Universidade do Minho
Escola de Ciências

Tânia Alícia Ribeiro Fernandes

The role of phospholipids and of the actin-binding protein cofilin in *Saccharomyces cerevisiae* acetic acid-induced apoptosis

Outubro de 2013

The role of phospholipids and of the actin-binding protein cofilin in *Saccharomyces cerevisiae* acetic acid-induced apoptosis
Tânia Alícia Ribeiro Fernandes
UMinho | 2013

Universidade do Minho
Escola de Ciências

Tânia Alícia Ribeiro Fernandes

The role of phospholipids and of the actin-binding protein cofilin in *Saccharomyces cerevisiae* acetic acid-induced apoptosis

Tese de Mestrado
Mestrado em Genética Molecular

Trabalho efetuado sob a orientação de
Professora Doutora Maria João Marques Ferreira Sousa Moreira
Professora Doutora Maria Manuela Sansonetty Gonçalves Côrte-Real

Outubro de 2013

DECLARAÇÃO

Nome: Tânia Alícia Ribeiro Fernandes

Endereço electrónico: taniafernandes.ba@gmail.com

Telefone: 918870242

N.º do Bilhete de Identidade: 13781412

Título da Tese de Mestrado:
The role of phospholipids and of the actin-binding protein cofilin in *Saccharomyces cerevisiae* acetic acid-induced apoptosis

Orientadores:
Professora Doutora Maria João Marques Ferreira Sousa Moreira
Professora Doutora Maria Manuela Sansonetty Gonçalves Côrte-Real

Instituição de Acolhimento:
Centro de Biologia Molecular e Ambiental (CBMA)

Ano de Conclusão: 2013

Designação do Mestrado:
Mestrado em Genética Molecular

Universidade do Minho, 31 de Outubro de 2013

Assinatura: _____

AGRADECIMENTOS

Às minhas orientadoras, Professora Maria João Sousa e Professora Manuela Côrte-Real primeiramente pela oportunidade em desenvolver a minha Tese de Mestrado neste grupo de investigação. A ambas, o meu mais sincero obrigada pelo apoio, preocupação e atenção que demonstraram ao longo deste ano. O carinho e segurança que me transmitiram, em todos os momentos mas, especialmente na fase final deste trabalho, jamais serão esquecidos. Obrigada pela enorme partilha de conhecimentos que faz de mim hoje uma aluna mais capaz.

À Doutora Susana Chaves, pela colaboração em momentos cruciais e pelas inúmeras palavras de reconhecimento e de incentivo.

A todos os colegas da Micro I obrigada por terem tornado tão agradáveis as minhas horas de trabalho. À Andreia, António, Dário, Flávio e Sara endereço o meu especial agradecimento pelos conhecimentos transmitidos e ajuda na parte experimental desta tese. À Ana Marta por ter sido a pessoa que me auxiliou inicialmente e por ao longo deste ano responder sempre prontamente às minhas questões. Aos amigos Lisandra, Marco, Sara, Selma e Vera agradeço pela colaboração e pela imensa vontade em ajudar que sempre demonstraram, um obrigada do tamanho da amizade que sinto por todos vocês. A estes e ainda, à Carla, Cláudia, Raquel e Suellen obrigada pela preocupação, presença e por me proporcionarem ao longo deste ano momentos de verdadeira amizade, conforto e felicidade.

Aos meus pais, pela educação, pela oportunidade em prosseguir os meus estudos, pela imensa colaboração nesta recta final, pelo orgulho que demonstram e por estarem sempre presentes, obrigada!

À minha irmã, Vera, pela amizade, por todo o apoio e interajuda. A ti e ao Zé Alberto, um obrigada especial pelo incentivo e por todo o orgulho que têm vindo a demonstrar no meu percurso.

Ao Emídio, por todo o companheirismo, pela compreensão na ausência, pela paciência, pela inigualável força sempre transmitida. Obrigada por fazeres parte da minha vida!

A todos os restantes amigos, técnicos e auxiliares do Departamento de Biologia, o meu mais sincero obrigada!

Por fim, gostaria de agradecer ao Centro de Biologia Molecular e Ambiental (CBMA), ao programa FEDER através do POFC–COMPETE e ao projecto da FCT PEst-C/BIA/UI4050/2011 e PTDC/AGR-ALI/102608/2008 pelo financiamento deste trabalho.

The role of phospholipids and of the actin-binding protein cofilin in *Saccharomyces cerevisiae* acetic acid-induced apoptosis

ABSTRACT

Several compounds and physiological conditions have been described to induce a programmed cell death (PCD) process in *Saccharomyces cerevisiae* that shares many of the morphological and biochemical hallmarks of mammalian apoptosis. This evidence makes this yeast a suitable model organism to solve some unanswered questions of apoptosis and of its regulation, namely in what regards the mitochondrial involvement.

In animal cells, cardiolipin (CL) and phosphatidylserine (PS) have been shown to bear a fundamental role in apoptosis signaling. Since there was no information reported on the role of these lipids in yeast cell death, the aim of the present study was to elucidate their involvement in apoptosis induced by acetic acid. Our results show, through inhibition of CL synthesis (*crd1*Δ), that the presence of CL is not required for apoptosis to occur. Oppositely, the deletion of *TAZ1*, involved in CL maturation, results in higher survival. However, this mutant displays an early loss of the plasma membrane integrity and metabolic activity, suggesting that it is dying later but by a necrotic process. An identical profile was observed in *cho1*Δ mutant, lacking PS synthesis, suggesting that either the presence of mature CL or PS are fundamental to trigger apoptosis in yeast cells. Curiously, the absence of Ups1p, involved in the translocation of phosphatidic acid (PA) to the inner mitochondria membrane, lead to a higher resistance associated with higher preservation of the plasma membrane integrity, suggesting that PA or the phospholipids synthetized from PA are essential for yeast apoptosis. Similarly, the deletion of *MDM10* or *MDM12*, encoding two proteins belonging to the ERMES (ER-Mitochondria Encounter Structure) complex which regulates the phospholipid trafficking between endoplasmic reticulum and mitochondria, results also in higher resistance. Although a decrease in levels of CL has been reported for all mutants, except *cho1*Δ, the differences observed suggest that these proteins can be involved in the synthesis or regulation of other phospholipids, whose levels inside the cell are able to mediate the cell death process.

Cofilin, an actin-binding protein, appears be able to regulate a mitochondrial-mediated mammalian apoptosis. Although in yeast cells there is no information about its apoptotic role, preliminary data revealed that a stress-specific interaction between cofilin and Por1p could exist. Our results appear to indicate that, after acetic acid treatment, cofilin is translocated to mitochondria where, by interaction with Por1p, prevents the mitochondrial outer membrane permeabilization, functioning like Por1p as a negative regulator of apoptosis. Curiously, depending on the mutated cofilin residues, it has an opposite impact on acetic acid induced-apoptosis.

In summary, our results show that the phospholipid composition of mitochondrial membranes and the cofilin translocation to this organelle seem to have an important role in the mediation of the apoptotic process induced by acetic acid. Identification of phospholipids or proteins involved in apoptosis regulation will provide novel strategies for the treatment of different pathologies associated with apoptosis dysfunctions such as cancer and neurodegenerative diseases.

O papel dos fosfolípidos e da cofilina, uma proteína de ligação à actina, na apoptose induzida por ácido acético em *Saccharomyces cerevisiae*

RESUMO

A levedura *Saccharomyces cerevisiae* na presença de diferentes compostos tóxicos ou em condições fisiológicas específicas desencadeia uma morte celular programada (PCD) que partilha muitas das características morfológicas e bioquímicas da apoptose em mamíferos tornando esta levedura um modelo adequado para desvendar alguns dos mecanismos ainda por esclarecer da apoptose e da sua regulação, nomeadamente no que se refere ao envolvimento mitocondrial.

Nas células animais, a cardiolipin (CL) e a fosfatidilserina (PS) mostraram ter um papel fundamental na sinalização da apoptose. Na levedura, no entanto, não existe informação relativa ao papel destes lípidos na morte celular, pelo que foi objectivo do presente trabalho estudar o envolvimento destas moléculas na apoptose induzida por ácido acético. Os nossos resultados mostram, através da inibição da síntese de CL (*crd1*Δ), que a presença de CL não é essencial para que apoptose ocorra. Contrariamente, a deficiência no gene *TAZ1*, envolvido na maturação da CL, resulta numa maior resistência. Contudo, este mutante perde a integridade da membrana plasmática e a actividade metabólica precocemente sugerindo a ocorrência de uma morte necrótica. Um perfil idêntico foi observado no mutante *cho1*Δ, onde não há síntese de PS, sugerindo que quer a presença de CL madura ou PS são fundamentais para desencadear apoptose na levedura. Curiosamente, a ausência da proteína Ups1p, envolvida na translocação de ácido fosfatídico (AP) para a membrana interna da mitocôndria, resulta numa maior resistência associada à preservação da integridade da membrana plasmática, sugerindo que o AP ou os fosfolípidos sintetizados a partir do AP são essenciais para este processo. Da mesma forma, a deficiência nos genes *MDM10* e *MDM12*, que codificam duas proteínas do complexo ERMES (*ER-Mitochondria Encounter Structure*), que regula o tráfico de fosfolípidos entre o retículo endoplasmático e a mitocôndria, resulta também numa maior resistência. Embora em todos os mutantes, à excepção do *cho1*Δ, tenha sido descrita uma redução nos níveis de CL, as diferenças observadas sugerem que estas proteínas podem estar envolvidas na síntese ou regulação de outros fosfolípidos cujos níveis dentro da célula são capazes de mediar o processo de morte celular.

A cofilina, uma proteína de ligação à actina, parece ser capaz de regular a apoptose mediada pela mitocôndria em mamíferos. Embora na levedura não exista qualquer informação acerca do seu papel na apoptose, dados preliminares mostraram que sob stresse podia existir uma interacção cofilina-Por1p. Os nossos resultados parecem indicar que, após tratamento com ácido acético, a cofilina é translocada para a mitocôndria onde, por interacção com o Por1p, inibe a permeabilização da membrana externa da mitocôndria, funcionando, tal como o Por1p, como um regulador negativo da apoptose. Curiosamente, dependendo dos resíduos da cofilina mutados, esta pode ter diferentes funções durante a apoptose induzida por ácido acético.

Em resumo, os nossos resultados mostram que a composição em fosfolípidos das membranas mitocondriais e a translocação de cofilin para este organelo parecem ter um papel importante na mediação do processo de apoptose induzida por ácido acético. A identificação de fosfolipídios ou proteínas envolvidas na regulação da apoptose fornecerá novas estratégias para o tratamento de diferentes patologias associadas a disfunções na apoptose, tais como o cancro e doenças neurodegenerativas.

TABLE OF CONTENTS

AGRADECIMENTOS iii

ABSTRACT v

RESUMO vii

LIST OF ABBREVIATIONS xi

CHAPTER 1 1

1.1 Apoptosis 3

1.2 Mitochondrial involvement in apoptosis 5

1.3 Programmed cell death in yeast 11

1.3.1 Acetic acid 14

1.3.1.1 Production and degradation 14

1.3.1.2. A stimulus of cell death 15

1.3.2 Mitochondrial-mediated apoptotic pathway induced by acetic acid 15

1.4 References 19

CHAPTER 2 27

2.1 Introduction 29

2.1.1 Cardiolipin biosynthetic pathway 30

2.1.2 Cardiolipin remodeling and the Barth Syndrome 31

2.1.3 Ups1p and the role of ERMES complex in the CL biosynthetic pathway: facts and hypotheses 32

2.1.4 Cardiolipin distribution within the mitochondria 35

2.1.5 The cardiolipin requirement in the respiratory chain and other functions 35

2.1.6 The role of cardiolipin in apoptosis 36

2.1.6.1 Cardiolipin peroxidation 36

2.1.6.2 Cardiolipin and cytochrome *c* 37

2.1.6.3 Cardiolipin as an activating platform for the mitochondrial tBid recruitment 38

2.1.6.4 Cardiolipin as a new model to explain caspase-8 activation 40

2.1.6.5 Requirement of cardiolipin for the functionality of PTP components 41

2.1.7 Phosphatidylserine – biosynthesis, intracellular localization and role in apoptosis 43

2.2 Main aims and study overview 45

2.3 Material and Methods 47
2.3.1 Yeast strains and plasmids 47
2.3.2 Growth conditions and cell death assays 48
2.3.3 Analysis of apoptotic markers 48
2.3.3.1 Assessment of the plasma membrane integrity / PI staining 48
2.3.3.2 Mitochondrial membrane potential 49
2.3.3.3 ROS production 49
2.3.3.4 Mitochondrial degradation 50
2.3.4 Flow cytometry 50
2.3.5 Fluorescence Microscopy 50
2.4 Results 51
2.5 Discussion 59
2.6 References 66

CHAPTER 3 73
3.1 Introduction 75
3.2 Main aims and study overview 81
3.3 Material and Methods 83
3.3.1 Yeast strains 83
3.3.2 Growth conditions and cell death assays 84
3.3.3 Preparation of mitochondria 85
3.3.4 Western blot analysis 85
3.3.5 Fluorescence microscopy 86
3.4 Results 87
3.5 Discussion 93
3.6 References 97

Concluding remarks and Future Prospects 101

LIST OF ABBREVIATIONS

7-AAD - 7-Aminoactinomycin

AAC - ADP/ATP Carrier

ABP - Actin-Binding Protein

ADF - Actin Depolymerizing Factor

AIF - Apoptosis Inducing Factor

AMID - AIF-homologous Mitochondrion-associated Inducer of Death

ANT - Adenine Nucleotide Translocator

Apaf-1 – Apoptotic Protease Activating Factor-1

ATP - Adenosine Triphosphate

BH - Bcl-2 Homology

BI - Bax Inhibitor

BTHS - Barth Syndrome

Caspase – Cystein-dependent aspartate-specific protease

CatD - Cathepsin D

CDP-DAG - Cytidinediphosphate-Diacylglycerol

CFU - Colony Forming Units

CL - Cardiolipin

CMP – Cytidine Monophosphate

CsA - Cyclosporin A

CyP-D - Cyclophilin D

cyt *c* - cytochrome *c*

DHE - Dihydroethidium

DiOC$_6$(3) - 3,3' Dihexyloxacarbocyanine iodide

DISC – Death Inducing Signaling Complex

DNA – Deoxyribonucleic Acid

Endo G - Endonuclease G

ER - Endoplasmic Reticulum

ERMES - ER-Mitochondria Encounter Structure

ETC - Electron Transport Chain

FADD - Fas-Associated Death Domain

FDA - Fluorescein Diacetate

FS - Forward Scatter

FUN-1 - 2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide-1

G-3-P - Glycerol-3-Phosphate

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GFP - Green Fluorescent Protein

H_2O_2 - Hydrogen Peroxide

Hk - Hexokinase

HtrA2/Omi - High temperature requirement A2

IAP - Inhibitor of Apoptosis

IMM - Inner Mitochondrial Membrane

IMS - Intermembrane Space

LB – Lysogeny Broth

LMP - Lysosomal Membrane Permeabilization

LPC – Lyso-Phosphatidylcholine

MAC - Mitochondrial Apoptosis-inducing Channel

MAM - Mitochondrial Associated Membrane

MES - 2-(N-Morpholino) Ethanesulfonic acid

MLCL - Monolysocardiolipin

MOMP - Mitochondrial Outer Membrane Permeabilization

mtGFP - matrix-targeted GFP

NADH - Nicotinamide Adenine Dinucleotide

NADPH - Nicotinamide Adenine Dinucleotide Phosphate-oxidase

Nma - Nuclear mediator of apoptosis

OD - Optical Density

OMM - Outer Mitochondrial Membrane

PA - Phosphatidic Acid

PBS - Phosphate Buffered Saline

PC - Phosphatidylcholine

PCD - Programmed Cell Death

PCR - Polymerase Chain Reaction

PE - Phosphatidylethanolamine

PEG - Polyethylene Glycol

PG - Phosphatidylglycerol

PGP - Phosphatidylglycerol Phosphate

Pi - Phosphate

PI - Phosphatidylinositol

PI - Propidium Iodide

PiC - Phosphate Carrier

PIP_2 - Phosphatidylinositol 4,5-bisphosphate

PS - Phosphatidylserine

PTP - Permeability Transition Pore

RFP - Red Fluorescent Protein

ROS - Reactive Oxygen Species

rpm - rotations per minute

SAM - Sorting and Assembly Machinery

SC - Synthetic Complete

SD - Standard Deviation

Smac/Diablo - Second mitochondria derived activator of caspase/Direct IAP binding protein with low pI

SS - Side Scatter

STS - Staurosporine

tBid - truncated Bid

TnCl - Taurine Chloramine

VDAC - Voltage-Dependent Anion Channel

Ybh3 - yeast BH3-only

Yca1 - Yeast caspase

Ysp - Yeast suicide protein

CHAPTER 1

General Introduction

1.1 Apoptosis

In 1972 Kerr and colleagues applied for the first time the term apoptosis to explain a morphologically distinct form of cell death that could occur in many different cells and organisms. It was described as an active form of cell death in which the cell uses its own machinery, in a manner highly specialized, to commit suicide (Kerr et al., 1972). Apoptosis is the most common form of programmed cell death (PCD) in eukaryotes and undoubtedly the best characterized mechanism of cell death due to its direct involvement in many fundamental biological events, such as development, differentiation, embryogenesis, and regulation of the immune system. Normally, the purpose of apoptosis is to remove damaged cells or cells representing a threat to the integrity of the organism, like in response to infectious agents, replacing them by new cells.

Today it is known that apoptosis involves a high number of proteins strictly regulated at multiple points that directly or indirectly are responsible for the cell death process. Thus, when the cell death regulatory mechanisms fail, the development of many human malignancies, such as neurodegenerative diseases, autoimmune disorders and cancer, among others, will be favored (Elmore, 2007).

Apoptosis can follow two important routes termed as the extrinsic (or death receptor) and the intrinsic (or mitochondrial) pathways (Figure 1).

The extrinsic pathway is mediated by a subgroup of the tumor necrosis factor receptors (TNFR) superfamily. After ligand-receptor (e.g. FasL/FasR) interaction, the death domain of the activated receptor on the cytosolic side of the plasma membrane binds to the corresponding domain in the adapter molecule FADD (Fas-Associated Death Domain), which in turn recruits the death protease procaspase-8, resulting in its auto-catalytic activation (Muzio et al., 1998).

The intrinsic pathway can be initiated in response to extracellular cues and/or internal injuries. The signals produced by these stimuli can act through loss of apoptotic suppression or by direct apoptosis activation. Absence of certain growth factors, hormones and cytokines can lead to failure of apoptosis suppression. On the other hand, some stimuli, such as radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals trigger directly the apoptosis activation (Elmore, 2007). Often, these stimuli converge at the mitochondria via pro-apoptotic Bcl-2 family proteins, such as Bax and Bak (Dewson et al., 2008; Wolter et al., 1997). Apoptosis is highly regulated by a series of pro- and anti-apoptotic proteins belonging to the Bcl-2 family, possessing one or more Bcl-2 homology (BH) domain. In healthy conditions, the anti-apoptotic members of this family, namely Bcl-2 and Bcl-X_L, prevent cell death through

inhibition of the release of lethal factors from the mitochondrial intermembrane space (IMS). However, under apoptotic conditions, Bax and/or Bak activation, directly or indirectly, lead to the cytochrome *c* (cyt *c*) release from the mitochondrial compartment. Once in the cytoplasm and in the presence of dATP or ATP, cyt *c* binds to the adaptor protein Apaf-1 to form the apoptosome, resulting in activation of procaspase-9 and other downstream caspases (Li et al., 1997). Like cyt *c*, other released mitochondrial proteins have an important role in apoptosis, namely the apoptosis inducing factor (AIF), endonuclease G (Endo G), high temperature requirement A2 (HtrA2/Omi) and second mitochondria derived activator of caspase/direct IAP binding protein with low pI (Smac/Diablo) (Bajt et al., 2006 ; Du et al., 2000; Suzuki et al., 2001). These proteins promote cell death mainly by two mechanisms: direct promoting chromatin condensation and DNA degradation, as in the case of AIF, Endo G and HtrA2/Omi, or by interaction with anti-apoptotic proteins nullifying their action, as in the case of Smac/Diablo (Saelens et al., 2004).

The extrinsic and intrinsic pathways converge at the level of caspase-3 activation, through caspase-8 or apoptosome action, respectively. However, procaspase-3 cleavage is antagonized by IAP (Inhibitor of Apoptosis), which itself can be inhibited by Smac/Diablo or HtrA2/Omi. Cross-talk and integration between the two pathways is provided by Bid which in its truncated form (tBid), produced by caspase-8, can result in Bax/Bak activation (Desagher et al., 1999; Li et al., 1998). After procaspase-3 cleavage, a set of events culminate in the ordered dismantling and removal of the cell.

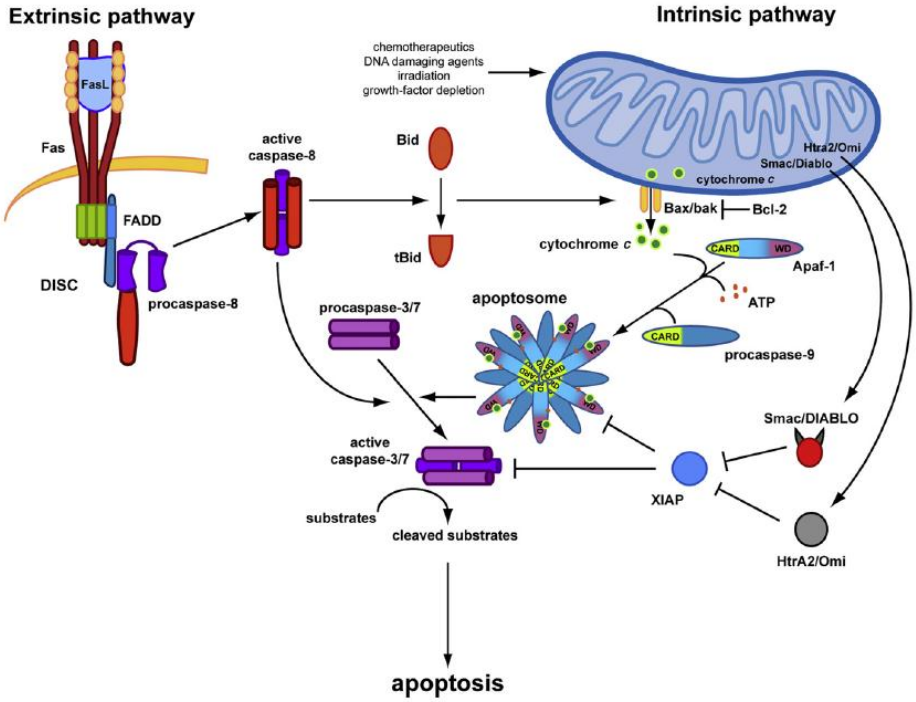

Figure 1 – The extrinsic and intrinsic apoptotic pathways and the cross-talk between them through the pro-apoptotic Bcl-2 family member Bid (scheme from Lamkanfi and Dixit, 2010).

As consequence of the activity of the internal machinery above described, cells exhibit some unique morphological and ultrastructural changes that allow distinguishing apoptosis from other processes of cell death, such as cell shrinkage, plasma membrane blebbing, exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane, chromatin condensation, nuclear fragmentation and finally, the formation of apoptotic bodies as result of cell fragmentation into compact membrane-enclosed structures (Saraste and Pulkki, 2000). Some of these changes underwent by the apoptotic cells, namely the PS externalization and apoptotic bodies formation allow the recognition and removal by phagocytic cells which are able to engulf and quickly remove them before the contents of the cell spill out onto surrounding cells and cause damage, preventing in this way an inflammatory response (Fadok et al., 1998).

1.2 Mitochondrial involvement in apoptosis

Mitochondria is a fundamental organelle in cellular homeostasis due to its implication in energy production, osmotic regulation, calcium homeostasis, modulation of redox status, inter-organelle communication, cell proliferation, senescence or even in apoptosis (reviewed in Rasola and Bernardi, 2007).

The mitochondrial mediated apoptotic pathway can involve the mitochondrial outer and/or inner membrane permeabilization due to the formation of pores, followed by rupture of the outer mitochondrial membrane (OMM) with release of pro-apoptotic factors localized in the IMS.

The mechanisms underlying the mitochondrial outer membrane permeabilization (MOMP) are still under debate and several models have been proposed, namely the formation of: i) a mitochondrial apoptosis-inducing channel (MAC) in the OMM, ii) a large conductance pore-forming complex, named permeability transition pore (PTP) in the inner mitochondrial membrane (IMM), iii) a lipid channel formed by ceramide or iv) oligomers of the voltage-dependent anion channel (VDAC).

The first model involves the pro-apoptotic proteins of the Bcl-2 family that can include the multidomain proteins Bax and Bak as well as the BH3-only proteins, like Bid and Bim (Kelekar and Thompson, 1998). Oppositely to Bak, that in healthy cells is present in the OMM, Bax and several BH3-only proteins are cytosolic and translocated to mitochondria only after an apoptotic stimulus. Under physiological conditions, Bax and Bak activation is inhibited by anti-apoptotic members of Bcl-2 family, such as Bcl-2 and Bcl-X_L. To become in their active form, either Bax or Bak, undergo conformational changes that can

be induced by tBid. When activated, they can form homo- or heterodimers in the OMM resulting in formation of a pore also called MAC that allows the release of IMS proteins (Dewson et al., 2012; Lovell et al., 2008; Suzuki et al., 2000).

However, the size of some crucial pro-apoptotic proteins largely exceeds the pore diameter (approximately 4 nm) of MAC, and therefore the formation of an IMM channel, named PTP, was also proposed (Pavlov et al., 2001). According to this model, the irreversibly opening of PTP leads to an increase of the mitochondrial matrix volume, rupture of the OMM and ultimately to the release of IMS pro-apoptotic components.

Usually the IMM possesses low permeability to ions and solutes maintaining the electrochemical potential difference ($\Delta\psi_m$) across the membrane. Thus, the primary consequence of PTP prolonged opening is a mitochondrial depolarization due to equilibrium of the proton concentration at the two sides of the IMM. Consequently, this leads to an inability of complex V (ATP synthase) to synthesize ATP and could result, as consequence of electron transport chain (ETC) loss of activity, in reactive oxygen species (ROS) generation due to direct transfer of electrons to molecular oxygen. The increase of IMM permeability to solutes with molecular masses below 1.5 KDa induces the massive release of the calcium (Ca^{2+}) stored in the matrix and its swelling due to osmotic pressure caused by the high concentration of proteins. Consequently, the unrestricted mitochondrial cristae unfolding leads to the OMM rupture and release of the IMS proteins, including pro-apoptotic factors, to the cytosol. The PTP prolonged opening is highly regulated and it only occurs when a set of important requirements are fulfilled, including low mitochondrial membrane potential ($\Delta\psi_m$), high mitochondrial Ca^{2+} concentration and low phosphate (Pi) concentration, among others. Oppositely, its opening is inhibited by high $\Delta\psi_m$, cyclosporin A (CsA), magnesium (Mg^{2+}) and through action of some recognized inhibitors, such as anti-apoptotic proteins of the Bcl-2 family (Bernardi, 1999). Interestingly, the transient PTP opening has a physiological role unrelated to death stimuli that can involve matrix volume and pH regulation, redox equilibrium, cristae remodeling, protein import and a fast Ca^{2+} release mechanism (Rasola and Bernardi, 2007).

The PTP molecular structure is only partially understood. The present consensus model proposes a supramolecular complex, where a subset of proteins has been proposed to be the core components of the channel. These proteins include the matrix chaperone cyclophilin D (CyP-D), the Adenine Nucleotide Translocator (ANT), VDAC, the mitochondrial Pi carrier (PiC) and the ATP synthase (Figure 2).

ANT, an ADP/ATP translocator, is a relative small protein (about 300 AA residues) that is inserted in the IMM. Although with much lower efficiency, ANT can also transport other solutes, such as

phosphoenolpyruvate, pyrophosphate and creatine phosphate (Shug and Shrago, 1973; Soboll et al., 1997). In addition, ANT has an important role in regulation of $\Delta\psi_m$ and ROS production (Korshunov et al., 1997). It can adopt two conformations shapes: *c* and *m*, that were associated to the PTP opening and closure, respectively (Schultheiss and Klingenberg, 1984). Although it is believed that ANT is one of the most important structural components of the PTP, some controversy has been raised about its role. It was shown that mouse liver cells lacking their two ANT isoforms remained competent to respond to various initiators of cell death, resulting in release of cyt *c*. Thus, it was proposed that ANT deficiency can be compensated in the IMM of these cells by other ANT-like channel of the same carrier family and that the ANT contribution in PTP formation may depends on specific conditions (Kokoszka et al., 2004). Although these results suggest that ANT is a non-essential component of PTP, due to the presence of another ANT isoform (ANT3) in human cells, the ANT role cannot be excluded.

CyP-D, a small mitochondrial water-soluble protein, was reported as an ANT modulator binding to its matrix side (Halestrap and Davidson, 1990). Experiments employing a CyP-D affinity column demonstrated a direct interaction between CyP-D and ANT. In addition, when the column was treated with CsA no binding between them was observed, proving that in fact CsA is an inhibitor of the PTP having CyP-D as target (Woodfield et al., 1998). Despite CyP-D regulatory role, PTP can be formed and opened in its absence (Basso et al., 2005).

VDAC is present in the OMM where it forms relatively large internal channels with a pore diameter of 2.5-3.0 nm that possess electrophysiological properties extremely similar to those of the PTP and which are affected by the same factors (Rasola and Bernardi, 2007; Szabo et al., 1993; Szabo and Zoratti, 1993). VDAC mediates the complex interaction between mitochondria and cytoplasm by transporting anions, cations, ATP, Ca^{2+} and metabolites, among others. During apoptosis, when PTP is in its open state, VDAC preferentially conducts anions. In mammalian cells, VDAC has three isoforms (VDAC1, VDAC2 and VDAC3) and it was described that the absence of one of these can be compensated, in some cases, by the presence of the other isoforms (Lemasters and Holmuhamedov, 2006). Curiously, the silencing of *VDAC1* affected cell growth and mitochondrial ATP synthesis, without changing the basic properties of the PTP. Thus, the participation of VDAC in PTP composition remains an open question (Abu-Hamad et al., 2006).

Hexokinase (Hk) was described as the main VDAC regulator through its binding at the OMM. Hk catalyzes the first step of glycolysis providing a functional link between mitochondria and the cytosol by governing the preferential utilization of mitochondrial ATP for glucose phosphorylation, coupling glycolysis

to oxidative phosphorylation (Robey and Hay, 2006). The Hk-VDAC association, mediated by the action of the serine/threonine kinase Akt, is directly related to the PTP state, in which the interaction of the two proteins implies the PTP closure and the Hk dissociation the PTP opening proving the metabolic link of the apoptotic process (Majewski et al., 2004).

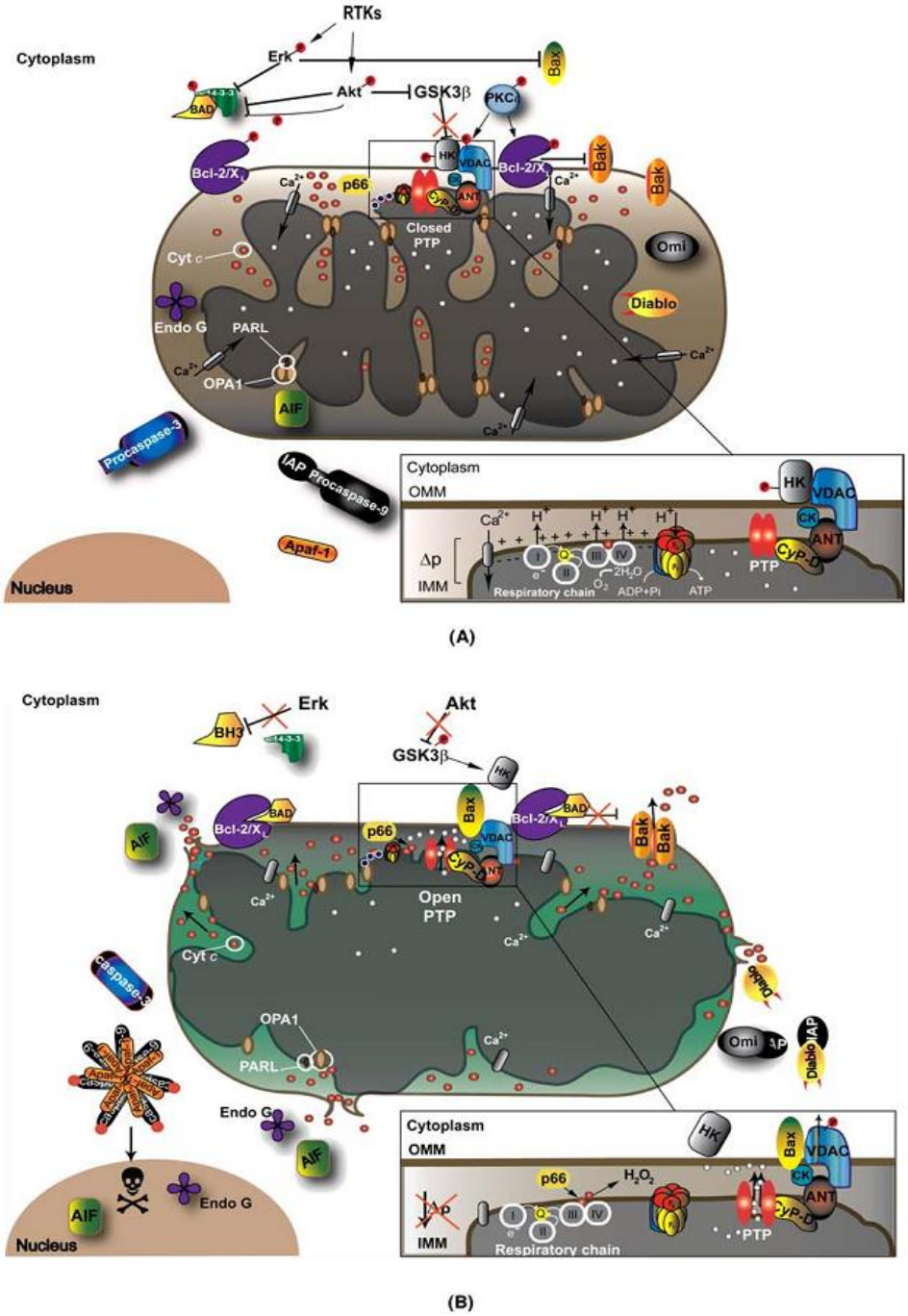

Figure 2 – Representation of the main molecules involved in the MOMP through PTP opening in the IMM during cell death. (A) Healthy cells with PTP closed and all pro-apoptotic machinery inactivated. (B) Pro-apoptotic molecules activation and the most important intracellular changes after an apoptotic stimulus (scheme from Rasola and Bernardi, 2007).

