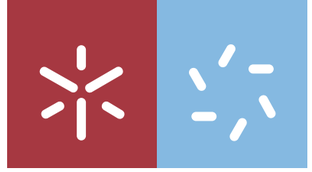
Exploring yeast as a tool to study the regulation of the human pro-apoptotic protein Bax

Vitória Baptista

UMinho | 2018



Universidade do Minho
Escola de Ciências

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Trabalho efetuado sob a orientação de
Professora Doutora Maria Manuela Sansonetty Gonçalves Côrte-Real
e de
Professora Doutora Susana Alexandra Rodrigues Chaves

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"Time and time again, truly basic studies of simple experimental organisms have proved directly relevant to human biology and human disease. An investment in such basic studies is an effective investment indeed."

H. Robert Horvitz

Exploring yeast as a tool to study the regulation of the human pro-apoptotic protein Bax

ABSTRACT

Cell death has long been considered crucial for proper tissue shaping during embryonic development and for normal turnover of the cells in several tissues. Moreover, mis-regulation of apoptosis has been implicated in a diversity of abnormal functions and in the progression and development of diseases. Indeed, a decrease in apoptosis has been associated with ageing and tumour progression, whereas an increase in apoptosis is mainly associated with ageing-related neurodegenerative diseases including Alzheimer's, Parkinson's and Huntington's diseases. The life and death switch is among others regulated by interactions between pro- and anti-apoptotic Bcl-2 family members, including Bax and Bcl-x_L, respectively. Bax plays a central role in apoptosis, as it is involved in the formation of pores within the mitochondria outer membrane through which apoptogenic factors, including cytochrome *c,* are released and originate a cascade of events that culminate in cell death. Given the importance of this pro-apoptotic protein in cell death, its potential as a therapeutic target was quickly recognized, and its mode of action and regulation have been studied extensively. However, many questions still remain, and thus further understanding of Bax regulation was the general purpose of this thesis. To accomplish this, we took advantage of the genetically tractable yeast *Saccharomyces cerevisiae,* whose genome is devoid of genes coding apparent homologues of the human Bcl-2 family. Therefore, the heterologous expression of human Bax allows its study without interference of the apoptotic network. To achieve our goal, we first optimized the conditions for expression of human Bax (Bax α) in yeast and characterized the phenotype of expression of Bax α and of an active form, Bax *c*-myc. Then, conditions that led to Bax α activation were found, and its regulation through different proteins was explored. Herein, we describe for the first time that sub-lethal concentrations of acetic acid trigger Bax α-mediated cell death without disturbing plasma membrane integrity and mitochondrial mass but increasing superoxide anion accumulation and release of cytochrome *c*, which is partially reverted by the anti-apoptotic protein Bcl-x_L. This finding allows us to mimic what happens in human cells, without the need to use non-natural mutants of Bax, such as phosphomimetic or non-phosphorylatable, or mitochondrial tagged versions. Thus, yeast continuously proves to be a valuable tool to express human Bax and perform studies regarding its function, regulation and interaction with other members of the Bcl-2 protein family and other partners.

Explorar a levedura como modelo para estudar a regulação da proteína pro-apoptótica humana Bax

RESUMO

A morte celular é um evento fundamental para a correta formação dos tecidos durante o desenvolvimento embrionário e para o normal funcionamento das células. Devido ao seu papel fundamental a nível fisiológico, desregulações neste processo estão associadas a uma grande diversidade de funções anormais e, consequentemente, ao desenvolvimento e progressão de várias doenças. De facto, uma diminuição da morte celular relaciona-se com envelhecimento e a progressão de tumores, enquanto que um aumento está associado a doenças neurodegenerativas incluindo a doença de Alzheimer, Parkinson e Huntington. O destino de uma dada célula é regulado, entre outras formas, por interações entre membros pro-apoptóticos e anti-apoptóticos da família de proteínas Bcl-2, nomeadamente a Bax e a Bcl-x_L, respetivamente. A proteína Bax está envolvida na formação de poros na membrana mitocondrial externa, através do qual são libertados fatores apoptóticos, incluindo o citocromo *c*, originando uma cascata de eventos que culmina na morte da célula. Considerando o papel importante desta proteína na morte celular, as suas capacidades como alvo terapêutico emergiram rapidamente. Por este motivo, o seu modo de ação e regulação têm sido extensivamente estudados. Contudo, várias questões permanecem eminentes e, por isso a melhor compreensão da sua regulação é o foco principal desta tese. Assim, escolhemos a levedura *Saccharomyces cerevisiae*, cujo genoma não possui genes codificantes de homólogos da família de proteínas humanas Bcl-2. Deste modo, a expressão heteróloga da proteína Bax humana na levedura permite o seu estudo sem interferência dos restantes membros da família. Para alcançarmos o nosso objetivo, primeiramente otimizamos as condições para expressão da proteína na levedura, e caracterizamos o seu fenótipo de expressão, bem como o de uma forma ativa, Bax *c*-myc. De seguida, determinamos condições que nos permitiram a ativação da proteína Bax humana e a sua regulação por outras proteínas. Nesta tese, descrevemos pela primeira vez que concentrações sub-letais de ácido acético desencadeiam um processo de morte celular mediado por Bax, sem provocar alterações na integridade da membrana plasmática e na massa mitocondrial, mas aumentando a acumulação de anião superóxido e causando a libertação de citocromo *c*, parcialmente revertida pela proteína anti-apoptótica Bcl-x_L. Estes resultados permitiram-nos mimetizar o que acontece em células humanas, sem a necessidade de usar mutantes de Bax artificiais, tais como os mutantes fosfomiméticos e não fosforiláveis ou versões endereçadas para a mitocôndria. Deste modo, a levedura continua a revelar o seu valor como modelo celular para expressar a proteína Bax humana e realizar estudos no que toca à sua função, regulação e interação com outros membros da família Bcl-2 e outros parceiros.

SCIENTIFIC OUTPUT

Panel Communications

1. Vitória Baptista, Cátia S. Pereira, Joana P. Guedes, Maria João Sousa, Susana Chaves and Manuela Côrte-Real (2018) "Yeast *Saccharomyces cerevisiae* as a model organism to exploit the regulation of the human pro-apoptotic protein Bax". IV Simpósio PDBMA, 25/10, Braga, Portugal.

2. Vitória Baptista, Cátia S. Pereira, Joana P. Guedes, Maria João Sousa, Susana Chaves and Manuela Côrte-Real (2018) "Exploring yeast as a tool to study the human pro-apoptotic protein Bax ". "XXI Jornadas de Biologia de Leveduras "Nicolau van Uden"", 8-9/6, Braga, Portugal.

3. Vitória Baptista, Cátia S. Pereira, Joana P. Guedes, Maria João Sousa, Susana Chaves and Manuela Côrte-Real (2018) "Yeast as a cell model to study the human pro-apoptotic protein Bax". IX Jornadas de Bioquímica "Da Investigação à Indústria",21/3, Braga, Portugal.

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LIST OF ABBREVIATIONS AND ACRONYMS

$\Delta\Psi_m$	Mitochondrial Membrane Potential
A	Alanine
A1	Bcl-2 related gene A1
AIF	Apoptosis Inducing Factor
APAF1	Apoptotic Protease-Activating Factor 1
ART	Apoptotic Regulation of Targeting
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 Associated protein-X
Bcl-2	B-Cell Lymphoma 2
Bcl-B	Bcl-2-like protein 10
Bcl-x_L	B-cell lymphoma-extra-large
Bcl-w	Bcl-2-like protein 2
BH	Homology domain
Bid	BH3-interacting death domain agonist
Bik	Bcl-2 interacting killer
Bim	Bcl-2-like protein 11
Bmf	Bcl-2 modifying factor
Bok	Bcl-2 related ovarian killer protein
CFU	Colony Forming Unit
CIVS	Cylindrical Intravacuolar Structures
COX	Cytochrome *c* oxidase
D	Aspartate
DHE	Dihydroethidium
Diablo	Direct IAP Binding Protein with a low pI
DISC	Death-Inducing Signalling Complex
DNA	Deoxyribonucleic acid
E	Glutamate

ER	Endoplasmic Reticulum
EV	Empty vector
HtrA2/Omi	High temperature requirement A2 protein
FADD	Fas-Associated Death Domain Protein
FASL	Fas ligand
FDA	Fluoresceine Diacetate
FLIP	FASS-like Apoptosis Regulator
G	Glycine
GSK3β	Glycogen Synthase Kinase 3β
Hrk	Activator of apoptosis hara-kiri
K	Lysine
Mcl-1	Myeloid cell leukaemia 1
MOM	Mitochondria outer membrane
MOMP	Mitochondria outer membrane permeabilization
NCCD	Nomenclature Committee on Cell Death
NATs	N-terminal acyltransferases
NMR	Nuclear Magnetic Resonance
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
P	Proline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PKA	Protein Kinase A
PKB/Akt	Protein Kinase B
PKC	Protein Kinase C
PUMA	p53 Upregulated Modulator of Apoptosis
RCD	Regulated Cell Death
ROS	Reactive Oxygen Species
RT	Room temperature
S	Serine
SCFA	Short-chain fatty acids

Smac	Second Mitochondrial Derived Activator of Caspases
tBid	Truncated Form of Bid
TNFR	Tumour Necrosis Factor Receptors
TRADD	TNFR- Associated Death Domain Protein
TRAIL	Related Apoptosis- Inducing Ligand
V	Valine
XIAP	X-linked inhibitor of apoptosis

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Chapter I

INTRODUCTION

I.1. Cell death

Cell death, a critical process to eliminate harmful cells, plays an important role in tissue homeostasis (Green and Llambi 2015) and in animal development and aging (Tower 2015), and is thus one of the most studied biological processes in the last decades.

Cell death can be classified based on morphological appearance, immunological characteristics, and enzymological and functional aspects (Kroemer *et al.* 2009). According to the Nomenclature Committee on Cell Death (NCCD), it can be subdivided in accidental, when completely unpreventable and caused by severe physical, chemical or mechanical stimuli, and regulated (RCD), when initiated by a genetically encoded molecular machinery. RCD can be influenced by specific pharmacological and genetic interventions and occurs as a consequence of microenvironmental changes. It can also occur in the context of embryonic development, immune responses and tissue homeostasis, and is in these cases known as programmed cell death (PCD) (Galluzzi *et al.* 2015; Galluzzi et al. 2018).

The term PCD was first introduced in 1964 by Lockshin and Williams, who observed that, during the metamorphosis of the silkworm, specific cells died in a programmed manner according to a "construction manual" for the insect (Lockshin and Williams 1965). Later on, in 1972, Kerr, Wyllie and Currie used the term apoptosis to describe a RCD type with specific morphological features that allows cells to commit suicide (Kerr et al. 1972).

I.2. Apoptosis: A Regulated Type of Cell Death

Apoptosis, a tightly regulated suicide program conserved throughout evolution, contributes to the correct formation of tissues and organs during embryonic development, and to the maintenance of vital physiological functions, namely immune and nervous systems in adult animals (Ashkenazi and Salvesen 2014). However, despite its beneficial biological role, it has been reported that excessive levels of apoptosis contribute to the development of neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, diabetes and immune deficiency, while insufficient apoptosis is associated with autoimmune diseases, cancer and viral infections (Thompson 1995; Elmore 2007; Ashkenazi and Salvesen 2014).

Cells committed into this well-characterized energy-dependent RCD process (**Figure I.1**), suffer particular morphological, biochemical and molecular changes, including cell shrinkage, nucleus and chromatin condensation (pyknosis), nucleus fragmentation (karyorrhexis), chromatin dissolution (karyolysis), cell membrane bleeding and exposure of phosphatidylserine (Kerr *et al.* 1972; Tower 2015). These events are followed by the budding of the dying cell into membrane-bound apoptotic bodies, which are engulfed by nearby cells through phagocytosis, resulting in minimal inflammation and damage to surrounding tissues and cells (Ashkenazi and Salvesen 2014; Tower 2015; Redza-Dutordoir

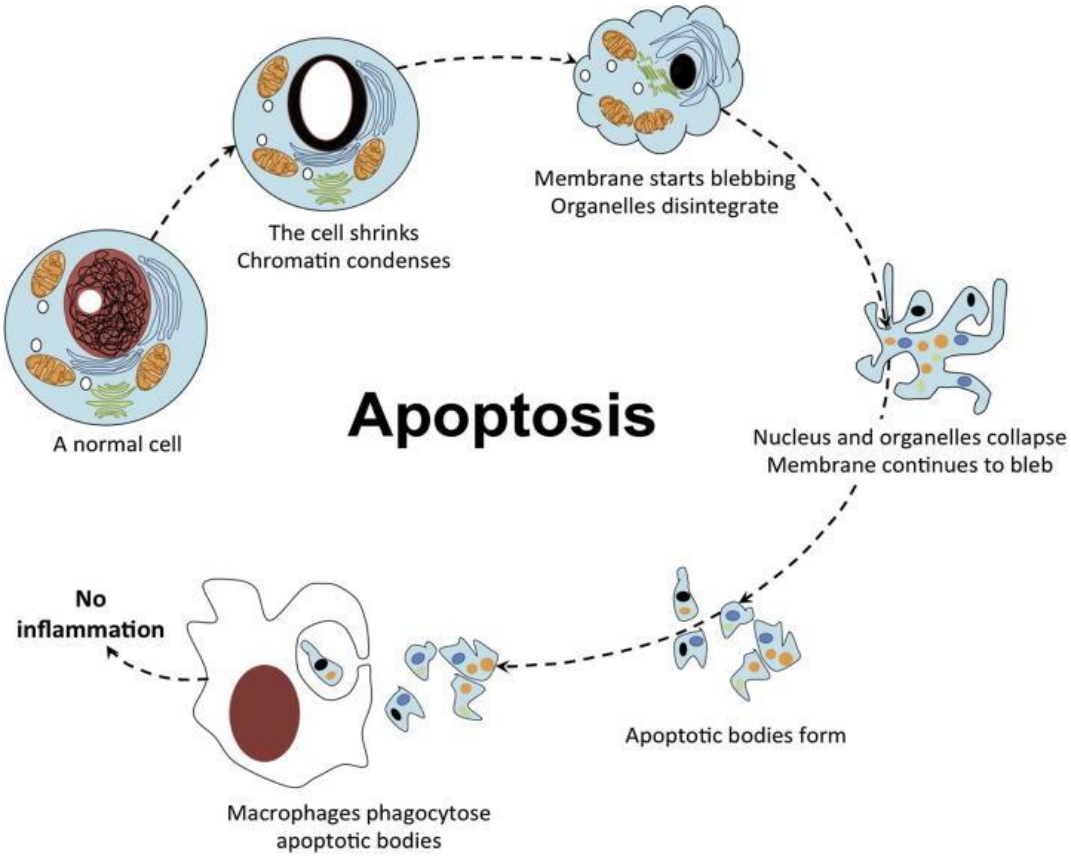

Figure I.1: Morphological events associated with the apoptotic process. (Abou-Ghali and Stiban 2015).

and Averill-Bates 2016).

