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THE ROLE OF CERAMIDE PATHWAY IN YEAST APOPTOSIS INDUCED BY ACETIC ACID

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Dissertação de Candidatura ao grau de Mestre em Contaminação e Toxicologia Ambientais submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Orientador – Professor Doutor Vítor Manuel Vieira da Costa
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Contents

Summary

The yeast *Saccharomyces cerevisiae* can undergo programmed cell death in response to different stimuli showing typical apoptotic markers, such as DNA fragmentation, chromatin condensation, cytochrome c release from mitochondria and production of reactive oxygen species (ROS). Acetic acid, a normal end product of the yeast fermentation, was already described as an agent capable to induce mitochondrial dependent apoptosis.

Sphingolipids are a ubiquitous class of biologically active lipids present in eukaryotic membranes and changes in their levels have been linked to apoptosis and oxidative stress in yeast and mammalian cells. Ceramide accumulates upon diverse stress treatments and increases the permeability of the mitochondria to cytochrome c, leading to the generation of ROS.

To characterize the relative contribution of de novo biosynthesis versus catabolism of ceramide in apoptotic cell death induced by acetic acid, yeast mutant cells affected in ceramide metabolism were used: *lag1*Δ and *lac1*Δ (unable to generate ceramide by de novo synthesis), *isc1*Δ (unable to generate ceramide by degradation of inositolphosphosphingolipids) and *ydc*1Δ and *ypc1*Δ (unable to breakdown ceramide). Our results showed that the *isc1*Δ and *lag1*Δ mutants exhibit a higher resistance to acetic acid that was correlated with lower levels of mitochondrial ROS production. However, acetic acid induced cell death was not associated with protein oxidation. Silencing of *YDC1* in ypc1Δ cells and of *LAC1* in lag1Δ cells, by using an antisense gene expression plasmid, did not affect stress resistance.

Structurally, the deletion of *ISC1* induced a severe alteration in lipid rafts distribution throughout the plasma membrane. Acetic acid also induces a rearrangement of these membrane microdomains. Acetic acid treatment decreased the rafts levels of Pma1p, the major membrane ATPase responsible for the maintenance of the intracellular pH. In untreated cells, Pma1p was less abundant in the *isc1*Δ mutant than in the wild type strain. However, Pma1p levels were higher in *isc1*Δ cells after treatment with acetic acid.

The *isc1*Δ cells also showed higher levels of phosphorylated (active) Hog1p. This mitogen activated protein kinase phosphorylates Fps1p, an aquaglyceroporine that also facilitates the entrance of acetic acid to the cell, and triggers its endocytosis and degradation.

The overall results suggest that ceramide production contributes to cell death induced by acetic acid especially through hydrolysis of inositolphosphosphingolipids catalyzed by Isc1p. The increase in acetic acid resistance of *isc1*Δ cells was correlated

with the stabilization of the Pma1p ATPase in lipid rafts and higher levels of active Hog1p that probably decrease acetic acid transport.

Resumo

A levedura *Saccharomyces cerevisiae* pode desenvolver apoptose como resposta a diferentes estímulos, apresentando os marcadores típicos de apoptose, tais como a fragmentação de ADN, condensação da cromatina, libertação do citocromo c da mitocondria e a produção de espécies reactivas de oxigénio (ROS). O ácido acético, um produto final da fermentação na levedura, foi já descrito como um agente capaz de induzir apoptose por uma via dependente da mitocôndria.

Os esfingolípidos são uma classe ubíqua de lípidos bioactivos presentes nas membranas eucarióticas e alterações nos seus níveis têm sido relacionados com a apoptose e stress oxidativo em células de levedura e mamíferos. A ceramide acumula-se após diversos tratamentos de stress e aumenta a permeabilidade da mitocôndria ao citocromo c, levando à formação de ROS.

Com o objectivo de caracterizar a contribuição da via de síntese de novo versus catabolismo da ceramida na apoptose induzida pelo ácido acético, foram usados mutantes de levedura afectados no metabolismo da ceramida: *lag1*Δ e *lac1*Δ (incapazes de sintetizar ceramida pela via de síntese de novo), *isc1*Δ (incapaz de sintetizar ceramida pela degradação dos inositolfosfoesfingolípidos) e *ydc*1Δ e *ypc1*Δ (incapazes de degradar a ceramida). Os nossos resultados mostraram que os mutantes *isc1*∆ e *lag1*Δ apresentam maior resistência ao ácido acético, o que foi relacionado com baixos níveis de produção de ROS mitocondriais. No entanto, a morte celular induzida pelo ácido acético não estava associada com a oxidação de proteínas. O silenciamento do gene *YDC1* no mutante *ypc1*∆ e do *LAC1* no mutante *lag1*∆, utilizando um plasmídeo de expressão com o gene antisense, não alterou a resistência ao stress.

Estruturalmente, a disrupção do gene *ISC1* induz uma alteração severa na distribuição das jangadas lipídicas na membrana plasmática. O ácido acético induz um rearranjo nestes microdomínios de membrana. O tratamento com ácido acético diminuiu os níveis nas jangadas da Pma1p, a principal ATPase de membrana responsável por manter o pH intracelular. Em células não tratadas, a Pma1p era menos abundante no mutante *isc1*Δ do que na estirpe selvagem. No entanto, os níveis de Pma1p eram superiores nas células *isc1*Δ após tratamento com ácido acético

As células *isc1*Δ também mostraram níveis superiores de Hog1p fosforilada (activa). Esta proteína cinase, activada por mitogénios, fosforila a Fps1, uma aquagliceroporina que facilita a entrada de ácido acético, e induz a sua endocitose e degradação.

Os resultados obtidos sugerem que a produção de ceramida contribui para a morte celular induzida por ácido acético, especialmente através da hidrólise dos

inositolfosfoesfingolípidos, catalisada pelo Isc1p. O aumento da resistência ao ácido acético nas células *isc1*Δ estava correlacionada com a estabilização da ATPase Pma1p nas jangadas lipídicas e com o aumento dos níveis de Hog1p activa que provavelmente diminui o transporte de ácido acético.

I - Introduction

I-1 Yeast as a model organism

The yeast *Saccharomyces cerevisiae* is a unicellular eukaryotic fungus of the ascomycete family. It is well established that yeast is an ideal model system, where cell architecture and fundamental cellular mechanisms can be successfully investigated and compared with those of higher eukaryotes, including humans. The utilization of *S. cerevisiae* combines several advantages. There is a high degree of conservation, among eukaryotes, of the basic cell structure and processes. The organization and function of molecules and organelles and the signaling pathways essential for the normal cell growth present a significant similarity, which allows their study in a simple organism, like yeast (Botstein *et al.*, 1997). Studies using yeast have contributed to the characterization of a wide range of cell processes, like cell cycle (Kaizu *et al.*, 2010), stress responses (Lushchak *et al.*, 2009), ageing (Barea *et al.* 2009) or even apoptosis (Ludovico *et al.*, 2002).

As a eukaryotic system, *S. cerevisiae* was the first organism to have the genome completely sequenced and available and, since then, several available online databases that compile all the known information about yeast genes and proteins have been developed. As a unicellular organism, it can grow in simple culture media with a short doubling time. It is also a microorganism easy to manipulate genetically and techniques and protocols for manipulation are well defined and optimized (Amberg *et al.*, 2005).

In 1997, it was described the first yeast strain carrying an apoptotic phenotype (Madeo *et al.*, 1997). Since then, yeast has been successfully used as a model system for the study of apoptosis. Yeast cells undergo apoptosis presenting the same characteristic markers of higher eukaryotes. Reactive oxygen species play a central role as regulators of the apoptotic pathway and several orthologues of crucial apoptotic regulators are also present in yeast cells (Madeo *et al.*, 2004).

Acetic acid is a normal end product of the fermentation carried out by *S. cerevisiae* and may be produced by some contaminating bacteria, being a familiar component in the yeast environment (Ludovico *et al.*, 2002). Acetic acid induces a mitochondrial-dependent cell death process in which yeast cells show the characteristic apoptotic markers (Ludovico *et al.*, 2001).

The structure and metabolism of sphingolipids is highly conserved among eukaryotes, from yeast to humans. *S. cerevisiae* cells have been used to study the formation and function of these molecules in eukaryotic cells and the characterization of many mammalian genes associated with sphingolipid metabolism was based on the homology to yeast counterparts (Cowart and Obeid, 2007). For these reasons, studies

using *S. cerevisiae* can provide relevant information about the role of sphingolipid metabolism in the response to acetic acid stress.

I-2 Sphingolipids

Sphingolipids are a ubiquitous class of biologically active lipids present in eukaryotic membranes. In *Saccharomyces cerevisiae*, they are mainly located in plasma membrane and represent about 30% of their total phospholipid content (Patton and Lester, 1991).

Structurally, different sphingolipids share a common base structure, the long chain base (LCB), which in yeast is usually a linear alkane of 18 or 20 carbons, linked by the hydroxyl group in C1 to a polar head group and an amine-linked fatty acid in C2 (Figure I-1). Depending of the sphingolipids and the organism, these components vary. Dihydrosphingosine (DHS) and phytosphingosine (PHS) are the two mains types of LCBs present in *S. cerevisiae* and the difference between them is an additional hydroxyl group on C4 on PHS. Mammals contain small amounts of both DHS and PHS, but their primary LCB is sphingosine, which is a DHS with a 4,5–double bound (Ozbayraktar and Ulgen, 2009).

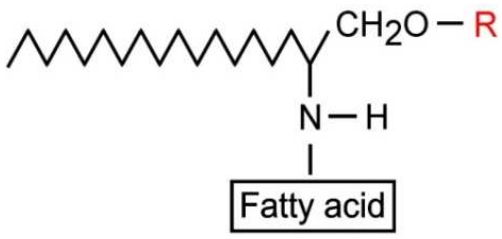

Figure I-1. General structure of sphingolipids. Sphingolipids have a long chain base linked to a polar head group (-R) and to a fatty acid.

Several evidences have been showing that sphingolipids are not only structural molecules, but they also play regulatory roles in cell. They play crucial roles in diverse biologic processes, like cell growth, apoptosis, angiogenesis, differentiation or senescence. Sphingolipids are also involved in several important human diseases, such as sphingolipidoses (Ozbayraktar and Ulgen, 2009), diabetes (Fox *et al.*, 2006), cardiovascular disease (Alewijnse and Peters, 2008) or Alzheimer (He *et al.*, 2010) and other neurological disorders (Ariga *et al.*, 1998).

Therefore, as sphingolipids play crucial roles in several cell processes and in important diseases, it is extremely important to understand how they develop their

functions, and for that it is necessary to understand how sphingolipids are regulated, their metabolism.

I-2.1 Sphingolipids metabolism in *S. cerevisiae*

S. cerevisiae sphingolipid metabolism is schematically represented in figure 2. The first step in yeast sphingolipid metabolism is the condensation of a fatty acyl-CoA, most commonly palmitoyl-CoA, and serine to yield 3–ketodihydrosphingosine. The reaction is catalyzed in the cytoplasmic side of the endoplasmic reticulum by serine palmitoyltransferase (SPT). SPT have two main subunits, encoded by two genes, *LCB1* and *LCB2*, and a small subunit, *TSC3*, that is only required for enzyme activation (Gable *et al.*, 1999).

3-ketodihydrosphingosine is a short lived metabolite and it is immediately converted to dihydrosphingosine (DHS) by 3–ketoreductase, encoded by *TSC10*. DSH is then converted to phytosphingosine (PHS) by the Sur2p/Syr2p C4-hydroxylase. Both DHS and PHS are called sphingoid bases and their functions are not yet distinguishable in yeast (Cowart and Obeid, 2007).

The two sphingoid bases can be phosphorylated to produce dihydro- or phytosphingosine-1-phosphate, in a reaction catalyzed by sphingoid base kinases, encoded by *LCB4* and *LCB5*. Evidences indicate that the phosphorylation takes place in the endoplasmic reticulum, but the kinases were already found in Golgi apparatus and endosome membranes (Ozbayraktar and Ulgen, 2009). The phosphorylated products are then cleaved by sphingoid base lyase Dpl1p to generate hexadecanal and ethanolamine-1-phosphate. This step is the only biochemical route by which sphingolipids are converted in non-sphingolipids molecules. Sphingoid base phosphates can be dephosphorylated by phosphatases to restore the sphingoid base, a process that involves Lcb3p and Ysr3p.

Alternatively to form phosphorylated derivatives, sphingoid bases can suffer an acylation to dihydro- or phytoceramide, which are the central and the most studied molecules of the sphingolipids metabolism. The acylation is catalyzed by ceramide synthase, encoded by two genes, *LAG1* and *LAC1*, and Lip1p, a protein required for ceramide synthase activity (Valleé and Riezman, 2005). Lag1p is known to regulate longevity in *S. cerevisiae*, and its deficiency prolongs the yeast replicative life-span (D´Mello *et al.*, 1994).

The ceramide synthase reaction can be reversed by two ceramidases: a dihydroceramidase, Ydc1p, and a phytoceramidase, Ypc1p. Yeast cells overexpressing both *YDC1* and *YPC1* present an increased breakdown of ceramide, resulting in accumulation of free long chain bases and their phosphates (Mao *et al.*, 2000).

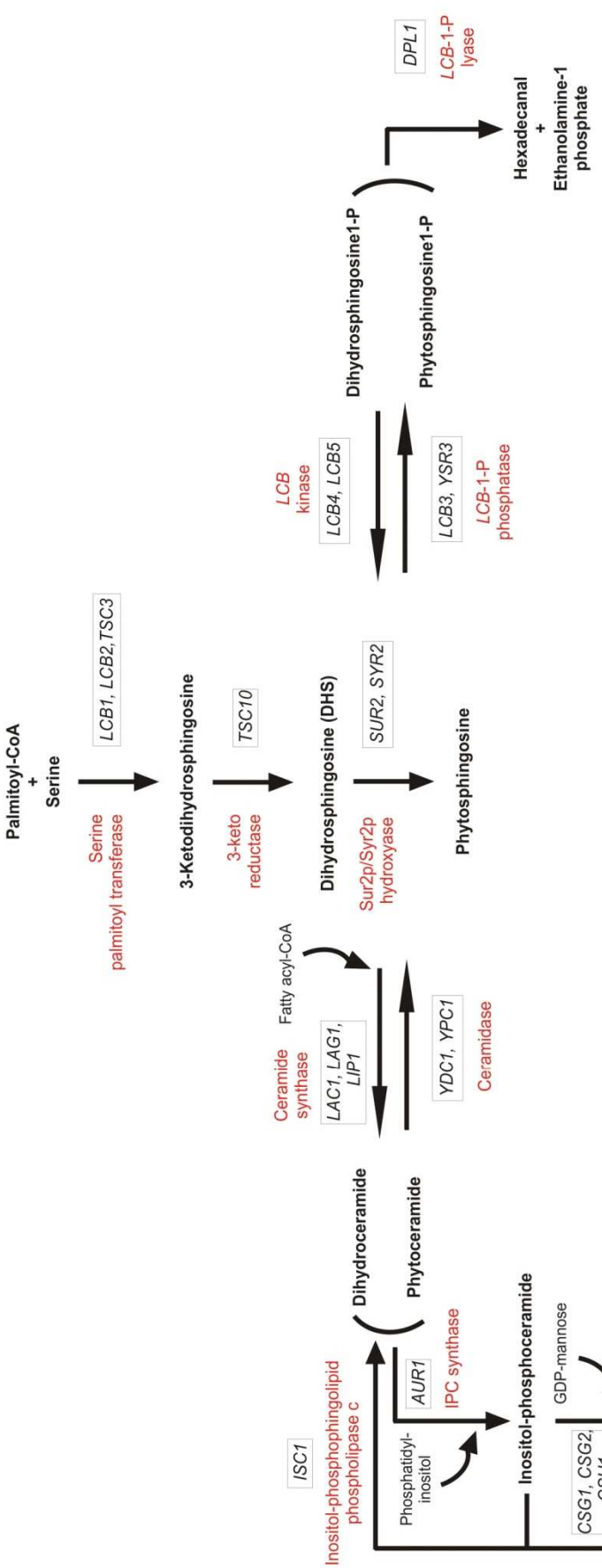

Figure I-2. Scheme of sphingolipids metabolism in *S. cerevisiae*. Enzymes are presented in red. The genes involved are represented close to the enzyme.

Ceramides are transported from the endoplasmic reticulum to the Golgi apparatus, where they are converted into complex sphingolipids by the addition of a polar head group. *S. cerevisiae* has only three types of complex sphingolipids. The enzyme Aur1p adds a *myo* – inositol phosphate to ceramides, forming inositol – phosphoceramide, IPC. IPC can be mannosylated into mannosylinositol phosphorylceramide (MIPC) and another myo – inositol phosphate is then transferred to MIPC, yielding mannosyldiinositol-phosphorylceramide ($M(IP)_2C$), the most abundant complex sphingolipid (Ozbayraktar and Ulgen, 2009).

Ceramide can also be produced through the degradation of the complex sphingolipids in a reaction catalyzed by Isc1p, an inositol-phosphosphingolipid phospholipase C. This enzyme hydrolyzes the head groups of IPC, MIPC and $M(IP)_2C$, producing both phyto– and dihydroceramides.

ISC1 gene was first discovered as codifying for inositol phosphosphingolipid phospholipase C based on homology to bacterial-secreted SMases. The enzyme overexpression resulted in increased levels of yeast ceramides, whereas its deletion causes a complete loss of phospholipase C and an accumulation of all complex sphingolipids. Isc1p activity was reconstructed in lipid-detergent mixed micelles and it was found that the activity is dependent of the presence of Mg^{2+} and it has an optimal pH of 7.5. Phosphatidylserine, cardiolipin and phosphatidylglycerol were also described as Isc1p activators (Sawai *et al.*, 2000). The enzyme has 30% of similarity with the mammalian neutral sphingomyelinase 2 (Sawai *et al.*, 2000). Isc1p has two transmembranar domains, a C terminus that associates with the membrane in the presence of the lipid activators, and an N terminus, with catalytic activity, that is in contact with the membrane to interact with the lipid substrates (Matmati and Hannun, 2008).

The *ISC1* gene deletion causes several phenotypes. *isc1*Δ strain presents a slow growth when compared with wild type under normal growth conditions (Vaena de Avalos *et al.*, 2004). *ISC1* deletion increases cellular resistance to high concentrations of NaCl and LiCl (Betz *et al.*, 2002), but this mutant is more sensitive to methanesulfonate (Chang *et al*, 2002). Almeida *et al.* (2008) demonstrated that Isc1p play a key role in oxidative stress resistance to hydrogen peroxide and in chronological lifespan, through the modulating of redox homeostasis and intracellular iron levels.

I-2.2 Cell signaling mediated by ceramide

Ceramide, the central molecule of the sphingolipids metabolism, acts as a second messenger for several cellular functions, from proliferation and differentiation to growth arrest and apoptosis. Depending of the cell type, ceramide can be linked to a variety of

receptors; moreover this molecule can engage different downstream effectors (Ruvolo, 2001).

