Universidade do Minho
Escola de Ciências

Dário Alexandre Martins Trindade

Contribution of the adenine nucleotide carrier, porin, and sphingolipid metabolism to mitochondria membrane permeabilization in *Saccharomyces cerevisiae*

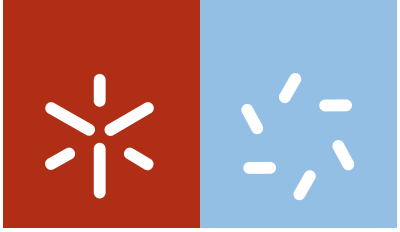
November 2013

Contribution of the adenine nucleotide carrier, porin, and sphingolipid metabolism to mitochondria membrane permeabilization in *Saccharomyces cerevisiae*

Dário Alexandre Martins Trindade

UMinho | 2013

This Thesis was developed under a cotutelle programme with the Institut de Biochimie et Génétique Cellulaires - IBGC, Université Bordeaux Segalen, Bordeaux, France.



Universidade do Minho
Escola de Ciências

Dário Alexandre Martins Trindade

Contribution of the adenine nucleotide carrier, porin, and sphingolipid metabolism to mitochondria membrane permeabilization in *Saccharomyces cerevisiae*

PhD Thesis on Molecular and Environmental Biology
Specialty of Cellular and Health Biology

Elaborated under the supervision of:
Professor Manuela Côrte-Real
Professor Maria João Sousa
Doctor Stéphen Manon

November 2013

Universidade do Minho, ___/___/_____

Assinatura: _____

Acknowledgements

Após uma longa aventura, que chega ao seu final com a apresentação deste trabalho, gostaria de destacar e agradecer aqueles com quem tive o prazer de privar, e que de alguma forma contribuíram para a realização deste trabalho.

Às professoras Maria João Sousa e Manuela Côrte-Real, as minhas mentoras, gostaria de lhes agradecer pela sua orientação, pela partilha de conhecimentos e pelo seu apoio ao longo destes anos. Particularmente, pela paciência (e que paciência) e dedicação demonstradas nesta recta final, as quais foram de resto uma grande força na preparação e entrega desta tese. Deixo aqui a minha gratidão.

To Doctor Stéphen Manon, with who I had the pleasure to work with, for the accepting me at his lab, for teaching me not only how to work but also how to think. He was truly important for my own evolution in science.

Gostaria também de agradecer a todos os meus colegas com quem tive o prazer de partilhar o laboratório. À Andreia A., ao António, à Dulce, à Elisabete, à Gabriela, à Helena, ao Jorge, à Lisandra, ao Marco, à Marlene, à Marta, à Natália, à Rita C., à Rita P., ao Rui, à Selma, à Susana, à Tânia, à Vera, entre outros colegas de laboratório mas também do departamento de Biologia. A todos eles deixo o meu agradecimento pelo companheirismo, pela partilha de conhecimentos, e acima de tudo pelos bons momentos.

To Nadine, Muriel, Florian and Axel for making my life easier at Bordeaux. A special acknowledge to Thibaud for his friendship and companionship.

Á Andreia, ao Flávio e à Sara, agradeço a amizade, o companheirismo e toda a motivação que sempre me deram.

À Fundação Calouste Gulbenkian, não só por ter tornado possível todo este processo mas também pela atitude excepcional com que sempre fui tratado.

À minha família, que eu tanto adoro, e que tudo faz por mim.

E por fim, à minha namorada. Sem a Joana nunca teria chegado a esta fase. Foste a minha constante, Joaninha... Obrigado por tudo.

Este trabalho é financiado por Fundos FEDER através do Programa Operacional Factores de Competitividade – COMPETE, por Fundos Nacionais através da FCT – Fundação para a Ciência e a Tecnologia no âmbito do projectoPEst-C/BIA/UI4050/2011, e pelo projecto FCT-ANR/BEX-BCM/0175/2012. D.T. usufruiu de uma bolsa de doutoramento da Fundação Calouste Gulbenkian (processo n.º 126474).

Abstract

A decisive event in the cell's life-or-death decision is the mitochondrial outer membrane permeabilization (MOMP). The biochemical events responsible for MOMP, considered by many investigators the "point-of-no-return" in cell death, are not entirely defined. Two major and very distinct mechanisms have been implicated in the control of MOMP: i) the action of Bcl-2 family proteins, which can directly engage the outer mitochondria membrane (OMM) and induce the opening of pores; and ii) the mitochondrial permeability transition pore (PTP), an inner membrane unselective channel that induces mitochondria swelling upon long term openings, and eventual rupture of the OMM.

The growing interest in cell death biology, fostered by the relevant contributions of yeast to the understanding of basic biological processes, brought the unicellular eukaryote *Saccharomyces cerevisiae* into the scene. Yeast cells lack some of the major regulators of apoptosis, such as Bcl-2 family proteins (though one related protein was recently identified), but still possess homologues of mitochondrially enclosed pro-apoptotic factors, as well as orthologues of the molecular components generally ascribed to PTP, including the ADP/ATP carrier (AAC) and Porin (Por1). These particular features of *S. cerevisiae*, along with the availability of genetic and molecular tools and easy manipulation, provided an excellent opportunity to study Bcl-2 family members in a "controlled" environment, or the contribution of the PTP and its components to cell death.

In this work, the particular contribution of AAC's thiol goups, its oxidation, Por1, and of a possible interaction between both proteins to cell death, using the well-established model of acetic acid-induced yeast death, was explored. It was observed that the cysteine residues of the Aac2p, when this AAC isoform was individually expressed, do not contribute to the mechanisms of acetic acid-induced death. This finding indicates that the oxidative modifications of Aac2p may not be implicated in the acetic acid-induced cell death program, at least not those involving thiol crosslinking. This idea is supported by the apparent absence of a particular Aac2p oxidation pattern. Nevertheless, the AAC was previously demonstrated to be required for acetic acid-induced cytochrome *c* release, and its absence promoted the survival of yeast cells. Deletion of Por1, on the other hand, decreased the viability of yeast cells treated with acetic acid. It was hypothesized that the two proteins could share the same pathway in

the regulation of cell death. To test this, cytochrome *c* release was evaluated in mitochondria isolated from *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* cells following acetic acid treatment. The data obtained suggest that absence of Por1 does not affect cytcochrome *c* release, hence MOMP, during acetic acid induced-death, but it may be important for its regulation. When both the AAC and Por1 were absent, yeast mitochondria could still release cytochrome *c*, raising the possibility of an AAC-independent mechanism. Furthermore, we found both proteins have distinct effects that regulate the cellular response to different stresses. Particularly, absence of the AACs somehow contributed to increased cell wall resistance and osmotic stress resistance.

Finally, *S. cerevisiae* was explored as a model to study mechanistic aspects relative to the function of Bcl-2 family proteins. Particularly, we assessed the role of sphingolipids, using cells lacking sphingolipid metabolism enzymes, in the action of the human pro-apoptotic regulator Bax. We found that absence of Isc1p, an inositol phospholipase C that degrades complex sphingolipids into ceramides in yeast, favored the viability of yeast cells expressing an active form of Bax. It was further revealed that this effect is not associated with changes in the action of Bax; rather, it might be related with the cellular consequences of Bax-action. Indeed, a parallel with the effect of Uth1p absence in yeast cells expressing Bax suggests that the absence of Isc1p could affect the selective degradation of mitochondria by mitophagy, and thus produce a different cell death response.

This work provides new insights into the physiological events underlying the contribution of mitochondrial proteins, previously associated with cell death responses, and sphingolipid metabolism to cell death induced by acetic acid and Bax, respectively. Once again, the yeast *S. cerevisiae* proved to be an excellent model in the particular field of cell life and death research.

Resumo

A permeabilização da membrana mitocondrial externa (MOMP) é considerado o evento decisivo para a sobrevivência celular. Os eventos bioquímicos subjacentes a este processo, considerado o "*ponto sem retorno*" na morte celular, não estão ainda completamente elucidados. Dois dos principais mecanismos apontados como responsáveis pelo controlo deste processo consistem na acção das proteínas da família Bcl-2, que podem interactuar directamente com a membrana mitocondrial externa induzindo a abertura de poros; e o poro de permeabilidade transitória (PTP), um canal não selectivo que ocorre na membrana mitocondrial interna e que, quando aberto por longos períodos, induz a tumefacção mitocondrial podendo culminar na eventual ruptura da membrana mitocondrial externa.

O crescente interesse no estudo da morte celular, potenciado pelas contribuições da levedura na elucidação de processos celulares fundamentais, despoletou a utilização da levedura enquanto modelo para a investigação da morte celular. Os principais reguladores da apoptose, nomeadamente as proteínas da família Bcl-2 (à excepção de um ortólogo recentemente identificado) não estão presentes na levedura. No entanto, este organismo possui ortólogos de vários factores apoptóticos, assim como dos constituintes moleculares do PTP, incluindo o transportador de ADP/ATP (AAC) e a porina de membrana mitocondrial externa (Por1p). Estas características da levedura *S. cerevisiae*, bem como a disponibilidade de ferramentas genéticas e moleculares aliadas a uma fácil manipulação, fazem deste sistema uma excelente ferramenta para o estudo da expressão de proteínas da família Bcl-2 num ambiente "controlado", ou do contributo do PTP e dos seus componentes para a morte celular.

Neste trabalho é estudada a contribuição dos grupos tiol da proteína AAC, e da sua oxidação assim como do papel das próprias proteínas AAC e Por1p, e uma possível interacção entre ambas, para a morte celular induzida por ácido acético. Nas condições testadas observou-se que os resíduos de cisteína do Aac2p, expresso como a única isoforma do AAC, não contribuem para os mecanismos de morte induzida por ácido acético. Este resultado sugere que o programa de morte celular induzido por este composto não depende de modificações oxidativas do Aac2p, pelo menos dependentes da oxidação de grupos tiol. Esta afirmação é suportada por aparente ausência oxidação do Aac2p. No entanto, o AAC já foi previamente descrito como elemento essencial para a libertação de citocromo *c* da mitocôndria durante a morte celular induzida por ácido

acético. Para além deste efeito, a deleção do AAC promove igualmente a sobrevivência das células de levedura. A ausência do gene *POR1*, por outro lado, diminui a viabilidade deste mutante quando exposto ao tratamento com ácido acético.

Em relação a uma possível interação do AAC e da porina Por, foi sugerido que as duas proteínas poderiam partilhar a mesma via na regulação do processo de morte celular em levedura. Para testar esta hipótese, a libertação de citocromo *c* foi avaliada em mitocôndrias isoladas dos mutantes *Δaac1/2/3*, *Δpor1* e *Δaac1/2/3Δpor1* após o tratamento com ácido acético. Os dados obtidos sugerem que a ausência da proteína Por1 não bloqueia a libertação de citocromo *c*, e consequentemente o MOMP, durante a morte induzida por ácido acético mas pode ser importante para a sua regulação. Na ausência dos genes *AACs* e *POR1* foi possível observar libertação de citocromo *c*, o que aponta para a existência de um mecanismo alternativo de regulação do MOMP nestas condições. Verificou-se também que estas proteínas têm respostas distintas quando sujeitas a diferentes stresses celulares. Em particular, a ausência dos AAC contribui, de alguma forma, para o aumento da resistência ao stress osmótico e da resistência da parede celular.

Por fim, a levedura *S. cerevisiae* foi usada como um modelo para estudo das proteínas da família Bcl-2. Especificamente, avaliou-se o papel dos esfingolípidos, na acção da proteína pro-apoptótica de mamíferos Bax usando estirpes mutadas no metabolismo dos esfingolípidos. Foi observado que a ausência da proteína Isc1p, uma inositol fosfolipase C que degrada esfingolípidos complexos em ceramidas, favorece a viabilidade de células que expressam uma forma activa da Bax. Mostrou-se ainda que este efeito parece não estar associado a uma alteração na acção da Bax, podendo, pelo contrário, estar relacionado com os defeitos subsequentes à acção desta proteína. Assim, e paralelamente ao que está descrito para ausência da proteína Uth1, a remoção do Isc1p poderá estar a influenciar a degradação selectiva de mitocôndrias através de um processo de mitofagia, conduzindo a um processo diferente na execução da morte celular.

Com este trabalho foi possível aprofundar os conhecimentos sobre os mecanismos fisiológicos subjacentes à contribuição de proteínas mitocondriais, anteriormente associadas a processos de morte celular, e do metabolismo de esfingolípidos na morte celular induzida por ácido acético e Bax, respectivamente. Mais uma vez, a levedura *S. cerevisiae* mostrou-se um excelente modelo para compreensão dos processos de morte celular.

Table of Contents

List of Abbreviations

AAC	ADP/ATP carrier
AIF	apoptosis inducing factor
ANT	adenine nucleotide translocator
CsA	cyclosporine A
CyP-D	cyclophilin D
cyt *c*	cytochrome c
Da (kDa)	dalton (kilodalton)
DHE	dihydroethidium
$DiOC_6(3)$	3,3′dihexyloxacarbocyanine iodide
IMM	inner mitochondrial membrane
IMS	inter membrane space
M	molar
mM	milimolar
μM	micromolar
nM	nanomolar
nm	nanometer
MOMP	mitochondrial outer membrane permeabilization
O.D.	optical density
OMM	outer mitochondrial membrane
PCD	programmed cell death
PI	propidium iodide
PT	permeability transition
PTP	permeability transition pore
PTPC	permeability transition pore complex
ROS	reactive oxygen species
VDAC	voltage-dependent anion channel
YMUC	yeast mitochondrial unselective channel
$\Delta\Psi_m$	mitochondrial membrane potential
$\Delta\Psi_p$	plasma membrane potential

Chapter I

General Introduction

This chapter comprises parts of the following publication:

Rego, A., Trindade, D., Chaves, S.R., Manon, S., Costa, V., Sousa, M.J., and Côrte-Real, M. (2013). The yeast model system as a tool towards the understanding of apoptosis regulation by sphingolipids. FEMS Yeast Res.

1. Life and Death

"Life is not an exact science, it is an art."

Samuel Butler

Life is indeed the most extraordinary creation of Nature. Despite the apparent delicacy, it was given the power to interact with and change the surrounding world, to adapt and evolve through the challenges it is confronted with. Like most elements in nature, life has its own antagonist, its complement, which goes by the name of Death. Importantly, one cannot be without the other. The balance between these two forces represents the very essence of life as we know it, determining the development of organisms and defining their lives.

The notion of regressive changes during development of multicellular organisms goes way back in science history, possibly to the time of Aristotle or Galen, and it was widely recognized in the early years of the 19th century (nicely reviewed in Clarke and Clarke, 1996). However, the association between those changes and the concept of naturally occurring cell death had to wait until the establishment of the cell theory by Schleiden (1842) and by Schwann (1839). Following the advances in the field of microscopy, the discovery of developmental cell death became possible in about 1840. Its first realization was published by Carl Vogt in 1842, when words such as "resorbed", "destroyed" and "disappearing" were used to report the elimination of the notochord and its replacement by the vertebrae during amphibian metamorphosis. Two years later, the work of Prévost and Lebert corroborated the observations of Vogt, again stating that the "simple globules" of the notochord are resorbed. By the end of 19th century several studies, mostly on insect and amphibian development, have provided enough evidences to ensure the continuation and prosperity of cell death investigation.

Since its very beginning, and throughout the 20th century, the field of cell death research has grown considerably, and cell death was found to occur in different organisms, from mammals to plants and even in unicellular organisms like yeasts. Advances in different scientific areas enabled a deeper look into this biological phenomenon, and it was eventually realized that cell death was not just a mere accident

from nature. Inevitably, a new concept was born. The term programmed cell death was used for the first time in a series of works published in 1964 and 1965 by Lockshin and Williams (Lockshin and Williams, 1965). In 1966, Saunders and colleagues observed that cell death could be prevented by substances released by other tissues, indicating that it is not an inevitable process and that it can be suppressed by signals from other cells (Saunders, 1966). Additionally, the finding that inhibitors of RNA and protein synthesis were able to inhibit cell death indicated that an active metabolism is necessary to carry out this process (Lockshin, 1969; Makman et al., 1971; Martin et al., 1988; Tata, 1966). These were the basis of the programmed cell death investigation that became a particular subject of interest in the following years (Häcker and Vaux, 1997).

2. Programmed Cell Death

The term Programmed Cell Death (PCD) generally defines all forms of death whose execution is under genetic control and is carried out in a regulated manner. This requires that the process follows a specific, orchestrated sequence of biochemical events, both ordered in time and space. Moreover, such process should implicate an advantage for the particular organism or cell population. Accordingly, PCD is a normal physiological form of cell death that plays a key role in multicellular organisms both during the embryonic development and in the maintenance of adult tissues.

During adult life, PCD is crucial for tissues homeostasis. It also constitutes a defense mechanism by which damaged and potentially dangerous cells can be eliminated for the benefit of the organism as a whole. This is the case, for instance, of virus-infected cells that undergo PCD to prevent the production of new virus particles, thus limiting the spread of the pathogen through the host organism. This biological process also eliminates cells carrying potentially harmful mutations resulting from DNA damage, an essential feature in the prevention of cancer. During development, PCD plays a key role by eliminating unwanted cells from a variety of tissues as, for example, the elimination of larval tissues during amphibian and insect metamorphosis, the elimination of tissue between the digits during the formation of fingers, and in the selection of neurons that have made the correct connections with their target cells during the development of the mammalian nervous system.

Besides its role in the development and homeostasis of cell populations, PCD is also a determinant aspect in pathology and aging. In fact, defective control of PCD is directly linked to several pathologies including chronic inflammatory and neurodegenerative diseases, autoimmunity and neoplasia, among others (Bellamy et al., 1995; Lockshin and Zakeri, 2001). By the end of the 20th century the interest on PCD had increased exponentially among the scientific community, and a great deal of studies have focused on the biochemical events that control and promote the cellular demise. Current knowledge reveals that the regulation of PCD is mediated by the integrated activity of a variety of signaling pathways, some acting to induce cell death and others to promote cell survival. The delicate balance between these cellular forces is the ultimate power that determines the fate of a cell.

2.1. Apoptosis

Apoptosis is probably the most studied and well understood form of PCD. The term apoptosis, resulting from the Greek word used to describe the "dropping off" or "falling off" of petals from flowers and leaves from trees, was first proposed by Kerr and his co-workers to describe a morphologically distinct mechanism of “controlled cell deletion”, which plays a complementary but opposite role to mitosis in the regulation of animal cell populations or during embryonic development (Kerr et al., 1972).

At the present time, apoptosis is considered a universal event in the normal development and aging of multicellular organisms, acting as a homeostatic mechanism that removes damaged cells and contributes to tissue normal development, maintenance and renewal (Elmore, 2007). Albeit its importance to the normal functioning of the organisms, a defective regulation of apoptosis, is usually connected to serious physiological consequences, resulting in the occurrence and progression of several different pathologies (Rudin and Thompson, 1997). For example, the inaccurate activation of apoptosis is responsible for different neurodegenerative disorders, such as Alzheimer’s disease (Smale et al., 1995) or Huntington’s disease (Hickey and Chesselet, 2003). On the other hand, suppression of apoptosis and the subsequent inability to remove potentially dangerous cells is commonly recognized as a determinant step in the development of cancers (Evan and Vousden, 2001; Lowe and Lin, 2000). This ambiguous feature of apoptosis has placed it under the spotlight of

scientific investigation in the last decades, particularly as a result of the increasing interest in apoptosis as a therapeutic target (Lockshin and Zakeri, 2007).

2.1.1. Apoptosis: main characteristics, molecular components and pathways involved

Kerr and colleagues generalized the idea that the mechanism of controlled cell deletion followed a specific pattern characterized by cell shrinkage, nuclear and cytoplasmic condensation, nuclear coalescence and margination of chromatin, and fragmentation of the cell and the nucleus into membrane-bound, and ultrastructurally well-preserved fragments. These fragments, named apoptotic bodies, were then taken up by other cells and rapidly degraded by lysosomal enzymes (Kerr et al., 1972). In 1977, using a genomic approach, Sulston and Horvitz demonstrated that ~13% of somatic cells in the embryo of *Caenorhabditis elegans* die predictably, shortly after appearing, thus establishing a genetic basis for cell death (Sulston and Horvitz, 1977). By 1982, the first genes involved in the control of cell death were revealed (Horvitz et al., 1983). Later on, apoptosis was associated to a specific pattern of internucleosomal DNA degradation, which provided a simple method of identifying and measuring the amount of apoptotic cell death (Arends et al., 1990).

Apoptosis has been suggested as an active, genetically predetermined and higly regulated phenomenon that can be triggered by a variety of environmental stimuli, both pathogenic and physiological. These include genotoxic agents (responsible for DNA damage), absence of survival factors and the specific activation of death receptors (Nagata, 2000). Apoptosis is now widely recognized and accepted as a distinct and important mode of programmed cell death, characterized by cell shrinking, chromatin condensation (pyknosis), DNA and nuclear fragmentation (karyorrhexis). At the final stage of apoptosis, organelles condensate into vesicles and lipid asymmetry is lost, resulting in the exposure of phosphatidylserine. Finally, dying cells fragment into small apoptotic bodies, thus preventing the uncontrolled release of intracellular components to the extracellular milieu, where it could be harmful to the surrounding cells. These critical morphological and biochemical changes are generally common to most processes of apoptosis, no matter how they were initiated.

Apoptosis may be mediated by several molecular pathways which respond to different stimuli and can be triggered by distinct mechanisms. Nevertheless, many of

the molecular regulators of apoptosis are shared among the different pathways that eventually intersect at some of point of the process. The best characterized and most prominent ones are called the extrinsic and intrinsic pathways, and their common regulators include caspases and Bcl-2 family proteins.

Caspases

The main effectors of apoptosis are the highly conserved cysteine-dependent aspartate-directed proteases, globally known as caspases. Over a dozen caspases have been identified in humans and about two-thirds of these have been suggested to function in apoptosis (Earnshaw et al., 1999). Based on their substrate preference, extent of sequence identity and structural similarities, caspases can usually be divided into two different groups: the initiator caspases (that include caspases-2, 8, 9 and 10) and the effector/executioners caspases (including caspases-3, 6 and 7) (Fig. 1).

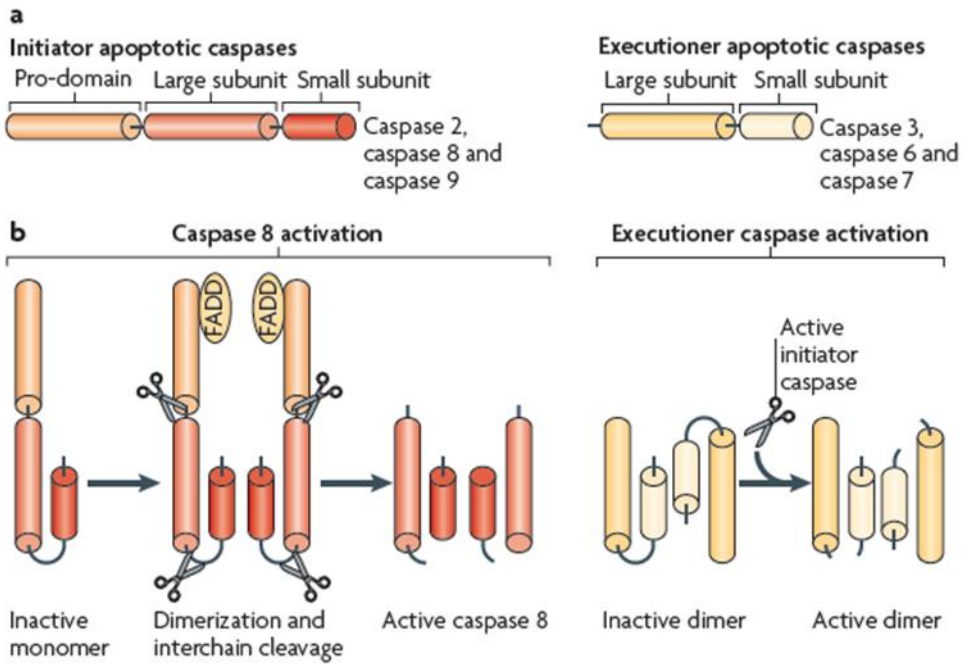


Fig. 1 – Schematic representation of caspase structure and processing. Caspases cleave substrates in a highly specific manner after the Asp residue in short tetrapeptid (X-X-X-Asp) motifs. **a**) Apoptotic caspases can be divided into two classes: initiator and executioner caspases. Initiator caspases are characterized by their long pro-domains, while the effector caspases possess shorter pro-domains. **b**). Caspases are synthesized as catalytically inactive zymogens. The inactive proteins, also called pro-caspases, are then activated by proteolytic cleavage of its pro-form. The smaller fragments then dimerise to form the active enzyme (from: Tait and Green, 2010).

In general, initiator caspases, which act at the apex of the signaling cascade, are characterized by their long pro-domains designated Death Effector Domain (DED, in caspases-8 and -10) and Caspase Recruitment Domain (CARD, in caspases-2 and -9), with more than 90 amino acid residues, while the effector caspases possess shorter pro-domains with only 20-30 residues (Budihardjo et al., 1999; Chowdhury et al., 2008).

These proteins, crucial to the execution of the controlled cell death program, are synthesized, and exist in cells as catalytically inactive zymogens. The inactive proteins, also called pro-caspases, are then activated by proteolytic cleavage of its pro-form, at specific aspartate residues. The smaller fragments then dimerise to form the active enzyme (Cohen, 1997; Shi, 2002). Besides cleaving several proteins, these enzymes can also act upon other caspases activating them and thus initiating a phenomenon known as the cascade of caspase activation (Chowdhury et al., 2008).

Although caspases are not strictly required for the execution of cell death activation they might be necessary for the acquisition of typical morphological markers of apoptosis (Kroemer and Martin, 2005; Kumar, 2007). Once caspases are initially activated, there seems to be an irreversible commitment towards cell death. Regulation of caspases is, therefore, vital to determine the cell's fate (Lamkanfi et al., 2007).

Bcl-2 Proteins Family

Apoptosis is a strictly controlled cellular process with a vast number of regulators. Besides caspases, the Bcl-2 protein family, which includes a set of proteins that can either promote or prevent cell death, plays a major role in the life-or-death decision that determines the fate of a cell (Adams and Cory, 1998, 2007; Chao and Korsmeyer, 1998; Cory and Adams, 2002; Nuñez and Clarke, 1994; Reed, 1994). The contribution of Bcl-2 family proteins to the regulation of apoptosis started to be unveiled when the *BCL2* (B-cell Lymphoma 2) gene product was found to inhibit cell death (McDonnell et al., 1990; Vaux et al., 1988). Since then, several Bcl-2-related proteins were found, all sharing at least one Bcl-2 Homology domain (BH), and classified according to their structure and function (Fig. 2).

Bcl-2, Bcl-x_L, Bcl-w, A1 and Mcl1 have four BH domains and constitute the anti-apoptotic members of the family. The pro-apoptotic members can be separated into multidomain promoters of cell death or effector proteins (Bax, Bak and possible Bok), and the BH3-only proteins that can act as direct activators (Bid, Bim and Puma), or

sensitizer/de-repressor proteins (Bad, Bik, Bmf, Hrk and Noxa). The effector proteins were initially described to have only three BH domains (BH1-3), but recent data revealed a BH4 domain (Kvansakul et al., 2008). The BH3-only direct activators interact with both the anti-apoptotic and the effector proteins, while the sensitizer/de-repressor members interact with the anti-apoptotic Bcl-2 repertoire exclusively (Chipuk et al., 2010). These proteins are responsible for the regulation of Mitochondria Outer Membrane Permeabilization (MOMP), an event that enables the release of apoptogenic factors, such as cytochrome *c* (cyt *c*), from mitochondria into the cytosol, eventually leading to the activation of caspases (Jürgensmeier et al., 1998; Kluck et al., 1997). The ability of Bcl-2 family proteins to form homo- or heterodimers suggests that there is a neutralizing competition between pro- and anti-apoptotic members, so that the ratio between the two subsets will determine the cells fate (Gross et al., 1999). These proteins are also subjected to posttranslational modifications that affect their function in promoting either death or survival of the cell. Phosphorylation/dephosphorylation by different kinases/phosphatases are among the regulation mechanisms of Bcl-2 family members through posttranslational modifications (for a review see Silva et al., 2011).

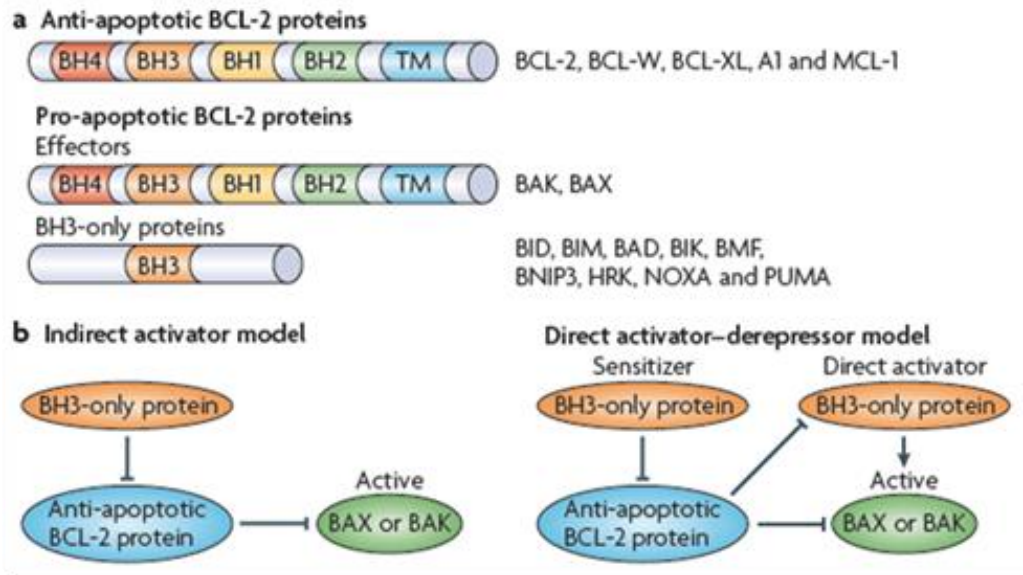


Fig. 2 – Schematic representation of the Bcl-2 family of proteins. **a)** The Bcl-2 family of proteins is divided into three groups based on their BH domain organization. The anti-apoptotic members (Bcl-2, Bcl-x_L, Bcl-w, A1 and Mcl1) have four BH. The pro-apoptotic members can be separated into effector proteins (Bax and Bak), which also contain four BH domains, and BH3-only proteins. The BH3-only proteins can act as direct activators (Bid, Bim and Puma), or sensitizer/de-repressor proteins (Bad, Bik, Bmf, Hrk and Noxa). **b)** Two models for Bax/Bak activation have been proposed. The indirect activator model consideres that competitive interactions of BH3-only with the anti-apoptotic Bcl-2 family members

disrupt the association of these proteins with Bax/Bak, which are releasing as activated forms. In the direct activator-derepressor model, Bax and Bak are activated via direct interactions with a subset of BH3-only proteins termed direct activators. Bcl-2 anti-apoptótic members may inhibit the action of activated Bax/Bak or sequester activating BH3-only proteins. A second subset of BH3-only proteins, named sensitizors, interacts exclusively with the anti-apoptotic Bcl-2 repertoire to neutralize it. (adapted from: Tait and Green, 2010).

Reactive oxygen species in Apoptosis

Over the past decades, Reactive Oxigen Species (ROS) have become recognized as common event in virtually all scenarios of PCD (Simon et al., 2000). Generation of oxygen derived free radicals such as superoxide, hydrogen peroxide as well as of other ROS, results from the capacity of molecular oxygen to readily accept electrons from other molecules. Most of these species are only moderately reactive with other biological molecules, but they can originate hydroxyl radicals, which are highly reactive and responsible for damages to DNA, lipids and proteins (Davies and Goldberg, 1987; Halliwell and Gutteridge, 1990; Schraufstätter et al., 1988). At low/physiological concentrations, ROS can act as chemical messengers mediating signaling pathways and transcriptional regulation (Huang and Philbert, 1996; Morel and Barouki, 1999; Thannickal and Fanburg, 2000), or provide a defense mechanism that can be used by cells of the immune system to destroy pathogens (Babior, 1984; Chanock et al., 1994; Morel et al., 1991). In excessive concentrations, however, ROS overcome the capacity of the cell's anti-oxidant defense mechanisms and induces oxidative stress, which might eventually lead to cell death (Buttke and Sandstrom, 1994). Several studies show that antioxidant agents and the over-activation of cellular defense mechanisms responsible for the elimination of ROS are actually capable of delaying or even preventing apoptosis (Ferrari et al., 1995; Verhaegen et al., 1995; Watson et al., 1997). Moreover, the inhibitory action of some anti-apoptotic proteins, such as Bcl-2, is partially associated with protection against oxidative stress (Hockenbery et al., 1993). The finding of active forms of cell death in plants and yeast that also involve ROS suggests the existence of an ancestral redox-sensitive death signaling pathway that is conserved throughout the eukaryotic domain (Levine et al., 1994; Madeo et al., 1999).

2.1.2. Apoptotic Pathways

The cascade of events leading to apoptosis can be sub-divided in activation and execution phase. During the activation phase, multiple signaling pathways lead to the central control of the cell death machinery and activate it. This is followed by the execution stage, in which the activated machinery acts on multiple cellular targets eventually promoting the destruction of the cell (Elmore, 2007). Currently, two well-understood mechanisms of apoptosis activation are documented: the extrinsic and the intrinsic apoptotic pathway (Fig. 3).

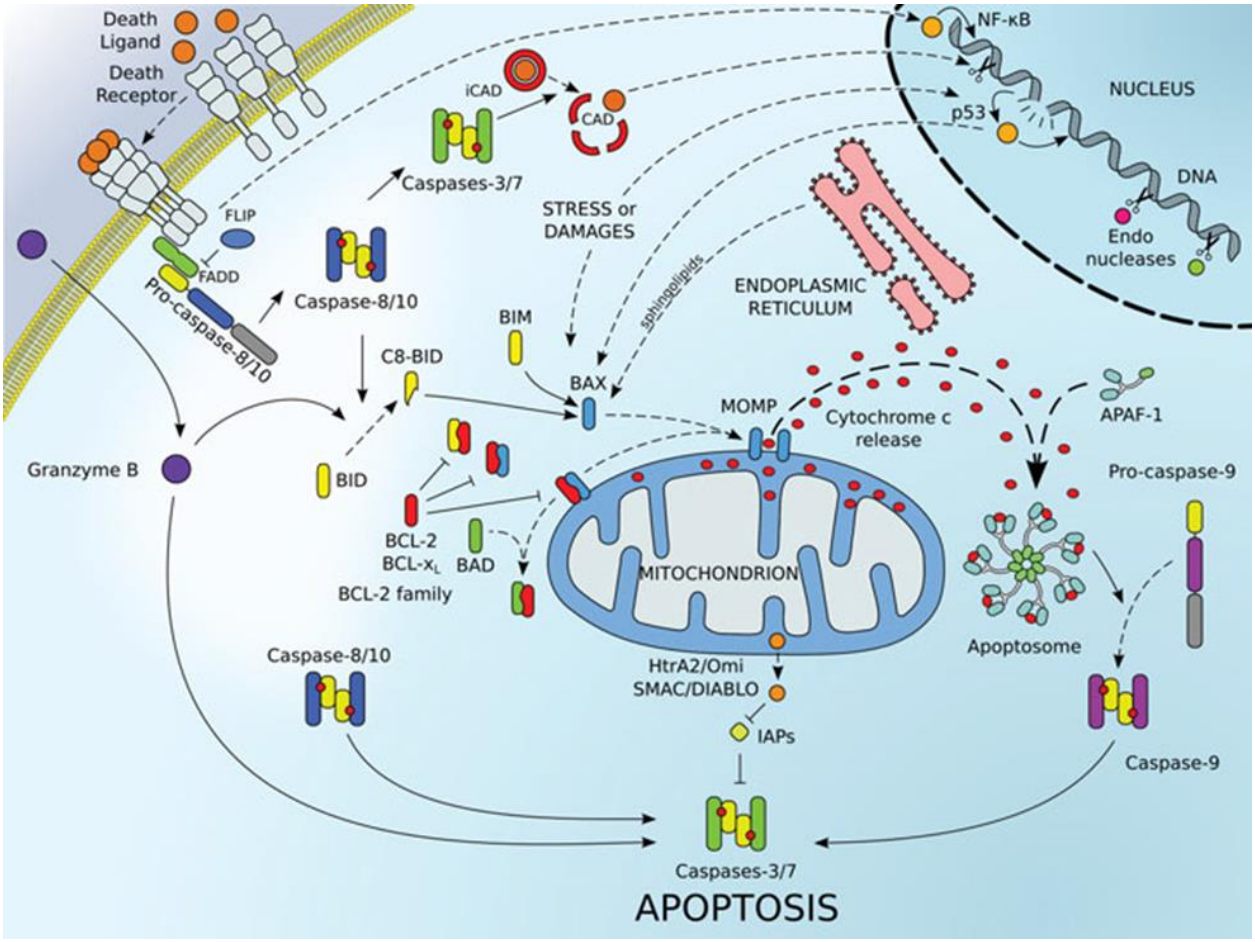


Fig. 3 – The major signaling pathways leading to cellular apoptosis in mammalians: extrinsic and intrinsic pathways. The extrinsic pathway is initiated through the stimulation of the death receptors. Association between the death receptor, the adaptor protein and the pro-caspase-8 forms the DISC, responsible for caspase-8 activation, and subsequent cascade of caspase activation. The intrinsic pathway is activated by internal stimuli and depends on MOMP. MOMP facilitates the release of pro-apoptotic proteins to the cytoplasm including cytochrome *c* that combines with Apaf-1 and pro-caspase 9 to form the apoptosome. Activated caspase-9 initiates the activation of other caspases. (image from Renault and Chipuk, 2013)

Both pathways converge on the same terminal, or execution phase (Hengartner, 2000). This final phase of apoptosis begins when the execution caspases are activated. Executiner caspases (-3, -6 and -7) activate a cytoplasmic endonuclease, which degrades nuclear material, as well as proteases that degrade the nuclear and cytoskeletal proteins, being responsible for the typical morphological and biochemical changes observed in apoptotic cells (Slee et al., 2001).

Extrinsic Pathway

The extrinsic pathway, also called death membrane receptor pathway, depends of transmembrane receptor-mediated interactions. This pathway involves death receptors that are members of the Tumor Necrosis Factor (TNF) receptor gene superfamily (Locksley et al., 2001), namely the death receptors Fas (also called Apo-1 or CD95), Tumor-Necrosis Factor Receptor (TNF-R) and TNF-Related Apoptosis-Inducing Ligand (TRAIL). The death receptors are activated by specific extracellular ligands that promote clustering of the receptors (Sartorius et al., 2001). The signal transduction into the cell is mediated by the cytoplasmic fraction of the death receptors, which contains two copies of a conserved domain called the Death Domain – DD. The receptors then recruit adaptor proteins such as Fas-Associated Death Domain protein (FADD) and TNF-R-Associated Death Domain protein (TRADD), which in turn also have two interaction domains: a DD and a Death Effector Domain (DED) (Chinnaiyan et al., 1995; Hsu et al., 1995). These proteins bind to the death receptors by interactions between the DDs, and to the pro-caspases via interactions between the DEDs. Association between the extracellular ligand, the death receptor, adaptor protein and the pro-caspase forms a complex called the Death Inducer Signaling Complex – DISC (Kischkel et al., 1995). The concentration of pro-caspase-8 in the DISC results in its cleavage and subsequent activation. Once active, caspase-8 initiates the cascade of caspase activation by processing caspases-3, -6 and -7, which will act upon different substrates (Chowdhury et al., 2008; Thorburn, 2004). The death receptors can be inhibited by a protein called cellular FLICE (FADD-Like IL-1β-Converting Enzyme)-inhibitory protein, which binds to FADD and caspase-8, rendering them ineffective (Krueger et al., 2001).

Alternatively, the extrinsic apoptotic pathway can be mediated by dependence receptors, such as the DCC and the UNC5B, which trigger the apoptotic signal in the

absence of their ligand, netrin-1. When DCC is activated, the pro-apoptotic signaling proceeds through the assembly of a DRAL- and TUCAN- (or NLRP1-) platform that binds and activates caspases-9 (Mille et al., 2009). Activation of UNC5B, triggers the apoptotic signal via PP2A-mediated dephosphorylation of DAP kinase, which can direct activation of executioner caspases or favor MOMP (Guenebeaud et al., 2010).

Intrinsic Pathway

The intrinsic pathway, also called mitochondrial pathway, initiates within the cell and is triggered by a wide variety of intracellular perturbations such as DNA damage (caused by radiation, toxins, hypoxia, hyperthermia, viral infections and free radicals), oxidative stress, cytosolic calcium (Ca^{2+}) overload or endoplasmic reticulum (ER) stress (Kroemer et al., 2007). Independently of the pathways responsible for sensing this stresses, all signals converge to the mitochondria and trigger MOMP, an event required for the activation of the intrinsic apoptotic pathway (Tait and Green, 2010). The several mechanisms underlying MOMP will be discussed in a different section. The MOMP, and consequent loss of the mitochondrial membrane potential, facilitates the release of several pro-apoptotic proteins into the cytoplasm, such as cytochrome *c* (cyt *c*), Apoptosis-Inducing Factor (AIF), Endonuclease G (Endo G), Smac/Diablo (Second Mitochondria-derived Activator of Caspases/Direct Inhibitor of Apoptosis Protein (IAP)-Binding protein with Low Pi) and High Temperature Requirement protein A2 (HtrA2/Omi) (Cai et al., 1998; Du et al., 2000; Garrido et al., 2006; van Loo et al., 2002).

After being released from the mitochondria, cyt *c* combines with Apoptotic protease activating factor-1 (Apaf-1) and deoxyadenosine triphosphate (dATP) to form a protein complex known as the apoptosome. This complex, in turn, activates caspase-9 thus initiating the cascade of caspase activation and subsequent cellular destruction (Acehan et al., 2002). Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting the activity of Inhibitors of Apoptosis Proteins (IAPs), which block the activity of executing caspases. (van Loo et al., 2002; Schimmer, 2004). AIF and Endo G, on the other hand, act in a caspase-independent manner and are translocated to the nucleus promoting an extensive DNA degradation (Enari et al., 1998; Joza et al., 2001; van Loo et al., 2001).

Apart from the release of pro-apoptotic factors, mitochondria membrane depolarization and its permeabiliation also induce the loss of cellular homeostasis: ATP synthesis stops, redox molecules such as NADH, NADPH and glutathione are oxidized, the expression of stress response genes is activated and production of ROS increases (Fleury et al., 2002; Kroemer et al., 2007).

2.2. Other Forms of Cell Death

For several years, apoptosis was wrongly assumed as synonymous for PCD. However, it must be kept in mind that although apoptosis is an abundant form of cell death observed in metazoans, and surely the best characterized one, dying cells may follow other forms of PCD, even during development (Chautan et al., 1999). Alternative pathways of cellular destruction include programmed necrosis, autophagic cell death and mitotic catastrophe as the most morphologically distinctive processes. Yet, unlike apoptosis these other forms of PCD are still difficult to distinguish unambiguously and remain poorly characterized (Galluzzi et al., 2007).

The term "autophagic cell death" has been widely used to define scenarios of cell death displaying a massive cytoplasmic vacuolization, which often indicates increased autophagy (Galluzzi et al., 2007). However, autophagic cell death is far from being a consensual terminology for this particular process. On the basis of this conflict resides the generalization of the term 'autophagic cell death' to imply that autophagy actually executes the cell demise, when in most scenarios it refers to a cell death program accompanied by autophagy (Kroemer and Levine, 2008). In fact, autophagy has been implicated as a mediator of cell death during the development of *Drosophila melanogaster* (Berry and Baehrecke, 2007; Denton et al., 2009), and considered responsible for the death of certain cancer cells in response to chemotherapeutic agents (Grandér et al., 2009), particularly when the apoptotic process is genetically compromised (Fazi et al., 2008). Nonetheless, overwhelming evidence suggests that, above all, autophagy constitutes a cytoprotective response activated by dying cells in the attempt to cope with stress, and its inhibition can actually accelerate cell death (Boya et al., 2005; Moreau et al., 2011). In summary, autophagic cell death refers only to a cell death process mediated by autophagy that can be prevented by the chemical or genetic inhibition of the autophagic machinery (Galluzzi et al., 2012).

Until recently, “Necrosis” was the term used to define a merely accidental cell death mechanism that was mostly recognized by the absence of typical morphological markers of apoptosis or autophagic cell death. Necrotic cells exhibit an increase in cell volume (oncosis) that eventually leads to the rupture of the plasma membrane and a disorganized dismantling of swollen organelles (Galluzzi et al., 2007). In mammalian biology, necrosis is considered a harmful process associated to the pathological loss of cells and the promotion of local inflammation (Vakkila and Lotze, 2004). Importantly, the inhibition of pathways responsible for apoptosis or autophagy has the ability to produce a necrotic phenotype from conditions that would otherwise result in apoptotic or autophagic cell death, respectively (Degenhardt et al., 2006; Kroemer and Martin, 2005). This simple definition of necrosis was radically changed when the receptor interacting proteins 1 and 3 (Rip1 and Rip3) were found to be required for the execution of necrosis, with the recognition that this form of cell death can occur in a regulated manner (He et al., 2009; Hitomi et al., 2008; Zhang et al., 2001). The discovery of biochemical pathways involved in the regulation of necrosis brought a new interest in this not-so-accidental form of cellular demise, and new concepts such as “Regulated Necrosis” or “Necroptosis” were introduced (Christofferson and Yuan, 2010; Vandenabeele et al., 2010).

The term “Mitotic Catastrophe” refers to a special case of cell death triggered by aberrant mitosis and occurring either during or shortly after mitosis. It is characterized by micro- and multi-nucleation events that precede cell death, and assumed to result from a combination of defects in cell cycle checkpoints and cellular damage (Castedo et al., 2004; Vakifahmetoglu et al., 2008a). However, the classification of mitotic catastrophe as an authentic cell death program is somewhat controversial, particularly since apoptotic and necrotic traits have also been detected in cells exhibiting multi-nucleation (Castedo et al., 2006; Vakifahmetoglu et al., 2008b), and mitotic aberrations were associated to cell senescence (Eom et al., 2005; Yun et al., 2009). Presently, mitotic catastrophe is considered an oncosuppressive mechanism initiated by perturbations of the mitotic apparatus, during the M phase of the cell cycle and paralleled by some degree of mitotic arrest, eventually triggering cell death or senescence (Galluzzi et al., 2012).

On the latest communication from the Nomenclature Committee for Cell Death (Galluzzi et al., 2012) it was established a biochemical classification for the different mechanisms of cell death, including some less-known and less-characterized forms that

await further investigations to determine if such processes constitute a truly independent form or a particular case of the better-known cell death mechanisms. The different modes of PCD mentioned on this communication are summarized in table 1.

Table 1 - Classification of the diferente modes of PCD according to their main biochemical features.

Mode of cell death	Cell death subroutine	Main biochemical features
Caspase-dependent intrinsic apoptosis	Apoptosis	MOMP Irreversible ΔΨm dissipation
Caspase-independent intrinsic apoptosis		Release of IMS proteins Respiratory chain inhibition
Extrinsic apoptosis by death receptors		Death receptor signaling Caspase-8 (-10) activation BID cleavage and MOMP (in type II cells) Caspase-3 (-6,-7) activation
Extrinsic apoptosis by dependence receptors		Dependence receptor signaling PP2A activation DAPK1 activation Caspase-9 activation Caspase-3 (-6,-7) activation
Necroptosis	Necrosis	Death receptor signaling Caspase inhibition RIP1 and/or RIP3 activation
Autophagic cell death	Autophagy	MAP1LC3 lipidation SQSTM1 degradation
Mitotic catastrophe	Mitotic catastrophe	Caspase-2 activation (in some instances) TP53 or TP73 activation (in some instances) Mitotic arrest
Anoikis	Intrinsic Apoptosis (?)	Downregulation of EGFR Inhibition of ERK1 signaling Lack of b1-integrin engagement Overexpression of BIM Caspase-3 (-6,-7) activation
Cornification	(?)	Activation of transglutaminases Caspase-14 activation
Entosis	(?)	RHO activation ROCK1 activation
Netosis	Autophagic cell death/ Regulated Necrosis (?)	Caspase inhibition NADPH oxidase activation NET release (in some instances)
Parthanatos	Regulated Necrosis (?)	PARP1-mediated PAR accumulation Irreversible ΔΨm dissipation ATP and NADH depletion PAR binding to AIF and AIF nuclear translocation
Pyroptosis	Intrinsic Apoptosis/ Regulated Necrosis (?)	Caspase-1 activation Caspase-7 activation Secretion of IL-1b and IL-18

Classification of different modalities of PCD according to their biochemical features, and predictede cell death subroutine. (?) = predicted or undefined. ATG, autophagy; BCN1, beclin 1; Dcm, mitochondrial transmembrane potential; CrmA, cytokine response modifier A; DAPK1, death-associated protein kinase 1; EGFR, epidermal growth factor receptor; ERK1, extracellular-regulated kinase 1; IL, interleukin; MAP1LC3, microtubule-associated protein 1 light chain 3; MOMP, mitochondrial outer membrane permeabilization; NET, neutrophil extracellular trap; PAD4, peptidylarginine deiminase 4; PAR, poly(ADP-ribose); PARP1, poly(ADP-ribose) polymerase 1; PP2A, protein phosphatase 2A; ROCK1, RHO-associated, coiled-coil containing protein kinase 1; SQSTM1, sequestosome 1; TG, transglutaminase; Z-VAD-fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Z-YVAD-fmk, N-benzyloxycarbonyl-Tyr-Val-Ala-DLAsp-fluoromethylketone. Adapted from Galluzzi et al., 2012; "Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012".

3. Yeast Cell Death

Until recently, apoptosis-like cell death was considered a metazoan-exclusive process, and the hypothesis of unicellular organisms, such as yeasts, committing a suicide program was considered illogical. Supporting this idea were the facts that, by that time, no homologues of the common mammalian apoptosis regulators were found in the yeast genome, and the difficulty to ascertain a physiological role to the cellular suicide in a unicellular organism. In the last two decades, however, it was found that cell death programs are not restricted to multicellular organisms, and can be observed in unicellular organisms such as the yeast *Saccharomyces cerevisiae.* The first evidence suggesting the presence of endogenous programmed cell death in yeast was obtained by Madeo and his coworkers, who described a cell cycle mutant cdc48^{S565G} of *S. cerevisiae* exhibiting a cell death process that displays typical characteristics of apoptosis (Madeo et al., 1997).

As aforementioned the physiological role of an apoptosis-like program in a unicellular organism was initially questioned by the lack of obvious reasons for a single-cell organism to commit suicide. However, yeast populations should not be interpreted just as a group of partitioned unicellular organisms that do not communicate among each other, but rather as a multicellular community of interacting individuals. In fact, several microorganisms cluster together, forming multicellular communities like colonies or biofilms (Váchová and Palková, 2005). A new concept now suggests that the sacrifice of individuals from a population of a unicellular organism can be advantageous for the whole population. Thus, it was proposed that a suicide-program in unicellular organisms serves an altruistic purpose, whereby unfitted, damaged or old cells self-sacrifice for the greater benefit of the population (Büttner et al., 2006; Fröhlich and Madeo, 2000).

As for metazoans, yeast cells undergoing an apoptotic-like cell death show characteristic morphological and biochemical markers such as: DNA fragmentation, chromatin condensation, exposure of phosphatidylserine on the outer leaflet of the plasma membrane (Ludovico et al., 2001; Madeo et al., 1997), mitochondrial fragmentation (Fannjiang et al., 2004) and cyt *c* release from mitochondria (Ludovico et al., 2002). The observation of an apoptotic-like program in yeast brought along the findings of several yeast orthologues of crucial apoptotic regulators, including caspases

(Madeo et al., 2002), AIF (Wissing et al., 2004), Omi/HtrA2 (Fahrenkrog et al., 2004), IAP (Walter et al., 2006) and Endo G (Büttner et al., 2007). Like cyt *c*, yeast Aif1p and Nuc1p, orthologues of mammalian AIF and Endo G, respectively, are localized in the mitochondria, and are released upon death stimuli. The yeast Nma111p, orthologue of Omi/HtrA2, is a serine dependent protease that, unlike its mammalian counterpart, is predominantly localized in the nucleus where it remains after a death stimulus (Fahrenkrog et al., 2004). A yeast caspase-related protease, Yca1p, has also been described as a mediator of yeast cell death, and its requirement for the death process depends on the nature of the insult (Liang et al., 2008; Mazzoni and Falcone, 2008). Depending on the experimental conditions, Yca1p may act both upstream, in a cyt *c*-independent manner (Mazzoni et al., 2005), and downstream of mitochondria, with a partially dependence on cyt *c* (Silva et al., 2005). Yeast Pep4p, an aspartate protease orthologue of mammalian cathepsin D, translocates from the vacuole into the cytosol during cell death induced by hydrogen peroxide (Mason et al., 2005), actin cytoskeleton stabilization (Gourlay and Ayscough, 2006) and acetic acid, being involved in mitochondrial degradation (Pereira et al., 2010). Other regulators such as Apaf-1 and of most members of the Bcl-2 family proteins, seem to be absent in yeast. Recently, a yeast BH3-only protein, the Ybh3p, was identified in yeast. This protein is translocated to the mitochondria, after a lethal stimulus, were it seems capable of mediating the mitochondrial pathway of cell death (Büttner et al., 2011).

Several different stimuli were found to induce an apoptotic-like cell death in yeast. Defects in several cellular processes such as N-glycosylation, chromatid cohesion, mRNA stability, and ubiquitination can trigger this form of cell death in yeast, as do DNA damage and replication failure (Carmona-Gutierrez et al., 2010). The apoptotic-like cell death program can be found during both replicative (Laun et al., 2001) and chronological (Fabrizio et al., 2004; Herker et al., 2004) aging, mating and pheromone exposure in the absence of the conjugating partner (Madeo and Fröhlich, 2002) and also occurs during the development of colonies on solid media (Váchová and Palková, 2005). Exogenous triggers in the form of chemical or physical stress and other natural triggers were also reported to induce an apoptotic-like cell death. These include H_2O_2 and acetic acid, the most common exogenous triggers used to induce an apoptotic-like yeast cell death (Ludovico et al., 2001; Madeo et al., 1999), ethanol, ceramide, hypochlorous acid, high salt concentrations, heavy metals, UV radiation, heat stress, and several pharmacological agents. Compounds, which normally constitute nutrients or

oligo-elements such as glucose, sorbitol, copper, manganese, and iron, can also trigger yeast cell death with apoptotic features when applied at toxic concentrations. The apoptotic-like cell death program may also be triggered by toxins from either non-clonal enemy strains or by higher eukaryotes in their defense against pathogenic fungi (reviewed in (Carmona-Gutierrez et al., 2010).

The human pro-apoptotic regulator Bax has been found to induce mortality in yeast cells (Greenhalf et al., 1996). Indeed, heterologous expression of the human pro-apoptotic Bax causes growth arrest and cell death in *S. cerevisiae*, accompanied by increased ROS production, and mitochondrial membrane permeabilization with subsequent cyt *c* release (Greenhalf et al., 1996; Kissová et al., 2006; Ligr et al., 1998; Polcic and Forte, 2003; Priault et al., 2003a, 2003b; Xu et al., 1999). Although these could be considered typical features of an apoptotic program, a study by Kissova and colleagues indicates that autophagy, and not an apoptotic-like cell death, is activated in Bax induced yeast cell death (Kissová et al., 2006). The contradictory observations regarding the mode of Bax-induced cell death in yeast may result from the different experimental conditions used. Importantly, certain physiological aspects of the functioning of Bcl-2 family proteins seem to be reproducible in yeast, which has been significantly used as a "cleaner" model to study mechanistic aspects of mammalian apoptotic regulators (Silva et al., 2011).

3.1. Acetic Acid in Yeast Cell Death

Acetic acid (systematically named ethanoic acid; CH_3COOH) is a weak organic acid commonly used in the food industry as a natural preservative. It can be formed as a byproduct of alcoholic fermentation by *S. cerevisiae* or as a product of the metabolism of acetic and lactic acid bacteria. Above a given concentration, acetic acid affects the fermentative performance of yeasts, and elevated concentrations induce yeast cell death. Its impact on yeast physiology is, therefore, relevant not only for cell research but also from a biotechnological perspective.

Acetic acid entry into the cells depends on the extracellular pH and growth conditions according to glucose regulation of its transporters. The anionic form is transported by an active transport process that involves a monocarboxylate permease encoded by *JEN1* (Casal et al., 1999), or a more specific acetate-proton symporter,

Ady2p (Paiva et al., 2004). In glucose-repressed yeast cells and at low pH, acetic acid is mainly in its undissociated form (pKa is 4.75), and it enters the cell essentially by simple diffusion (Casal et al., 1996) and through the aquaglyceroporin Fps1p (Mollapour and Piper, 2007). Fps1p is a channel that has an important role in the control of acetic acid transport across the plasma membrane, and is regulated by the high osmolarity glyecerol (HOG) mitogen-activated protein kinase (MAPK), Hog1p. In the presence of acetic acid, Hog1p is activated and phosphorylates Fps1p, targeting this channel for degradation in the vacuole. This adaptive response decreases the entry of acetic acid into the cell, protecting yeast from its toxic effects (Mollapour and Piper, 2007). After entering the cells in the undissociated form, and since the intracellular pH is usually close to neutrality, acetic acid dissociates into acetate anion and H^+, which leads to intracellular acidification, anion accumulation (if the extracellular pH is lower than the intracellular one) (Casal et al., 1996), and inhibition of cellular metabolic activity, namely, fermentation (Pampulha and Loureiro-Dias, 2000). Studies on enzymatic activities showed that enolase is the glycolitic enzyme most affected by acetic acid, leading to alteration of glycolysis (Pampulha and Loureiro-Dias, 2000). High concentrations of acetic acid can also compromise the viability of yeast cells (Pinto et al., 1989), eventually resulting in yeast cell death (Ludovico et al., 2001).

In 2001, Ludovico and colleagues characterized the acetic acid-induced cell death in *S. cerevisiae* and identified different processes of cellular demise that were dependent on the concentration of acid used in those experiments. Treatment with high concentrations of acetic acid was followed by ultrastructural changes typical of necrosis, while lower concentrations of acid produced a cell death phenotype sharing some features with mammalian apoptosis (Ludovico et al., 2001). Chromatin condensation along the nuclear envelope, exposure of phosphatidylserine on the surface of the cytoplasmic membrane and DNA strand breaks were observed during acetic acid induced cell death (Ludovico et al., 2001).

In the last decade, acetic acid has been commonly used as an inducer of apoptosis, in studies that unravel some of the processes and mechanisms behind this type of cell death in yeast. It was demonstrated that yeast undergoes a programmed cell death that can be mediated by mitochondria, when exposed to lethal concentrations of acetic acid. Acetic acid-treated yeast cells present accumulation of ROS, transient hyperpolarization followed by loss of mitochondrial membrane potential, decrease in cytochrome oxidase activity, affecting mitochondrial respiration, and release of cyt *c* (Ludovico et al., 2002).

Acetic acid also induces different ultrastructural mitochondrial alterations in yeast, including mitochondrial swelling, reduction in cristae number and formation of myelinic bodies as well (Ludovico et al., 2003). Apart from cyt *c*, Aif1p was also found to exit mitochondria in response to acetic acid being translocated to the nucleus (Wissing et al., 2004). Other mitochondrial proteins also affect the final cell fate, including those involved in mitochondrial fission/fusion like Fis1p, Dnm1p, Mdv1p (Fannjiang et al., 2004), Ysp2p (Sokolov et al., 2006) Nuc1p, the yeast ortholog of the mammalian Endonuclease G (Büttner et al., 2007), and homologues of the putative components of mammalian permeability transition pore (PTP), the ADP/ATP carrier (AAC) and Por1p (Pereira et al., 2007). Mitochondrial degradation has also been associated to yeast cells undergoing apoptosis ((Fannjiang et al., 2004) in a process that seems to involve the vacuolar protease Pep4p together with the AAC proteins (Pereira et al., 2010).

The metacaspase Yca1p is activated in cells undergoing acetic acid-induced apoptosis in a growth phase dependent manner (Pereira et al., 2007). Nevertheless, in the absence of this Yca1p cells were still able to undergo apoptosis, although at lower rate than wild-type cells, which suggest that a caspase-independent pathway may also exist in yeast (Guaragnella et al., 2006). Besides Yca1p, the protease Kex1p, already known to participate in PCD caused by defective N-glycosylation, was also associated to the active cell death program induced by acetic acid (Hauptmann et al., 2006). The study of the yeast protein expression profile during acetic acid-induced apoptosis allowed the observation of alterations in the levels of proteins directly or indirectly linked with the target of rapamycin (TOR) pathway (Almeida et al., 2009).

In spite of great efforts, many of the players involved in acetic acid induced yeast cell death and the exact mechanisms by which this process is executed remain unknown. A major challenge in the future will be to understand how yeast cells signal and execute death programs and how different molecular components might interact to decide the cell's fate.

4. Mitochondria in Cell Death

Until the final years of the 20th century mitochondria (a term derived from the Greek words “mitos” meaning threads and “chondros” to mean granule (Benda, 1898)) were considered to have one major purpose within cells: energy production. This perspective of purely metabolic mitochondria suffered a dramatic change with the discovery of another crucial biological function of mitochondria: the control of cell death. Progressively, different fields of cell research, including mitochondria bioenergetics and cell death, were revitalized, and our understanding of cellular physiology evolved under the premise that mitochondria are pluripotent organelles controlling life and death of eukaryotic cells.

4.1. Physiology of Mitochondria

Mitochondria are peculiar organelles with distinctive structural and biochemical properties, and frequently designated as the cell’s power plants due to their critical role in the generation of metabolic energy in eukaryotic cells. Indeed, mitochondria are responsible for most of the useful energy derived from the breakdown of carbohydrates and fatty acids, which is converted to ATP by the process of oxidative phosphorylation.