Most of the morphological changes observed during apoptosis are caused by a set of cysteine-aspartic proteases called caspases (Thornberry and Lazebnik 1998). Conserved throughout evolution, these proteases have a key role in the regulation of apoptosis and inflammation (Shalini *et al.* 2015) and, as suggested by their name, they hydrolyse peptide bonds only after specific aspartic acid residues of the substrate, depending on the active-site cysteine residue (McIlwain *et al.* 2013).

I.2.1. Two distinct but convergent pathways to apoptosis

Apoptotic cell death can be triggered by a wide variety of stimuli that can act through an intrinsic or extrinsic signalling pathway (**Figure I.2**), which often reinforce one another (Ashkenazi and Salvesen 2014).

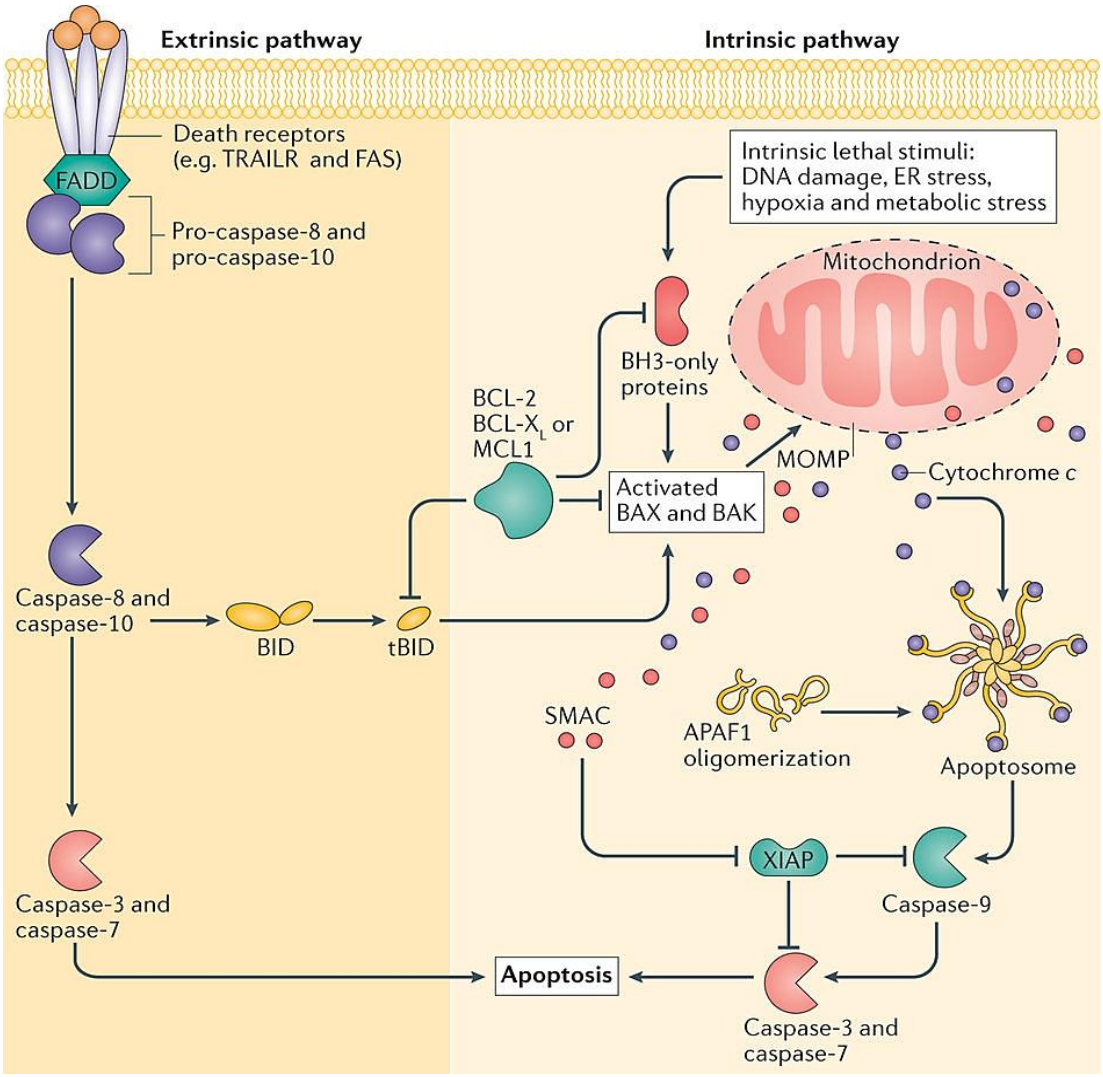

Figure I.2: Extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is initiated by the binding of death receptors with their ligands (FASL- FAS ligand; TRAIL- TNF related apoptosis inducing ligand), resulting in the activation of initiator caspases via Fas-associated death domain protein (FADD), that then activate the effector caspases leading to apoptosis. In this pathway, the initiator caspases can also cleave the BH3-only protein Bid (BH3-interacting death domain agonist), originating truncated Bid (tBid) that enables the crosstalk between the two pathways. In the intrinsic pathway activated BH3-only members supress the action of pro-survival Bcl-2 proteins, stimulating the activity of Bax and Bak. These pro-apoptotic proteins lead to mitochondria outer membrane permeabilization (MOMP), allowing the release of cytochrome *c*, Smac (second mitochondria-derived activator of caspases) and other apoptotic factors to the cytosol. The apoptosome, composed by cytochrome *c* and Apoptotic Protease-Activating Factor 1 (APAF1), recruits and activates pro-caspase 9, which in turn activates the effector caspases, leading to apoptosis. Smac inhibits the caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP). ER, endoplasmic reticulum; MCL1, myeloid cell leukaemia 1. (Ichim and Tait 2016).

The extrinsic or death receptor pathway involves the binding of a ligand to a plasma membrane death receptor. Fas, TNFR1 and TNF-related apoptosis-inducing ligand (TRAIL) receptors are some of the best characterized death receptors, and bind to FasL, TNF-α and TRAIL, respectively (Ashkenazi and Dixit 1998; Elmore 2007). In this pathway, the binding of the ligand to its receptor triggers the recruitment of the adaptor protein Fas-associated death domain (FADD) and pro-caspase 8 to form the multiprotein death-inducing signalling complex (DISC) (Ashkenazi and Salvesen 2014; Nagata and Tanaka 2017). Pro-caspase 8 is matured into caspase 8 by homodimerization in the DISC and, when activated, processes effector caspases, typically caspases 3 and 7, which induce the degradative phase of apoptosis (Tait and Green 2010; Portt *et al*. 2011; Cuda *et al*. 2016). In addition, Fas can also lead to cell death through the crosstalk between the extrinsic and intrinsic pathways. This is mediated by the activation of B-cell lymphoma 2 (Bcl-2) homology (BH) domain 3-only protein Bid (BH3-interacting death domain agonist), a pro-apoptotic protein that is cleaved by caspase 8. The truncated form of Bid (tBid) is then recruited to the mitochondria, leading to the intrinsic pathway via Bax (Bcl-2 Associated protein-X) and Bak (Bcl-2 homologous antagonist/killer) (Ashkenazi and Salvesen 2014; Cuda *et al*. 2016).

The intrinsic pathway of apoptosis, also known as mitochondrial pathway, is triggered by several cellular stresses, including activated oncogenes, deoxyribonucleic acid (DNA) damage, oxidative stress, irradiation, hypoxia and growth factor deprivation (Green and Llambi 2015; Tower 2015). In this pathway, a pro-apoptotic member of the Bcl-2 family is activated, inducing mitochondrial outer membrane permeabilization (MOMP). This allows the pro-apoptotic factors, namely cytochrome *c*, Smac/Diablo (second mitochondrial derived activator of caspase/ direct IAP binding protein with a low pI), Omi/HtrA2 (High temperature requirement A2 protein), Nuclease G and AIF (Apoptosis inducing factor) to be release into the cytosol, instead of being sequestered in the intermembrane space of mitochondria (Green and Llambi 2015). When in the cytosol, cytochrome *c* promotes the assembly of a multiprotein complex called apoptosome, which is composed by itself, oligomerized apoptotic protease-activating factor 1 (APAF1) and pro-caspase 9. Caspase 9, which is activated in the apoptosome, induces apoptosis by cleavage and activation of the executioner caspases (Cuda *et al*. 2016; Nagata and Tanaka 2017). Smac and Omi bind and, consequently, inactivate the caspase inhibitor X-linked inhibitor of apoptosis (XIAP), promoting caspases activation by the apoptosome (Green and Llambi 2015), and Nuclease G along with AIF mediate chromatin condensation and DNA fragmentation.

I.2.3. Hallmarks of apoptosis

As mentioned above, apoptosis occurs via a tightly regulated signalling cascade of events that results in a sequence of morphological, molecular and biochemical changes within the cell, which ultimately leads to its death. According to the NCCD, a cell is considered dead when it has lost its plasma membrane integrity, when itself and its content undergo fragmentation into apoptotic bodies or when these apoptotic bodies are phagocyted. Nevertheless, a "point of no return" into death has been considered to exist and is characterized by MOMP along with the release of cytochrome *c*, hence, massive caspase activation and loss of mitochondrial membrane potential ($\Delta\Psi_m$), as well as exposure of phosphatidylserine (Kroemer *et al*. 2009).

Several timepoints and hallmarks of apoptosis (**Table I**), such as caspases activation, cytochrome *c* release, reactive oxygen species (ROS) accumulation, chromatin condensation and DNA fragmentation can be used to identify this type of cell death. However, some apoptotic features overlap with those of other types of cell death, namely of necrosis, and it is thus crucial to use different assays to avoid misinterpretation (Elmore 2007).

Table I.1: Hallmarks of apoptosis and its detection methods.

Apoptotic marker	Detection methods	Reference
Caspases activation	Western-blot, Fluorometric assay, Fluorescence microscopy and flow cytometry	(Elmore 2007; Krysko *et al*. 2008)
Cytochrome *c* release	Western-blot, Fluorescence microscopy and Redox Spectrophotometry	(Renault *et al*. 2015; Büttner *et al*. 2011; Krysko *et al*. 2008; Ludovico *et al*. 2002)
Chromatin condensation	DAPI	(Madeo *et al*. 1999)
Phosphatidylserine exposure	Anexin V/ Propidium Iodide (PI)	(Madeo *et al*. 1999; Ludovico *et al*. 2001; Renault *et al*. 2015; Büttner *et al*. 2011)
Loss of $\Delta\Psi m$	Fluorescence probes Imaging or flow cytometry	(Pringle *et al*. 1989; Büttner *et al*. 2011)
ROS accumulation	Fluorescence probes	(Madeo *et al*. 1999; Büttner *et al*. 2011)
DNA fragmentation	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick end Labelling (TUNEL) assay	(Madeo *et al*. 1999; Büttner *et al*. 2011; Ludovico *et al*. 2001; Krysko *et al*. 2008)

I.3. The Bcl-2 protein family: Central players in apoptosis

The Bcl-2 protein family members play a major role in the regulation of apoptosis (Adams and Cory 2007; Chipuk *et al.* 2010), as they are involved in the engagement of cells into the death process, in regulating MOMP (Shamas-Din *et al.* 2013) and in conferring the appropriate apoptotic characteristics that thereafter lead to cell death (Renault *et al.* 2017). In addition, they control other cellular processes, namely mitochondrial dynamics, levels of calcium in the endoplasmic reticulum (ER) and autophagy (Chipuk *et al.* 2010).

The Blc-2 gene was discovered in 1984 during the study of a chromosome translocation in human follicular B cell lymphoma (Tsujimoto *et al.* 1984), and soon after it was found to be involved in cell survival (Vaux *et al.* 1988; Tsujimoto 1989). Years later, genomic analysis of the nematode *Caenorhabditis elegans* revealed an apoptotic molecular pathway regulated by this protein family, demonstrating cell death as an active and fundamental biological process conserved among species (Horvitz 1999). Afterwards, based on sequence similarity and capability to bind to the Bcl-2 protein, other members have been identified, expanding the Bcl-2 family (**Figure I.3**) (Adams and Cory 1998; Tsujimoto 1998).

All anti-apoptotic proteins, namely Bcl-2, BCL-x_L (B-cell lymphoma-extra-large), Blc-w (Bcl-2-like protein 2), Bcl-B (Bcl-2-like protein 10), Mcl-1 (Myeloid cell leukaemia 1) and A1 (Bcl-2 related gene A1), possess the four BH domains (BH1 to BH4) and prevent MOMP by antagonizing their pro-apoptotic counterparts. The pro-apoptotic proteins comprise either the multidomain-containing effector proteins Bax, Bak and Bok (Bcl-2 related ovarian killer protein), which form pores in the mitochondria outer membrane (MOM), leading to its permeability to a set of apoptotic factors, or BH3-only proteins. BH3-only proteins can be further classified as sensitizers [Noxa (Phorbol-12-myristate-13-acetate-induced protein 1), Bmf (Bcl-2 modifying factor), Bik (Bcl-2 interacting killer), Bad (Bcl-2-associated death promoter) and Hrk (Activator of apoptosis harakiri)] or activators [Bid, Bim (Bcl-2-like protein 11) and PUMA (p53 upregulated modulator of apoptosis)] based on their ability to bind to anti-apoptotic proteins, or both anti-apoptotic and effector proteins, respectively. These proteins are structurally different; they only encompass the conserved BH3 domain, and the rest of the sequence is diverse from the other members of the Bcl-2 family (Adams and Cory 1998; Chipuk *et al.* 2010; Kale *et al.* 2018).

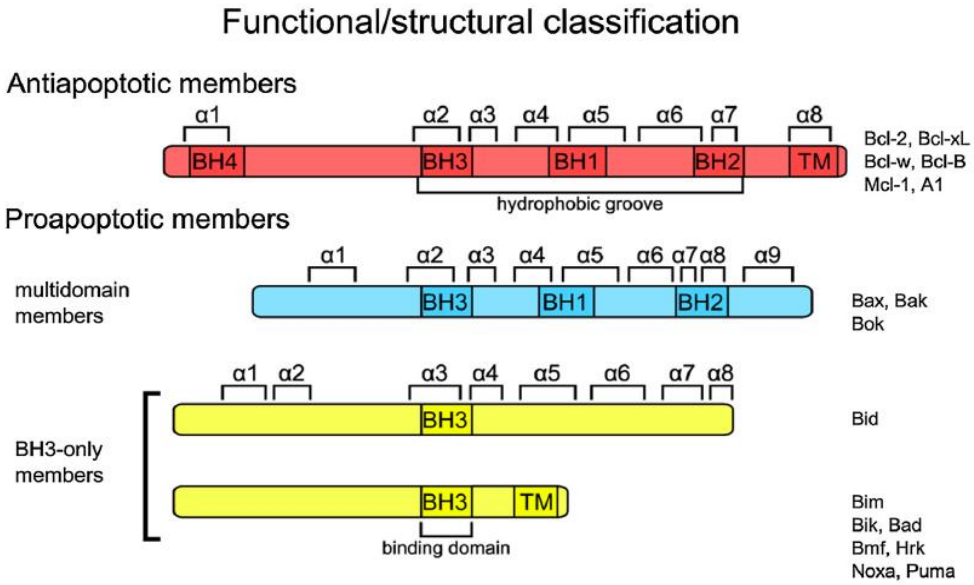

Figure I.3: The Bcl-2 family members. The Bcl-2 proteins family was subdivided based on structure and function into anti-apoptotic and pro-apoptotic members. The anti-apoptotic members contain the four Bcl-2 homology domains (BH1 to BH4) and a transmembrane domain (TM), while the pro-apoptotic ones are further subdivided into multidomain members, which encompass three BH domains including BH1, BH2 and BH3, and BH3-only members. Figure adapted from (Renault et al. 2017).