Ceramide is produced by the *de novo* synthesis or by the degradation of complex sphingolipids in a response to a wide range of stimulus that induce cellular stress, like ultraviolet light (Dai *et al.*, 2004), ionizing radiation (Vit and Rosselli, 2003), heat shock (Plesofsky *et al.*, 2008) or several chemotherapeutic drugs (Struckhoff *et al.*, 2004). Ceramide-mediated cell transduction acts by activating a number of protein kinases, such as the jun kinases (JNKs), kinase suppressor of Ras (KSR) or the protein kinase PKC-ξ. Conversely ceramide can inhibit some kinases, such as other PKC isoforms and PKB (Ruvolo, 2001).

Protein phosphatases PP1A and PP2A, which are oligomeric holoenzymes, are potent targets of ceramide. PP1 was shown to be inhibited by phosphatidic acid, blocking the dephosphorylation of retinoblastoma product gene (Rb) and cleavage of Poly ADP-ribose polymerase (PARP) (Kishikawa *et al.*, 1999), two tumor suppressor proteins. PP2A targets PKC (Lee *et al.*, 2000) and Akt proteins (Garcia *et al.*, 2003), inactivating them, in response to ceramide. Additionally, ceramide directly targets PKC and Akt substrates, as Raf-1 (Zhou *et al.*, 2002) or caspase-9, respectively (Chalfant *et al.*, 2002)

Ceramide induced apoptosis often involves the SAPK/JNK signaling pathway (Chen *et al.*, 2008). Jarvis *et al.* (1997) reported that, in U937 cells, ceramide mediated cell death is primarily associated with a strong stimulation of SAPK pathway, whereas MAPK/ERK suffers a weak inhibition. The ceramide activation of SAPK is associated to a cell cycle arrest and to an inhibition of cell proliferation (Bourbon *et al.*, 2000).

Ceramide is also known to activate a number of other kinases, including KSR (Zhang *et al.*, 1997) and PKC-ξ (Bourbon *et al.*, 2000). KSR, the kinase suppressor of RAS, seem to mediate pro-inflammatory response, only in the absence of the Bcl2 family member BAD. KSR is required for the ceramide-induced activation of the ERK1/ERK2 kinases in intestinal epithelial cells (Basu *et al.*, 1998), a mechanism that involves the Raf-1 activation (Yan and Polk, 2001). In turn, an inhibition of ERK1/ERK2 kinases was already correlated with the cell growth arrest induced by ceramide in smooth muscle pericytes (Bourbon *et al.*, 2000) and in epidermal melanocytes (Kim *et al.*, 2002).

Thus, strategies to promote ceramide metabolism alterations or the use of ceramide analogs directly may become useful in the treatment of several diseases, but some gaps in signaling mechanism need to be filled.

I-2.3 Sphingolipids and apoptosis

Apoptosis is a highly regulated form of cell death that results in the removal of dispensable cells. In unicellular organisms, apoptosis occurs to release nutrient sources for the fittest and younger individuals in stressful times (Herke *et al.*, 2004). In yeast, apoptosis can be induced by several exogenous agents, like H_2O_2, hyperosmotic stress or acetic acid (Ribeiro *et al.*, 2006), or endogenous agents, resulting from defects on cellular processes, such as DNA damage or disturbed DNA repair mechanisms (Madeo *et al.*, 2004).

Several sphingolipids have been shown to play crucial roles in both cell death signaling and survival, and the relative amounts of each particular sphingolipid determines if cell follows to apoptosis or if it survives. Interestingly, different sphingolipids exert opposite functions in the regulation of cell death and survival. Ceramide is the sphingolipid most intensively studied in relation to cell death induction and it is described that this sphingolipid, together with sphingosine, are proapoptotic molecules, mediating cell cycle arrest and differentiation. In contrast sphingosine-1-phosphate has been associated with proliferation, survival and inhibition of apoptosis (Taha *et al.*, 2006).

Ceramide accumulates in cells after treatment with some apoptotic agents and has been linked with some key mediators of the tumor developing (Radin, 2003). For these reasons, ceramide is designated as a “tumor suppressor lipid” (Chada and Ramesh, 2007) and the intersection between tumor suppressor therapeutics and ceramide signaling was already pointed to be used in chemotherapeutic drugs (Liu *et al.*, 2006).

The effects of ceramide seem to depend of the levels of some members of the classical modulators of apoptosis, namely the Bcl-2 proteins family. Sawada *et al.* (2000) showed that the overexpression of Bcl-2 and Bcl-xL in glioma cells attenuates ceramide accumulation following DNA damage stimuli. In prostate and colorectal cancer cell lines, ceramide induces apoptosis only when Bax is overexpressed (Haefen *et al.*, 2002). It is known that Bcl-2 and Bcl-xL have anti-apoptotic properties and Bax is a proapoptotic molecule, but it is not clear if the accumulation of ceramide is a cause or a consequence of the signal transduction mediated by these proteins. Other regulatory proteins are altered by this proapoptotic molecule, including caspase and non-caspase proteases that are activated in response to ceramide (Taha *et al.*, 2006).

Mitochondria are also affected by ceramide. Ceramide can induce fission in mitochondria of rat cardiomyocytes (Parra *et al.*, 2008), inhibits the complex III of the electron transport system (Gudz *et al.*, 1997) and increases the permeability of the mitochondrial membrane to several proteins, including cytochrome c (Siskind *et al.*, 2002).

Sphingosine, the other described proapoptotic sphingolipid, mediates apoptosis via the caspase-dependent pathway (Taha *et al.*, 2006). Sphingosine induces Bid cleavage, cytochrome c release from the mitochondria and activation of several caspases (Cuvillier *et al.*, 2000) and decreases the levels of both Bcl-2 and Bcl-xL (Shirahama *et al.*, 1997).

Sphingosine-1-phosphate has a role in apoptosis opposite to that of sphingosine and ceramide. It attenuates cell death and growth inhibitory effects caused by several stress factors. In agreement, knockdown of sphingosine kinase causes accumulation of ceramide that leads to cell death (Taha *et al.*, 2005). In human leukemia cells, sphingosine-1-phosphate inhibits the release of mitochondrial proapoptotic factors caused by serum deprivation, anti-Fas, TNF or ceramide (Cuvillier *et al.*, 2001).

The interconversion of these three sphingolipids has a versatile role in the regulation of cell functions and a decisive function in cell fate. Therefore, the modulation of sphingolipid metabolism can be exploited for therapeutic purposes.

I-2.3.1 Sphingolipids and apoptosis in yeast

Despite of being described as a useful model organism, fewer studies were performed in yeast to investigate the involvement of sphingolipid in yeast apoptosis.

Laun *et al.* (2005) characterized the transcriptome of *S. cerevisiae* apoptotic cells and some genes associated with the sphingolipids metabolism were differentially expressed, namely *YPC1*, *YSR3*, *LCB5*, *LAG1* and *IPT1*.

Oxidative stress conditions and ageing induce cell death in yeast by apoptosis. Alterations in sphingolipids metabolism affect cell death under these conditions. It was already shown that mutants lacking the complex sphingolipid $M(IP)_2C$ are more resistant to oxidative stress and have an increased chronological lifespan, whereas yeast mutants with increased levels of $M(IP)_2C$ are hypersensitive to oxidative stress and display a shorter lifespan (Aerts *et al.*, 2006). In addition, overexpression of the yeast ceramidase Ydc1p triggers fragmentation and dysfunction of mitochondria and vacuoles, increasing apoptotic cell death induced by oxidative stress or associated with ageing. Exogenous addition of ceramide restores organelle function (Aerts *et al.*, 2008). The deficiency in Isc1p, the yeast orthologue of mammalian neutral sphingomyelinase 2, also increases apoptotic cell death associated with oxidative stress markers and mitochondrial dysfunction (Almeida *et al.*, 2008).

I-3 Membrane rafts

The plasma membrane of eukaryotic cells has a large variety of different lipid species and the quantities of these lipids exceed the levels required to form a simple bilayer, transcending the classic mosaic fluid model and raising the idea that lipids are organized in microdomains (Munro, 2003). These microdomains are called rafts and many studies show their involvement in several important biological processes, like signal transduction (Pike, 2003), apoptosis (Legembre *et al.*, 2006), cell adhesion (Huang *et al.*, 2006) or exocytosis (reviewed by Salaun *et al.*, 2004) and endocytosis (Nabi and Le, 2003). In addition to roles in normal cellular function, rafts have also been suggested to be the point of cellular entry of a wide range of viruses (Chazal and Gerlier, 2003), bacteria (Hartlova *et al.*, 2010) and toxins (Hasen *et al.*, 2005).

Lipid rafts were already identified in several fungal species, including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and the pathogens *Cryptococcus neoformans* and *Candida albicans* (Wachtler and Balasubramanian, 2006). In *S. pombe* and *C. neoformans* the lipid rafts microdomains are concentrated in a specific region of plasma membrane, whereas in *S. cerevisiae* they are distributed uniformly throughout the plasma membrane (Bagnat and Simons, 2002).

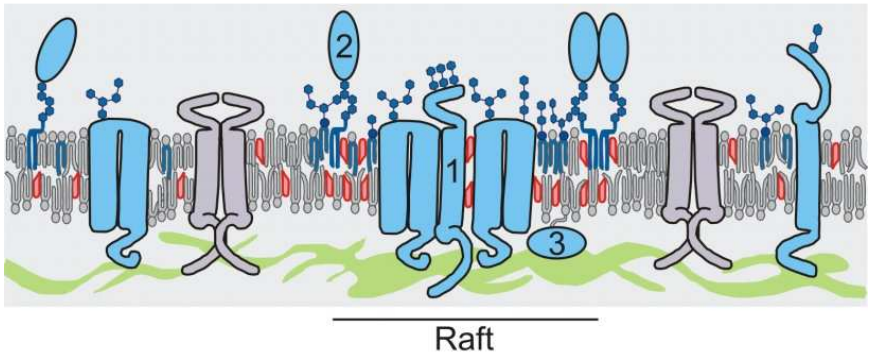

Figure I-3. Lipid rafts organization (Adapted from Lingwood and Simons, 2010). Lipid rafts are microdomains inserted in the plasma membrane (phospholipids in grey) constituted by the lateral association of sphingolipids (dark blue) and cholesterol (red). Several proteins can be associated (light blue): transmembrane proteins (1), GPI-anchored proteins (2) and acylated proteins (3).

As shown in figure I-3, membrane rafts are small microdomains (10–200 nm) constituted by the lateral association of sphingolipids and cholesterol (Lohr and Kersten, 2010). Sphingolipids are higher packed due to the saturated hydrocarbon chains when compared with the unsaturated fatty acids of phospholipids in the non-raft phase (Simons and Vaz, 2004). The empty spaces between them are filled by cholesterol molecules via hydrogen bonds and van der Waals interactions between the 3-OH groups of cholesterol

and the amide groups of sphingolipids (Filippov *et al*., 2006). The fatty-acid chains of the phospholipids present in lipid rafts are more saturated than those in the surrounding membrane and this allows close packing with the saturated acyl chains of sphingolipids (London and Brown, 2000). The interaction between cholesterol and sphingolipids promotes the formation of a liquid ordered phase, which excludes itself from the surrounding phospholipid bilayer that exists in a liquid disordered phase (McMullen *et al.*, 2004).

A useful and common approach for studying lipid rafts and their putative role in biological systems is their biochemical isolation and subsequent identification of common localized signaling components. Lipid raft membranes are traditionally defined as being insoluble in cold (4ºC) non-ionic detergents, such as Triton X-100 (Allen *et al.*, 2007). The fractions of detergent resistant membranes (DRMs) can be separated from the soluble membranes based on their low density (Simons and Toomre, 2000) since they float in a density gradient after ultracentrifugation (Willhite and Wrigh, 2009).

I-3.1 Rafts associated proteins

Lipid rafts contain not only lipids, but they can also bind GPI-anchored proteins, transmembrane proteins and doubly acylated tyrosine kinases of the Src family (Simons and Ikonen, 1997; Figure I-3). This association allows rafts to function as signaling platforms, coupling events on the outside of the cell with signaling pathways inside the cell.

The mechanism of protein association with lipid rafts is not well understood. Proteins associated with raft domains are acyl linked on the N-terminal amino group or on cysteine residues (Wong and Schlichter, 2000), through GPI linkage (Sharma *et al.*, 2004), and covalent attachment with cholesterol (Karpen *et al.*, 2001). The transmembrane segment is another determinant feature for targeting proteins to lipid rafts. A smooth and uniform surface of a transmembrane helix can more readily mix with rigid cholesterol-rich membrane domains (Epand, 2006). Some lipid raft-associated proteins have also a region described as cholesterol recognition amino acid consensus, the tetrapeptide motif Tyr-Ile-Tyr-Phe, which is frequently found near the end of a transmembrane helix of a sterol-sensing domain. Although this sequence is not required to bind cholesterol, it contributes to cholesterol sequestration since the peptide is efficient in recruiting cholesterol (Epand, 2006).

The rafts associated proteins are structural and regulatory diverse. Caveolin is a transmembrane raft protein linked by an acyl group to cholesterol with structural functions. It makes a hairpin loop in the membrane, promoting the formation of small invaginations of

rafts with important regulatory functions, the caveola (Simons and Ikonen, 1997). Dynamin, another protein found in rafts, is a regulatory GTPase implicated in endocytosis that allows the entry of some viruses (Pelkmans and Helenius, 2002). Dynamin is also involved in intracellular protein trafficking, and organelle partitioning (Hinshaw, 2000).

I-3.1.1 The raft associated protein Pma1p

ATPases are another group of proteins present in rafts. Yeast ATPases are important proteins that pump protons out of the cell, creating a pH and an electrochemical gradient that drives the transport of some important molecules, like aminoacids, sugars or inorganic ions (Scarborough, 2000).

The first evidence for the existence of the yeast membrane ATPase was provided by Pena *et al*. (1969), through physiological studies. Other evidences proved the existence of the protein, such as measures of enzymatic activity (Scarborough, 1976) and enzyme purification (Dufour and Goffeau, 1978).

The gene encoding for the plasma-membrane H^+-ATPase, *PMA1*, was cloned and sequenced in *S. cerevisiae* (Serrano *et al.*, 1986), *S. pombe* (Ghislain *et al.*, 1987) and some other fungal species (Addison, 1986). The Pma1p protein is the major membrane ATPase, constituting 25–50% of the total plasma membrane proteins (Roberg *et al.*, 1999). In addition to its role in the transport of some important nutrients, Pma1p has a second physiological function that consists in the maintenance of the neutral pH of the cytosol. Portillo and Serrano (1989) demonstrated that the ATPase is rate-limiting for growth and that a decrease in ATPase activity is correlated with a decrease intracellular pH.

Structurally, the ATPase consists in a 100 kDa polypeptide subunit with 918 amino acid residues (Serrano *et al.*, 1986), linked to the lipid layer by four transmembrane segments at the N-terminal end of the molecule and six at the C-terminal end. The ten transmembrane segments are mainly α-helical in structure, except for two pieces in the middle of two segments, which are directly involved in the transport of calcium ions. The cytoplasmic portion of the molecule is arranged into three distinct domains: N, containing the ATP-binding site; P, containing the phosphorylation site, and A, an anchor domain (Morsomme *et al.*, 2000).

Pma1p, as the sphingolipids, is biosynthesized in the ER membrane, where it homo-oligomerizes to form a 1.8 MDa complex that is also resistant to extraction by detergents (Gaigg *et al.*, 2005). This protein-lipid complex is then packaged into transport vesicles (Roberg *et al.*, 1999) to be transported to Golgi apparatus. From there, Pma1p is transported to the cell surface incorporated in the secretory pathway, but it does not

intersect with endosomes (Harsay and Schekman, 2002). At the cell surface, Pma1p becomes stabilized to perform its functions.

I-4 Acetic acid

The continuous changing of the environmental conditions faced by *S. cerevisiae* during alcoholic fermentation can result in an incomplete fermentation with the release of some unwanted metabolites. Acetic acid is one of these metabolites and its adverse effects, as well as those of other weak acids, such as benzoic or lactic acid among others, are well described in yeast. These compounds are also often used as preservatives in food or drinks (Narendranath *et al.* 2001; Kresnowati *et al.*, 2008).

The production of acetic acid during alcoholic fermentation is linked to osmotic stress (Erasmus *et al.*, 2003; Figure I-4). To counter-balance osmotic stress, yeast increases the production of intracellular glycerol which is accompanied by the oxidation of NADH to NAD^+. To maintain de redox balance, NAD^+ has to be reduced and this occurs with an increase in the oxidation of acetaldehyde to acetate, and consequently, an increase in acetic acid production (Nevoigt and Stahl, 1997). A study with *S. cerevisiae* submitted to sugar-induced osmotic stress showed an increase in the expression of genes that encode the enzymes involved in the production of acetic acid (Erasmus *et al.*, 2003).

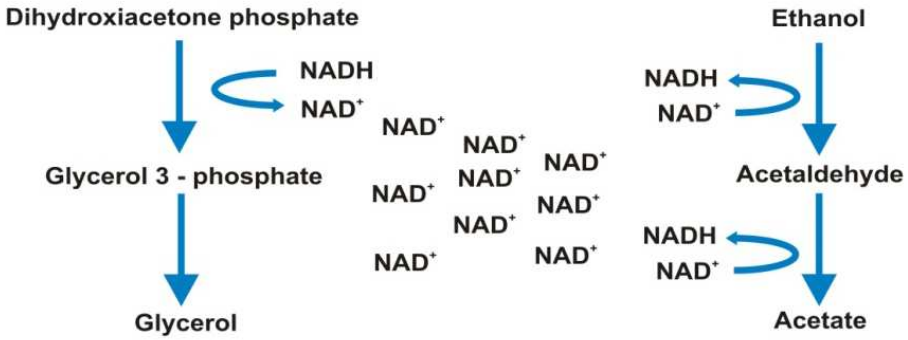

Figure I-4. Production of acetic acid due to osmotic stress. To counter-balance the NAD^+ produced in the synthesis of glycerol, yeast cells oxidize acetaldehyde.

The presence of acetic acid during fermentation can also result from other sources, such as contaminating organisms. In wine, *Brettanomyces,* a genus from *Saccharomycetaceae* family, are a known yeast contaminant that can accumulate acetic acid during industrial fermentation (Aguilar-Uscanda *et al.*, 2000). The contamination can also be due to bacteria, the genus *Gluconobacter* from *Acetobacteriaceae* family is a well known bacteria that produces acetic acid (Bartowsky and Henschke, 2008).

S. cerevisiae is also capable of use acetic acid and other weak acids as the only carbon source (Casal *et al.*, 1996). After the dissociation of acetic acid in acetate, this compound is converted by acetyl-CoA synthetase in acetyl-CoA. Acetyl-CoA then enters in the Krebs cycle to be oxidized to carbon dioxide (Berg *et al.*, 1996).

