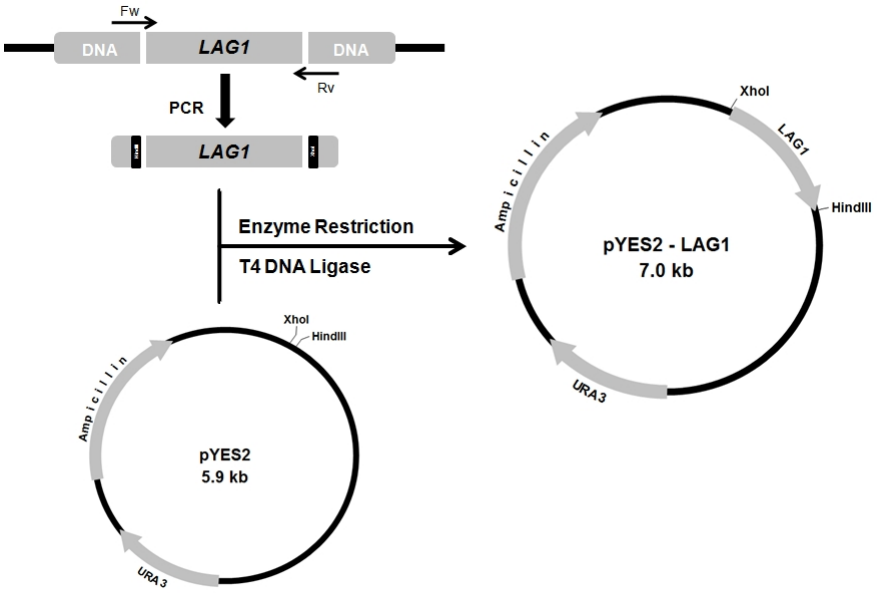

Figure 13. General scheme of the strategy used for construction of pYES2-*LAG1*.

The pYES2-as*LAC*1 and pYES2-as*YDC1* vectors, used for silencing of *LAC1* and *YDC1* genes, were constructed by amplification of *LAC1* and *YDC1* genes from genomic DNA by PCR, using a forward primer that introduces a stop codon and a *Hind*III restriction site and a reverse primer that introduces a start codon and a *Xho*I restriction site in the flanks (oligonucleotides number 24-27), and cloning into the *Hind*III and *Xho*I sites of pYES2.

All plasmids were amplified in *Escherichia coli* DH5α by transformation using standard procedures and selected on Luria Bertani medium [LB; 1% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 1% (w/v) NaCl and 2% (w/v) Agar] supplemented with 100 µg/mL Ampicillin. Plasmids were then extracted from *E. coli* using the GenElute Plasmid Miniprep kit (Sigma-Aldrich) and correct integration of the insert was confirmed by restriction analysis.

3.3. Genomic DNA isolation

Cells from a 10 mL overnight culture were pelleted, washed with sterile water and suspended in 100 µL of Lysis Buffer [2% (v/v) Triton X-100, 1% (v/v) SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)]. About 100 µL of glass beads, 50 µL phenol and 50 µL chloroform/isoamyl alcohol [48:2 (v/v)] were added to the cells and the tubes vortexed for 3 min and centrifuged for 5 min at 4000 rpm. The upper phase was transferred to a new tube, and 100 µL chloroform and 100 µL TE [10

mM Tris and 1 mM EDTA (pH 8.0)] were added. The tubes were vortexed and centrifuged for 5 min at 14000 rpm. The new upper phase was transferred to a new tube containing 100% ethanol and centrifuged for 3 min at 14000 rpm. The pellet was suspended in 400 µL TE and 30 µL 1 mg/mL RNase A. The samples were incubated for 5 min at 37ºC and then 10 µL 4 M ammonium acetate and 1 mL ethanol 100% were added. After centrifuging 3 min at 14000 rpm, the DNA pellets were washed twice with ice-cold 70% (v/v) ethanol, air-dried, and resuspended in water.

3.4. Yeast electroporation

Cells grown to exponential phase on 50 mL of YPD medium were pelleted at 4°C and resuspended in 5 mL of TE and 5 mL of 0.1 M lithium acetate (pH 7.5). After shaking 45 min at 30°C, 250 µL of 1 M dithiothreitol (DTT) was added to the suspension and samples incubated an additional 15 min at 30ºC with shaking. Next, the suspension was sequentially washed with 50 mL of sterile water, 25 mL of ice-cold sterile water, 2 mL of ice-cold 1 M sorbitol and resuspended in 50 µL of 1 M sorbitol. 40 µL of electrocompetent cells were then mixed with 5 µL of DNA (approximately 0.1 µg), incubated for 5 min on ice, and transferred to a sterile 0.2 cm electroporation cuvette. An electric pulse [1.5 kV (E_0=7.5 kV/cm), 25 µ F, 200 Ω in parallel (τ =5 msec)] was applied using a Pulse Controller (Bio-Rad). YPD medium containing 1 M of sorbitol was immediately added into the cuvette and the cells recovered by incubation at 26°C for 1 h. After recovery, cells were plated on selective medium with the appropriate antibiotic or lacking the appropriate selective markers for selection, and grown at 26°C for 3-5 days.

3.5. Growth conditions and treatments

Strains were grown in Synthetic Complete Galactose medium [SC Gal; 2% (w/v) Galactose, 0.67% (w/v) Yeast nitrogen base without aminoacids, 0.14% (w/v) Drop-out mixture lacking histidine, leucine, tryptophan and uracil, 0.008% (w/v) Histidine, 0.04% (w/v) Leucine, 0.008% (w/v) Tryptophan and 0.008% (w/v) Uracil] to early exponential phase (OD_{600} = 0.5 - 0.6) at 26°C in an orbital shaker at 140 rpm, with a ratio of flask volume/medium of 5:1. Strains transformed with plasmids were grown in the same medium lacking the appropriate amino acids. Solid media were prepared by adding 2% (w/v) agar. For acetic acid treatment, strains were cultured under the condition described above, harvested and suspended in the treatment medium consisting of SC Gal at pH 3.0 (set with HCl) containing 180 mM of acetic acid and incubated for 200

min at 26°C, in an orbital shaker at 140 rpm. Cell viability was measured as a percentage of colony forming units (c.f.u.) on YPD medium.

3.6. Analysis of oxidative stress and apoptotic markers

3.6.1. Assessment of plasma membrane integrity/PI staining

Plasma membrane integrity was assessed by flow cytometry using propidium iodide (PI) (Sigma-Aldrich) staining. PI was added to yeast cell suspensions (10^6 cells/mL) to a final concentration of 5 µg/mL and incubated for 10 min at room temperature. Cells with red fluorescence [FL-3 channel (488/620 nm)] were considered to contain plasma membrane disruption.

3.6.2. ROS

Intracellular superoxide anion and mitochondrial ROS were detected by flow cytometry using Dihydroethidium (DHE) and MitoTracker Red CM-H_2XRos (Molecular Probes, Eugene, U.S.A.) as probes, respectively. For DHE staining, untreated or acetic acid-treated cells (180 mM) of wild-type and mutant cells were harvested by centrifugation, resuspended in 500 µL PBS [80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl)] and incubated with 5 µg/mL DHE for 30 min in the dark. For MitoTracker Red CM-H_2XRos staining, untreated or acetic acid treated cells (180 mM) of wild-type and mutant cells were harvested, resuspended in PBS and incubated with 0.4 µg/mL MitoTracker Red CM-H_2XRos at 37°C for 20 min in the dark. Cells with red fluorescence [FL-3 channel (488/620 nm)] were considered to contain superoxide anion or mitochondrial ROS.

