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Mestrado em Genética Molecular

Trabalho realizado sob orientação do
Professora Doutora Maria João Marques Ferreira Sousa Moreira
e da
Professora Doutora Maria Manuela Sansonetty Gonçalves Côrte-Real

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DECLARAÇÃO

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

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Abstract

Title: The role of endoplasmic reticulum-mitochondria contact sites and mitochondrial phospholipid composition in *Saccharomyces cerevisiae* acetic acid-induced apoptosis

Endoplasmic reticulum-mitochondria contact sites (ERM-CS), found from yeast to mammals, are interface structures where membranes of the two organelles are maintained in close proximity by tethering protein complexes. These structures play a prominent role in the communication between the two organelles, including the transport of molecules and transmission of regulatory signals, and have been implicated in apoptotic signaling, though the exact mechanism is still unclear. In the present work we questioned if perturbation of ERM-CS could have a role in *Saccharomyces cerevisiae* acetic acid-induced apoptosis. We also aimed to assess if perturbation of mitochondrial phospholipid content resulting from deficiency in ERM-CS components or in proteins involved in phospholipid synthesis or transport could influence acetic acid-induced apoptosis. Cell viability assays and assessment of cytochrome *c* (cyt *c*) release carried in *S. cerevisiae* and in mutants affected in the above mentioned processes show that all the mutant strains, except those involved in cardiolipin biosynthesis and remodeling, display a delay in apoptotic cell death in response to acetic acid treatment related with an hindrance of cyt *c* release. Analysis of the phospholipid composition of mitochondria isolated from the strains under study, grown in glucose or in a less repressive carbon source, and treated or not with acetic acid, show different profiles between carbon sources and the different strains, and also after treatment with acetic acid. Knowing the important role of the ERMES complex in the mitochondrial metabolism, and to uncover if this complex could be involved in Bax mediated cell death, we transformed mutant strains deficient in each protein of this complex with Bax. Assessment of cell death induced by expression of Bax, suggested that there is no alteration in any of the transformed strains in comparison with the BY4741 phenotype, with the exception of one mutant, which showed a slight decrease in cell survival. However, further analysis is necessary to confirm these results.

Resumo

Título: O papel dos locais de contacto entre o retículo endoplasmático e a mitocôndria, e da composição fosfolipídica mitocondrial na apoptose induzida por ácido acético em *Saccharomyces cerevisiae*.

Os locais de contacto entre o retículo endoplasmático e a mitocôndria (ERM-CS), presentes na levedura e conservados em células de mamífero, estabelecem uma interface de proximidade entre estes dois compartimentos celulares através de complexos proteicos específicos (ex. ERMES em leveduras). Estas estruturas têm um papel proeminente na comunicação entre estes dois organelos, incluindo o transporte de moléculas e sinais regulatórios, e foram também implicados na sinalização apoptótica, embora este mecanismo ainda seja elusivo. Neste trabalho questionamos se perturbações dos ERM-CS poderiam ter implicações na apoptose induzida por ácido acético na levedura *S. cerevisiae*. Foi ainda objetivo perceber se perturbações do perfil fosfolipídico mitocondrial, causado por deficiências em componentes do ERMES ou em proteínas envolvidas na síntese ou transporte de fosfolípidos afectavam a apoptose induzida por ácido acético. Ensaios de viabilidade celular e avaliação da libertação de citocromo *c* (cit *c*) realizados na estirpe selvagem de *S. cerevisiae* e em mutantes deficientes nos processos acima mencionados mostraram que todas as estirpes mutantes, exceto as afectadas na biossíntese e remodelação de cardiolipina, exibem um atraso na morte celular relacionado com uma inibição da liberação cit *c*. A análise da composição de fosfolípidos de mitocôndrias isoladas a partir das estirpes em estudo, cultivadas em glucose ou em numa fonte de carbono menos repressora, e após tratamento com ácido acético, mostrou diferentes perfis fosfolipídicos. Conhecendo o papel importante do complexo ERMES no metabolismo mitocondrial, e com vista a descobrir se este complexo pode estar envolvido na morte celular mediada por Bax, as estirpes mutantes deficientes em cada proteína deste complexo foram transformadas com Bax. Avaliação da morte celular induzida por expressão da Bax, sugere que não há nenhuma alteração em qualquer das estirpes transformadas em comparação com a estirpe selvagem, com a exceção de um mutante, que mostrou um ligeiro decréscimo na sobrevivência de células. No entanto, uma análise mais aprofundada é necessária para confirmar estes resultados.

List of Abbreviations

αH α-helixes

AAC ADP/ATP Carrier

AIF Apoptosis Inducing Factor

ANT Adenine Nucleotide Translocator

BH Bcl-2 Homolog

BMP Bis(monoacylglycero)phosphate

BSA Bovine Serum Albumin

Calpains Ca^{2+} Sensitive Cysteine Proteases

Caspases Cys-Asp acid Proteases

CDP-DG Cytidine Diphosphate-diacylglycerol

CFU Colony Forming Units

Cho-P Choline-phosphate

cyt *c* cytochrome *c*

CL Cardiolipin

CoA Coenzyme A

DG Diacylglycerol

DNA Deoxyribonucleic Acid

DISC Death Inducing Signaling Complex

Drp1 Dynamin-related Protein 1

EDTA Ethylenediaminetetraacetic Acid

EMC Endoplasmic Reticulum Membrane Complex

Endo G Endonuclease G

ER Endoplasmic Reticulum

ERMES ER-Mitochondria Encounter Structure

Etn-P Ethanolamine-phosphate

FA Fatty Acids

FacI4 Fatty acid CoA ligase 4

Hex Hexadecenal

HOG High Osmolarity Glycerol

IAP Inhibitors of Apoptosis Protein

IMM Inner Mitochondrial Membrane

IP3R IP3 Receptor

LC Liquid Chromatography

MAC Mitochondrial Apoptosis Channel

MAM Mitochondria Associated Membrane

MAPK Mitogen-activated Protein Kinase

MCU Mitochondrial Calcium Uniporter

Mfn Mitofusin

MLCL Monolysocardiolipin

MOMP Mitochondrial Outer Membrane Permeabilization

MS Mass Spectrometry

mtDNA Mitochondrial DNA

OMM Outer Mitochondrial Membrane

OD Optical Density

PA Phosphatidic acid

PC Phosphatidylcholine

PE Phosphatidylethanolamine

PEG Polyethylene Glycol

PG Phosphatidylglycerol

PGK1 Phosphoglycerate Kinase

PI Phosphatidylinositol

Pis1 Phosphatidylinositol Synthase

PS Phosphatidylserine

PSS Phosphatidylserine Synthase

PTP Permeability Transition Pore

PVDF Polyvinylidene Difluoride

RNA Ribonucleic Acid

ROS Reactive Oxygen Species

SAM S-adenosylmethionine

Smac/Diablo Second Mitochondria Derived Activator of Caspase/Direct IAP Binding protein with low pI

tBid Truncated Bid

TCA Trichloroacetic Acid

TG Triglycerides

TIM Translocase of the Inner Mitochondrial Membrane

TLC Thin-Layer Chromatography

TNF Tumor Necrosis Factor

TOM Translocase of the Outer Mitochondrial Membrane

VDAC Voltage Dependent Anion Channe

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Chapter I – General Introduction

1. Apoptosis

The term apoptosis was coined by Kerr et al. in 1972 as an active form of programmed cell death triggered by an environmental stimulus (Kerr, Wyllie, & Currie, 1972). Hitherto, apoptosis is the most studied form of programmed cell death, and is characterized by the ordered appearance of several morphological or biochemical features. It occurs primarily as a mean of controlling cell population in a given tissue, during development or aging, but it can also occur as a defense mechanism by eliminating cells damaged by deleterious agents. This process is highly coordinated, normally genetically determined and energy-dependent (Elmore, 2007).

This type of programmed cell death involves single or small cells clusters. Its predominant morphological features are, in an early stage, cell shrinkage and pyknosis (irreversible chromatin condensation) and, in later stages, blebbing of the plasma membrane, along with karyorrhexis and karyolysis (fragmentation and dissolution of the nucleus), loss of plasma membrane phospholipid asymmetry and budding. The fragmentation of the cell into apoptotic bodies occurs without loss of the cell membrane integrity, these structures being then phagocytized and degraded in cells such as macrophages (Elmore, 2007). This feature distinguishes apoptotic from necrotic cell death where loss of membrane integrity occurs with the subsequent release of several cell constituents that cause an inflammatory response.

There are two main apoptotic pathways, the extrinsic and the intrinsic pathways. The extrinsic pathway, also known as the death receptor pathway, acts mainly as a response to external stimuli. This pathway acts by involvement of death receptors from the Tumor Necrosis Factor (TNF) receptor protein superfamily (such as FasR or TNFR1), which upon binding of the respective ligands, trimerizes and stimulates the Death Inducing Signaling Complex (DISC), leading to the recruitment and activation of the Caspase pathway (Elmore, 2007; Gómez-Sintes, Hernández, Lucas, & Avila, 2011; Zacks, Zheng, Han, Bakhru, & Miller, 2004).

The Caspase pathway involves the activation of a cascade of different caspases (Cys Asp acid proteases), which are divided into two groups, the initiators and the executioners (Figure 1). Upon activation, the initiators caspases (like C8, C9 and C10) cleave certain substrates, the more important of which are the executioner caspases, (C3, C6 and C7). After cleavage, these activated caspases are translocated to their cellular targets, leading to several of the phenotypic characteristics of apoptosis (Tait & Green, 2010).

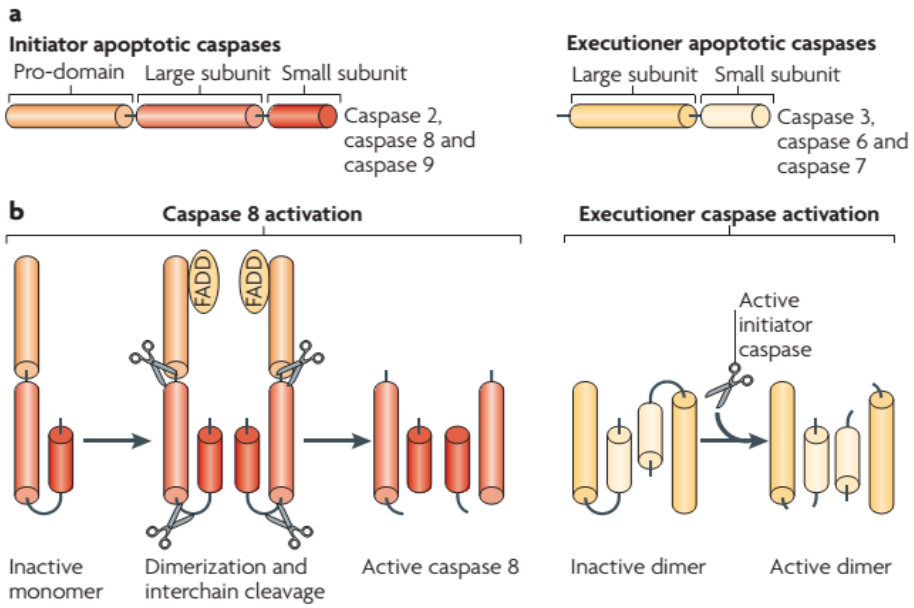


Figure 1 - Caspase (Cys Asp acid proteases) classification and activation. a) caspases are divided into two classes – initiators and executioners; b) caspase activation (Tait & Green, 2010)

The intrinsic pathway, also known as the mitochondrial pathway, is activated by intracellular stimuli caused by cell stress (e.g. DNA damage, increase of cytosolic calcium concentration, oxidative stress). This pathway is mitochondria-dependent, contrary to the extrinsic pathway, in which the mitochondria may be involved solely in the amplification of cell death. The activation of this pathway leads to the permeabilization of the mitochondrial outer membrane (MOMP), causing the release of pro-apoptotic proteins, such as cytochrome *c* (cyt *c*) and Smac/Diablo (Second Mitochondria Derived Activator of Caspase/Direct IAP Binding protein with low pI). These factors will activate the caspase-dependent mitochondrial pathway by activating Apaf-1 and procaspase-9, resulting in the formation of the apoptosome. In later stages of apoptosis there is also the release of AIF, endonuclease G (Nuc1) and CAD, that will cause nuclear DNA fragmentation and condensation (Elmore, 2007).

Despite the differences between them, the two pathways are linked, sharing several molecules, in a way that the activation of one can enhance and amplify, or even provoke the activation of the other (Figure 2) (Crompton, 2000; Elmore, 2007). Cells can be divided into two types, according to the necessity of involving the intrinsic pathway. In Type I cells, such as lymphocytes, the caspase pathway is enough to effectively execute cell death, whereas in type II cells, such as hepatocytes, there is a need to provoke MOMP through Bid activation (Crompton, 2000; Galluzzi, Kepp, Trojel-Hansen, & Kroemer, 2012; Hao & Mak, 2010).

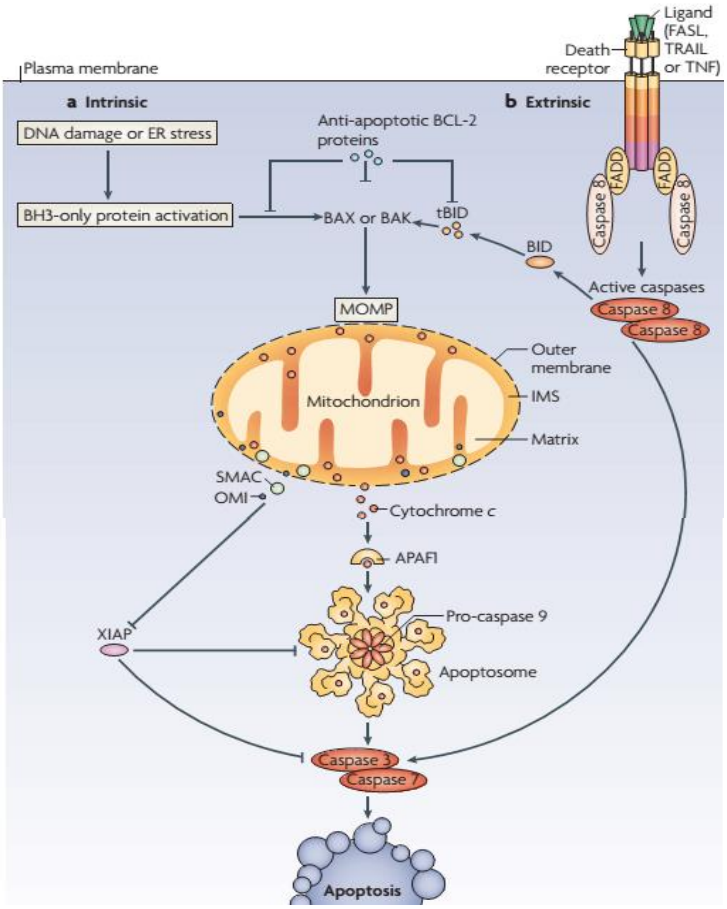


Figure 2 - Intrinsic and extrinsic pathways of apoptosis. a) Intrinsic pathway, initiated by internal stimuli; b) extrinsic pathway, initiated by death receptor activation (Tait & Green, 2010).

1.1 The BCL-2 protein family

1.1.1 Pro-apoptotic *vs* anti-apoptotic members

Apoptosis is a well-regulated process, and key intervenients in that regulation, particularly in MOMP, are several proteins of the Bcl-2 family. According to the activity and the number of Bcl-2 homolog (BH) domains, the family members are divided into three classes: anti-apoptotic, pro-apoptotic, and BH3-only proteins (Figure 3). The first class includes proteins that have four BH-domains, from BH1 to BH4. Bcl-2, Bcl-xL and Mcl-1, among others, are part of this class. Pro-apoptotic proteins can also have the four BH-domains, but BH4 domain is less conserved in this class. Bax, Bak and Bcl-Xs are included in the pro-apoptotic class (Adams & Cory, 2007; Andersen & Kornbluth, 2013; Chipuk, Moldoveanu, Llambi, Parsons, & Green, 2011; Er et al., 2006; García-Sáez, 2012; Ghibelli & Diederich, 2010).

The last class is the most heterogeneous class and includes proteins that only have the BH3 domain, such as Bid, Bad, Noxa and Puma. The BH3-only proteins are sub-divided further into two

subgroups: the activators, which directly bind to the pro-apoptotic proteins, activating them, and the sensitizers, which bind to the anti-apoptotic proteins, liberating the pro-apoptotic proteins attached to them, thus blocking the inhibitors (Adams & Cory, 2007; Andersen & Kornbluth, 2013; Chipuk et al., 2011; Er et al., 2006; García-Sáez, 2012; Ghibelli & Diederich, 2010).

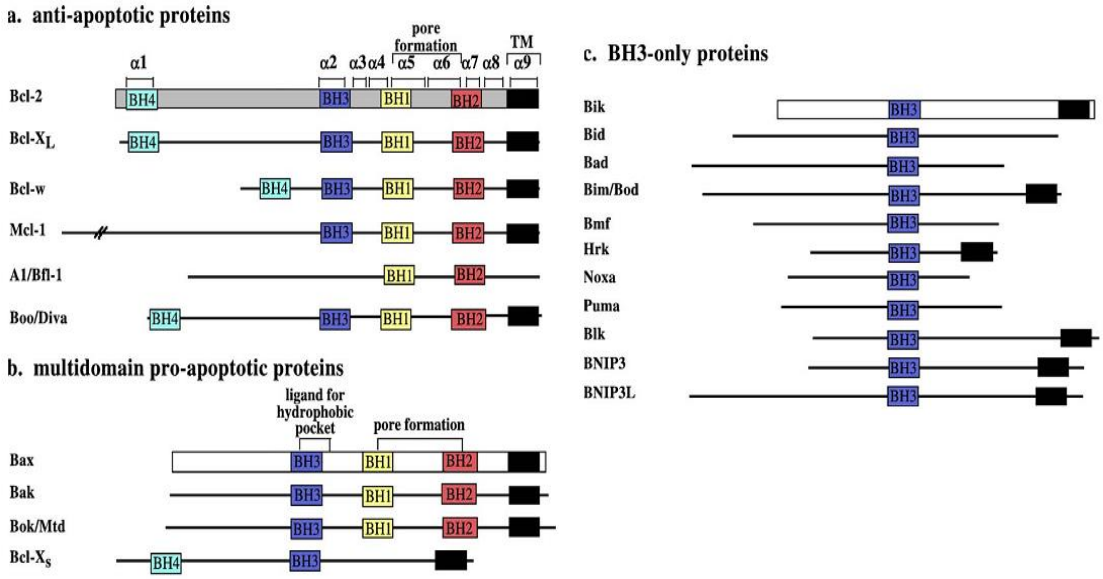


Figure 3 - Mammalian Bcl-2 family members, with highlighted BH domains, and important secondary structures. A) anti-apoptotic proteins; b) multi domain pro-apoptotic proteins; c)BH3-only proteins (Er et al., 2006)

1.2 Role of mitochondria in apoptosis

Mitochondria have an essential role "deciding" if the cell lives or dies when subjected to stress. These organelles, which are believed to originate from a symbiotic relation between prokaryotes and early eukaryotes, are the energy production base of the cell. Their shapes vary between several vesicular structures to tubular networks with ramifications across the cell (Cosentino & García-Sáez, 2014; Friedman & Nunnari, 2014).

Mitochondria present two membranes: the outer mitochondrial membrane (OMM), which forms a smooth envelope, rich in lipids that provide fluidity, and transmembrane channels that allow the passage of molecules of small size (3-5 kDa); and the inner mitochondrial membrane (IMM) that presents several invaginations, the cristae, is rich in proteins essential for mitochondrial functions, and is impermeable even to small molecules. Between both membranes, in the intermembrane space, lie several proteins involved in both the respiratory chain and apoptosis. The most abundant protein in this region is cyt *c*. Inside the IMM, the matrix is where many catabolic and anabolic pathways occur, such

as Krebs cycle or amino acids synthesis, and also where the mitochondrial DNA (mtDNA) is located, which encodes the genes for some of the enzymes involved in oxidative phosphorylation, and RNA and proteins necessary for the synthesis of those enzymes (Cosentino & García-Sáez, 2014; Friedman & Nunnari, 2014; Herrmann & Riemer, 2010; Horvath & Daum, 2013).

Upon apoptotic stimuli, several alterations of the mitochondria lead to cell death. Some of these alterations include: MOMP; alterations of the mitochondrial membranes phospholipidic composition; loss of mitochondrial membrane potential, which consequently blocks oxidative phosphorylation and leads to an accumulation of reactive oxygen species (ROS); cristae remodeling; and mitochondrial fragmentation (Cosentino & García-Sáez, 2014).

MOMP is considered a point of no return in the cell death process. Although there has been a lot of breakthroughs in the past few years, there are still many theories about the mechanism responsible for this permeabilization, and the means by which it works are still elusive.