I-4.1 Transport of acetic acid across the plasma membrane

Acetic acid is partially dissociated in aqueous systems, establishing equilibrium between the undissociated form and its anionic form, acetate. The degree of dissociation depends on the pKa of acetic acid (4.75) and the pH of the solution (Mollapour and Piper, 2007). This property is extremely important because it determines how acetic acid is transported across the membranes and the effects caused by acetic acid. Even its utilization as carbon source is dependent on its transport across the plasma membrane.

Several studies contributed to the characterization of the mechanisms involved in the transport of acetic acid through the plasma membrane (Casal *et al.*, 1996; Cardoso and Leão, 1992). The anionic form is transported by active transport in a process that involves an acetate-proton symport, encoded by *JEN1* (Casal *et al.*, 1999), or by a more general monocarboxylate carrier, an acetate-propionate-formate permease (Paiva *et al.*, 1998). Both mechanisms are inhibited by ethanol and subjected to glucose repression (Casal *et al.*, 1996; Paiva *et al.*, 1998).

The undissociated acetic acid enters the cell by passive transport through a recently discovered aquaglyceroporin, Fps1p (Mollapour and Piper, 2007). Inside the cell, the acid dissociates as a function of the pH differences and acidifies the neutral cytosol (Casal *et al.*, 1998; Figure I-5).

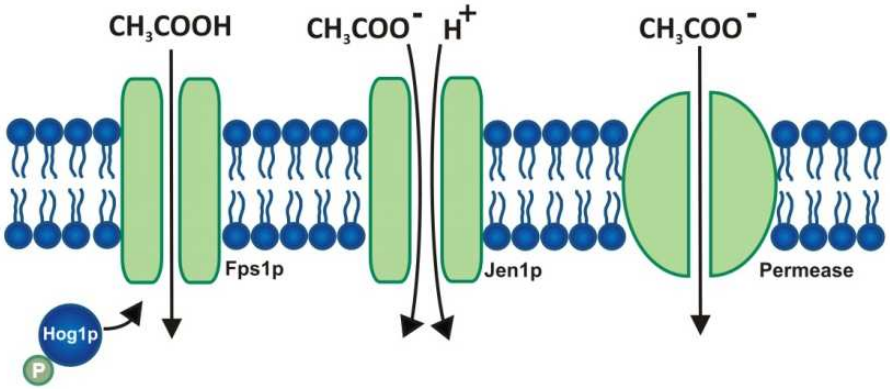

Figure I-5. Transport of acetic acid across the plasma membrane. The undissociated molecule can cross the membrane through Fps1 channel. The dissociated form of acetic acid is transported by Jen1p or by an acetate-propionate-formate permease.

I-4.1.1 The acetic acid channel Fps1p

The plasma membrane aquaglyceroporin Fps1p was the first example of a channel for carboxylic acids to be reported in yeasts. Fps1p is also involved in the regulation of the intracellular glycerol concentration. Increased external osmolarity induces Fps1p closure to prevent the release of glycerol. In contrast, a decrease in the external osmolarity causes channel opening, allowing a rapidly export of glycerol and preventing bursting (Tamás *et al.*, 1999). The channel is also required for controlling turgor pressure during fusion of mating yeast cells (Philips and Herskowitz, 1999).

The pathway responsible for the regulation of Fps1p in response to changes in osmolarity appears to involve the Hog1p (High Osmolarity Glycerol response) MAP kinase (Chen and Thorner, 2007). Cells use the HOG pathway to stimulate transcriptional responses in order to counter-balance osmotic stress. The Hog1p phosphorylation leads to its translocation into the nucleus where it activates four different transcription factors (Msn2p, Sko1p, Hot1p and Spm1p), modulating more than 10% of the total yeast genome (O´Rourke and Herskowitz, 2004; Figure I-6).

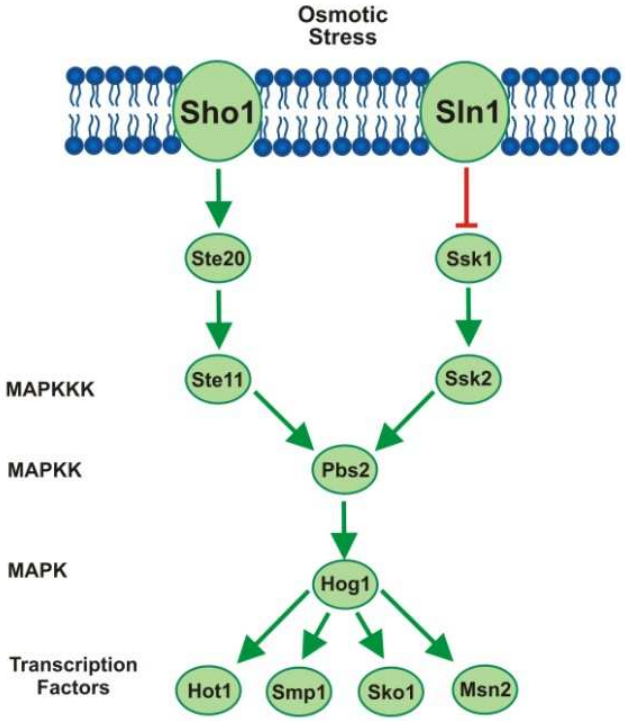

Figure I-6. Schematic diagram of the yeast HOG pathway. Pbs2 integrates signals from two major independent upstream osmosensing mechanisms, which leads to the activation of specific MAPKKKs.

When yeast cells are exposed to acetic acid, Hog1p is activated and phosphorylates Fps1p, targeting this channel for endocytosis and consequent degradation

in the vacuole. This adaptive response decreases the uptake of acetic acid, protecting yeast cells from its toxic effects. In agreement, mutants in the Fps1p channel are more resistant to acetic acid and accumulate lower levels of acetic acid than wild type cells and the activation of Hog1p is abolished with the loss of Fps1p (Mollapour and Piper, 2007).

I-4.2 Acetic acid as an inducer of apoptosis

A large variety of stress conditions and compounds are able to induce yeast cell death with apoptotic features. H_2O_2 (Ribeiro *et al.*, 2006), aspirin (Sapienza *et al.*, 2008), HOCl (King *et al.*, 2004), or acetic acid (Ludovico *et al.*, 2001), have already been employed in order to stimulate apoptosis in yeast. As acetic acid is a normal end product of the yeast alcoholic fermentation, acetic acid-induced apoptosis could also be considered as a close natural scenario of yeast apoptotic cell death. This seems to be the reason why the role of this agent in yeast apoptosis has been extensively studied (Ludovico *et al.*, 2001).

In fact, acetic acid is an agent capable of compromise cell viability (Pinto *et al.*, 1989) and it induces apoptosis in yeast cells, showing the typical apoptotic markers, such as chromatin condensation along the nuclear envelope, exposure of phosphatidylserine at the outer surface of the plasma membrane and formation of DNA strand breaks (Ludovico *et al.*, 2001).

Several orthologues of key mammalian apoptotic regulators were associated to yeast apoptosis induced by acetic acid. Ludovico *et al.* (2002) demonstrated that acetic acid-induced cell death was mediated by a mitochondria-dependent apoptotic pathway, since the depletion of mitochondrial DNA prevents cell apoptosis. The programmed cell death after treatment with acetic acid also resulted in cytochrome c release from the mitochondria and ROS production (Ludovico *et al.*, 2002).

In mammalian apoptosis, caspases are the principal executers of this pathway and some metacaspase proteases have been characterized as apoptotic regulators in plants, fungi and protozoa supporting the concept of a widely conserved cell death pathway (Uren *et al.*, 2000). In yeast, it was already described an orthologue of mammalian caspases, Yca1p (Madeo *et al.*, 2002), that showed to be involved in acetic acid induced apoptosis (Guaragnella *et al.*, 2006).

The yeast orthologue of mammalian AIF1 (Apoptosis-inducing factor), Aif1p, is a flavoprotein with oxidoreductase activity localized in the mitochondrial intermembrane space that has an important function in the apoptotic pathway. Upon apoptosis induction, AIF translocates to the nucleus, where it leads to chromatin condensation and DNA

degradation. In yeast cells treated with acetic acid, Aif1p is released from mitochondria and migrates to the nucleus (Wissing *et al.*, 2004).

Acetic acid-induced apoptosis has also been associated with mitochondrial fragmentation and degradation. It was recently shown that Pep4, an orthologue of the mammalian cathepsin D, is released from the vacuole into the cytosol during acetic acid induced apoptosis to act in mitochondrial degradation. The mitochondrial ATP/ADP carrier also affects mitochondrial degradation, possibly through its involvement in mitochondrial permeabilization (Pereira *et al.*, 2010).

Yeast has also been used to study the role of mammalian protein kinase C (PKC) isoforms in the regulation of programmed cell death induced by acetic acid (Saraiva *et al.*, 2006). The PKC-α, -δ, -ξ or -□ isoforms are pro-apoptotic molecules and PKC isoforms modulate the Bcl-xL anti-apoptotic activity differently, through interference with its phosphorylation state.

II – Aim of the work

II - Aim of the work

The major goal of this thesis was to elucidate the role of ceramide pathway in yeast apoptosis induced by acetic acid. The following approaches were used:

- Identification of enzymes of ceramide metabolism with a role in cell death induced by acetic acid;
- Characterization of cellular and molecular mechanisms underlying changes in cellular resistance to acetic acid in yeast mutant cells affected in ceramide metabolism. The involvement of lipid rafts and its major ATPase, Pma1p, and the role of Hog1p, the MAP kinase of the HOG signaling pathway that plays a key role in cellular adaptation to acetic acid stress, were investigated.

III - **Material and methods**

III-1 Yeast strains, plasmids and growth conditions

Saccharomyces cerevisiae strains used in this study are listed in table III-1. Wild type strain (CG379), *lac1*Δ, *lag1*Δ, *ydc1*Δ, *ypc1*Δ and *isc1*Δ mutants were grown in YPD (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose) or in Synthetic Complete (SC) - Galactose medium (2% (w/v) galactose, 0.67% (w/v) yeast nitrogen base without aminoacids, 0.14% (w/v) drop-out medium 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). The strains transformed with plasmids were grown in the same medium, lacking uracil. Solid media were prepared by adding 1.5% (w/v) agar.

Cells were grown early to exponential phase (OD_{600}=0.5-0.6) in an orbital shaker at 140 rpm, at 26ºC, with a ratio of flask volume / medium of 5:1.

Table III-1. *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] carrying pYES2 (*URA3*, GAL promoter)	This study
*lac1*Δ	[CG379] *lac1*Δ :: *KanMx4*	Rego, A.
*lag1*Δ	[CG379] *lag1*Δ :: *KanMx4*	Rego, A.
*lag1*Δ pYES2	[*lag1*Δ] carrying pYES2 (*URA3*)	This study
*lag1*Δ pYES2-*LAG1*	[*lag1*Δ] carrying pYES2-*LAG1* (*URA3*)	This study
*lag1*Δ pYES2-AS-*LAC1*	[*lag1*Δ] carrying pYES2-AS-*LAG1* (*URA3*)	This study
*ydc1*Δ	[CG379] *ydc1*Δ :: *KanMx4*	Rego, A.
*ypc1*Δ	[CG379] *ypc1*Δ :: *KanMx4*	Rego, A.
*ypc1*Δ pYES2	[*ypc1*Δ] carrying pYES2 (*URA3*)	This study
*ypc1*Δ pYES2-AS-*YDC1*	[*ypc1*Δ] carrying pYES2-AS-*YDC1* (*URA3*)	This study
*isc1*Δ	[CG379] *isc1*Δ :: *KanMx4*	Rego, A.
*isc1*Δ pYES2	[*isc1*Δ] carrying pYES2 (*URA3*)	This study
*isc1*Δ pYES2-*ISC1*	[*isc1*Δ] carrying pYES2-*ISC1* (URA3)	This study

The plasmids used in this study are described in table III-2. For the construction of the pYES2-*LAG1* plasmid, the DNA fragment corresponding to the *LAG1*-coding region was amplified by PCR, using *S. cerevisiae* CG379 genomic DNA (54 ºC as annealing temperature and 1 min 20 sec as elongation time) and primers *LAG1_Hin*dIII_Fw and

*LAG1_Xho*I_Rv (oligonucleotide sequences in table III-3), which introduces *Hin*dIII and *Xho*I restriction sites at each end of the fragment.

Table III-2. Plasmids used in this study

Plasmids	Source or reference
pYES2	Invitrogen
pYES2-*LAG1*	This study
pYES2-*ISC1*	Y. Hannun, Medical University of South Carolina, Charleston, USA
pYES2-AS-*LAC1*	This study
pYES2-AS-*YDC1*	This study

Table III-3. Oligonucleotides used in this study (restriction sites underlined)

Name	Oligonucleotide Sequence
*LAG1_Hin*dIII_Fw	5´-ACGACAAGCTTAACATGACATCAGCTACGGACAAAT-3´
*LAG1_Xho*I_Rv	5´-AGATACTCGAGCGTTTATTCACACTTTTCCTTAGAT-3´
*LAC1*_anti_Fw	5´-TAAAAGCTTGCTTCATCGACAATAAGCCAAG-3´
*LAC1*_anti_Rv	5´-CACCTCGAGCCTATGAATATCCTTTTTCGTTGGAGTA-3´
*YDC1*_anti_Fw	5´-GAAAAGCTTCAATTACTGTTCAGCTGGCCTTATCCA-3´
*YDC1*_anti_Rv	5´-CAACTCGAGTCCATGGTTATTCTTTTTTGTTTCATCATC-3´

The plasmid used for attempted antisense inhibition of *LAC1* gene expression was constructed as follows. *LAC1*-coding region was amplified by PCR (55 ºC as annealing temperature and 1 min 20 sec as elongation time) using *S. cerevisiae* CG379 genomic DNA and primers *LAC1*_anti_Fw and *LAC1*_anti_Rv. The primers were designed to generate DNA fragments flanked by *Hin*dIII and *Xho*I restriction sites and with the start and the termination codons exchanged. The plasmid used for antisense inhibition of *YDC1* gene expression was constructed using the primers *YDC1*_anti_Fw and *YDC1*_anti_Rv in a similar strategy.

The DNA fragments obtained by PCR were cloned into pYES2 *Hin*dIII and *Xho*I restriction sites. *E. coli* DH5α was transformed by standard procedures (Froger and Hall, 2007) with the generated plasmids or with pYES2 (empty vector) and the transformants were selected on solid LB medium (1% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 1.0% (w/v) NaCl, 2% (w/v) agar) supplemented with 100 µg/mL ampicillin. Plasmids were extracted from *E. coli* using GenElute™ Plasmid Miniprep kit (Sigma-Aldrich) and their insertion was confirmed by digestion with *Xho*I and *Hin*dIII restriction enzymes, followed by electrophoresis in agarose gels.

III-2 Yeast electroporation

Yeast cells were transformed with the plasmids by electroporation. Electroporation is a method used to introduce polar molecules into a host cell through the cell membrane by electric pulses. A high-voltage electric field is applied to the cells, producing transient holes in the cell membrane through which plasmid DNA.

The *lag1*Δ strain was transformed with pYES2 (empty vector), pYES2-*LAG1* or pYES2-AS-*LAC1* (gene expression under the galactose promoter). The *isc1*Δ strain was transformed with pYES2 or pYES2-*ISC1*. The *ypc1*Δ strain was transformed with pYES2 or pYES2-AS-*YDC1*. The wild type strain was transformed with pYES2.

III-2.1 Preparation of electrocompetent cells

Cells were grown in 50 mL of YPD medium to exponential phase (OD_{600} = 1.3 – 1.5), harvested, resuspended in 10 mL of TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and 0.1 M lithium acetate pH 7.5 and gently shacked during 45 min at 30 ºC. It was added 250 µL of 1M DTT and cells were shacked 15 min at 30 ºC. Ice-cold sterile water was added for a final volume of 50 mL and cells were centrifuged at 4 ºC. Cells were washed with 25 mL of ice-cold sterile water, 2 mL of 1 M sorbitol (4 ºC), and resuspended in 50 µL of 1 M sorbitol (4 ºC).

III-2.2 Electro-transformation and plating

Electrocompetent cells (40 µL) were mixed with 0.1 µg of plasmid and incubated on ice for 30 min. These cells were then transferred to a sterile 2 mm electroporation cuvette that was mounted into the Pulse controller (Bio-Rad) between the anode and cathode. An electric pulse was applied in parallel (1.5 kV, 25 µF, and 200 Ω). After the pulse delivery, the cuvette was removed and 1 mL of SC - Glucose medium supplemented with 1 M sorbitol was immediately added. Cells were incubated at 26 °C for 1h at 140 rpm for recovering. Different dilutions of the cells were spread into selective SC plates lacking uracil and grown at 26ºC for 3 days.

III-3 Acetic acid treatment and viability assays

Cells were grown near to early exponential phase (OD_{600}=0.5 - 0.6) in SC - Galactose medium. The culture was centrifuged (5,000 rpm, 5 minutes) and the medium was replaced by SC – Galactose medium with the pH adjusted to 3.0 (set with HCl). Cells

were treated with 180 mM acetic acid for 200 min at 26°C in an orbital shaker (140 rpm). Treated and control (incubated for 200 minutes in SC – Galactose medium with pH adjusted to 3.0) cells were diluted in YPD medium and plated on YPD medium containing 1.5 % (w/v) agar.

Cell viability was determined by colony forming unit (c.f.u.) counts after 3 days incubation at 26°C. No further colonies appeared after that incubation period. The percentage of viable cells was estimated considering 100% survival the number of c.f.u. obtained after 200 minutes incubation in the absence of acetic acid.

III-4 Analysis of cell cycle by flow cytometry

Staining of yeast cells with propidium iodide (PI), coupled to flow cytometry, represents a useful technique to quantify the proportion of cells in each phase of the cell cycle.

Single mutants *lac1*Δ, *lag1*Δ, *ydc1*Δ, *ypc1*Δ and *isc1*Δ and wild type strains were used in this test. Cells (1×10^7) were harvested, resuspended in 1mL of 70% ethanol and agitated at room temperature, during 1 hour, to fix. The cells were then washed with 50 mM sodium citrate pH 7.0 and resuspended in 1 mL of the same solution. RNAse A was added to a final concentration of 0.25 mg/mL and the samples were incubated for 1 h at 50 ºC. Cells were washed with water and resuspended in sodium citrate buffer. The samples were sonicated for 30 seconds at output 2, duty cycle 30 %, centrifuged and resuspended in 1 mL of the same solution. Propidium iodide was then added to a final concentration of 16 µg/mL and incubated for 30 minutes at the room temperature in the dark. Fluorescence was measured on the FL-2 channel of a Becton-Dickinson FACSort flow cytometer (excitation and emission 488 and 585 nm, respectively). The data was analyzed using the FlowJo software (Tree Star).

III-5 Determination of ROS levels

The production of reactive oxygen species (ROS) by mitochondria was monitored by flow cytometry using a mitochondria-selective probe, MitoTracker Red CM-H_2XROS (Molecular Probes™), as described by Ludovico *et al.* (2002). The reduced probe does not fluoresce until entering an actively respiring cell, where it is oxidized by ROS to a red fluorescent compound sequestered in the mitochondria.