One mitochondrial phosphate carrier, PiC, was also demonstrated to have an important role in PTP conformation. It was shown that PiC induces cyt *c* release and caspase-9-dependent cell death. However, in its absence cyt *c* release as well as other apoptotic events were delayed but not totally extinguished. Thus, it suggested that PiC can be involved in the PTP opening although it is not fundamental for this process (Alcala et al., 2008). It was reported that PiC can bind to ANT1 and VDAC1 or CyP-D in the channel, making unclear its real function (Alcala et al., 2008; Leung et al., 2008). Therefore, it is needed to clarify whether the PiC is a component or regulator of the PTP. Interestingly, Pi is a PTP inhibitor and the regulation of PiC by this substrate could affect the pore opening.

Differently to the mentioned above new evidences indicated that PTP is formed by dimers of the ATP synthase, which curiously is regulated by the PTP modulators (such as Ca^{2+}, Mg^{2+}, adenine nucleotides and Pi). Interestingly, this new complex is independent of the presence of OMM components suggesting a dual function for complex V in the ATP synthesis and PTP formation (Bonora et al., 2013; Giorgio et al., 2013).

Another mechanism, associated with ceramide channels formation in the OMM, has also been implicated in the release of apoptogenic factors from mitochondria. Ceramide is a sphingosine-based signaling lipid that can regulate several cellular processes being involved in differentiation, proliferation, and apoptosis. One of the first evidences of ceramide pro-apoptotic function was the blockage of its generation lead to the apoptosis inhibition (Alphonse et al., 2002). Though ceramide is produced in different cell compartments it was shown that ceramide induces cell death specifically when generated in mitochondria in response to a number of apoptosis-inducing factors (Mullen and Obeid, 2012). Oppositely to other membrane lipids, the intermolecular structure of ceramide, namely the hydrogen bonds, allows the production of columns of ceramide residues that result in formation of channels able to transport apoptogenic factors to the cytoplasm. Moreover, its increased synthesis is associated with ROS generation, ATP depletion, loss of the mitochondrial $\Delta\psi_m$, among other alterations that facilitate cell death (Siskind, 2005). So, the inhibition of ceramide channels formation could be integrated in some strategies of apoptosis prevention, namely in neurodegenerative diseases.

Finally, another mechanism involving VDAC oligomerization has also been described to explain the mitochondrial release of pro-apoptotic factors during apoptosis. As referred to above, VDAC is more commonly associated to PTP formation although its function has been found dispensable. Additionally, some studies have suggested that VDAC can mediate the activity of the pro-apoptotic proteins Bak and Bax (Roy et al., 2009; Yamagata et al., 2009). Nevertheless, VDAC oligomerization appears itself to be a

mechanism of apoptosis induction in many cell types and in response to a high number of apoptotic inducers. The clear correlation between the levels of VDAC oligomerization and apoptosis was, in part, demonstrated when an apoptosis inhibitor prevented VDAC oligomerization. However, the presence of a caspase inhibitor did not change VDAC oligomerization and cyt *c* release. Curiously, the pore size formed by VDAC oligomerization is not large enough to allow the transport of some apoptogenic factors, including cyt *c.* Therefore, the formation of VDAC homo- and hetero-oligomers containing VDAC1 and the pro-apoptotic proteins Bax and Bak was suggested (Keinan et al., 2010; Shoshan-Barmatz et al., 2008; Shoshan-Barmatz et al., 2010).

Independently of the mechanism whereby the release of apoptogenic factors happens, mitochondria undergo significant ultrastructural changes that can contribute to cell death. In this context, Drp1/Mfn2, involved in fission/fusion, frequently show an altered expression through Bax/Bak action. Most often, mitochondrial morphology changes from a tubular network to a punctate structure in the course of apoptosis suggesting a relation between this form of regulated cell death and mitochondrial fission/fragmentation. In this way, Bax/Bak indirectly facilitates MOMP and cell death by destabilizing the OMM (Breckenridge et al., 2003; Reis et al., 2012). Oppositely, it was recently demonstrated that Bax regulates the sensitivity of cells to undergo primary necrosis through a Bax-driven mitochondria fusion mechanism that lowers the threshold for PTP opening (Whelan et al., 2012). This is distinct from the role of Bax in apoptosis and is in accordance with some studies, which provided hints that Bcl-2 proteins may regulate cell death in situations where necrosis was thought to be involved (Hochhauser et al., 2003).

Additionally, the remodeling of mitochondrial cristae might also contribute to the release of apoptogenic factors. The intra-cristae regions, that contain approximately 85% of the total cyt *c* pool, can form a barrier to the free diffusion of this or other proteins (Detmer and Chan, 2007). Therefore, remodeling of mitochondrial cristae, at least partially controlled by PARL and Opa1 protein interaction, to bring the apoptogenic factors into the IMS, mitochondrial fission to help the membrane destabilization and either formation of channels into the OMM or its rupture by PTP opening are crucial events for triggering the apoptotic cascade (Cipolat et al., 2006).

More recently, it was uncovered a lysosome-mitochondria crosstalk during apoptosis in which lysosomes have an important regulatory role. Depending on the apoptotic stimulus not only the OMM, but also the lysosomal membrane can suffer permeabilization and release proteins that directly or indirectly interact with others in the cytosol amplifying the death signal. However, only the moderate lysosomal membrane permeabilization (LMP), with release of some cathepsins, is responsible to trigger this type of

cell death, while extensive lysosomal permeabilization can lead to necrosis (Kirkegaard and Jaattela, 2009). Cathepsin D (CatD) has been considered as a central player in this response although its presence could have contrasting effects depending on the cell line and cellular context. In most cases, CatD regulates the intrinsic pathway through stimulation of cyt *c* release from mitochondria. For this reason, CatD has been explored as an anti-tumor chemotherapeutic target (Benes et al., 2008).

In conclusion, although much information is still necessary for a more complete understanding of many of the mechanisms underlying the intrinsic apoptotic pathway, it seems that it is mainly regulated by Bcl-2 family members through its association to other proteins, or by the formation of channels on the OMM. In addition, the reported lysosome involvement in apoptosis proves that this cell death mode can be mediated by an intrinsic network of organelles and proteins, which act together to fully trigger the death process.

1.3 Programmed cell death in yeast

Over the last years, yeast has become the favorite research tool in several areas of cell biology. This can be explained based on its easy handling and technical tractability together with the functional advantage of this organism being a eukaryote. Furthermore, it has a high level of phylogenetic conservation of biochemical pathways and regulators when compared with mammals.

Although nowadays yeast has become a biological model to study PCD, the first studies reporting an apoptotic program in yeast generated considerable controversy (Skulachev, 2002). It was questioned why a unicellular organism should have developed and conserved a suicide program during evolution once the demise of a single cell would imply the organism death. However, in the last years this classical point of view has been discarded because many works proved that yeast populations should be understood as a multicellular community of interacting individuals, and not only as a group of independent unicellular organisms. In this context, the death of a particular cell might therefore be beneficial for the clone, promoting its survival. So, this altruistic yeast behavior can occur to eliminate infertile or damaged cells after failed mating, genetic recombinants non-adapted to the environment or old cells (Büttner et al., 2006; Gourlay et al., 2006).

The definition of PCD and its nomenclature applied to the yeast exacerbated the controversies. Although it was shown that yeast PCD plays a role in responding to virus, it has not been proven that this

PCD process in yeast cells is indeed true apoptosis and therefore many researches prefer the term apoptotic-like cell death or, more generally, PCD (Breinig et al., 2006; Ivanovska and Hardwick, 2005).

The apoptotic markers observed in a mutant strain caring a mutation in the AAA-ATPase gene *CDC48* were the first evidence of this type of PCD in yeast (Madeo et al., 1997). Nowadays, the occurrence of an apoptotic process is also supported by the fact that yeast encodes several orthologues of mammalian cell death regulators, although they also lack many others.

It was reported that the disruption of the metacaspase Mca1p/Yca1p, orthologue of mammalian caspases, reduced the occurrence of cell death in aged cultures showing that, similar to caspases, the yeast metacaspase plays an important role in the execution of cell death (Madeo et al., 2002). Despite of functional conservation, they have different cleavage specificity raising doubts about a common source of both proteins. Caspases cleave their substrates after aspartate (acid residue) whereas metacaspases cleave after arginine or lysine (basic residues). However, TSN (Tudor Staphylococcal Nuclease) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) are common biological substrates for metacaspases and caspases during cell death (Silva et al., 2011; Sundstrom et al., 2009). Thereby, it was possible to conclude that, although caspases and metacaspases have phylogenetic distance and differences in their proteolytic mechanisms, they have some common target molecules in cell death indicating that they constitute functional orthologues.

Curiously, the uncovering that yeast apoptosis can occur independently of Yca1p activity suggested the intervention of other proteins with caspase-like activity or, like in mammals, an alternative pathway independent of metacaspases (Madeo et al., 2009).

In yeast, orthologues of cyt *c*, AIF, Endo G and Htr2/Omi were equally uncovered and the first three proteins have the same subcellular localization as their mammalian conterparts (Büttner et al., 2007; Fahrenkrog et al., 2004; Wissing et al., 2004). Oppositely, Nma111p (Nuclear mediator of apoptosis), yeast orthologue of the mammalian mitochondrial Htr2/Omi, is found in the nucleus, where it remains upon apoptotic induction, killing cells via its serine protease activity (Fahrenkrog et al., 2004).

Although cyt *c* release from mitochondria has been observed and suggested to be associated to the yeast apoptotic-like PCD, it remains unclear if there is an apoptosome-like structure activated by this protein, and what is the causal role of cyt *c* release. Curiously, the involvement of cyt *c* in metacaspase activation was suggested after exposition to hyperosmotic stress (Silva et al., 2005).

AIF and AIF-homologous mitochondrion-associated inducer of death (AMID) are both mitochondrial flavoproteins that trigger caspase-independent apoptosis. In yeast it was demonstrated that

overexpression of the internal NADH dehydrogenase Ndi1p, the yeast AMID homologue, can be responsible for triggering a PCD process. So, this protein, which catalyzes the oxidation of intramitochondrial NADH and is localized in the IMM, appears to be another mitochondrion-associated protein implicated in yeast cell death. Curiously, this effect was repressed by increased respiration on glucose-limited media suggesting that there is an interaction between the regulatory network of energy metabolism and the Ndi1p-induced yeast cell apoptosis (Li et al., 2006).

Yeast encodes a protein containing an N-terminal domain analogous to the IAP protein in mammals, termed Bir1p, which is antagonized by Nma111p. Similarly to the homologue IAP in mammals, Bir1p has been reported to protect cells against apoptosis because yeast cells lacking *BIR1* are more sensitive to apoptosis induced by oxidative stress. In addition, the *BIR1* overexpression resulted in apoptosis inhibition (Walter et al., 2006).

For many years it was believed that yeast constituted a cellular environment devoid of orthologues of members of the Bcl-2 protein family, namely Bax or Bak and thus it was used for the expression of these human proteins simplifying the interpretation of functional analyses. However, recently it was reported the unexpected finding that yeast genome encodes a protein containing a functional BH3 domain, which was termed yeast BH3-only protein (Ybh3p). It displays a high degree of similarity with BH3 domains from higher eukaryotes and it was shown to mediate the intrinsic apoptotic pathway in acetic acid- and hydrogen peroxide (H_2O_2)-induced cell death. Ybh3p after activation undergoes a Bcl-X_L-inhibitable translocation from the cytosol to mitochondria where it leads to breakdown of mitochondrial membrane potential (Büttner et al., 2011). On the other hand, parallel studies by Cebulski and colleagues provided evidences that the *S. cerevisiae* protein encoded by the same open reading frame as Ybh3p, and renamed Bxi1p, is a bona fide homolog for the mammalian Bax-inhibitor (BI1). These authors showed that Bxi1p is an ER-localized protein that links the unfolded protein response and PCD (Cebulski et al., 2011).

As stated earlier in human cells, mitochondrial fragmentation, regulated by Drp1, has an important role in apoptosis favouring MOMP and the release of apoptogenic factors. In yeast cells undergoing apoptosis the same mitochondrial morphology transition was observed (Fannjiang et al., 2004). Additionally, in pheromone or amiodarone-induced cell death two other mitochondrial proteins were identified that also affect the fragmentation of the mitochondrial compartment in the course of apoptosis, named yeast suicide protein 1 (Ysp1p), and yeast suicide protein 2 (Ysp2p) (Pozniakovsky et al., 2005;

Sokolov et al., 2006). Nevertheless, more studies about the PCD in yeast are needed to clarify some important steps of this process.

PCD in yeast exhibits many of the morphological and biochemical hallmarks of mammalian apoptosis. These similarities include PS externalization to the outer layer of cytoplasmic membrane, chromatin condensation, nuclear fragmentation and ROS production (Madeo et al., 1997). Moreover, many of the basic components of the mammalian apoptotic machinery are conserved in yeast. This makes yeast a suitable model organism to solve some unanswered questions of apoptosis and its regulation.

Though apoptosis induction has been described in several yeast species, this process has been more explored in *Saccharomyces cerevisiae*. In this budding yeast species, PCD can be triggered in response to cell aging or under several stress conditions as UV exposure or oxidative (H_2O_2), saline and hyperosmotic stress, treatment with broad-spectrum compounds, such as acids (e.g. acetic acid), or by addition of specific compounds, including anti-fungal agents (Sharon et al., 2009).

1.3.1 Acetic acid

1.3.1.1 Production and degradation

Acetic acid is frequently an end product of the alcoholic fermentation carried out by *S. cerevisiae*. In this process, the production of this acid is usually proportional to the sugar concentration in the medium, and occurs to counterbalance the hyperosmotic stress of the medium through the generation of reducing equivalents (NADH and NADPH). This also allows the yeast cell to maintain the redox balance (Remize et al., 2000).

In the yeast *S. cerevisiae,* acetic acid transport and metabolism is repressed by glucose, which is the preferential carbon and energy source. So, when acetic acid is added to a medium with glucose, a diauxic growth will occur with the consumption of acetic acid taking place only after glucose depletion. As consequence of acetic acid accumulation in the medium, the yeast fermentative performance is negatively affected. The mechanism of acetic acid transport depends on the extracellular pH and growth conditions, as well as on the carbon source used for growth. When cells are growing in a medium with glucose and acetic acid at low pH, e.g. pH 3.5, this acid is essentially undissociated (pK_a=4.75) and crosses the plasma membrane by simple diffusion (Casal et al., 1996). In this process the acetic acid passive influx can be enhanced by ethanol presence. Thus, under these conditions, the acid accumulates quickly inside the cell negatively affecting its metabolic activity and viability. Yeast cells are also able to

grow on acetic acid medium as the sole carbon and energy source. At pH 5.5 to 6.5 the dissociated form predominates and it is transported by acetate-proton symports. In this process, the presence of ethanol or buthanol inhibits the acetate transport in a noncompetitive way (Casal et al., 1998). Some proteins has been associated to acetate transport, such as Ady2p, Jen1p, a yeast transporter required for lactate uptake and Fps1p, a plasma membrane aquaglyceroporin channel (Casal et al., 1999; Paiva et al., 2004; Mollapour and Piper, 2007). When acetic acid is the sole carbon and energy source, it is metabolized to acetyl Co-A by the acetyl-CoA synthase 1 (Acs1p). Then, it is oxidized in the tricarboxylic acid cycle inside the mitochondria or can be channeled to the anaplerotic glyoxylate cycle and gluconeogenic pathway, both of which are repressed by the presence of glucose (Santos et al., 2003).

1.3.1.2. A stimulus of cell death

When acetic acid is not degraded its accumulation results in intracellular acidification, anion accumulation and inhibition of some cellular metabolic activities, including fermentation (Pampulha and Loureiro, 1989). Thus, the exposure of exponential-phase cells, growing in a glucose medium at pH 3.0, to acetic acid can compromise their survival. However, the cell death process induced by acetic acid depends on its concentration. Exposition to low acid concentrations (20-80 mM) results in a process similar to mammalian apoptosis. Oppositely, high acetic acid concentrations (120-200 mM) lead to alterations typical of necrosis with extensive cell disorganization and most of the intracellular structures destroyed (Ludovico et al., 2001). However, the acetic acid effect is also dependent on cell growth phase and stationary-phase cells exposed to high acetic acid concentrations (>120 mM) can also trigger an apoptotic like-PCD process (Ludovico et al., 2002). The morphological and biochemical analyses of these cells, namely the results of pulsed field gel electrophoresis of chromosomal DNA showed that DNA is cleaved into fragments of several hundred kilobases, and highlighted the similarities between this process and mammalian apoptosis (Ribeiro et al., 2006).

1.3.2 Mitochondrial-mediated apoptotic pathway induced by acetic acid

The crucial role of mitochondria in mammalian apoptosis has been extensively described (Eisenberg et al., 2007). More recently, the involvement of mitochondria in PCD triggered by different stimuli in yeast cells was also shown (Pereira et al., 2008).

The use of acetic acid as an inducer of apoptosis started in our Lab about twelve years ago and nowadays it has been extensively exploited in the yeast apoptosis field. In *S. cerevisiae*, it was demonstrated that apoptotic-like PCD induced by acetic acid (140 mM), that implies cyt *c* release from mitochondria to cytosol, is not affected by the loss of oxidative phosphorylation (oligomycin treatment). Curiously, in *ATP10* (coding for an ATPase assembly factor) mutant cells no release of cyt *c* was detected. Thus, it was thought that the PCD process could be related to the inhibition of ATP synthesis, but when associated with the absence of oligomycin effect, it was suggested that ATPase complex itself is essential for *S. cerevisiae* to undergo a PCD through its involvement in the mechanism of cyt *c* release. At the same time, acetic acid-induced cell death was not triggered in the respiratory deficient (*rho0Δ*) cells and this process was, at least partially, inhibited in cells that are unable to synthesize mature cyt *c*. The yeast apoptotic-like PCD induced by acetic acid is associated with reduced oxygen consumption, mitochondrial membrane potential and cytochrome *c* oxidase (COX) activity, as well as with accumulation of mitochondrial ROS and cyt *c* release (Ludovico et al., 2002). It was shown that besides cyt *c* other mitochondrial proteins are release. Indeed Aif1p (yeast AIF) and Nuc1p (yeast EndoG) undergo a mitochondrial-nuclear shuttling and that both are potent cell-death inducers (Wissing et al., 2004; Büttner et al., 2007). Oppositely to mammalian cells, the *YCA1* disruption attenuates the effect of *AIF1* overexpression in apoptosis stimulation (Wissing et al., 2004). On the other hand, the Nuc1p-mediated death is independent of Yca1p or Aif1p (Büttner et al., 2007). More recently, it was shown that Ndi1p is also involved in the acetic acid-induced PCD, in a process in which its apoptotic activity is dependent of the N-terminal cleavage (Cui et al., 2012).

In the early studies on yeast apoptosis the formation of a yeast PTP-like channel was hypothesized as the mechanism underlying the release of apoptogenic factors. This possibility, supported by the presence of orthologous of the mammalian PTP putative components, offered the possibility to use yeast to clarify the molecular nature of the PTP as well as to understand its regulatory and functional mechanisms. Yeast genetic approaches revealed that deletion of *POR1* (yeast VDAC) enhances the apoptotic-like PCD triggered by acetic acid, suggesting that Por1p may promote cell survival. On the other hand, yeast cells devoid of ADP/ATP carrier (AAC) protein (yeast ANT) were more resistant to this treatment which was associated with a delay in the emergence of important features of apoptosis, such as, chromatin condensation, DNA strand breaks and cyt *c* release. It was shown that this effect was not related to strain defects in oxidative phosphorylation or ATP synthesis, including ADP/ATP translocation activity. Furthermore, a normal pattern of caspase activity was observed in this strain suggesting that, under

these conditions, caspase activation is not associated with cyt *c* release. Although this process was delayed in *AAC* deficient strain it was not fully prevented which led the authors to propose that an alternative death pathway, which does not involve MOMP and cyt *c* release can occur. In addition, it was verified that the absence of Cpr3p (yeast orthologue of CyP-D) has no effect on acetic acid-induced cell death (Pereira et al., 2007). Curiously, Yca1p, whose caspase-like activity was shown under these conditions, demonstrated to be dispensable for acetic acid induced cell death (80 mM; 200 min). The occurrence of PCD in *yca1*Δ mutant suggested that, in the wild-type, it can happen through a non-caspase route (Guaragnella et al., 2006).

To better understand the mechanism underlying cyt *c* release, this process was investigated over the time course of acetic acid-induced cell death. The western blot analysis showed that its release happens early through coupled mitochondria still without MOMP suggesting that an alternative pathway not involving rupture of the OMM could explain cyt *c* release in yeast cells (Giannattasio et al., 2008).

Some results demonstrated that, during acetic acid induced-PCD, caspase activation is not dependent of the release of cyt *c*, but curiously in *yca1*Δ mutant cells no cyt *c* release was found (Pereira et al., 2007; Guaragnella et al., 2010a). Under these conditions, the absence of cyt *c* or metacaspase did not prevent the cell death, suggesting that an alternative pathway, that appears to imply H_2O_2 generation, can be activated (Giannattasio et al., 2005; Guaragnella et al., 2010a). Indeed, the presence of an antioxidant (NAC - N-acetyl-L-cysteine) prevented the induction of PCD by acetic acid in a wild-type strain. However, cells lacking Yca1p and/or cyt *c* were not protected by the presence of NAC suggesting that cell death when independent of cyt *c* release and Yca1p activation occurs in a ROS-independent manner (Guaragnella et al., 2010b).

The yeast BH3-only protein (Ybh3p) appears also to have an important role in acetic acid-induced apoptotic-like PCD. Upon treatment, Ybh3p enhanced PS externalization and DNA fragmentation whereas its BH3 domain deletion or complete deficiency resulted in reduced cell death occurrence (Büttner et al., 2011).

During apoptosis, mitochondria suffer a set of important structural and functional changes, which culminate in its degradation. Under acetic acid treatment, this process occurs through an autophagy-independent pathway involving the vacuolar protease Pep4p, the orthologue of the human CatD, located in the vacuole. This protease is translocated from vacuole to cytosol and it works together with AAC proteins that were proposed to relay a signal of mitochondrial dysfunction targeting its destruction. Interestingly, the deletion and overexpressing of *PEP4* promote sensitivity and resistance to the acetic

acid, respectively (Pereira et al., 2010). Thus, these results demonstrated that the apoptotic-like PCD induced by acetic acid in yeast does not only imply the involvement of mitochondria but can also be mediated by the vacuole, suggesting a complex regulation and interaction between these organelles.

S. cerevisiae such as previously mentioned is surely the most common yeast model for the study of PCD. Its completely sequenced genome and the corresponding databases available enhance the interest in this yeast species for the development of genomic technology. Therefore, a screening using the Euroscarf haploid mutant collection was recently performed in order to uncover genes involved in resistant and sensitive phenotypes under acetic acid-inducing apoptotic conditions. The genes identified with this study were clustered according to their biological function and known physical and genetic interactions providing this way a huge amount of information for future studies (Sousa et al., 2013).

1.4 References

Abu-Hamad, S., Sivan, S., and Shoshan-Barmatz, V. (2006). The expression level of the voltage-dependent anion channel controls life and death of the cell. Proceedings of the National Academy of Sciences *103*, 5787-5792.

Alcala, S., Klee, M., Fernandez, J., Fleischer, A., and Pimentel-Muinos, F. X. (2008). A high-throughput screening for mammalian cell death effectors identifies the mitochondrial phosphate carrier as a regulator of cytochrome *c* release. Oncogene *27*, 44-54.

Alphonse, G., Aloy, M., Broquet, P., Gerard, J., Louisot, R., Rousson, R., and Rodriguez-Lafrasse, C. (2002). Ceramide induces activation of the mitochondrial/caspases pathway in Jurkat and SCC61 cells sensitive to gamma-radiation but activation of this sequence is defective in radioresistant SQ20B cells. International Journal of Radiation Biology *78*, 821-835.

Bajt, M. L., Cover, C., Lemasters, J. J., and Jaeschke, H. (2006). Nuclear translocation of endonuclease G and apoptosis-inducing factor during acetaminophen-induced liver cell injury. The Journal of Toxicological Sciences *94*, 217-225.

Basso, E., Fante, L., Fowlkes, J., Petronilli, V., Forte, M. A., and Bernardi, P. (2005). Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. The Journal of Biological Chemistry *280*, 18558-18561.

Benes, P., Vetvicka, V., and Fusek, M. (2008). Cathepsin D-many functions of one aspartic protease. Critical Reviews in Oncology/Hematology *68*, 12-28.

Bernardi, P. (1999). Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiological Reviews *79*, 1141-1145.

Bonora, M., Bononi, A., Marchi, E. D., Giorgi, C., Lebiedzinska, M., Marchi, S., Patergnani, S., Rimessi, A., Suski, J. M., Wojtala, A., Wieckowski, M. R., Kroemer, G., Galluzzi, L., and Pinton, P. (2013). Role of the c subunit of the F_0 ATP synthase in mitochondrial permeability transition. Cell Cycle *12*, 674-683.

Breckenridge, D. G., Stojanovic, M., Marcellus, R. C., and Shore, G. C. (2003). Caspase cleavage product of BAP31 induces mitochondrial fission through endoplasmic reticulum calcium signals, enhancing cytochrome *c* release to the cytosol. The Journal of Cell Biology *160*, 1115-1127.

Breinig, F., Sendzik, T., Eisfeld, K., and Schmitt, M. J. (2006). Dissecting toxin immunity in virus-infected killer yeast uncovers an intrinsic strategy of self-protection. Proceedings of the National Academy of Sciences of the United States of America *103*, 3810-3815.

Büttner, S., Eisenberg, T., Carmona-Gutierrez, D., Ruli, D., Knauer, H., Ruckenstuhl, C., Sigrist, C., Wissing, S., Kollroser, M., Fröhlich, K.-U.*, et al.* (2007). Endonuclease G Regulates Budding Yeast Life and Death. Molecular Cell *25*, 233-246.

Büttner, S., Eisenberg, T., Herker, E., Carmona-Gutierrez, D., Kroemer, G., and Madeo, F. (2006). Why yeast cells can undergo apoptosis: death in times of peace, love, and war. The Journal of Cell Biology *175*, 521-525.

Büttner, S., Ruli, D., Vogtle, F. N., Galluzzi, L., Moitzi, B., Eisenberg, T., Kepp, O., Habernig, L., Carmona-Gutierrez, D., Rockenfeller, P.*, et al.* (2011). A yeast BH3-only protein mediates the mitochondrial pathway of apoptosis. The EMBO Journal, 1-14.

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

Casal, M., Cardoso, H., and Leão, C. (1998). Effects of ethanol and other alkanols on transport of acetic acid in *Saccharomyces cerevisiae*. Applied and Environmental Microbiology *64*, 665-668.

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

Cebulski, J., Malouin, J., Pinches, N., Cascio, V., and Austriaco, N. (2011). Yeast Bax Inhibitor, Bxi1p, is an ER-Localized Protein That Links the Unfolded Protein Response and Programmed Cell Death in *Saccharomyces cerevisiae*. PloS ONE *6*, e20882.

Cipolat, S., Rudka, T., Hartmann, D., Costa, V., Serneels, L., Craessaerts, K., Metzger, K., Frezza, C., Annaert, W., D'Adamio, L.*, et al.* (2006). Mitochondrial rhomboid PARL regulates cytochrome *c* release during apoptosis via OPA1-dependent cristae remodeling. Cell *126*, 163-175.

Cui, Y., Zhao, S., Wu, Z., Dai, P., and Zhou, B. (2012). Mitochondrial release of the NADH dehydrogenase Ndi1 induces apoptosis in yeast. Molecular biology of the cell *23*, 4373-4382.

Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J. C. (1999). Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome *c* release during apoptosis. The Journal of Cell Biology *144*, 891-901.

Detmer, S. A., and Chan, D. C. (2007). Functions and dysfunctions of mitochondrial dynamics. Nature Reviews Molecular Cell Biology *8*, 870-879.

Dewson, G., Kratina, T., Sim, H. W., Puthalakath, H., Adams, J. M., Colman, P. M., and Kluck, R. M. (2008). To trigger apoptosis, Bak exposes its BH3 domain and homodimerizes via BH3:groove interactions. Molecular Cell *30*, 369-380.

Dewson, G., Ma, S., Frederick, P., Hockings, C., Tan, I., Kratina, T., and Kluck, R. M. (2012). Bax dimerizes via a symmetric BH3:groove interface during apoptosis. Cell Death & Differentiation *19*, 661-670.

Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell *102*, 33-42.

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

Elmore, S. (2007). Apoptosis: a review of programmed cell death. Toxicologic Pathology *35*, 495-516.

Fadok, V. A., Bratton, D. L., Frasch, S. C., Warner, M. L., and Henson, P. M. (1998). The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell Death & Differentiation *5*, 551-562.

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

Fannjiang, Y., Cheng, W. C., Lee, S. J., Qi, B., Pevsner, J., McCaffery, J. M., Hill, R. B., Basanez, G., and Hardwick, J. M. (2004). Mitochondrial fission proteins regulate programmed cell death in yeast. Genes & Development *18*, 2785-2797.

Giannattasio, S., Atlante, A., Antonacci, L., Guaragnella, N., Lattanzio, P., Passarella, S., and Marra, E. (2008). Cytochrome c is released from coupled mitochondria of yeast en route to acetic acid-induced programmed cell death and can work as an electron donor and a ROS scavenger. FEBS Letters *582*, 1519-1525.

Giannattasio, S., Guaragnella, N., Côrte-Real, M., Passarella, S., and Marra, E. (2005). Acid stress adaptation protects Saccharomyces cerevisiae from acetic acid-induced programmed cell death. Gene *354*, 93–98.

Giorgio, V., von Stockum, S., Antoniel, M., Fabbro, A., Fogolari, F., Forte, M., Glick, G. D., Petronilli, V., Zoratti, M., Szabo, I.*, et al.* (2013). Dimers of mitochondrial ATP synthase form the permeability transition pore. Proceedings of the National Academy of Sciences of the United States of America *110*, 5887-5892.

Gourlay, C. W., Du, W., and Ayscough, K. R. (2006). Apoptosis in yeast-mechanisms and benefits to a unicellular organism. Molecular Microbiology *62*, 1515-1521.

Guaragnella, N., Bobba, A., Passarella, S., Marra, E., and Giannattasio, S. (2010a). Yeast acetic acid-induced programmed cell death can occur without cytochrome *c* release which requires metacaspase YCA1. FEBS Letters *584*, 224-228.

Guaragnella, N., Passarella, S., Marra, E., and Giannattasio, S. (2010b). Knock-out of metacaspase and/or cytochrome c results in the activation of a ROS-independent acetic acid-induced programmed cell death pathway in yeast. FEBS Letters *584*, 3655-3660.

Guaragnella, N., Pereira, C., Sousa, M. J., Antonacci, L., Passarella, S., Côrte-Real, M., Marra, E., and Giannattasio, S. (2006). YCA1 participates in the acetic acid induced yeast programmed cell death also in a manner unrelated to its caspase-like activity. FEBS Letters *580*, 6880-6684.

Halestrap, A. P., and Davidson, A. M. (1990). Inhibition of Ca^{2+}-induced large-amplitude swelling of liver and heart mitochondria by cyclosporinis probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl *cis-trans* isomerase and preventing it interacting with the adenine nucleotide translocase. Biochemical Journal *268*, 153-160.

Hochhauser, E., Kivity, S., Offen, D., Maulik, N., Otani, H., Barhum, Y., Pannet, H., Shneyvays, V., Shainberg, A., Goldshtaub, V.*, et al.* (2003). Bax ablation protects against myocardial ischemia-reperfusion injury in transgenic mice. American Journal of Physiology Heart and Circulatory Physiology *284*, 2351-2359.

Ivanovska, I., and Hardwick, J. M. (2005). Viruses activate a genetically conserved cell death pathway in a unicellular organism. The Journal of Cell Biology *170*, 391-399.

Keinan, N., Tyomkin, D., and Shoshan-Barmatz, V. (2010). Oligomerization of the mitochondrial protein voltage-dependent anion channel is coupled to the induction of apoptosis. Molecular and Cellular Biology *30*, 5698-5709.

Kelekar, A., and Thompson, C. B. (1998). Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends in Cell Biology *8*, 324-330.

Kerr, J., Wyllie, A., and Currie, A. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue. British Journal of Cancer *26*, 239-257.

Kirkegaard, T., and Jaattela, M. (2009). Lysosomal involvement in cell death and cancer. Biochimica et Biophysica Acta *1793*, 746-754.

Kokoszka, J. E., Waymire, K. G., Levy, S. E., Sligh, J. E., Cai, J., Jones, D. P., MacGregor, G. R., and Wallace, D. C. (2004). The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. Nature *427*, 461-465.

Korshunov, S. S., Skulachev, V. P., and Starkov, A. A. (1997). High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Letters *416*, 15-18.

Lamkanfi, M., and Dixit, V. M. (2010). Manipulation of host cell death pathways during microbial infections. Cell Host &Microbe Review *8*, 44-54.

Lemasters, J. J., and Holmuhamedov, E. (2006). Voltage-dependent anion channel (VDAC) as mitochondrial governator-thinking outside the box. Biochimica et Biophysica Acta *1762*, 181-190.

Leung, A. W. C., Varanyuwatana, P., and Halestrap, A. P. (2008). The mitochondrial phosphate carrier interacts with cyclophilin D and may play a a key role in the permeability transition. The Journal of Biological Chemistry *283*, 26312-26323.

Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell *94*, 491-501.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479-489.

Li, W., Sun, L., Liang, Q., Wang, J., Mo, W., and Zhou, B. (2006). Yeast AMID Homologue Ndi1p Displays Respiration-restricted Apoptotic Activity and Is Involved in Chronological Aging. Molecular Biology of the Cell *17*, 1802-1811.

Lovell, J. F., Billen, L. P., Bindner, S., Shamas-Din, A., Fradin, C., Leber, B., and Andrews, D. W. (2008). Membrane binding by tBid initiates an ordered series of events culminating in membrane permeabilization by Bax. Cell *135*, 1074-1084.