Structurally, mitochondria are defined by a double-membrane system, consisting of an inner (IMM) and an outer mitochondrial membranes (OMM), separated by an intermembrane space (IMS). The inner membrane has a larger surface area and forms numerous hollow flattened or tubular structures designated by the name of cristae and invaginate into the interior of the organelle, called matrix, which contains the mitochondrial genetic system as well as the enzymes responsible for central reactions of oxidative metabolism (Frey and Mannella, 2000). The inner membrane invaginations form a functionally distinct compartment of the IMS: the cristal lumen. Indeed, it is in the cristae membranes that the different complexes from the electron transport chain are found. Accordingly, it is estimated that 85% to 97% of the cyt *c* resides within the cristal lumen, with the remainder localized in the IMS (Bernardi et al., 1999; Scorrano et al., 1999). Cytochrome *c* is electrostatically associated with cytochrome *c* reductase and cytochrome oxidase, and is also associated with the anionic phospholipid cardiolipin, an important component of the IMM with an asymmetrical distribution

across the lipid bilayer. The F_0F_1 ATP synthase is also concentrated in the cristae of the IMM, facing the matrix. The cristae joins the rest of the inner membrane in dynamic structures designated crista junctions (Perkins et al., 1997), that under stress conditions can actually "close" cristae and cut the communication with the remaining IMS (Mannella et al., 2001).

The two membranes from mitochondria have considerably different properties. Under physiological conditions, the IMM is nearly impermeable to solutes and ions, which allows the respiratory chain complexes (I to IV) to build up the proton gradient required for oxidative phosphorylation (Mitchell and Moyle, 1965a, 1965b). The charge imbalance generated by the chemical prtoton gradient across the IMM constitutes the basis of the inner mitochondrial membrane potential ($\Delta\Psi_m$). The movement of protons, back into the matrix, through the F_0F_1 ATP synthase drives the syntheses of ATP from ADP and inorganic phosphate. As a nearly impermeable barrier, the IMM possesses a series of highly selective ion channels and transporters required to ensure the exchange of metabolites between the matrix and cytosol. The energy required for these transports can be provided by the electrochemical gradient. Transient loss of the $\Delta\Psi_m$ may occur in physiological circumstances through the "flickering" of one or several IMM pores, (Zoratti and Szabò, 1995). Long-lasting or permanent $\Delta\Psi_m$ dissipation has major consequences for mitochondrial function and is often associated with cell death (Marchetti et al., 1996).

The OMM, in contrast to its interior partner, is considered freely permeable to solutes and small molecules (< 1 kDa). This leaky behavior of the OMM is generally ascribed to the presence of a very abundant protein, the Voltage-Dependent Anion Channel (VADC), which promotes the flux of metabolites between the cytosol and the IMS. The permeability of the OMM is a tightly regulated process not only in normal "life" but also during cellular death, when different signals converge into the OMM to determine the fate of the cell (Kroemer and Reed, 2000).

The IMM and OMM interact with each other at the contact sites, where the two membranes are closely juxtaposed. This contact sites are dynamic structures that might provide a platform for functional or structural interactions between proteins of the IMM and OMM (Kottke et al., 1988). A fairly known example is the interaction between VDAC, in the OMM, and the Adenine Nucleotide Translocator (ANT) in the IMM, two proteins that can form complexes *in vitro*, and are co-purified with cytosolic hexokinase and the benzodiazepine receptor (Brdiczka et al., 2006; McEnery, 1992). These

interactions at contact sites might facilitate the exchange of metabolites between the matrix and the cytosol, as well as the import of proteins to mitochondria (Adams et al., 1989). Additionally, contact sites were proposed to mediate the process of mitochondria membrane permeabilization during cell death, providing physical support for the formation of a Permeability Transition Pore (Beutner et al., 1996; Crompton et al., 2002).

Mitochondria, crucial organelles for cellular bioenergetics, also contain several crucial regulators of the cell death programs, creating an authentic switch between normal life and a resolute death.

4.2. Mitochondria Membrane Permeabilization

As in normal cellular life, the permeability of the OMM is tightly regulated during the cell death. Mitochondria outer membrane permeabilization (MOMP) is probably the most significant biochemical event in the mitochondrial pathway of apoptosis enabling the release of several apoptotic factors, normally confined to mitochondria within the IMS (Liu et al., 1996). Considered by some as the "point of no return" in the cascade of events that promotes cell death, inappropriate regulation of MOMP has major consequences for health and disease (Green and Reed, 1998).

The biochemical and biophysical mechanisms underlying MOMP are complex, distinct and in some cases debatable, probably involving the coordination of several independent steps (Tait and Green, 2010). In response to intracellular stress signals, pro- and anti-apoptotic signals are generated and converge at mitochondria. When a death signal prevails, the OMM loses its integrity and becomes permeable to soluble IMS proteins, namely the pro-apoptotic proteins cyt *c*, Smac/DIABLO, Omi/HTRA2, AIF and Endo G. Along with the release of mitochondrial proteins, MOMP leads to uncoupling of the respiratory chain, which results in increased ROS production and the dissipation of the $\Delta\Psi_m$, interrupting ATP production and $\Delta\Psi_m$-deriven transport activities (Kroemer et al., 2007). Once MOMP has occurred, caspases are activated in the cytosol, through the association of cyt *c* with Apaf1 and dATP, to form the apoptosome and activate caspases-9, as referred above (Li et al., 1997; Zhou and Ling, 1997). When this pathway is inhibited, a caspases-independent cell death (CIDC) program, which probably involves ROS production, mitochondrial dysfunction and IMS

proteins such as AIF and Endo G, ensures the execution of cell destruction (Chipuk and Green, 2005).

MOMP can be triggered directly at the OMM by the action of pro-apoptotic members of the Bcl-2 family, such as Bax and Bak, which have the ability to form pores (von Ahsen et al., 2000; Kluck et al., 1997). Apart from the contribution of proteinaceous channels involving the Bcl-2 family members, the formation of lipidic pores in OMM may constitute another model for MOMP (Siskind et al., 2005), and recent evidences suggest that these mechanisms may actually collaborate in the permeabilization of mitochondria during cell death (Ganesan et al., 2010) (Fig. 4). Alternatively, MOMP can be triggered at the IMM, as a "secondary" effect of Mitochondria Permeability Transition (MPT); a phenomenon consisting of an abrupt increase in the permeability of the IMM to water and small solutes (Kroemer et al., 2007). The different proposed mechanisms accounting for MOMP are discussed in the following sections.

4.2.1. Bax/Bak-mediated MOMP

As discussed in a previous section, the Bcl-2 family proteins are central regulators of apoptosis in multicellular organisms. Bax and Bak are the direct effectors of apoptosis acting downstream of other Bcl-2 family members to induce MOMP, and cells lacking both proteins fail to undergo MOMP and apoptosis in response to different intrinsic apoptotic stimuli (Wei et al., 2001). The exact molecular mechanisms underlying Bax/Bak mediated permeabilization, however, remain a matter of debate.

In healthy cells, Bax is mostly cytosolic, where it awaits for activation signals that trigger its translocation to mitochondria and subsequent activation (Hsu et al., 1997). Bak, on the other hand, has a constitutive mitochondrial localization. Upon apoptotic stimulation, Bax and Bak can be activated by direct interaction with truncated Bid (tBid) in a process that involves extensive conformational changes, required for both translocation/insertion of Bax and the homo-oligomerization of Bak and Bax (Eskes et al., 2000; Hsu et al., 1997; Wei et al., 2000). These conformational changes are a critical and complex step on the activation of Bak and particularly Bax, whose relocation to mitochondria is required but may not be sufficient to induce its permeabilizing activity (Renault et al., 2012; Westphal et al., 2011). Indeed, the ability of Bax or Bak to oligomerize is apparently associated to the abilty of both proteins to induce MOMP,

suggesting that this event is also required to activate the apoptotic activity of these proteins (Dewson et al., 2008; George et al., 2007).

In a classical model of Bax activation, tBid has been demonstrated to associate with membranes where it interacts with Bax, promoting its insertion and subsequent oligomerization in a coordinated sequence of events that culminate in membrane permeabilization. Anti-apoptotic members such as Bcl-x_L can prevent Bax activation by inhibiting its association with tBid (Lovell et al., 2008). Other BH3-only proteins, such as Bim and Puma can also interact directly with Bax and Bak to promote MOMP, and the absence of these two regulators prevents ER stress-induced Bax/Bak activation (Kim et al., 2009). A model for Bak activation by tBid, proposes that the association between both proteins exposes the BH3 domain of Bak allowing symmetrical Bak dimers to form through reciprocal BH3 domain-interactions. The resulting homodimers of Bak might then interact via their α6 helices, close to the BH1 domain, to form high-order Bak homo-oligomers that promote MOMP (Dewson et al., 2008, 2009) (Fig. 4). An identical process is predicted to mediate the formation of Bax homo-oligomers, which also depends on the BH3 and BH1 domains of Bax (George et al., 2007). The exact number of Bax or Bak molecules that must oligomerize to successfully induce MOMP remains uncertain. The mechanisms by which high-order oligomers of Bax or Bak promote MOMP also remain unclear, although it is believed to depend on the formation of proteinaceous channels or on the destabilization of lipid membranes to form lipidic pores (Fig. 4) (Tait and Green, 2010).

The structural similarities with bacterial pore-forming toxins were an early suggestion that Bax and Bak could insert in OMM and form pores (Muchmore et al., 1996; Petros et al., 2004; Suzuki et al., 2000). In fact, several studies demonstrated that Bcl-2 family proteins are capable of forming pores in artificial membranes, a feature that is not exclusive to the pro-apoptotic members (Antonsson et al., 1997; Schendel et al., 1997). Indeed, Bid and Bax were able to produce membrane openings in reconstituted vesicles in a process that was inhibited by Bcl-x_L and required cardiolipin, thus suggesting that a BH3/Bax/lipid interaction might be enough to permeabilize membranes (Kuwana et al., 2002). Patch clamping experiments on isolated mitochondria, and using tBID to induce MOMP, revealed that Bax or Bak must be present to enable the formation of a cyt *c*-translocating OMM channel (Martinez-Caballero et al., 2009). The stepwise increase in the conductance of this channel, with similar kinetics to MOMP, is consistent with pore assembly by sequential recruitment of

activated Bax or Bax homodimers (Martinez-Caballero et al., 2009). Furthermore, the link between Bax/Bak oligomerization and their ability to promote MOMP (Antonsson et al., 2000; Dejean et al., 2005), supports the hypothesis that Bax/Bak oligomers form proteinaceous pores large enough to allow the release of cyt *c* (≈ 12.5 kDa), but not of a higher molecular weight protein having similar physico-chemical properties, such as hemoglobin tetramer (63 kDa) (Dejean et al., 2005). This evidences support the hypothesis of MOMP mediated by Bax or Bak pores, and a proteinaceous pore implies that Bax/Bak could form a "ring-like" structure fully spanning OMM (or artificial membranes) (Martinez-Caballero et al., 2009).

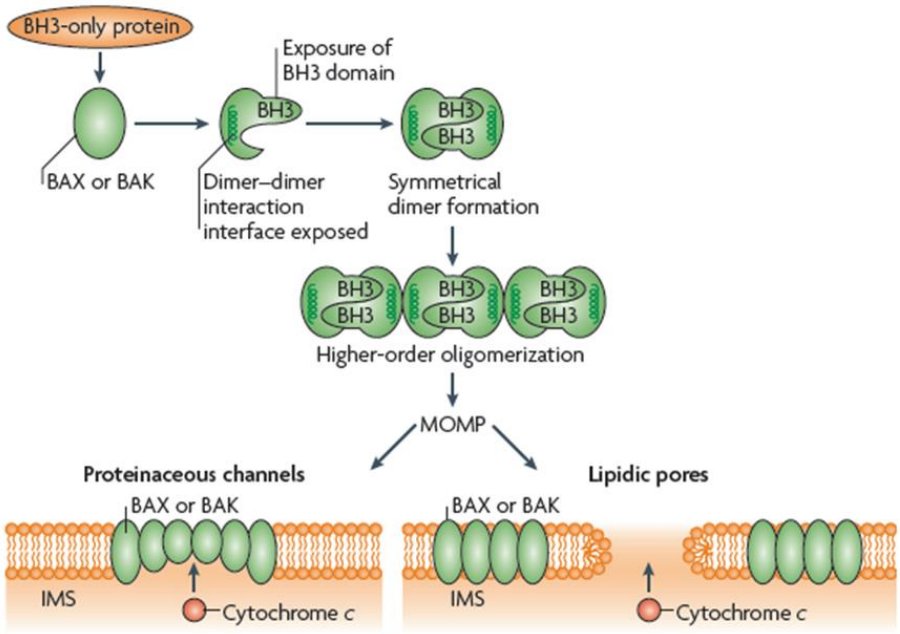


Fig. 4 – Bax/Bak activation and pore formation. Activated BH3-only proteins, such as tBid, bind to Bax/Bak inducing extensive conformational changes that are required for their activation. Exposure of the BH3 domain and hydrophobic cleft of Bax/Bak drives the formation of dimmers between symmetrical Bax/Bak moledules, through BH3 domain-cleft interactions. During activation, a dimer-dimer interaction surface also is exposed, allowing high-order oligomers to form. MOMP might result from the formation of proteinaceous channels by Bax/Bak oligomers (bottom left image), or from the formation of lipidic pores (bottom right image) resulting form lipid membrane destabilization by Bax/Bak oligomers. (image from: Tait and Green, 2010).

An alternative to the model of pores formed by Bax or Bak, is the regulation of existing mitochondrial channels by these proteins. In this model, instead of forming pores by themselves, Bax and Bak would regulate the opening of mitochondrial channels, such as the mitochondrial permeability transition pore complex (which will be

discussed below), to mediate MOMP. This cooperation is supported by electrophysiological studies with purified recombinant Bax and purified ANT or VDAC, two proposed members of the PTP (Green and Kroemer, 2004). Indeed, a Bax-VDAC interaction has been described (Pastorino et al., 2002), and different models supporting a direct collaboration between Bax and VDAC in the control of mitochondrial cyt *c* release were proposed (Banerjee and Ghosh, 2004; Shimizu et al., 1999, 2000). Meanwhile, Bid, but not Bax, has been demonstrated to regulate VDAC channels (Rostovtseva et al., 2004), and evidences obtained from the genetic manipulation of mice clearly rule out a possible contribution of VDAC for Bax-mediated MOMP (Baines et al., 2007). Other studies support a regulatory role of VDAC2 in the action of Bak (Cheng et al., 2003; Roy et al., 2009). Similarly, Bax has been co-purified with ANT suggesting a possible interaction between both proteins that was previously proposed to form a channel that could facilitate cyt *c* release (Brenner et al., 2000; Marzo et al., 1998a). In fact, Bax and ANT can physically interact and efficiently form pores in reconstituted liposomes or planar lipid bilayers (Belzacq et al., 2002). Using the genetic manipulation of mice, once again, it has been demonstrated that inactivation of two ANT isoforms (ANT1 and ANT2) had no significant consequences for cell death in response to various inducers, and ANTs were considered dispensible for mitochondrial cyt *c* release (Kokoszka et al., 2004). Shortly after this particular work, however, a novel isoform of murine ANT was identified (Rodić et al., 2005). The contribution of cyclophilin D (Cyp-D), a proposed element of the PTP, for cell death was also tested. Cyp-D-less cells were found to undergo normal apoptosis with no signifficant impact on cyt *c* release, which constitutes another evidence supporting that Bax does not seem to require the PTP components to induce MOMP (Baines et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Yeast has also been used as a biological model to study the functioning of Bax and its putative collaboration with mitochondrial proteins, providing evidence that Bax is able to release cyt *c* without the collaboration of the PTP or any similar system (Priault et al., 2003b). A work with a cell-free system actually revealed that the only requirements for Bax-induce membrane permeabilization seem to be the activation by Bid and a specific lipid mileu (Kuwana et al., 2002). Instead, two distinct mechanisms leading to cyt *c* release, Bax-dependent and -independent, may participate in specific processes leading to cell death and eventually coexisting in special circumstances (Eskes et al., 1998).

4.2.2. Contribution of Lipidic Pores

An alternative model of MOMP proposes the formation of lipidic pores, catalysed and/or stabilized by Bax/Bak oligomers. In this model, activated Bax or Bak act directly by destabilizing the lipid bilayer structure of the OMM, promoting membrane bending and the eventual formation of pores large enough to allow release of cytochrome *c* from mitochondria (Basañez et al., 1999, 2002; Hardwick and Polster, 2002). Accordingly, Bax-induced pores have been described as large round holes with diameters of 25–100 nm, with smooth edges at least partially framed by lipids (Qian et al., 2008; Schafer et al., 2009).

For a long time, the mitochondria-specific phospholipid cardiolipin (diphosphatidylglycerol) has been a candidate of choice for this model. Indeed, it is able to form inverted micelles structures (hexagonal phase HII) that reorganize the lipid bilayers around contact sites between the outer and inner membranes and change membrane permeability properties (Schug and Gottlieb, 2009). Furthermore, it is well-documented that cardiolipin is absolutely required for the mitochondrial localization of the BH3-only protein tBid (Lutter et al., 2000), which itself favours the oligomerization of Bax (Eskes et al., 2000). However, other experiments showed that cardiolipin is not required for Bax pore-forming activity, and might be more likely involved in the detachment of cytochrome *c* (and other apoptogenic factors) from the outer face of the inner mitochondrial membrane (Gonzalvez et al., 2005a; Kagan et al., 2005). It is noteworthy that the utilization of cardiolipin synthase-less yeast mutants has been very useful for these studies (Gonzalvez et al., 2005b; Iverson et al., 2004).

Experiments performed by the groups of Siskind and Colombini delivered significant support for the role of ceramide-formed channels in Bcl-2 family proteins-induced MOMP. Indeed, in vitro experiments have demonstrated that physiological concentrations of ceramides are capable of forming large stable pores in membranes, an ability that seems to be specific of ceramide since its natural occurring precursor, dihydroceramide (lacking the 4–5 trans double bound present in ceramide), does not present this biochemical feature (Siskind and Colombini, 2000). These ceramide channels increase the permeability of planar lipid bilayers and of the OMM enabling the release of IMS proteins with an estimated molecular weight cut-off of 60 kDa (Siskind et al., 2002). Experiments on isolated mitochondria have demonstrated that cytochrome *c* can pass through these channels. Unrelated proteins of the IMS having a comparable

size, such as adenylate kinase (≈ 17 kDa) are also released, but matricial proteins such as fumarase (≈ 53 kDa) are not. Other apoptogenic factors of the IMS known to be released during apoptosis, such as Smac/Diablo (≈ 53 kDa for the dimer), Omi/HtrA2 (≈ 48 kDa) and endonuclease G (≈ 28 kDa) might potentially pass through ceramide channels. Only AIF (≈ 61 kDa) has a size bigger than the cut-off of these channels. Interestingly, the cut-off of ceramide channels is about the same as the one of Bax channels (≈ 60 kDa). Moreover, it was demonstrated that Bax and ceramide can act synergistically in channel formation, in way that activated Bax would favour the growth of ceramide channels up to a maximal/optimal size determined by the structure of this pro-apoptotic regulator (Ganesan et al., 2010).

Ceramides are definitely molecules of interest, particularly since several different studies have implicated ceramide in mitochondria-mediated apoptosis (Pettus et al., 2002). First, cellular ceramide levels increase prior to the activation of the mitochondrial pathway of apoptosis (Rodriguez-Lafrasse et al., 2001). Second, ceramide has been shown to interact with and inhibit components of the mitochondrial respiratory chain in isolated mitochondria (Gudz et al., 1997), to increase ROS production in whole cells (France-Lanord et al., 1997) and isolated mitochondria (García-Ruiz et al., 1997), to induce mitochondrial depolarization and dysfunction (Hearps et al., 2002), and to facilitate the release of pro-apoptotic proteins (Di Paola et al., 2004; Zhang et al., 2008). Third, the discovery that mitochondria contain the enzymes involved in ceramide synthesis and the observation that several agents such as TNF, UV radiation and Fas increase the levels of ceramide in isolated mitochondria, confirmed that apoptosis occurs via an increase in mitochondrial ceramide levels (Siskind et al., 2005). Together, this evidences support a model of MOMP resulting from lipid channels induced and regulated by the action of Bcl-2 family proteins.

4.2.3. Contribution of the IMM for MOMP

One of the earlier mechanisms proposed to induce MMP is the formation of a mitochondrial pore designated PTP. Permeability transition defines the sudden increase in permeability of the IMM, which allows the passage of solutes and metabolites up to 1.5 kDa, caused by the reversible opening of a voltage-dependent and high-conductance channel (≈ 2.3 nm diameter) (Crompton and Costi, 1988; Crompton et al., 1987a). Opening of the PTP can induce several physiological changes including the dissipation

of $\Delta\Psi_m$, resulting from the loss of IMM impermeability, and a massive swelling of the matrix, due to the osmotic-driven entering of water, which may leads to the rupture of the OMM and release of IMS proteins into the cytosol (Crompton et al., 1999; Halestrap, 1999). Mitochondrial permeability transition (MPT) has been described to occur under conditions of elevated matrix Ca^{2+} concentrations, and is sensitized by oxidative stress, high phosphate (Pi) concentrations and depletion of adenine nucleotides, although it can also be modulated by a series of substances, which change the Ca^{2+} sensitivity of the pore (Bernardi et al., 1999; Crompton and Costi, 1988; Crompton et al., 1987, 1987; Halestrap et al., 2002; Haworth and Hunter, 1979; Hunter and Haworth, 1979) the numerous efforts to define its molecular composition, the structure of the PTP remains indefinable. Several proteins normally participating in different metabolic functions have been proposed as either components or regulators of the PTP. The core components initially ascribed to the mammalian PTP included the ANT, VDAC and Cyp-D (Crompton et al., 1998; Halestrap et al., 1997), all of which are highly conserved among eukaryotic organisms. Other proteins such as the cytosolic hexokinase, the matricial creatine kinase or the OMM TSPO (a.k.a. Bz receptor) were at some point associated to PTP formation (Zamzami et al., 2001). Several models have also been proposed each introducing new elements, such as the inorganic phosphate carrier or the F0F1 ATPase into the history of the PTP (Bernardi, 2013; Halestrap, 2010; Siemen and Ziemer, 2013).

Mitochondrial unselective channels are not exclusive to mammalian cells, but rather widely distributed in many species, exhibiting both similarities and differences in their regulation and physiological role (Uribe-Carvajal et al., 2011). One such example is Yeast Unselective Mitochondrial Channel (YMUC) (Azzolin et al., 2010).

Adenine Nucleotide Translocator – ANT

The ADP/ATP carrier (AAC) is the most abundant member of the Mitochondrial Carrier Family (MCF), which consists of a set of proteins responsible for approximately 20 different transport functions involved in the fluxes of metabolites through the IMM. These mitochondrial carriers are implicated in energy-generating pathways, amino acid synthesis and degradation, and also in mitochondrial DNA replication (Nury et al., 2006). The AAC exchanges cytosolic ADP for ATP produced in mitochondria, and seems to be indispensible to this organelle.

The involvement of ANT in the mitochondrial PTP is based on the effects that different ANT-interacting compounds produce on MPT (Halestrap, 2009). Indeed, PTP opening is stimulated by adenine nucleotide depletion and carboxyatractyloside (CAT), a specific ANT ligand, and inhibited by ATP, ADP and bongkekric acid (BKA), another ligand of the ANT that locks the translocator in a different conformation from that induced by CAT. These compounds act by changing the Ca^{2+} sensitivity of mitochondrial PTP. An interaction between the ANT and Cyp-D was also found (Crompton et al., 1998; Woodfield et al., 1998).

The ANT has also been implicated in MPT as a target for oxidative stress and thiol reagents, two inducers of PTP opening that reduce the ability of adenine nucleotides to inhibit PT (Halestrap et al., 1997). This effect resulted from the cross-linking of Cys160 with Cys 257 of the ANT and could be reproduced by eosin 5-maleimide, which only modifies Cys 160 (McStay et al., 2002). Additionally, the increased matrix Ca^{2+} concentrations can induce the 'c' conformation of the ANT (with the ATP binding site facing the cytsol), suggesting it could contain the Ca^{2+} triggering site of the PTP (Halestrap, 1987; Pebay-Peyroula et al., 2003).

Experiments with reconstituted bovine ANT or the translocator from *Neuspora crassa* demonstrated the ability of these proteins to form non-specific channels induced by Ca^{2+} (Brustovetsky et al., 2002; Halestrap and Brenner, 2003). In the case of NcANT, opened pore probability was increased by oxidative stress at high membrane potential, in a cyclophilin-dependent manner. Studies using mice that were genetically inactivated for two ANT isoforms revealed that mitochondria could still undergo a CsA-sensitive permeability transition which was less sensitive to Ca^{2+} than wild-type mitochondria, and insensitive to ligands of the ANT such as ADP, CAT and BKA (Kokoszka et al., 2004). Another ANT isoform was found shortly after in mice, which could account for the presence of some ANT in the manipulated mice mitochondria (Rodić et al., 2005). Still this would unlikely account for the observed adenine nucleotide-insensitive MPTP opening. Similarly the AAC, a *S. cerevisiae* homologue of mammalian ANT, is not required for yeast mitochondria to undergo MPT (Ballarin and Sorgato, 1995). Such evidences suggested that the ANT is not an indispensable component of the mitochondrial PTP, instead it might contribute with regulatory functions.

Voltage-Dependent Anion Chanel – VDAC

VDAC (also called porin) is a small (≈30 kDa) highly-conserved protein that presumably exists in the OMM of all eukaryotic organisms. Its main function as an OMM channel is to facilitate and regulate the flow of metabolites between the cytosol and the mitochondrial IMS. To do so, VDAC forms a pore with an estimated internal diameter of 2.5 nm.

VDAC was included in the PTP history after being co-purified in a complex with ANT, under specific conditions (McEnery, 1992). Later, VDAC was also found to bind ANT and Cyp-D from detergent-solubilized mitochondria (Crompton et al., 1998). However, genetically manipulated mice mitochondria, lacking the all three isoforms of VDAC, still exhibited MPT thus suggesting that VDACs are not essential components of the PTP. In *S. cerevisiae* the VDAC homologue, Porin1 (Por1p), also seems dispensable for the formation of YMUC (Lohret and Kinnally, 1995), nevertheless the absence of this porin changed the physiological properties of YMUC, suggesting a possible regulatory role (Gutierrez-Aguilar et al., 2007; Lohret and Kinnally, 1995).

Other PTP components/models

The involvement of Cyp-D in the PTP originates from the observation that submicromolar concentrations of the immunosuppressant drug cyclosporine A (CsA) could inhibit MPT, a critical finding for the elucidation of the molecular mechanism of the pore (Crompton et al., 1998). The mitochondrial Cyp-D was subsequently identified as a ≈ 18 kDa nuclear encoded cycliphilin isoform (Connern and Halestrap, 1992; Griffiths and Halestrap, 1991). Several CsA analogues, and other compounds, bind Cyp-D and inhibit PTP opening (Waldmeier et al., 2002). Mitochondria from mice in which Cyp-D was genetically inactivated no longer exhibited a CsA-sensitive PTP, and required a much higher Ca^{2+} loading to undergo permeability transition (Baines et al., 2005; Basso et al., 2005). These findings reveal an important role of Cyp-D in the regulation of MPT, probably by facilitating a Ca^{2+}-triggered conformational change in a membrane protein component of the PTP.

Pi is a long-known inducer of mitochondria swelling (Azzi and Azzone, 1965; Crompton et al., 1988), a process that, more recently, has been proposed to involve the inorganic phosphate carrier (PiC) as the pore's Cyp-D binding component or the channel itself (Alcalá et al., 2007; Leung et al., 2008; Varanyuwatana and Halestrap,

2012). In the model of PiC as a member of the PTP, pore opening would be favored by Ca^{2+} and Cyp-D binding, possibly involving the formation of a heterodimer with the ANT (Leung et al., 2008). Contrasting reports on the ability of Pi to activate or inhibit the PTP, however, originated some skepticism around this model (Basso et al., 2008; Herick et al., 1997).

An alternative and distinct model, proposes that PTP could be formed by the aggregation of misfolded integral membrane proteins damaged by stress, rather than being formed by specific proteins (He and Lemasters, 2002; Kowaltowski et al., 2001). In this model, PTP opening would be blocked by chaperone-like proteins, including CyP-D, modulated by Ca^{2+} and CsA, and occur when the protein aggregates exceeded the available chaperones (He and Lemasters, 2002). The involvement of the ANT and PiC would merely reflect the abundance of these proteins in the IMM and their susceptibility to oxidative damage. Yet, this model does not seem to account for typical features of the PTP such as the absolute Ca^{2+} requirement or the regulation by voltage, pH or adenine nucleotides, as well as a defined diameter.

Recently, a new protein was brought into the picture and the PTP proposed to be constituted by dimers of the F_OF_1 ATP synthase (Giorgio et al., 2013). In fact, Cyp-D is able to bind the lateral stalk of the F_OF_1 ATP synthase in a process that requires Pi and partially inhibits its enzymatic activity. CsA could stimulate this activity suggesting that it blocked Cyp-D binding to the enzyme (Giorgio et al., 2009). Cyp-D is proposed to form an electrostatic interaction with the OSCP subunit of F_OF_1 ATP synthase, more precisely in a region over-lapping with helices 3 and 4 of this component (Giorgio et al., 2013). Importantly this is also the binding site of Bz-423, an inhibitor of the F_OF_1 ATP synthase (Johnson et al., 2005), subsequently found to sensitize PTP opening to Ca^{2+}, an effect that is opposed by Pi-dependent Cyp-D binding to OSCP (Giorgio et al., 2013). Moreover, decreased amounts of OSCP affected the Ca2+ dependence of the pore, suggesting the F_OF_1 ATP synthase as good candidate for PTP component.

4.3. Mitochondrial morphology and cell death

Another possible contribution of mitochondria to cell death may rely on their dynamic behaviour, as mitochondria continually fuse and divide to yield a dynamic interconnected network that spreads throughout the cell. Massive fission of the

mitochondrial network is a frequent event observed in apoptotic cells, with mitochondria changing from a thread organization into small, round and numerous grain-like organelles during apoptosis (reviewed in Youle and Karbowski, 2005). This event can be preceded by an elongation and hyperfusion of mitochondria, a process that is interpreted as a cell defence mechanism (Tondera et al., 2009). The physiological relevance of mitochondrial fragmentation for the apoptotic cell death program, however, is controversial issue (Landes and Martinou, 2011).

Importantly, changes in the dynamics of mitochondria morphology are actually able to modulate cell death. Inhibition of Drp1p and Fis1p, two proteins necessary for mitochondrial fission, is able to delay or even prevent apoptosis (Frank et al., 2001; Lee et al., 2004), while overexpression of Fis1p has been demonstrated to promote cell death (James et al., 2003). Conversely, overexpression of Mfn1 and Mfn2 mitofusins prevents apoptosis (Sugioka et al., 2004) while the loss of Opa1, a dynamin-related mitochondrial GTPase required for mitochondrial fusion, induces fragmentation of mitochondria, cyt *c* release and apoptosis (Olichon et al., 2003). Similar relations between mitochondrial morphology and cell death are also found in *C. elegans* and yeast (Fannjiang et al., 2004; Jagasia et al., 2005).

As for mammals, yeast proteins involved in mitochondria morphology can also modulate cell death. Accordingly, deletion of the fission proteins Dnm1p, homologue of mammalian Drp1p, and Mvd1p delays the fragmentation of the mitochondrial network and promotes cell survival following a death stimulus or in ageing cells (Fannjiang et al., 2004; Scheckhuber et al., 2007). Dnm1p-mediated mitochondrial fragmentation and yeast cell death can be prevented by the human anti-apoptotic proteins Bcl-2 and Bcl-x_L (Fannjiang et al., 2004). Other proteins associated with cell death in yeast can also contribute to mitochondrial morphology. This is the case for Yca1p (yeast caspase 1), a Ca^{2+}-dependent cysteine protease whose deletion has been associated to a decrease in mitochondria fission and increased survival in response to different death stimuli (Mazzoni et al., 2005). Two mitochondrial proteins named yeast suicide protein 1 and 2, Ysp1p and Ysp2p, have also been identified as necessary elements for mitochondrial fission associated to amiodarone-induced cell death in yeast (Pozniakovsky et al., 2005; Sokolov et al., 2006).

5. Sphingolipid Metabolism and Cell Death

5.1. Sphingolipid Metabolism

Sphingolipids are a class of lipids composed typically by an 18-carbon amino alcohol backbone, the sphingosine long-chain base, attached to one long-chain fatty acid and, in most cases, to a polar head group. Modifications in the type of sphingosine long-chain, fatty acid and polar head group are what give rise to the vast family of sphingolipids (Hannun and Obeid, 2011; Ozbayraktar and Ulgen, 2009).

In mammalian cells, the sphingolipid metabolic pathway displays a complex network of reactions resulting in the formation of a multitude of sphingolipids in which ceramide can be considered a metabolic hub, occupying a central position in sphingolipid biosynthesis and catabolism. In fact, sphingolipid metabolism comprises a *de novo* biosynthesis pathway as well as a sphingolipid turnover pathway (Fig 5).

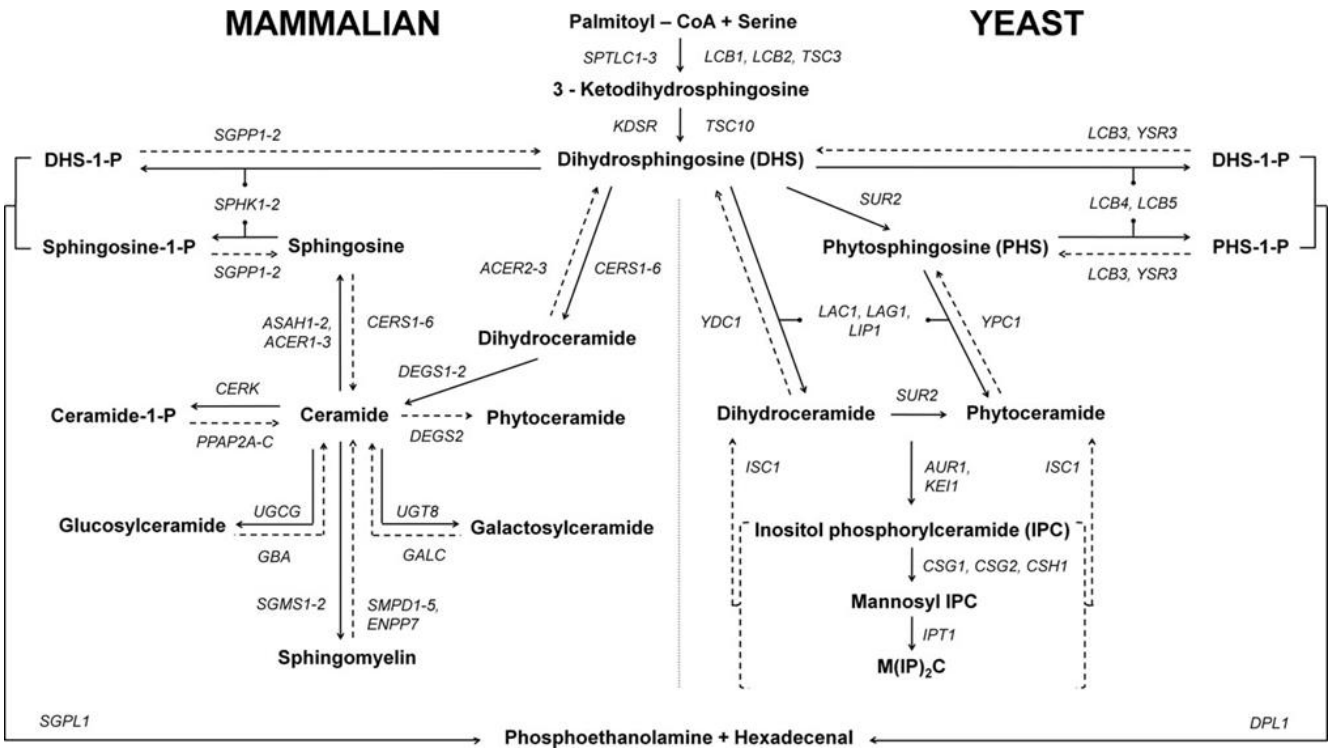


Fig. 5 – Overview of mammalian (left) and yeast (right) sphingolipid metabolism. Ceramide, which occupies a central position in the sphingolipid network, can be generated by *de novo* syntesis or by the turnover of complex sphingolipids. The pathways and constituents underlying the metabolism of sphingolipids are highly conserved in yeast (image from Rego et al., 2013).

Sphingolipid biosynthesis is initiated in the ER with the condensation of serine and palmitoyl-CoA, a reaction catalyzed by a serine palmitoyltransferase, to yield 3-keto-dihydrosphingosine. In turn, a 3-ketodihydrosphingosine reductase reduces 3-keto-dihydrosphingosine to dihydrosphingosine, which is subsequently acylated to dihydroceramide by the action of one of six distinct ceramide synthases. Dihydroceramide can be desaturated by dihydroceramide desaturase to form ceramide. In the following sphingolipid biosynthetic reactions, ceramide is used to generate complex sphingolipids such as glucosylceramide, galactosylceramide and sphingomyelin. Sphingomyelin, the most abundant complex sphingolipid, is produced by transferring a phosphocholine headgroup from phosphatidylcholine to ceramide through the action of sphingomyelin synthase. Ceramide can also be glycosylated by glucosyl or galactosyl ceramide synthases thus generating glucosylceramide and galactosylceramide, respectively, or can be phosphorylated to form ceramide-1-phosphate.

An alternative important source of ceramide is provided by the turnover of complex sphingolipids. The conversion of glucosylceramide and galactosylceramide to ceramide occurs predominantly through hydrolysis of the monosaccharide residues from these lipids by the action of specific hydrolases. The catabolism of sphingomyelin into ceramide is catalyzed by one of the acid, neutral or alkaline shingomyelinases (SMases). The yielded ceramide can be broken down by ceramidases leading to the formation of sphingosine, which in turn can be phosphorylated to sphingosine-1-phosphate. The sphingosine-1-phosphate can be recycled into the biosynthestic pathway or instead it can be irreversibly cleaved to ethanolamine phosphate and hexadecenal, thus exiting the sphingolipids metabolism (for a review see Bartke and Hannun, 2009; Gault et al., 2010; Hannun and Obeid, 2008).

The pathways and constituents underlying the metabolism of sphingolipids are highly conserved in yeast (Fig 5). Phytoceramide and dihydroceramide are assumed to be the yeast counterparts of mammalian ceramides. The steps to the generation of dihydroceramide in yeast are identical to what was described for mammalian cells. Phytoceramide, the yeast ceramide counterpart, is generated by the hydroxylation of dihydrosphingosine to form phytosphingosine, which is subsequently acylated. Phytoceramide and dihydroceramide are first converted into inositol phosphorylceramide, by the transfer of a phosphorylinositol group to ceramide, and then mannosylated to form mannosylinositol phosphorylceramide. The final step in yeast

sphingolipid synthesis is the formation of mannosyldiinositol phosphorylceramide, the most abundant complex lipid in this organism. As in mammalian cells, yeast ceramides can also be produced by the catabolism of the complex sphingolipids. This reaction is catalyzed by the inositol phosphosphingolipid phospholipase C (Isc1p), which has phospholipase-C-type activity and hydrolyses the polar head groups from complex sphingolipids, releasing dihydroceramide and phytoceramide. (for a review see Dickson, 2010; Rego et al., 2013).

5.2. Sphingolipids in cell death signalling

First discovered by Johann Thudichumin in the 1870s, sphingolipids represent the second major class of lipids constituents of membranes in eukaryotes, and for a long time, they were considered to be merely structural components of the biological membranes. However, intensive research on sphingolipids' metabolism and function has revealed that they can act as bioactive molecules in a variety of signaling pathways, controlling diverse cellular processes such as differentiation (Okazaki et al., 1989), senescence (Venable et al., 1995), proliferation (Adam et al., 2002), cell cycle progression (Cowart and Obeid, 2007), mitogenesis, inflammation (Lamour and Chalfant, 2005), migration and angiogenesis (Hannun and Obeid, 2008), necrosis (Hetz et al., 2002), and apoptosis (Obeid et al., 1993). In fact, a vast number of scientific works were published in the last decades connecting the dynamic equilibrium of the levels of sphingolipids, particularly ceramide, sphingosine and sphingosine-1-phosphate, to the processes of PCD, particularly apoptosis. In mammalian cells, the increase of ceramide and sphingosine levels has been shown to induce cell growth arrest and apoptosis, whereas the increase of sphingosine-1-phosphate levels promotes cell proliferation and inhibits apoptosis (Futerman and Hannun, 2004).

The increase of ceramide levels during apoptosis was observed in response to several stimuli such as different chemotherapeutic agents (Quintans et al., 1994), cytokines (TNF-α; IL-1b and FasL) (Dressler et al., 1992; Gulbins et al., 1995; Mathias et al., 1993), environmental stresses (like heat shock, oxidative stress, ionizing and UV radiation and growth factor withdrawal (Chang et al., 1995; Obeid et al., 1993), and other agents of stress, injury or infection (HIV or bacteria) (Grassmé et al., 2003; De Simone et al., 1996). Generation of ceramide after these stimuli can occur either by *de*

novo synthesis (slow accumulation) and/or by complex sphingolipids turnover (fast accumulation) (Zhang et al., 2009). The elevation of ceramide levels, by inducing the endogenous expression of ceramide metabolic enzymes or through direct exogenous delivery, has also been reported to induce apoptosis (Veldman et al., 1998; Zhang et al., 1997). Ceramide-induced apoptosis is a very specific process since dihydroceramide, the natural precursor of ceramide, is not able to induce apoptosis, and the blockage of ceramide generation is able to prevent cell death mediated by pro-apoptotic agents (Bielawska et al., 1993; Obeid et al., 1993) (Fig. 6).

The effects of ceramide in apoptosis appear to be mediated by diverse pathways including: protein kinases and phosphatases, such as ceramide-activated protein kinase (CAPK), protein kinase C (PKC), mitogen-activated protein kinases (MAPKs) and ceramide-activated protein phosphatases (CAPPs), or through the interaction with caspases and mitochondria (Hannun and Obeid, 2008; Mathias et al., 1998; Nikolova-Karakashian and Rozenova, 2010). Indeed, ceramide-mediated cell death seems to depend, at least in part, on the activation of PP1 and PP2A CAPPs, promoting their phosphorylation and consequent inactivation of AKT/PKB, an important element in the regulation of cell survival (Chalfant et al., 1999). It has also been proposed that ceramide regulates PKC ζ (Bourbon et al., 2000; Lozano et al., 1994), Kinase Suppressor of Ras (KSR) (Zhang et al., 1997), JNK pathway (Nica et al., 2008; Verheij et al., 1996; Westwick et al., 1995), and p38 (Willaime et al., 2001) by significantly changing the phosphorylation levels of various key substrates. Another binding target for ceramide is the endossomal acidic aspartate protease cathepsin D, which directly affects the regulation of MOMP by Bcl-2 family proteins (Heinrich et al., 2004).

Ceramides have also direct effects on mitochondrial function, thus interfering with its role in mitochondria-mediated apoptosis. For instance, ceramide inhibits components of the mitochondrial respiratory chain in isolated mitochondria (Gudz et al., 1997), induces mitochondrial dysfunction (Hearps et al., 2002) and the release of pro-apoptotic proteins (Di Paola et al., 2004; Zhang et al., 2008), and increases the production of ROS in isolated mitochondria (García-Ruiz et al., 1997). Additionally, it was also described that cellular ceramide levels increase prior to the activation of the mitochondrial pathway of apoptosis (Rodriguez-Lafrasse et al., 2001), as do the mitochondrial ceramide levels (Siskind et al., 2005).

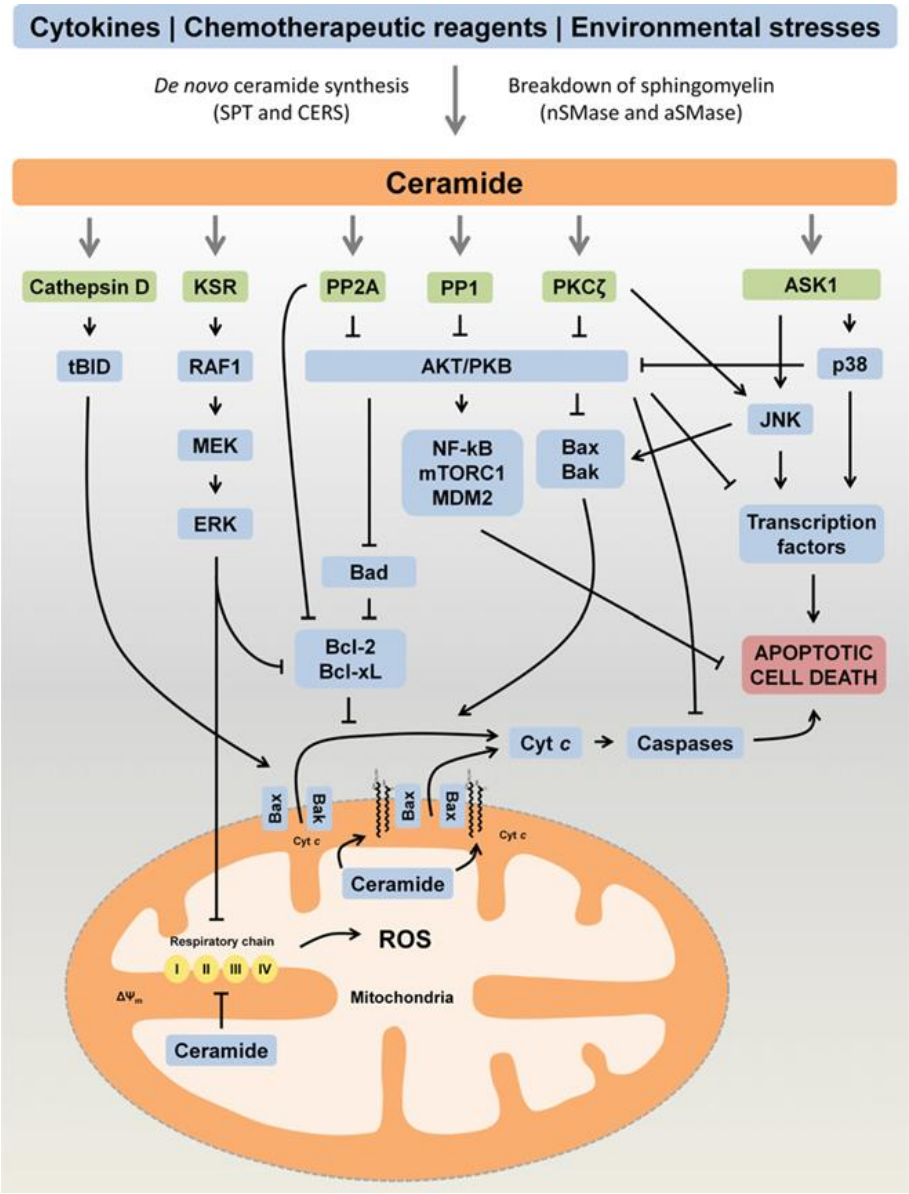


Fig. 6 – Ceramide signaling in Apoptosis. Different stimuli, including environmental stresses, citokines and diverse chemotherapeutic agents, induce ceramide generation through *de novo* synthesis or breakdown of complex sphingolipids. Generated ceramide accumulates and activates a series of proteins responsible for multiple signaling events, thus amplifying the scope of ceramide signaling. Downstream targets of ceramide include: ceramide-activated protein kinases (CAPKs), ceramide-activated protein phosphatases (CAPPs), Protein Kinase C (PKC) and mitogen-activated protein kinases (MAPKs). Ceramide mediated signaling events target several players directly or indirectly involved in the control of apoptosis including Bcl-2 family proteins and mitochondria (image from Rego et al., 2013).

These overwhelming and increasing evidences place sphingolipids in the front line of the cellular battle for life and death. Through signaling events or direct interference in mitochondria, ceramide in particular, and sphingolipids in general might truly have the power to change the balance and decide the cellular fate. The intersection of

sphingolipid metabolism with the Bcl-2 family function, key regulators of MOMP and cell death, will be discussed in the following sections.

5.2.1. Sphingolipids and Bcl-2 family proteins in the regulation of MOMP

The concept of cooperation between ceramides and Bcl-2 family members during MOMP arises from the observation that anti-apoptotic proteins Bcl-2 and Bcl-x_L are capable of preventing ceramide-induced cytochrome *c* release from mitochondria (see for example (Geley et al., 1997; Ghafourifar et al., 1999; Wiesner et al., 1997), although the specific molecular mechanisms regulating these actions remain unclear. It has been shown that Bcl-2 and Bcl-x_L can inhibit ceramide-induced apoptosis by direct action on ceramide generation, reducing or even preventing its accumulation (Sawada et al., 2000; Tepper et al., 1999). Recently, it has been demonstrated that the pro-apoptotic protein Bak is required for long-chain ceramide generation during irradiation-induced apoptosis, a mechanism that seems to depend on post-translational modifications of ceramide synthase (Siskind et al., 2010). Due to the complexity of sphingolipid metabolism (Hannun and Obeid, 2008), the mechanisms regulating the role of Bcl-2 family members in ceramide-induced apoptosis may rely on the different metabolic pathways and subcellular locations regarding ceramide generation, involving a complex interplay between sphingolipid metabolism proteins and Bcl-2 family members.

Nonetheless, it has also been established that over-expression of either Bcl-2 or Bcl-x_L can protect different cell lines against ceramide-induced apoptosis without preventing ceramide generation and/or accumulation (Allouche et al., 1997; Wiesner et al., 1997; Zhang et al., 1997). These findings suggest that inhibition of ceramide-induced apoptosis by anti-apoptotic Bcl-2 proteins occurs downstream of ceramide generation. In fact, addition of ceramide analogues to isolated mitochondria induces cytochrome *c* release, but this process is completely prevented by preincubation of mitochondria with Bcl-2 (Ghafourifar et al., 1999).

The formation of protein-permeable ceramide channels in the OMM does not require Bcl-2 family proteins; however, both human Bcl-x_L and CED-9 (a Bcl-2 homologue from *C. elegans*) are capable of disassembling ceramide channels in mitochondria isolated from rat liver and yeast (Siskind et al., 2008). The same phenomenon is observed in the defined system of planar phospholipid membranes, suggesting that

channel disassembly might involve a direct interaction with the anti-apoptotic Bcl-2 members (Siskind et al., 2008).

Ceramide also collaborates with pro-apoptotic members of the Bcl-2 family, since it has been reported to induce a Bax-dependent cell death process in a variety of cell lines (von Haefen et al., 2002; Kim et al., 2001; Sawada et al., 2000). In fact, Bax translocates to mitochondria of cells undergoing ceramide-mediated apoptosis (Kim et al., 2001), and a direct interaction between ceramide and Bax has been demonstrated in isolated yeast mitochondria and planar phospholipid membranes, revealing that these molecules act synergistically on mitochondria to induce MOMP (Ganesan et al., 2010). An *in vitro* experiment with bacterial SMase-treated mitochondria showed that generation of ceramide in this organelle might be sufficient to induce Bax translocation to mitochondria (Birbes et al., 2005). Recent studies have identified ceramide-enriched detergent-resistant microdomains in the mitochondrial membrane, with which Bax preferentially associates (Martínez-Abundis et al., 2009). Additionally, Bax isolated from these mitochondrial ceramide-rich domains (MCRDs), which are virtually non-existent in healthy cells, is in the form of high-molecular weight oligomers compatible with pore formation, while the non-MCRD membranes contain the monomeric form of Bax exclusively (Lee et al., 2011). A deeper look into the relation between sphingolipids and the pro-apoptotic Bcl-2 family members revealed that mitochondrial ceramide serves as precursor of sphingosine-1-phosphate and hexadecenal, which cooperate specifically with Bak and Bax, respectively (Chipuk et al., 2012). Indeed, overexpression of mouse sphingomyelin synthase-1, altering sphingolipid metabolism to favour SM accumulation, was able to decrease the sensitivity of yeast cells to the action of Bax (Yang et al., 2006). Such observations support the idea that ceramide accumulation in the OMM of apoptotic cells creates a specific micro-environment that might be essential for the action of pro-apoptotic Bcl-2 proteins.

5.2.2. Sphingolipids and phosphoregulation of Bcl-2 family members

The collaboration between sphingolipids and Bcl-2 family members is not limited to the direct interaction of ceramide and these apoptotic regulators in the mitochondrial membrane. Ceramide, like other sphingolipids, can act as a signalling molecule in a variety of physiological responses, and several proteins have been shown to interact with ceramide both *in vitro* and in cells (Hannun and Obeid, 2008). In fact, protein

phosphatases 1 (PP1) and 2A (PP2A) are stereospecific ceramide-activated protein phosphatases (CAPP) that can bind to ceramide *in vitro* (Chalfant et al., 1999, 2004), and mediate two distinct ceramide signalling pathways. In cancer cells, exogenously added or *de novo* generated ceramide induced dephosphorylation of Serine/Arginine-rich (SR) proteins (Chalfant et al., 2001), known modulators of mRNA splicing and specific substrates of PP1 (Mermoud et al., 1994). Consistently, ceramide has been shown to regulate the alternative splicing of Bcl-x and caspase-9 mRNA (Boise et al., 1993; Srinivasula et al., 1999) through a PP1-dependent mechanism (Chalfant et al., 2002). Treatment of lung cancer cells with ceramide, or ceramide-inducing agents, downregulates the mRNA levels of the anti-apoptotic proteins Bcl-x_L and caspase-9b, and increases the levels of the pro-apoptotic forms Bcl-x_S and caspase-9 (Chalfant et al., 2002). Ceramide is also a specific activator of PP2A (Dobrowsky and Hannun, 1992, 1993), a protein that directly interacts with Bcl-2 to dephosphorylate this anti-apoptotic regulator at serine 70 and promote its binding to p53, therefore inhibiting Bcl-2's survival functions (Deng and Ma, 1998; Deng et al., 2009; Ruvolo et al., 1999). PP2A is able to regulate the phosphorylation state of Bax in response to ceramide as well, since it co-localizes and interacts with Bax in intact cells, and directly dephosphorylates it *in vitro* (Xin and Deng, 2006). Ceramide-induced Bax dephosphorylation causes a conformational change that promotes the insertion and oligomerization of Bax in the OMM, and also disrupts the Bcl-2/Bax association to liberate Bax from the heterodimeric complex (Xin and Deng, 2006). Additionally, PP2A activates the pro-apoptotic function of Bad, in a mechanism that involves dissociation of Bad from 14-3-3 proteins (Chiang et al., 2001), a family of multifunctional phosphoserine binding molecules that can serve as effectors of survival signalling (Tzivion and Avruch, 2002). The 14-3-3 protein associates with Bad, facilitating the phosphorylation of Ser155 by survival promoting kinases, and thus controlling Bad's pro-apoptotic activity (Datta et al., 2000).

Other targets for ceramide mediated-dephosphorylation include for example the retinoblastoma gene product RB, PKCα (Lee et al., 1996) and protein kinase B (PKB or AKT) (Schubert et al., 2000). The serine/threonine protein kinase AKT is recruited to the plasma membrane and activated through PI(3)K signalling in response to various growth factors (Burgering and Coffer, 1995). AKT is considered a central player in the regulation of metabolism, mobility, transcription, cell-cycle progression and cell survival (Fayard et al., 2005). The pro-survival role of AKT is associated, at least in

part, with the inhibition of apoptosis (Dudek et al., 1997; Kauffmann-Zeh et al., 1997), and once activated this kinase interacts with different proteins, acting both upstream and downstream from MOMP (Franke et al., 2003). Among other cellular targets, AKT is able to regulate the action of both anti- and pro-apoptotic Bcl-2 family members. The first evidence of this relation emerged from the observation that growth factor activation of the PI(3)K/AKT signalling pathway induces phosphorylation of Bad, and that AKT is actually able to phosphorylate this pro-apoptotic regulator both *in vitro* and *in vivo*, thus promoting cell survival (Datta et al., 1997; del Peso et al., 1997). Since then, several studies have reported a role of AKT in the regulation of other Bcl-2 family proteins such as Bcl-2 (Pugazhenthi et al., 2000), Bcl-x_L (Jones et al., 2000) Bax (Gardai et al., 2004), Bak and Bik (Kennedy et al., 1999). Interestingly, downregulation of PI(3)K activity by stress-induced ceramide results in the inhibition of AKT and decreased phosphorylation of Bad (Zundel and Giaccia, 1998), indicating that the action of AKT is negatively regulated by this sphingolipid. The regulation of AKT by ceramide might comprise two independent mechanisms: blockage of AKT translocation to the plasma membrane, and stimulation of AKT dephosphorylation (Stratford et al., 2004). Experiments in rat adipocytes revealed that PP2A is the primary regulator of AKT dephosphorylation, establishing a direct pathway for ceramide to control AKT activity (Resjö et al., 2002). Another key element involved in ceramide-mediated AKT inactivation is the atypical PKC isoform ζ, which associates with AKT in vivo and acts as negative regulator of this kinase (Doornbos et al., 1999). Ceramide can specifically bind PKCζ and has been shown to activate this kinase *in vitro*, and *in vivo* by treating cells with exogenous SMase (Bourbon et al., 2000; Lozano et al., 1994), a process that involves recruitment and phosphorylation of PKCζ within structured lipid microdomians (Fox et al., 2007). Ceramide-activated PKCζ increasingly associates with AKT, leading to diminished AKT activation and consequent growth arrest (Bourbon et al., 2002). PKCζ phosphorylates AKT at Thr34 inhibiting its ability to bind to phosphatidylinositol 3,4,5-triphosphate (PIP3) at the plasma membrane, thereby disrupting AKT activation by PI(3)K signalling (Powell et al., 2003). Curiously, PKCζ can also promote cell survival through the inhibition of acid SMase in response to UV-C irradiation (Charruyer et al., 2007) and the phosphorylation of Bax at Ser184, sequestering Bax in cytoplasm (Xin et al., 2007). Such observations establish a new mechanism for the regulation of cell death by sphingolipids that comprises an interplay between ceramide, PI(3)K/AKT signalling and Bcl-2 family members.

Another direct effector of ceramide is the lysosomal aspartyl protease cathepsin D. Endosomal ceramide, resulting from the activity of an acid SMase, binds to and promotes the autocatalytic proteolysis of procathepsin D to originate its enzymatically active isoforms (Heinrich et al., 1999). Remarkably, cathepsin D colocalizes with the BH3-only protein Bid in HeLa cells, and directly cleaves this apoptotic regulator *in vitro* (Heinrich et al., 2004). Stimulation of acid SMase activity by TNF-α or chemotherapeutic drugs leads to the accumulation of endosomal and lysosomal ceramide, which in turn enhances the cathepsin D-mediated cleavage of Bid, activating it to promote the insertion of Bax in the OMM and trigger the mitochondrial pathway of apoptosis (Heinrich et al., 2004).

Other sphingolipids can also participate in the regulation of Bcl-2 family proteins. Sphingosine, which unlike ceramide is not exclusively confined to membrane fractions, is able to activate sphingosine dependent kinases (SDK) such as SDK1 – a caspase-cleaved fragment of PKCδ (Hamaguchi et al., 2003; Megidish et al., 1998) – and PKA, both of which phosphorylate a serine residue at the interface of the dimeric 14-3-3 proteins. This reaction results in disruption of dimeric 14-3-3 into the monomeric form, potentially modulating several biological functions (Woodcock et al., 2003). Indeed, 14-3-3 proteins have been shown to block apoptosis (Xing et al., 2000) in a process that involves the inhibition of Bad (Masters et al., 2001). Therefore, sphingosine-induced monomerization of 14-3-3 protein may promote an apoptotic pathway via disruption of 14-3-3's anti-apoptotic function. Additionally, sphingosine can also activate 3-Phosphoinositide-Dependent Kinase 1 (PDK1), a kinase acting upstream of AKT (King et al., 2000). Another sphingolipid, sphigosine-1-phosphate, generally associated with pro-survival effects, enhances activation of the PI(3)K/AKT pathway with an associated up-regulation of the anti-apoptotic Bcl-2 and down-regulation of the pro-apoptotic Bim (Limaye et al., 2005).

5.2.3. Sphingolipids and the heterologous expression of Bcl-2 family members in yeast

Generation of ceramide in response to different stresses has also been observed in yeasts, suggesting that ceramide signalling is an "old" response conserved from yeasts to mammals (Jenkins et al., 1997; Mathias et al., 1998). Interestingly, addition of ceramide to the yeast *Saccharomyces cerevisiae* induces a cell cycle arrest in G1 phase,

an effect partially mediated by a ceramide-activated protein phosphatase (Nickels and Broach, 1996). Recent developments on the putative role of ceramide in yeast cell death implicate mitochondria and the organization of the plasma membrane as targets for ceramide action and consequent loss of viability (Carmona-Gutierrez et al., 2011; Pacheco et al., 2013; Rego et al., 2012). These evidences argue in favour of exploring yeast as a model to understand the basis of ceramides' cytotoxicity

In vitro experiments with isolated yeast mitochondria have been a useful tool to show the regulation of ceramide channel by recombinant Bcl-2 family proteins (Siskind et al., 2008). However, *in vivo* experiments in yeast would be an interesting alternative to gain more insights in the mechanistic support of this process. Although yeast does not contain a family of Bcl-2-like proteins, only one putative member being described so far (Büttner et al., 2011), the heterologous expression of pro-apoptotic Bax or Bak in yeast is able to induce MOMP (Marzo et al., 1998b; Priault et al., 1999), eventually leading to growth arrest and death. For that reason, yeast has been used to identify proteins capable of suppressing the growth-inhibitory effects of Bax (Yang et al., 2006). This study reported the involvement of sphingolipid metabolism in the effects of Bax and showed that mouse sphingomyelin synthase 1 (SMS1) expression can prevent Bax-mediated cell death as well as the effects of several stresses, namely hydrogen peroxide, osmotic stress, elevated temperature and exogenous ceramides. Different mutants of Bcl-2 family proteins can be expressed in yeast, and they resume the properties expected from experiments in mammalian cells (see for example Arokium et al., 2004; Cartron et al., 2005).

