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Study of the influence of lipid rafts in acetic acid-induced apoptosis

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Summary

Lipid rafts are sphingolipid-sterol rich micro-domains of the plasma membrane that have been associated with different cellular processes, including apoptosis. During the past years, yeast has been successfully established as a model to study mechanisms of programmed cell death in mammalian cells. *Saccharomyces cerevisiae* commits to cell death showing typical hallmarks of metazoan apoptosis in response to different stimuli. Gup1p, a pleiotropic *O*-acyltransferase first associated with glycerol metabolism and transport, is involved in lipid metabolism, rafts integrity and assembly, as well as in GPI anchor remodeling, among a wide range of cellular processes. While Gpd1p/Gpd2p are the two isoforms of glycerol-3-phosphate dehydrogenase that have a prime role in the production of glycerol, Rvs161p is a lipid raft protein mainly involved in the regulation of actin cytoskeleton polarization and secretory vesicle trafficking. Actin cytoskeleton together with vesicle trafficking have been suggested to play a crucial role in yeast apoptosis. Therefore, it is conceivable that lipid rafts may also play a role in yeast cell death and understanding their function in such process in yeast may provide further insights on their role in mammalian apoptosis. For this purpose we used two known apoptosis inducing conditions, chronological aging and acetic acid, and analyzed several apoptotic markers in Δ*gup1*, Δ*gpd1*Δ*gpd2* and Δ*rvs161* mutant strains. We found that Δ*gup1* presents a significantly reduced chronological life span as compared to Wt and is also highly sensitive to acetic acid treatment. According to the apoptotic markers analyzed, cells lacking *GUP1* seem to be incapable of undergoing apoptosis. Instead this mutant appears to be experiencing a necrotic cell death process. The Δ*gpd1*Δ*gpd2* (but not single mutants) and Δ*rvs161* mutant strains are also sensitive to acetic acid. However, the apoptotic markers examined suggest that these mutants die by apoptosis, differently from that we observed for *Δgup1* mutant. The ergosterol distribution in Wt and in the acetic acid-sensitive mutant strains *Δgup1*, Δ*gpd1*Δ*gpd2* and Δ*rvs161* was subsequently observed by filipin staining. An altered ergosterol distribution was visualized in all mutants studied, particularly in *Δgup1* mutant. We also observed that acetic acid induces a rearrangement in the distribution of ergosterol.

Altogether, our results indicate that lipid rafts seems to be a key component in apoptotic induction/signaling, probably even essential in some circumstances for the correct development of apoptosis in yeast.

Sumário

Os *rafts*, domínios da membrana plasmática ricos em esfingolípidos e ergosterol, têm sido associados com diversos processos celulares, incluindo a apoptose. Nos últimos anos, a levedura tem sido aceite como um bom modelo para o estudo dos mecanismos de regulação da morte celular programada. Vários estudos demostraram que, em resposta a diferentes estímulos, a levedura *Saccharomyces cerevisiae* apresenta uma morte celular com os fenótipos típicos da apoptose de mamíferos. A proteína Gup1p, uma *O*-aciltransferase anteriormente associada com o metabolismo e transporte de glicerol, está envolvida, de entre vários processos, no metabolismo lipídico, na arquitectura/integridade dos *rafts*, assim como na remodelação das âncoras GPI. As proteínas Gpd1p/Gpd2p são duas isoformas da glicerol-3-fosfatodesidrogenase, enzima responsável pela produção do glicerol. O Rvs161p é uma proteína dos *rafts* envolvida na regulação da polarização da actina e no tráfego de vesículas. Os filamentos de actina assim como o trafego de vesículas têm sido apontados como importantes intervenientes na apoptose em leveduras. Assim, é possível que os rafts exerçam um papel importante na morte celular em leveduras. O conhecimento da sua função neste processo poderá ajudar a compreender a importância dos *rafts* neste tipo de morte celular em mamíferos.
Para estudar esta hipótese, foram analisados diferentes marcadores apoptóticos nos mutantes Δgup1, Δ*gpd1*Δ*gpd2* e Δ*rvs161*, em duas condições indutoras de apoptose, o envelhecimento cronológico e o tratamento com ácido acético. Verificamos que, quando comparado com a Wt, este mutante apresenta uma redução significativa na longevidade sendo também extremamente sensível ao ácido acético. Os marcadores apoptóticos estudados indicam que as células deletadas no gene *GUP1* são incapazes de morrer por apoptose. Nas condições mencionadas, a estirpe Δ*gup1* parece morrer por necrose. Os mutantes Δ*gpd1*Δ*gpd2* (mas não os mutantes simples) e Δ*rvs161* são também sensíveis ao ácido acético. Contrariamente ao que se observou para o mutante *Δgup1*, estes mutantes morrem por apoptose. Por último, examinou-se a distribuição do ergosterol nos mutantes sensíveis ao ácido acético. Observou-se alterações na sua distribuição em todos os mutantes, particularmente o mutante *Δgup1*. Verificou-se também que o tratamento com ácido acético induz um reorganização da distribuição do ergosterol.
De um modo geral, os resultados obtidos sugerem que os *rafts* exercem uma função determinante na indução/sinalização da apoptose, sendo portanto, a sua integridade essencial no desencadear do processo apoptótico.

Table of Contents

Chapter I

INTRODUCTION

I - 1 Cell death

Soon after being recognized that organisms are made of cells, it was discovered that cell death is an important part of development. Cell death was first observed during amphibian metamorphosis (Vogt, 1842). Several years later, inhibitors of RNA and protein synthesis were found to inhibit cell death, indicating that an active metabolism is necessary to carry out this process. The term programmed cell death (PCD) was first used by Lockshin and Williams in 1964 to describe the cell death that occurs in predictable places and at predictable times during mammalian development, emphasizing that death is somehow programmed into the development plan of the organism (Lockshin and Williams, 1964). It is clear, however, that cell death can be prevented by substances released by other tissues, indicating that the death is not inevitable and apparently can be suppressed by signals from other cells (Saunders, 1966).

Accordingly, the term PCD is used nowadays to define all modes of death whose execution is carried out in a regulated manner and is under molecular control. This requires that the process follows a specific, orchestrated choreography, in which one event is triggered by another. Moreover, such process should implicate an advantage for the particular organism or cell population. Thus, PCD is a normal physiological form of cell death that plays a key role in both the maintenance of adult tissues and embryonic development. In adult tissues, for instance, PCD is involved in the maintenance of homeostasis, in the control of cell number, and also provides a defense mechanism by which damaged and potentially dangerous cells can be eliminated. During development, PCD plays a key role by eliminating unwanted cells from a variety of tissues, like the elimination of the tissue between the digits during the formation of fingers, and in the selection of neurons that present correct connections with their target cells (Galluzzi *et al.*, 2007).

For several years, apoptosis was wrongly understood as synonymous for PCD. However, it must be kept in mind that although apoptosis is the most common form, dying cells may follow other types of PCD, including programmed necrosis, autophagic cell death and mitotic catastrophe (Table I-1). Yet, unlike apoptosis the other types of

PCD are still difficult to distinguish unambiguously and remain poorly characterized (Galluzzi *et al.*, 2007).

Table I-1. Morphological aspects of different types of cell death. Adapted from Galluzzi *et al.*, 2007.

Cell death type	Characteristic morphological aspects
Apoptosis	- Rounding up the cell - Reduction of cellular and nuclear volume - Retraction of pseudopods - Nuclear fragmentation - Little modification of cytoplasmic organelles - Plasma membrane blebbing
Necrosis	- Cytoplasmic swelling - Rupture of plasma membrane - Swelling of cytoplasmic organelles - Moderate chromatin condensation
Autophagy	- Lack of chromatin condensation - Massive vacuolization of the cytoplasm (double-membrane autophagic vacuoles)
Mitotic catastrophe	- Micronucleation - Multinucleation

Besides its role in the development and homeostasis of cell populations, PCD has also been associated with the occurrence of several human and mammalian diseases. Since then, the study of different types of PCD has increased exponentially among the scientific community, aiming to use the emerging knowledge in the treatment of several pathologies.

I - 2 Apoptosis

The term apoptosis was first proposed in 1972 by Kerr, Wyllie, and Currie to describe a morphologically distinct mechanism of "controlled cell deletion", which plays a complementary but opposite role to mitosis in the regulation of animal cell populations (Kerr *et al.*, 1972). Since then, apoptosis has been recognized and accepted as an

important physiological process of PCD. Nowadays, apoptosis is considered a universal event in the normal development and aging of multicellular organisms, acting as a homeostatic mechanism that removes mutated, infected or dispensable cells and contributes to tissue growth, maintenance and renewal. Albeit its importance to the normal functioning of the organisms, a defective regulation of apoptosis is, usually, associated to the occurrence and progression of several pathologies (Rudin and Thompson, 1997). The inappropriate activation of apoptosis may cause or contribute to a variety of diseases including acquired immunodeficiency syndrome (AIDS) (Ameisen *et al.*, 1995), ischemic strokes (Raff *et al.*, 1993), and neurodegenerative disorders, such as Alzheimer's (Smale *et al.*, 1995) or Huntington's diseases (Hickey and Chesselet, 2003). Conversely, a defective activation of apoptosis is responsible for some autoimmune diseases (Tan, 1994) and is commonly recognized as a determinant step in the development of cancers (Lowe and Lin, 2000; Evan and Vousden, 2001; Reed, 2003). Apoptosis has been placed under the spotlight of scientific investigation in the last years, mainly due to the growing understanding of its important role on the emergence and prevention of diseases, increasing the interest in apoptosis as a therapeutic target.

I - 2.1 The morphology of apoptosis

Apoptosis has been suggested as an active programmed phenomenon that can be triggered by a variety of stimuli, which include genotoxic agents (responsible for DNA damage), loss of extracellular survival factors or the specific activation of death receptors (Nagata, 2000). Despite the process responsible for triggering apoptosis, the ultimate morphological and biochemical characteristics of the process are similar. Thus, apoptotic cells are distinguished by membrane blebbing, cell shrinkage, chromatin condensation and intranucleosomal cleavage of DNA, followed by cell fragmentation into membrane-bound apoptotic bodies, and the loss of lipid asymmetry with phosphatidylserine exposure on the surface of the plasma membrane. Phosphatidylserine externalization is known to be responsible for 'calling' neighboring cells to phagocyte apoptotic cells, preventing the uncontrolled release of intracellular

components to the extracellular milieu (secondary necrosis) and inflammation (Kerr *et al.*, 1972; Fleury *et al.*, 2002).

I - 2.2 The biochemistry of apoptosis

Many of the genes that control apoptosis have been identified, and the molecular mechanisms underlying these processes were proved to be evolutionarily conserved (Metzstein *et al.*, 1998). Central to the execution of the suicide program are the cysteine-dependent aspartate-specific proteases, so called caspases (Shi, 2002). The Bcl-2 family, a second set of apoptotic regulators, is also important to the regulation of apoptosis (Adams and Cory, 1998).

I - 2.2.1 Caspases

Most of the morphological changes that were observed during apoptosis are caused by a set of cysteine proteases that are activated specifically in apoptotic cells. These death proteases are homologous to each other, and are part of a large protein family known as the caspases (Cysteine dependent Aspartate-specific Proteases) (Shi, 2002). Caspases are highly conserved through evolution. Over a dozen caspases have been identified in humans; about two-thirds of these have been suggested to function in apoptosis (Earnshaw *et al.*, 1999).

All known caspases have cysteine residues at their active sites crucial for its proteolytic activity, that selectively cleave target proteins in specific positions (after an aspartate residue). As for most proteases, caspases are synthesized as enzymatically inert zymogens called pro-caspases. These inactive proteins are then activated by proteolytic cleavage of its pre-form, at the aspartate residues. The smaller fragments dimerise to form the active enzyme (caspases). Besides cleaving several proteins, these enzymes can also act upon other caspases activating them and leading to a phenomenon known as the cascade of caspase activation (Chowdhury *et al.*, 2008). Regulation of caspases is, therefore, central to determine the cell's fate. Once caspases are initially activated, there seems to be an irreversible commitment towards cell death.

Caspases have been divided into subfamilies based on their substrate preference, extent of sequence identity and structural similarities. Apoptotic caspases are usually divided into two different groups: the initiator caspases (that include caspases-2, 8, 9 and 10) and the effector caspases (caspasese-3, 6 and 7) (Rai *et al.*, 2005). In general, initiator caspases, which act at the apex of the signaling cascade, are characterized by its long pro-domains (Death Effector Domain (DED) in caspases-8 and 10 and Caspase Recruitment Domain (CARD) in caspases-2 and 9), with more than 90 amino acid residues, while the effector caspases possess shorter pro-domains with only 20-30 residues. (Budihardjo *et al.*, 1999; Chowdhury *et al.*, 2008).

I - 2.2.2 Bcl-2 family

The Bcl-2 family proteins are also key regulators of apoptosis. All proteins of the Bcl-2 family share homologue regions known as BH domains. Thus, at the first time, this family was divided into three groups based on structural similarities: Anti-apoptotic members (share sequence similarity in all four conserved BH domains (BH1–4)); the pro-apoptotic members (have similarity in three domains (BH1–3)); and the ‘‘BH3-only’’ subfamily (display sequence similarity only in the BH3 domain) (Adams and Cory, 2001; Borner, 2003). Presently, with new results obtained for the sub-group “BH3-only” proteins, they are divided into four categories accordingly to their function: the anti-apoptotic Bcl-2 proteins (A1, Bcl-2, Bcl-w, Bcl-xL and Mcl-1); Bcl-2 effector proteins, (Bak and Bax); direct activators BH3 only proteins (Bid, Bim and Puma); and sensitizers/de-repressors proteins (Bad, Bik, Bmf, Hrk and Noxa) (Chipuk *et al.*, 2010).

Bcl-2 family members are critical for the regulation of the mitochondrial pathway of apoptosis (Roset et al., 2007). However, the molecular actions of Bcl-2 family members are still controversial. The ability of these proteins to form homo- or heterodimers suggests that there is a neutralizing competition between both members, therefore the ratio between the two subsets will determine the cell’s fate (Gross *et al.*, 1999). These proteins are also subjected to post-translational modifications, for instance phosphorylation, that affect their function. Such modifications can be mediated by the interaction with other cytoplasmic and mitochondrial proteins, such as the protein kinase C (PKC) isoforms (Gutcher *et al.*, 2003).

I - 2.3 Mechanisms of apoptosis

The cascade of events leading to this PCD process can be sub-divided in activation and execution phase. During the activation phase, multiple signaling pathways lead to the central control of the cell death machinery and activate it. This is followed by the execution stage, in which the activated machinery acts on multiple cellular targets resulting on the destruction of the cell (Elmore, 2007).

I - 2.3.1 Activation phase

Currently, two well-understood mechanisms of apoptosis activation are documented: the extrinsic apoptotic pathway and the intrinsic apoptotic pathway (Fig I-1). Moreover, there is evidence that the two pathways are linked and that the molecules of one pathway can influence the other (Igney and Krammer, 2002).

I - 2.3.1.1 Extrinsic apoptotic pathway

The extrinsic pathway, also called death receptor pathway, depends of transmembrane receptor-mediated interactions. This pathway involves death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily (Locksley *et al.*, 2001). All members of the TNF receptor family share similar cysteine-rich extracellular domains and a cytoplasmic domain of about 80 amino acids called the death domain (DD) (Ashkenazi and Dixit, 1998). This DD plays a crucial role in spreading the death signal from the cell surface to the intracellular signaling pathways.

The death receptors are activated by specific extracellular ligands that promote clustering of the receptors. After ligand binding, cytoplasmic adapter proteins such as FADD (Fas-Associated protein with Death Domain) or TRADD (Tumor necrosis factor Receptor type 1-Associated Death Domain) are recruited, which in turn also have a DD and an additional interaction domain, the death effector domain (DED). These adapter proteins are responsible for connecting the death receptor, through the interaction between the DDs, to pro-caspase-8, via dimerization of the DEDs. Association between

the extracellular ligand, the death receptor, the adaptor protein and the pro-caspase 8 forms a complex called the Death Inducer Signaling Complex – DISC. The concentration of pro-caspase-8 in the DISC results in its cleavage and subsequent activation (Kischkel *et al.*, 1995). Once active, caspase-8 initiates the cascade of caspase activation by processing caspases-3, -6 and -7, which will act upon different substrates (Thorburn, 2004; Chowdhury *et al.*, 2008). The death receptors can be inhibited by a protein called c-FLIP, which binds to FADD and caspase-8, rendering them ineffective (Kataoka *et al.*, 1998; Scaffidi *et al.*, 1999).

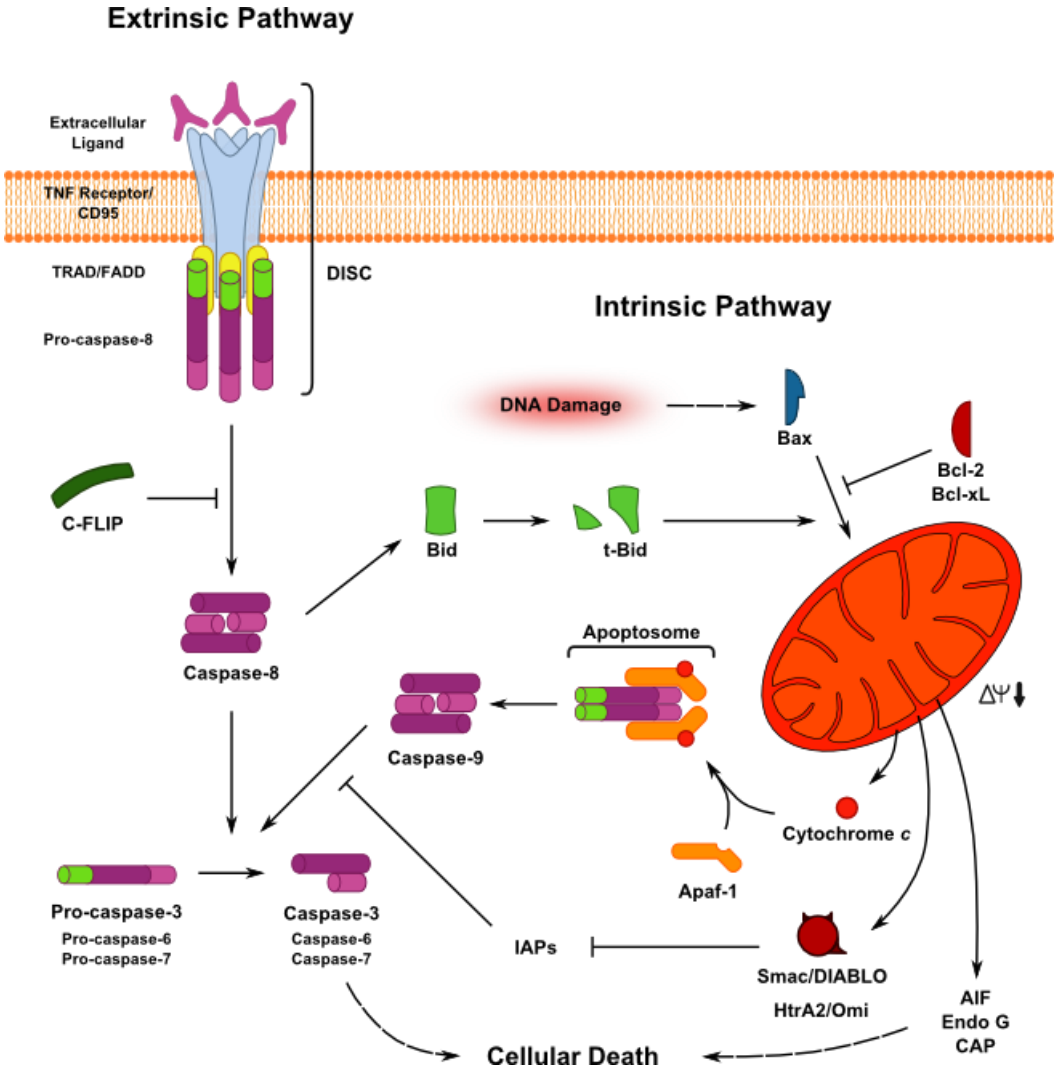

Fig. I-1. Apoptotic pathways. The extrinsic pathway is initiated through the stimulation of the death receptors. Association between the death receptor, the adaptor protein and the pro-caspase 8 forms the DISC, responsible for the caspases 8 activation. The intrinsic pathway is activated by internal stimuli that depend on the MMP. The MMP facilitates the release of pro-apoptotic proteins to the cytoplasm, such as cytochrome *c* that combines with Apaf-1 and pro-caspase 9 to form the apoptosome, resulting in caspases 9 activation.