3.6.3. Protein carbonylation

Protein oxidation was measured in wild-type and mutant cells by immunodetection of protein carbonyls, using an anti-dinitrophenyl (DNP) antibody, following modification of protein carbonyls with 2,4-dinitrophenylhydrazine (DNPH) (Figure 14).

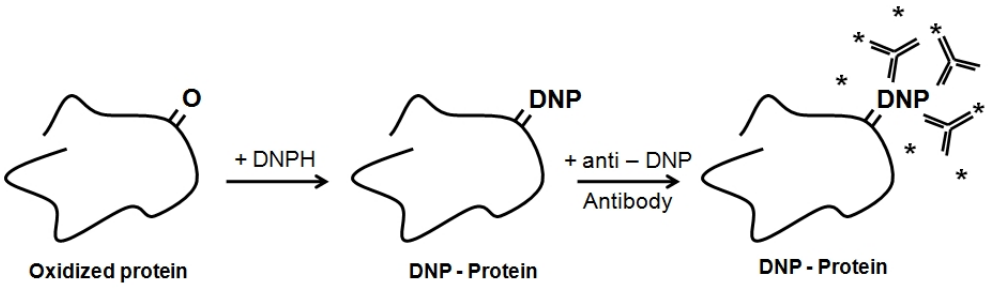

Figure 14. General scheme of the strategy used for detection of protein carbonyls. First the proteins are derivatized to incorporate the DNPH group and later identified by immunodetection using an anti-DNP antibody.

3.6.3.1. Preparation of protein extracts

For detection of protein oxidation by Western blot of total cellular extracts, 25 mL of untreated or acetic acid treated cells (180 mM) of wild-type and mutant cells were harvested, resuspended in 100 µL of Phosphate Buffer [50 mM Na_2HPO_4 and 0.1 mM EDTA (pH 7.0)] supplemented with 5 µL of Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany) and lysed with glass beads by vortexing (5 cycles of 1 min). After centrifugation at 14000 rpm for 15 min, the supernatant was collected and protein concentration estimated by the Lowry method using bovine serum albumin (BSA) as a standard (Lowry *et al*., 1951).

3.6.3.2. Derivatization

For derivatization, 40 µg of total protein from each cellular extract were mixed with one volume of 12% (v/v) Sodium Dodecyl Sulfate (SDS) and two volumes of 20 mM DNPH and 10% (v/v) Trifluoroacetic acid. After incubation for 30 min in the dark, samples were neutralized with 1.5 volumes of Neutralizing Solution [2 M Tris, 30% (v/v) Glycerol and 19% (v/v) β-mercaptoethanol].

3.6.3.3. SDS gel electrophoresis/Western blot

Proteins (12 µg) were separated by SDS gel electrophoresis on a 12.5% SDS-poly-acrylamide gel at 16 mA and transferred to a Hybond-P Polyvinylidene Difluoride Membrane (PVDF) (Hybond-ECL, GE Healthcare) at 0.8 mA/cm^2 during 1 h. Membranes were blocked for 1 h in PBS-T [PBS with 0.05% (v/v) Tween-20] containing

5 % (w/v) non-fat dry milk, washed in PBS-T and then incubated for 2 h with the primary antibody rabbit IgG anti-DNP (1:1500; Sigma Aldrich). After washing twice with PBS-T for 15 min, membranes were incubated with the secondary antibody anti-rabbit IgG-peroxidase (1:5000; Sigma Aldrich) and washed twice, with PBS-T and PBS, for 15 min. Immunodetection of bands was revealed by chemiluminescence (ECL, GE Healthcare).

3.6.3.4. Silver staining

After SDS electrophoresis, a replica gel was fixed in 30% (v/v) ethanol and 10% (v/v) acetic acid for 30 min, and then washed twice with 20% (v/v) ethanol and twice with deionized water for 10 min (each wash). The gels were then soaked in 0.2 g/L sodium thiosulfate for 1 min, washed with deionized water and incubated with 2 g/L silver nitrate for 30 min. After rinsing with deionized water, gels were transferred to Developer Solution [0.026% (v/v) Formaldehyde, 3% (v/v) Sodium carbonate and 0.001% (v/v) Sodium thiosulfate]. When an adequate degree of staining was achieved, gels were transferred to Stop Solution [50 g/L Tris and 2.5% (v/v) Glacial acetic acid] for 30 min and washed twice in deionized water for 30 min.

3.6.4. Mitochondrial fragmentation and degradation

Mitochondrial morphology changes were observed using cells transformed with a plasmid expressing of mitochondrial GFP (pYES2-*mtGFP*). After exposure to acetic acid, images of the mitochondrial network were acquired in an Olympus BX61 microscope equipped with a confocal Olympus FLUOVIEW microscope with an Olympus PLAPON 60X/oil objective with a numerical aperture of 1.42, and using the Olympus FLUOVIEW software. Mitochondria degradation was also determined in these cells by assessing the percentage of cells that still exhibit mtGFP fluorescence after acetic acid exposure, using an Epics® XL™ (Beckman Coulter) flow cytometer. The percentage of cells that exhibit GFP fluorescence was determined in biparametric histograms [ratio (FL-1 area (log) / FS (log)) x GFP fluorescence (FL-1 Peak)] to eliminate variations in fluorescence due to cell size and to discriminate between the cells with intense spots of mitochondrial-GFP and cytosolic-GFP resultant from mitochondrial degradation.

3.6.5. Mitochondrial membrane potential

Mitochondrial membrane potential was measured by flow cytometry, using cells labeled 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% (w/v) Glucose, pH 6.0 (set with $Ca(OH)_2$)]. After addition of 1 nM $DiOC_6$, cells were incubated at 30°C for 30 min in the dark. For double staining, cells were subsequently incubated with PI as described above. Detection of $DiOC_6$ fluorescence was determined using the monoparametric histograms [ratio (FL-1 area (log) / FS (log)] to eliminate variations in fluorescence due to cell size in cells which preserved plasma membrane integrity, i.e. without PI fluorescence [FL-3 channel (488/620 nm)].

3.6.6. Cytochrome *c* detection

3.6.6.1. Subcellular fractionation/preparation of yeast mitochondria

One liter of cells were grown and treated under the same conditions as described above, harvested at the end of exponential phase (OD_{600} = 1.4 - 1.6) and resuspended in 15 mL of Suspension Buffer [60% (v/v) 2 M Sorbitol, 6% (v/v) 1 M Sodium phosphate (pH 7.5) and 2% (v/v) 0.5 M EDTA]. Cells were then digested with 50 mg Zymolyase 20T (ImmunO, MP Biomedicals) to obtain spheroplasts, washed twice with 1.2 M sorbitol, and suspended in Lysis Buffer [0.5 M Sorbitol, 20 mM Tris/HCl (pH 7.5) and 1 mM EDTA]. Spheroplasts were lysed with a few strokes in a glass Dounce homogenizer (tight fitting piston) with care to avoid mitochondrial lysis. Homogenates were centrifuged at 2500 rpm for 10 min and the supernatant then centrifuged at 15000 rpm for 15 min. The supernatant constitutes the cytosolic fraction. The pellet, containing the mitochondrial fraction, was suspended in Lysis Buffer and both fractions frozen in liquid nitrogen and stored at -80ºC. Estimation of the protein concentration of the fractions was determined by the Bradford method using BSA as standard (Bradford, 1976).