Yeast cells (single mutants *lac1*Δ, *lag1*Δ, *ydc1*Δ, *ypc1*Δ and *isc1*Δ and wild type strains) untreated or treated with 180 mM acetic acid were harvested (1 mL of the culture), washed and resuspended in 1 mL of phosphate-buffered saline solution PBS (80 mM

Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.4). The culture was then divided in two parts and 500 µL were incubated with 0.4 µg/ml MitoTracker Red CM-H_2XROS for 20 minutes at 37°C, in the dark. The other 500 µL of cells were submitted to the same conditions, in the absence of the Mitotracker dye (negative control). Fluorescence was measured on the FL-3 channel of a Becton-Dickinson FACSort flow cytometer. The data was analyzed using the FlowJo software (Tree Star).

III-6 Analysis of protein carbonylation

Protein carbonylation is another oxidative stress marker and was analysed by western-blot followed by immunodetection. This method is based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to form a 2,4-dinitrophenylhydrazone (DNP), which can then be detected by chemiluminescence using an anti-DNP primary antibody.

III-6.1. Samples preparation

Yeast cells (single mutants *lac1*Δ, *lag1*Δ, *ydc1*Δ, *ypc1*Δ and *isc1*Δ and wild type strains) untreated or treated with 180 mM acetic acid were harvested (25 mL of the culture) for 5 minutes at 5,000 rpm, washed with water and resuspended in 100 µL of phosphate buffer (50 mM sodium phosphate, 0.1 mM EDTA, pH 7.0). Glass beads and protease inhibitor cocktail (Complete™ Mini EDTA-free tablets, Roche) were added and cells were lysed by vortexing for 5 cycles of 1 min, with 1 min intervals on ice. The supernatant was collected after centrifugation at 13,000 rpm for 15 minutes. Protein content of cellular extracts was estimated by the method of Lowry (Lowry *et al*., 1951), using bovine serum albumin as standard.

III-6.2 Western-blot and immunodetection

Cellular extracts (10 µL) were mixed with the same volume of 12% SDS and 20 µL volumes of 20 mM DNPH, 10% trifluoroacetic acid. The samples were incubated for 30 minutes at room temperature in the dark and neutralized with 15 µL of neutralizing solution (2 M Tris, 30 % glycerol, 19 % β-mercaptoethanol). Proteins (12 µg) were separated by SDS-PAGE. Polyacrylamide gels were prepared as described in table III-4. Electrophoresis was performed at 12 mA during the stacking gel and 16 mA during the running gel, using 0.025 M Tris pH 8.3, 0.192 M glycine, 0.1 % SDS as buffer.

After electrophoresis, proteins were transferred to a hydrated nitrocellulose membrane (Hybond-ECL, GE Healthcare), using a semi-dry system and a transfer buffer (39 mM glycine, 48 mM Tris, 0.0375 % SDS, 20 % methanol). Blotting was performed at 0.8 mA/cm^2 during 1 h. The membrane was blocked with 5 % of milk powder (low fat) in TPBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.05 % Tween) for at least 1 h and incubated 2 h with the primary antibody rabbit IgG anti-DNP (1:1,500; Sigma Aldrich). The membrane was than washed twice with TPBS during 15 minutes and incubated with the secondary antibody anti-rabbit IgG-peroxidase (1:5,000; Sigma Aldrich). The membrane was washed twice with TPBS during 15 minutes and twice with PBS during 15 min. Immunodetection was performed by chemiluminescence, using a kit from GE Healthcare. The membranes were exposed to a Hyperfilm-ECL (GE Healthcare) for 1-3 minutes, and the film was developed.

Table III-4. Reagents used in the preparation of a poliacrylamide gel.

Reagents	Running gel (12.5%)	Running gel (7.5%)	Stacking gel
30 % Acrylamide	2.9 mL	1.73 mL	250 µL
Running Buffer (1.5 M Tris-HCl pH 8.8 0.4 % SDS)	1.64 mL	1.64 mL	-
Stacking Buffer (0.5 M Tris-HCl pH 6.8 0.4 % SDS)	-	-	625 µL
H_2O	2.31 mL	2.18 mL	1.6 mL
10 % Ammonium Persulfate	52 µL	52 µL	18.8 µL
TEMED	5.5 µL	5.5 µL	2.5 µL

All the procedures were performed with two replicas and the second gel was silver stained as a control for protein loading.

III-6.3 Silver staining

Silver staining is a highly sensitive method for detecting proteins and nucleic acids in polyacrylamide gels. The staining was performed as previously described (Chevallet *et al.*, 2006). After electrophoresis, the gels were fixed twice in 30% ethanol, 10% acetic acid for 30 min and rinsed in 20% ethanol, for 10 min and then water, for 10 min. The gel was soaked for one minute in 0.2 g/L sodium thiosulfate, washed in water for 1 min and incubated with 2 g/L silver nitrate during 15 to 30 min. The developing solution (0.026% Formaldehyde, 3% Sodium carbonate, 1×10^{-3} % Sodium tiosulfate) was added to the gel

until bands developed. The gel was immediately transferred to the stop solution (50 g/L Tris and 2.5 % Glacial acetic acid) and stored in water at 4ºC.

III-7 Filipin staining

Sterol-lipid distribution was assessed in vivo using filipin, which is a useful probe for determining, by fluorescent microscopy, the relative levels of unesterified ergosterol in cells. The filipin staining was performed basically as previously described (Beh and Rine, 2004). One ml of 37.5% formaldehyde was added to 9 ml of cell culture grown in SC-Galactose medium (DO_{600}=0.5-0.6). After 10 minutes of constant mixing at 26°C, the fixed cells were centrifuged 5 min at 3,000 rpm (4ºC) and the pellet was washed twice with 10 ml of distilled water. The washed cells were resuspended in 1 ml of water from which 0.4 ml was mixed with 8 µl of 5 mg/mL filipin solution in DMSO. After incubation in the dark with filipin for 15 minutes at 26°C, cells were mounted on polylysine coated slides, sealed under coverslips with nail polish spotted directly on slides and filipin fluorescence was observed with a UV filter set on an Zeiss Axiovert 200M microscope (Carl Zeiss, Germany) equipped with a CoolSnap HQ digital camera (Roper).

III-8 Analysis of rafts

The isolation of membrane rafts was based in their characteristic property, the insolubility in cold (4ºC) non-ionic detergents such as Triton X-100.

Membrane rafts were isolated from wild type and *isc1*∆ cells untreated or treated with 180 mM acetic acid and the Pma1p protein was analyzed by Western-blotting followed by immunodetection. These assays were performed as previous described (Willhite and Wrigh, 2009) with some alterations.

III-8.1 Isolation of membrane fractions

Yeast cells (250 mL, DO_{600} = 0,5-0,6) were pelleted at 5,000 rpm, 4ºC and washed with 50 mL of cold water. Cells were resuspended with 500 µL of TNE buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5), 25 µL of protease inhibitor cocktail (Complete™ Mini EDTA-free tablets, Roche) and 50 µL of PMSF 100 mM.

Mechanical lysis was accomplished using a FastPrep24 homogenizer (MP Biomedicals), by vigorous shacking of the cell suspension in the presence of glass beads. Five cycles of 1 min were used, with 1 min intervals on ice. The homogenate was cleared

by centrifugation at 3,000 rpm for 5 min at 4ºC. Membranes were isolated from the cleared lysate by centrifugation at 13,000 rpm for 45 min at 4ºC.

The membrane pellet was resuspended in 250 µL TNE buffer containing a protease inhibitor cocktail and PMSF. Suspension was aided by vortexing and repeated pipetting. Protein content of membrane extracts was estimated by the method of Lowry *et al*. (1951) using bovine serum albumin as the standard.

III-8.2 Isolation of membrane rafts and analysis of Pma1p

Membrane proteins (1.2 mg in a final volume of 418 µL TNE) were treated with 1% (v/v) Triton X-100 on ice for 30 minutes. An Optiprep solution (Sigma-Aldrich™) was then added to a final concentration of 40% (v/v) and the mix was loaded at the bottom of an ultracentrifuge tube. The sample was overlaid with 6.6 mL of 30% (v/v) Optiprep in TNE buffer and the tube was filled with TNE buffer (Figure III-1).

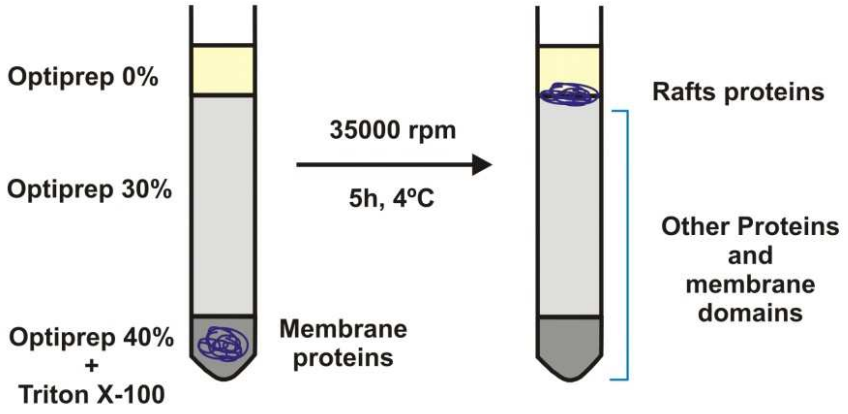

Figure III-1. Figure III-1. Schematic representation of the Optiprep Gradient and the rafts floating. Membrane proteins were treated with 1% (v/v) Triton X-100 and an Optiprep solution was added to a final concentration of 40% (v/v). The mix was loaded at the bottom of an ultracentrifuge tube and overlaid with 6.6 mL of 30% (v/v) Optiprep in TNE buffer. The tube was filled with TNE buffer. Samples were spun at 35,000 rpm at 4ºC for 5 h, leading to the separation of rafts (dark blue) from the other membrane domains.

Samples were spun at 35,000 rpm in a Sorvall ultra Pro80 ultracentrifuge using the SW41 rotor, at 4ºC for 5 h. The upper fraction (about 2 mL) containing raft proteins (interface between TNE and 30% (v/v) Optiprep), was pippetted to a new ultracentrifuge tube that was filled with TNE buffer containing PMSF. Samples were centrifuged at 35,000 rpm, at 4ºC for 1 h. The supernatant was discarded and the pellet resuspended in 15 µL of 4x sample buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol), 15 µL of 8M urea, 2 µL of protease inhibitor cocktail and PMSF (0.1 mM, final concentration). Samples were incubated at 37ºC for 15 min and loaded on a

7.5% polyacrylamide gel. Proteins were separated by SDS-PAGE and blotted into Hybond-ECL (GE Healthcare) or silver stained, as described above. Immunodetection of Pma1p was performed as described above, using rabbit IgG anti-Pma1p (1:4,000; kindly provided by Prof. Ramon Serrano, Universidad Politécnica de Valencia, Spain), as primary antibody, and anti-rabbit IgG-peroxidase (1:5,000; Sigma Aldrich), as secondary antibody.

To test if Pma1p was phosphorylated, membrane rafts isolated by ultracentrifugation were treated with λ-phosphatase before SDS-PAGE and immunobloting. Briefly, the raft pellets were resuspended in 19.6 µL of phosphatase buffer (500 mM Tris-HCl pH 7.5, 1 M NaCl, 20 mM DTT, 0.1% Brij 35), 2.5 µL of 10 mM $MnCl_2$ and 1.9 µL of water, and incubated at 30ºC for 30 min, in the presence of 400 U of λ-phosphatase (λ-PPase; New England BioLabs) or buffer (control). The reaction was stopped by adding 25 µL of 4x sample buffer and 50 µL of 8M urea.

III-9 Analysis of phospho-Hog1p

For the analysis of phospho-Hog1p levels, protein extracts were prepared as described in III-6., using 100 µL of phosphate buffer, 5 µL of protease inhibitor cocktail and phosphatase inhibitors: 12.5 µL of 50 mM sodium fluoride, 4 µL of 5 mM sodium pyrophosphate and 4 µL of 1 mM sodium orthovanadate. Proteins were separated by SDS-PAGE, using 12.5% polyacrylamide gels, and stained with 0.5% Coomassie blue R-250, 10% acetic acid, 40% methanol, or blotted into a Hybond-ECL membrane. Immunodetection of phospho-Hog1p was performed as described in III-6.2 with some alterations. The membrane was blocked with 5% BSA in TTBS (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.05% Tween) and washed with TTBS and TBS. Rabbit IgG anti-phospho-p38 MAPK (1:500; Cell Signalling Technology) and anti-rabbit IgG-peroxidase (1:5,000; Sigma Aldrich) were used as primary and secondary antibodies, respectively. Immunodetection of Act1p (loading control) was performed using rabbit IgG anti-actin (1:500; Sigma Aldrich) as primary antibody.

III-10 Statistical analysis

Data were expressed as mean values ± SD of at least three independent experiments. Values were compared by Student´s *t*-test. The 0.001 probability level was chosen as the point of statistical significance throughout.

IV – **Results and discussion**

1. Alterations in ceramide metabolism modulates acetic acid stress resistance

Sphingolipids metabolism has an important role in yeast programmed cell death, survival and proliferation (Taha *et al.*, 2006). Acetic acid, a normal end product of the yeast fermentation, induces a mitochondrial-dependent apoptotic pathway (Ludovico *et al.*, 2001; Ludovico *et al.*, 2002). In this work, we investigated if ceramide, a pro-apoptotic molecule, modulates apoptosis triggered by acetic acid.

To characterize the relative contribution of de novo biosynthesis versus catabolism of sphingolipids in apoptotic cell death induced by acetic acid, the following mutants were previously generated in the *S. cerevisiae* CG379 strain, by homologous recombination (Rego *et al*., IBMC, Porto, unpublished): *lac1*Δ and *lag1*Δ (mutants unable to produce ceramide by *de novo* synthesis), *ydc1*Δ and *ypc1*Δ (mutants unable to breakdown ceramide into sphingosine) and *isc1*Δ (mutant unable to produce ceramide by degradation of complex sphingolipids). Wild type and mutant cells were grown in SC – Galactose medium and treated with 180 mM acetic acid during 200 min. The analysis of cell viability (Figure IV-1) showed that *LAG1* and *ISC1* gene deletion increased acetic acid stress resistance: 6.0 ± 3.8 % of wild type cells remained viable, whereas 33.1 ± 3.7 % of *lag1*Δ cells and 51.8 ± 9.2 % of *isc1*Δ mutants survived. The viability of the other mutant strains analyzed was similar to that of wild type cells.

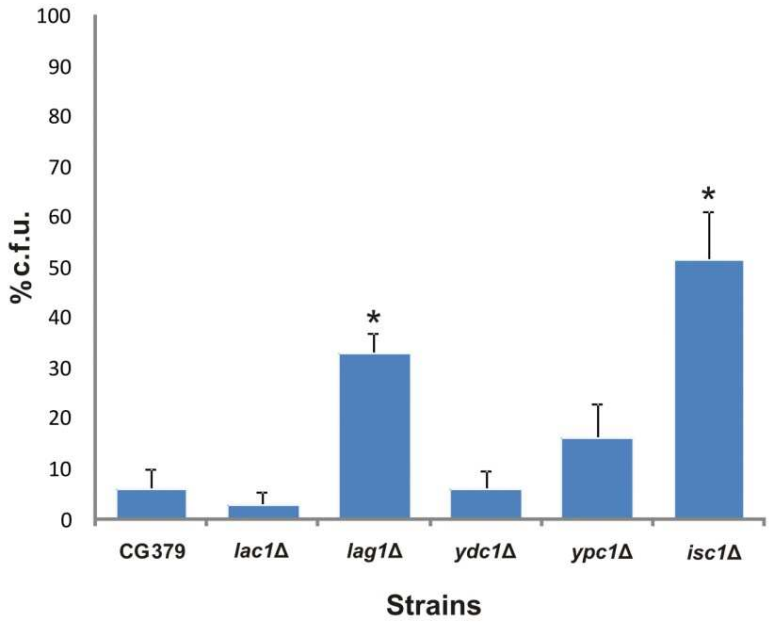

Figure IV-1. The role of the genes associated with ceramide metabolism in acetic acid stress resistance. *S. cerevisiae* CG379, *lac1*Δ, *lag1*Δ, *ydc1*Δ, *ypc1*Δ and *isc1*Δ were grown in SC- Galactose medium to the exponential phase ($O.D._{600}$ = 0.5-0.6) and exposed to 180 mM of acetic acid for 200 minutes in SC - Galactose medium with pH=3.0. Cell viability is expressed as the percentage of the colony-forming units. The % c. f. u. in

control cells (untreated but maintained in pH 3.0 medium) was 100 % (data not shown). Values are means ± SD of at least three experiments. *p<0,01.

To confirm that the acetic acid resistance phenotype observed in *lag1*Δ and *isc1*Δ mutants was due to the disruption of these genes and not to secondary mutations generated during genetic manipulation of wild type cells, both strains were transformed with pYES2 (empty vector), *lag1*Δ mutants with pYES2-*LAG1* and *isc1*Δ mutants with pYES2-*ISC1* (genes expressed under the *GAL1* promoter). For the generation of pYES2-*LAG1* plasmid, the *LAG1* gene was amplified by PCR and cloned in pYES2 (Figure IV-2). The pYES2-*ISC1* plasmid was kindly provided by Prof. Yusuf Hannun (Medical University of South Carolina, USA). Wild type cells were also transformed with pYES2 (control).

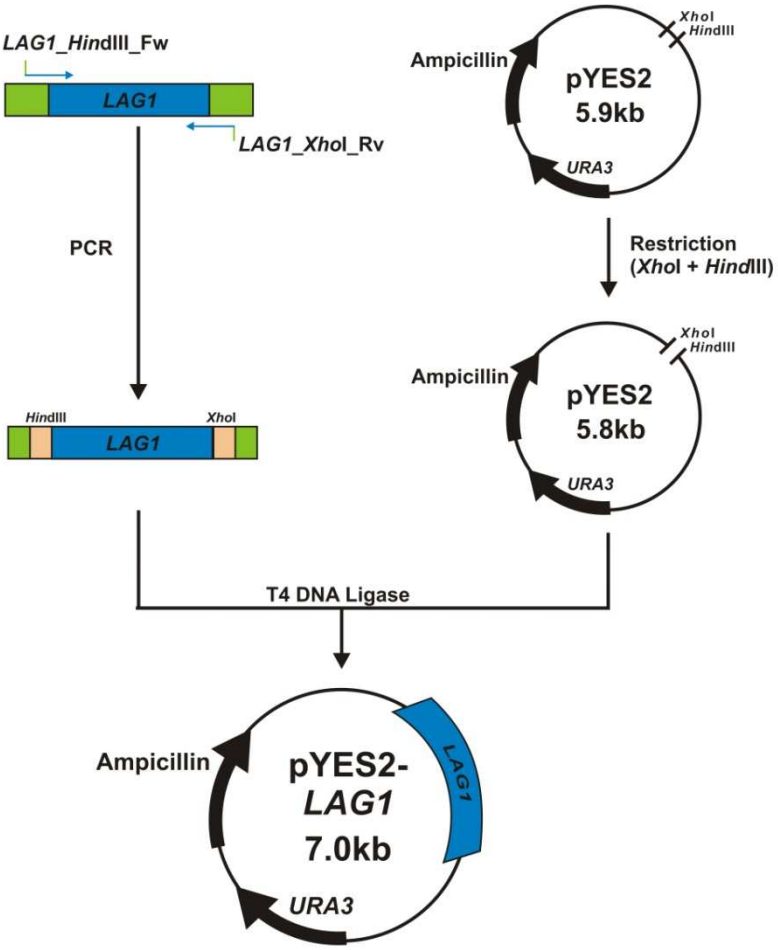

Figure IV-2. Strategy of pYES2-*LAG1* construction. *LAG1* gene was amplified by PCR and cloned in pYES2 plasmid linearized.