Ludovico, P., Rodrigues, F., Almeida, A., Silva, M. T., Barrientos, A., and Côrte-Real, M. (2002). Cytochrome c release and mitochondria involvement in programmed cell death induced by acetic acid in *Saccharomyces cerevisiae*. Molecular Biology of the Cell *13*, 2598-2606.

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

Madeo, F., Carmona-Gutierrez, D., Ring, J., Büttner, S., Eisenberg, T., and Kroemer, G. (2009). Caspase-dependent and caspase-independent cell death pathways in yeast. Biochemical and Biophysical Research Communications *382*, 227-231.

Madeo, F., Frohlich, E., and Frohlich, K. U. (1997). A yeast mutant showing diagnostic markers of early and late apoptosis. The Journal of Cell Biology *139*, 729-734.

Madeo, F., Herker, E., Maldener, C., Wissing, S., Lachelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S. J., Wesselborg, S., and Frohlich, K. U. (2002). A caspase-related protease regulates apoptosis in yeast. Molecular Cell *9*, 911-917.

Majewski, N., Nogueira, V., Bhaskar, P., Coy, P. E., Skeen, J. E., Gottlob, K., Chandel, N. S., Thompson, C. B., Robey, R. B., and Hay, N. (2004). Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. Molecular Cell *16*, 819-830.

Mollapour, M., and Piper, P. W. (2007). Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. Molecular and Cellular Biology *27*, 6446-6456.

Mullen, T., and Obeid, L. (2012). Ceramide and apoptosis: exploring the enigmatic connections between sphingolipid metabolism and programmed cell death. Anti-Cancer Agents in Medicinal Chemistry *12*, 340-363.

Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998). An Induced Proximity Model for Caspase-8 Activation. The Journal of Biological Chemistry *273*, 2926-2930.

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

Pampulha, M. E., and Loureiro, V. (1989). Interaction of the effects of acetic acid and ethanol on inhibition of fermentation in *Saccharomyces cerevisiae*. Biotechnology Letters *11*, 269-274.

Pavlov, E. V., Priault, M., Pietkiewicz, D., Cheng, E. H., Antonsson, B., Manon, S., Korsmeyer, S. J., Mannella, C. A., and Kinnally, K. W. (2001). A novel, high conductance channel of mitochondria linked to apoptosis in mammalian cells and Bax expression in yeast. The Journal of Cell Biology *155*, 725-731.

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

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

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

Pozniakovsky, A. I., Knorre, D. A., Markova, O. V., Hyman, A. A., Skulachev, V. P., and Severin, F. F. (2005). Role of mitochondria in the pheromone- and amiodarone-induced programmed death of yeast. The Journal of Cell Biology *168*, 257-269.

Rasola, A., and Bernardi, P. (2007). The mitochondrial permeability transition pore and its involvement in cell death. Apoptosis *12*, 815-833.

Reis, Y., Bernardo-Faura, M., Richter, D., Wolf, T., Brors, B., Hamacher-Brady, A., Eils, R., and Brady, N. R. (2012). Multi-Parametric Analysis and Modeling of Relationships between Mitochondrial Morphology and Apoptosis. PLoS ONE *7*, e28694.

Remize, F., Andrieu, E., and Dequin, S. (2000). Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae*: role of the cytosolic Mg^{2+} and mitochondrial K^{+} Acetaldehyde Dehydrogenases Ald6p and Ald4p in acetate formation during alcoholic fermentation. Applied and Environmental Microbiology *66*, 3151-3159.

Ribeiro, G. F., Côrte-Real, M., and Johansson, B. (2006). Characterization of DNA Damage in Yeast Apoptosis Induced by Hydrogen Peroxide, Acetic Acid, and Hyperosmotic Shock. Molecular Biology of the Cell *17*, 4584-4591.

Robey, R. B., and Hay, N. (2006). Mitochondrial hexokinases, novel mediators of the antiapoptotic effects of growth factors and Akt. Oncogene *25*, 4683-4696.

Roy, S. S., Ehrlich, A. M., Craigen, W. J., and Hajnoczky, G. (2009). VDAC2 is required for truncated BID-induced mitochondrial apoptosis by recruiting BAK to the mitochondria. EMBO Reports *10*, 1341-1347.

Saelens, X., Festjens, N., Walle, L. V., Gurp, M. v., Loo, G. v., and Vandenabeele, P. (2004). Toxic proteins released from mitochondria in cell death. Oncogene *23*, 2861-2874.

Santos, M. M. d., Gombert, A. K., Christensen, B., Olsson, L., and Nielsen, J. (2003). Identification of in vivo enzyme activities in the cometabolism of glucose and acetate by *Saccharomyces cerevisiae* by using ^{13}C-labeled substrates. Eukaryotic Cell *2*, 599-608.

Saraste, A., and Pulkki, K. (2000). Morphologic and biochemical hallmarks of apoptosis. Cardiovasc Research *45*, 528-537.

Schultheiss, H. P., and Klingenberg, M. (1984). Immunochemical characterization of the adenine nucleotide translocator. Organ specificity and conformation specificity. European Journal of Biochemistry *143*, 599-605.

Sharon, A., Finkelstein, A., Shlezinger, N., and Hatam, I. (2009). Fungal apoptosis: function, genes and gene function. FEMS Microbiology Reviews *33*, 833-854.

Shoshan-Barmatz, V., Keinan, N., Abu-Hamad, S., Tyomkin, D., and Aram, L. (2010). Apoptosis is regulated by the VDAC1 N-terminal region and by VDAC oligomerization: release of cytochrome c, AIF and Smac/Diablo. Biochimica et Biophysica Acta (BBA) - Bioenergetics *1797*, 1281-1291.

Shoshan-Barmatz, V., Keinan, N., and Zaid, H. (2008). Uncovering the role of VDAC in the regulation of cell life and death. Journal of Bioenergetics and Biomembranes *40*, 183-191.

Shug, A. L., and Shrago, E. (1973). Inhibition of phosphoenolpyruvate transport via the tricarboxylate and adenine nucleotide carrier systems of rat liver mitochondria. Biochemical and Biophysical Research Communications *53*, 659-665.

Silva, A., Almeida, B., Sampaio-Marques, B., Reis, M. I. R., Ohlmeier, S., Rodrigues, F., Vale, A. d., and Ludovico, P. (2011). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a specific substrate of yeast metacaspase. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1813*, 2044-2049.

Silva, R. D., Sotoca, R., Johansson, B., Ludovico, P., Sansonetty, F., Silva, M. T., Peinado, J. M., and Côrte-Real, M. (2005). Hyperosmotic stress induces metacaspase- and mitochondria-dependent apoptosis in *Saccharomyces cerevisiae*. Molecular Microbiology *58*, 824-834.

Siskind, L. (2005). Mitochondrial ceramide and the induction of apoptosis. Journal of Bioenergetics and Biomembranes *37*, 143-153.

Skulachev, V. P. (2002). Programmed death in yeast as adaptation? FEBS Letters *528*, 23-26.

Soboll, S., Conrad, A., Eistert, A., Herick, K., and Krämer, R. (1997). Uptake of creatine phosphate into heart mitochondria: a leak in the creatine shuttle. Biochimica et Biophysica Acta (BBA) - Bioenergetics *1320*, 27-33.

Sokolov, S., Knorre, D., Smirnova, E., Markova, O., Pozniakovsky, A., Skulachev, V., and Severin, F. (2006). Ysp2 mediates death of yeast induced by amiodarone or intracellular acidification. Biochimica et Biophysica Acta *1757*, 1366-1370.

Sousa, M., Duarte, A. M., Fernandes, T. R., Chaves, S. R., Pacheco, A., Leão, C., Côrte-Real, M., and Sousa, M. J. (2013). Genome-wide identification of genes involved in the positive and negative regulation of acetic acid-induced programmed cell death in *Saccharomyces cerevisiae*. BMC Genomics (*article under revision*).

Sundstrom, J. F., Vaculova, A., Smertenko, A. P., Savenkov, E. I., Golovko, A., Minina, E., Tiwari, B. S., Rodriguez-Nieto, S., Zamyatnin, A. A., Valineva, T.*, et al.* (2009). Tudor staphylococcal nuclease is an evolutionarily conserved component of the programmed cell death degradome. Nature Cell Biology *11*, 1347-1354.

Suzuki, M., Youle, R. J., and Tjandra, N. (2000). Structure of Bax: coregulation of dimer formation and intracellular localization. Cell *103*, 645-654.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001). A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Molecular Cell *8*, 613-621.

Szabo, I., and Zoratti, M. (1993). The mitochondrial permeability transition pore may comprise VDAC molecules. I. Binary structure and voltage dependence of the pore. FEBS letters *330*, 201-205.

Szabo, I., De Pinto, V., and Zoratti, M. (1993). The mitochondrial permeability transition pore may comprise VDAC molecules. II. The electrophysiological properties of VDAC are compatible with those of the mitochondrial megachannel. FEBS letters *330*, 206-210.

Walter, D., Wissing, S., Madeo, F., and Fahrenkrog, B. (2006). The inhibitor-of-apoptosis protein Bir1p protects against apoptosis in *S. cerevisiae* and is a substrate for the yeast homologue of Omi/HtrA2. Journal of Cell Science *119*, 1843-1851.

Whelan, R. S., Konstantinidis, K., Wei, A.-C., Chen, Y., Reyna, D. E., Jha, S., Yang, Y., Calvert, J. W., Lindsten, T., Thompson, C. B.*, et al.* (2012). Bax regulates primary necrosis through mitochondrial dynamics. Proceedings of the National Academy of Sciences, 1-6.

Wissing, S., Ludovico, P., Herker, E., Büttner, S., Engelhardt, S. M., Decker, T., Link, A., Proksch, A., Rodrigues, F., Côrte-Real, M.*, et al.* (2004). An AIF orthologue regulates apoptosis in yeast. The Journal of Cell Biology *166*, 969-974.

Wolter, K. G., Hsu, Y.-T., Smith, C. L., Nechushtan, A., Xi, X.-G., and Youle, R. J. (1997). Movement of Bax from the cytosol to mitochondria during apoptosis. The Journal of Cell Biology *139*, 1281-1292.

Woodfield, K., Ruck, A., Brdiczka, D., and Halestrap, A. P. (1998). Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition. Biochemical Journal *336*, 287-290.

Yamagata, H., Shimizu, S., Nishida, Y., Watanabe, Y., Craigen, W., and Tsujimoto, Y. (2009). Requirement of voltage-dependent anion channel 2 for pro-apoptotic activity of Bax. Oncogene *28*, 3563-3572.

CHAPTER 2

The role of phospholipids in *Saccharomyces cerevisiae* acetic acid-induced apoptosis

2.1 Introduction

Yeast cells have been extensively used as a model to study some issues of interest in mammalians. Specific phospholipids, namely cardiolipin (CL) and phosphatidylserine (PS), have a well-characterized role in animal apoptosis. The great similarity between their cellular function in both yeast and mammalian cells reinforces the yeast application in the study of the role of these phospholipids in apoptosis regulation.

Cardiolipin (CL) (1,3-diphosphatidyl-sn-glycerol) is structural and functionally a unique phospholipid that is predominantly present in the IMM. Oppositely to the other phospholipids, it has four acyl chains linked to a backbone of three glycerol moieties conferring it acidic and hydrophobic features, respectively (Figure 1A) (Lecocq and Ballou, 1964). Although CL is mostly present in the heart, where it was first identified, it can be found in all mammalian tissues (Pangborn, 1942).

Phosphatidylserine (PS) is also a glycerophospholipid that has a glycerol backbone with two fatty acyl chains linked on the *sn*-1 and *sn*-2 carbons and one phosphate group on *sn*-3. Additionally, the attachment of a serine to the phosphate gives it a negative net charge that is the distinguishing feature of this phospholipid (Figure 1B). Although not exclusively, it is predominantly localized in the inner leaflet of the plasma membrane (Leventis and Grinstein, 2010). Importantly, PS is the primary precursor phospholipid and its transport from the endoplasmic reticulum, where it is synthesized, to mitochondria allows regulating the levels of other fundamental phospholipids inside the cell.

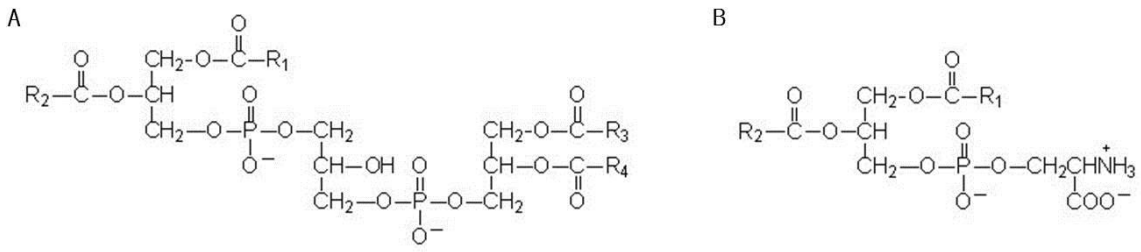

Figure 1 – Molecular structure of two phospholipids, cardiolipin (A) and phosphatidylserine (B). Cardiolipin, an anionic phospholipid exclusively present in mitochondria, is formed by four acyl chains (R1-R4) linked to a backbone of three glycerol moieties linked by two phosphates. Phosphatidylserine, like most of other phospholipids has two acyl chains (R1-R2) linked to a glycerol backbone that exclusively has a serine molecule linked to the phosphate group (Adapted from *The Medical Biochemistry Page*).

2.1.1 Cardiolipin biosynthetic pathway

In contrast to most other phospholipids, CL is not synthetized in the endoplasmic reticulum (ER) and great part of its biosynthetic process happens in the mitochondria. Commonly to the other phospholipids, the first step of CL synthesis is the acylation of glycerol-3-phosphate (G-3-P) through a G-3-P acyltransferase yielding acylglycerol-3-phosphate (Voelker, 2004). This last phospholipid suffers another acylation resulting in phosphatidic acid (PA) synthesis. PA is converted to cytidinediphosphate-diacylglycerol (CDP-DAG) through CDP synthase action. The phosphatidylglycerol phosphate (PGP) synthase catalyzes, in a committed and rate-limiting step in the CL biosynthetic pathway, the synthesis of PGP from CDP-DAG and G-3-P with release of CMP (Chang et al., 1998b). After this, PGP is dephosphorylated by PGP phosphatase yielding phosphatidylglycerol (PG) (Kelly and Greenberg, 1990).

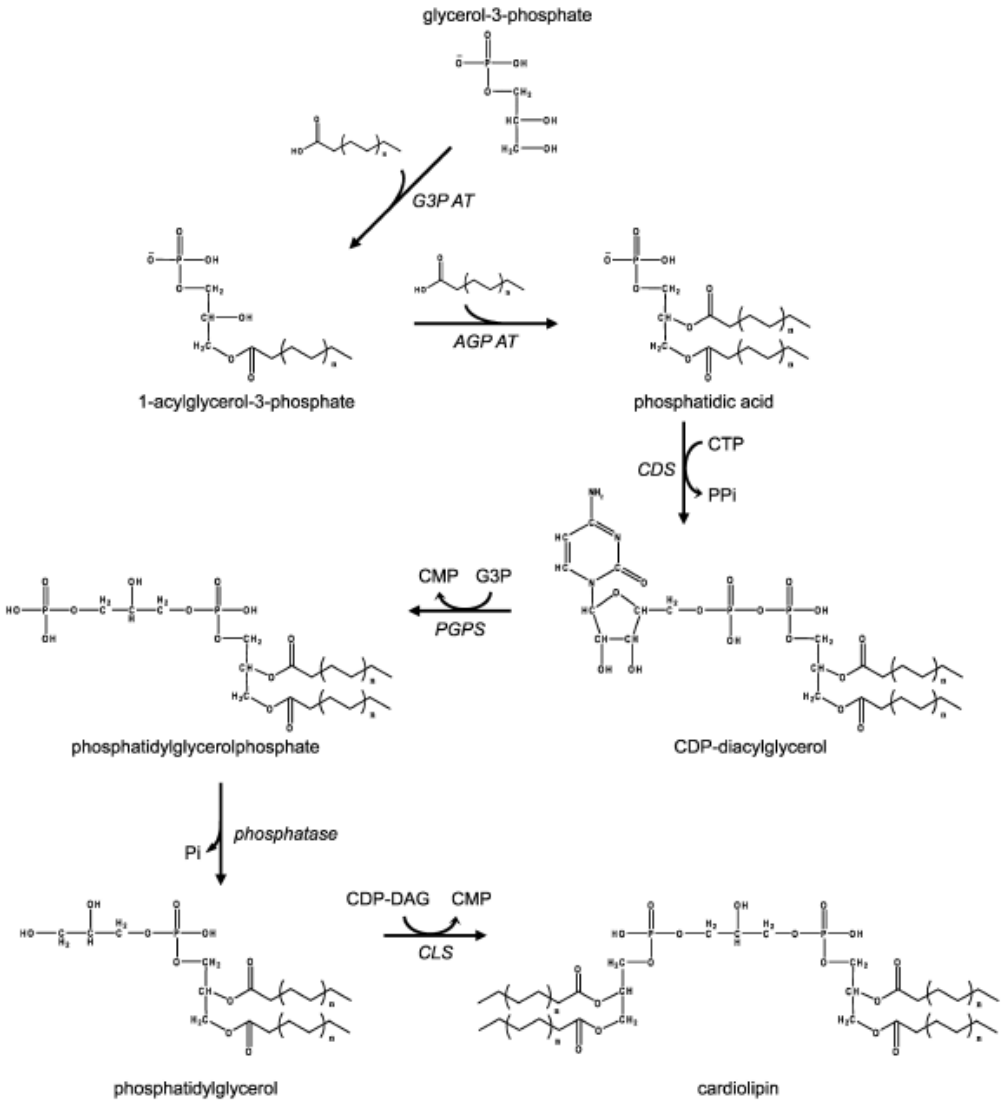

Figure 2 – The cardiolipin (CL) biosynthetic pathway in eukaryotic cells. The final step of CL generation involves the condensation of one molecule of phosphatidylglycerol (PG) and one molecule of CDP-diacylglycerol (CDP-DAG) catalysed by the enzyme CL synthase (Cls, yeast Crd1p) (Scheme from Houtkooper and Vaz, 2008).

The cardiolipin synthase (yeast Crd1p) catalyses an irreversible condensation between two molecules, PG and CDP-DAG, leading to CL synthesis with release of CMP (Figure 2) (Chang et al., 1998a). The evaluation of PG amount in the mitochondrial membrane is assumed as a reference of CL synthesis effectiveness. The CL biosynthetic process is common among fungi (including *S. cerevisiae*), plant and mammalian cells suggesting a common eukaryotic mechanism of CL formation. Oppositely, in prokaryotic cells the CL synthesis happens through condensation of two molecules of PG (Schlame et al., 1993).

2.1.2 Cardiolipin remodeling and the Barth Syndrome

When compared with other phospholipids, CL is enriched in unsaturated acyl chains, which differ between organisms and even among tissues and cell types within one organism. These findings proved that after CL synthesis, process common for all eukaryotic organisms, an additional mechanism involves the acyl chain remodeling which is required to obtain the CL final composition (Houtkooper et al., 2006). Interestingly, among various organisms and tissues only one or two types of fatty acids are present in CL that leads to a high degree of structural uniformity and molecular symmetry among cardiolipins (Schlame et al., 2005).

The biosynthesis and remodeling of CL have been extensively studied in the yeast *S. cerevisiae*. After CL synthesis, it suffers deacylation, forming monolysocardiolipin (MLCL), followed by reacylation achieving its unsaturated composition, in a process that involves the yeast homologue of tafazzin encoded by the *TAZ1* gene (Gu et al., 2004). Taz1p is a CoA-independent transacylase that transfers unsaturated acyl chains preferentially from phosphatidylcholine (PC) to CL, yielding at the final mature CL and lyso-PC (LPC) (Figure 3) (Schlame and Ren, 2006).

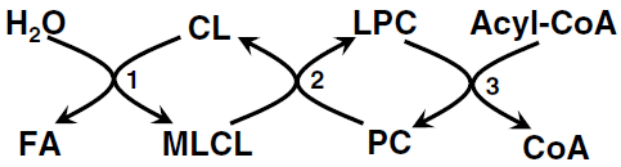

Figure 3 – The Taz1p involvement in the CL remodeling. After CL deacylation (loss of one fatty acid (FA)) and MLCL generation (1), Taz1p catalyzes the PC-CL transacylation, yielding CL mature and lyso-PC (LPC) (2), which is reacylated to produce PC (3) (Scheme from Schlame and Ren, 2006).

It was established that CL can only fully perform its cellular functions after remodeling. In fact, the loss of tafazzin activity in Barth Syndrome (BTHS) patients and the complications of this disease underscored

the importance of CL remodeling. At the same time, the decrease of CL in its mature form and the accumulation of MLCL in yeast and human tafazzin-deficient cells proved the existence of the two-step remodeling pathway described before (Gu et al., 2004; Valianpour et al., 2005).

In the last decade, some interest has been dedicated to BTHS, a rare X-linked recessive disorder, clinically characterized by cardiomyopathy, skeletal myopathy, neutropenia and growth retardation. The biochemical mechanisms that are responsible for these alterations are not yet fully understood but it is known that this happens generically due to abnormal mitochondria and defective oxidative phosphorylation (Barth et al., 1999).

2.1.3 Ups1p and the role of ERMES complex in the CL biosynthetic pathway: facts and hypotheses

Ups1p, a yeast member of a conserved family of intermembrane space (IMS) proteins, was initially associated to the Mgm1p processing, a conserved dynamin-like GTPase that has a central role in the yeast mitochondrial fusion machinery. In this way, it was reported that Ups1p regulates the mitochondrial shape and number in a carbon source-dependent manner being as well involved in the regulation of cell growth (Sesaki et al., 2006).

The import of Ups proteins is mediated by Mdm35p, another IMS protein, in an unusual mechanism in which both proteins are stably associated leading either to the formation of functional protein complexes that drives mitochondrial protein import or Ups1p accumulation at the IMS (Tamura et al., 2010). Beyond this, it was demonstrated that Ups1p-Mdm35p association prevents Ups1p degradation by the mitochondrial peptidases Yme1p and Atp23p (Potting et al., 2010).

Curiously, the *UPS1* deletion resulted in CL decreased levels suggesting a role of this protein in CL metabolism or accumulation (Osman et al., 2009; Potting et al., 2010; Tamura et al., 2009). These changed levels of CL led to some defects in the mitochondrial protein import as consequence of a conformational change of Tim23p, a dynamic complex in the IMM. On the other hand, the protein import driven by Tim22p, such as of AAC and PiC, both putative components of the PTP complex, was not affected. However, similarly to the *crd1*Δ and *taz1*Δ mutants, in *ups1*Δ cells the AAC protein assembly was impaired evidencing once again that Ups1p can be involved in the CL metabolism (Tamura et al., 2009).

While most phospholipids are synthesized in the ER and then transported to mitochondria, CL and phosphatidylethanolamine (PE) are produced within the mitochondria. In both situations, the physical

connection between the ER and the mitochondria, established by the ERMES (ER-mitochondria encounter structure) complex in the yeast *S. cerevisiae*, is crucial (Kornmann et al., 2009). The PE synthesis involves the PS translocation from the ER to the OMM and then to the IMM (Simbeni et al., 1993). After PS decarboxylation and PE synthesis, it returns to the OMM and then to the ER, where it is methylated and converted in PC (Figure 4) (Kodaki and Yamashita, 1987). In the CL synthesis process the PA translocation from the ER to the OMM was also described (Connerth et al., 2012).

The ERMES complex is part of a well differentiated sub-compartment of the ER, identified in all eukaryotic cell systems from yeast to human and called the mitochondria-associated membrane (MAM) (Achleitner et al., 1999). MAMs are enriched in enzymes involved in phospholipid biosynthesis, and calcium handling proteins (Raturi and Simmen, 2013). At MAM, the proximity between the membrane of the ER and OMM (10–25 nm) allows direct contact of proteins and lipids (Csordas et al., 2006). Although in mammals a great number of proteins had been identified as part of MAMs, in yeast cells, the ERMES complex is the only machinery reported so far to form ER-OMM contact sites (Raturi and Simmen, 2013; Tamura et al., 2012). The ERMES complex is composed at least by the proteins Mmm1p, Mdm10p, Mdm12p, Mdm34p (Mmm2p) and Gem1p. In this complex, Mmm1p is an integral ER protein glycosylated on its N-terminal side, which interacts with Mdm10p, an OMM β-barrel protein. In turn, Mdm34p at the OMM and the cytosolic Mdm12p promote this interaction probably by direct association (Figure 4) (Kornmann et al., 2009). Mdm10p is simultaneouly a component of ERMES and of the Sorting and Assembly Machinery (SAM) complex which is responsible for the assembly of membrane β-barrel proteins in the OMM. It was reported that Mdm10p-SAM interaction specially assists Tom40p, the central translocase of TOM complex, assembly (Meisinger et al., 2007; Meisinger et al., 2004). The Ca^{2+}-binding Miro (mitochondrial rho-like) GTPase Gem1p is a regulatory component of the ERMES complex showing that it is not a passive conduit for interorganellar lipid exhange (Kornmann et al., 2011). The existence of this complex was discovered a short time ago, although individualized information for some of the ERMES proteins has been described. Considering all this information, it seems plausible to associate this particular complex to other mitochondrial functions, including morphology manintenance, calcium exchange, coordinated protein import and mitochondrial DNA replication (Kornmann and Walter, 2010).

Curiously, the loss of ERMES complex and Ups1p result in similar defects in mitochondrial phospholipid metabolism, namely with a decrease in the CL synthesis rate simultaneously to an increase in PS levels. Therefore, as ERMES complex was demonstrated to be important for organelles communication and phospholipids exchange, the Ups1p localization at the IMS suggests a function in

phospholipid trafficking between the two mitochondrial membranes (Tamura et al., 2012). Altogether this information appears to suggest that Ups1p and ERMES proteins act at similar steps in phospholipid metabolism.

Considering the information above, it was recently demonstrated that Ups1p is essential for the PA translocation from the OMM to the IMM thereby justifying its role in the CL biosynthetic pathway. During this process, the presence of negatively charged phospholipids facilitates the interaction of the Ups1p-Mdm35p complex with the IMM, which happens simultaneously to the Ups1p dissociation of Mdm35p and release of PA. When the physiological levels of CL are present at the IMM (10-20%), a regulatory feedback mechanism is triggered. In this process an irreversible association of Ups1p to the IMM results in its proteolysis by Yme1p action preventing the PA translocation and consequently, the CL synthesis (Connerth et al., 2012).

Although the loss of ERMES complex lead to reduced levels of CL inside the cell, until now it was not clarified the mechanism by which this complex is able to regulate the levels of this phospholipid. However, considering the last finding about Ups1p role in CL synthesis, through PA translocation to the IMM, it appears plausible that ERMES complex can be fundamental for PA or other CL precursors translocation from the ER, where they are generated, to mitochondria justifying the similar profile between ERMES and Ups1p mutants.

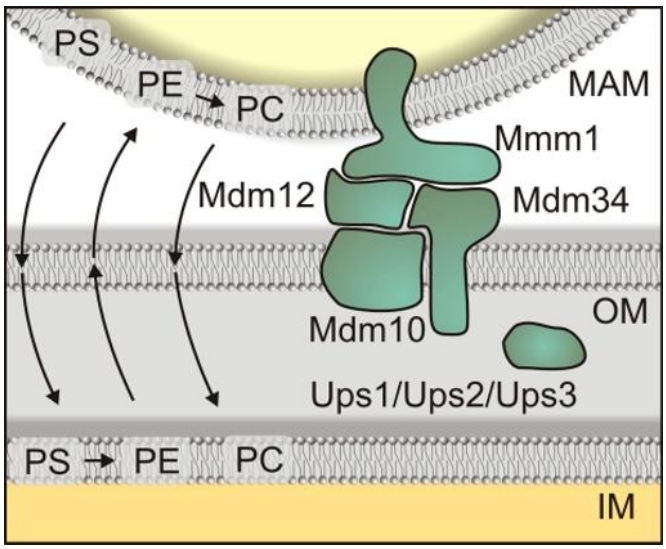

Figure 4 - As consequence of MAMs enrichment in enzymes and precursors involved in phospholipid biosynthesis, the mitochondria and ER connection is fundamental. In yeast, it is promoted by the ERMES complex, which is composed by an integral ER glycoprotein (Mmm1p) and three mitochondria-associated proteins (Mdm34p, Mdm10p and Mdm12p). On the other hand, Ups1p at the IMS is crucial for the PA translocation from the OMM to the IMM promoting the CL synthesis (Scheme from Osman et al., 2011).

2.1.4 Cardiolipin distribution within the mitochondria

The CL chemical composition, with four acyl chains linked to a diphosphatidylglycerol group, confers it a dimeric nature that results in a specific conical structure when in the IMM (Cullis et al., 1986).

The CL localization has been essentially associated to the IMM, where it has a fundamental role in the integrity and activity of some complexes of the respiratory chain, however it is not exclusively present there. Although less significantly, it was proposed that the conical shape acquired by CL favours a hexagonal phase of the membrane that allows the connection of the inner and the outer mitochondrial membrane at the contact sites (Figure 5) (Ardail et al., 1990; de Kroon et al., 1997). Thus, the CL localization at the OMM can elucidate its role in the mitochondrial membrane curvature and morphology.

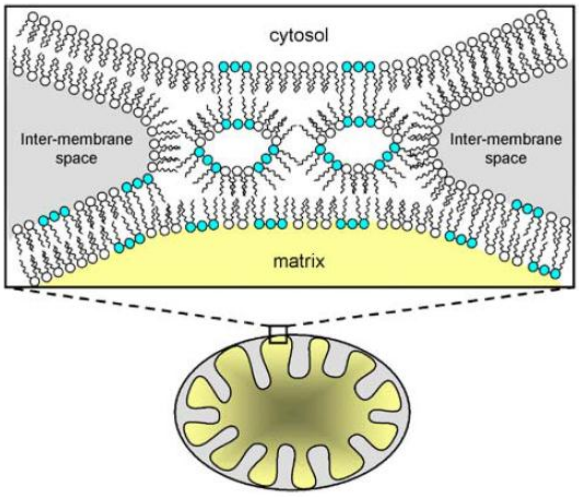

Figure 5 – The non-bilayer hexagonal structure adopted by CL at the mitochondrial contact sites (scheme from Gonzalvez and Gottlieb, 2007).

2.1.5 The cardiolipin requirement in the respiratory chain and other functions

At the IMM, where it is predominantly present, CL due to its nature interacts with a large number of mitochondrial proteins (reviewed in Schlame et al., 2000). CL is fundamental for the stability and activity of the respiratory chain once it is required for the optimal functioning of the complex I (NADH:ubiquinone oxido-reductase), complex III (ubiquinone:cytochrome *c* oxido-reductase), complex IV (cytochrome *c* oxidase) and complex V (ATP synthase) (Acehan et al., 2011; Fry and Green, 1981; Pfeiffer et al., 2003). Furthermore, the CL structure, specifically its headgroup with negative charge, has been suggested to function as a trap of protons in the IMS promoting the effectiveness of complex V activity and, at the same time, minimizing the changes in pH in this cellular location (Haines and Dencher, 2002). Despite the referred above, under normal conditions, yeast cells lacking CL retained the capacity to perform

oxidative phosphorylation, albeit at a reduced rate (Jiang et al., 2000). In the IMM, CL interacts strongly with cyt *c*, a mobile electron carrier that mediate transport between complex III and IV (Ott et al., 2002). This interaction has been studied in more depth since cyt *c* detachment has serious implications in apoptosis. Other studies reported that CL is required for the activity of some substrate carriers, including ANT and PiC (Bisaccia and Palmieri, 1984; Hoffmann et al., 1994). Furthermore, due to exclusive CL localization in the mitochondria, it has an important role in mitochondrial structure, governing the relationship between mitochondria volume and function (Koshkin and Greenberg, 2002).

Curiously, it was demonstrated that the effect of CL absence depends on growth conditions, which justifies some controversial results on the role of CL in the bioenergetics. When grown in non-fermentable carbon sources, the yeast *crd1*Δ mutant accumulates the precursor lipid PG that can, at least in part, compensate the CL loss in some cellular processes. Oppositely, under fermentable conditions (medium containing high glucose concentrations), it was demonstrated that the specific activity of the enzyme PGP synthase is decreased and the PG levels are not detectable when compared with aerobic conditions (Jiang et al., 2000).

Additionally, CL appears also to be required for protein and phospholipid import, cell wall biogenesis, translational regulation of electron transport chain (ETC), aging and apoptosis (Joshi et al., 2009).

2.1.6 The role of cardiolipin in apoptosis

Nowadays it is well established that mitochondria plays a determinant role in apoptosis. The study of CL, whose location is exclusive to this organelle, has also demonstrated that it is directly or indirectly important for the conduction of the PCD process.

2.1.6.1 Cardiolipin peroxidation

It was reported that in the early stages of apoptosis there is a decrease in the mature CL levels simultaneously with an increase of both MLCL and CL that suffer peroxidation (Matsko et al., 2001; Sorice et al., 2004). Until now, there is no information about an increase of MLCL levels in yeast cells during apoptosis, however it was shown that CL peroxidation enhances the death process, favouring cyt *c* release (Korytowski et al., 2011). It is known that both CL forms are important in the execution of apoptosis but oppositely to the well understood CL hydrolysis event during apoptosis, the process by which CL suffer peroxidation has generated some controversy.

Under physiological conditions, the process of CL maturation protects, at least in part, the cells against oxidative stress due to the deacylation-reacylation cycles. It is known that the leaked electrons present at the IMS can easily react with oxygen molecules leading to ROS production, and that the respiratory chain complexes stability requires the presence of mature CL at the IMM. So, an important consequence of the decrease in CL levels is obviously an accumulation of electrons, due to the inactivity of the complexes, which yields an increase in ROS production. Consequently, the location of CL near the ROS generation site conjugated with its composition in unsaturated acyl groups make this phospholipid more susceptible to the oxidative stress resulting, simultaneously, in CL peroxidation and a decrease in oxidative phosphorylation (Paradies et al., 2000; Petrosillo et al., 2001). On the other hand, it was shown that CL peroxidation and unbinding from the IMM leads to loss of respiratory chain complexes activity that results in an increase of ROS production that can further increase CL peroxidation. So, it is not clear if ROS are responsible for CL peroxidation and its decreased levels in the IMM or whether CL peroxidation results in an increased ROS production due to loss of respiratory complexes activity and presence of free electrons in the IMS (Gonzalvez and Gottlieb, 2007). More recently, it was reported that cyt *c* may itself be responsible for the CL peroxidation. During apoptosis, the partial loss of the interaction between cyt *c* and CL induces the cyt *c* unfolding leading to exposure of its catalytic domain that results in CL peroxidation. Importantly, the peroxidase activity of the CL-cyt *c* complex depends on unsaturated acyl chains of CL once its activity appears to be activated by the presence of H_2O_2 (Belikova et al., 2006; Kagan et al., 2005). The cyt *c* peroxidase activity has not been addressed in yeast yet. In mammalians, CL peroxidation has a relevant role in the apoptotic process since it was shown that CL distribution toward the OMM, caused by its peroxidation, could be important for the anchoring of fundamental pro-apoptotic proteins.