There are several channels known to open during apoptosis. The mitochondrial permeability transition pore (PTP), is believed to be a complex of several proteins such as VDAC (Voltage Dependent Anion Channel), a channel that allows the passage of several cytosolic molecules and ions, ANT (Adenine nucleotide translocator), and possibly cyclophilin D and other factors, though the lack of any of these proteins does not appear to impair the formation of this complex (Büttner et al., 2011; Vieira et al., 2000; Vyssokikh & Brdiczka, 2003). Although studies report opening of these channels, they do not directly allow the passage of cyt *c* or other pro-apoptotic factors, due to the small opening and only communicating between the matrix and the cytosol (Er et al., 2006; García-Sáez, 2012; Ghibelli & Diederich, 2010).

It was believed that the opening of these channels could lead to swelling of the mitochondrial matrix with the rupture of the OMM, freeing the pro-apoptotic factors present in the intermembrane space into the cytosol, but further studies determined that this permeabilization could occur due to the opening of newly formed pores in the OMM, the MACs (Kuwana et al., 2002; Lin et al., 2011; Peixoto et al., 2011). Studies showed that these channels are formed through oligomerization of Bax and/or Bak. These channels would be big enough to allow the efflux of molecules such as cyt *c*, SMAC and AIF to the cytosol (Gillies & Kuwana, 2014; Peixoto et al., 2011; Tait & Green, 2010).

Even with the knowledge that Bax is involved in the formation of these channels, the process by which this protein is stimulated to oligomerize and form channels is still unknown, although some theories have been proposed.

1.2.1 Bax role in apoptosis and MOMP

Bax is a pro-apoptotic protein of 21kD, with 192 amino-acids. In 2000, Suzuki et al. defined the three-dimensional structure of this protein, identifying several domains important in its characterization (Figure 4) (Suzuki, Youle, & Tjandra, 2000). The localization of this protein is mainly dictated by its activation. Although normally cytosolic when inactive, a small part of the protein is constitutively bound to the mitochondria by a weak link through a C-terminal transmembrane domain, possibly interacting with VDAC (Li & Dewson, 2015). Upon binding of the activator, Bax undergoes conformational changes, exposing certain domains such as the α-helixes (αH) 5/6, that are putative pore-forming transmembrane domains, and αH 9, an anchoring domain. All three are hydrophobic and involved in the translocation and insertion of the protein in the mitochondrial membrane (Suzuki et al., 2000). Other relevant domains of this protein are: αH 1, a site of interaction with the activators truncated Bid (tBid) and Puma; and the N-terminal, an unstructured domain used in assays to discriminate between the active/inactive form of Bax, because it hides the αH 9 in the inactive form of the protein, and exposes it when Bax is activated (Suzuki et al., 2000).

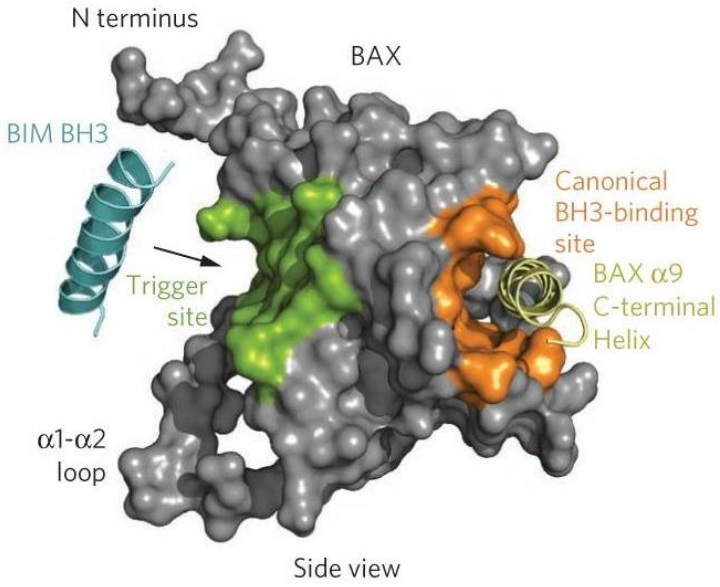


Figure 4 - Modelling of Bax protein with highlighted central domains for its activity, along with the Bim protein. (Gavathiotis & Reyna, 2012)

Bax has several functions within the cell. It is implicated in the dynamics of mitochondria, more precisely in mitochondrial fission, due to its presence in the membrane sites of fission and capacity to

bind to the proteins involved, increasing the fission rate and leading to the collapse of the mitochondrial network (Er et al., 2006; Ghibelli & Diederich, 2010).

Along with fission, Bax promotes MOMP, through the activation of MAC (Mitochondrial Apoptosis Channel), a channel formed by its homo- or hetero-oligomerization– this subject will be further analyzed below in this introduction (Ghibelli & Diederich, 2010). MOMP causes the release of mitochondrial proteins such as SMAC/Diablo, a protein dimer that competes with Inhibitors of Apoptosis Proteins (IAPs) bound to caspases, liberating them; Apoptosis Inducing Factor (AIF), a protein that promotes the activation of caspase-independent apoptotic pathways, along with Endo G, an endonuclease that is included in this pathway; and more importantly, cyt *c* (Ghibelli & Diederich, 2010).

Besides contributing to the release of cyt *c* by MOMP, Bax also indirectly amplifies its release. After activation, Bax is translocated to the ER membrane, where Bcl-2 is inhibiting IP3R (IP3 receptor). Once there, Bax interferes with this inhibition, increasing the Ca^{2+} efflux to the cytosol (Decuypere et al., 2011). In the zones of close proximity between the ER and mitochondria, the high concentrations of Ca^{2+} induce the oxidation of cardiolipin, which leads to further release of cyt *c* (Er et al., 2006; García-Sáez, 2012; Ghibelli & Diederich, 2010).

Bax is activated by various signals both from the intrinsic and the extrinsic apoptosis pathways, and also by physicochemical alterations. After activation from the extrinsic pathway, Bid is recruited to the mitochondria via PACS-2, a multifunctional sorting protein, and is cleaved into tBid by the activated caspase-8. Then, tBid recruits Bax from the cytosol, and by binding to the αH 1 of the protein, it will activate it, and expose the domains required for targeting the protein to the mitochondrial membrane (Ghibelli & Diederich, 2010; Korsmeyer et al., 2000; Ott, Norberg, Zhivotovsky, & Orrenius, 2009). Bax can also be activated by other BH3-only proteins such as Bim (Er et al., 2006).

Bax activation can be triggered by alterations of the physicochemical properties of the cytosol. In the case of oxidative stress, the MAP kinases JNK and p38 are activated, causing up-regulation of Bax, and its liberation from chaperones like Ku70 or 14-3-3 by acetylation and phosphorylation, respectively. These proteins are able to sequestrate cytosolic Bax. The 14-3-3 chaperone is also important for Bax translocation to the mitochondria. Oxidative stress may also activate Bax, directly, through the oxidation of the protein and consequent dimerization. The dimer would acquire the ability to translocate and insert into the mitochondrial membrane (Er et al., 2006; Ghibelli & Diederich, 2010; Smaili et al., 2003).

The increase of Ca^{2+} concentrations in the cytosol indirectly causes Bax activation, through the action of calpains (Ca^{2+} sensitive cysteine proteases), which gain proteolytic activity under high Ca^{2+}

concentrations, cleaving Bax's N-terminal region and exposing the domains of the protein required for its activity. Calpains also cleave Bid, and though in a different site than it is normally cleaved, it maintains its role as a Bax activator (Ghibelli & Diederich, 2010; Smaili et al., 2003).

Differences in the pH, can also activate this protein, by either exposing the BH3 domain, allowing for heterodimerization (pH 6.0-6.8 and 7.6-8.0), or exposing the N-terminal, resulting in translocation of the protein to the mitochondria (pH <6 and >8). Similarly, heat induces the conformational changes necessary for this translocation (Er et al., 2006; Kelekar & Thompson, 1998). In cancer cells there is expression of a glycoprotein, clusterin, that is able to specifically bind to the active Bax, inhibiting its oligomerization, an essential step for its role in apoptosis (Er et al., 2006).

One of the models currently being discussed for the activation of the MOMP channels is the conjugation of two previously proposed models, the direct activator model and the depressor model, and thus is referred to as "embedded together" model. This model discusses the role of the sub-division of the BH3-only proteins, where the roles of the activators and the sensitizers work together to allow activation and insertion of Bax in the outer mitochondrial membrane, where oligomerization takes place (Figure 5) (Bender & Martinou, 2013; Hagberg & Mallard, 2009; Hollville & Martin, 2012; Korsmeyer et al., 2000; Ripple, Abajian, & Springett, 2010; Walensky & Gavathiotis, 2011).

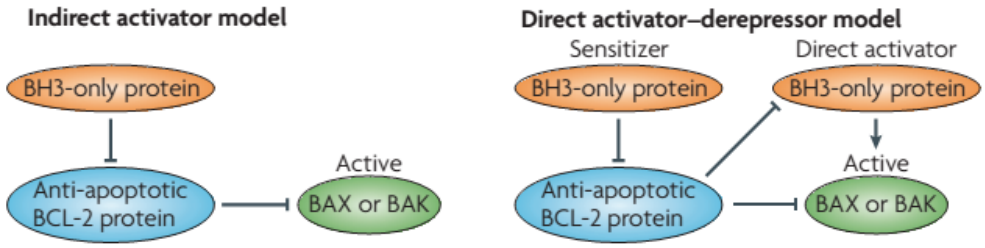


Figure 5 – Bax activation by BCL-2 family proteins (Tait & Green, 2010)

Studies show that the activation of Bax leads to the exposure of the BH3 domain. There are two models for its oligomerization, the asymmetric autoactivation and the symmetric dimer formation. The first model believes that the Bax's exposed BH3 domain could, along with the BH3-only activators, recruit more Bax, through an autoactivating mechanism, which would be propagated linearly throughout the oligomer. The second and more accepted model, proposes the formation of dimers by disulfide cross-linking. These dimers would then connect between them, by dimer oligomerization (Bender & Martinou, 2013; Chipuk et al., 2008; Kelekar & Thompson, 1998; Korsmeyer et al., 2000).

The channel that is formed, MAC, shows an increase in the conductance according to the increased number of Bax homodimers that become part of the structure. Also, it was reported that lipids are involved in the formation of this channel, since the observation of these structures with electron microscopy showed that the edges of the channel do not present proteins and are, in fact, of phospholipidic nature. One of the lipids most discussed in this subject is cardiolipin (CL), as it was shown that its presence is required for the formation of MAC (Bender & Martinou, 2013; Tait & Green, 2010). Also, there are several studies that link MACs not to CL, but to a sphingolipid, ceramide, due to its capacity to form channels through phospholipidic bilayers. Studies show that after apoptotic stimuli, the ceramide concentrations in the OMM increase, and that this lipid after being translocated to the mitochondria is transformed into hexadecenal (hex) that acts synergistically with Bax forming a channel big enough for the release of the pro-apoptotic factors (Chipuk et al. 2012; Stiban & Perera 2015). There are also studies that suggest the formation of pores composed only by ceramide, or that ceramide acts solely as Bax activators (Chipuk et al., 2012; Patwardhan et al., 2015). Other studies suggest that VDAC oligomerizes when induced by phospholipids present in mitochondrial membrane, specially phosphatidylglycerol (PG), when the ratio PG/CL is high, forming channels big enough to allow cyt *c* release (Betaneli, Petrov, & Schwille, 2012; Zheng et al., 2010).

Cyt *c* is a protein of around 12 kD, found in mitochondria, more specifically bound to the outer face of the Inner Mitochondrial Membrane (IMM) by cardiolipin (CL), where it has an important role in the production of energy, as an intermediate of the electron transport chain (Ascenzi, Polticelli, Marino, Santucci, & Coletta, 2011). Since 85% of this molecule is present within the cristae, disorganization and loosening of these structures caused by the disruption of the Opa1 (Mgm1 in yeast) complexes is needed for its significant release from mitochondria. The dysfunction of these complexes is known to impair replication of mtDNA and the electron transport chain. The disassembly of Opa1 complexes is induced by pro-apoptotic BH3-only proteins, while still requiring the presence of Bax (Otera & Mihara, 2012; Yamaguchi, Lartigue, Perkins, & Scott, 2008) and is also aided by the decreased mitochondrial membrane potential. Although essential, this remodeling of the cristae is still not enough to release cyt *c*, further events regarding CL being necessary (Cosentino & García-Sáez, 2014).

Along with the pro-apoptotic factors, other enzymes present in the intermembrane space are released, such as adenylate cyclase, a controller of the adenine levels (Burkart, Shi, Chouinard, & Corvera, 2011), whereas proteins from the matrix remain in the mitochondria (such as citrate synthase) (Leek, Mudaliar, Henry, Mathieu-Costello, & Richardson, 2001), indicating that the IMM remains intact.

In later stages of apoptosis, fragmentation of this organelle occurs by overexpression and translocation of Drp1 (dynamin-related protein 1) (Dnm1 in yeast) to the OMM. The Ca^{2+} accumulation in the cytoplasm due to mitochondrial and ER dysfunction leads to the dephosphorylation of Drp1 by Ca^{2+}-activated calcineurin, inducing its translocation to the mitochondria, where it interacts with Fis1 to carry out mitochondrial fragmentation (Cosentino & García-Sáez, 2014; Scorrano, 2013). Although the mechanism of the MOMP is not yet well understood, its impact in apoptosis is undoubtedly of great importance (Belizário, Alves, Occhiucci, Garay-Malpartida, & Sesso, 2007; Bender & Martinou, 2013; Montero et al., 2010; Tait & Green, 2010).

1.3 Yeast as a model of apoptosis

Until less than 20 years ago, the idea that unicellular organisms could undergo apoptosis was discarded because it was considered illogical for a cell to willingly commit suicide. However, Madeo and his colleagues, in 1997 found proof that a possible form of apoptosis was exhibited by a cell cycle mutant $cdc48^{S565G}$ of *S. cerevisiae*. This mutant presented exposure of phosphatidylserine, DNA fragmentation and chromatin condensation and fragmentation, all text-book markers of programmed cell death (Madeo, 1997). This created controversy, but ultimately, the idea that yeast populations should not be thought of as a group of independent unicellular organisms but as a multicellular community, with interaction between its members has prevailed. With this idea in mind, the thought of a cell committing to a programmed cell death process because it is damaged or old, and it would be of greater benefit as a resource for healthy cells in the colony, is not so odd.

Still, yeast lacks several of the major apoptotic regulators. However, this fact can have its advantages, since yeast can become a basic and more simple model that can be used to study not only yeast apoptosis, but also interactions between main mammalian apoptosis regulators through heterologous expression (Fröhlich, Madeo, & Frohlich, 2000; Weinberger, Ramachandran, & Burhans, 2003).

Some of the orthologue proteins important for yeast apoptosis are the metacaspase Yca1, the mitochondrial proteins Nuc1 and Aif1 (orthologues of mammalian Endo G and AIF respectively) and the protease Nma111 (orthologue of HtrA2/Omi) (Carmona-Gutierrez et al., 2010; Mazzoni & Falcone, 2008). Yca1 is the only known orthologue of mammalian caspases, and is involved in apoptosis induced by several triggers (Carmona-Gutierrez et al., 2010; Mazzoni & Falcone, 2008). Although it seems to be an important protein for yeast apoptosis, it is not essential. Studies show that apoptotic cell

death mediated by Nuc1 and Aif1 does not require Yca1, which could imply that, as for mammalian, yeast apoptosis may not always require caspases (Carmona-Gutierrez et al., 2010).

Another protein that might be involved in yeast apoptosis is Nma111, orthologue of HtrA2/Omi, a mitochondrial protease in humans, that in yeast is located in the nucleus and acts through cleavage of Bir1, a yeast inhibitor of apoptosis (AIP). Besides Nuc1 and Aif1, there are other mitochondrial proteins involved in yeast apoptosis, such as Ndi1, a NADH dehydrogenase located in the IMM; cyt *c*, although its mammalian role as an activator of caspase function is still unclear in yeast; and Drp1, which, as in mammalian apoptosis, causes mitochondrial fragmentation upon death stimuli (Carmona-Gutierrez et al., 2010). The mitochondrial ADP/ATP carrier (AAC) and the vacuolar protease Pep4, the yeast orthologue of human cathepsin D, have also been linked to yeast apoptosis, enhancing mitochondrial degradation (Pereira et al., 2010).

As previously stated, proteins from the Bcl-2 family are essential as apoptosis regulators in mammalian cells. Until recently, obvious orthologues of these proteins have not been found in yeast, which made yeast the perfect model for their study upon heterologous expression, and for the elucidation of their involvement in apoptosis. For example, heterologous expression of the human pro-apoptotic protein Bax has been found to induce yeast cell death (Greenhalf, Stephan, & Chaudhuri, 1996), with impaired growth, accumulation of ROS, MOMP and cyt *c* release (Greenhalf et al., 1996; Priault, Camougrand, Kinnally, Vallette, & Manon, 2003). Other apoptosis markers, such as plasma membrane blebbing, phosphatidylserine exposure at the outer leaflet of the plasma membrane, chromatin condensation, DNA fragmentation, characteristic of apoptosis have been identified (Ligr et al., 1998). Also, a study using a patch clamping technique for analysis of a high conductance channel, in a Bax expressing strain, found a mitochondrial channel in the OMM with MAC characteristics, which would allow the release of the pro-apoptotic factors from mitochondria (Pavlov et al., 2001; Polcic, Jaka, & Mentel, 2015; Priault et al., 2003). These channels differed from the ones artificially made solely from Bax proteins in *in vitro* systems, a fact that would lead to believe the MAC channels require a component other than Bax, found in both yeast and mammalian mitochondria (Polcic et al., 2015). Still, further studies using the same system suggest that the cell death induced by Bax expression may not be yeast apoptosis, but instead, this protein activates autophagy that culminate in cell death (Kiššova et al., 2006).

In 2011, Büttner et al. found a yeast protein with a BH3 domain, the Ybh3, which has been shown to regulate apoptosis in yeast, more specifically the mitochondria mediated pathway of apoptosis. It was demonstrated that this protein, when overexpressed, sensitizes the cells to apoptotic stimuli, by

translocating to the mitochondria and disrupting mitochondrial membrane potential. It was also shown that this protein is inhibited by Bcl-X_L and that, once in the mitochondria, through interaction with Mir1p and Cor1p, it may physically interact with putative components of PTP, although this contact is not essential for cell death mediated by Ybh3p (Büttner et al., 2011).

Yeast apoptosis has been studied extensively since it was first described, and it has been shown to be induced by several factors, including oxygen stress (such as H_2O_2), valproic and acetic acid, hyperosmotic stress, arsenic, mRNA instability, loss of ubiquitination control, impaired DNA replication and chronological aging, among others (Carmona-Gutierrez et al., 2010).

1.4 Acetic acid as an inducer of yeast apoptosis

Acetic acid (CH_3COOH) is a byproduct of alcoholic fermentation carried by *S. cerevisiae*. It was previously described that in certain concentrations it can lead to an impaired fermentation. In most *S. cerevisiae* strains repression by glucose inhibits acetic acid metabolism. In this condition, at low pH, its non-dissociated form (pK_a = 4.75) enters the cell by simple diffusion and through the aquaglyceroporin channel Fps1, and its dissociation when inside may cause intracellular acidification, which in turn affects several pathways essential for the cell activity. However, in the presence of acetic acid, this channel is phosphorylated by Hog1 (high osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK)), leading to the translocation and degradation of this channel in the vacuole, as a way of protecting the cell from stress. The MAPK Slt2, a protein that is part of the PKC pathway, which has a role in maintaining cell wall integrity, is also activated by acetic acid stress. However, Fps1-facilitated entry of acetic acid inhibits the activation of Slt2 phosphorylation (Mollapour, Shepherd, & Piper, 2009). The effect acetic acid has in the cell depends on the concentration used, and can cause cell death either by necrosis, when at high concentrations, or with some of the characteristic features of mammalian apoptosis. Some of the cell alterations occurring during acetic acid-induced apoptotic cell death are exposure of phosphatidylserine in the outer leaflet of the cytoplasmic membrane, chromatin condensation and DNA fragmentation (P. Ludovico, Sousa, Silva, Leão, & Côrte-Real, 2001; M. J. Sousa, Ludovico, Rodrigues, Leão, & Côrte-Real, 2012). Also, this cell death process involved mitochondrial dysfunction including accumulation of reactive oxygen species (ROS), initial hyperpolarization followed by loss of mitochondrial membrane potential, impaired mitochondrial respiration and cyt *c* release (Paula Ludovico et al., 2002).