The analysis of cell viability showed that *LAG1* and *ISC1* expression suppressed the increased acetic acid stress resistance of *lag1*Δ and *isc1*Δ strains, respectively (Figure IV-3). As expected, the viability of the mutant cells transformed with the empty vector (27.0 ± 8.4 % in *lag1*Δ-pYES2 cells and 37.3 ± 11.1 % in *isc1*Δ-pYES2 cells) was higher

compared with that of wild type cells (6.6 ± 2.6 %). In addition, the reintroduction of the genes decreased acetic acid resistance to levels similar to the observed in wild type cells: cellular viability was 7.5 ± 3.3 % in *lag1*Δ-pYES2-*LAG1* and 10.2 ± 2.7 % in *isc1*Δ-pYES2-*ISC1* cells.

These results suggest that ceramide generated by the *de novo* biosynthesis mediated by Lag1p and through hydrolysis of inositolphosphosphingolipids catalysed by Isc1p contributes to cell death induced by acetic acid.

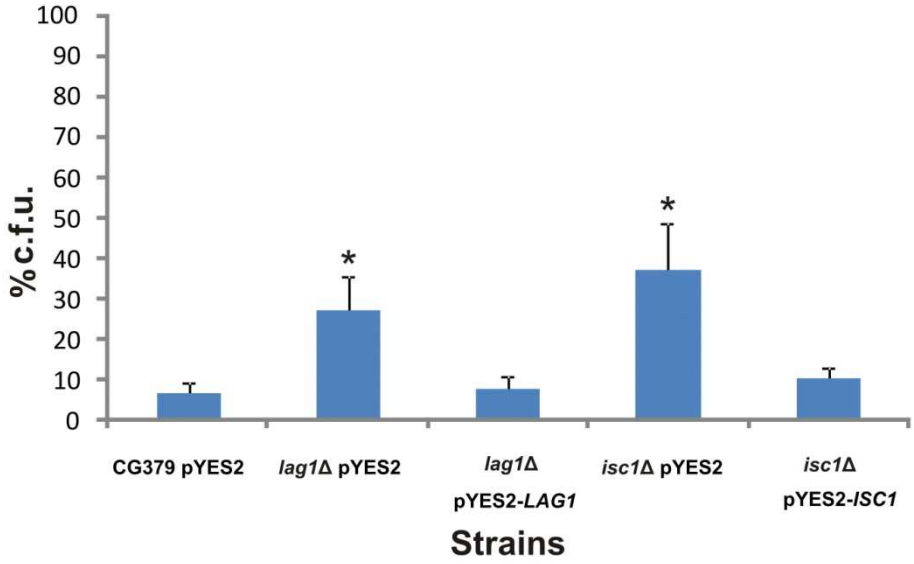

Figure IV-3. *ISC1* and *LAG1* genes suppress the increased resistance of *isc1*Δ and *lag1*Δ mutant cells to acetic acid. Yeast cells were grown in SC-Galactose medium lacking uracil to exponential phase ($O.D._{600}$ = 0.5-0.6) and exposed to 180 mM of acetic acid for 200 minutes in SC-Galactose medium with pH=3.0. Cell viability is expressed as the percentage of the colony-forming units. The % c. f. u. in control cells (untreated but maintained in pH 3.0 medium) was 100 % (data not shown). Values are means ± SD of at least three experiments. *p<0,01.

In sphingolipids metabolism, dihydrosphingosine and phytosphingosine are acylated on the amine group to generate dihydroceramide and phytoceramide, respectively. Ceramide synthase catalyses the acylation reaction and the enzyme activity requires two proteins with redundant functions, Lac1p and Lag1p (Dickson and Lester, 2002). Ceramides generated by ceramide synthase or via hydrolysis of complex sphingolipids can be cleaved back into sphingoid bases and free fatty acids by the ceramidases Ypc1p and Ydc1p. Ypc1p is more specific for phytoceramide and Ydc1p is more specific to dihydroceramide (Cowart and Obeid, 2007).

Considering this putative redundancy, we postulated that the deficiency in both enzymes associated with a similar function (Lac1p and Lag1p for ceramide synthase; Ypc1p and Ydc1p for ceramidase) could exacerbate the phenotypes. To test this

hypothesis, a RNA silencing technique was used. Plasmids for antisense inhibition of *LAC1* or *YDC1* gene expression were constructed in the pYES2 vector and introduced in *lag1*Δ and *ypc1*Δ cells, respectively. When cells express the antisense gene, an antisense mRNA, which is complementary to the RNA generated from the genomic DNA (sense mRNA), is produced. The sense and the antisense mRNAs hybridize, blocking the translation and silencing the respective gene (Figure IV-4).

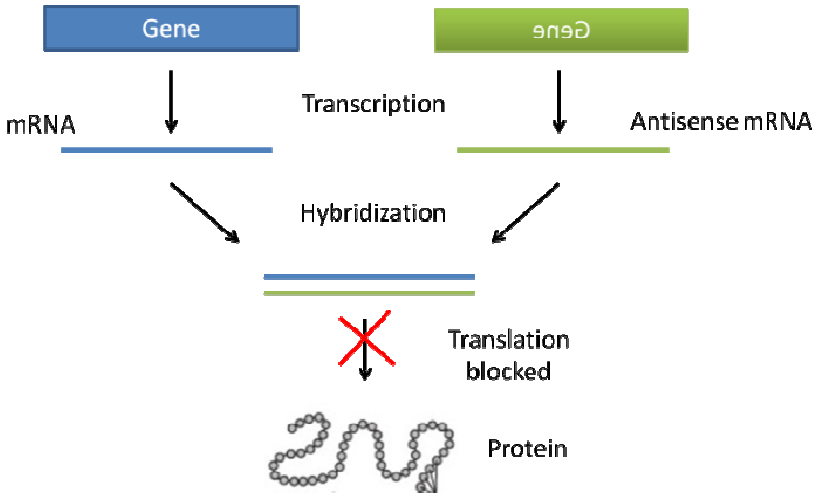

Figure IV-4. RNA silencing technique used to construct double mutants When cells express the antisense gene, it is produced an antisense mRNA that hybridize with the sense RNA, blocking the translation and silencing the respective gene.

As shown in Figure IV-5, when the *LAC1* gene was silenced in *lag1*Δ, the resistance to acetic acid was similar to the observed in the wild type strain. Schorling *et al.* (2001) described that the deletion of both *LAC1* and *LAG1* genes strongly reduces the levels of complex sphingolipids, which have important functions in cell. The decrease in acetic acid resistance associated with *LAC1* gene silencing in *lag1*Δ mutants, when compared with that of *lag1*Δ mutants, is probably due to the almost complete loss of complex sphingolipids. These results suggest that ceramide produced by de novo biosynthesis may not be a major determinant of acetic acid induced cell death.

When the *YDC1* gene was silenced in *ypc1*Δ mutants, cell viability remained unchanged, suggesting that yeast ceramidase activity is not an important factor in acetic acid resistance.

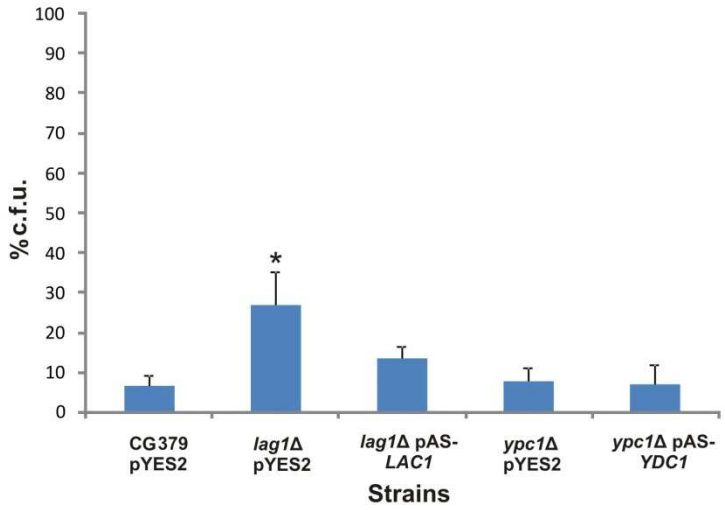

Figure IV-5. Effect of antisense inhibition of *LAC1* and *YDC1* on acetic acid stress resistance. Yeast cells were grown in SC-Galactose medium lacking uracil to exponential phase ($O.D._{600}$ = 0.5-0.6) and exposed to 180 mM of acetic acid for 200 minutes in SC-Galactose medium with pH=3.0. Cell viability is expressed as the percentage of the colony-forming units. The % c. f. u. in control cells (untreated but maintained in pH 3.0 medium) was 100 % (data not shown). Values are means ± SD of at least three experiments. *p<0,01.

Interestingly, ceramide accumulation in strains lacking ceramidases don´t increases the acetic acid sensibility; however, the loss in the production in ceramide by deficiency in *ISC1* gene increases the acetic acid stress resistance. These differences can be related with the intracellular enzymes location.

Isc1p is a transmembranar protein located in the outer membrane of the mitochondria (Kitagaki *et al.*, 2007), whereas Ydc1p and Ypc1p ceramidases are located in endoplasmic reticulum (Bartke and Hannun, 2009). Mitochondria supports the oxidative pathways, where are involved various redox carriers that can potentially release single electrons and oxidize several surrounding molecules (Andreyev *et al.*, 2005) and then, a single alteration in a mitochondrial membrane protein can lead to modifications in the normal behavior of the oxidative pathways. This would explain the differences obtained in our results for stress resistance to acetic acid.

2. Alterations in ceramide metabolism do not affect the cell cycle

Several apoptotic agents can promote death at specific phases of the cell cycle indicating that the cell-cycle and apoptosis are intimately linked (Coquelle *et al.*, 2006). The observation of apoptotic cells arrested in G2/M is common in the literature (DiPaola, 2002). In *Candida albicans*, it was verified that acetic acid induces cell cycle arrest in

G2/M phase (Phillips *et al.*, 2003). In *S. cerevisiae*, acetic acid induces cell cycle arrest in the G0/G1 phases (Almeida *et al.*, 2009).

Aiming to verify if the effect of acetic acid in the cell cycle is altered in the mutant strains of ceramide metabolism, cells untreated or treated with acetic acid were incubated with propidium iodide and analyzed by flow cytometry. Propidium iodide (PI) can cross the plasma membrane of fixed cells and binds to DNA.

Figure IV-6 shows the results obtained with PI staining. In all assays, the autofluorescence was measured and it was found to be negligible when compared with the cells incubated with propidium iodide. In all the strains studied, the profile of the cells treated with acetic acid was also similar to that of control cells, suggesting that both acetic acid and ceramide metabolism did not affect the cell cycle. The fact that acetic acid did not induce a G0/G1 growth arrest, as described by Almeida *et al*. (2009), may be due to different carbon sources used in the assay.

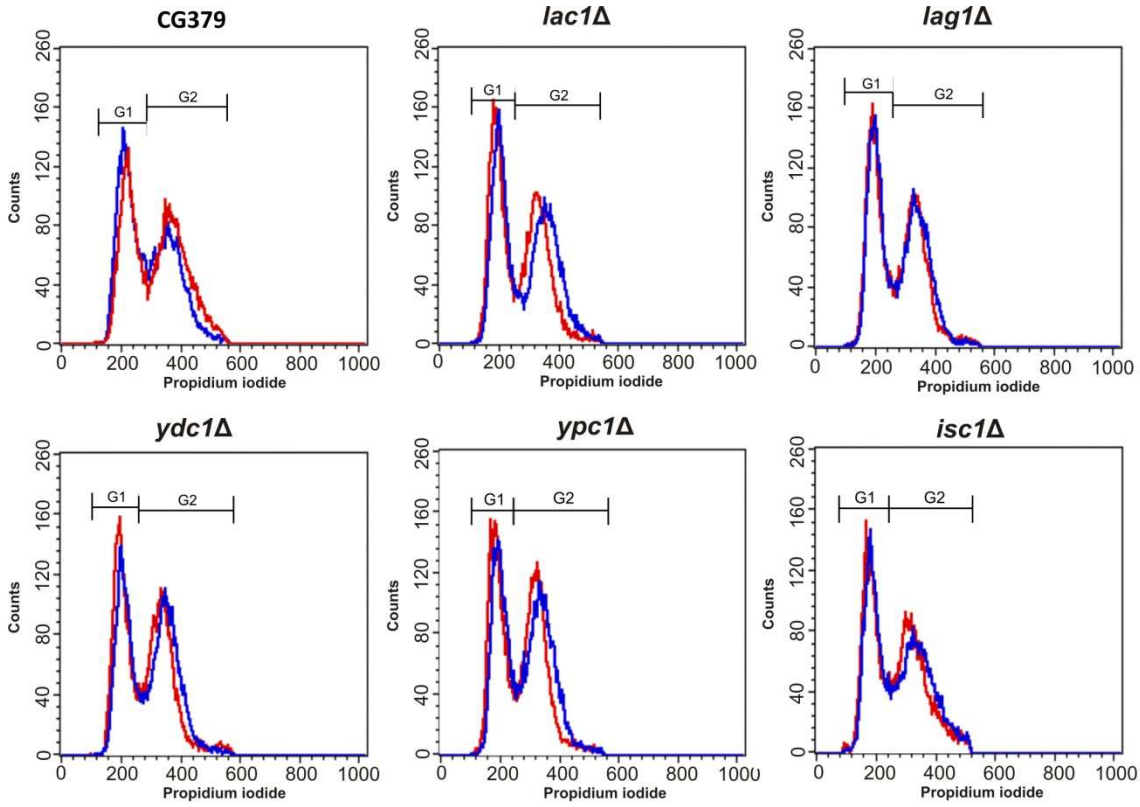

Figure IV-6. The role of ceramide and acetic acid in cell cycle. 1×10^7 cells treated (red) and non-treated (blue) with acetic acid were fixed in ethanol 70%, 1 hour, at the room temperature. Cells were washed and resuspended in sodium citrate 50 mM pH 7.0 + RNAse A 0.25 mg/mL and incubated 1 hour to 50 ºC. Cells were sonicated (30%) and incubated with propidium iodide 16 µg/mL in the absence of light, 30 minutes, at the room temperature. Samples were analysed by flow cytometry.

3. Oxidative stress markers

Acetic acid induces a mitochondrial-dependent apoptotic pathway (Ludovico et al., 2001; Ludovico *et al.*, 2002) and reactive oxygen species act as central regulators of yeast apoptosis (Madeo *et al.*, 2004). In mammalian cells, it has been shown that apoptosis inducing stimuli such as FAS activation (Tepper *et al.*, 1997) and anticancer drugs (Bose *et al.*, 1995) increase the levels of ceramide, which can regulate apoptosis by transcriptional-dependent or -independent mechanisms. The later involves release of mitochondria intermembrane space proteins (Elrick *et al.*, 2006) and generation of reactive oxygen species and is inhibited by anti-apoptotic members of the Bcl-2 family (Mathias *et al.*, 1998). Reactive oxygen species oxidize biomolecules, such as proteins, nucleic acids and lipids (Finkel and Holbrook, 2000). Therefore, we decided to test the correlation between changes in ceramide metabolism and the production of mitochondrial ROS and protein carbonylation during cell death induced by acetic acid.

3.1. Acetic acid-induced mitochondrial ROS production decreases in *isc1*∆ cells

To determine the amount of mitochondrial ROS production, cells untreated or treated with acetic acid were labeled with MitoTracker Red CM-H_2XRos, a molecular probe sensitive to ROS that accumulates in mitochondria, and the percentage of fluorescent cells was determined by flow cytometry (Figure IV-7). In control cells, mitochondrial ROS levels were very low in all strain, although *isc1*∆ cells showed significantly higher levels. This is consistent with previous data showing that this mutant strain displays a mitochondrial dysfunction associated to higher levels of ROS (Almeida *et al.*, 2008). In the wild type strain, the percentage of ROS positive cells after treatment with acetic acid was 77.0 ± 5.8 %. This is in agreement with published data showing that acetic acid induces mitochondrial ROS production (Ludovico *et al.*, 2002). The *lac1*Δ, *lag1*Δ, *ydc1*Δ and *ypc1*Δ mutant cells showed similar production of mitochondrial ROS. However, *isc1*∆ mutants presented a lower percentage of ROS positive cells (55.5 ± 3.8 %). These results are consistent with cellular viability, with the *isc1*∆ strain that is more resistant to acetic acid showing less production of mitochondrial ROS.

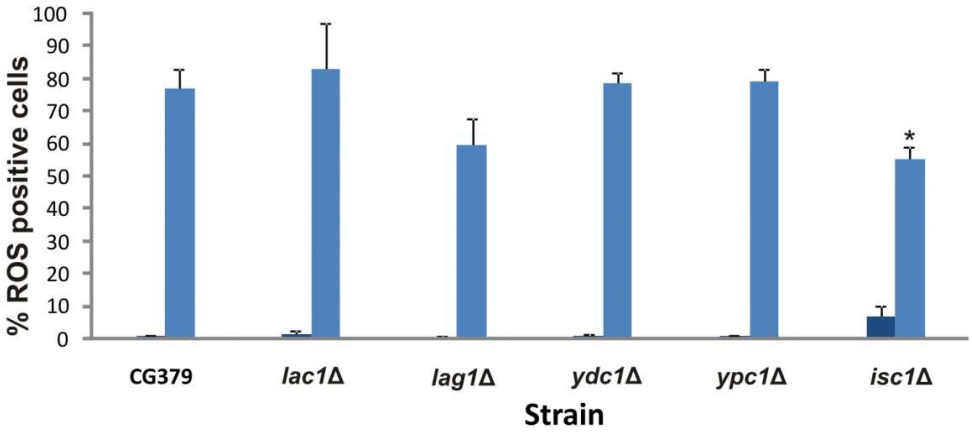

Figure IV-7. Mitochondrial ROS production. Yeast cells, untreated (dark blue) or treated with 180 mM acetic acid (light blue), were harvested, washed and resuspended in phosphate-buffered saline solution (PBS). Cell were then incubated with 0.4 µg/ml MitoTracker Red CM-H_2XRos for 20 minutes at 37 °C, in the dark, and analyzed by flow cytometry. Values are means ± SD of at least three experiments. *p□0,001

3.2. Effect of acetic acid on protein carbonylation

In cells, the amount of proteins oxidized increase as a consequence of the decline in the antioxidant defense system, the diminished capacity for removal of oxidized proteins, or to the increase in the susceptibility of proteins to oxidative attack (Nystrom, 2005). All of these factors are a consequence of the increase in the production of ROS due to stress inducing agents.