I.3.1. The role of Bcl-2 protein hydrophobic groove in the cell fate

Despite the different roles and some divergence in the primary structure, all Bcl-2 family members share a common tertiary structure (Renault and Manon 2011). Indeed, the BH1, BH2 and BH3 domains of anti- and multidomain pro-apoptotic proteins form a hydrophobic groove where the amphipathic BH3 domain of another family member can bind (Muchmore *et al.* 1996; Sattler *et al.* 1997). The presence of this binding groove explains the ability of Bcl-2 family members to form homodimers and heterodimers, and since heterodimerization between pro-apoptotic and anti-apoptotic members inhibits the biological activity of their partners (Oltvai *et al.* 1993), interactions within this family govern the cell fate decision (Kale *et al.* 2018).

I.4. The pro-apoptotic protein Bax: the switch between life and death

As previously mentioned, Bax is a member of the Bcl-2 family whose function is crucial for the commitment of cells into the mitochondrial pathway of apoptosis. Oltvai and colleagues discovered this 21 kDa pro-apoptotic protein back in 1963, during their study concerning interactions of Bcl-2 with other proteins. Through co-immunoprecipitation, the authors were able to identify Bax as an interacting

partner of Bcl-2 and then, using gene alignment, they found out that these two proteins share extensive homology, particularly in their BH1 and BH2 domains. They therefore hypothesized and proved that Bax was also capable of modulating cell death, contributing to it when overexpressed (Oltvai *et al*. 1993).

I.4.1. Bax structure

In healthy cells, the pro-apoptotic protein Bax is mainly localized in the cytosol in a soluble monomeric conformation, although a small fraction of the protein can also be found loosely attached to the MOM (Hsu *et al*. 1997; Hsu and Youle 1998) and in the ER (Zong *et al*. 2003). Upon a cell death stimulus, Bax translocates from the cytosol and inserts into mitochondria, where it may adopt a fully active conformation, leading to cell dismantling (Wolter *et al*. 1997; Renault and Manon 2011).

The structure of cytosolic Bax (**Figure I.4**) was unveiled by nuclear magnetic resonance (NMR), which showed that this globular protein, as other members of the Bcl-2 family (Muchmore *et al*. 1996), encompasses nine α-helices: a hydrophobic α-helix core (α-helix 5) surrounded by eight amphipathic α-helices, connected by loops (Suzuki *et al*. 2000). As stated above, Bax BH1 (portion of α-helices 4 and 5), BH2 (α-helices 7 and 8) and BH3 (α-helix 2) domains comprise a hydrophobic groove where another family member can bind. However, in Bax native status, α-helix 9, which consists of a C-terminal transmembrane domain responsible for the anchoring of Bax in the MOM under apoptotic scenarios, occupies this groove, masking its BH3 domain and explaining its predominantly cytosolic location (Lalier *et al*. 2007; Westphal *et al*. 2011). In spite of its importance, domains other than α-helix 9 can also modulate Bax localization. Indeed, the N-terminal domain of Bax, specifically a 20 amino acid sequence known as "Apoptotic Regulation of Targeting" (ART), is also capable of locking the protein in a soluble inactive conformation, and its movement is required for mitochondrial translocation (Goping *et al*. 1998). Indeed, when several ART residues are replaced, mitochondrial translocation is favoured in yeast (Arokium *et al*. 2004) and human cells (Cartron *et al*. 2002). Thus, both the N- and C-termini of Bax are required for it to maintain a stable inactive conformation, which is stabilized by ionic interactions between different domains of the protein. The disruption of these interactions culminates in Bax activation.

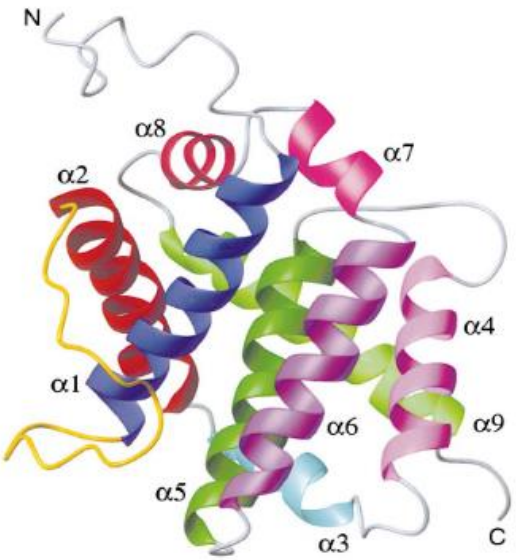

Figure I.4: Bax tertiary structure. Structure of human Bax with coloured α helices. BH1 (portion of α-helices 4 and 5), BH2 (α-helices 7 and 8) and BH3 (α-helix 2) domains define a hydrophobic groove occupied by the C-terminal transmembrane region of α-helix 9. (Suzuki et al. 2000)

I.4.2. Bax activation: the commitment into cell death

During apoptosis, activation of Bax results from a multistep process in which the pro-apoptotic protein undergoes conformational transitions, switching from a globular cytosolic structure to an extended membrane-embedded protein. In this MOM activated status, Bax hydrophobic domains, otherwise hidden, are exposed, increasing its affinity for biological membranes, where it oligomerizes conducting to MOMP and, consequently, cell death (Lalier *et al.* 2007). This commitment into cell death (**Figure I.5**) can be initiated when an activator BH3-only protein transiently interacts with Bax (Kim *et al.* 2009) through binding to either its "rear pocket", composed by α-helices 1 and 6 (Gavathiotis *et al.* 2008; Gavathiotis *et al.* 2010), or to its hydrophobic groove (Peter E. Czabotar *et al.* 2013), thereby releasing the C-terminal α-helix 9 that can further insert into MOM. This first step is preceded by exposure of the N-terminal and partial unfolding of α-helices 5 and 6, which cause the protein to dissociate into a core, α-helices 2-5, and a latch, α-helices 6-9. Thus, together, these rearrangements allow the exposure of the BH3 domain, which is crucial for Bax dimerization (Wang *et al.* 1998). The last step known as BH3-in-groove dimerization is characterized by the binding of the Bax BH3 domain to a canonical groove of another Bax molecule in order to create a "symmetric" dimer. Bax "symmetric" dimers self-assemble into higher oligomers that form large ring-like structures in the MOM,

permeabilizing it, in a process that is still poorly characterized (Cosentino and García-Sáez 2017; Peña-Blanco and García-Sáez 2018).

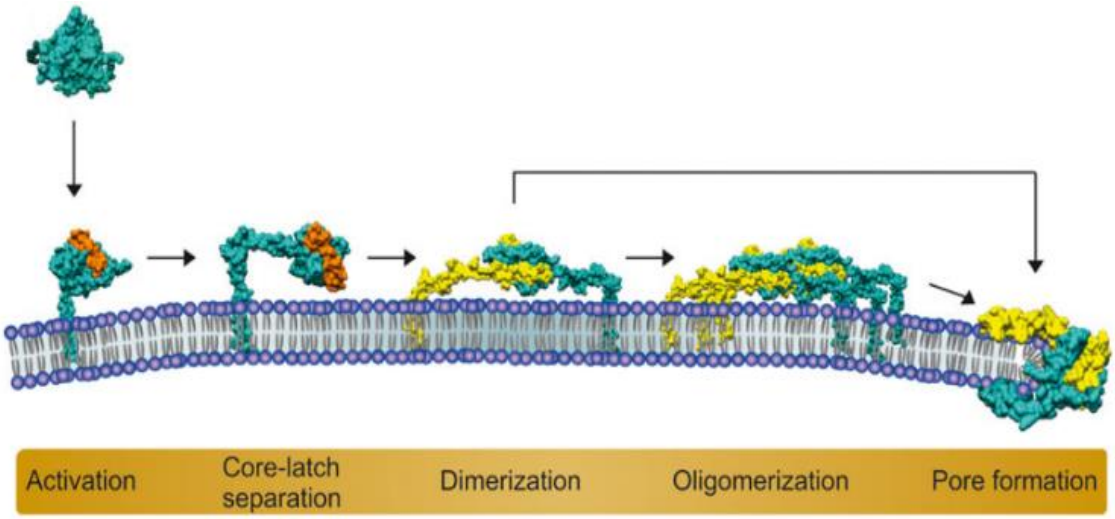

Figure I.5: Step-by-step process characterizing Bax activation, oligomerization and pore formation. In its inactive form, Bax (cyan) presents a soluble, globular conformation with its α-helix 9 masked into its groove. Upon activation by a BH3-only protein (orange), Bax's α-helix 9 is displaced, enabling interaction with the mitochondria membrane. This is followed by the unfolding of α-helices 5 and 6 creating a core and a latch, which allow the exposure of Bax BH3 domain needed for dimerization with another Bax molecule (yellow). The assembly of several dimers create oligomers responsible for membrane permeabilization. (Peña-Blanco and García-Sáez 2018)

I.4.3. Regulation of Bax activity: to die or not to die?

Bax regulation must be a well-coordinated process, since it has a key role in the decision of cell fate. This pro-apoptotic protein must be tightly regulated in order to maintain cell homeostasis, otherwise several disorders can develop. Bax can be regulated through several mechanisms, namely through its degradation and synthesis, by protein modifications and by interactions with other proteins.

I.4.3.1 Bax regulation by protein modifications

Protein modifications, particularly phosphorylation, might influence the function of Bcl-2 family proteins, through modulation of their structure, stability, binding partners, degradation rates and alterations in their subcellular localization (Billen *et al.* 2008; Kutuk and Letai 2008).

Phosphoregulation of the Bcl-2 proteins results from the balance of kinases and phosphatases that act on serine, threonine and tyrosine residues (Kutuk and Letai 2008). Phosphorylation of Bax mainly occurs in residues of both its N- and C-terminal, affecting its binding properties and the commitment into cell death (Billen *et al.* 2008). The foremost point of Bax phosphorylation is its S184

(Serine 184), localized in the C-terminal α-helix 9, whose movement is vital for Bax activation (Suzuki *et al*. 2000). The phosphorylation of this residue by Protein Kinase B (PKB/Akt) in neutrophils (Gardai *et al*. 2004) and Protein Kinase Cζ (PKCζ) in a MEF cell line (Xin *et al*. 2007) induces conformational changes that inhibit Bax translocation and, thereby its apoptotic function. These results were sustained by Wang *et al.* that reported that a non-phosphorylated Bax mutant S184A (S184Alanine) functions as an active form of Bax, while a phosphomimetic mutant S184E (S184 Glutamate) is not able to insert into the MOM (Wang *et al*. 2010). In accordance, this same group discovered that nicotine favours Bax phosphorylation in A549 human lung cancer cells by activation of Akt and abrogation of cisplatin-induced Bax translocation and MOM insertion, thereby increasing cell survival and chemoresistance (Xin and Deng 2005). Inversely, Bax dephosphorylation at S184 by Protein phosphatase 2A improves its pro-apoptotic function (Xin and Deng 2006). Additionally, Bax point mutations driven by site-direct mutagenesis showed that changes of S184 to a charged residue [(Aspartate (D), E or Lysine (K)] block Bax translocation, whereas substitutions to an hydrophobic residue [A or Valine (V)] increase Bax mitochondrial localization (Nechushtan *et al*. 1999). However, unexpectedly, in yeast cells, heterologous co-expression of Akt and human Bax resulted in an increased Bax mitochondrial and cellular content, associated with a greater capacity to release cytochrome *c* (Simonyan *et al*. 2016). In the same study, it was reported that the point mutation S184D results in activation of Bax, indicating that any movement of α-helix 9 is associated with an active conformation, but not necessarily with translocation (Renault and Manon 2011). PKCα, another kinase, modulates mitochondrial translocation and insertion of Bax *c*-myc, an active form of Bax, and induces cell death, when co-expressed in yeast cells (Silva *et al*. 2011). However, the authors clarified that this pro-apoptotic output induced by PKCα is not due to Bax *c*-myc phosphorylation, and the increased cell death is independent of its kinase activity (Silva *et al*. 2011). Another Bax residue that is phosphorylated was found by Linseman *et al*. using cultures of cerebellar neurons, who demonstrated that S163, localized in the loop between α-helices 8 and 9, is also a target of phosphorylation by Glycogen Synthase Kinase 3β (GSK3β). Phosphorylation of S163 by this kinase favours the activation of Bax and its movement towards mitochondria (Linseman *et al*. 2004). The non-phosphorylated (S163A) and the phosphomimetic (S163D) Bax mutants were also tested in yeast, and neither led to any increment in Bax activity and mitochondrial localization (Wang *et al*. 2010). However, these features increased when conjugating the S163D mutation with the substitution of P168 (Proline 168), a residue involved in the movement of α-helix 9 (Cartron *et al*. 2005), to A (P168A), and S60A, which cause a higher movement of α-helix 9. This suggests that, apart

from S163 phosphorylation, other conformational changes may occur (Arokium *et al.* 2007; Renault and Manon 2011). Nevertheless, GSK3β can also undergo phosphorylation and consequently inactivation by Akt (**Figure I.6**), which suggests that Akt can inhibit Bax directly and indirectly through GSK3β (Arokium *et al.* 2007; Renault and Manon 2011). After S163 phosphorylation, α-helix 9 moves away from the hydrophobic groove, exposing S60, localized in the Bax BH3 domain. Due to the existence of a consensus sequence for Protein Kinase A (PKA), S60 is also predicted to be a target of phosphorylation. This residue is close to D33 and K64, which are stabilizers of α-helices 1 and 2 in cytosolic Bax; phosphorylation of S60 is therefore thought to destabilize this interaction, allowing the interaction of these domains with other Bax partners, namely Tom 22 (Arokium *et al.* 2007; Renault and Manon 2011). The S60A substitution leads to the movement of α-helix 9, preventing Bax activation even in the presence of the activating mutations P168A or P13G (P13Glycine), whereas a mutation to D leads to a not fully activated Bax accumulation on mitochondria (Arokium *et al.* 2007; Billen *et al.* 2008). Finally, Kim *et al.* (2006) described that in hepatocytes, under stress conditions, c-Jun NH2-terminal kinase or p38 kinase promote Bax phosphorylation at Threonine 167, leading to a conformational modification that activates Bax and exposes its N- and C- terminal domains, increasing Bax translocation to MOM, and consequently its killing activity.