The yeast metacaspase, Yca1, is also activated by acetic acid stress, and this activation is dependent of culture growth phase, being maximal at the very beginning of exponential growth phase

and decreasing along growth (Pereira, Camougrand, Manon, Sousa, & Côrte-Real, 2007). Also, acetic acid-induced apoptosis can occur without cyt *c* release, though to a smaller extent (Guaragnella, Bobba, Passarella, Marra, & Giannattasio, 2010). The presence of an apoptosome is still unclear, as are any events downstream of cyt *c* release. Acetic acid-induced apoptosis also provokes the release of the other pro-apoptotic factors from the intermembrane space, such as Nuc1 and Aif1, which undergo translocation to the nucleus to exert their functions (Guaragnella et al., 2012; M. J. Sousa et al., 2012). Mitochondrial dynamics is also of great importance in acetic acid-induced cell death in yeast, as deletion of *DRP1* gene increases acetic acid resistance, and impairs mitochondrial fragmentation (Giannattasio, Guaragnella, Zdralević, & Marra, 2013; M. J. Sousa et al., 2012).

2. Mitochondrial Phospholipids

Lipids are a group of organic molecules which are hydrophobic, that is soluble in organic solvents but not in water. These compounds can be characterized according to their structure and functions, being divided into 8 classes, as established by the International Lipid Classification and Nomenclature Committee in 2005: fatty acids (FA), glycerolipids, glycerophospholipids, sphingolipids, prenol lipids, glycolipids, sterols and its derivatives, and polyketides (Fahy E, Subramaniam S, Brown HA, Glass CK, Merrill AH Jr, Murphy RC, Raetz CRH et al., 2005; Klug & Daum, 2014).

The endoplasmic reticulum, along with the Golgi apparatus and the mitochondria, are the main lipid synthesizing organelles. As some of the newly synthesized lipids are needed in other locations within the cell, the cell's phospholipid transport systems are also of major importance in lipid metabolism. In this context, mitochondria associated membranes (MAMs), an ER membrane sub fraction, play key functions in the synthesis of some phospholipids. Yeast, besides being used as a model for the study of programmed cell death, is also used as a research model for the elucidation of lipid metabolism, since apart from the more practical advantages already covered, the metabolic pathways are very well-conserved among all eukaryotes. (Daum, Lees, Bard, & Dickson, 1998; Klug & Daum, 2014; Santos & Riezman, 2012; Zhang et al., 2014)

2.1 Endoplasmic Reticulum - Mitochondrial (ER-M) contact sites: MAMs, ERMES and EMC

There are different optimal conditions for the several metabolic pathways within the cell, which evolutionarily shaped the organelles, each with a specific controlled milieu. Due to this

compartmentalization of the cell, a way of connecting the different organelles, such as vesicular trafficking, is needed for exchange of nutrients and metabolites. The connection between the ER and the mitochondria, however, does not use this form of communication, but rather a tether between their membranes that can be observed by electron microscopy, and is also evidenced in mitochondria isolates with ER membrane contamination. Tethering of the mitochondrial to the ER is achieved by several protein complexes and is present in about 5-20% of the mitochondrial surface. In this zone of close apposition, both organelles are within 30 nm of each other (De Vos et al., 2012; Kornmann, 2013; Michel & Kornmann, 2012; Raturi & Simmen, 2013). This zone of close apposition is controlled by proteins like PACS-2 in mammals, where the lack of this protein results in disruption of the tethering complexes and fragmentation of the mitochondria (Simmen et al., 2005).

There are several protein complexes that are involved in the interactions between ER and mitochondria in metazoans (Figure 6):

- In the ER membrane and the OMM there is a protein Mmr1, that can dimerize and anchor both organelles during budding, allowing proper mitochondrial inheritance after their delivery through Myo2, across the actin cables to the bud (Kornmann, 2013; McBride, 2011);
- The mitochondrial Fis1 binds to the ER protein Bap31, and the activation of this complex through the recruitment of procaspase-8 forming an ARCosome leads to the release of Ca^{2+}. This release amplifies the death signals, inducing apoptosis (Iwasawa, Mahul-Mellier, Datler, Pazarentzos, & Grimm, 2011; Kornmann, 2013; Vance, 2014);
- The ER protein VAPB interacts with the mitochondrial PTPIP51, a complex involved in the regulation of intracellular Ca^{2+} homeostasis (De Vos et al., 2012; Kornmann, 2013);
- The mitochondrial VDAC, a nonselective pore for ions and hydrophilic molecules, and the IP3R localized in ER membrane are bound by the chaperone Grp75. This chaperone also regulates Ca^{2+} flux between these organelles, by catalyzing its release from the ER by IP3R, and its transport through VDAC into the mitochondria (Decuypere et al., 2011; Hayashi, Rizzuto, Hajnoczky, & Su, 2009; Kornmann, 2013; Malhotra & Kaufman, 2011);
- In mammalian cells, the mitochondrial proteins mitofusin (Mfn) 1 and 2, bind to the ER Mfn2, linking the two organelles (Kornmann, 2013);
- The PACS-2 is also required for the close apposition of the organelles, possibly through links with IP3R (Simmen et al., 2005).

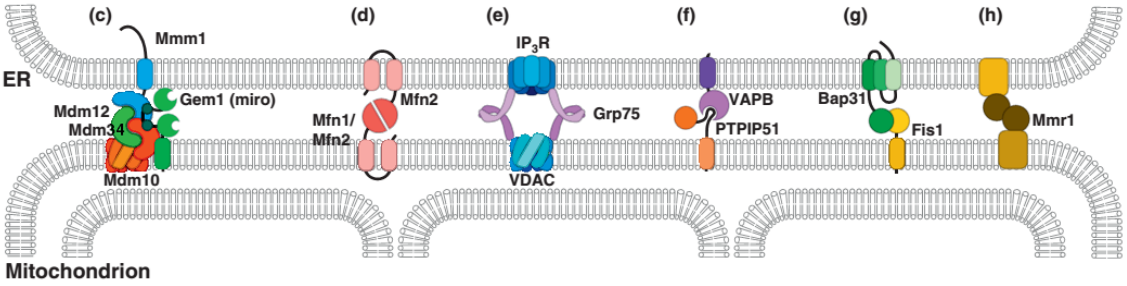


Figure 6 - Representation of the several protein interactions in MAMs in yeast (c) and metazoans ((d) to (h)) (Kornmann, 2013).

The contact between mitochondria and the endoplasmic reticulum has been associated with several functions. One of those functions is the involvement in mitochondrial dynamics and its inheritance. Studies showed that the mitochondrial fission events are in greater numbers in the sites where there is close apposition between the two organelles, possibly because ER plays a role in the constricting stages. Evidence of ER-determined mitochondria inheritance during division of yeast and mammalian cells was also provided. In yeasts, the ERMES complex may also be involved in replication of the mtDNA (mitochondrial DNA), as strains deficient in proteins from this complex showed loss of mtDNA (English & Voeltz, 2013; Kornmann, 2013; Michel & Kornmann, 2012; Otera & Mihara, 2012; Raturi & Simmen, 2013).

Facilitating calcium (Ca^{2+}) exchange is also a function of ER-mitochondria contact sites. This ion is essential in cell metabolism, being involved in several processes, such as regulation of cellular bioenergetics, intracellular signaling or motility. Ca^{2+} ions entering mitochondria through these ER-M contact sites serve various proposes, acting as co-factors for enzymes of the citric acid cycle in the mitochondrial matrix and other proteins related to the mitochondrial division and motility. In addition, high Ca^{2+} concentrations in the mitochondrial matrix enhances the onset of apoptosis, by activation of the PTP, and possibly amplifying pro-apoptotic factors leakage to the cytosol (English & Voeltz, 2013; Kornmann, 2013; Malhotra & Kaufman, 2011; Michel & Kornmann, 2012).

As previously described, the chaperone protein Grp75 binds to the IP3R in the ER membrane, and to the VDAC channel in the OMM, so that both proteins are "mouth-to-mouth". This way, when the IP3R is activated by a physiological stimulus, the Ca^{2+} transported to the cytosol flows directly through the channel into the mitochondrial intermembrane space, and then through the mitochondrial calcium uniporter (MCU) to enter the matrix. The release of this ion from the ER is regulated by several

mechanisms, namely by the cytosolic calcium concentration: low or very high Ca^{2+} concentrations inhibit the IP3R, emphasizing the control of the ER-M contact sites in the calcium homeostasis (Decuypere et al., 2011; Kornmann, 2013; Raturi & Simmen, 2013).

One of the first functions described for the connections between ER and mitochondria was its role in lipid metabolism, more precisely in its synthesis. ER-M contact sites are enriched with both enzymes involved in lipid biosynthesis and in proteins responsible for its transport between the membranes of the two organelles. This allows the use of these proteins as reliable marker proteins, such as fatty acid coenzyme A (CoA) ligase 4 (Facl4), an enzyme that mediates the catalysis of the bond between fatty acids and CoA for triacylglycerol synthesis (Kornmann, 2013; Osman, Voelker, & Langer, 2011; Raturi & Simmen, 2013).

In yeast, the best characterized tethering complex is ERMES. The ERMES complex includes 4 different proteins: Mmm1, an ER Protein, Mdm12, a cytosolic protein, and Mdm34 and Mdm10, both mitochondrial proteins (Figure 7). This complex has an array of functions, from aiding the lipid exchange, regulation of mtDNA replication and assisting mitochondrial protein import (Kornmann, 2013; Lahiri et al., 2014; Michel & Kornmann, 2012).

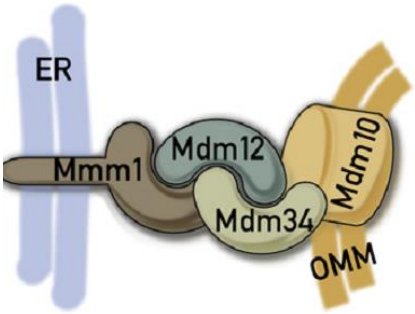


Figure 7 - The ERMES complex at the ER–mitochondria connection (Michel & Kornmann, 2012)

The wide range of processes in which this complex is involved implies the presence of regulation of some sort for it to respond correctly to the right stimuli. During the purification of this complex, a new protein was discovered, identified later as the Miro GTPase Gem1. Gem1 has two GTPase domains, being suggested that they have different roles, the first domain focusing in localization, and the second regulating the ERMES, specifically in the lipid exchange and synthesis (Michel & Kornmann, 2012).

Until recently, ERMES was the only characterized tethering complex between ER and the mitochondria in yeast, but the deletion of this complex only reduced the phospholipid exchange present in the mutants, and the transfer of PS to the mitochondria was not impaired (Lahiri et al., 2014; Zinser et al., 1991). These studies suggested that another complex would exist tethering the two organelles, since the proximity between them seems vital to the cell.

In 2014, Lahiri et. al showed a relation between the Endoplasmic Reticulum Membrane Complex (EMC) and Cho2, responsible by transport of PE from the mitochondria to the ER. The yeast EMC is formed of 6 proteins (EMC1-6), and is involved in ER-associated degradation (Wideman, 2015). The

genetic analysis of the cluster uncovered a possible function associated with mitochondria and lipid metabolism. The authors also tested PS transfer from ER to mitochondria in mutants without EMC proteins 1/2/3/5 and 6, which showed a decreased transport (about 50%), and found that the mitochondria in these mutants are abnormal, showing a 50% decrease in PE levels, an increase of PA and CL, and as it was previously demonstrated for cells with *MMM1* deletion, these cells cannot grow in nonfermentable carbon sources. Ultimately the same group showed that cells missing either EMC or ERMES complex display decreased ER-mitochondrial tethering, and that cells missing both EMC and Mmm1p were not viable (Lahiri et al., 2014).

2.2 Yeast Phospholipid Metabolism

Although there is not one molecular monomer for lipids, fatty acids (FA) are basic structures common to the majority of complex lipids. They are composed of a carboxyl group attached to a linear hydrocarbon chain, which can vary in length and saturation. Usually used as energy storage, they can also regulate transcription, be part of the signaling pathways, or be involved in the synthesis of several other lipids species, such as membrane lipids. This great demand calls for several ways for the cell to produce them, either by *de novo* synthesis which takes place in the cytosol and mitochondria, with consequent elongation in the ER; by uptake from the outside of the cell; or by hydrolysis of complex lipids within the cell. The FA species present in yeast vary according to the growth conditions and the subcellular membrane of focus (Daum et al., 1998; Klug & Daum, 2014). Due to their toxicity at high concentrations, they are stored in lipid droplets in the cytosol, and are rapidly consumed in phospholipid formation, explaining why there is always a steady state low concentration of this lipids (Cosentino & García-Sáez, 2014).

Glycerophospholipids, or as they are most commonly called phospholipids, are formed by a polar hydrophilic head and a hydrophobic tail which provide an amphipathic character and help the formation of the characteristic bilayer composition of most cell membranes. The tail is composed of a diacylglycerol (DG) backbone, formed by a glycerol molecule to which fatty acids link through ester bonds in the sn1 and sn2 carbons. The glycerol sn3 carbon is where the head forms a bond through a phosphate group, which links with an alcohol. The most common head groups, which separate phospholipids in several classes, are serine, ethanolamine, choline, glycerol or inositol, and form respectively phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylinositol (PI). Also, phospholipids differ according to the saturation of the fatty acid chain (Figure 8) (Daum et al., 1999; Horvath, Wagner, Steyrer, & Daum,

2011; Klug & Daum, 2014). The phospholipid membrane composition depends on the organelle, and is different in both inner and outer layer of the membrane, these phospholipid specificity having an important function in membrane structure (Horvath et al., 2011; Klug & Daum, 2014).

2.2.1 Phospholipid biosynthesis in *S. cerevisiae*

The central metabolite of the biosynthesis pathways for the formation of the several phospholipid classes is phosphatidic acid (PA). This lipid is the divergence point of two major pathways of lipid biosynthesis: first, its cleavage into DG allows its entry into the storage pathway, where it is transformed in triglycerides (TG) and is then stored in lipid droplets; secondly, its conversion to cytidine diphosphate-diacylglycerol (CDP-DG), by Cds1 in the ER and Tam41 in the mitochondria, contributes to the formation of most phospholipids as their precursor. From this precursor, all other phospholipids are formed, by either the CDP-DG pathway or the Kennedy pathway (Horvath et al., 2011; Klug & Daum, 2014; Zhang et al., 2014).

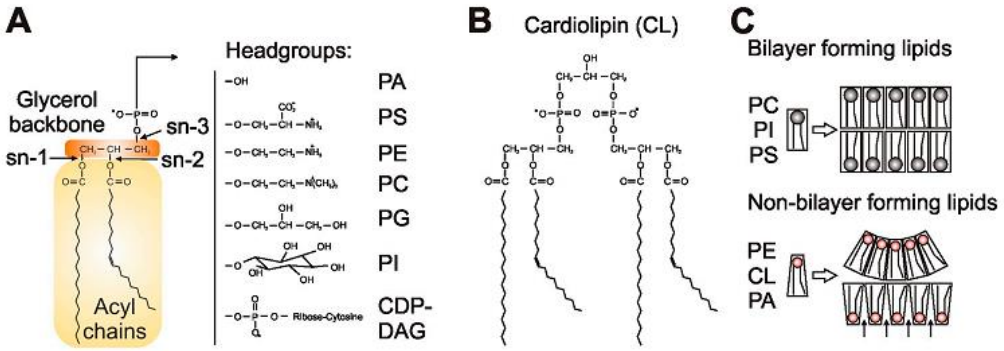


Figure 8 - Phospholipids in mitochondrial membranes. (A) The central structural element of phospholipids is a glycerol backbone with different hydrophilic head groups (B) CL structure (C) Bilayer and non-bilayer phospholipids have different shapes. (Osman et al., 2011)

2.2.2 The Kennedy pathway

The Kennedy pathway is one of the ways to produce PC and PE, two of the most abundant phospholipids in yeast. It occurs in the ER, and the *de novo* synthesis happens through a series of reactions catalyzed by enzymes that are significantly conserved on eukaryotes (Figure 9) (Gibellini & Smith, 2010; Zhang et al., 2014).

The first step consists of a phosphorylation of choline and ethanolamine transported from external sources through Hnm1 (a plasma membrane transporter), by ATP-dependent choline and ethanolamine kinases (CKi1 and EKi1, respectively), which form choline-phosphate (Cho-P) and ethanolamine-phosphate (Etn-P). These enzymes differ in specificity, since CKi1 can use both substrates (although

choline preferably), where EKi1 can only use ethanolamine (Gibellini & Smith, 2010; Klug & Daum, 2014; Zhang et al., 2014). Afterwards, cytidylyltransferases specific to both Cho-P and Etn-P (PCT1 and ECT, respectively) combine CTP to form CDP-choline and CDP-ethanolamine. These enzymes are considered rate limiting, and thus control PC and PE synthesis through this pathway.

The final step of this pathway involves the ER-enzymes DAG-cholinephosphotransferase (Cpt1) and DAG-ethanolaminephosphotransferase (Ept1), which catalyze the transfer of head groups choline-phosphate and ethanolamine-phosphate to DAG, produced by the dephosphorylation of PA by Pah1, forming PC and PE. PC can be further transformed in PS in the ER through the enzymes Pss1 and Pss2 (phosphatidylserine synthase) (Gibellini & Smith, 2010; Klug & Daum, 2014; Zhang et al., 2014).

2.2.3 The CDP-DG pathway

The CDP-DG pathway is the other branch of phospholipid biosynthesis, by which PI, PS, PG, and consequently CL, PE and PC, are formed, in a pathway that includes both the ER and mitochondria (Figure 9). The CDP-DG present at the ER membrane can be transformed into PI through the ER protein phosphatidylinositol synthase (Pis1) that places an inositol group where the cytidine monophosphate (CMP) is inserted. In the mitochondria, CDP-DG is transformed into PGP, through the phosphatidylglycerophosphate synthase (Pgs1), which is in turn transformed in PG by the phosphatidylglycerophosphate phosphatase, Gep4. The phospholipid PG can be further transformed into cardiolipin through a process described further in this introduction (Klug & Daum, 2014; Zhang et al., 2014).

The same precursor (CDP-DG) also allows the formation of PS, through a reaction catalyzed by the ER enzyme PS synthase Cho1, which replaces the CMP with a serine group. This phospholipid is then translocated to the mitochondrial inner membrane, where it suffers decarboxylation into PE in a reaction catalyzed by Psd1 or Psd2 (Flis & Daum, 2013; Klug & Daum, 2014; Tatsuta, Scharwey, & Langer, 2014; Zhang et al., 2014). PE is essential for mitochondrial function, due to its capacity to generate negative membrane curvature and form nonbilayer structures (Aguilar et al., 1999; Osman et al., 2011).

For the formation of PC, the transport of PE back to the ER is required, and the PE head group undergoes sequential methylations, using methyl groups from S-adenosylmethionine (SAM). The first reaction is catalyzed by phosphatidylethanolamine methyltransferase Cho2 and the other two by the phospholipid methyltransferase, Opi3. As the enzymes required for this pathway are intercalated between the two membranes, the flipping the phospholipids back and forth needs to be assured, not

only to obtain the final products, but also to disperse these important phospholipids throughout the various cell membranes (Gaynor et al., 1991; Klug & Daum, 2014; Osman et al., 2011; Zhang et al., 2014).

Evidence was found in yeast that this phospholipid exchange between the two membranes happens mostly in the ER-mitochondria contact sites, due to the reduced phospholipid transport present in strains with deletions in proteins of the tethering complexes already described (Kornmann, 2013; Osman et al., 2011; Prinz, 2010; Tamura, Sesaki, & Endo, 2014; Tatsuta et al., 2014). In mammals, the levels of these enzymes are highly controlled by other proteins, such as PACS-2, that mediates the membrane associated quantities of Facl4 and Pss1 (Simmen et al., 2005).

2.2.4 Cardiolipin synthesis and remodeling

Cardiolipin (CL), is a phospholipid that makes up to 20% of the mitochondrial membranes, mostly present in the IMM, with a role in the stability and activity of some of IMM proteins, and involved in apoptosis. The cascade of enzymes required for its synthesis is found in the IMM, but the precursor of this lipid, phosphatidic acid (PA), can be found either in the ER membrane, or, to a lesser extent, be synthesized *de novo* in mitochondria by phospholipases, as already described (Figure 9) (Ascenzi et al., 2011; Osman et al., 2011).

Synthesis of CL is catalyzed by the Crd1, the cardiolipin synthase, that uses PG and CDP-DG as substrates. Then, CL enters a cycle of remodeling, but the pathways involved in mammalians are still rather unclear, and the proteins involved still unknown. Firstly, CL is remodeled into monolysocardiolipin (MLCL), by Cld1 through removal of an acyl chain, and MLCL in again transformed in CL through a transacylation reaction, where Taz1 transfers an acyl chain from another phospholipid (Baile, Lu, & Claypool, 2014; Baile, Sathappa, et al., 2014; Klug & Daum, 2014; Zhang et al., 2014). Although this mechanism is observed in both eukaryotes and prokaryotes, remodeled and unremodeled CL appear to be indistinguishable in yeast, and remodeling may only be a repair mechanism of CL acyl chain damages caused by reaction with ROS (Baile, Sathappa, et al., 2014).