To investigate if acetic acid induced ROS production leads to oxidative damages and its correlation with stress resistance, protein carbonylation was analyzed in wild type cells and in the different mutants affected in ceramide metabolism stressed with acetic acid (Figure IV-8).

In all the strains analyzed, the results show that acetic acid did not induce protein carbonylation. In contrast, oxidative stress triggered by H_2O_2 significantly increased the levels of protein carbonyls, which is consistent with published data (Cabiscol *et al*., 2000; Costa *et al*., 2002). Although the analysis of protein carbonyls profile after acetic acid treatment showed 3 bands that are more intense, the relative quantity of these proteins also increased in treated cells (see silver staining). The enrichment in some proteins seems to be associated with the disappearance of many proteins when cells are stressed, probably due to degradation. In fact, Valenti *et al*. (2008) have already reported a role of protein degradation by proteasome in acetic acid induced apoptosis. Acetic acid has also been shown to block the uptake of aromatic amino acids (Bauer *et al*., 2003) and to induce severe amino acids starvation (Almeida *et al*., 2009). Therefore, the intracellular protein degradation can be activated to provide the necessary amino acids.

These results suggest that, despite the induction of mitochondrial ROS production, the accumulation of oxidized proteins does not contribute to apoptotic cell death induced by acetic acid.

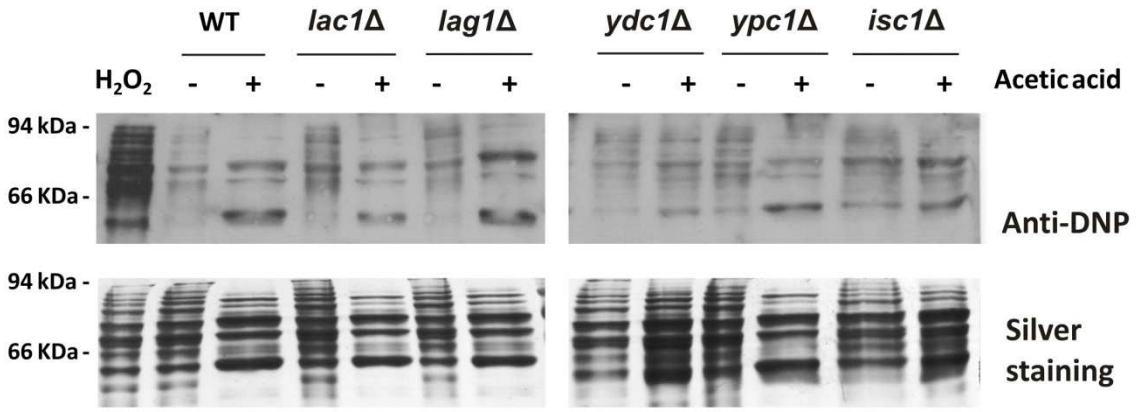

Figure IV-8. Effect of acetic acid on protein carbonylation. (A) Protein extracts were prepared from yeast cells, untreated or treated with acetic acid, derivatized with DNPH, separated by SDS-PAGE and blotted into a nitrocellulose membrane. Immunodetection was performed using an anti-DNP antibody, as described in material and methods. Wild type cells treated with 1.5 mM H_2O_2 were used as a positive control. A representative experiment is shown (out of three experiments with similar results). B) Silver staining of a replica gel.

4. Effect of acetic acid on lipid rafts

Lipid rafts are microdomains within the plasma membrane, formed by lateral association of sphingolipids to cholesterol (ergosterol in yeast) that makes them resistant to some detergents (Willhite and Wright, 2009). Rafts play a key role in connecting the plasma membrane to the cytoskeleton, ER and Golgi apparatus, for correct sorting and trafficking through exocytosis or endocytosis (Bagnat *et al.*, 2000). Rafts segregate signaling molecules such as sphingolipids, phosphatidylinositol 4,5-biphosphate, kinases and GPI-anchored proteins that are attached to the plasma membrane via a lipid anchor containing either a ceramide or diacylglycerol (Simons and Ikonen, 1997). As *isc1*Δ mutants lack a protein involved in the generation of ceramide, we raised the hypothesis that the increased resistance to acetic acid observed in this strain may result from structural and functional alterations in their lipid rafts. Two approaches were used in these studies: the analysis of lipid rafts in vivo, using filipin, a fluorescent dye that binds to ergosterol; isolation of detergent-resistant microdomains and analysis of Pma1p.

4.1. Filipin staining

Sterol-rich membrane domains have been identified in several fungus species, including the budding yeast *S. cerevisiae*. The sterol binding fluorescent dye filipin has been used to detect regions with high sterol content in their plasma membrane (Beh and Rine, 2004). Although the size of individual lipid rafts is still debated, they seem to be much smaller than the filipin stained domains, which might represent clusters of lipid rafts. Thus, filipin staining indicates the relative distribution of lipid rafts in the membrane (Wachtler and Balasubramanian, 2006).

Our results show a uniform distribution of the sterols throughout the membrane and a polarized localization in the tip of the mating projection in control wild type (CG379) cells (Figure IV-9). This kind of sterol distribution was already reported in *S. cerevisiae* (Bagnat and Simons, 2002). After treatment with acetic acid, cells presented an even (less punctuated) sterol distribution, indicating that the integrity or assembly of lipid rafts was altered.

Notably, most of the *isc1*Δ mutant cells showed an atypical heterogeneous, more punctuated filipin-stain distribution at the level of the plasma membrane. These findings indicate that *ISC1* deletion interferes with the maintenance and assembly of sphingolipid-sterol-ordered domains. The structural consequences that results from the deficiency in Isc1p, a crucial enzyme in the ceramide production, may be related with the higher resistance to acetic acid observed in this mutant strain.

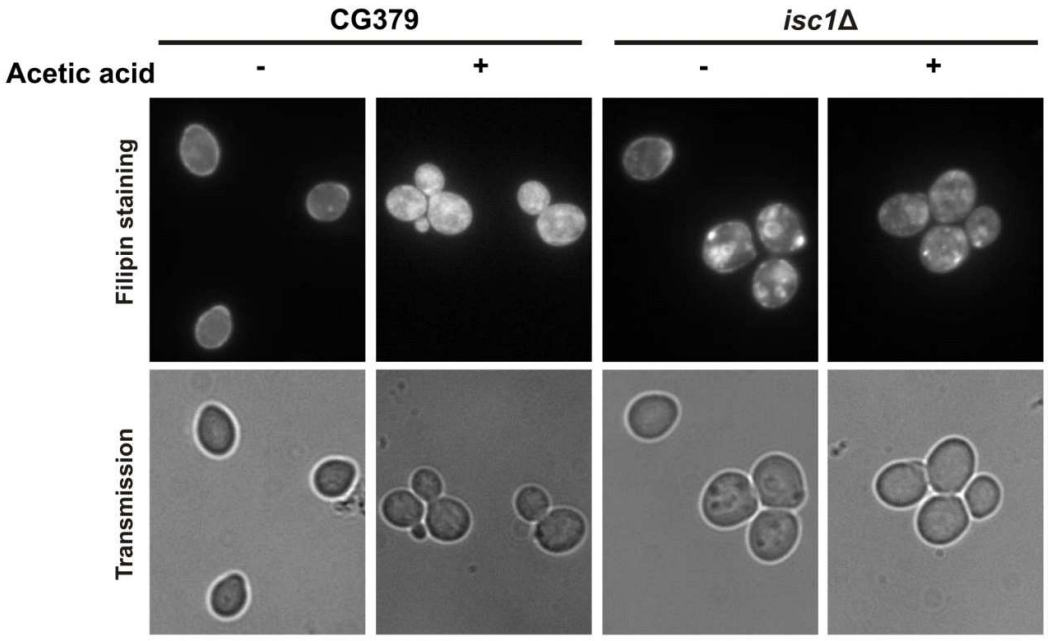

Figure IV-9. Sterol rich domains distribution. CG379 and *isc1*Δ mutant cells were grown in SC- Galactose and treated or not with acetic acid. Cells were fixed in 37.5% formaldehyde, washed with water, fixed with filipin 5 mg/mL, and observed on an Zeiss Axiovert 200M microscope. Representative data are shown from three independent experiments.

4.2. Isc1p deficiency decreases Pma1p depletion in rafts induced by acetic acid

Pma1p is an essential 100 kDa protein that contains 10 membrane-embedded domains and is associated with rafts (Morsomme *et al.*, 2000). It is the major plasma membrane H^+-ATPase in *S. cerevisiae* that couples the hydrolysis of ATP to the pump of H^+ ions out of the cell, creating an electrochemical proton gradient that regulates proper cytoplasmic pH and drives the uptake of nutrients across the plasma membrane (Morsomme *et al.*, 2000).

When cells are treated with acetic acid, they need to pump the protons out of the cell to maintain intracellular pH. Therefore, we postulated that Pma1p levels in lipid rafts may increase after treatment with acetic acid and Isc1p deficiency may enhance this effect. To test this hypothesis, cellular membranes were isolated from wild type and *isc1*Δ strains, untreated or treated with acetic acid, solubilized with Triton X-100 and centrifuged in an Optiprep Gradient. The upper fractions that contained the detergent-resistant membranes and, thus, proteins associated with lipid rafts, were collected and Pma1p was analyzed by western-blot followed by immunodetection.

Unexpectedly, the levels of Pma1p associated with lipid rafts decreased in wild type cells treated with acetic acid (Figure IV-10). This decrease seems to be unspecific since the analysis of total proteins (silver staining) showed a drastic decrease in all proteins associated with rafts. However, Pma1p levels in total cellular membranes was not affected by acetic acid, suggesting that the decrease of the protein in the rafts may result from a decreased integrity or assembly of these microdomains.

The Pma1p associated with rafts was less abundant in untreated *isc1*Δ cells than in wild type cells. This probably results from alterations in *isc1*Δ rafts due to changes in ceramide that may decrease the association of Pma1p with these microdomains. However, the decrease in Pma1p present in rafts (but not in total cellular membranes) induced by acetic acid was partially suppressed in *isc1*Δ cells. It is likely that this stabilization of Pma1p contributes to the higher resistance to acetic acid observed in this mutant strain.

The immunoblot showed two bands recognized by the Pma1p antibody, one with approximately 100 kDa (the molecular weight of this protein) and another band with a higher molecular weight. It is known that Pma1p can be phosphorylated and activated in a

serine residue at its C-terminus mediated by Ptk2p (Eraso *et al*., 2006) or it can be phosphorylated by serine/threonine kinases (Yck1p and Yck2p), resulting in a decreased proton pump activity (Estrada *et al*., 1996). To test if the higher molecular band detected was a phosphorylated form of Pma1p, the total cellular membranes were treated with a phosphatase, □-PPase, before the western blotting (Figure IV-10C). Both bands were not affected by the phosphatase treatment. Therefore, the upper band may result from another post-translational modification of the protein.

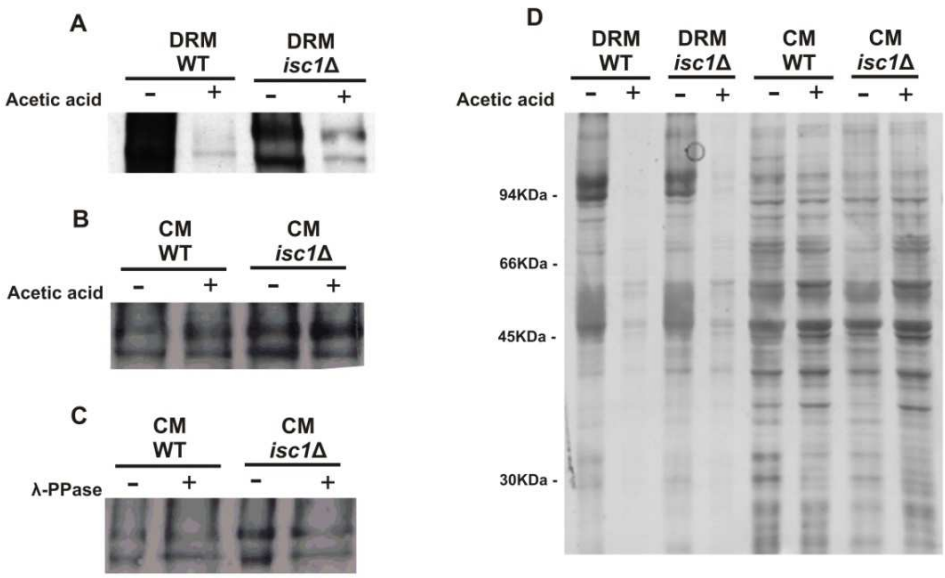

Figure IV-10. Effect of acetic acid on Pma1p present in rafts. Cellular membranes (CM) were isolated form wild type and *isc1*Δ cells, untreated or treated with acetic acid, solubilized with 1% Triton X-100 and centrifuged in an Optiprep gradient. A) The upper fractions containing the detergent-resistance membranes (DRM) were collected and analyzed by western-blot followed by immunodetection, using rabbit IgG anti-Pma1p as primary antibody. B) Analysis of Pma1p in total CM . C) Analysis of Pma1p in total CM prepared from control cells and treated or not with λ-phosphatase. D) Silver staining of DRM and CM fractions. A representative experiment is shown (out of three experiments with similar results).

5. Phospho-Hog1p levels increase after treatment with acetic acid

The Hog1p MAPK is involved in the regulation of the acetic acid channel Fps1p. Mollapour and Piper (2007) proposed that, when cells are exposed to acetic acid, Hog1p is activated by phosphorylation on Thr174 and Tyr176 by the MAPKK Pbs2p and phosphorylates Fps1p, targeting the channel for degradation. Thus, we raised the hypothesis that Hog1p might be activated in *isc1*Δ cells, leading to a faster endocytosis of Fps1p channel for degradation. This would decrease the transport of acetic acid into the cell, explaining the higher resistance to acetic acid.

As expected, the results showed an increase in phospho-Hog1p levels in cells treated with acetic acid (Figure IV-11). In this experiment, actin was used as loading control, but the results indicate that this protein was degraded during the treatment with acetic acid. Similar results were obtained using glyceraldehyde 3-phosphate dehydrogenase, another loading control frequently used (data not show).

In the *isc1*Δ strain, Hog1p phosphorylation levels were higher to those observed in the wild type strain, both in untreated cells and after treatment with acetic acid. This increased activation of the HOG pathway is consistent with the higher stress resistance observed in the *isc1*Δ strain. Whether this is associated to a decrease in Fps1p levels and acetic acid uptake remains to be demonstrated. After *S. cerevisiae* exposure to hyperosmotic stress, Hog1p is phosphorylated on Thr174 and Tyr176 by the MAPKK Pbs2 (Brewster *et al.*, 1993).

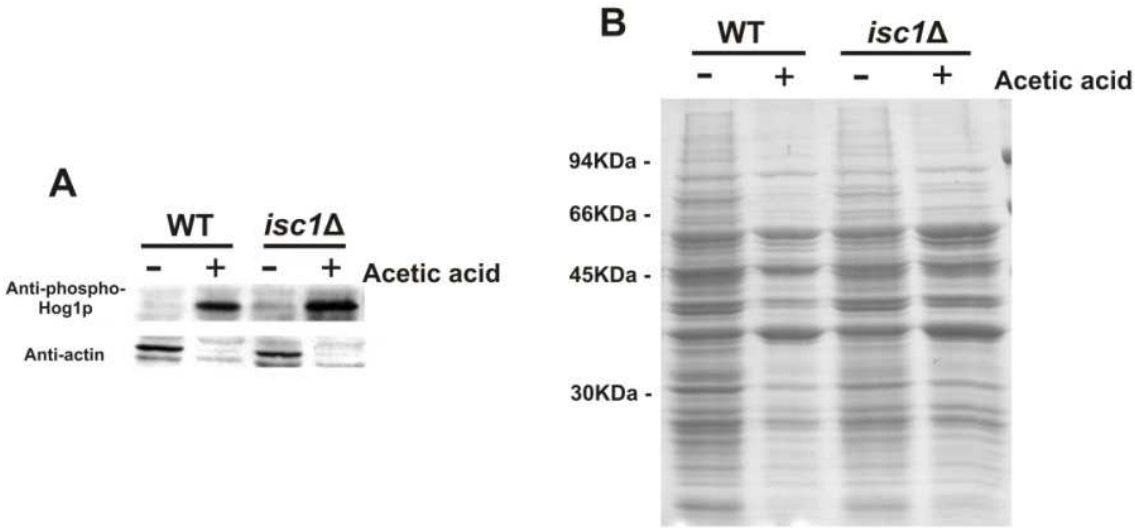

Figure IV-11. The role of Hog1p in *isc1*Δ acetic acid stress resistance. Proteins from WT and *isc1*Δ cells, untreated or treated with acetic acid were separated by SDS-PAGE and blotted into a nitrocellulose membrane. A) Phospho-Hog1p was detected with rabbit anti-phospho-Hog1 as primary antibody and anti-rabbit IgG-peroxidase as a secondary antibody. Actin levels were also analyzed using rabbit anti-actin as primary antibody (loading control). B) A replica gel was stained with Coomasie blue. A representative blot/gel is shown (out of 3 independent experiments with similar results).

V – Conclusions and future perspectives

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Apoptosis is a complex mechanism of cell death that is programmed and controlled by specific biochemical pathways (Taha *et al.*, 2006). Several agents have been used as external inducers of apoptosis, most commonly low doses of hydrogen peroxide or acetic acid (Ribeiro *et al.*, 2006). In the last few years acetic acid has received an emerging attention as it is a normal end product of the alcoholic fermentation carried out by *S. cerevisiae* and, therefore, a natural apoptosis inducer (Ludovico *et al.*, 2001).
Ceramide is the most studied sphingolipid and it is implicated in several cell processes, as differentiation, cell cycle arrest or senescence (Taha *et al.*, 2006) and plays an important role in apoptotic cell death. The programmed cell death regulated by ceramide is crucial in cancer development and progression, and this knowledge is a powerful tool in the efficacy of anti-cancer therapeutics (Ogretmen, 2006).