Besides phosphorylation, other protein modifications have been explored in order to understand their role in Bax regulation. In fact, our group recently demonstrated that Bax is also a target of N-terminal acetylation by N-terminal acyltransferases (NATs). These enzymes target N-terminal specific

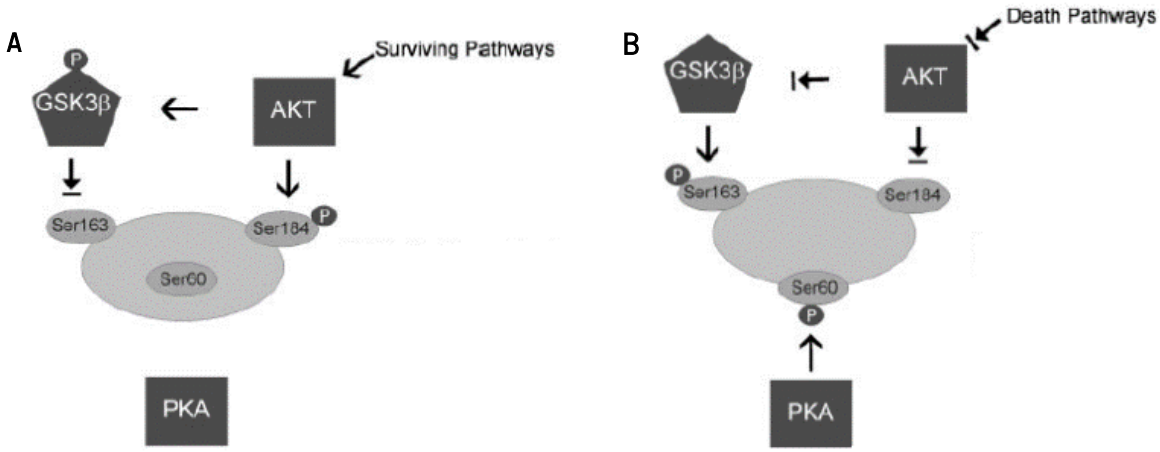

Figure I.6: A possible model of Bax regulation by phosphorylation. (A) In healthy cells, Protein Kinase B (Akt/PKB) phosphorylates Serine (Ser) 184. This kinase is also able to phosphorylate Glycogen Synthase Kinase 3β (GSK3β), inactivating and preventing it to phosphorylate Ser163. In this way, Bax apoptotic function is inhibited. **(B)** Upon death stimuli, Akt/PKB does not phosphorylate Ser184 neither GSK3β. Thus, GSK3β can phosphorylate Ser163, moving α-helix 9 from the hydrophobic groove, making Ser60 accessible to Protein Kinase A (PKA) phosphorylation. Figure adapted from Arokium et al.(2007).

residues sequences, one of which (Met-Asp) is present in the N-terminal of Bax and is acetylated by NatB. NatB, specifically its catalytic subunit NAA20, acetylates Bax, keeping it in its cytosolic and inactive conformation in yeast and MEF cells (Alves *et al.* 2018). When exploring the importance of N-terminal acetylation by addressing Bax location and extent of cytochrome *c* release in yeast cells lacking Naa20p, the authors found that this protein modification regulates Bax targeting to mitochondria, but its absence is not sufficient to trigger Bax activation and the subsequent cytochrome *c* release (Alves *et al.* 2018).

I.4.3.2 Bax regulation by interactions with other members of the Bcl-2 protein family

Bcl-2 protein family members are also key regulators of Bax function, dictating the fate of the cell (**Figure I.7**). In fact, it is nowadays clear that the two main partners of Bax, the anti-apoptotic proteins Bcl-2 and Bcl-x_L, inhibit its activity (Renault and Manon 2011), and that interaction with BH3-only proteins is required to induce apoptosis (Zong *et al.* 2001). The pro-apoptotic BH3-only proteins execute their function by neutralizing the pro-survival Bcl-2 family members and/or by directly activating the pro-apoptotic ones, Bax and Bak (Kuwana *et al.* 2005).

The neutralization of the anti-apoptotic Bcl-2 proteins that is achieved through binding of the BH3 domain of sensitizer BH3-only proteins to the hydrophobic groove of pro-survival ones has already been observed in the yeast *Saccharomyces cerevisiae* when using the BH3-only proteins Noxa, Bik, Bmf and tBid (Gérecová *et al.* 2013). Nevertheless, due to some differences in the BH3 domains and in the binding groove, some BH3-only proteins (namely Bad and Noxa) are selective, binding stronger to anti-apoptotic proteins, contrary to others (such as Bim, tBid and Puma) (van Delft and Huang 2006; Peter E. Czabotar *et al.* 2013). The BH3-only proteins Bim, tBid and possibly PUMA (Gallenne *et al.* 2009) are also capable of directly activating Bax and Bak (Kuwana *et al.* 2005). Although, instead of interacting directly with the Bax BH3 domain, Bid and PUMA interact with its α-helix 1, resulting in the movement of α-helix 9 and in mitochondrial insertion (Cartron *et al.* 2004; Kim *et al.* 2009). tBid is localized in the MOM, where it competes with the anti-apoptotic proteins for Bax binding. The interaction between tBid and Bax results in Bax oligomerization and insertion into the MOM, which causes cytochrome *c* release (Eskes *et al.* 2000; Renault and Manon 2011). Similar to tBid, PUMA has emerged as a Bax activator, since it helps in Bax targeting to mitochondria and cytochrome *c* release, both in mammals and yeast (Gallenne *et al.* 2009; Kim *et al.* 2009; Renault and Manon 2011).

The manner through which the anti-apoptotic proteins inhibit Bax is not well established yet. However, two hypothesis have been put forward: the anti-apoptotic proteins can inhibit cell death through interactions with the BH3 domains of Bax and Bak (Renault and Manon 2011), or they can neutralize the BH3-only proteins that can directly activate the pro-apoptotic proteins (Peter E. Czabotar *et al.* 2013; Renault *et al.* 2013). Bax presence in the mitochondrial membrane is not sufficient to trigger MOMP, suggesting that Bax must suffer further activation. Thus, despite being controversial, it is acceptable to think that the MOM proteins Bcl-2 and Bcl-x_L can serve as receptors towards Bax activation (Renault and Manon 2011). Another hypothesis of Bax regulation through pro-survival Bcl-2 proteins is the competition between Bcl-x_L and Bax for binding to MOM and tBid, in liposomes or in isolated mitochondria. However, as summarized by Renault *et al.* (2013), despite inhibiting Bax function, Bcl-x_L is able to trigger its translocation to mitochondria. Furthermore, it is now known that overexpression of Bcl-x_L stimulates not only Bax translocation to the mitochondria, but also its retrotranslocation to the cytosol (Renault *et al.* 2015; Renault *et al.* 2017), suggesting that Bcl-x_L plays a fundamental role in Bax localization. In yeast, it was found that the co-expression of Bax and Bcl-x_L leads to a higher localization of Bax in the mitochondria than when Bax was expressed alone (Renault *et al.* 2015). Nevertheless, the interaction between these two proteins is dependent on their localization and Bax phosphorylation (Garenne *et al.* 2016). In addition to Bcl-x_L, overexpression of Bcl-2 results in the same effect of triggering Bax localization to mitochondria (Teijido and Dejean 2010) and, under both conditions, Bax function remains blocked by the partner. However, they induce different conformational changes: Bcl-2 leads to Bax oligomerization while inhibiting its insertion into the membrane (Renault *et al.* 2013), whereas Bcl-x_L does not (Renault *et al.* 2017).

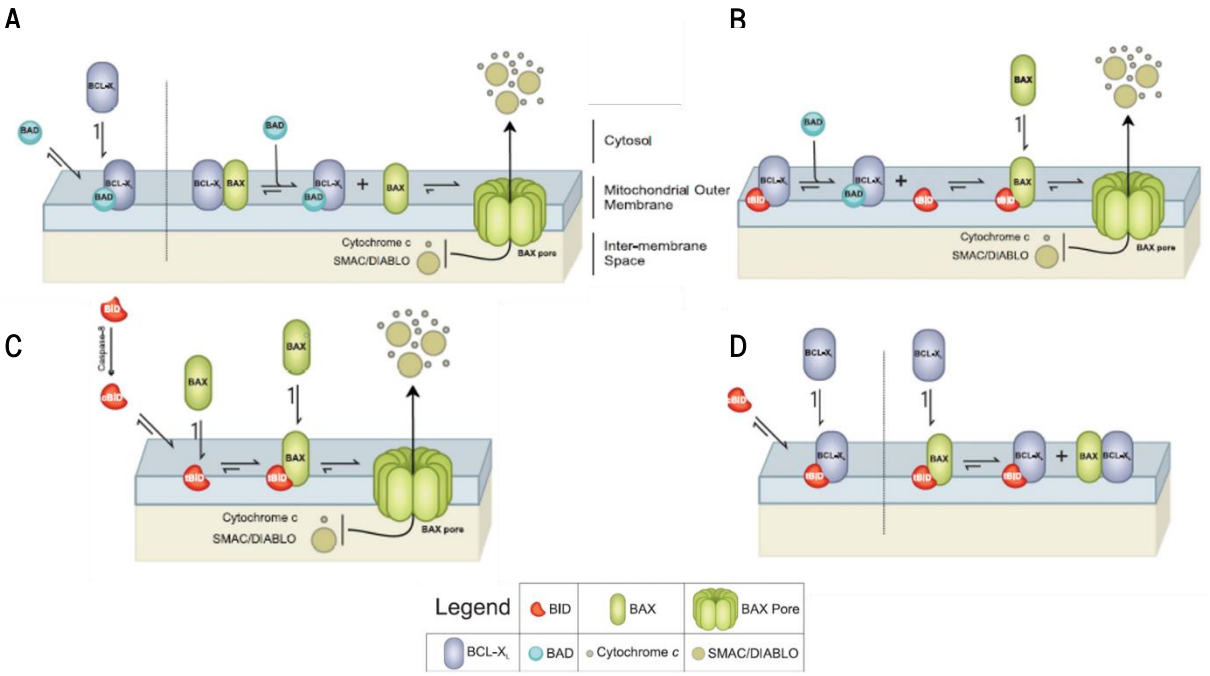

Figure I.7: Bcl-2 proteins interaction and their outcome in cell survival. (A) Sensitizers BH3-only proteins inhibit the pro-survival proteins resulting in Bax activation and release of cytochrome *c* from the mitochondria inter-membrane space to the cytosol. **(B)** Bad binds strongly to anti-apoptotic proteins than tBid. **(C)** Activator BH3-only proteins recruit Bax from the cytosol to mitochondria outer membrane, where they interact and cause cell death. **(D)** Anti-apoptotic proteins may interact with activator BH3-only proteins disabling their interaction with the effector pro-apoptotic proteins, which stay free to interact with other anti-apoptotic proteins and prevent cell death. Image adapted from Kale et al. 2018.

I.5. Yeast as a model organism to study Bax function and regulation

Yeast is an unicellular eukaryotic organism that, due to its rapid growth rate, non-pathogenicity, easy storage and genetic analysis tools, has been established as a cell model in several areas of cell biology, namely in the elucidation of the molecular mechanisms underlying apoptosis (Carmona-Gutierrez *et al.* 2010; Carmona-Gutierrez *et al.* 2018). Yeast apoptosis remained controversial until 1997 when Madeo *et al.* demonstrated that yeast carrying a mutation in the gene *CDC48*, coding for a type II AAA ATPase, die exhibiting an apoptotic phenotype with several morphological and molecular characteristics of mammalian apoptosis, including the exposure of phosphatidylserine at the plasma membrane, chromatin condensation, chromatin and DNA fragmentation and formation of equivalents to apoptotic bodies (Madeo *et al.* 1997). Henceforward, several reports using different physiological scenarios, gene alterations and different exogenous compounds described an apoptotic phenotype in *S. cerevisiae* (Carmona-Gutierrez *et al.* 2010). The study of acetic acid as trigger of cell death (Ludovico *et al.* 2001) left no doubt of the existence of a mitochondrial-dependent pathway in yeast resembling the

mammalian-intrinsic pathway, with cells showing production of ROS, cytochrome *c* release into the cytosol, and mitochondrial dysfunctions including decrease in cytochrome *c* oxidase (COX) activity and mitochondrial depolarization (Ludovico *et al.* 2002). Over time, further studies uncovered additional regulators and mediators of yeast apoptosis, whose mammalian homologues are also involved in apoptosis (Pereira *et al.* 2008), except for members of the Bcl-2 family. The study of this protein family in yeast arose accidentally, when the yeast-two hybrid system was used to assess possible interactions among the anti-apoptotic proteins Bcl-2 and Bcl-x_L with the pro-apoptotic Bax (Sato *et al.* 1994). Unexpectedly, Sato *et al.* (1994) verified that the LexA-Bax fusion protein was able to kill yeast, and its lethal phenotype could be prevented by co-expression of anti-apoptotic Bcl-2 family members. Since this mimic the regulation described in the metazoan counterparts, Bcl-2 family proteins most likely conserve their function, generating a similar biochemical and physiological output in yeast cells. As such, several groups employed yeast as model to express these proteins to assess their function and discover new mammalian apoptotic regulators (Priault *et al.* 2003; Pereira *et al.* 2008). The fact that it is devoid of homologues of the Bcl-2 family proteins (Polčic *et al.* 2015) therefore became one of the greatest advantages of yeast in apoptosis-related studies, since it became possible to use heterologous expression to provide new insights into their structure, function, regulation and interactions, without interference of the other members. This, along with the easy manipulation of yeast, makes this unicellular eukaryote an essential research tool to elucidate mammalian cell death pathways, providing information into human pathologies, such as cancer, ageing, neurodegenerative disorders and others.

Chapter II

AIMS

Cell death has long been considered crucial for proper tissue and organ formation during embryonic development, tissue homeostasis and removal of harmful cells. Moreover, mis-regulation of apoptosis levels has been implicated in a diversity of abnormal functions and in the progression and development of diseases. Thus, understanding the underlying molecular mechanisms of this process is of high interest and will impact the design of novel therapeutic strategies. The Bax protein is a key player in the apoptotic-cell death process, governing the cell fate decision. Although several reports highlight the mechanisms of regulation of this pro-apoptotic protein, the manner in which it remains inactive and becomes activated is still elusive. Thus, continuing previous studies from our group, the scope of this thesis was to use the budding yeast *S. cerevisiae* and heterologously express the human pro-apoptotic protein Bax in order to provide further insights in its regulation. To accomplish this goal, preliminary optimizations were performed:

- Determination of the appropriate conditions for a suitable expression of the human pro-apoptotic protein Bax in yeast;
- Characterization of the yeast phenotypes expressing the native form, Bax α, or the active form, Bax *c*-myc.