3.6.6.2. Mitochondrial integrity

The integrity of mitochondria during the procedure was evaluated by measuring citrate synthase activity. Citrate synthase is an enzyme of the Krebs cycle exclusively localized in the mitochondrial matrix that catalyzes the reaction of acetyl-CoA with oxaloacetate to form citrate and regenerating CoA. Detection of citrate synthase activity

in the cytosolic fraction reflects rupture of the inner mitochondrial membrane. Citrate synthase activity was measured by following the formation of CoA, which reacts with 5,5′-dithiobis (2-nitrobenzoic acid) (DNTB) and forms the absorbing substance thionitrobenzoic acid with absorption at 412 nm. Briefly, 10 µL of protein samples were mixed with 150 µL of water, 20 µL 1 mM DTNB, 5 µL 12.2 mM acetyl-CoA, 5 µL 10% (v/v) Triton X-100 and 10 µL 10 mM oxaloacetate, and the citrate synthase activity determined by following the absorbance of samples for 3 min at 412 nm in a Secomam S1000 spectrophotometer.

3.6.6.3. SDS gel electrophoresis/Western blot

Mitochondrial and cytosolic fractions were separated electrophoretically on a 12.5% SDS-poly-acrylamide gel and transferred to PVDF as describe above. Membranes were cut into strips and incubated with the primary antibodies mouse monoclonal anti-yeast phosphoglycerate kinase (PGK1) antibody (1:5000, Molecular Probes), mouse monoclonal anti-yeast porin (POR1) antibody (1:5000, Molecular Probes) and rabbit polyclonal anti-yeast cytochrome *c* (CYC1) antibody (1:1000, custom-made by Millegen), followed by incubation with secondary antibodies against mouse or rabbit IgG-peroxidase (1:5000; Sigma Aldrich). Pgk1p and Por1p were used as loading control for cytosolic and mitochondrial fractions, respectively. Immunodetection of bands was revealed by chemiluminescence (ECL, GE Healthcare).

3.7. Flow cytometric assays

All the flow cytometric assays were performed in an Epics® XL™ (Beckman Coulter) flow cytometer, equipped with an argon-ion laser emitting a 488-nm beam at 15mW. The population of cells with high homogeneity and frequency was gated in a histogram of Side Scatter (SS) x Forward Scatter (FS). Twenty thousand cells per sample were analyzed. The resulting data were analyzed with WinMDI 2.8 software.

3.8. Reproducibility and statistic analysis of the results

The results obtained are represented by mean and standard deviation (SD) values of at least three independent experiments. Statistical analyses were carried out using GraphPad Prism Software v5.00 (GraphPad Software, California, USA). P-values lower than 0.05 were assumed to represent a significant difference.

4. RESULTS

4.1. Acetic acid stress response

Changes in sphingolipid metabolism have been linked to apoptosis and oxidative stress in both yeast and mammalian cells. It has been reported that an increase of ceramide and sphingosine levels leads to apoptosis, whereas an increase of sphingosine-1-phosphate levels delays/abrogates cell death (Futerman and Hannun, 2004). Since exposure of *S. cerevisiae* cells to acetic acid results in mitochondria-dependent apoptosis with features of mammalian apoptosis, we aimed to determine the involvement of ceramide pathways in this cell death process.

In order to characterize the relative contribution of *de novo* biosynthesis *versus* catabolism of sphingolipids to acetic acid-induced apoptotic cell death, yeast strains lacking Lag1p, Lac1p (unable to generate ceramides by *de novo* synthesis), Isc1p (unable to generate ceramides by degradation of inositolphosphosphingolipids), Ydc1p and Ypc1p (unable to breakdown ceramides) were constructed in the CG379 background by homologous recombination. Strains were grown in SC Gal medium, exposed to 180 mM acetic acid, pH 3.0, for 200 min and the cell viability determined. In Figure 15, these results are expressed as the percentage of colony forming units (c.f.u).

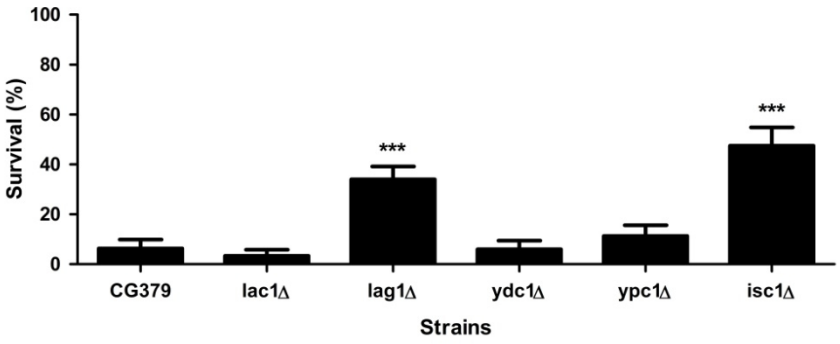

Figure 15. Survival of the indicated *S. cerevisiae* strains exposed to 180 mM acetic acid, pH 3.0, for 200 min. Cell viability was determined by standard dilution plate counts and expressed as a percentage of c.f.u on YPD plates. Values are mean ± SD of at least three independent experiments. Values significantly different from CG379 strain: *** P<0.001, One-way ANOVA and Turkey Test.

It had previously been shown that 180 mM of acetic acid compromises yeast viability and promotes apoptosis (Ludovico *et al*., 2002). In accordance with these studies, exposure of wild-type cells to acetic acid under the conditions referred above resulted in cell death, and only 6.3 ± 3.6 % of wild-type cells remained viable. The *lac1*Δ, *ydc1*Δ and *ypc1*Δ mutant strains exhibited a similar percentage of viability. However, 33.9 ± 5.2 % of *lag1*Δ and 47.5 ± 7.4 % of *isc1*Δ cells were viable under the

same conditions, indicating that deletion of the *LAG1* or *ISC1* genes increases resistance to acetic acid stress. In addition, acetic acid-induced cell death in the different strains was not associated with significant loss of plasma membrane integrity as measured by propidium iodide (PI) staining, indicating that yeast strains die by apoptosis (Figure 16). After 200 min of acetic acid-treatment, more than 95% of cells in all strains are PI-negative.

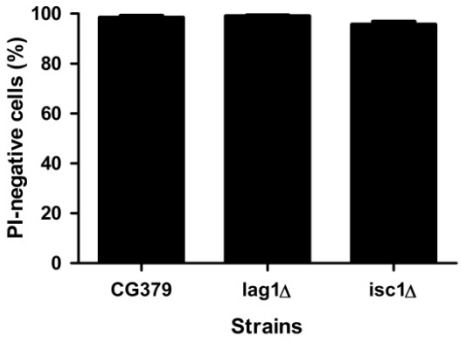

Figure 16. Percentage of PI-negative cells of the indicated *S. cerevisiae* strains exposed to 180 mM acetic acid, pH 3.0, for 200 min. PI fluorescence was determined by flow cytometry. Values are mean ± SD of at least three independent experiments.

Correct integration of the different *KanMX4* deletion cassettes in the genome of the CG379 strain had been confirmed by PCR (described in Materials and Methods). However, in order to confirm the acetic acid resistance observed in *lag1*Δ and *isc1*Δ cells was not due to secondary mutations generated in the process of strain construction, *lag1*Δ and *isc1*Δ strains were transformed with plasmids expressing the respective wild-type genes, and treated under the conditions described above (Figure 17).