I - 2.3.1.2 Intrinsic apoptotic pathway

The intrinsic pathway (also called mitochondrial pathway) initiates within the cell and is triggered by stress stimuli such as DNA damage, caused by radiation, toxins, hypoxia, hyperthermia, viral infections and free radicals, or by absence of certain growth factors, such as hormones and cytokines. The activation of this pathway depends on the mitochondrial membrane permeabilization (MMP). Several mechanisms underlying MMP have been proposed including the formation of pores in the mitochondrial membrane mediated by pro-apoptotic members of the Bcl-2 family, such as Bax and Bak (Dejean *et al.*, 2005). The MMP, and consequent loss of the mitochondrial membrane potential, facilitates the release of several pro-apoptotic proteins to the cytoplasm. The first pro-apoptotic proteins that are released from mitochondria include cytochrome *c*, Smac/DIABLO (Second Mitochondria derived Activator of Caspases/Direct Inhibitor of Apoptosis Protein (IAP)-Binding protein with Low PI), and the serine protease HtrA2/Omi (Cai *et al.*, 1998; Du *et al.*, 2000; van Loo *et al.*, 2002; Garrido *et al.*, 2006). Latter, the pro-apoptotic proteins AIF (Apoptosis-Inducing Factor), endonuclease G and CAD (Caspase-Activated Deoxyribonuclease) are also released.

The first group of proteins activates the caspase dependent mitochondrial pathway. After being released from the mitochondria, cytochrome *c* combines with Apaf-1 and dATP to form a protein complex known as the apoptosome. This complex, in turn, activates caspase-9 thus initiating the cascade of caspase activation which leads to cellular death (Acehan *et al.*, 2002). Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting IAP (Inhibitors of Apoptosis Proteins) activity (van Loo *et al.*, 2002; Schimmer, 2004).

The second group of pro-apoptotic proteins is released only after the cell has committed to die. AIF and Endonuclease G are both translocated to the nucleus causing nuclear fragmentation and condensation (Joza *et al.*, 2001; Li *et al.*, 2001) in the so-called “stage I” (Susin *et al.*, 2000). Both proteins function in a caspase-independent manner. CAD is subsequently released from the mitochondria and also translocates to the nucleus where, after cleavage by caspase-3, it leads to DNA fragmentation and a more pronounced and advanced chromatin condensation (Enari *et al.*, 1998), referred to as “stage II” condensation (Susin *et al.*, 2000).

Besides allowing the release of pro-apoptotic factors, loss of mitochondrial membrane potential and mitochondria permeabilization also induces the loss of cellular homeostasis: ATP synthesis stops, redox molecules such as NADH, NADPH and glutathione are oxidized, and the production of reactive oxygen species (ROS) increases (Fleury *et al.*, 2002).

I - 2.3.2 Execution phase

Both the extrinsic and intrinsic pathways converge on the same terminal, or execution phase. This final phase of apoptosis begins when the execution caspases are activated. Execution caspases (3, 6 and 7) activate cytoplasmic endonuclease, which degrades nuclear material, and active as well proteases that degrade the nuclear and cytoskeletal proteins, being responsible for the typical morphological and biochemical changes seen in apoptotic cells (Slee *et al.*, 2001).

Caspase-3 is considered to be the most important of the execution caspases. As mentioned above, it is responsible for specific activation of endonuclease CAD. Caspase-3 also induces the cytoskeletal reorganization and the disintegration of the cell into apoptotic bodies. This caspase cleaves gelsolin, an actin binding protein, and the fragments of gelsolin, in turn, cleave actin filaments resulting in disruption of the cytoskeleton, intracellular transport, cell division, and signal transduction (Kothakota *et al.*, 1997). The phagocytosis of apoptotic cells is the last stage of apoptosis. Externalization of phosphatidylserine on the surface of apoptotic cells is the hallmark of this phase.

I - 3 Apoptosis in yeast *Saccharomyces cerevisiae*

The first evidence suggesting the presence of an endogenous PCD in yeast was obtained by Madeo and his co-workers, in 1997, who described a cell cycle cdc48^{S565G} mutant of *Saccharomyces cerevisiae* exhibiting a cell death process that presented typical characteristics of apoptosis (Madeo *et al.*, 1997). The finding that yeast can undergo apoptosis made possible to investigate this process of PCD in a genetically tractable

eukaryotic organism, constituting an ideal model to study the cell death regulatory network of higher organisms, including the switches between apoptotic, autophagic, and necrotic pathways. Since this discovery, several orthologous of crucial mammalian apoptotic proteins have been identified in yeast. For example, the metacaspase Yca1p (Madeo *et al.*, 2002), an HtrA2/Omi homologue, Nma111p (Fahrenkrog *et al.*, 2004), an IAP (Walter *et al.*, 2006), Bir1p and two AIF/AMID homologues, Aif1p and Ndi1p (Wissing *et al.*, 2004; Li *et al.*, 2006) have been described. Although each of these proteins has been shown to function in yeast apoptosis under certain conditions, the pathways in which they are involved remain to be elucidated.

I - 3.1 Yeast apoptotic triggers

Several stimuli have been described as initiators of yeast apoptosis. Such stimuli can be divided into: a) exogenous triggers in the form of chemical or physical stress, natural triggers like mating pheromone exposure and via heterologous expression of human pro-apoptotic proteins; and b) endogenous triggers as part of lethal signal transduction pathways (Carmona-Gutierrez *et al.*, 2010).

H_2O_2 and acetic acid are the major exogenous triggers commonly used to induce apoptosis in yeast (Madeo *et al.*, 1999; Ludovico *et al.*, 2001). However, a variety of additional agents have also been reported to induce an apoptotic phenotype, namely ethanol, hypochlorous acid, high salt concentrations, heavy metals, UV radiation and heat stress. Several pharmacological agents, such as aspirin, paclitaxel, edelfosine, arsenic, and bleomycin can also induce yeast apoptosis. In addition, several compounds, which normally constitute nutrients or oligo-elements, can also trigger apoptosis when they are applied at toxic concentrations. This includes glucose, sorbitol (hyper-osmotic stress), copper, manganese, and iron. Apoptotic death may also be triggered by toxins from either non-clonal enemy strains in competition for nutrients (killer strain attack), or by higher eukaryotes in their defense against pathogenic fungi (plant or animal attack) (Carmona-Gutierrez *et al.*, 2010). Heterologous expression of human pro-apoptotic proteins, essentially proteins from the Bcl-2 family, leads to an apoptotic cell death as well. The expression of the pro-apoptotic protein Bax in yeast resulted in cell death with an apoptotic nature, which could be prevented by the co-expression of the

anti-apoptotic Bcl-2 or Bcl-xL proteins, suggesting that the apoptotic pathways might be conserved from yeast to mammalians (Madeo *et al.*, 2004). In contrast to the previous studies, other authors showed the absence of typical apoptotic markers in Bax induced yeast cell death. Instead they revealed that autophagy is activated (Kissova *et al.*, 2006).

Endogenous triggers have also been associated with apoptotic death. Defects in several cellular processes such as N-glycosylation, chromatid cohesion, mRNA stability, and ubiquitination can trigger cell death in yeast (Carmona-Gutierrez *et al.*, 2010). Moreover, DNA damage (resulting from oxygen metabolism and ROS generation) and replication failure can stimulate the activation of yeast cell death programs. Apoptosis may also play a role in both replicatively (Laun *et al.*, 2001) and chronologically (Fabrizio *et al.*, 2004; Herker *et al.*, 2004) aged cells. In addition, apoptosis has also been demonstrated to occur during development of colonies on solid media (Vachova and Palkova, 2005).

I - 3.2 Benefits to a unicellular organism

Although being the most extensively studied, *S. cerevisiae* is not the only unicellular organism that can exhibit typical markers of apoptosis. In fact, apoptosis has been described for several other model organisms such as, *Schizosaccharomyces pombe*, *Dictyostelium discoideum*, *Trypanosoma cruzi*, and *Tetrahymena thermophile* (Frohlich and Madeo, 2000). Thus, the question arises: why a unicellular organism should have developed and conserved a suicide program during evolution in a context in which the demise of a single cell can be viewed as the death of the organism.

Yeast populations should not be interpreted just as a group of partitioned unicellular organisms that do not communicate among each other, but rather as a multicellular community of interacting individuals. Several microorganisms cluster together to survive nutrient depletion, forming multicellular communities called biofilms (Vachova and Palkova, 2005). In such community, the benefit of a cellular suicide program seems evident: the self-destruction of virus infected, damaged, and old cells, which consume dwindling nutrients or spread an infection, contributes to the viability and reproductive

success of fitted members of the community harboring similar genomes (Buttner *et al.*, 2006).

It has been hypothesized that apoptosis in unicellular organisms, even in a liquid culture, serves an altruistic purpose, whereby old and sick cells self-sacrifice for the good of the population. Under certain circumstances, the death of a single cell might be beneficial for the whole population, thus promoting the survival of other cells (Frohlich and Madeo, 2000; Gourlay *et al.*, 2006). Several physiological scenarios in which altruistic death of single cells promotes survival of the population as a whole support this idea (Buttner *et al.*, 2006). For instance, a) the pheromone signaling leads to the apoptotic death of cells that fail mating, therefore eliminating infertile or damaged cells (Severin and Hyman, 2002); b) death of old cells within the colony center feeds the young cells at the colony margin (Vachova and Palkova, 2005); c) the death of chronologically old cells preserves resources, releases nutrients, and allows adaptive "re-growth", whereas replicatively old cells die for the good of young cells, which inherit the undamaged cellular material upon cellular division. Such findings reinforce the idea of an altruistic behavior in yeast, as a reasonable explanation for the self-sacrifice of unicellular organisms.

I - 4 Chronological aging in yeast

In the past 50 years, the yeast *S. cerevisiae* has been widely used as a model to study cellular aging (Mortimer and Johnston, 1959; Bitterman *et al.*, 2003; Kaeberlein, 2006; Piper, 2006). Since then, two forms of aging have been described: replicative aging and chronological aging. Replicative aging was defined as the number of daughter cells produced by each mother cell before senescence, whereas chronological aging is the length of time that a yeast cell can survive in a non-dividing state (Fabrizio and Longo, 2003). Studies in both yeast aging models demonstrated a relation between environmental nutrients and longevity. Reduction of the number of calories consumed is known to increase both replicative life span (RLS) and chronological life span (CLS) (Lin *et al.*, 2002; Fabrizio and Longo, 2003). Under the CLS model, calorie restriction

is induced either by incubation on water, or by growing the cells in synthetic medium containing low doses of glucose (0.05% to 0.5%) (Smith *et al.*, 2007). In fact, calorie restriction promotes longevity in a variety of organisms other than yeast, including worms, flies and rodents (Masoro, 2005), yet the anti-aging molecular mechanisms activated by this practice are still not completely understood.

Studies in *S. cerevisiae* CLS led to the discovery of three life span-regulatory pathways, namely the Sch9, TOR, and Ras/PKA pathways (Fabrizio *et al.*, 2001; Powers *et al.*, 2006; Fabrizio and Longo, 2008), which are partially conserved in higher eukaryotes (Longo and Fabrizio, 2002) (Fig I-2).

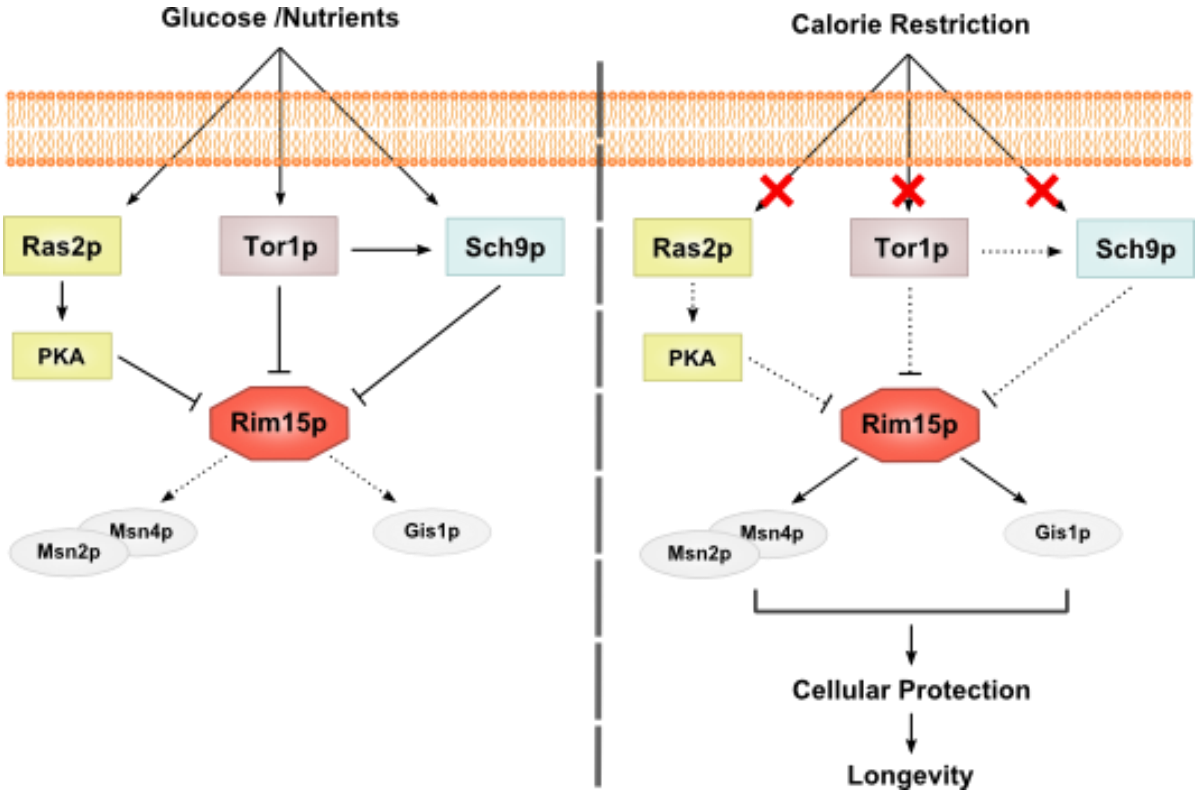

Fig. I-2. Pathways that regulate life span in *S. cerevisiae*. Nutrients activate the Sch9, TOR, and Ras/PKA pathways, which in turn promote the down-regulation of Msn2p/Msn4p and Gis1p-dependent stress resistance systems, through repressing Rim15p. In calorie restriction conditions occurs the down-regulation of the Sch9, TOR, and Ras/PKA pathways and consequent activation of Rimp15p-controlled Msn2p/Msn4p and Gis1p protection system.

These pathways are highly regulated by the availability of nutrients, such as glucose. Therefore, when glucose or other nutrients are present, these pathways are activated and generate signaling cascades that promote cell growth and division. Otherwise, if the nutrients are scarce, the reduction of the Sch9, TOR, and Ras/PKA signaling causes cell division arrest and the activation of mechanisms responsible for cellular protection. Deletion of the gene *SCH9* gene, coding a serine/threonine kinase, extends the yeast CLS and promotes stress resistance (Fabrizio *et al.*, 2001). Likewise to Sch9p, the serine–threonine activity of Tor1p is promoted by the presence of nutrients and its inactivation, as well as the inactivation of other proteins from TOR pathway, leads to yeast CLS extension and also promotes protection against stress (Powers *et al.*, 2006). Moreover, protein Tor1p, an element of the TORC1 complex, has been described as the protein that directly phosphorylates and promotes the activation of Sch9p (Urban *et al.*, 2007). This information indicates that the pro-aging role of Tor1p is, at least in part, due to stimulation of Sch9p (Fabrizio and Longo, 2008). Similarly, lack of Ras2p causes CLS extension and stress resistance. Together, these results confirm the association between longevity extension and ability to respond to stress. This association has been observed in a variety of organisms, from yeast to flies and mice, suggesting that increasing cellular protection against damage and possibly increasing repair may be conserved molecular strategies to delay aging (Longo, 1999; Longo and Fabrizio, 2002).

The Sch9, TOR, and Ras/PKA pathways have been intensively studied and some of the main mediators of these life span-regulatory pathways have been discovered. A recent study by Wei and collaborators revealed that the chronological life span extension caused by *RAS2*, *TOR1* and *SCH9* gene deletions or by calorie restriction is totally dependent on the activity of Rim15p (Wei *et al.*, 2008). Lack of Rim15p causes the total reversion of the longevity extension phenotype observed the on three mentioned long-lived mutants indicating that the pro-aging pathways controlled by Sch9p, Tor1p, and Ras2p converge on this protein (Wei *et al.*, 2008). Rim15p, a serine/threonine kinase, is responsible for activation of the stress resistance transcription factors Msn2p, Msn4p and Gis1p. These transcription factors stimulate the expression of stress resistance proteins. Up-regulation of these transcription factors therefore leads to an increase of superoxide dismutase (SOD) and catalase levels, thereby minimizing oxidative stress and cellular damage (Gorner *et al.*, 1998).