Following these, specific tasks regarding Bax α regulation were defined:

- Ascertain the role of the yeast kinase Rim11p (the orthologue of the human GSK3β) in Bax α function;
- Assess the effect of sub-lethal concentrations of acetic acid in Bax α activation;
- Characterize the phenotype of acetic acid-induced cell death through Bax α activation;
- Identify the role of PKCε in Bax regulation;
- Investigate if under acetic acid-induced Bax α-cell death conditions, Bcl-x_L is able to interact with Bax α and modulate the fate of the cell.

Chapter III

MATERIALS AND METHODS

III.1. Yeast strains and plasmids

All *S. cerevisiae* strains used in this study are listed in **Table III.1**, along with their genotype. Apart from the wild-type strain W303-1A, that was kindly provided by Stéphen Manon, all the strains were constructed during this study.

Table III.1: *S. cerevisiae* strains used in this study.

Strain	Genotype
W303-1A	*Mat a; ade2-1; his3-11,15; leu2-3,112; trp1-1; ura3-1*
EV	W303-1A harbouring pYES3 EV
Bax α	W303-1A harbouring pYES3 Bax α
Bax *c*-myc	W303-1A harbouring pCM189 Bax *c*-myc
*rim11*Δ	W303-1A *rim11*Δ::*KanMX4*
*rim11*Δ EV	W303-1A *rim11*Δ harbouring pYES3 EV
*rim11*Δ Bax α	W303-1A *rim11*Δ harbouring pYES3 Bax α
EV + EV	W303-1A harbouring pYES3 EV and pYES2EV
EV + Bcl-x_L	W303-1A harbouring pYES3 EV and pYES2 Bcl-x_L
EV + Bcl-x_LΔC	W303-1A harbouring pYES3 EV and pYES2 Bcl-x_LΔC
Bax α + EV	W303-1A harbouring pYES3 Bax α and pYES2 EV
Bax α + Bcl-x_L	W303-1A harbouring pYES3 Bax α and pYES2 Bcl-x_L
Bax α + Bcl-x_LΔC	W303-1A harbouring pYES3 Bax α and pYES2 Bcl-x_LΔC
EV + EV	W303-1A harbouring pYES3 EV and YEplac181 EV
EV + PKCε	W303-1A harbouring pYES3 EV and YEplac181 PKCε
Bax α + EV	W303-1A harbouring pYES3 Bax α and YEplac181 EV
Bax α + PKCε	W303-1A harbouring pYES3 Bax α and YEplac181 PKCε

All mutant strains were constructed using the wild-type *S. cerevisiae* strain W303-1A, provided by Stéphen Manon, using the plasmids listed in **Table III.2** and the protocol for yeast transformation using lithium acetate described in section III.1.3. The *rim11*Δ mutant was constructed in the wild-type strain by homologous recombination (**Figure III.1**) with a disruption cassette (*KanMX4*) amplified by Polymerase Chain Reaction (PCR), using genomic DNA extracted (described on section III.1.1.) from the respective Euroscarf (EUROSCARF, Institute of Molecular Biosciences Johann Wolfgang Goethe-

University Frankfurt, Germany) mutant strain as template and the oligonucleotides listed in **Table III.3**. The correct integration of the disruption cassette was confirmed by colony PCR (described on section III.1.2.) using the oligonucleotides listed in Table III.3.

Table III.2: List of plasmids used in this study.

Plasmid	Genotype	Source
pYES3 EV	TRP1::GAL1/10; AmpR	Invitrogen
pYES3 Bax α	pYES3 harbouring Bax α	Manon S. (Arokium *et al.* 2007)
pCM189 Bax *c*-myc	TRP1::Tet-Off; AmpR	Silva R. (Priault *et al.* 1999)
pYES2 EV	URA3::GAL1/10; AmpR	Invitrogen
pYES2 Bcl-x_L	pYES2 harbouring Bcl-xL	Manon S. (Renault *et al.* 2015)
pYES2 Bcl-x_LΔC	pYES2 harbouring Bcl-xLΔC	Manon S. (Renault *et al.* 2015)
YEplac181 EV	LEU2::GAL1/10; AmpR	(Gietz and Akio 1988)
YEplac181 PKCε	YEplac181 harbouring PKCε	Silva R.

III.1.1. Genomic DNA Extraction

Cells from a 1 mL culture grown overnight in YPD [1 % of yeast extract, 2 % of peptone and 2 % of glucose] were pelleted, washed twice with deionized water (dH_2O) and resuspended in 100 µL of a solution of 1 M sorbitol and 100 mM pH=7.5 EDTA. To promote yeast cell wall digestion, the samples were treated with 2 µL of 20 mM Zymolyase (GRiSP Research Solutions) and incubated for 1 hour at 37 °C. The resulting spheroplasts were mixed with 100 µL of a solution containing 50 mM Tris-HCl and 20 mM pH=7.4 EDTA, and 50 µL of 10% SDS (Sodium Dodecyl Sulphate). After incubation at 65 °C for 5 minutes, 80 µL of 5 M potassium acetate were added and the samples incubated on ice for 5 minutes. Then, the samples were centrifuged at maximum speed at 4 °C for 30 minutes and the supernatant transferred to a new microtube containing with the same volume of isopropanol. Following incubation at room temperature (RT) for 5 minutes or 30 minutes in ice, DNA was pelleted by centrifugation at maximum speed for 15 minutes, washed with ethanol and air dried. Finally, the pellet was resuspended in 20 µL of 1X TE (100 mM Tris, 10 mM EDTA pH=8) buffer.

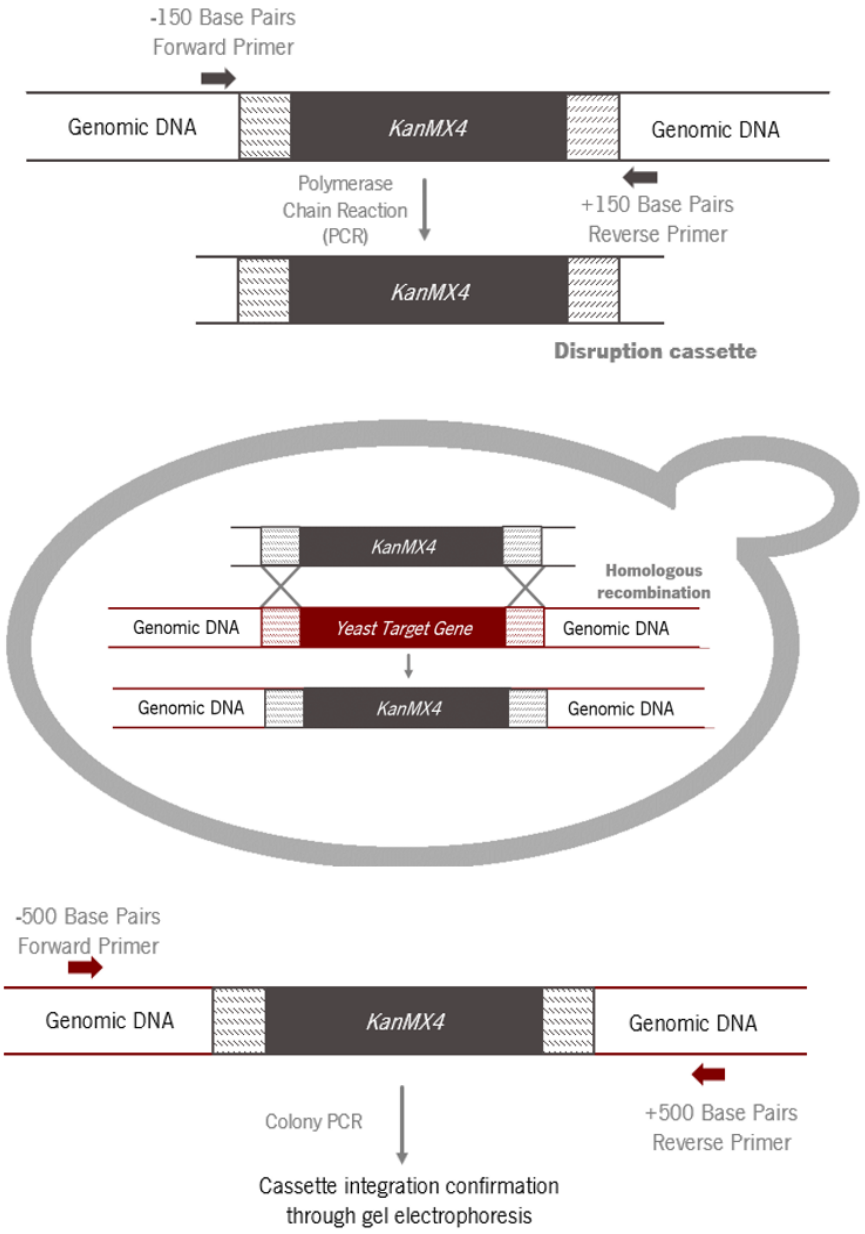

Figure III.1: Schematic representation of the construction of the *rim11*Δ mutant strain. First, the disruption cassette was amplified by PCR and then used for yeast homologous recombination. The correct insertion of the disruption cassette was assessed by colony PCR and the PCR product visualized by agarose gel electrophoresis.

III.1.2. Colony PCR

Isolated yeast colonies were resuspended in 25 µL of 20 mM NaOH and incubated at 100 °C for 15 minutes. After vortexing 1 minute, the DNA was collected by centrifuging samples at maximum speed and collecting the supernatant, which was stored at -20 °C.

Table III.3: List of oligonucleotides used in this study. A and B correspond to the oligonucleotides used to amplify the disruption cassette, while C and D were used to assess correct cassette integration.

		Oligonucleotides sequence
A	-150 Base Pairs Forward Primer	5'- TTCCAGAATAGCCAACCACC- 3'
B	+150 Base Pairs Reverse Primer	3'- AAAGCCGTATTCTTTGCCAG- 3'
C	-500 Base Pairs Forward Primer	5'- TGATTTTGATGACGAGCGTAAT- 3'
D	+500 Base Pairs Reverse Primer	3'- CTGCAGCGAGGAGCCGTAAT- 3'

III.1.3. Yeast transformation using lithium acetate

For each transformation, *S. cerevisiae* cells were grown overnight in YPD medium or in Synthetic Complete medium [SC; 2%(w/v) Glucose, 0.175% (w/v) Yeast nitrogen base without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 0.1% potassium phosphate, 0.2% (w/v) Drop-out mixture, and 0.01% auxotrophic requirements; pH=5.5] without tryptophan, diluted the following day to an optical density at 640 nm (OD_{640nm}) of 0.2 in YPD 2 times glucose or SC 2 times glucose without tryptophan and incubated until mid-exponential phase ($OD_{640nm}\approx$ 0.8). The cells were then harvested, centrifuged and washed to remove any remaining medium. Then, the transformation mix, which is composed of 240 µl of 50 % (w/v) Polyethylene Glycol, 36 µl of 1 M Lithium Acetate, 10 µl of 2 mg/ml salmon sperm DNA, 32 µL of sterile H_2O and 2 µL of plasmid DNA, was added to 100 µL of cellular suspension resuspended in sterile H_2O. After incubation at 42 °C for 40 minutes, the cells were pelleted at maximum speed, resuspended in 50 µL of sterile water and plated on the appropriate selective medium. Finally, the plates were incubated at 30 °C until the appearance of transformants. Solid media was prepared by adding 2% (w/v) Agar to the medium.

III.2. Growth conditions

S. cerevisiae strains were inoculated (**Figure III.2**) to a starting OD_{640nm}=0.05 in SC glucose medium and grown overnight. The yeast cultures were then transferred to SC medium containing the non-fermentable carbon source lactate instead of glucose, to an OD_{640nm}=0.2. After approximately 24 hours, the cultures were diluted to OD_{640nm}=0.5 in fresh SC lactate medium. Afterwards, 1% galactose was added to trigger the expression of Bax, and the cultures were grown for 32 hours.

Bax *c*-myc is under the control of the bacterial tetracycline-repressive promoter (Tet-Off promoter), which is repressed by doxycycline (Sigma) (Priault *et al*. 1999). In this way, to provide an adequate growth, the promoter was repressed in the strain expressing Bax *c*-myc by adding 2 µg/mL of doxycycline to the medium; in the last step, to allow Bax expression, 0.1 µg/mL of doxycycline were used. For survival assays, cells were spread on YPD medium containing 10 µg/mL of doxycycline. All the incubations were performed at 30 °C, 200 rpm.

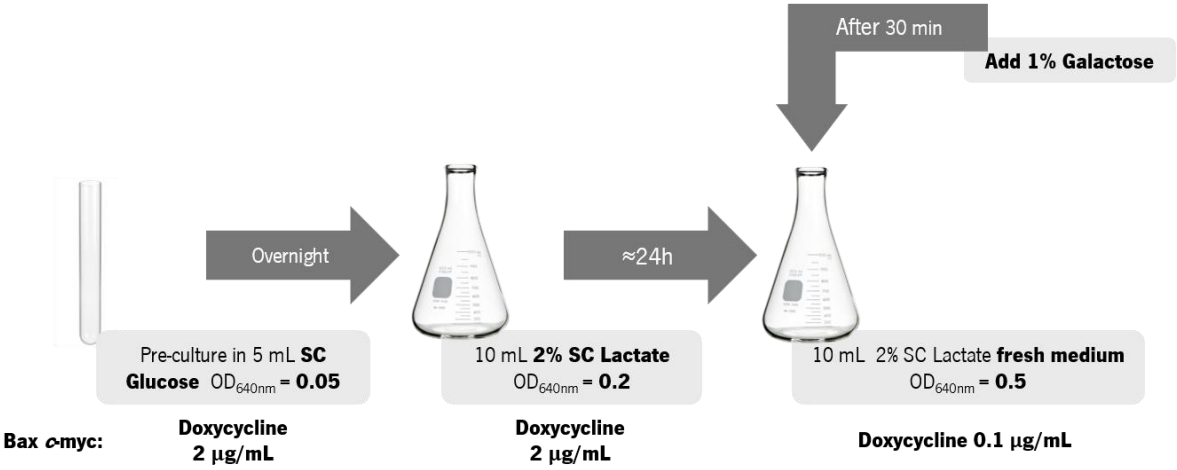

Figure III.2: Schematic representation of yeast cells growth conditions. In each medium, the correct auxotrophic requirements were applied. Doxycycline was just used for Bax *c*-myc. This strain is under the control of a Tet-Off promoter; thus, the addition of doxycycline is needed to repress its expression.