2.1.6.2 Cardiolipin and cytochrome *c*

Although the physiological levels of CL are essential for the stability of some respiratory chain complexes, the loss of CL-cyt *c* association, due to cyt *c* role in the execution of apoptosis through activation of the caspase cascade, has earned greater visibility. It was demonstrated that either in isolated membranes or synthetic vesicles, cyt *c* binds strongly to CL-rich domains. Firstly, this association happens through electrostatic interactions since, at physiological pH, cyt *c* has the net charge of +8 and can bind, through its A-site, to the negative headgroups of CL in the outer leaflet of the IMM. Additionally,

cyt *c* has also a hydrophobic cavity, named the C-site, which allows the interaction with the acyl chains of CL. Whereas the A-site is loosely bound to CL, the C-site is tightly bound (Tuominen et al., 2002).

As described above, it has been proposed that MOMP and the subsequent release of cyt *c* and other apoptogenic factors can be catalyzed by cyt *c*. In this process, the C-site of cyt *c* catalyzes CL peroxidation and thus cyt *c* can dissociate from the IMM since it has lower affinity for peroxidized than normal CL (Gonzalvez and Gottlieb, 2007). Therefore, catalyzed by cyt *c* or not, the simultaneous increase of CL peroxidation and ROS production, associated to decreased mature CL levels, appear to be the promoters of an efficient release of apoptotic proteins. Reinforcing this idea, in cyt *c*-deficient HeLa cells the release of the pro-apoptotic protein Smac/Diablo was inhibited during apoptosis and this phenotype was altered when CL peroxidation was induced (Kagan et al., 2005).

Despite the importance of CL-cyt *c* association, it is interesting to notice that only around 15% of cyt *c* is linked to the IMM and that less than 5% is associated with CL (Schug and Gottlieb, 2009).

Over the years, it has been suggested that cyt *c* release occurs in two steps. The first step involves its detachment from CL in the IMM followed by its relocalization in the IMS, and release to the cytosol through permeabilization of the OMM (Figure 6) (Ott et al., 2002). In this point of view, the CL-cyt *c* association is of great importance and could possibly function as an important therapeutic target.

2.1.6.3 Cardiolipin as an activating platform for the mitochondrial tBid recruitment

As mentioned above, the CL chemical structure ensures it a hexagonal form that favours its location at the contact sites between the IMM and the OMM. After Bid cleavage, its truncated form is translocated and interacts with mitochondria specifically at the contact sites, which are rich in CL (Lutter et al., 2001). Using yeast isolated mitochondria the CL connection with the cytosol was suggested to mediate the recruitment of tBid to mitochondria, and consequently Bax and/or Bak oligomerization and activation that results in OMM permeabilization with release of apoptotic factors (Figure 6) (Gonzalvez et al., 2005b; Kim et al., 2004). Therefore, tBid functions as a bifunctional molecule: firstly its helix αH6 allows the connection with CL destabilizing the mitochondrial structure and function, and then the activation and oligomerization of Bax/Bak is achieved by exposure of its BH3 domain (Gonzalvez et al., 2010). It was shown that after tBid translocation, independently of Bax or Bak activation, the oxidative phosphorylation is inhibited in both mice and yeast isolated mitochondria. In CL-deficient yeast cells, tBid is not able to establish connection with the mitochondria, and under these conditions the mitochondrial bioenergetics or their complexes activities are not disturbed. This proved that the lack of tBid binding is not caused by

the effect of CL deficiency in the respiratory chain and, at the same time, highlighted that tBid binds exclusively to mitochondrial membranes that have at least the physiological levels of CL (Gonzalvez et al., 2005a; Gonzalvez et al., 2005b).

Unlike mammalian model, in yeast cells tBid does not promote Bax to acquire an active form but surprisingly the tBid interaction with mitochondria was increased when it was co-expressed with Bax. At the same time, in the presence of Bax-*c-myc* the expression of tBid enhanced cyt *c* release mediated by this active form of Bax suggesting that tBid in yeast cells, like in mammalians, can be fundamental but not absolutely required for Bax-mediated apoptosis in a process in which tBid is not responsible for the increased amounts of Bax bound to the mitochondria (Priault et al., 2003).

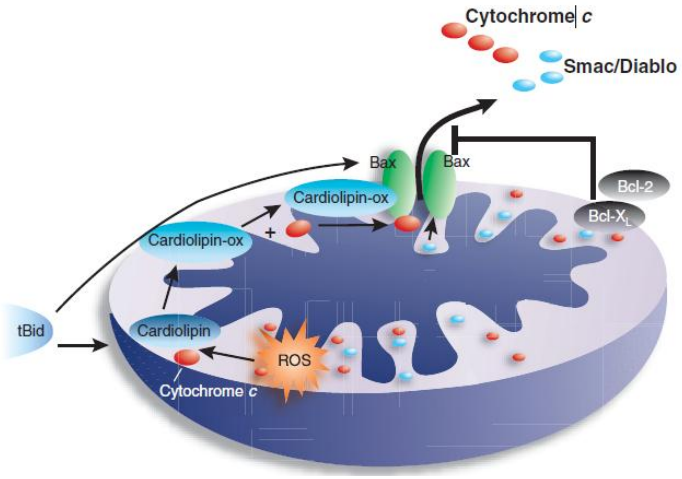

Figure 6 – After apoptosis induction, the increased production of ROS can lead to CL peroxidation and translocation to the contact sites, where it acts like an activating platform for the tBid recruitment. The detachment of cyt *c* from CL simultaneously to Bax recruitment to the OMM induced by tBid anchorage lead to MOMP with release of apoptotic factors, such as cyt *c* and Smac/Diablo (Scheme from Orrenius and Zhivotovsky, 2005).

In yeast cells, some doubts were raised about the requirement of CL for Bax activation. It was shown by heterologous expression of Bax in wild-type and *crd1*Δ yeast strains that CL is not only unnecessary as also its absence favours Bax-mediated cyt *c* release (Iverson et al., 2004). More recently, it was reported that Bax insertion was inhibited in CL-deficient yeast mitochondria growing under fermentable conditions (Lucken-Ardjomande et al., 2008). Although these two studies have been done under different growth conditions, it was hypothesized that in Iverson and colleagues work, the treatment of recombinant Bax with a detergent that promotes artificial Bax oligomerization probably prevented the evaluation of the dependence of Bax activation on CL (Lucken-Ardjomande et al., 2008).

In animal cells, it was shown that tBid has the same affinity for MLCL as for mature CL, supporting the hypothesis of CL degradation in apoptotic cells and MLCL localization at the contact sites (Esposti et

al., 2003; Liu et al., 2005; Sorice et al., 2004). Additionally, it was reported that tBid binding, Bax recruitment and cyt *c* release are increased by the presence of hydroperoxide CL species at the OMM either in yeast or mice cells (Gonzalvez et al., 2010; Korytowski et al., 2011). In this last model, CL peroxidation and tBid association contributes to mitochondrial morphology changes favouring the process of PCD, namely through cyt *c* repositioning in the IMS (Epand et al., 2002; Kim et al., 2004). In the same way, in animal cells, tBid appears to regulate this process through its involvement in the formation of protein complexes that favour both the mitochondrial cristae remodeling and the formation of mitochondrial fission sites (Garofalo et al., 2005). Therefore, some authors proposed that the tBid-CL association leads primarily to cristae remodeling as well as inhibition of oxidative phosphorylation, followed by activation of the Bcl-2 pro-apoptotic proteins and MOMP (Gonzalvez and Gottlieb, 2007).

2.1.6.4 Cardiolipin as a new model to explain caspase-8 activation

During apoptosis, CL clustering acts like an apoptotic signaling platform in the OMM not only for tBid but also for caspase-8. It was reported that in the mitochondrial mediated apoptosis, CL at the contact sites has a role in caspase-8 recruitment, oligomerization and processing resulting in its activation and amplification of the apoptotic signal. It was shown that caspase-8 accumulation at the mitochondria is critical for the release of the apoptotic factors from the IMS (Gonzalvez et al., 2008; Scorrano, 2008). However, it is not clear if procaspase-8 auto-processing happens before activated caspase-8 insertion into the OMM or if procaspase-8 is activated by proximity after OMM insertion to generate its active form (Zhang and Saghatelian, 2013). Additionally, the mechanism whereby caspase-8 interacts with CL in mitochondria remains unclear. It was demonstrated that there is colocalization between tBid and caspase-8 in the CL-rich domains at the contact sites. Gonzalvez and colleagues in 2008 hypothesized that this protease translocation happens together with its substrate Bid, and that the interaction between caspase-8 and CL can be stabilized through its insertion in the unsaturated acyl chains of CL, resulting in an incomplete insertion on the OMM. However, after Fas apoptotic induction in Bid knockdown cells, caspase-8 translocation was not affected (Gonzalvez et al., 2008). Other authors have previously shown that the interaction of caspase-8 with mitochondria is mediated by other proteins (Stegh et al., 2002). Therefore, it was proposed that additionally to a protein-protein interaction role in caspase-8 translocation, like for tBid, the protein accumulation is CL-dependent (Gonzalvez et al., 2008).

The order in which the events mentioned above occur is not yet well understood and many doubts remain to be clarified. Recently, the integration of the different evidences from the literature described above led to the proposal that in the mitochondrial-mediated apoptotic pathway, procaspase-8 is translocated and accumulated in the mitochondrial CL-rich contact sites, with consequent autoprocessing to active caspase-8. Then, caspase-8 interacts and cleaves Bid in its truncated form. At the same time, tBid is responsible for Bax/Bak recruitment and activation, and the increased levels of produced ROS leads to CL peroxidation. Consequently, CL is translocated to the OMM simultaneously promoting the detachment of cyt *c* that is then released through the pore formed by the Bcl-2 pro-apoptotic proteins in the OMM (Figure 7) (Zhang and Saghatelian, 2013).

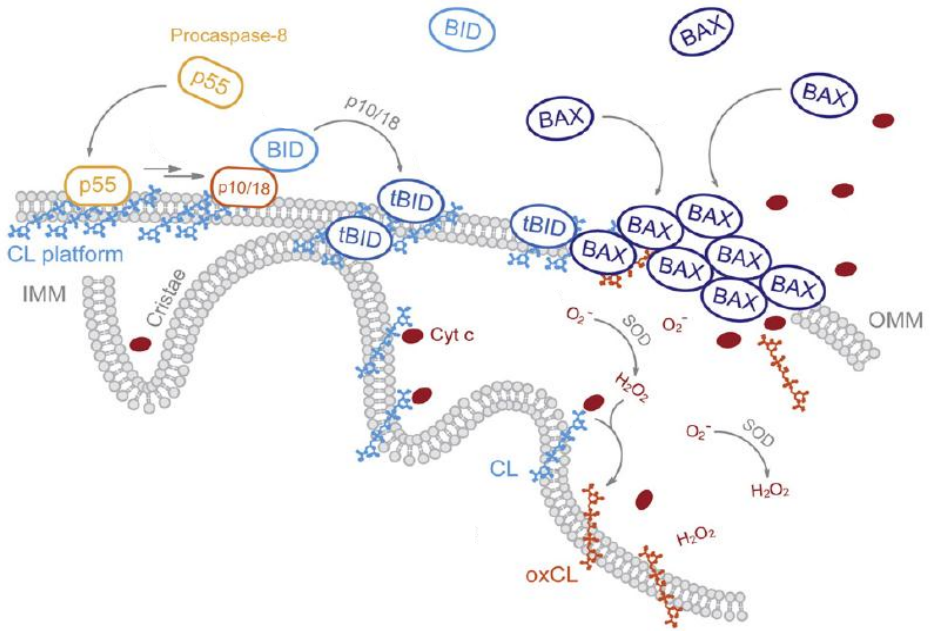

Figure 7 – The CL requirement for the sequential order of events in the mitochondrial-mediated apoptotic pathway. During apoptosis, CL localization in the contact sites is fundamental for caspase-8 accumulation and activation, as for tBid generation. The simultaneous ROS production, CL peroxidation and cyt *c* repositioning in the IMS favour the release of apoptotic factors through Bax/Bak pores at the OMM, whose formation was enhanced by tBid and translocation of peroxided CL (Scheme adapted from Zhang and Saghatelian, 2013).

2.1.6.5 Requirement of cardiolipin for the functionality of PTP components

The high sequence and functional similarity between the human ANT and the yeast AAC proteins provided the use of the yeast as a cell model to study the CL-ADP/ATP carrier association. In yeast cells, it was demonstrated that CL is important for AAC assembly, structure, function and interaction with other proteins (Claypool, 2009; Claypool et al., 2008b; Jiang et al., 2000). Nevertheless, yeast cells lacking CL are able to grow in respiratory conditions oppositely to *aac2*Δ mutants indicating that this translocator

does not absolutely depends on the presence of CL or that PG increased levels can substitute the role of CL in AAC function (Claypool, 2009).

During mammalian apoptosis, it was reported that tBid inhibits the ADP-stimulated respiration by indirect inhibition of the ANT activity, mediated by CL reorganization into the mitochondria (Gonzalvez et al., 2005b). The CL-AAC interaction appears also to be involved in the PTP opening. It was proposed that the Ca^{2+} taken up into the matrix has high affinity to the headgroups of CL tightly bound to the AAC. Therefore, during apoptosis the increase of Ca^{2+} concentration in the mitochondrial matrix was suggested to displace CL from AAC exposing the ion positive charges at the matrix side of the AAC. So, the removal of CL can destabilize the AAC rearrangement due to electrostatic repulsion leading to the PTP opening (Figure 8) (Klingenberg, 2009).

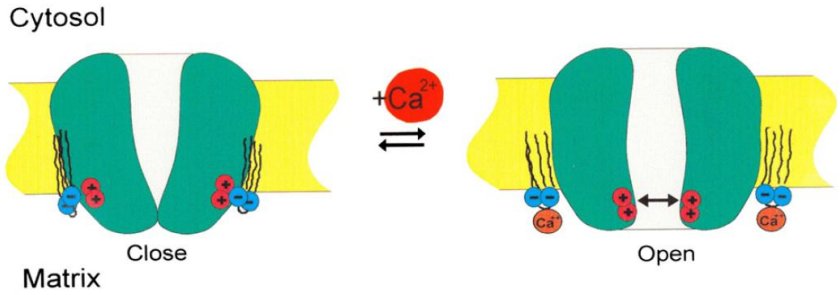

Figure 8 – Schematic representation of calcium role in the PTP opening through its interaction with cardiolipin and subsequent destabilization of CL-AAC interaction and irreversible AAC opening (Scheme from Klingenberg, 2009).

Although not so well described, CL has also been attributed a role in the regulation of the PiC activity through a direct interaction. Curiously, AAC and PiC are absolutely indispensable for oxidative phosphorylation where they provide the substrates for the ATP synthase whose oligomerization is not affected in yeast mitochondria lacking CL (Claypool, 2009; Klingenberg, 2009).

The relationship between CL and VDAC has been less emphasized since this channel is not on the IMM. However, it was shown that VDAC is localized predominantly at the contact sites where it colocalizes with CL (Crompton, 1999).

A study done with liposomes proved that PG, phosphatidylinositol (PI), PS and CL interact with VDAC, a protein with a positive charge at neutral pH. Whereas the other phospholipids induce VDAC oligomerization, CL disrupts this process. This happens probably due to different hydrophobic structures of these phospholipids since CL has four rather than two acyl chains. Interestingly, when cells are growing in respiratory conditions, the decrease in CL levels and the increase in PG during apoptosis favour VDAC oligomerization at the contact sites. On the other hand, in normal conditions (non-apoptotic),

CL interacts with VDAC in a competitive way with PG not allowing VDAC oligomerization whose increase is closely associated to apoptosis (Betaneli et al., 2012). So, the asymmetry of VDAC gating, an intrinsic property of this channel, as well as its oligomeric status can be either catalyzed or suppressed by the membrane lipid composition.

Altogether these results show that CL is a central intermediate in the mitochondrial apoptotic program, controlling this process at different levels. Therefore, CL is a potentially interesting target for therapeutical intervention in diseases in which cell death is deregulated, either in cancer or degenerative diseases. The considerable amount of work done using yeast as a model system reinforces its application to elucidate and better characterize the molecular mechanisms of action of CL during apoptosis for subsequent analysis and validation in higher eukaryotes.

2.1.7 Phosphatidylserine – biosynthesis, intracellular localization and role in apoptosis

Phosphatidylserine is a negatively charged phospholipid that contains two acyl chains linked to a glycerol moiety. Like in CL, the PS acyl chains composition can present some differences between cell types and organelles. However, in almost all cases, saturated fatty acids of 16 or more carbons are linked to the *sn*-1 position, whereas unsaturated fatty acids are found at the *sn*-2 position of the glycerol (Pike et al., 2002). Undoubtedly the feature that allows distinguishing PS from other phospholipids is the linkage of serine at the phosphate on position *sn*-3.

In yeast, the PS biosynthetic pathway involves the association of serine to CDP-DAG, where the preceding process of synthesis is common to the one described for CL (Steiner and Lester, 1972). This process appears to be catalyzed by Cho1p, yeast PS synthase, since *CHO1* deletion significantly reduces the cell levels of PS (Atkinson et al., 1980). On the other hand, in mammalian cells PS synthesis can imply two different pathways. In both cases the CDP-DAG molecule is not involved and PS is produced by exchanging the headgroup of PC or PE by serine (Figure 9) (Vance, 2008). At the beginning, the PS synthase localization within the animal cell was not clear, but today it is known that it resides in the MAMs, where other phospholipid catalytic enzymes are also present (Saito et al., 1996). In yeast cells, although not completely proved, it is expected that PS synthase has a similar localization (Zinser et al., 1991). To reinforce this hypothesis, after PS synthesis in the ER, it is decarboxylated in mitochondria leading to PE formation, providing a convenient marker of the transfer of PS from ER to mitochondria. PS

is surely a key phospholipid within the cell since after its conversion into PE, this last suffers methylation to generate PC.

PS has an asymmetric transbilayer distribution as it predominates at the inner leaflet of the plasma membrane. Nevertheless, it can also be present at different ratios in the ER, Golgi complex, endosomes and mitochondria (Leventis and Grinstein, 2010). Although PS has low abundance in biological membranes, its unique physical and biochemical properties gives it a physiological importance.

In animal cells, the PS externalization with translocation to the outer leaflet of the plasma membrane after an apoptotic stimulus, mediated by some already identified proteins, allows the recognition of the apoptotic cells for subsequent engulfment by phagocytic cells (Balasubramanian et al., 2007). In yeast, oppositely to the mammalian cells, PS is greatly enriched in the plasma membrane comprising 34% of the total phospholipids (Vance and Steenbergen, 2005). The biological significance of this representativeness as well as the role of PS externalization during apoptosis in yeast are not yet known although in both yeast and animal cells, this phenomenon has been a hallmark of this type of cell death (Madeo et al., 1997).

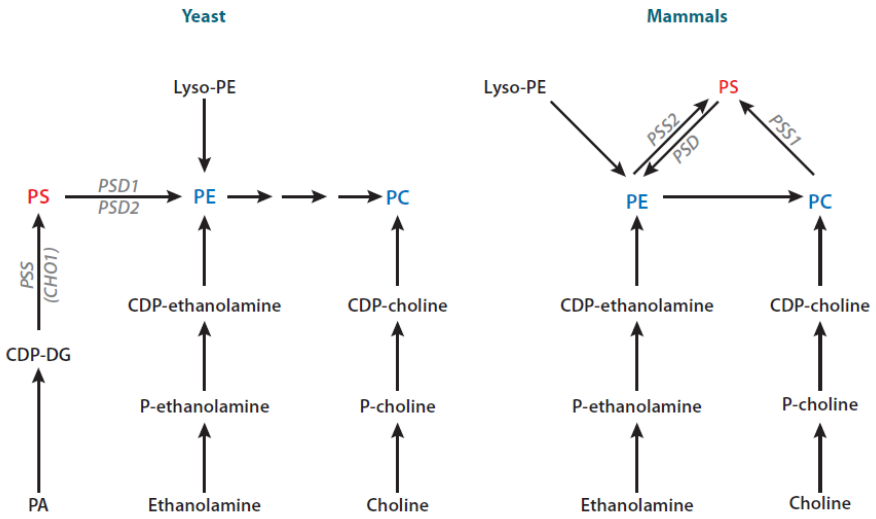

Figure 9 – The crosstalk between PS, PE and PC metabolism in yeast and mammalian cells (Scheme from Leventis and Grinstein, 2010).

2.2 Main aims and study overview

As aforementioned, a genome-wide screening in yeast identified nonessential genes whose deletion is associated with resistance or sensitivity to acetic acid-induced cell death (Sousa et al., 2013). The information provided by this study revealed a considerable number of genes coding for proteins with mitochondrial function, and confirmed the indubitable role of mitochondria in the apoptotic process induced by acetic acid in yeast. Among the Euroscarf collection of knockout mutants used, the mutant strains *crd1*Δ, *taz1*Δ, *ups1*Δ, *mdm10*Δ and *mdm12*Δ, that exhibit decreased levels of CL due to the lack of enzymes involved in different steps of CL synthesis or of its intermediates, or to the absence of components of the ERMES, and the mutant strain *cho1*Δ, lacking phosphatidylserine synthase, displayed a phenotype. This is in accordance with exclusive localization of CL in mitochondria and the recognized role of this phospholipid and of PS in apoptosis and its regulation, as reviewed above. In yeast cells, apart from studies on the heterologous expression of mammalian proteins of the Bcl-2 family, there is no information about the CL role in the mitochondrial-mediated apoptotic pathway. Though PS externalization is clearly a hallmark of apoptosis in both animal and yeast cells, the relevance of this event was not yet clarified in yeast. Therefore, we aimed to further understand the role of CL as well of the effect of inhibition of PS externalization in the apoptotic-like PCD induced by acetic acid. To this end we used different mutant strains that exhibit decreased levels of CL (*crd1*Δ, *taz1*Δ, *ups1*Δ, *mdm10*Δ, *mdm12*Δ) or of PS (*cho1*Δ). In particular the *ups1*Δ mutant, which displays reduced levels of the CL biosynthetic intermediate PA and is thus affected in CL levels, and the *mdm10*Δ and *mdm12*Δ mutants, affected in the ERMES complex engaged in the connection of the ER with mitochondria will provide additional insights on the role of CL in apoptosis.

This work was accomplished through an integrated approach involving tools and methodologies already established in the lab, focusing on the characterization of cell death markers for selected mutants.

2.3 Material and Methods

2.3.1 Yeast strains and plasmids

The wild-type strain of *Saccharomyces cerevisiae* used in this study was BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). This strain and all mutants studied belong to the EUROSCARF knockout mutant collection (EUROSCARF, Frankfurt, Germany) and were constructed by replacing the non-essential open reading frame of the respective gene by the *KanMX4* cassette in the wild-type.

To monitor the effect of acetic acid in the mitochondrial morphology and degradation, a new plasmid containing a sequence encoding a matrix-targeted GFP protein (mtGFP) was constructed. Briefly, both the plasmids YX232-mtGFP (*TRP1*; Westermann and Neupert, 2000) and YX242 (*LEU2*; Rosenblum et al., 1998) were digested with the restriction enzymes *EcoR*I and *Xho*I at 37 ˚C overnight. After extraction of DNA fragments of interest from the agarose gel (GenElute Extraction Kit, Sigma-Aldrich), the ligation between the mtGFP sequence and the YX242 plasmid was performed during two hours at the room temperature. Thereafter, *Escherichia coli* XL1 Blue competent cells were transformed with the ligation product and grown, in LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl and 2% (w/v) agar) supplemented with 100 μg/mL of Ampicillin (Formedium), at 37 ˚C overnight. The effectiveness of this transformation was assessed by colony PCR using the primers pYX forward (ATCTATAACTACAAAAAACACATACAGGAATTCGGGCCCATGACA) and the GFP reverse (AGCGTCGACGTTACCTTATTTGTACAATTCATCCATATCCATGGG). The same colonies were simultaneously grown in LB plates and when positives, they were grown overnight in LB liquid medium (with the before mentioned Ampicillin concentration) and the plasmid pYX242-mtGFP was purified using a Miniprep kit (GenElute Plasmid Miniprep kit, Sigma-Aldrich) according to the manufacturer's instructions. Finally, the correct construction of the plasmid was confirmed by digestion, with the same restriction enzymes, in an agarose gel using both pYX242 (empty vector) and pYX232-mtGFP as control.

The yeast strains transformation with the constructed pYX242-mtGFP was carried out essentially as previously described in the Lithium acetate/Single Stranded carrier DNA/Polyethylene Glycol (PEG) method (Gietz and Woods, 2006), with some differences in the events order. A thermal shock was done to increase the process efficiency, with the sequential events: 30 minutes at 30 ˚C, 200 rpm; 30 minutes in a bath at 42 ˚C and one hour in the ice. After cell growth in a selective medium lacking leucine, the effectiveness of transformation was evaluated through visualization and counting of GFP-positive cells in the fluorescence microscope and flow cytometer, respectively.

2.3.2 Growth conditions and cell death assays

Yeast cells were maintained and grown in rich medium (YEPD; 0.5% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) glucose). Exceptionally, strains transformed with the plasmid were selected and grown in synthetic complete media (SC; 0.17% (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) uracil, adenine, histidine, lysine and tryptophan) lacking leucine.

For cell death assays induced by acetic acid, cells were grown overnight aerobically in YEPD medium or SC medium, for the transformants, until exponential phase (OD_{640nm}=0.5-0.7) in an orbital shaker at 200 rpm, 30 ˚C. Then, cells were harvest by centrifugation, suspended in YEPD at pH 3.0 (set with HCl) to a final concentration of $2x10^7$cell/mL and treated with 120 mM of acetic acid (Panreac) for up to 200 minutes at 30 °C. Samples were rigorously collected at specific time points (0, 60, 120, 180 and 200 minutes). For the cell survival assays, in these times four 1:10 serial dilutions in deionized sterile water were done and seven drops (30 μL each) of the last dilution were spotted on YEPD plates. The colony forming units (CFU) were counted after 48 hours of incubation at 30 ˚C. Cell viability was calculated over the time as percentage of CFU in relation to the time zero (considered 100%).

2.3.3 Analysis of apoptotic markers

2.3.3.1 Assessment of the plasma membrane integrity / PI staining

The plasma membrane integrity was assessed in untreated and acetic acid-treated cells by staining with the impermeable dye propidium iodide (PI, Sigma-Aldrich). Therefore, wild-type and mutant cells before and along the treatment, at the same time points used in the survival assay, were harvest, washed and suspended in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH adjusted to 7.4) at the final concentration of 10^6 cell/mL. The yeast cells were incubated at room temperature in the dark during 10 minutes with 1 μg/mL of PI.

Cells stained were analyzed by flow cytometry where the monoparametric detection of PI fluorescence was performed using FL-4 channel (488/675 nm). PI is an intercalating agent that binds to the nuclei acids in cells whose plasma membrane integrity was lost. Thus, cells with red fluorescence were considered to exhibit plasma membrane disruption and to be non-viable.

2.3.3.2 Mitochondrial membrane potential

To monitor the acetic acid effect on the mitochondrial membrane potential, cells were stained with the green fluorescent dye 3,3'dihexyloxacarbocyanine iodide ($DiOC_6(3)$, Molecular Probes). At the initial time and throughout the treatment, cells were collected, washed and suspended in the $DiOC_6(3)$ buffer (10 mM MES (2-(N-morpholino) ethanesulfonic acid), 0.1 mM $MgCl_2$, 2% (w/v) glucose, pH adjusted to 6.0 with $Ca(OH)_2$) at the final concentration of 10^6 cell/mL. Then, cells were incubated in the dark with 1 nM $DiOC_6(3)$ for 30 minutes at 30 ˚C. Additionally, 10 minutes before cytometer analysis, PI was also added at the concentration of 1 μg/mL.

$DiOC_6(3)$ is a cationic, lipophilic and permeable molecule that at the concentration and incubation time used binds to the mitochondrial membrane, where it accumulates according with the electrical potential.

In the flow cytometer, the detection of $DiOC_6(3)$ and PI was performed using the FL-1 (488/525 nm) and FL-4 (488/675 nm) channels, respectively. A monoparametric histogram [ratio (FL-1 area (log)/FS (log))] was performed in order to eliminate variations in fluorescence due to cell size. Furthermore, for the analysis of $DiOC_6(3)$ stained cells only PI negative cells were considered (through the used of a gate limited to these cells), analyzing in this way, the acetic acid effect in the mitochondrial membrane potential only in cells that maintain plasma membrane integrity.

2.3.3.3 ROS production

To assess the ROS production the probe dihydroethidium (DHE, Sigma-Aldrich) was applied. For flow cytometer analysis, untreated and acetic acid-treated cells were harvest, washed and suspended in PBS at the final concentration of 10^6 cell/mL. Subsequently, cells were incubated at room temperature in the dark for 40 minutes with 2 μg/mL DHE.

The red fluorescence in cells stained with DHE is an indicator of superoxide anion production. DHE, that is a chemically reduced form of ethidium bromide, displays a blue fluorescence in cell cytoplasm. In the presence of superoxide anion, DHE suffers oxidation and its ethidium form intercalates the cell's DNA staining the nucleus with a bright red fluorescence. Therefore, monoparametric detection of DHE was performed using the FL-4 channel (488/675 nm) and cells with red fluorescence were considered to have accumulated superoxide anions.

2.3.3.4 Mitochondrial degradation

To monitor the mitochondrial degradation, wild-type and mutant strains were transformed with the plasmid YX242 encoding a matrix-targeted GFP protein (pYX242-mtGFP). Such as in the assessment of the apoptotic markers exposed before, cells exactly at the same time points were collected and suspended in PBS at the final concentration of 10^6 cell/mL. Detection of GFP was assessed using the channel FL-1 (488/525 nm) and a biparametric histogram [ratio (FL-1 area (log)/FS (log)) x GFP fluorescence (FL-1 Peak)] was performed in order to eliminate variations in fluorescence due to cell size and to discriminate between the cells with intense spots of mitochondrial-GFP and cytosolic-GFP resultant from mitochondrial degradation.

The mitochondria degradation was calculated through the decrease of the percentage of GFP-positive cells over the time in relation to the percentage exhibited, individually for each strain, at time zero (considered 100%).

2.3.4 Flow cytometry

Flow cytometry analysis was performed in an Epics® XL-MCL™ (Beckman COULTER®) flow cytometer, equipped with an argon-ion laser emitting a 488-nm beam at 15 mW. The population of cells displayed a high homogeneity and frequency was gated in a histogram of Side Scatter (SS) x Forward Scatter (FS). Twenty thousand cells were analyzed per sample and experiments were independently reproduced at least three times. Data were analyzed in Flowing Software 2.

2.3.5 Fluorescence Microscopy

To assess to the mitochondrial morphology, cells transformed with the plasmid YX242-mtGFP (500 μL) were harvest by centrifugation before and after 10 minutes of acetic acid treatment and suspended in PBS at a final concentration of 10^7 cell/mL. Cells were visualized and images acquired in a Leica Microsystems DM-5000B epifluorescence microscope coupled to a Leica DCF350FX digital camera.

2.4 Results

Nowadays, the role of mitochondria in apoptosis is unquestionable although there are still some mechanisms to clarify. Therefore, it was aimed to study the role of CL, a phospholipid exclusively present in mitochondria, in the acetic acid-induced apoptotic PCD. For this purpose, we used yeast strains deleted in genes with a well-established role in the CL biosynthetic pathway, such as *CRD1* (CL synthase), *TAZ1* (CL remodeling), *UPS1* (responsible for PA translocation to the IMM) and others that we speculated that could also be important for CL synthesis, such as *MDM10* and *MDM12*, and whose deletion could also lead to decreased CL levels. At the same time, we intended to study the effect of acetic acid in the *cho1*Δ mutant, lacking the enzyme responsible for PS synthesis, in order to better characterize the involvement of PS in acetic acid-induced PCD.

The effect of acetic acid on all the mutants was initially evaluated on yeast survival by treatment of the cells with 120 mM of this acid for 200 minutes (Figure 10). The results showed that all deleted strains are resistant to acetic acid when compared to the wild-type (BY4741), although for *crd1*Δ this was only observed for the 60 minutes time point. A similar situation was observed for *ups1*Δ mutant that for the last two time points did not differed from the wild-type strain. *taz1*Δ and *mdm10*Δ mutants strains revealed the most resistant of the tested strains maintaining a cell survival of 54.83±5.9% and 64.97±4.5%, respectively, after 200 minutes. The deletion of the genes *CHO1* and *MDM12* led to similar survival percentages throughout the treatment, presenting approximately 20% of survival at 200 minutes.