I - 4.1 Apoptosis in chronological aging

Chronologically aged yeast cells die exhibiting markers of apoptosis, such as nuclear condensation/fragmentation, phosphatidylserine exposure, caspase activation, cytochrome *c* release and accumulation of ROS (Fabrizio and Longo, 2003; Herker *et al.*, 2004). However, the process that determines which cells have to die in a chronologically aged population is not well understood. Allen and collaborators showed that only older cells, harboring two or more bud scars, kill themselves in times of starvation, demonstrating that during chronological aging, apoptosis selectively removes older individuals from the population (Allen *et al.*, 2006). Furthermore, dying aged yeast cells stimulate directly the survival of the fittest cells by releasing substances into the medium (Fabrizio *et al.*, 2004; Herker *et al.*, 2004). In fact, several lines of evidence have demonstrated that cellular suicide represents a survival strategy for the group (Fabrizio *et al.*, 2004; Longo *et al.*, 2005). Some studies, reported a phenomenon referred to as "adaptive re-growth", in which a "re-growth" is observed after the majority of population is dead (Fabrizio *et al.*, 2004). Its major features are the dependency on DNA mutations that accumulate during aging and the requirement of the nutrients released by dead cells. Both features are dependent of superoxide, which accelerates cell death and consequent nutrients accumulation, and causes DNA damage that facilitates the appearance of genetic mutants with the ability to reentry the cell cycle in conditions that normally do not promote growth (Fabrizio *et al.*, 2004).

Further analysis of yeast apoptosis during chronological aging has revealed other aspects that influence cell survival. During chronological aging (in glucose), cells initially ferment glucose to ethanol, which accumulates in the extracellular environment. After glucose depletion, cells begin to metabolize ethanol by mitochondrial respiration (Kaeberlein, 2010). This results in an increased of ROS that contributes to the chronological aging process. As cells continue to use ethanol as a secondary carbon source, acetic acid is secreted into the extracellular milieu, leading to acidification of the growth medium (Kaeberlein, 2010). A recent study from Burtner and co-workers pointed to the importance of acetic acid as a mediator of yeast apoptosis during aging. The authors showed that buffering the aging culture to a higher pH or removing acetic acid from the expired medium is sufficient to extend CLS. Moreover, transferring post-mitotic yeast to water containing acetic acid, suppresses this life span extension,

indicating that acetic acid is both necessary and sufficient to promote chronological aging (Burtner *et al.*, 2009).

I - 5 Acetic acid in *Saccharomyces cerevisiae*

Acetic acid is a normal end product of the alcoholic fermentation carried out by *S. cerevisiae*. This yeast is capable to use acetate, resulting from dissociation of acetic acid, as the only carbon source. Acetate metabolism, as for most of yeast carbon sources, is subjected to glucose repression. Acetate can be metabolized to acetyl Co-A which is oxidized in the tricarboxylic acid cycle. It is also used to produce succinate replenishing the cell with biosynthetic precursors by entering in the glyoxylate cycle. Moreover, acetyl Co-A is used for the synthesis of macromolecules, which requires active gluconeogenesis (Kruckberg and Dickinson, 2004).

Several studies have contributed to the characterization of the mechanisms involved in the transport of acetic acid through the plasma membrane (Casal *et al.*, 1996; Casal *et al.*, 1999; Paiva *et al.*, 1999; Mollapour and Piper, 2007) (Fig. I-3). Acetic acid entry into the cells depends on the extracellular pH and growth conditions according to glucose regulation of the transporters.

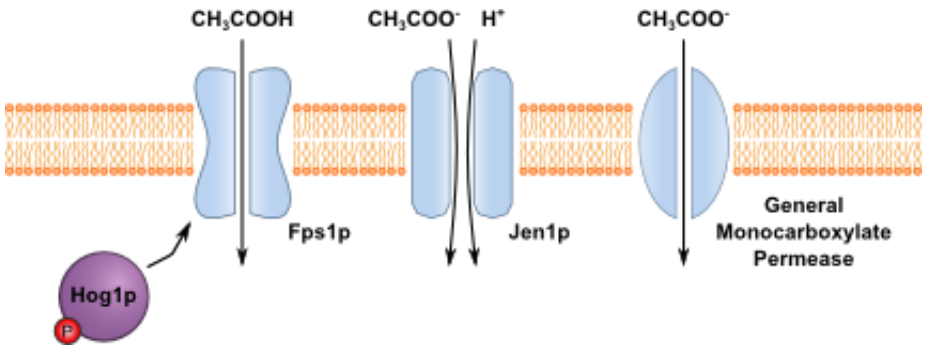

Fig I-3. Proteins involved in acetic acid transport across the plasma membrane. The undissociated form enters through Fps1p channel, which is regulated by Hog1p. The dissociated form of acetic acid is transported by Jen1p or by a more general monocarboxylate permease.

The anionic form is transported by an active transport process that involves an acetate-proton symporter, encoded by *JEN1* (Casal *et al.*, 1999), or by a more general monocarboxylate permease (Paiva *et al.*, 1999). The protein Ady2p was found to be essential for acetate transport activity as well (Paiva *et al.*, 2004). At low pH, when acetic acid is mainly in its undissociated form (pKa is 4.75), it enters essentially by simple diffusion (Casal *et al.*, 1996) through the aquaglyceroporin Fps1p (Mollapour and Piper, 2007). Fps1p is a channel that has an important role in the control of acetic acid movement, and is regulated by Hog1p, a mitogen-activated protein kinase (MAPK) from the HOG pathway. When yeast cells are subject 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 entry of acetic acid into the cell, protecting yeast from its toxic effects (Mollapour and Piper, 2007).

Once inside the cell, acetic acid dissociates leading to an intracellular acidification, an anion accumulation (Casal *et al.*, 1996), and the inhibition of metabolic activity (Pampulha and Loureiro-Dias, 1989). Generated acetate induces osmotic stress that activates both the Hog1p and Slt2p stress-activated MAP kinases. Nevertheless, only active Hog1p, not Slt2p, is needed for the acquisition of acetate resistance (Mollapour and Piper, 2006). Moreover, the activity of several glycolytic enzymes such as hexokinase, phosphofructokinase and, mainly, enolase is also affected by acetic acid (Pampulha and Loureiro-Dias, 1990). It is well established now that when present in high concentrations, acetic acid can also compromise the viability of yeast cells, resulting in cell death (Pinto *et al.*, 1989; Ludovico *et al.*, 2001).

I - 5.1 Acetic acid-induced apoptosis

In *S. cerevisiae*, acetic acid-induced apoptosis was primarily described by Ludovico and co-workers in 2001. The authors showed that low doses of acetic acid trigger PCD in exponentially grown cells exhibiting typical markers of mammalian apoptosis, namely, chromatin condensation along the nuclear envelope, exposure of phosphatidylserine on the outer leaflet of the cytoplasmic membrane, and occurrence of DNA strand breaks. On the other hand, high doses of acetic acid lead to a necrotic death (Ludovico *et al.*, 2001). Subsequent studies demonstrated that, like in mammalian cells, the yeast

apoptosis triggered by acetic acid can be mediated by mitochondria. Yeast cells exposed to this compound present accumulation of ROS, transient hyperpolarization followed by loss of mitochondrial membrane potential, decrease in cytochrome oxidase activity affecting mitochondrial respiration, and release of lethal factors like cytochrome *c* (Ludovico *et al.*, 2001).

The mitochondrial cytochrome *c* release seems to be dependent on the presence of the yeast orthologues of adenine nucleotide translocator ADP/ATP carrier (AAC), a protein involved in the mitochondrial outer membrane permeabilization through the so-called permeability transition pore (PTP) (Pereira *et al.*, 2007). In mammalian it has been proposed that major components of the PTP are the adenine nucleotide transporter (ANT), the voltage dependent anion channel (VDAC), and cyclophilin D (Crompton, 1999; Martinou *et al.*, 2000; Bras *et al.*, 2005). Yeast genetic approaches revealed that, while deletion of *POR1* (VDAC yeast homologue) enhances apoptosis triggered by acetic acid, absence of ADP/ATP carrier (ANT yeast homologue) protects cells exposed to acetic acid (Pereira *et al.*, 2007).

On the other hand, a recent study has proven that the majority of cytochrome *c* release in acetic acid-induced apoptosis occurs from intact coupled mitochondria in the absence of mitochondrial PTP (Giannattasio *et al.*, 2008). So far, the exact process by which cytochrome *c* is released from mitochondria and its function in yeast remains unclear. Disruption of cytochrome *c* partially prevents acetic acid-induced cell death, which is linked to increased mitochondrial membrane potential and loss of cytochrome *c* oxidase activity (Ludovico *et al.*, 2002). Consistently, cells that lack mitochondrial DNA, which are respiration-deficient, display resistance against acetic acid (Ludovico *et al.*, 2002). Besides, it has been described that yeast acetic acid-induced PCD can occur without cytochrome *c* release (Pereira *et al.*, 2007; Guaragnella *et al.*, 2010). Other mitochondrial proteins have been associated with apoptosis induced by acetic acid, such as, Fis1p, Dnm1p and Mdv1p involved in fission/fusion (Fannjiang *et al.*, 2004), Nuc1p (yeast endonuclease G) (Buttner *et al.*, 2007) and Ysp2p (Sokolov *et al.*, 2006).

Mitochondrial fragmentation and degradation has also been observed in response to acetic acid but, unlike mammalian cells, this is not dependent on the autophagy/mitophagy process. Instead, the vacuolar protease Pep4p (cathepsin D yeast homologue) is translocated to the cytosol and, together with the AAC proteins plays an

important role in mitochondrial degradation (Pereira *et al.*, 2010). Another work also provides evidence of the vacuole involvement in the PCD process. Deletion of class C vacuolar protein sorting genes results in a drastically enhanced sensitivity to treatment with acetic acid and leads to a necrotic death (Schauer *et al.*, 2009). These results unveil a complex interplay between mitochondria and the vacuole in yeast PCD.

The only yeast orthologue of mammalian caspases identified so far, the metacaspase Yca1p, is activated in cells undergoing acetic acid-induced apoptosis. This activation seems to be strongly dependent of the growth phase (Pereira *et al.*, 2007). Still, while studying the response to acetic acid in yeast cells lacking Yca1p, Guaragnella and co-workers verified that these cells undergo apoptosis, although at lower rate than wild-type cells. Considering this, the authors proposed that a caspase-independent pathway may exist in yeast (Guaragnella *et al.*, 2006).

In the past few years, numerous studies have also demonstrated that many different proteins are involved in apoptosis caused by acetic acid. The protease Kex1p, already known to participate in PCD caused by defective N-glycosylation, was also associated to the active cell death program induced by acetic acid (Hauptmann *et al.*, 2006). Furthermore, the glycolytic enzyme phosphoglycerate kinase, Pgk1p, was found to act as a multicopy suppressor of apoptotic phenotypes, including in the death induced by acetic acid (Mazzoni *et al.*, 2009). Another work, in which the yeast protein expression profile during acetic acid-induced apoptosis was analyzed, described alterations in the levels of proteins directly or indirectly linked with the target of rapamycin (TOR) pathway (Almeida *et al.*, 2009). Transient proteasome activation is also necessary for protein degradation during acetic acid-induced apoptosis (Valenti *et al.*, 2008), contrarily to what was observed in mammalian and plant cells where PCD is induced by proteasome inhibition (Shinohara *et al.*, 1996; Kim *et al.*, 2003).

Although acetic acid has been shown to induce PCD in *S. cerevisiae*, the players involved and the exact mechanisms by which this process is executed remain unknown. A major challenge in the future will be to understand how yeast cells signal and execute death programs and how the different players interact to decide the cell's fate.

I - 6 Lipid rafts

In 1972, Singer and Nicolson described the plasma membrane as a fluid mosaic, with a homogeneous phospholipid bi-layer in which proteins diffuses freely (Singer and Nicolson, 1972). However, 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 fluid mosaic model and raising the idea that lipids are organized in micro-domains (Bretscher, 1973). Since then, several studies that were designed to elucidate the temporal and spatial architecture of the plasma membrane have provided a new picture (Simons and van Meer, 1988; Simons and Ikonen, 1997). Many of these studies indicate that the plasma membrane is a mosaic of compartments that is maintained by an active cytoskeleton mesh. The membrane raft hypothesis has proposed a dynamic and heterogeneous compartmentalization in which specific lipids may associate with each other to form platforms commonly referred as lipid micro-domains or rafts (Simons and Ikonen, 1997).

Lipid rafts are regions of membranes with a distinct and, characteristic structural composition that appear to act as platforms to co-localize proteins involved in intracellular signaling pathways. Rafts are particularly rich in sphingolipids and cholesterol (yeast have ergosterol instead of cholesterol) (Fig I-4). The fatty-acid side-chains of the phospholipids present in lipid rafts tend to be more highly saturated than those in the surrounding membrane. This allows close packing with the saturated acyl chains of sphingolipids, and probably leads to phase separation (Brown and London, 2000). The empty spaces between sphingolipids are filled by cholesterol molecules (or ergosterol in yeast) 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). Due to the presence of cholesterol, a lipid-ordered domain is formed exhibiting less fluidity than the surrounding plasma membrane (McMullen *et al.*, 2004). This tight packing of lipids and phase separation is probably responsible for the principal property of lipid rafts: their insolubility in nonionic detergents at 4 °C (Brown and London, 1998).

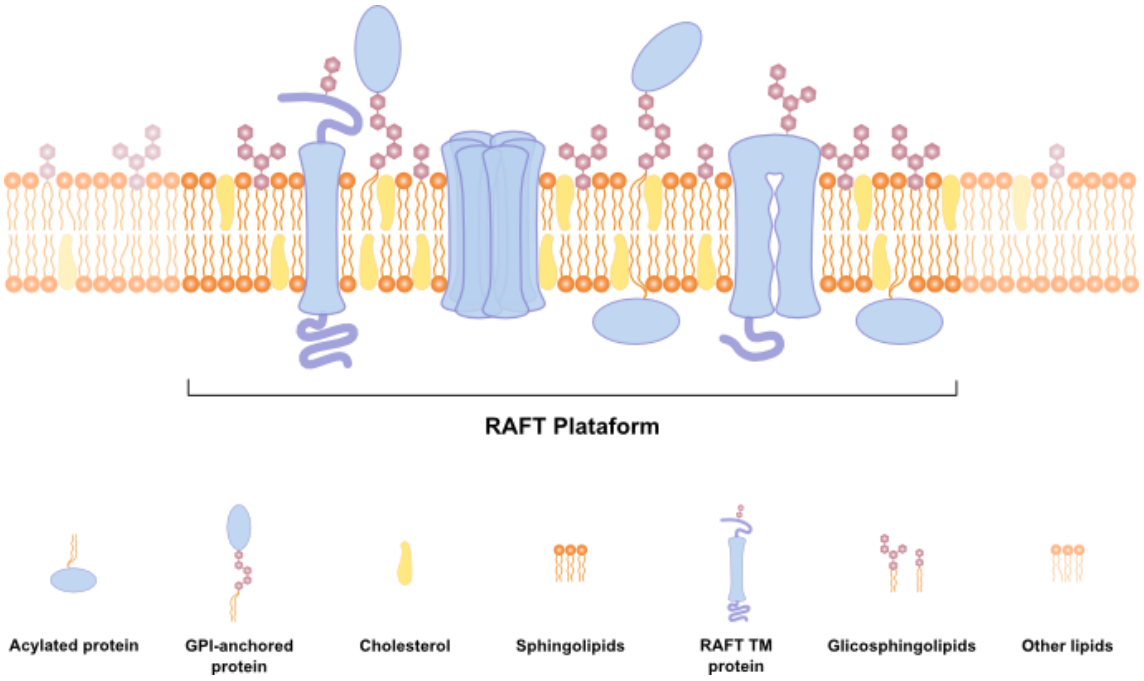

Fig. I-4. Schematic representation of lipid rafts structure. Lipid rafts are specialized membrane micro-domains containing high concentrations of sphingolipids and cholesterol. This composition results in lateral phase separation and the generation of a liquid-ordered domain. Bulk plasma membrane contains less cholesterol and more phospholipids with unsaturated acyl chains, being more fluid than lipid rafts. A variety of proteins position themselves into lipid rafts: glycosylphosphatidylinositol (GPI)-anchored proteins; transmembrane proteins (TM) and acylated proteins.

Another important characteristic of lipid rafts is their ability to include or exclude proteins to variable extents. A large number of studies suggest that several protein modifications such as palmitoylation, myristoylation, dual N-terminal acyl-modification or glycosylphosphatidylinositol (GPI)-anchor can target proteins to lipid rafts (Zacharias *et al.*, 2002; Smotrys and Linder, 2004; Pike, 2009). In contrast, proteins with transmembrane segments have been shown to be targeted to rafts by amino acid sequences in their extracellular (Yamabhai and Anderson, 2002), transmembrane (Scheiffele *et al.*, 1997), or intracellular (Crossthwaite *et al.*, 2005) domains. It has also been hypothesized that "lipid shells" surrounding transmembrane segments of proteins give them an enhanced affinity for cholesterol-enriched lipid rafts (Anderson and Jacobson, 2002). Thus, it was not surprising the finding that lipid rafts are enriched, for instance, in (GPI)-anchor proteins (Brown and London, 1998), doubly acylated proteins,

such as tyrosine kinases of the src family or the α-subunits of heterotrimeric G proteins (Resh, 1999), cholesterol-linked and palmitoylated proteins such as Hedgehog (Rietveld *et al.*, 1999), and palmitoylated transmembrane proteins (Brown and London, 1998). Moreover, cytoskeletal and adhesion proteins were also identified in lipid rafts. Included in this group of proteins are actin, myosin, vinculin, cofilin, cadherin, filamin, and ezrin (Pike, 2009).

Various studies about lipid rafts have demonstrated their involvement in a wide range of important biological processes, including numerous signal transduction pathways (Pike, 2003), cell adhesion and migration (Huang *et al.*, 2006), protein sorting during exocytosis (Salaun *et al.*, 2004) and endocytosis (Nabi and Le, 2003), as well as in apoptosis (Legembre *et al.*, 2006). In fact, recent publications have showed that several proteins involved in apoptosis regulation and signaling are also localized in lipid rafts, including Fas/CD95 receptor (Gajate and Mollinedo, 2001) and the pro-apoptotic protein of the Bcl-2 family, Bad (Ayllon *et al.*, 2002). Protein Bad, known to be associated with mitochondria in apoptotic cells, presents itself linked to lipid rafts in proliferative cells. This suggests a dynamic involvement of rafts in Bad-dependent regulation of apoptosis (Ayllon *et al.*, 2002). Gajate and Mollinedo also propose a role for lipid rafts in regulation of apoptosis. They showed that the Fas/CD95 receptor translocates to lipid rafts in order to amplify the Fas signaling (Gajate and Mollinedo, 2001; Hueber *et al.*, 2002; Scheel-Toellner *et al.*, 2002; Legembre *et al.*, 2006).