The availability of yeast mutants deleted for genes encoding enzymes involved in ceramide metabolism offers the opportunity to test the effects of these enzymes on the capacity of Bax, and other Bcl-2 family members, to interact with OMM and to regulate MOMP. For example, one might expect that a yeast mutant with low ceramides content might be less sensitive to Bax-induced permeabilization of the OMM to cytochrome *c*. Conversely, a lower expression of Bcl-2/Bcl-x_L would be required to disassemble ceramide channels and prevent the release of cytochrome *c* induced by a yeast cell death stimulus such as acetic acid (Pereira et al., 2007). Combining the utilization of ceramide metabolism mutants with Bcl-2 proteins might provide a powerful system to accurately define the molecular determinants underlying their interactions during cell death.

Numerous kinases and phosphatases are conserved between yeast and mammals. Several yeast kinases, such as Sch9p, the yeast potential homolog of AKT (Fabrizio et

al., 2001), are able to phosphorylate Bax (Simonyan et al., unpublished data). Alternatively, mammalian kinases and phosphatases can be expressed in yeast, keep their function, and eventually compensate the genetic absence of the endogenous yeast homologs. The heterologous co-expression of mammalian kinases/phosphatases of interest with Bcl-2 family members in yeast mutants for ceramide metabolism might be a sophisticated and powerful tool to investigate the connection between ceramides, kinases/phosphatases signalling pathways and the capacity of Bcl-2 family proteins to regulate MOMP. As an example, one can envisage the heterologous expression of AKT and Bax in ceramide metabolism mutant strains as a tool to investigate how the ceramide-regulation of the kinase may have consequences on Bax ability to promote MOMP.

Together, the reports mentioned in the last sections and the promise from future experimentation, provide a nice perspective on what might be the intricate network of cellular events that connects sphingolipids, Bcl-2 family proteins and mitochondria in the regulation of cell death.

6. Scope of the thesis

The yeast *S. cerevisiae*, while easy-to-manipulate and genetically defined model, has been widely and successfully used to study different aspects of cell death biology, either through the heterologous expression of know regulators from mammalian cell death programs, or through the examination of possibly conserved cell death mechanisms mediating the response of yeast cells to different stress stimuli (Priault et al., 2003; Carmona-Gutierrez et al., 2010). On this work, and once more, we explore the potential of yeast to investigate distinct processes involved in cell death, through a set of different physiological and biochemical assays. Accordingly, the following chapters of this thesis will describe the knowledge and experimental approaches used to: i) evaluate the role of Aac2p cysteine residues, and Aac2p oxidation in the acetic acid-induced apoptotic-like death of *S. cerevisiae* ii) assess the role of the AAC and Por1 in acetic acid-induced MOMP, during yeast cell death; iii) and study the influence of sphingolipid metabolism in the action of structurally and functionally different forms of Bax, as well as in the pro-apoptotic activity of Bcl-x_L. The observations documented during our experimentations are presented and discussed in light of contemporary knowledge, followed by the major conclusions and future perspectives into what may follow in each particular subject.

7. References

Acehan, D., Jiang, X., Morgan, D.G., Heuser, J.E., Wang, X., and Akey, C.W. (2002). Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol. Cell *9*, 423–432.

Adam, D., Heinrich, M., Kabelitz, D., and Schütze, S. (2002). Ceramide: does it matter for T cells? Trends Immunol. *23*, 1–4.

Adams, J.M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science *281*, 1322–1326.

Adams, J.M., and Cory, S. (2007). Bcl-2-regulated apoptosis: mechanism and therapeutic potential. Curr. Opin. Immunol. *19*, 488–496.

Adams, V., Bosch, W., Schlegel, J., Wallimann, T., and Brdiczka, D. (1989). Further characterization of contact sites from mitochondria of different tissues: topology of peripheral kinases. Biochim. Biophys. Acta *981*, 213–225.

Von Ahsen, O., Renken, C., Perkins, G., Kluck, R.M., Bossy-Wetzel, E., and Newmeyer, D.D. (2000). Preservation of mitochondrial structure and function after Bid- or Bax-mediated cytochrome c release. J. Cell Biol. *150*, 1027–1036.

Alcalá, S., Klee, M., Fernández, J., Fleischer, A., and Pimentel-Muiños, F.X. (2007). 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.

Allouche, M., Bettaieb, A., Vindis, C., Rousse, A., Grignon, C., and Laurent, G. (1997). Influence of Bcl-2 overexpression on the ceramide pathway in daunorubicin-induced apoptosis of leukemic cells. Oncogene *14*, 1837–1845.

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.

Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J.J., Mazzei, G., et al. (1997). Inhibition of Bax channel-forming activity by Bcl-2. Science *277*, 370–372.

Antonsson, B., Montessuit, S., Lauper, S., Eskes, R., and Martinou, J.C. (2000). Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. Biochem. J. *345 Pt 2*, 271–278.

Arends, M.J., Morris, R.G., and Wyllie, A.H. (1990). Apoptosis. The role of the endonuclease. Am. J. Pathol. *136*, 593–608.

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 alpha-helix is a second ART sequence and plays a role in the interaction with anti-apoptotic BCL-xL. J. Biol. Chem. *279*, 52566–52573.

Azzi, A., and Azzone, G.F. (1965). Swelling and shrinkage phenomena in liver mitochondria. I. Large amplitude swelling induced by inorganic phosphate and by ATP. Biochim. Biophys. Acta *105*, 253–264.

Azzolin, L., von Stockum, S., Basso, E., Petronilli, V., Forte, M.A., and Bernardi, P. (2010). The mitochondrial permeability transition from yeast to mammals. FEBS Lett. *584*, 2504–2509.

Babior, B.M. (1984). Oxidants from phagocytes: agents of defense and destruction. Blood *64*, 959–966.

Baines, C.P., Kaiser, R.A., Purcell, N.H., Blair, N.S., Osinska, H., Hambleton, M.A., Brunskill, E.W., Sayen, M.R., Gottlieb, R.A., Dorn, G.W., et al. (2005). Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature *434*, 658–662.

Baines, C.P., Kaiser, R.A., Sheiko, T., Craigen, W.J., and Molkentin, J.D. (2007). Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. Nat. Cell Biol. *9*, 550–555.

Ballarin, C., and Sorgato, M.C. (1995). An electrophysiological study of yeast mitochondria. Evidence for two inner membrane anion channels sensitive to ATP. J. Biol. Chem. *270*, 19262–19268.

Banerjee, J., and Ghosh, S. (2004). Bax increases the pore size of rat brain mitochondrial voltage-dependent anion channel in the presence of tBid. Biochem. Biophys. Res. Commun. *323*, 310–314.

Bartke, N., and Hannun, Y.A. (2009). Bioactive sphingolipids: metabolism and function. J. Lipid Res. *50 Suppl*, S91–96.

Basañez, G., Nechushtan, A., Drozhinin, O., Chanturiya, A., Choe, E., Tutt, S., Wood, K.A., Hsu, Y., Zimmerberg, J., and Youle, R.J. (1999). Bax, but not Bcl-xL, decreases the lifetime of planar phospholipid bilayer membranes at subnanomolar concentrations. Proc. Natl. Acad. Sci. U. S. A. *96*, 5492–5497.

Basañez, G., Sharpe, J.C., Galanis, J., Brandt, T.B., Hardwick, J.M., and Zimmerberg, J. (2002). Bax-type apoptotic proteins porate pure lipid bilayers through a mechanism sensitive to intrinsic monolayer curvature. J. Biol. Chem. *277*, 49360–49365.

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. J. Biol. Chem. *280*, 18558–18561.

Basso, E., Petronilli, V., Forte, M.A., and Bernardi, P. (2008). Phosphate is essential for inhibition of the mitochondrial permeability transition pore by cyclosporin A and by cyclophilin D ablation. J. Biol. Chem. *283*, 26307–26311.

Bellamy, C.O., Malcomson, R.D., Harrison, D.J., and Wyllie, A.H. (1995). Cell death in health and disease: the biology and regulation of apoptosis. Semin. Cancer Biol. *6*, 3–16.

Belzacq, A.-S., Vieira, H.L.A., Kroemer, G., and Brenner, C. (2002). The adenine nucleotide translocator in apoptosis. Biochimie *84*, 167–176.

Bernardi, P. (2013). The mitochondrial permeability transition pore: a mystery solved? Front. Physiol. *4*, 95.

Bernardi, P., Scorrano, L., Colonna, R., Petronilli, V., and Di Lisa, F. (1999). Mitochondria and cell death. Mechanistic aspects and methodological issues. Eur. J. Biochem. FEBS *264*, 687–701.

Berry, D.L., and Baehrecke, E.H. (2007). Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. Cell *131*, 1137–1148.

Beutner, G., Ruck, A., Riede, B., Welte, W., and Brdiczka, D. (1996). Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore. FEBS Lett. *396*, 189–195.

Bielawska, A., Crane, H.M., Liotta, D., Obeid, L.M., and Hannun, Y.A. (1993). Selectivity of ceramide-mediated biology. Lack of activity of erythro-dihydroceramide. J. Biol. Chem. *268*, 26226–26232.

Birbes, H., Luberto, C., Hsu, Y.-T., El Bawab, S., Hannun, Y.A., and Obeid, L.M. (2005). A mitochondrial pool of sphingomyelin is involved in TNFalpha-induced Bax translocation to mitochondria. Biochem. J. *386*, 445–451.

Boise, L.H., González-García, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nuñez, G., and Thompson, C.B. (1993). bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell *74*, 597–608.

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

Bourbon, N.A., Sandirasegarane, L., and Kester, M. (2002). Ceramide-induced inhibition of Akt is mediated through protein kinase Czeta: implications for growth arrest. J. Biol. Chem. *277*, 3286–3292.

Boya, P., González-Polo, R.-A., Casares, N., Perfettini, J.-L., Dessen, P., Larochette, N., Métivier, D., Meley, D., Souquere, S., Yoshimori, T., et al. (2005). Inhibition of macroautophagy triggers apoptosis. Mol. Cell. Biol. *25*, 1025–1040.

Brdiczka, D.G., Zorov, D.B., and Sheu, S.-S. (2006). Mitochondrial contact sites: their role in energy metabolism and apoptosis. Biochim. Biophys. Acta *1762*, 148–163.

Brenner, C., Cadiou, H., Vieira, H.L., Zamzami, N., Marzo, I., Xie, Z., Leber, B., Andrews, D., Duclohier, H., Reed, J.C., et al. (2000). Bcl-2 and Bax regulate the channel activity of the mitochondrial adenine nucleotide translocator. Oncogene *19*, 329–336.

Brustovetsky, N., Tropschug, M., Heimpel, S., Heidkämper, D., and Klingenberg, M. (2002). A large Ca2+-dependent channel formed by recombinant ADP/ATP carrier from Neurospora crassa resembles the mitochondrial permeability transition pore. Biochemistry (Mosc.) *41*, 11804–11811.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999). Biochemical pathways of caspase activation during apoptosis. Annu. Rev. Cell Dev. Biol. *15*, 269–290.

Burgering, B.M., and Coffer, P.J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature *376*, 599–602.

Buttke, T.M., and Sandstrom, P.A. (1994). Oxidative stress as a mediator of apoptosis. Immunol. Today *15*, 7–10.

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. J. Cell Biol. *175*, 521–525.

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. Mol. Cell *25*, 233–246.

Büttner, S., Ruli, D., Vögtle, 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. EMBO J. *30*, 2779–2792.

Cai, J., Yang, J., and Jones, D.P. (1998). Mitochondrial control of apoptosis: the role of cytochrome c. Biochim. Biophys. Acta *1366*, 139–149.

Carmona-Gutierrez, D., Eisenberg, T., Büttner, S., Meisinger, C., Kroemer, G., and Madeo, F. (2010). Apoptosis in yeast: triggers, pathways, subroutines. Cell Death Differ. *17*, 763–773.

Carmona-Gutierrez, D., Reisenbichler, A., Heimbucher, P., Bauer, M.A., Braun, R.J., Ruckenstuhl, C., Büttner, S., Eisenberg, T., Rockenfeller, P., Fröhlich, K.-U., et al. (2011). Ceramide triggers metacaspase-independent mitochondrial cell death in yeast. Cell Cycle Georget. Tex *10*, 3973–3978.

Cartron, P.-F., Arokium, H., Oliver, L., Meflah, K., Manon, S., and Vallette, F.M. (2005). Distinct domains control the addressing and the insertion of Bax into mitochondria. J. Biol. Chem. *280*, 10587–10598.

Casal, M., Cardoso, H., and Leão, C. (1996). Mechanisms regulating the transport of acetic acid in Saccharomyces cerevisiae. Microbiol. Read. Engl. *142 (Pt 6)*, 1385–1390.

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

Castedo, M., Perfettini, J.-L., Roumier, T., Andreau, K., Medema, R., and Kroemer, G. (2004). Cell death by mitotic catastrophe: a molecular definition. Oncogene *23*, 2825–2837.

Castedo, M., Coquelle, A., Vivet, S., Vitale, I., Kauffmann, A., Dessen, P., Pequignot, M.O., Casares, N., Valent, A., Mouhamad, S., et al. (2006). Apoptosis regulation in tetraploid cancer cells. EMBO J. *25*, 2584–2595.

Chalfant, C.E., Kishikawa, K., Mumby, M.C., Kamibayashi, C., Bielawska, A., and Hannun, Y.A. (1999). Long chain ceramides activate protein phosphatase-1 and protein phosphatase-2A. Activation is stereospecific and regulated by phosphatidic acid. J. Biol. Chem. *274*, 20313–20317.

Chalfant, C.E., Ogretmen, B., Galadari, S., Kroesen, B.J., Pettus, B.J., and Hannun, Y.A. (2001). FAS activation induces dephosphorylation of SR proteins; dependence on the de novo generation of ceramide and activation of protein phosphatase 1. J. Biol. Chem. *276*, 44848–44855.

Chalfant, C.E., Rathman, K., Pinkerman, R.L., Wood, R.E., Obeid, L.M., Ogretmen, B., and Hannun, Y.A. (2002). De novo ceramide regulates the alternative splicing of caspase 9 and Bcl-x in A549 lung adenocarcinoma cells. Dependence on protein phosphatase-1. J. Biol. Chem. *277*, 12587–12595.

Chalfant, C.E., Szulc, Z., Roddy, P., Bielawska, A., and Hannun, Y.A. (2004). The structural requirements for ceramide activation of serine-threonine protein phosphatases. J. Lipid Res. *45*, 496–506.

Chang, Y., Abe, A., and Shayman, J.A. (1995). Ceramide formation during heat shock: a potential mediator of alpha B-crystallin transcription. Proc. Natl. Acad. Sci. U. S. A. *92*, 12275–12279.

Chanock, S.J., Benna, J. el, Smith, R.M., and Babior, B.M. (1994). The respiratory burst oxidase. J. Biol. Chem. *269*, 24519–24522.

Chao, D.T., and Korsmeyer, S.J. (1998). BCL-2 family: regulators of cell death. Annu. Rev. Immunol. *16*, 395–419.

Charruyer, A., Jean, C., Colomba, A., Jaffrézou, J.-P., Quillet-Mary, A., Laurent, G., and Bezombes, C. (2007). PKCzeta protects against UV-C-induced apoptosis by inhibiting acid sphingomyelinase-dependent ceramide production. Biochem. J. *405*, 77–83.

Chautan, M., Chazal, G., Cecconi, F., Gruss, P., and Golstein, P. (1999). Interdigital cell death can occur through a necrotic and caspase-independent pathway. Curr. Biol. CB *9*, 967–970.

Cheng, E.H.Y., Sheiko, T.V., Fisher, J.K., Craigen, W.J., and Korsmeyer, S.J. (2003). VDAC2 inhibits BAK activation and mitochondrial apoptosis. Science *301*, 513–517.

Chiang, C.W., Harris, G., Ellig, C., Masters, S.C., Subramanian, R., Shenolikar, S., Wadzinski, B.E., and Yang, E. (2001). Protein phosphatase 2A activates the proapoptotic function of BAD in interleukin- 3-dependent lymphoid cells by a mechanism requiring 14-3-3 dissociation. Blood *97*, 1289–1297.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M., and Dixit, V.M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505–512.

Chipuk, J.E., and Green, D.R. (2005). Do inducers of apoptosis trigger caspase-independent cell death? Nat. Rev. Mol. Cell Biol. *6*, 268–275.

Chipuk, J.E., Moldoveanu, T., Llambi, F., Parsons, M.J., and Green, D.R. (2010). The BCL-2 family reunion. Mol. Cell *37*, 299–310.

Chipuk, J.E., McStay, G.P., Bharti, A., Kuwana, T., Clarke, C.J., Siskind, L.J., Obeid, L.M., and Green, D.R. (2012). Sphingolipid metabolism cooperates with BAK and BAX to promote the mitochondrial pathway of apoptosis. Cell *148*, 988–1000.

Chowdhury, I., Tharakan, B., and Bhat, G.K. (2008). Caspases - an update. Comp. Biochem. Physiol. B Biochem. Mol. Biol. *151*, 10–27.

Christofferson, D.E., and Yuan, J. (2010). Necroptosis as an alternative form of programmed cell death. Curr. Opin. Cell Biol. *22*, 263–268.

Clarke, P.G., and Clarke, S. (1996). Nineteenth century research on naturally occurring cell death and related phenomena. Anat. Embryol. (Berl.) *193*, 81–99.

Cohen, G.M. (1997). Caspases: the executioners of apoptosis. Biochem. J. *326 (Pt 1)*, 1–16.

Connern, C.P., and Halestrap, A.P. (1992). Purification and N-terminal sequencing of peptidyl-prolyl cis-trans-isomerase from rat liver mitochondrial matrix reveals the existence of a distinct mitochondrial cyclophilin. Biochem. J. *284 (Pt 2)*, 381–385.

Cory, S., and Adams, J.M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. Nat. Rev. Cancer *2*, 647–656.

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

Crompton, M., and Costi, A. (1988). Kinetic evidence for a heart mitochondrial pore activated by Ca2+, inorganic phosphate and oxidative stress. A potential mechanism for mitochondrial dysfunction during cellular Ca2+ overload. Eur. J. Biochem. FEBS *178*, 489–501.

Crompton, M., Costi, A., and Hayat, L. (1987). Evidence for the presence of a reversible Ca2+-dependent pore activated by oxidative stress in heart mitochondria. Biochem. J. *245*, 915–918.

Crompton, M., Ellinger, H., and Costi, A. (1988). Inhibition by cyclosporin A of a Ca2+-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. Biochem. J. *255*, 357–360.

Crompton, M., Virji, S., and Ward, J.M. (1998). Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. Eur. J. Biochem. FEBS *258*, 729–735.

Crompton, M., Virji, S., Doyle, V., Johnson, N., and Ward, J.M. (1999). The mitochondrial permeability transition pore. Biochem. Soc. Symp. *66*, 167–179.

Crompton, M., Barksby, E., Johnson, N., and Capano, M. (2002). Mitochondrial intermembrane junctional complexes and their involvement in cell death. Biochimie *84*, 143–152.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231–241.

Datta, S.R., Katsov, A., Hu, L., Petros, A., Fesik, S.W., Yaffe, M.B., and Greenberg, M.E. (2000). 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol. Cell *6*, 41–51.

Davies, K.J., and Goldberg, A.L. (1987). Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. J. Biol. Chem. *262*, 8220–8226.

Degenhardt, K., Mathew, R., Beaudoin, B., Bray, K., Anderson, D., Chen, G., Mukherjee, C., Shi, Y., Gélinas, C., Fan, Y., et al. (2006). Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell *10*, 51–64.

Dejean, L.M., Martinez-Caballero, S., Guo, L., Hughes, C., Teijido, O., Ducret, T., Ichas, F., Korsmeyer, S.J., Antonsson, B., Jonas, E.A., et al. (2005). Oligomeric Bax is a component of the putative cytochrome c release channel MAC, mitochondrial apoptosis-induced channel. Mol. Biol. Cell *16*, 2424–2432.

Deng, Z.-L., and Ma, Y. (1998). Aflatoxin sufferer and p53 gene mutation in hepatocellular carcinoma. World J. Gastroenterol. WJG *4*, 28–29.

Deng, X., Gao, F., and May, W.S. (2009). Protein phosphatase 2A inactivates Bcl2's antiapoptotic function by dephosphorylation and up-regulation of Bcl2-p53 binding. Blood *113*, 422–428.

Denton, D., Shravage, B., Simin, R., Mills, K., Berry, D.L., Baehrecke, E.H., and Kumar, S. (2009). Autophagy, not apoptosis, is essential for midgut cell death in Drosophila. Curr. Biol. CB *19*, 1741–1746.

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. Mol. Cell *30*, 369–380.

Dewson, G., Kratina, T., Czabotar, P., Day, C.L., Adams, J.M., and Kluck, R.M. (2009). Bak activation for apoptosis involves oligomerization of dimers via their alpha6 helices. Mol. Cell *36*, 696–703.

Dickson, R.C. (2010). Roles for sphingolipids in Saccharomyces cerevisiae. Adv. Exp. Med. Biol. *688*, 217–231.

Dobrowsky, R.T., and Hannun, Y.A. (1992). Ceramide stimulates a cytosolic protein phosphatase. J. Biol. Chem. *267*, 5048–5051.

Dobrowsky, R.T., and Hannun, Y.A. (1993). Ceramide-activated protein phosphatase: partial purification and relationship to protein phosphatase 2A. Adv. Lipid Res. *25*, 91–104.

Doornbos, R.P., Theelen, M., van der Hoeven, P.C., van Blitterswijk, W.J., Verkleij, A.J., and van Bergen en Henegouwen, P.M. (1999). Protein kinase Czeta is a negative regulator of protein kinase B activity. J. Biol. Chem. *274*, 8589–8596.

Dressler, K.A., Mathias, S., and Kolesnick, R.N. (1992). Tumor necrosis factor-alpha activates the sphingomyelin signal transduction pathway in a cell-free system. Science *255*, 1715–1718.

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.

Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.A., Kaplan, D.R., and Greenberg, M.E. (1997). Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science *275*, 661–665.

Earnshaw, W.C., Martins, L.M., and Kaufmann, S.H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. *68*, 383–424.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. Toxicol. Pathol. *35*, 495–516.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature *391*, 43–50.

Eom, Y.-W., Kim, M.A., Park, S.S., Goo, M.J., Kwon, H.J., Sohn, S., Kim, W.-H., Yoon, G., and Choi, K.S. (2005). Two distinct modes of cell death induced by doxorubicin: apoptosis and cell death through mitotic catastrophe accompanied by senescence-like phenotype. Oncogene *24*, 4765–4777.

Eskes, R., Antonsson, B., Osen-Sand, A., Montessuit, S., Richter, C., Sadoul, R., Mazzei, G., Nichols, A., and Martinou, J.C. (1998). Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg2+ ions. J. Cell Biol. *143*, 217–224.

Eskes, R., Desagher, S., Antonsson, B., and Martinou, J.C. (2000). Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol. Cell. Biol. *20*, 929–935.

Evan, G.I., and Vousden, K.H. (2001). Proliferation, cell cycle and apoptosis in cancer. Nature *411*, 342–348.

Fabrizio, P., Pozza, F., Pletcher, S.D., Gendron, C.M., and Longo, V.D. (2001). Regulation of longevity and stress resistance by Sch9 in yeast. Science *292*, 288–290.

Fabrizio, P., Pletcher, S.D., Minois, N., Vaupel, J.W., and Longo, V.D. (2004). Chronological aging-independent replicative life span regulation by Msn2/Msn4 and Sod2 in Saccharomyces cerevisiae. FEBS Lett. *557*, 136–142.

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

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

Fayard, E., Tintignac, L.A., Baudry, A., and Hemmings, B.A. (2005). Protein kinase B/Akt at a glance. J. Cell Sci. *118*, 5675–5678.

Fazi, B., Bursch, W., Fimia, G.M., Nardacci, R., Piacentini, M., Di Sano, F., and Piredda, L. (2008). Fenretinide induces autophagic cell death in caspase-defective breast cancer cells. Autophagy *4*, 435–441.

Ferrari, G., Yan, C.Y., and Greene, L.A. (1995). N-acetylcysteine (D- and L-stereoisomers) prevents apoptotic death of neuronal cells. J. Neurosci. Off. J. Soc. Neurosci. *15*, 2857–2866.

Fleury, C., Pampin, M., Tarze, A., and Mignotte, B. (2002). Yeast as a model to study apoptosis? Biosci. Rep. *22*, 59–79.

Fox, T.E., Houck, K.L., O'Neill, S.M., Nagarajan, M., Stover, T.C., Pomianowski, P.T., Unal, O., Yun, J.K., Naides, S.J., and Kester, M. (2007). Ceramide recruits and activates protein kinase C zeta (PKC zeta) within structured membrane microdomains. J. Biol. Chem. *282*, 12450–12457.

France-Lanord, V., Brugg, B., Michel, P.P., Agid, Y., and Ruberg, M. (1997). Mitochondrial free radical signal in ceramide-dependent apoptosis: a putative mechanism for neuronal death in Parkinson's disease. J. Neurochem. *69*, 1612–1621.

Frank, S., Gaume, B., Bergmann-Leitner, E.S., Leitner, W.W., Robert, E.G., Catez, F., Smith, C.L., and Youle, R.J. (2001). The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev. Cell *1*, 515–525.

Franke, T.F., Hornik, C.P., Segev, L., Shostak, G.A., and Sugimoto, C. (2003). PI3K/Akt and apoptosis: size matters. Oncogene *22*, 8983–8998.

Frey, T.G., and Mannella, C.A. (2000). The internal structure of mitochondria. Trends Biochem. Sci. *25*, 319–324.

Fröhlich, K.U., and Madeo, F. (2000). Apoptosis in yeast--a monocellular organism exhibits altruistic behaviour. FEBS Lett. *473*, 6–9.

Futerman, A.H., and Hannun, Y.A. (2004). The complex life of simple sphingolipids. EMBO Rep. *5*, 777–782.

Galluzzi, L., Maiuri, M.C., Vitale, I., Zischka, H., Castedo, M., Zitvogel, L., and Kroemer, G. (2007). Cell death modalities: classification and pathophysiological implications. Cell Death Differ. *14*, 1237–1243.

Galluzzi, L., Vitale, I., Abrams, J.M., Alnemri, E.S., Baehrecke, E.H., Blagosklonny, M.V., Dawson, T.M., Dawson, V.L., El-Deiry, W.S., Fulda, S., et al. (2012). Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ. *19*, 107–120.

Ganesan, V., Perera, M.N., Colombini, D., Datskovskiy, D., Chadha, K., and Colombini, M. (2010). Ceramide and activated Bax act synergistically to permeabilize the mitochondrial outer membrane. Apoptosis Int. J. Program. Cell Death *15*, 553–562.

García-Ruiz, C., Colell, A., Marí, M., Morales, A., and Fernández-Checa, J.C. (1997). Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. J. Biol. Chem. *272*, 11369–11377.

Gardai, S.J., Hildeman, D.A., Frankel, S.K., Whitlock, B.B., Frasch, S.C., Borregaard, N., Marrack, P., Bratton, D.L., and Henson, P.M. (2004). Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils. J. Biol. Chem. *279*, 21085–21095.

Garrido, C., Brunet, M., Didelot, C., Zermati, Y., Schmitt, E., and Kroemer, G. (2006). Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. Cell Cycle Georget. Tex *5*, 2592–2601.

Gault, C.R., Obeid, L.M., and Hannun, Y.A. (2010). An overview of sphingolipid metabolism: from synthesis to breakdown. Adv. Exp. Med. Biol. *688*, 1–23.

Geley, S., Hartmann, B.L., and Kofler, R. (1997). Ceramides induce a form of apoptosis in human acute lymphoblastic leukemia cells that is inhibited by Bcl-2, but not by CrmA. FEBS Lett. *400*, 15–18.

George, N.M., Evans, J.J.D., and Luo, X. (2007). A three-helix homo-oligomerization domain containing BH3 and BH1 is responsible for the apoptotic activity of Bax. Genes Dev. *21*, 1937–1948.

Ghafourifar, P., Klein, S.D., Schucht, O., Schenk, U., Pruschy, M., Rocha, S., and Richter, C. (1999). Ceramide induces cytochrome c release from isolated mitochondria. Importance of mitochondrial redox state. J. Biol. Chem. *274*, 6080–6084.

Giorgio, V., Bisetto, E., Soriano, M.E., Dabbeni-Sala, F., Basso, E., Petronilli, V., Forte, M.A., Bernardi, P., and Lippe, G. (2009). Cyclophilin D modulates mitochondrial F0F1-ATP synthase by interacting with the lateral stalk of the complex. J. Biol. Chem. *284*, 33982–33988.

Giorgio, V., von Stockum, S., Antoniel, M., Fabbro, A., Fogolari, F., Forte, M., Glick, G.D., Petronilli, V., Zoratti, M., Szabó, I., et al. (2013). Dimers of mitochondrial ATP synthase form the permeability transition pore. Proc. Natl. Acad. Sci. U. S. A. *110*, 5887–5892.

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

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

Gourlay, C.W., and Ayscough, K.R. (2006). Actin-induced hyperactivation of the Ras signaling pathway leads to apoptosis in Saccharomyces cerevisiae. Mol. Cell. Biol. *26*, 6487–6501.

Grandér, D., Kharaziha, P., Laane, E., Pokrovskaja, K., and Panaretakis, T. (2009). Autophagy as the main means of cytotoxicity by glucocorticoids in hematological malignancies. Autophagy *5*, 1198–1200.

Grassmé, H., Jendrossek, V., Riehle, A., von Kürthy, G., Berger, J., Schwarz, H., Weller, M., Kolesnick, R., and Gulbins, E. (2003). Host defense against Pseudomonas aeruginosa requires ceramide-rich membrane rafts. Nat. Med. *9*, 322–330.

Green, D.R., and Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. Science *305*, 626–629.

Green, D.R., and Reed, J.C. (1998). Mitochondria and apoptosis. Science *281*, 1309–1312.

Greenhalf, W., Stephan, C., and Chaudhuri, B. (1996). Role of mitochondria and C-terminal membrane anchor of Bcl-2 in Bax induced growth arrest and mortality in Saccharomyces cerevisiae. FEBS Lett. *380*, 169–175.

Griffiths, E.J., and Halestrap, A.P. (1991). Further evidence that cyclosporin A protects mitochondria from calcium overload by inhibiting a matrix peptidyl-prolyl cis-trans isomerase. Implications for the immunosuppressive and toxic effects of cyclosporin. Biochem. J. *274 (Pt 2)*, 611–614.

Gross, A., McDonnell, J.M., and Korsmeyer, S.J. (1999). BCL-2 family members and the mitochondria in apoptosis. Genes Dev. *13*, 1899–1911.

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 Lett. *580*, 6880–6884.

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

Guenebeaud, C., Goldschneider, D., Castets, M., Guix, C., Chazot, G., Delloye-Bourgeois, C., Eisenberg-Lerner, A., Shohat, G., Zhang, M., Laudet, V., et al. (2010). The dependence receptor UNC5H2/B triggers apoptosis via PP2A-mediated dephosphorylation of DAP kinase. Mol. Cell *40*, 863–876.

Gulbins, E., Bissonnette, R., Mahboubi, A., Martin, S., Nishioka, W., Brunner, T., Baier, G., Baier-Bitterlich, G., Byrd, C., and Lang, F. (1995). FAS-induced apoptosis is mediated via a ceramide-initiated RAS signaling pathway. Immunity *2*, 341–351.

Gutierrez-Aguilar, R., Benmezroua, Y., Balkau, B., Marre, M., Helbecque, N., Charpentier, G., Polychronakos, C., Sladek, R., Froguel, P., and Neve, B. (2007). Minor contribution of SMAD7 and KLF10 variants to genetic susceptibility of type 2 diabetes. Diabetes Metab. *33*, 372–378.

Häcker, G., and Vaux, D.L. (1997). A chronology of cell death. Apoptosis Int. J. Program. Cell Death *2*, 247–256.

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

Halestrap, A.P. (1987). The regulation of the oxidation of fatty acids and other substrates in rat heart mitochondria by changes in the matrix volume induced by osmotic strength, valinomycin and Ca2+. Biochem. J. *244*, 159–164.

Halestrap, A.P. (1999). The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury. Biochem. Soc. Symp. *66*, 181–203.

Halestrap, A.P. (2009). What is the mitochondrial permeability transition pore? J. Mol. Cell. Cardiol. *46*, 821–831.

Halestrap, A.P. (2010). A pore way to die: the role of mitochondria in reperfusion injury and cardioprotection. Biochem. Soc. Trans. *38*, 841–860.

Halestrap, A.P., and Brenner, C. (2003). The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death. Curr. Med. Chem. *10*, 1507–1525.

Halestrap, A.P., Woodfield, K.Y., and Connern, C.P. (1997). Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. J. Biol. Chem. *272*, 3346–3354.

Halestrap, A.P., McStay, G.P., and Clarke, S.J. (2002). The permeability transition pore complex: another view. Biochimie *84*, 153–166.

Halliwell, B., and Gutteridge, J.M. (1990). Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol. *186*, 1–85.

Hamaguchi, A., Suzuki, E., Murayama, K., Fujimura, T., Hikita, T., Iwabuchi, K., Handa, K., Withers, D.A., Masters, S.C., Fu, H., et al. (2003). Sphingosine-dependent protein kinase-1, directed to 14-3-3, is identified as the kinase domain of protein kinase C delta. J. Biol. Chem. *278*, 41557–41565.

Hannun, Y.A., and Obeid, L.M. (2008). Principles of bioactive lipid signalling: lessons from sphingolipids. Nat. Rev. Mol. Cell Biol. *9*, 139–150.

Hannun, Y.A., and Obeid, L.M. (2011). Many ceramides. J. Biol. Chem. *286*, 27855–27862.

Hardwick, J.M., and Polster, B.M. (2002). Bax, along with lipid conspirators, allows cytochrome c to escape mitochondria. Mol. Cell *10*, 963–965.

Hauptmann, P., Riel, C., Kunz-Schughart, L.A., Fröhlich, K.-U., Madeo, F., and Lehle, L. (2006). Defects in N-glycosylation induce apoptosis in yeast. Mol. Microbiol. *59*, 765–778.

Haworth, R.A., and Hunter, D.R. (1979). The Ca2+-induced membrane transition in mitochondria. II. Nature of the Ca2+ trigger site. Arch. Biochem. Biophys. *195*, 460–467.

He, L., and Lemasters, J.J. (2002). Regulated and unregulated mitochondrial permeability transition pores: a new paradigm of pore structure and function? FEBS Lett. *512*, 1–7.

He, S., Wang, L., Miao, L., Wang, T., Du, F., Zhao, L., and Wang, X. (2009). Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell *137*, 1100–1111.

Hearps, A.C., Burrows, J., Connor, C.E., Woods, G.M., Lowenthal, R.M., and Ragg, S.J. (2002). Mitochondrial cytochrome c release precedes transmembrane depolarisation and caspase-3 activation during ceramide-induced apoptosis of Jurkat T cells. Apoptosis Int. J. Program. Cell Death *7*, 387–394.

Heinrich, M., Wickel, M., Schneider-Brachert, W., Sandberg, C., Gahr, J., Schwandner, R., Weber, T., Saftig, P., Peters, C., Brunner, J., et al. (1999). Cathepsin D targeted by acid sphingomyelinase-derived ceramide. EMBO J. *18*, 5252–5263.

Heinrich, M., Neumeyer, J., Jakob, M., Hallas, C., Tchikov, V., Winoto-Morbach, S., Wickel, M., Schneider-Brachert, W., Trauzold, A., Hethke, A., et al. (2004). Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation. Cell Death Differ. *11*, 550–563.

Hengartner, M.O. (2000). The biochemistry of apoptosis. Nature *407*, 770–776.

Herick, K., Krämer, R., and Lühring, H. (1997). Patch clamp investigation into the phosphate carrier from Saccharomyces cerevisiae mitochondria. Biochim. Biophys. Acta *1321*, 207–220.

Herker, E., Jungwirth, H., Lehmann, K.A., Maldener, C., Fröhlich, K.-U., Wissing, S., Büttner, S., Fehr, M., Sigrist, S., and Madeo, F. (2004). Chronological aging leads to apoptosis in yeast. J. Cell Biol. *164*, 501–507.

Hetz, C.A., Hunn, M., Rojas, P., Torres, V., Leyton, L., and Quest, A.F.G. (2002). Caspase-dependent initiation of apoptosis and necrosis by the Fas receptor in lymphoid cells: onset of necrosis is associated with delayed ceramide increase. J. Cell Sci. *115*, 4671–4683.

Hickey, M.A., and Chesselet, M.F. (2003). Apoptosis in Huntington's disease. Prog. Neuropsychopharmacol. Biol. Psychiatry *27*, 255–265.

Hitomi, J., Christofferson, D.E., Ng, A., Yao, J., Degterev, A., Xavier, R.J., and Yuan, J. (2008). Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell *135*, 1311–1323.

Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L., and Korsmeyer, S.J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell *75*, 241–251.

Horvitz, H.R., Sternberg, P.W., Greenwald, I.S., Fixsen, W., and Ellis, H.M. (1983). Mutations that affect neural cell lineages and cell fates during the development of the nematode Caenorhabditis elegans. Cold Spring Harb. Symp. Quant. Biol. *48 Pt 2*, 453–463.

Hsu, H., Xiong, J., and Goeddel, D.V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell *81*, 495–504.

Hsu, Y.T., Wolter, K.G., and Youle, R.J. (1997). Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc. Natl. Acad. Sci. U. S. A. *94*, 3668–3672.

Huang, J., and Philbert, M.A. (1996). Cellular responses of cultured cerebellar astrocytes to ethacrynic acid-induced perturbation of subcellular glutathione homeostasis. Brain Res. *711*, 184–192.

Hunter, D.R., and Haworth, R.A. (1979). The Ca2+-induced membrane transition in mitochondria. III. Transitional Ca2+ release. Arch. Biochem. Biophys. *195*, 468–477.

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. J. Biol. Chem. *279*, 1100–1107.

Jagasia, R., Grote, P., Westermann, B., and Conradt, B. (2005). DRP-1-mediated mitochondrial fragmentation during EGL-1-induced cell death in C. elegans. Nature *433*, 754–760.

James, D.I., Parone, P.A., Mattenberger, Y., and Martinou, J.-C. (2003). hFis1, a novel component of the mammalian mitochondrial fission machinery. J. Biol. Chem. *278*, 36373–36379.

Jenkins, G.M., Richards, A., Wahl, T., Mao, C., Obeid, L., and Hannun, Y. (1997). Involvement of yeast sphingolipids in the heat stress response of Saccharomyces cerevisiae. J. Biol. Chem. *272*, 32566–32572.

Johnson, K.M., Chen, X., Boitano, A., Swenson, L., Opipari, A.W., Jr, and Glick, G.D. (2005). Identification and validation of the mitochondrial F1F0-ATPase as the molecular target of the immunomodulatory benzodiazepine Bz-423. Chem. Biol. *12*, 485–496.

Jones, R.G., Parsons, M., Bonnard, M., Chan, V.S., Yeh, W.C., Woodgett, J.R., and Ohashi, P.S. (2000). Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels in vivo. J. Exp. Med. *191*, 1721–1734.

Joza, N., Susin, S.A., Daugas, E., Stanford, W.L., Cho, S.K., Li, C.Y., Sasaki, T., Elia, A.J., Cheng, H.Y., Ravagnan, L., et al. (2001). Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature *410*, 549–554.

Jürgensmeier, J.M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., and Reed, J.C. (1998). Bax directly induces release of cytochrome c from isolated mitochondria. Proc. Natl. Acad. Sci. U. S. A. *95*, 4997–5002.

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. Nat. Chem. Biol. *1*, 223–232.

Kauffmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J., and Evan, G. (1997). Suppression of *c-myc*-induced apoptosis by Ras signalling through PI(3)K and PKB. Nature *385*, 544–548.

Kennedy, S.G., Kandel, E.S., Cross, T.K., and Hay, N. (1999). Akt/Protein kinase B inhibits cell death by preventing the release of cytochrome c from mitochondria. Mol. Cell. Biol. *19*, 5800–5810.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer *26*, 239–257.

Kim, H., Hsieh, J.J.-D., and Cheng, E.H.-Y. (2009). Deadly splicing: Bax becomes Almighty. Mol. Cell *33*, 145–146.

Kim, H.J., Mun, J.Y., Chun, Y.J., Choi, K.H., and Kim, M.Y. (2001). Bax-dependent apoptosis induced by ceramide in HL-60 cells. FEBS Lett. *505*, 264–268.

King, C.C., Zenke, F.T., Dawson, P.E., Dutil, E.M., Newton, A.C., Hemmings, B.A., and Bokoch, G.M. (2000). Sphingosine is a novel activator of 3-phosphoinositide-dependent kinase 1. J. Biol. Chem. *275*, 18108–18113.

Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H., and Peter, M.E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. *14*, 5579–5588.

Kissová, I., Plamondon, L.-T., Brisson, L., Priault, M., Renouf, V., Schaeffer, J., Camougrand, N., and Manon, S. (2006). Evaluation of the roles of apoptosis, autophagy, and mitophagy in the loss of plating efficiency induced by Bax expression in yeast. J. Biol. Chem. *281*, 36187–36197.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R., and Newmeyer, D.D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science *275*, 1132–1136.

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.

Kottke, M., Adam, V., Riesinger, I., Bremm, G., Bosch, W., Brdiczka, D., Sandri, G., and Panfili, E. (1988). Mitochondrial boundary membrane contact sites in brain: points of hexokinase and creatine kinase location, and control of Ca2+ transport. Biochim. Biophys. Acta *935*, 87–102.

Kowaltowski, A.J., Castilho, R.F., and Vercesi, A.E. (2001). Mitochondrial permeability transition and oxidative stress. FEBS Lett. *495*, 12–15.

Kroemer, G., and Levine, B. (2008). Autophagic cell death: the story of a misnomer. Nat. Rev. Mol. Cell Biol. *9*, 1004–1010.

Kroemer, G., and Martin, S.J. (2005). Caspase-independent cell death. Nat. Med. *11*, 725–730.

Kroemer, G., and Reed, J.C. (2000). Mitochondrial control of cell death. Nat. Med. *6*, 513–519.

Kroemer, G., Galluzzi, L., and Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. Physiol. Rev. *87*, 99–163.

Krueger, A., Schmitz, I., Baumann, S., Krammer, P.H., and Kirchhoff, S. (2001). Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. J. Biol. Chem. *276*, 20633–20640.

Kumar, S. (2007). Caspase function in programmed cell death. Cell Death Differ. *14*, 32–43.

Kuwana, T., Mackey, M.R., Perkins, G., Ellisman, M.H., Latterich, M., Schneiter, R., Green, D.R., and Newmeyer, D.D. (2002). Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell *111*, 331–342.

Kvansakul, M., Yang, H., Fairlie, W.D., Czabotar, P.E., Fischer, S.F., Perugini, M.A., Huang, D.C.S., and Colman, P.M. (2008). Vaccinia virus anti-apoptotic F1L is a novel Bcl-2-like domain-swapped dimer that binds a highly selective subset of BH3-containing death ligands. Cell Death Differ. *15*, 1564–1571.

Lamkanfi, M., Festjens, N., Declercq, W., Vanden Berghe, T., and Vandenabeele, P. (2007). Caspases in cell survival, proliferation and differentiation. Cell Death Differ. *14*, 44–55.

Lamour, N.F., and Chalfant, C.E. (2005). Ceramide-1-phosphate: the "missing" link in eicosanoid biosynthesis and inflammation. Mol. Interv. *5*, 358–367.

Landes, T., and Martinou, J.-C. (2011). Mitochondrial outer membrane permeabilization during apoptosis: the role of mitochondrial fission. Biochim. Biophys. Acta *1813*, 540–545.

Laun, P., Pichova, A., Madeo, F., Fuchs, J., Ellinger, A., Kohlwein, S., Dawes, I., Fröhlich, K.U., and Breitenbach, M. (2001). Aged mother cells of Saccharomyces cerevisiae show markers of oxidative stress and apoptosis. Mol. Microbiol. *39*, 1166–1173.

Lee, H., Rotolo, J.A., Mesicek, J., Penate-Medina, T., Rimner, A., Liao, W.-C., Yin, X., Ragupathi, G., Ehleiter, D., Gulbins, E., et al. (2011). Mitochondrial ceramide-rich macrodomains functionalize Bax upon irradiation. PloS One *6*, e19783.

Lee, J.Y., Hannun, Y.A., and Obeid, L.M. (1996). Ceramide inactivates cellular protein kinase Calpha. J. Biol. Chem. *271*, 13169–13174.

Lee, Y., Jeong, S.-Y., Karbowski, M., Smith, C.L., and Youle, R.J. (2004). Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol. Biol. Cell *15*, 5001–5011.

Leung, A.W.C., Varanyuwatana, P., and Halestrap, A.P. (2008). The mitochondrial phosphate carrier interacts with cyclophilin D and may play a key role in the permeability transition. J. Biol. Chem. *283*, 26312–26323.

Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. (1994). H2O2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell *79*, 583–593.

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.

Liang, Q., Li, W., and Zhou, B. (2008). Caspase-independent apoptosis in yeast. Biochim. Biophys. Acta *1783*, 1311–1319.

Ligr, M., Madeo, F., Fröhlich, E., Hilt, W., Fröhlich, K.U., and Wolf, D.H. (1998). Mammalian Bax triggers apoptotic changes in yeast. FEBS Lett. *438*, 61–65.

Limaye, V., Li, X., Hahn, C., Xia, P., Berndt, M.C., Vadas, M.A., and Gamble, J.R. (2005). Sphingosine kinase-1 enhances endothelial cell survival through a PECAM-1-dependent activation of PI-3K/Akt and regulation of Bcl-2 family members. Blood *105*, 3169–3177.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell *86*, 147–157.

Lockshin, R.A. (1969). Programmed cell death. Activation of lysis by a mechanism involving the synthesis of protein. J. Insect Physiol. *15*, 1505–1516.

Lockshin, R.A., and Williams, C.M. (1965). Programd Cell Death I. Cytology of Degeneration in the Intersegmental Muscles of the Pernyi Silkmoth. J. Insect Physiol. *11*, 123–133.

Lockshin, R.A., and Zakeri, Z. (2001). Programmed cell death and apoptosis: origins of the theory. Nat. Rev. Mol. Cell Biol. *2*, 545–550.

Lockshin, R.A., and Zakeri, Z. (2007). Cell death in health and disease. J. Cell. Mol. Med. *11*, 1214–1224.

Locksley, R.M., Killeen, N., and Lenardo, M.J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell *104*, 487–501.

Lohret, T.A., and Kinnally, K.W. (1995). Multiple conductance channel activity of wild-type and voltage-dependent anion-selective channel (VDAC)-less yeast mitochondria. Biophys. J. *68*, 2299–2309.

Van Loo, G., Schotte, P., van Gurp, M., Demol, H., Hoorelbeke, B., Gevaert, K., Rodriguez, I., Ruiz-Carrillo, A., Vandekerckhove, J., Declercq, W., et al. (2001). Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. Cell Death Differ. *8*, 1136–1142.

Van Loo, G., van Gurp, M., Depuydt, B., Srinivasula, S.M., Rodriguez, I., Alnemri, E.S., Gevaert, K., Vandekerckhove, J., Declercq, W., and Vandenabeele, P. (2002). The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. Cell Death Differ. *9*, 20–26.

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.

Lowe, S.W., and Lin, A.W. (2000). Apoptosis in cancer. Carcinogenesis *21*, 485–495.

Lozano, J., Berra, E., Municio, M.M., Diaz-Meco, M.T., Dominguez, I., Sanz, L., and Moscat, J. (1994). Protein kinase C zeta isoform is critical for kappa B-dependent promoter activation by sphingomyelinase. J. Biol. Chem. *269*, 19200–19202.

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

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. Mol. Biol. Cell *13*, 2598–2606.

Ludovico, P., Sansonetty, F., Silva, M.T., and Côrte-Real, M. (2003). Acetic acid induces a programmed cell death process in the food spoilage yeast Zygosaccharomyces bailii. FEMS Yeast Res. *3*, 91–96.

Lutter, M., Fang, M., Luo, X., Nishijima, M., Xie, X., and Wang, X. (2000). Cardiolipin provides specificity for targeting of tBid to mitochondria. Nat. Cell Biol. *2*, 754–761.

Madeo, F., and Fröhlich, K.U. (2002). Comment on Severin, F.F., and Hyman, A.A. (2002). Pheromone induces programmed cell death in S. cerevisiae. Curr. Biol. 12, R233-R235. Curr. Biol. CB *12*, R445.

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

Madeo, F., Fröhlich, E., Ligr, M., Grey, M., Sigrist, S.J., Wolf, D.H., and Fröhlich, K.U. (1999). Oxygen stress: a regulator of apoptosis in yeast. J. Cell Biol. *145*, 757–767.

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

Makman, M.H., Dvorkin, B., and White, A. (1971). Evidence for induction by cortisol in vitro of a protein inhibitor of transport and phosphorylation processes in rat thymocytes. Proc. Natl. Acad. Sci. U. S. A. *68*, 1269–1273.

Mannella, C.A., Pfeiffer, D.R., Bradshaw, P.C., Moraru, I.I., Slepchenko, B., Loew, L.M., Hsieh, C.E., Buttle, K., and Marko, M. (2001). Topology of the mitochondrial inner membrane: dynamics and bioenergetic implications. IUBMB Life *52*, 93–100.

Marchetti, P., Castedo, M., Susin, S.A., Zamzami, N., Hirsch, T., Macho, A., Haeffner, A., Hirsch, F., Geuskens, M., and Kroemer, G. (1996). Mitochondrial permeability transition is a central coordinating event of apoptosis. J. Exp. Med. *184*, 1155–1160.

Martin, D.P., Schmidt, R.E., DiStefano, P.S., Lowry, O.H., Carter, J.G., and Johnson, E.M., Jr (1988). Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J. Cell Biol. *106*, 829–844.

Martínez-Abundis, E., Correa, F., Pavón, N., and Zazueta, C. (2009). Bax distribution into mitochondrial detergent-resistant microdomains is related to ceramide and cholesterol content in postischemic hearts. FEBS J. *276*, 5579–5588.

Martinez-Caballero, S., Dejean, L.M., Kinnally, M.S., Oh, K.J., Mannella, C.A., and Kinnally, K.W. (2009). Assembly of the mitochondrial apoptosis-induced channel, MAC. J. Biol. Chem. *284*, 12235–12245.

Marzo, I., Brenner, C., Zamzami, N., Jürgensmeier, J.M., Susin, S.A., Vieira, H.L., Prévost, M.C., Xie, Z., Matsuyama, S., Reed, J.C., et al. (1998a). Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science *281*, 2027–2031.

Marzo, I., Brenner, C., Zamzami, N., Jürgensmeier, J.M., Susin, S.A., Vieira, H.L., Prévost, M.C., Xie, Z., Matsuyama, S., Reed, J.C., et al. (1998b). Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science *281*, 2027–2031.

Mason, D.A., Shulga, N., Undavai, S., Ferrando-May, E., Rexach, M.F., and Goldfarb, D.S. (2005). Increased nuclear envelope permeability and Pep4p-dependent degradation of nucleoporins during hydrogen peroxide-induced cell death. FEMS Yeast Res. *5*, 1237–1251.

Masters, S.C., Yang, H., Datta, S.R., Greenberg, M.E., and Fu, H. (2001). 14-3-3 inhibits Bad-induced cell death through interaction with serine-136. Mol. Pharmacol. *60*, 1325–1331.

Mathias, S., Younes, A., Kan, C.C., Orlow, I., Joseph, C., and Kolesnick, R.N. (1993). Activation of the sphingomyelin signaling pathway in intact EL4 cells and in a cell-free system by IL-1 beta. Science *259*, 519–522.

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

Mazzoni, C., and Falcone, C. (2008). Caspase-dependent apoptosis in yeast. Biochim. Biophys. Acta *1783*, 1320–1327.

Mazzoni, C., Herker, E., Palermo, V., Jungwirth, H., Eisenberg, T., Madeo, F., and Falcone, C. (2005). Yeast caspase 1 links messenger RNA stability to apoptosis in yeast. EMBO Rep. *6*, 1076–1081.

McDonnell, T.J., Nunez, G., Platt, F.M., Hockenberry, D., London, L., McKearn, J.P., and Korsmeyer, S.J. (1990). Deregulated Bcl-2-immunoglobulin transgene expands a resting but responsive immunoglobulin M and D-expressing B-cell population. Mol. Cell. Biol. *10*, 1901–1907.

McEnery, M.W. (1992). The mitochondrial benzodiazepine receptor: evidence for association with the voltage-dependent anion channel (VDAC). J. Bioenerg. Biomembr. *24*, 63–69.

McStay, G.P., Clarke, S.J., and Halestrap, A.P. (2002). Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. Biochem. J. *367*, 541–548.

Megidish, T., Cooper, J., Zhang, L., Fu, H., and Hakomori, S. (1998). A novel sphingosine-dependent protein kinase (SDK1) specifically phosphorylates certain isoforms of 14-3-3 protein. J. Biol. Chem. *273*, 21834–21845.

Mermoud, J.E., Cohen, P.T., and Lamond, A.I. (1994). Regulation of mammalian spliceosome assembly by a protein phosphorylation mechanism. EMBO J. *13*, 5679–5688.

Mille, F., Thibert, C., Fombonne, J., Rama, N., Guix, C., Hayashi, H., Corset, V., Reed, J.C., and Mehlen, P. (2009). The Patched dependence receptor triggers apoptosis through a DRAL-caspase-9 complex. Nat. Cell Biol. *11*, 739–746.

Mitchell, P., and Moyle, J. (1965a). Stoichiometry of proton translocation through the respiratory chain and adenosine triphosphatase systems of rat liver mitochondria. Nature *208*, 147–151.

Mitchell, P., and Moyle, J. (1965b). Evidence discriminating between the chemical and the chemiosmotic mechanisms of electron transport phosphorylation. Nature *208*, 1205–1206.

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. Mol. Cell. Biol. *27*, 6446–6456.

Moreau, K., Ravikumar, B., Renna, M., Puri, C., and Rubinsztein, D.C. (2011). Autophagosome precursor maturation requires homotypic fusion. Cell *146*, 303–317.

Morel, Y., and Barouki, R. (1999). Repression of gene expression by oxidative stress. Biochem. J. *342 Pt 3*, 481–496.

Morel, F., Doussiere, J., and Vignais, P.V. (1991). The superoxide-generating oxidase of phagocytic cells. Physiological, molecular and pathological aspects. Eur. J. Biochem. FEBS *201*, 523–546.

Muchmore, S.W., Sattler, M., Liang, H., Meadows, R.P., Harlan, J.E., Yoon, H.S., Nettesheim, D., Chang, B.S., Thompson, C.B., Wong, S.L., et al. (1996). X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature *381*, 335–341.

Nagata, S. (2000). Steering anti-cancer drugs away from the TRAIL. Nat. Med. *6*, 502–503.

Nakagawa, Y., Aoki, N., Aoyama, K., Shimizu, H., Shimano, H., Yamada, N., and Miyazaki, H. (2005). Receptor-type protein tyrosine phosphatase epsilon (PTPepsilonM) is a negative regulator of insulin signaling in primary hepatocytes and liver. Zoolog. Sci. *22*, 169–175.

Nica, A.F., Tsao, C.C., Watt, J.C., Jiffar, T., Kurinna, S., Jurasz, P., Konopleva, M., Andreeff, M., Radomski, M.W., and Ruvolo, P.P. (2008). Ceramide promotes apoptosis in chronic myelogenous leukemia-derived K562 cells by a mechanism involving caspase-8 and JNK. Cell Cycle Georget. Tex *7*, 3362–3370.

Nickels, J.T., and Broach, J.R. (1996). A ceramide-activated protein phosphatase mediates ceramide-induced G1 arrest of Saccharomyces cerevisiae. Genes Dev. *10*, 382–394.

Nikolova-Karakashian, M.N., and Rozenova, K.A. (2010). Ceramide in stress response. Adv. Exp. Med. Biol. *688*, 86–108.

Nuñez, G., and Clarke, M.F. (1994). The Bcl-2 family of proteins: regulators of cell death and survival. Trends Cell Biol. *4*, 399–403.

Nury, H., Dahout-Gonzalez, C., Trézéguet, V., Lauquin, G.J.M., Brandolin, G., and Pebay-Peyroula, E. (2006). Relations between structure and function of the mitochondrial ADP/ATP carrier. Annu. Rev. Biochem. *75*, 713–741.

Obeid, L.M., Linardic, C.M., Karolak, L.A., and Hannun, Y.A. (1993). Programmed cell death induced by ceramide. Science *259*, 1769–1771.

Okazaki, T., Bell, R.M., and Hannun, Y.A. (1989). Sphingomyelin turnover induced by vitamin D3 in HL-60 cells. Role in cell differentiation. J. Biol. Chem. *264*, 19076–19080.

Olichon, A., Baricault, L., Gas, N., Guillou, E., Valette, A., Belenguer, P., and Lenaers, G. (2003). Loss of OPA1 perturbates the mitochondrial inner membrane structure and integrity, leading to cytochrome c release and apoptosis. J. Biol. Chem. *278*, 7743–7746.

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

Pacheco, A., Azevedo, F., Rego, A., Santos, J., Chaves, S.R., Côrte-Real, M., and Sousa, M.J. (2013). C2-Phytoceramide Perturbs Lipid Rafts and Cell Integrity in Saccharomyces cerevisiae in a Sterol-Dependent Manner. PloS One *8*, e74240.

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 Chichester Engl. *21*, 201–210.

Pampulha, M.E., and Loureiro-Dias, M.C. (2000). Energetics of the effect of acetic acid on growth of Saccharomyces cerevisiae. FEMS Microbiol. Lett. *184*, 69–72.

Di Paola, M., Zaccagnino, P., Montedoro, G., Cocco, T., and Lorusso, M. (2004). Ceramide induces release of pro-apoptotic proteins from mitochondria by either a Ca2+ -dependent or a Ca2+ -independent mechanism. J. Bioenerg. Biomembr. *36*, 165–170.

Pastorino, J.G., Shulga, N., and Hoek, J.B. (2002). Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis. J. Biol. Chem. *277*, 7610–7618.

Pebay-Peyroula, E., Dahout-Gonzalez, C., Kahn, R., Trézéguet, V., Lauquin, G.J.-M., and Brandolin, G. (2003). Structure of mitochondrial ADP/ATP carrier in complex with carboxyatractyloside. Nature *426*, 39–44.

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. Mol. Microbiol. *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. Mol. Microbiol. *76*, 1398–1410.

Perkins, G., Renken, C., Martone, M.E., Young, S.J., Ellisman, M., and Frey, T. (1997). Electron tomography of neuronal mitochondria: three-dimensional structure and organization of cristae and membrane contacts. J. Struct. Biol. *119*, 260–272.

Del Peso, L., González-García, M., Page, C., Herrera, R., and Nuñez, G. (1997). Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science *278*, 687–689.

Petros, A.M., Olejniczak, E.T., and Fesik, S.W. (2004). Structural biology of the Bcl-2 family of proteins. Biochim. Biophys. Acta *1644*, 83–94.

Pettus, B.J., Chalfant, C.E., and Hannun, Y.A. (2002). Ceramide in apoptosis: an overview and current perspectives. Biochim. Biophys. Acta *1585*, 114–125.

Pinto, I., Cardoso, H., Leão, C., and van Uden, N. (1989). High enthalpy and low enthalpy death in Saccharomyces cerevisiae induced by acetic acid. Biotechnol. Bioeng. *33*, 1350–1352.

Polcic, P., and Forte, M. (2003). Response of yeast to the regulated expression of proteins in the Bcl-2 family. Biochem. J. *374*, 393–402.

Powell, D.J., Hajduch, E., Kular, G., and Hundal, H.S. (2003). Ceramide disables 3-phosphoinositide binding to the pleckstrin homology domain of protein kinase B (PKB)/Akt by a PKCzeta-dependent mechanism. Mol. Cell. Biol. *23*, 7794–7808.

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. J. Cell Biol. *168*, 257–269.

Priault, M., Camougrand, N., Chaudhuri, B., Schaeffer, J., and Manon, S. (1999). Comparison of the effects of bax-expression in yeast under fermentative and respiratory conditions: investigation of the role of adenine nucleotides carrier and cytochrome c. FEBS Lett. *456*, 232–238.

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

Priault, M., Camougrand, N., Kinnally, K.W., Vallette, F.M., and Manon, S. (2003b). Yeast as a tool to study Bax/mitochondrial interactions in cell death. FEMS Yeast Res. *4*, 15–27.

Pugazhenthi, S., Nesterova, A., Sable, C., Heidenreich, K.A., Boxer, L.M., Heasley, L.E., and Reusch, J.E. (2000). Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. J. Biol. Chem. *275*, 10761–10766.

Qian, S., Wang, W., Yang, L., and Huang, H.W. (2008). Structure of transmembrane pore induced by Bax-derived peptide: evidence for lipidic pores. Proc. Natl. Acad. Sci. U. S. A. *105*, 17379–17383.

Quintans, J., Bonn, G.A., McShan, C.L., and Gottschalk, A.R. (1994). Studies on the induction of apoptosis in WEHI 231 cells by pharmacological agents and lipid mediators. Sphingosine and ceramide induce apoptosis in WEHI 231. Cell Death Differ. *1*, 93–99.

Reed, J.C. (1994). Bcl-2 and the regulation of programmed cell death. J. Cell Biol. *124*, 1–6.