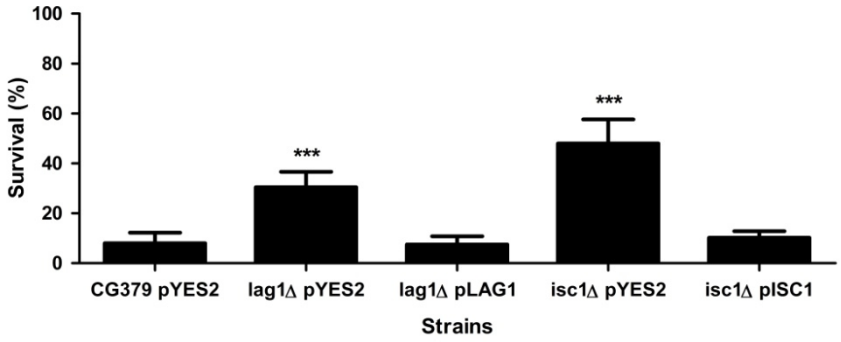

Figure 17. Survival of *lag1*Δ and *isc1*Δ mutants transformed with pYES2-*LAG1* and pYES2-*ISC1*, respectively, or with pYES2 (empty vector) after exposure to 180 mM acetic acid, pH 3.0, for 200 min. Cell viability was determined by standard dilution plate counts and expressed as a percentage of c.f.u. on YPD plates. Values are mean ± SD of at least three independent experiments. Values significantly different from CG379 pYES2 strain: *** P<0.001, One-way ANOVA and Turkey Test.

As expected, expression of the empty vector had no effect on cell viability. On the other hand, expression of *LAG1* and *ISC1* (pYES2-*LAG1* and pYES2-*ISC1*) suppressed the acetic acid resistance of *lag1*Δ (7.5 ± 3.3 %) and *isc1*Δ (10.2 ± 2.7 %) cells, confirming the observed phenotype is due to disruption of these genes.

In sphingolipid metabolism, there are several enzymes involved in the same step of the pathway. This is the case for Lag1p and Lac1p, two ceramide synthases responsible for the conversion of DHS and PHS to ceramide, and for Ydc1p and Ypc1p, two alkaline ceramidases involved in ceramide turnover. Therefore, double mutants were constructed in order to determine whether deletion of both genes involved in these two steps increased resistance to acetic acid. Silencing of the *YDC1* gene in *ypc1*Δ and of the *LAC1* gene in *lag1*Δ mutants was performed using the antisense vectors pYES2-as*YDC1* and pYES2-as*LAC1*, respectively. The antisense mRNA of the genes expressed in these vectors hybridizes with the sense mRNA of the genes expressed from the genomic DNA, blocking gene expression and thus silencing the genes.

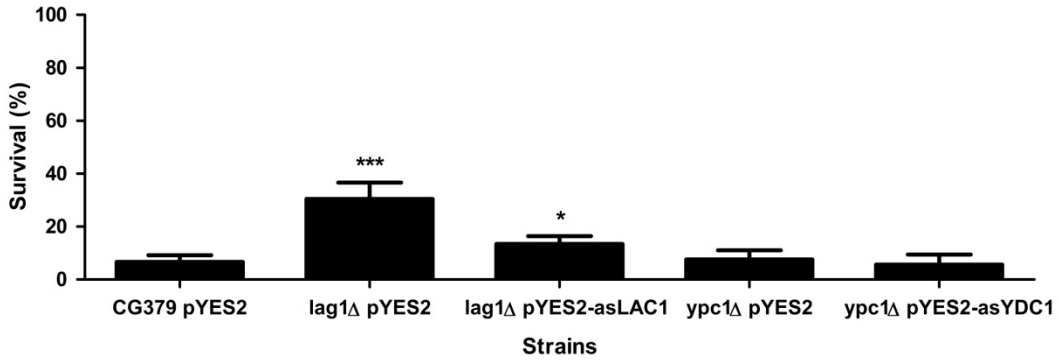

Figure 18. Survival of *lag1*Δ and *ypc1*Δ mutants transformed with pYES2-as*LAC1* and pYES2-as*YDC1*, respectively, or with pYES2 (empty vector) after exposure to 180 mM acetic acid, pH 3.0, for 200 min. Cell viability was determined by standard dilution plate counts and expressed as a percentage of c.f.u. on YPD plates. Values are mean ± SD of at least three independent experiments. Values significantly different from CG379 pYES2 strain: * P<0.05 and *** P<0.001, One-way ANOVA and Turkey Test.

As seen in Figure 18, neither double mutant strain was more resistant to acetic acid than the single mutants *lag1*Δ or *ydc1*Δ. Indeed, simultaneous depletion of *LAC1* and *LAG1* (*lag1*Δ pYES2-as*LAC1* strain) decreased acetic acid resistance of *lag1*Δ (13.4 ± 3.0 %), whereas depletion of *YDC1* and *YPC1* (*ypc1*Δ pYES2-as*YDC1* strain) did not alter the phenotype of the individual mutants (5.7 ± 3.8 %), which was

comparable to that of wild-type cells. In fact, previous studies have shown that cells become very sick or nonviable when both *LAC1* and *LAG1* genes are deleted, depending on the genetic background (Guillas *et al*., 2003), and that ceramides and complex lipids are drastically reduced to levels that could compromise cell viability under stress conditions (Guillas *et al*., 2001). This could also be the reason for the decrease in resistance observed in this study for the *lag1*Δ pYES2-as*LAC1* strain. The results obtained with the depletion of both *YDC1* and *YPC1* suggest that ceramidases are not important for acetic acid resistance. Previous studies have shown that deletion of both genes does not affect cell growth, suggesting they are not essential to the cell (Mao, 2000).

4.2. Oxidative markers

Apoptosis is in many cases associated with the production of ROS in a wide variety of organisms, including yeast (Madeo *et al*., 1999). These include the superoxide anion, which is mainly generated in mitochondria, hydroxyl radicals and hydrogen peroxide. When the levels of ROS exceed the antioxidant capacity of the cells, homeostasis is disrupted and molecules such as lipids, proteins and nucleic acids are oxidized and compromise survival (Finkel and Holbrook, 2000). For that reason, we determined the involvement of sphingolipid metabolism in the production of ROS produced in response to acetic acid treatment.

4.2.1. Intracellular ROS

To determine the levels of ROS, untreated and cells treated with acetic acid were labeled with MitoTracker Red CM-H_2XRos and Dihydroethidium (DHE). The MitoTracker Red CM-H_2XRos probe is a rosamine derivative used to detect mitochondrial free radicals. The reduced version of MitoTracker Red CM-H_2XRos does not fluoresce until entering an actively respiring cell, where it is oxidized by ROS to a red fluorescent compound, which is sequestered in the mitochondria. Dihydroethidium is a neutral probe capable of penetrating the membrane of living cells and intercalate into DNA after it is dehydrogenated (oxidized by superoxide anions generated in mitochondria) to ethidium. Intracellular ROS were assessed by flow cytometry using both ROS-sensitive probes, and results expressed as percentage of ROS-positive cells (Figure 19).

When MitoTracker Red CM-H_2XRos was used as a probe, the percentage of wild-type ROS-positive cells after acetic acid treatment was 77.0 ± 10.9 %. The *lac1*Δ,

*lag1*Δ, *ydc1*Δ and *ypc1*Δ mutants also showed a similar production of mitochondrial ROS. On the other hand, the *isc1*Δ mutant had a lower percentage of cells with mitochondrial ROS, only 55.5 ± 3.8 % when compared to wild-type, in agreement with its resistance phenotype. When DHE was used as a probe, the superoxide anion was detected in 50.0 ± 8.7 % of wild-type cells after 200 min of treatment. Again, in agreement with their resistance phenotypes, *lag1*Δ and *isc1*Δ mutants displayed lower levels of superoxide anion, 28.8 ± 1.1 % and 12.8 ± 3.6 %, respectively. The results obtained confirm the involvement of ROS in cell death and suggest sphingolipids play a role in ROS production, since the mutants most resistant to acetic acid had lower levels of ROS.