III.2.1. Acetic acid treatment

For acetic acid treatment (**Figure III.3**) cells were cultivated as described above. After 16 hours of Bax expression, cells were harvested to an OD_{640nm}=0.5 in the appropriate medium at pH=3.0 (pH 3.0), set with HCl, so the acid could enter the cell in its undissociated form (CH_3COOH), by simple diffusion (Casal *et al*. 1996). Cells were then treated with 50, 80, 100 or 120 mM of acetic acid, followed by 180 minutes incubation at 30 °C at 200 rpm. Time course changes of cell viability were assessed as described below. For the negative control, cells were treated in the same way but without the addition of acetic acid. For acetic acid treatment cells were cultivated as described above.

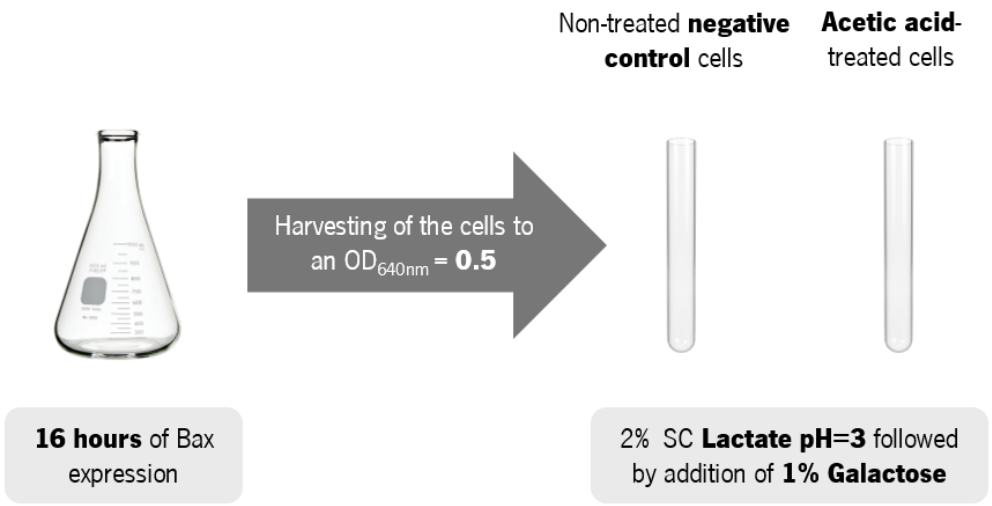

Figure III.3: Schematic representation of acetic acid treatment. Cells 16h after induction of Bax expression were collected to a final OD_{640nm}=0.5 and resuspended in SC Lac pH 3, with the appropriate auxotrophic requirements.

III.3. Assessment of cell growth and survival

Cells were grown and treated with acetic acid as described above, and their growth evaluated (30 °C, 200 rpm) at different times by measuring OD at 640 nm. For survival assays, OD_{640nm} was measured at each timepoint and the cells counted in a Neubauer chamber (Hirschmann® Technicolor) and after plating a fixed volume of an appropriate culture dilution, as referred below, plates were incubated at 30 °C for 2-3 days. Yeast cell viability was then evaluated as percentage of colony-forming units (CFU) through normalization to either OD_{640nm} or to the cell number as counted in the Neubauer chamber.

For cells counting, 10 µL from a 1:20 dilution of the 10^{-1} dilution were placed in the Neubauer chamber and counted under 400x magnification in an optical microscope (HM-LUX3, Leitz). The concentration of cells (cells/mL) was determined using the formula $\text{Cells/mL} = \frac{\text{Number of cells x 10000}}{\text{Number of counted squares x Dilution}}$. For CFU assays, five drops of 40 µL from the 10^{-4} dilution were plated on YPD plates and incubated at 30 °C for 2-3 days.

III.4. Flow cytometry analysis

Flow cytometry analyses were performed using a CytoFLEX (Beckman Coulter Inc.) flow cytometer equipped with three solid state lasers with emission of a 488 nm beam at 50 mW, a 405 nm beam at 80 mW and a 635 beam at 50 mW. Yeast cell populations with high frequency and

homogeneity were gated in a histogram of Side Scatter (SS) x Forward Scatter (FS) for fluorescence analysis. For each sample, about 30 000 events were evaluated and further analysed using FlowJo 7.6.1.

III.4.1. Metabolic activity as a measure of cell survival using FDA staining

Yeast cell viability and vitality were analysed by measuring the metabolic activity of the cell, based on the assumption that metabolically active cells are alive whereas metabolic inactive cells are dead. For this purpose, Fluorescein Diacetate (FDA) was used. FDA is an esterase substrate that can enter the cell, where it is hydrolysed to fluorescein and acetate. This highly pH-dependent probe serves as a viability probe since it measures the enzymatic activity and the cell membrane integrity, which is required for the retention of fluorescein (Prudêncio *et al.* 1998). After 16 hours of Bax expression, EV, Bax α and Bax *c*-myc carrying-strains were harvested and resuspended in 500 µL of phosphate buffer saline (PBS; 37 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4). Then the cells were stained with 4 µM FDA (Sigma) for 20 minutes at RT in the dark. The detection of the fluorescence by flow cytometry was achieved by collecting green fluorescence through a band-pass filter corresponding to the FITC channel.

III.4.2. Assessment of plasma membrane integrity by PI staining

Propidium Iodide (PI) has been used in order to assess the plasma membrane integrity, which allows discriminating cell death types. This fluorescent nucleic acid intercalating agent is not able to enter the cell unless its plasma membrane is compromised. Yeast cells expressing EV and Bax α were grown and treated as described in sections III.2 and III.2.1, respectively. Then, cells were harvested and resuspended in 500 µL of PBS with 2 µM PI (Sigma-Aldrich) and incubated for 10 minutes at RT in the dark. The detection of the fluorescence was achieved by flow cytometry by collecting red fluorescence through a band-pass filter corresponding to the PE channel.

III.4.3. Measurement of mitochondrial mass by MitoTracker Green staining

MitoTracker Green stains mitochondria in live cells independently of the membrane potential, providing information regarding mitochondrial mass. Yeast cells expressing EV and Bax α were grown and treated as described in section III.2 and III.2.1, respectively. Then, cells were harvested and

resuspended in 500 μL of PBS with 0.4 μM of MitoTracker Green FM (Molecular Probes) and incubated for 30 minutes at 37 °C in the dark. The detection of the fluorescence was achieved by flow cytometry by collecting green fluorescence through a band-pass filter corresponding to the FITC channel.

III.4.4. Evaluation of superoxide anion accumulation using DHE and MitoSOX staining

To detect whole-cells superoxide anion accumulation, the Dihydroethidium (DHE) probe was used. DHE is a superoxide-specific dye that exhibits blue fluorescence in the cytosol; in the presence of this ROS species it oxidizes to ethidium, which intercalates within DNA, staining the nuclei with a bright red fluorescence. Additionally, to monitor this ROS species specifically in mitochondria, the MitoSOX™ Red indicator was used. MitoSOX™ Red is a reagent able to permeabilize live cells, selectively targeting mitochondria. Like the DHE probe, MitoSOX™ Red is oxidized by superoxide anion and exhibits red fluorescence upon binding to nucleic acids. Cells were harvested and resuspended in 500 μL PBS. Afterwards, cells were separately incubated with 2 μM DHE (Sigma-Aldrich) for 30 minutes, or with 5 μM MitoSOX™ Red (Life Technologies) for 45 minutes, at RT in the dark. The detection of the fluorescence was achieved by flow cytometry by collecting red fluorescence through a band-pass filter corresponding to the PE channel.

III.5. Fluorescence microscopy

For this purpose, a Leica Microsystems DM-5000B epifluorescence microscope coupled to a Leica DCF350 FXR2-193510309 digital camera was used or Olympus BX63 microscope. The photomicrographs obtained were acquired and processed using Leica Application Suite (LAS) AF 6000LX Microsystems software along with a 100x oil-immersion objective and the appropriate filter settings for differential phase contrast (DIC), red fluorescence (N21) and green fluorescence (GFP). Photomicrographs were further managed using GIMP 2.10.6. software.

III.5.1. Metabolic activity as a measure of cell survival using FUN-1 staining

Yeast cells expressing EV, Bax α and Bax *c*-myc were collected for viability assessment with the FUN-1 dye. When the plasma membrane is intact, FUN-1 dye diffuses into cells independently of their

viability status, and results in a bright yellow-green fluorescence of the cytosol. Metabolic active (vital) cells are able to process and concentrate the probe within the vacuole producing red-orange fluorescent structures named red cylindrical intravacuolar structures (CIVS) (Millard *et al.* 1997). However, metabolic inactive (non-vital) cells are not able to transport the probe into the vacuole and it thus remains in the cytosol, where it causes a diffuse red fluorescence (Prudêncio *et al.* 1998). The selected yeast strains were grown as described in section III.2 and, 16 hours after Bax expression, were harvested and resuspended in 500 μL of PBS. Afterwards, cells were incubated with 4 μM FUN-1 (Invitrogen) for 30 minutes at 30 °C in the dark, centrifuged and resuspended in dH_2O and analysed by fluorescence microscopy.

III.6. Mitochondrial isolation for Bax and cytochrome *c* localization assessment

Yeast cells were grown and treated as described above in sections III.2 and III.2.1, respectively, however using higher volumes. After 16 hours of Bax expression, followed by 60 minutes of 80 mM of acetic acid treatment, cells were harvested and washed twice with dH_20. Then, cells were resuspended in suspension buffer (1.2 M Sorbitol, 60 mM Sodium Phosphate and 1 mM EDTA), in a proportion of 15 mL of buffer per 10 g of cells and incubated with 30 mM dithiothreitol (DTT; Sigma-Aldrich) for 20 minutes at 30 °C, 200 rpm. After centrifugation at 5000 rpm, cells were again resuspended in suspension buffer and converted to spheroplats by zymolyase-treatment (15 mL of suspension buffer per 30 mg of zymolyase) for 1 hour at 30 °C, 200 rpm. Afterwards, spheroplasts were washed twice with ice-cold 1.2 M Sorbitol and resuspended in lysis buffer (2 M Sorbitol, 1 M Tris, 0.5 mM EDTA and H_2O). The suspension was then transferred to an ice-cold hand-potter and carefully homogenized to preserve mitochondria. Lysed cells and free organelles were observed in the optical microscope, and cell debris along with whole cells were pelleted by centrifuging two times at 2500 rpm for 10 minutes. Finally, the supernatant was recovered and centrifuged at 15000 rpm for 15 minutes in order to separate fractions. The resulting pellet corresponded to mitochondria and the supernatant held the cytosol. Subsequently, both fractions were washed to be contaminant-free by multiple 15 minutes 15000 rpm centrifugations and, at the end, the mitochondrial fraction was resuspended in lysis buffer and stored at -20 °C.

III.7. Analysis of protein expression

III.7.1. Preparation of total protein extracts

To prepare total protein extracts, cells were grown as described above, and at each time point 1 mL of cell culture at OD_{640nm}=1 was collected by centrifugation. Briefly, after washing the cells, the pellet was resuspended in 500 µL of dH_2O and 50 µl of lysis solution [3.5% (v/v) β- mercaptoethanol in 2 M NaOH] and incubated for 15 minutes in ice. Lysis was followed by protein precipitation with 50 µL of 50% (w/v) trichloroacetic acid and, after incubation for 15 minutes, the samples were centrifuged at maximum speed for 10 minutes at 4 °C. The pellet was resuspended in 60 µl of Laemmli buffer [2% de β-mercaptoethanol, 2% SDS, 0.1M Tris pH 8.8, 20% Glycerol and 0.02% Blue Bromophenol] and then, 3 µl of 1 M Tris pH 9.8 was added to adjust the pH. Finally, samples were denatured by boiling at 95 °C for 5 minutes and stored at -20 °C.

III.7.2. Preparation of mitochondrial and cytosolic samples

Protein concentration was determined by the Bradford method, using bovine serum albumin (BSA) as a standard (Bradford 1976) and absorbance was read at 595 nm in a Synergy HT microplate reader (BIO-TEK). Then, approximately 50 µg of mitochondria and 100 µg of cytosol were precipitated. For this purpose, the respective sample volumes were collected, and 50% TCA and lysis buffer were added. After incubation on ice for 15 minutes, samples were centrifuged at 15000 rpm for 10 minutes and resuspended in Laemmeli buffer. As stated for total protein extracts, 1 M Tris pH 9.8 was added to adjust the pH. Denaturation was performed by incubation at 37 °C for 15 minutes. The samples were stored at -20 °C until use.

III.7.3. Western-blot

10 µL of protein extract and 1 µL of a reference molecular weight marker (NZY Colour Protein Marker II) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% resolving gel [375 mM Tris-HCl pH 8.8, 0.4% SDS, 15% glycerol, 0.1% TEMED and 0.05% APS] and 5% stacking gel [125 mM Tris-HCl pH 6.8, 0.4% SDS, 0.1% TEMED and 0.05% APS], at 20 mA per gel. Subsequently, proteins were transferred to Polyvinylidene Difluoride (PVDF) membranes, in a semi-dry Trans-Blot Electrophoretic Transfer system, at 60 mA per membrane. In order to prevent non-specific binding, the membranes were blocked in 5% non-fat milk in PBS-T (PBS with 0.1% Tween-

20), for 1h, at RT with agitation. Then, the membranes were incubated overnight with agitation at 4 °C with the appropriate primary antibodies listed in **Table V**. After washing with 1% PBS-T for 15 minutes, the membranes were incubated for 1 hour at RT with agitation with the corresponding secondary antibodies (**Table V**). Afterwards, the membranes were washed 4 times with 1% PBS-T for 15 minutes and signals detected by chemiluminescence with the ECL detection system (Enhanced ChemiLuminescence Millipore-Merck), which detects the activity of the peroxidase coupled to secondary antibodies and visualized with a ChemiDoc XRS image system with the Quantity One software (BioRad).

Table III.4: List of primary and secondary antibodies used in this study.

Antibody	Antigen size (kDa)	Dilution	Temperature (°C)	Incubation time	Manufacturer
Monoclonal anti-yeast phosphoglycerate kinase (**Pgk-1**) (mouse)	45	1:5000	4	Overnight	Invitrogen
Monoclonal anti-yeast porin (**Por-1**) (mouse)	31	1:10000	4	Overnight	Invitrogen
Polyclonal anti-human Bax (**Bax**) (rabbit)	21	1:5000	4	Overnight	Sigma
Monoclonal anti-human Bcl-xL (**Bcl-xL**) (mouse)	30	1:1000	4	Overnight	Santa Cruz Biotechnology
Polyclonal anti-human PKCε (**PKCε**) (rabbit)	84	1:100	4	Overnight	Santa Cruz Biotechnology
Polyclonal anti-yeast cytochrome *c* (**Cyt-1**) (rabbit)	13	1:1000	4	Overnight	Millegen
Secondary anti-mouse (Goat)	-	1:5000	RT	1h	Jackson ImmunoResearch
Secondary anti-rabbit (Goat)	-	1:5000	RT	1h	Jackson ImmunoResearch

III.8. Statistical analysis

GraphPad Prism 6.0 (Graph-Pad Software, Inc., La Jolla, CA, USA) was used to perform statistical analysis. The analyses were made between groups and the corresponding used test is referred in figure legends. The results are exhibited in form of mean ± S.E.M, and significance was recognized for $P \leq 0.05$.