Rego, A., Costa, M., Chaves, S.R., Matmati, N., Pereira, H., Sousa, M.J., Moradas-Ferreira, P., Hannun, Y.A., Costa, V., and Côrte-Real, M. (2012). Modulation of mitochondrial outer membrane permeabilization and apoptosis by ceramide metabolism. PloS One *7*, e48571.

Rego, A., Trindade, D., Chaves, S.R., Manon, S., Costa, V., Sousa, M.J., and Côrte-Real, M. (2013). The yeast model system as a tool towards the understanding of apoptosis regulation by sphingolipids. FEMS Yeast Res.

Renault, T.T., Grandier-Vazeille, X., Arokium, H., Velours, G., Camougrand, N., Priault, M., Teijido, O., Dejean, L.M., and Manon, S. (2012). The cytosolic domain of human Tom22 modulates human Bax mitochondrial translocation and conformation in yeast. FEBS Lett. *586*, 116–121.

Resjö, S., Göransson, O., Härndahl, L., Zolnierowicz, S., Manganiello, V., and Degerman, E. (2002). Protein phosphatase 2A is the main phosphatase involved in the regulation of protein kinase B in rat adipocytes. Cell. Signal. *14*, 231–238.

Rodić, N., Oka, M., Hamazaki, T., Murawski, M.R., Jorgensen, M., Maatouk, D.M., Resnick, J.L., Li, E., and Terada, N. (2005). DNA methylation is required for silencing of ant4, an adenine nucleotide translocase selectively expressed in mouse embryonic stem cells and germ cells. Stem Cells Dayt. Ohio *23*, 1314–1323.

Rodriguez-Lafrasse, C., Alphonse, G., Broquet, P., Aloy, M.T., Louisot, P., and Rousson, R. (2001). Temporal relationships between ceramide production, caspase activation and mitochondrial dysfunction in cell lines with varying sensitivity to anti-Fas-induced apoptosis. Biochem. J. *357*, 407–416.

Rostovtseva, T.K., Antonsson, B., Suzuki, M., Youle, R.J., Colombini, M., and Bezrukov, S.M. (2004). Bid, but not Bax, regulates VDAC channels. J. Biol. Chem. *279*, 13575–13583.

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

Rudin, C.M., and Thompson, C.B. (1997). Apoptosis and disease: regulation and clinical relevance of programmed cell death. Annu. Rev. Med. *48*, 267–281.

Ruvolo, P.P., Deng, X., Ito, T., Carr, B.K., and May, W.S. (1999). Ceramide induces Bcl2 dephosphorylation via a mechanism involving mitochondrial PP2A. J. Biol. Chem. *274*, 20296–20300.

Sartorius, U., Schmitz, I., and Krammer, P.H. (2001). Molecular mechanisms of death-receptor-mediated apoptosis. Chembiochem Eur. J. Chem. Biol. *2*, 20–29.

Saunders, J.W., Jr (1966). Death in embryonic systems. Science *154*, 604–612.

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

Schafer, B., Quispe, J., Choudhary, V., Chipuk, J.E., Ajero, T.G., Du, H., Schneiter, R., and Kuwana, T. (2009). Mitochondrial outer membrane proteins assist Bid in Bax-mediated lipidic pore formation. Mol. Biol. Cell *20*, 2276–2285.

Scheckhuber, C.Q., Erjavec, N., Tinazli, A., Hamann, A., Nyström, T., and Osiewacz, H.D. (2007). Reducing mitochondrial fission results in increased life span and fitness of two fungal ageing models. Nat. Cell Biol. *9*, 99–105.

Schendel, S.L., Xie, Z., Montal, M.O., Matsuyama, S., Montal, M., and Reed, J.C. (1997). Channel formation by antiapoptotic protein Bcl-2. Proc. Natl. Acad. Sci. U. S. A. *94*, 5113–5118.

Schimmer, A.D. (2004). Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. Cancer Res. *64*, 7183–7190.

Schinzel, A.C., Takeuchi, O., Huang, Z., Fisher, J.K., Zhou, Z., Rubens, J., Hetz, C., Danial, N.N., Moskowitz, M.A., and Korsmeyer, S.J. (2005). Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. Proc. Natl. Acad. Sci. U. S. A. *102*, 12005–12010.

Schraufstätter, I., Hyslop, P.A., Jackson, J.H., and Cochrane, C.G. (1988). Oxidant-induced DNA damage of target cells. J. Clin. Invest. *82*, 1040–1050.

Schubert, K.M., Scheid, M.P., and Duronio, V. (2000). Ceramide inhibits protein kinase B/Akt by promoting dephosphorylation of serine 473. J. Biol. Chem. *275*, 13330–13335.

Schug, Z.T., and Gottlieb, E. (2009). Cardiolipin acts as a mitochondrial signalling platform to launch apoptosis. Biochim. Biophys. Acta *1788*, 2022–2031.

Scorrano, L., Petronilli, V., Colonna, R., Di Lisa, F., and Bernardi, P. (1999). Chloromethyltetramethylrosamine (Mitotracker Orange) induces the mitochondrial permeability transition and inhibits respiratory complex I. Implications for the mechanism of cytochrome c release. J. Biol. Chem. *274*, 24657–24663.

Shi, Y. (2002). Mechanisms of caspase activation and inhibition during apoptosis. Mol. Cell *9*, 459–470.

Shimizu, S., Narita, M., and Tsujimoto, Y. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature *399*, 483–487.

Shimizu, S., Shinohara, Y., and Tsujimoto, Y. (2000). Bax and Bcl-xL independently regulate apoptotic changes of yeast mitochondria that require VDAC but not adenine nucleotide translocator. Oncogene *19*, 4309–4318.

Siemen, D., and Ziemer, M. (2013). What is the nature of the mitochondrial permeability transition pore and what is it not? IUBMB Life *65*, 255–262.

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. Mol. Microbiol. *58*, 824–834.

Silva, R.D., Manon, S., Gonçalves, J., Saraiva, L., and Côrte-Real, M. (2011). The importance of humanized yeast to better understand the role of bcl-2 family in apoptosis: finding of novel therapeutic opportunities. Curr. Pharm. Des. *17*, 246–255.

Simon, H.U., Haj-Yehia, A., and Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis Int. J. Program. Cell Death *5*, 415–418.

De Simone, C., Grazia Cifone, M., Roncaioli, P., Moretti, S., Famularo, G., Alesse, E., Boschini, A., and Testi, R. (1996). Ceramide, AIDS and long-term survivors. Immunol. Today *17*, 48.

Siskind, L.J., and Colombini, M. (2000). The lipids C2- and C16-ceramide form large stable channels. Implications for apoptosis. J. Biol. Chem. *275*, 38640–38644.

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

Siskind, L.J., Fluss, S., Bui, M., and Colombini, M. (2005). Sphingosine forms channels in membranes that differ greatly from those formed by ceramide. J. Bioenerg. Biomembr. *37*, 227–236.

Siskind, L.J., Feinstein, L., Yu, T., Davis, J.S., Jones, D., Choi, J., Zuckerman, J.E., Tan, W., Hill, R.B., Hardwick, J.M., et al. (2008). Anti-apoptotic Bcl-2 Family Proteins Disassemble Ceramide Channels. J. Biol. Chem. *283*, 6622–6630.

Siskind, L.J., Mullen, T.D., Romero Rosales, K., Clarke, C.J., Hernandez-Corbacho, M.J., Edinger, A.L., and Obeid, L.M. (2010). The BCL-2 protein BAK is required for long-chain ceramide generation during apoptosis. J. Biol. Chem. *285*, 11818–11826.

Slee, E.A., Adrain, C., and Martin, S.J. (2001). Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. J. Biol. Chem. *276*, 7320–7326.

Smale, G., Nichols, N.R., Brady, D.R., Finch, C.E., and Horton, W.E., Jr (1995). Evidence for apoptotic cell death in Alzheimer's disease. Exp. Neurol. *133*, 225–230.

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. Biochim. Biophys. Acta *1757*, 1366–1370.

Srinivasula, S.M., Ahmad, M., Guo, Y., Zhan, Y., Lazebnik, Y., Fernandes-Alnemri, T., and Alnemri, E.S. (1999). Identification of an endogenous dominant-negative short isoform of caspase-9 that can regulate apoptosis. Cancer Res. *59*, 999–1002.

Stratford, S., Hoehn, K.L., Liu, F., and Summers, S.A. (2004). Regulation of insulin action by ceramide: dual mechanisms linking ceramide accumulation to the inhibition of Akt/protein kinase B. J. Biol. Chem. *279*, 36608–36615.

Sugioka, R., Shimizu, S., and Tsujimoto, Y. (2004). Fzo1, a protein involved in mitochondrial fusion, inhibits apoptosis. J. Biol. Chem. *279*, 52726–52734.

Sulston, J.E., and Horvitz, H.R. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev. Biol. *56*, 110–156.

Suzuki, T., Yokozaki, H., Kuniyasu, H., Hayashi, K., Naka, K., Ono, S., Ishikawa, T., Tahara, E., and Yasui, W. (2000). Effect of trichostatin A on cell growth and expression of cell cycle- and apoptosis-related molecules in human gastric and oral carcinoma cell lines. Int. J. Cancer J. Int. Cancer *88*, 992–997.

Tait, S.W.G., and Green, D.R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. Nat. Rev. Mol. Cell Biol. *11*, 621–632.

Tata, J.R. (1966). Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. Dev. Biol. *13*, 77–94.

Tepper, A.D., de Vries, E., van Blitterswijk, W.J., and Borst, J. (1999). Ordering of ceramide formation, caspase activation, and mitochondrial changes during CD95- and DNA damage-induced apoptosis. J. Clin. Invest. *103*, 971–978.

Thannickal, V.J., and Fanburg, B.L. (2000). Reactive oxygen species in cell signaling. Am. J. Physiol. Lung Cell. Mol. Physiol. *279*, L1005–1028.

Thorburn, A. (2004). Death receptor-induced cell killing. Cell. Signal. *16*, 139–144.

Tondera, D., Grandemange, S., Jourdain, A., Karbowski, M., Mattenberger, Y., Herzig, S., Da Cruz, S., Clerc, P., Raschke, I., Merkwirth, C., et al. (2009). SLP-2 is required for stress-induced mitochondrial hyperfusion. EMBO J. *28*, 1589–1600.

Tzivion, G., and Avruch, J. (2002). 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. J. Biol. Chem. *277*, 3061–3064.

Uribe-Carvajal, S., Luévano-Martínez, L.A., Guerrero-Castillo, S., Cabrera-Orefice, A., Corona-de-la-Peña, N.A., and Gutiérrez-Aguilar, M. (2011). Mitochondrial Unselective Channels throughout the eukaryotic domain. Mitochondrion *11*, 382–390.

Váchová, L., and Palková, Z. (2005). Physiological regulation of yeast cell death in multicellular colonies is triggered by ammonia. J. Cell Biol. *169*, 711–717.

Vakifahmetoglu, H., Olsson, M., and Zhivotovsky, B. (2008a). Death through a tragedy: mitotic catastrophe. Cell Death Differ. *15*, 1153–1162.

Vakifahmetoglu, H., Olsson, M., Tamm, C., Heidari, N., Orrenius, S., and Zhivotovsky, B. (2008b). DNA damage induces two distinct modes of cell death in ovarian carcinomas. Cell Death Differ. *15*, 555–566.

Vakkila, J., and Lotze, M.T. (2004). Inflammation and necrosis promote tumour growth. Nat. Rev. Immunol. *4*, 641–648.

Vandenabeele, P., Galluzzi, L., Vanden Berghe, T., and Kroemer, G. (2010). Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat. Rev. Mol. Cell Biol. *11*, 700–714.

Varanyuwatana, P., and Halestrap, A.P. (2012). The roles of phosphate and the phosphate carrier in the mitochondrial permeability transition pore. Mitochondrion *12*, 120–125.

Vaux, D.L., Cory, S., and Adams, J.M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with *c-myc* to immortalize pre-B cells. Nature *335*, 440–442.

Veldman, R.J., Klappe, K., Hoekstra, D., and Kok, J.W. (1998). Metabolism and apoptotic properties of elevated ceramide in HT29rev cells. Biochem. J. *331 (Pt 2)*, 563–569.

Venable, M.E., Lee, J.Y., Smyth, M.J., Bielawska, A., and Obeid, L.M. (1995). Role of ceramide in cellular senescence. J. Biol. Chem. *270*, 30701–30708.

Verhaegen, S., McGowan, A.J., Brophy, A.R., Fernandes, R.S., and Cotter, T.G. (1995). Inhibition of apoptosis by antioxidants in the human HL-60 leukemia cell line. Biochem. Pharmacol. *50*, 1021–1029.

Verheij, M., Bose, R., Lin, X.H., Yao, B., Jarvis, W.D., Grant, S., Birrer, M.J., Szabo, E., Zon, L.I., Kyriakis, J.M., et al. (1996). Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature *380*, 75–79.

Waldmeier, P.C., Feldtrauer, J.-J., Qian, T., and Lemasters, J.J. (2002). Inhibition of the mitochondrial permeability transition by the nonimmunosuppressive cyclosporin derivative NIM811. Mol. Pharmacol. *62*, 22–29.

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. J. Cell Sci. *119*, 1843–1851.

Watson, R.W., Rotstein, O.D., Jimenez, M., Parodo, J., and Marshall, J.C. (1997). Augmented intracellular glutathione inhibits Fas-triggered apoptosis of activated human neutrophils. Blood *89*, 4175–4181.

Wei, M.C., Lindsten, T., Mootha, V.K., Weiler, S., Gross, A., Ashiya, M., Thompson, C.B., and Korsmeyer, S.J. (2000). tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev. *14*, 2060–2071.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B., and Korsmeyer, S.J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science *292*, 727–730.

Westphal, D., Dewson, G., Czabotar, P.E., and Kluck, R.M. (2011). Molecular biology of Bax and Bak activation and action. Biochim. Biophys. Acta *1813*, 521–531.

Westwick, J.K., Bielawska, A.E., Dbaibo, G., Hannun, Y.A., and Brenner, D.A. (1995). Ceramide activates the stress-activated protein kinases. J. Biol. Chem. *270*, 22689–22692.

Wiesner, D.A., Kilkus, J.P., Gottschalk, A.R., Quintáns, J., and Dawson, G. (1997). Anti-immunoglobulin-induced apoptosis in WEHI 231 cells involves the slow formation of ceramide from sphingomyelin and is blocked by bcl-XL. J. Biol. Chem. *272*, 9868–9876.

Willaime, S., Vanhoutte, P., Caboche, J., Lemaigre-Dubreuil, Y., Mariani, J., and Brugg, B. (2001). Ceramide-induced apoptosis in cortical neurons is mediated by an increase in p38 phosphorylation and not by the decrease in ERK phosphorylation. Eur. J. Neurosci. *13*, 2037–2046.

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

Woodcock, J.M., Murphy, J., Stomski, F.C., Berndt, M.C., and Lopez, A.F. (2003). The dimeric versus monomeric status of 14-3-3zeta is controlled by phosphorylation of Ser58 at the dimer interface. J. Biol. Chem. *278*, 36323–36327.

Woodfield, K., Rück, 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. Biochem. J. *336 (Pt 2)*, 287–290.

Xin, M., and Deng, X. (2006). Protein phosphatase 2A enhances the proapoptotic function of Bax through dephosphorylation. J. Biol. Chem. *281*, 18859–18867.

Xin, M., Gao, F., May, W.S., Flagg, T., and Deng, X. (2007). Protein kinase Czeta abrogates the proapoptotic function of Bax through phosphorylation. J. Biol. Chem. *282*, 21268–21277.

Xing, H., Zhang, S., Weinheimer, C., Kovacs, A., and Muslin, A.J. (2000). 14-3-3 proteins block apoptosis and differentially regulate MAPK cascades. EMBO J. *19*, 349–358.

Xu, Q., Jürgensmeier, J.M., and Reed, J.C. (1999). Methods of assaying Bcl-2 and Bax family proteins in yeast. Methods San Diego Calif *17*, 292–304.

Yang, Z., Khoury, C., Jean-Baptiste, G., and Greenwood, M.T. (2006). Identification of mouse sphingomyelin synthase 1 as a suppressor of Bax-mediated cell death in yeast. FEMS Yeast Res. *6*, 751–762.

Youle, R.J., and Karbowski, M. (2005). Mitochondrial fission in apoptosis. Nat. Rev. Mol. Cell Biol. *6*, 657–663.

Yun, M., Han, Y.-H., Yoon, S.H., Kim, H.Y., Kim, B.-Y., Ju, Y.-J., Kang, C.-M., Jang, S.H., Chung, H.-Y., Lee, S.-J., et al. (2009). p31comet Induces cellular senescence through p21 accumulation and Mad2 disruption. Mol. Cancer Res. MCR *7*, 371–382.

Zamzami, N., Maisse, C., Métivier, D., and Kroemer, G. (2001). Measurement of membrane permeability and permeability transition of mitochondria. Methods Cell Biol. *65*, 147–158.

Zhang, G., Park, M.A., Mitchell, C., Walker, T., Hamed, H., Studer, E., Graf, M., Rahmani, M., Gupta, S., Hylemon, P.B., et al. (2008). Multiple cyclin kinase inhibitors promote bile acid-induced apoptosis and autophagy in primary hepatocytes via p53-CD95-dependent signaling. J. Biol. Chem. *283*, 24343–24358.

Zhang, M., Li, Y., Zhang, H., and Xue, S. (2001). BAPTA blocks DNA fragmentation and chromatin condensation downstream of caspase-3 and DFF activation in HT-induced apoptosis in HL-60 cells. Apoptosis Int. J. Program. Cell Death *6*, 291–297.

Zhang, P., Liu, B., Jenkins, G.M., Hannun, Y.A., and Obeid, L.M. (1997). Expression of neutral sphingomyelinase identifies a distinct pool of sphingomyelin involved in apoptosis. J. Biol. Chem. *272*, 9609–9612.

Zhang, Y., Ranta, F., Tang, C., Shumilina, E., Mahmud, H., Föller, M., Ullrich, S., Häring, H.-U., and Lang, F. (2009). Sphingomyelinase dependent apoptosis following treatment of pancreatic beta-cells with amyloid peptides Abeta(1-42) or IAPP. Apoptosis Int. J. Program. Cell Death *14*, 878–889.

Zhou, Y., and Ling, E.A. (1997). Effects of acute complete outlet obstruction on the NADPH-diaphorase reactivity in the intramural ganglia of the guinea pig urinary bladder: light and electron microscopic studies. J. Urol. *158*, 916–923.

Zoratti, M., and Szabò, I. (1995). The mitochondrial permeability transition. Biochim. Biophys. Acta *1241*, 139–176.

Zundel, W., and Giaccia, A. (1998). Inhibition of the anti-apoptotic PI(3)K/Akt/Bad pathway by stress. Genes Dev. *12*, 1941–1946.

Chapter II

Assessment of oxidative modifications of the Aac2p, and its contribution to mitochondria membrane permeabilization in yeast.

Trindade, D., Manon, S., Sousa, M.J., Côrte-Real, M. - Assessment of oxidative modifications of the Aac2p, and its contribution to mitochondria membrane permeabilization in yeast.

Manuscript in preparation

1. Introduction

Mitochondrial outer membrane permeabilization (MOMP) is considered a crucial event in the intrinsic mammalian apoptotic pathway, enabling the release of apoptogenic molecules, such as cytochrome *c* (cyt *c*), from mitochondria to the cytosol (Tait and Green, 2010). In spite of its importance for apoptosis, the molecular mechanisms responsible for MOMP are still a matter of debate. Different mechanisms underlying such mitochondrial events have been suggested involving the pro-apoptotic members of the Bcl-2 protein family, putative components of the permeability transition pore (PTP) and the formation of ceramide channels, all of which might be involved in a complex interplay that determines the fate of a cell (Desagher and Martinou, 2000; Siskind, 2005).

In mammals, the formation of a mitochondrial pore designated PTP, which induces mitochondria swelling and subsequent rupture of the outer mitochondrial membrane (OMM), is considered a key step in mitochondria permeabilization and cell death (Kroemer and Reed, 2000). Mitochondrial PTP is a non-specific pore that allows the passage of virtually any molecule with a molecular mass inferior to 1500 Da, occurring in the inner mitochondrial membrane (IMM) under conditions of elevated matrix Ca^{2+} concentrations, especially when this is accompanied by oxidative stress and depletion of adenine nucleotides (Halestrap et al., 2002). The exact molecular composition of the PTP is not entirely defined, although it is generally accepted that opening of PTP implicates a multicomponent protein complex (PTPC) and not a single protein (Siemen and Ziemer, 2013). PTPC assembly is thought to occur at the contact sites of the inner and outer mitochondrial membranes (Beutner et al., 1996), and several different proteins have been considered as either structural or regulatory components of the pore. The first candidates included the voltage-dependent anion channel (VDAC) in the OMM (Szabo et al., 1993; Szabo and Zoratti, 1993), the adenine nucleotide translocator (ANT) in the IMM (Halestrap and Davidson, 1990), and cyclophylin D (CyP-D) in the matrix (Griffiths and Halestrap, 1991; Halestrap and Davidson, 1990). Other proteins such as hexokinase (Beutner et al., 1998; Beutner et al., 1996), the translocator protein TSPO (a.k.a. peripheral benzodiazepine (Bz) receptor) (Hirsch et al., 1998) and Bcl-2 family members (Marzo et al., 1998; Zamzami and Kroemer, 2001) have been proposed to participate in the PTPC assembly and/or regulation. This original model of PTPC has

evolved. While the contribution of some proteins to the PTPC was questioned, new elements were introduced as possible members of the pore. In 2008, the mitochondrial phosphate (Pi) carrier was brought into this issue and suggested as an important component of the PTPC, an hypothesis based on the interactions of the carrier with CyP-D and the ANT (Leung et al., 2008). Recently, Giorgio and colleagues observed that purified dimers of the F_1F_0 ATP synthase reconstituted into lipid bilayers where able to trigger the opening of a channel with a conductance identical to that of the PTP, and thus proposing a new model for the pore (Giorgio et al., 2013).

The hypothesis of ANT's involvement in the PTP probably arose from the observation that Ca^{2+}-induced MOMP can be modulated by ligands of the ANT, such as the ANT's inhibitors atractyloside and bongkrekic acid or its own substrate ADP (Haworth and Hunter, 1979; Hunter and Haworth, 1979). Supporting this idea, a highly purified and functional ANT was incorporated in, and successfully permeabilized asolectin/cardiolipin vesicles in the presence of Ca^{2+} (Ruck et al., 1998). This effect was inhibited by ADP, promoted by atractyloside and insensitive to cyclosporine A (which binds to CyP-D), driving the authors to conclude that ANT itself is capable of adopting a pore-like structure under conditions known to induce PTP opening (Ruck et al., 1998). The concept of the ANT as a core component of the PTP was seriously questioned when Kokoszka and colleagues observed that mouse liver mitochondria lacking the two isoforms of ANT could still undergo PT induced by different promoters of cell death (Kokoszka et al., 2004). Under such experimental conditions, PTP opening simply required higher concentrations of Ca^{2+}, and could no longer be regulated by the ANT ligands (Kokoszka et al., 2004). Meanwhile, a novel ANT gene, *ANT4*, was identified is mouse stem cells casting some doubts on the idea that the ANT would be irrelevant for PTP opening (Rodic et al., 2005). It seems reasonable to assume that, even if it is not a core component of the PTP, the ANT might play important regulatory functions in some scenarios of cell death.

The ADP/ATP carrier (AAC), a yeast orthologue of mammalian ANT, has been identified as a necessary element to MOMP, and consequent release of cyt *c*, during cell death induced by acetic acid in yeast (Pereira et al., 2007). Absence of AAC proteins increases the resistance of *Saccharomyces cerevisiae* to acetic acid, yet the biochemical events underlying such process remain poorly understood (Pereira et al., 2007). Deletion of Aac2p, the major isoform of the AAC expressed under derepressed conditions (Kolarov et al., 1990), is associated with a massive reduction in cyt *c* content

and respiration (Gawaz et al., 1990). The role of Aac2p in acetic acid-induced cell death, however, does not depend on the carrier's capacity to translocate adenine nucleotides since op1, a mutated form of Aac2p affected on its transport activity, was still able to promote MOMP (Pereira et al., 2007). Opening of the PTP has been associated with several distinct mechanisms including the chemical modification of PTPC proteins, such as protein oxidation. ADP/ATP translocases are known to be sensitive to oxidative stress, especially in the thiol groups of cysteine residues (McStay et al., 2002). Thiol reagents and oxidative stress, known inducers of MPT, are able to modify thiol groups in the ANT (Halestrap et al., 1997), and the cross-linking of ANT thiols has been proposed as a mechanism capable of inducing PTP opening (Costantini et al., 2000). In yeast, it has been proposed that ROS-mediated Aac2p modifications are responsible for reduced proliferation rates of *S. cerevisiae* cells in response to an over-activation of RAS/protein kinase A (PKA) signaling (Hlavata et al., 2008). Such effect was independent of the ADP/ATP transport activity, leading the authors to suggest that oxidative modifications to the Aac2p might contribute to a PTP-like function in yeast mitochondria.

The present work aimed to understand whether oxidative modifications of the Aac2p may contribute to MOMP and consequent release of cyt *c*, during acetic acid induced programmed cell death (PCD) in *S. cerevisiae*. For such purpose, *S. cerevisiae* strain JL1-3, lacking the three isoforms of the AAC (*AAC1*, *2* and *3*), was transformed with two different copies of the *AAC2* gene, a wild-type (*wt*) and a cysteine-less *AAC2* (*CL*), in which the 4 cysteine residues were replaced by alanines. Different aspects of the acetic acid-induced cell death including survival, plasma membrane integrity, ROS production and mitochondrial membrane potential, were evaluated. Data collected during this study suggest that the cysteines in the Aac2p are not responsible, *per se*, for promoting a biochemical change capable of leading the Aac2p to a PTP-like function in yeast mitochondria. Moreover, the mechanisms involved in acetic acid-induced cell death do not seem to depend on the oxidation of the Aac2p.

2. Materials and Methods

2.1. Yeast strains, plasmids and growth conditions

The yeast strains and plasmids used in this study are listed in Table 1:

Table 2 – List of strains used in the experiments described further ahead.

Name	Strain/Plasmid	Source/Reference
Δaac1/2/3	JL1-3 (MATa, *ade2*, *his3*, *leu2*, *trp1*, *ura3*, *can1*, *aac1*::*LEU2*, *aac2*::*HIS3*, *aac3*::*URA3*)	Kissova *et al.*, 2000
Aac2 *wt*	JL1-3 + pYES-P*pic2-aac2*	This study
Aac2 *CL*	JL1-3 + pYES-P*pic2-aac2-cystein-less*	This study
Aac2 *wt*-V5-His6	JL1-3 + pYES-P*pic2-aac2*-V5-His6	This study
Aac2 *CL*-V5-His6	JL1-3 + pYES-P*pic2-aac2-cystein-less*-V5-His6	This study
His6-Aac2	JL1-3 + pYES-P*pic2*-His6-*aac2*	This study

Saccharomyces cerevisiae JL1-3 (*MATa, ade2, his3, leu2, trp1, ura3, can1, Δaac1::LEU2, Δaac2::HIS3, Δaac3::URA3*), which is a derivative of W303-1B lacking the three isoforms of the AAC (*AAC1*, *AAC2* and *AAC3*) (Kissova et al., 2000), was transformed with the plasmids pYES-P*pic2-aac2*, pYES-P*pic2-aac2-CL* (*cysteine-less*) and pYES-P*pic2*-His6-*aac2*, kindly provided by Doctor Edmund Kunji, to originate a strain expressing a normal form of the Aac2p (*wt*), a cysteine less form of the carrier (*CL*) or a His6 tagged Aac2p protein (His6-Aac2p) respectively. JL1-3 was also transformed with plasmids pYES-P*pic2-aac2*-V5-His6 and pYES-P*pic2-aac2-cystein-less*-V5-His6. To construct the Aac2-V5-His6, a mutated sequences of the AAC2 *wt* and *CL* gene were amplified by PCR, using a set of primers designed to change the stop codon for a serine (TAA into TCA) and to include the restriction sites *Nho*I and *Xho*I at both ends of the mutated gene. This fragment was digested and then inserted into the pYES3-P*pic2* plasmid which carries the sequences of the V5 epitote and His6 tag followed by a stop codon. The resulting construction consisted of the *AAC2* or *AAC2* cystein-less sequence with a V5-His6 double tag attached to its C-terminal. All plasmids were amplified in *E. coli* XL-Blue, extracted using a Sigma GenElute^{TM} Plasmid extraction kit and verified by sequencing. Transformation of *S. cerevisiae* cells with the

above mentioned plasmids was done by lithium acetate general protocol (Ito et al., 1983). Transformed cells were selected and kept in SC-glucose –W selective medium (0.175% YNB, 2% Glucose, 0.5% $(NH_4)_2SO_4$, (2% agar for solid medium)) supplemented with adenine (final concentration of 40 mg/L) and without tryptophan. All strains were pre-grown in SC-glucose –W at 30°C, 200 rpm.

2.2. Acetic acid treatment

S. cerevisiae cells were grown until exponential growth phase ($O.D._{640}$ = 0.8 – 1.0; 30 °C, 200 rpm) in synthetic minimal medium with 2% glucose or 2% galactose as carbon sources, supplemented with adenine (40 mg/L). The cultures were collected and resuspended to a final concentration of 10^7 cells per mL in fresh medium adjusted to pH 3.0 with HCl and containing 160 or 180 mM acetic acid. Incubation took place for 180 min at 30 °C as previously described. At different time points, samples were collected from these cultures and diluted to 10^{-4} cell/mL. 40µL from the diluted suspensions were inoculated onto YPDA medium (2% glucose, 1% yeast extract, 1% bactopeptone and 2% agar) and colony forming units (c.f.u.) were counted after 48 h incubation at 30 °C. The percentage of viable cells was estimated considering 100% survival the number of c.f.u. obtained at time zero.

2.3. PI exclusion, $DiOC_6$ Staining and ROS production

Propidium Iodide (PI) exclusion, $DiOC_6(3)$ staining and ROS production were monitored by flow cytometry analysis. The membrane integrity of Aac2 *wt* and *CL* strains was assessed by PI (Sigma) exclusion. Cells were harvested at different time points during acetic acid treatment (160 mM), washed and resuspended in PBS (137 mM NaCl; 2.7 mM KCl; 100 mM Na2HPO4; 2 mM KH2PO4; pH 7.4) containing PI (4 µg/ml). The samples were incubated for 10 minutes at room temperature in the dark before analysis. The fluorescent probe 3,3′dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (Invitrogen) was used to evaluate how acetic acid influences the mitochondrial potential in Aac2 *wt* and *CL* mutants. $2x10^6$ acetic acid-treated cells were harvested, washed, and resuspended in a buffer (10 mM MES; 0.1mM MgCl2; 2% (wt/v) glucose, adjusted to pH 6 with $Ca(OH)_2$) containing $DiOC_6(3)$ (1 nM) and PI (4 µg/ml). Cells were analyzed by flow cytometry after 30 minutes of incubation at room temperature and protected from light. To study ROS production, $1x10^6$ Aac2 *wt* and *CL* cells were collected,

resuspended in PBS in the presence of DHE (2 μg/ml) and incubated for 30 minutes at 30 °C, in the dark. Samples were analyzed by flow cytometry.

Flow cytometry analysis was performed in an Epics® XLTM (BeckmanCoulter) flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Red fluorescence, emitted by DHE and PI, was collected through FL-3 (605/635 nm) and FL-4 (660/700 nm) sensors, respectively. Green fluorescence emitted by $DiOC_6(3)$ was collected through FL-1 (505/545 nm) sensor. The obtained data were further analyzed by WinMDI 2.8 software or Flowing Software version 2.5.0.

2.4. Mitochondrial isolation and Western-blot analysis

Mitochondria isolation was performed by differential centrifugation, after enzymatic digestion of the cell wall with zymoliase, essentially as previously described by Daum and colleagues (Daum et al., 1982). Some minor modifications were introduced to the protocol; spheroplasts were suspended in sorbitol 0.5 M, Tris/HCl pH 7.5 20 mM, EDTA 1 mM, and a Dounce homogenizer was used to obtain an even suspension. Assessment of cyt *c* release from mitochondria was performed by Western-blotting. Protein lysates were precipitated with 0.3 M trichloroacetic acid (TCA), solubilized in Laemmli buffer (2% SDS), separated on 12.5% SDS-PAGE gels and then blotted into polyvinylidene fluoride (PVDF) membranes (hybond-P; Amersham). Membranes were blocked with 5% non-fat milk in phosphate buffered saline (PBS) containing 0.05% Tween 20, for at least 1 h at room temperature. Membranes were then incubated overnight at 4 °C with primary antibodies: mouse monoclonal anti yeast phosphoglycerate kinase (*PGK1*) antibody (1:5000, Molecular Probes), mouse monoclonal anti yeast porin (*POR1*) antibody (1:5000, Molecular Probes), rabbit polyclonal anti yeast cytochrome *c* (*CYC1*) antibody (1:1000, custom-made by Millegen), rabbit polyclonal anti yeast F1F0 ATP synthase subunit β (*ATP2*) antibody (1:30000, homemade by Jean Velours, IBGC, Bordeaux - France), mouse monoclonal anti yeast cytochrome *c* oxidase subunit II (*COX2*) antibody (1:5000, Molecular Probes), rabbit polyclonal anti yeast translocase of outer mitochondrial membrane 40 (*TOM40*) antibody (1:2000, home made by Carla Koehler, UCLA, Los Angeles - USA), mouse monoclonal anti V5 epitope antibody (1:5000, Invitrogen), rabbit polyclonal anti His6 Tag antibody (1:5000, Bethyl Laboratories, Inc). Membranes were incubated for 1 h at room temperature with secondary antibodies against mouse or rabbit IgGs

(1:10000), coupled to horse radish peroxidase (Jackson Laboratories) and revealed by chemiluminescence (ECL+Amersham). Protein quantifications were performed by the Lowry method, using Folin-Ciocalteau's reagent (Lowry et al., 1951). For total protein extracts approximately $2x10^6$ cells were collected, suspended in 500 µL of water and disrupted with 50 µL of a mixture of 3.5% β-mercaptoethanol in 2 M NaOH, for 15 minutes on ice. Proteins were precipitated with 50 µL of 3 M TCA for 15 min on ice and deposited by a rapid centrifugation. The resulting pellet was washed with acetone, and resuspended in Laemmli buffer for SDS-PAGE (Laemmli, 1970). Membrane blotting and developing was performed as described above.

2.5. Redox Spectrophotometry

Cytochrome content of whole cells was measured by differential redox spectrophotometry. Cells were collected during exponential growth, and concentrated in a cellular suspension of 2 mL at ≈ 40 $O.D._{.640nm}$. The concentrated cell suspension was divided by two cuvettes and analyzed in a double beam spectrophotometer, Varian Cary 4000. The reference cuvette was oxidized with hydrogen peroxide (H_2O_2), and the sample cuvette reduced with sodium dithionite. Spectra were acquired between 500 and 650 nm and cytochromes $c+c_1$, b and $a+a_3$ were quantified by the O.D. differences, 550 nm minus 540 nm, 561 nm minus 575 nm, and 603 nm minus 630 nm, respectively, with molar extinction coefficient of 18000, 18000 and 24000 M^{-1}, respectively.

2.6. Immunoprecipitation of His6-Aac2p

Immunoprecipitation of the His6-Aac2p was performed using Pierce Protein A/G Plus Agarose from the Pierce Classic IP Kit (Thermo Scientific). All steps from this protocol were executed at 4°C, unless otherwise indicated. Briefly, mitochondria from His6-Aac2 control and acetic acid-treated cells were isolated as described above and 2 mg of mitochondrial protein were transferred to an IP column and solubilized in 500 µL of cold IP Lysis/Wash Buffer (0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP-40 (tergitol), 5% glycerol, pH 7.4), for 30 minutes with gentle mixing. To prepare the immune complex 1 µg of anti-His6 antibody was added, and the mixture incubated overnight at 4°C. Pierce Protein A/G Agarose resin was added to the antibody/lysate sample, and mixture was incubated with gentle end-over-end mixing for 6 hours, to allow the capture of the immune complex. The column was placed in a collection tube

and centrifuged for 60 seconds at 1000 x *g*. The resin was washed three times with 200 µL of cold IP Lysis/Wash Buffer, and once with 100µL of 1X Conditioning Buffer (Pierce Classic IP Kit). To elute the captured proteins, 50µL of Elution Buffer (pH 2.8, Pierce Classic IP Kit) were added to the resin and the mixture incubated for 10 minutes at room temperature, before centrifugation.

Samples of purified His6-Aac2p and mitochondria from control and acetic-acid treated cells were separated by SDS-PAGE, blotted and probed with the anti-His6 antibody. The remaining samples of purified His6-Aac2p were used for OxyBlot determinations.

2.7. Detection of protein oxidation by OxyBlot

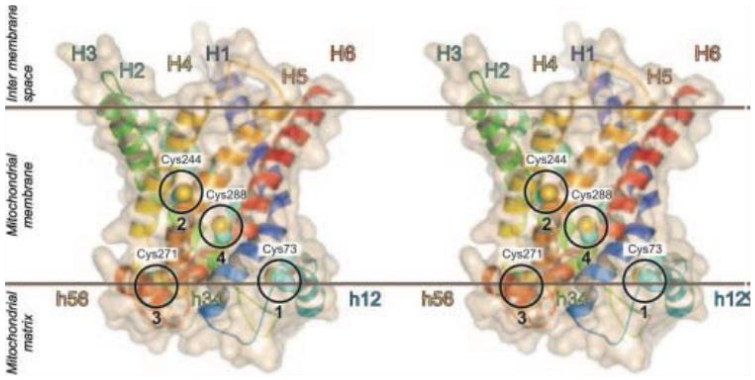
For OxyBlot determinations, samples of purified His6-Aac2p and mitochondria from control and acetic-acid treated cells were prepared according to manufacturer's instructions (OxyBlot™ Protein Oxidation Detection Kit, Millipore). Shortly, 30 µg of mitochondrial protein or 10 µL of purified His6-Aac2p were denaturated with 6% SDS and derivatized with 10 or 20 µL of 1 x 2,4-Dinitrophenylhydrazine (DNPH) solution, respectively. A control reaction was performed for the mitochondrial samples by adding 10 µL of 1 x Derivatization-Control solution to a second protein solution (30 µg of mitochondrial protein denaturated with 6% SDS). Samples were incubated at room temperature for 15 minutes, and the reaction stopped by the addition of 7.5 or 15 µL of Neutralization Solution to the mitochondrial or purified protein samples, respectively. Samples were loaded onto a polyacrylamide gel, electrophoretically separated and blotted onto a PVDF membrane. The membrane was incubated in Blocking/Dilution Buffer (1% BSA in PBS-T) for 1 hour with gentle shaking. 1° Antibody (anti-DNP) was diluted 1:150 in Blocking/Dilution Buffer, added to the membrane and incubated for 1 hour at room temperature under agitation. The membrane was rinsed twice with 1 x PBS-T, and then washed in 1 x PBS-T once for 15 minutes and twice for 5 minutes. A horseradish peroxidase-conjugated secondary Antibody was diluted 1:300, added to membrane and incubated for 1 hour at room temperature with gentle shaking. The washing steps described previously were repeated before revealing the membrane by chemiluminescence (ECL+Amersham) with a G-Box imaging system (Syngene).

3. Results

3.1. Acetic acid tolerance of Aac2p-wt and Aac2-cysteine-less mutants

A recent study from our group has identified the Aac2p as an important element in MMP, and consequent release of cyt *c*, during yeast cell death. In the absence of AAC proteins, yeast cells exhibit increased resistance to acetic acid-induced death, a phenotype that is not related to the impairment of oxidative phosphorylation (Pereira et al., 2007). This observation emphasizes the important role of mitochondria in yeast cell death.

A



B

```
1   MSSNAQVKTP LPPAPAPKKE SNFLIDFLMG GVSAAVAKTA ASPIERVKLL
51  IQNQDEMLKQ GTLDRKYAGI LDCFKRTATQ EGVISFWRGN TANVIRYFPT
101 QALNFAFKDK IKAMFGFKKE EGYAKWFAGN LASGGAAGAL SLLFVYSLDY
151 ARTRLAADSK SSKKGGARQF NGLIDVYKKT LKSDGVAGLY RGFLPSVVGI
201 VVYRGLYFGM YDSLKPLLLT GSLEGSFLAS FLLGWVVTTG ASTCSYPLDT
251 VRRRMMMTSG QAVKYDGAFD CLRKIVAAEG VGSLFKGCGA NILRGVAGAG
301 VISMYDQLQM ILFGKKFK*
```

Fig. 1 – (A) Stereoview of the structural model of the yeast ADP/ATP carrier, AAC2, presented in Bamber et al., 2007. Trans-membrane (TM) α-helices (H1, H2, H3, H4, H5 and H6) are numbered according to their order of appearance in the sequence. Two matrix helices are also identified by h12 and h56, the numbers corresponding to the two TM α-helices they connect. The four cysteines of the AAC2 are encircled and represented as van der Waals spheres. (B) Aminoacid sequence of the *S. cerevisiae* AAC2. Each one of the four cysteine residues, Cys^{73}, Cys^{244}, Cys^{271} and Cys^{288}, identified by black arrows, were replaced by alanines to produce the cysteine-less AAC2.

To understand whether oxidative modifications are contributing to the role of Aac2p in MMP, *S. cerevisiae* strains carrying either a *wt* or a cysteine-less (*CL*) Aac2p, in which all four cysteine residues were replaced by alanine residues (Fig. 1A and B), were studied in response to acetic acid. The *CL* mutant of yeast Aac2p functions like the native (*wt*) form of the carrier, with the native conformation in the mitochondrial membrane, and an initial velocity of ADP uptake to mitochondria as high as 84% that of a *wt* Aac2p, showing that these cysteines are not required for adenine nucleotide transport (Bamber et al., 2007a; Bamber et al., 2007b; Hatanaka et al., 2001). Aac2 *wt* and *CL* cultures grown in glucose or galactose as carbon sources were treated with 160 mM acetic acid, and the viability was evaluated by c.f.u. counting. In both cases, no significant differences were observed between the survival profile of *wt* and *CL* mutants (Fig. 2A). The platting efficiency of both strains decreases progressively with the exposure to acetic acid, reaching values of approximately 40% after 120 min of incubation in glucose medium (Fig. 2A). Growth and treatment in the presence of galactose as the sole carbon source, contributes to an increased toxicity of acetic acid, reaching levels as low as 10% survival after 120 min (Fig. 2A). The following experiments, characterizing the death process in the Aac2 *wt* and *CL* strains, were performed in galactose medium since it is less effective in the repression of the respiratory metabolism and allows the cells to possess a higher mitochondrial mass (Herrero et al., 1985).

To characterize the mechanisms responsible for acetic acid-induced cell death in Aac2 *wt* and *CL* strains, the preservation of plasma membrane integrity, commonly used as an indicator of cell viability and a tool to differentiate necrotic, apoptotic and normal cells in PCD assays, was evaluated by propidium iodide (PI) exclusion. The plasma membrane is impermeable to PI, a fluorescent molecule and an intercalating agent that can bind to double stranded nucleic acids and is generally excluded from healthy cells. Aac2 *wt* and *CL* cells collected during the acetic acid treatment were incubated with PI, and analysed by flow cytometry. The amount of PI positive cells, i.e. cells which have lost their plasma membrane integrity, increased along with the exposure to acetic acid in both cell types (Fig. 2B). This increase, however, does not match the loss of cell viability observed by c.f.u. counting indicating that not all dying cells have lost plasma membrane integrity, or that this event is delayed when compared to loss of plating efficiency. Interestingly, *CL* cells display a tendency to accumulate less PI than *wt* cells,

particularly upon a longer exposure to acetic acid (Fig. 2B), suggesting that the Aac2 cysteine-less mutation may result in a different cellular response to this death stimulus.

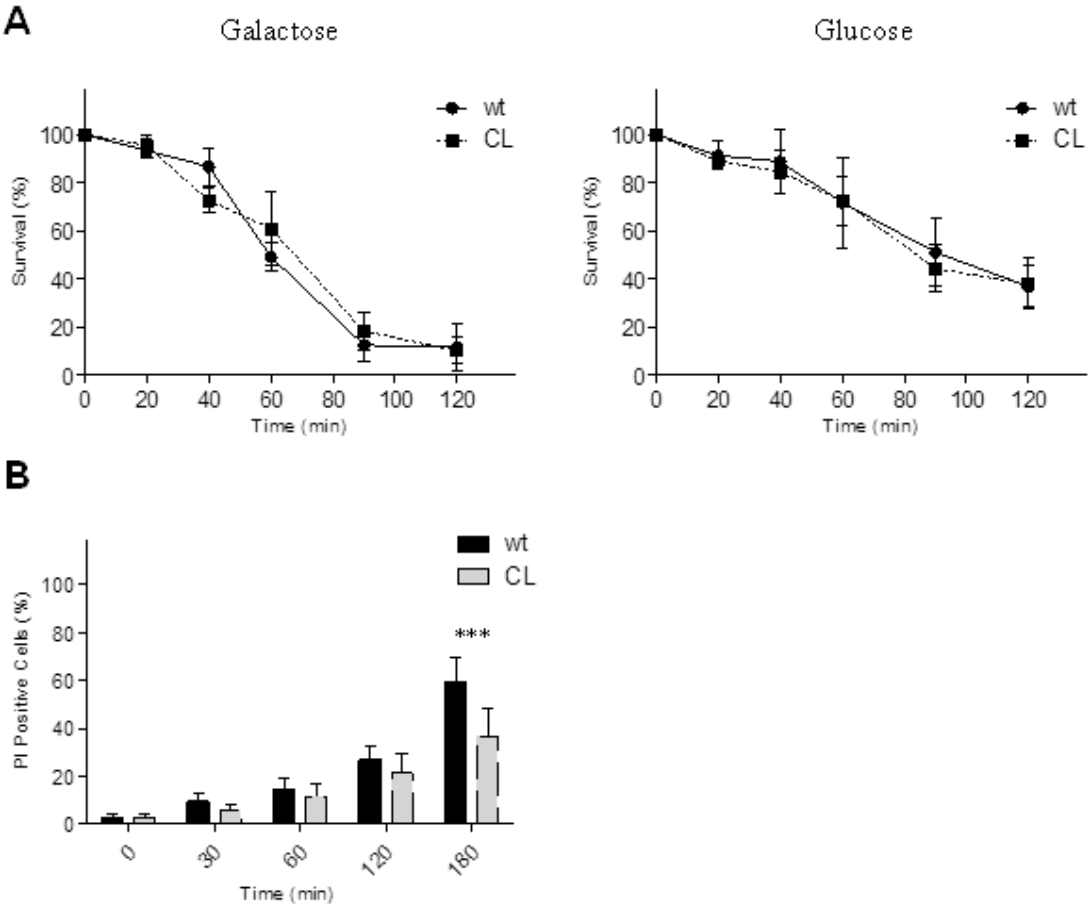


Fig. 2 – Cell death of Aac2 *wt* and Aac2 *CL* cell cultures during acetic acid treatment. A) Survival curve of cell cultures pre-grown in synthetic minimal medium, pH 3.0, with galactose (A – left panel) or glucose (A – right panel) as the sole carbon source and treated in the same medium with acetic acid (160 mM). Viability was determined by c.f.u. counts at the indicated time points (100 % survival corresponds to the total c.f.u. number at time zero). Both strains are more sensitive to acetic acid when galactose was used. Still, loss of cell viability occurs at identical rates for both strains in the two tested scenarios. B) Loss of plasma membrane integrity was assessed by PI staining in both Aac2 *wt* and Aac2 *CL* strains. Acetic acid-treated cells were collected at indicated time points, resuspended in PBS and incubated with PI (4 µg/mL), for 10 minutes in the dark, prior to flow cytometric analysis. Loss of plasma membrane integrity enhances with increasing time of exposure to acetic acid in both strains. Upon longer exposures (180 min) a difference between both strains is observed ($P<0.001$). Data represent mean ± SD of at least 3 independent experiments.

3.2. $\Delta\Psi_m$ and ROS production in Aac2p-mediated MOMP

As a central player in PCD, mitochondria are subjected to several changes in their structure and biochemical properties, some of which are universally recognized as hallmarks of the different scenarios of cell death. In healthy cells, the IMM acts as a nearly impermeable barrier to all ions, allowing the respiratory chain complexes to build up the proton gradient required for oxidative phosphorylation (Mitchell and Moyle, 1965). The charge imbalance resulting from the generation of this gradient across the IMM constitutes the inner mitochondrial membrane potential ($\Delta\Psi_m$). Since the disruption of $\Delta\Psi_m$ will affect ATP synthesis, its maintenance is vital for cellular bioenergetics (Mitchell and Moyle, 1965). Long-lasting or permanent loss of $\Delta\Psi_m$ is commonly associated with cell death and the opening of a PTP (Zamzami et al., 1995). As observed in some scenarios of mammalian apoptosis, the $\Delta\Psi_m$ of *S. cerevisae* also dissipates upon yeast exposure to a death stimulus, such as toxic concentrations of acetic acid (Ludovico et al., 2002). For the *Δaac1/2/3* strain, a possible relation between $\Delta\Psi_m$ and the increased resistance to acetic acid has been proposed, considering that the $\Delta\Psi_m$ dissipation profile from this mutant differs from that of *wt* cells (Pereira et al., 2010). $\Delta\Psi_m$ can be estimated by the capacity of mitochondria to incorporate the lipophilic cationic fluorescent probe $DiOC_6(3)$ assessed by flow cytometry (Petit et al., 1990). This probe can freely enter the cell and is concentrated in the cytoplasm driven by the plasma membrane potential ($\Delta\Psi_p$). It then enters mitochondria driven by $\Delta\Psi_m$ and accumulates on the inner surface of IMM. Since the relative cell volume affects the fluorescence measurements without revealing a real difference in $\Delta\Psi_m$, all the cytometric data were expressed as a ratio (R) between the mean of green fluorescence intensity (FL-1 channel) and the mean of frontal scatter (FS), in an attempt to normalize the fuorescence intensity for the relative cell volume. Additionally, the determinations of $\Delta\Psi_m$ in Aac2 *wt* and *CL* cells collected during acetic acid treatment were monitored in the cell population with preserved plasma membrane integrity, i.e. PI-negative cells. Fluorescence values were also normalized to time zero. The results suggest that $\Delta\Psi_m$ of both *wt* and *CL* drops shortly after exposure to acetic acid (≈ 30 min) (Fig. 3A). This effect is apparently transient, since the $\Delta\Psi_m$ of both cell types seems to recover after 1 hour of treatment. When comparing *wt* and *CL* cells, no significant differences were observed regarding the $\Delta\Psi_m$ dissipation profile.

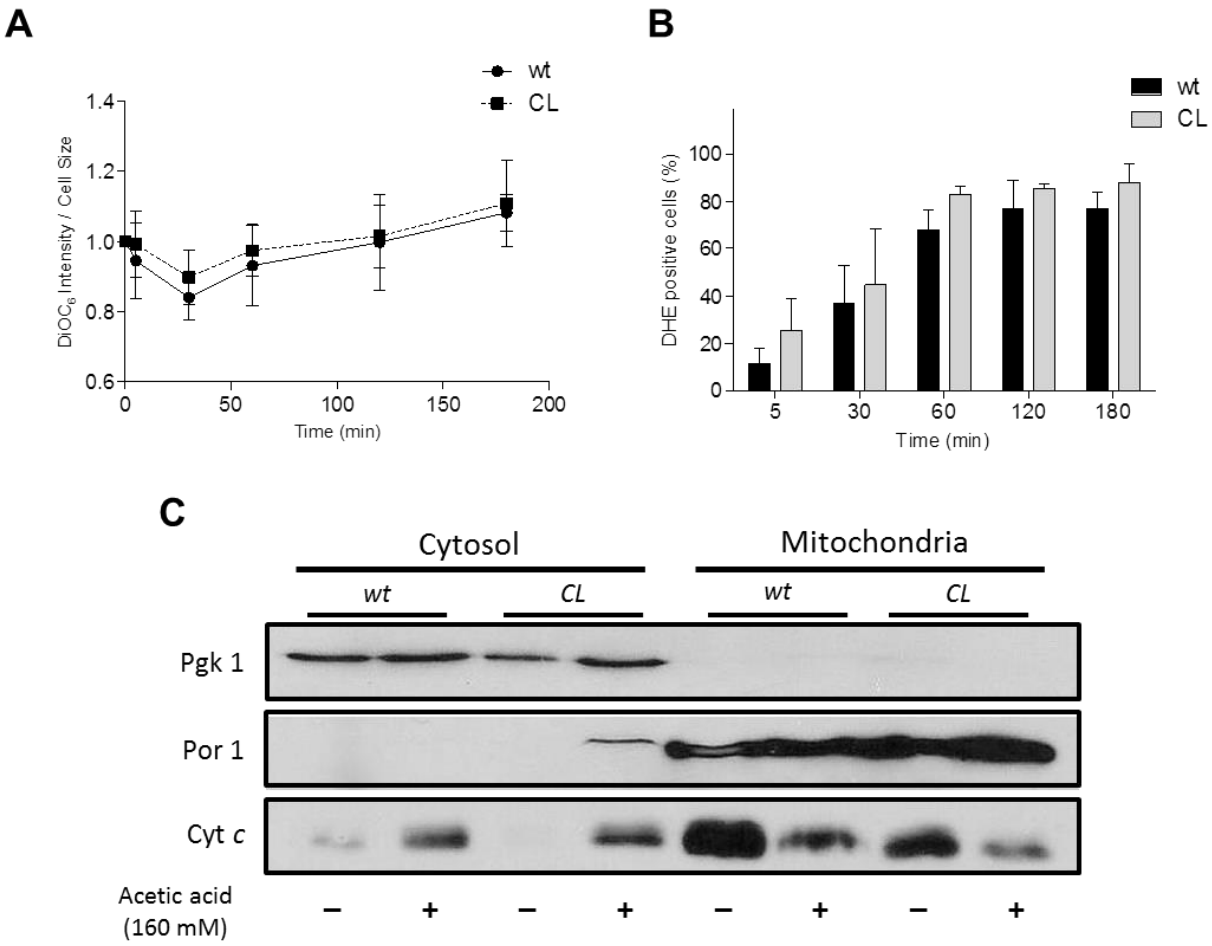


Fig. 3 – Cell death markers of Aac2 *wt* and Aac2 *CL* strains in response to 160 mM of acetic acid for 180 min in medium containing galactose. A) Variation of the mitochondrial membrane potential of Aac2 *wt* and Aac2 *CL* cells treated with acetic acid, assessed by flow cytometry using the fluorescent probe $DiOC_6(3)$. Cells were incubated in $DiOC_6(3)$ buffer containing 1 nM of $DiOC_6(3)$, for 30 minutes in the dark. Fluorescence at time zero was considered as 100%. Membrane potential changes were identical in both *wt* and *CL* cells. B) Accumulation of ROS in acetic acid-treated cells was evaluated by DHE staining. Cells were collected and incubated in PBS containing 2 µg/mL of DHE, for 30 minutes in the dark, prior to flow cytometric analysis. ROS accumulation occurs progressively in both strains. No statistical difference is observed. C) Cytochrome *c* detection by Western blot in mitochondria and corresponding cytosolic fractions obtained from Aac2 *wt* and Aac2 *CL* cells treated with acetic acid. Mitochondria from cells without any treatment were used as control. The same amount of mitochondrial and post-mitochondrial protein was loaded into the gel. Porin (Por1p) from the outer mitochondrial membrane and the cytosolic phosphoglycerate kinase (Pgk1p) were also evaluated as control for mitochondria and cytosolic fractions, respectively. Upon treatment with acetic acid cyt *c* is released from mitochondria into the cytosol. Such phenomenon was similarly observed in both strains. Data from A and B panels represent mean ± SD of at least 3 independent experiments. In panel C one representative experiment is shown. For flow cytometry assays a minimum of 35,000 cells were counted in an Epics XL Flow Cytometer.

Another common feature to numerous scenarios of PCD is the accumulation of ROS (Simon et al., 2000), and acetic acid-induced death has also been associated with ROS production by yeast cells (Ludovico et al., 2002; Pereira et al., 2007). The exact role of ROS in this process, however, remains undefined. To study ROS accumulation in Aac2 *wt* and *CL* strains, acetic acid-treated cells were stained with DHE, a probe that can react with superoxide and other oxidants to generate oxidative red fluorescent products (Zhao et al., 2003). The fluorescence resulting from oxidation of DHE was then quantified by flow cytometry. The overall result indicates that there is no significant difference in ROS accumulation between *wt* and *CL* cells treated with acetic acid (Fig. 3B). In both mutants, red fluorescence increases considerably with the exposure to the acid, showing a 5-fold greater intensity after just 1 hour of treatment. Superoxide production increases for another hour, until it finally stabilizes. This observation indicates that the *cysteine-less* form of the Aac2p is not affecting the production/accumulation of ROS during acetic acid-induced yeast cell death.

3.3. Cytochrome c release from Aac2 wt and CL mitochondria

Cyt *c* release from mitochondria during MOMP, is probably one of the most decisive events during programmed cell death. Once the OMM is permeabilized, several apoptogenic factors, including cyt *c*, are released into the cytosol, triggering a sequence of biochemical events ultimately leading to cell death (Kluck et al., 1997; Liu et al., 1996; Yang et al., 1997). In yeast cells, cyt *c* release from mitochondria was first reported by Manon and colleagues in a strain expressing the human pro-apoptotic protein Bax (Manon et al., 1997). Since then, mitochondrial release of cyt *c* has been found in different scenarios of yeast cell death, including acetic acid-induced death (Ludovico et al., 2002). Interestingly, the absence of AAC proteins impairs MOMP and cyt *c* release in *S. cerevisiae* cells treated with acetic acid, revealing an essential role of the AAC in this particular process (Pereira et al., 2007).

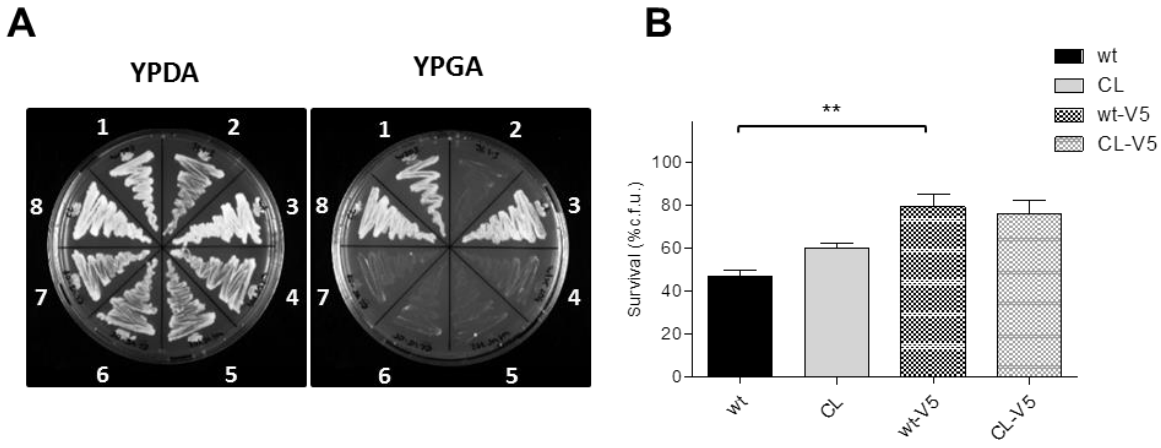
In order to test if the cysteine residues of the Aac2p, and a possible thiol crosslinking, are responsible for the Aac2p-dependent acetic acid-induced cyt *c* release, mitochondria from *wt* and *CL* cells were prepared before and after treatment with acetic acid. Mitochondria isolation was performed by differential centrifugation, after enzymatic digestion of the cell wall with zymoliase and cell disruption with a Dounce homogenizer. Cyt *c* release in both cell types was analysed by Western-blot using

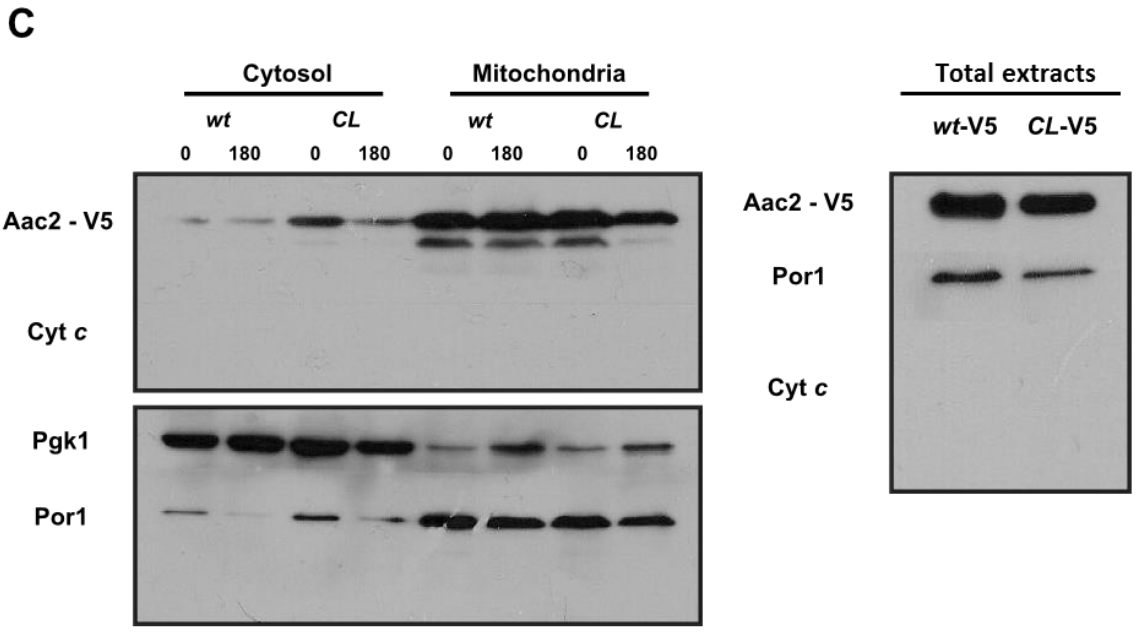
specific antibodies to detect cyt *c* in the mitochondrial extracts and the corresponding post-mitochondrial fractions. As shown in Fig. 3C, cyt *c* was released from mitochondria of *wt* cells after exposure to acetic acid, which is in agreement with previous descriptions from our group (Ludovico et al., 2002; Pereira et al., 2007). This observation is supported by the appearance of cyt *c* in the post-mitochondrial fraction of treated cells, contrasting with the cyt *c*-free fraction of untreated cells (Fig. 3C). As for *CL* mutant, the result was similar to the one of *wt* strain, displaying a drop in cyt *c* content of mitochondria from treated cells, and an increase in its post-mitochondrial fraction (Fig. 3C). These observations suggest that the cysteine residues of Aac2p, and therefore the possibility of a PTP-like function mediated by thiol crosslinking, are not associated to the role of this carrier in acetic acid-induced MOMP.