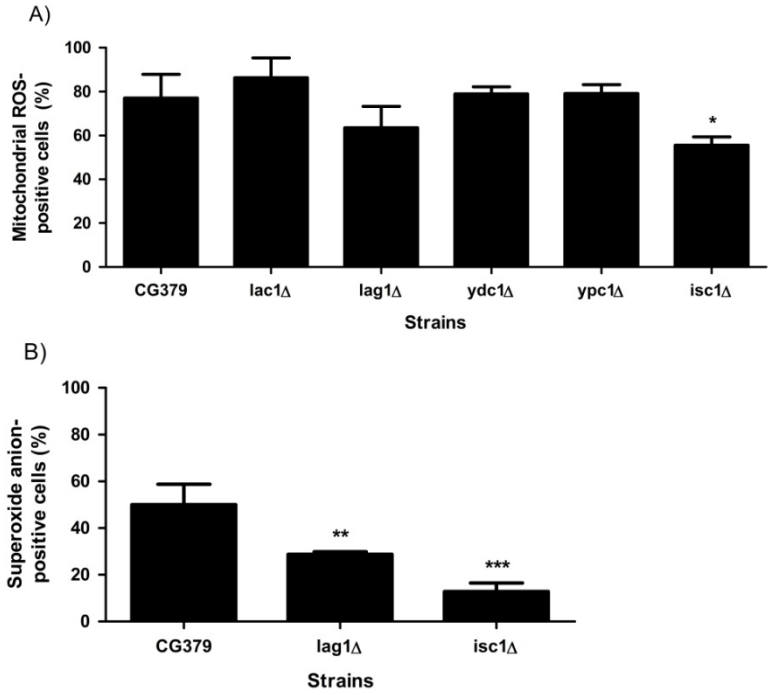

Figure 19. Levels of mitochondrial ROS (A) and superoxide anion (B) in the indicated *S. cerevisiae* strains exposed to 180 mM acetic acid, pH 3.0, for 200 min, using MitoTracker Red CM-H_2XRos and DHE, respectively. Values are mean + SD of at least three independent experiments. Values significantly different from CG379: * P<0.05, ** P<0.01 and *** P<0.001, One-way ANOVA and Turkey Test.

4.2.2. Protein oxidation

In order to explore whether the acetic acid phenotypes observed above were associated with oxidative damage, protein carbonylation was analyzed in wild-type and mutant cells by immunodetection (Figure 20A).

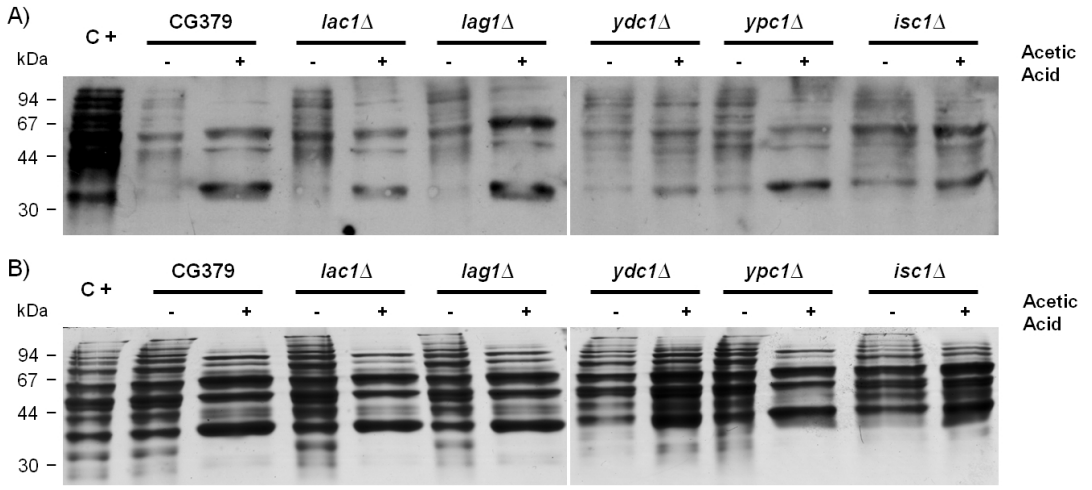

Figure 20. Protein oxidation of the indicated *S. cerevisiae* strains before (-) and after (+) exposure to 180 mM acetic acid, pH 3.0, for 200 min. (A) Immunodetection of protein carbonyls; (B) Silver staining. C+, CG379 cells treated with 1.5mM H_2O_2 for 200 min. A representative experiment of at least two independent experiments with similar results is shown.

The levels of protein carbonylation in untreated and acetic acid-treated cells were similar, and much lower than those observed in cells treated with H_2O_2. Hydrogen peroxide was used as positive control, since there is a correlation between H_2O_2-induced apoptosis and accumulation of carbonylated proteins (Mohammed *et al*., 2005). However, cells treated with H_2O_2 had the same protein profile as untreated cells, whereas cells treated with acetic acid did not. In the latter case, there was enrichment in three protein bands, while others were greatly decreased or disappeared altogether (Figure 20B). One explanation may come from a previous report indicating transient proteosome activation is required for acetic acid-induced cell death (Valentini *et al*., 2008). In summary, the results indicate that acetic acid-induced cell death was not associated with protein oxidation in any of the strains tested.

4.3. Mitochondrial dynamics

Mitochondria are essential organelles that exist in dynamic networks, and often change their localization and shape during stress conditions (Karbowski and Youle, 2003). In past years, it has been shown acetic acid triggers a mitochondria-dependent apoptotic pathway in yeast, associated with typical mitochondrial markers such as mitochondrial fragmentation, degradation, hyperpolarization and release of cytochrome *c* to the cytosol (Ludovico *et al*., 2002). Since the *lag1*Δ and *isc1*Δ mutants were more

resistant to acetic acid than wild-type cells, we assessed whether they still exhibited these apoptotic markers.

4.3.1. Mitochondrial fragmentation

During apoptosis, the typical mitochondrial morphology, normally providing an efficient form to deliver energy to all areas of cell, changes from the tubular network to a punctuate pattern, a process called mitochondrial fragmentation. This process had previously been described for acetic acid-induced apoptosis, and we thus set out to characterize the relative contribution of sphingolipids to this process. Mitochondrial morphological changes were observed through confocal microscopy using cells transformed with pYES2-*mtGFP*, a vector that expresses *GFP* fused to a mitochondrial presequence of the ATPase subunit 9 that targets GFP to mitochondria (Figure 21).