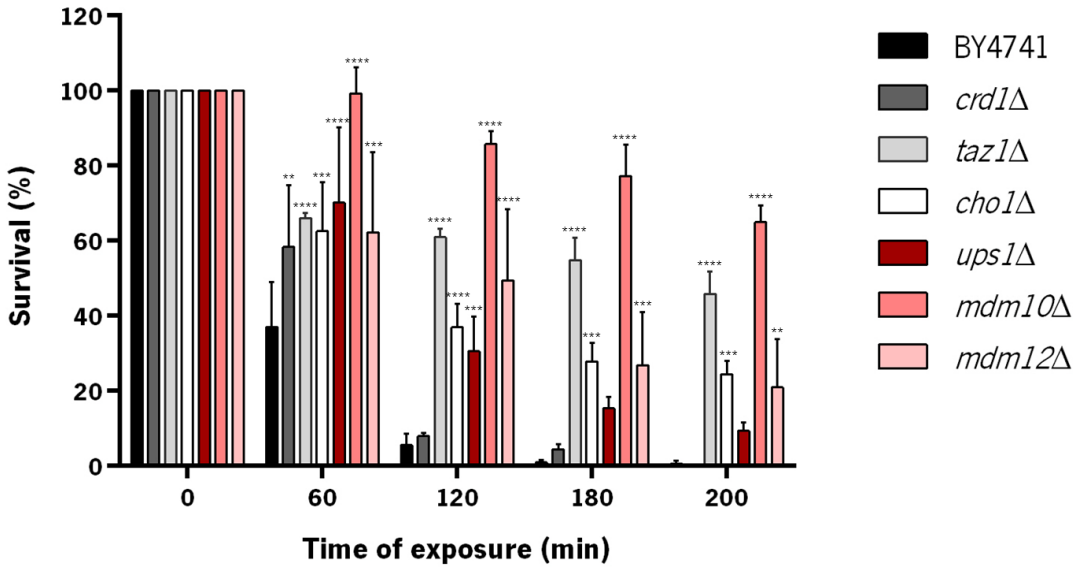

Figure 10 - Relative cell survival, determined by CFU counts on YEPDA plates, of exponential phase yeast cells treated with 120 mM of acetic acid during 200 minutes. The number of colonies obtained, individually for each strain, was normalized to the time zero, which was considered 100%. Reported values are the mean of at least three independent experiments with standard deviation. A two-way ANOVA test was employed to compare the survival between wild-type (BY4741) and deleted strains, for each time of treatment. Statistical analysis: *p<0,05, **p<0,01, ***p<0,001 and ****p<0,0001.

To better characterize these phenotypes, the plasma membrane integrity was assessed by flow cytometer analysis of PI stained cells (Figure 11). In general, the mutant strains presented lower percentages of PI-positive cell along treatment, consistent with their higher resistance. However, there was not a strict correlation between the loss of CFU and increase of PI-positive cells. For example, in the case of *cho1*Δ mutant, although this mutant revealed as one of the most resistant strains evaluated by CFU counts, after 120 minutes of treatment with acetic acid, the percentage of PI-positive cells was higher in this strain than in wild-type, and it still increased considerably for the two last time, being the highest after the wild-type strain. A similar situation was observed for *taz1*Δ mutant. On the other hand, *crd1*Δ and *ups1*Δ mutants, although being only slightly more resistant than the wild-type when evaluated by CFU, were able to retain the membrane integrity much more efficiently, reaching the end of the assay only with 8.03±4.04% and 4.19±2.5% of PI-positive cells, respectively.

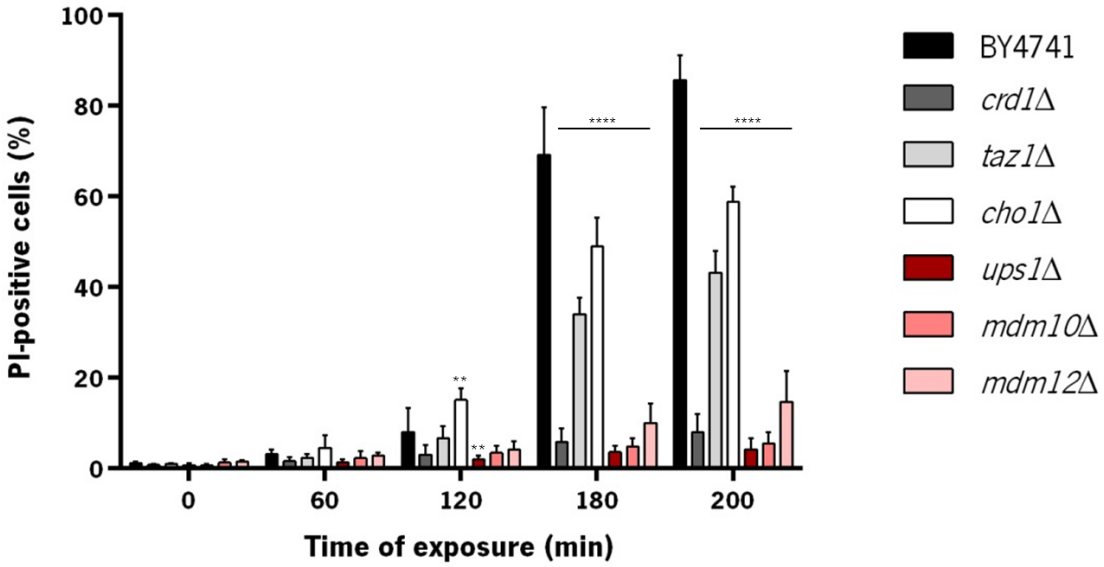

Figure 11 - Percentage of cells treated with 120 mM of acetic acid during 200 minutes displaying loss of plasma membrane integrity. Yeast cells at exponential phase were, for each time point, harvest and stained with PI and the percentage of cells incorporating this dye was evaluated by flow cytometry. Reported values are the mean of at least three independent experiments with standard deviation. A two-way ANOVA test was employed to compare the percentage of PI-positive cells between wild-type (BY4741) and deleted strains, for each time of treatment. Statistical analysis: *p<0,05, **p<0,01, ***p<0,001 and ****p<0,0001.

To monitor the effect of acetic acid on the mitochondrial membrane potential, cells were collected at the same time points, stained with $DioC_6(3)$ and analyzed by flow cytometry (Figure 12). Differently from the other probes analyzed, in this assay it was not evaluated the percentage of stained cells but instead the mean of the fluorescence intensity, corrected for the relative size of the cells. The results showed that the wild-type strain suffers a hyperpolarization at 60 minutes followed by a loss of the mitochondrial membrane potential that is gradual until the end of the treatment, as previously described (Ludovico et al., 2002). Generically all mutant strains exhibited a constant mitochondrial membrane potential over treatment time, although some of these have a slight increase that may indicate some perturbation caused by the treatment. During the acetic acid treatment, *ups1*Δ mutant displayed the lowest membrane potential when compared with the wild-type. In *taz1*Δ, *cho1*Δ and *mdm12*Δ the mitochondrial membrane potential appears to increase initially remaining thereafter almost unchanged. On the other hand, *crd1*Δ and *mdm10*Δ strains have a slight increase to 180 minutes followed by a significant decrease.

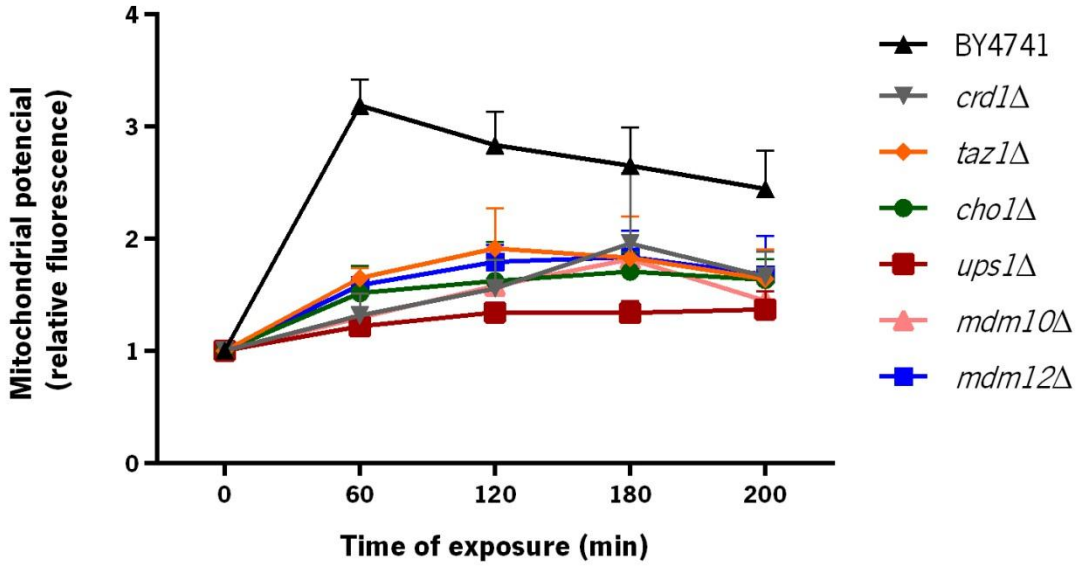

Figure 12 – Changes of the mitochondrial membrane potential (relative fluorescence values) in yeast strains treated with 120 mM of acetic acid during 200 minutes. Cells, at each time point, were harvest, stained with $DioC_6(3)$ and after incubation analyzed by flow cytometry. The values obtained were, for each strain, normalized to the time zero. Reported values are the mean of at least three independent experiments.

To address the intracellular levels of ROS, cells under the same conditions were labeled with DHE. At the initial time point, the superoxide levels inside the cells did not vary significantly among strains (Figure 13). Subsequently, it was observed that BY4741, *taz1*Δ and *mdm12*Δ present an early increase in the superoxide anion accumulation, which was evident for almost 100% of the cells in the two last time points. Although with a delay at the initial time points, the same was verified for *crd1*Δ mutant. *cho1*Δ, *ups1*Δ and *mdm10*Δ mutants have lower ROS levels than BY4741, even at the final of the treatment.

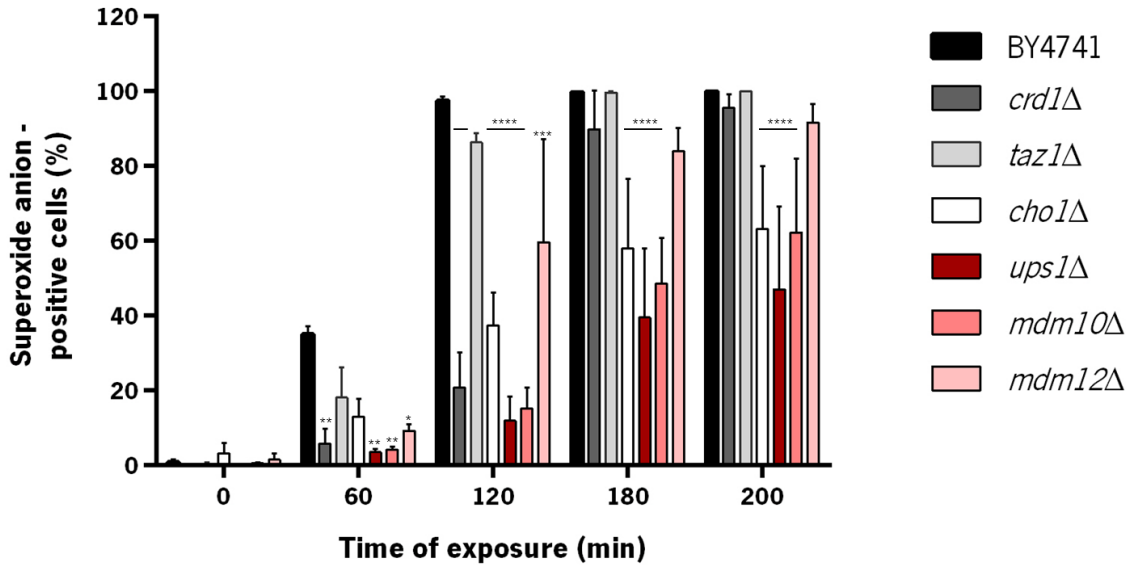

Figure 13 - Percentage of yeast cells displaying superoxide anion accumulation. During the treatment with 120 mM of acetic acid, exponential yeast cells were harvest and stained with DHE with subsequent analyzes by flow cytometry. Reported values are the mean of at least three independent experiments with standard deviation. A two-way ANOVA test was employed to compare the percentage of DHE-positive cells between wild-type (BY4741) and deleted strains, for each time point. Statistical analysis: *p<0,05, **p<0,01, ***p<0,001 and ****p<0,0001.

To examine the effect of acetic acid in the mitochondrial morphology and degradation, cells were transformed with a plasmid containing a sequence that encodes a matrix target protein coupled to GFP (mtGFP). To achieve this, we constructed a new plasmid with the appropriate selective marker for these *S. cerevisiae* strains that allowed maintaining the growth and treatment conditions. So, the purpose of this construction was to change the insert coding for mtGFP protein from YX232 plasmid to YX242 plasmid that had the selective marker of interest (Figure 14A). Both the plasmids YX232-mtGFP (*TRP1*) and YX242 (*LEU2*) were digested with the restriction enzymes *EcoR*I and *Xho*I. Thereafter the fragments of interest were extracted from the agarose gel at the expected molecular weights (Figure 14B) and the ligation was promoted. *E. coli* competent cells were transformed with the resulting product and the effectiveness of this process was assessed by colony PCR using two primers that flanks the mtGFP

sequence (Figure 14C). After plasmid purification from the positive *E. coli* colony, the construction was confirmed by digestion (Figure 14D). Once validated, the yeast strains were transformed with the new plasmid. After construction of all the strains carrying the plasmid expressing mtGFP, flow cytometry analysis allowed to evaluate the transformation efficiency (not shown).

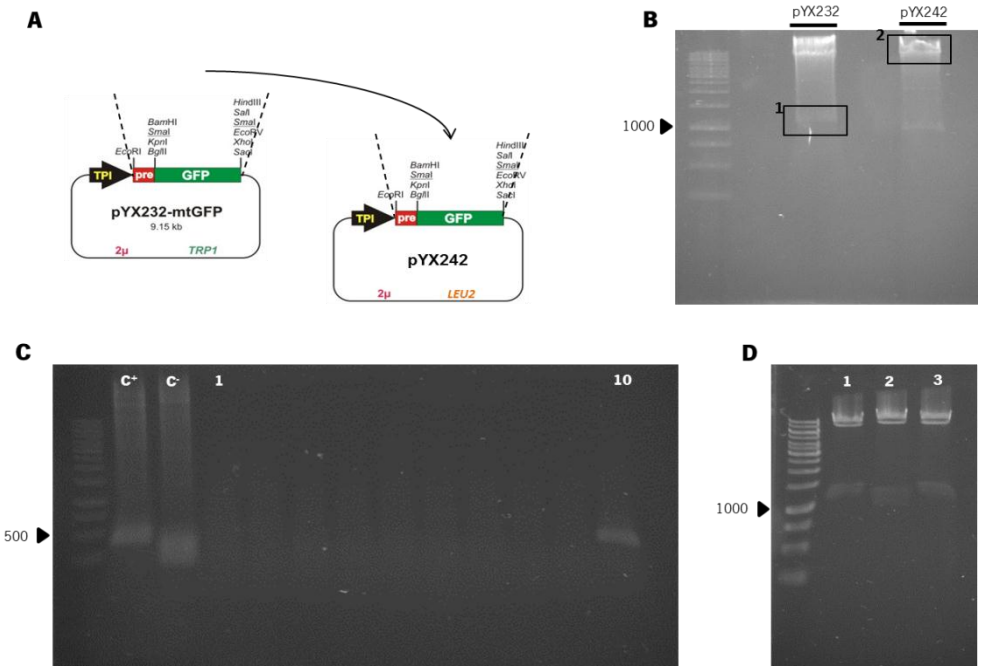

Figure 14 – Schematic representation of the most important steps for pYX242-mtGFP construction. (A) The purpose was to insert the mtGFP sequence of the YX232 plasmid in pYX242 that has leucine as selective marker. (B) Both plasmids were digested and the insert (1) and pYX242 (2) were extracted from an agarose gel. (C) After ligation, *E. coli* cells were transformed with the product and the positive colonies assessed by colony PCR, using at the same time a positive (pYX232-mtGFP) and negative (pYX242) control. (D) After plasmid purification of the positive colony (number 10), the right construction was confirmed by digestion comparing with the pYX232-mtGFP (1) and pYX242 (2), having the new construction, as expected, (3) an insert with same molecular weight of 1, and plasmid like 2.

It has been previously shown that acetic acid can lead to a mitochondrial morphology change from a tubular network to a punctate morphology that subsequently can be degraded by vacuolar proteases, like Pep4p (Pereira et al., 2010). Therefore, we evaluated the changes in the mitochondrial morphology by fluorescence microscopy and its degradation by the decrease of GFP-positive cells in the flow cytometer.

In this study, it was observed that after acetic acid treatment, the *taz1*Δ mutant strain exhibited a substantial loss of the number of GFP-positive cells when compared with the wild-type strain. However, both strains displayed less than 2% of GFP-positive cells after 200 minutes of treatment. Although at 120 minutes *cho1*Δ exhibited a significant higher percentage of cells without mitochondrial degradation, for higher treatment times it did not differ from the *crd1*Δ, *mdm10*Δ and *mdm12*Δ mutants. Curiously,

*ups1*Δ deleted strain, that displayed the lowest production of superoxide anions, exhibited the highest number of GFP-positive cells in all time points of the treatment (Figure 15).

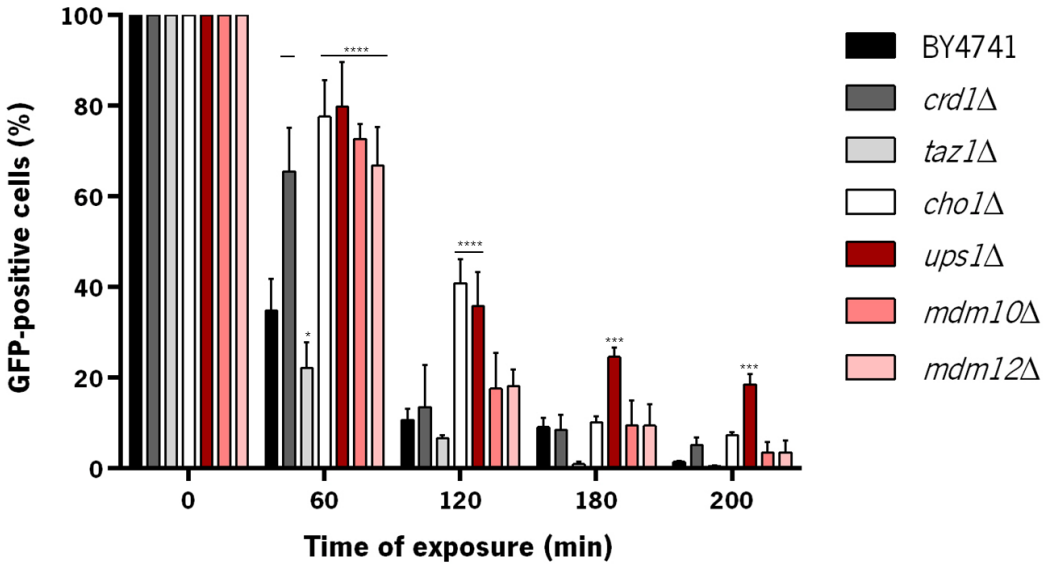

Figure 15 – Exponential yeast cells transformed with the plasmid YX242-mtGFP were treated with 120 mM of acetic acid during 200 minutes. At each time point, cells were harvest and the percentage of GFP-positive cells monitored by flow cytometry, in which the fluorescence at the initial time, for each strain, was considered 100%. In this assay, the decrease of the percentage of GFP-positive cells was assumed as mitochondrial degradation. Reported values are the mean of at least three independent experiments with standard deviation. A two-way ANOVA test was employed to compare the percentage of GFP-positive cells between wild-type (BY4741) and deleted strains, for each time of treatment. Statistical analysis: *p<0,05, **p<0,01, ***p<0,001 and ****p<0,0001.

Analysis of the transformed strains by fluorescence microscopy showed that acetic acid had a fast effect on the mitochondria morphology (after 10 minutes). In the wild-type, the clearly defined mitochondrial network in cells without treatment is changed to a punctate form after acetic acid treatment. The same happened for *crd1*Δ and *taz1*Δ mutants, although at the beginning they presented a not so well-defined network, when compared with the wild-type, which is associated with clustered forms. Surprisingly, *cho1*Δ presented the greatest number of mitochondrial networks that is equally fragmented in many punctate forms after treatment. Oppositely, *ups1*Δ displays a punctate form before the treatment, with few and big rounded forms that, after acetic acid, are converted into a high number of small units that appears clustered. On the other hand, *mdm10*Δ and *mdm12*Δ mutant strains present among them, before and after the death stimulus, similar profiles characterized by elongated structures that turned into few and large rounded structures along treatment (Figure 16).

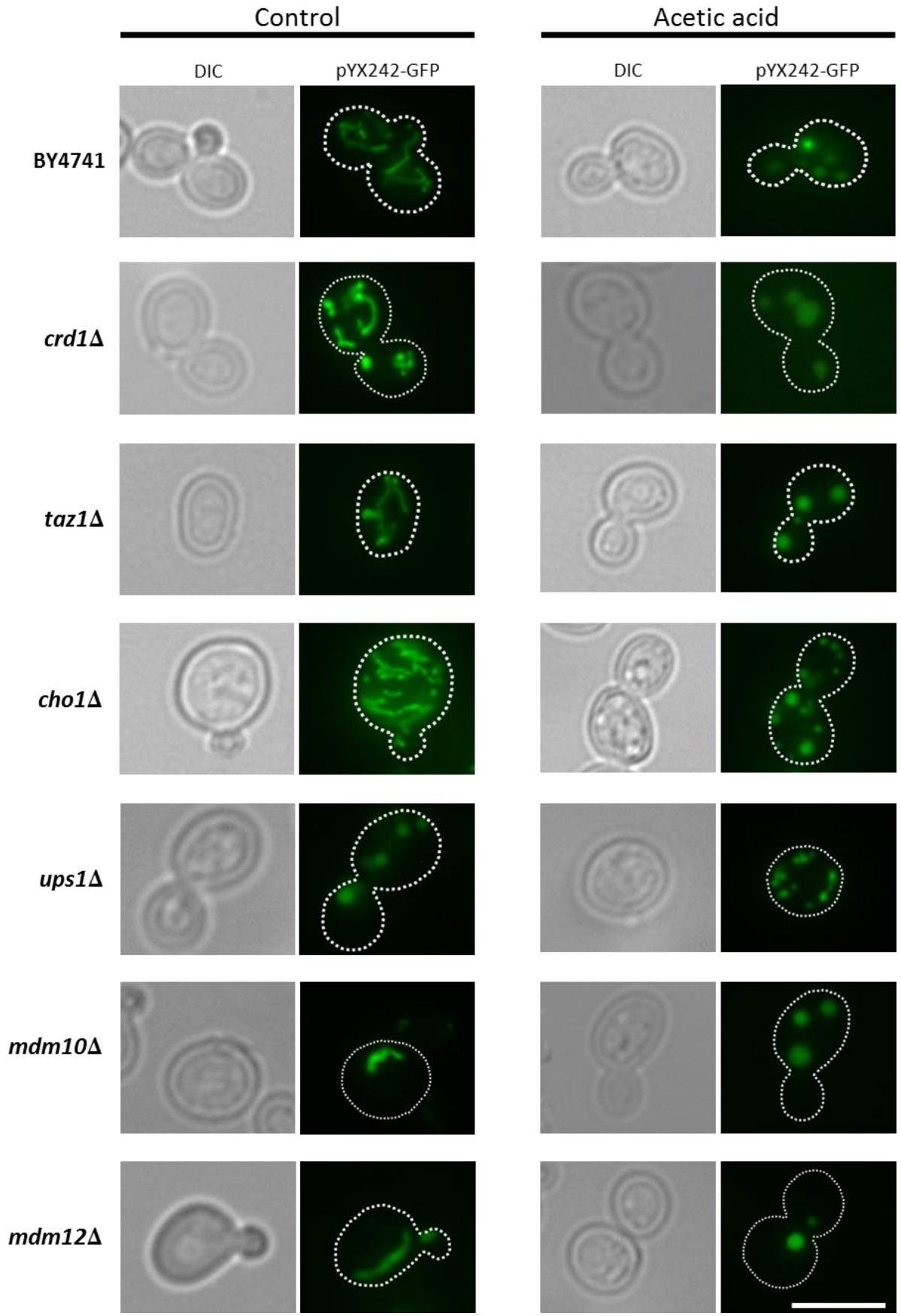

Figure 16 - Analysis of mitochondrial morphology in exponential yeast cells transformed with the plasmid YX242-mtGFP. Cells were visualized through a fluorescence microscopy at time zero and after 10 minutes of acetic acid treatment (120 mM). Pictures collected are representative of the results obtained in at least three independent experiments. Bar, 7.5µm

2.5 Discussion

Once the occurrence of PCD in the yeast *S. cerevisiae* was established, this cellular model has been extensively used to investigate the mechanisms of PCD in animal cells that have not yet been addressed or completely understood. In both models, the mitochondrial mediated-apoptotic pathway has been demonstrated. Convinced that yeast may further contribute to highlight the mitochondria involvement in this process, the main goal of this thesis was to study the role of selected genes, which code for mitochondrial proteins directly or indirectly involved in the biosynthesis of the phospholipids CL and PS, in acetic acid-induced apoptotic cell death. Indeed the reduced information about the function of phospholipids in the yeast apoptotic-like PCD, prompt us to characterize this process of cell death in mutants with altered phospholipid profiles, in particular with reduced levels of CL and PS.

In animal cells, it was demonstrated that CL is surely fundamental to trigger apoptosis. Besides its interaction with cyt *c* in the IMM, the peroxidation and/or degradation that occur during apoptosis favour the mitochondrial recruitment and activation of apoptotic molecules, such as tBid, caspase-8 and Bax/Bak, which finally lead to MOMP and release of mitochondrial proteins with apoptotic functions.

The relevance of the present study is stressed by the absence of information on the role of CL in the yeast apoptotic-like PCD. We found that inhibition of CL synthesis, through *CRD1* deletion, lead to a survival percentage similar to that of wild-type strain. This suggests that, oppositely to the well-established role of CL in animal apoptosis, this phospholipid is not fundamental to trigger apoptosis in acetic acid-yeast treated cells. As aforementioned, it was reported that the yeast *crd1*Δ mutant, when grown in non-fermentable carbon sources, accumulates the precursor lipid PG that can, at least in part, compensate the effect of CL loss in some cellular processes, namely apoptosis. Oppositely, under fermentable conditions the specific activity of the enzyme PGP synthase is decreased, and the PG levels are not detectable when compared with aerobic conditions (Jiang et al., 2000). In our study conditions, cells were grown under a high glucose concentration (fermentative metabolism), and it is therefore expectable that the decreased levels of CL in the *crd1*Δ mutant were not compensated. However, it was never reported, in yeast or animal cells, the effect of acetic acid in the relative levels of phospholipids inside the cell. So, under our study conditions, the hypothesis that another phospholipid can compensate the decreased CL levels inside the cells, and consequently its role in apoptosis, cannot be ruled out. Curiously, during acetic acid treatment, in the *crd1*Δ mutant, the loss of the plasma membrane integrity and ROS accumulation were delayed when compared with the wild-type. Furthermore, as described in

other studies performed in yeast cells (Jiang et al., 2000), *crd1*Δ strain presented a mitochondrial membrane potential lower than the wild-type during acetic acid treatment. In addition, in accordance with animal cells with RNA interference (RNAi) silenced CL synthase (Choi et al., 2007), we found that CL is required for the maintenance of the mitochondrial structure, even in cells without treatment. Altogether these results suggest that, even in the presence of altered levels of some phospholipids (e.g. PG) that may assure an identical cell survival of *crd1*Δ and wild-type, the physiological consequences of CL loss cannot be completely restored. Oppositely to our study, it was reported that there is no mitochondrial morphology changes in a *crd1*Δ yeast mutant with increased levels of PG (Chang et al., 1998a). Therefore, our results appear to indicate that CL deceased levels are not compensated by altered levels of other phospholipids and that instead, under acetic acid treatment, CL is not fundamental to trigger apoptosis.

As previously mentioned, it has been described that CL after being synthesized by Crd1p undergoes a process of maturation triggered by Taz1p whose relevance was underlined after the discovery of its involvement in the BTHS. When this transacylase is deleted there is no synthesis of mature CL and at the same time, the MLCL levels increase inside the cell. In this study, it was evaluated the response of *taz1*Δ mutant to acetic acid inducing apoptotic conditions in order to understand the role of CL remodeling and the consequence of increased MLCL levels in this process of cell death. During the treatment, *taz1*Δ mutant revealed to be significantly resistant presenting about 50% of viable cells at 200 minutes, compared with 1.0% of the wild-type cells. Curiously, *taz1*Δ mutant at 200 minutes treatment displayed a percentage of PI-positive cells (50%) identical to the percentage of nonviable cells, which appears to demonstrate that in the absence of mature CL, cells are dying later but by a necrotic process. An identical profile was observed in *cho1*Δ mutant, which was resistant to acetic acid, maintaining a relatively high percentage of viable cells at the end of the treatment, associated with a similar increase in the percentage of PI-positive cells. This is in contrast to the observed with the mutants *ups1*Δ, *mdm10*Δ and *mdm12*Δ, in which the increase of cell survival was associated with a low percentage of PI-positive cells. Taking into account that both Taz1p and Cho1p are involved in the synthesis of mature CL and PS, respectively, it is conceivable that the increased percentage of PI-positive cells may reflect a higher PI permeability of the plasma membrane caused by the loss of these phospholipids, rather than the real percentage of non-viable cells with compromised plasma membrane. Considering this hypothesis, cells were stained with 10 μg/mL of 7-aminoactinomycin (7-AAD, Molecular Probes) that like PI, only permeates cells with compromised plasma membrane integrity, but whose molecular weight is 1270.45,

almost the double of PI. However, the number of the 7-AAD positive cells was very similar to the PI staining (data not shown). To further characterize the cell death process in these mutants the metabolic activity was evaluated by FUN-1 (2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide, Molecular Probes) and FDA (fluorescein diacetate, Sigma-Aldrich) staining. The processing of FUN-1 by viable cells results in the formation of cylindrical intra-vacuolar structures (CIVS) which display a very regular form. However, when cells lose their metabolic capacity, the non-processing of FUN-1 leads to its accumulation in cytosol that is perceptible due to an increase in red fluorescence. The monitoring of FUN-1 staining in the mutant strains *taz1*Δ and *cho1*Δ through fluorescence microscopy showed that, oppositely to well-defined CIVS in the vacuole at time zero, cells presented a diffuse red staining throughout the cell for the final periods of treatment (data not shown). Accordingly, in these mutant strains after 200 minutes of treatment, an increase in the mean of the red fluorescence intensity of about two-fold, when compared with the time zero, was assessed by flow cytometry (data not shown). Staining with FDA is an alternative to monitor metabolic activity as assessed by esterase activity but only in cells which preserved their plasma membrane integrity. Cells with active esterases cleave FDA to fluorescein and acetate, and exhibited green fluorescence if fluorescein is retained in the cytosol. Under our study conditions, the FDA staining demonstrated clearly that in *taz1*Δ mutant 50% of the cells lost their metabolic activity after 200 minutes (data not shown). In *taz1*Δ and *cho1*Δ strains, the identical percentage of non-viable cells and of cells with loss of plasma membrane integrity and metabolic activity suggest that, after 200 minutes of acetic acid treatment, these mutants are dying by necrosis. Since some authors proposed that the yeast Taz1p, like CL, interacts with multiple proteins, including the ATP synthase (Claypool et al., 2008a), it is likely that in the absence of this protein the ATP synthesis and the metabolic activity are decreased. This interpretation may explain the results obtained with FUN-1 and FDA staining.

In animal cells, PS translocation to the outer leaflet of the plasma membrane during apoptosis is fundamental to allow the recognition and clearance of apoptotic cells by phagocytes, preventing an inflammatory response (Balasubramanian et al., 2007). Although, in yeast cells, the PS externalization had also been reported during apoptotic-like PCD (Madeo et al., 1997), the consequences of the impairment of this phenomenon, namely through PS synthase inhibition, was never described. Curiously, in our study the appearance of some features typical of necrosis, such as loss of plasma membrane integrity and metabolic activity, in *cho1*Δ mutant appears to indicate that the presence of PS is, like in mammalian cells, fundamental to trigger an apoptotic death. However our results may not be directly

associated with the loss of PS externalization and instead with the decreased intracellular levels of PS. Thus, more studies are needed to clarify this process.

In our study, the observed resistance of *taz1*Δ mutant to acetic acid treatment appears to be in agreement with the decreased tafazzin expression levels in B cell lymphoma, suggesting that tafazzin loss may contribute to tumor progression (Kobayashi et al., 2003). On the other hand, also in accordance with our results, lymphoblast cells of BTHS patients (deficient in Taz1p activity) demonstrated equally high levels of cells whose plasma membrane integrity was lost when treated with stimuli that induce the mitochondrial-mediated apoptotic pathway, such as etoposide and cisplatin (Gonzalvez et al., 2008). In our treatment conditions, the high and early ROS production associated with an increase in mitochondrial degradation demonstrated an altered mitochondrial function in *taz1*Δ mutant strain. Accordingly with these results, in animal cells it was reported that *TAZ1* deficient cells have serious mitochondrial abnormalities associated also with energy metabolism impairment, including the adhesion of opposing membranes that results in intracrista space deformation (Acehan et al., 2007; Xu et al., 2005). Therefore, it is possible that in our study after three hours of acetic acid treatment, *taz1*Δ mutant cells trigger a necrotic pathway once the plasma membrane integrity and the metabolic activity were impaired, although it cannot be ruled out that up to two hours of treatment cells are dying by an initial apoptotic process. In sum, our results appear to suggest that in yeast cells, like for mammalians, the Taz1p and consequently the presence of mature CL are fundamental to trigger an apoptotic pathway.

Analysis of the results with *crd1*Δ and *taz1*Δ mutants, shows that CL is fundamental to maintain the mitochondrial membrane potential, morphology and integrity independently of its degree of maturation, as previously described (Brandner et al., 2005; Chen et al., 2008). The increase of ROS production has been reported to happen in very different apoptotic scenarios, however the similar ROS levels in *taz1*Δ and *crd1*Δ, whose response to acetic acid is very different, indicate that the increase of oxidative stress cannot be always correlated with loss of cell viability, like described in other studies (Pereira et al., 2007).

In the absence of Ups1p, PA is not translocated to the IMM and therefore, the process of CL synthesis, as well as of its intermediates, is blocked. The results show that *UPS1* deletion confers resistance to acetic acid-induced PCD. Interestingly, among the mutants studied *ups1*Δ presented the lower mitochondrial membrane potential associated with the lowest percentage of ROS production and mitochondrial degradation. It has already been reported, in accordance with the present study, that when grown in fermentable carbon sources, the mitochondrial potential is reduced in *ups1*Δ mutant that additionally exhibits an altered mitochondrial morphology characterized by short tubules, small fragments

and aggregates (Sesaki et al., 2006; Tamura et al., 2009). Our results now suggest that the PA presence in the IMM, probably due synthesis of CL and of its precursors, is fundamental for the apoptotic process. Curiously, the *ups1*Δ phenotype appears not to result simply from a decrease in CL levels once *ups1*Δ and *crd1*Δ mutant strains exhibit significant differences in response to acetic acid inducing apoptotic conditions.