3.4. Characterization of the Aac2 wt- and CL-V5-His6 mutants

To understand if oxidative modifications are capable of shifting the Aac2p into a structure/function that can mediate MOMP, two V5-His6 double tagged Aac2p (*wt* and *CL*) were constructed. The goal was to isolate these proteins from cells treated with acetic acid, and search for carbonylated amino acid residues. Yeast strains carrying the tagged proteins, Aac2 *wt*-V5-His6 or Aac2 *CL*-V5-His6, were tested for their respiratory ability in order to estimate the impact from the tag in the capacity of Aac2 *wt* and *CL* to transport adenine nucleotides. Both *wt*-V5-His6 and *CL*-V5-His6 have impaired respiration as shown by the lack of growth on YPGA medium (2% glycerol) (Fig. 4A), indicating that the physiological role of the Aac2p is compromised in these constructions. In parallel, these strains were treated with acetic acid (160 mM) and the survival, assessed by c.f.u. counting, was compared with that of the untagged *wt* and *CL* strains. Interestingly, the V5-His6 double tagged strains exhibit an increased resistance to acetic acid when compared with the untagged *wt* and *CL* (Fig. 4B). To test if this increased resistance is related to the inhibition of acetic acid-induced cyt *c* release in the *wt*-V5-His6 and CL-V5-His6 mutants, and to confirm the mitochondrial localization of the tagged proteins, mitochondria were isolated from both cell types after exposure to acetic acid (160 mM for 3h). Using an anti-V5 antibody, the *wt*-V5-His6 and *CL*-V5-His6 forms of the carrier were detected in the obtained mitochondrial fractions at the expected size, suggesting a correct addressing of these proteins to mitochondria (Fig.

4C). Surprisingly, no cyt *c* was detected in any sample, including the ones of untreated mitochondria (Fig. 4C).





D

	Cytochrome (nmol/mg d.w.)	
	Aac2	Aac2-V5-His6
Cyt c+c_1	39,58	16,68
Cyt b	17,16	20,11
Cyt c/Cyt b Ratio	2,31	0,83

Fig. 4 – Characterization of JL1-3 strain carrying a V5-His6 double tagged Aac2 *wt* or Aac2 *CL*. A) Growth of W303 (1), JL 1-3 (2), Aac2 *wt* (3), Aac2 *wt*-V5-His6 (4 and 5), Aac2 *CL*-V5-His6 (6 and 7) and Aac2 *CL* (8) in Glucose (left panel) or Glycerol (right panel). Aac2 *wt*-V5-His6 and Aac2 *CL*-V5-His6 strains were not able to grow on medium containing glycerol as carbon source. B) Survival of Aac2 *wt*-V5-His6 and Aac2 CL-V5-His6 cell cultures during acetic acid treatment in synthetic minimal medium with galactose. Exponential cells were treated with 160 mM acetic acid for 180 min and viability determined by c.f.u. counts (100 % survival corresponds to the total c.f.u. number at time zero, values represent % c.f.u. after 180 minutes). Aac2 *wt*-V5-His6 strain was more resistance to acetic acid treatment then Aac2 *wt* ($p<0.01$). C) Western Blot analysis of mitochondria extracted from Aac2 *wt*-V5-His6 and Aac2 *CL*-V5-His6 cells before and after exposure to acetic acid (160 mM, 3 hours) (left panel). Cyt *c* and Aac2-V5-His6 proteins were detected using anti-cyt *c* and anti-V5 antibodies, respectively. Por1p and Pgk1p were used as controls. The same amount of mitochondrial and post-mitochondrial protein was loaded into the gel. No cyt *c* was detected for both strains. An identical result was obtained for total extracts of Aac2 *wt*-V5-His6 and Aac2 *CL*-V5-His6 cells (right panel). One representative experiment is shown, on each panel. D) Cytochrome content of Aac2 *wt* and Aac2 *wt*-V5-His6 cells assessed by redox spectrum analysis. Cytochrome quantities were calculated considering the maximum and minimum absorbance values for each cytochrome, ($\Delta O.D._{550nm} - \Delta O.D._{540nm}$) for cyt *c* and ($\Delta O.D._{561nm} - \Delta O.D._{575nm}$) for cyt *b*, respectively, per mg of dry weight. Aac2 *wt*-V5-His6 exhibits a lower cyt $c+c_1$ content when compared to Aac2 *wt*.

The same phenomenon was observed in total protein extracts from *wt*-V5-His6 and *CL*-V5-His6 cells (Fig. 4C), indicating that for some reason cyt *c* appears to be absent in these mutants. To clear out this possibility, the cytochrome content in the presence of a tagged and untagged Aac2p was compared by redox spectrophotometry. Cells from *wt* and *wt*-V5-His6 strains were collected during exponential growth, concentrated and divided into two cuvettes, to prepare an oxidized or reference sample and a reduced sample. When compared to the Aac2 *wt*, cells expressing the tagged carrier presented a remarkably low content of cyt *c*, resulting in a lower cyt *c*/*cyt b* ratio as well (Fig. 4D). Such evidence suggests that the insertion of the V5-His6 double tag in the C-terminal of Aac2p not only compromised the physiological role of the carrier but also resulted in the loss of cyt *c* cellular content.

3.5. Immunoprecipitation of His6-Aac2

Since the insertion of a V5-His6 double tag at the C-terminal of Aac2p affected its normal function, even resulting in the loss of cyt *c* in *wt*-V5-His6 and *CL*-V5-His6 cells, a new construction consisting of a six-histidine tag attached to the N-terminal of Aac2p

was used to immunoprecipitate the carrier. Cells containing the His6-Aac2p, were collected before and after acetic acid treatment (160 mM for 3h), and mitochondria were isolated as described above. The presence of His6-Aac2p in the mitochondrial samples was confirmed by WB using an anti-His6 antibody.

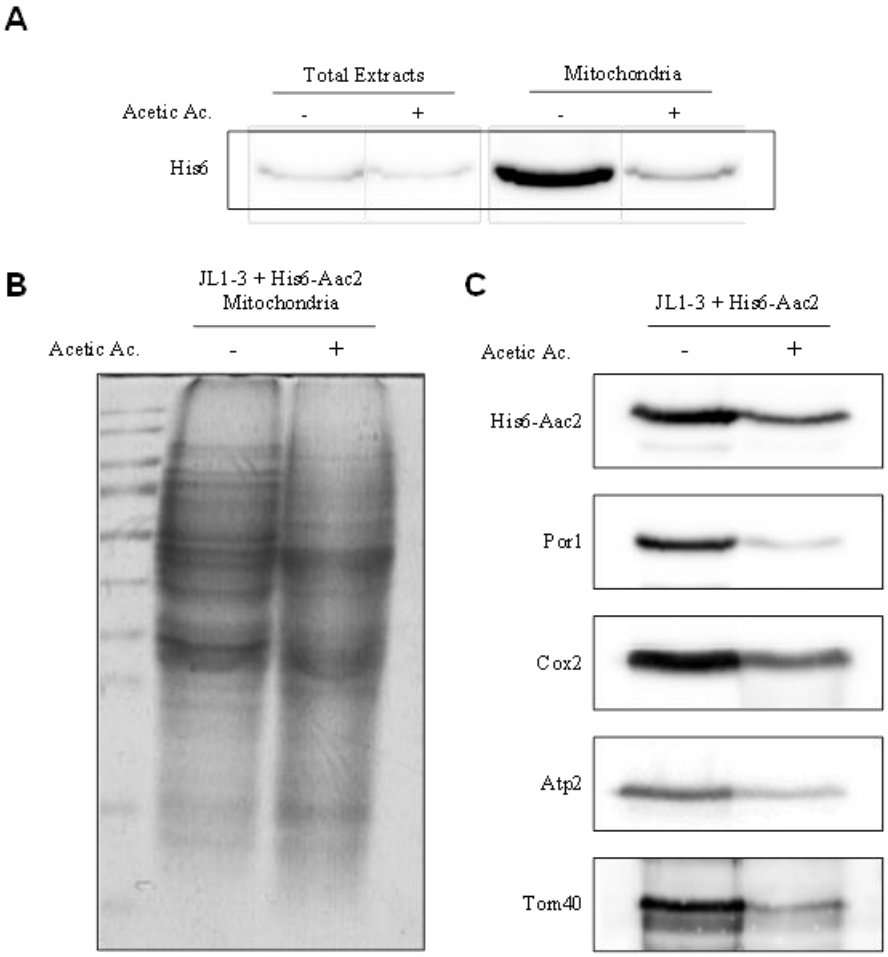


Fig. 5 – Effect of acetic acid treatment on mitochondria extracted from His6-Aac2 cells. A) Detection of His6-Aac2p in total extracts and mitochondria isolated from control and acetic acid treated cells, by Western Blot using an anti-His6 antibody. 50 µg of protein were loaded into the gel. The His6-Aac2p content in mitochondria from acetic acid-treated cells is considerably lower than that of control mitochondria. B) Mitochondria protein profile of control and acetic acid treated cells revealed by coomassie staining. Some mitochondrial proteins appear to be degraded and/or downregulated in cells exposed to acetic acid, as may be concluded by the absence or smoothness of some bands. Other proteins are expressed or upregulated under these conditions, resulting in the appearance or increased thickness of some bands. C) Detection of different mitochondrial proteins in mitochondria extracts from control and acetic acid treated His6-Aac2 cells. Both IMM proteins (His6-Aac2p, Cox2p, Atp2p) and OMM proteins (Por1p and Tom40p) are degraded or downregulated after exposure to acetic acid. For each panel, one representative experiment is shown.

Although an equivalent amount of protein from both control and acetic acid treated mitochondria was loaded into the polyacrylamide gel and blotted, mitochondria from acetic acid-treated cells have clearly less His6-Aac2p than those isolated from control cells (Fig. 5A). In fact, acetic acid exposure induces drastic changes in the mitochondrial protein profile, as observed by Coomassie staining of mitochondrial extracts from control and acetic acid-treated cells (Fig. 5B). IMM proteins such as Cox2p and Atp2 and OMM proteins such as Por1p and Tom40p seem to be degraded and/or down regulated during acetic acid treatment, suggesting that mitochondrial proteins are targeted by acetic acid (Fig. 5C). To immunoprecipitate the His6-Aac2p, 2 mg of mitochondrial protein isolated from control and acetic-acid treated cells were solubilized and incubated over-night with an Anti-His6 antibody. The immune complex was captured with Pierce Protein A/G Plus Agarose (Thermo Scientific) and the protein recovered by low-pH elution. The presence of His6-Aac2p in the purified samples was verified by Western-Blotting (Fig. 6A).

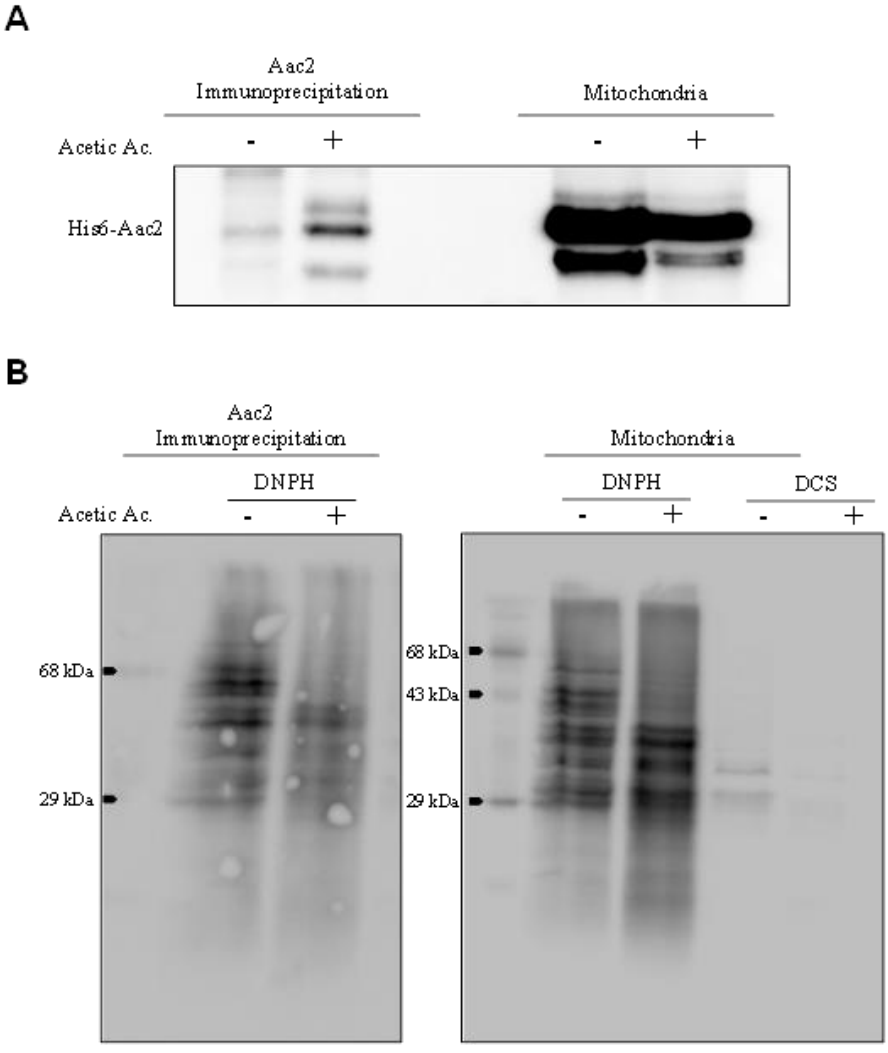


Fig. 6 – Immunoprecipitation of His6-Aac2p. A) Western Blot detection of His6-Aac2 in purified and mitochondrial samples from control cells and cells exposed to acetic acid. B) Oxyblot determination of protein carbonylation in His6-Aac2p purified samples and mitochondria extracted from control and acetic acid-treated cells. Control and acetic acid-treated samples were derivatized with DNPH. A control with Derivatization Control Solution (DCS) was performed for the mitochondrial samples.

To find out if acetic acid is able to induce the oxidation of Aac2p, the carbonylation state of purified His6-Aac2p from control and acetic acid-treated cells was evaluated using an OxyBlot kit (Milipore). Samples of purified His6-Aac2p and mitochondria extracts were derivatized with a DNPH solution, according to manufacturer's instructions, separated in a polyacrylamide gel, blotted into a PVDF membrane and the carbonyl groups detected with the provided antibodies. When comparing the protein oxidation profile of control and acetic acid-treated mitochondria, it seems that acetic acid was responsible for an increased oxidation of some proteins (Fig. 6B). This result, however, must take into consideration the fact that acetic acid is responsible for a considerable change in the mitochondrial protein profile of treated cells, as mentioned above. While some proteins might actually be more oxidized after acetic acid-treatment, others are degraded or even down- or up-regulated. As for the purified His6-Aac2p protein sample, no particular differences were observed (Fig. 6B).

4. Discussion

Since Crompton and Costi hypothesized the involvement of MPT in cell death almost 3 decades ago (Crompton and Costi, 1988), many different investigators have focused on studying the mechanisms responsible for this phenomenon. MPT is now attributed to the opening of a proteinaceous pore in the IMM, whose scaffold structure was originally thought to rely on the dynamic interactions between VDAC, ANT and CyP-D. Several studies support a role of the ANT in PTP opening, but its exact contribution for PCD is somewhat blurry (Leung and Halestrap, 2008). MOMP has also been associated to yeast cell death induced by acetic acid (Ludovico et al., 2002). Pereira and colleagues have demonstrated that AAC proteins, the yeast orthologues of mammalian ANTs, are required for stability and release of cyt *c* during acetic acid-induced cell death in *S. cerevisiae* (Pereira et al., 2007). Additionally, oxidative stress has been proposed as a possible trigger of the Aac2-mediated mitochondrial permeabilization (Hlavata et al., 2008). To understand if the role of Aac2p in acetic acid induced-MOMP is controlled by oxidative modifications to this carrier, yeast strains lacking the three isoforms of the AAC (*AAC1*, *2* and *3*) were transformed with a normal (Aac2 *wt*) or a cysteine-less Aac2p (Aac2 *CL*), and different aspects of cell death were tested.

Our data clearly indicate that the absence of cysteine residues from the Aac2p has no effect in the resistance of *S. cerevisiae* cells to acetic acid, as observed by the survival profile of the Aac2 *wt* and Aac2 *CL* strains. The plating efficiency of both strains decreases progressively and similarly upon exposure to acetic acid, an effect that is aggravated when cells were pre-grown in medium with galactose, instead of glucose, as the sole carbon source and suspended in the same medium to assess acetic acid-induced cell death. Mitochondria play a central role in acetic acid induced death, and mitochondrial proteins/function seem to be determinant for the capacity of *S. cerevisiae* to cope with the toxic effect of the acid (Sousa *et al.*, 2013; accepted for publication). Some mitochondrial proteins protected while others sensitised cells to acetic acid induced cell death. In this particular case, both strains have altered mitochondria which may affect their resistance to acetic acid. The higher mitochondrial mass of cells grown in the presence of galactose as the sole carbon source (Herrero et al., 1985) may therefore represent an increase in a cellular target for acetic acid. Accordingly,

heterologous expression of the human pro-apoptotic regulator Bax in *S. cerevisiae* demonstrated that Bax is more efficient to kill yeast cells grown on mannose, a fermentable but not repressive carbon source, than on glucose grown cells (Priault et al., 1999). This is in contrast to the higher resistance of *S. cerevisiae* W303-1A stationary cells relative to exponential cells grown in YEPD medium containing glucose (Ludovico et al., 2002). The differences in the used medium composition and culture stage as well as in strains with expression plasmids may underlie these apparent opposite results.

Other features of cell death were also evaluated and compared between both strains, including plasma membrane integrity, $\Delta\Psi_m$, cyt *c* release from mitochondria and ROS accumulation. The evaluation of plasma membrane integrity by PI exclusion revealed a lower accumulation of PI in Aac2 *CL* cells, particularly upon prolonged exposure to acetic acid. Since there are no differences in the survival profile, such effect could indicate that the apoptotic-like cell death and subsequent secondary necrosis is delayed in Aac2 *CL* strain. Considering the importance of Aac2p in such process, it is possible that the absence of cysteine residues could modulate, but not prevent, the physiological role of this carrier during cell death. Further assays of cell survival for longer acetic acid treatments, and assessment of cell death markers, are required to elucidate this hypothesis. A similar delay in the loss of plasma membrane integrity has already been observed by Pereira and colleagues in *Δaac1/2/3* cells, although it was accompanied by an increased resistance to acetic acid as well as a delay in the occurrence of other cellular events associated with acetic acid-induced death (Pereira et al., 2007).

During cell death, opening of PTP induces the dissipation of mitochondrial $\Delta\Psi_m$ (Marchetti et al., 1996), an event associated to a matrix remodelling that might facilitate the release of cyt *c* from mitochondrial intra cristae space, its exposure to the IMS and subsequent release (Gottlieb et al., 2003). Under optimal experimental conditions, an increase in $DiOC_6(3)$ fluorescence intensity could represent a mitochondrial hyperpolarization since more polarized mitochondria would accumulate more cationic probe. On the other hand, mitochondrial depolarization would reduce the capacity of mitochondria to retain the fluorescent probe, hence resulting in decreased fluorescence intensity. Still, one must also consider the limitations and conditionings that are intrinsic to this approach in order to ensure a meaningful interpretation of the results obtained. Ultimately, the amount of dye loaded into mitochondria will depend on several factors including; the concentration of dye in the medium, $\Delta\Psi_p$, $\Delta\Psi_m$, mitochondrial size or

mass and loading/equilibrium time (Perry et al., 2011). All these variables should be controlled or accounted for, in order to enable a proper interpretation of the obtained results. For instance, optimizing the correlation between cell fluorescence and the magnitude of $\Delta\Psi_m$ might require the use of ultra-low concentrations of $DiOC_6(3)$ and the lowest possible dye/cell ratio, thus avoiding unspecific staining (Rottenberg and Wu, 1998; Wilson et al., 1985). Creating an artificial $\Delta\Psi_m$, for instance with a valinomycin-induced K^+ diffusional potential, should allow such optimization. Also, changes in mitochondrial mass affect accumulation of the probe and the fluorescence signal must therefore be normalized for this cell parameter so that the changes in fluorescence only reflect changes in $\Delta\Psi_m$. Previous works, using either rhodamine 123 (Rh123) (Ludovico et al., 2002) or $DiOC_6(3)$ (Pereira et al., 2007) have addressed this issue and proposed the normalization of the fluorescence signal by calculating the ratio between the green fluorescence (FL1, log) and the relative size (FS, log) to correct for changes in mitochondrial mass. Both studies report that wild-type yeast cells undergo a transient hyperpolarization followed by progressive depolarization, during acetic acid treatment. Our results suggest that $\Delta\Psi_m$ of Aac2 *wt* and *CL* cells slightly drops during the first 30 minutes of acetic acid treatment. After this initial period, the fluorescence intensity increases in both strains, reaching values higher than those recorded for time zero. The distinct evolution of $\Delta\Psi_m$ observed during our experiments could be a consequence of the genetic manipulation performed on the studied strains, which lack *AAC1* and *AAC3*, and express *AAC2* exclusively. In fact, deletion of the three AAC isoforms also produces an altered $\Delta\Psi_m$ phenotype in response to acetic acid (Pereira et al., 2007).

Production of ROS during acetic acid-induced cell death in Aac2 *wt* and *CL* strains was also evaluated. ROS play an important physiological role, particularly in signal transduction events associated to vital cell functions. However, uncontrolled ROS production or inappropriate anti-oxidant defence mechanisms may result in serious damages to the cell's biomolecules (Simon et al., 2000). As for mammalian cells, ROS accumulation is also considered a common event in most scenarios of yeast cell death (Ludovico et al., 2005). The exact role of ROS during PCD in yeast is not defined and conflicting observations suggest that ROS accumulation does not always correlate with loss of viability (Trancíková et al., 2004). Indeed, Pereira and colleagues observed that the increased resistance of *Δaac1/2/3* strain to acetic acid was not accompanied by diminished ROS production/accumulation, even though other cellular events associated

to yeast PCD were also delayed in this particular mutant (Pereira et al., 2007). The accumulation of ROS in Aac2 *wt* and *CL* strains increases steadily and equally after exposure to acetic acid, reaching what appears to be a threshold (approx. 80% of the cells with red fluorescence) after 2 hours of treatment. Since it was not possible to observe any statistically significant differences, it is logical to assume that the absence of cysteine residues from the Aac2p had no impact on ROS generation during acetic acid treatment. Such result is in agreement with the previous observation that the role of AAC proteins in acetic acid-induced death seems to be independent from ROS accumulation (Pereira et al., 2007), which in turn might be a consequence of acetic acid-induced mitochondrial dysfunction.

One final feature of yeast cell death evaluated in Aac2 *wt* and *CL* strains was the release of cyt *c* from mitochondria. As for mammalian cells, cyt *c* can be translocated from mitochondria to the cytosol of yeast cells undergoing death induced by different stimuli, including the heterologous expression of the pro-apoptotic protein Bax, absence of the histone chaperone *ASF1*/*CIA1*; acetic acid or H_2O_2 (Ludovico et al., 2002; Manon et al., 1997; Pereira et al., 2007; Yamaki et al., 2001). The exact role of cyt *c* release in *S. cerevisiae* cell death, however, remains to be clarified. Immunodetection of cyt *c* in mitochondrial and post-mitochondrial fractions isolated from Aac2 *wt* and *CL* cells revealed an acetic acid-induced cyt *c* release from mitochondria of both strains. The decrease on mitochondrial cyt *c* was accompanied by an equivalent enrichment of this protein in the corresponding post-mitochondrial fractions, indicating that both *wt* and *CL* forms of the Aac2p were able to restore the AAC-mediated MMP on a *Δaac1/2/3* background. Considering the obtained results, it is reasonable to say that even if the cysteine residues of the Aac2p were being targeted and modified by ROS, they do not contribute to the role of Aac2p in MOMP during acetic acid-induced cell death. It is possible that other protein modifications could be responsible for this particular feature of the Aac2p in cell death.

To understand if acetic acid is able to promote changes in the Aac2 protein structure, an experiment was devised in order to isolate this carrier before and after acetic acid treatment, and to study eventual protein modifications. The idea was to purify the Aac2 *wt* and *CL* proteins using a V5-His6 double tag attached to the C-terminus of these carriers. Unexpectedly, the presence of this double tag produced an Aac2p that has lost its ability to translocate adenine nucleotides, judging by the lack of growth in non-fermentable carbon sources. Additionally, both *wt*- and *CL*-V5-His6 tagged Aac2p lead

to an increased resistance of *S. cerevisiae* to acetic acid. A similar resistance phenotype has previously been reported for respiratory deficient mutants such as ρ^0, *Δatp10* and *Δcyc3* (Ludovico et al., 2002). Yet, the resistance phenotype of *Δaac1/2/3* mutant, which also lacks the ability to grow on non-fermentable carbon sources, is not related to its respiratory deficiency since the presence of a mutated form of Aac2p with reduced respiration capacity (Gawaz et al., 1990), is able to reverse this resistance phenotype (Pereira et al., 2007). As the role of the AAC in yeast cell death is not dependent on its carrier activity, we wondered if the presence of a double tag in Aac2p could affect the protein's structure disturbing its role in MMP. This hypothesis was tested by the evaluation of cyt *c* release form Aac2 *wt*-V5-His6 and *CL*-V5-His6 mitochondria during acetic acid treatment, only to find out that apparently there is no cyt *c* present in these strains. The Aac2 *wt*-V5-His6 and *CL*-V5-His6 constructions were addressed to mitochondria and had the expected size, but for some reason cyt *c* was absent or significantly reduced in these mitochondria, possibly degraded. This absence/reduction was confirmed by immunodetection of cyt *c*, in total and mitochondrial extracts, and also by redox spectrophotometry in the case of Aac2 *wt*-V5-His6. One possible explanation resides in the implications of a C-terminus tag for the Aac2 protein's biochemistry, with consequences to mitochondrial bioenergetics and function as well as to other mitochondrial proteins. It is possible that a C-terminus tagged Aac2p, although normally produced and imported to mitochondria, may not be properly inserted and/or folded into the IMM, an idea that has previously been suggested (Fiore et al., 2000).

The import of Aac2p into mitochondria depends on the cooperation of its three modules (each containing a pair of membrane-spanning domains), and their interaction with the receptor protein Tom70p, which then transfers the Aac2 precursor to the TOM import complex (Wiedemann et al., 2001). Removal of the first (N-terminal) and third (C-terminal) modules strongly impairs the stable accumulation of Aac2 precursor in the TOM complex (Wiedemann et al., 2001). Although neither the N- or C-terminus are required for initiation of the protein transfer to the IMM (Wiedemann et al., 2001), the third module is able to stall the translocation arresting the precursor at the TOM complex, presumably a mechanism to prevent misfolding and aggregation of the precursor in the IMS (Endres et al., 1999). The import and insertion in IMM, which requires several members of the TIM import machinery, depends on $\Delta\Psi_m$ and is regulated by a signal contained in the C-terminal part of the Aac2p (Endres et al., 1999). The presence of a C-terminus V5-His6 double tag could be enough to affect such a

delicate process, eventually causing inappropriate insertion or folding of the carrier. It should be mentioned, however, that a covalent tandem dimmer of the Aac2 was found to be stable and active *in vivo* (Trézéguet et al., 2000). On the other hand, the absence of cyt *c* in the Aac2 *wt*/*CL*-V5-His6 strains suggests that, for some reason, these proteins completely destabilize the mitochondrial import systems, including the one for cyt *c*. The peculiar transport of cyt *c* into mitochondria is driven by haem assembly and involves the mitochondrial general import pore Tom40p and the receptor Tom22p (Diekert et al., 2001; Wiedemann et al., 2003). Following the transport across the OMM, apocytochrome *c* binds to a holocytochrome *c* synthase (HCCS) in the IMS and is trapped inside mitochondria by conversion to holocytochrome *c* (Dumont et al., 1991). The TOM complex is both required and sufficient for the import of HCCS to the IMS, and the absence of this enzyme prevents the mitochondrial accumulation of apocytchrome *c* (Diekert et al., 1999; Dumont et al., 1988).

Because the V5-His6 tag affected the function of Aac2p, its use to purify this protein and assess the role of oxidative modifications in its ability to permeabilize mitochondria was hindered. Isolation of Aac2p was accomplished using a construction that consists of a six histidine tag attached to the N-terminal of the Aac2p. Although the expression levels of His6-Aac2p were lower than those of the untagged carrier, the presence of an N-terminal affinity tag does not significantly affect the protein's translocation to mitochondrial membranes neither its capacity to transport adenine nucleotides (Bamber et al., 2007b). Immunodetection of His6-Aac2p in total protein or mitochondrial extracts, from control and acetic acid treated cells, revealed a significant reduction in the total amount of carrier after exposure to acetic acid. The same phenomenon was observed for several other mitochondrial proteins, including outer and inner membrane proteins, and the general protein profile of acetic acid treated mitochondria is considerably different from that of control mitochondria. This reduced amount of mitochondrial proteins such as Aac2p, Por1p or Cox2p may be in part explained by mitochondrial degradation during acetic acid treatment, an event that has already been reported (Ludovico et al., 2002; Pereira et al., 2010). On the other hand, some these proteins might be specifically regulated during acetic acid induced death. For example, cyt *c* release from mitochondria, triggered by heterologous expression of Bax in yeast, has been associated to Cox2p degradation by the mitochondrial AAA-type protease Yme1p (Manon et al., 2001). Similarly, the levels of Cox2p were also decreased in yeast cells undergoing acetic-acid induced death (Ludovico et al., 2002). In a wider

perspective, genes involved in transcription and in mitochondrial functions are essential for the adaptation of *S. cerevisiae* to acetic acid (Mira et al., 2010); Sousa *et al.*, unpublished data), and changes in gene expression may also account for the different mitochondrial protein profile of control and treated cells. This profile is also evident during the determination of protein carbonylation in mitochondria isolated from control and treated cells, making it somewhat difficult to analyse the results. Looking at protein oxidation in the mitochondrial extracts, it is fair to say that some proteins appear to be more carbonylated in the acetic acid treated samples, comparing to control mitochondria. Still, the expression levels of these proteins during exposure to acetic acid must also be accounted for the analysis of their carbonylation state. Importantly, detection of carbonylated proteins in the His6-Aac2p immunoprecipitated samples raises some doubts on the hypothesis of an acetic acid-induced Aac2p oxidation. In fact, by comparing the His6-Aac2p purified samples, from control and acetic acid-treated cells, it is not possible to stress out any increase on protein carbonylation after exposure of yeast cells to the acid, even though the Aac2p was present in both purified samples.

The contribution of ANT to MOMP has generated quite a controversial discussion in in the field of cell death. Early studies have identified the ANT as an important regulator of mitochondria permeability transition in response to high [Ca^{2+}] (Hunter and Haworth, 1979), and this carrier was later proposed as an important component of the PTP (Bauer et al., 1999; Marzo et al., 1998). The exact contribution of the ANT to cell death was thoroughly studied by many different groups in the past decades, yet several questions remain to be answered. Oxidative stress was long identified as an activator of the PTP (Crompton et al., 1987), an effect attributed to the oxidation/reduction state of mitochondrial thiol groups (Costantini et al., 1995, 1996; Petronilli et al., 1994). It was later proposed that oxidative stress and thiol crosslinking agents could act on the ANT, causing structural modifications that would eventually lead to MMP (Costantini et al., 2000; Halestrap et al., 1997). Indeed, the crosslinking of ANT's cysteine residues might stabilize the carrier in a "c-state" conformation (the ADP binding site facing the cytosol) (McStay et al., 2002), which is required to sensitize the IMM permeability to Ca^{2+} (Lê Quôc and Lê Quôc, 1988). In yeast, the finding that AAC also plays an essential role in cell death induced by acetic acid and diamide (a known thiol crosslinking agent), raised the possibility of using *S. cerevisiae* as a model system to study the role of this carrier in MMP (Pereira et al., 2007). As for other scenarios of cellular demise, acetic acid-induced yeast death is characterized by ROS production,

and the AAC was proposed to be a target for oxidative modifications. Our results suggest that, in the scenario of acetic acid-induced yeast cell death, the oxidation of thiol groups in the Aac2p is unlikely related to the role of this carrier in MOMP. Mitochondrial dysfunction associated with a reduction of cytochrome *c* oxidase (COX) activity, and a decrease in the amounts of Cox2p and of cytochromes $a+a_3$ is accompanied by ROS accumulation during acetic acid induced apoptosis (Ludovico et al., 2002). The results obtained in the context of the present study suggest that ROS accumulation is not necessarily the trigger of AAC-mediated MOMP. To rule out this hypothesis it should be interesting to test the effect of diamide, a true thiol crosslinking agent, on yeast cell death in the presence of a cysteine-less Aac2p, and compare it to acetic acid. A different possibility is that the Aac2p could experience other modifications than thiol oxidation. Also, it might be worth to study the influence of the AAC's conformational states in acetic acid-death, by blocking the carrier with specific ligands.

Although the importance of the ANT and its thiol groups in cell death has been questioned (Kokoszka et al., 2004), it is undeniable that its counterpart in yeast plays a crucial role in some scenarios of cell death (Pereira et al., 2007). And even if it is not the case during acetic acid-induced death, post-translational modifications of the ANT might actually affect its role in MMP (Queiroga et al., 2010). Several recent reports connect the effect of ROS, thiol groups and anti-oxidant defence mechanisms or reducing agents with the regulation of PTP opening, thus strengthening the concept of oxidation/reduction events as an important mechanism of MMP regulation (Greco et al., 2011; Singh et al., 2011; Sloan et al., 2012). The finding that a *C. elegans* orthologue of mammalian ANT is a central player of PCD (Shen et al., 2009) suggests a conserved dual role for the carrier in cellular physiology, and as for the mechanisms regulating this process one may say the game is still on.

5. Supplementary Data

5.1. Acetic acid tolerance of wt and CL mutants

The viability of Aac2 *wt* and *CL* exposed to 180 mM of acetic acid was evaluated by c.f.u. counting. Cells were grown and treated in medium with glucose or galactose as carbon sources and in both cases no significant differences were observed in Aac2 *wt* and *CL* (Fig. 7). Galactose seems affect the capacity of Aac2 *wt* and *CL* cells to respond to acetic treatment, since the survival these cultures was lower than that of glucose-grown cultures (Fig. 7).

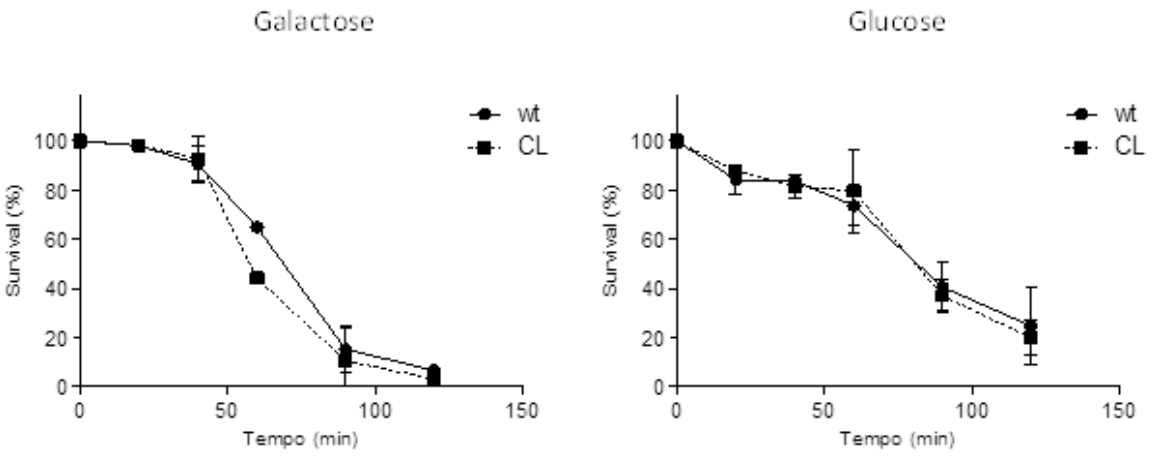


Fig. 7 – Cell death of Aac2 *wt* and Aac2 *CL* strains during acetic acid treatment. Survival curve of cell cultures during acetic acid treatment (180 mM) in synthetic complete media with galactose (left panel) or glucose (right panel). Data represent mean ± SD of at least 2 independent experiments.

One possible explanation for such occurrence may be related with in the fact that dissociation of acetic acid in the cytosol leads to intracellular acidification, acetate anion accumulation (Casal et al., 1996) and inhibition of cellular metabolic activity, namely, fermentation (Pampulha and Loureiro-Dias, 1989). This event could affect the cell's energetic capacity, and in the presence of galactose as the sole carbon source it would be a very important limiting factor for the cellular reaction to acetic acid stress. This idea might find some support on the observation that carbohydrate metabolism genes are determinant for maximal tolerance to acetic acid (Mira et al., 2010).

5.2. The effect of two anti-oxidants during acetic acid treatment

To understand if the acetic acid-induced cell death in *S. cerevisiae* is being regulated by ROS, *wt* cells were incubated with two different anti-oxidants, N-acetylcysteine (NAC) and resveratrol, prior to the exposure to acetic acid. NAC is a thiol, a mucolytic agent and a precursor of L-cysteine and reduced glutathione. It is also a source of sulfhydryl groups in cells, acting as a scavenger of free radicals such as hydrogen peroxide and hydroxyl radicals. It can also increase the glutathione levels in cells. NAC has been extensively used to investigate the role of ROS in several biological and pathological processes, although there is some controversy rising from the ability of NAC to act either as a pro-apoptotic or pro-survival agent (Zafarullah et al., 2003). Resveratrol (3,4',5-trihydroxystilbene) is a phytoalexin found in various plants, including grapes, berries and peanuts, as well as in wines (especially red wines). Resveratrol has several biological attributes, including antioxidant and anti-inflammatory activities, anti-platelet aggregation effects, anti-atherogenic properties, oestrogen-like growth-promoting effects, growth-inhibiting activities, immunomodulation and chemoprevention. Recently, resveratrol has been identified as an anti-carcinogenic and anti-ageing agent. However, and depending on the concentration, resveratrol can also exhibit pro-oxidant activities, leading to oxidative breakage of DNA in the presence of transition metal ions such as copper (de la Lastra and Villegas, 2007).

Both antioxidants were added to the culture medium 1 hour before the addition of acetic acid. The effect of NAC (5 mM) or resveratrol (100 μM) in the survival of *wt* cells exposed to different acetic acid concentrations (40, 80 and 160 mM), was tested after 3 hours of exposure. In both cases the survival of cells treated in the presence of the antioxidant is similar to that of control cells (treated only with the acid), having no significant differences between both treatments (Fig. 8). In our experimental conditions, it seems that the presence of an anti-oxidant could affect the cell's capacity to resist the stress induced by lower concentrations (40 mM) of acetic acid.

It is possible that the concentrations of each anti-oxidant used in this assay, were not enough to actually prevent the generation/accumulation of ROS induced by acetic acid treatment. To test this hypothesis, ROS accumulation during these experimental conditions should be controlled using, for an instance, a fluorescent probe to react with superoxide and other oxidative molecules. It should be mentioned that antioxidants such

as NAC have already been successfully used to promote yeast survival during acetic acid treatment (Guaragnella et al., 2010).

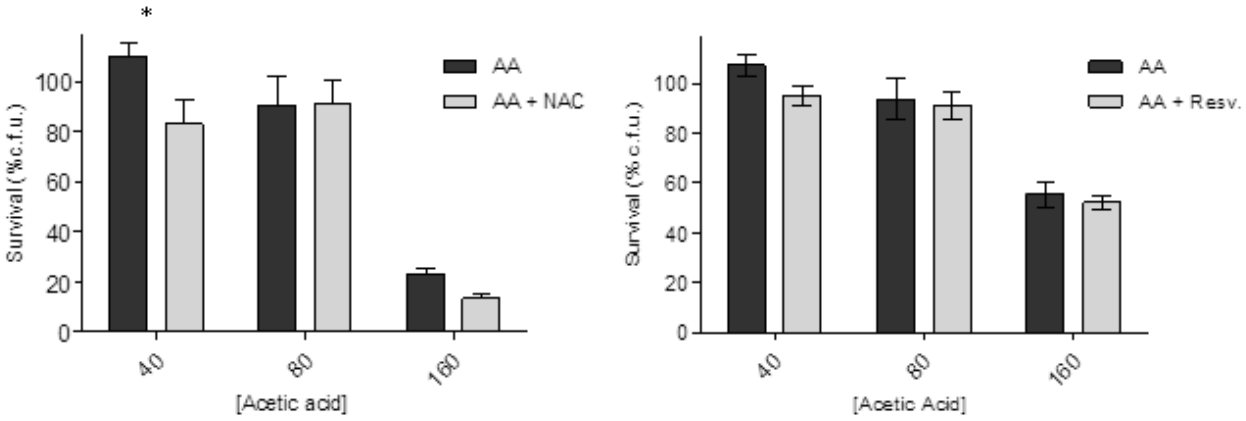


Fig. 8 – Cross effect of the antioxidants NAC (5 mM), on the left, and Resveratrol (100 µM), on the right, in the acetic acid treatment of Aac2 *wt* cultures exposed to three different concentrations of acetic acid (40, 80 and 160 mM) for 180 minutes. Viability was determined by c.f.u. counting after 48 hours at 30°C. 100 % survival corresponds to the total c.f.u. number at time zero. Statistical analysis indicates that the presence of the antioxidants is not able to promote cell survival upon acetic acid treatment. For cells treated with 40 mM of acetic, the combined effect of NAC was in fact deleterious for cell survival ($P < 0.05$). Data represent mean ± SD of at least 3 independent experiments.

Acknowledgements

We would like to thank Doctor Edmund Kunji, from Mitochondria Biology Unit, Medical Research Centre, Cambridge, UK, for kindly providing the plasmids pYES-P*pic2-aac2*, pYES-P*pic2-aac2-CL* (*cysteine-less*) and pYES-P*pic2*-His6-*aac2* used in this study. Work at the Institut de Biochimie et Génétique Cellulaires (IBGC), CNRS/Université Bordeaux Segalen was supported by Fundação Calouste Gulbenkian. D. Trindade was the recipient of a fellowship from Fundação Calouste Gulbenkian, Portugal.

6. References

Bamber, L., Harding, M., Monne, M., Slotboom, D.J., and Kunji, E.R. (2007a). The yeast mitochondrial ADP/ATP carrier functions as a monomer in mitochondrial membranes. Proceedings of the National Academy of Sciences of the United States of America *104*, 10830-10834.

Bamber, L., Slotboom, D.J., and Kunji, E.R. (2007b). Yeast mitochondrial ADP/ATP carriers are monomeric in detergents as demonstrated by differential affinity purification. Journal of molecular biology *371*, 388-395.

Bauer, M.K., Schubert, A., Rocks, O., and Grimm, S. (1999). Adenine nucleotide translocase-1, a component of the permeability transition pore, can dominantly induce apoptosis. The Journal of cell biology *147*, 1493-1502.

Beutner, G., Ruck, A., Riede, B., and Brdiczka, D. (1998). Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases. Biochimica et biophysica acta *1368*, 7-18.

Beutner, G., Ruck, A., Riede, B., Welte, W., and Brdiczka, D. (1996). Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore. FEBS letters *396*, 189-195.

Casal, M., Cardoso, H., and Leao, C. (1996). Mechanisms regulating the transport of acetic acid in Saccharomyces cerevisiae. Microbiology *142 (Pt 6)*, 1385-1390.

Costantini, P., Belzacq, A.S., Vieira, H.L., Larochette, N., de Pablo, M.A., Zamzami, N., Susin, S.A., Brenner, C., and Kroemer, G. (2000). Oxidation of a critical thiol residue of the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. Oncogene *19*, 307-314.

Costantini, P., Chernyak, B.V., Petronilli, V., and Bernardi, P. (1995). Selective inhibition of the mitochondrial permeability transition pore at the oxidation-reduction sensitive dithiol by monobromobimane. FEBS letters *362*, 239-242.

Costantini, P., Chernyak, B.V., Petronilli, V., and Bernardi, P. (1996). Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. The Journal of biological chemistry *271*, 6746-6751.

Crompton, M., and Costi, A. (1988). Kinetic evidence for a heart mitochondrial pore activated by Ca^{2+}, inorganic phosphate and oxidative stress. A potential mechanism for mitochondrial dysfunction during cellular Ca^{2+} overload. European journal of biochemistry / FEBS *178*, 489-501.

Crompton, M., Costi, A., and Hayat, L. (1987). Evidence for the presence of a reversible Ca^{2+}-dependent pore activated by oxidative stress in heart mitochondria. The Biochemical journal *245*, 915-918.

Daum, G., Bohni, P.C., and Schatz, G. (1982). Import of proteins into mitochondria. Cytochrome b_2 and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria. The Journal of biological chemistry *257*, 13028-13033.

de la Lastra, C.A., and Villegas, I. (2007). Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. Biochemical Society transactions *35*, 1156-1160.

Desagher, S., and Martinou, J.C. (2000). Mitochondria as the central control point of apoptosis. Trends in cell biology *10*, 369-377.

Diekert, K., de Kroon, A.I., Ahting, U., Niggemeyer, B., Neupert, W., de Kruijff, B., and Lill, R. (2001). Apocytochrome c requires the TOM complex for translocation across the mitochondrial outer membrane. The EMBO journal *20*, 5626-5635.

Diekert, K., Kispal, G., Guiard, B., and Lill, R. (1999). An internal targeting signal directing proteins into the mitochondrial intermembrane space. Proceedings of the National Academy of Sciences of the United States of America *96*, 11752-11757.

Dumont, M.E., Cardillo, T.S., Hayes, M.K., and Sherman, F. (1991). Role of cytochrome c heme lyase in mitochondrial import and accumulation of cytochrome c in Saccharomyces cerevisiae. Molecular and cellular biology *11*, 5487-5496.

Dumont, M.E., Ernst, J.F., and Sherman, F. (1988). Coupling of heme attachment to import of cytochrome c into yeast mitochondria. Studies with heme lyase-deficient mitochondria and altered apocytochromes c. The Journal of biological chemistry *263*, 15928-15937.

Endres, M., Neupert, W., and Brunner, M. (1999). Transport of the ADP/ATP carrier of mitochondria from the TOM complex to the TIM22-54 complex. The EMBO journal *18*, 3214-3221.

Fiore, C., Trezeguet, V., Roux, P., Le Saux, A., Noel, F., Schwimmer, C., Arlot, D., Dianoux, A.C., Lauquin, G.J., and Brandolin, G. (2000). Purification of histidine-tagged mitochondrial ADP/ATP carrier: influence of the conformational states of the C-terminal region. Protein expression and purification *19*, 57-65.

Gawaz, M., Douglas, M.G., and Klingenberg, M. (1990). Structure-function studies of adenine nucleotide transport in mitochondria. II. Biochemical analysis of distinct *AAC1* and *AAC2* proteins in yeast. The Journal of biological chemistry *265*, 14202-14208.

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.

Gottlieb, E., Armour, S.M., Harris, M.H., and Thompson, C.B. (2003). Mitochondrial membrane potential regulates matrix configuration and cytochrome *c* release during apoptosis. Cell death and differentiation *10*, 709-717.

Greco, T., Shafer, J., and Fiskum, G. (2011). Sulforaphane inhibits mitochondrial permeability transition and oxidative stress. Free radical biology & medicine *51*, 2164-2171.

Griffiths, E.J., and Halestrap, A.P. (1991). Further evidence that cyclosporin A protects mitochondria from calcium overload by inhibiting a matrix peptidyl-prolyl cis-trans isomerase. Implications for the immunosuppressive and toxic effects of cyclosporin. The Biochemical journal *274 (Pt 2)*, 611-614.

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

Halestrap, A.P., Connern, C.P., Griffiths, E.J., and Kerr, P.M. (1997). Cyclosporin A binding to mitochondrial cyclophilin inhibits the permeability transition pore and protects hearts from ischaemia/reperfusion injury. Molecular and cellular biochemistry *174*, 167-172.

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

Halestrap, A.P., McStay, G.P., and Clarke, S.J. (2002). The permeability transition pore complex: another view. Biochimie *84*, 153-166.

Hatanaka, T., Kihira, Y., Shinohara, Y., Majima, E., and Terada, H. (2001). Characterization of loops of the yeast mitochondrial ADP/ATP carrier facing the cytosol by site-directed mutagenesis. Biochemical and biophysical research communications *286*, 936-942.

Haworth, R.A., and Hunter, D.R. (1979). The Ca^{2+}-induced membrane transition in mitochondria. II. Nature of the Ca^{2+} trigger site. Archives of biochemistry and biophysics *195*, 460-467.

Herrero, P., Fernandez, R., and Moreno, F. (1985). Differential sensitivities to glucose and galactose repression of gluconeogenic and respiratory enzymes from *Saccharomyces cerevisiae*. Archives of microbiology *143*, 216-219.

Hirsch, T., Decaudin, D., Susin, S.A., Marchetti, P., Larochette, N., Resche-Rigon, M., and Kroemer, G. (1998). PK11195, a ligand of the mitochondrial benzodiazepine receptor, facilitates the induction of apoptosis and reverses Bcl-2-mediated cytoprotection. Experimental cell research *241*, 426-434.

Hlavata, L., Nachin, L., Jezek, P., and Nystrom, T. (2008). Elevated Ras/protein kinase A activity in *Saccharomyces cerevisiae* reduces proliferation rate and lifespan by two different reactive oxygen species-dependent routes. Aging cell *7*, 148-157.

Hunter, D.R., and Haworth, R.A. (1979). The Ca^{2+}-induced membrane transition in mitochondria. I. The protective mechanisms. Archives of biochemistry and biophysics *195*, 453-459.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. Journal of bacteriology *153*, 163-168.

Kissova, I., Polcic, P., Kempna, P., Zeman, I., Sabova, L., and Kolarov, J. (2000). The cytotoxic action of Bax on yeast cells does not require mitochondrial ADP/ATP carrier but may be related to its import to the mitochondria. FEBS letters *471*, 113-118.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R., and Newmeyer, D.D. (1997). The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science *275*, 1132-1136.

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.

Kolarov, J., Kolarova, N., and Nelson, N. (1990). A third ADP/ATP translocator gene in yeast. The Journal of biological chemistry *265*, 12711-12716.

Kroemer, G., and Reed, J.C. (2000). Mitochondrial control of cell death. Nature medicine *6*, 513-519.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lê Quôc, K., and Lê Quôc, D. (1988). Involvement of the ADP/ATP carrier in calcium-induced perturbations of the mitochondrial inner membrane permeability: importance of the orientation of the nucleotide binding site. Archives of biochemistry and biophysics *265*, 249-257.

Leung, A.W., and Halestrap, A.P. (2008). Recent progress in elucidating the molecular mechanism of the mitochondrial permeability transition pore. Biochimica et biophysica acta *1777*, 946-952.

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

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell *86*, 147-157.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. The Journal of biological chemistry *193*, 265-275.

Ludovico, P., Madeo, F., and Silva, M. (2005). Yeast programmed cell death: an intricate puzzle. IUBMB life *57*, 129-135.

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.

Manon, S., Chaudhuri, B., and Guérin, M. (1997). Release of cytochrome *c* and decrease of cytochrome *c* oxidase in Bax-expressing yeast cells, and prevention of these effects by coexpression of Bcl-x_L. FEBS letters *415*, 29-32.

Manon, S., Priault, M., and Camougrand, N. (2001). Mitochondrial AAA-type protease Yme1p is involved in Bax effects on cytochrome *c* oxidase. Biochemical and biophysical research communications *289*, 1314-1319.

Marchetti, P., Castedo, M., Susin, S.A., Zamzami, N., Hirsch, T., Macho, A., Haeffner, A., Hirsch, F., Geuskens, M., and Kroemer, G. (1996). Mitochondrial permeability transition is a central coordinating event of apoptosis. The Journal of experimental medicine *184*, 1155-1160.

Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J.M., Susin, S.A., Vieira, H.L., Prevost, M.C., Xie, Z., Matsuyama, S., Reed, J.C., *et al.* (1998). Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science *281*, 2027-2031.

McStay, G.P., Clarke, S.J., and Halestrap, A.P. (2002). Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. The Biochemical journal *367*, 541-548.

Mira, N.P., Palma, M., Guerreiro, J.F., and Sá-Correia, I. (2010). Genome-wide identification of *Saccharomyces cerevisiae* genes required for tolerance to acetic acid. Microbial cell factories *9*, 79.

Mitchell, P., and Moyle, J. (1965). Stoichiometry of proton translocation through the respiratory chain and adenosine triphosphatase systems of rat liver mitochondria. Nature *208*, 147-151.

Pampulha, M.E., and Loureiro-Dias, M.C. (1989). Combined effect of acetic acid, pH and ethanol on intracellular pH of fermentating yeast. Appl Microbiol Biotechnol *31*, 547-550.

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.

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.

Perry, S.W., Norman, J.P., Barbieri, J., Brown, E.B., and Gelbard, H.A. (2011). Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. BioTechniques *50*, 98-115.

Petit, P.X., O'Connor, J.E., Grunwald, D., and Brown, S.C. (1990). Analysis of the membrane potential of rat- and mouse-liver mitochondria by flow cytometry and possible applications. European journal of biochemistry / FEBS *194*, 389-397.

Petronilli, V., Costantini, P., Scorrano, L., Colonna, R., Passamonti, S., and Bernardi, P. (1994). The voltage sensor of the mitochondrial permeability transition pore is tuned by the oxidation-reduction state of vicinal thiols. Increase of the gating potential by oxidants and its reversal by reducing agents. The Journal of biological chemistry *269*, 16638-16642.

Priault, M., Camougrand, N., Chaudhuri, B., Schaeffer, J., and Manon, S. (1999). Comparison of the effects of bax-expression in yeast under fermentative and respiratory conditions: investigation of the role of adenine nucleotides carrier and cytochrome *c*. FEBS letters *456*, 232-238.

Queiroga, C.S., Almeida, A.S., Martel, C., Brenner, C., Alves, P.M., and Vieira, H.L. (2010). Glutathionylation of adenine nucleotide translocase induced by carbon monoxide prevents mitochondrial membrane permeabilization and apoptosis. The Journal of biological chemistry *285*, 17077-17088.

Rodic, N., Oka, M., Hamazaki, T., Murawski, M.R., Jorgensen, M., Maatouk, D.M., Resnick, J.L., Li, E., and Terada, N. (2005). DNA methylation is required for silencing of *Ant4*, an adenine nucleotide translocase selectively expressed in mouse embryonic stem cells and germ cells. Stem Cells *23*, 1314-1323.

Rottenberg, H., and Wu, S. (1998). Quantitative assay by flow cytometry of the mitochondrial membrane potential in intact cells. Biochimica et biophysica acta *1404*, 393-404.

Ruck, A., Dolder, M., Wallimann, T., and Brdiczka, D. (1998). Reconstituted adenine nucleotide translocase forms a channel for small molecules comparable to the mitochondrial permeability transition pore. FEBS letters *426*, 97-101.

Shen, Q., Qin, F., Gao, Z., Cui, J., Xiao, H., Xu, Z., and Yang, C. (2009). Adenine nucleotide translocator cooperates with core cell death machinery to promote apoptosis in *Caenorhabditis elegans*. Molecular and cellular biology *29*, 3881-3893.

Siemen, D., and Ziemer, M. (2013). What is the nature of the mitochondrial permeability transition pore and what is it not? IUBMB life *65*, 255-262.

Simon, H.U., Haj-Yehia, A., and Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis : an international journal on programmed cell death *5*, 415-418.

Singh, B.K., Tripathi, M., Pandey, P.K., and Kakkar, P. (2011). Alteration in mitochondrial thiol enhances calcium ion dependent membrane permeability transition and dysfunction in vitro: a cross-talk between mtThiol, Ca^{2+}, and ROS. Molecular and cellular biochemistry *357*, 373-385.

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

Sloan, R.C., Moukdar, F., Frasier, C.R., Patel, H.D., Bostian, P.A., Lust, R.M., and Brown, D.A. (2012). Mitochondrial permeability transition in the diabetic heart: contributions of thiol redox state and mitochondrial calcium to augmented reperfusion injury. Journal of molecular and cellular cardiology *52*, 1009-1018.

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.

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.

Tait, S.W., and Green, D.R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. Nature reviews Molecular cell biology *11*, 621-632.

Trancíková, A., Weisová, P., Kissová, I., Zeman, I., and Kolarov, J. (2004). Production of reactive oxygen species and loss of viability in yeast mitochondrial mutants: protective effect of Bcl-x_L. FEMS yeast research *5*, 149-156.

Trézéguet, V., Le Saux, A., David, C., Gourdet, C., Fiore, C., Dianoux, A., Brandolin, G., and Lauquin, G.J. (2000). A covalent tandem dimer of the mitochondrial ADP/ATP carrier is functional *in vivo*. Biochimica et biophysica acta *1457*, 81-93.

Wiedemann, N., Kozjak, V., Prinz, T., Ryan, M.T., Meisinger, C., Pfanner, N., and Truscott, K.N. (2003). Biogenesis of yeast mitochondrial cytochrome c: a unique relationship to the TOM machinery. Journal of molecular biology *327*, 465-474.

Wiedemann, N., Pfanner, N., and Ryan, M.T. (2001). The three modules of ADP/ATP carrier cooperate in receptor recruitment and translocation into mitochondria. The EMBO journal *20*, 951-960.

Wilson, H.A., Seligmann, B.E., and Chused, T.M. (1985). Voltage-sensitive cyanine dye fluorescence signals in lymphocytes: plasma membrane and mitochondrial components. Journal of cellular physiology *125*, 61-71.

Yamaki, M., Umehara, T., Chimura, T., and Horikoshi, M. (2001). Cell death with predominant apoptotic features in *Saccharomyces cerevisiae* mediated by deletion of the histone chaperone *ASF1/CIA1*. Genes to cells : devoted to molecular & cellular mechanisms *6*, 1043-1054.

Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P., and Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. Science *275*, 1129-1132.

Zafarullah, M., Li, W.Q., Sylvester, J., and Ahmad, M. (2003). Molecular mechanisms of N-acetylcysteine actions. Cellular and molecular life sciences : CMLS *60*, 6-20.

Zamzami, N., and Kroemer, G. (2001). The mitochondrion in apoptosis: how Pandora's box opens. Nature reviews Molecular cell biology *2*, 67-71.

Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J.L., Petit, P.X., and Kroemer, G. (1995). Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death *in vivo*. The Journal of experimental medicine *181*, 1661-1672.

Zhao, H., Kalivendi, S., Zhang, H., Joseph, J., Nithipatikom, K., Vasquez-Vivar, J., and Kalyanaraman, B. (2003). Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. Free radical biology & medicine *34*, 1359-1368.

Chapter III

The role of Por1p in AAC-mediated mitochondria membrane permeabilization (MMP) and release of cytochrome *c*

Trindade, D., Manon, S., Côrte-Real, M., Sousa, M.J.- The role of Por1p in AAC-mediated mitochondria membrane permeabilization (MMP) and release of cytochrome c

Manuscript in preparation

1. Introduction

The contribution of mitochondrial proteins to the different mechanisms of cell death has taken a great deal of attention, particularly since mitochondria were found to be more than just the cell's "power station", also playing a central role in programmed cell death (PCD). One such protein is the Voltage Dependent Anion Channel (VDAC), presumably the most abundant protein of the outer mitochondrial membrane (OMM) that functions as a low-specificity molecular sieve with a cut-off at approximately 1 kDa. VDAC is considered to be responsible for the permeability of the OMM to several small molecules, and its voltage-dependence regulates the flow of metabolites between the cytoplasm and the inner mitochondrial membrane (IMM) (Colombini, 1979).

Different models have been proposed considering the role of VDAC in mitochondria outer membrane permeabilization (MOMP), a determinant step in most scenarios of cell death (Desagher and Martinou, 2000). One of these models associates VDAC to the opening of the permeability transition pore (PTP), an idea originated from electrophysiological studies in which the voltage dependence of the pore resembled that of VDAC (Szabo et al., 1993; Szabo and Zoratti, 1993). This hypothesis found some support in the work of Beutner and colleagues, who observed that, when reconstituted into liposomes, hexokinase-enriched fractions found in mitochondrial contact sites, also containing VDAC and the adenine nucleotide translocator (ANT), were able to form channels with the conductance expected for the PTP (Beutner et al., 1996). A different model, proposes an association between pro-apoptotic Bcl-2 family proteins and VDAC as the mechanism responsible for cytochrome (cyt *c*) release during apoptosis (Shimizu et al., 1999). Supporting this idea, Shimizu and colleagues proposed that Bax and Bak accelerated the opening of VDAC in reconstituted proteoliposomes, whereas Bcl-x_L directly interacted with VDAC to close it (Shimizu et al., 1999). An alternative model proposes that apoptosis induction favours the closed conformation of VDAC, resulting in the inability to exchange cytosolic ADP for mitochondrial ATP, which would inhibit the F_1F_0 ATPase and prevent the flux of H^+ back into the matrix (Vander Heiden et al., 1999). Hyperpolarization of the inner mitochondrial membrane (IMM) would then promote osmotic swelling of the matrix and subsequent rupture of the outer mitochondrial membrane (OMM) (Vander Heiden et al., 1997). On this model, anti-

apoptotic Bcl-2 family members such as Bcl-x_L would prevent cell death by promoting VDAC opening and facilitating the ADP/ATP exchange (Vander Heiden et al., 2001). Meanwhile, the model of VDAC regulation by Bax was questioned and instead, truncated Bid, another pro-apoptotic regulator, was found to modulate voltage gating of VDAC by inducing channel closure (Rostovtseva et al., 2004). Nevertheless, it should be mentioned that the typical "closed" VDAC conformation actually corresponds to a lower-conductivity state with only 50 % reduction in channel conductance and an estimated pore radius of 0.9 nm (Colombini et al., 1987; Mannella and Guo, 1990). Along with a decrease in pore size, the shift from an open to a "closed" state changes the selectivity of the channel lowering its specificity to anions in favour of a more cation selective form (Hodge and Colombini, 1997).