Cardiolipin, found predominantly in the mitochondria, is, as mentioned above, important for several processes within this organelle. The presence of this cross-linked dimer in the IMM acts as an integrity stabilizer, which helps protecting the membrane from damage. This stabilization is essential for the activity of the respiratory complexes III and IV of the respiratory chain and also of the AAC (ATP/ADP carrier) (Houtkooper & Vaz, 2008; Osman et al., 2011).

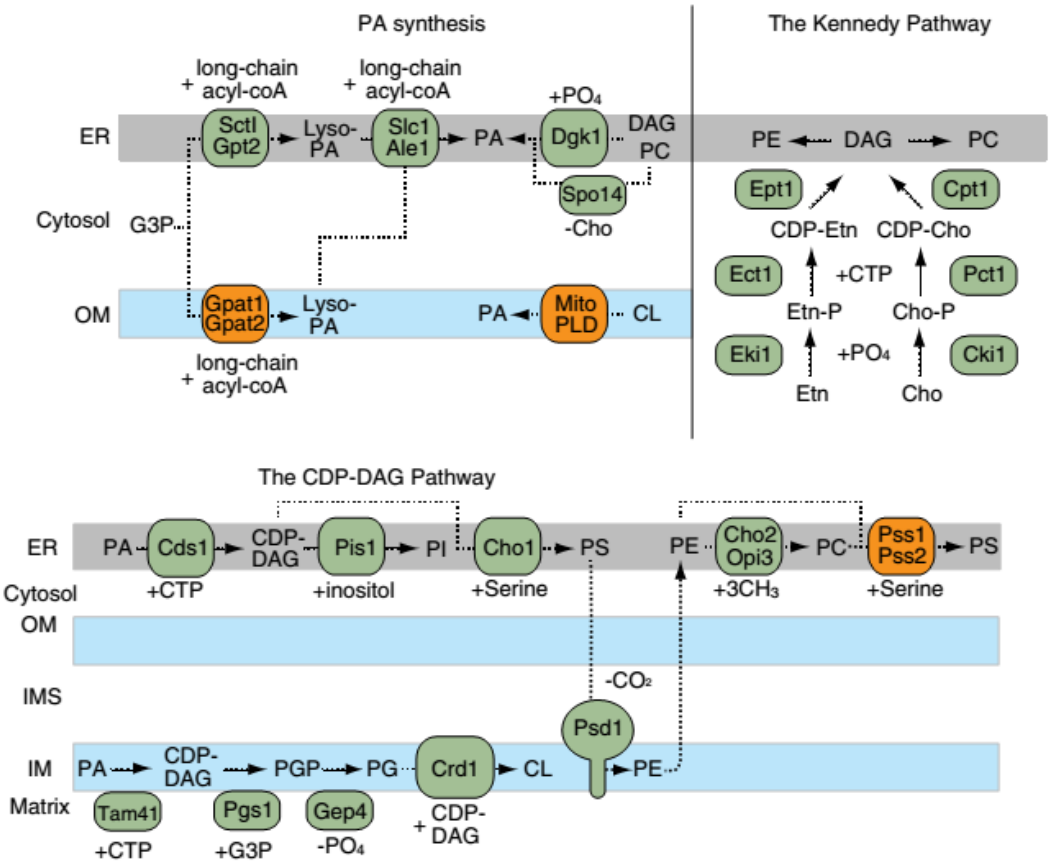


Figure 9 - Biosynthetic pathways for mitochondrial phospholipids. The major precursor PA is synthesized in the ER and mitochondrial outer membranes. The CDP-DAG pathway is located in the ER and mitochondrial inner membranes and produces a variety of phospholipids including PI, PS, PE, PC and CL. The ER-located Kennedy pathway generates PE and PC (Zhang et al., 2014).

This important mitochondrial phospholipid also has a role in protein import from the cytosol into the mitochondrial matrix. TIM (translocase of the inner mitochondrial membrane) and TOM (translocase of the outer mitochondrial membrane) are the complexes responsible for the protein transport, and studies show that cells lacking the proteins responsible for the CL synthesis have an impaired protein import into mitochondria (Osman et al., 2011). It is also believed that CL is involved in regulation of fusion/fission of mitochondria. This phospholipid binds to the positively charged lysines in inter-mitochondrial membranes Opa1 and its yeast orthologue Mgm1, stimulating its fusion activity. Also, translocation of Opa1/Mgm1 to the IMM is dependent in cardiolipin concentration (Gonzalvez & Gottlieb, 2007; Houtkooper & Vaz, 2008; Zhang et al., 2014).

2.3 Phospholipids during apoptosis: role and alterations

Some of the events that occur during apoptosis lead to changes in lipid composition, of both the plasma and intracellular membranes. One of the phospholipids identified as having a role in this form of

cell death is PS, since, as already described in this introduction, its externalization to the outer leaflet of the plasmatic membrane is the signal for clearance by the macrophages (Fadok & Henson, 2003). In the mitochondrial membrane other alterations regarding the oxidation of the phospholipids or of their ratio among the two mitochondrial membranes may be at play. MOMP, as already described, may be aided by these alterations, since changes in membrane organization facilitate the formation of the pores through which the pro-apoptotic proteins may be released. Peroxidation of mitochondrial phospholipids by ROS may even change the favored curvatures of certain phospholipids, such as PA. Also, the increase of certain phospholipid species, namely LPC and DG, which favor opposite membrane curvatures, is observed during apoptosis (Cosentino & García-Sáez, 2014; Crimi & Esposti, 2011). The release of Ca^{2+} by the ER, and the consequent increased concentration in ER-M contact sites will also aid converting the bilayered disposition into hexagonal phases, due to the conical shape of CL and PE (Cosentino & García-Sáez, 2014; Crimi & Esposti, 2011; Flis & Daum, 2013; Osman et al., 2011).

Cardiolipin has an important role in different stages of apoptosis. Firstly, this lipid can activate the Fas receptor in the plasma membrane, thus being capable of inducing apoptosis. Also, as previously stated, cyt *c* is linked to CL in the IMM, and when the degree of unsaturation of the CL acyl chains is high, cyt *c* is more susceptible to undergo a conformational change and act as a CL-specific peroxidase. The modified cyt *c*, along with the elevated ROS levels, will result in CL oxidation, which leads to a reorganization of the lipids in microdomains with hexagonal HII configuration, with a core of polar phospholipid heads (Firsov, Kotova, Korepanova, Osipov, & Antonenko, 2015; Montero et al., 2010; Osman et al., 2011; Unsay, Cosentino, Subburaj, & García-Sáez, 2013). This alteration in CL structure causes an alteration of the curvature of the membrane. This may be involved in the increase of specificity in the recruitment of truncated-Bid to the inner and outer mitochondrial membranes, in the efficiency of membrane insertion and oligomerization of Bax, and in the formation of MAC (Gonzalvez & Gottlieb, 2007; Hollville & Martin, 2012; Kagan et al., 2005; Lutter et al., 2000; Montero et al., 2010; Osman et al., 2011; Raemy & Martinou, 2014). Also, cyt *c* itself may be able to cause disorganization of the OMM and cause MOMP, but only upon translocation of CL to the OMM (Xu, Vanderlick, & Beales, 2013).

3. Main aims and study overview

In 2013, Sousa et al. performed a genome-wide screening in *S. cerevisiae* to uncover genes involved in the regulation of apoptosis induced by acetic acid. Besides reinforcing the exploitation of

yeast as a model to study apoptosis, and the use of acetic acid as a cell death inducing agent, since it identified the involvement of several genes expressing recognized pro- and anti-apoptotic proteins, it also revealed the important role of the mitochondria in acetic acid induced-apoptosis, as mitochondrial function was the category depicting more genes which deletion resulted in altered cell responses to this cell death inducer (M. J. M. Sousa et al., 2013). Among the strains identified in the screening as more resistant to acetic acid induced cell death and that grouped under the term "mitochondrial function", were mutants deleted in genes coding for several OMM proteins, and specifically *Δmdm10*. Since, as referred above, permeabilization of the OMM is responsible for the release of several pro-apoptotic factors that lead to the commitment of the cell to apoptosis, we aimed to study the potential involvement of ERMES components in the regulation of OMM permeabilization. Some of the proteins identified were also involved in phospholipid metabolism, and due to the role of ERMES in phospholipid metabolism, the aim was to further comprehend the importance of phospholipids in acetic acid induced apoptosis.

Mutant strains from the Euroscarf knockout collection *Δmdm10* and *Δmdm12,* deleted in ERMES complex components, were selected to study a possible role in MOMP, by either direct regulation or by alteration of the mitochondrial phospholipid profile. Also to more directly study the potential involvement of phospholipids in acetic acid induced cell death, *Δcrd1, Δtaz1, Δups1* and *Δcho1*, key proteins of mitochondrial phospholipid biosynthesis, were studied. Cell death assays, revealed that all mutants, but specially *Δtaz1* and *Δmdm10,* were resistant to 120 mM acetic acid treatment. Also, assessment of plasma membrane integrity by propidium iodide staining showed that all strains displayed a low percentage of stained cells. Mitochondrial alterations in response to acetic acid-induced cell death were also evaluated, namely, ROS accumulation, mitochondrial membrane potential and mitochondrial morphology and degradation. These analysis revealed that all mutants displayed alterations in mitochondrial response during acetic acid induced apoptosis, although with differences among them (Fernandes, 2013).

Following these results, the present study, aimed to access cyt *c* release in the selected strains, using techniques previously established in the lab.

Since we wanted to verify if the effect in the phenotype caused by the deletion of the genes under study could be due to an altered phospholipidic composition of the mitochondrial membrane, other of our aims was to evaluate the mitochondrial phospholipidic content of the mutants used in this work, before and after acetic acid treatment.

Furthermore, knowing that ER-M contact sites have a role in mammalian apoptosis, we also aimed to uncover if disturbance of these structures could be involved in Bax mediated cell death. For such we expressed an active and an inactive form of this protein in yeast strains deficient in each protein from ERMES complex (*Δmdm10, Δmdm12, Δmdm34* and *Δmmm1)* and assessed cell death induced by Bax expression.

Chapter II - Materials and Methods

1. Assessment of cyt c release during acetic acid-induced cell death in the strains *Δcrd1, Δtaz1, Δups1, Δmdm10, Δmdm12* and *Δcho1*

1.1 Yeast strains

All the strains used in this study belong to the EUROSCARF knockout mutant collection (EUROSCARF, Frankfurt, Germany). The wild-type strain of *Saccharomyces cerevisiae* used was BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), alongside with deletion strains *Δcrd1*, *Δtaz1*, *Δups1*, *Δmdm10*, *Δmdm12* and *Δcho1*.

1.2 Culture Media and Growth Conditions

All strains were maintained in rich medium (YEPD; 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% agar) plates. For growth in liquid medium, the strains were grown in YPGal (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) galactose), YEPD (YEPD; 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) or YPLac (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) lactate) in aerobic conditions, and incubated overnight at 30 ˚C and 200 rpm.

1.3 Medium Selection by Western Blot analysis

For medium selection for posterior analysis, all the strains were grown in YPGal, YEPD and YPLac in aerobic conditions, and incubated overnight at 30 ˚C and 200 rpm. The cells were collected by centrifugation (1 mL at an optical density (O.D.) at 640nm of 1) and washed with deionized water. Then, they were disrupted using a Lysis Buffer (3.5% β-mercaptoethanol in 2 M NaOH) and incubated for 15 minutes in ice. The proteins present in the lysate were precipitated adding 50% trichloroacetic acid (TCA) and incubated another 15 minutes on ice. After centrifugation and resuspension in SDS 2%, samples were heated at 95 °C for 5 minutes. They were then separated by SDS-PAGE, on a 15% SDS polyacrylamide gel at 25 mA, and transferred to a HybondP Polyvinylidene difluoride membrane (PVDF; GE Healthcare) at 60 mA for 90 minutes. The membrane was blocked with 5% (w/v) non-fat dry milk for one hour, cut into strips and incubated with the primary antibodies overnight at 4 ˚C: mouse monoclonal anti-yeast phosphoglycerate kinase (PGK1) antibody (1:5000, Molecular Probes) for total protein control and mouse monoclonal anti-yeast porin (Por1) antibody as a marker of mitochondrial fraction (1:5000, Molecular Probes). Afterwards, the strips were washed with PBS-T and incubated with

the secondary antibodies against mouse (1:5000; Sigma-Aldrich). The bands were revealed by chemiluminescence (Immobilon, Millipore) in a ChemiDoc™ XRS.

1.4 Growth curves and cell death assays

Growth of the wild-type and mutant strains in YPGal –at a $O.D._{640nm}$ of 0.5, was monitored by $O.D._{640nm}$ measurements performed periodically. The specific growth rate was estimated from the slope of the linear regression of the natural logarithm of the $O.D._{640nm}$ values correspondent to the exponential phase as a function of time.

For the cell death assays with acetic acid, all strains were grown overnight in YPGal in aerobic conditions, at 30 ˚C and 200 rpm, until an $O.D._{640nm}$ of 0.8. The cells were then collected by centrifugation, resuspended in YPGal at pH 3.0 (set with HCl) to a final $O.D._{640nm}$ of 0.7, and treated with 120 mM of acetic acid at 30 ˚C. Samples were then collected at different time points (0, 20, 40, 60, 90 and 120 minutes) and went through 4 serial dilutions of 1:10 with sterile water. Five drops of 30 µL from the last dilution were spotted in YEPD plates, which were then incubated at 30 ˚C. Colony forming units were counted 48 hours after, and cell viability was calculated considering time zero as 100%.

1.5 Assessment of total cytochrome *c* content

Cytochrome *c* cell content was assessed in total protein extracts prepared from 1 mL of culture at $O.D._{640nm}$ 1. The proteins of the extracts were separated by SDS-PAGE, transferred to a PVDF membrane and blocked for an hour, as previously described. After, the membrane was incubated with the primary antibodies overnight at 4˚C: rabbit polyclonal anti-yeast cytochrome *c* (CYC1) antibody (1:2500 custom-made by Millegen), mouse monoclonal anti-yeast porin (POR1) antibody for mitochondrial fraction control (1:5000, Molecular Probes) and mouse monoclonal anti-yeast phosphoglycerate kinase (PGK1) antibody (1:5000, Molecular Probes) for cytosolic fraction control. Afterwards, the strips were incubated with the secondary antibodies against mouse (1:5000; Sigma-Aldrich) or rabbit IgG-peroxidase (1:5000; Sigma-Aldrich). The bands were revealed by chemiluminescence (Immobilon, Millipore) in a ChemiDoc™ XRS.

1.6 Mitochondria isolation

For mitochondria isolation, cells were grown in 3 L of YPGal until an $O.D._{640nm}$ of 1.5 to 1.8. Half the culture was then collected to use as control (t0), and the rest was treated with 120 mM of acetic acid at

pH 3.0 (adjusted with HCl) then incubated for 90 minutes at 30 ˚C and 200 rpm. The control cells were washed with deionized water, resuspended in a Suspension Buffer containing 1.2 M sorbitol, 60 mM sodium phosphate pH 7.5 and 1 mM ethylenediaminetetraacetic acid (EDTA), according to the wet weight. The cells were converted to spheroplasts through enzymatic digestion with zymolyase (Zymolyase® 20T from GRiSP) (20 mg for 10 g of wet weight) and 1% β-mercaptoethanol, with incubation for 40 minutes in a 32 ˚C bath.

From our experience we know that cells exposed to acetic acid strength their cell wall rendering the cell wall digestion more difficult, which also depends on the strain genetic background. We had therefore to optimize the cell wall digestion of treated cells, and for that we used the wild-type strain BY4741 after 90 minutes of treatment (t90). Before digestion cells were resuspended in a pre-incubation buffer (0.5 M β-mercaptoethanol; 0.1 M Tris pH 7.0) to reduce the disulfide bonds and facilitate digestion of the wall, and incubated at 30 ˚C for 15 minutes. Cells were then washed twice with a 10 mM Tris/0.5M KCl buffer at pH 7, before being resuspended in the Suspension Buffer, and subjected to digestion with zymolyase. For the same quantity of zymolyase (100 mg for 10 g wet weight) and 1% β-mercaptoethanol, incubation for 40 minutes and two different temperatures (32˚C or 45˚C) and, for each temperature, two different pHs (without and with adjustment, around pH 6.5 and at 7.5, respectively) were tested. Cell wal digestion was monitored by phase contrast microscopy, through evaluation of the consequent loss of refringency. These four samples were then processed as described below.

The spheroplasts of both the control and treated cells were then washed twice with cold 1.2 M Sorbitol. From this point on, all the steps were performed on ice. The spheroplasts were ressuspended in a Lysis buffer, containing 0.5 M sorbitol, 20 mM Tris pH 7.5 and 1 mM EDTA. The concentration of sorbitol induces an osmotic shock that, along with hand-potter homogenization, caused the rupture of the plasma membrane with preservation of mitochondrial membrane integrity. After several differential centrifugations both the mitochondrial and post-mitochondrial fractions were stored at -80 ˚C.

1.7 Assessment of cyt *c* release upon treatment with acetic acid

The release of cytochrome *c* in cells undergoing acetic acid-induced apoptosis was evaluated by western blot analysis of the mitochondrial fraction and post-mitochondrial fraction obtained after mitochondrial isolation. Firstly, the protein quantification of each fraction was performed using the Bradford method with bovine serum albumin (BSA) as a standard. From the fractions, a certain amount of protein (75 μg for the mitochondrial fraction and 150 μg for the post-mitochondrial fraction) was

precipitated with 50% TCA and incubated in ice for 15 minutes. After being ressuspended in SDS 2% and heated at 95 °C for 5 minutes, the samples were separated on SDS-PAGE, and transferred to a PVDF membrane and blocked for an hour. After, the membrane was incubated with both primary (anti-yeast Por1, PGK and cyt *c*) and secondary antibodies as previously described. The bands were revealed by chemiluminescence (Immobilon, Millipore) in a ChemiDoc™ XRS.

1.8 Assessment of cell wall resistance

Cell wall resistance was assessed through enzymatic digestion. Cells were cultivated in YPGal and then collected, washed and resuspended in PBS to an $O.D._{640nm}$ of 0.5. Then, after the addition of zymolyase to a final concentration of 20 µg/mL, cells were incubated at 30 ˚C with 200 rpm agitation, and the $O.D._{800nm}$ was measured periodically. Absorbance at time zero was considered 100%.

2. Assessment of alterations in the mitochondiral phospholipid composition of *Δcrd1, Δtaz1, Δups1, Δmdm10, Δmdm12* and *Δcho1* strains with and without acetic acid treatment

2.1 Yeast strains

Both the wild type and all the strains used in the previous section were analysed for changes in mitochondrial phospholipid composition.

2.2 Mitochondria isolation

For mitochondria fractioning, cells were grown in 1.5 L of YPGal and 2 L of YEPD until an $O.D._{640nm}$ of 1.5 to 1.8. The cells from strains BY4741, Δ*crd1*, Δ*taz1*, Δ*ups1*, Δ*mdm10*, Δ*mdm12* and Δ*cho1,* grown in YPGal underwent the same protocol as the control culture used for cytochrome *c* evaluation. From the strains BY4741, Δ*crd1*, Δ*taz1*, Δ*ups1*, Δ*mdm10* and Δ*mdm12*, grown in YEPD, the mitochondria were isolated using the same protocol as above, without using the pre-incubation step, but instead incubating the cells with zymolyase (for 10 g humid pellet weight was added 20 mg for untreated cells and 1000mg for treated cells) and 1% β-mercaptoethanol, with incubation for 40 minutes in a 32 ˚C bath. To evaluate the purity of the mitochondria pellet and discard possible ER membrane contamination, wild-type and Δ*crd1* strains were further purified with a sucrose gradient to

compare with the same strains without gradient purification. This purification consisted in the resuspension of the mitochondrial pellet from the last step of the isolation procedure in SEM Buffer (10 mM MOPS/KOH (pH 7.2), 250 mM sucrose, 1 mM EDTA) and application of this suspension on top of a sucrose gradient (60% (w/v), 32% (w/v), 23% (w/v) and 15% (w/v)). Then, the tubes were centrifuged at 33000 rpm for one hour at 4 ˚C which caused the mitochondria to locate in the interface between the 60% and 32% concentrations. The layers below 32% above the mitochondria were then carefully removed, and the mitochondria were collected, resuspended in SEM Buffer and centrifuged again at 31000 rpm for 30 minutes at 4 ˚C. The supernatant was discarded and mitochondria were stored at -80 ˚C for later analysis.