In this study we also addressed for the first time the role of ERMES complex components in the yeast apoptotic-like PCD. Although the deletion of both genes confer resistance to the acetic acid, *mdm10*Δ presented after 200 minutes higher percentage of survival and lower number of PI- and DHE- positive cells, than the *mdm12*Δ mutant. On the other hand, the acetic acid treatment led to an identical profile of mitochondrial degradation in both mutant strains, associated with also similar morphological changes. It was reported that mitochondria from ERMES complex mutants can exhibit large, spherical or oblong shapes, which is in accordance with our observations (Jensen, 2005). Indeed, the ERMES complex has been extensively associated to the mitochondrial morphology and it is possible that the continuous trafficking of phospholipids may be critical for maintaining the morphology and function of this compartment (Tamura et al., 2012).

Like before hypothesized, ERMES complex, especially Mdm10p at OMM, due to the reported CL decreased levels could be important for PA translocation from ER to mitochondria. If this is the explanation for the observed results, the consequence of *MDM10* or *UPS1* deletion should be identical. Although both mutants present similar percentage of PI and DHE-positive cells, the survival was enhanced in the *mdm10*Δ mutant demonstrating that Mdm10p can be involved in additional mechanisms fundamental for the apoptotic process. It was previously hypothesized that ERMES complex can have a fundamental role in the calcium cross-talk between ER and mitochondria. It is known that, during apoptosis, the high levels of this ion within the mitochondria lead to its swelling and permeabilization. So, the mitochondria calcium overload inhibition can, at least in part, justify this phenotype. Moreover, the involvement of Mdm10p and Mdm12p in the β-barrel protein assembly machinery at OMM, that can themself be important for MOMP, may also justify the resistance observed in the *mdm10*Δ mutant or even Mdm10p can itself be involved in the mechanism of release of mitochondrial apoptotic proteins, such as cyt *c*.

In our study it was aimed to characterize the role of CL in the apoptotic-like PCD induced by acetic acid, using for this end different mutant strains that exhibit altered levels of CL. Curiously, our results

demonstrated that, for example, the CL synthesis abrogation (*crd1*Δ mutant) and the absence of mature CL (*taz1*Δ mutant) result in significantly different responses to acetic acid treatment.

In animal cells, mature CL can suffer hydrolysis during apoptosis increasing the levels of MLCL inside the cell (Liu et al., 2005). It was reported that the deletion of *TAZ1* gene in yeast cells lead also to increased levels of MLCL (Gu et al., 2004), mimicking animal cells during apoptosis. However, under our conditions the yeast *taz1*Δ appears to dye by a necrotic process suggesting that the increased levels of MLCL may in yeast and animal cells trigger different processes of cell death. On the other hand, the *crd1*Δ mutant results appear to suggest that CL is not fundamental to trigger apoptosis. However, as discussed above, the presence of altered levels of other(s) phospholipid(s), which may to explain the similar phenotype of *crd1*Δ mutant and the wild-type, should not be discarded. So, some questions arise including if the complete absence of CL (*crd1*Δ mutant) or the loss of mature CL (*taz1*Δ mutant), with subsequent increase of MLCL levels, establishes the response and/or the type of cell death triggered.

Beyond that, it is important to clarify if in these mutants additionally to the reported phospholipid changes, there are others that could explain the results. Curiously, in all these mutants the phospholipid profile has already been identified, however, there is some contradictory information between different experiments, which suggest that the phospholipid ratio inside the mitochondria can differ with the strain, growth conditions and carbon source. It was reported that *crd1*Δ and *taz1*Δ mutants growing in YEPD presented decreased levels of PA (Gu et al., 2004; Zhong et al., 2004). Like it was explained before, PA is only associated to the membranes which contain negatively charged phospholipids, therefore the absence of CL can, at least in part, justify this result. It is possible that the decreased rate of PA translocation to the IMM can, through a feedback mechanism, results in its import inhibition. On the other hand, the increased levels of PE, PS and PC in the *taz1*Δ mutant strain appear to suggest that increased levels of MLCL results in a shift of CL biosynthetic pathway to the synthesis of other phospholipids thus justifying the high levels within the mitochondria and perhaps the decreased PA levels (Gu et al., 2004). Interestingly, the decreased levels of PE in ERMES complex mutants, suggest that this complex can be in fact fundamental for phospholipids trafficking between ER and mitochondria (Tamura et al., 2012).

Although in all mutants here studied had been reported decreased levels of CL, the different responses obtained in our study underlie that these proteins must be involved in the synthesis or regulation of other phospholipids or even proteins with fundamental function in the apoptotic process. Also the resistance verified in *cho1*Δ mutant cannot exclusively be associated to PS decreased levels

once this phospholipid was demonstrated to be increased in the *ups1*Δ, *mdm10*Δ and *mdm12*Δ mutants that also display a resistant phenotype. Therefore, it is possible that the different responses to the acetic acid treatment are caused by alterations in the levels of multiple phospholipids or their relative proportions within the cell membranes.

Although this work requires a phospholipid characterization that may allow establishing a model for the involvement of the different phospholipids species, the role of the proteins encoded by the genes studied are certainly fundamental for maintaining the mitochondrial integrity, emphasizing the importance of these proteins in the cell death mediated by this organelle.

2.6 References

Acehan, D., Malhotra, A., Xu, Y., Ren, M., Stokes, D. L., and Schlame, M. (2011). Cardiolipin affects the supramolecular organization of ATP synthase in mitochondria. Biophysical Journal *100*, 2184-2192.

Acehan, D., Xu, Y., Stokes, D. L., and Schlame, M. (2007). Comparison of lymphoblast mitochondria from normal subjects and patients with Barth syndrome using electron microscopic tomography. Laboratory Investigation *87*, 40-48.

Achleitner, G., Gaigg, B., Krasser, A., Kainersdorfer, E., Kohlwein, S. D., Perktold, A., Zellnig, G., and Daum, G. (1999). Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact. European Journal of Biochemistry *264*, 545-553.

Ardail, D., Privat, J. P., Egret-Charlier, M., Levrat, C., Lerme, F., and Louisot, P. (1990). Mitochondrial contact sites. Lipid composition and dynamics. Journal of Biological Chemistry *265*, 18797-18802.

Atkinson, K., Fogel, S., and Henry, S. A. (1980). Yeast mutant defective in phosphatidylserine synthesis. The Journal of Biological Chemistry *255*, 6653-6661.

Balasubramanian, K., Mirnikjoo, B., and Schroit, A. J. (2007). Regulated externalization of phosphatidylserine at the cell surface: implications for apoptosis. The Journal of Biological Chemistry *282*, 18357-18364.

Barth, P. G., Wanders, R. J., Vreken, P., Janssen, E. A., Lam, J., and Baas, F. (1999). X-linked cardioskeletal myopathy and neutropenia (Barth syndrome) (MIM 302060). Journal of Inherited Metabolic Disease *22*, 555-567.

Belikova, N. A., Vladimirov, Y. A., Osipov, A. N., Kapralov, A. A., Tyurin, V. A., Potapovich, M. V., Basova, L. V., Peterson, J., Kurnikov, I. V., and Kagan, V. E. (2006). Peroxidase activity and structural transitions of cytochrome c bound to cardiolipin-containing membranes. Biochemistry *45*, 4998-5009.

Betaneli, V., Petrov, E. P., and Schwille, P. (2012). The role of lipids in VDAC oligomerization. Biophysical Journal *102*, 523-531.

Bisaccia, F., and Palmieri, F. (1984). Specific elution from hydroxylapatite of the mitochondrial phosphate carrier by cardiolipin. Biochimica et Biophysica Acta *766*, 386-394.

Brandner, K., Mick, D. U., Frazier, A. E., Taylor, R. D., Meisinger, C., and Rehling, P. (2005). Taz1, an Outer Mitochondrial Membrane Protein, Affects Stability and Assembly of Inner Membrane Protein Complexes: Implications for Barth Syndrome. Molecular Biology of the Cell *16*, 5202-5214.

Chang, S., Heacock, P. N., Mileykovskaya, E., Voelker, D. R., and Dowhan, W. (1998a). Isolation and Characterization of the Gene (CLS1) Encoding cardiolipin Synthase in Saccharomyces cerevisiae. The Journal of Biological Chemistry *273*, 14933-14941.

Chang, S. C., Heacock, P. N., Clancey, C. J., and Dowhan, W. (1998b). The PEL1 gene (renamed PGS1) encodes the phosphatidylglycero-phosphate synthase of Saccharomyces cerevisiae. The Journal of Biological Chemistry *273*, 9829-9836.

Chen, S., He, Q., and Greenberg, M. L. (2008). Loss of tafazzin in yeast leads to increased oxidative stress during respiratory growth. Molecular Microbiology *68*, 1061-1072.

Choi, S. Y., Gonzalvez, F., Jenkins, G. M., Slomianny, C., Chretien, D., Arnoult, D., Petit, P. X., and Frohman, M. A. (2007). Cardiolipin deficiency releases cytochrome c from the inner mitochondrial membrane and accelerates stimuli-elicited apoptosis. Cell Death & Differentiation *14*, 597-606.

Claypool, S. M. (2009). Cardiolipin, a critical determinant of mitochondrial carrier protein assembly and function. Biochimica et Biophysica Acta *1788*, 2059-2068.

Claypool, S. M., Boontheung, P., McCaffery, J. M., Loo, J. A., and Koehler, C. M. (2008a). The cardiolipin transacylase, tafazzin, associates with two distinct respiratory components providing insight into Barth syndrome. Molecular Biology of the Cell *19*, 5143-5155.

Claypool, S. M., Oktay, Y., Boontheung, P., Loo, J. A., and Koehler, C. M. (2008b). Cardiolipin defines the interactome of the major ADP/ATP carrier protein of the mitochondrial inner membrane. The Journal of Cell Biology *182*, 937-950.

Connerth, M., Tatsuta, T., Haag, M., Klecker, T., Westermann, B., and Langer, T. (2012). Intramitochondrial transport of phosphatidic acid in yeast by a lipid transfer protein. Science *338*, 815-818.

Crompton, M. (1999). The mitochondrial permeability transition pore and its role in cell death. The Biochemical Journal *341* 233-249.

Csordas, G., Renken, C., Varnai, P., Walter, L., Weaver, D., Buttle, K. F., Balla, T., Mannella, C. A., and Hajnoczky, G. (2006). Structural and functional features and significance of the physical linkage between ER and mitochondria. The Journal of Cell Biology *174*, 915-921.

Cullis, P. R., Hope, M. J., and Tilcock, C. P. S. (1986). Lipid polymorphism and the roles of lipids in membranes. Chemistry and Physics of Lipids *40*, 127-144.

de Kroon, A. I. P. M., Dolis, D., Mayer, A., Lill, R., and de Kruijff, B. (1997). Phospholipid composition of highly purified mitochondrial outer membranes of rat liver and *Neurospora crassa*. Is cardiolipin present in the mitochondrial outer membrane? Biochimica et Biophysica Acta *1325*, 108-116.

Epand, R. F., Martinou, J. C., Fornallaz-Mulhauser, M., Hughes, D. W., and Epand, R. M. (2002). The apoptotic protein tBid promotes leakage by altering membrane curvature. The Journal of Biological Chemistry *277*, 32632-32639.

Esposti, M. D., Cristea, I. M., Gaskell, S. J., Nakao, Y., and Dive, C. (2003). Proapoptotic Bid binds to monolysocardiolipin, a new molecular connection between mitochondrial membranes and cell death. Cell Death & Differentiation *10*, 1300-1309.

Fry, M., and Green, D. E. (1981). Cardiolipin requirement for electron transfer in complex I and III of the mitochondrial respiratory chain. The Journal of Biological Chemistry *256*, 1874-1880.

Garofalo, T., Giammarioli, A. M., Misasi, R., Tinari, A., Manganelli, V., Gambardella, L., Pavan, A., Malorni, W., and Sorice, M. (2005). Lipid microdomains contribute to apoptosis-associated modifications of mitochondria in T cells. Cell Death & Differentiation *12*, 1378-1389.

Gietz, R. D., and Woods, R. A. (2006). Yeast transformation by the LiAc/SS Carrier DNA/PEG method. Methods in Molecular Biology *313*, 107-120.

Gonzalvez, F., Bessoule, J. J., Rocchiccioli, F., Manon, S., and Petit, P. X. (2005a). Role of cardiolipin on tBid and tBid/Bax synergistic effects on yeast mitochondria. Cell Death & Differentiation *12*, 659-667.

Gonzalvez, F., and Gottlieb, E. (2007). Cardiolipin: setting the beat of apoptosis. Apoptosis : an International Journal on Programmed Cell Death *12*, 877-885.

Gonzalvez, F., Pariselli, F., Dupaigne, P., Budihardjo, I., Lutter, M., Antonsson, B., Diolez, P., Manon, S., Martinou, J. C., Goubern, M.*, et al.* (2005b). tBid interaction with cardiolipin primarily orchestrates mitochondrial dysfunctions and subsequently activates Bax and Bak. Cell Death & Differentiation *12*, 614-626.

Gonzalvez, F., Pariselli, F., Jalmar, O., Dupaigne, P., Sureau, F., Dellinger, M., Hendrickson, E. A., Bernard, S., and Petit, P. X. (2010). Mechanistic issues of the interaction of the hairpin-forming domain of tBid with mitochondrial cardiolipin. PloS ONE *5*, e9342.

Gonzalvez, F., Schug, Z. T., Houtkooper, R. H., MacKenzie, E. D., Brooks, D. G., Wanders, R. J. A., Petit, P. X., Vaz, F. M., and Gottlieb, E. (2008). Cardiolipin provides an essential activating platform for caspase-8 on mitochondria. The Journal of Cell Biology *183*, 681-696.

Gu, Z., Valianpour, F., Chen, S., Vaz, F. M., Hakkaart, G. A., Wanders, R. J., and Greenberg, M. L. (2004). Aberrant cardiolipin metabolism in the yeast taz1 mutant: a model for Barth syndrome. Molecular Microbiology *51*, 149-158.

Haines, T. H., and Dencher, N. A. (2002). Cardiolipin: a proton trap for oxidative phosphorylation. FEBS Letters *528*, 35-39.

Hoffmann, B., Stockl, A., Schlame, M., Beyer, K., and Klingenberg, M. (1994). The reconstituted ADP/ATP carrier activity has an absolute requirement for cardiolipin as shown in cysteine mutants. The Journal of Biological Chemistry *269*, 1940-1944.

Houtkooper, R. H., Akbari, H., van Lenthe, H., Kulik, W., Wanders, R. J., Frentzen, M., and Vaz, F. M. (2006). Identification and characterization of human cardiolipin synthase. FEBS Letters *580*, 3059-3064.

Houtkooper, R. H., and Vaz, F. M. (2008). Cardiolipin, the heart of mitochondrial metabolism. Cellular and Molecular Life Sciences *65*, 2493-2506.

Iverson, S. L., Enoksson, M., Gogvadze, V., Ott, M., and Orrenius, S. (2004). Cardiolipin Is Not Required for Bax-mediated Cytochrome c Release from Yeast Mitochondria. Journal of Biological Chemistry *279*, 1100-1107.

Jensen, R. E. (2005). Control of mitochondrial shape. Current Opinion in Cell Biology *17*, 384-388.

Jiang, F., Ryan, M. T., Schlame, M., Zhao, M., Gu, Z., Klingenberg, M., Pfanner, N., and Greenberg, M. L. (2000). Absence of cardiolipin in the crd1 null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function. The Journal of Biological Chemistry *275*, 22387-22394.

Joshi, A. S., Zhou, J., Gohil, V. M., Chen, S., and Greenberg, M. L. (2009). Cellular functions of cardiolipin in yeast. Biochimica et Biophysica Acta *1793*, 212-218.

Kagan, V. E., Tyurin, V. A., Jiang, J., Tyurina, Y. Y., Ritov, V. B., Amoscato, A. A., Osipov, A. N., Belikova, N. A., Kapralov, A. A., Kini, V.*, et al.* (2005). Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. Nature Chemical Biology *1*, 223-232.

Kelly, B. L., and Greenberg, M. L. (1990). Characterization and regulation of phosphatidylglycerolphosphate phosphatase in Saccharomyces cerevisiae. Biochimica et Biophysica Acta *1046*, 144-150.

Kim, T. H., Zhao, Y., Ding, W. X., Shin, J. N., He, X., Seo, Y. W., Chen, J., Rabinowich, H., Amoscato, A. A., and Yin, X. M. (2004). Bid-cardiolipin interaction at mitochondrial contact site contributes to mitochondrial cristae reorganization and cytochrome c release. Molecular Biology of the Cell *15*, 3061-3072.

Klingenberg, M. (2009). Cardiolipin and mitochondrial carriers. Biochimica et Biophysica Acta *1788*, 2048-2058.

Kobayashi, T., Yamaguchi, M., Kim, S., Morikawa, J., Ogawa, S., Ueno, S., Suh, E., Dougherty, E., Shmulevich, I., Shiku, H., and Zhang, W. (2003). Microarray reveals differences in both tumors and vascular specific gene expression in de novo $CD5^+$ and $CD5^-$ diffuse large B-cell lymphomas. Cancer Research *63*, 60-66.

Kodaki, T., and Yamashita, S. (1987). Yeast phosphatidylethanolamine methylation pathway. Cloning and characterization of two distinct methyltransferase genes. The Journal of Biological Chemistry *262*, 15428-15435.

Kornmann, B., Currie, E., Collins, S. R., Schuldiner, M., Nunnari, J., Weissman, J. S., and Walter, P. (2009). An ER-mitochondria tethering complex revealed by a synthetic biology screen. Science *325*, 477-481.

Kornmann, B., Osman, C., and Walter, P. (2011). The conserved GTPase Gem1 regulates endoplasmic reticulum–mitochondria connections. Proceedings of the National Academy of Sciences *108*, 14151-14156.

Kornmann, B., and Walter, P. (2010). ERMES-mediated ER-mitochondria contacts: molecular hubs for the regulation of mitochondrial biology. Journal of Cell Science *123*, 1389-1393.

Korytowski, W., Basova, L. V., Pilat, A., Kernstock, R. M., and Girotti, A. W. (2011). Permeabilization of the mitochondrial outer membrane by Bax/truncated Bid (tBid) proteins as sensitized by cardiolipin hydroperoxide translocation: mechanistic implications for the intrinsic pathway of oxidative apoptosis. Journal of Biological Chemistry *286*, 26334-26343.

Koshkin, V., and Greenberg, M. L. (2002). Cardiolipin prevents rate-dependent uncoupling and provides osmotic stability in yeast mitochondria. Biochemical Journal *364*, 317-322.

Lecocq, J., and Ballou, C. (1964). On the structure of cardiolipin. Biochemistry *3*, 976-980.

Leventis, P. A., and Grinstein, S. (2010). The distribution and function of phosphatidylserine in cellular membranes. Annual Review of Biophysics *39*, 407-427.

Liu, J., Durrant, D., Yang, H. S., He, Y., Whitby, F. G., Myszka, D. G., and Lee, R. M. (2005). The interaction between tBid and cardiolipin or monolysocardiolipin. Biochemical and Biophysical Research Communications *330*, 865-870.

Lucken-Ardjomande, S., Montessuit, S., and Martinou, J. C. (2008). Contributions to Bax insertion and oligomerization of lipids of the mitochondrial outer membrane. Cell Death & Differentiation *15*, 929-937.

Ludovico, P., Rodrigues, F., Almeida, A., Silva, M. T., Barrientos, A., and Côrte-Real, M. (2002). Cytochrome c release and mitochondria involvement in programmed cell death induced by acetic acid in *Saccharomyces cerevisiae*. Molecular Biology of the Cell *13*, 2598-2606.

Lutter, M., Perkins, G., and Wang, X. (2001). The pro-apoptotic Bcl-2 family member tBid localizes to mitochondrial contact sites. BMC Cell Biology *2*, 1-9.

Madeo, F., Frohlich, E., and Frohlich, K. U. (1997). A yeast mutant showing diagnostic markers of early and late apoptosis. The Journal of Cell Biology *139*, 729-734.

Matsko, C. M., Hunter, O. C., Rabinowich, H., Lotze, M. T., and Amoscato, A. A. (2001). Mitochondrial lipid alterations during Fas- and radiation-induced apoptosis. Biochemical and Biophysical Research Communications *287*, 1112-1120.

Meisinger, C., Pfannschmidt, S., Rissler, M., Milenkovic, D., Becker, T., Stojanovski, D., Youngman, M. J., Jensen, R. E., Chacinska, A., Guiard, B.*, et al.* (2007). The morphology proteins Mdm12/Mmm1 function in the major beta-barrel assembly pathway of mitochondria. The EMBO journal *26*, 2229-2239.

Meisinger, C., Rissler, M., Chacinska, A., Szklarz, L. K., Milenkovic, D., Kozjak, V., Schonfisch, B., Lohaus, C., Meyer, H. E., Yaffe, M. P.*, et al.* (2004). The mitochondrial morphology protein Mdm10 functions in assembly of the preprotein translocase of the outer membrane. Developmental Cell *7*, 61-71.

Orrenius, S., and Zhivotovsky, B. (2005). Cardiolipin oxidation sets cytochrome c free. Nature Chemical Biology *1*, 188-189.

Osman, C., Haag, M., Potting, C., Rodenfels, J., Dip, P. V., Wieland, F. T., Brugger, B., Westermann, B., and Langer, T. (2009). The genetic interactome of prohibitins: coordinated control of cardiolipin and phosphatidylethanolamine by conserved regulators in mitochondria. The Journal of Cell Biology *184*, 583-596.

Osman, C., Voelker, D. R., and Langer, T. (2011). Making heads or tails of phospholipids in mitochondria. The Journal of Cell Biology *192*, 7-16.

Ott, M., Robertson, J. D., Gogvadze, V., Zhivotovsky, B., and Orrenius, S. (2002). Cytochrome c release from mitochondria proceeds by a two-step process. Proceedings of the National Academy of Sciences *99*, 1259-1263.

Pangborn, M. C. (1942). Isolation and purification of a serologically active phospholipid from beef heart. Journal of Biological Chemistry *143*, 247-256.

Paradies, G., Petrosillo, G., Pistolese, M., and Ruggiero, F. M. (2000). The effect of reactive oxygen species generated from the mitochondrial electron transport chain on the cytochrome c oxidase activity and on the cardiolipin content in bovine heart submitochondrial particles. FEBS Letters *466*, 323-326.

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

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

Petrosillo, G., Ruggiero, F. M., Pistolese, M., and Paradies, G. (2001). Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from beef-heart submitochondrial particles via cardiolipin peroxidation. Possible role in the apoptosis. FEBS Letters *509*, 435-438.

Pfeiffer, K., Gohil, V., Stuart, R. A., Hunte, C., Brandt, U., Greenberg, M. L., and Schagger, H. (2003). Cardiolipin stabilizes respiratory chain supercomplexes. The Journal of Biological Chemistry *278*, 52873-52880.

Pike, L. J., Han, X., Chung, K. N., and Gross, R. W. (2002). Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis. Biochemistry *41*, 2075-2088.

Potting, C., Wilmes, C., Engmann, T., Osman, C., and Langer, T. (2010). Regulation of mitochondrial phospholipids by Ups1/PRELI-like proteins depends on proteolysis and Mdm35. The EMBO journal *29*, 2888-2898.

Priault, M., Cartron, P. F., Camougrand, N., Antonsson, B., Vallette, F. M., and Manon, S. (2003). Investigation of the role of the C-terminus of Bax and of tc-Bid on Bax interaction with yeast mitochondria. Cell Death & Differentiation *10*, 1068-1077.

Raturi, A., and Simmen, T. (2013). Where the endoplasmic reticulum and the mitochondrion tie the knot: the mitochondria-associated membrane (MAM). Biochimica et Biophysica Acta *1833*, 213-224.

Rosenblum, J. S., Pemberton, L. F., Bonifaci, N., and Blobel, G. (1998). Nuclear import and the evolution of a multifunctional RNA-binding protein. The Journal of Cell Biology *143*, 887-899.

Saito, K., Kuge, O., Akamatsu, Y., and Nishijima, M. (1996). Immunochemical identification of the pssA gene product as phosphatidylserine synthase I of Chinese hamster ovary cells. FEBS Letters *395*, 262-266.

Schlame, M., Brody, S., and Hostetler, K. Y. (1993). Mitochondrial cardiolipin in diverse eukaryotes. Comparison of biosynthetic reactions and molecular acyl species. European Journal of Biochemistry *212*, 727-735.

Schlame, M., and Ren, M. (2006). Barth syndrome, a human disorder of cardiolipin metabolism. FEBS Letters *580*, 5450-5455.

Schlame, M., Ren, M., Xu, Y., Greenberg, M. L., and Haller, I. (2005). Molecular symmetry in mitochondrial cardiolipins. Chemistry and Physics of Lipids *138*, 38-49.

Schlame, M., Rua, D., and Greenberg, M. L. (2000). The biosynthesis and functional role of cardiolipin. Progress in Lipid Research *39*, 257-288.

Schug, Z. T., and Gottlieb, E. (2009). Cardiolipin acts as a mitochondrial signalling platform to launch apoptosis. Biochimica et Biophysica Acta *1788*, 2022-2031.

Scorrano, L. (2008). Caspase-8 goes cardiolipin: a new platform to provide mitochondria with microdomains of apoptotic signals? The Journal of Cell Biology *183*, 579-581.

Sesaki, H., Dunn, C. D., Iijima, M., Shepard, K. A., Yaffe, M. P., Machamer, C. E., and Jensen, R. E. (2006). Ups1p, a conserved intermembrane space protein, regulates mitochondrial shape and alternative topogenesis of Mgm1p. The Journal of Cell Biology *173*, 651-658.

Simbeni, R., Tangemann, K., Schmidt, M., Ceolotto, C., Paltauf, F., and Daum, G. (1993). Import of phosphatidylserine into isolated yeast mitochondria. Biochimica et Biophysica Acta *1145*, 1-7.

Sorice, M., Circella, A., Cristea, I. M., Garofalo, T., Di Renzo, L., Alessandri, C., Valesini, G., and Esposti, M. D. (2004). Cardiolipin and its metabolites move from mitochondria to other cellular membranes during death receptor-mediated apoptosis. Cell Death & Differentiation *11*, 1133-1145.

Sousa, M., Duarte, A. M., Fernandes, T. R., Chaves, S. R., Pacheco, A., Leão, C., Côrte-Real, M., and Sousa, M. J. (2013). Genome-wide identification of genes involved in the positive and negative regulation of acetic acid-induced programmed cell death in *Saccharomyces cerevisiae*. BMC Genomics (*article under revision*).

Stegh, A. H., Barnhart, B. C., Volkland, J., Algeciras-Schimnich, A., Ke, N., Reed, J. C., and Peter, M. E. (2002). Inactivation of Caspase-8 on Mitochondria of Bcl-xL-expressing MCF7-Fas Cells: Role for the bifunctional apoptosis regulator protein. Journal of Biological Chemistry *277*, 4351-4360.

Steiner, M. R., and Lester, R. L. (1972). In vitro studies of phospholipid biosynthesis in *Saccharomyces cerevisiae*. Biochimica et Biophysica Acta *260*, 222-243.

Tamura, Y., Endo, T., Iijima, M., and Sesaki, H. (2009). Ups1p and Ups2p antagonistically regulate cardiolipin metabolism in mitochondria. The Journal of Cell Biology *185*, 1029-1045.

Tamura, Y., Iijima, M., and Sesaki, H. (2010). Mdm35p imports Ups proteins into the mitochondrial intermembrane space by functional complex formation. The EMBO Journal *29*, 2875-2887.

Tamura, Y., Onguka, O., Hobbs, A. E., Jensen, R. E., Iijima, M., Claypool, S. M., and Sesaki, H. (2012). Role for two conserved intermembrane space proteins, Ups1p and Ups2p, in intra-mitochondrial phospholipid trafficking. The Journal of Biological Chemistry *287*, 15205-15218.

The Medical Biochemistry Page. URL:http://themedicalbiochemistrypage.org/lipid-synthesis.php, accessed in August of 2013.

Tuominen, E. K., Wallace, C. J., and Kinnunen, P. K. (2002). Phospholipid-cytochrome c interaction: evidence for the extended lipid anchorage. The Journal of Biochemical Chemistry *277*, 8822-8826.

Valianpour, F., Mitsakos, V., Schlemmer, D., Towbin, J. A., Taylor, J. M., Ekert, P. G., Thorburn, D. R., Munnich, A., Wanders, R. J., Barth, P. G., and Vaz, F. M. (2005). Monolysocardiolipins accumulate in Barth syndrome but do not lead to enhanced apoptosis. The Journal of Lipid Research *46*, 1182-1195.

Vance, J. E. (2008). Phosphatidylserine and phosphatidylethanolamine in mammalian cells: two metabolically related aminophospholipids. Journal of Lipid Research *49*, 1377-1387.

Vance, J. E., and Steenbergen, R. (2005). Metabolism and functions of phosphatidylserine. Progress in Lipid Research *44*, 207-234.

Voelker, D. R. (2004). Lipid synthesis and transport in mitochondrial biogenesis. Topics in Current Genetics *8*, 267-291.

Westermann, B., and Neupert, W. (2000). Mitochondria-targeted green fluorescent proteins: convenient tools for the study of organelle biogenesis in *Saccharomyces cerevisiae*. Yeast *16*, 1421-1427.

Xu, Y., Sutachan, J. J., Plesken, H., Kelley, R. I., and Schlame, M. (2005). Characterization of lymphoblast mitochondria from patients with Barth syndrome. Laboratory Investigation *85*, 823-830.

Zhang, T., and Saghatelian, A. (2013). Emerging roles of lipids in BCL-2 family-regulated apoptosis. Biochimica et Biophysica Acta.

Zhong, Q., Gohil, V. M., Ma, L., and Greenberg, M. L. (2004). Absence of cardiolipin results in temperature sensitivity, respiratory defects, and mitochondrial DNA instability independent of pet56. The Journal of Biological Chemistry *279*, 32294-32300.

Zinser, E., Sperka-Gottlieb, C. D., Fasch, E. V., Kohlwein, S. D., Paltauf, F., and Daum, G. (1991). Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*. Journal of Bacteriology *173*, 2026-2034.

CHAPTER 3

The role of the actin-binding protein cofilin in *Saccharomyces cerevisiae* acetic acid-induced apoptosis

3.1 Introduction

The actin cytoskeleton plays a fundamental role in many cellular functions, including endocytosis, motility, organelle and vesicle trafficking, cytokinesis and signal–response coupling (Franklin-Tong and Gourlay, 2008). The dynamic changes in the actin cytoskeleton, such as severing and polymerization of actin filaments, can predominantly explain the diversity and the specificity of some of these functions. The actin-binding proteins (ABPs) are associated with actin and promote the cycles of assembly/disassembly. Among the ABPs, cofilin, which belongs to the cofilin/actin depolymerizing factor (ADF) family, is a small protein that affects both actin polymerization and depolymerization, functioning as a key regulator in the dynamic reorganization of the actin cytoskeleton. In this process, the pH-dependent actin-severing activity of cofilin increases the number of free barbed ends, which consequently promote the elongation/polymerization of the newly generated actin filaments (Bailly and Jones, 2003; Ichetovkin et al., 2002; Moriyama and Yahara, 2002). Cofilin can be inactivated upon phosphorylation through LIM or TES kinases or upon binding to phosphoinositides (of which phosphatidylinositol 4,5-bisphosphate (PIP_2) is the most frequent). On the other hand, the cofilin activity can be reestablished by some phosphatases (including Slinghot) or through PIP_2 hydrolysis (Figure 1) (Bernard, 2007; Bailly and Jones, 2003; Zhao et al., 2010).

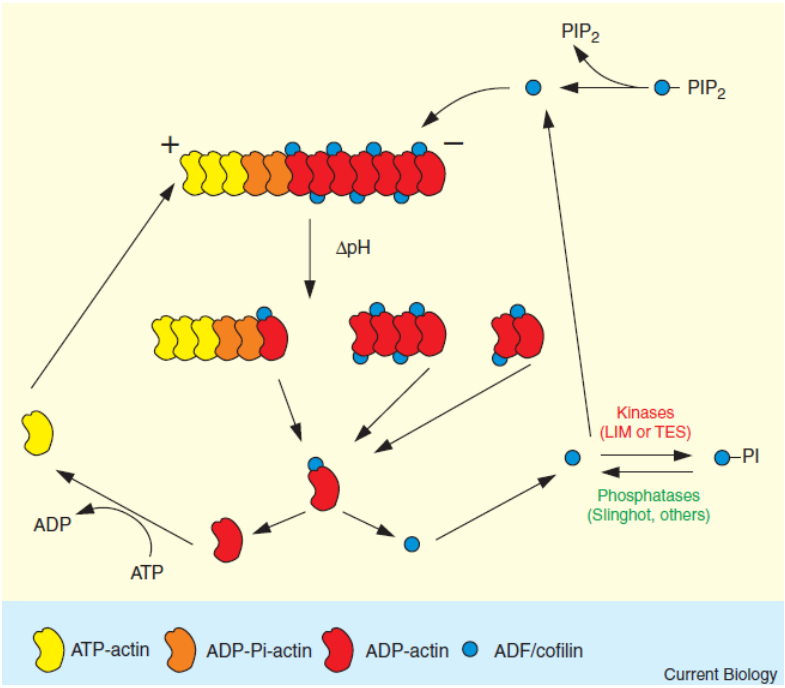

Figure 1 – The role of cofilin in actin dynamics. Cofilin binds to the ADP-actin and its actin-severing activity is promoted by transient pH variation. After ADP-actin monomers dissociation, cofilin can be inactivated by addition of one phosphate group (promoted by kinases activity) or through PIP_2 binding. At the same time, the actin monomers released undergo nucleotide exchange and reinsertion at the barbed (+) end of actin filaments promoting their elongation. The cofilin actin-binding activity can be restored by phosphatases or PIP_2 hydrolysis (Scheme from Bailly and Jones, 2003).

The control of the redox environment inside the cell is of the great importance since high levels of oxidants have the capacity to induce cell death. Although dependent on the chemical nature of the oxidant, these compounds can generally damage proteins, lipids and DNA with many functional consequences.

Some research has been done to identify proteins that in addition to undergo oxidation in these conditions have a role in apoptosis induction. Proteomic approaches allowed the identification of some target proteins, including the actin-binding protein cofilin. The cofilin translocation from the cytosol to the mitochondria in HL60 cells treated with staurosporine (STS), that stimulates the production of intracellular ROS, was the first evidence that this protein could be fundamental for the occurrence of MOMP and consequently apoptosis (Chua et al., 2003).