So far, the pro-apoptotic role of ceramide in acetic acid induced apoptosis has not accessed. Therefore, we used *S. cerevisiae* as a biological model to study this effect. Our results suggest that ceramide produced by *de novo* biosynthesis mediated by the Lag1p ceramide synthase and degradation of complex sphingolipids catalyzed by Isc1p is involved in acetic acid induced cell death. It will be important to test if Isc1p and Lag1p act through the same or an independent pathway. It is possible that a *lag1*Δ*isc1*Δ double mutant is inviable since ceramide deprivation is lethal. Indeed, it was previously shown that the deletion of *ISC1* in the lcb1-100 mutant (affected in the first step of sphingolipids biosynthesis) is lethal (Cowart *et al.*, 2006). Thus, *lag1*Δ cells could be transformed with the centromeric plasmid pCM184 expressing *ISC1* under the control of Tet promoter (which is repressed by the addition of doxycycline), as described by García-Rubio *et al.* (2003), before *ISC1* gene deletion. The analysis of acetic acid resistance in these cells treated or not with doxycycline, will clarify if the deficiency in both Isc1p and Lag1p has a synergistic effect.

Acetic acid induces a mitochondria-dependent apoptotic pathway in *S. cerevisiae* (Ludovico *et al.*, 2002). The increased acetic acid resistance of *isc1*Δ cells was correlated with lower levels of mitochondrial reactive oxygen species. Our results also showed that the increase in ROS was not sufficient to provoke protein oxidation, as assessed by measuring protein carbonylation, another marker of oxidative stress (Nystrom, 2005). We cannot exclude the hypothesis that oxidized proteins accumulate specifically inside mitochondria, the organelle where ROS production occurs. This could be tested by doing similar studies in isolated mitochondria. However, it may be difficult to do this analysis since it was previously shown that acetic acid induces a Pep4-dependent mitochondrial degradation (Pereira *et al*., 2010).These studies could be complemented with the assays

for apoptotic markers, namely chromatin condensation along the nuclear envelope, detected by transmission electron microscopy, the exposure of phosphatidylserine at the outer surface of the membrane, measured with fluorescein-isothiocyanate (FITC) staining, and the formation of DNA strand breaks, verified by TUNEL reaction, all as described in Ludovico *et al.* (2001).

Membrane rafts are plasma membrane microdomains constituted by the lateral association of sphingolipids and ergosterol. Our results showed that acetic acid induced cell death was associated with changes in the integrity or assembly of lipid rafts. Moreover, the deletion of *ISC1* resulted in an atypical, more punctuated ergosterol distribution at the plasma membrane, a structural consequence that may be related to the higher resistance to acetic acid observed in *isc1*∆ mutant cells. Pma1p, located in lipid rafts microdomains, is the major membrane ATPase with an important function on the maintenance of intracellular pH. As acetic acid decreases intracellular pH, we analyzed the levels of Pma1p after treatment with this agent. The protein levels were decreased in lipid rafts, but not in total cellular membranes. This is consistent with a decrease in the integrity of rafts, although it is possible that the protein is also being endocytosed for degradation. This hypothesis could be tested by the observation of yeast cells expressing a Pma1-GFP construct. Notably, Isc1p deficiency decreased the depletion of Pma1p in rafts. The stabilization of Pma1p probably accounts for the higher resistance observed in *isc1*∆ cells. It will be important to measure Pma1p activity in wild type and *isc1*∆ cells in order to understand how these changes correlate with stress resistance. The ATPase activity could be measured through the calculation of intracellular pH, as described by Brett *et al.* (2005). To measure the activity, strains would be transformed with pCB901YpHc, containing the pHluorin gene. pHluorin is a pH-sensitive green fluorescent protein, which can be detected in a fluorometer.

It was previous described that when cells are exposed to acetic acid, the Hog1p MAP kinase is activated and phosphorylates Fps1p channel, leading to its endocytosis and degradation (Mollapour and Piper, 2007). In agreement, our results show that acetic acid increased phospho-Hog1p levels. Moreover, the levels of phospho-Hog1p were higher in *isc1*Δ, both in untreated cells and after exposure to acetic acid. Thus, Isc1p deficiency seems to increase acetic acid resistance by a mechanism associated with the induction of Hog1p, which probably increases the destabilization of the Fps1p channel and, therefore, decreases the transport of acetic acid into the cell. To test this hypothesis, the uptake of acetic acid (radiolabeled) and the levels of Fps1p in the membrane should be measured. The later may be assessed using a Fps1-GFP construct. The key role of Hog1p in this mechanism may be confirmed by showing that the deletion of *HOG1* gene in *isc1*Δ cells abolishes its increased resistance to acetic acid.

The overall results support the utilization of yeast as a useful model organism to study in vivo the involvement of ceramide in apoptosis induced by acetic acid, but it is clear that much more work is needed to characterize the mechanisms involved.

VI – References

VI – References

Addison, R. (1986) Primary structure of the *Neurospora* plasma membrane H^+-ATPase deduced from the gene sequence. *J. Biol. Chem.*, 261(32): 14896-14901.

Aerts, A., Francois, I., Bammens, L., Cammue, B., Smets, B., Winderickx, J., Accardo, S., Vosc, D., Thevissen, K. (2006) Level of $M(IP)_2C$ sphingolipid affects plant defensin sensitivity, oxidative stress resistance and chronological life-span in yeast. *FEBS Lett*. 580: 1903–1907.

Aerts, A., Zabrocki, P., François, I., Carmona-Gutierrez, D., Govaert, G., Mao, C., Smets, B., Madeo, F., Winderickx, J., Cammue, B., Thevissen, K. (2008) Ydc1p ceramidase triggers organelle fragmentation, apoptosis and accelerated ageing in yeast. *Cell. Mol. Life Sci.*, 65: 1933 – 1942.

Aguilar-Uscanda, M., Delia, M., Strahaiano, P. (2000) Nutricional requirements of Brettanomyces bruxellensis: growth and physiology in batch and chemostat cultures. *Can. J. Microbiol.*, 46: 1046-1050.

Alewijnse, A., Peters, S. (2008) Sphingolipid signalling in the cardiovascular system: Good, bad or both? *Eur. J. Pharmacol.*, 585: 292-302.

Allen, J., Halverson-Tamboli, R., Rasenick, M. (2007) Lipid raft microdomains and neurotransmitter signalling. *Nature Rev. Neurosci.*, 8: 128-140.

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

Almeida, T., Marques, M., Mojzita, D., Amorim, M., Silva, R., Almeida, B., Rodrigues, P., Ludovico, P., Hohmann, S., Moradas-Ferreira, P., Côrte-Real, M., Costa, V. (2008) Isc1p plays a key role in hydrogen peroxide resistance and chronological lifespan through modulation of iron levels and apoptosis. *Mol. Biol. Cell.*, 19: 865–876.

Amberg, D., Burke, D., Strathern, J. (2005) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Course Manual.

Andreyev, A., Kushnareva, Y., Starkov, A. (2005) Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Mosc)*, 70(2): 200-214.

Ariga, T., Jarvis, W., Yu, R. (1998) Role of sphingolipid-mediated cell death in neurodegenerative diseases. *J. Lipid Res.*, 39: 1-16.

Bagnat, M. and Simons, K. (2002) Cell surface polarization during yeast mating. *Proc. Natl. Acad. Sci. U. S. A.*, 99: 14183–14188.

Bagnat, M., Keränen, S., Shevchenko, A., Shevchenko, A., Simons, K. (2000) Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. *PNAS U S A*, 97(7): 3254-3259.

Barea, F., Bonatto, D., (2009) Aging defined by a chronologic–replicative protein network in Saccharomyces cerevisiae: An interactome analysis, *Mech. Ageing Dev.*, 130: 444–460.

Bartke, N., and Hannun, Y. (2009) Bioactive sphingolipids: metabolism and function. *J. Lipid Res.*, 50: S91-S96.

Bartowsky, E., Henschke, P. (2008) Acetic acid bacteria spoilage of bottled red wine - A review. *Int. J. Food Microbiol.*, 125: 60–70.

Basu, S., Bayoumy, S., Zhang, Y., Lozano, J., Kolesnick, R. (1998) BAD enables ceramide to signal apoptosis via Ras and Raf-1. *J. Biol. Chem.*, 273: 30419–30426.

Bauer, B., Rossington, D., Mollapour, M., Mamnun Y., Kuchler, K., Piper, P. (2003) Weak organic acid stress inhibits aromatic amino acid uptake by yeast, causing a strong influence of amino acid auxotrophies on the phenotypes of membrane transporter mutants. *Eur. J. Biochem.*, 270: 3189–3195.

Beh, C. and Rine, J. (2004) A role for yeast oxysterol-binding protein homologs in endocytosis and in the maintenance of intracellular sterol-lipid distribution. *J. Cell Sci.*, 117(14): 2983:2996.

Berg, M., Jong-Gubbels, P., Kortland, C., Dijken, J., Pronk, J., Steensma, H. (1996) The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. *J. Biol. Chem.*, 271(46): 28953–28959.

Betz, C., Zajonc, D., Moll, M., Schweizer, E. (2002) ISC1-encoded inositol phosphosphingolipid phospholipase C is involved in Na1/Li1 halotolerance of Saccharomyces cerevisiae. *Eur. J. Biochem.*, 269: 4033–4039.

Bose, R., Verheij, M., Haimovitz-Friedman, A., Scottob, K., Fuksc, Z., Kolesnick, R. (1995) Ceramide synthase mediates daunorubicin-induced apoptosis: An alternative mechanism for generating death signals. *Cell*, 82(3): 405-414.

Botstein, D., Chervitz, S., Cherry, J. (1997) Yeast as a model organism. *Science*, 277: 1259-1260.

Bourbon, N., Yun, J., Kester, M. (2000) Ceramide directly activates protein kinase C ξ to regulate a stress-activated protein kinase signaling complex. *J. Biol. Chem.*, 275(45): 35617–35623.

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

Brewster, J., de Valoir, T., Dwyer, N., Winter, E., Gustin, M. (1993) An osmosensing signal transduction pathway in yeast. *Science*, 259(5102): 1760-1763.

Cabiscol, E., Piulats, E., Echave, P., Herrero, E., Ros, J. (2000). Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, 275: 27393 – 27398.

Cardoso, H., Leão, C. (1992) Mechanisms underlying the low and high enthalpy death induced by short-chain monocarboxylic acids and ethanol in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.*, 38: 388-392.

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

Casal, M., Cardoso, H., Leão, C. (1998) Effects of ethanol and other alkanols on transport of acetic acid in *Saccharomyces cerevisiae*. *Appl. Envir. Microbiol.*, 64(2): 665–668.

Casal, M., Paiva, S., Andrade, R., Gancedo, C., Leão, C. (1999) The lactate-proton symport of Saccharomyces cerevisiae is encoded by *JEN1*. *J. Bacteriol.*, 181(8): 2620-2623.

Chada, S., Ramesh, R. (2007) Apoptin studies illuminate intersection between lipidomics and tumor suppressors. *Mol.Ther.*, 15(1): 7-9.

Chalfant, C., Rathman, K., Pinkerman, R., Wood, R., Obeid, L., Ogretmen, B., Hannun, Y. (2002) *De novo* ceramide regulates the alternative splicing of caspase 9 and Bcl-x in A549 lung adenocarcinoma cells. *J. Biol. Chem.*, 277(15): 12587–12595.

Chang, M., Bellaoui, M., Boone, C., Brown, G. (2002) A genome-wide screen for methyl methanesulfonate-sensitive mutants reveals genes required for S phase progression in the presence of DNA damage. *Proc. Natl. Acad. Sci.*, 99: 16934–16939.

Chazal, N. and Gerlie, D. (2003) Virus entry, assembly, budding, and membrane rafts. *Microbiol. Mol. Biol. Rev.*, 67(2): 226–237.

Chen, C., Lin, C., Chang, W., Huang, W., Teng, C., Lin, Y. (2008) Ceramide induces p38 MAPK and JNK activation through a mechanism involving a thioredoxin-interacting protein-mediated pathway. *Blood*, 111(8): 4365-4374.

Chen, R., Thorner, J. (2007) Function and regulation in MAPK signaling pathways: Lessons learned from the yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta*, 1773: 1311-1340.

Chevallet, M., Luche, S., Rabilloud, T. (2006) Silver staining of proteins in polyacrylamide gels. *Nat Protoc.*, 1(4): 1852–1858.

Coquelle, A., Mouhamad, S., Pequignot, M., Braun, T., Carvalho, G., Vivet, S., Métivier, D., Castedo, M., Kroemer, G. (2006) Enrichment of non-synchronized cells in the G1, S and G2 phases of the cell cycle for the study of apoptosis. *Biochem. Pharmacol.*, 72: 1396–1404.

Costa, V., Amorim, M. A., Quintanilha, A., Moradas-Ferreira, P. (2002) Hydrogen peroxide-induced carbonylation of key metabolic enzymes in Saccharomyces cerevisiae: the involvement of the oxidative stress response regulators Yap1 and Skn7. *Free Radic. Biol. Med.*, 33: 1507 – 1515.

Cowart A., Obeid, L. (2007) Yeast sphingolipids: Recent developments in understanding biosynthesis, regulation, and function. *Biochim. Biophys. Acta*, 1771: 421–431.

Cuvillier, O., Edsall, L., Spiegel, S. (2000) Involvement of sphingosine in mitochondria-dependent Fas-induced apoptosis of type II Jurkat T cells. *The J. Biol. Chem.*, 275(21) 15691–15700.

Cuvillier, O., Levade, T. (2001) Sphingosine 1-phosphate antagonizes apoptosis of human leukemia cells by inhibiting release of cytochrome c and Smac/DIABLO from mitochondria. *Blood*, 98(9): 2828-2836.

D'Mello, N., Childress, A., Franklin, D., Kale, S., Pinswasdi, C. and Jazwinski, S. (1994) Cloning and characterization of LAG1, a longevity-assurance gene in yeast, *J. Biol. Chem.*, 269: 15451–15459.

Dai, Q., Liu, J., Chen, J., Durrant, D., McIntyre, T., Lee, R. (2004) Mitochondrial ceramide increases in UV-irradiated HeLa cells and is mainly derived from hydrolysis of sphingomyelin. *Oncogene*, 23: 3650–3658.

Dickson, R. and Lester, R. (2002) Sphingolipid functions in Saccharomyces cerevisiae. *Biochim. Biophys. Acta*, 1583: 13-25.

DiPaola, R. (2002) To arrest or not to G2-M Cell-cycle arrest : commentary re: A. K. Tyagi et al., Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth inhibition, G(2)-M arrest, and apoptosis. *Clin. Cancer Res.*, 8: 3512-3519.

Dufour, J., Goffeau, A. (1978) Solubilization by lysolecithin and purification of the plasma membrane ATPase of the yeast *Schizosaccharomyces pombe. J. Biol. Chem.,* 253(19): 7026-7032.

Elrick, M., Fluss, S., Colombini, M. (2006) Sphingosine, a product of ceramide hydrolysis, influences the formation of ceramide channels. *Biophys J.*, 91(5): 1749-1756.

Epand, R. (2006) Cholesterol and the interaction of proteins with membrane domains. *Prog. Lipid Res.*, 45: 279-294.

Erasmus, D., Merwe, G., Vuuren, H. (2003) Genome-wide expression analyses: Metabolic adaptation of Saccharomyces cerevisiae to high sugar stress. *FEMS Yeast Res.*, 3(4): 375-399.

Eraso, P., Mazóna, M., Portillo, F. (2006) Yeast protein kinase Ptk2 localizes at the plasma membrane and phosphorylates in vitro the C-terminal peptide of the H^+-ATPase. *Biochim. Biophys. Acta*, 1758(2): 164-170.

Estrada, E., Agostinis, P., Vandenheede, J., Goris, J., Merlevede, W., François, J., Goffeau, A., Ghislain, M. (1996) Phosphorylation of yeast plasma membrane H+-ATPase by casein kinase I. *J. Biol. Chem.*, 271(50): 32064-72.

Filippov, A., Oradd, G., Lindblom, G. (2006) Sphingomyelin structure influences the lateral diffusion and raft formation in lipid bilayers. *Biophys. J.*, 90: 2086-2092.

Finkel, T., Holbrook, N. (2000) Oxidants, oxidative stress and the biology of ageing. *Nature*, 408: 239–247.

Fox, T., Han, X., Kelly, S., Merrill, A., Martin, R., Anderson, R., Gardner, T., Kester, M. (2006) A potential mechanism of cell death in diabetic retinopathy. *Diabetes*, 55: 3573-3580.

Froger A., Hall J.E. (2007). Transformation of Plasmid DNA into E. coli Using the Heat Shock Method. *Vis Exp.*, 6: 253.

Gable, K., Slife, H., Bacikova, D., Monaghan, E., Dunn, T., 1999, Tsc3p Is an 80-Amino Acid Protein Associated with Serine Palmitoyltransferase and Required for Optimal Enzyme Activity, *J. Biol. Chem.*, 275: 7597–7603.

Gaigg, B., Timischl, B., Corbino, L., Schneiter, R. (2005) Synthesis of sphingolipids with very long chain fatty acids but not ergosterol is required for routing of newly synthesized plasma membrane ATPase to the cell surface of yeast. *J. Biol. Chem.*, 280(23): 22515–22522.

Garcia, A., Cayla, X., Guergnon, J., Dessuge, F., Hospital, V., Rebollo, M., Fleischer, A., Rebollo, A. (2003) Serine/threonine protein phosphatases PP1 and PP2A are key players in apoptosis. *Biochimie*, 85(8): 721-726.

Garcia-Rubio M., Huertas P., Gonzalez-Barrera S., Aguilera A. (2003) Recombinogenic effects of DNA-damaging agents are synergistically increased by transcription in Saccharomyces cerevisiae. New insights into transcription-associated recombination. *Genetics*, 165: 457–466.

Ghislain, M., Schlesserg, A., Groeffeau, A. (1987) Mutation of a conserved glycine residue modifies the vanadate sensitivity of the plasma membrane H^+-ATPase from *Schizosaccharomyces pombe*. *J. Biol. Chem.*, 262(36): 17549-17555.

Guaragnella, N., Pereira, C., Sousa, M., Antonaccia, L., Passarella, S., Côrte-Real, M., Marra, E., Giannattasio, S. (2006) YCA1 participates in the acetic acid induced yeast programmed cell death also in a manner unrelated to its caspase-like activity. *FEBS Lett.*, 580: 6880–6884.

Gudz, T., Tserng, K., Hoppel, C. (1997) Direct inhibition of mitochondrial respiratory chain complex III by cell-permeable ceramide. *J. Biol. Chem.*, 272(39): 24154–24158.

Haefen, C., Wieder, T., Gillissen, B., Starck, L., Graupner, V., Dorken, B., Daniel, P. (2002) Ceramide induces mitochondrial activation and apoptosis via a Bax-dependent apthway in human carcinoma cells. *Oncogene*, 21: 4009-4019.

Hansen, G., Dalskov, S., Rasmussen, C., Immerdal, L., Niels-Christiansen, L., Danielsen, E. (2005) Cholera toxin entry into pig enterocytes occurs via a lipid raft- and clathrin-dependent mechanism. *Biochemistry*, 44: 873-882.

Harsay, E. and Schekman, R. (2002) A subset of yeast vacuolar protein sorting mutants is blocked in one branch of the exocytic pathway. *J. Cell Biol.*, 156: 271–285.