2.3 Lipid extraction

For the phospholipid analysis, the lipid fraction was extracted from the mitochondrial fraction (containing at least 4 µg of protein) using the Bligh & Dyer method. The sample was resuspended in 1 mL of water, transferred to refrigerated glass tubes, added 3.75 mL $CHCl_3$:MeOH (1:2 (v/v)) and after vortexing, incubated in ice for 30 minutes. After another 30 seconds vortexing period, 1.25 mL $CHCl_3$ and 1.25 mL water were added, vortexing after each step. Centrifugation for 5 minutes at 1000 rpm followed, in order to separate the aqueous and organic phase, being the last collected to another glass tube. 1.88 mL of $CHCl_3$ were added to the remaining aqueous phase and new vortexing cycle and new centrifugation allowed to recover the remaining lipids and add to the previous organic phase sample. $CHCl_3$ was then evaporated from organic phase through nitrogen stream. To transfer to a smaller tube for storage at 20˚C, the sample was resuspended in a small amount of $CHCl_3$, vortexed, transferred and again evaporated.

2.4 Phospholipid quantification

Phosphorus measurement was performed to determine the amount of phospholipids in each sample. After the addition of 125 µL of 70% perchloric acid and heating at 170 ˚C for 40-60 minutes, 2.5% $NaMoO_4.H_2O$ and 10% ascorbic acid were added to the samples and to the standards of several concentrations of NaH_2PO_4. Then, they were incubated for 10 minutes in boiling water, and subsequently cooled in cold water. The absorbance of the samples and standards was then measured at 797 nm, and through the construction of a calibration curve, the concentration of phospholipids was calculated.

2.5 Separation and quantification of phospholipid classes

For the separation of the several classes of phospholipids of the different samples, Thin-Layer Chromatography (TLC) was used. The TLC silica plate was pre-washed with chloroform/methanol (1:1, v/v), and after drying, sprinkled with boric acid 2.3%. After drying for 5-10 minutes, the plate is subjected to the temperature of 100 ˚C for 30 minutes. Once cooled, samples of about 30 µg (resuspended in $CHCl_3$) and standards were applied, and the plate was placed, samples down, in a glass chamber saturated with a solution of chloroform/ethanol/water/triethylamine (30:35:7:35 v/v). The plate was removed once the migration front was about 1 cm below the top of the plate, which took about 3-4 hours, and then was left to dry in the fume hood. Afterwards, the plate was sprinkled with primuline (50 µg/ml) acetone/water (80:20), and revealed under UV light. Each band was scraped from the plate to a glass tube. Subsequently a phosphorus measurement was performed, in order to quantify the amount of phospholipids in each band, through the same method previously described, with the small difference of an extra centrifugation right before the $O.D._{797nm}$ measurement in order to pellet the silica.

2.6 LC-MS characterization of cardiolipin and phosphatidylglycerol species in BY4741 and *Δcrd1* strains

To evaluate the presence of cardiolipin and PG from the BY and *Δcrd1* (treated and un-treated) mitochondrial lipids extracts prepared as previously described, a sample of 25 µg was analyzed by LC-MS (liquid chromatography - mass spectrometry). The characterization of individual molecular species of these phospholipids was achieved by mass spectrometry (MS), using a linear ion trap (LXQ; Thermo Finnigan, San Jose, CA, USA) mass spectrometer after separation by liquid chromatography (LC). HPLC system (Waters Alliance 2690) was used with an Ascentis®Si column (15 cm × 1 mm, 3 µm) and a precolumn split (Acurate, LC Packings, USA) in order to obtain a flow rate of 40 $\mu L.min^{-1}$. The solvent system consisted of two mobile phases as follows: mobile phase A (acetonitrile:methanol:water; 50:25:25 (v/v/v) with 2.5 mM ammonium acetate) and mobile phase B (acetonitrile:methanol 60:40 (v/v) with 2.5 mM ammonium acetate). Initially, 0% of mobile phase A was held isocratically for 8 minutes, followed by linear increase to 60% of phase A within 7 min and maintained for 22 minutes. The LXQ was operated in the negative mode (electrospray voltage − 4.7 kV), the capillary temperature was 275 ˚C and the sheath gas flow was 8 U. Data acquisition was carried out on Xcalibur data system (V2.0).

3. Assessment of the cell survival in the strains *Δups1, Δmdm10, Δmdm12, Δmdm34* and *Δmmm1* upon expression of human Bax α and Bax P168A

3.1 Yeast strains and plasmids

The strains used for this study belong to the EUROSCARF knockout mutant collection (EUROSCARF, Frankfurt, Germany). The wild-type strain of *Saccharomyces cerevisiae* used was BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), alongside with mutants deleted in Ups1, Mdm10, Mdm12, Mdm34 and Mmm1. These strains were maintained and grown in rich medium (YEPD; 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, with 2% agar for solid medium). The transformed strains were maintained in a synthetic complete media (SCGlu-Ura; 0.175% (w/v) yeast nitrogen base without amino acids and ammonium sulfate, 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose, 0.2% (w/v) Drop-out mix, plus 0.01% (w/v), adenine, histidine, lysine and tryptophan, with 2% agar for solid medium) lacking uracil.

For the Bax expression studies, the wild-type and mutated strains were transformed with pYES2 expression vector with or without the human Bax sequence, with yeast codon bias. Besides the wild-type Bax (Bax α), a mutated form of Bax, Bax P168A, was also inserted in the plasmid, and transformed in the same mutant strains. This mutant form of Bax, has the proline 168 in the C-terminal substituted by an alanine, that results in a constitutively activated protein.

For transformation, cells were pre-treated with lithium acetate, by the addition of lithium acetate, Polyethylene Glycol (PEG) and ssDNA, with incubations of 30 minutes at 30 ˚C, 200 rpm; 30 minutes in a bath at 42 ˚C and 30 minutes in ice. The thermal shock increases the efficiency of the transformation (Gietz & Woods, 2006).

For selection of transformed strains, isolated transformed colonies grown in medium lacking uracil, were selected transferred to induction medium and expression of Bax was assessed through Western Blot analysis. For each strain, three different colonies were selected, grown in SC Glu -Ura, inoculated in SCLac –Ura pH5.5 (SCLac-Ura; 0.175% (w/v) yeast nitrogen base without amino acids and ammonium sulfate, 0.5% (w/v) ammonium sulfate, 0.5% (w/v) lactate, 0.2% (w/v) Drop-out mix, plus 0.01% (w/v), adenine, histidine, lysine and tryptophan) lacking uracil and 0.5% ethanol at $D.O._{600nm}$=

0.2 and incubated for 24 hours at 30 ˚C and 200 rpm. Afterwards galactose was added to a final concentration of 0.5% to induce Bax expression. The cells were collected four hours later and disrupted using a Lysis Buffer (3.5% de β-mercaptoetanol em 2 M de NaOH). The proteins present in the lysate were precipitated with 50% TCA, and separation of the different proteins through SDS-Page ensued as previously described. The strips were incubated with mouse monoclonal anti-yeast phosphoglycerate kinase (PGK1) antibody (1:5000, Molecular Probes) and rabbit monoclonal anti-Bax antibody (1:5000, Sigma-Aldrich) overnight at 4 °C, and incubated with the secondary antibodies for 1 hour at room temperature (RT). The immunochemical reaction was revealed as previously described.

3.2 Growth conditions and cell death assays

The strains used in cell death assays, first were grown overnight in aerobic conditions and in SCGlu –Ura at 30 ˚C and 200 rpm and then collected through centrifugation, resuspended in SCLac – Ura pH 5.5 at a $D.O._{640}$=0.2 and incubated at 3 0˚C for 24 h. Afterwards, galactose was added at a 0.5% concentration for Bax induction, the samples were collected at two time points (0 and 4 hours) and went through 4 serial dilutions of 1:10 with water. Five drops of 30 µL from the last dilution were then spotted in YEPD plates, which were then incubated at 30 ˚C. Colony forming units were counted 72 h after, and cell viability was calculated having time zero as 100%.

Chapter III – Results

1. Assessment of cyt *c* release during acetic acid-induced apoptosis in the strains *Δcrd1, Δtaz1, Δups1, Δmdm10, Δmdm12* and *Δcho1*

Previous work conducted at our laboratory, showed that *S. cerevisiae* strains deleted in *MDM10* and *MDM12* genes, that code for components of the ERMES complex, displayed higher resistant to acetic acid-induced apoptosis. Also, strains deleted in genes involved in phospholipid biosynthesis and maturation pathways linked to mitochondria, namely, *CRD1*, *TAZ1*, *UPS1* and *CHO1,* showed altered sensitivity in response to acetic acid-induced apoptosis (Fernandes, 2013). These strains also displayed differences in the emergence of cellular alterations in response to acetic acid, such as in ROS accumulation and in mitochondrial membrane potential.

Since we now aimed to assess if the release of cytochrome *c* (cyt *c*), usually observed during acetic acid-induced apoptosis, is altered in the mutant strains under study, we first tested different culture conditions that ensured a high mitochondrial content.

1.1. Assessment of mitochondrial content in Δ*crd1*, Δ*taz1*, Δ*ups1*, Δ*mdm10*, Δ*mdm12* and Δ*cho1* strains

For this purpose, the amount of the outer mitochondrial membrane Por1 was used to compare mitochondrial mass in total extracts prepared from BY4741 and Δ*crd1* cells grown in media with glucose, galactose or lactate as the sole carbon source (Figure 10). As expected, the western blots revealed a remarkable difference in the Por1 amount when comparing the three carbon sources. Indeed, the medium with glucose showed a little amount of this protein in both BY4741 and Δ*crd1* strains, while the medium with lactate presented the highest amount. However, taking into consideration that some of the strains used in this work have impaired respiration metabolism, we chose galactose as the carbon source, since a good amount of Por1 could also be obtained in medium with this sugar.

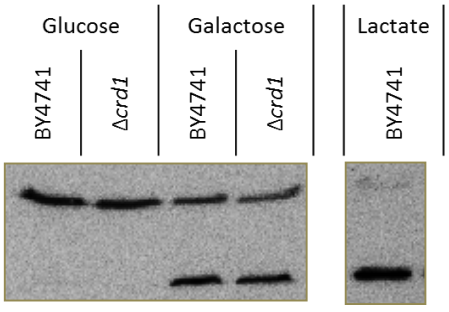
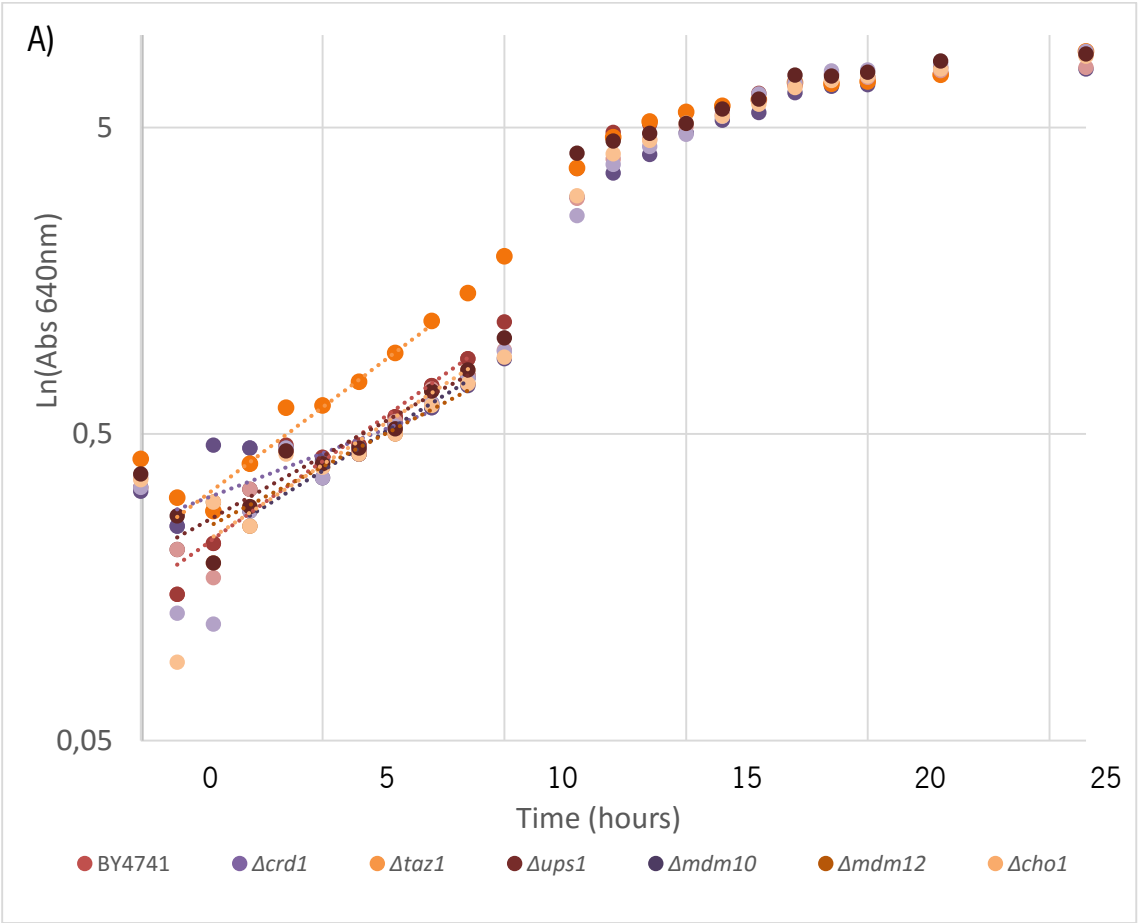


Figure 10 –Selection of the growth medium for mitochondrial isolation and assessment of cytochrome c release. Western blot analysis in total protein extracts from S. cerevisiae *BY4741 and the mutant* Δcrd1*. The amount of Por1 from cultures grown in glucose, galactose and lactate was compared, using Pgk1 as protein loading control. The samples grown in glucose and in galactose were prepared from 1 mL of culture at an O.D.$_{640nm}$ of 2. The sample from the culture in lactate medium was prepared from 1 mL of culture at an O.D.$_{640nm}$ of 1.*

1.2 Growth of Δ*crd1*, Δ*taz1*, Δ*ups1*, Δ*mdm10*, Δ*mdm12* and Δ*cho1* strains in galactose medium

As the previous work with the mutants under study was performed with glucose grown cells, we checked whether the mutant strains exhibited growth differences in the selected medium (above section). All strains achieved similar maximum $O.D._{640nm}$ values (Figure 11) in galactose medium though exhibiting different specific growth rates, with *Δtaz1 and* Δ*crd1* strain showing the highest and lowest specific growth rate, respectively. All the other strains displayed specific growth rates similar to the wild type strain, BY4741.

A)



B)

Strain	Specific growth Rate (min^{-1})
BY4741	0.081
Δ*crd1*	0.042
Δ*taz1*	0.123
Δ*ups1*	0.068
Δ*mdm10*	0.080
Δ*mdm12*	0.064
Δ*cho1*	0.078

Figure 11 - Growth of BY4741, Δcrd1, Δtaz1, Δups1, Δmdm10, Δmdm12 *and* Δcho1 *strains in a medium containing galactose as the carbon and energy source. A) The Abs 640 values were represented in a semilogarithmic plot to built the growth curve. B) Table of the specific growth rate of the strains, estimated from the slope value of the linear regression applied to the natural logarithm of absorbance values as function of time corresponding to the exponential phase.*

1.3 Survival of galactose grown cells of Δ*crd1*, Δ*taz1*, Δ*ups1*, Δ*mdm10*, Δ*mdm12* and Δ*cho1* strains during acetic acid-induced apoptosis

Survival rates of the BY4741, Δ*crd1*, Δ*taz1*, Δ*ups1*, Δ*mdm10*, Δ*mdm12* and Δ*cho1* strains in response to acetic acid were previously evaluated in the lab with cells grown in medium with glucose as the carbon and energy source (Fernandes, 2013). Since in the present study we changed the carbon source to galactose we assessed if the mutant resistant phenotypes to acetic acid-induced apoptosis previously observed were also present under these new growth conditions. The results showed that only the Δ*ups1*, Δ*mdm10*, Δ*mdm12* and Δ*cho1* strains presented a resistant phenotype in response to the treatment with 120 mM acetic acid, when compared with the wild-type strain (Figure 12). The Δ*ups1* and Δ*cho1* strains were the most resistant, with around 70% of survival after 120 minutes of treatment, while the strains mutated in the ERMES proteins had a survival rate of about 30%, in comparison with the strains Δ*crd1* and Δ*taz1,* which like the BY4741 strain, showed almost no survival.

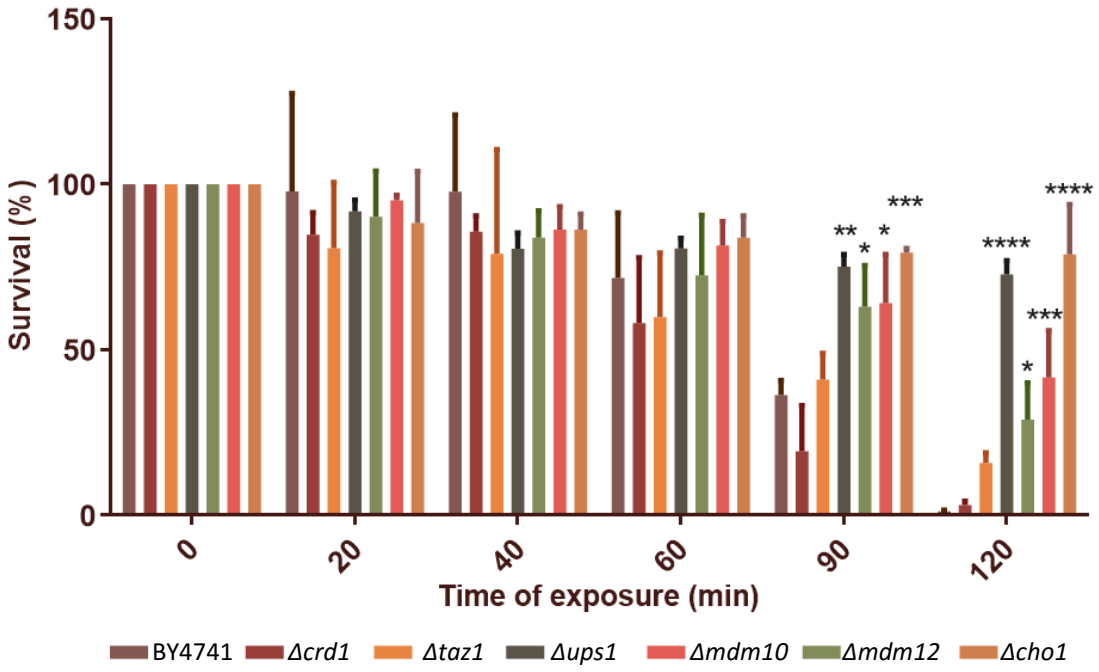


Figure 12 – Survival of the strains BY4741, Δcrd1, Δtaz1, Δups1, Δmdm10, Δmdm12 *and* Δcho1 *grown in galactose, upon treatment with 120 mM of acetic acid. Time points were taken at t0, t20, t40, t60, t90 and t120, and survival percentage was determined by colony forming unit counts, considering t0 values as 100% survival. Error bars represent standard deviation for n = 3; p<0.001.*

1.4 Assessment of cytochrome *c* release in galactose grown cells of Δ*crd1*, Δ*taz1*, Δ*ups1*, Δ*mdm10*, Δ*mdm12* and Δ*cho1* during acetic acid-induced apoptosis

1.4.1 Assessment of cyt c content *in galactose grown cells of Δ*crd1, *Δ*taz1, *Δ*ups1, *Δ*mdm10, *Δ*mdm12 *and Δ*cho1 strains

Since the strains Δ*crd1*, Δ*mdm10* and Δ*mdm12* do not grow in lactate medium due to a decreased respiratory metabolism, and as one of the goals of this work was to assess the release of cyt *c* upon acetic acid-induced apoptosis, we next assessed if the mutants present large differences in the content of this protein. Western blot analysis of total protein extracts showed that all strains express cyt *c*, though the mutants Δ*ups1* and Δ*mdm12* show lower amounts when compared with wild type (Figure 13).