Chapter IV

RESULTS

IV.1. Bax α decreases the specific growth rate of yeast cells without compromising their viability

The mechanisms underlying Bax activation have not been completely elucidated, though it is known that this pro-apoptotic protein needs to be inserted into the MOM and activated to induce cell death. Heterologous expression of Bax in yeast has been previously used to understand Bax regulation, but mainly by assessing the effect of point mutations or of mitochondrial-directed versions on its activity. Here, we sought to further understand how this protein can be regulated using wild-type Bax α. For this purpose, Bax α was heterologously expressed in yeast under the control of a *GAL10* promoter. We first optimised the conditions for expression of Bax α in yeast, so that its expression does not lead to a loss in cell survival (since in healthy human cells and also in yeast cells under non-apoptotic conditions Bax is cytosolic and inactive). Cells transformed with a plasmid expressing Bax α or the respective empty vector (EV) were grown in SC-glucose overnight and then transferred to SC-lactate. After approximately 24 hours, the lactate medium was refreshed and Bax α expression was induced by addition of 1% galactose. Then, cell growth was assessed by measuring the OD_{640nm} of the cultures for up to 32 hours. Expression of Bax α slightly extended the lag phase and significantly decreased the specific growth rate without affecting the cell concentration at stationary phase (**Figure IV.1.1**).

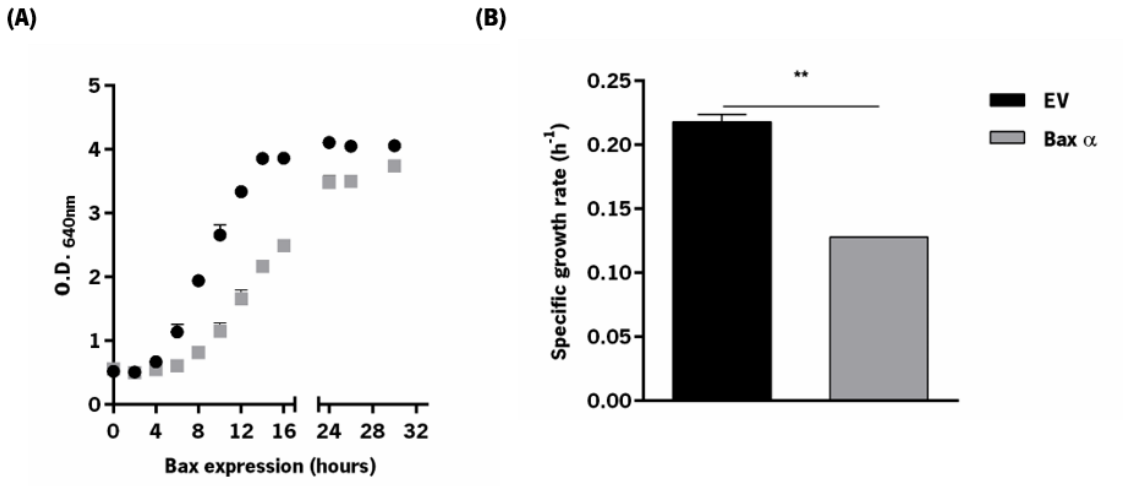

Figure IV.1.1: Expression of Bax α in yeast decreases cell growth rate. (A) Growth curves of control cells (EV) and Bax α. **(B)** Specific growth rate (h^{-1}) of both strains calculated using the formula $\mu = \frac{\ln X_t - \ln X_{t0}}{t - t_0}$, and using exponential phase optical densities (ODs) at 640 nm. These data resulted from two independent experiments and the significant differences were analysed by an unpaired Student's *t*test, where **p≤0.01.

We next performed clonogenic survival assays, by plating a fixed culture volume after appropriate dilution before and 16 hours and 24 hours after induction of Bax α expression. CFUs were then normalized to either OD_{640nm} (**Figure IV.1.2A**) or to cell concentration by counting in a Neubauer chamber (**Figure IV.1.2B**) and expressed as a percentage of cell viability in relation to time 0 (T0) and control cells. Both approaches led to similar results and indicate that Bax α expression does not significantly decrease cell viability. Moreover, since CFU counting normalized to OD_{640nm} is not as laborious as cell counting in the Neubauer chamber, it was the methodology selected for normalization in subsequent cell survival assays. As a control, total cells extracts were collected at each time point for western-blot analysis. The results show that Bax α expression levels at 16 hours were higher than at 24 hours of induction (**Figure IV.1.2C and IV.1.2D**). Thus, this induction time was selected for further experiments.

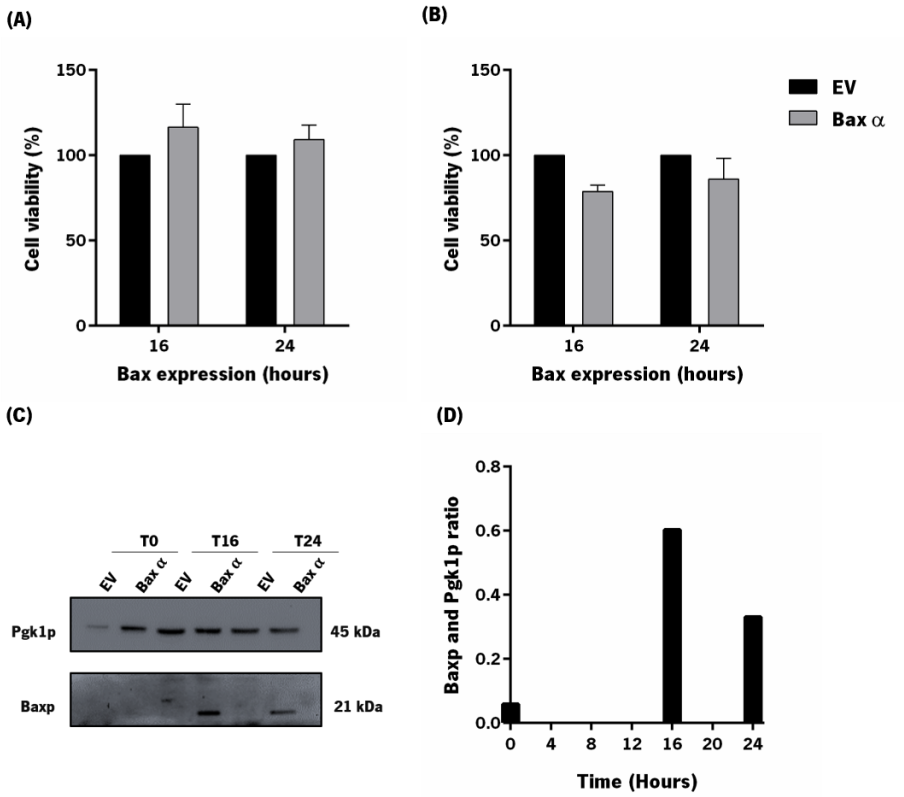

Figure IV.1.2: Expression of Bax α in yeast cells does not compromise cell viability. Percentage of cell viability evaluated by counting colony forming units (CFU) normalized to **(A)** OD_{640nm} and **(B)** cells numbers as determined by counting in a Neubauer chamber $(Concentration\ (cells/mL) = \frac{Number\ of\ cells \times 10000}{Number\ of\ squares \times Dilution}$. Cell viability of Bax α-expressing cells is normalized to time 0 (T0) and to control cells. Data are mean of three independent experiments analysed by two-way ANOVA. **(C)** Immunodetection of Bax in control and Bax α-expressing cells along 24 hours of expression. Pgk1p was used as a loading control **(D)** Quantification of western-blot signals using the ImageJ 1.51j8 software. Data is from a single experiment.

Taken together, these results indicate that the growth conditions tested are suitable for Bax α expression, and are in agreement with literature data that report that Bax is inactive and cytosolic in non-apoptotic cells and does not affect yeast cell viability (Gallenne *et al*. 2009). These assays also indicate that the decrease in the specific growth rate is not due to cell death, but due to a decrease in cell growth.

IV.2. An active form of human Bax compromises cell growth and induces yeast cell death

Since Bax α needs to be activated to cause MOMP and, consequently, cell death, we also optimized conditions for expression of an active form of Bax, Bax *c*-myc, as a positive control. This variant is not natural but encompasses a C-terminal *c*-myc tag that results in Bax localization in mitochondria, conferring it a cytotoxic phenotype in yeast cells (Priault *et al*. 1999). Bax *c*-myc was expressed under the control of a Tet-Off promoter so its expression was regulated by the use of doxycycline (Garí *et al*. 1997). Confirmation of Bax *c*-myc expression levels was performed by western-blot and normalized qualitatively by band intensity to a loading control (**Figure IV.2.1A**). We also confirmed by semi-quantitative spot assay that, after 16 hours of Bax α expression, the number of viable yeast cells was decreased in the Bax *c*-myc-expressing strain (**Figure IV.2.1B**). Quantitative CFU counting demonstrated that Bax *c*-myc decreased cell survival by approximately 75% (**Figure IV.2.1C**).

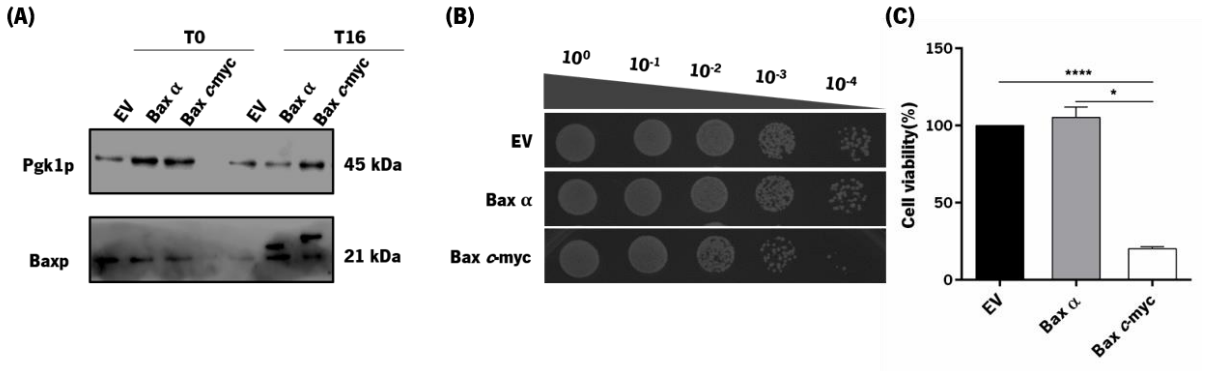

Figure IV.2.1: Expression of an active form of human Bax decreases cell viability in yeast cells. (A) Immunodetection of Bax in cells expressing Bax α or Bax *c*-myc for 16 hours. For Bax *c*-myc expression, 0.1 µg/mL doxycycline were used. Pgk1p was used as a loading control. Assessment of **(B)** cell growth by spot assay and **(C)** percentage of cell viability by CFU counting of yeast cells expressing Bax α or Bax *c*-myc. Cell viability of Bax-expressing cells was normalized to T0 and to control cells (EV). Data are the mean of three independent experiments analysed by two-way ANOVA, where *p<0.05; ****p<0.0001.

IV.3. Expression of an active form of human Bax induces superoxide anion accumulation without disturbing metabolic activity

As previously reported (Priault *et al*. 1999; Silva *et al*. 2011), we found that Bax *c*-myc decreased cell viability, as measured by the ability of cells to reproduce and form colonies, even after expression of Bax *c*-myc is turned off. We therefore sought to determine whether cell death had already been induced after the 16 hours of expression. To accomplish this, we used the two-colour fluorescent dye FUN-1, which diffuses into cells and stains the cytosol with green fluorescence, regardless of their viability status. Cells that have their plasma membrane intact and are metabolically active are able to further process the dye and produced CIVS (Millard *et al*. 1997). The results obtained with this dye (**Figure IV.3.1**) partially support those obtained with growth and clonogenic assays. Indeed, control cells and cells expressing Bax α present clear CIVS, meaning that these cells are metabolically active. Unexpectedly, cells expressing Bax *c*-myc also presented clear CIVS, suggesting that those cells are metabolically active. Since boiled cells presented only diffuse red fluorescence (**Figure VIII.1**), showing a loss of metabolic activity, we confirmed that the probe is working properly.

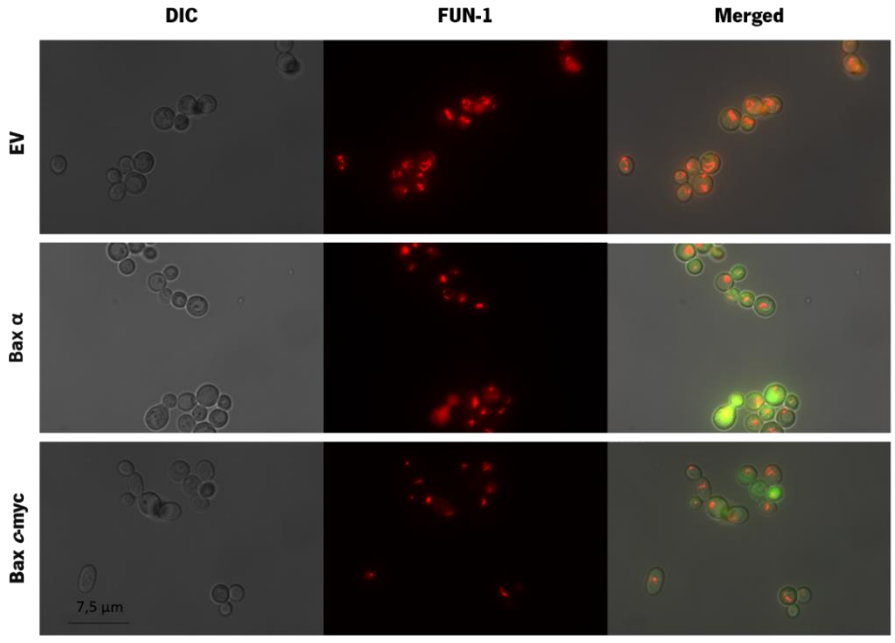

Figure IV.3.1: Yeast cells expressing human Bax α and Bax *c*-myc process the FUN-1 dye into red cylindrical intravacuolar structures (CIVS). Fluorescence microscopy images of the transformed cells 16 hours after Bax expression and stained with 4 µM FUN-1 for 30 minutes at 30 °C, in the dark. The photomicrographs shown are representative of several microscope fields. Bar, 7.5 µm.

Another way to determine cell vitality is by measuring the activity of specific enzymes. Thus, we used the FDA probe, an esterase substrate that when hydrolysed to fluorescein emits green fluorescence. This probe allows the measurement of the esterase enzymatic activity and the simultaneous assessment of the plasma membrane integrity, which is required for retention of the probe. Even though flow cytometry results using this dye are somehow ambiguous due to the different possible interpretations, the results show that 16 hours of Bax expression resulted in just a slight decrease in the percentage of FDA-positive cells (**Figure IV.3.2**), corroborating the results obtained with the FUN-1 probe.