Lipid rafts have been observed in several organisms, spanning mammalian cells and yeast (Kubler *et al.*, 1996; Brown and London, 1998). In fact, lipid rafts have been identified in several fungal species, including the budding yeast *S. cerevisiae*, the fission yeast *Schizosaccharomyces pombe* as well as the pathogens *Cryptococcus neoformans* and *Candida albicans*. In yeast, lipid rafts, constituted by sphingolipids and ergosterol, are mainly involved in biosynthetic delivery of proteins to the yeast plasma membrane (Bagnat *et al.*, 2000), cell polarity (Bagnat and Simons, 2002), signaling (Bagnat and Simons, 2002), as well as protein anchoring and for connection of the membrane to cytoskeleton, endoplasmic reticulum and Golgi apparatus.

I - 7 Scope of the thesis

In mammalian cells, lipid rafts have been intensively studied and recent pieces of evidence showed that, besides its importance in a wide range of vital biological process, these micro-domains plays a key role in apoptosis as well (Ayllon *et al.*, 2002). However, to the date, its importance in yeast apoptosis is not known. Thus, the major aim of this thesis was to elucidate the influence of lipid rafts in yeast apoptosis. In order to accomplish this goal, we assessed apoptosis in several mutant strains, and rafts integrity/functionality was further analysed on the mutant strains in which apoptosis revealed affected. The mutants analyzed were the Δ*gup1*, Δ*gpd1*Δ*gpd2* and Δ*rvs161*.

- Gup1p is a membrane-bound O-acyltransferase belonging to the MBOAT family (Hofmann, 2000; Neves *et al.*, 2004) with multiple localizations at the plasma membrane and ER (Holst *et al.*, 2000; Bleve *et al.*, 2005), and that was associated to glycerol metabolism and transport (Holst *et al.*, 2000). Gup1p was further implicated in a vast number of distinct cell structure and functional processes, like cell wall structure and biogenesis (Ferreira *et al.*, 2006), lipid metabolism and sphingolipid-sterol rich domain's integrity and assembly (Oelkers *et al.*, 2000; Ferreira and Lucas, 2008), GPI anchor remodeling (Bosson *et al.*, 2006; Jaquenoud *et al.*, 2008), anaerobic sterol uptake (Reiner *et al.*, 2006), secretory/endocytic pathway (Bonangelino *et al.*, 2002), cytoskeleton polarization and bud site selection (Casamayor and Snyder, 2002), and telomere length (Askree *et al.*, 2004). Recently, Abe and co-workers demonstrated that the mammalian Gup1p acts as a negative regulator for N-terminal palmitoylation of sonic hedgehog protein (Abe *et al.*, 2008).

- Gpd1p and Gpd2p are the two isoforms of glycerol 3-phosphate dehydrogenase, glycerol formation rate-controlling enzyme. These isoforms have different physiological roles: the Gpd1p is mainly involved in glycerol production during osmotic stress, whereas the Gpd2p is primarily involved in adjusting the NADH–NAD redox balance under anaerobic conditions (Albertyn *et al.*, 1994; Ansell *et al.*, 1997). Furthermore, they have distinct intracellular localization that explains their different roles. Gpd1p is partly cytosolic and partly peroxisomal, while Gpd2p is located in mitochondria (Valadi *et al.*, 2004).

- Rvs161p is a lipid rafts protein belonging to N-BAR (for Bin, amphiphysin, Rvs) family (Ren *et al.*, 2006). Rvs161p is involved in the regulation of cell polarity (Durrens *et al.*, 1995), actin cytoskeleton polarization (Sivadon *et al.*, 1995), endocytosis (Munn *et al.*, 1995), and secretory vesicle trafficking (Gammie *et al.*, 1998; Breton *et al.*, 2001).

Chapter II

MATERIALS AND METHODS

II - 1 Strains and growth conditions

The *Saccharomyces cerevisiae* strains used in this study are listed in Table II - 1.

Table II - 1. Strains used in the present work.

Strain	Genotype	Origin
W303-1A	MATa *leu2-3 leu2-112 ura3-1 trp1-1 his3-11 his3-15 ade2-1 can1-100*	Thomas and Rothstein, 1989
BHY54	Isogenic to W303-1A but gup1::HIS5^{+}	Holst *et al.*, 2000
YFA1	Isogenic to W303-1A but gpd1::KanMX4	Azevedo F., unpublished results
YFA2	Isogenic to W303-1A but gpd2::KanMX4	Azevedo F., unpublished results
YSH642	Isogenic to W303-1A but gpd1::TRP1 gpd2::URA3	Holst *et al.*, 2000
YFA3	Isogenic to W303-1A but rvs161::KanMX4	Azevedo F., unpublished results

Yeast batch cultures were grown aerobically in minimal medium (0.67% (wt/v) YNB (Difco)) with 2% (wt/v) glucose and adequate quantities of auxotrophic requirements (Sherman, 2002). Incubation was performed at 30 °C, 200 rpm, orbital shaking and air/liquid ratio 3/1. Yeast strains maintenance was done on rich medium (YPD (Difco) with 2% agar), grown at 30 ºC for 48 h and kept at 4 ºC up to 5 days.

II - 2 Sensitivity to acetic acid

Drop tests were performed from mid-exponential YNB cultures containing approximately 1×10^{6} cells/ml. Ten-fold serial dilutions were made, and 5 µl of each suspension was applied on YNB medium supplemented with different acetic acid concentrations (50, 80 and 100 mM). Results were scored after 2 days of incubation at 30 °C.

II - 3 Acetic acid treatment

Yeast strains were grown until exponential phase (OD_{600} = 0.5–0.6) on YNB medium. Then the cultures were collected and resuspended to a final concentration of 10^7 cells ml^{-1} in fresh YNB adjusted to pH 3.0 with HCl and containing 160 mM acetic acid. Incubation took place for 180 min at 30 ºC as previously described (Ludovico *et al.*, 2001; Guaragnella *et al.*, 2006). At determined time points, 40 µl from a 10^{-4} cell suspension were inoculated onto YPD agar plates and colony forming units (c.f.u.) were counted after 48 h incubation at 30 °C. The percentage of viable cells was estimated considering 100% survival the number of c.f.u. obtained in time 0.

II - 4 Chronological aging

For aging experiments, pre-inoculum cultures grown overnight on YNB were used to start batch cultures at 0.05 (OD_{600nm}). At the stipulated time points, culture aliquots were taken to assess growth through OD_{600} and c.f.u., and for apoptotic assays. c.f.u. were determined plating 1,000 cells, counted on a Neubauer chamber, on YPD agar, at previously described (Herker *et al.*, 2004). Colonies were counted after 48 h incubation at 30 °C. No further colonies appeared after that incubation period.

II - 5 Apoptotic markers

PI, Annexin V, DAPI and DiOC6 staining were performed both in cells treated with acetic acid and in aging cells as previously described, with some modifications (Madeo *et al.*, 1997; Madeo *et al.*, 1999; Ludovico *et al.*, 2001; Gourlay and Ayscough, 2005).

II - 5.1 PI staining

Membrane integrity was assessed by PI (Propidium Iodide) staining. Cells were harvested, washed and resuspended in PBS (137 mM NaCl; 2.7 mM KCl; 100 mM Na_2HPO_4; 2 mM KH_2PO_4; pH 7.4) containing PI (4 µg/ml) (Sigma). The samples were

incubated for 10 min at room temperature in the dark and analyzed in an Epics® XL™ (Beckman Coulter) flow cytometer. At least 20,000 cells from each sample were analyzed.

II - 5.2 FITC-coupled Annexin V staining

Phosphatidylserine exposure was detected by an FITC-coupled Annexin V reaction with the ApoAlertAnnexin V Apoptosis Kit (CLONTECH Laboratories). For that, cells were primarily harvested and washed in digesting buffer (1.2 M sorbitol; 0.5 mM $MgCl_2$; 35 mM K_2HPO_4; pH 6.8). To promote the drug course through cell wall, an incubation step with Zymolyase (20T) at 30 °C was performed. Phase-contrast microscopy was used to monitor that step, preventing this way damage to the unfixed spheroplasts. Cells were subsequently centrifuged (10 min at 1500 rpm) and resuspended in 200 µl of binding buffer (1.2 M sorbitol; 10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM $Cacl_2$). To 40 µl of this cell suspension, 2 µl Annexin V (1 µg/ml) and 1 µl PI (4 µg/ml) were added, and the mixture incubated for 20 min at room temperature in the dark. Finally, extra 400 µl of binding buffer were added to the mixture just prior to analysis in an Epics® XL™ (Beckman Coulter) flow cytometer. At least 20,000 cells from each sample were analyzed.

II - 5.3 $DiOC_6$ staining

For evaluation of mitochondrial potential the probe $DiOC_6$ (3,3′dihexyloxacarbocyanine iodide) (Invitrogen) was used. Cells were harvested, washed, and resuspended in $DiOC_6$ buffer (10 mM MES; 0.1mM $MgCl_2$; 2% (wt/v) glucose, adjusted to pH 6 set with $Ca(OH)_2$) containing $DiOC_6$ (20 ng/ml). Cells were visualized by light microscopy (LM) after 30 min at room temperature in the dark. Stained cells were visualized in a Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings using a 100x oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software. At least 300 cells were counted per sample.

II - 5.4 DAPI staining

Chromatin condensation was assessed by DAPI (4,6-diamino-2-phenylindole dihydrochloride) (Sigma) staining. Cells were harvested, washed, fixed for 45 min with 3.7% formaldehyde, permeabilized with a solution of 70% (v/v) ethanol for 30 min, sonicated for 5 sec and afterwards stained with DAPI (1 μg/ml). Cells were visualized by LM after 5 min at room temperature in the dark. Stained cells were visualized in a Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings using a 100x oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software. At least 300 cells were counted per sample.

II - 6 Assessment of ROS

To visualize accumulation of ROS cells were harvested by centrifugation, resuspended in PBS in the presence of DHE (Dihydroethidium) (4μg/ml), and further incubated in the dark for 30 min at room temperature. To quantify the number of cells displaying high ROS levels, at least 20,000 cells were counted in an Epics® XL™ (Beckman Coulter) flow cytometer.

II - 7 Filipin staining

Sterol-lipid distribution was assessed in vivo using filipin staining. This was performed basically as previously described (Beh and Rine, 2004; Ferreira and Lucas, 2008; Ferreira *et al.*, 2010). A volume of 10 ml of treated and untreated cells were harvested and resuspended in 10 ml of 3.7% formaldehyde for 10 min, with constant mixing, at 30 °C. Subsequently, the fixed cells were spun down and the pellet was washed twice with 10 ml of distilled water. The washed cells were resuspended in 1 ml of water. From this solution a volume of 200 μl was mixed with 4 μl of freshly made 5 mg/ml filipin (Sigma) in ethanol (on ice), and incubated in the dark for 10 min. For fluorescence microscopy, cells were mounted directly on slides with a 10 μl drop of anti-fading agent VectaShield (Vector Laboratories) and observed in a Leica Microsystems DM-5000B

epifluorescence microscope with appropriate filter settings using a 100x oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

Chapter III

RESULTS AND DISCUSSION

This chapter comprises parts from the following publication:

Joana Tulha, Fábio Faria-Oliveira, Cândida Lucas and Célia Ferreira (2011) Programmed cell death in *Saccharomyces cerevisiae* is hampered by the deletion of *GUP1* gene. Submitted

0 - 1 Programmed cell death in *Saccharomyces cerevisiae* is hampered by the deletion of *GUP1* gene.

0 - 1.1 Abstract

Yeast has been successfully established as a model to study mechanisms of programmed cell death regulation. *S. cerevisiae* commits to cell death showing typical hallmarks of metazoan apoptosis in response to different stimuli. The *O*-acyltransferase Gup1p is required for several cellular processes that are related to apoptosis, such as rafts integrity and stability, lipid metabolism, GPI anchor correct remodeling, mitochondrial function, vacuole morphology, bud site selection and actin dynamics. In the present work we used two known apoptosis inducing conditions, chronological aging and acetic acid, to assess several apoptotic markers in Δ*gup1* mutant strain. We found that this mutant presents a significantly reduced chronological life span as compared to Wt and it is also highly sensitive to acetic acid treatment. In addition, it presents extremely high levels of ROS. There were notorious differences between the levels/types of apoptotic markers in each strain, which suggest that the mutant, under either condition, probably dies of necrosis and not from apoptosis. This result is compatible with the mentioned phenotypes, in particular with the changes in plasma membrane lipid composition and the absence of lipid rafts, which are being increasingly implicated in apoptosis triggering and signaling.

0 - 1.2 Results

GUP1 is involved in a wide range of cellular processes, some of which are associated directly or indirectly with apoptosis, such as rafts integrity and lipids metabolism (Oelkers *et al.*, 2000; Laun *et al.*, 2005; Bosson *et al.*, 2006; Aerts *et al.*, 2008; Almeida *et al.*, 2008; Ferreira and Lucas, 2008), cytoskeleton polarization (Ni and Snyder, 2001;

Gourlay and Ayscough, 2005), and telomere length (Askree *et al.*, 2004, Qi *et al.*, 2003). Here, we assess apoptotic markers for *Δgup1* mutant strain and compare them with Wt, in two different conditions documented to induce apoptosis in yeast: chronological aging and acetic acid treatment (Herker *et al*., 2004; Ludovico *et al.*, 2001).

0 - 1.2.1 *Δgup1* mutant cells exhibit a reduction in chronological life span

Yeast chronological life span is described as the length of time a population remains viable in the post-diauxic and stationary phases (Fabrizio and Longo, 2003; Rockenfeller and Madeo, 2008). Chronologically aged yeast cells die exhibiting specific markers of apoptosis (Fabrizio and Longo, 2003; Herker *et al.*, 2004). We checked the survival of *Δgup1* chronologically aged cells in comparison to Wt, continuously for 30 days throughout stationary phase until complete death of the culture.

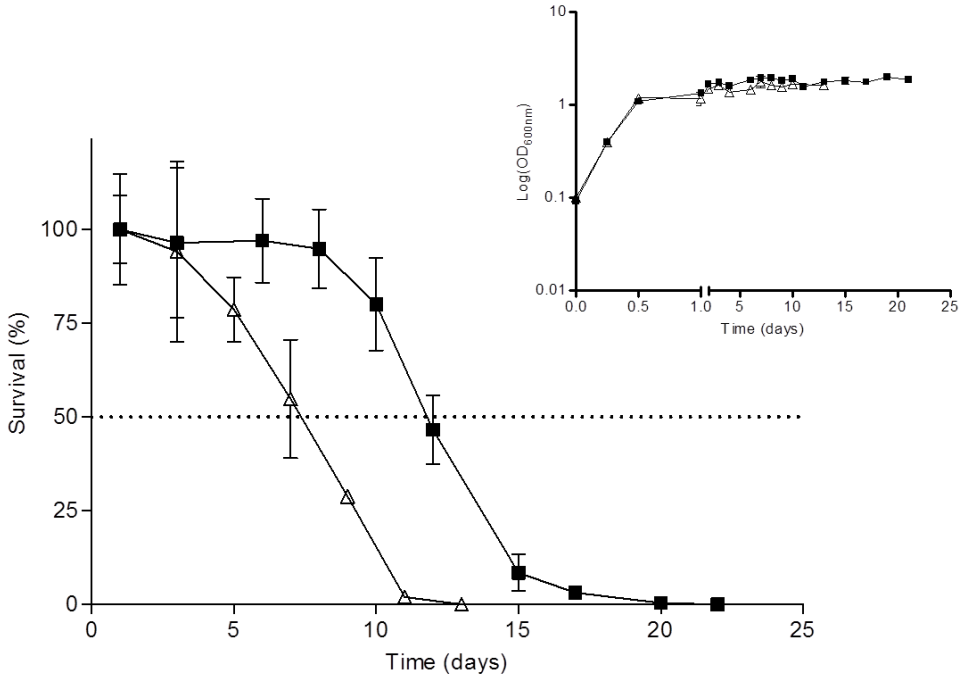

Fig. III-1. Deletion of *GUP1* decreases chronological life span. Wt (■) and Δ*gup1* (△) cells were inoculated in YNB medium and survival monitored by c.f.u. for 30 days, after exponential phase (100% represents the 1,000 plated cells counting using a Neubauer chamber). The growth curve in YNB for both strains is presented in the insert. Data represent mean ± SD of at least 3 independent experiments.

The growth curve (Fig. III - 1 insert) showed an apparent similar growth rate for both strains during exponential phase, as well as an almost coincident transition to diauxic and stationary phases. On the other hand, the survival curve (Fig. III - 1) showed that *Δgup1* mutant cells die considerably sooner than Wt. After day 3 the survival rate of *Δgup1* mutant started to decrease, reaching 50% around day 7, and in day 11 we observed that only a small number of *Δgup1* mutant cells stayed alive. Conversely, Wt strain begins to die around day 8, reaches 50% survival at day 12 and on day 19 the percentage of viable cells is almost zero.

0 - 1.2.2 Chronological aged *Δgup1* mutant seems to be incapable of dying by apoptosis

In order to investigate whether chronologically aged Wt and *Δgup1* mutant cells die by apoptosis, we analyzed several apoptotic markers in exponentially growing and chronologically aged cultures in both strains (Herker *et al.*, 2004; Schauer *et al.*, 2009). We chose 6 hours growth to assess exponential cells, and day 7 or day 12 to test chronologically aged cells of *Δgup1* mutant and Wt, respectively.

In yeast, as in mammalian cells, the maintenance of plasma membrane integrity during cell death is an indicator of PCD. In this work, we evaluated the integrity of plasma membrane, in exponential and aged Wt and *Δgup1* mutant strains, by PI staining. In Δ*gup1* mutant, we observed a substantial increase in the number of cells stained with PI over time, until every cell presented PI positive. Still, although the pattern is identical, in Wt the percentage of PI positive cell is proximally 2-fold less (Fig. III – 2 A). Yet, the percentages of necrotic cells can be over evaluated since apoptotic cells can become leaky during further cultivation, increasing PI positives. To distinguish this secondary necrosis from primary necrosis further tests were performed.

Phosphatidylserine has an asymmetric distribution in the lipid bilayer of the cytoplasmic membrane (Cerbon and Calderon, 1991). The exposure of phosphatidylserine at the outer surface of the cytoplasmic membrane occurs at the early stages of apoptosis (Martin *et al.*, 1995), when membrane integrity is still retained. We checked this through the FITC-coupled Annexin V reaction followed by flow cytometry of co-

labeled Annexin V/PI cells. Exponential grown *Δgup1* mutant population displayed around 3.5% of the cells with exposure of phosphatidylserine (Annexin V positive, but PI negative). No significant difference was observed when *Δgup1* mutant has aged, being this percentage approximately 3%. In contrast, in Wt cells the exposure of phosphatidylserine increases in aged cells (less than 1% to ~12%) (Fig. III - 2 B).