Studies with mitochondria extracted from mice in which the VDAC isoforms were genetically inactivated, seriously questioned the contribution of VDAC to MOMP and cell death. The first evidences were obtained by Krauskopf and collegues who observed that the basic properties of the PTP in *VDAC1*$^{-/-}$ mitochondria were identical to those of wild-type mitochondria, demonstrating that VDAC1 isoform is not required for the opening and regulation of PTP (Krauskopf et al., 2006). Additional confirmation was obtained in the work of Baines and colleagues, demonstrating that *wild-type* and VDAC-deficient mitochondria or whole cells were still able to undergo mitochondrial permeability transition (MPT) induced by Ca^{2+} overload and oxidative stress (Baines et al., 2007). Furthermore, the absence of VDAC isoforms did not affected cyt *c* release, caspase cleavage and cell death induced by the pro-apoptotic Bcl-2 proteins Bax and Bid (Baines et al., 2007). Such evidences discredited the hypothesis of a VDAC mediated cell death mechanism involving the participation of this protein in the PTP or the collaboration with the Bcl-2 family proteins (Galluzzi and Kroemer, 2007).

Although most of the mammalian apoptotic regulators are absent in yeast, the original candidates proposed to build up the scaffold structure of mammalian PTP, appear to be conserved among eukaryotic organisms. In fact, *Saccharomyes cerevisiae* possesses three isoforms of the ADP/ATP carrier (*AAC1*, *AAC2* and *AAC3*), orthologues of the mammalian ANTs (Kolarov et al., 1990; Lawson and Douglas, 1988), a VDAC gene *POR1* (Blachly-Dyson et al., 1997; Mihara and Sato, 1985), and a mitochondrial cyclophilin, *CPR3* (Matouschek et al., 1995). As for mammalians, a large-conductance unselective channel has also been found in yeast mitochondria and was later named "yeast PTP" (Jung et al., 1997). The properties of this yeast mitochondrial unselective

channel (YMUC) were summarized in a review by Manon and colleagues in 1998, and a comparison with the features of mammalian PTP was made (Manon et al., 1998). Based on the available knowledge by then, it was concluded that even if YMUC presents some functional analogies with mammalian PTP, its regulation was different enough to consider YMUC a controversial counterpart of the mammalian PTP (Manon et al., 1998). However, recent data indicates that the apparently big differences between these two channels might only be due to different sensitivities of yeast and mammalian mitochondrial cyclophilin to phosphate and/or Ca^{2+}, and Ca^{2+}-induced permeability transition can be observed in yeast suggesting that the YMUC and mammalian PT may be the expression of very similar events (Azzolin et al., 2010; Yamada et al., 2009). The exact composition of the YMUC remains to be elucidated, and some evidences argue against a putative contribution of the AAC or Porin (Ballarin and Sorgato, 1995; Lohret et al., 1996). Nevertheless, the absence of Por1p is enough to alter its voltage dependence and desensitize it to Ca^{2+} regulation (Gutierrez-Aguilar et al., 2007; Lohret and Kinnally, 1995).

Interestingly, both AAC and Por1p have been implicated in yeast cell death induced by different stimuli. Absence of AAC proteins increased the resistance of yeast cells to acetic acid and diamide, preventing acetic acid-induced cyt *c* release from mitochondria (Ludovico et al., 2002; Pereira et al., 2007). On the other hand, deletion of Por1p increases the sensitivity of yeast cells to acetic acid, a process that is associated to a faster DNA degradation. The *Δpor1* mutant yeast is also sensitive to hydrogen peroxide and diamide (Pereira et al., 2007), exhibiting mitochondrial network fragmentation and increased $\Delta\Psi_m$, two events that are apparently not related to the acetic acid sensitive phenotype (Pereira, 2008; Pereira et al., 2007).

The aim of this work was to study the contribution of the AAC and Por1p, and a potential interaction of both proteins, to MMP and yeast cell death, particularly during acetic acid-induced death in which these proteins apparently have different regulatory roles. To understand the impact of Por1p in AAC-mediated MMP, we studied cyt *c* release from mitochondria of cells lacking Por1p and the three isoforms of the AAC, and compared it to *wt*, *Δaac1/2/3* and *Δpor1* mitochondria.

2. Materials and Methods

2.1. Strains and Growth Conditions

The yeast strains and plasmids used in this study are listed in Table 1:

Table 3 – List of strains used on the experiments described in the following sections.

Name	Strain/Plasmid	Source/Reference
wt	W303-1B (*MATα; ura3; trp1; leu2; his3; ade2; canR*)	Gift from Alexander Tzagoloff
Δaac1/2/3	JL1-3Δ2Δ3 (*MATa, ade2, his3; leu2; trp1; ura3; can1; aac1::LEU2; Δaac2::HIS3; Δaac3::URA3*)	Postis *et al.*, 2005
Δpor1	W303-1B; *Δpor1::kanMX4*	This study
Δaac1/2/3Δpor1	JL1-3Δ2Δ3; *Δpor1::kanMX4*	This study
Atp3	W303-1B (*MATα; ura3; trp1; leu2; his3; ade2; canR Δatp3*::*kanMX4*) + pCM189-ATP3	Gift from Emmanuel Tétaud

Saccharomyces cerevisiae strains W303-1B and JL1-3Δ2Δ3, which is a derivate of W303 lacking the three isoforms of the AAC (*AAC1*, *2* and *3*; (Postis et al., 2005)), were transformed with a *Δpor1*::kanMX4 interruption cassette, amplified by PCR from genomic DNA of BY4741 *Δpor1* EUROSCARF deletion strain (EUROSCARF, Institute of Molecular Biosciences Johann Wolfgang Goethe-University Frankfurt, Germany), to generate *Δpor1* and *Δaac1/2/3Δpor1* strains, respectively. Yeast cells were transformed by the lithium acetate method (Ito et al., 1983), after which transformants were selected in medium containing geneticin (200 µg/µL) and confirmed by PCR. In the experiments described below, cells were pre-grown in YPD medium (2% Glucose, 1% yeast extract, 1% bactopeptone), transferred to YPGal medium (2% Galactose, 1% yeast extract, 1% bactopeptone) and incubated over-night (O.N.) at 30°C (200 r.p.m.) until an optical density of 1.5-2.0 was reached (Pereira et al., 2007). A *Δatp3* strain was transformed with plasmid pCM189-ATP3, containing the *ATP3* sequence under control of a Tet-off promoter and kindly provided by Doctor Emmanuel Têtaud (IBGC, Bordeaux), and selected in YNB medium (0.175% YNB, 2% Glucose, 0.5% (NH4)2SO4, 2% agar) without uracil. The Atp3 strain was pre-grown in synthetic complete medium (0.175% YNB, 2% Glucose, 0.5% $(NH_4)_2SO_4$) without uracil. Doxycycline, 10 µg/mL, was used whenever inhibition of *ATP3* expression was desired.

2.2. Acetic acid treatment

For acetic acid tolerance and mitochondria extraction assays, acetic acid was added at a final concentration of 180 mM to cultures grown over-night until exponential growth phase ($O.D._{640nm}$ = 1.5 – 2.0) in YPGal medium. Survival of *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* cells to acetic acid treatment was evaluated by c.f.u. counting from samples collected at different time points during a 3 hours period, plated onto YPDA (YPD supplemented with 2% Agar; 200 µl from a $1.25x10^3$ cell/mL suspension) and grown for 2 days at 30°C. Percentage of viable cells was estimated considering 100% survival the number of c.f.u. at time zero minutes.

2.3. Mitochondria Isolation and Western-blot analysis

For mitochondria isolation, approximately 5 L of *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* cultures were grown O.N. in YPGal as previously described. Half of each culture was used as control (T0), and the remaining was subjected to acetic acid treatment (180 mM) for 90 or 180 minutes. Control and acetic acid treated cells were converted into spheroplasts by enzymatic digestion with zymolyase (Zymolyase 20T, Seikagaku Biobusiness Corporation), disrupted by hand-potter or mechanical homogenization, and the mitochondrial fraction recovered after a series of differential centrifugations (Law et al., 1995). Mitochondrial suspensions were frozen in liquid nitrogen and stored at -80°C. For characterization of the mitochondrial and post-mitochondrial fractions by Western-blot, 50 µg of proteins were precipitated with TCA, and solubilized in 2% SDS before being separated on SDS–PAGE (Laemmli, 1970). Proteins were then blotted into PVDF membranes. Characterization of both fractions was carried out with antibodies directed against cytochrome *c* (rabbit polyclonal Anti-*CYC1* antibody; 1:1000, custom-made by Millegen); phosphoglycerate kinase (mouse monoclonal Anti-*PGK1* antibody; 1:5000, Molecular Probes), and the beta subunit of the F1 sector of mitochondrial F_1F_0 ATP synthase (rabbit polyclonalclonal Anti-*ATP2* antibody; 1:20000, home made by Jean Velours, IBGC, Bordeaux). All protein quantifications were performed by the Lowry method (Lowry et al., 1951).

2.4. Redox Spectrophotometry

For cytochrome quantification, 9.6 mg of thawed mitochondrial proteins were diluted in recuperation buffer (0.6 M Mannitol; 10 mM Tris-maleate; 2 mM EGTA; pH 6.8) to

a final volume of 1.6 mL, producing a mitochondria suspension with the final concentration of 6 mg/mL. The suspension was equally divided into two glass cuvettes and the reference and sample cuvettes were oxidized and reduced with potassium ferricyanide and sodium dithionite, respectively. Samples were then analysed in a double beam spectrophotometer, Varian Cary 4000. Alternatively, 200 µl of a 10 mg/mL mitochondria preparation were analysed in a micro-plate spectrophotometer. For whole cell cytochrome quantification, cells were collected during exponential growth, and concentrated in a cellular suspension of 2 mL at ≈ 40 $O.D._{640nm}$. This suspension was divided in two cuvettes and analyzed in a double beam spectrophotometer, Varian Cary 4000. Hydrogen peroxide (2 µl) was used instead of potassium ferricyanide for whole cell cytochrome quantifications. Spectra were acquired between 500 and 650 nm and cytochromes $c+c_1$, *b* and $a+a_3$ were quantified by the O.D. at differences 550 nm minus 540 nm, 561 nm minus 575 nm, and 603 nm minus 630 nm, respectively (Fig. 1).

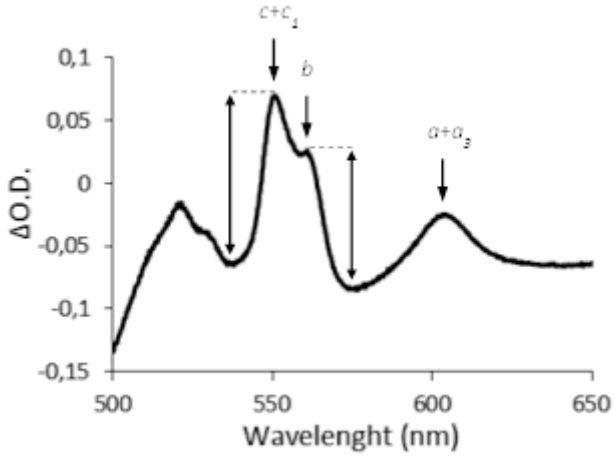


Fig. 1 - The amounts of cytochromes *c* and *b* in isolated mitochondria were measured by redox difference spectrophotometry. Mitochondria were diluted in 1.8 mL of recuperation buffer (6 mg/mL) and divided by two glass cuvettes, one oxidized with ferricyanide (refrence) and the other reduced with sodium dithionite (sample). Difference spectra were acquired in a Varian Cary 4000 spectrophotometer.

2.5. Cell wall resistance evaluated by enzymatic digestion

Wt, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* cultures were grown O.N. on either YPD or YPGal medium, as previously described. Cells were then collected, washed and resuspended in PBS to obtain a cellular suspension with an $O.D._{640nm}$ of 0.5. Zymolyase was added to a final concentration of 20 µg/mL and the cellular suspensions incubated

at 30°C, with agitation for one hour. Cell wall digestion was followed by periodic measures of the suspension's absorbance (A_{800}). $\Delta O.D._{.800nm}$ was estimated considering 100% the absorbance measured at time zero minutes.

2.6. Cell wall, osmotic and heat stress assays

For spot assays, samples from exponentially grown cultures of *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* strains were collected and adjusted to a 1.0 $O.D._{640nm}$ suspension. These samples were diluted 5 times in a 1:10 dilution series, and 5 µl of each dilution were plated in YPDA medium supplemented with different stress agents: NaCl (1M), KCl (1.5 M), Caffeine (7.5 mM) or Caffeine (7.5 mM) + Sorbitol (1M); or YPGA medium (2% glycerol). To test the effect of these stresses in the *Δatp3* mutant, doxycycline was added to a final concentration of 10 µg/mL. Plates were incubated for 5 days at 30°C. The growth ability of theses strains in YPDA medium at 37°C, was also tested.

3. Results

3.1. Acetic Acid tolerance of wt, Δaac1/2/3, Δpor1 and Δaac1/2/3Δpor1 strains

Pereira and colleagues have previously demonstrated that the absence of the three AAC isoforms (*AAC1*, *2* and *3*) increases the resistance of *S. cerevisiae* to acetic acid, while deletion of *POR1* greatly sensitizes yeast cells acting as a pro-survival molecule (Pereira et al., 2007). The authors also found that AAC proteins are required for acetic acid-induced cyt *c* release from mitochondria to the cytosol (Pereira et al., 2007). Considering these observations, we questioned how the absence of both AAC and Por1p, two members of the originally proposed pore structure, would affect acetic acid-induced MOMP. To answer this question, the survival of wt, *Δpor1, Δaac1/2/3* and *Δaac1/2/3Δpor1* strains to acetic acid was evaluated by c.f.u. counting.

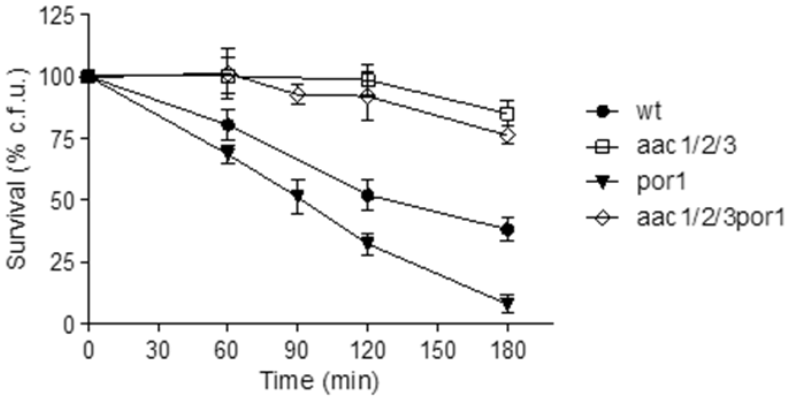


Fig. 2 – Survival of *S. cerevisiae wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* cells during acetic acid treatment. Cell cultures were pre-grown in YPD, transferred to YPGal and grown O.N. until an $O.D._{640nm}$ of 1.5-2.0 was reached. Acetic acid was then added to a final concentration of 180 mM. Viability was determined by c.f.u. counts at the indicated time points (100 % survival corresponds to the total c.f.u. number at time zero). Deletion of Por1p results in higher sensitivity of *S. cerevisiae* to acetic acid, while the absence of the AAC proteins, independently of the presence or absence of Por1p, is responsible for increased resistance to the deleterious effects of the acid. Data represent mean ± SD of at least 3 independent experiments.

The simultaneous absence of AAC and Por1p produces a resistance phenotype similar to *Δaac1/2/3* (Fig. 2). Both *Δaac1/2/3* and *Δaac1/2/3Δpor1* strains exhibited a high plating efficiency after 3 hours of exposure to acetic acid (approximately 85% and

75%, respectively). On the other hand, the *Δpor1* strain was more sensitive to acetic acid than the *wt*, exhibiting less than 10% survival against approximately 40% of the *wt* (Fig. 2). It seems that in the absence of both proteins, the resistance phenotype resulting from deletion of the AAC isoforms prevails over the sensitivity conferred by Por1p deletion, suggesting that the sensitivity resulting from Por1p deletion depends on the presence of the AAC proteins. It is possible that Por1p acts on the AAC to regulate MOMP during acetic-induced death.

3.2. Mitochondria Isolation and Cytochrome Quantification

In multicellular organisms, release of cyt *c* from mitochondria to the cytosol is considered a point of no return during apoptosis. Once released, cyt *c* combines with cytosolic Apaf-1 and pro-caspase 9 to form the apoptosome, which activates caspase 9 thus propagating the death signal (Li et al., 1997). Although these proteins lack obvious homologs in yeast, cyt *c* release from mitochondria is a common event to some scenarios of yeast cell death, including deletion of the histone chaperone *ASF1*/*CIA1*, pheromone- and amiodarone-induced cell death, *CDC48* mutation, and also H_2O_2 or acetic acid treatments (Braun et al., 2006; Ludovico et al., 2002; Pereira et al., 2007; Pozniakovsky et al., 2005; Yamaki et al., 2001). Pereira and colleagues found that MOMP and cyt *c* release during acetic acid-induced death of *S. cerevisiae* requires the presence of the AAC, an observation that correlates with the increased resistance of the *Δaac1/2/3* mutant to acetic acid. Since the absence of Por1p sensitized yeast cells to this acid and this effect was reversed in the absence of AAC proteins, we decided to evaluate the impact of Por1p removal in the AAC-mediated MOMP and cyt *c* release during acetic acid treatment. The content of cytochromes $c+c_1$ and *b* was evaluated by redox spectra of whole cells (Fig. 3A). The higher cyt *c*/cyt *b* ratios estimated for *Δaac1/2/3* and *Δaac1/2/3Δpor1* cells might result from slight differences in the content of cyt *c* and/or *b* in these cells (Fig. 3A and 3B). Mitochondria were extracted from *wt* and *Δpor1* cells, before and after a 3 hours exposure to acetic acid (180 mM), and the cytochrome content was measured by redox spectrophotometry. Mitochondria from *wt* and *Δpor1* untreated cells produced classical spectra, with the estimated cyt *c*/cyt *b* ratios of 2.35 and 2.12, respectively (Fig. 3C). As for acetic acid-treated cells, mitochondria extraction proved to be much more difficult and the mitochondrial mass obtained by the end of the procedure was significantly lower. Nevertheless, we were

able to quantify the cytochromes in mitochondria from acetic acid-treated *wt* cells and observed a cyt *c*/cyt *b* ratio of approximately 1.60, suggesting the release of cyt *c* during this experiment (Fig. 3C). Such result is in agreement with the previously reported by Pereira and colleagues (Pereira et al., 2007). The same was not true for *Δpor1* cells treated with acetic acid, from which the amount of mitochondrial mass obtained was very low. The resulting spectra only proved the poor quality of these mitochondria and so it was not possible to properly measure the cytochrome content in these samples (Fig. 3C).

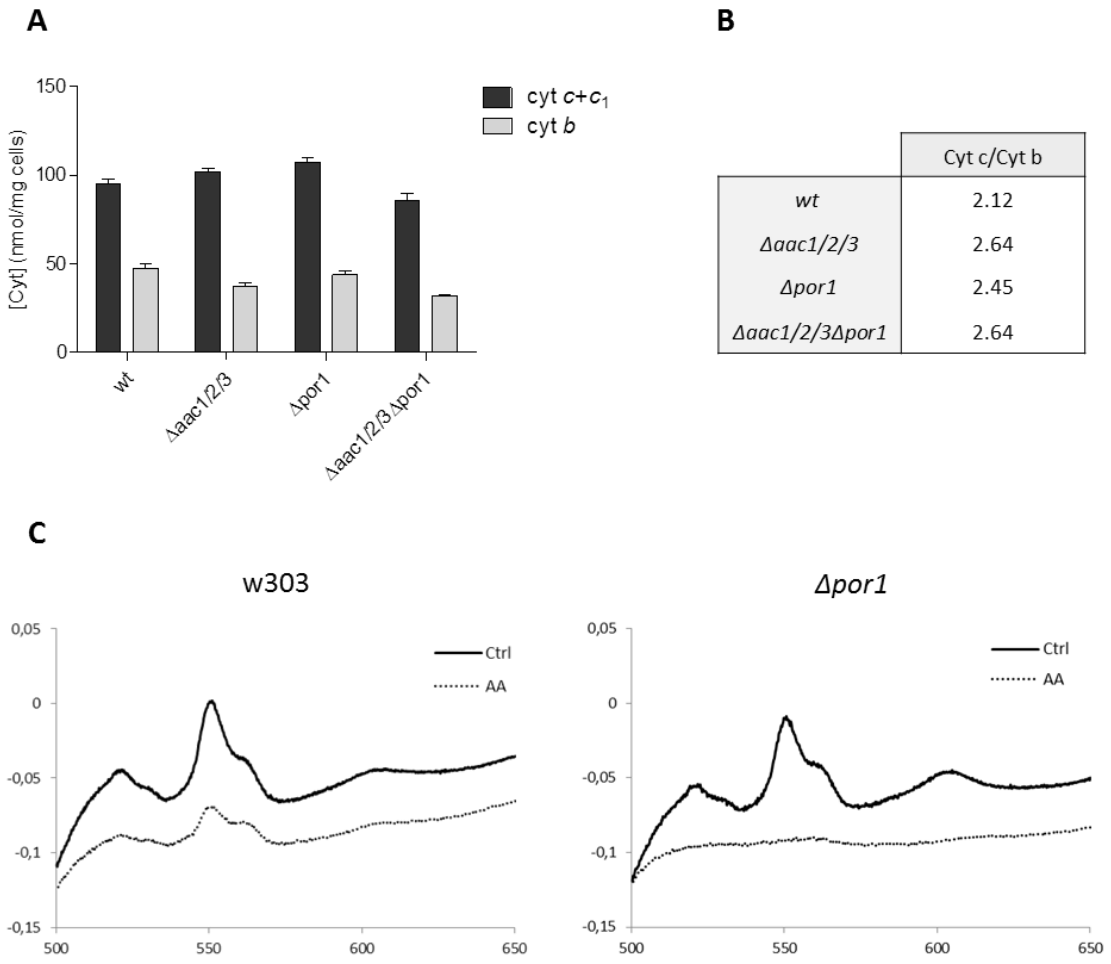
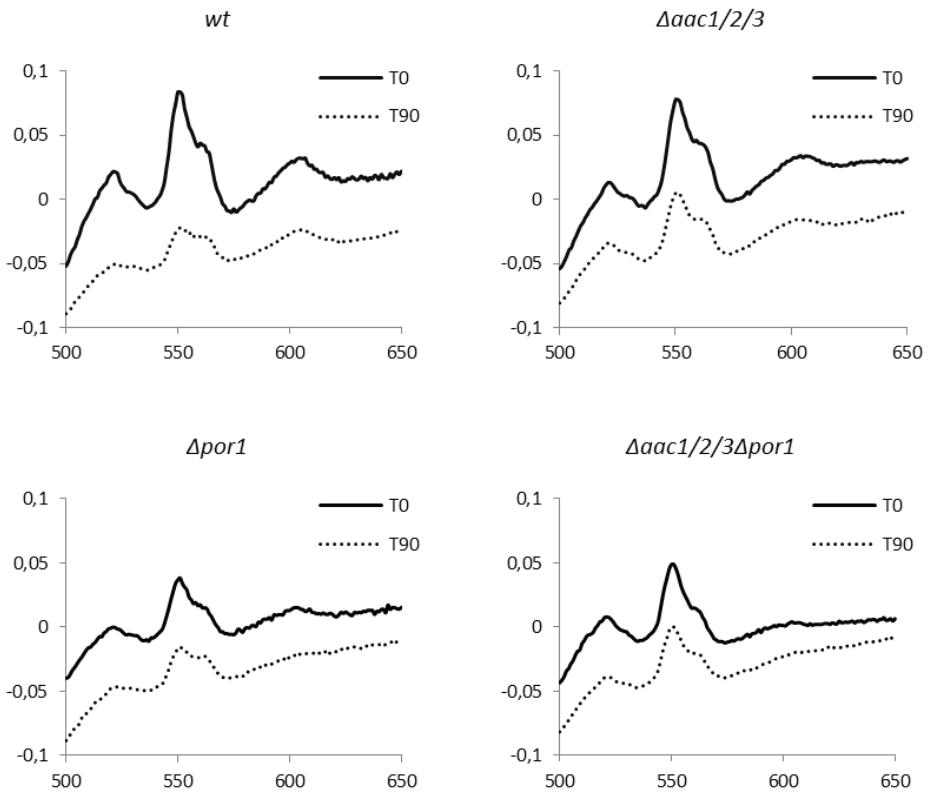


Fig. 3 – Cytochrome quantification in *S. cerevisiae wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* mutants. (A) Quantification of cytochromes $c+c_1$ and *b* in *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* cells, by redox spectra. Cells were collected, resuspended to a final $O.D._{640nm}$ of 40.0 and divided in two cuvettes. The reference cuvette was oxidized with hydrogen peroxide (H_2O_2), and the sample reduced with sodium dithionite. Spectra were acquired in a double beam spectrophotometer, and the content of cytochromes $c+c_1$ and *b* was estimated by the O.D. differences 550 nm minus 540 nm and 561 nm minus 575 nm, respectively. The *Δaac1/2/3Δpor1* mutant apparently possesses less cyt *c* and *b* when compared with the other strains. Data represent mean ± SD of at least 3 independent experiments. (B) Cyt *c*/cyt *b*

ratio estimated for *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* cells. (C) Redox spectra of *wt* and *Δpor1* mitochondria isolated from control and acetic acid treated cells. Cells were collected before (control) and after the exposure to acetic acid, 180 mM for 3 hours, and the mitochondria preparations obtained after enzymatic digestion of the cell wall with zymolyase followed by mechanical disruption of spheroplasts and differential centrifugations. For redox spectrophotometry, mitochondria preparations (6 mg/mL) were oxidized with ferrycianide and reduced with sodium dithionite. Difference spectra were acquired in a double beam spectrophotometer, and the content of cytochromes c+c_1 and *b* was estimated as described above. One representative experiment is shown for each mitochondria sample.

Since the *Δpor1* strain is particularly sensitive to acetic acid, presenting less than 10% of plating efficiency after 3 hours of treatment with acetic acid (180 mM), we decided to examine cyt *c* release from mitochondria upon a shorter exposure to acetic acid, thus with a higher survival percentage of *Δpor1* cells. After 90 minutes of treatment with acetic acid (180 mM), *Δpor1* strain exhibits a viability of about 50%, and therefore mitochondria extractions from *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* were performed after a 90 minutes exposure to the acid. Mitochondria from *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* control cells produced good spectra, with estimated cyt *c*/cyt b ratios of approximately 2.0 (Fig. 4A and 4B). In spite of a recurrent low yield of mitochondrial mass, it also possible to obtain enough mitochondria from acetic acid-treated cells to analyse the cytochrome's content after 90 minutes of treatment. During this period, the cytochrome ratio of *wt* mitochondria decreased from an average value of 2.03 to 1.39 (Fig. 4B), while the *Δaac1/2/*3 mitochondria presented a slighter variation from 1.71 to 1.55 (Fig. 4B). These observations reproduce the results of Pereira and colleagues (Pereira et al., 2007), indicating an impaired release of cyt *c* from *Δaac1/2/*3 mitochondria. The cyt *c*/cyt *b* ratio from *Δpor1* mitochondria varied from 1.97 in control cells to 1.52 after the 90 minutes exposure to acetic acid, indicating a rate of cyt *c* release similar to that observed in *wt* mitochondria. Again, mitochondria extraction from *Δpor1* cells treated with acetic acid was problematic, and obtaining good mitochondria preparations proved to be truly challenging. In some cases, the resulting spectra were not suitable for cytochrome quantification. As for the quadruple mutant, the average values obtained for mitochondrial cyt *c*/cyt *b* ratio in control and acetic acid-treated cells, revealed a small variation from 2.12 to 2.03 (Fig. 4B). Therefore, it seems that cyt *c* release in *Δaac1/2/3Δpor1* cells might be compromised, as observed in the *Δaac1/2/*3 mutant.

A



B

Cyt c/Cyt b	Control	90' A.A.
wt	2.03 ± 0.28	1.39 ± 0.08
Δaac1/2/3	1.71 ± 0.07	1.55 ± 0.30
Δpor1	1.97 ± 0.19	1.52 ± 0.05
Δaac1/2/3Δpor1	2.12 ± 0.22	2.03 ± 0.56

Fig. 4 – Evaluation of mitochondrial cytochrome content from *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* control and acetic acid treated cells. Cells were collected before and after exposure to acetic acid, 180 mM for 90 minutes, and mitochondria isolated after enzymatic digestion of the cell wall with zymolyase followed by mechanical disruption of spheroplasts and differential centrifugations. (A) Redox spectra from *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* mitochondria isolated from control and acetic treated cells. 400 µL of mitochondria preparations (10 mg/mL) were divided in two wells from a 96-well micro-plate, one sample oxidized with ferrycianide and the other reduced with sodium dithionite. Difference spectra were acquired between 500 and 650 nm in a in a micro-plate spectrophotometer, and the content of cytochromes c+c_1 and b was estimated by the O.D. differences 550 nm minus 540 nm and 561 nm minus 575 nm, respectively. A decrease in the content of cyt *c* can be observed in mitochondrial preparations from *wt* and *Δpor1* acetic acid-treated cells. Mitochondria from *Δaac1/2/3* and

Δaac1/2/3Δpor1 treated cells seem to retain most of their cyt *c*. One representative experiment is shown for each mitochondria sample. (B) Cyt *c*/cyt *b* ratio estimated for *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* mitochondria from control and acetic acid treated cells (180 mM, 90 minutes). Mitochondria from *wt* and *Δpor1* cells exposed to acetic acid present the larger decrease in cyt *c*/cyt *b* ratios, contrasting with those of *Δaac1/2/3* and *Δaac1/2/3Δpor1* mitochondria. Data represent mean ± SD of at least 3 independent experiments.

3.3. Cytochrome c release evaluated by Western-blot

To confirm the predicted effects from the absence of AAC, Por1p or both proteins in MOMP and cyt *c* release, mitochondria and their corresponding post-mitochondrial fractions were collected, and 50 µg of protein were precipitated for immunodetection of cyt *c*. Western-blot analyses of *wt* mitochondrial and post-mitochondrial samples confirmed the cyt *c* release predicted by redox spectra analysis. Comparing to control samples, there is a significant decrease in the mitochondrial content of cyt *c* in acetic acid-treated *wt* cells (Fig. 5), as previously described by Pereira and colleagues (Pereira et al., 2007). In contrast, the absence of AAC proteins allows mitochondria from treated cells to retain most of their cyt *c* (Fig. 5).

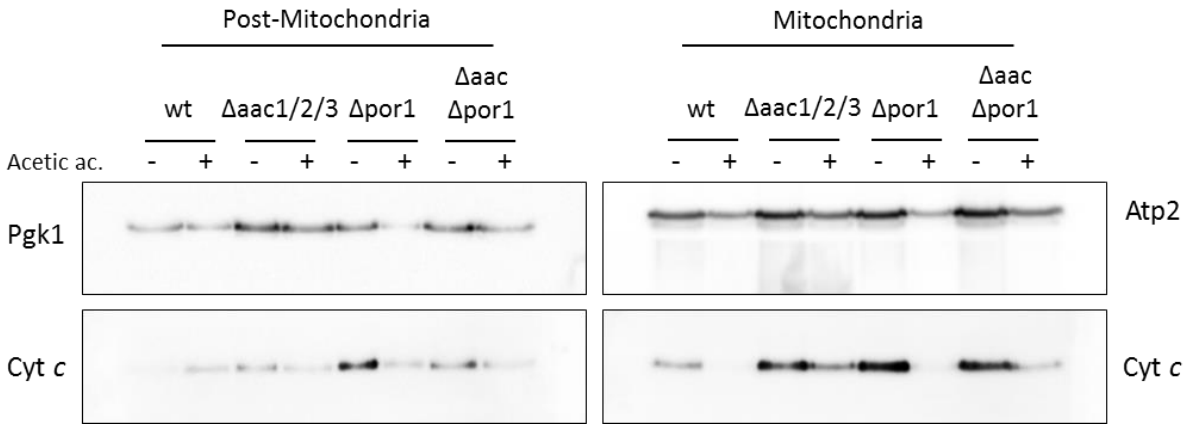


Fig. 5 – Immunodetection of cyt *c* in *wt*, *Δaac1/2/3*, *Δpor1*, *Δaac1/2/3Δpor1* mitochondrial and post mitochondrial fractions isolated from control and acetic-treated cells. Cytosolic 3-phosphoglycerate kinase (Pgk1) and the beta subunit of the F1 sector of mitochondrial F_1F_0 ATP synthase (Atp2) were used as controls for the post- and mitochondrial fractions, respectively. Cyt *c* detection on mitochondrial extracts suggests that *wt* and *Δpor1* mitochondria readily release cyt *c* during acetic acid treatement, whereas *Δaac1/2/3* mitochondria are able to retain a significant amount of this cytochrome. *Δaac1/2/3Δpor1* mitochondria appear to lose more cyt *c* than *Δaac1/2/3* mitochondria, and possibly to a lesser extent than *wt* mitochondria. One representative experiment is shown.

In the set of our results, it was not possible to observe a significant increase of cyt *c* in the post-mitochondrial fractions of *Δaac1/2/*3 cells. On the other hand, the absence of Por1p does not appear to compromise MOMP since the mitochondrial content of cyt *c* decreases after treatment with acetic acid (Fig. 4). Interestingly, we could still observe cyt *c* release from mitochondria of acetic acid-treated *Δaac1/2/3Δpor1* cells, in what appears to be a lesser extent than the release observed for *wt* or *Δpor1* cells (Fig. 5). This observation suggests that MOMP might still occur in the absence of AAC proteins, although at a significantly reduced rate, and suggests an important role of Por1p in the regulation of this process. In a general manner, the results from Western-blot analysis matched the profile of cyt *c* release obtained by redox spectrophotometry, with an exception for *Δaac1/2/3Δpor1*, which presented some differences between both results. These differences might be related to the low content of cyt *b* in *Δaac1/2/*3*Δpor1* acetic acid-treated cells that would affect the estimated cyt *c*/cyt *b* ratio in these mitochondria.

3.4. Effect of AAC and Por1p in the resistance of cell wall to enzymatic digestion

During mitochondria extraction experiments we found that the enzymatic digestion of the cell wall from *Δaac1/2/3* and *Δaac1/2/3Δpor1* cells was much slower and less efficient when compared to the other strains. As a consequence less spheroplasts were obtained from these strains, which eventually contributed to a lower yield in mitochondrial mass. Upon such observations, we decided to evaluate the effects of AAC and Por1p deletions on the resistance of the cell wall to enzymatic digestion with zymolyase. Cell wall digestion by zymolyase was evaluated through variation of the $O.D._{800nm}$ from cellular suspensions, incubated for 60 minutes at 30°C. Our results indicate that *Δaac1/2/3* and *Δaac1/2/3Δpor1* cells have increased resistance to digestion by zymolyase when compared to *wt* or *Δpor1* cells, which show no significant differences among them (Fig. 6). For cells grown in the presence of glucose as carbon source, *Δaac1/2/3* and *Δaac1/2/3Δpor1* mutants exhibit a small O.D. variation after a 60 minutes digestion, reaching values of 62% and 81% of the cells with intact cell wall, respectively. In the same conditions, the $\Delta O.D._{800nm}$ of *wt* and *Δpor1* cells is considerably higher, decreasing to values of nearly 15% of cell wall integrity. When the same cells were grown in YPGal, *Δaac1/2/3* and *Δaac1/2/3Δpor1* mutants presented no significant variation. Once again cells lacking both AAC and Por1p were the most resistant to enzymatic digestion of the cell wall, while the resistance of *wt* and *Δpor1*

cells slightly increased (≈ 25%) (Fig. 6). These results suggest a direct connection between the absence of AAC proteins and the observed changes in cell wall resistance to enzymatic digestion, revealing what could be a new aspect of mitochondrial signalization.

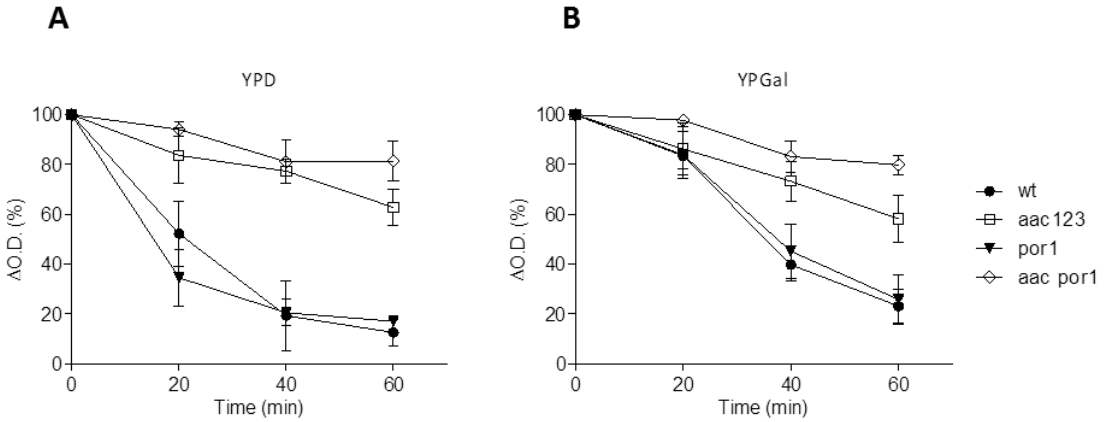


Fig. 6 – Enzymatic digestion of *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* cell wall with zymolyase (20 µg/mL). Cells grown O.N. in YPD or YPGal until exponential phase (approximately 1.0 $O.D._{640nm}$), were collected, washed and resuspended in PBS to a final concentration of 0.5 $O.D._{640nm}$. Digestion was followed by periodic measures of the suspension's absorbance at 800 nm. For each strain, values were normalized for the A_{800nm} at time zero, allowing the assessment of cell wall digestion measured as a variation in absorbance ($\Delta O.D._{800nm}$). Digestion of the cell wall from *wt* and *Δpor1* cells is much more efficient and faster than that of *Δaac1/2/3* and *Δaac1/2/3Δpor1* cells, particularly in glucose-grown cells. This effect is attenuated by the growth in the presence of galactose instead of glucose. *Δaac1/2/3Δpor1* cells are apparently the most resistant to cell wall digestion by zymolyase. Data represent mean ± SD of at least 3 independent experiments.

3.5. Cell wall, osmotic and thermotolerance assays

Since AAC proteins and mitochondria might be involved in signalling events capable of changing the structure of cell wall, particularly evident during acetic acid stress, we decided to evaluate the response of the selected strains to other kinds of stresses. For such purpose, we tested the ability of *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* cells to grown under conditions of osmotic, cell wall and heat stress, by dropout assay. Exponentially grown cells were collected and resuspended to obtain cellular suspensions of 1.0 $O.D._{640nm}$, which were further diluted in a series of 1:10 dilutions. Samples from each suspension were plated in YPDA supplemented with different stress

agents and grown for 5 days at 30°C. Thermotolerance of each strain was tested by the ability of yeast cells to grow at 37°C. Growth in YPGA was used as control.

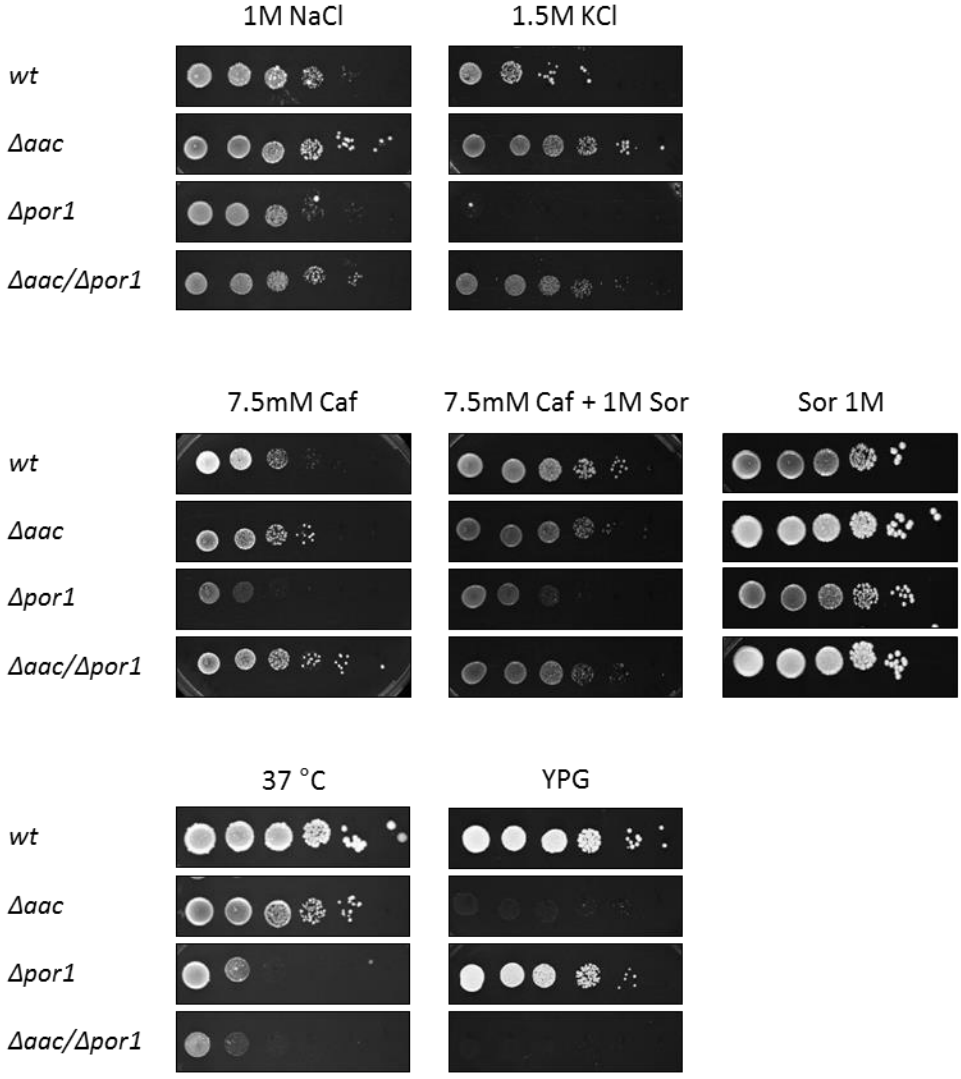


Fig. 7 – Cell wall stress, osmotic stress and thermotolerance of *S. cerevisiae* wt, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* strains. Exponentially grown cells were collected, diluted in a series of 1:10 dilutions and plated in YPDA medium, YPDA supplemented with different stress agents, or YPGA. Osmotic stress was tested in the presence of 1 M NaCl (upper left panel) or 1.5 M KCl (upper right panel). Response to cell wall stress was tested in the presence of 7.5 mM caffeine alone (middle left panel) or 7.5 mM caffeine with 1 M sorbitol (middle centre panel). Sorbitol 1M was used as a control (middle right panel). Thermotolerance was tested by the capacity of each strain to grown on YPDA medium at 37°C (lower left panel). The ability to grow on glycerol was used as control for the respiratory capacity of the tested strains (lower right panel). Absence of the AAC proteins seems to increase the cell's capacity to tolerate osmotic and cell wall stresses, contrasting with the absence of por1, which increases the sensitivity of yeast cells to the mentioned stresses. The phenotype resulting from the absence of AAC proteins overcomess the sensitivity conferred by Por1 deletion, with an exception for the thermotolerance. One representative experiment is presented.

Our results suggest that *Δaac1/2/3* and *Δaac1/2/3Δpor1* cells are more tolerant to osmotic stress than *wt* cells, as observed by growth in the presence of NaCl (1M), and particularly KCl (1.5M) (Fig. 7). On the other hand, the absence of Por1p greatly sensitizes yeast cells to osmotic stress induced by these agents, a phenotype that is completely reversed by the absence of AAC proteins. We also tested the ability of these strains to grow in the presence of the cell wall-perturbing agent caffeine (7.5 mM). Caffeine is a purine analogue that elicits pleiotropic and varied effects on cells, and used to induce cell wall stress for long (Bode and Dong, 2007; Martin et al., 1996). Among its known targets, caffeine inhibits Tor1p-regulated cellular functions and the kinase activity of the ATR and ATM DNA damage checkpoint kinases, interferes with Sit4-mediated signalling and ubiquitin-dependent protein sorting, and promotes a dual phosphorylation of Mpk1/Slt2p, the mitogen-activated protein kinase from (MAPK) from the cell wall integrity (CWI) pathway, preventing the formation of a complex with the transcription factor Swi4 (Hood-DeGrenier, 2011; Kuranda et al., 2006; Reinke et al., 2006; Sarkaria et al., 1999; Truman et al., 2009).

The *Δaac1/2/3* and, particularly, *Δaac1/2/3Δpor1* mutants were able to grow better than the *wt* strain in the presence of caffeine alone (Fig. 7). Once again, *Δpor1* cells exhibited a sensitivity phenotype (Fig. 7). The interference of caffeine in cell wall stability of *wt* cells was evident since its deleterious effect on the growth of this strain was significantly reversed by osmotic stabilization with sorbitol (Fig. 7). On the other hand, only a slight improvement was observed for the other three strains when grown in the presence of caffeine and sorbitol (Fig. 7). This observation suggests that AAC and Por1p deletions modulate the response of *S. cerevisiae* to osmotic stress. Sorbitol alone had no impact in the growth of these cells (Fig. 7). Finally, we tested the response of *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* to heat stress, by measuring their growth ability at 37°C. Our results demonstrate that *wt* cells were the fittest to grow under a heat stress situation (Fig. 7). Absence of AAC proteins had a minor impact on the thermotolerance of *S. cerevisiae*, while deletion of Por1p greatly sensitized yeast cells (Fig. 7). Unlike for osmotic and cell wall stresses, the *Δaac1/2/3Δpor1* mutant exhibited decreased thermotolerance when compared to the *wt* strain, a phenotype similar to that of *Δpor1* mutant (Fig. 7). This was the only tested situation in which the effect of Por1p deletion overcame that of AAC absence, reinforcing the important role of Por1p inº the adaptation of yeast cells to heat stress situations (Blachly-Dyson et al., 1997).

The absence of AAC proteins from yeast cells compromises oxidative phosphorylation resulting in the incapacity of *Δaac1/2/3* mutants to grow on non-fermentable carbon sources (Gawaz et al., 1990).

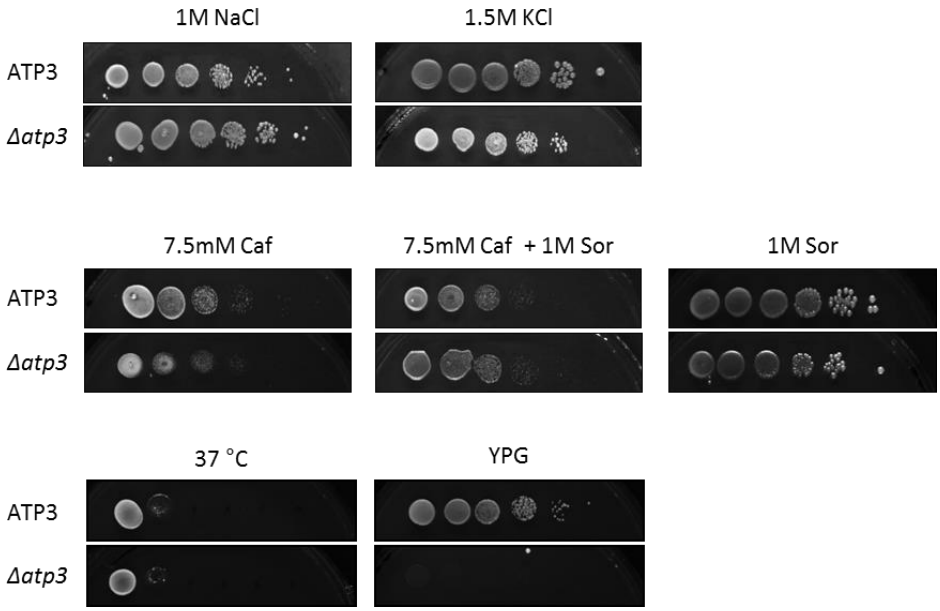


Fig. 8 - Cell wall stress, osmotic stress and thermotolerance of a *S. cerevisiae Δatp3* strain carrying a plasmid with the *ATP3* sequence under control of a Tet-off promoter, in the presence (*Δatp3*) or absence (ATP3) of doxycycline to inhibit or allow expression of *ATP3*, respectively. Cells were grown O.N. in synthetic complete medium without uracil until exponential phase, collected and diluted in a series of 1:10 dilutions. Samples were then plated in YPDA medium, YPDA supplemented with different stress agents, or YPGA, in the presence or absence of doxycycline (10 mg/mL). Osmotic stress was tested in the presence of 1 M NaCl (upper left panel) or 1.5 M KCl (upper right panel). Response to cell wall stress was tested in the presence of 7.5 mM caffeine alone (middle left panel) or 7.5 mM caffeine with 1 M sorbitol (middle centre panel). Sorbitol 1 M was used as a control (middle right panel). Heat stress was tested by the growth capacity on YPDA medium at 37°C (lower left panel). The ability to grow on glycerol was used as control for the respiratory capacity, and to validate this system (lower right panel). The response of yeast cells in the presence or absence of Atp3p was identical, in the different stress situations. One representative experiment is presented.

Considering the results described above, we decided to test if the effect of AAC deletion in cell integrity and the osmotic response was correlated to the respiratory deficiency of *Δaac1/2/3* mutants. For such purpose, we tested the ability of a *Δatp3* mutant, lacking the gamma subunit of the mitochondrial F_1F_0 ATP synthase, to grow on the different stress situations in which the AAC and Por1p mutants were tested. This

strain was transformed with a plasmid containing the *ATP3* sequence under control of a tet-off promoter, allowing us to stop *ATP3* expression by growing these cells in the presence of doxycycline. This way, we were able to compare the effect of Atp3p presence or absence in the capacity of yeast cells to withstand osmotic and cell wall stresses and their thermotolerance, thus measuring the impact of energetic metabolism in such process. Our results indicate that absence of Atp3p does not affect the cell's response to NaCl, KCl, caffeine or growth at 37°C, considering the identical behaviour observed in the presence (*Δatp3*) or absence (*ATP3*) of doxycycline (Fig. 8). Growth on YPGA medium was used as control and validated this system since growth was inhibited in the presence of doxycycline (*Δatp3* mutant) (Fig. 8). Altogether, it seems that the increased resistance of *Δaac1/2/3* mutants to stress induced by NaCl, KCl and caffeine is not correlated to their respiratory deficiency, suggesting a direct role of the AAC proteins in such phenomenon.

4. Discussion

The mechanisms underlying mitochondria permeabilization have been the subject of extensive studies for the last decades. Throughout this period a variety of new concepts and ideas were postulated while others were questioned or even discarded. A good example of such evolution is the contribution of mitochondrial proteins such as the ANT or VDAC to permeability transition (PT) and MOMP. Originally thought to compose the scaffold structure of the PTP, the role of ANT and VDAC in cell death is still controversial. Genetic and molecular studies have seriously questioned their contribution to such an important cellular process, eventually throwing these proteins into the backstage of cell death research (Baines et al., 2007; Kokoszka et al., 2004).

The yeast *S. cerevisiae* has played an important role towards the understanding of mitochondria permeabilization during cell death. Yeast was early considered to be devoid of the major regulators of cell death in mammals, such as the Bcl-2 family members, and so, it has been was widely used in studies involving, for instance, the heterologous expression of the mammalian pro-apoptotic regulator Bax, helping to explain mechanistic aspects from its cellular actions (Kissova et al., 2006; Ligr et al., 1998; Manon et al., 1997; Priault et al., 2002; Priault et al., 2003a; Priault et al., 2003b). In addition, and since yeast possesses homologues for the components previously ascribed to mammalian PTP including three AAC isoforms, Porin and a mitochondrial cyclophilin, it has also been used to understand the involvement of putative components of the PTP in Bax-induced cell death (Gross et al., 2000; Kissova et al., 2000; Marzo et al., 1998; Priault et al., 1999a; Priault et al., 1999b; Shimizu et al., 2000). Furthermore, in the absence of heterologous expression of Bax, yeast cells also exhibit a mitochondrial swelling, resulting from the flow of metabolites across the IMM, which is capable of inducing structural damage to the mitochondrial membranes (Velours et al., 1977). Such swelling, considered to be responsible for the rupture of the OMM in some scenarios of cell death (Vander Heiden et al., 1997), has been attributed to the opening of a large-conductance unselective channel in yeast mitochondria, or YMUC. The size and absence of selectivity of YMUC led some researchers to consider it the “yeast PTP” (Jung et al., 1997). Nevertheless, the peculiar regulatory properties of this yeast channel make it a controversial counterpart of mammalian PTP (Manon et al., 1998), and a

putative contribution of AAC and porin has been discarded (Ballarin and Sorgato, 1995; Lohret et al., 1996).

Interestingly, both the AAC and Por1p have been identified as important elements during acetic acid-induced cell death and mitochondria permeabilization in *S. cerevisiae*, apparently with opposite effects. The absence of AAC proteins increases the resistance of yeast cells to acetic acid-induced death, impairs the mitochondrial release of cyt *c* and delays the outcome of several cellular events associated to an apoptotic-like cell death. On the other hand, Por1p deletion results in increased sensitivity to acetic acid and accelerates other processes such as DNA degradation and loss of plasma membrane integrity (Pereira et al., 2007). A similar effect was found for diamide-induced stress, again with increased resistance in the absence of AAC proteins and a higher sensitivity promoted by *POR1* deletion (Pereira et al., 2007). Characterization of a *Δpor1* mutant revealed that the absence of Por1p affects the dynamics of mitochondrial morphology, favouring the fragmentation of the mitochondrial network. Also, *Δpor1* mutant cells exhibit an increased mitochondrial membrane potential ($\Delta\Psi_m$) when compared to *wt* cells, a phenotype that is not related to the mitochondrial fragmentation (Pereira, 2008). A previous work from our group revealed that the sensitivity of *Δpor1* mutant cells to acetic acid is apparently dependent on the presence of AAC proteins, thus suggesting a common pathway for cell death (Cardoso et al., 2008; Pereira, 2008). Here, we tested the response of *S. cerevisiae* cells lacking the AAC proteins, Por1p or both, to lethal concentrations of acetic acid and measured the impact of each mutation on MOMP and consequent release of cyt *c*, aiming to elucidate putative interactions between these proteins.

Our results confirmed the observations of Pereira and colleagues in which the absence of all three AAC isoforms promotes an increased resistance to acetic acid treatment, whereas the absence of Por1p sensitizes yeast cells (Pereira et al., 2007). When AAC and Por1p are simultaneously absent, yeast cells exhibit an acetic acid-resistance phenotype, and the survival of *Δaac1/2/3Δpor1* closely matches that of the *Δaac1/2/3* mutant, suggesting that Por1 is not involved in the mechanism responsible for the resistance associated to AAC deletion. Accompanying the resistance phenotype, cyt *c* release from mitochondria during acetic acid-induced death is impaired in *Δaac1/2/3* mutant cells (Pereira et al., 2007). These observations support the hypothesis of a possible contribution of AAC and Por1p to the formation of a pore in mitochondria, although with apparent distinct effects. Since Por1p is not involved in the resistance

phenotype of the *Δaac1/2/3* mutant, we decided to evaluate the impact of Por1p, and its putative collaboration with the AAC, on MOMP during acetic acid treatment.

Mitochondria were extracted from the different cell types, before and after exposure to acetic acid, to assess MOMP measured by the ability to release cyt *c*. Our results showed that after 3h of treatment, it is very difficult to obtain good mitochondria preparations from acetic acid treated cells, and the final outcome in mitochondrial mass was considerably lower when compared to control cells. This was particularly evident for the *Δpor1* mutant, most likely because of its reduced survival capacity (< 10% after 3 hours). Considering this limitation, we changed our experimental conditions to evaluate cyt *c* release after a shorter exposure to acetic acid (90 min), thus ensuring a higher survival of *Δpor1* cells (~50%). Importantly, at 90 min of treatment the survival propensity of each strain was similar to that observed after 3 hours, again with increased survival of *Δaac1/2/3* and *Δaac1/2/3Δpor1* cells, and lower cell viability of the *Δpor1* mutant.

After 90 minutes of acetic acid exposure, we could still isolate enough mitochondria for redox spectra analysis of cyt *c* relase from *wt*, *Δaac1/2/3*, *Δpor1* and *Δaac1/2/3Δpor1* mitochondria. As expected, acetic acid treatment induced the release of cyt *c* in *wt* cells, confirmed by a significant decrease (about 30%) on cyt *c*/cyt *b* ratio and by immunodetection of cyt *c* in mitochondrial and post-mitochondrial fractions. Conversely, the absence of AAC proteins seems to impair this process resulting, at least in our experimental conditions, in smaller release (about 10%) of the cyt *c*. Apart from the observation that cyt *c* was not released form mitochondria in *Δaac1/2/3* acetic acid-treated cells, Pereira and colleagues reported what seems to be a selective degradation of this cytchrome along with other mitochondrial proteins (Pereira et al., 2007). This may not be the case in our experiments, still it should be noted that the treatment periods used in both works are considerably different (90 or 200 minutes), and the shorter acid exposure used in this work could be insufficient to discriminate the mentioned protein degradation.

The absence of Por1p does not seem to impair MOMP during acetic acid treatment, as suggested by the absence of cyt *c* from *Δpor1* mitochondrial fractions, and the variation in cyt *c*/cyt *b* ratio observed for this strain (≈ 25%) which is similar to that of *wt* cells. Still, the fragility of *Δpor1* mitochondria was a constant limitation during these procedures, making it hard to obtain good mitochondrial preparations and to define a direct relationship between cause and effect. Interestingly, in the absence of both AAC

and Por1p, cyt *c* release is still observed in mitochondria isolated from acetic acid-treated yeast cells. The loss of mitochondrial cyt *c* in the *Δaac1/2/3Δpor1* mutant could be classified as greater than that observed in *Δaac1/2/3* cells, and similar or slightly inferior to the release of cyt *c* from *wt* mitochondria. This result is divergent from that observed by redox spectrophotometry, in which no significant variance was observed in cyt *c*/cyt *b* ratio, probably because of the simultaneous decrease in the content of both cytochromes (c+c_1 and b). The release of cyt *c* in the absence of both AAC and Por1p might lead to a new perspective on the contribution of these proteins to MOMP.

If MOMP during acetic acid-induced death was completely dependent on the AAC proteins, and regulated by Por1p, it would be expected to observe no permeabilization of *Δaac1/2/3* mitochondria, contrasting to unregulated permeabilization of *Δpor1* mitochondria. Following this hypothesis, if both proteins are removed, the absence of AAC proteins should be enough to prevent the uncontrolled MOMP of cells lacking Por1p as well. Although the absence of AAC isoforms is able to completely convert the sensitive phenotype resulting from Por1p deletion into a resistant one, it is apparently insufficient to prevent the release of cyt *c* from mitochondria of acetic acid treated cells. It should be noted, however, that cyt *c* release and cell death do not necessarily correlate with each other. The contradictory observations from redox spectra and Western-blot analyses do not provide a solid answer to the initial question, and two possible scenarios might be speculated: either the AAC and Por1p are "just" two regulatory players of a more complex mechanism mediating the yeast MOMP, a scenario that has also been hypothesized in mammalian cells (Siemen and Ziemer, 2013), or a different mechanism could be accountable for the results described above.

In mammalian cells, the contribution of AAC and Por1p orthologues, ANT and VDAC, to MOMP and cyt *c* release during cell death has been discarded. Mitochondria lacking either ANT or VDAC isoforms could still undergo permeability transition induced by Ca^{2+}, and only a regulatory role was attributed to the ANT (Baines et al., 2007; Kokoszka et al., 2004). Likewise, the AAC and Por1p are apparently dispensable for the YMUC (Ballarin and Sorgato, 1995; Lohret et al., 1996). Considering these evidences, it would not be strange to envisage an acetic acid-induced MOMP in the absence of these proteins, as well. Nevertheless, the impact from AAC deletion in the cell survival of *S. cerevisiae* to acetic acid treatment cannot be ignored and cyt *c* release is definitely impaired in *Δaac1/2/3* cells. In *Δaac1/2/3Δpor1* cells the resistance phenotype "provided" by the absence of AAC proteins actually overcomes the

sensitivity phenotype resulting from Por1p deletion. Therefore, it is challenging to explain how two mutant strains, both lacking the AAC proteins and exhibiting identical resistance to acetic acid would present different mitochondrial permeabilization. The distinctive trait between these two strains is the absence of Por1p a protein that, among other functions, has been associated to the maintenance of redox sate, mitochondrial DNA import and even cytoskeleton rearrangements (D'Souza-Schorey et al., 1997; Galganska et al., 2008; Weber-Lotfi et al., 2009). Additionally, the absence of Por1p, or inhibition of this channel with the VDAC inhibitor 4´-diisothiocyano-2,2´-disulfonic acid stilbene (DIDS), induces the fragmentation of the mitochondrial network, a phenotype that is not reversed by the absence of AAC proteins (Pereira, 2008). This contribution of Por1p to mitochondrial morphology might be of outmost importance to understand the role of this protein in cyt *c* release from mitochondria.