It was demonstrated that during STS, etoposide and taurine chloramine (TnCl)-induced apoptosis, the oxidation of cofilin in its four cysteine residues and dephosphorylation at serine residue 3 are early steps in apoptosis induction and are indispensable to trigger this type of cell death (Chua et al., 2003; Klamt et al., 2009). After cofilin activation, that includes oxidation and dephosphorylation, it dissociates from actin filaments and is translocated to the mitochondria where it induces the loss of mitochondrial membrane potential, non-physiological swelling and release of apoptotic factors (Figure 2) (Klamt et al., 2009; Wabnitz et al., 2010).

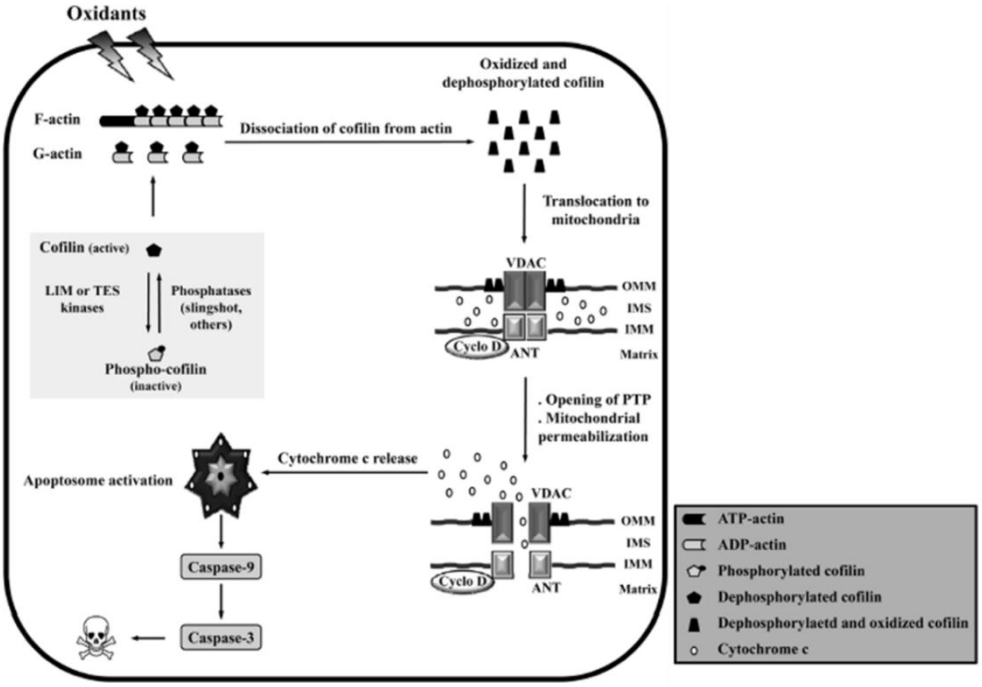

Figure 2 – The cofilin role in apoptosis. Under physiological conditions cofilin severing activity is regulated by phosphorylation/dephosphorylation. In the presence of some oxidants, the dephosphorylated cofilin is oxidized, losing the affinity to depolymerize the actin filaments. Once activated, cofilin is translocated to the OMM, where it induces MOMP through PTP opening that results in cell death (Scheme from Zdanov et al., 2010).

As aforementioned, the cofilin dephosphorylation happens, under physiological conditions, in response to many stimuli that imply a change in the actin organization. However, it is only translocated to mitochondria when simultaneously oxidized in the cysteine residues resulting in a conformational change that renders it incapable to depolymerize the actin filaments (Bamburg, 1999; Chua et al., 2003; Endo et al., 2003). It was proposed that these structural modifications in cofilin could lead to a stiff actin cytoskeleton further affecting the oxidative stress response (Klemke et al., 2008).

It was demonstrated by the use of PTP blocking agents (CsA and bongkrekic acid) that the oxidized cofilin causes mitochondria swelling through PTP opening. Curiously, under these conditions, the cofilin translocation was not inhibited whereby the association with the OMM, unlike its pro-apoptotic function, appears to be independent of the PTP function. On the other hand, when the cofilin cysteine residues were exchanged by alanines its oxidation and translocation were inhibited and apoptosis was not triggered, showing its requirement for the MOMP (Klamt et al., 2009).

Curiously, the cofilin oxidized-induced cell death was not repressed by the broad-range caspase inhibitor zVAD-fmk or by Bcl-x_L. Additionally, it was shown that the PTP opening induced by cofilin translocation was independent of Bax/Bak activation in TnCI treated cells (Klamt et al., 2009). However, the involvement of these pro-apoptotic Bcl-2 family proteins appears to be cell or stimulus-dependent, since Methyl Antcinate A is able to induce apoptosis in human liver cancer cells through oxidative stress in a process mediated simultaneously by cofilin and Bax translocation to the mitochondria (Hsieh et al., 2010). Furthermore, it was recently demonstrated that during excitotoxic neuronal death, Bax translocation to mitochondria is triggered by cofilin dephosphorylation, where this actin-binding protein might physically interact and transport Bax. Therefore, in this cell line and treatment conditions, was proposed that the knock down of cofilin had a protective effect due to inhibition of MOMP mediated by Bax (Posadas et al., 2012).

Cofilin is not obviously the only activated protein during apoptosis able to induce MOMP, but it is assuredly important for this process once the knockdown of endogenous cofilin reduced significantly the cell death (Chua et al., 2003; Klamt et al., 2009). However, the type of cell death triggered by cofilin oxidation appears to be also dependent of the cell line and stimulus that lead to the oxidative stress. In T cells, the long-term oxidative stress induced by H_2O_2 led to cofilin oxidation and translocation to the mitochondria in a process that is independent of caspases activation and not considered an apoptotic but instead a necrotic-like PCD (Wabnitz et al., 2010). Although at the beginning PTP had been essentially associated to an apoptotic cell death, nowadays the induction of MOMP through PTP opening has been

increasingly associated to a necrotic PCD pathway (Vaseva et al., 2012). Wabnitz and colleagues findings together with the reported role of cofilin independent of the Bcl-2 pro-apoptotic proteins, suggest that this protein can lead to the cyt *c* release through PTP opening. To reinforce this hypothesis, it was observed that after cofilin translocation it interacts with the OMM, where it was conjectured to induce permeabilization through VDAC association (Chua et al., 2003; Franklin-Tong and Gourlay, 2008; Zdanov et al., 2010). Controversially, it was proposed that HSC70 is responsible for mitochondrial import of oxidized cofilin suggesting that it does not stay at the OMM (Wabnitz et al., 2010).

Oppositely to the heretofore mentioned, it was very recently shown in mouse embryonic fibroblast cells that the translocation of dephosphorylated cofilin to mitochondria, which under these conditions occurs together with actin, can itself not imply a role in the cell death induced by STS or H_2O_2 suggesting again that cell type-specific functions for cofilin in the cell death signaling must exist (Rehklau et al., 2012). Furthermore, depending on concentration, exposure time and microenvironment, the effects of oxidants can be very distinct.

Anyway, the pro-death function of cofilin under some oxidative stress conditions seems to make it a good therapeutic target for cancer. Indeed, the stimulation of cofilin oxidation when associated with known antitumor drugs decreased the resistance of some cancer cells to the treatment (Hsieh et al., 2010; Li et al., 2013; Zhu et al., 2012). However, essentially in the most resistant and invasive cancer cells *COF1* gene that encodes cofilin is overexpressed, which suggest that in non-physiological conditions such as cancer environment, this increase can be explained based in the cofilin role in actin dynamics and cell motility. So, the high levels of this protein can, through increased actin filaments generation, promote the metastasis and cells migration making the severity of the cancer cells further increased (Jiang et al., 2011; Peng et al., 2011; Polachini et al., 2012; Steller et al., 2011; Wei et al., 2012). Therefore, in these situations, the most prominent treatment must imply the cofilin phosphorylation inhibiting the actin dynamics. In sum, cofilin can have a dual function in either control the progression of certain forms of cancer or confer resistance of some tumors to chemotherapy.

Abnormal regulation of cofilin levels has also been associated to neurodegenerative pathologies, such as Alzheimer or Huntington's disease. Dephosphorylated and activated cofilin was found in inclusions of human Alzheimer's brain where it forms rod-shaped actin bundles (named rods). These structures result in loss of synaptic connections between neurons and cognitive decline (Maloney and Bamburg, 2007; Minamide et al., 2000).

Considering the above, the use of cofilin as a new therapeutic target to treat some diseases must have into account the physiological levels of this protein, as the expression levels variation along time and its subcellular distribution determining which diseases result from the oxidative stress-induced pro-death function of cofilin, and which mirror its role in the regulation of actin dynamics and cell motility.

The cofilin sequence, structure and function are highly conserved among eukaryotic cells. The budding yeast *S. cerevisiae*, that has a single and essential cofilin encoded by the *COF1* gene, is nowadays used as a model system to explore new functions of this actin-binding protein. Oppositely to the reported mammalian cells, the *COF1* gene is indispensable for the yeast survival and it is not possible to make a complete disruption of this gene (Moon et al., 1993). At the same way, yeast cells overexpressing *COF1* are unable to grow showing that this protein is essential under physiological expression levels (Iida and Yahara, 1999). Thus, systematic mutagenesis was used to generate a library of isogenic yeast strains that express mutant forms of cofilin, in which the charged residues on the surface of cofilin were changed by alanine (Figure 3) (Lappalainen et al., 1997). Yeast cofilin, differently from mammalians, has only a single cysteine residue. It was demonstrated that the mutation of this residue interferes with the mitochondrial function but it remains unclear if it is responsible for mitochondrial translocation under oxidative stress conditions (Kotiadis et al., 2012).

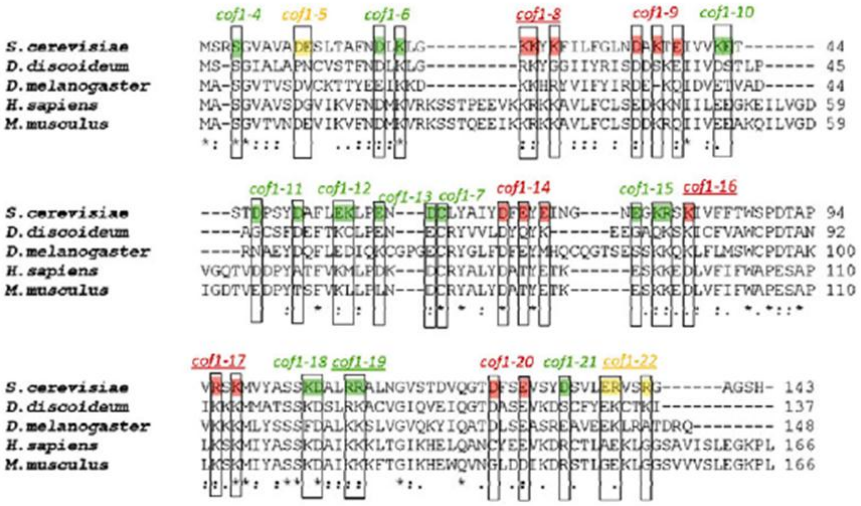

Figure 3 - Alignment of cofilin sequence from yeast (*Saccharomyces cerevisiae*), slime mold (*Dictyostelium discoideum*), fruit fly (*Drosophila melanogaster*), human (*Homo sapiens*) and mouse (*Mus musculus*). The red coloured residues are essential for actin binding and viability in yeast cells. The residues that also affect the actin dynamics but are non-essential for yeast cells are labeled in yellow and those not involved in yeast growth are coloured in green (Scheme from Kotiadis et al., 2012).

The yeast mutant strains in which the charged residues on the cofilin surface were replaced by alanine were grouped accordingly with the analysis of their performance in YEPD solid medium

supplemented with H_2O_2 or glycerol, respiratory activity, ROS production and mitochondrial morphology. Mutants were clustered in Class I (*cof1-4*, *cof1-6*, *cof1-7*, *cof1-11*, *cof1-12*, *cof1-15, cof1-18*, *cof1-19* and *cof1-21*), Class II (*cof1-10 and cof1-13*) and Class III (*cof1-5* and *cof1-22*). The cofilin mutants of Class I displayed higher respiratory rate than the wild-type. In Class II, the cofilin mutants were the most sensitive to oxidative stress and they displayed no functional mitochondria, which appear fragmented and aggregated during the diauxic shift caused by cell growth during 24 hours in YEPD medium. Finally, the cofilin mutants grouped in the Class III shown some sensitivity to oxidative stress and also partially fragmented mitochondria during exponential phase of growth (Kotiadis et al., 2012).

It was demonstrated that the exchanged residues in *cof1-5* mutant (Class III), associated with the actin binding, lead to tolerated effects in the actin cytoskeleton (Lappalainen et al., 1997). Oppositely, the cellular function of residues not associated with the actin or PIP_2 binding, such as in *cof1-6* and *cof1-7* mutants (Class I), remains to be elucidated (Kotiadis et al., 2012).

Although until the moment there is no information about the cofilin role in yeast cell death, considering the high similarity of this protein between yeast and mammalian cells, an identical mechanism of cofilin action may be expected.

3.2 Main aims and study overview

Like previously exposed, in mammalians there are some evidences of oxidized cofilin interaction with the OMM but the mechanism by which cofilin interacts and leads to PTP opening in this system model remains to be clarified. Until the moment, in yeast cells, it was not demonstrated that under oxidative stress cofilin undergoes oxidation and translocation to the mitochondria. However, recent preliminary data suggest that a stress-specific interaction between cofilin and Por1p (yeast VDAC) can exist in yeast cells (Kotiadis et al., 2012). Since it has been previously described that Por1p is involved in acetic acid induced apoptotic cell death in yeast (Pereira et al., 2007) we raised the hypothesis that an interaction between Por1p and cofilin could have a role in this cell death process, namely through modulation of MOMP. To address this hypothesis we aimed to characterize the process of cell death induced by acetic acid in yeast strains expressing the *cof1-5*, *cof1-6* or *cof1-7* mutant forms of cofilin. It has previously been demonstrated that these strains display similar respiratory activity and identical percentage of ROS-positive cells when compared with the wild-type having no expectable interference on mitochondrial function under physiological conditions (Kotiadis et al., 2012). Our initial results through evaluation of the cell survival, plasma membrane integrity, ROS production and mitochondrial membrane potential in response to acetic acid induced cell death allowed to identify differences in the response of the different cofilin mutants when compared with the wild-type strain.

Considering the reported information, in the present work it was intended to evaluate, throughout acetic acid treatment, the cofilin subcellular distribution and the Por1p interference in this process. Furthermore, it was aimed to determine the relationship between the cofilin pro-death function and the Por1p activity.

3.3 Material and Methods

3.3.1 Yeast strains

The parental strain *S. cerevisiae* CGY384 (*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 COF1::LEU2*) was used in this study as the wild-type. The wild-type and the cofilin mutant strains (*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-x::LEU2*, in which *x* means *5,6 or 7*) were kindly provided by Professor Campbell Gourlay (Kotiadis et al., 2012) and they were constructed by replacing some surface charged residues by alanine (Lappalainen et al., 1997). In this study, the *POR1* gene was deleted from all strains with the PCR product amplified from the *por1*Δ::*KanMX4* cassette. All oligonucleotides used for amplification of the *KanMX4* cassette (iPor1, iPor2) or confirmation of the disruption are listed in the Table I. Yeast strains were transformed using the Lithium acetate/Single Stranded carrier DNA/PEG method previously described (Gietz and Woods, 2006) with some differences in the events order. Briefly, a thermal shock proceeded by a recovery time was done to increase the process efficiency, with the sequential events: 30 minutes at 30 ˚C, 200 rpm, followed by 30 minutes in a bath at 42 ˚C, one hour in the ice and growth during four hours (two generations) in YEPD medium. The recombinant clones were selected for the Geneticin antibiotic. After 48 hours, the colonies grown were sub-cultured simultaneous, but independently, in YEPD medium containing Geneticin and in SC Glucose medium lacking leucine, in order to select the double mutants. After this, the effectiveness of transformation was confirmed by colony PCR, using the primers Por1A and Por1D that bind upstream and downstream of insertion, respectively, alone or in conjugation with the primers Kan327 forward and Kan326 reverse that anneal within of the kanamycin gene.

Table I – List of nucleotides used for amplification and confirmation of *por1*Δ::*KanMX4* disruption

Name	**Oligonucleotide sequence (5' – 3')**
iPor1	TTATAGCCAGCAGAGCACGA
iPor2	ATGATTATGAGAACCAGCCG
Por1A	TTCCAACAAGTTTAATGGTCAGAAT
Por1D	AATGTTCGAAACCAATCTGAAAATA
Kan327	CTCGGGCAATCAGGTGCGACA
Kan326	CCGAGGCAGTTCCATAGGATGGC

To monitor the cofilin subcellular localization before and during the acetic acid treatment, the wild-type and *por1*Δ strains were transformed with the plasmid pRS416 (*URA3*) expressing a wild-type Cof1-RFP (Red Fluorescent Protein) fusion protein kindly provided by Professor Campbell Gourlay. Firstly, this plasmid was amplified by transforming *E. coli* XL1 Blue competent cells with subsequent selection in LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl and 2% (w/v) agar) supplemented with Ampicillin (Formedium). One of the positive colonies were grown in the same medium overnight and the plasmid was purified using a Miniprep kit (GenElute Plasmid Miniprep kit, Sigma-Aldrich) according to the manufacturer's instructions. Like for *POR1* deletion, the yeast strains transformation with the plasmid pRS416 was carried out essentially as previously described in the Lithium acetate/Single Stranded carrier DNA/PEG method (Gietz and Woods, 2006), with the same differences in the events order described before. Thereafter, cells were grown in a selective medium lacking uracil and the positive yeast colonies were further grown in medium lacking uracil and leucine to select the double mutants and thus the correct construction. At the end, the effectiveness of transformation was evaluated through visualization with the fluorescence microscope.

3.3.2 Growth conditions and cell death assays

Yeast cells were maintained in rich medium (YEPD; 0.5% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) glucose). To select the strains with correct insertion of the *por1*Δ::*KanMX4* cassette, isolated colonies were grown in YEPD medium containing 200 μg/mL Geneticin (Sigma-Aldrich) and, at the same time, in SC medium lacking leucine (0.17% (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) uracil, adenine, histidine, lysine and tryptophan).

The yeast strains transformed with the plasmid pRS416 were selected and grown in SC medium lacking uracil (0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate, 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose/galactose, 0.2% (w/v) Drop-Out mix, plus 0.01% (w/v) leucine, adenine, histidine, lysine and tryptophan).

For acetic acid treatment, cells were firstly grown in SC medium with 2% (w/v) of glucose (without uracil for the transformants with the plasmid) at pH 5.5 (set with NaOH) in an orbital shaker at 200 rpm, 30 ^{0}C. One day after, cells were transferred to SC medium with 2% (w/v) of galactose (without uracil for the plasmid transformants) at pH 3.0 (set with HCl) and grown under the same conditions until the

exponential phase (OD_{600nm}=0.8-1.1). Thereafter, cells were harvest by centrifugation, suspended to the same cellular density (OD_{600nm}=0.6) in all strains with the last medium and treated with 150 mM acetic acid (Panreac), pH 3.0, for up to 180 minutes at 30 °C. Samples were rigorously collected at specific time points (0, 30, 60, 120 and 180 minutes), where the OD was measured and the number of cells adjusted to the initial cellular density in order to avoid overestimation of the percentage of cell survival. For the cell survival assay, in each time point four 1:10 seriated dilutions in deionized sterile water were done and seven drops of the last dilution were spotted on YEPD plates. The counting of the CFU was performed after 48 hours of incubation at 30 °C. Cell viability over the time was calculated as percentage of CFU in relation to the time zero (100%).

3.3.3 Preparation of mitochondria

For mitochondria preparation, cells were firstly grown twenty-four hours in 25 mL of SC Glucose medium pH 5.5 and posteriorly inoculated in 600 mL of SC Galactose medium pH 3.0, maintaining the volume ratio flask/growth medium equal to 5:1. Cells were grown until the exponential phase (OD_{600}=1.5) and directly harvested (time zero) or treated with acetic acid (150 mM) during 90 minutes.

The mitochondria fraction was isolated by differential centrifugation essentially as previously described (Arokium et al., 2004). After cells harvesting and washing with deionized water, they were suspended, proportionally to the cellular mass, with the suspension buffer containing 1.2 M sorbitol, 60 mM sodium phosphate pH 7.5 and 1 mM ethylenediaminetetraacetic acid (EDTA). The cell wall was digested through simultaneous addition of zymolyase (10 mg for non-treated and 50 mg for the acetic acid-treated cells) and 1% (v/v) of β-mercaptoethanol followed by incubation in a bath at 32 °C during 30 and 45 minutes for untreated and acetic acid treated cells, respectively. Spheroplasts were suspended in a lyse buffer containing 0.5 M sorbitol, 20 mM Tris pH 7.5 and 1 mM EDTA and they were disrupted simultaneously by an osmotic shock caused by the decreased sorbitol concentration and a hand-potter homogenization aiming to preserve the outer mitochondrial membrane integrity. Finally, the mitochondrial fraction was collected after some differential centrifugations.

3.3.4 Western blot analysis

To evaluate the amount of cytochrome *c* in the mitochondrial fraction, the protein concentration was estimated with the Bradford method using bovine serum albumin (BSA) as standard (Bradford, 1976).

An equal amount of protein (50 μg) was collected in all strains untreated or treated with acetic acid, precipitated with trichloroacetic acid (TCA) 50% and heated at 95 °C for 5 minutes. The samples were separated electrophoretically on a 12.5% SDS-polyacrylamide gel at 25 mA and transferred to a Hybond-P Polyvinylidene difluoride membrane (PVDF; GE Healthcare) at 54 mA for one hour. The membranes were cut into strips, blocked with 5% (w/v) non-fat dry milk to prevent unspecific bindings and incubated overnight at 4 °C with the primary antibodies: rabbit polyclonal anti-yeast cytochrome *c* (CYC1) antibody (1:3000, custom-made by Millegen), mouse monoclonal anti-yeast porin (POR1) antibody (1:10000, Molecular Probes) and mouse monoclonal anti-yeast phosphoglycerate kinase (PGK1) antibody (1:5000, Molecular Probes). Posteriorly, they were incubated with secondary antibodies against mouse (1:5000; Sigma-Aldrich) or rabbit IgG-peroxidase (1:3000; Sigma-Aldrich). The detection of the proteins Por1p and Pgk1p was applied as controls for mitochondrial and cytosolic fractions, respectively. The immunodetection of the bands was revealed by chemiluminescence (Immobilon, Millipore) and the intensity individually measured through the Image J Software (NIH Website). The amount of cyt *c* in the mitochondrial fraction was normalized, in each sample loaded, to the correspondent amount of Por1p (cyt c/Por1p).

3.3.5 Fluorescence microscopy

The effect of acetic acid in cofilin intracellular distribution, namely in its translocation to the mitochondria, was evaluated in the wild-type and *por1*Δ strains transformed with the pRS416 plasmid and stained with the green fluorescent dye $DiOC_6(3)$ to visualize the mitochondrial networks. At each time point, cells (200 μL) were harvest by centrifugation, suspended in $DiOC_6(3)$ buffer and stained with 1 μM $DiOC_6(3)$ with subsequent incubation in the dark for 30 minutes at 30 °C. At least 300 cells were individually analyzed at each time point to evaluate and categorize the cofilin subcellular distribution. Cells were visualized on a Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings using a 100x oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

3.4 Results

The study of the cofilin involvement in the cell death started to be investigated only in the last decade. In mammalian cells it was shown that under oxidative stress, the increased ROS generation lead to oxidation of dephosphorylated cofilin that results in its dissociation of actin filaments, translocation to mitochondria leading to the release of some apoptotic proteins to the cytosol. However, until the moment, it remains unclear the mechanism whereby cofilin interacts and leads to permeabilization of mitochondria.

Cofilin is a protein highly conserved among eukaryotic organisms with high functional similarity among yeast and mammalian cells. So we aimed to study, in the budding yeast *S. cerevisiae*, the role of this actin binding protein (ABP) in the apoptotic-like PCD induced by acetic acid. Because cofilin is essential for the yeast cell viability, strains with charged residues (exchanged to alanine) mutations were employed to uncover its function (Lappalainen et al., 1997).

Preliminary studies obtained in our research group revealed the acetic acid (180 mM) effect in cell survival (CFU counting), as well as in the plasma membrane integrity, mitochondrial membrane potential and ROS production (flow cytometry analysis). The results demonstrated that the cofilin mutant strains, *cof1-5*, *cof1-6* and *cof1-7* are resistant to the acetic acid treatment when compared with the wild-type strain (CGY384) (Figure 4A). The reduced percentage of PI-positive cells demonstrated that in all strains the plasma membrane integrity was not disrupted even after three hours of treatment (Figure 4B). The *cof1-6* and *cof1-7* mutants displayed higher percentage of ROS generation (DHE-positive cells) whereas *cof1-5* mutant, equally resistant to this treatment, exhibited lower production of ROS than the wild-type (Figure 4C). Curiously, there were no statistically significant differences in the mitochondrial depolarization among the strains (Figure 4D).

Altogether these results suggested that the exchange of these charged residues could be involved in the cofilin apoptotic role during the yeast acetic acid-induced apoptotic-like PCD. Therefore, it was then evaluated in this study if cofilin mutations could influence the mitochondrial outer membrane permeabilization, through quantification of cytochrome *c* amount in mitochondria isolated from wild-type and *cof1-6* strains before and after acetic acid treatment (Figure 5A). Accordingly with the resistance demonstrated by CFU counting, the results suggest that the decrease in the amount of mitochondrial cyt *c* after treatment was much more pronounced in the wild-type than in the *cof1-6* mutant which amount remained unchanged (Figure 5B).

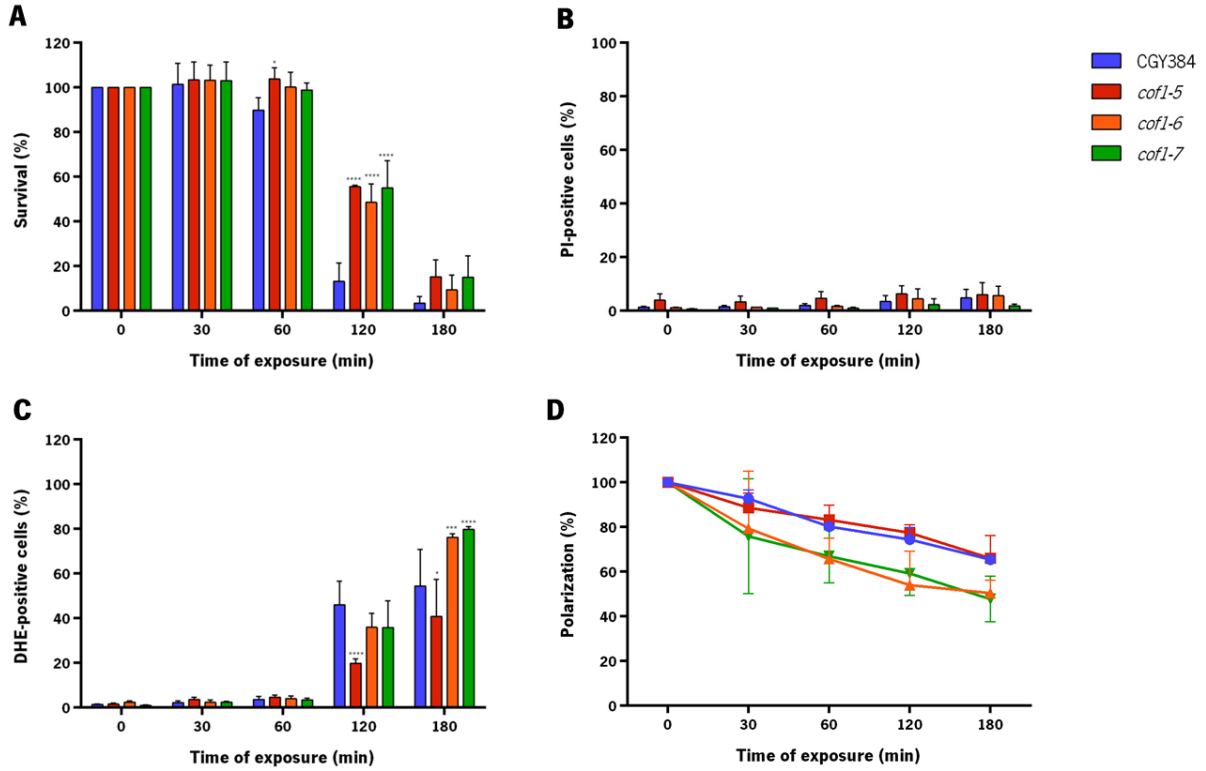

Figure 4 – Effect of acetic acid treatment (180 mM) in exponential wild-type (CGY384) and cofilin mutant strains during three hours, evaluated through (A) relative cell survival (CFU counts), (B) plasma membrane integrity (PI staining), (C) ROS accumulation (DHE staining) and (D) mitochondrial membrane potential ($DioC_6(3)$ dye). The reported values are the mean of at least three independent experiments with SD. A two-way ANOVA test was employed to compare wild-type *versus* mutant strains, for each time of treatment. Statistical analysis: $*p<0,05$, $**p<0,01$, $***p<0,001$ and $****p<0,0001$. (Rui Silva, unpublished results)

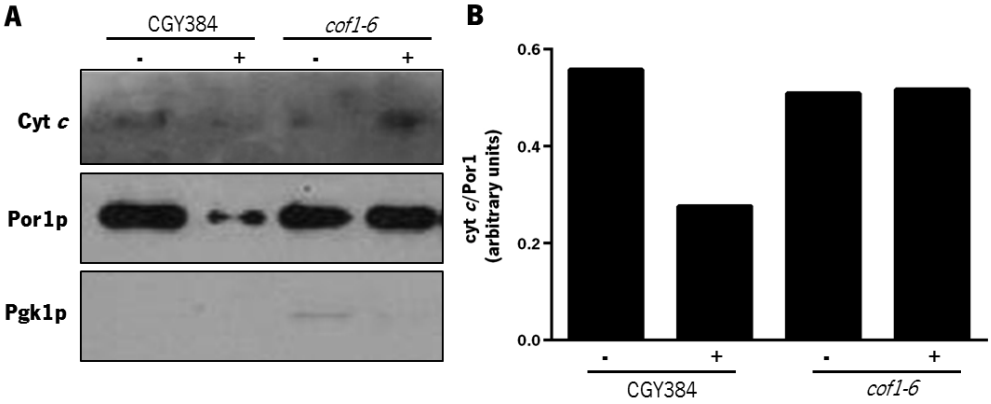

Figure 5 – Western blot analysis of cytochrome *c* relative amount in mitochondria isolated from wild-type (CGY384) and *cof1-6* mutant before (-) and after (+) 90 minutes of acetic acid treatment (150 mM). The mitochondrial porin (Por1p) and cytosolic phosphoglycerate kinase (Pgk1p) levels were used as control of mitochondrial and cytosolic fractions, respectively (A). Considering that the different levels of Por1p means different amount of protein loaded, the amount of cyt *c* was normalized accordingly with the respective levels of protein control (B).

As mentioned before, in mammalian cells some evidences appears to suggest that oxidized cofilin interacts with the OMM via VDAC association. The yeast Por1p presents high functional similarity with the mammalian VDAC, namely it also seems to be involved in the regulation of MOMP (Pereira et al., 2007). Therefore, it was aimed to determine if under acetic acid treatment cofilin is translocated to mitochondria and to understand the Por1p involvement in this process, as well as its role in the resistance verified in cofilin mutant strains.

For this purpose, the *POR1* gene had to be disrupted in the wild-type and cofilin mutants through transformation with the amplified product of the *por1*Δ::*KanMX4* cassette. Transformant cells were selected in medium containing geneticin and the colonies that grew were posteriorly, at the same time, subcultured in the same medium or in SC glucose lacking leucine to allow the selection of double mutants. The correct insertion of the cassette inside the genome was confirmed, in the colonies grown in both culture media, by colony PCR using the pairs of primers: Por1A – Kan326 (Figure 6A), Kan327-Por1D (Figure 6B) and Por1A-Por1D (Figure 6C).

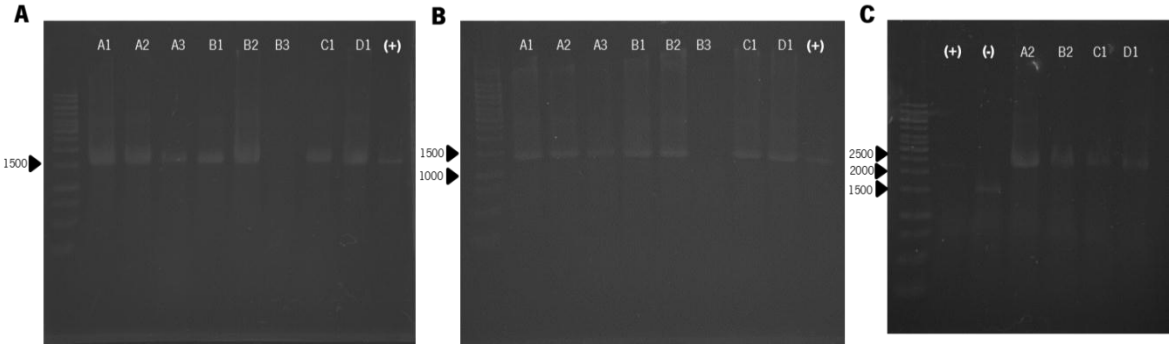

Figure 6 – Agarose gel electrophoresis for confirmation of *POR1* deletion in wild-type and cofilin mutant strains. The colony PCR was performed in the positive colonies (three colonies for the wild-type (A1,A2,A3) and *cof1-5* (B1,B2,B3), and one for *cof1-6* (C1) and *cof1-7* (D1)) using the pairs of primers Por1A–Kan326 (1513bp) (A) and Kan327-Por1D (1321bp) (B). To confirm the correct insertion of the *por1*Δ::KanMX4 cassette in the genome a PCR was performed with pair of primers Por1A-Por1D (2210bp) in the previously demonstrated positive colonies (C). In all situations the *por1*Δ strain (Euroscarf collection) was used as positive control (+) and in the last PCR, the genome of the non-transformed wild-type was applied as negative control (-).