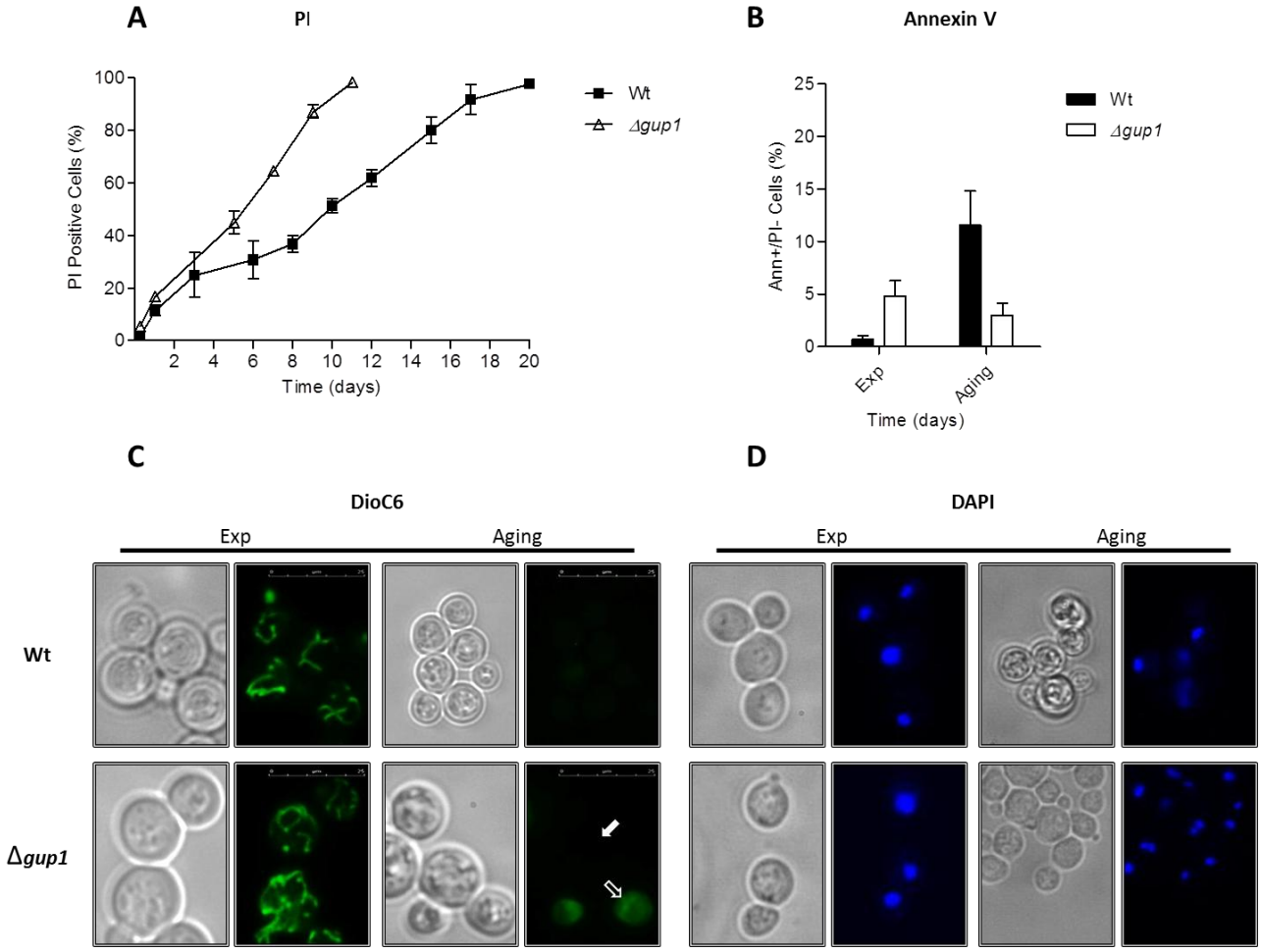

Fig. III – 2. Analysis of apoptotic markers in Wt and Δ*gup1* chronologically aged cells. (A) Graphic representation of the percentage of cells displaying positive PI staining. (B) Phosphatidylserine externalization assessed by cytometric analysis of Annexin V labeling. Graphic representation of the percentage of cells displaying Ann V(+)/PI (–). (C) Representative photos of $DiOC_6$ staining exponential phase and aged cells. Filled arrows indicate cells with depolarized mitochondrial membrane, whereas open arrow indicate cells with an unspecific $DiOC_6$ staining. (D) Representative photos of DAPI staining of exponential phase and aged cells. For flow cytometry and fluorescence microscopy assays a minimum of 35,000 and 300 cells were counted, respectively. Data represent mean ± SD of 3 independent experiments.

In order to evaluate the mitochondrial membrane depolarization, $DiOC_6$ was used. At a concentration of 20 ng/ml this dye accumulates specifically at mitochondrial membranes and can be observed by fluorescence microscopy. Nonetheless, cells that have low mitochondrial membrane potential will fail to accumulate $DiOC_6$ (Gourlay and Ayscough, 2005). Both *gup1*Δ mutant and Wt exponential cells stained with $DiOC_6$ revealed intact mitochondrial networks, confirming a normal polarization of mitochondrial membranes (Fig. III - 2 C left panels). Aged cells (7 and 12 days in Wt and gup1Δ mutant, respectively), showed a decrease in green fluorescence in approximately 40% of Wt cells and in 50% of gup1Δ mutant cells, demonstrating a reduction in mitochondrial membrane potential (Fig. III - 2 C right panels). Moreover, some cells exhibited a strong green fluorescence all over the cell, mainly in *gup1*Δ mutant strain, suggesting that these cells possibly had the plasma membrane altered, which in turn resulted in the accumulation of $DiOC_6$ on the cytosol (Fig. III - 2 C right panels).

Finally, we evaluated chromatin condensation through DAPI staining (Fig. III - 2 D). Moderate chromatin condensation upon DAPI staining was observed in 80% of old Δ*gup1* mutant cells, which can be visualized by the fluorescent semicircles formed by the chromatin fragments (Fig. III - 2 D right panels). Regarding Wt aged cells, we observed some cells with chromatin condensation, but we also detected cells without stained nucleus or even with multiples nucleus (Fig. III - 2 D right panels). These are probably due to an endomitosis process (Pichova *et al.*, 1997; Laun *et al.*, 2001). In contrast, in exponentially growing cultures, both Wt and Δ*gup1* cells presented integral chromatin mirrored as single round fluorescent circles in the middle of the cell (Fig. III - 2 D left panels).

0 - 1.2.3 *Δgup1* mutant cells are sensitive to acetic acid

In a previous work, it was described that *Δgup1* mutant cells are sensitive to weak acids (Ferreira *et al.*, 2006). However, the concentrations of acetic acid that induce apoptosis in yeast are considerably higher than the ones studied at that time (50 mM). Therefore, we investigated *Δgup1* mutant and Wt sensitivity to a wide range of acid concentrations (50, 80 and 100 mM).

With the lowest concentration of acetic acid (50 mM), no effect was observed; however, when the concentration was increased both strains were affected, being Δ*gup1* mutant the most sensitive one. At the highest concentration tested, 100 mM of acetic acid, the difference between the two strains was more obvious, with Δ*gup1* mutant showing growth only until the second dilution, whereas Wt presents growth up to the fourth dilution (Fig. III - 3 A).

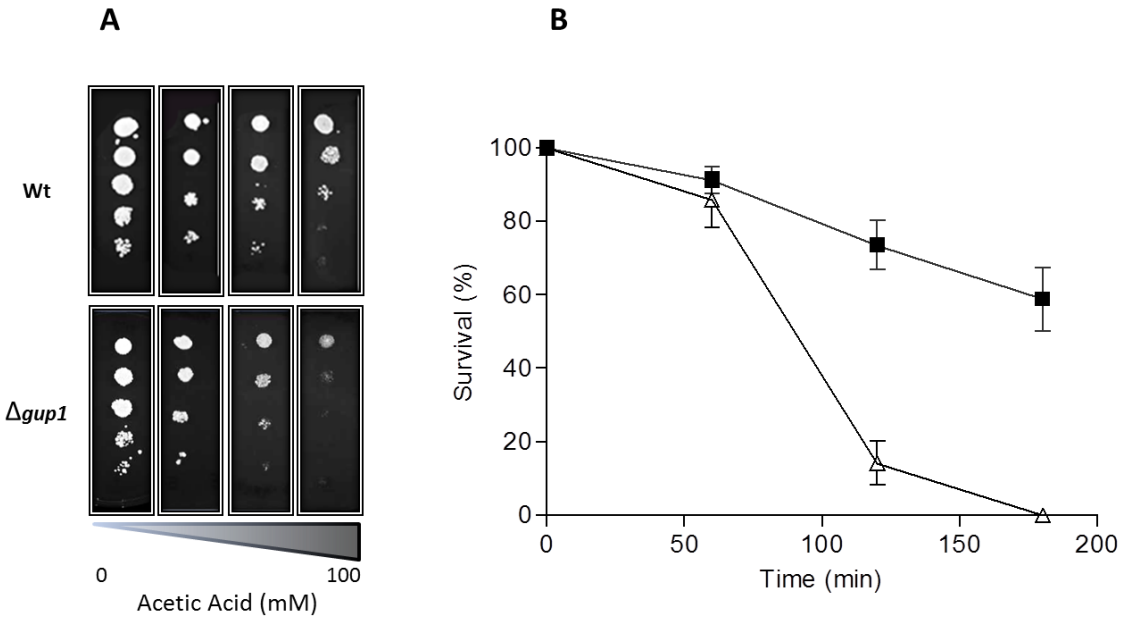

Fig. III – 3. Loss of *GUP1* confers sensitivity and reduces survival in presence of acetic acid. (A) Sensitivity of Wt and Δ*gup1* mutant cells to several increasing concentrations of acetic acid by Drop assay. Cultures were grown to mid-exponential phase in YNB medium, and ten-fold serial dilutions were spotted onto YNB plates (pH 3) supplemented with acetic acid. All plates were incubated at 30 °C for 48 h. (B) Survival curve of Wt (■) and Δ*gup1* (△) cultures during acetic acid treatment. Exponential cells were treated with 160 mM acetic acid in YNB medium(pH 3) for 180 min and viability determined by c.f.u. at the indicated time points (100% survival corresponds to the total c.f.u. at time zero). Data represent mean ± SD of at least 3 independent experiments.

Additionally we determined the death kinetics of both strains treated with 160 mM of acetic acid (Fig. III - 3 B). For that, Wt and *Δgup1* mutant cells at exponential growth phase were exposed to acetic acid, and the survival rate measured by c.f.u. counts. In both cases, the yeast cells died in response to acetic acid, but the cell death patterns were different. Until 60 min of acetic acid treatment, no significant difference was

founded between Wt and *Δgup1* strains, presenting around 90% and 85% cell viability, respectively. These percentages progressively decreased in both strains, being more accentuated in Δ*gup1* mutant. After 120 min in the presence of acetic acid, only 15% of *Δgup1* mutant cells remained alive, whereas Wt presented a survival rate of around 75%. At the last time-point analyzed, 180 min, the dissimilarity among strains sharpened up; only a few cells of *Δgup1* mutant were viable, whereas Wt strain displayed a survival rate of around 55% (Fig. III - 3 B).

0 - 1.2.4 Acetic acid induces cell death similar to that triggered by chronological aging

In order to assess, in *Δgup1* mutant cells, whether cell death induced by acetic acid treatment followed a programmed process of apoptosis, we analyze several apoptotic markers. The first marker analyzed was PI staining to estimate the loss of membrane integrity. Acetic acid treatment leads to a pronounced increase of *Δgup1* mutant PI positive cells, reaching nearly 100% after 180 min of treatment, while in the Wt strain this percentage didn't exceed 10% (Fig. III - 4 A).

In addition, we examined the phosphatidylserine exposure by simultaneously FITC-coupled Annexin V/PI staining (Fig. III – 4 B). Similarly to what was observed with the aging experiment, no significant difference was detected in *Δgup1* mutant Annexin V/PI stained cells before and after apoptosis induction with acetic acid (180 min treatment). In both cases, the percentage of apoptotic cells didn't exceed 3%. In opposition, Wt strain presents trace amounts of Annexin V+/PI- stained cells before apoptosis induction that increase after the treatment with acetic acid, reaching up to 8% (Fig. III - 4 B).

Yeast mitochondria undergo both structural and functional changes after the incubation with acetic acid, including mitochondrial membrane depolarization (Ludovico *et al.*, 2002). In order to evaluate this phenomenon, $DiOC_6$ staining was used to visualize mitochondrial membranes (Fig. III – 4 C). Just before apoptosis induction with acetic acid, most of Wt and Δ*gup1* mutant cells presented intact mitochondrial networks (Fig. III – 4 C left panels). After the treatment, it was possible to visualize depolarization of mitochondrial membranes in approximately 40% and 30% of *Δgup1* and Wt cells,

respectively, mirrored by the absence of fluorescence (Fig. III – 4 C right panels). Furthermore, we observed a considerable number of *Δgup1* mutant cells displayed an increase in $DiOC_6$ green fluorescence, similarly to the results obtained when the apoptotic inducer was chronological aging (Fig. III - 4 C right panels).

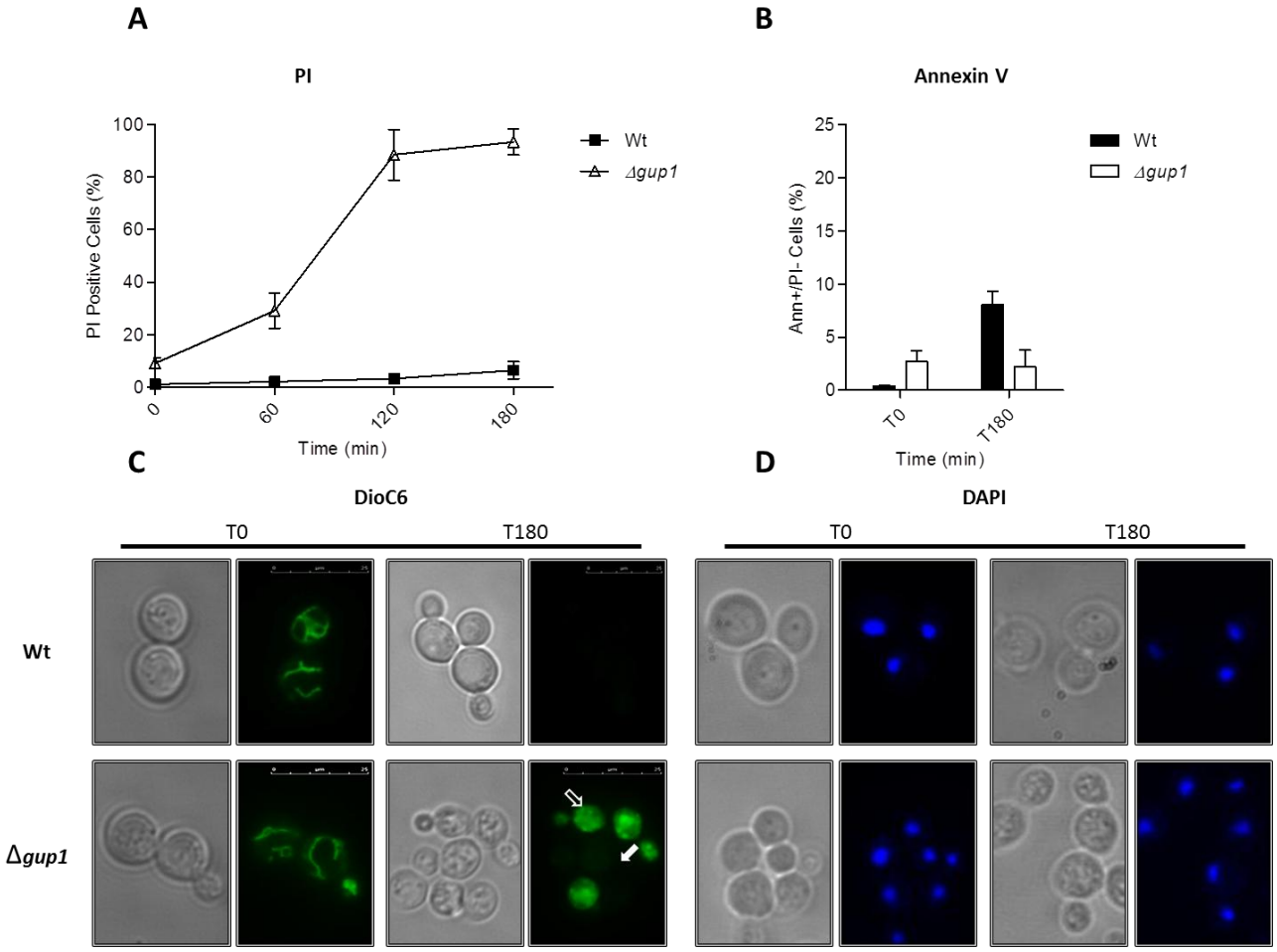

Fig. III – 4. Analysis of apoptotic markers in Wt and Δ*gup1* mutant cells treated with 160 mM acetic acid for 180 min. (A) Graphic representation of the percentage of cells displaying positive PI staining. (B) Phosphatidylserine externalization assessed by cytometric analysis of Annexin V labeling. Graphic representation of the percentage of cells displaying Ann V(+)/PI (–) (C) Representative photos of $DiOC_6$ staining untreated cells and cells after 180 min acetic acid treatment. Filled arrows indicate cell with depolarized mitochondrial membrane, whereas open arrow indicate cells with an unspecific $DiOC_6$ staining. (D) Representative photos of DAPI staining untreated cells and after 180 min acetic at acid treatment. For flow cytometry and fluorescence microscopy assays a minimum of 35,000 and 300 cells were counted, respectively. Data represent mean ± SD of 3 independent experiments.

Additionally, we checked for chromatin condensation during acetic acid treatment by staining cells with DAPI (Fig. III – 4 D). Nearly no chromatic condensation was observed in both *Δgup1* mutant and Wt untreated cells, as reflected by the single round fluorescent circles in the center of the cells (Fig. III – 4 D left panels). Yet, after the treatment with acetic acid, we observed a significant increase in *Δgup1* mutant cells exhibiting moderate chromatin condensation along the nuclear envelope (~90%). In Wt, ~25% of cells present chromatin condensation (Fig. III - 4 D right panels).

0 - 1.2.5 *Δgup1* mutant cells accumulate large amounts of ROS during chronological aging and acetic acid treatment.