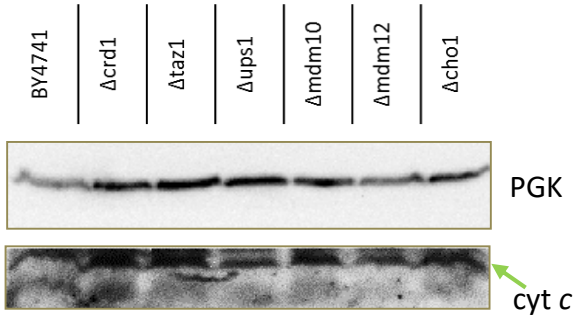


Figure 13 – Cytochrome c content in BY4741, Δcrd1, Δtaz1, Δups1, Δmdm10, Δmdm12 *and* Δcho1 *strains evaluated through western blot analysis, using PGK as control. Cyt* c *revelation required more exposure time, hence the grainy blot. The total protein extracts used were prepared from cell cultures grown in galactose, using 1mL at an O.D.*$_{640nM}$ *of 1.*

1.4.2 Optimizatin of the cell wall enzymatic digestion step of the mitochondrial isolation protocol

When performing the mitochondrial fractioning protocol of cultures treated for 90 minutes with 120 mM of acetic acid a low efficiency of cellular lysis was observed, which resulted in an inability to isolate mitochondria. To optimize the digestion step, mitochondrial isolation of wild-type treated cells was performed testing several cell wall digestion conditions and then assessing Por1 protein amount through western blot analysis. The conditions differed in the pH of the solution upon addition of the enzyme (pH 7.5 or pH 6.5) and temperature of incubation, either 32 °C or 45 °C. Though the latter may be considered high for yeast, and could cause stress for the cell, this temperature was tested because the manufacturer manual of the zymolyase used indicates such temperature and pH 6.5 as the optimal conditions for hydrolysis of yeast glucans. Looking at the results, it is noticeable that the samples at pH 6.5 (in which the pH was not corrected) present higher amounts of Por1, and between the two temperatures tested, the sample that was incubated at 45 °C during digestion presents higher

amounts of Por1 (Figure 14). Thus, the best cell wall digestion was achieved with zymolyase (100 mg for 10 g wet weight) for 40 minutes at 45 ˚C and without pH correction.

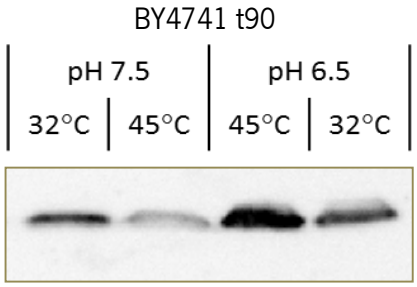


Figure 14 – Western blot analysis of total cell extracts prepared from cells subject to different enzymatic cell wall digestion conditions. Using the mitochondria isolation protocol, digestion of BY4741 treated cells was performed under different conditions, at temperatures 32 °C and 45 °C, and with corrected pH to 7.5 or uncorrected pH of about 6.5. The analysis was performed using Por1 as a mitochondrial marker to compare mitochondrial mass present in the isolates from the different samples.

1.4.3 Evaluation of cytochrome c release

Mitochondrial fractions were prepared from Δ*crd1*, Δ*taz1*, Δ*ups1*, Δ*mdm10*, Δ*mdm12* and Δ*cho1* cells using the optimized cell wall digestion conditions, before and after 90 minutes exposure to 120 mM acetic acid to assess cyt *c* release by Western blot analysis. All mitochondrial fractions obtained exhibited no or or very low levels of PGK (phosphoglycerate kinase, used as a cytosolic marker) indicating that they were almost free of cytosolic contamination, while they were enriched in mitochondria as assessed by the levels of Por1. Regarding the post-mitochondrial fractions, some samples were free of mitochondrial contaminations while others were not or even corresponded to low digestion yields since no PGK was detected at all. Although the results are not very clear, it is visible that in the untreated samples cyt *c* was detected in the mitochondrial fractions from all strains (Figure 15).

In the treated samples, the results show that the BY4741 strain releases cyt *c* from the mitochondrial into the cytosol. The same was not seen in the Δ*ups1*, Δ*mdm10*, Δ*mdm12* and Δ*cho1* strains, where cyt *c* is still in the mitochondrial fraction (Figure 15). In the treated samples from the strains Δ*crd1* and Δ*taz1*, there is no cyt *c* band in either fraction. While a good amount of Por1, indicative of an enriched mitochondrial content, is visible in the mitochondrial fraction of these strains, PGK is barely visible in the corresponding post-mitochondrial fractions. These observations together with the fact that all strains possess similar levels of this pro-apoptotic factor, support the notion that the release of cyt *c* occurs, but is not visible due to the low protein concentration of the post-mitochondrial fraction. Thus, to properly conclude about this, further replicas of these assays are required.

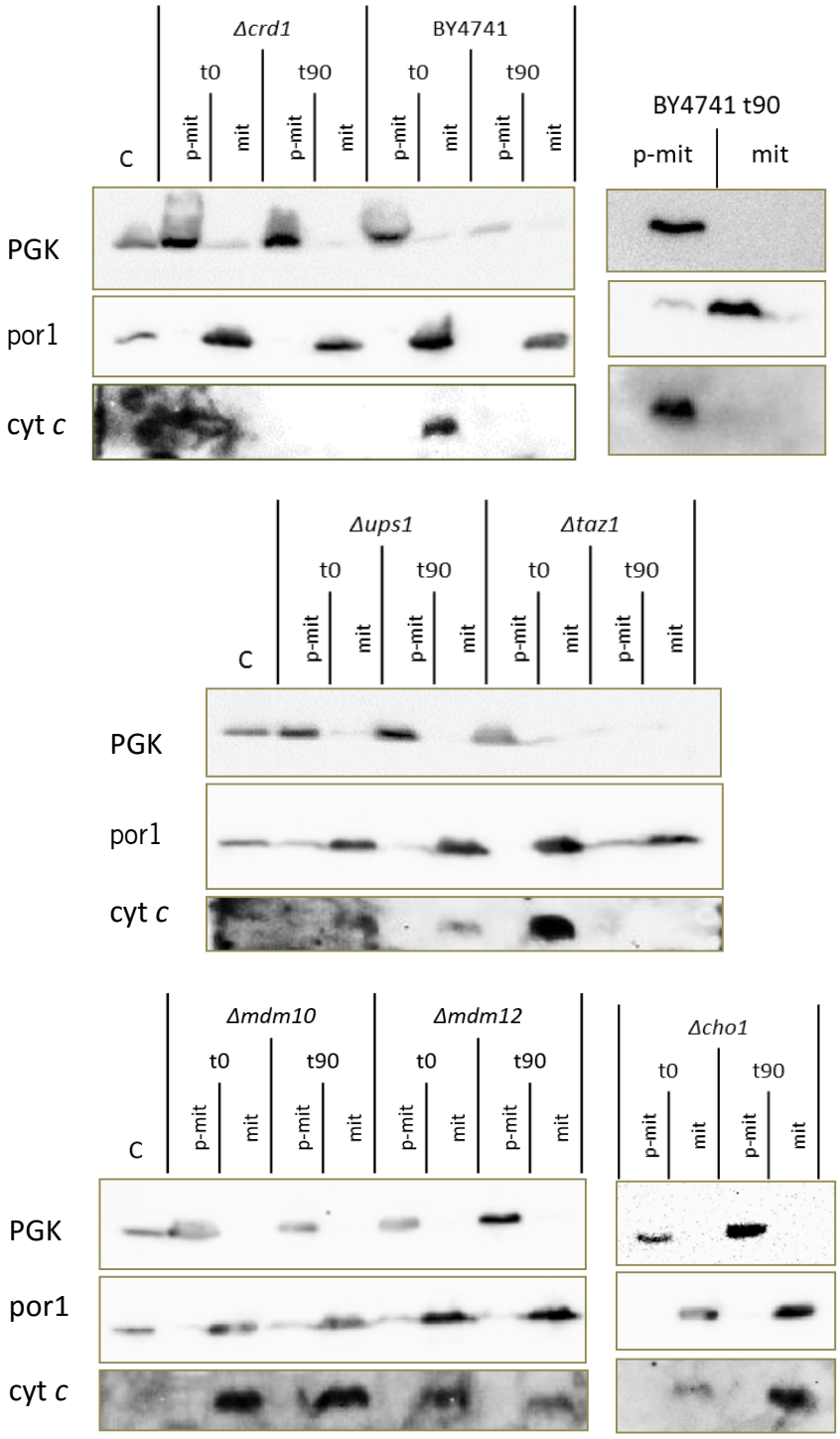


Figure 15 - Assessment of cytochrome c release during acetic acid-induced apoptosis. Mitochondrial and post-mitochondrial fractions, from both treated (t90) and untreated (t0) cultures of BY4741, Δcrd1, Δtaz1, Δups1, Δmdm10, Δmdm12 *and* Δcho1*, were subjected to western blot analysis, using PGK and Por1 as markers for post-mitochondrial and mitochondrial fractions respectively. Cytochrome c release is determined by its presence in the post-mitochondial fraction. A total protein extract from BY4741 was used as control. (C - control; p-mit – post-mitochondrial fraction; mit – mitochondrial fraction)*

1.5 Assessment of the cell wall resistance of Δ*crd1*, Δ*taz1*, Δ*ups1*, Δ*mdm10*, Δ*mdm12* and Δ*cho1* strains after acetic acid treatment

The low efficiency of cell lysis previously observed when performing the mitochondrial fractionation protocol of mutant cultures treated for 90 minutes with 120 mM of acetic acid suggested alterations of the cell wall.

To evaluate whether resistance to cell wall digestion of the mutated strains treated with acetic acid treatment was linked to alteration in the cell wall composition, variation of $O.D._{800nm}$ upon addition of zymolyase to cell suspensions and incubation at 30 °C was measured. All the mutant strains showed resistance to digestion when compared to the wild-type strain, which showed a rapid decrease in absorbance, higher than 60% in the first 20 minutes, and with only 17% of initial OD after one hour (Figure 16). The Δ*ups1* mutant was the less resistant strain, with around 35% of initial absorvance at the end of the experiment, while the Δ*crd1* and Δ*mdm12* strains showed very similar digestion rates, with around 60% of the initial OD after 60 minutes. The strains with the highest resistance to cell wall digestion were Δ*taz1,* Δ*mdm12* and Δ*cho1*, with about 80% of initial OD after 60 min.

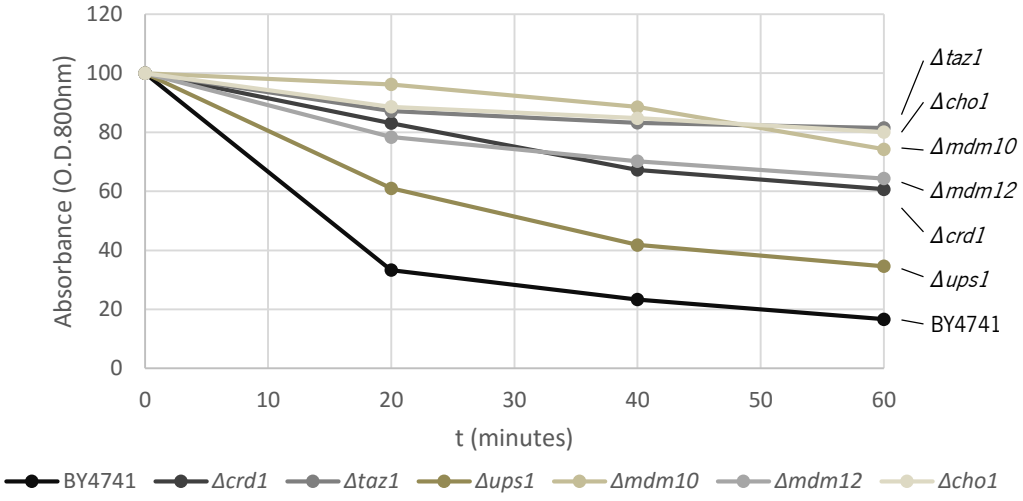


Figure 16 - Enzymatic digestion of the cell wall of BY4741, Δcrd1, Δtaz1, Δups1, Δmdm10, Δmdm12 *and* Δcho1 *strains. Cells grown in galactose were collected and resuspended in PBS, zymolyase was added and the measurements at an* $O.D._{800nm}$ *were taken at time point t0, t20, t40 and t60. Absorbance at* t_0 *was considered as 100%. (n=1)*

2. Assessment of alterations in the mitochondrial phospholipid composition of *Δcrd1, Δtaz1, Δups1, Δmdm10, Δmdm12* and *Δcho1* strains in response to acetic acid treatment

Phospholipids, in particular cardiolipin (CL), have an important role in mitochondria metabolism and in its role in apoptosis. Therefore, to study if alterations in mitochondrial phospholipid content can

underlie the increased resistance to acetic acid-induced apoptosis observed for the strains deleted in genes *MDM10* and *MDM12*, deficient in ERMES complex, analysis of mitochondrial phospholipids from these strains was performed. In addition, and for comparison, mitochondrial phospholipids were also analyzed in strains deleted in genes involved in phospholipid biosynthesis and maturation pathways, namely *CRD1*, *TAZ1*, *UPS1* and *CHO1*. These studies were conducted in cells cultivated in two different media using either glucose or galactose as the sole carbon source. For glucose grown cells, mitochondrial membrane phospholipid analysis was compared between treated and non-treated cells. For galactose grown cells mitochondrial membrane phospholipid analysis was performed only for untreated cells.

Regarding glucose grown cells, five different phospholipidic classes were identified, namely, phosphatidylcholine (PC), phosphatidylinositol/lysophosphatidylethanolamine (PI/LPE), phosphatidylserine (PS); phosphatidylethanolamine (PE) and cardiolipin (CL) (Figure 19). From the different phospholipids known to be present in the mitochondrial membranes, the only one that seemed to be lacking is phosphatidic acid (PA). For the different classes present, it is possible to observe that there is not much variation in their relative percentages between strains, each class amounting to about 20%. This was observed either for treated and untreated cells with only a few statistically significant variations being measured. Regarding the untreated samples, and compared with wild type, only Δ*mdm12* strain shows a slight increase in PE class, and Δ*crd1* strain shows a slight decrease in the PC class, no other apparent alterations in the phospholipid composition of the mitochondrial membranes being observed (Figure 19).

The effect of 90 min. treatment with 120 mM acetic acid on phospholipid composition for each strain is shown in Figure 18. While Δ*crd1* and Δ*taz1* strains did not show any significant alteration in response to acetic acid treatment, the other mutants exhibited significant alterations in CL and/or PI/LPE, PE, PC and PS classes. Indeed, the wild type strain exhibited an increase in the PE and PI/LPE classes, and a decrease in the CL class, whereas Δ*ups1* strain only had an increase in the PC class. On the other hand, while Δ*mdm12* strain displayed a decrease in the PE class and a comparable increase in the PS class, Δ*mdm10* displayed a decrease in the CL and a comparable increase in the PC class.

To ascertain if the mitochondrial fractions used for the phospholipid analysis were contaminated with membranes from other origins, a comparison between phospholipid profiles of the mitochondrial fractions without any further purification or purified through a sucrose gradient was performed. The results evidenced that for the untreated samples the purified mitochondria only showed a small increase of the PE class. For samples treated with acetic acid, the purified mitochondria displayed an

increase of not only PE but also CL and PC, and a decrease in PS and PI/LPE (Figure 19). These results indicated that while the mitochondria from untreated cells do not seem significantly contaminated with other membranes, in the case of mitochondria from treated samples some contamination with membranes richer in PS and PI/LPE may be present. This result may be related with the fact that good mitochondria yields are difficult to obtain from cells treated with acetic acid and so potential contaminations may be more relevant, namely with ER membranes.

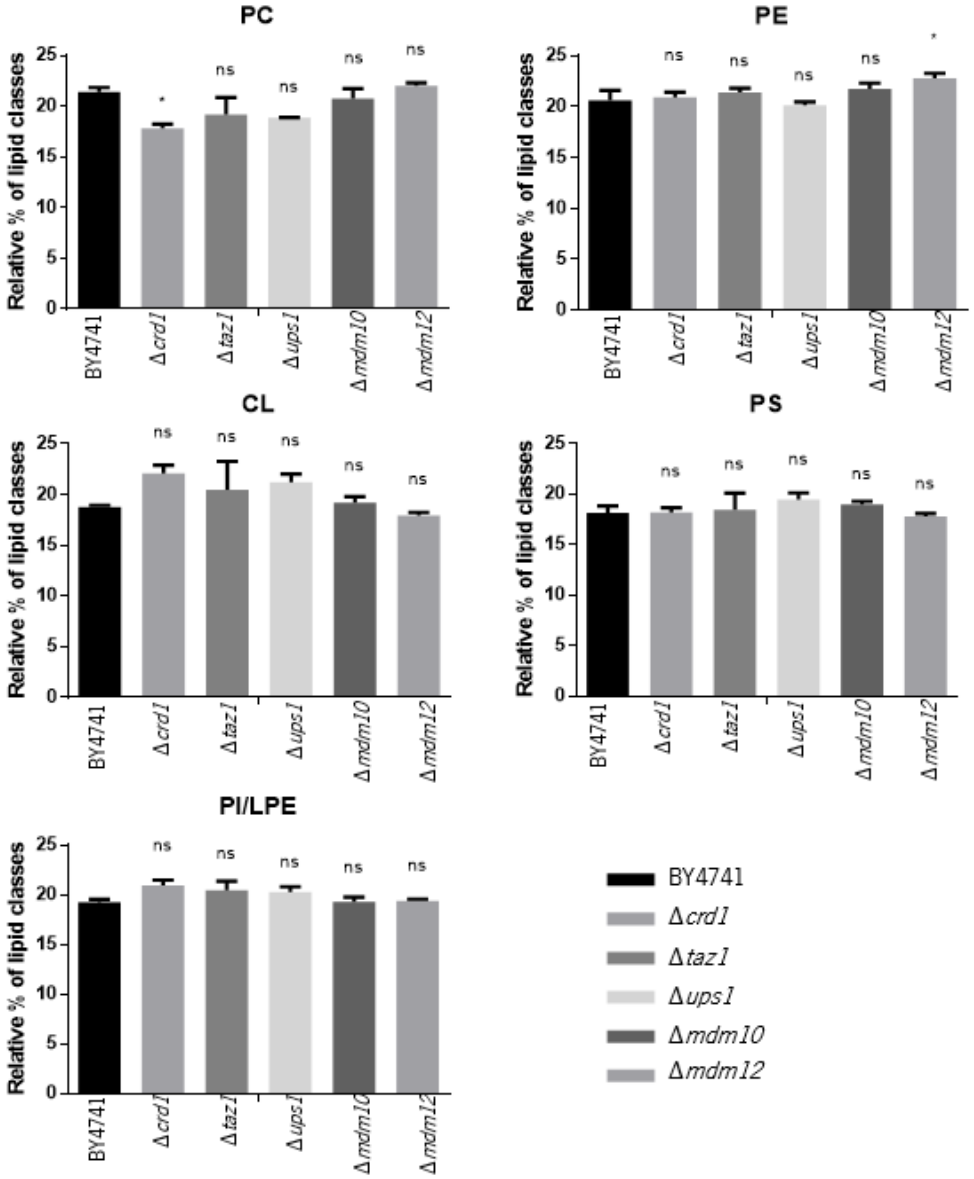


Figure 17 - Analysis of mitochondrial phospholipid profile, through separation of the phospholipid classes using TLC. Mitochondrial isolates from BY4741, Δcrd1, Δtaz1, Δups1, Δmdm10 *and* Δmdm12 *strains, grown in glucose, before (t0) and after 90 min. treatment (t90) with 120 mM acetic acid. PC – posphatidylcholine; PI/LPE – phosphatidylinositol/ lysophosphatidylethanolamine; PS – phosphatidylserine; PE – phosphatidylethanolamine; CL – cardiolipin). Error bars represent standard deviation for n = 3; p<0.001.*