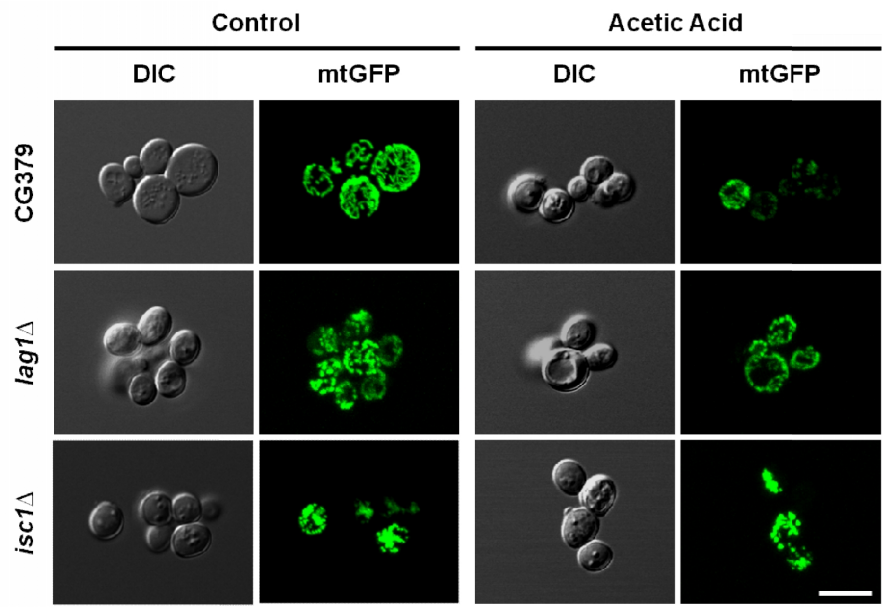

Figure 21. Mitochondrial morphology changes observed in *S. cerevisiae* strains CG379, *lag1*Δ and *isc1*Δ expressing mitochondrial GFP before (control) and after exposure to 180mM acetic acid, pH 3.0, for 200 min. A representative experiment is shown. Bar, 5μm.

As expected, under normal conditions, the mitochondrial morphology of the wild-type strain consists of perfect mitochondrial networks. After acetic acid treatment, mitochondrial networks are destabilized, leading to the formation of the typical punctuate pattern that is normally observed in apoptotic cell death. On the other hand, untreated *lag1*Δ and *isc1*Δ mutants exhibit a mitochondrial morphology distinct from

that observed in the wild-type strain: in these mutant strains, mitochondria form aggregates under normal conditions, more visible in the *isc1*Δ strain, which were not destabilized by exposure to acetic acid, suggesting that deletion of these genes may hinder mitochondrial fragmentation. Sphingolipid metabolism had previously been associated with mitochondrial morphology. Overexpression of *YDC1* results in a decrease of ceramide levels and mitochondrial fragmentation (Aerts *et al.*, 2008). Isc1p is localized in mitochondria and has a role in the generation of ceramide in mitochondria, and its deletion increases the levels of α-hydroxylated phytoceramides (Kitagaki *et al.*, 2007). Thus, the morphologic alterations we observed confirm the contribution of ceramide to the normal morphology of mitochondria.

4.3.2. Mitochondrial degradation

Mitochondrial degradation has previously been described in yeast cells exposed to apoptotic stimuli such as acetic acid (Pereira *et al.*, 2010). In order to address whether deletion of *LAG1* or *ISC1* affected mitochondrial degradation, cells were transformed with pYES2-*mtGFP* and the loss of mtGFP fluorescence, representative of the loss of mitochondrial mass, was monitored by flow cytometry during 240 min of exposure to acetic acid (Figure 22).

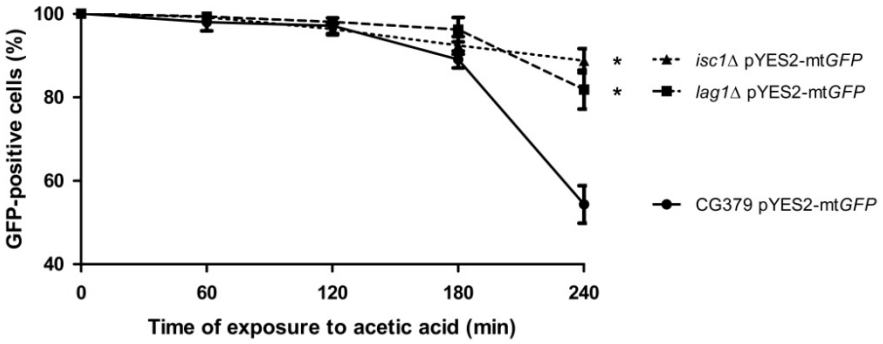

Figure 22. Mitochondrial degradation, measured by loss of mtGFP fluorescence in *S. cerevisiae* strains CG379, *isc1*Δ and *lag1*Δ expressing pYES2-*mtGFP* after exposure to 180 mM acetic acid, pH 3.0, during 240 min. Values are mean ± SD of at least three independent experiments. Values significantly different from CG379 pYES2-*mtGFP* strain: * P<0.05, One-way ANOVA and Turkey Test.

Up to 180 min of treatment, about 90% of cells of all strains still exhibited GFP fluorescence, indicating the levels of mitochondria degradation are low. However, after 240 min of acetic acid treatment, only 54.34 ± 4.5 % of wild-type cells exhibited GFP

fluorescence, whereas GFP fluorescence was still present in 81.85 ± 4.7 % and 88.74 ± 2.9 % of *lag1*Δ and *isc1*Δ mutant cells, respectively. These results suggest that deletion of *ISC1* or *LAG1* delays mitochondrial degradation in response to acetic acid.

4.3.3. Mitochondrial membrane potential

Mitochondrial membrane potential is an important aspect of mitochondria function. It is responsible for the generation of ATP and is generated by the electron transport chain. Some studies in yeast apoptosis reported a transient hyperpolarization followed by a depolarization of mitochondria after acetic acid stimuli, a process associated with the release of cytochrome *c* from mitochondria to the cytosol (Ludovico *et al.*, 2002; Pereira *et al.*, 2010). We therefore investigated the role of sphingolipids on the mitochondrial potential. Variations in mitochondrial potential were analyzed using the membrane potential-sensitive probe $DiOC_6$, which accumulates in the mitochondria as a function of its membrane potential. Mitochondrial potential was assessed by the ratio between the mean of green fluorescence intensity (FL-3 channel) and the mean of the FS (both measured as log values) of the subpopulation which preserved plasma membrane integrity (gated for PI-negative cells) (Figure 23). The changes of mitochondrial potential were expressed in relative values comparatively with time zero.

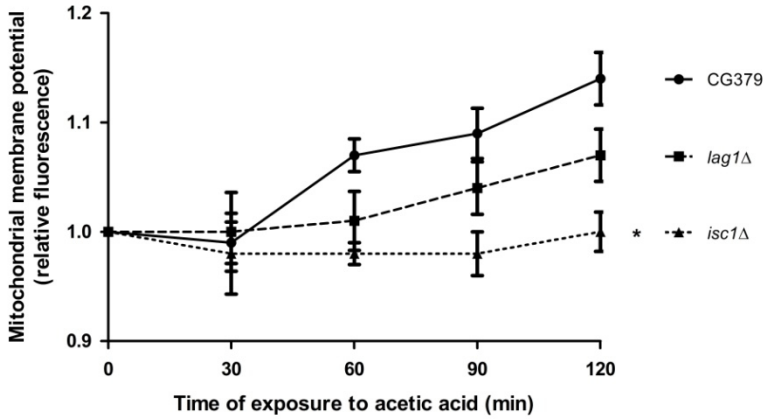

Figure 23. Mitochondrial membrane potential (relative fluorescence) assessed by flow cytometry using the probe $DiOC_6$ in *S. cerevisiae* strain CG379, *isc1*Δ and *lag1*Δ expressing mitochondrial GFP after exposure to 180 mM acetic acid, pH 3.0, for 120 min. Values are mean ± SD of at least three independent experiments. Values significantly different from CG379 strain: * P<0.05, One-way ANOVA and Turkey Test.