Changes in the dynamics of mitochondria morphology are actually able to modulate cell death in several organisms, including yeasts. Accordingly, deletion of yeast's fission proteins Dnm1p or Mvd1p delays the fragmentation of the mitochondrial network and, in case of cells lacking Mvd1p, promotes cell survival following a death stimulus or in ageing cells (Fannjiang et al., 2004; Scheckhuber et al., 2007). Other proteins associated with cell death in yeast can also contribute to mitochondrial morphology. This is the case of Yca1p (yeast caspase 1), a Ca^{2+}-dependent cysteine protease whose deletion has been associated to a decrease in mitochondria fission of a yeast strain lacking *LSM4*, a gene involved in mRNA decapping whose deletion leads to an apoptotic-like cell death, and increased survival in response to different death stimuli. (Mazzoni et al., 2005; Mazzoni et al., 2003). An analogy could be established for the role of Por1p in yeast cell death. The absence of Por1 affects mitochondrial morphology leading to the fragmentation of these organelles and promoting cell death associated to cyt *c* release, somewhat explaining the results obtained for the *Δpor1* mutant, and also the possible loss of mitochondrial cyt *c* observed in *Δaac1/2/3Δpor1* cells, which are also unable to form mitochondrial networks (Pereira, 2008).

The increased mitochondrial fragmentation (Pereira, 2008), or reduced osmotic stability resulting from the absence of Por1p (Sanchez et al., 2001), could be responsible for the observed cyt *c* release in *Δaac1/2/3Δpor1* cells during acetic acid treatment, thus explaining an eventual bypass of the impaired MOMP in the absence of AAC. Further experimentation could help to clarify this hypothesis. For instance, determining the activities of mitochondrial proteins such as the IMS adenylate kinase

and the matrix citrate synthase, in mitochondrial and post mitochondrial fractions of untreated and acid-treated cells, would help to monitor the integrity of OMM and IMM, respectively, and hence to properly evaluate MOMP in each strain. This would allow measuring the real impact of AAC, Por1p or both, in cyt *c* release and to exclude a possible contribution from mitochondrial lability and damage resulting from the extraction procedure. It should also be interesting to manipulate the dynamics of mitochondrial morphology to test its influence in the sensitivity of yeast cells to acetic acid, and evaluate the role of mitochondrial fusion/fission proteins in the AAC-mediated MOMP. Alternatively, *S. cerevisiae POR1* has a paralog, *POR2*, which has been demonstrated to complement the inability of *Δpor1* cells to grow at 37°C when it is overexpressed. In spite of the resemblance with Por1p (50%), Por2p is not a real porin and does not seem to significantly contribute for the permeability of the OMM (Blachly-Dyson et al., 1997; Lee et al., 1998). It could be worthy to test the effect of Por2p in acetic acid-induced MOMP and cyt *c* release, particularly since Por2p does not seem to have channel properties in yeast mitochondria, and its deletion is associated to increased mitochondrial branching without affecting the acetic acid tolerance or $\Delta\Psi_m$ of yeast cells (Blachly-Dyson et al., 1997; Pereira, 2008).

The effect of AAC deletion in the acetic acid-induced cell death may also be associated to a different process, possibly unrelated to MOMP. Indeed, we observed that obtaining spheroplasts from strains lacking the AAC proteins was much harder when compared to *wt* cells, even without any treatment. Upon such observation, we wondered if the absence of AAC proteins could somehow promote an increased resistance of yeast cells through changes in their cell wall. In fact, the absence of AAC results in increased resistance to cell wall digestion by zymolyase, while deletion of Por1p had no effect. When both proteins are absent we observed a resistance phenotype similar to that of *Δaac1/2/3* cells. As for acetic acid treatment, the increased resistance seems to depend exclusively on the absence of AAC. Considering these observations, we decided to evaluate the response of each strain to several stress conditions by their ability to grown in the presence of different stress agents.

Our results indicate that the absence of AAC proteins increases the resistance of yeast cells to osmotic stress and a cell wall disturbing agent. This increased resistance is independent of Por1p, since the *Δaac1/2/3Δpor1* mutant preserves the phenotype resulting from AAC deletion. *Δpor1* cells, on the other hand, are more sensitive to both kinds of stresses. Recently, it was demonstrated that Por1p and Por2p contribute to the

positive control of Snf1 protein kinase, the central activator of respiratory metabolism responsible for the transcription of a large set of genes, including many involved in mitochondrial functions (Hardie, 2007; Strogolova et al., 2012; Young et al., 2003). Snf1p had previously been proposed as an essential element for the adaptation to salt stress through the activation of mitochondrial function (Pastor et al., 2009), thus accounting, at least in part, for the higher sensitivity of *Δpor1* cells. Curiously, the absence of AAC does not significantly affect the response to heat stress and in this case the sensitivity phenotype resulting from Por1p deletion is also exhibited by the *Δaac1/2/3Δpor1* mutant. These evidences implicate two different processes; (i) the response to heat stress highly influenced by the presence of Por1p and unrelated to the AAC, (ii) and a pathway leading to increased resistance to osmotic stress which involves alterations in the cell wall integrity and depends on the absence of AAC proteins. To test a possible connection between the increased resistances of AAC-less cells to osmotic and cell wall stresses and the eventual limitations on ATP production, we used a biological system which enabled us to switch off the expression of *ATP3*. Our data suggests that this resistance phenotype, resulting from the absence of AAC, cannot be attributed to the impaired respiration and its consequences for the energetic metabolism.

We can conclude that, the absence of AAC proteins leads to increased resistance of *S. cerevisiae* cells to osmotic and cell wall stresses, a process associated to changes in the cell wall structure and/or composition. This mechanism does not seem to depend on the presence of Por1p. To our knowledge, no direct connection has been established between the AAC proteins and the cell wall biogenesis/composition in *S. cerevisiae*. Still, it is possible to find some associations between proteins or processes involved in cell wall assembly/remodelling and mitochondria. For instance, the defective cell wall of *Candida albicans ccr4/pop2* mutants is linked to mitochondrial dysfunction and phospholipid imbalance (Dagley et al., 2011). Proteins associated to the cell wall integrity pathway, namely Pkc1p, Bck1p and Mkk1, were found to relocate to mitochondria of yeast cells treated with farnesol (Fairn et al., 2007). Sun4p and Uth1p, two members from the SUN family, are localized in the cell wall and mitochondria (Velours et al., 2002). The latter, has been associated to the biogenesis of both structures (Camougrand et al., 2000; Ritch et al., 2010). Alternatively, the ADP/ATP carrier from *Pseudozima antarctica* is apparently involved in the biosynthesis of mannosylerythriol lipids (Morita et al., 2010).

We can further propose that the changes in the cell wall of *Δaa1/2/3* strains could be a preconditioning favouring the capacity of these cells to respond and survive to acetic acid. Different screenings have identified genes associated to cell wall functions, such as the synthesis of cell wall components, cell wall assembly or remodelling, and even genes required for the mannosylation of proteins, as essential for the resistance of *S. cerevisiae* cells to acetic acid (Sousa et al., 2013, accepted for publication) (Mira et al., 2010). Additionally, acetic acid treatment induces the activation of two yeast MAP kinases; Hog1p MAPK from the osmotic stress signalling pathway, and Slt2p from the cell wall integrity (CWI) pathway (Mollapour and Piper, 2006). A recent work from our group actually implicates the CWI pathway in the signalling of acetic acid-induced death, and blocking this signal transduction renders cells more resistant to the acid (Azevedo, 2011). Furthermore, changes in the plasma membrane and cell wall resulting from the adaptation of yeast cells exposed to weak acids have also been proposed to limit the diffusional entry of the acids (Ullah et al., 2013). It is possible that the cell wall changes induced by the absence of AAC proteins actually promote the increased resistance of yeast cells to acetic acid. Measuring the uptake of acetic acid in these strains should provide important clues on this hypothesis.

In this work, we assessed the role of AAC and Por1p in MOMP during acetic acid-induced cell death. Although the exact contribution of each protein to MOMP requires further investigations, we found that the presence of Por1p is not necessary for cyt *c* release form mitochondria of acetic acid treated cells. Mitochondria from cells lacking both AAC and Por1p are apparently able to undergo acetic acid-induced cyt *c* release, which raises the possibility that AAC proteins may not be indispensable for this process as well. In the past few years, the inorganic phosphate carrier (PiC) and the F_1F_0 ATP synthase have been proposed has putative elements of the PTP in yeast mitochondria, and it should be interesting to test the impact of both proteins in this working model (Giorgio et al., 2013; Leung et al., 2008). It is also possible that the absence of Por1p *per se* weakens mitochondria and compromises their integrity during acetic acid treatment. Both scenarios should be explored. Additionally, we reveal a possible connection between the AAC proteins and the structure/composition of the cell wall, which is well documented as an important element in the resistance of yeast cells to different acids. This unexpected link between two very different cellular components might also account for the resistance of *Δaac1/2/3* strains to acetic acid.

Acknowledgements

We would like to thank Doctor Alexander Tzagoloff, from Columbia University, Biological Sciences, New York, USA, for providing the yeast strain W303. We also thank Doctor Emmanuel Tétaud from Institute de Biochimie et Génétiques Celulaires (IBGC), CNRS – Université Bordeaux Segalen, Bordeaux, France, for providing the *Δatp3* mutant and plasmid pCM189-ATP3. Work at the Institut de Biochimie et Génétique Cellulaires (IBGC), CNRS/Université Bordeaux Segalen was supported by Fundação Calouste Gulbenkian. D. Trindade was the recipient of a fellowship from Fundação Calouste Gulbenkian, Portugal.

5. References

Azevedo, F. (2011). The role of MAPK signaling pathways in acetic acid-induced cell death of *Saccharomyces cerevisiae*. In Departamento de Biologia (Escola de Ciências: Universidade do Minho). Master thesis.

Azzolin, L., von Stockum, S., Basso, E., Petronilli, V., Forte, M.A., and Bernardi, P. (2010). The mitochondrial permeability transition from yeast to mammals. FEBS letters *584*, 2504-2509.

Baines, C.P., Kaiser, R.A., Sheiko, T., Craigen, W.J., and Molkentin, J.D. (2007). Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. Nature cell biology *9*, 550-555.

Ballarin, C., and Sorgato, M.C. (1995). An electrophysiological study of yeast mitochondria. Evidence for two inner membrane anion channels sensitive to ATP. The Journal of biological chemistry *270*, 19262-19268.

Beutner, G., Ruck, A., Riede, B., Welte, W., and Brdiczka, D. (1996). Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore. FEBS letters *396*, 189-195.

Blachly-Dyson, E., Song, J., Wolfgang, W.J., Colombini, M., and Forte, M. (1997). Multicopy suppressors of phenotypes resulting from the absence of yeast VDAC encode a VDAC-like protein. Molecular and cellular biology *17*, 5727-5738.

Bode, A.M., and Dong, Z. (2007). The enigmatic effects of caffeine in cell cycle and cancer. Cancer letters *247*, 26-39.

Braun, R.J., Zischka, H., Madeo, F., Eisenberg, T., Wissing, S., Buttner, S., Engelhardt, S.M., Buringer, D., and Ueffing, M. (2006). Crucial mitochondrial impairment upon *CDC48* mutation in apoptotic yeast. The Journal of biological chemistry *281*, 25757-25767.

Camougrand, N.M., Mouassite, M., Velours, G.M., and Guerin, M.G. (2000). The "SUN" family: *UTH1*, an ageing gene, is also involved in the regulation of mitochondria biogenesis in *Saccharomyces cerevisiae*. Archives of biochemistry and biophysics *375*, 154-160.

Cardoso, T., Pereira, C., Pacheco, A., Côrte-Real, M., and Sousa, M.J. (2008). Absence of ADP/ATP carrier rescues the apoptotic sensitive phenotype of *Saccharomyces cerevisiae POR1* deletion strain. Personal communication.

Colombini, M. (1979). A candidate for the permeability pathway of the outer mitochondrial membrane. Nature *279*, 643-645.

Colombini, M., Yeung, C.L., Tung, J., and Konig, T. (1987). The mitochondrial outer membrane channel, VDAC, is regulated by a synthetic polyanion. Biochimica et biophysica acta *905*, 279-286.

D'Souza-Schorey, C., Boshans, R.L., McDonough, M., Stahl, P.D., and Van Aelst, L. (1997). A role for POR1, a Rac1-interacting protein, in ARF6-mediated cytoskeletal rearrangements. The EMBO journal *16*, 5445-5454.

Dagley, M.J., Gentle, I.E., Beilharz, T.H., Pettolino, F.A., Djordjevic, J.T., Lo, T.L., Uwamahoro, N., Rupasinghe, T., Tull, D.L., McConville, M., *et al.* (2011). Cell wall integrity is linked to mitochondria and phospholipid homeostasis in *Candida albicans* through the activity of the post-transcriptional regulator Ccr4-Pop2. Molecular microbiology *79*, 968-989.

Desagher, S., and Martinou, J.C. (2000). Mitochondria as the central control point of apoptosis. Trends in cell biology *10*, 369-377.

Fairn, G.D., Macdonald, K., and McMaster, C.R. (2007). A chemogenomic screen in *Saccharomyces cerevisiae* uncovers a primary role for the mitochondria in farnesol toxicity and its regulation by the Pkc1 pathway. The Journal of biological chemistry *282*, 4868-4874.

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.

Galganska, H., Budzinska, M., Wojtkowska, M., and Kmita, H. (2008). Redox regulation of protein expression in *Saccharomyces cerevisiae* mitochondria: possible role of VDAC. Archives of biochemistry and biophysics *479*, 39-45.

Galluzzi, L., and Kroemer, G. (2007). Mitochondrial apoptosis without VDAC. Nature cell biology *9*, 487-489.

Gawaz, M., Douglas, M.G., and Klingenberg, M. (1990). Structure-function studies of adenine nucleotide transport in mitochondria. II. Biochemical analysis of distinct AAC1 and AAC2 proteins in yeast. The Journal of biological chemistry *265*, 14202-14208.

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.

Gross, A., Pilcher, K., Blachly-Dyson, E., Basso, E., Jockel, J., Bassik, M.C., Korsmeyer, S.J., and Forte, M. (2000). Biochemical and genetic analysis of the mitochondrial response of yeast to BAX and BCL-X_L. Molecular and cellular biology *20*, 3125-3136.

Gutierrez-Aguilar, M., Perez-Vazquez, V., Bunoust, O., Manon, S., Rigoulet, M., and Uribe, S. (2007). In yeast, Ca^{2+} and octylguanidine interact with porin (VDAC) preventing the mitochondrial permeability transition. Biochimica et biophysica acta *1767*, 1245-1251.

Hardie, D.G. (2007). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nature reviews Molecular cell biology *8*, 774-785.

Hodge, T., and Colombini, M. (1997). Regulation of metabolite flux through voltage-gating of VDAC channels. The Journal of membrane biology *157*, 271-279.

Hood-DeGrenier, J.K. (2011). Identification of phosphatase 2A-like Sit4-mediated signalling and ubiquitin-dependent protein sorting as modulators of caffeine sensitivity in S. cerevisiae. Yeast *28*, 189-204.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. Journal of bacteriology *153*, 163-168.

Jung, D.W., Bradshaw, P.C., and Pfeiffer, D.R. (1997). Properties of a cyclosporin-insensitive permeability transition pore in yeast mitochondria. The Journal of biological chemistry *272*, 21104-21112.

Kissova, I., Plamondon, L.T., Brisson, L., Priault, M., Renouf, V., Schaeffer, J., Camougrand, N., and Manon, S. (2006). Evaluation of the roles of apoptosis, autophagy, and mitophagy in the loss of plating efficiency induced by Bax expression in yeast. The Journal of biological chemistry *281*, 36187-36197.

Kissova, I., Polcic, P., Kempna, P., Zeman, I., Sabova, L., and Kolarov, J. (2000). The cytotoxic action of Bax on yeast cells does not require mitochondrial ADP/ATP carrier but may be related to its import to the mitochondria. FEBS letters *471*, 113-118.

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.

Kolarov, J., Kolarova, N., and Nelson, N. (1990). A third ADP/ATP translocator gene in yeast. The Journal of biological chemistry *265*, 12711-12716.

Krauskopf, A., Eriksson, O., Craigen, W.J., Forte, M.A., and Bernardi, P. (2006). Properties of the permeability transition in VDAC1($^{-/-}$) mitochondria. Biochimica et biophysica acta *1757*, 590-595.

Kuranda, K., Leberre, V., Sokol, S., Palamarczyk, G., and Francois, J. (2006). Investigating the caffeine effects in the yeast Saccharomyces cerevisiae brings new insights into the connection between TOR, PKC and Ras/cAMP signalling pathways. Molecular microbiology *61*, 1147-1166.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Law, R.H., Manon, S., Devenish, R.J., and Nagley, P. (1995). ATP synthase from *Saccharomyces cerevisiae*. Methods in enzymology *260*, 133-163.

Lawson, J.E., and Douglas, M.G. (1988). Separate genes encode functionally equivalent ADP/ATP carrier proteins in *Saccharomyces cerevisiae*. Isolation and analysis of *AAC2*. The Journal of biological chemistry *263*, 14812-14818.

Lee, A.C., Xu, X., Blachly-Dyson, E., Forte, M., and Colombini, M. (1998). The role of yeast VDAC genes on the permeability of the mitochondrial outer membrane. The Journal of membrane biology *161*, 173-181.

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

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.

Ligr, M., Madeo, F., Frohlich, E., Hilt, W., Frohlich, K.U., and Wolf, D.H. (1998). Mammalian Bax triggers apoptotic changes in yeast. FEBS letters *438*, 61-65.

Lohret, T.A., and Kinnally, K.W. (1995). Multiple conductance channel activity of wild-type and voltage-dependent anion-selective channel (VDAC)-less yeast mitochondria. Biophysical journal *68*, 2299-2309.

Lohret, T.A., Murphy, R.C., Drgon, T., and Kinnally, K.W. (1996). Activity of the mitochondrial multiple conductance channel is independent of the adenine nucleotide translocator. The Journal of biological chemistry *271*, 4846-4849.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. The Journal of biological chemistry *193*, 265-275.

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.

Mannella, C.A., and Guo, X.W. (1990). Interaction between the VDAC channel and a polyanionic effector. An electron microscopic study. Biophysical journal *57*, 23-31.

Manon, S., Chaudhuri, B., and Guerin, M. (1997). Release of cytochrome c and decrease of cytochrome *c* oxidase in Bax-expressing yeast cells, and prevention of these effects by coexpression of Bcl-x_L. FEBS letters *415*, 29-32.

Manon, S., Roucou, X., Guerin, M., Rigoulet, M., and Guerin, B. (1998). Characterization of the yeast mitochondria unselective channel: a counterpart to the mammalian permeability transition pore? Journal of bioenergetics and biomembranes *30*, 419-429.

Martin, H., Castellanos, M.C., Cenamor, R., Sanchez, M., Molina, M., and Nombela, C. (1996). Molecular and functional characterization of a mutant allele of the mitogen-activated protein-kinase gene SLT2(MPK1) rescued from yeast autolytic mutants. Current genetics *29*, 516-522.

Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J.M., Susin, S.A., Vieira, H.L., Prevost, M.C., Xie, Z., Matsuyama, S., Reed, J.C., *et al.* (1998). Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science *281*, 2027-2031.

Matouschek, A., Rospert, S., Schmid, K., Glick, B.S., and Schatz, G. (1995). Cyclophilin catalyzes protein folding in yeast mitochondria. Proceedings of the National Academy of Sciences of the United States of America *92*, 6319-6323.

Mazzoni, C., Herker, E., Palermo, V., Jungwirth, H., Eisenberg, T., Madeo, F., and Falcone, C. (2005). Yeast caspase 1 links messenger RNA stability to apoptosis in yeast. EMBO reports *6*, 1076-1081.

Mazzoni, C., Mancini, P., Verdone, L., Madeo, F., Serafini, A., Herker, E., and Falcone, C. (2003). A truncated form of KlLsm4p and the absence of factors involved in mRNA decapping trigger apoptosis in yeast. Molecular biology of the cell *14*, 721-729.

Mihara, K., and Sato, R. (1985). Molecular cloning and sequencing of cDNA for yeast porin, an outer mitochondrial membrane protein: a search for targeting signal in the primary structure. The EMBO journal *4*, 769-774.

Mira, N.P., Palma, M., Guerreiro, J.F., and Sa-Correia, I. (2010). Genome-wide identification of *Saccharomyces cerevisiae* genes required for tolerance to acetic acid. Microbial cell factories *9*, 79.

Mollapour, M., and Piper, P.W. (2006). Hog1p mitogen-activated protein kinase determines acetic acid resistance in *Saccharomyces cerevisiae*. FEMS yeast research *6*, 1274-1280.

Morita, T., Ito, E., Kitamoto, H.K., Takegawa, K., Fukuoka, T., Imura, T., and Kitamoto, D. (2010). Identification of the gene PaEMT1 for biosynthesis of mannosylerythritol lipids in the basidiomycetous yeast Pseudozyma antarctica. Yeast *27*, 905-917.

Pastor, M.M., Proft, M., and Pascual-Ahuir, A. (2009). Mitochondrial function is an inducible determinant of osmotic stress adaptation in yeast. The Journal of biological chemistry *284*, 30307-30317.

Pereira, C. (2008). Involvement of mitochondrial proteins in yeast apoptosis. In Escola de Ciências, Universidade do Minho (Braga, Portugal: Universidade do Minho). PhD thesis.

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.

Postis, V., De Marcos Lousa, C., Arnou, B., Lauquin, G.J., and Trezeguet, V. (2005). Subunits of the yeast mitochondrial ADP/ATP carrier: cooperation within the dimer. Biochemistry *44*, 14732-14740.

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.

Priault, M., Bessoule, J.J., Grelaud-Coq, A., Camougrand, N., and Manon, S. (2002). Bax-induced cell death in yeast depends on mitochondrial lipid oxidation. European journal of biochemistry / FEBS *269*, 5440-5450.

Priault, M., Camougrand, N., Chaudhuri, B., Schaeffer, J., and Manon, S. (1999a). Comparison of the effects of bax-expression in yeast under fermentative and respiratory conditions: investigation of the role of adenine nucleotides carrier and cytochrome *c*. FEBS letters *456*, 232-238.

Priault, M., Camougrand, N., Kinnally, K.W., Vallette, F.M., and Manon, S. (2003a). Yeast as a tool to study Bax/mitochondrial interactions in cell death. FEMS yeast research *4*, 15-27.

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

Priault, M., Chaudhuri, B., Clow, A., Camougrand, N., and Manon, S. (1999b). Investigation of bax-induced release of cytochrome c from yeast mitochondria permeability of mitochondrial membranes, role of VDAC and ATP requirement. European journal of biochemistry / FEBS *260*, 684-691.

Reinke, A., Chen, J.C., Aronova, S., and Powers, T. (2006). Caffeine targets TOR complex I and provides evidence for a regulatory link between the FRB and kinase domains of Tor1p. The Journal of biological chemistry *281*, 31616-31626.

Ritch, J.J., Davidson, S.M., Sheehan, J.J., and Austriaco, N. (2010). The *Saccharomyces* SUN gene, *UTH1*, is involved in cell wall biogenesis. FEMS yeast research *10*, 168-176.

Rostovtseva, T.K., Antonsson, B., Suzuki, M., Youle, R.J., Colombini, M., and Bezrukov, S.M. (2004). Bid, but not Bax, regulates VDAC channels. The Journal of biological chemistry *279*, 13575-13583.

Sanchez, N.S., Pearce, D.A., Cardillo, T.S., Uribe, S., and Sherman, F. (2001). Requirements of Cyc2p and the porin, Por1p, for ionic stability and mitochondrial integrity in *Saccharomyces cerevisiae*. Archives of biochemistry and biophysics *392*, 326-332.

Sarkaria, J.N., Busby, E.C., Tibbetts, R.S., Roos, P., Taya, Y., Karnitz, L.M., and Abraham, R.T. (1999). Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer research *59*, 4375-4382.

Scheckhuber, C.Q., Erjavec, N., Tinazli, A., Hamann, A., Nystrom, T., and Osiewacz, H.D. (2007). Reducing mitochondrial fission results in increased life span and fitness of two fungal ageing models. Nature cell biology *9*, 99-105.

Shimizu, S., Narita, M., and Tsujimoto, Y. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. Nature *399*, 483-487.

Shimizu, S., Shinohara, Y., and Tsujimoto, Y. (2000). Bax and Bcl-x_L independently regulate apoptotic changes of yeast mitochondria that require VDAC but not adenine nucleotide translocator. Oncogene *19*, 4309-4318.

Siemen, D., and Ziemer, M. (2013). What is the nature of the mitochondrial permeability transition pore and what is it not? IUBMB life *65*, 255-262.

Strogolova, V., Orlova, M., Shevade, A., and Kuchin, S. (2012). Mitochondrial porin Por1 and its homolog Por2 contribute to the positive control of Snf1 protein kinase in Saccharomyces cerevisiae. Eukaryotic cell *11*, 1568-1572.

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.

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.

Truman, A.W., Kim, K.Y., and Levin, D.E. (2009). Mechanism of Mpk1 mitogen-activated protein kinase binding to the Swi4 transcription factor and its regulation by a novel caffeine-induced phosphorylation. Molecular and cellular biology *29*, 6449-6461.

Ullah, A., Chandrasekaran, G., Brul, S., and Smits, G.J. (2013). Yeast adaptation to weak acids prevents futile energy expenditure. Frontiers in microbiology *4*, 142.

Vander Heiden, M.G., Chandel, N.S., Schumacker, P.T., and Thompson, C.B. (1999). Bcl-x_L prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Molecular cell *3*, 159-167.

Vander Heiden, M.G., Chandel, N.S., Williamson, E.K., Schumacker, P.T., and Thompson, C.B. (1997). Bcl-x_L regulates the membrane potential and volume homeostasis of mitochondria. Cell *91*, 627-637.

Vander Heiden, M.G., Li, X.X., Gottleib, E., Hill, R.B., Thompson, C.B., and Colombini, M. (2001). Bcl-x_L promotes the open configuration of the voltage-dependent anion channel and metabolite passage through the outer mitochondrial membrane. The Journal of biological chemistry *276*, 19414-19419.

Velours, G., Boucheron, C., Manon, S., and Camougrand, N. (2002). Dual cell wall/mitochondria localization of the 'SUN' family proteins. FEMS microbiology letters *207*, 165-172.

Velours, J., Rigoulet, M., and Guerin, B. (1977). Protection of yeast mitochondrial structure by phosphate and other H^+-donating anions. FEBS letters *81*, 18-22.

Weber-Lotfi, F., Ibrahim, N., Boesch, P., Cosset, A., Konstantinov, Y., Lightowlers, R.N., and Dietrich, A. (2009). Developing a genetic approach to investigate the mechanism of mitochondrial competence for DNA import. Biochimica et biophysica acta *1787*, 320-327.

Yamada, A., Yamamoto, T., Yoshimura, Y., Gouda, S., Kawashima, S., Yamazaki, N., Yamashita, K., Kataoka, M., Nagata, T., Terada, H., *et al.* (2009). Ca2+-induced permeability transition can be observed even in yeast mitochondria under optimized experimental conditions. Biochimica et biophysica acta *1787*, 1486-1491.

Yamaki, M., Umehara, T., Chimura, T., and Horikoshi, M. (2001). Cell death with predominant apoptotic features in Saccharomyces cerevisiae mediated by deletion of the histone chaperone *ASF1/CIA1*. Genes to cells : devoted to molecular & cellular mechanisms *6*, 1043-1054.

Young, E.T., Dombek, K.M., Tachibana, C., and Ideker, T. (2003). Multiple pathways are co-regulated by the protein kinase Snf1 and the transcription factors Adr1 and Cat8. The Journal of biological chemistry *278*, 26146-26158.

Chapter IV

Influence of sphingolipid metabolism on the activity of the pro-apoptotic regulator Bax, in yeast cells

Trindade, D., Sousa, M.J., Côrte-Real, M. Manon, S. - Influence of sphingolipid metabolism on the activity of the pro-apoptotic regulator Bax, in yeast cells

Manuscript in preparation

1. Introduction

Along with the Bcl-2 family members, sphingolipid metabolism also regulates apoptosis, although only recently we began to understand how these particular type of lipids mechanistically intersect with Bcl-2 family function (Zhang and Saghatelian, 2013). Alterations in sphingolipid profiles, in particular ceramide profiles, are a common event in many scenarios of apoptosis (Taha et al., 2006a). Furthermore, the addition of exogenous ceramide analogs to cells is able to trigger apoptosis (Obeid et al., 1993), and the modulation of ceramide production can actually regulate this cell death process (see for example: Alphonse et al., 2004; Rodriguez-Lafrasse et al., 2002; Taha et al., 2006b). One of the mechanisms proposed for the ceramide control of apoptosis is the formation of ceramide channels (Colombini, 2010; Siskind et al., 2006). Indeed, ceramides are able to form large stable channels in membranes, a phenomenon that seems to be exclusive for this sphingolipid since the biologically inactive dihydroceramides cannot form such structures (Siskind and Colombini, 2000). Although Bcl-2 family proteins are not required for the assembly of these channels, the anti-apoptotic members Bcl-x_L and CED-9 (the *C. elegans* Bcl-2 homologue) disassemble ceramide channels on isolated rat liver and yeast mitochondria, as well as in planar phospholipid membranes (Siskind et al., 2008). On the other hand, the pro-apoptotic protein Bax seems to act synergistically with ceramide channels to permeabilize the outer mitochondrial membrane (OMM) (Ganesan et al., 2010). The contribution of sphingolipid metabolism to cell death, however, is not limited to ceramide. Sphingosine, like ceramide, mediates apoptosis whereas sphingosine-1-phosphate promotes cell survival and inhibits apoptosis (Cuvillier, 2002; Spiegel and Milstien, 2003). These antagonistic roles of closely related sphingolipid species create the concept of the so-called sphingolipid rheostat, according to which the relative levels of these lipids are important determinants of cell fate, and the sphingolipid interconversions enables cells to orchestrate different cellular responses (Hannun and Obeid, 2008). Recently it has been proposed that mitochondria actively maintain a specific sphingolipid milieu that favors Bak/Bax function and apoptosis (Chipuk et al., 2012). Still, a challenge remains to determine how specific sphingolipids cooperate with pro- and anti-apoptotic proteins *in vivo*.

The connection between sphingolipids and Bcl-2 family members is not restricted to the direct interaction of ceramide and these apoptotic regulators in OMM. Ceramide has diversified downstream targets including protein kinases and phosphatases, such as ceramide activated protein kinases (CAPK), protein kinase C (PKC), mitogen-activated-protein kinases (MAPKs) and ceramide activated protein phosphatases (CAPPs). Several of this kinases and phosphatases can actually modulate the activity of different members of the Bcl-2 family through phosphorylation/dephosphorylation events.

In the past decades, yeast has been extensively used to study the regulation and function of different Bcl-2 family proteins. Additionally, the simple and genetic tractable unicellular eukaryotic organism *S. cerevisiae* has also been essential for the investigation of sphingolipid metabolism. Together these two features make yeast an advantageous model system to further address the connections of Bcl-2 family members and sphingolipids. The purpose of this work was to further elucidate the influence of shingolipid metabolism on the activity of the pro-apoptotic regulator Bax. For such purpose, several *S. cerevisiae* sphingolipid mutants were tested in response to the heterologous expression of Bax. Among these mutants, we focused on the effect of *ISC1* deletion on the action of different forms of Bax exhibiting distinct functional properties and cellular effects. Our data bring a new emphasis to the role of ceramide in PCD, and how this sphingolipid might contribute to the action of Bax in yeast cells.

2. Materials and Methods

2.1. Strains and Plasmids

The strains and plasmids used in this work are listed in table 1.

Table 1 – List of strains and plasmids used on this work.

Name	Strain/Plasmids	Source/Reference
BY *wt*	BY4742 (*MATα; his3Δ 1; leu2Δ 0; lys2Δ 0; ura3Δ 0*)	Euroscarf
BY *Δifa38*	BY4742 *Δifa38 (MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YBR159w::kanMX4)*	Euroscarf
BY *Δisc1*	BY4742 *Δisc1 (MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YER019w::kanMX4)*	Euroscarf
BY *Δydc1*	BY4742 *Δydc1 (MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YPL087w::kanMX4)*	Euroscarf
BY *Δypc1*	BY4742 *Δypc1 (MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YBR183w::kanMX4)*	Euroscarf
BY *Δymr1*	BY4742 *Δypc1 (MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YJR110w::kanMX4)*	Euroscarf
W303	W303 1B *(MATα ; ura3-1; trp1Δ 2; leu2-3,112; his3-11,15; ade2-1; can1-100)*	IBGC - CNRS
Δisc1	W303 1B *(MATα ; ura3-1; trp1Δ 2; leu2-3,112; his3-11,15; ade2-1; can1-100; ISC1::kanMX4)*	This study
Bax *c-myc*	pCM189-Bax *c-myc*	Priault et al., 1999
Bax-*wt*	pYES3-Bax	Arokium et al., 2004
Bax-P168A	pYES3-Bax-*P168A*	Arokium et al., 2004
Bcl-x_L	pYES2-Bcl-x_L	Arokium et al., 2004
	pFL39-Bcl-x_L	Priault et al., 2002
Empty vector	pYES3	Invitrogen

W303 *Δisc1* mutant was generated by homologous recombination with *Δisc1::kanMX4* fragment in *S. cerevisiae* W303 cells. The transformation products were confirmed by PCR, and are referred to as *Δisc1*. The Bax gene referred ahead is a chemically synthesized version of the human Bax cDNA, carrying substitutions at the third position of several codons to improve its expression in yeast (Greenhalf et al., 1996). All *BY4742* strains mentioned in table 1 (*wt*, *Δifa38*, *Δisc1*, *Δydc1*, *Δymr1* and *Δypc1*) were transformed with the plasmid pCM189-Bax *c-myc* (*URA3*), carrying the Bax cDNA, fused to a 5'-DNA sequence encoding the *c-myc* epitope (EQKLOSEEDLNG), under control of a tet-promoter (as described in Priault et al., 1999). W303 and *Δisc1* cells were subsequently transformed with three different forms

of Bax: (i) pCM189-Bax *c-myc*, containing the above described Bax gene fused to a *myc* epitope (Priault et al., 1999); (ii) pYES3-*Bax-P168A,* containing a Bax gene in which proline 168 was replaced by an alanine under control of the GAL1/10 promoter (as described in Arokium et al., 2004); (iii) and pYES3-Bax containing a "wild-type" version of the human Bax, and referred to as Bax-*wt*, under control of the GAL1/10 promoter (Arokium et al., 2004). W303 and *Δisc1* strains were also transformed with empty vector pYES3 (*TRP1*, Invitrogen). For co-expression of Bcl-x_L two different plasmids, both carrying the cDNA of human Bcl-x_L under control of a GAL1/10 promoter, were used. The vector pYES2-Bcl-x_L (*URA3*) was used to co-express Bcl-x_L with Bax-*wt* or Bax-P168A (Arokium et al., 2004), while pFL39-Bcl-x_L (*TRP1*) was used to co-express Bcl-x_L with Bax *c-myc* (Priault et al., 2002), in W303 and *Δisc1* strains. Transformations of *S. cerevisiae* cells with the above mentioned plasmids were performed by lithium acetate general protocol (Ito et al., 1983).

2.2. Growth conditions

The BY mutants transformed with pCM189-Ba*x c-myc* were selected in YNB plates (2% glucose) without uracil (-Ura), in the presence of 5 μg/mL of doxycycline to inhibit the expression of Bax *c-myc*. The BY + Bax *c-myc* mutants were pre-grown in synthetic complete medium (SC) with glucose (0.175% yeast nitrogen base w/o amino acids (Difco), 0.5% ammonium sulfate, 0.1% potassium phosphate, 2% glucose, with dropout) and without uracil (-U), in the presence of 2 μg/mL of doxycycline, thus preventing the expression of Bax *c-myc* .

W303 and *Δisc1* strains transformed with pYES3, pYES3-*Bax* or pYES3-*Bax-P168A* were selected in YNB plates (2% glucose) without tryptophan (-W), while cells co-transformed with either of the Bax-containing plasmids and pYES2-*Bcl-x_L* were selected in YNB plates (2% glucose) without tryptophan (-W) and uracil (-U). These mutants were pre-grown in SC glucose medium (0.175% yeast nitrogen base w/o amino acids (Difco), 0.5% ammonium sulfate, 0.1% potassium phosphate, 2% glucose, with dropout) without tryptophan (-W), for the strains carrying pYES3, pYES3-*Bax* or pYES3-*Bax-P168A* alone, or without tryptophan and uracil (-UW) for the strains co-transformed with pYES2-*Bcl-x_L*.

W303 and *Δisc1* cells transformed with pCM189-*Bax c-myc* alone, or together with pFL39-*Bcl-x_L* were selected in YNB plates (2% glucose) without uracil (-U) or without

uracil and tryptophan (-UW), respectively, both supplemented with doxycycline (5 µg/mL) to prevent Bax *c-myc* expression. These mutants were pre-grown in SC glucose medium (0.175% yeast nitrogen base w/o amino acids (Difco), 0.5% ammonium sulfate, 0.1% potassium phosphate, 2% glucose, with dropout) without uracil (-U), for the strains carrying pCM189-*Bax c-myc* alone, or without uracil and tryptophan (-UW) for the strains co-transformed with pFL39-*Bcl-x_L*. Doxycycline (2 µg/mL) was added to prevent Bax *c-myc* expression. For mitochondria extractions, cells were transferred to SC lactate medium (2% DL-Lactate, pH adjusted to 5.5 with NaOH) without uracil or without uracil and tryptophan, accordingly. Expression of Bax *c-myc* was induced after transferring the cells into fresh medium supplemented with 0.1 µg/mL of doxycycline. Co-expression of Bcl-x_L was induced by the addition of 0.5% galactose to the medium.

2.3. Survival assays of Bax c-myc mutants

To test the viability of BY + Bax *c-myc* mutants, cells were pre-grown as mentioned above, collected, washed and transferred to SC lactate -U medium (2% DL-Lactate, pH adjusted to 5.5 with NaOH), supplemented with 2 µg/mL of doxycycline. To induce Bax *c-myc* expression, exponentially grown cells were collected, washed twice with sterile water, and transferred to fresh SC lactate -U medium with 0.1 or 0.2 µg/mL of doxycycline, to have a 0.5 $O.D._{640nm}$ culture. For each strain, a control with 2 µg/mL of doxycycline was also prepared. These cultures were then incubated for 14h at 30°C, after which a sample of each culture was extracted, diluted, and plated in YPDA (1% yeast extract, 1% bactopeptone, 2% Glucose, 2% Agar) supplemented with 10 µg/mL of doxycycline (250 cells per plate). The number of c.f.u. was counted after 48h incubation at 30°C, and the c.f.u. values obtained in control cultures were used as reference. Expression of Bax-*c-myc* was confirmed by WB.

2.4. Mitochondria Isolation and Western-blot analysis

For mitochondria extraction, BY *wt* and BY *Δisc1* + Bax *c-myc* strains were pre-grown in SC glucose -U medium, and then transferred to SC medium with glycerol (2%) and ethanol (2%) as carbon sources and without uracil, to obtain fully differentiated mitochondria. Bax *c-myc* expression was achieved as described above. 2 L cultures (1.0 $O.D._{640nm}$) were incubated for 14 hours at 30°C, prior to mitochondria extraction. W303 and *Δisc1* cells transformed with pYES3, pYES3-*Bax* or pYES-Bax-

P168A were grown in SC lactate -W medium (2% DL-Lactate, pH adjusted to 5.5 with NaOH). Cells co-transformed with pYES2-Bcl-x_L were grown in SC lactate -UW medium. Expression of Bax, Bax-P168A and Bcl-x_L in these strains was accomplished by the addition of 0.5% galactose to the culture medium. 2 L cultures were set at 0.5 $O.D._{640nm}$ an incubated for 14 hours.

Cells were collected, converted into spheroplasts by enzymatic digestion with zymolyase (Zymolyase 20T, Seikagaku Biobusiness Corporation), disrupted by hand-potter or mechanical homogenization, and the mitochondrial fraction recovered after a series of differential centrifugations (adapted from Law et al., 1995). Mitochondrial suspensions were frozen in liquid nitrogen and stored at -80 °C. For characterization of the mitochondrial and post-mitochondrial fractions by Western-blot, 50 μg of proteins were precipitated with TCA, washed with acetone, and solubilized in 2% SDS before being separated on SDS–PAGE (adapted from Laemmli, 1970). When referred, the microsomal fraction was isolated after centrifuging the post-mitochondrial fraction at 100,000 g. Protein samples were then blotted on PVDF membranes (hybond-P; Amersham). Characterization of the different cellular fractions was carried out with antibodies directed against mitochondrial cytochrome *c* (rabbit polyclonal anti-*CYC1* antibody; 1:1000, custom-made by Millegen) and cytochrome *c* oxidase subunit II (mouse monoclonal anti-*COX2* antibody; 1:1000, Molecular Probes); cytosolic phosphoglycerate kinase (mouse monoclonal anti-*PGK1* antibody; 1:5000, Molecular Probes); and the endoplasmic reticulum protein dolichol phosphate mannose synthase (mouse monoclonal anti-*DPM1*, Molecular Probes). Immunodetection of Bax and Bcl-x_L was performed with the antibodies anti-*Bax* (N20) (rabbit polyclonal from Santa-Cruz) and anti-*Bcl-x_L* (rabbit polyclonal, BD Transduction Laboratories).

2.5. Carbonate treatment

For carbonate extraction, 1 mg of protein from freshly isolated mitochondria (before storage at -80 °C) was resuspended in Hepes 1 mM buffer and incubated with Na_2CO_3 (0.1 M, pH 10.0) or Triton X-100 (1%) for 10 minutes at 4 °C, to remove loosely bound or integrated proteins respectively. Samples were then centrifuged for 15 minutes at 100,000 g. Both pellet and supernatant were collected, and the proteins precipitated with TCA, as described above, prior to Western-blot analyses.

2.6. Redox Spectrophotometry

For cytochrome quantification, 10 mg of thawed mitochondrial proteins were diluted in recuperation buffer (0.6 M Mannitol; 10 mM Tris-maleate; 2 mM EGTA; pH 6.8) to a final volume of 1.6 mL, producing a mitochondria suspension with the final concentration of 6.24 mg/mL. The suspension was equally divided into two glass cuvettes and the reference and sample cuvettes were oxidized and reduced with potassium ferricyanide and sodium dithionite, respectively. Samples were then analyzed in a double beam spectrophotometer, Varian Cary 4000. Alternatively, 200 µl of a 10 mg/mL mitochondria preparation were analyzed in a micro-plate spectrophotometer. For whole cell cytochrome quantification, cells were collected during exponential growth, and concentrated in a cellular suspension of 2 mL at ≈ 40 $O.D._{640nm}$. This suspension was divided in two cuvettes and analyzed in a double beam spectrophotometer, Varian Cary 4000. Difference spectra were acquired between 500 and 650 nm and cytochromes $c+c_1$, b and $a+a_3$ were quantified by the O.D. differences 550 nm minus 540 nm, 561 nm minus 575 nm, and 603 nm minus 630 nm, respectively.

2.7. PI staining

Propidium Iodide (PI) exclusion in W303 and *Δisc1* cells expressing Bax *c-myc* or co-expressing Bax *c-myc* and Bcl-x_L, was monitored by flow cytometric analysis. Cells were transferred to SC lactate medium -U or -UW, accordingly, grown to exponential phase, collected, washed and transferred into fresh medium containing 0.1 µg/mL of doxycycline and 0.5% galactose, to induce Bax *c-myc* and Bcl-x_L expression. Control cultures were prepared with 2 µg/mL of doxycycline. Cells were harvested after 14h and 40h, washed and resuspended in PBS (137 mM NaCl; 2.7 mM KCl; 100 mM Na2HPO4; 2 mM KH2PO4; pH 7.4) containing 4 µg/ml of PI (Sigma). The samples were incubated for 10 minutes at room temperature, in the dark, before analyzed. The survival of each strain after 14 hours was also tested by c.f.u. counting, as described previously. Flow cytometric analysis was performed in an Epics® XLTM (BeckmanCoulter) flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Red fluorescence was collected through FL-4 (660/700 nm) sensor. The obtained data were further analyzed by Flowing Software version 2.5.0.

3. Results

3.1. Screening sphingolipid mutants for resistance to the action of Bax c-myc

To understand how sphingolipids might influence the action of human apoptotic regulators of the Bcl-2 family, namely Bax, using the yeast *S. cerevisiae* as a model system, we started by screening a set of selected strains, mutated in genes involved in sphingolipid metabolism. This set, obtained from the EUROSCARF collection, included the following mutants: *Δifa38* (missing the major microsomal 3-ketoreductase of the elongase system of enzymes required for the synthesis of very long-chain fatty acid; accumulates high levels of dihydrosphingosine, phytosphingosine and medium-chain ceramides) (Beaudoin et al., 2002; Han et al., 2002), *Δisc1* (missing a inositol phosphosphingolipid phospholipase C that can be localized in the mitochondrial membrane and hydrolyzes complex sphingolipids to yield both phyto- and dihydroceramides) (Sawai et al., 2000), *Δydc1* and *Δypc1* (lacking the alkaline dihydroceramidase and phytoceramidase, respectively, that are responsible for the cleavage of dihydro- and phytoceramide into the corresponding sphingoid bases (Mao et al., 2000a, 2000b), and *Δymr1* (which lacks a phosphatidylinositol 3-phosphate (PI3P) phosphatase, involved in several protein sorting pathways (Taylor et al., 2000). All the mentioned strains were transformed with plasmid pCM189 carrying Bax *c-myc* under control of a *tet-off* expression system. The presence of a *c-myc* epitope in the C-terminal of Bax results in an active conformation of this protein with enhanced insertion in the mitochondrial membrane that very efficiently induces yeast cell death (Greenhalf et al., 1996; Manon et al., 1997). Full Bax expression is observed after 14h incubation in inducible conditions (Fig. 1A). Therefore, the survival of BY mutants (*wt*, *Δifa38*, *Δisc1*, *Δydc1*, *Δymr1*, *Δypc1*) expressing Bax *c-myc* was evaluated by c.f.u. counting, following a period of 14 hours in the absence of doxycycline, to induce Bax *c-myc* expression. Control cultures were prepared in the same way, and 2 μg/mL of doxycycline were added, to inhibit Bax expression. Under these conditions, Bax *c-myc* induced a high rate of cell death and only a residual amount of cells were able to retain their plating efficiency. No differences were observed among the different strains, which presented survival values below 10% (data not shown).

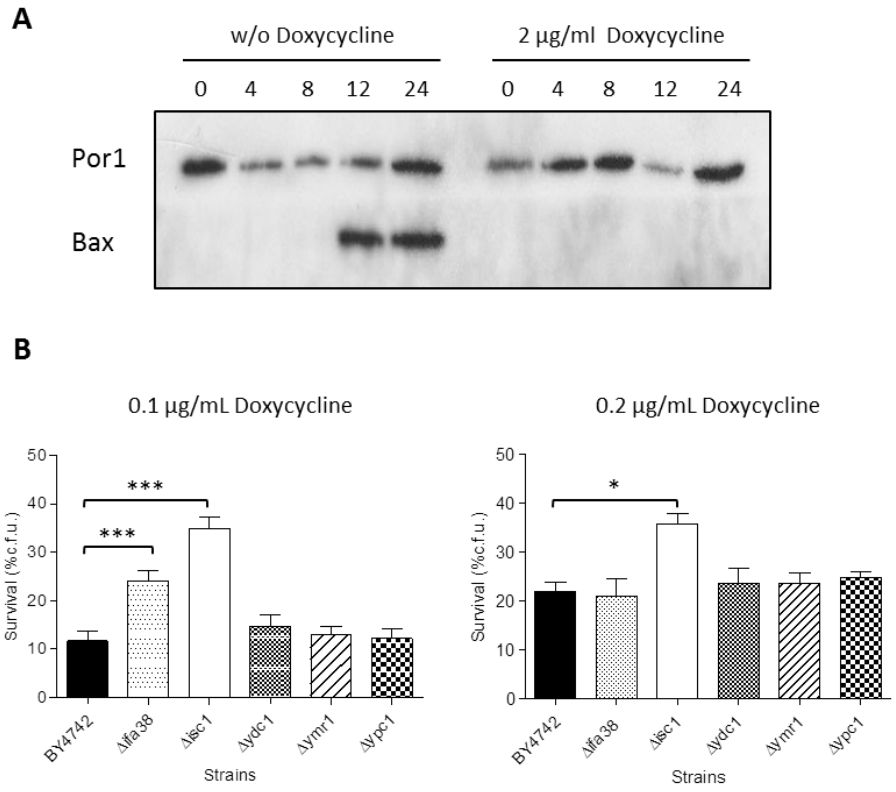


Fig. 1 – Tolerance of sphingolipid metabolism mutants to the action of Bax *c-myc*. A) Representative Western-blot for detection of Bax *c-myc* in cultures grown with or without doxycycline. Samples were collected at 0, 4, 8, 12 and 24 hours. Porin (Por1p) from the outer mitochondrial membrane was used as control. Full Bax expression is obtained after 12 hours incubating in the absence of doxycycline. In the presence of 2 µg/mL doxycycline, there is no expression of Bax *c-myc*. B) Tolerance of BY4742 wt, *Δifa38*, *Δisc1*, *Δydc1*, *Δymr1* and *Δypc1* strains to the action of Bax *c-myc*. Cultures were incubated in the presence of 0.1 µg/mL or 0.2 µg/mL of doxycycline to allow Bax *c-myc* expression and 2 µg/mL to prevent it. Samples were collected after a 14h incubation, plated onto YPDA with 10 µg/mL of doxycycline, and the c.f.u. counted after 2 days incubating at 30°C. Survival was estimated using the c.f.u. obtained for control cultures as reference. When the mutants were incubated in the presence of 0.1 µg/mL of Doxycycline, *Δisc1* and *Δifa38* strains exhibited an increased tolerance to the action of Bax *c-myc*, compared to the wild-type BY4742 (*** $P < 0.001$). *Δisc1* mutant retained this tolerance phenotype when incubated in the presence of 0.2 µg/mL of doxycycline (* $P<0.05$).

Considering the high effectiveness of Bax *c-myc* in killing yeast cells, we decided to incubate each strain for 14h in the presence of 0.1 or 0.2 µg/mL of doxycycline, which should enable moderate expression levels of this pro-apoptotic regulator. Under these conditions, we observed a different response among the BY-sphingolipid mutants. In the presence of 0.1 µg/mL of doxycycline, the survival of the BY *wt* strain is slightly higher than 10%, a value similar to the ones obtained for BY *Δydc1*, *Δymr1* and *Δypc1*

mutants (Fig. 1B). The BY *Δifa38* and *Δisc1* mutants, however, have an increased resistance to the action of Bax *c-myc*, with 20% and 30% survival, respectively (Fig. 1B). When Bax *c-myc* expression was induced in the presence of 0.2 μg/mL of doxycycline, the survival of BY *wt* practically doubled compared to the induction with 0.1 μg/mL of doxycycline (Fig. 1B). The same was true for BY *Δydc1*, *Δymr1* and *Δypc1* mutants whose survival was similar to that of the *wt* strain (Fig. 1B). Curiously, the survival of either BY *Δifa38* and *Δisc1* mutants was not significantly improved in the presence of a higher concentration of doxycycline, and the resistance phenotype of BY *Δifa38* was no longer evident. Nevertheless, the BY *Δisc1* mutant exhibited the highest survival to the action of Bax *c-myc* (Fig. 1B). These observations suggest that the physiological changes resulting from the absence of Isc1p may affect the action of the human pro-apoptotic regulator Bax. Therefore, we focused on the role of Isc1p on the activity of the pro-apoptotic regulator Bax.

Isc1p, a yeast homologue of the mammalian neutral sphingomyelinases, shares significant structural and functional homology with mammalian neutral sphingomyelinases, and its activity is not limited to yeast complex sphingolipids since it can also hydrolyze sphingomyelin, the mammalian counterpart of these complex sphingolipids which does not exist in yeast (Sawai et al., 2000). Isc1p has also been associated to essential cell processes including growth, cell division, sporulation, ion protection, heat shock response, genotoxic protection, oxidative stress response, and aging (reviewed in (Matmati and Hannun, 2008). Although it is usually localized to the ER, Isc1p is activated and translocates to mitochondria in the post-diauxic growth phase (Vaena de Avalos et al., 2004). Isc1p also influences mitochondrial related functions, such as the regulation of cellular redox homeostasis (Almeida et al., 2008; Kitagaki et al., 2007). Furthermore, loss of Isc1p leads to a shortening in chronological life span, associated to the modulation of iron metabolism and apoptosis (Almeida et al., 2008), an effect that is directly associated to the altered sphingolipid profile (Barbosa et al., 2011).

3.2. Bax c-myc addressing to mitochondria and cyt c release

Since Bax *c-myc* is already an active form of Bax (Priault et al., 2003), we wondered if the increased survival observed for BY *Δisc1* mutant could be related to a reduced addressing of Bax *c-myc* to OMM of these cells. To test this hypothesis, mitochondria

of BY *wt* and *Δisc1* were extracted after 14h incubating in Bax-expressing conditions, and 1 mg of freshly isolated mitochondria was submitted to carbonate extraction. This treatment removes proteins that are loosely bound to the mitochondrial membrane, leaving only the proteins that are fully inserted. The carbonate treated mitochondria and corresponding supernatant fraction were processed for western-blot analysis.

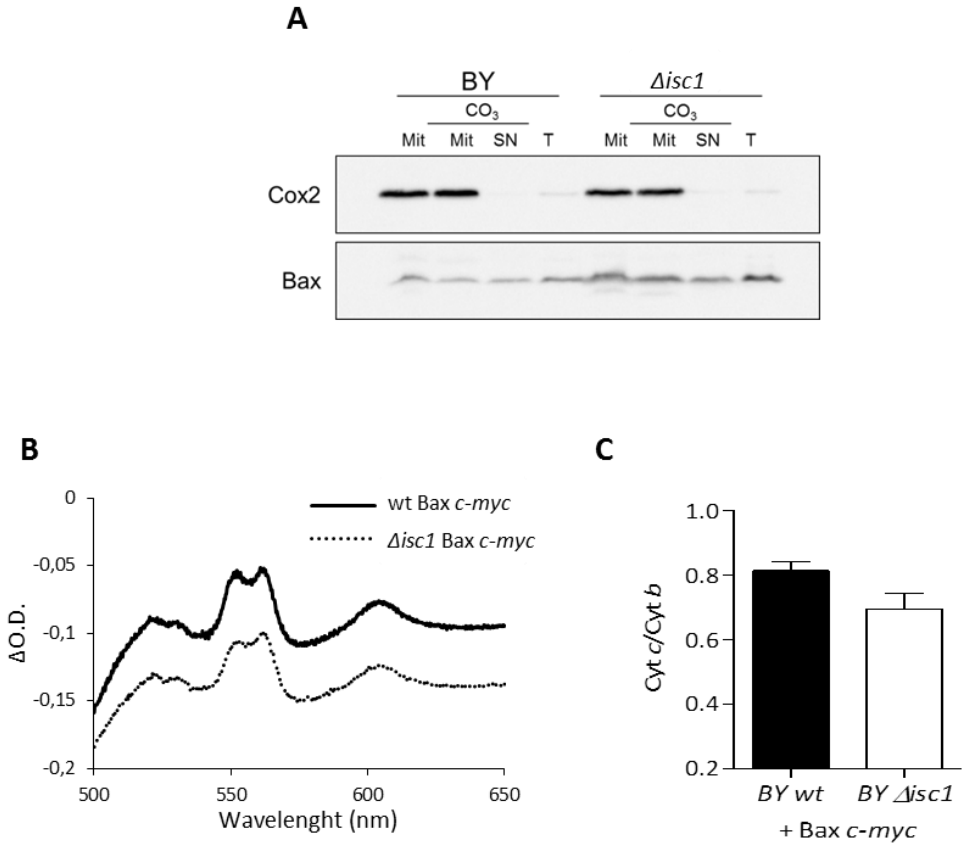


Fig. 2 – Bax *c-myc* addressing to, and permeabilization of BY *wt* and *Δisc1* mitochondria. Cells transformed with Bax *c-myc* were grown for 14 hours in the absence of doxycycline, enabling full Bax *c-myc* expression. A) Bax *c-myc* addressing to mitochondria was assessed by Western-blot, after treatment of mitochondria (1 mg) with sodium carbonate (Na_2CO_3, 0.1 M, pH 10.0) to evaluate the insertion of Bax *c-myc* in the OMM. The mitochondrial fractions (CO3 Mit), recovered by high speed centrifugation, and corresponding supernatants (CO_3 SN) were evaluated for the presence of Bax. Bax *c-myc* and Cox2p were also detected in isolated mitochondria (Mit) and total protein extracts (T). B) Cytochrome spectra from BY *wt* and *Δisc1* + Bax *c-myc* isolated mitochondria. The *ISC1* mutation did not disturbe the action of Bax *c-myc*, which efficiently permeabilized the mitochondrial membrane of both strains. A representative spectrum from each strain is shown. C) Cyt *c*/cyt *b* ratios from BY *wt* and *Δisc1* mitochondria (0.82 and 0.70, respectively). A small difference is observed, probably resulting from a decreased cyt *c* content of *Δisc1* cells. (Data represent the mean of at least 3 independent experiments and the corresponding)

Bax *c-myc* is mostly localized to the OMM of both strains, and the carbonate extraction did not reveal any significant differences (Fig. 2A). Bax *c-myc* seems to be equally inserted in the OMM of *Δisc1* cells when compared with the BY *wt* cells, since no difference was found between the pellet and the supernatant of carbonate-treated mitochondria of both strains (Fig. 2A). Since there are no differences in the insertion of Bax *c-myc* in the OMM of both cell types, we evaluated its capacity to permeabilize mitochondria, by redox spectrophotometry. Bax *c-myc* was able to induce a significant release of cyt *c* from mitochondria of BY *wt* and *Δisc1* cells, as observed by the small cyt *c* peak on both spectra and reduced cyt *c*/cyt *b* ratios (Fig. 2B and C). A small difference was observed since the cyt *c*/cyt *b* ratio of BY *Δisc1* mutant (0.70) is inferior to that of BY *wt* mitochondria (0.82) (Fig, 2C). A similar difference was also observed in spectra obtained from whole cells (not shown), indicating that *Δisc1* mutant could already have a decreased amount of cyt *c*.

3.3. Effect of Δisc1 mutation in Bax-wt addressing and activity

The absence of Isc1p results in altered mitochondrial sphingolipid profile with a decrease in α-HO C_{26}-phytoceramides, and an accumulation of other species such as α-HO C_{14}-phytoceramide and non-HO C_{26}-phytoceramides (Kitagaki et al., 2007). The altered sphingolipid profile of *Δisc1* cells has been associated to the activation of a yeast type 2A ceramide-activated protein phosphatase (CAPP), suggesting that Isc1p might act as its upstream regulator (Barbosa et al., 2011). Importantly, the mammalian protein phosphatase 2A (PP2A) was found to regulate the phosphorylation state of Bax in response to ceramide (Xin and Deng, 2006). To test if the absence of Isc1p could lead to the activation of Bax, we evaluated the capacity of Bax-*wt* to insert in the mitochondrial membrane and induce the release of cyt *c*. W303 and *Δisc1* cells were grown in SC lactate -W for 14 hours in the presence of 0.5% galactose to induce the expression of Bax. Our observations suggest that Bax-*wt* has a microsomal localization rather than a mitochondrial one, which is consistent with the inactive conformation of this protein (Fig. 3A). No significant differences are apparent in the cellular localization of Bax-*wt* in W303 and *Δisc1* strains (Fig. 3A). The redox spectra are also identical for both cell types, and the cyt *c*/cyt *b* ratios (1.57 and 1.45, respectively) are identical to those observed for mitochondria from control cells (without Bax expression).