It is well documented that the loss of mitochondrial membrane potential can lead to increased production of ROS in higher eukaryotes, which is seen as an apoptotic-related process in yeasts (Madeo *et al.*, 1999; Laun *et al.*, 2001). On the other hand, several points of evidence indicate that, in yeast, the accumulation of ROS is a major factor determining aging (Longo *et al.*, 1996; Nestelbacher *et al.*, 2000), and triggering PCD (Madeo *et al.*, 1999; Buttner *et al.*, 2006; Carmona-Gutierrez *et al.*, 2010). The accumulation of ROS is commonly measured by incubating cells with dihydroethidium (DHE), which is oxidized (by ROS) to the ethidium. ROS were measured in both chronologically aged *Δgup1* mutant and Wt cells at different time points, covering exponential, stationary and death phases (Fig. III - 5 A). A significantly higher increase of ROS levels over time was observed in *Δgup1* mutant in comparison to Wt cells. The biggest difference was on day 6, when the percentage of *Δgup1* cells exhibiting ROS accumulation was the twice (~80%) that of Wt cells (~40%). The mutant reached 100% of cells with ROS accumulation on day 10, while Wt took 17 days to reach that state (Fig. III - 5 A).

The difference between Wt and *Δgup1* mutant strains was also extremely notorious in acetic acid treated cells (Fig. III - 5 B). Soon after acetic acid addition, *Δgup1* mutant exhibited ROS accumulation in ~ 8% of the cells, whereas Wt presented less than 1%. The difference between the strains was accentuated with time. At one hour treatment *Δgup1* cells with ROS accumulation was higher than 30% and Wt cells less than 5%. Two hours treatment led to a substantial rise of ROS positive *Δgup1* mutant cells

(~85%) compared with only ~10% of Wt. At the end of the treatment, almost all Δ*gup1* mutant cells exhibited ROS accumulation, in clear contrast with the ~15% of ROS accumulation displayed by Wt strain (Fig. III - 5 B).

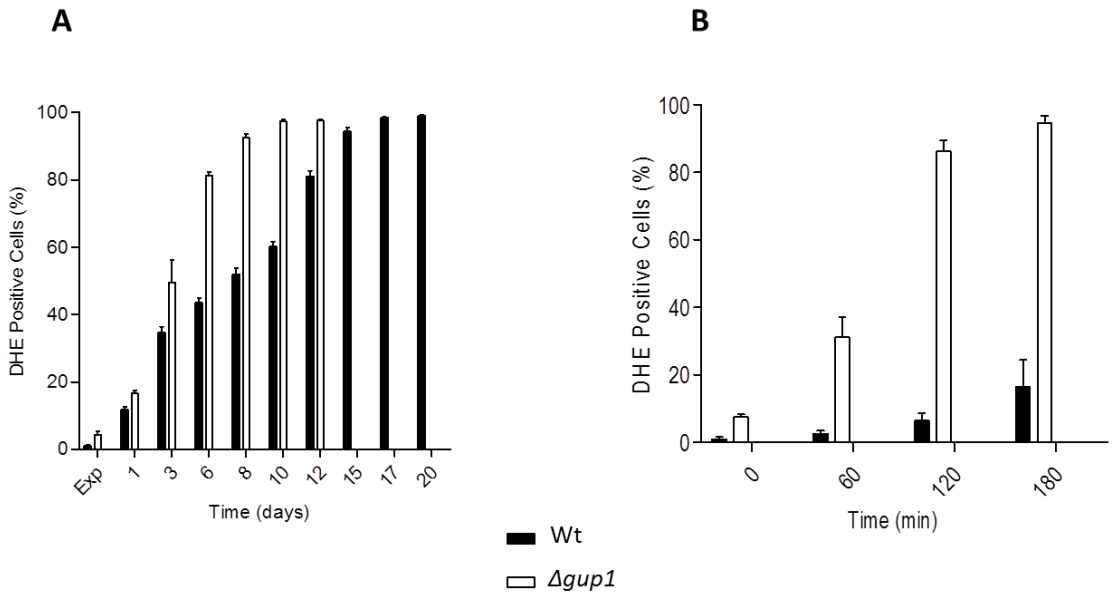

Fig. III – 5. *GUP1* deletion promotes substantial ROS accumulation. Cells from chronological aging assay (A) and from acetic acid treatment (B) were analyzed for accumulation of ROS using DHE staining by flow cytometry. At least 35,000 cells were analyzed. Data represent mean ± SD of at least 3 independent experiments.

0 - 1.3 Discussion

During the past years, yeast has been successfully established as a model to study mechanisms of apoptotic regulation. The finding of an endogenous PCD process with an apoptotic phenotype has turned yeast into a powerful model for apoptosis research (Fleury *et al.*, 2002; Madeo *et al.*, 2002; Carmona-Gutierrez *et al.*, 2010). In fact, *S. cerevisiae* commits to cell death showing typical features of mammalian apoptosis, in response to different stimuli. However, how cell compounds participate in the processes leading to cell death in yeast remains to be established. Gup1p, an *O*-acyltransferase, is required for several cellular processes that are related to apoptosis development, namely, rafts integrity and stability, lipid metabolism including GPI anchor correct

remodeling, proper mitochondrial and vacuole function, and actin dynamics (Ni and Snyder, 2001; Dickson and Lester, 2002; Garcia *et al.*, 2003; Gourlay and Ayscough, 2005; Bosson *et al.*, 2006; Ferreira *et al.*, 2006; Ferreira and Lucas, 2008; Pereira *et al.*, 2008; dos Santos and Sa-Correia, 2009; Schauer *et al.*, 2009).

In this work we used two known apoptosis-inducing conditions, chronological aging (Herker *et al.*, 2004) and acetic acid (Ludovico *et al.*, 2001), to assess several apoptotic markers in *Δgup1* mutant strain. We found that, when compared with Wt, *Δgup1* mutant presents a significant reduced chronological life span, showing almost no viability after 11 days incubation. Chronologically aged yeast cultures were shown to die exhibiting typical apoptotic markers (Herker *et al.*, 2004). In accord, we showed that chronologically aged Wt cells predominantly commit to an apoptotic death, as revealed by a) PI negative cells; b) phosphatidylserine externalization; b) depolarization of mitochondrial membrane; and c) chromatin condensation. Yet, while chronologically aged Wt cells die exhibiting apoptotic markers, *Δgup1* aged cells seems to be incapable of undergoing apoptosis. Instead, these cells appeared to be experiencing a necrotic cell death process. The Δ*gup1* aged culture exhibited a higher number of cells with loss of membrane integrity, and didn't reveal an increase of phosphatidylserine exposure on the surface of the plasma membrane. Such observations discredit the possibility that this cells are dying through an apoptotic process, being more likely that the reduction in life span is due to a necrotic death. Furthermore, both loss of mitochondrial membrane potential and moderate chromatin condensation that we observed in this mutant have already been described in necrotic phenotypes (Galluzzi *et al.*, 2007; Sripriya *et al.*, 2009). Lately, several points of evidence, suggest that necrotic cell death also occurs in yeast. Moreover, that can occur under normal physiological conditions or in the presence of cell death inducing substances, and not necessarily resulting from brutal chemical or physical damage, as previously thought (Buttner *et al.*, 2007).

We also used acetic acid as an apoptotic inducer of cell death in both Wt and *Δgup1* mutant strain. Our results revealed that acetic acid induces a cell death process similar to that observed in aging cultures. These results are in accordance with the hypothesis proposed in a previous work, in which the toxicity of acetic acid produced during aging was suggested as the major cause of chronological aging in yeast (Burtner *et al.*, 2009). Reinforcing such idea are the acidified cultures that we observed during aging, probably

resulting from acetic acid production and release to the medium (data not shown). Moreover, it was also reported that the signaling of acetic acid-induced apoptosis is linked to amino-acid metabolism as well as to the TOR pathway (Almeida *et al.*, 2009), as it happens in the aging process (Powers *et al.*, 2006).

A necrotic death induced by acetic acid was already observed in other mutants, namely in mutants in class C VPS genes that code for proteins essential for vacuolar and endossomal vesicle function (Schauer *et al.*, 2009).

Accumulation of ROS has predominantly been associated to yeast apoptosis under numerous conditions (Pozniakovsky *et al.*, 2005; Braun *et al.*, 2006; Eisenberg *et al.*, 2007). Some studies have addressed a fundamental role of ROS in the execution apoptotic death, after treatment with low doses of hydrogen peroxide (Madeo *et al.*, 1999) or the superoxide-mediated altruistic program of aging (Fabrizio *et al.*, 2004). Interestingly, however, many studies have suggested a crucial involvement of ROS during necrosis of mammalian cells (Festjens *et al.*, 2006) as well as in yeast necrosis (Eisenberg *et al.*, 2007; Schauer *et al.*, 2009). This evidence is in accordance with our results. We observed a significant difference in ROS accumulation between Wt and *Δgup1* mutant strain in both chronological aging and acetic acid treatment. *Δgup1* cells, which present a necrotic phenotype, have an extremely higher accumulation of ROS. If ROS can contribute, apart from its role on apoptosis, to the necrotic cell death in yeast as well, or if it is rather a byproduct that accumulates as a result of cellular demise, remains to be elucidated.

Gup1p has been described to have an important function on lipid rafts assembly/integrity (Ferreira and Lucas, 2008). In the literature, rafts have been increasingly implicated on regulation of apoptotic signaling in mammalian cells (Garcia *et al.*, 2003; Mollinedo and Gajate, 2010). In response to intra or extracellular stimuli, lipid rafts can include or exclude proteins to variable extents. This favors specific protein–protein interactions and modulates the activity of signaling apoptotic cascades. Moreover, in mammalian cells a number of proteins involved in apoptotic signals have been found to locate in lipid rafts, namely Fas/CD95 receptor (Gajate and Mollinedo, 2001) and the pro-apoptotic protein of Bcl-2 family, Bad (Ayllon *et al.*, 2002). Our results showed that the PDC processes in *S. cerevisiae* is altered by *GUP1* deletion and reinforce the importance of lipid rafts on the regulation of apoptotic signaling in yeast.

Moreover, our findings point to that these membrane domains seem to be indispensable for a proper development of PCD, under aging and acetic acid conditions, namely in the switch from a necrotic to an apoptotic death phenotype.

0 - 2 Involvement of rafts in acetic acid-induced apoptosis

0 - 2.1 Abstract

Recent evidence has revealed the occurrence of an apoptotic phenotype in *S. cerevisiae* that is inducible by acetic acid. However, the exact apoptotic mechanisms underlying this process remain unknown. Lipid rafts, micro-domains in the plasma membrane particularly rich in sphingolipids and ergosterol, have been associated to a wide range of important biological processes, including numerous signal transduction pathways, cell adhesion and migration, protein sorting during exocytosis and endocytosis, as well as in apoptosis. In this work, we used mutants affected in processes directly or indirectly related to apoptosis, namely Δ*gup1*, Δ*gpd1*Δ*gpd2* (and single mutants) and Δ*rvs161* to evaluate the involvement of these micro-domains in acetic acid-induced apoptosis. The *Δgup1* mutant is affected on lipid synthesis/GPI-anchor remodeling, cytoskeleton polarization, and protein sorting, among other processes. The *Δgpd1*Δ*gpd2* mutant is defective in glycerol synthesis and intracellular regulation. The *Δrvs161* mutant is a lipid raft protein involved in the regulation of actin cytoskeleton polarization, polarized growth, endocytosis and secretory vesicle trafficking.

We found that Δ*gup1*, Δ*gpd1*Δ*gpd2* and Δ*rvs161* mutants were more sensitive to treatment with acetic acid. Yet, the death process is different among each other. Although, the Δ*gpd1*Δ*gpd2* and Δ*rvs161* mutants died exhibiting apoptotic markers,

acetic acid treatment seems induce necrosis in *Δgup1* mutant. Filipin staining revealed an altered lipid rafts distribution in all mutants studied, particularly in *Δgup1* mutant. We also observed that acetic acid induces a rearrangement of these membrane micro-domains.

Altogether, our results indicate that lipid rafts seem to be a key component in apoptotic signaling, possibly essential in some circumstances.

0 - 2.2 Results

Recent studies have proposed that lipid rafts play a central role in apoptosis signaling (Gajate and Mollinedo, 2001; Ayllon *et al.*, 2002; Garcia *et al.*, 2003). In this work we used several mutants to further assess rafts distribution. These include: a) pleiotropic *Δgup1* mutant affected on lipid synthesis/GPI-anchor remodeling (Bosson *et al.*, 2006; Ferreira and Lucas, 2008), cytoskeleton polarization (Oelkers *et al.*, 2000) and protein sorting (Bonangelino *et al.*, 2002) among many other processes; b) mutants defective in glycerol synthesis and intracellular regulation, namely *Δgpd1*Δ*gpd2* mutant, and the single mutants *Δgpd1 and Δgpd2* (Valadi *et al.*, 2004), as well as c) *Δrvs161* mutant, affected in the regulation of actin cytoskeleton polarization (Sivadon *et al.*, 1995), cell polarity (Durrens *et al.*, 1995) and secretory vesicle trafficking (Gammie *et al.*, 1998; Breton *et al.*, 2001).

0 - 2.2.1 *Δgup1, Δrvs161* and *Δgpd1*Δ*gpd2* mutants are very sensitive to acetic acid

The sensitivity to acetic acid (50, 80 e 100mM) evaluated for Wt and *Δgup1* mutant strain in the previous section (Chapter III, Section 1.2.3) was determined in *Δgpd1*Δ*gpd2*, *Δgpd1*, *Δgpd2* and *Δrvs161* mutants strains as well (Fig. III - 6 A). With the lowest concentration, 50mM, no effect was observed between strains. When we used 80mM of acetic acid, *Δgpd1*Δ*gpd2* strain showed some sensitivity, more accentuated than the one observed with *Δgup1* mutant strain. With *Δrvs161* mutant no alteration were noticed. At the highest concentration (100mM), *Δgpd1*Δ*gpd2* showed an elevated sensitivity to acetic acid, whereas *Δrvs161* presented very low sensitivity (Fig.

III - 6 A). Again, the behavior of *Δgpd1Δgpd2* strain was similar to the one observed before for *Δgup1* mutant strain. In addition, the single *Δgpd1* and *Δgpd2* mutants were also tested but no significant phenotype was observed when compared with Wt (data not shown).

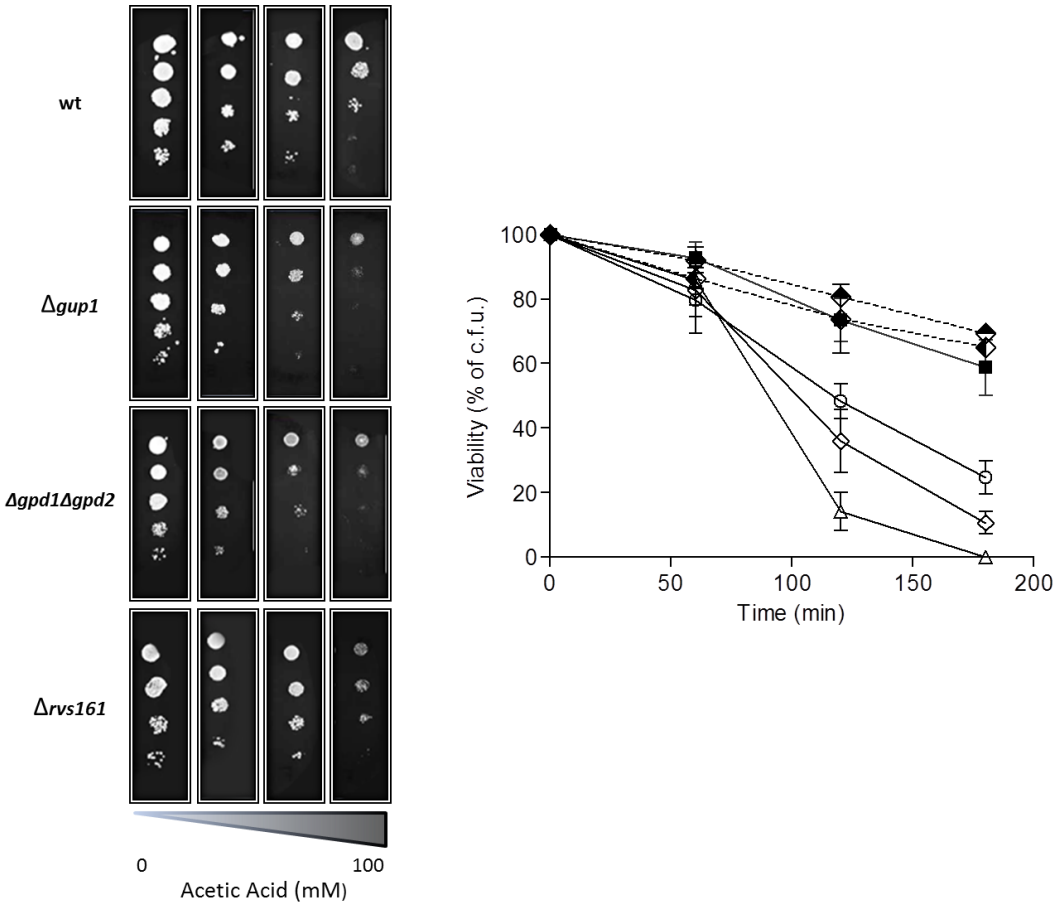

Fig III – 6. (A) Sensitivity of Wt, Δ*gup1*, Δ*gpd1*Δ*gpd2* and Δ*rvs161* mutant cells to several increasing concentrations of acetic acid by Drop assay. Cultures were grown to mid-exponential phase in YNB medium, and ten-fold serial dilutions were spotted onto YNB plates (pH 3) supplemented with acetic acid. All plates were incubated at 30 °C for 48 h. (B) Survival curve of Wt (■), Δ*gup1* (△), Δ*gpd1*Δ*gpd2* (◇), Δ*gpd1* (◓), Δ*gpd2* (◧) and Δ*rvs161* (○) cultures during acetic acid treatment. Exponential grown cells were treated with 160 mM acetic acid in YNB medium(pH 3) for 180 min and viability determined by c.f.u. at the indicated time points (100% survival corresponds to the total c.f.u. at time 0). Data represent mean ± SD of at least 3 independent experiments.

Additionally, the same mutants were submitted to acetic acid treatment (160 mM) during 180 min, and we measured the survival rate by c.f.u. counts (Fig. III - 6 B) (Ludovico *et al.*, 2001). The *Δgpd1* and *Δgpd2* single mutants showed a percentage of survival similar to Wt, whereas *Δgup1*, *Δgpd1Δgpd2* and *Δrvs161* are considerably more sensitive. Until 60 min of acetic acid treatment, no significant difference was visible between Wt and mutants. The dissimilarities among the strains increase with time of treatment, being more significant after 180 min. The *Δgpd1Δgpd2* and *Δrvs161* strains presented a viability of approximately to 10% and 20% of c.f.u., respectively, in opposite to ~58% survival of Wt and to ~0% survival of the *Δgup1* mutant strain (Chapter III, Section 1.2.3) (Fig. III - 6 B).