To examine whether cofilin subcellular localization is changed under acetic acid treatment and if Por1p has some role in this process, the wild-type and *por1*Δ strains were transformed with the pRS416 plasmid coding a Cof1-RFP fusion protein. To visualize simultaneously the cofilin localization and the mitochondrial networks, transformant cells were stained with the mitochondrial membrane potential dye $DioC_6$(3). The acquired images showed that in cells without treatment, cofilin is arranged in round and well-defined small structures, that are distributed homogeneously inside the cell, and do not particularly

co-localize with mitochondria. After treatment, cofilin displays a diffuse distribution that, due to the similarity with the $DioC_6(3)$ staining, appears to suggest that in fact it could be, under these apoptotic conditions, translocated to the mitochondria (yellow staining; Figure 7A).

The same alterations in cofilin rearrangement was visualized in wild-type and *por1*Δ mutant, in which the number of cells that display red fluorescence totally diffuse inside the cell increases throughout the acetic acid treatment. However, it was observed a delay in the transition from punctate to diffuse distribution in *por1*Δ mutant. Curiously, after 3 hours of acetic acid treatment *por1*Δ mutant still display some cells with cofilin distribution unchanged, while in the wild-type after 2 hours of treatment the number of cells with cofilin under punctate form is almost inexistent (Figure 7B).

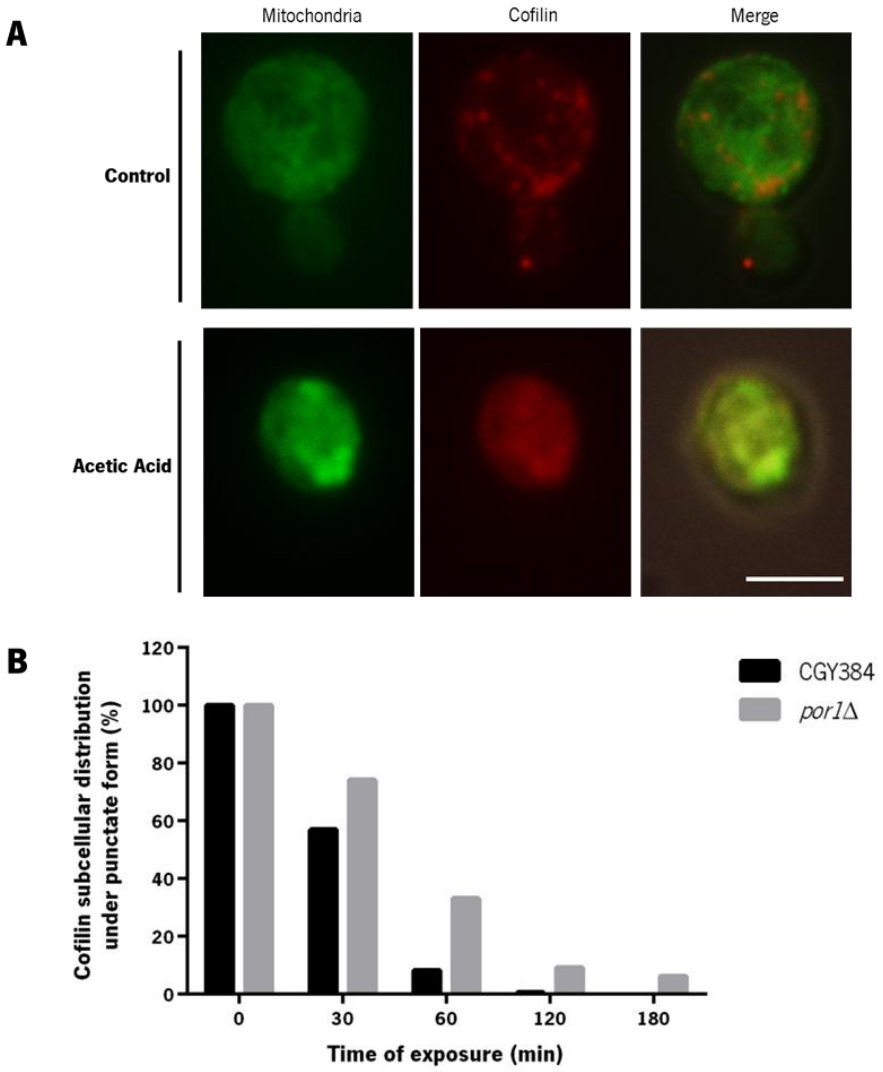

Figure 7 – The cofilin subcellular localization in the wild-type strain, transformed with the pRS416 plasmid (Cof1-RFP), without (control) and after acetic acid treatment (150 mM). The mitochondrial network was visualized by $DioC_6(3)$ staining and cofilin-mitochondria colocalization was monitored with a fluorescence microscope. Bar, 7.5μm (A). Evaluation of acetic acid effect in cofilin morphology change from punctate to diffuse form in both wild-type and *por1*Δ mutant (B).

Following the fluorescence microscopy data, showing the interference of Por1p in cofilin intracellular distribution, it was intended to investigate if the cofilin pro-apoptotic role is also mediated by Por1p activity. So, cell survival was evaluated in all strains with or without *POR1* deletion under acetic acid treatment (150 mM). The results confirmed that, as in the preliminary results previously mentioned, these mutations in the *COF1* gene confer resistance to acetic acid. The percentage of cell survival in *cof1-7* mutant was surprisingly high even after three hours of treatment. The deletion of *POR1* in the wild-type, *cof1-5* and *cof1-6* strains resulted in higher sensitivity to the acetic acid treatment than the same strains without deletion of this gene. The results also showed that in the absence of Por1p, *cof1-5* and *cof1-6* mutants did not give raise to higher resistance to acetic acid, when compared with the wild-type. On the other hand, the deletion of *POR1* in *cof1-7* mutant strain did not lead to a change in the phenotype, with the exception for the two hours of treatment where it was slightly more resistant (Figure 8).

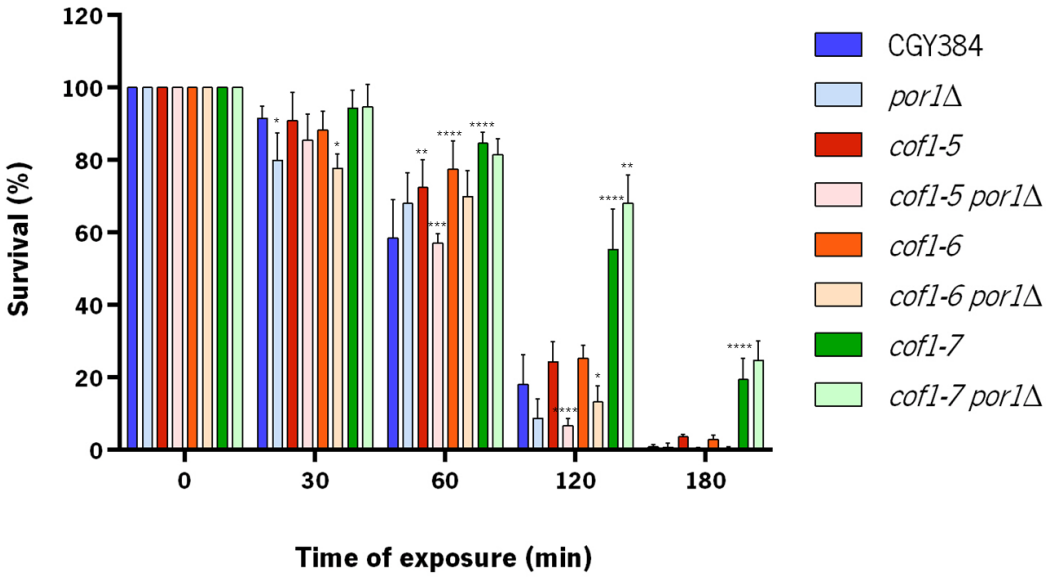

Figure 8 - Effect of cofilin mutation and/or *POR1* deletion on viability (CFU counts) of *S. cerevisiae* CGY384 exponential cells treated with 150 mM of acetic acid during three hours. The number of colonies obtained, individually for each strain, was normalized to the time zero, which was considered 100%. Reported values are the mean of at least five independent experiments with SD. A two-way ANOVA test was employed to compare both the wild-type *versus* cofilin mutant strains (single mutants) as well as wt and cofilin mutant strains *versus* these strains with *POR1* gene deleted (*por1*Δ and double mutant strains) for each time of treatment. Statistical analysis: * p<0,05, ** p<0,01, *** p<0,001 and **** p<0,0001.

3.5 Discussion

In animal cells it has been described that cofilin has the ability to mediate a mitochondrial-dependent apoptotic process. During this process, some evidences appear to indicate that oxidized cofilin interacts with the OMM, however the mechanism by which this interaction occurs and leads to PTP opening remains to be clarified. In yeast cells, a similar role of cofilin during apoptotic-like PCD was not been reported. However, recent preliminary data suggests that a stress-specific interaction between cofilin and Por1p (yeast VDAC) can exist in yeast cells (Kotiadis et al., 2012). Considering the previously described Por1p involvement in yeast apoptosis (Pereira et al., 2007), it was intended to determine in yeast cells firstly the role of cofilin in the acetic acid-induced cell death and then the involvement of Por1p activity in this process.

The resistance displayed by the cofilin mutants during acetic acid treatment immediately suggested that in yeast cells, like in mammalians, cofilin can regulate the cell death process. Although in mammalians the cell death process triggered by oxidized cofilin has not been clearly defined, appearing to be cell type and context-dependent, after acetic acid treatment the reduced number of PI-positive cells displayed by all yeast strains suggest that, under these conditions, they are dying by an apoptotic process that seems to be delayed by the mutations under study. Consistent with the observed resistance of the cofilin mutant strains evaluated by cell viability assay, after 90 minutes of acetic acid treatment, the decrease in the amount of mitochondrial cyt *c* was more pronounced in the wild-type than in the *cof1-6* mutant which amount seems to remain unchanged. Therefore, the results obtained suggest that *cof1-6* can be involved in the regulation of MOMP through its inhibition.

Underlying the proposed role of yeast cofilin in the regulation of MOMP, the fluorescence microscopy data suggest that, after acetic acid treatment, cofilin could be translocated to the mitochondria. In addition, our results show that the cofilin redistribution is delayed in the *por1*Δ mutant, when compared with the wild-type, suggesting that Por1p at the OMM can be required for the cofilin intracellular redistribution. Furthermore, since it was previously shown that the absence of Por1p sensitized cells to death in response to acetic acid (Pereira et al., 2007), our results suggest that the redistribution of cofilin may work as a negative regulator of yeast apoptosis.

In our study, the CFU assay revealed that the *POR1* deletion in *cof1-5* and *cof1-6*, but not in *cof1-7*, mutants leads to a similar percentage of survival when compared with the *por1*Δ single mutant showing that the increased resistance of *cof1-5* and *cof1-6* mutants is dependent on Por1p presence. As *cof1-6*

mutant displays lower cyt *c* release under acetic acid treatment, the results suggest that cofilin holds a regulatory function in the permeabilization of OMM that depends on Por1p presence.

Altogether these results seems to indicate that, after acetic acid treatment, cofilin is translocated to mitochondria, where by interaction with Por1p at the OMM, prevents MOMP and consequently the cyt *c* release, functioning like Por1p as a negative regulator of apoptosis, such as outlined below (Figure 9).

In animal cells, it was reported that cofilin mitochondrial translocation is triggered by its oxidation that occurs as a consequence of an increased oxidative stress inside the cell (Chua et al., 2003; Klamt et al., 2009). Curiously, under our study conditions, it was observed that the redistribution of cofilin occurs early when compared with the ROS accumulation. Therefore, it is possible that, during acetic acid treatment, cofilin redistribution and hypothetically its translocation to the mitochondria is not triggered by an increased oxidative stress but instead by other structural change(s) that allow its dissociation of actin filaments and consequently translocation to mitochondria. It has been reported that yeast cells treated with acetic acid exhibit altered levels of proteins directly or indirectly associated with the pathways: target of rapamycin (TOR) and RAS/cAMP/cAMP-dependent protein kinase (PKA), as consequence of severe intracellular amino-acid starvation or intracellular acidification, respectively (Almeida et al., 2009; Zdralevic et al., 2011). Curiously, these pathways have been associated with actin cytoskeleton rearrangement (Jacinto et al., 2004; Gerits et al., 2007). Therefore, it is possible that after acetic acid treatment, one or even both pathways lead to a reorganization of actin cytoskeleton that consequently makes cofilin unable to associate with the actin filaments, resulting in its translocation to the mitochondrial membrane (Figure 9). However, further experiments are still need to test this hypothesis, or to unravel other mechanisms that may explain the cofilin translocation in our treatment conditions.

Like discussed above, in our study conditions, *cof1-5* and *cof1-6* appears to display an antiapoptotic role during the acetic acid-induced PCD. In mammalians, it was demonstrated that the apoptosis-inducing ability of cofilin, but not its mitochondrial localization, is dependent on the functional actin-binding domain (Chua et al., 2003). The exchanged residues in the *cof1-5* mutant, although non-essential for the yeast survival, affect the actin dynamics. In turn, it was shown that the exposure of the N-terminal sequence (15-30 amino acid residues) of the human cofilin is fundamental for its mitochondrial translocation (Chua et al., 2003). The *cof1-6* mutant has exchanged residues in this sequence and, if a similar process happens in yeast cells, although cofilin may be translocated to the mitochondria, the different conformational change acquired, could influence its insertion at the OMM and consequently its apoptotic function. Thereby, it is conceivable that the exchanged residues in *cof1-5* and

cof1-6 can be in fact required for an apoptotic role of cofilin, justifying the high resistance and the antiapoptotic role of these mutations when compared with the wild-type.

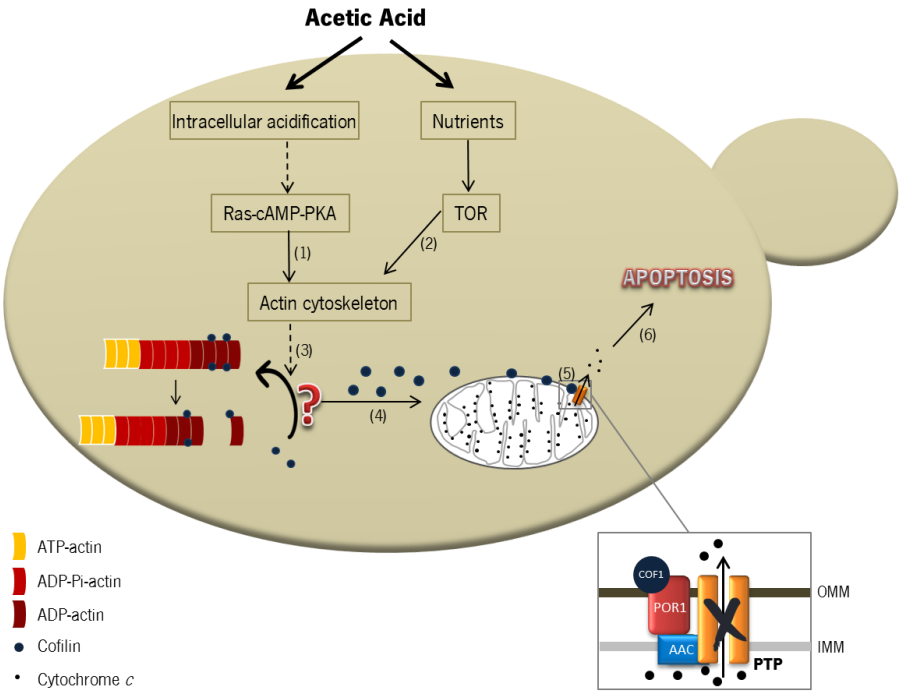

Figure 9 – Scheme of the hypothesized role of cofilin in a yeast mitochondrial-mediated apoptotic pathway. The addition of acetic acid to the medium, due to its passive influx, leads quickly to intracellular alterations, namely acidification that can result in Ras-cAMP-PKA pathway activation (1). In the same way, acetic acid can lead to intracellular amino-acid starvation triggering TOR pathway activation (2). Each of these pathways, individually or together can lead to actin cytoskeleton reorganization that can perturb the cofilin-actin association (3) promoting cofilin translocation to mitochondria (4). At the OMM, cofilin interacts with Por1p, inhibiting the MOMP (5) and subsequently the release of cyt *c* and apoptosis (6).

The yeast *S. cerevisiae* cofilin has only a single cysteine residue but until the present, its importance or the effect of its potential oxidation has not been reported. In the *cof1-7* mutant the cofilin cysteine residue was exchanged by alanine. Despite the resistance of this mutant suggesting, like for *cof1-5* and *cof1-6* mutants, an antiapoptotic function, preliminary results obtained in our investigation group demonstrated an increased amount of cyt *c* release into the cytosolic fraction after acetic acid treatment, when compared with the wild-type (Rui Silva, unpublished data). Furthermore, the deletion of *POR1* in *cof1-7* mutant did not result in increased sensitivity to acetic acid, suggesting that the higher resistance of the mutant is independent of the mechanisms mediated by Por1p. As a whole the results suggest that *cof1-7* exchanged residues induce resistance to apoptosis by a mechanism independent of its potential role in MOMP. In agreement with our results, in human cells the exchange of cofilin cysteine residues by alanine inhibits apoptosis (Klamt et al., 2009), however these results are insufficient to propose a role for

cysteine residues in the yeast cofilin because in *cof1-7* mutant there is additionally other exchanged residue.

Differently from the *POR1* deleted strains, in the *cof1-7* mutant the increased release of cyt *c* did not result in a sensitive phenotype when evaluated the cell survival. This supports the notion of, particularly in the *cof1-7* mutant, the existence of some mechanisms of resistance that are triggered downstream to cyt *c* release. Reinforcing a role for *cof1-7* mutant independent from MOMP, preliminary results provided by Professor Campbell Gourlay group show that this cofilin mutant strain exhibit increased levels of Ste12p, a transcriptional factor that is activated by a MAP kinase signaling cascade. Curiously, in a genome-wide screening it was shown that the deletion of *DIG2*, gene that encodes an inhibitor of Ste12p activity, resulted in higher resistance than the wild-type strain to the acetic acid treatment (Sousa et al., 2013). Although the screen has been done in another wild-type strain, this result appears to evidence the Ste12p anti-apoptotic function during the acetic acid-induced cell death.

Some of the regulatory mechanisms of PTP opening have been described to be mediated by VDAC (yeast Por1p) activity. As described in the Chapter 1, Hexokinase (Hk) was reported to regulate the VDAC activity through its binding at the OMM (Robey and Hay, 2006). Additionally, it was described that Gelsolin, another ABP, also prevents apoptosis by binding to VDAC and promoting a closed conformation (Koya et al., 2000; Kusano et al., 2000). Our results suggest that yeast cofilin is also involved in apoptosis inhibition by interaction with Por1p and that the residues mutated in *cof1-5* and *cof1-6* have a role in this mechanism. Additional studies are, however, required to confirm this proposed mechanism.

Curiously, the results here presented suggest that depending of the cofilin residues mutated, it may have different functions during the apoptotic-like PCD process induced by acetic acid. Therefore, further investigation of the role of cofilin can effectively make this protein a good target for the development of new drugs for the treatment of pathologies with the apoptotic machinery deregulated.

3.6 References

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

Arokium, H., Camougrand, N., Vallette, F. M., and Manon, S. (2004). Studies of the interaction of substituted mutants of Bax with yeast mitochondria reveal that the C-terminal hydrophobic α-helix is a second ART sequence and plays a role in the interaction with anti-apoptotic Bcl-x_L. The Journal of Biological Chemistry *279*, 52566-52573.

Bailly, M., and Jones, G. E. (2003). Polarised Migration: Cofilin Holds the Front. Current Biology *13*, 128-130.

Bamburg, J. R. (1999). Proteins of the ADF/cofilin family: essential regulators of actin dynamics. Annual Review of Cell and Developmental Biology *15*, 185-230.

Bernard, O. (2007). Lim kinases, regulators of actin dynamics. The International Journal of Biochemistry & Cell Biology *39*, 1071-1076.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram of protein utilizing the principle of protein-dye binding. Analytical Biochemistry *7*, 248-254.

Chua, B. T., Volbracht, C., Tan, K. O., Li, R., Yu, V. C., and Li, P. (2003). Mitochondrial translocation of cofilin is an early step in apoptosis induction. Nature Cell Biology *5*, 1083-1089.

Endo, M., Ohashi, K., Sasaki, Y., Goshima, Y., Niwa, R., Uemura, T., and Mizuno, K. (2003). Control of growth cone motility and morphology by LIM kinase and Slingshot via. The Journal of Neuroscience *23*, 2527-2537.

Franklin-Tong, V. E., and Gourlay, C. W. (2008). A role for actin in regulating apoptosis/programmed cell death: evidence spanning yeast, plants and animals. Biochemical Journal *413*, 389-404.

Gerits, N., Mikalsen, T., Kostenko, S., Shiryaev, A., Johannessen, M., and Moens, U. (2007). Modulation of F-actin rearrangment by the cyclic AMP/cAMP-dependent protein kinase (PKA) pathway is mediated by MAPK-activated Protein Kinase 5 and requires PKA-induced nuclear export of MK5. The Journal of Biological Chemistry *282*, 37232-37243.

Gietz, R. D., and Woods, R. A. (2006). Yeast transformation by the LiAc/SS Carrier DNA/PEG method. Methods in Molecular Biology *313*, 107-120.

Hsieh, Y. C., Rao, Y. K., Wu, C. C., Huang, C. Y., Geethangili, M., Hsu, S. L., and Tzeng, Y. M. (2010). Methyl antcinate A from Antrodia camphorata induces apoptosis in human liver. Chemical Research in Toxicology *23*, 1256-1267.

Ichetovkin, I., Grant, W., and Condeelis, J. (2002). Cofilin produces newly polymerized actin filaments that are preferred for dendritic nucleation by the Arp2/3 Complex. Current Biology *12*, 79-84.

Iida, K., and Yahara, I. (1999). Cooperation of two actin-binding proteins, cofilin and Aip1, in *Saccharomyces cerevisiae*. Genes to Cells *4*, 21-32.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M. A., Hall, A., and Hall, M. N. (2004) Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nature Cell Biology *6*, 1122-1131.

Jiang, N., Kham, S. K., Koh, G. S., Lim, J. Y. S., Ariffin, H., Chew, F. T., and Yeoh, A. E. (2011). Identification of prognostic protein biomarkers in childhood acute lymphoblastic (ALL). Journal of Proteomics *74*, 843-857.

Klamt, F., Zdanov, S., Levine, R. L., Pariser, A., Zhang, Y., Zhang, B., Yu, L. R., Veenstra, T. D., and Shacter, E. (2009). Oxidant-induced apoptosis is mediated by oxidation of the actin-regulatory protein cofilin. Nature Cell Biology *11*, 1241-1246.

Klemke, M., Wabnitz, G. H., Funke, F., Funk, B., Kirchgessner, H., and Samstag, Y. (2008). Oxidation of cofilin mediates T cell hyporesponsiveness under oxidative stress conditions. Immunity *29*, 404-413.

Koya, R. C., Fujita, H., Shimizu, S., Ohtsu, M., Takimoto, M., Tsukimoto, Y., and Kuzumaki, N. (2000). Gelsolin inhibits apoptosis by blocking mitochondrial membrane potental loss and cytochrome c release. The Jounal of Biological Chemistry *275*, 15343-15349.

Kotiadis, V. N., Leadsham, J. E., Bastow, E. L., Gheeraert, A., Whybrew, J. M., Bard, M., Lappalainen, P., and Gourlay, C. W. (2012). Identification of new surfaces of cofilin that link mitochondrial function to the control of multi-drug resistance. Journal of Cell Science *125*, 2288-2299.

Kusano, H., Shimizu, S., Koya, R. C., Fujita, H., Kamada, S., Kuzumaki, N., and Tsujimoto, Y. (2000). Human gelsolin prevents apoptosis by inhibiting apoptotic mitochondrial changes via closing VDAC. Oncogene *19*, 4807-4814.

Lappalainen, P., Fedorov, E. V., Fedorov, A. A., Almo, S. C., and Drubin, D. G. (1997). Essential functions and actin-binding surfaces of yeast cofilin revealed by systematic mutagenesis. The EMBO Journal *16*, 5520–5530.

Li, G., Cheng, Q., Liu, L., Zhou, T., Shan, C. Y., Hu, X. Y., Zhou, J., Liu, E. H., Li, P., and Gao, N. (2013). Mitochondrial translocation of cofilin is required for allyl isothiocyanate-mediated cell death via ROCK1/PTEN/PI3K signaling pathway. Cell Communication and Signaling *11*, 50-63.

Maloney, M. T., and Bamburg, J. R. (2007). Cofilin-mediated neurodegeneration in Alzheimer's disease and other amyloidopathies. Molecular Neurobiology *35*, 21-44.

Minamide, L. S., Striegl, A. M., Boyle, J. A., Meberg, P. J., and Bamburg, J. R. (2000). Neurodegenerative stimuli induce persistent ADF/cofilin-actin rods that disrupt distal neurite function. Nature Cell Biology *2*, 628-636.

Moon, A. L., Janmey, P. A., Louie, K. A., and Drubin, D. G. (1993). Cofilin is an essential component of the yeast cortical cytoskeleton. The Journal of Cell Biology *120*, 421-435.

Moriyama, K., and Yahara, I. (2002). The actin-severing activity of cofilin is exerted by the interplay of three distinct sites on cofilin and essential for cell viability. Biochemical Journal *365*, 147-155.

Peng, X., Gong, F., Zhao, Y., Zhou, L., Xie, Y., Liao, H., Lin, H., Li, Z., Tang, M., and Tong, A. (2011). Comparative Proteomic Approach Identifies Pkm2 and Cofilin-1 as Potential Diagnostic, Prognostic and Therapeutic Targets for Pulmonary Adenocarcinoma. PLoS ONE *6*, e27309.

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

Polachini, G. M., Sobral, L. M., Mercante, A. M., Paes-Leme, A. F., Xavier, F. C., Henrique, T., Guimarães, D. M., Vidotto, A., Fukuyama, E. E., Gois-Filho, J. F.*, et al.* (2012). Proteomic approaches identify members of cofilin pathway involved in oral tumorigenesis. PLoS ONE *7*, e50517.

Posadas, I., Perez-Martinez, F. C., Guerra, J., Sanchez-Verdu, P., and Cena, V. (2012). Cofilin activation mediates Bax translocation to mitochondria during excitotoxic neuronal death. Journal of Neurochemistry *120*, 515-527.

Rehklau, K., Gurniak, C. B., Conrad, M., Friauf, E., Ott, M., and Rust, M. B. (2012). ADF/cofilin proteins translocate to mitochondria during apoptosis but are not generally required for cell death signaling. Cell Death & Differentiation *19*, 958-967.

Robey, R. B., and Hay, N. (2006). Mitochondrial hexokinases, novel mediators of the antiapoptotic effects of growth factors and Akt. Oncogene *25*, 4683-4696.

Steller, E. J., Ritsma, L., Raats, D. A., Hoogwater, F. J., Emmink, B. L., Govaert, K. M., Laoukili, J., Rinkes, I. H., Rheenen, J. v., and Kranenburg, O. (2011). The death receptor CD95 activates the cofilin pathway to stimulate tumour cell invasion. EMBO Reports *12*, 931-937.

Sousa, M., Duarte, A. M., Fernandes, T. R., Chaves, S. R., Pacheco, A., Leão, C., Côrte-Real, M., and Sousa, M. J. (2013). Genome-wide identification of genes involved in the positive and negative regulation of acetic acid-induced programmed cell death in *Saccharomyces cerevisiae*. BMC Genomics (*article under revision*).

Vaseva, A. V., Marchenko, N. D., Ji, K., Tsirka, S. E., Holzmann, S., and Moll, U. M. (2012). p53 opens the mitochondrial permeability transition pore to trigger necrosis. Cell *149*, 1536-1548.

Wabnitz, G. H., Goursot, C., Jahraus, B., Kirchgessner, H., Hellwig, A., Klemke, M., Konstandin, M. H., and Samstag, Y. (2010). Mitochondrial translocation of oxidized cofilin induces caspase-independent necrotic-like programmed cell death of T cells. Cell Death and Disease *1*, e58.

Wei, R., Zhang, Y., Shen, L., Jiang, W., Li, C., Zhong, M., Xie, Y., Yang, D., He, L., and Zhou, Q. (2012). Comparative proteomic and radiobiological analyses in human lung adenocarcinoma cells. Molecular and Cellular Biochemistry *359*, 151–159.

Zdanov, S., Klamt, F., and Shacter, E. (2010). Importance of cofilin oxidation for oxidant-induced apoptosis. Cell Cycle *9*, 1675- 1677.

Zdralevic, M., Guaragnella, N., Antonacci, L., Marra, E., and Giannattasio, S. (2011). Yeast as a tool to study signaling pathways in mitochondrial stress response and cytoprotection. The Scientific World Journal *2012*, 1-11.

Zhao, H., Hakala, M., and Lappalainen, P. (2010). ADF/cofilin binds phosphoinositides in a multivalent manner to act as a PIP2-Density Sensor. Biophysical Journal *98*, 2327-2336.

Zhu, J. S., Ouyang, D. Y., Shi, Z. J., Xu, L. H., Zhang, Y. T., and He, X. H. (2012). Cucurbitacin B induces cell cycle arrest, apoptosis and autophagy associated with G Actin Reduction and Persistent Activation of Cofilin in Jurkat Cells. Pharmacology *89*, 348-356.

Concluding remarks and Future prospects

Apoptosis is the most widely studied process of PCD due to its involvement in many fundamental biological events. *Saccharomyces cerevisiae* undergoes a PCD process in response to different stimuli and exhibits many of the morphological and biochemical hallmarks of mammalian apoptosis. Many studies, including of our research group, have shown that the yeast *S. cerevisiae* is able to trigger a mitochondrial-mediated apoptotic pathway in response to acetic acid treatment. The similarities to the mammalian intrinsic pathway led to an increased interest in exploiting this simple model to solve some unanswered questions of apoptosis and its regulation. Therefore, this thesis aimed to contribute to a better understanding of this process through the study of the role of phospholipids and of the actin-binding protein cofilin in acetic acid-induced PCD.

Relocalization of CL at the mitochondrial membranes and PS externalization to the outer leaflet of the plasma membrane are surely fundamental to trigger apoptosis in mammalian cells. Despite this reported fundamental role of CL and PS in mammalian apoptosis the effect of their decreased levels in yeast apoptotic-like PCD was never assessed. We found that:

- CL is not essential to trigger acetic acid-induced apoptosis but is required to maintain the mitochondrial function and morphology;
- Mature CL is required for the preservation of mitochondrial function and in determining an apoptotic cell death. In its absence death, though delayed, mainly occurs through necrosis;
- PS appears essential for cells to commit into an apoptotic-like PCD since in its absence death is delayed and exhibits features of necrosis;
- Phospholipid trafficking between ER and mitochondria mediated by components of the ERMES complex, confined to MAMs, and also the translocation of PA from the OMM to the IMM mediated by Ups1p, appear to positively regulate the yeast apoptosis.

Although a decrease in CL levels has been reported for all mutants here studied, except *cho1*Δ, the observed differences between mutants suggest that these proteins can be involved in the synthesis or regulation of other phospholipids, whose levels inside the cell are able to mediate the cell death process. Therefore, a characterization of the phospholipid profiles in all mutants, before and after acetic acid treatment is needed. For this purpose, the phospholipid composition of mitochondria from these strains

might be determined by separating the lipids extracted from mitochondria by thin layer chromatography, and then by scraping and quantifying the different spots by UV absorbance, as described (Gonzalvez et al., 2005a).

Considering the purpose of the present study that focuses on a deeper characterization of mitochondrial involvement in this process of cell death, it would be also fundamental to evaluate cyt *c* release, through western blot analysis in non-treated and acetic acid treated-cells, and compare the relative levels of cyt *c* in the mitochondrial and cytosolic factions, to understand the role of these proteins in MOMP. In addition, considering the reported role of CL in the AAC structure and function, and in VDAC oligomerization, it would also be interesting to delete the *AAC1/2/3* and *POR1* genes in all mutants strains to verify if the interaction of CL with these proteins can have an effect in cell death revealing in this way a new mechanism of yeast PTP regulation.

During mammalian apoptosis, the actin-binding protein cofilin appears to regulate the mitochondrial-mediated apoptotic pathway. However, the mechanism by which cofilin interacts with the OMM and leads to MOMP is not clarified. Considering the high phylogenetic conservation of this protein in both animal and yeast cells, as well as preliminary data suggesting that, under stressful conditions, cofilin is able to interact with Por1p (yeast VDAC) at the OMM, we used yeast as a model to evaluate the role of cofilin in yeast apoptosis and the involvement of Por1p in cofilin-mediated apoptosis. Since cofilin is indispensable for yeast survival we used mutant forms of this protein. Our results appear to suggest that in cells undergoing apoptosis in response to acetic acid:

- Cofilin is translocated to mitochondria where, by interaction with Por1p at the OMM, prevents MOMP, and consequently the release of cyt *c*, functioning like Por1p as a negative regulator of apoptosis (*cof1-5* and *cof1-6* evidences);

  Despite our results, further research will be required to get a full picture on the role of cofilin in yeast apoptosis regulation. To test our hypothesis it will be required to assess the Por1p-cofilin interaction at the OMM through an immunoprecipitation assay. Additionally, it would be interesting to evaluate the translocation of cofilin to mitochondria through western blot. The simultaneous detection of cofilin and cyt *c* in the mitochondrial and cytosolic fractions of all strains will allow us to evaluate not only the relationship between cofilin translocation and MOMP in the wild-type, and

the Por1p involvement in this process, as well as the requirement of the exchanged residues in the mutants to achieve a similar response.

- In *cof1-7* mutant the higher resistance of the mutant is independent of its potential role in MOMP.

  Considering the reported increased levels of Ste12p, a transcriptional factor that is activated by a MAP kinase signaling cascade, in this cofilin mutant strain, it would be interesting to evaluate the effect of *STE12* deletion in all mutants.

In conclusion, the results of this thesis sustain that phospholipids play a decisive role on the nature of yeast PCD, and on the regulation of the cell death process triggered by acetic acid. Additionally, a role of yeast cofilin in the regulation of MOMP induced by acetic acid is also proposed. Since the core apoptotic machinery is conserved in yeast, the exploitation of this simple eukaryotic model to the elucidate the role of cofilin and of the phospholipids here studied in mitochondrial-mediated apoptosis will offer new insights towards the development of novel targeted drugs for therapeutic intervention against pathologies associated with apoptosis dysfunctions, including cancer and neurodegenerative diseases.