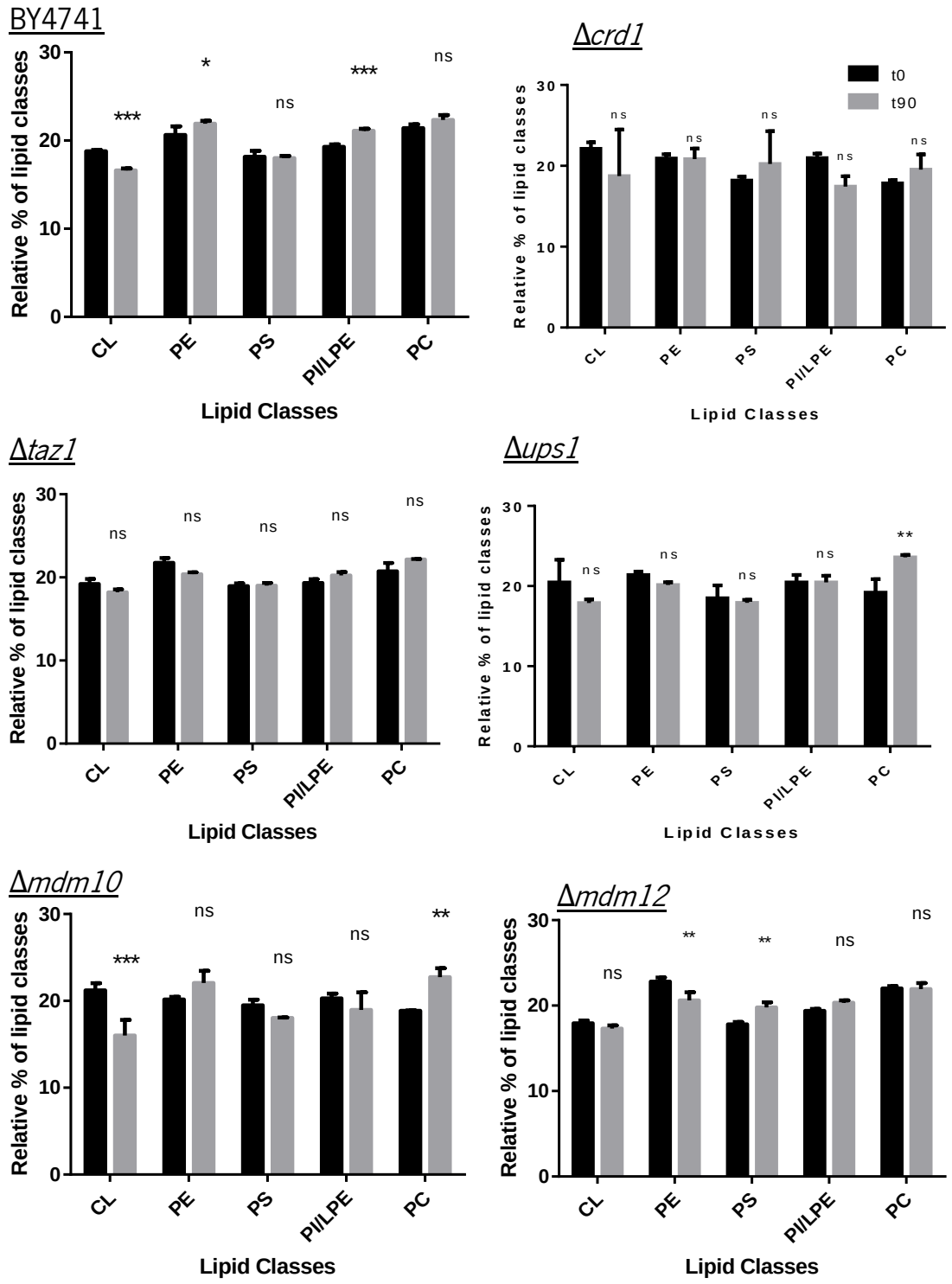


Figure 18- Analysis of mitochondrial membrane phospholipid profile, through separation of the phospholipid classes using TLC. Mitochondrial isolates from BY4741, Δcrd1, Δtaz1, Δups1, Δmdm10 *and* Δmdm12 *strains, grown in glucose, at t0 and t90 of 120 mM acetic acid treatment. (PC – phosphatidylcholine; PI/LPE – phosphatidylinositol/ lysophosphatidylethanolamine; PS – phosphatidylserine; PE – phosphatidylethanolamine; CL – cardiolipin). Error bars represent standard deviation for n = 3; p<0.001.*

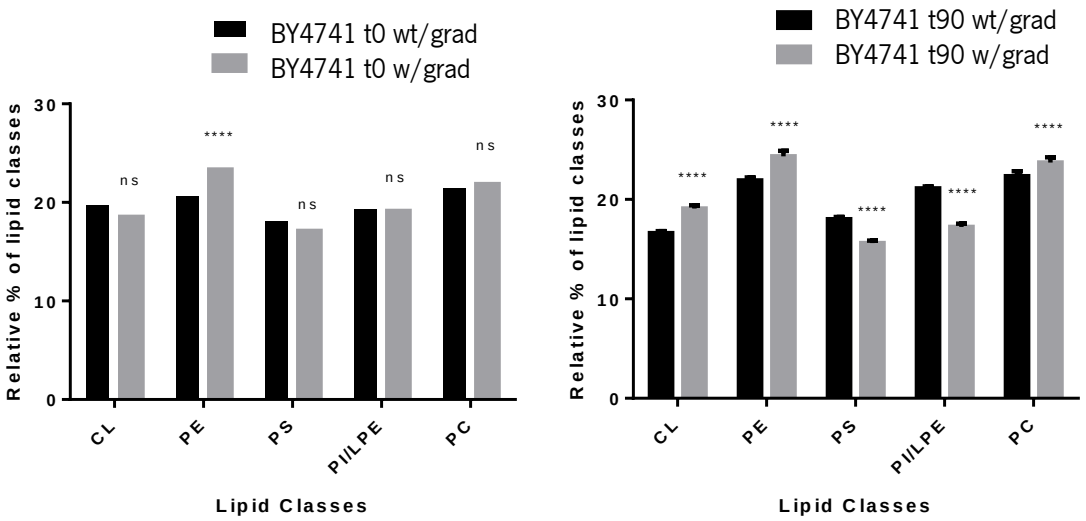


Figure 19 – Comparison of the phospholipid profile of mitochondria isolated from BY4741 cells, untreated and treated with 120 mM acetic acid, and subject or not to purification by sucrose gradient. (PC – phosphatidylcholine; PE/LPE – phosphatidylinositol/ lysophosphatidylethanolamine; PS – phosphatidylserine; PE – phosphatidylethanolamine; CL – cardiolipin; wt/grad – without sucrose gradient purification; w/grad – with sucrose gradient purification). Error bars represent standard deviation for n = 3; p<0.001.

Since the TLC assays displayed a band on the position corresponding to CL in the Δ*crd1* strain, that is deleted in the CL synthase protein, and has been described not to present this phospholipid, additional LC-MS analysis were performed in both wild type and Δ*crd1* strains, to clarify if the phospholipid present in the band collected was indeed CL. In the graphics obtained, it is possible to see that in the spectrum of the band from the mitochondria of untreated wild type strain, several species of CL were separated, identified by each peak, and some of them are present in a considerable amount (Figure 21). Interestingly, in the treated sample, there is a decrease of the number of species and total amount of CL. Regarding the Δ*crd1* samples, the band from untreated mitochondria show a low number of small peaks, showing residual CL presence, that were almost absent in the treated sample.

Since CL is synthesized from PG by Crd1, the absence of CL in the Δ*crd1* strains could lead to an accumulation of PG in the mitochondrial membrane. To evaluate this hypothesis LC-MS analysis was performed to identify PG species in BY4741 and Δ*crd1* strains, in both treated and untreated conditions. Comparing the spectra of mitochondria isolated from treated and non-treated wild type strain, it can be observed that even though some species show a decrease in relative abundance after acetic acid treatment, most species, particularly those of higher m/z ratio, have a higher concentration at t90, indicating that the total amount of PG increases (Figure 21). In the Δ*crd1* strain we can see that there are very few species of PG present before treatment, and that there is only a slight increase of PG relative abundance caused by the treatment with acetic acid.

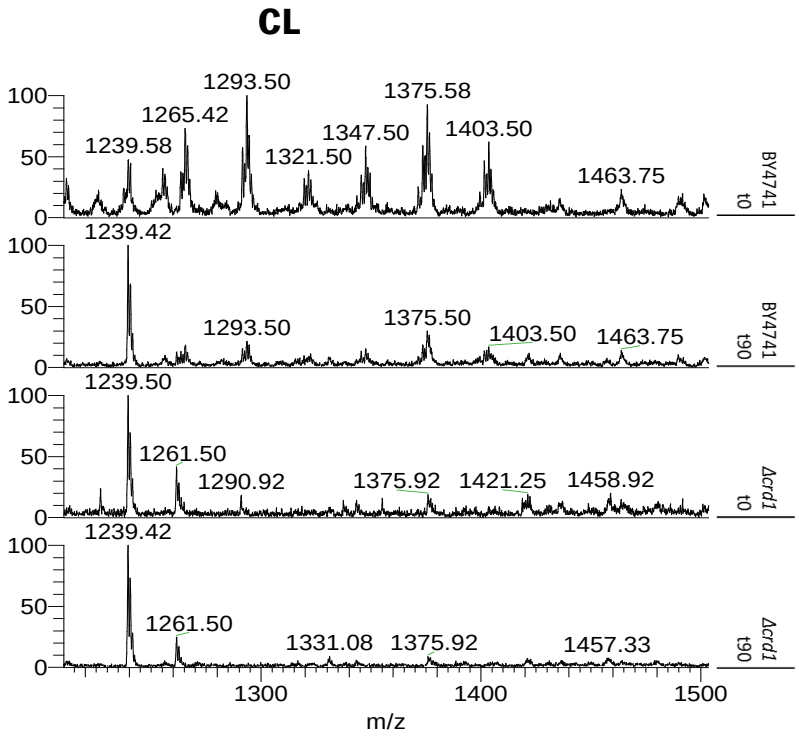


*Figure 21 – Spectrum of mitochondrial membrane cardiolipin relative abundance, obtained by LC-MS technique, in acetic acid treated and untreated BY4741 and Δ*crd1 *strain grown in medium with glucose. Each peak represents a different CL species, and the peak identified with m/z of 1239 is an internal standard.*

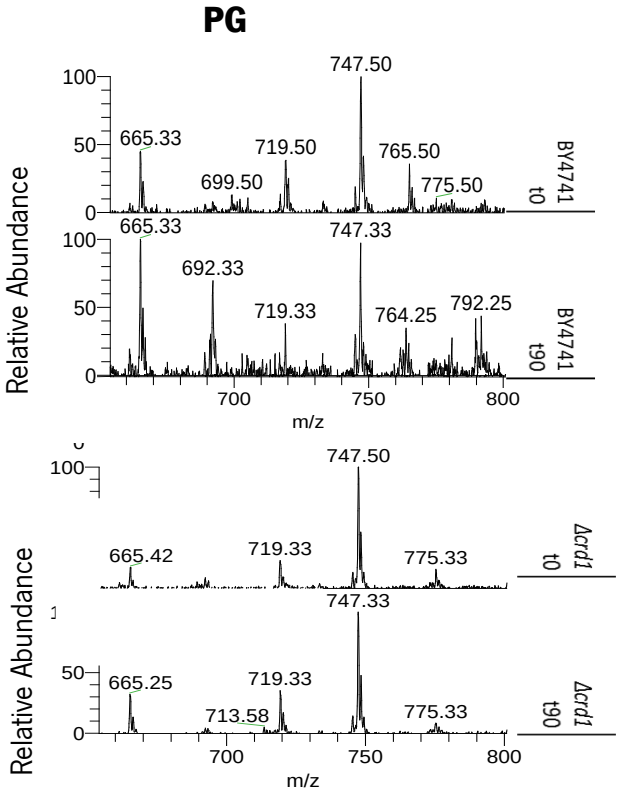


*Figure 21 - Spectrum of mitochondrial membrane PG relative abundance, obtained by LC-MS technique, for acetic acid treated and untreated cells of BY4741 and Δ*crd1 *strains grown in medium with glucose. Each peak represents a different PG species, and the peak identified with m/z of 665 is an internal*

The mitochondrial phospholipidic profiles of the Δ*crd1,* Δ*taz1,* Δ*ups1* Δ*mdm10,* Δ*mdm12 and* Δ*cho1* strains were also analyzed in cells cultured in galactose medium. As referred above this medium was used in order to obtain cells with higher mitochondrial mass and more active respiration since galactose has a less efficient catabolic repression effect than glucose (Herrero, Fernández, & Moreno, 1985). From the TLC plates it was already clear that there were differences in the amounts of phospholipids of the different strains, and that Δ*crd1* presented a band of PG, that was absent in the other strains (Figure 22). Also, at the position expected for CL band, it can be seen that most of the strains show two bands, while Δ*crd1* has only one, and interestingly Δ*ups1* has three. It is possible that the phospholipid whose band was absent in Δ*crd1* is CL, and that the other band present in all samples is the unidentified phospholipid present in the same mutant grown in glucose, which formed the band where CL would migrate. To be able to compare with the previous analysis of glucose cells, all bands were scraped together for quantification.

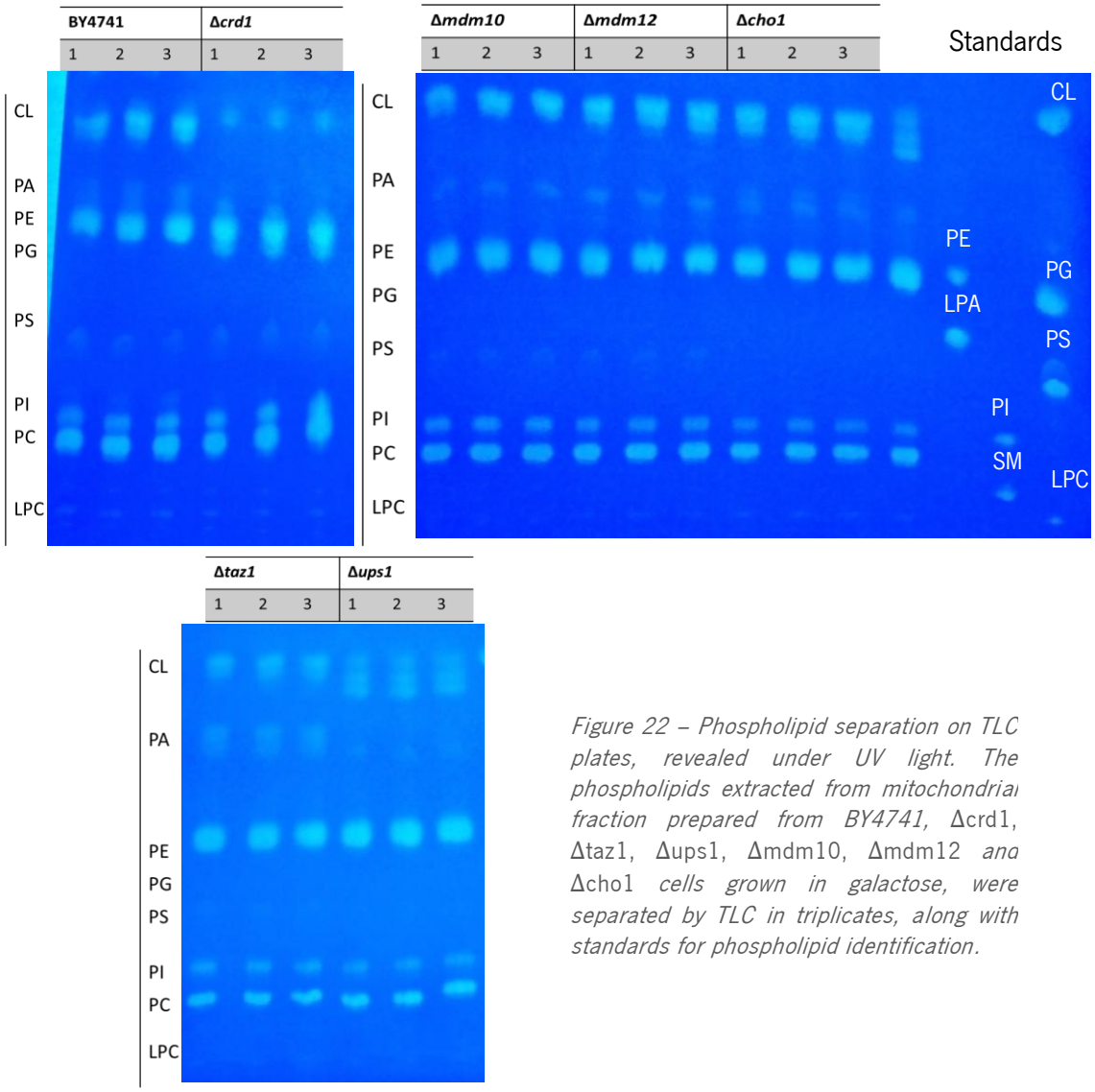


Figure 22 – Phospholipid separation on TLC plates, revealed under UV light. The phospholipids extracted from mitochondrial fraction prepared from BY4741, Δcrd1, Δtaz1, Δups1, Δmdm10, Δmdm12 *and* Δcho1 *cells grown in galactose, were separated by TLC in triplicates, along with standards for phospholipid identification.*

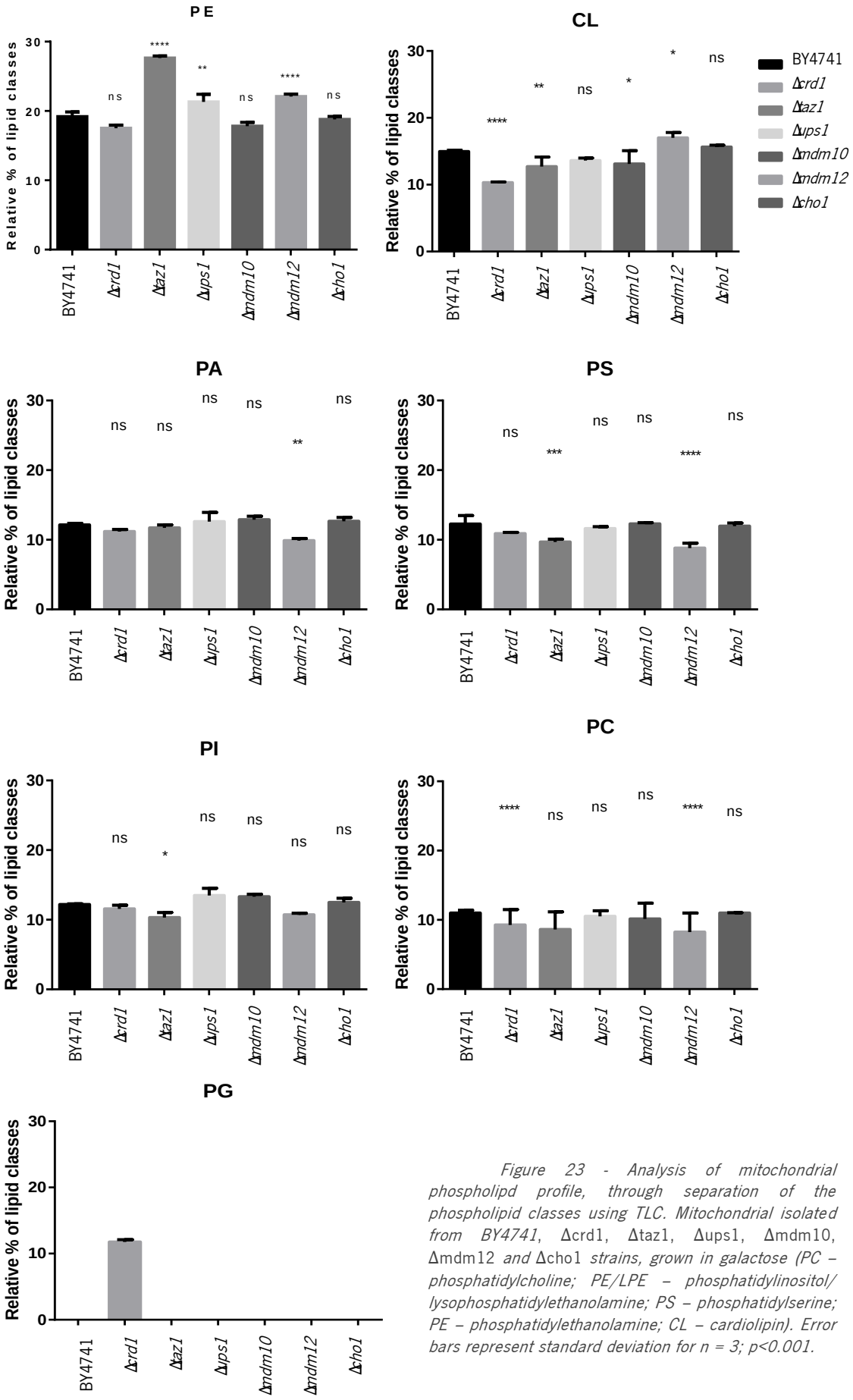


Figure 23 - Analysis of mitochondrial phospholipd profile, through separation of the phospholipid classes using TLC. Mitochondrial isolated from BY4741, Δcrd1, Δtaz1, Δups1, Δmdm10, Δmdm12 *and* Δcho1 *strains, grown in galactose (PC – phosphatidylcholine; PE/LPE – phosphatidylinositol/ lysophosphatidylethanolamine; PS – phosphatidylserine; PE – phosphatidylethanolamine; CL – cardiolipin). Error bars represent standard deviation for n = 3; p<0.001.*

In this assay, the alterations of the several phospholipid classes in the mutant strains were more significant than in the assay with glucose-grown cells, and further separation of phospholipids was observed (Figure 23). It is possible to see that in comparison to the wild type, the Δ*cho1* strain does not show any statistically significant alterations, and that Δ*ups1* only shows an increase in the PE class. The more remarkable alteration is the increase of about 9% in Δ*taz1* PE class. Also, the Δ*crd1* strain was the only one having a TLC band for the PG class, of about 12% of total phospholipids.