0 - 2.2.2 Acetic acid-induced death displays characteristic markers of apoptosis in *Δgpd1Δgpd2* and *Δrvs161* strains, but not in *Δgup1* mutant

In order to assess whether cell death induced by acetic acid treatment followed a programmed process of apoptosis, we analyze several apoptotic markers in the mutants that prove to be sensitive to acetic acid. The first marker analyzed was the integrity of plasma membrane, by PI staining. Acetic acid treatment leads to a pronounced increase of Δ*gup1* mutant PI positive cells as mentioned before (Chapter III, Section 1.2.4), reaching nearly 100% after 180 min of treatment, while Δ*gpd1*Δ*gpd2* and Δ*rvs161* strains this percentage didn't exceed 39% and 37% respectively. In Wt culture only 7% of cells loosed the membrane integrity after acetic acid treatment (Fig. III - 7 A).

Additionally, translocation of phosphatidylserine from the inner leaflet to the extracellular side of the plasma membrane was also examined by FITC-coupled Annexin V/PI co-staining (Fig. III – 7 B). Wt and Δ*gpd1*Δ*gpd2* and Δ*rvs161* mutants, presents trace amounts of Annexin V/PI stained cells before apoptosis induction, that increase after the treatment with acetic acid, reaching up to 8%, 7% and 6% respectively. This is in clear opposition to the previously observed for Δ*gup1* mutant strain (Chapter III, Section 1.2.4), in which no significant difference was detected in Annexin V/PI stained cells before and after apoptosis induction with acetic acid (180 min treatment). In both cases, the percentage of apoptotic cells didn't exceed 3% (Fig. III – 7 B).

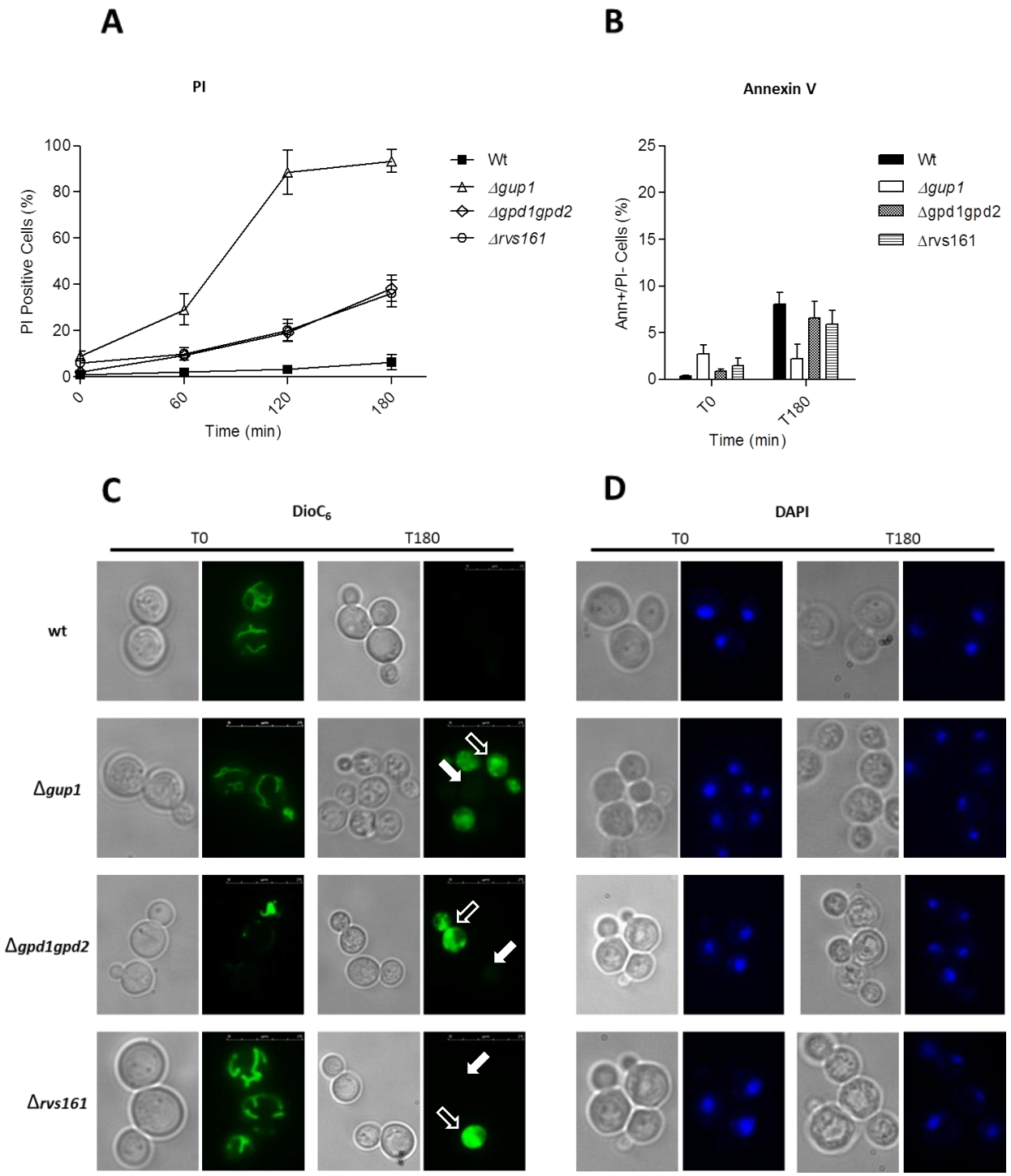

Fig. III – 7. Analysis of apoptotic markers in Wt, Δ*gup1*, Δ*gpd1*Δ*gpd2* and Δ*rvs161* mutant cells treated with 160 mM acetic acid for 180 min. (A) Graphic representation of the percentage of cells displaying positive PI staining. (B) Graphic representation of the percentage of cells displaying Ann V(+)/PI (–). (C) Representative photos of $DiOC_6$ staining untreated cells and cells after 180 min of acetic acid treatment. Filled arrows indicate cells with depolarized mitochondrial membrane, whereas open arrow indicate cells with an unspecific $DiOC_6$ staining. (D) Representative photos of DAPI staining untreated cells after 180 min acetic acid treatment. For flow cytometry and fluorescence microscopy assays a minimum of 35,000 and 300 cells were counted, respectively. Data represent mean ± SD of 3 independent experiments.

Yeast mitochondria undergo both structural and functional changes in the apoptotic death process activated by acetic acid (Ludovico *et al.*, 2002), that involved mitochondrial membrane depolarization. In order to evaluate the mitochondrial membrane depolarization, $DiOC_6$ was used. At a concentration of 20 ng/ml, this dye accumulates specifically at mitochondrial membranes and can be observed by fluorescence microscopy. Nonetheless, cells that have low mitochondrial membrane potential will fail to accumulate $DiOC_6$ (Gourlay and Ayscough, 2005) (Fig. III – 7 C). Just before apoptosis induction with acetic acid, most of Wt and mutants cells presented intact mitochondrial networks, with the exception of Δ*gpd1*Δ*gpd2* double mutant that didn't presented any fluorescence (Fig. III – 7 C left panels). This is probably due to probe inability to proper signaling in this mutant. After the treatment (180 min), it was possible to visualize depolarization of mitochondrial membranes in approximately 35% of the Δ*rvs161* cells, in comparison to 30% and 40% of wt and *Δgup1* depolarized cells mentioned before (Chapter III Section 1.2.4) (Fig. III – 7 C right panels). Furthermore, some cells displayed an increase in $DiOC_6$ green fluorescence suggesting a possibly altered plasma membrane that results in the accumulation of $DiOC_6$ on the cytosol (Fig. III - 7 C right panels).

Additionally, we checked for chromatin condensation during acetic acid treatment, by staining cells with DAPI (Fig. III – 7 D). No chromatin condensation was observed in untreated cells, as reflected by the single round fluorescent circles in the center of the cells (Fig. III – 7 D left panels). Yet, after treatment we observed an increase in Wt, Δ*gpd1*Δ*gpd2* and Δ*rvs161* cells (~25%, ~45% and ~40% respectively) exhibiting chromatin condensation which can be visualized by the fluorescent semicircles formed by the chromatin fragments. As mentioned in the previous section (Chapter III Section1.2.4), moderate chromatin condensation upon DAPI staining was observed in 93% of treated Δ*gup1* mutant cells (Fig. III - 7 D right panels).

0 - 2.2.3 Δ*gup1*, Δ*gpd1*Δ*gpd2* and Δ*rvs161* mutants present different ROS accumulations during acetic acid treatment

Accumulation of ROS is an event commonly linked to apoptosis induction. Moreover, ROS has been shown to be sufficient to induce apoptosis in yeast (Madeo *et al.*, 1999).

In this work we used the dye dihydroethidium (DHE) to measure the ROS accumulation in Δ*gpd1*Δ*gpd2* and Δ*rvs161* cultures cells treated and untreated with acetic acid (160 mM), and compared it with the previous attained results for Wt and Δ*gup1* strains (Chapter III, Section 1.2.5) (Fig. III - 8). In the presence of the superoxide anion, DHE is oxidized to ethidium that intercalates within nucleic acids, staining the cell with a bright red fluorescence that can be quantified by flow cytometry. As soon as time 0, it was possible to visualize a difference between Wt and the diverse mutants. All mutants present more ROS accumulation than Wt.

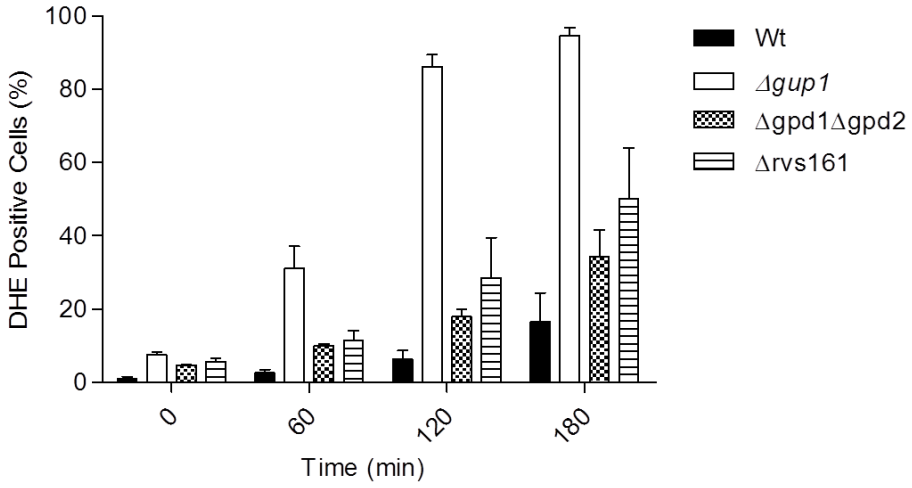

Fig. III – 8. Accumulations of ROS. Cells from acetic acid treatment were analyzed for accumulation of ROS using DHE staining by flow cytometry. At least 35,000 cells were analyzed. Data represent mean ± SD of at least 3 independent experiments.

Throughout the acetic acid treatment these differences become more pronounced, yet not as much as seen with Δ*gup1* mutant that already had ~30% of cells with accumulation of ROS after 1 hour of treatment (Chapter III, Section1.2.5). Both Δ*gpd1*Δ*gpd2* and Δ*rvs161* mutants presented ~10%, whereas Wt displayed less than 3% of DHE positive cells. After 2 hours of incubation with acetic acid we observed an increase of ROS accumulation in all strains. The differences between strains were extremely notorious after 3 hours of treatment. Wt exhibited ~15% of DHE positive cells, whereas the Δ*gpd1*Δ*gpd2* and Δ*rvs161* mutant strains presented ~35% and ~50%,

respectively. In clear contrast Δ*gup1* mutant strain displayed ~95% DHE positive cells (Chapter III Section1.2.5). (Fig. III - 8).

0 - 2.2.4 Δ*gup1,* Δ*gpd1*Δ*gpd2* and Δ*rvs161* mutants are affected in sterol lipid distribution

In mammalian cells, the distribution of free cholesterol can be visualized by fluorescence microscopy using the cholesterol probe, filipin (Severs, 1997). Although filipin binds cholesterol with greater affinity than ergosterol (Bittman *et al.*, 1974), filipin fluorescence was successfully adapted for analyzing yeast sterol-lipid distribution. Therefore, filipin staining has been used to detect regions with high sterol content in yeast plasma membrane (Beh and Rine, 2004). Importantly, 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).

In this work we analyzed the sterol-domains in Wt, *Δgup1*, *Δgpd1*Δ*gpd2* and *Δrvs161* mutant cells treated or untreated with acetic acid (160 mM) (Fig. III – 9). It should be noted that before being stained with filipin these cultures were fixed with 3.7% formaldehyde.

As expected, the plasma membrane of untreated Wt cells was not stained homogeneously, but rather in distinct patches, as observed in other works (Grossmann *et al.*, 2007; Ferreira and Lucas, 2008). Contrarily, most of untreated Δ*gup1* cells showed an atypical homogeneous distribution, after filipin staining, at the plasma membrane. This profile has previously been described by our group (Ferreira and Lucas, 2008) and indicates that *GUP1* deletion interferes with the maintenance and distribution of sterol. Compared with filipin-stained Wt cells, filipin fluorescence in untreated cells of Δ*gpd1*Δ*gpd2* mutant showed some differences in the distribution of sterol-domains, being especially punctuated at the level of the plasma membrane. The Δ*rvs161* mutant presented a more homogeneous sterol distribution, similar to Δ*gup1* mutant, but it was still possible to see some punctuated structures. Furthermore, in a great number of untreated cells, fluorescence was also observed in small cytoplasmic spots, which might

correspond to internal membranes or sterol-rich endocytic vesicles (Beh and Rine, 2004; Alvarez-Vasquez *et al.*, 2007; Ferreira and Lucas, 2008) (Fig III – 9, left panels).

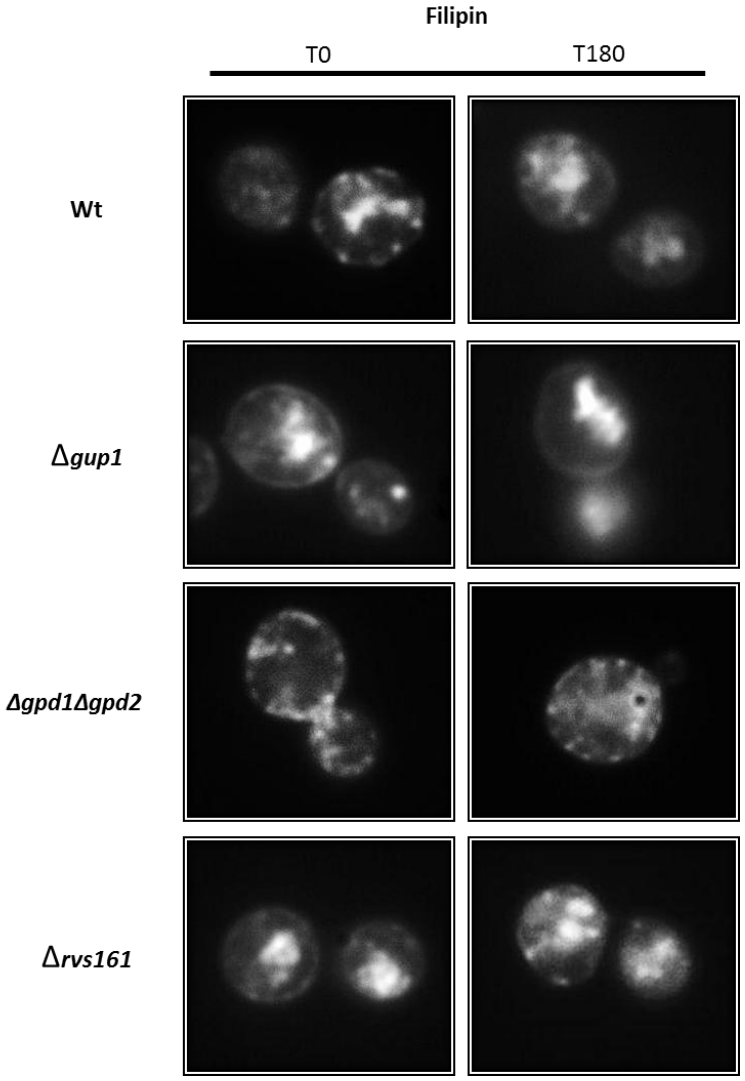

Fig. III – 9. Sterol-rich domains distribution. Wt, *Δgup1*, *Δgpd1Δgpd2* and *Δrvs161* mutant cells treated or untreated with acetic acid (160mM) were fixed in 3.7% formaldehyde, stained with filipin (5 mg/ml) and stabilized onto slides with a drop of an anti-fading agent.

After 3 hours of acetic acid treatment, all cells presented a different sterol distribution pattern (Fig III – 9, right panels). In Wt and Δ*gpd1*Δ*gpd2* treated cells it is possible to observe a drop on the number of sterol patches in plasma membrane. On the other hand,

in Δ*gup1* and Δ*rvs161* cells incubated with acetic acid the differences were more subtle. We observed a reduction in filipin fluorescence at the level of the plasma membrane in both mutants. For all strains, we visualize an increase in cytoplasmic fluorescence after acetic acid treatment.

0 - 2.3 Discussion

Lipid rafts, micro-domains of the plasma membrane especially rich in sphingolipids and ergosterol, have the capability to include or exclude proteins to variable extents. This allows specific protein–protein interactions and modulates the activity of several signaling cascades. In the literature, rafts have been increasingly implicated on the regulation of apoptotic signaling in mammalian cells (Garcia *et al.*, 2003; Mollinedo and Gajate, 2010). Moreover, in mammalian cells a number of proteins involved in apoptotic signals have been found to localize in lipid rafts, namely the pro-apoptotic protein of Bcl-2 family, Bad (Ayllon *et al.*, 2002) and the Fas/CD95 receptor (Gajate and Mollinedo, 2001). However, in yeast, the information about the involvement of lipid rafts in apoptosis remains unclear. As previously demonstrated, cells with deletion in *GUP1* gene are incapable of committing to apoptosis when incubated with acetic acid, dying by necrosis instead (Chapter III, Section 1). Gup1p is a membrane-bound *O*-acyltransferase that is involved in a wide range of crucial process for cell preservation and functioning, including in lipid metabolism and in rafts integrity/assembly (Ferreira and Lucas, 2008). These finding suggest a possible involvement of lipid rafts in yeast apoptosis as well. To gain further insight into the possible involvement of lipid rafts in apoptotic signaling, we assessed apoptosis in several mutants affected in process that are directly or indirectly related to apoptosis, namely *Δgpd1gpd2* and the single mutants *Δgpd1* and *Δgpd2*, and *Δrvs161*. We then analyzed rafts distribution in the mutants that presented altered apoptosis. Acetic acid was used in this work as an inducer of apoptosis (Ludovico *et al.*, 2001).