Hartlova, A., Cerveny, L., Hubalek, M., Krocova, Z., Stulik, J. (2010) Membrane rafts: a potential gateway for bacterial entry into host cells. *Microbiol. Immunol.*, 54: 237–245.

He, X., Huangb, Y., Li, B., Gong, C., Schuchman, E. (2010) Deregulation of sphingolipid metabolism in Alzheimer's disease. *Neurobiol. Aging*, 31: 398–408.

Herker, E., Jungwirth, H., Lehmann. K., Maldener, C., Frohlich, K., Wissing, S., Buttner, S., Fehr, M., Sigrist, S., Madeo, F. (2004) Chronological aging leads to apoptosis in yeast, *J. Cell Biol.*, 164: 501-507.

Hinshaw, J. (2000) Dynamin and its role in membrane fission. *Annu. Ver. Cell Dev. Biol.*, 16: 483-519.

Huang, Q., Shen, H., Shui, G., Wenk, M., Ong, C. (2006) Emodin inhibits tumor cell adhesion through disruption of the membrane lipid raft-associated integrin signaling pathway, *Cancer Res.*, 66: 5807-5815.

Jarvis, W., Fornari, F., Auer, K., Freemerman, A., Szabo, E., Birrer, M., Johnson, C., Barbour, S., Dent, P., Grant, S. (1997) Coordinate regulation of stress- and mitogen-activated protein kinases in the apoptotic actions of ceramide and sphingosine. *Mol. Pharmacol.*, 52(6): 935-947.

Kaizu, K., Moriya, H., Kitano, H., (2010) Fragilities Caused by Dosage Imbalance in Regulation of the Budding Yeast Cell Cycle, *PLoS Genet.*, 6 (5): e1000919.

Karpen, H., Bukowski, J., Hughes, T., Gratton, J., Sessa, W., Gailani, M. (2001) The sonic hedgehog receptor patched associates with caveolin-1 in cholesterol-rich microdomains of the plasma membrane. *J. Biol. Chem.*, 276: 19503-19511.

Kim, D., Kim, S., Chung, J., Kim, K., Eun, H., Park, K. (2002) Delayed ERK activation by ceramide reduces melanin synthesis in human melanocytes. *Cell. Signal.*, 14: 779–785.

King, D., Hannum, D., Qi, J., Hurst, J. (2004) HOCl-mediated cell death and metabolic dysfunction in the yeast *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.*, 423: 170–181.

Kishikawa, K., Chalfant, C., Perry D., Bielawska, A., Hannun, Y. (1999) Phosphatidic acid is a potent and selective inhibitor of protein phosphatase 1 and an inhibitor of ceramide-mediated responses. *J. Biol. Chem.*, 274: 21335–21341.

Kitagaki, H., Cowart, A., Matmati, N., Vaena de Avalos, S., Novgorodov, S., Zeidan, Y., Bielawski, J., Obeid, L., Hannun, Y. (2007) Isc1 regulates sphingolipid metabolism in yeast mitochondria. *Biochim. Biophys. Acta.*, 1768(11): 2849–2861.

Kresnowati, M., Winden, W., Gulik, W., Heijnen, J. (2008) Energetic and metabolic transient response of Saccharomyces cerevisiae to benzoic acid. *FEBS J.*, 275: 5527–5541.

Laun, P., Ramachandran, L., Jarolim, S., Herker, E., Liang, P., Wang, J., Weinberger, M., Burhans, D., Suter, B., Madeo, F., Burhans, W., Breitenbach, M. (2005) A comparison of the aging and apoptotic transcriptome of *Saccharomyces cerevisiae*. *FEMS Yeast Res.*, 5: 1261–1272.

Lee, J., Hannun, Y., Obeid, L. (2000) Functional dichotomy of protein kinase C (PKC) in tumor necrosis factor-alpha (TNF-alpha) signal transduction in L929 cells. *J. Biol. Chem.*, 275: 29290–29298.

Legembre, P., Daburon, S., Moreau, P., Moreau, J., Taupin, J. (2006) Cutting edge: modulation of Fas-mediated apoptosis by lipid rafts in T lymphocytes. *J. Immunol.*, 176: 716-720.

Lingwood, D. and Simons, K. (2010) Lipid rafts as a membrane-organizing principle. *Science*, 327: 36-50.

Liu, X. Zeidan, Y., Elojeimy,S., Holman, D., El-Zawahry, A., Guo, G., Bielawska, A., Bielawski, J., Szulc, Z., Rubinchik, S., Dong, J., Keane, T., Tavassoli, M., Hannun, Y., Norris, J. (2006) Involvement of sphingolipids in apoptin-induced cell killing. *Mol. Ther.*, 14: 627–636.

Lohr, N. and Kersten, J. (2010) Man overboard! Rescuing myocardium with membrane rafts, *Anesthesiology*, 112: 1076 – 1078.

London, E. and Brown, D. (2000) Insolubility of lipids in triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). *Biochim. Biophys. Acta.*, 1508: 182-195.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.

Ludovico, P., Rodrigues, F., Almeida, A., Silva, M., Barrientos, A., Côrte-Real, M. (2002) Cytochrome c Release and Mitochondria Involvement in Programmed Cell Death Induced by Acetic Acid in Saccharomyces cerevisiae. *Mol. Biol. Cell*, 13: 2598–2606.

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

Lushchak, O., Nykorak, N., Ohdate, T., Inoue, Y., Lushchak, V. (2009) Inactivation of Genes Encoding Superoxide Dismutase Modifies Yeast Response to S-NitrosoglutathioneInduced Stress, *Biochemistry (Mosc)*, 74: 445-451.

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

Madeo, F., Herker, E., Maldener, C., Wissing, S., Lächelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S., Wesselborg, S., Fröhlich, K. (2002) A caspase-related protease regulates apoptosis in yeast. *Mol. Cell*, 9(4): 911-917.

Madeo, F., Herker, E., Wissing, S., Jungwirth, H., Eisenberg, T., Frohlich, K. (2004) Apoptosis in yeast, *Curr. Opin. Cell. Biol*., 7: 655-660.

Mao, C., Xu, R., Bielawska, A., Obeid, L. (2000) Cloning of an alkaline ceramidase from Saccharomyces. *J. Biol. Chem.*, 275: 6876-6884.

Mathias, S., Peña, L., Kolesnick, L. (1998) Signal transduction of stress via ceramide. *Biochem. J.*, 335(3): 465–480.

Matmati, N. and Hannun, Y. (2008) ISC1 (inositol phosphosphingolipid-phospholipase C), the yeast homologue of neutral sphingomyelinases. *J. Lipid Res.*. 49: 922-928.

McMullen, T.Lewis, R., McElhaney, R. (2004) Cholesterol–phospholipid interactions, the liquid-ordered phase and lipid rafts in model and biological membranes. *Curr. Op. Coll. Int. Sci.*, 8: 459–468.

Mollapour, M. and Piper, P. (2007) Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. *Mol. Cell Biol.*, 27: 6446–6456.

Morsomme, P., Slayman, C., Goffeau, A. (2000) Mutagenic study of the structure, function and biogenesis of the yeast plasma membrane H^+-ATPase, *Biochim. Biophys. Acta*, 1469: 133-157.

Munro, S. (2003) Lipid rafts: elusive or illusive? *Cell*, 115:377-388.

Nabi, I. and Le, P. (2003) Caveolae/raft-dependent endocytosis, *J. Cell Biol.*, 161(4): 673-677.

Narendranath, N, Thomas, K., Ingledew, W. (2001) Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium. *J. Ind. Microbiol. Biotechnol.*, 26:171-177.

Nevoigt, E. and Stahl, U. (1997) Osmoregulation and glycerol metabolism in the yeast Saccharomyces cerevisiae. *FEMS Microbiol. Rev.*, 21: 231-241.

Nystrom, T. (2005) Role of oxidative carbonylation in protein quality control and senescence. *EMBO J.*, 24: 1311–1317.

O´Rourke, S. and Herskowitz, I. (2004) Unique and redundant roles for HOG MAPK pathway components as revealed by whole-genome expression analysis. *Mol. Biol. Cell*, 15: 532-542.

Ogretmen, B. (2006) Sphingolipids in cancer: Regulation of pathogenesis and therapy. *FEBS Lett.*, 580: 5467–5476.

Ozbayraktar, F. and Ulgen, K. (2009) Molecular facets of sphingolipids: Mediators of diseases, *Biotechnol. J.*, 4: 1028–1041.

Paiva, S., Althoff, S., Casal, M., Leão, C. (1998) Transport of acetate in mutants of *Saccharomyces cerevisiae* defective in monocarboxylate permeases. *FEMS Microbiol. Lett.*, 170(2): 301-306.

Parra, V., Eisner, V., Chiong, M., Criollo, A., Moraga, F., Garcia, A., Hartel, S., Jaimovich, E., Zorzano, S., Hidalgo, C., Lavandero (2008) Changes in mitochondrial dynamics during
Patton, J. and Lester, R. (1991) The Phosphoinositol Sphingolipids of Saccharomyces cerevisiae Are Highly Localized in the Plasma Membrane, *J. Bacteriol.*, 173: 3101-3108.

Pelkmans, L., and Helenius, A. (2002) Endocytosis via caveolae. *Traffic*, 3: 311-320.
Pena, A., Cinco, G., A., Puyou, Tuena, M. (1969) Studies on the mechanism of the stimulation of glycolysis and respiration by K^+ in *Saccharomyces cerevisiae. Biochim. Biophys. Acta*, 180(1): 1-8.

Pereira, C., Chaves, S., Alves, S., Salin, B., Camougrand, N., Manon, S., Sousa, M., Côrte-Real, M. (2010) Mitochondrial degradation in acetic acid-induced yeast apoptosis: the role of Pep4 and the ADP/ATP carrier. *Mol. Microbiol.*, 76(6): 1398-1410.

Philips, J., Herskowitz, I. (1997) Osmotic balance regulates cell fusion during mating in Saccharomyces cerevisiae. *J. Cell Biol.*, 138: 961–974.

Phillips, A., Sudbery, I., Ramsdale, M. (2003) Apoptosis induced by environmental stresses and amphotericin B in *Candida albicans*. *Proc. Natl. Acad. Sci. U S A*, 100(24): 14327-32.

Pike, L (2003) Lipid rafts: Bringing order to chaos. *J. Lipid Res.,* 44, 655–667.

Plesofsky, N., Levery, S., Castle, S., Brambl, R. (2008) Stress-induced cell death Is mediated by ceramide synthesis in *Neurospora crassa*. *Eukaryot. Cell*, 7(12): 2147–2159.

Portillo, F., and Serrano, R. (1989) Growth control strength and active site of yeast plasma membrane ATPase studied by site-directed mutagenesis. *Eur. J. Biochem.*, 186: 501–507.

Radin, N. (2003) Killing tumours by ceramide-induced apoptosis: a critique of available drugs, *Biochem. J.*, 371: 243-256.

Ribeiro, G., Côrte-Real, M., Johansson, B. (2006) Characterization of DNA damage in yeast apoptosis induced by hydrogen peroxide, acetic acid, and hyperosmotic shock. *Mol. Biol. Cell*, 17: 4584–4591.

Roberg, K., Crotwell, M., Espenshade, P., Gimeno, R., Kaiser, C. (1999) LST1 is a SEC24 homologue used for selective export of the plasma membrane ATPase from the endoplasmic reticulum. *J. Cell Biol.*, 145(4): 659-672.

Ruvolo, P. (2001) Ceramide regulates cellular homeostasis via diverse stress signaling pathways. *Leukemia*, 15: 1153-1160.

Salaun, C., James, D., Chamberlain, L. (2004) Lipid rafts and the regulation of exocytosis. *Traffic*, 5(4): 255-264.

Sapienza, K., Bannister, W., Balzan, R. (2008) Mitochondrial involvement in aspirin-induced apoptosis in yeast. *Microbiology*, 154: 2740–2747.

Saraiva, L., Silva, R., Pereira, G., Gonçalves, J., Côrte-Real, M. (2006) Specific modulation of apoptosis and Bcl-xL phosphorylation in yeast by distinct mammalian protein kinase C isoforms. *J. Cell Sci.*, 119(15): 3171-3181.

Sawada, M., Nakashima, S., Banno, Y., Yamakawa, H., Takenaka, K., Shinoda, J., Nishimura, Y., Sakai, N., Nozawa, Y. (2000) Influence of Bax or Bcl-2 overexpression on the ceramide-dependent apoptotic pathway in glioma cells, *Oncogene*, 19: 3508-3520.

Sawai, S., Okamoto, Y., Luberto, C., Maoi, C., Bielawska, C., Domae, N., Hannun, Y. (2000) Identification of ISC1 (YER019w) as inositol phosphosphingolipid phospholipase C in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, 275(50): 39793–39798.

Scarborough, G. (1976) *In vitro* demonstration of biological role of *Neurospora* plasma-membrane ATPase, *Federation Proceedings*, 35(7): 1587-1597.

Scarborough, G. (2000) The plasma membrane proton-translocating ATPase. *Cell Mol. Life Sci.*, 57: 871–883.

Schorling, S., Vallée, B., Barz, W., Riezman, H., Oesterhelt, D. (2001) Lag1p and Lac1p are essencial for the acyl-CoA-dependent ceramide synthase reaction in Saccharomyces cerevisiae. *Mol. Biol. Cell*, 12: 3417-3427.

Serrano, R., Kielland-Brandt, M., Fink, G. (1986) Yeast plasma membrane ATPase is essential for growth and has homology with (Na^+ + K^+), K^+ and Ca^{2+}-ATPase. *Nature*, 319: 689–693.

Sharma, P., Varma, R., Sarasij, R. , Ira, Gousset, K., Krishnamoorthy, G., Rao, M., Mayor, S. (2004) Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell*, 116: 577-589.

Shirahama, T., Sakakura, C., Sweeney, E., Ozawa, M., Takemoto, M., Nishiyama, K., Ohia, Y., Igarashi, Y. (1997) Sphingosine induces apoptosis in androgen-independent human prostatic carcinoma DU-145 cells by suppression of bcl-XL gene expression. *FEBS Lett.*, 407(1): 97-100.

Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes, *Nature*, 387: 569-572.

Simons, K. and Toomre, D. (2000) Lipid rafts and signal transduction. *Nature Rev. Mol. Cell Biol.*, 1: 31-39.

Simons, K. and Vaz, W. (2004). Model systems, lipid rafts, and cell membranes. *Annu. Rev. Biophys. Biomol. Struct.*, 33: 269-295.

Siskind, L., Kolesnick, R., Colombini, M. (2002) Ceramide channels increase the permeability of the mitochondrial outer membrane to small proteins, *J. Biol. Chem.*, 277(30): 26796–26803.

Struckhoff, A., Bittman, R., Burow, M., Clejan, S., Elliott, S., Hammond, T., Tang, Y., Beckman, B. (2004) Novel ceramide analogs as potential chemotherapeutic agents in breast cancer. *J. Pharmacol. Exp. Ther.*, 209(2): 523-532.

Taha, T., Kitatani, K., El-Alwani, M., Bielawski, J., Hannun, Y., Obeid, L. (2005) Loss of sphingosine kinase-1 activates the intrinsic pathway of programmed cell death: modulation of sphingolipid levels and the induction of apoptosis. *FASEB J.*, 20: 482–484.

Taha, T., Mullen, T., Obeid, L. (2006) A house divided: ceramide, sphingosine, and sphingosine-1- phosphate in programmed cell death, *Biochim. Biophys. Acta*, 1758: 2027–2036.

Tamás, M., Luyten, K., Sutherland, F., Hernandez, A., Albertyn, J., Valadi,H., Li, H., Prior, B., Kilian, S., Ramos, J., Gustafsson, L., Thevelein, J., Hohmann, S. (1999) Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Mol. Microbiol.*, 31: 1087–1104.

Tepper, A., Cock, J., Vries, E., Borst, J., Blitterswijk, W. (1997) CD95/Fas-induced ceramide formation proceeds with slow kinetics and is not blocked by caspase-3/CPP32 inhibition. *J. Biol. Chem.*, 272(39): 24308–24312.

Uren, A., O'Rourke, K., Aravind, L., Pisabarro, M., Seshagiri, S., Koonin, E., Dixit, V. (2000) Identification of paracaspases and metacaspases: Two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Mol. Cell*, 6: 961–967.

Vaena de Avalos, S., Okamoto, Y., Hannun, Y. (2004) Activation and localization of inositol phosphosphingolipid phospholipase C, Isc1p, to the mitochondria during growth of Saccharomyces cerevisiae. *J. Biol. Chem.*, 279(12):11537-45.

Valenti, D., Vacca, R., Guaragnella, N., Passarella, S., Marra, E., Giannattasio, S. (2008) A transient proteasome activation is needed for acetic acid-induced programmed cell death to occur in Saccharomyces cerevisiae. *FEMS Yeast Res.*, 8: 400–404.

Valleé, B. and Riezman, H. (2005) Lip1p: a novel subunit of acyl-CoA ceramide synthase, *EMBO J.*, 24: 730–741.

Vit, J., Rosselli, F. (2003) Role of the ceramide-signaling pathways in ionizing radiation-induced apoptosis. *Oncogene*, 22: 8645–8652.

Wachtler, V. and Balasubramanian, M. (2006) Yeast lipid rafts? – An emerging view. *Trends Cell Biol.*, 16(1): 1-4.

Willhite, D. and Wrigh, S. (2009) Detergent-based isolation of yeast membrane rafts: An Inquiry-Based Laboratory Series for the Undergraduate Cell Biology or Biochemistry Lab. *BAMBED*, 37(6) 349–354.

Wissing, S., Ludovico, P., Herker, E., Buttner, S., Engelhardt, S., Decker, T., Link, A., Proksch, A., Rodrigues, F., Côrte-Real, M., Frohlich, K., Manns, J., Cande, C, Sigrist, S., Kroemer, G., Madeo, F. (2004) An AIF orthologue regulates apoptosis in yeast. *J. Cell Biol.*, 166: 969-974.

Wong, W. and Schlichter, L. (2004) Differential recruitment of Kv1.4 and Kv4.2 to lipid rafts by PSD-95. *J. Biol. Chem.*, 279(1): 444–452.

Yan, F. and Polk, D. (2001) Kinase suppressor of ras is necessary for tumor necrosis factor alpha activation of extracellular signal-regulated kinase/mitogen-activated protein kinase in intestinal epithelial cells. *Cancer Res.*, 61: 963–969.

Zhang, Y., Yao, B., Delikat, S., Bayoumy, S., Lin, X., Basu, S., McGinley, M., Chan-Hui, P., Lichenstein, H., Kolesnick, R. (1999) Kinase suppressor of Ras is ceramide-activated protein kinase. *Cell*, 89: 63–72.

Zhou, M., Horita, D., Waugh, D., Byrd, R., Morrison, D. (2002) Solution structure and functional analysis of the cysteine-rich C1 domain of kinase suppressor of Ras (KSR). *J. Mol. Biol.*, 315: 435-446.