Under our experimental conditions, acetic acid treatment led to an increase of mitochondrial membrane potential in both wild-type and *lag1*Δ mutant strains, while in *isc1*Δ mutant the mitochondrial membrane potential was not affected along 120 min.

4.3.4. Cytochrome *c* release

As described above, a crucial event in yeast apoptosis induced by acetic acid is the translocation of cytochrome *c* from mitochondria to the cytosol. Since we found *isc1*Δ and *lag1*Δ mutant strains were more resistant to acetic acid, we next determined whether acetic acid still triggered release of cytochrome *c* in these strains. Cytosolic and mitochondrial fractions were isolated by differential centrifugation. The integrity of the inner mitochondrial membrane after the procedure was assessed through determination of the activity of citrate synthase, a protein exclusively localized in the mitochondrial matrix. Western blot was then performed in order to detect the levels of cytochrome *c* in the different fractions (Figure 24).

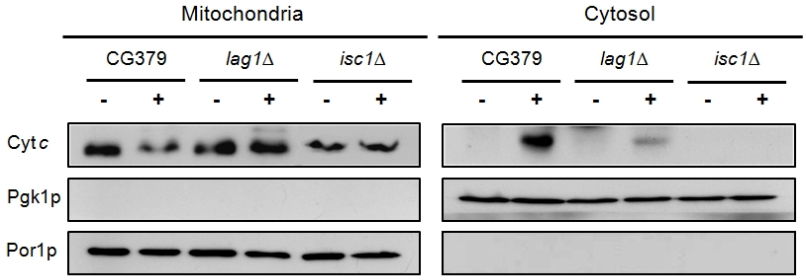

Figure 24. Western blot analysis of cytochrome *c* in *S. cerevisiae* strains CG379, *isc1*Δ and *lag1*Δ before (-) and after (+) exposure to 180 mM acetic acid, pH 3.0, for 200 min, in both mitochondrial and cytosolic fractions. Cytosolic phosphoglycerate kinase (Pgk1p) and mitochondrial porin (Por1p) levels were used as loading control of cytosolic and mitochondrial fractions, respectively. A representative experiment is shown of at least two independent experiments with similar results.

Western blot analysis revealed that, under normal conditions, cytochrome *c* is exclusively localized in the mitochondria of all strains. Treatment of the wild-type strain with acetic acid resulted in a decrease of the cytochrome *c* content in mitochondria, and consequent detection in the cytosol. On the other hand, and in correlation with the phenotype of acetic acid resistance, the *lag1*Δ mutant exhibited less translocation of cytochrome *c* and *isc1*Δ cells did not exhibit cytochrome *c* release to the cytosol. Western blot analysis also revealed that *isc1*Δ cells have a lower overall content of cytochrome *c* when compared with the other strains.

5. DISCUSSION AND FUTURE PERSPECTIVES

Sphingolipids are lipid second messengers generated in response to different physiological signals and stress stimuli. They affect multiple aspects of cellular function, including apoptosis (Hannun and Obeid, 2008). In mammalian cells, an increase of ceramide and sphingosine levels promotes apoptosis, whereas an increase of sphingosine-1-phosphate levels inhibits apoptosis (Futerman and Hannun, 2004). Apoptosis is an evolutionary conserved type of cell death that is crucial for normal tissue homeostasis and development. Deregulation of apoptosis contributes to the pathogenesis of several diseases, including cancer (unwanted cell proliferation), autoimmune diseases (failure to eliminate aberrant cells) and neurodegenerative disorders (excessive loss of cells) (Fischer and Schulze-Osthoff, 2005). Given the important role of sphingolipids in regulation of apoptosis and survival, the study of the sphingolipid metabolism has a considerable impact in resolution of several diseases and development of therapies.

The yeast *S. cerevisiae* is an excellent model system for research. It is a genetically tractable organism amenable to modifications, and its entire genome sequence has already been sequenced (Mager and Winderickx, 2005). Since it possesses an endogenous apoptotic machinery, and sphingolipid metabolism is similar to its mammalian counterpart, we aimed to characterize the role of sphingolipids in the yeast mitochondrial apoptotic pathway. Acetic acid-induced cell death has been extensively characterized in the yeast *S. cerevisiae.* In previous studies, it has been shown that acetic acid triggers a mitochondria-mediated apoptotic pathway associated with mitochondrial ROS accumulation, decrease in cytochrome *c* oxidase (COX) activity and subsequent release of cytochrome *c* (Ludovico *et al*., 2002). Additionally, other studies implicated the ADP/ATP carrier in the mitochondrial outer membrane permeabilization and cytochrome *c* release (Pereira *et al*., 2007) and the vacuolar protease Pep4p in mitochondrial degradation (Pereira *et al*., 2010).

For the first time, this study shows that the sphingolipid pathway plays a role in the mitochondrial yeast apoptotic pathway induced by acetic acid. Absence of *LAG1,* ortholog of mammalian longevity assurance gene (LASS1) or *ISC1,* ortholog of mammalian neutral sphingomyelinases, enhanced the cell survival of yeast cells exposed to acetic acid. Characterization of the phenotype of a *lag1*Δ*isc1*Δ double mutant would be necessary to ascertain whether Isc1p and Lag1p act through the same pathway or independently.

Several studies have shown that ROS are key signaling molecules in mammalian cells (D'Autréaux and Toledano, 2007). In comparison with wild-type cells, *lag1*Δ and *isc1*Δ mutant strains accumulated fewer ROS in response to acetic acid. Accumulation of ROS is directly related with mitochondrial dysfunction and promotion of yeast apoptosis (Madeo *et al*., 1999; Perrone *et al*., 2008). Accordingly, *lag1*Δ and

*isc1*Δ mutant strains exhibited lower levels of ROS and less mitochondrial dysfunction. Our results also showed that the increase of ROS production was not sufficient to cause protein oxidation. In the future, it would be interesting to investigate the role of Isc1p and Lag1p in oxidative stress elicited during acetic acid treatment. The levels of lipid peroxidation and antioxidant defenses such as glutathione, superoxide dismutase and catalase activities may be correlated with the viability of the wild type and *lag1*Δ and *isc1*Δ strains. A previous study showed that exposure to acetic acid did not alter catalase or superoxide dismutase activity in wild-type cells. However, acetic acid cell death decreased in cells overexpressing catalase T and increased when Cu,Zn-superoxide dismutase is overexpressed, suggesting that hydrogen peroxide contributes to acetic acid induced cell death (Guaragnella *et al*., 2008).

Ceramides play a role in ROS production and MOMP in mammalian cells, as ceramides increased ROS production by directly inhibiting the mitochondrial complex III (Gudz *et al*., 1997) and increased the permeability of mitochondrial membranes to cytochrome *c* through the formation of pores (Siskind, 2005). Notably, previous studies showed that acetic acid elicits similar mitochondrial dysfunctions affecting specifically mitochondrial complex III activity (Ludovico *et al*., 2002) and triggering MOMP (Pereira *et al*., 2007). Indeed, in the wild-type strain, we detect high levels of intracellular ROS and cytochrome *c* release into the cytosol. However, in *lag1*Δ and *isc1*Δ mutants, which we presume contain lower levels of certain ceramides or cannot increase the levels at same rate as the wild-type strain under acetic acid stress, the levels of ROS and cytochrome *c* in the cytosol were much lower than those observed in the wild-type strain. Cytochrome *c* release is directly related to the promotion of apoptosis in mammalian cells due to its involvement in the formation of the apoptosome (Acehan *et al*., 2002) and also occurs in acetic-acid yeast apoptosis (Ludovico *et al*., 2002). The results obtained suggest that, as described in mammalian cells, ceramide has an active role in cytochrome *c* release in yeast apoptosis, possibly through the formation of ceramide pores.