3. Assessment of the cell survival of *Δups1, Δmdm10, Δmdm12, Δmdm34* and *Δmmm1* strains upon expression of human Bax α and Bax P168A

As referred before Ups1, is responsible for the transport of phosphatidic acid between the two mitochondrial membranes, and Mdm10, Mdm12, Mdm34 and Mmm1, are the four components of the ERMES complex. To ascertain whether the function of the pro-apototic protein Bax could be affected by phospholipid metabolism or by the interaction with the zones of close apposition between the ER and the mitochondria, mutant strains lacking each of these proteins were transformed with either Bax α, the inactive and mainly cytosolic form of Bax, or with Bax P168A mutant, a mitochondrial and active form of Bax. For selection of the clones, cultures of the transformed cells were induced to express Bax by addition of galactose. Bax expression was evaluated by western blot analysis of total extracts prepared from cultures after four hours of induction (t4) (Figure 24). PGK amount was used for the control of total protein, and transformants with the most similar Bax/PGK ratios were selected. Analysis of Bax/PGK ratios showed higher levels of Bax P168A than of Bax α in Δ*ups1,* Δ*mdm12* and Δ*mdm34* strains (Figure 24).

Following the selection of the transformed strains, cell viability assays were conducted to evaluate cell survival rates upon Bax expression. While, expression of Bax α in the wildtype strain had an effect on cell survival, unexpectedly Bax P168A did not. The results also showed that, despite the existence of small variations in cell survival of the mutant strains transformed with Bax in comparison with the corresponding mutants transformed with the empty vector, these differences were not statistically significant, excepting the Δ*mdm34* strain transformed with Bax α, which showed a significant decrease in cell survival (Figure 25). Further analysis by Western blot revealed that the transformed strains Δ*mdm12* with Bax α or Bax P168A, were lacking the encoded protein, probably due to loss of the plasmid (not shown).

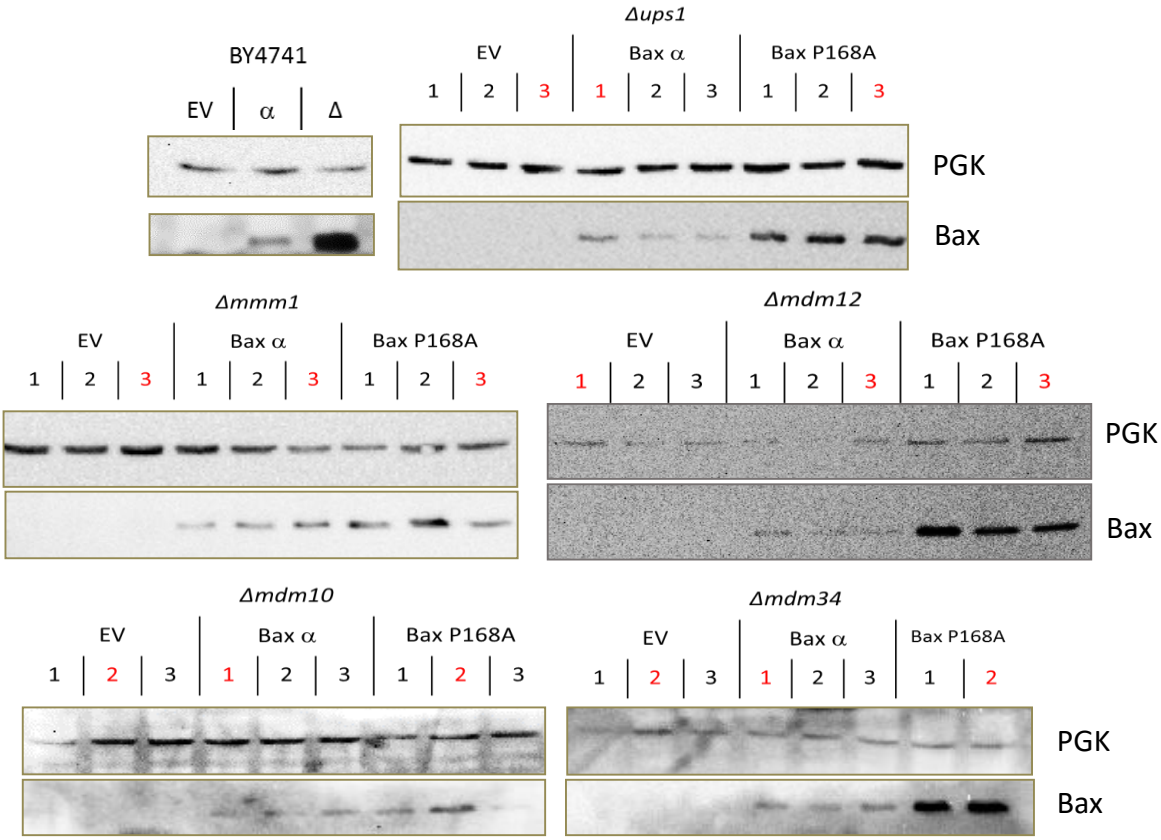


Figure 24 – Selection of mutants transformed with plasmids for expression of Bax α and Bax P168A through western blot analysis, using PGK as control for protein expression. Wild type was already transformed from previous work in the laboratory. Three isolated colonies from each transformation were selected for this assay, and the selected strains are highlighted in red.

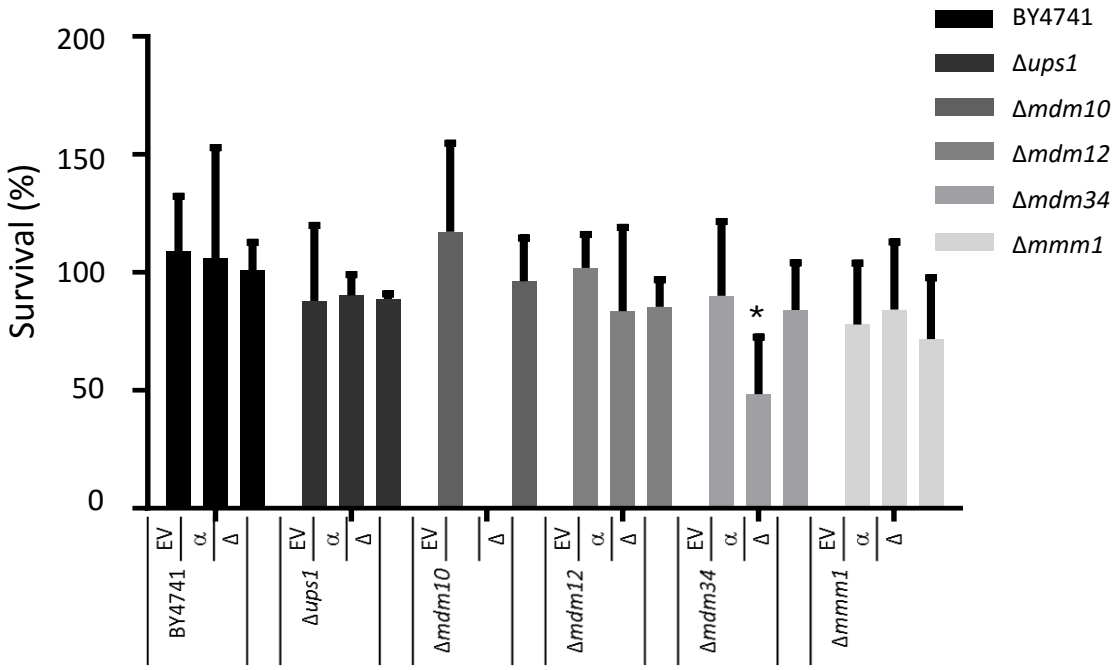


Figure 25 - Cell death assay of the strains BY4741, Δups1, Δmdm10, Δmdm12, Δmdm34 and Δmmm1, upon induction of Bax expression by addition of galactose to the medium. Time points were taken at t0 and t4 (hours), and survival percentage was determined by colony forming unit counts, considering t0 values as 100% survival. Error bars represent standard deviation for n = 2; p<0.001.

Chapter IV – Discussion

A characterization of the cell death of Δ*crd1,* Δ*taz1,* Δ*ups1,* Δ*mdm10,* Δ*mdm12* and Δ*cho1* strains in response to acetic acid showed higher cell survivals for all the mutant strains in comparison to the wild type (Fernandes, 2013). In the present study we aimed to ascertain whether these resistance phenotypes were related with a reduced permeabilization of the outer mitochondrial membrane. In order to do so, we evaluated the release of cyt *c*, in the mutant strains after 90 minutes exposure to 120 mM acetic acid treatment.

This study, first carried with cells grown on glucose, revealed the need to grow cells in a non or less repressible carbon to overcome the low mitochondrial yield obtained with the isolation protocol. Since some of the strains, namely Δ*crd1*, Δ*mdm10* and Δ*mdm12,* do not grow in lactate medium, growth on galactose was selected. This change in the carbon source resulted in a different survival phenotype of the strains to the treatment with 120 mM of acetic acid. The wild type strain showed a delay in the loss of cell survival with less than 40% of survival at 60 minutes when grown in glucose, in comparison with about 75% at the same time point in galactose. However, after 120 minutes, cells grown in both carbon sources exhibit a low survival (about 5% in glucose and 1% in galactose).

As for the mutant strains, their resistance phenotype is altered since at time point 60 minutes, in galactose, in contrast to glucose, all mutant strains display survival rates similar to the wild type. However, in galactose medium after 120 minutes of acetic acid treatment while Δ*ups1,* Δ*mdm10,* Δ*mdm12* and Δ*cho1* preserve their resistance phenotype observed in glucose medium*,* Δ*taz1* and Δ*crd1* exhibited survival rates identical to the wild type. Furthermore, Δ*ups1*, along with Δ*cho1,* which in glucose only show about 30% of survival at this time point, in galactose, are the most resistant strains, with an increase of about 50% of the survival rates. Contrasting with this increase, the high resistance observed for Δ*mdm10* in glucose decreases to less than half in galactose, along with a less noticeable decrease in the Δ*mdm12* strain. These results are consistent with the higher resistance to acetic acid-induced apoptosis of mutants lacking genes involved in glucose repression (Sousa M et 2013), and with a study with W303-B1 cells grown raffinose which exhibit higher survival rates upon treatment with this cell death inducer (Guaragnella et al., 2013). The last study also provided evidence that this resistance is associated to an activation of the retrograde pathway (RTG), which has been implicated in the increase of yeast longevity and replicative ability, possibly through activation of a defense mechanism to mitochondrial stress.

The release of cyt *c* upon acetic acid treatment was already studied for both the wild type strains W303 and BY4741 by either western blot or analysis of the redox spectra (Giannattasio et al.,

2008; Guaragnella, Bobba, Passarella, Marra, & Giannattasio, 2010; Paula Ludovico et al., 2002; Pereira et al., 2007; Rego et al., 2014; Z. et al., 2002). The analysis of cyt *c* release in the mutants under study, allowed us to infer that the release of this pro-apoptotic factor after acetic acid treatment only occurs in the Δ*crd1* and Δ*taz1* strains most likely due to MOMP. This is consistent with the similar survival rates of these two mutants and the wild type, since MOMP has a well-known role in apoptosis. The fact that both Δ*crd1* and Δ*taz1* strains are involved in CL metabolism, may also suggest that neither CL nor Crd1 or Taz1 proteins would be involved in apoptosis induced by acetic acid in galactose grown cells. Furthermore, studies show that in yeast, CL and MLCL are indistinguishable (Baile, Sathappa, et al., 2014), which is in accordance with the similar phenotypes of these two mutants. As previously explained, cyt *c* binds to CL present in the IMM, and upon increase of the ROS levels during apoptosis, cyt *c* acts as a CL-specific oxidase, oxidizing CL and freeing itself. In the case of Δ*crd1* and Δ*taz1* strains, the CL levels are absent and diminished respectively, and hence the release of cyt *c* may be even easier upon MOMP.

The lack of cyt *c* release in the Δ*ups1,* Δ*mdm10,* Δ*mdm12* and Δ*cho1* strains could imply that the phospholipidic profile of the mitochondrial membranes is involved in MOMP. Although it could also be said that the missing proteins in the mutant strains could have a more direct role in cyt *c* release from mitochondria into the cytosol, the increased resistance of Δ*cho1* to acetic acid-induced apoptosis could undermine such conclusion due to its ER localization.

On the other hand, the phospholipid profile of the mitochondrial membranes can vary according to the carbon source in the culture medium (Tuller, Nemec, Hrastnik, & Daum, 1999). This may justify the differences in the observed phenotype of these strains grown on glucose and galactose as carbon sources. In this context, and in order to assess the potential influence of altered phospholipid compositions on the phenotypes observed, we characterized the phospholipid profiles of mitochondria isolated from the wild type and mutant strains grown in glucose or galactose as well as after acetic acid treatment.

The general phospholipidic profile of the subcellular membranes present in yeast, more specifically of *S. cerevisiae* cells grown in glucose medium, was established by Zinser et al. in 1991 (Zinser et al., 1991). In this study the author assessed the phospholipidic content of the several subcellular membranes, such as plasma membrane, vacuoles, nucleus and mitochondria, and went as far as discriminating the phospholipidic composition of each mitochondrial membrane (Table 1 and 2) (Zinser et al., 1991).

Table 2 - Phospholipid composition of organelles from Saccharomyces cerevisiae*. Taken from* (Klug & Daum, 2014)

	Mol% of total phospholipids						
Cell fraction	PC	PE	PI	PS	CL	PA	Others
Homogenate	51.0	25.0	11.4	5.1	3.7	1.1	2.7
Plasma membrane	11.3	24.6	27.2	32.2	nd	3.3	1.4
Endoplasmic reticulum	38.9	18.6	22.4	6.4	0.3	3.4	10.0
Mitochondria	33.4	22.7	20.6	3.3	7.2	1.7	10.1
Peroxisomes	39.8	17.4	22.0	2.5	2.7	6.1	10.5

Phospholipid composition of yeast cells and organelles are shown as summarized previously by Zinser *et al.* (1991) and Horvath *et al.* (2011).

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; PA, phosphatidic acid; others, other phospholipids such as lyso-phospholipids, phosphatidylglycerol, dimethylphosphatidylethanolamine; nd, not detectable.

Table 1 - Lipid composition of the outer and Inner mitochondrial membrane. Taken from (Zinser et al., 1991)

	Yeast* (*Saccharomyces cerevisiae*)	
	OMM	IMM
Phospholipid (mg/mg protein)	0.91	0.15
Sterols (mg/mg protein)	<0.01	0.03
Phosphatidylcholine	46	38
Phosphatidylethanolamine	33	24
Phosphatidylinositol	10	16
Phosphatidylserine	1	4
Cardiolipin	6	16
Phosphatidic acid	4	2

The results obtained in the present work for glucose-grown cells showed that there were no significant differences between the mitochondrial phospholipidic composition of wild type and the mutants strains. Comparing the mitochondrial phospholipid profiles obtained in this work with the results from Zinser et al. using D273-10B and X-2180 wild type strains, it can be observed that our results reveled lower PC content (around 20% *versus* 33.4%), higher PS (around 20% *versus* 3.3%), and higher CL (around 20% *versus* 7.2%). Although a contamination could explain these differences, this does not seem to be the case as according to comparison with the purified mitochondria from wild type strain only the treated samples appear to present signals of such an occurrence, and this contamination may originate from the tougher isolation method used in treated cells. Regarding the mitochondrial phospholipid profile of galactose grown cells, the results are also different, showing around 20% of PE, 15% of CL and about 10-12% PA, PS, PI and PC. These disparities could be explained by the different backgrounds of strains used (Daum et al. 1999), or by differences in the medium composition. In particular, a study that used different media, reported an increase in PC and PE of about 4-6% of total phospholipids of the mitochondrial membranes from glucose-grown cells in comparison to those grown in lactate or ethanol, a decrease of PI and PS, and an increase in CL from 7.2% to about the double (Tuller et al., 1999). Comparing these results with the ones obtained in the present work analyzing the mitochondrial phospholipidic profile of the untreated samples in both carbon sources, glucose and galactose, it can be observed that the change from glucose to a non-repressible

substrate resulted in a decrease of PS in both studies while CL and PC levels varied in opposite directions.

Also regarding the mitochondrial phospholipid analysis, the comparison between the different strains evidences some differences in the profiles obtained. The strain Δ*crd1* shows a slight decrease of PC in the glucose grown cells, and of more notice, appears to present a good percentage (about 20%) of a phospholipid that co-migrates with CL. Since this strain is known to be devoid of CL (Gohil, Thompson, & Greenberg, 2005; Iverson, Enoksson, Gogvadze, Ott, & Orrenius, 2004), and we also proved its absence by LC-MS, the lack of this phospholipid may be compensated with an increased synthesis of another phospholipid, possibly bis(monoacylglycero)phosphate (BMP). Although BMP is supposedly of mammalian origin, reports of its presence in prokaryotes have been published (Nishihara, Morii, & Koga, 1982). Also, PG is a precursor of *de novo* synthesis of BMP (Hullin-Matsuda et al., 2007), and its potential accumulation in Δ*crd1*, due to the blockage in its conversion to CL, could lead to synthesize BMP. The results also indicate that this potential conversion of PG to BMP would be more complete in glucose-grown cells than in cells grown in galactose where PG was found in a considerable amount. This strain also shows a decrease of PC, which could be the result of the lack of CL, since both phospholipids are substrate to Taz1 activity (Hullin-Matsuda et al., 2007). As for the Δ*taz1* mutant, the results in galactose show that there is a decrease in CL, which was expected, since the biosynthesis of CL in not impaired, but only its remodulation, decreasing the amount of MLCL that is transformed back to CL. This strain also shows a decrease of PS and an increase of PE in the mitochondria, suggesting an interaction with Psd1. Δ*cho1* does not show any alterations in the mitochondrial phospholipid composition and that is probably be due to a compensation mechanism, in which PS is synthetized by Pss1/2 in the ER and then transported to the mitochondria. As for the ERMES mutants, both displayed different alterations of their mitochondrial phospholipidic profile. While the Δ*mdm10* strain presented a decrease in CL (Osman et al., 2009), Δ*mdm12* displayed an increase in this phospholipid (contrary to what has been published (Kornmann et al., 2010)) along with PE, and a decrease in PS and PC. Lastly, Δ*ups1* shows an increase of PE, while another study described that this mutant has decreased amounts of CL (Herrmann, 2010). Δ*ups1* also presents a third band in the proximity of the CL spot, but its identification would need further investigation.

Finally, the analysis of the results from the cell death assays performed with the mutants deficient in each of the proteins from ERMES complex transformed with both active and inactive forms of Bax, suggest some differences in survival rate upon Bax expression. However, these results are still

preliminary and need further confirmation, since the phenotype of the wild type strain transformed with the active form of Bax is not in accordance with the expected results.

Summarizing, from this work we can conclude that lack of CL or of its remodeling did not impair release of cyt *c* in galactose-grown cells treated with acetic acid, but lack of Mdm10, Mdm12, Cho1 and Ups1 significantly inhibited this release. Furthermore, the absence of cyt *c* release was associated with increased resistance to acetic acid-induced cell death, while the strains in which cyt *c* was release did not displayed changes in resistance whencompared to wild type cells. The changes in the resistance phenotypes were also accompanied by altered mitochondrial phospholipidic profiles. However, a correlation between both phenotypes is not evident, since strains presenting the most pronounced alteration in lipid composition were not the ones presenting the higher resistance. The mitochondrial phospholipidic profiles were also dependent on the carbon source used, and when grown in galactose, the mutant strains presented a more diverse profile and higher differences in comparison with the wild type than when grown in glucose. The result also showed that there isn't a significant influence of the mitochondrial phospholipidic composition on the resistance to acetic acid induced cell death, in mutant strains grown in glucose, as these cells displayed increased resistance when compared to wild type strains but only slight differences in the mitochondrial phospholipidic profiles.

To further understand the mechanism that are taking part, more detailed lipidic analysis and new replicas are needed. To extract any subjectivity from the mitochondrial phospholipid profiling, LC-MS should be performed in all the conditions, if possible to obtain enough purified mitochondria. Also, the identification of the unknown phospholipids present in the proximity of the CL spot in the TLC plates would be interesting – both the band present across all the strains, and the one only present in the Δ*ups1*, and a repeat of the cyt *c* release analysis for the strains Δ*crd1* and Δ*taz1*, to confirm the presence of this pro-apoptotic factor in the post-mitochondrial fraction. In case the isolation of mitochondria reveals itself difficult, exploration of other methods analysis of cyt *c* release, such as redox spectra (mitochondria or even whole cells), could be employed.

As for the analysis of Bax mediated cell death, confirmation of the transformants and/or the phenotypes is needed to assure the conclusions taken from our results. Furthermore, the analysis of the cell death observed should be completed, by methods used previously (such as assessment of cyt c release, plasma membrane integrity, ROS accumulation, etc.).

Chapter V - References

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