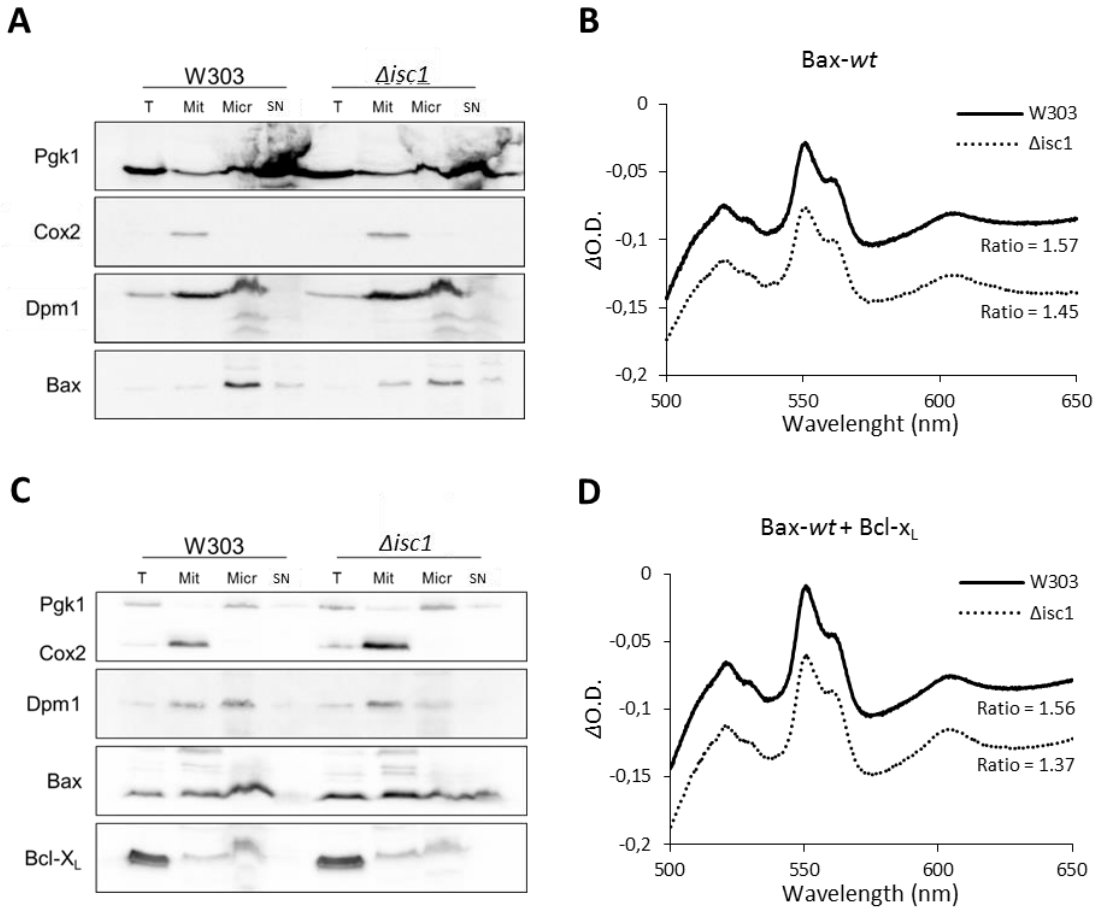


Fig. 3 – Expression of Bax-*wt*, or co-expression of Bax-*wt* and Bcl-x_L, in W303 *wt* and *Δisc1* cells. Western-Blot and redox spectra analysis of W303 *wt* and *Δisc1* mitochondria preparations from cells expressing Bax-*wt*, A) and B), or co-expressing Bax-*wt* and Bcl-x_L, C) and D). Cells were grown for 14h in the presence of 0.5% galactose to enable Bax and Bcl-x_L expression. A) Western-blot detection of Bax-*wt* in total protein extracts (T), isolated mitochondria (Mit), microsomes (Micr) and the post-mitochondrial fraction (SN). Cox2, Dpm1 and Pgk1 were used as controls for mitochondria, microsomal and post-mitochondrial fractions, respectively. When expressed alone, Bax-*wt* is mostly localized in the microsomal fraction. B) Cytochrome spectra of W303 and *Δisc1* mitochondria isolated from cells expressing Bax-*wt*. Bax-*wt* does not induce the release of cyt *c* from mitochondria of both strains. C) Western-blot detection of Bax-*wt* and Bcl-x_L in the equivalent cellular fractions as in A. Bcl-x_L is mostly localized in the mitochondrial and microsomal fractions of W303 and *Δisc1* cells, and promotes the addressing of Bax-*wt* to mitochondria. D) Cytochrome spectra of W303 and *Δisc1* mitochondria isolated from cells co-expressing Bax-*wt* and Bcl-x_L. The increased mitochondrial localization of Bax-*wt* is not associated with the activation of this protein, since no cyt *c* is released from mitochondria of either strain.

Simultaneously, we tested the effect of Bcl-x_L co-expression in W303 and *Δisc1* strains. Cells carrying Bax-*wt* and Bcl-x_L were transferred to SC lactate -UW, supplemented with 0.5% galactose to induce the expression of both proteins. Mitochondria were extracted after 14h incubation. The cellular localization of Bcl-x_L

seems to be divided between the mitochondrial and the microsomal fractions, and the co-expression of this protein actually increases the amount of Bax-*wt* localized in mitochondria from both W303 and *Δisc1* cells (Fig. 3C), an effect that has been observed before (Renault et al., 2013). Redox spectra indicate that no cyt *c* was released from W303 and *Δisc1* mitochondria under these conditions as well, and cyt *c*/cyt *b* ratios of mitochondria co-expressing Bax-*wt* and Bcl-x_L are identical to those of cells expressing Bax-*wt* alone (Fig. 3D). These data are consistent with the nearly inactive state of Bax-*wt*, a condition that is apparently not affected by the absence of Isc1p.

3.4. Effect of Δisc1 mutation on the action of Bax-P168A mutant

So far, the higher survival of *Δisc1* cells to the action of Bax *c-myc* cannot be attributed to a reduced addressing/insertion of this form of Bax in the OMM or impaired cyt *c* release, and the absence of Isc1p does not seem to change the inactive state of a *wild-type* Bax as well. Since Bax *c-myc* is a highly active form of Bax, in matters of addressing to and permeabilization of mitochondria, we tested the effect of *Δisc1* mutation in the action of Bax-P168A mutant protein. Proline 168 is localized in region between helixes α8 and α9, and is assumed to play an important role in the conformational change responsible for the movement of α9, which is required for the mitochondrial addressing/insertion of Bax. The substitution of proline 168 for an alanine facilitates a conformational change that mimics the physiological active form of Bax that is addressed to mitochondria and permeabilizes the OMM (Arokium et al., 2004). W303 and *Δisc1* cells carrying the pYES3-*Bax-P168A* were grown in SC lactate -W for 14h, in the presence of 0.5% galactose to induce Bax-P168A expression. Bax-P168A is found in the mitochondrial fractions of both strains, and is not significantly removed by carbonate treatment in either case, which is expected for a protein inserted in the mitochondrial membrane (Fig. 4A). It also has the capacity to induce cyt *c* release from mitochondria of W303 and *Δisc1* cells, as revealed by redox spectra (Fig. 4B). The resulting cyt *c*/cyt *b* is identical for both strains (1.15 and 1.14) and, unlike the experiments with Bax *c-myc* and Bax-*wt*, not even a small difference is observed.

The influence of *Δisc1* mutation in the action of Bcl-x_L was also tested. W303 and *Δisc1* cells transformed with pYES3-*Bax-P168A* and pYES2-*Bcl-x_L* were grown in SC lactate -UW for 14h. Expression of both proteins was induced in 0.5% galactose. Once

again, Bcl-x_L is localized in the mitochondrial fraction of W303 and *Δisc1* cells, and its insertion is not affected by Isc1p mutation (Fig. 4C).

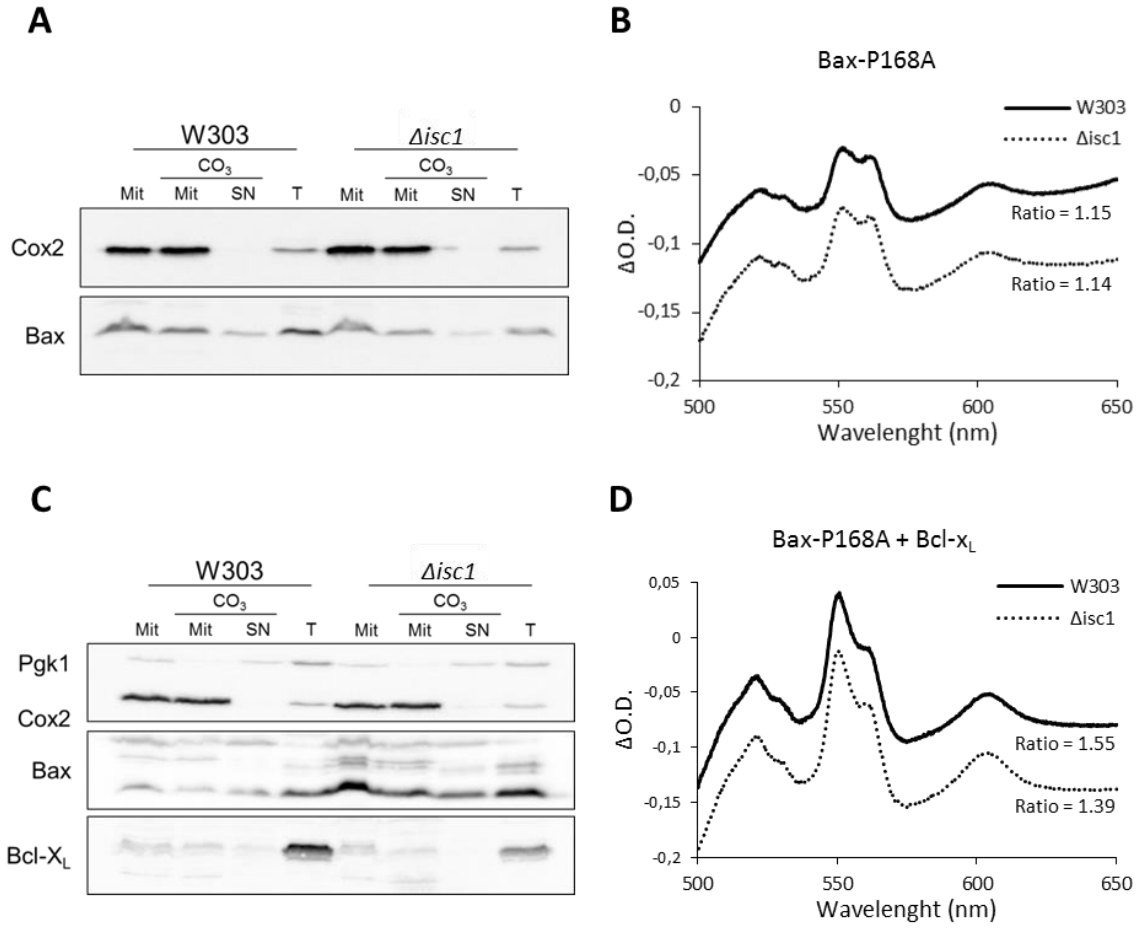


Fig. 4 – Expression of Bax-P168A, and co-expression of Bax-P168A and Bcl-x_L, in W303 *wt* and *Δisc1* cells, grown for 14h in the presence of 0.5% galactose to enable the expression of both proteins. Western-Blot and redox spectra analysis of W303 *wt* and *Δisc1* mitochondria preparations from cells expressing Bax-P168A, A) and B), or co-expressing Bax-P168A and Bcl-x_L, C) and D). A) Western-blot detection of Bax-P168A in total protein extracts (T), isolated mitochondria (Mit) or mitochondria treated with sodium carbonate, and respective supernatant, to evaluate the insertion of Bax-P168A in the OMM (CO_3 Mit and CO_3 SN). Cox2 was used as control. Bax-P168A is localized in mitochondria and properly inserted in the OMM. B) Cytochrome spectra of W303 and *Δisc1* mitochondria isolated from cells expressing Bax-P168A, which efficiently induces the release of cyt *c*. The action of Bax-P168A does not seem to be affected by the *ISC1* mutation. C) Western-blot detection of Bax-P168A and Bcl-x_L in total protein extracts (T), isolated mitochondria (Mit) or mitochondria treated with sodium carbonate (CO_3 Mit) and respective supernatant (CO_3 SN). Bax-P168A and Bcl-x_L are localized in mitochondria and inserted in the OMM of both strains. D) Cytochrome spectra of W303 and *Δisc1* mitochondria isolated from cells co-expressing Bax and Bcl-x_L. The absence of Isc1p does not affect the pro-survival role of Bcl-x_L, which is able to prevent Bax-P168A-induced mitochondria permeabilization and cyt *c* release.

Co-expression of Bcl-x_L does not change the capacity of Bax-P168A to insert itself in the OMM of either strain (Fig. 4C). Furthermore, Bcl-x_L successfully prevented cyt *c* release from W303 and *Δisc1* mitochondria, as demonstrated by the redox spectra and the cyt *c*/cyt *b* ratios obtained for both strains (1.55 and 1.39, respectively). It seems fair to say that the antagonizing activity of Bcl-x_L, towards the pro-apoptotic regulator Bax, is not affected by the physiological changes resulting from the absence of Isc1p.

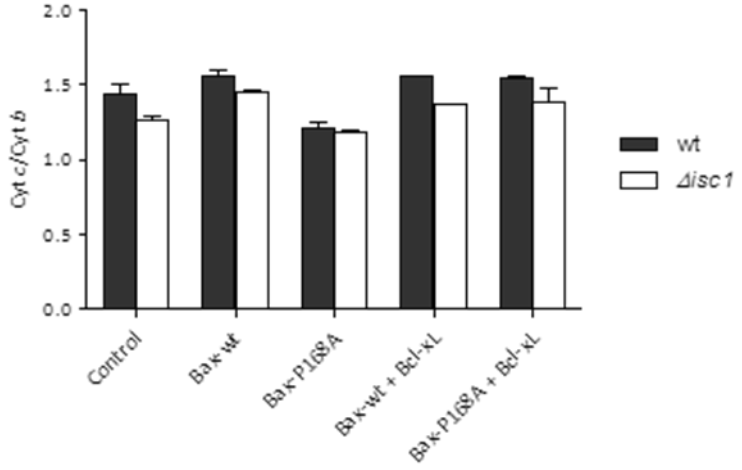


Fig. 5 – Graphic representation of the cyt *c*/cyt *b* ratio of W303 *wt* and *Δisc1* mitochondria isolated from cells without Bax or Bcl-x_L (control), and cells expressing Bax-*wt*, Bax-P168A, or co-expressing these forms of Bax with Bcl-x_L. W303 and *Δisc1* control mitochondria show a cyt *c*/cyt *b* ratio of 1.45 and 1.27, respectively. Mitochondria from cells expressing Bax-*wt* exhibit a cyt *c*/cyt *b* ratio of 1.57 and 1.45, supporting the fact that this protein has an inactive conformation and therefore cannot induce cyt *c* release. Bax-P168A, which has an active conformation, is able to induce cyt *c* release, and ratios of W303 and *Δisc1* mitochondria from cells expressing this form of Bax are very similar; 1.22 and 1.18, respectively. Co-expression with Bcl-x_L prevents this process resulting in cyt *c*/cyt *b* ratios identical to those observed for control or Bax-*wt* expressing cells, 1.55 and 1.39. In all the conditions tested the differences observed between W303 and *Δisc1* cells are not statistically significant.

Comparing the cyt *c*/cyt *b* ratios of mitochondria isolated from W303 and *Δisc1* cells expressing different forms of Bax, co-expressing Bcl-x_L, or carrying an empty vector allowed us to evaluate the impact of Isc1p in the action of these apoptotic regulators, measured by the capacity to induce cyt *c* release. Bax-*wt* is able to induce only a marginal cyt *c* release from W303 or *Δisc1* mitochondria, when we compare the ratios of these cells with those of W303 and *Δisc1* cells transformed with pYES3 empty vector (Fig. 5). Co-expression of Bcl-x_L with this form of Bax has no effect in the mitochondrial cyt *c*/cyt *b* ratios. Expression of Bax-P168A lowered the cyt *c*/cyt *b* ratios

from W303 and *Δisc1* mitochondria, which is consistent with cyt *c* release. This effect can be prevented by co-expression of Bcl-x_L, which restores the cyt *c*/cyt *b* ratios of both types of mitochondria to values identical to those observed for control and for Bax-*wt* or Bax-*wt* + Bcl-x_L expressing cells (Fig. 5). Though a small difference between the ratios of W303 and *Δisc1* mitochondria is observed in most of the tested conditions referred above, presumably due to a lower content of cyt *c* in *Δisc1* cells, it is no longer distinguishable after 14h expressing Bax-P168A.

3.5. Bax-induced loss of plasma membrane integrity in W303 and Δisc1 cells

A work from Camougrand and colleagues revealed that, in yeast cells, the Bax-induced loss of platting efficiency is associated with the maintenance of plasma membrane integrity (Camougrand et al., 2003). Moreover, yeast cells expressing Bax display autophagic features, and the selective degradation of altered mitochondria seems to be crucial for a regulated death in which mitochondria remained relatively organized in the form of orderly distributed spots and plasma membrane intigrety is maintained (Kissová et al., 2006). Since we did not observed any major differences in Bax addressing to mitochondria or its capacity to induce mitochondrial cyt *c* release in the absence of Isc1p, we decided to evaluate the loss of plasma membrane integrity in cells expressing Bax *c-myc*, through their ability to exclude propidium iodide (PI). For this experiment, the W303 and *Δisc1* cells expressing Bax *c-myc* alone, or co-expressed with Bcl-x_L, were used.

Expression of Bax *c-myc* in W303 and *Δisc1* revealed a survival profile identical to that of the BY4742 counterparts, in which the absence of Isc1p promotes an increased resistance to the action of Bax (Fig. 6A). The loss of platting efficiency in both strains could be rescued by co-expression with Bcl-x_L, supporting the idea that the anti-apoptotic role of this protein is not affected by the absence of Isc1p (Fig. 6A). In spite of a higher resistance to Bax *c-myc* the percentage of cells with compromised membrane integrity (PI positive cells) is significantly higher in the *Δisc1* mutant. After 14h expressing Bax *c-myc*, about 20% of W303 cells are stained with PI, contrasting with almost 40% registered for the *Δisc1* mutant (Fig. 6B). This difference subsists after a 40h incubation in inducible conditions (Fig. 6B). Co-expression of Bcl-x_L successfully prevented the loss of plasma membrane integrity of W303 and *Δisc1* cells (Fig. 6B). Although the deletion of Isc1p is able to promote some level of resistance of yeast cells

to the action of Bax on cell viability, death in *Δisc1* cells is accompanied by an increased loss of plasma membrane integrity.

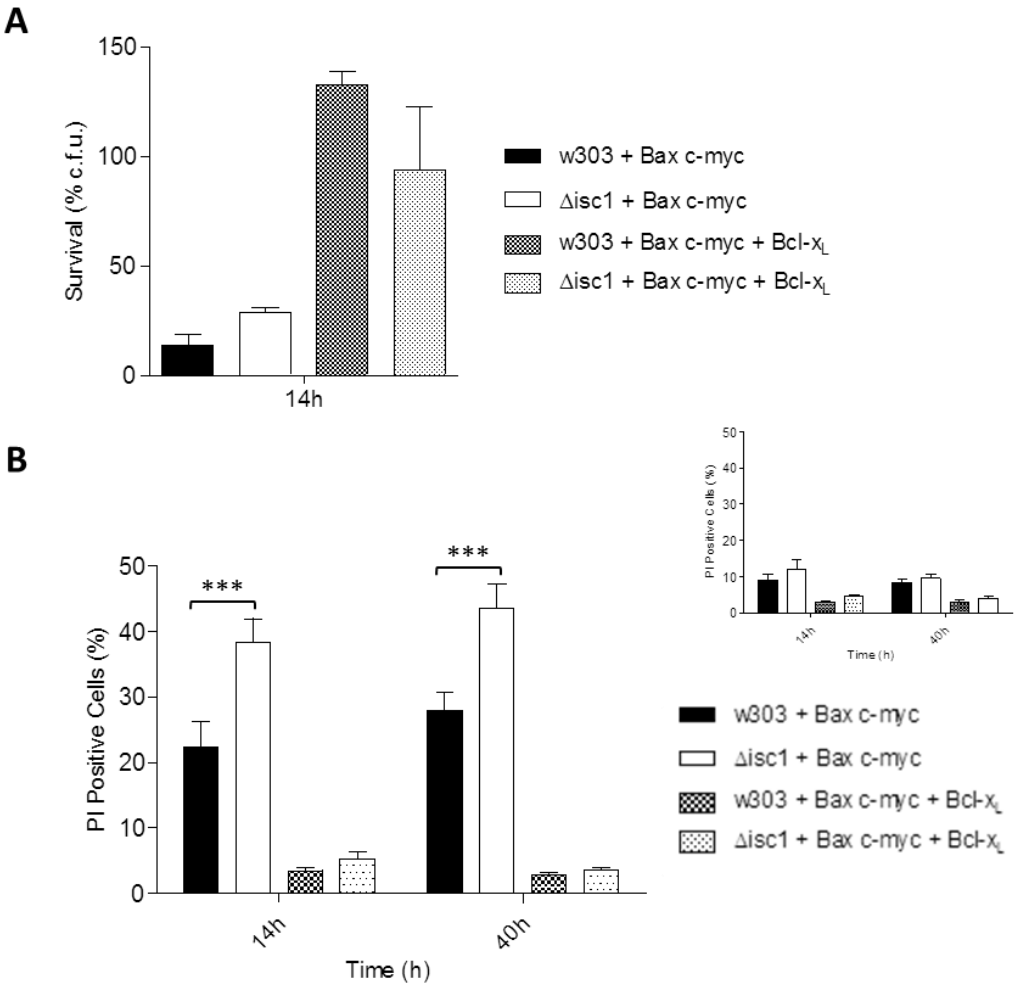


Fig. 6 – Expression of Bax *c-myc* or co-expression of Bax *c-myc* and Bcl-x_L in W303 *wt* and *Δisc1* cells. A) Survival of W303 and *Δisc1* cells to the action of Bax *c-myc*, in the absence or presence of Bcl-xL. Cells were grown in the presence of 0.1 µg/mL of doxycycline and 0.5% galactose to induce the expression of Bax *c-myc* and Bcl-x_L, respectively. Cells were collected after 14h incubation and platted onto YPDA containing 10 µg/mL of doxycycline, thus stopping the expression of Bax *c-myc*. c.f.u. were counted after two days incubating at 30°C. The survival of W303 and *Δisc1* expressing Bax *c-myc* is similar to that observed for the BY4742 strains, and the absence of Isc1p favors cell survival. Bcl-x_L reverts Bax-induced loss of platting efficiency in both strains. B) Plasma membrane integrity, assessed by the ability to exclude PI, of W303 *wt* and *Δisc1* cells expressing Bax *c-myc* alone or co-expressed with Bcl-x_L. Cells were cultured as described above, and samples collected after 14 and 40h incubation. Cells were resuspended in PBS containing PI, incubated for 10 minutes protected from light. PI staining was evaluated by flow cytometry. In *Δisc1* cells the loss of plasma membrane integrity is higher than in W303 cells (*** $P<0.001$). This effect is prevented by co-expression of Bcl-x_L. A control using cultures grown in the presence of 2 µg/mL of doxycycline, inhibiting Bax *c-myc* expression was performed (insert).

4. Discussion

Although the interplay between sphingolipids and members of the Bcl-2 family in the regulation of apoptosis has been extensively studied the connections between these two distinct types of known apoptotic regulators have not been completely clarified. To obtain further insights into the role of sphingolipid metabolism in cell death regulation by Bcl-2 family proteins we exploit the yeast *S. cerevisiae*. We were particularly interested to determine the impact of sphingolipid manipulation on the action of the human pro-apoptotic regulator Bax. The pathways and enzymes responsible for sphingolipid metabolism are highly conserved among fungi, plant and animal kingdoms, and model organisms such as *S. cerevisiae* greatly contributed to our understanding of the sphingolipids' biology. In fact sphingolipid studies in yeast have allowed the identification and cloning of most of the known enzymes of sphingolipid metabolism, which eventually led to the identification of their mammalian orthologues. It also uncovered cellular targets of sphingolipids which include pathways of stress and cell regulation such as cell cycle, heat stress, ubiquitin-mediated proteolysis, endocytosis, and protein trafficking (Obeid et al., 2002). In this work, three different forms of human Bax, namely Bax *c-myc* , Bax *wild-type* (Bax-*wt*) and Bax-P168A were heterologously expressed in both wild type and yeast mutant cells deficient in sphingolipid metabolism enzymes, alone or together with Bcl-x_L. Expression of Bax-*wt*, a nearly inactive form localized in the cytosol, has almost no effect on yeast growth, whereas Bax-P168A and Bax *c-myc* are active forms localized in mitochondria and capable of inducing MOMP. Using these different forms of Bax allowed us to study the impact of enzymes from sphingolipid metabolism in the distinct steps of Bax activation.

Expression of a Bax *c-myc* protein in the *S. cerevisiae* mutants (*Δifa38*, *Δisc1*, *Δydc1*, *Δypc1*, and *Δymr1*), allowed the identification of Isc1p as involved in Bax-induced cell death. In fact, Isc1p is crucial for a normal mitochondrial function, and actually regulates the sphingolipid profile of these organelles (Kitagaki et al., 2007), evidences that qualify Isc1p as a potential candidate for the regulation of Bcl-2 family members. The presence of a *c-myc* tag added to the C-terminal of Bax promotes the mitochondrial localization of this protein and induces cyt *c* release from mitochondria and yeast cell death (Priault et al., 1999, 2003).

Considering that the sphingolipid profile of mitochondria from *Δisc1* cells is significantly different from that of *wt* cells, and that mitochondrial sphingolipids are

determinant for the action of Bax, we tested the ability of Bax *c-myc* to insert in the OMM and induce cyt *c* release. Our hypothesis was that the sphingolipid alterations resulting from Isc1p absence could affect the addressing/insertion of Bax in mitochondria and impair cyt *c* release. Comparison of mitochondria samples from *Δisc1* and *wt* cells expressing Bax *c-myc*, however, revealed no obvious differences. In both strains Bax *c-myc* is equally capable of inserting in OMM as revealed by carbonate treatment. The capacity of this protein to trigger cyt *c* release is not impaired in the absence of Isc1p either. In fact, redox spectra of mitochondria from both cell types clearly demonstrate the cyt *c*/cyt *b* ratio of *Δisc1* + Bax *c-myc* mitochondria is even lower than that of *wt* mitochondria. This difference in not attributed to a facilitated cyt *c* release from *Δisc1* mitochondria, since an identical difference between *wt* and *Δisc1* cyt *c*/cyt *b* ratios was also observed in spectra of whole cells and mitochondrial preparations from control experiments. Since Isc1p has a role in mitochondrial function, the observed difference might be explained by the observed reduced cyt *c* content of *Δisc1* cells in accordance with previous studies (Rego et al., 2012). In addition, loss of Isc1p has previously been associated to reduced levels of mitochondrial cytochrome oxidase (COX) and decreased capacity to grow on non-fermentable carbon sources (Vaena de Avalos et al., 2005).

An identical analysis was performed with the two other different forms of Bax; a native Bax (Bax-*wt*), and a mutant of native Bax in which proline 168 was substituted by an alanine (Bax-P168A). These two proteins have distinct physiological behaviors. Native Bax is inactive and mostly cytosolic, waiting for an activation signal that eventually triggers its translocation to mitochondria and subsequent activation (Hsu et al., 1997). Additionally, a small population of native Bax can also be found in the endoplasmic reticulum (ER) (Zong et al., 2003). On the other hand, the P168A mutant of native Bax is strongly addressed to mitochondria of yeast cells, inserted in the OMM and has the capacity to promote cyt *c* release (Arokium et al., 2004). Since native Bax and Bax P168A have different intracellular localization and activity, the impact of Isc1p deletion in mitochondrial addressing/insertion and in cyt *c* release could be different.

Bax activity is also modulated by phosphorylation/dephosphorylation, which can be regulated by ceramide. For instance, mammalian protein phosphatases 1 (PP1) and 2A (PP2A) are ceramide-activated protein phosphatases (CAPPs) that bind to ceramide and regulate two distinct signaling pathways (Chalfant et al., 1999, 2004). Ceramide-activated PP2A can actually regulate the phosphorylation state of Bax, and the PP2A-

mediated Bax dephosphorylation is responsible for a conformational change that promotes its insertion and oligomerization in the OMM and also disrupts the Bcl-2/Bax association, liberating the pro-apoptotic regulator from this heterodimeric complex (Xin and Deng, 2006). Recently, Isc1p has been proposed as an upstream regulator of Sit4p, whose activation is implicated in mitochondrial dysfunction associated to shortened lifespan and oxidative stress sensitivity of *Δisc1* mutants (Barbosa et al., 2011). Sit4p is a PP2A-like protein phosphatase that shares structural homology to the catalytic subunit of mammalian PP2A, and functional homology with the mammalian protein serine/threonine phosphatase 6 (PP6) (Arndt et al., 1989; Bastians and Ponstingl, 1996). Moreover, Sit4p has been considered a component of a yeast ceramide-activated protein phosphatase, and is actually involved in ceramide stress response in yeast (Nickels and Broach, 1996; Woodacre et al., 2013). Although, there are no reports on a possible connection between mammalian PP6 and the Bcl-2 family members, this phosphatase regulates the sensitivity of glioblastoma cells to radiation (Shen et al., 2011). It seems, therefore, plausible to assume that the changes on sphingolipid profile of *Δisc1* cells, namely the decreased amount of α-HO C_{26}-phytoceramides and the increase of its non-HO form, could modulate a cellular response capable of affecting the action of the nearly inactive or less active forms Bax-*wt* and Bax P168A, respectively.

According to our results, Bax-*wt* is mostly localized to the microsomal and cytosolic fractions of *wt* and *Δisc1* cells, and barely found in mitochondria. These observations are consistent with the inactive status of Bax, and suggest that the absence of Isc1p does not contribute to Bax activation. Furthermore, redox spectra and cyt *c*/cyt *b* ratios indicate that there is no cyt *c* release under these conditions. Indeed, the small difference between the ratios of both strains is identical to that observed upon expression of Bax *c-myc* and might be equally explained. Considering these findings we can say that the sphingolipid profile resulting from the absence of Isc1p does not change the inactive state of native Bax. Co-expression of Bax-*wt* with the anti-apoptotic regulator Bcl-x_L enhanced the translocation of Bax to mitochondria of both cell types. Although it may seem a contradictory idea, given that Bcl-x_L promotes cell survival by blocking Bax-induced MOMP, its ability to promote Bax mitochondrial localization while inhibiting cyt *c* release has already been observed in HeLa cells and yeast cells expressing these human proteins (Llambi et al., 2011; Renault et al., 2013). Redox spectra of mitochondria isolated from W303 or *Δisc1* cells co-expressing Bax-*wt* and Bcl-x_L, confirmed the absence of cyt *c* release, even if Bax is more addressed to mitochondria.

Once more, a slight difference between the cyt *c*/cyt *b* ratios of both strains was observed, and the values were identical to those detected under conditions where only Bax-*wt* was expressed.

Our results show that the absence of Isc1p does not affect the activity of Bax- *c-myc* neither of native Bax. The impact of Isc1p deletion on the action of a pre-active mutant Bax-P168A form was then tested. Results demonstrated that Bax-P168A is addressed to mitochondria and inserted in the OMM, definitely excluding the hypothesis of impaired insertion of Bax in mitochondria of *Δisc1* cells. Bax-P168A also induces mitochondrial cyt *c* release in both strains, consistent with an active state. Interestingly, the cyt *c/*cyt *b* ratios of W303 and *Δisc1* cells expressing Bax-P168A are pretty much identical (approximately 1.22 and 1.18, respectively). This means that the small differences between the *wt* and *Δisc1* cyt *c*/cyt *b* ratios observed in cells expressing Bax *c-myc*, Bax-*wt*, co-expressing Bax-*wt* and Bcl-x_L or carrying an empty vector (with a magnitude between 0.12 and 0.19), are practically non-existent in the presence of Bax-P168A (with a mean difference of 0.04). Co-expression of Bcl-x_L, which does not change the mitochondrial localization of Bax-P168A, is able to prevent Bax-induced cyt *c* release, and the ratios of W303 and *Δisc1* cells under these conditions (1.55 and 1.39, respectively), are similar to those of control, Bax-*wt* or Bax-*wt* + Bcl-x_L cells. Restoring the cyt *c*/cyt *b* ratios to values consistent with the absence of cyt *c* release also restores the difference between W303 and *Δisc1* ratios. These findings suggest that cyt *c* release might be somehow delayed in *Δisc1* cells, but such hypothesis would require further experimentation. The lower amount of cyt *c* in *Δisc1* cells may not be a limiting factor in this case, since upon expression of Bax *c-myc* both *wt* and *Δisc1* mitochondria loose high amounts of cyt *c*, and the final difference between the ratios of both strains was still present. It should be worthy to test a shorter time of incubation in conditions were Bax is expressed. If the absence of Isc1p somehow affects Bax-P168A-induced cyt *c* release, this effect should be measurable shortly after Bax expression as well.

Since the increased survival observed for *Δisc1* cells expressing active forms of Bax could not be easily correlated to defects on Bax addressing and insertion in the OMM, neither to changes of the activation state of Bax, we tried to characterize the process of cell death in the absence of Isc1p. Interestingly, the delayed Bax-induced cell death of *Δisc1* cells is followed by increased loss of plasma membrane integrity, when compared to *wt* cells. This curious result is not entirely surprising since a similar situation has

previously been described for Bax-induced cell death in a yeast strain lacking the *UTH1* gene (Kissová et al., 2006).

The yeast *Δuth1* mutant was previously identified in a screen for extended longevity and discovered to be resistant to the action of Bax. Shortly after, it was found to be required for mitochondrial autophagy, also known as mitophagy (Camougrand et al., 2003; Kissová et al., 2004). Importantly, the absence of Uth1p does not affect the mitochondrial localization of Bax *c-myc*, nor its ability to induce MOMP and cyt *c* release (Camougrand et al., 2003). Later on, Kissova and colleagues observed that, unlike *wt* cells, the *Δuth1* mutant was unable to maintain plasma membrane integrity upon expression of Bax *c-myc*. They describe the slow loss of *Δuth1* platting efficiency as a necrotic form of cell death similar to that observed for acute stresses, and propose that the "apparent" resistance of *Δuth1* to the effects of Bax *c-myc* is actually caused by the slow kinetics of this form of cell death, contrasting with a regulated loss of proliferation capacity (Kissová et al., 2004). It was also observed that Bax *c-myc* induces the fragmentation of the mitochondrial network, a common and early, but not exclusive, event during mammalian apoptosis, although differently in *wt* and *Δuth1* strains. Mitochondria from *wt* cells expressing Bax *c-myc* were fragmented into relatively organized punctuated structures with a somewhat ordered distribution, while *Δuth1* mitochondria were fragmented into disorganized structures of different sizes and random cellular distribution (Kissová et al., 2004). Moreover, mitochondrial degradation was impaired in *Δuth1* cells suggesting the inactivation of mitophagy in the absence of Uth1p. The authors concluded that mitophagy is required for a rapid and regulated form of cell death, probably through the elimination of mitochondria damaged by the action of Bax, thus preventing a necrotic-like cell death (Kissová et al., 2004).

The resemblances shared by the effect of Isc1p deletion on the action of Bax, and that described for a yeast *Δuth1* mutant under identical circumstances, are tempting enough for us to establish a possible parallelism between both mutations. We did observed an increased resistance of *Δisc1* cells to the effects of an active Bax that cannot be correlated to the insertion of this pro-apoptotic protein in the OMM, and may be independent of its ability to induce mitochondrial cyt *c* release. Nevertheless, this resistance phenotype is accompanied by increased loss of plasma membrane integrity. The absence of Isc1p drastically changes the mitochondria morphology of yeast cells from the thread-like network, typical of *w*t cells, into what appears to be randomly distributed aggregates, even in the absence of any stress. Importantly, mitochondrial

degradation is also compromised in *Δisc1* cells during acetic acid treatment, and the absence of Isc1p increases the survival of yeast cells to acetic acid-induced death (Rego et al., 2012). Relevant for this line of evidences, recent studies associate sphingolipid metabolism, particularly ceramides, to mitochondria dynamics and mitophagy (Parra et al., 2008; Sentelle et al., 2012; Smith et al., 2013). Therefore, it should be enlightening to evaluate mitochondria degradation in *Δisc1* cells expressing an active form of Bax, and test the effect of this particular mutation in the ability of yeast cells to remove damaged mitochondria.

Recently, Chipuk and colleagues described that hexadecenal, a product of the irreversible cleavage of sphingosine-1-phophate, can associate with Bax to promote its activation (Chipuk et al., 2012). In yeast sphingolipid metabolism, the phosphate lyase Dpl1p is responsible for the cleavage of both dihydro- and phytosphingosines-1-phosphate (Saba et al., 1997). Manipulation of Dpl1p should provide an interesting opportunity to test and eventually validate this kind of *in vivo* studies in yeast. It should also be interesting to expand this approach to other proteins involved in sphingolipid metabolism. For instance, yeast cell lacking *LAG1*, a catalytic subunit of ceramide synthase, have altered mitochondria morphology and exhibit increased resistance to acetic acid associated to impaired mitochondrial degradation, somewhat similar to *Δisc1* cells (Rego et al., 2012). Overexpression of Ydc1, another protein of interest, has been associated to mitochondrial fragmentation and cell death (Aerts et al., 2008). This system could also be applied to study the effects of exogenously added sphingolipids in yeast cells transformed with mammalian Bax.

The sphingolipid metabolism and the Bcl-2 family function might intersect at different levels of cell death regulation. Among these, sphingolipid signaling has been shown to regulate several members of the Bcl-2 family by interfering with signaling cascades, or even through the regulation of DNA splicing. It has also been proposed that a specific milieu of sphingolipids is required for the proper function of Bcl-2 family members in mitochondria (Chipuk et al., 2012). Finally, ceramides are able to form lipidic channels which can be disassembled by anti-apoptotic Bcl-2 proteins such as Bcl-x_L, or enlarged/stabilized by the pro-apoptotic Bax (Ganesan et al., 2010; Siskind et al., 2008). Our work demonstrates that genes involved in sphingolipid metabolism can interfere with the effects of Bax in yeast. The absence of Isc1p increased the tolerance of yeast cells to the action of an active recombinant form of Bax, a phenomenon that is not associated with reduced addressing or insertion of this pro-apoptotic regulator in

OMM. It is possible that changes in the mitochondrial sphingolipid profile could affect the dynamics of Bax-induced cyt *c* release, but the mechanism behind such hypothesis remains unknown and further testing is required to validate this scenario. Alternatively, the resistance phenotype of *Δisc1* cells could be related to a direct effect of this mutation in the regulation of mitophagy. This work provides another insight on the vital action of sphingolipids as modulators of the cell death processes, emphasizing the valuable contribution of yeast as a model to understand the physiology of life and death.

5. References

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

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

Alphonse, G., Bionda, C., Aloy, M.-T., Ardail, D., Rousson, R., and Rodriguez-Lafrasse, C. (2004). Overcoming resistance to gamma-rays in squamous carcinoma cells by poly-drug elevation of ceramide levels. Oncogene *23*, 2703–2715.

Arndt, K.T., Styles, C.A., and Fink, G.R. (1989). A suppressor of a HIS4 transcriptional defect encodes a protein with homology to the catalytic subunit of protein phosphatases. Cell *56*, 527–537.

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 alpha-helix is a second ART sequence and plays a role in the interaction with anti-apoptotic BCL-xL. J. Biol. Chem. *279*, 52566–52573.

Barbosa, A.D., Osório, H., Sims, K.J., Almeida, T., Alves, M., Bielawski, J., Amorim, M.A., Moradas-Ferreira, P., Hannun, Y.A., and Costa, V. (2011). Role for Sit4p-dependent mitochondrial dysfunction in mediating the shortened chronological lifespan and oxidative stress sensitivity of Isc1p-deficient cells. Mol. Microbiol. *81*, 515–527.

Bastians, H., and Ponstingl, H. (1996). The novel human protein serine/threonine phosphatase 6 is a functional homologue of budding yeast Sit4p and fission yeast ppe1, which are involved in cell cycle regulation. J. Cell Sci. *109 (Pt 12)*, 2865–2874.

Beaudoin, F., Gable, K., Sayanova, O., Dunn, T., and Napier, J.A. (2002). A Saccharomyces cerevisiae gene required for heterologous fatty acid elongase activity encodes a microsomal beta-keto-reductase. J. Biol. Chem. *277*, 11481–11488.

Camougrand, N., Grelaud-Coq, A., Marza, E., Priault, M., Bessoule, J.-J., and Manon, S. (2003). The product of the UTH1 gene, required for Bax-induced cell death in yeast, is involved in the response to rapamycin. Mol. Microbiol. *47*, 495–506.

Chalfant, C.E., Kishikawa, K., Mumby, M.C., Kamibayashi, C., Bielawska, A., and Hannun, Y.A. (1999). Long chain ceramides activate protein phosphatase-1 and protein phosphatase-2A. Activation is stereospecific and regulated by phosphatidic acid. J. Biol. Chem. *274*, 20313–20317.

Chalfant, C.E., Szulc, Z., Roddy, P., Bielawska, A., and Hannun, Y.A. (2004). The structural requirements for ceramide activation of serine-threonine protein phosphatases. J. Lipid Res. *45*, 496–506.

Chipuk, J.E., McStay, G.P., Bharti, A., Kuwana, T., Clarke, C.J., Siskind, L.J., Obeid, L.M., and Green, D.R. (2012). Sphingolipid metabolism cooperates with BAK and BAX to promote the mitochondrial pathway of apoptosis. Cell *148*, 988–1000.

Colombini, M. (2010). Ceramide channels and their role in mitochondria-mediated apoptosis. Biochim. Biophys. Acta *1797*, 1239–1244.

Cuvillier, O. (2002). Sphingosine in apoptosis signaling. Biochim. Biophys. Acta *1585*, 153–162.

Ganesan, V., Perera, M.N., Colombini, D., Datskovskiy, D., Chadha, K., and Colombini, M. (2010). Ceramide and activated Bax act synergistically to permeabilize the mitochondrial outer membrane. Apoptosis Int. J. Program. Cell Death *15*, 553–562.

Greenhalf, W., Stephan, C., and Chaudhuri, B. (1996). Role of mitochondria and C-terminal membrane anchor of Bcl-2 in Bax induced growth arrest and mortality in Saccharomyces cerevisiae. FEBS Lett. *380*, 169–175.

Han, G., Gable, K., Kohlwein, S.D., Beaudoin, F., Napier, J.A., and Dunn, T.M. (2002). The Saccharomyces cerevisiae YBR159w gene encodes the 3-ketoreductase of the microsomal fatty acid elongase. J. Biol. Chem. *277*, 35440–35449.

Hannun, Y.A., and Obeid, L.M. (2008). Principles of bioactive lipid signalling: lessons from sphingolipids. Nat. Rev. Mol. Cell Biol. *9*, 139–150.

Hsu, Y.T., Wolter, K.G., and Youle, R.J. (1997). Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc. Natl. Acad. Sci. U. S. A. *94*, 3668–3672.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. *153*, 163–168.

Kissová, I., Deffieu, M., Manon, S., and Camougrand, N. (2004). Uth1p is involved in the autophagic degradation of mitochondria. J. Biol. Chem. *279*, 39068–39074.

Kissová, I., Plamondon, L.-T., Brisson, L., Priault, M., Renouf, V., Schaeffer, J., Camougrand, N., and Manon, S. (2006). Evaluation of the roles of apoptosis, autophagy, and mitophagy in the loss of plating efficiency induced by Bax expression in yeast. J. Biol. Chem. *281*, 36187–36197.

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

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Law, R.H., Manon, S., Devenish, R.J., and Nagley, P. (1995). ATP synthase from Saccharomyces cerevisiae. Methods Enzymol. *260*, 133–163.

Llambi, F., Moldoveanu, T., Tait, S.W.G., Bouchier-Hayes, L., Temirov, J., McCormick, L.L., Dillon, C.P., and Green, D.R. (2011). A unified model of mammalian BCL-2 protein family interactions at the mitochondria. Mol. Cell *44*, 517–531.

Manon, S., Chaudhuri, B., and Guérin, M. (1997). Release of cytochrome c and decrease of cytochrome c oxidase in Bax-expressing yeast cells, and prevention of these effects by coexpression of Bcl-xL. FEBS Lett. *415*, 29–32.

Mao, C., Xu, R., Bielawska, A., Szulc, Z.M., and Obeid, L.M. (2000a). Cloning and characterization of a Saccharomyces cerevisiae alkaline ceramidase with specificity for dihydroceramide. J. Biol. Chem. *275*, 31369–31378.

Mao, C., Xu, R., Bielawska, A., and Obeid, L.M. (2000b). Cloning of an alkaline ceramidase from Saccharomyces cerevisiae. An enzyme with reverse (CoA-independent) ceramide synthase activity. J. Biol. Chem. *275*, 6876–6884.

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

Nickels, J.T., and Broach, J.R. (1996). A ceramide-activated protein phosphatase mediates ceramide-induced G1 arrest of Saccharomyces cerevisiae. Genes Dev. *10*, 382–394.

Obeid, L.M., Linardic, C.M., Karolak, L.A., and Hannun, Y.A. (1993). Programmed cell death induced by ceramide. Science *259*, 1769–1771.

Obeid, L.M., Okamoto, Y., and Mao, C. (2002). Yeast sphingolipids: metabolism and biology. Biochim. Biophys. Acta *1585*, 163–171.

Parra, V., Eisner, V., Chiong, M., Criollo, A., Moraga, F., Garcia, A., Härtel, S., Jaimovich, E., Zorzano, A., Hidalgo, C., et al. (2008). Changes in mitochondrial dynamics during ceramide-induced cardiomyocyte early apoptosis. Cardiovasc. Res. *77*, 387–397.

Priault, M., Camougrand, N., Chaudhuri, B., Schaeffer, J., and Manon, S. (1999). Comparison of the effects of bax-expression in yeast under fermentative and respiratory conditions: investigation of the role of adenine nucleotides carrier and cytochrome c. FEBS Lett. *456*, 232–238.

Priault, M., Bessoule, J.-J., Grelaud-Coq, A., Camougrand, N., and Manon, S. (2002). Bax-induced cell death in yeast depends on mitochondrial lipid oxidation. Eur. J. Biochem. FEBS *269*, 5440–5450.

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 Differ. *10*, 1068–1077.

Rego, A., Costa, M., Chaves, S.R., Matmati, N., Pereira, H., Sousa, M.J., Moradas-Ferreira, P., Hannun, Y.A., Costa, V., and Côrte-Real, M. (2012). Modulation of mitochondrial outer membrane permeabilization and apoptosis by ceramide metabolism. PloS One *7*, e48571.

Renault, T.T., Teijido, O., Antonsson, B., Dejean, L.M., and Manon, S. (2013). Regulation of Bax mitochondrial localization by Bcl-2 and Bcl-x(L): keep your friends close but your enemies closer. Int. J. Biochem. Cell Biol. *45*, 64–67.

Rodriguez-Lafrasse, C., Alphonse, G., Aloy, M.-T., Ardail, D., Gérard, J.-P., Louisot, P., and Rousson, R. (2002). Increasing endogenous ceramide using inhibitors of sphingolipid metabolism maximizes ionizing radiation-induced mitochondrial injury and apoptotic cell killing. Int. J. Cancer J. Int. Cancer *101*, 589–598.

Saba, J.D., Nara, F., Bielawska, A., Garrett, S., and Hannun, Y.A. (1997). The BST1 gene of Saccharomyces cerevisiae is the sphingosine-1-phosphate lyase. J. Biol. Chem. *272*, 26087–26090.

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

Sentelle, R.D., Senkal, C.E., Jiang, W., Ponnusamy, S., Gencer, S., Selvam, S.P., Ramshesh, V.K., Peterson, Y.K., Lemasters, J.J., Szulc, Z.M., et al. (2012). Ceramide targets autophagosomes to mitochondria and induces lethal mitophagy. Nat. Chem. Biol. *8*, 831–838.

Shen, Y., Wang, Y., Sheng, K., Fei, X., Guo, Q., Larner, J., Kong, X., Qiu, Y., and Mi, J. (2011). Serine/threonine protein phosphatase 6 modulates the radiation sensitivity of glioblastoma. Cell Death Dis. *2*, e241.

Siskind, L.J., and Colombini, M. (2000). The lipids C2- and C16-ceramide form large stable channels. Implications for apoptosis. J. Biol. Chem. *275*, 38640–38644.

Siskind, L.J., Kolesnick, R.N., and Colombini, M. (2006). Ceramide forms channels in mitochondrial outer membranes at physiologically relevant concentrations. Mitochondrion *6*, 118–125.

Siskind, L.J., Feinstein, L., Yu, T., Davis, J.S., Jones, D., Choi, J., Zuckerman, J.E., Tan, W., Hill, R.B., Hardwick, J.M., et al. (2008). Anti-apoptotic Bcl-2 Family Proteins Disassemble Ceramide Channels. J. Biol. Chem. *283*, 6622–6630.

Smith, M.E., Tippetts, T.S., Brassfield, E.S., Tucker, B.J., Ockey, A., Swensen, A.C., Anthonymuthu, T.S., Washburn, T.D., Kane, D.A., Prince, J.T., et al. (2013). Mitochondrial fission mediates ceramide-induced metabolic disruption in skeletal muscle. Biochem. J.

Spiegel, S., and Milstien, S. (2003). Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat. Rev. Mol. Cell Biol. *4*, 397–407.

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

Taha, T.A., Kitatani, K., El-Alwani, M., Bielawski, J., Hannun, Y.A., and Obeid, L.M. (2006b). Loss of sphingosine kinase-1 activates the intrinsic pathway of programmed cell death: modulation of sphingolipid levels and the induction of apoptosis. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *20*, 482–484.

Taylor, G.S., Maehama, T., and Dixon, J.E. (2000). Myotubularin, a protein tyrosine phosphatase mutated in myotubular myopathy, dephosphorylates the lipid second messenger, phosphatidylinositol 3-phosphate. Proc. Natl. Acad. Sci. U. S. A. *97*, 8910–8915.

Vaena de Avalos, S., Okamoto, Y., and Hannun, Y.A. (2004). Activation and localization of inositol phosphosphingolipid phospholipase C, Isc1p, to the mitochondria during growth of Saccharomyces cerevisiae. J. Biol. Chem. *279*, 11537–11545.

Vaena de Avalos, S., Su, X., Zhang, M., Okamoto, Y., Dowhan, W., and Hannun, Y.A. (2005). The phosphatidylglycerol/cardiolipin biosynthetic pathway is required for the activation of inositol

phosphosphingolipid phospholipase C, Isc1p, during growth of Saccharomyces cerevisiae. J. Biol. Chem. *280*, 7170–7177.

Woodacre, A., Lone, M.A., Jablonowski, D., Schneiter, R., Giorgini, F., and Schaffrath, R. (2013). A novel Sit4 phosphatase complex is involved in the response to ceramide stress in yeast. Oxid. Med. Cell. Longev. *2013*, 129645.

Xin, M., and Deng, X. (2006). Protein phosphatase 2A enhances the proapoptotic function of Bax through dephosphorylation. J. Biol. Chem. *281*, 18859–18867.

Zhang, T., and Saghatelian, A. (2013). Emerging roles of lipids in BCL-2 family-regulated apoptosis. Biochim. Biophys. Acta *1831*, 1542–1554.

Zong, W.-X., Li, C., Hatzivassiliou, G., Lindsten, T., Yu, Q.-C., Yuan, J., and Thompson, C.B. (2003). Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J. Cell Biol. *162*, 59–69.

Chapter V

Final Considerations

1. Concluding Remarks and Future Perspectives

Mitochondria hold the power to determine life and death of each eukaryotic cell. More than "just" containing all the essential elements to generate significant amounts of energy and promote life, mitochondria also encloses proteins that can potentially change the biological balance and promote cell death. The mechanisms and pathways that involve mitochondria permeabilization and the commitment to cell death are complex and far from fully comprehended.

Several years ago, it was suggested that the adenine nucleotide translocator (AAC in yeast) was involved in cell death by participating in the formation of an inner membrane pore, the PTP (Halestrap and Davidson, 1990). Other studies established a possible collaboration between the ANT and the Bcl-2 family members, major regulators of cell death in mammals (Marzo et al., 1998). In both cases the contribution of the ANT was questioned, and its role in cell death was doubted. While studying the contribution of putative components of the PTP to cell death induced by acetic acid in *S. cerevisiae*, Pereira and colleagues revealed an important role of the AAC, the yeast homologue of mammalian ANT, in mediating both MOMP and cell death (Pereira et al., 2007).

Considering that: i) yeast is deprived of the major regulators of mammalian cell death, the Bcl-2 family members (only recently a BH3-only protein was identified), ii) acetic acid can trigger different cell death phenotypes and is accompanied by ROS in yeast production, iii) ROS are known sensitizers of the PTP and mediators of cell death, iv) the thiol groups are preferentially oxidized by ROS, and the AACs are particularly sensitive to their action, v) binding of compounds to the cysteine residues of the AAC locks the translocator in specific conformations, and vi) absence of the AAC protects cells from acetic acid-induced death, we decided to test the contribution of AAC cysteine residues to cell death in yeast. Our most relevant observations were:

- The cysteine residues of Aac2p do not influence acetic acid-induced yeast cell death;
- Yeast cells containing an Aac2p protein lacking the four cysteine residues, responds in a similar manner to Aac2p wild-type cells in respect to ROS production, loss of plasma membrane integrity and MOMP;
- The Aac2p is not particularly oxidized following acetic acid treatment.

In light of this evidence we can exclude the contribution of the Aac2p cysteine residues to acetic acid-induced MOMP. It has been proposed that oxidative stress-mediated crosslinking of thiol groups locks Aac2p in the "c" conformation, which would then favour the binding of CyP-D, over ADP, greatly sensitizing the mitochondrial PTP to Ca^{2+} (Crompton et al., 1987; McStay et al., 2002). If acetic acid induces an identical conformational change in Aac2p, and Aac2p is involved in PTP opening, we should observe changes in cytochrome *c* release. Moreover, analysis of protein carbonylation does not suggest that this carrier is particularly oxidized during acetic acid treatment. Definite evidence for this hypothesis could be provided by mass spectrometry analysis. Nevertheless, diamide, a true pro-oxidant agent, should offer a more suitable stimulus to test the putative participation of Aac2p, its thiol groups and conformational changes, in PTP opening. Like for acetic acid, yeast cells lacking the three AAC isoforms exhibit increased tolerance to diamide treatment (Pereira et al., 2007). Recently, the inorganic phosphate carrier (PiC) and the F_OF_1 ATP synthase have been suggested as components of the mammalian PTP (Alcala et al., 2008; Giorgio et al., 2013; Leung et al., 2008). Therefore, it should be interesting to test the putative contribution of both proteins to the acetic acid-induced cytochrome *c* release, and yeast provides excellent genetic tools to do so. Finally, the addition of a V5-His6 double tag to the C-terminus of Aac2p had major implications for normal mitochondrial functions, in particular for energetic metabolism. This construction was found in mitochondria but fails to restore the respiratory competence of *Δaac1/2/3* cells. This effect is associated with a drastic loss of cytochrome *c*. One hypothesis is that the TOM-TIM-mediated translocation of Aac2p to mitochondria is affected by the presence of the double tag, and that this protein could actually destabilize the mitochondrial protein import systems. It would be worthwhile to isolate mitoplasts from cells expressing Aac2p and V5-His6 double tagged Aac2p, and compare the levels of the carrier with those of whole mitochondria, to confirm a proper addressing to the IMM. In conclusion, Aac2p participates in acetic acid-induced cytochrome *c* release from mitochondria, but this effect does not depend on its cysteine residues and possible on oxidative stress as well.

Yeast cells lacking another putative component of the mitochondrial PTP, Por1 (VDAC), were found to be more sensitive to acetic acid than wild-type cells. This effect could be completely converted into a resistance phenotype by the simultaneous absence of the AAC proteins, suggesting a shared pathway to the promotion of cell death, in which Por1p could have a regulatory effect over an AAC-mediated pore formation

(Pereira et al., 2007). This relation was tested by comparing the impact of each protein, alone or combined, in the capacity of mitochondria to undergo acetic acid-induced cytochrome *c* release. We observed that:

- Acetic acid has increased cytotoxicity in yeast strains lacking Por1p, which apparently possess debilitated mitochondria, difficult to isolate;
- Absence of Por1p does not impair acetic acid-induced cytochrome *c* release from mitochondria;
- Absence of AAC proteins impairs mitochondrial cytochrome *c* release induced by acetic acid;
- When both AAC and Por1p are absent mitochondria apparently can still release cytochrome *c*;
- Absence of AAC contributes to increased cell wall resistance, while Por1 deletion has no significant effect;
- AAC and Por1p are, like for acetic acid, implicated in the resistance of yeast cells to osmotic and cell wall stresses (the absence of AACs increases resistance while absence of Por1p sensitizes cells);
- Absence of AAC reverts the sensitivity of *Δpor1* cells to osmotic and cell wall stress, but does not rescue the decreased thermotolerance resulting from Por1 deletion;
- The effect of AAC's absence in the resistance of yeast to osmotic and cell wall stresses does not seem to depend on the bioenergetic limitations of *Δaac1/2/3* strains.

These results indicate that both the AAC proteins and Por1p can influence the yeast cell's response to the acetic acid insult. It is possible that these proteins act in the same pathway mediating yeast cell death, with AAC as a facilitator of mitochondrial cytochrome *c* release and Por1p as negative regulator. Nevertheless, both proteins seem to mediate different physiological processes. Indeed, Por1p is required for mitochondrial stability, normal mitochondria dynamics, and for the capacity of yeast cells to cope with osmotic and cell wall stress as well as growth at over-optimal temperatures. In future experiments with mitochondria isolated from these strains, it will be important to control the inner mitochondria membrane integrity, in order to ensure that the observed cytochrome *c* release truly reflects MOMP. Although localized

to the IMM, the AAC is able to influence the cell wall resistance of yeasts, which could suggest its involvement, direct or not, in signalling events. The mechanism by which the AAC influences the cell wall resistance of yeast, however, remains uncertain.

In a considerably different approach, we exploited the potential of yeast as a model system to study the influence of sphingolipid metabolism on the action of human apoptotic regulators. Sphingolipids are widely recognized as crucial signalling molecules in a variety of cellular processes, including the pathways of cell death (Hannun and Obeid, 2008), and can directly influence the action of the Bcl-2 family proteins, particularly that of the pro-apoptotic regulator Bax (addressed in Chapter 1 – "Sphingolipids in cell death signalling"). The conservation in yeast of the sphingolipid metabolism in yeast, in combination with the ability to express of different forms of human Bax, namely the native form of Bax (nearly inactive form localized in the cytosol), the pre-active Bax P168A and the active Bax *c-myc* (both localized in mitochondria), provides an advantageous approach to identify which enzymes ofinvolved in sphingolipid metabolism play a roleinvolved in the distinct steps of human Bax activation.A screen with a set of yeast strains mutated in sphingolipid metabolism enzymes, revealed a possible contribution of Isc1p, responsible to the degradation of complex sphingolipids into ceramides, for the regulation of Bax *c-myc*-induced loss of cell viability. From the heterologous expression of different forms of Bax in *Δisc1* yeast cells, we can conclude that:

- The resistance of *Δisc1* cells to the action of Bax *c-myc*, cannot be attributed to reduced addressing and/or insertion of this active form into the OMM;
- The resistance of *Δisc1* cells to the action of Bax *c-myc* cannot be attributed to changes in cytochrome *c* release;
- The resistance phenotype observed in *Δisc1* cells expressing Bax *c-myc*, is likely related to the consequences of Bax *c-myc* effects in mitochondria;
- This resistance phenotype of *Δisc1* cells is probably related to the regulation of mitophagy.
- Absence of Isc1p does not influence the activation of Bax-*wt* with respect to addressing/insertion into mitochondria or induced cytochrome c release;
- Absence of Isc1p does not influence the function of the pre-active form Bax-P168A with respect to addressing/insertion into mitochondria, though a slight delay in cytochrome c release is apparent

Our results suggest that sphingolipids, and genes involved in sphingolipid metabolism, have the ability to regulate the effects of Bax in yeast cells. In fact, the absence of Isc1p improved the viability of yeast cells expressing an active form of Bax. This effect, however, does not seem to be related with the regulation of Bax action in mitochondria, i.e. its ability to be addressed and inserted in the OMM, or to induce cytochrome *c* release when fully activated. Rather it seems to depend on downstream events. Supporting this hypothesis, we provide evidences suggesting that the increased viability of *Δisc1* somewhat resembles that of a *Δuth1* mutant, in which mitophagy is severely compromised and cell death progression changes from that observed for wild-type cells. From our experiments, we cannot completely discard an effect of Isc1p deletion on the dynamics of Bax-induced cytochrome *c* release, and kinetics of cytochrome *c* release could be performed to validate this scenario. Nevertheless, it might be worth exploring a possible contribution of Isc1p to the process of mitophagy.

Yeast has been extensively, and successfully, used as a model to study the role of proteins and evolutionary conserved systems involved in cell death. Here, once more, the yeast *S. cerevisiae* has proven its great value providing interesting clues to different biological processes. Consisting of an epistemological approach, this work represents another small step towards the understanding of the vast and highly complex network of biological events that strongly control cell death, a natural element of life.

"Life is pleasant. Death is peaceful. It's the transition that's troublesome."

Isaac Asimov

2. References

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.

Crompton, M., Costi, A., and Hayat, L. (1987). Evidence for the presence of a reversible Ca^{2+}-dependent pore activated by oxidative stress in heart mitochondria. The Biochemical journal *245*, 915-918.

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.

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

Hannun, Y.A., and Obeid, L.M. (2008). Principles of bioactive lipid signalling: lessons from sphingolipids. Nature reviews Molecular cell biology *9*, 139-150.

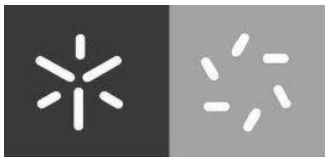
Leung, A.W., Varanyuwatana, P., and Halestrap, A.P. (2008). The mitochondrial phosphate carrier interacts with cyclophilin D and may play a key role in the permeability transition. The Journal of biological chemistry *283*, 26312-26323.

Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J.M., Susin, S.A., Vieira, H.L., Prevost, M.C., Xie, Z., Matsuyama, S., Reed, J.C., *et al.* (1998). Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science *281*, 2027-2031.

McStay, G.P., Clarke, S.J., and Halestrap, A.P. (2002). Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. The Biochemical journal *367*, 541-548.

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.

This thesis performed under a cotutelle program between:



Centro de Biologia Molecular e Ambiental (CBMA)

Universidade do Minho

And

Institut de Biochimie et Génétique Cellulaires

Université Bordeaux Segalen

CNRS - Centre national de la recherche scientifique

Dário Trindade was the recipient of a scholarship from Gulbenkian Science Institute, Fundação Calouste Gulbenkian (process n.º 126474).

FUNDAÇÃO
CALOUSTE
GULBENKIAN

This work was supported by FEDER through POFC – COMPETE and by national funds from FCT through the project PEst-C/BIA/UI4050/2011.