It has been suggested that mitochondrial fragmentation is required for MOMP and cytochrome *c* release. Drp1p and Fis1p, proteins involved in mitochondrial fission, have been implicated in ceramide-induced apoptosis in cardiomyocytes. Parra and coworkers have shown that ceramide increases the mitochondrial content of Drp1p and Fis1p and promotes fragmentation of the mitochondrial network (Parra *et al*., 2008). The yeast ortholog of human Drp1p promotes mitochondrial fragmentation/degradation and cell death following treatment with several death stimuli, namely acetic acid (Fannjiang *et al*., 2004). In accordance with this previous work, we observed that acetic acid leads to mitochondrial fragmentation in wild-type cells. However this was not observed in the *lag1*Δ and in *isc1*Δ strains. These results suggest that ceramide

generated by Isc1p and Lag1p is implicated in mitochondrial fragmentation and consequently in cytochrome *c* release to cytosol. In fact, *ISC1* deletion leads to the formation of mitochondrial aggregates, which are resistant to mitochondrial fragmentation and it does not exhibit cytochrome *c* release. We therefore hypothesize that acetic acid induces mitochondria fragmentation through an increase of ceramide and activation/recruitment of Drp1p and Fis1p to mitochondria. To test this hypothesis, the levels and distribution of Drp1p and Fis1p should be determined by Western blot and immunofluorescence, respectively.

In mammalian cells, ceramides specifically bind to and activate the endossomal acidic aspartate protease cathepsin D. This interaction induces the autocatalytic proteolysis of the pro-enzyme to the active form of cathepsin D and depends on acid sphingomyelinase, indicating that CatD is a target of ceramide (Heinrich *et al*., 1999). The yeast Pep4p, with homology to CatD, translocates from the vacuole to the cytosol in response to several stress conditions; it is essential for the removal of oxidized proteins after oxidative damage induced by H_2O_2 and in chronological ageing (Marques *et al*., 2006), and is involved in mitochondrial degradation in acetic acid-induced apoptosis (Pereira *et al*., 2010). Absence of *PEP4* results in a cell survival sensitive phenotype in response to acetic acid that apparently is not due to an accumulation of oxidized proteins. We found that *lag1*Δ and *isc1*Δ cells, which are more resistant to acetic acid, have less mitochondrial degradation than the wild-type strain. We therefore hypothesize that these mutants have less release/activation of Pep4p by ceramides, and therefore exhibit lower levels of mitochondrial degradation. In the future, it would be interesting to assess whether the acetic acid resistance phenotype of *lag1*Δ and *isc1*Δ mutant cells correlates with alterations in Pep4p cellular re-localization/activation in response to acetic acid. Release and localization of EGFP-Pep4p and Pep4p activity in cells undergoing acetic acid-induced death should be determined. Finally, and in order to determine whether Lag1p, Isc1p and Pep4p act independently or in the same pathway, *lag1*Δ*pep4*Δ and *isc1*Δ*pep4*Δ mutants should be constructed and characterized regarding acetic acid-induced apoptosis.

Collapse of the mitochondrial membrane potential is often considered an event of apoptosis that precedes the release of pro-apoptotic proteins such as cytochrome *c*. Opening of the mitochondrial PTP results in loss of the mitochondrial membrane potential, uncoupling of oxidative phosphorylation, ATP depletion, and apoptosis (Kroemer *et al*., 2007). In previous studies, it was shown that acetic acid induces a transient hyperpolarization of mitochondrial membrane potential that precedes cytochrome *c* release (Ludovico *et al*., 2002). However, we could not reproduce these results under our experimental conditions. This may be explained by the growth phase and differences in mitochondrial mass of the cells before exposure to acetic acid that

influence signal/background fluorescence of the probes used for mitochondrial potential measurements, such as $DiOC_6$. Indeed we reproduce the previous reported transient hyperpolarization followed by depolarization in late exponential phase cells but not in exponential cells. Acetic acid treatment increased the mitochondrial membrane potential of wild-type and *lag1*Δ mutant strains, while in *isc1*Δ mutant the mitochondrial membrane potential was not affected. Mitochondrial hyperpolarization has already been observed in other studies. For instance, heterologous expression of BAX in yeast induces hyperpolarization of mitochondria, production of ROS and cell death (Gross *et al*., 2000). Since hyperpolarization is related to uncoupling of oxidative phosphorylation and ROS production, the results obtained are in agreement with the literature. Indeed, the strains with lower intracellular ROS levels had less variation in mitochondrial potential and lower levels of cytochrome *c* release. It has been suggested that Isc1p plays a role in mitochondrial function, since it localizes to mitochondria in the fermentative phase and *ISC1*-deleted strains are defective in aerobic respiration due to an inability to up-regulate genes required for growth in non-fermentable carbon sources (Kitagaki *et al*., 2009). It has been shown that respiratory-deficient mutants are more resistant to acetic acid-induced apoptosis (Ludovico *et al*., 2002). This is consistent with the observed resistant phenotype of *isc1*Δ cells to acetic acid-induced apoptosis. However, it is not clear whether deleting Isc1p renders cells more resistant to acetic acid-induced apoptosis because cells have increased mitochondrial deficiency. To address this issue, a respiratory deficient mutant should be constructed in the *isc1*Δ strain and the double mutant characterized regarding acetic acid-induced apoptosis.

It is possible that the acetic acid resistance and fewer mitochondrial dysfunctions observed in *lag1*Δ and *isc1*Δ mutant cells result from higher activation of Hog1p and destabilization of Fps1p in the plasma membrane. Mollapour and Piper demonstrated that Hog1p directly phosphorylates Fps1p, targeting the channel for endocytosis and degradation in the vacuole. In addition, they observed that deletion of *FPS1* abolishes the accumulation of undissociated acetic acid in the cell (Mollapour and Piper, 2007). Different approaches may be used to test this hypothesis, namely: i) determination of the uptake and accumulation of acetic acid (radiolabeled) in wild-type and mutant cells grown at pH 3.0 and 6.8 (at this pH most of the acetic acid is dissociated and will be used as a control); ii) determination of the activity of the HOG1 pathway in control and acetic acid-treated cells in both strains by Western blot using antibodies specific for dually phosphorylated Hog1p (anti-phospho-p38 MAPK); iii) visualization of changes in plasma membrane Fps1p by epifluorescence microscopy, using wild-type and mutant cells transformed with pUG23-FPS1-GFP.

Our preliminary results indicate the *lag1*Δ and *isc1*Δ mutants may have lower levels of the ceramides necessary to signal acetic acid-induced apoptosis. Adding exogenous C_2-phytoceramide to acetic acid-treated cells suppressed the resistance phenotype observed when *lag1*Δ and *isc1*Δ mutants were treated with only acetic acid (preliminary results, not shown). However, these results need further confirmation, as well as validation through a lipidomic analysis to assess changes in bioactive sphingolipids (long chain sphingoid bases and its phosphates, dihydroceramides, phytoceramides and α–hydroxylated-phytoceramides) during acetic acid stress.

In conclusion, our results indicate acetic acid elicits production of ceramides, especially through hydrolysis of complex lipids and *de novo* synthesis catalyzed by Isc1p and Lag1p, respectively, leading to mitochondrial dysfunction and consequently apoptosis.

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