We found that *Δgup1*, *Δgpd1gpd2* and *Δrvs161* were more sensitive to treatment with acetic acid than Wt. Gpd1p/Gpd2p proteins are two isoforms of glycerol-3-phosphate dehydrogenase, responsible for the glycerol production (Albertyn *et al.*, 1994; Ansell *et*

al., 1997). Hog1 MAPK is transiently activated in yeast exposed to high levels of acetic acid (Mollapour and Piper, 2007). The activated Hog1p generates a stress response that culminates in the expression of *GPD1* gene. However, in a previous work, Mollapour and Piper showed that in low pH cultures (pH 4.5), the acetate-activated Hog1p, although conferring acetic acid resistance, does not promote the *GPD1* gene overexpression or intracellular glycerol inductions (Mollapour and Piper, 2006). This can be due to inhibition of cell metabolic activity that occur following acetic acid accumulation (Pampulha and Loureiro-Dias, 1989). This is in agreement with our results. We only observed an increase in the acetic acid sensibility in the double mutant Δ*gpd1*Δ*gpd2* but not in the single mutants Δ*gpd1* or Δ*gpd2*. This result suggests that the double Δ*gpd1*Δ*gpd2* mutant is possible affected in other cellular processes, besides glycerol production, important for acetic acid resistance.

The Δ*rvs161* mutant has also proved to be more sensitive to acetic acid than Wt cells. Rvs161p is a lipid raft protein involved in the regulation of cell polarity (Durrens *et al.*, 1995), actin cytoskeleton polarization (Sivadon *et al.*, 1995), endocytosis (Munn *et al.*, 1995), and secretory vesicle trafficking (Gammie *et al.*, 1998; Breton *et al.*, 2001). As mentioned before, cells respond to acetic acid by activating the Hog1p. Hog1p activated also phosphorylates Fps1p targeting this channel for endocytosis and consequent degradation in the vacuole. This an adaptive mechanism to decrease the uptake of acetic acid and thus protecting yeast cells from its toxic effects (Mollapour and Piper, 2007). Certainly, other proteins are endocytosed from plasma membrane as well. Therefore, is not surprising that a mutant affected in endocytosis and secretory vesicle trafficking is more sensitive to acetic acid.

Wt cells treated with acetic acid predominantly died exhibiting apoptotic markers, namely a) maintenance of plasma membrane integrity; b) phosphatidylserine externalization; b) depolarization of mitochondrial membrane; and c) chromatin condensation. These results are in agreement with the literature (Ludovico *et al.*, 2001). The studied mutants present different death processes. While the Δ*gpd1*Δ*gpd2* and Δ*rvs161* mutants died exhibiting apoptotic markers similar to Wt, acetic acid treatment seems to induce a necrotic cell death in *Δgup1* mutant, as mentioned before (Chapter III, Section 1).

Sterol distribution was further assessed in the mutant strains which revealed to be sensitivity to acetic acid. Our results with filipin staining showed that both Δ*gpd1*Δ*gpd2* and Δ*rvs161* have an altered rafts distribution. Still, their sterol distribution does not seem to be as altered as the one of the Δ*gup1* mutant (that presented the distribution of ergosterol completely homogeneous in plasma membrane). This is in accordance with Ferreira and Lucas work (Ferreira and Lucas, 2008). The Δ*gpd1*Δ*gpd2* mutant displayed an especially punctuated pattern of filipin staining at the level of the plasma membrane, while Δ*rvs161* mutant showed a pattern less punctuated than Wt. These results indicate that the mutants with deletion in the *GPD1/GPD2* and *RVS161* genes have an altered rafts distribution, possibly affecting rafts integrity/assembly.

After acetic acid treatment, all cells presented a different sterol distribution pattern. We observed a reduction in fluorescence at the level of the plasma membrane and an increase in cytoplasmic fluorescence. This can be a result of increased vesicle trafficking between plasma membrane and ER and Golgi (Beh and Rine, 2004). Conversely, these findings seem to indicate that cell death induced by acetic acid is associated with a rearrangement of lipid rafts. Yet, the possibility that filipin may not function properly in the intracellular low pH, that was generated by acetic acid dissociation inside the cell, has to be considered. Further experiments must be done in other to elucidate such events. Altogether, these results indicate that lipid rafts seem to be a key component in apoptotic signaling, possibly essential in some circumstances.

Chapter IV

FINAL CONSIDERATIONS

Final Considerations

Apoptosis is the most common process of PCD in eukaryotic cells, vital for the fast elimination of useless or injured cells, and for the differential development of tissues and organs. It is, therefore, indispensable for homeostasis of the organisms, but the malfunction of this process can lead to serious diseases. The role of apoptosis in the prevention and progress of several pathologies turned it into an attractive therapeutic target for the treatment of conditions such as cancer and neurodegenerative disorders, over the past decades. However, the high complexity of mammalian cells has made it difficult to study the molecular mechanisms underlying apoptosis.

The existence of PCD processes in unicellular organisms was for long disregarded due to the absence of clear benefits of such process for a single cell. Nonetheless, numerous works contributed to the finding that PCD can occur in unicellular organisms (Madeo *et al.*, 1997; Ludovico *et al.*, 2001; Herker *et al.*, 2004), establishing yeast as a good model to study mechanisms of apoptotic regulation (Frohlich *et al.*, 2007). Since then, several inducers of yeast apoptosis have been identified, including chronological aging (Herker *et al.*, 2004) and acetic acid (Ludovico *et al.*, 2001). However, the exact mechanisms and the essential players to this process remain poorly understood. In mammalian cells, lipid rafts have been intensively associated to apoptosis. Thus, the major aim of this thesis was to understand the importance of these lipid micro-domains in yeast apoptosis.

We found that Δ*gup1* mutant is incapable of committing to apoptosis during chronological aging or during treatment with acetic acid. Instead, this mutant appears to undergo a necrotic cell death. Confirmation of the necrotic death of Δ*gup1* mutant might be accomplished by monitoring HMBG1/Nhp6p localization (HMBG1 is released from the nucleus during necrosis) or the disintegration of subcellular structures using electron microscopy.

Being Δ*gup1* mutant affected in lipid rafts integrity/assembly (Ferreira and Lucas, 2008) lipid metabolism (Oelkers *et al.*, 2000; Ferreira and Lucas, 2008), and GPI anchor remodeling (Bosson *et al.*, 2006; Jaquenoud *et al.*, 2008), we suggest that the integrity of rafts may be essential for apoptosis induction and/or signaling. However, we must keep in mind that Gup1p are involved in several processes, besides raft's dynamics, that could also be affecting this mutant's phenotype after apoptosis induction. These process

include: cell wall structure and biogenesis (Ferreira *et al.*, 2006), secretory/endocytic pathways (Bonangelino *et al.*, 2002), cytoskeleton polarization and bud site selection (Ni and Snyder, 2001; Casamayor and Snyder, 2002), mitochondrial and vacuole morphology (Bonangelino *et al.*, 2002) and telomere length (Askree *et al.*, 2004).

We also verified that mutants sensitive to apoptosis induced by acetic acid, namely Δ*gpd1*Δ*gpd2* and Δ*rvs161*, have an altered sterol distribution as visualized by filipin staining. In fact, our results indicate that acetic acid induces rearrangements in the sterol distribution. This hypothesis may be tested by extraction of detergent resistant membrane domains (DRMs) in order to monitor membrane lipid rafts. Lipid rafts integrity can also be assessed by plasma membrane proton ATPase (Pma1p) or Gas1p detection. These proteins have been extensively used as markers for raft integrity since Pma1p requires raft association for proper localization and Gas1p is a GPI anchor protein (Bagnat *et al.*, 2001; Balguerie *et al.*, 2002; Ferreira and Lucas, 2008).

Another strategy similarly worthy to explore, is the study of apoptosis under conditions where lipid rafts formation is affected. For instance, it might be interesting to use compounds known to disturb rafts, like myriocin (a sphingolipid inhibitor) and ketoconazole (ergosterol inhibitor), and assess whether they influence acetic acid-induced apoptosis.

Taken together, our results show that lipid rafts seem to be important in yeast apoptosis, giving new insights into the molecular mechanisms underlying this particular process of PCD, and highlighting the complex network of cellular structures that interact, cooperate and compete to regulate cell death. Once more, the yeast model *S. cerevisiae* has proven to be a powerful tool to study the fascinating world of cellular death, in this particular case, the relation between apoptosis and lipid rafts.

Chapter V

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Chapter VI

SUPPLEMENTARY DATA

VI - 1 Treatment with different acetic acid concentrations

Acetic acid is a weak organic acid produced during yeast fermentation that becomes toxic when present at high concentrations, hence affecting several aspects of cellular physiology. In *S. cerevisiae*, specific concentrations of acetic acid are able to induce a process of cell death that shares common features with mammalian apoptosis, (Ludovico *et al.,* 2001). In order to determine the suitable concentration of acetic acid, Wt strain was tested for a wide range of concentrations (100, 120, 140, 160 and 180 mM) (Fig.VI - 1).

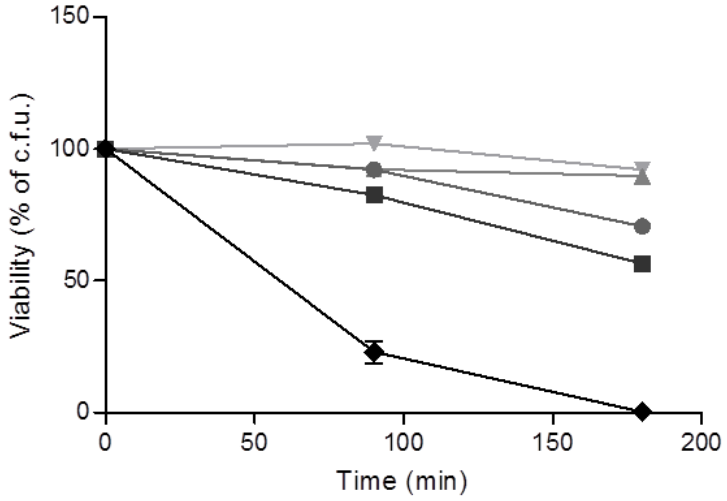

Fig. VI – 1. Survival curve of Wt cultures in the presence of different acetic acid concentrations (100 mM (▼), 120 mM (▲) 140 mM (●), 160 mM (■) and 180 mM (◆)). Exponential cells were treated with acetic acid for 180 min in YNB medium (pH 3) and viability determined by c.f.u. at the indicated time points (100% survival corresponds to the total c.f.u. at time zero). Data represent mean ± SD of at least 3 independent experiments.

Cultures treated with 100 and 120 mM of acetic acid showed an elevated survival rate. With 140 mM of acetic acid the survival was lower than that obtained with the concentrations of 100 and 120 mM, however it was still considerably high. In opposite, we verified that Wt cells were extremely sensitive to 180 mM of acetic acid. Therefore, the concentration of 160 mM was chosen, since it caused a viability of ~50% after 180 min of treatment.

VI - 2 Ceramide C2 treatment

Ceramide is a sphingosine-based lipid signaling molecule that has been associated to different cellular process, including apoptosis (Ogretmen and Hunnun, 2004; Thevissen *et al.*, 2006). The strains, Δ*gup1*, Δ*gpd1*Δ*gpd2*, Δ*gpd1*, Δ*gpd2* and Δ*rvs161* were treated with ceramide C2 (45mM) during 180 min preceding c.f.u. determination (Fig IV – 2).

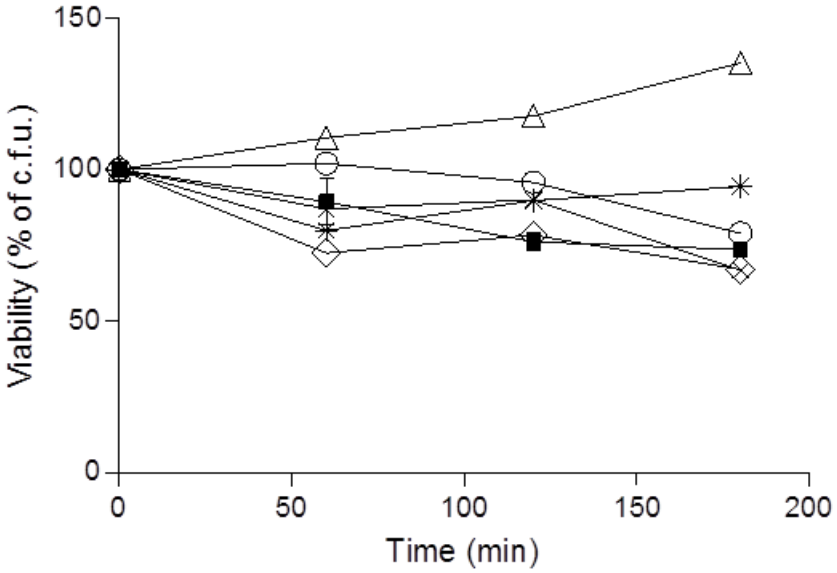

Fig. VI – 2. Survival curve of Wt (■), Δ*gup1* (△), Δ*gpd1*Δ*gpd2* (◇), Δ*gpd1* (∗), Δ*gpd2* (×) and Δ*rvs161* (○) cultures in the presence of ceramide (C2). Exponential cells were treated with ceramide (C2) for 180 min, and viability was determined by c.f.u. at the indicated time points (100% survival corresponds to the total c.f.u. at time zero). Data represent mean ± SD of at least 3 independent experiments.

After 180 min of treatment, Wt strain presented a viability of ~70%, the same that was observed for the all mutant strains, with the exception of Δ*gup1* and Δ*gpd1*. The latter was slightly more resistant than Wt, displaying ~95% survival. On the other hand, ceramide treatment did not cause any dead on Δgup1, only a minor growth inhibition.

VI - 3 Sensitivity to myriocin

It is well documented that the depletion of sphingolipids in membranes results in a loss of lipid rafts (Martin and Konopka, 2004). Thus, in order to promote loss of lipid rafts, we used myriocin, an inhibitor of serine palmitoyltransferase which catalyzes the first step of sphingolipid biosynthesis (Miyake *et al.,* 1995). Two different concentrations of myriocin (5 µg/ml and 7.5 µg/ml) were used, according to the literature (Ferreira and Lucas, 2008) (Fig. VI - 3).

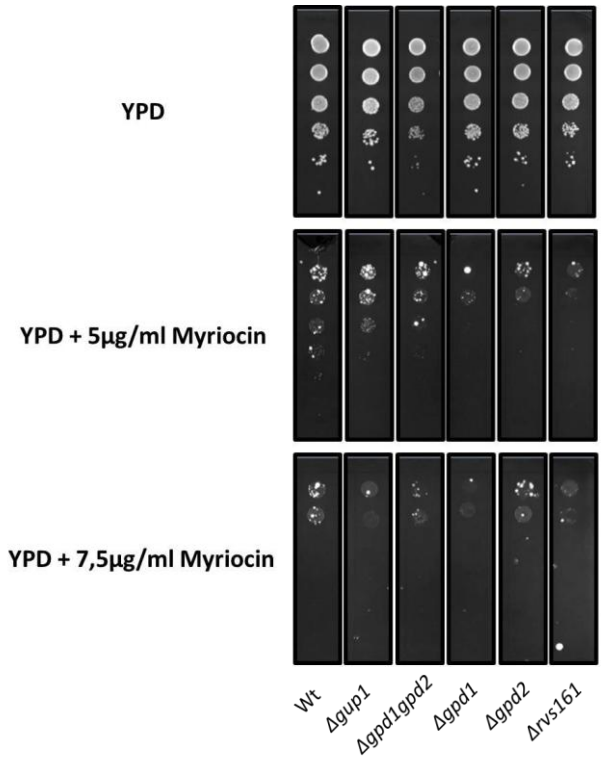

Fig VI – 3. Rafts-related mutants are more sensitive to sphingolipid biosynthesis inhibition. Wt and *Δgup1*, *Δgpd1gpd2, Δgpd1, Δgpd2* and *Δrvs161*mutants were grown to mid-exponential phase in YPD medium, and ten-fold serial dilutions were spotted onto YPD plates or YPD supplemented with myriocin with the indicated dosage. All plates were incubated at 30 °C and photographed after 3 days of incubation.

When exposed to 5 µg/ml of myriocin all mutant strains present higher sensitivity than the wt. This phenotype was more evident in the single mutants *Δgpd1* and *Δgpd2,* and in *Δrvs161* mutant, whereas *Δgpd1Δgpd2* and *Δgup1* mutants were slightly sensitive.

Regarding exposition to 7.5 µg/ml of myriocin no differences were observed between strains.

VI - 4 DRMs isolation

DRMs (Detergent Resistant Membrane domains) were isolated essentially as described before (Ferreira and Lucas, 2008) with some modifications. Treated and untreated cells were grown in 600 ml YNBD medium until exponential phase (DO600 = 0.5-0.6), collected by centrifugation and washed once in 50 ml of cold water. Cell pellet was resuspended in 500 µl cold TNE buffer (50 mM Tris– HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) with 2.5 µg/ml pepstatin, 5 M PMFS, 1 µg/ml aprotinin and 1 µg/ml leupeptin (ROCHE) (TNEi). Subsequently, cells were disrupted mechanically in the same buffer, using 425 µmØ glass beads in a Kaiser homogenizer (B. Braun) equipped with a cooler system. Cell extracts were separated from glass beads by centrifugation at 500 g for 5 min at 4 °C. The cleared lysate was then centrifuged at 18,000 g for 45 min at 4 °C. The pellet (total membranes) was resuspended in 500 µl TNEi buffer. Total quantity of protein was determined by Lowry method (Lowry *et al.,* 1951). To 2.5 mg of protein was added 418 µl of TNEi buffer. The mixture was then incubated with Triton X100 (1% final concentration) for 30 min on ice. An Optiprep solution (Sigma) 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 carefully overlaid with 8,6 mL of 30% (v/v) Optiprep (in TNEi), filled with TNE buffer and subjected to a centrifugation at 245,000 g for 5 h at 4 °C, in a TH641 rotor (Sorval Ultracentrifuge). The interface obtained between the 30% Optiprep and TNEi was carefully separated and transferred to a new centrifuge tube. To this 11 ml of TNEi was added and the suspension centrifuged again at 245,000 g for an extra hour, at 4 °C. Fractions were precipitated with TCA, and analyzed by SDS-PAGE**.**

The protein profiles of the DMRs of all strains are under study.

VI - 5 Supplementary references

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