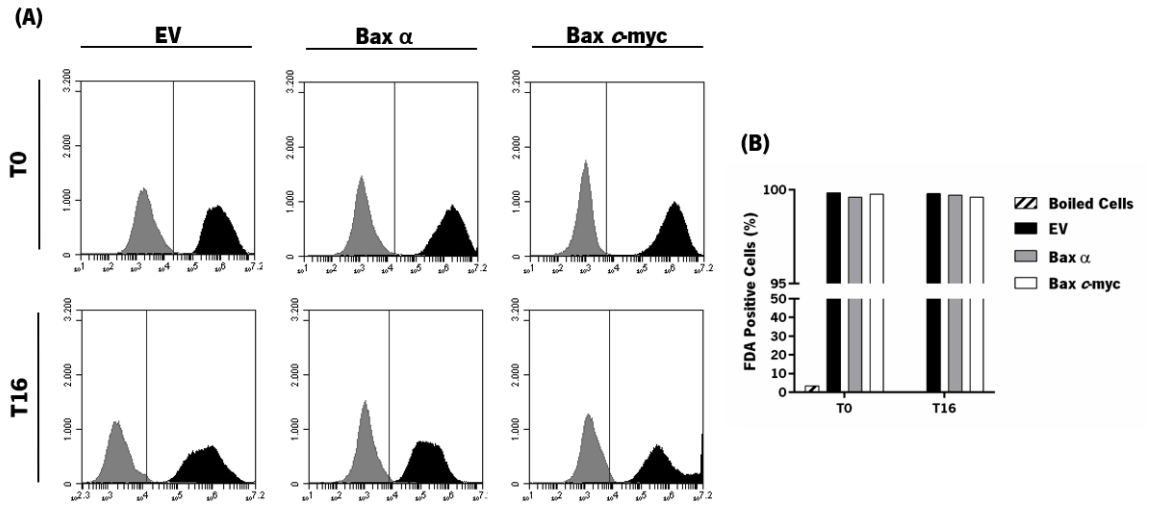

Figure IV.3.2: Yeast cells expressing Bax α and Bax *c*-myc retain FDA staining. (A) Monoparametric histograms of FDA stained cells green fluorescence. Counts presented in the right side of the histogram are considered FDA positive. Grey histograms correspond to cell's autofluorescence, while black histograms correspond to FDA stained cells. **(B)** Percentage of cells displaying esterase activity evaluated by FDA staining, for the indicated yeast strains 16 hours after Bax expression. Boiled cells were used as a positive control of the dye. Data correspond to a single experiment.

Taken together, the results indicate that expression of Bax *c*-myc for 16 hours does not decrease metabolic activity of the cell, as it remains capable of producing CIVS and maintain esterase activity. Nonetheless, one should keep in mind that the observed metabolic activity might be the result of basal or residual activity. However, since Bax expression is turned off when cells are plated for viability assays, a process resulting in cell death must have already been triggered and death occurs after this time point. One possibility was that ROS accumulated in the cells. Indeed, yeast cell death is usually associated with oxidative damage, mainly due to ROS accumulation. We therefore evaluated the levels of superoxide anion in yeast cells by flow cytometry using the DHE probe. Results show a slight increase in superoxide anion accumulation in Bax α-expressing cells when compared with the control,

but a considerable increase in the case of cells expressing Bax *c*-myc (**Figure IV.3.3**), in agreement with a previous report (Silva *et al.* 2011).

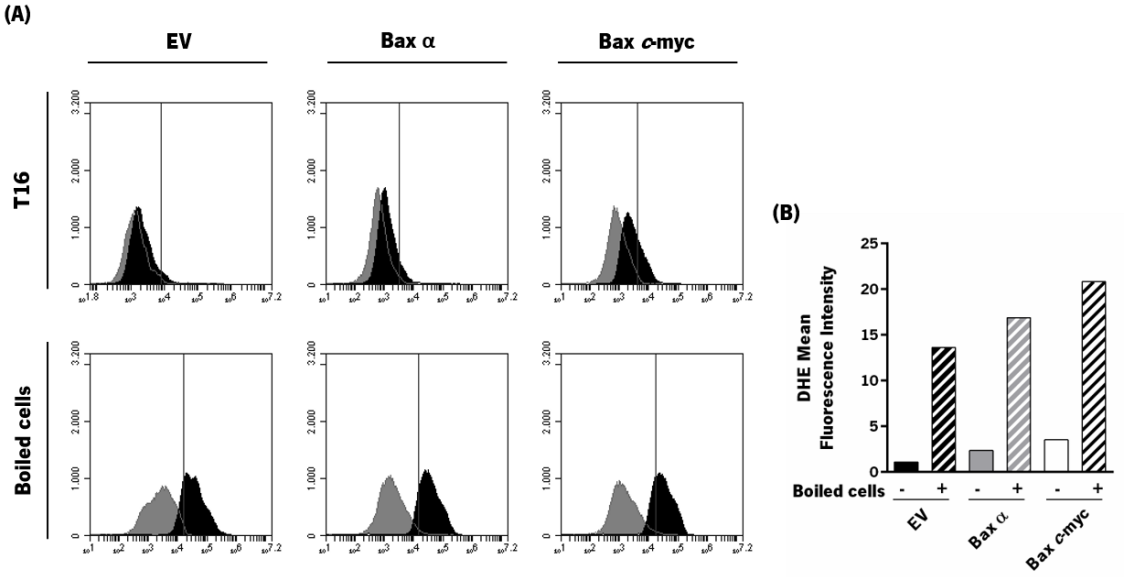

Figure IV.3.3: Expression of Bax α and Bax *c*-myc in yeast results in superoxide anion accumulation. (A) Histograms of monoparametric detection of DHE stained cells fluorescence intensity at each time point for the indicated strains. Counts presented in the right side of the histogram are considered DHE positive. Grey histograms correspond to autofluorescence, while black histograms indicate staining. **(B)** Mean of the positive red fluorescence intensity provided by DHE staining for the indicated yeast strains, 16 hours after Bax expression. The results correspond to a single flow cytometry experiment and are normalized to autofluorescence. For each strain, boiled cells were used as a positive control.

In summary, we optimized conditions to express Bax α, as well as a positive control for Bax activity, Bax c-myc. We therefore next sought to uncover the role of several proteins/stimuli on Bax α activity.

IV.4. The yeast orthologue of GSK3β does not activate Bax α in yeast

We first investigated whether Bax α could be regulated by Rim11p, the yeast orthologue of the human GSK3β. Preliminary results from our group suggested that deletion of *RIM11* decreased the specific growth rate of Bax-expressing cells, although not statistically significantly (Mendes 2017). However, Bax α localized to mitochondria in *rim11*Δ cells and led to the release of cytochrome *c*, consistent with an active Bax (Mendes 2017). However, Bax α also seemed to localize to mitochondria of the respective wild-type control, raising questions of possible secondary mutations in this strain, and therefore a definitive role for Rim11p could not be ascertained. To further characterize the effect of this

kinase in yeast cells expressing Bax α, we deleted *RIM11* in W303 cells by homologous recombination (described in Materials and Methods). The cell viability of the *rim11*Δ strain transformed with the empty vector (control cells) or with Bax α was evaluated by CFU counting and compared with the wild type-expressing strains (**Figure IV.4.1**). We found that deletion of *RIM11* did not affect viability of cells expressing Bax α, suggesting that the absence of this protein *per se* is not sufficient to activate Bax α.

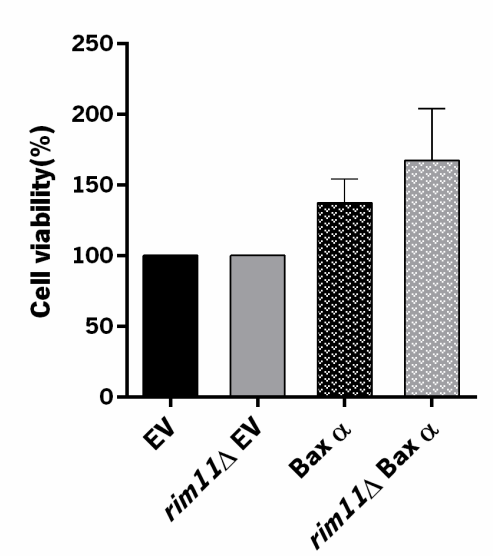

Figure IV.4.1: Absence of the yeast orthologue of GSK3β does not affect Bax α activity in yeast cells. Assessment of cell survival by CFU counting of *rim11*Δ cells expressing Bax α and respective control cells (expressing EV). Cell survival of Bax α-expressing cells is normalized to T0 and to control cells and values are the mean of three independent experiments analysed by one-way ANOVA.

IV.5. Sub-lethal concentrations of acetic acid induce cell death of yeast cells expressing Bax α

An alternative way to activate Bax α in yeast, as in mammalian cells, is by using an exogenous trigger. When testing for a possible role of yeast cathepsin D in Bax α activation, Pereira *et al.* (2015) found that sub-lethal doses of acetic acid (120 mM for 360 minutes in that strain) were able to induce cell death in yeast expressing inactive Bax α. Thus, we sought to understand if exposure to acetic acid could activate Bax α and whether increased cytotoxicity is associated with Bax α translocation to mitochondria and cytochrome *c* release. We started by monitoring the cytotoxic effect of acetic acid in control and Bax α-expressing cells after exposure to 50, 80, 100 or 120 mM of acid along 180 minutes, at pH 3.0. We found that exposure of Bax α-expressing cells to 80 mM of acetic acid led to a drastic decrease in cell viability within 60 minutes, with a reduction of approximately 75%, while the

control strains remained viable (**Figure IV.5.1**). For lower concentrations (50 mM) some decrease in cell survival was observed, however it was not significant in comparison with control cells. In contrast, for acid concentrations higher than 80 mM (100 and 120 mM), both control and Bax α-expressing strains displayed an abrupt decline in cell survival. These results suggest that 60 minutes of exposure to a sub-lethal concentration of acetic acid (80 mM) acts as an exogenous trigger of Bax α activation. Thus, these conditions were selected for further analysis.

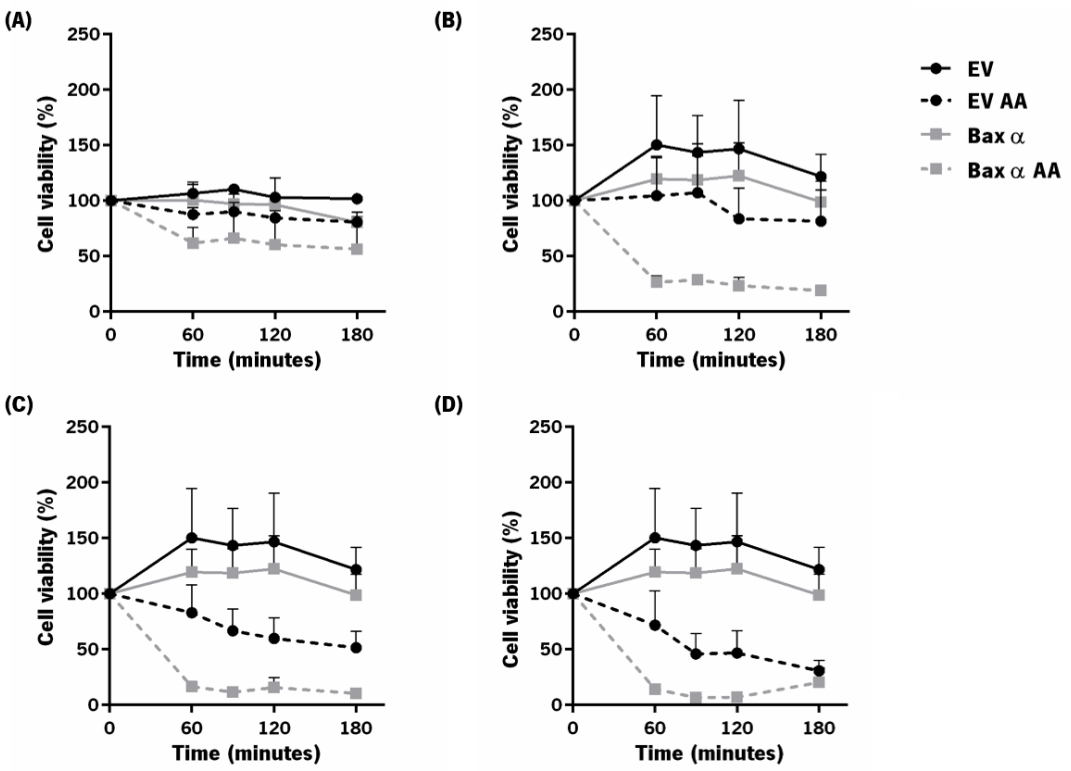

Figure IV.5.1: Sub-lethal concentrations of acetic acid activate Bax α in yeast cells. The percentage of cell survival of yeast cells expressing human Bax was analysed along 180 minutes of treatment with **(A)** 50 mM, **(B)** 80 mM, **(C)** 100 mM or **(D)** 120 mM of acetic acid (AA), at pH 3.0. For each concentration non-treated control cells were also subjected to the same growth conditions. Cell survival of Bax α-expressing cells was normalized to T0 and the values are the mean of three independent experiments analysed by two-way ANOVA.

IV.6. Acetic acid-induced cell death of yeast expressing Bax α occurs without compromising plasma membrane integrity

In order to characterize acetic acid-induced cell death in cells expressing Bax α, plasma membrane integrity was evaluated by flow cytometry. The drastic decrease in cell survival of Bax α-expressing cells treated with sub-lethal concentrations of acetic acid could be the result of a necrotic or of an apoptotic cell death. Knowing that apoptotic cells maintain their plasma membrane intact, while necrotic cells do not (Kroemer *et al.* 2009), we used PI staining to evaluate the plasma membrane integrity. PI is able to enter the plasma membrane of disrupted cells, thus any fluorescence signal with this dye is indicative of cells with a compromised plasma membrane. We found that before acetic acid treatment (but after 16 hours of Bax α expression) approximately 2% and 0% of Bax-expressing and control cells, respectively, are stained with PI, consistent with the existence of a negligible dead cell population (**Figure VIII.2**). After 60 minutes of exposure to 80 mM acetic acid, both Bax α-expressing and control (EV) cells display an increase in PI-positive cells (19% and 12%, respectively), when compared to non-treated control cells, which was statistically significant in the case of Bax α (**Figure IV.6.1**). Nevertheless, clonogenic assays demonstrate that 75% of cells died, while only 19% stained positive with PI (possibly due to loss of plasma membrane integrity). This suggests that acetic acid-induced cell death of yeast cells expressing Bax α is an apoptotic process.

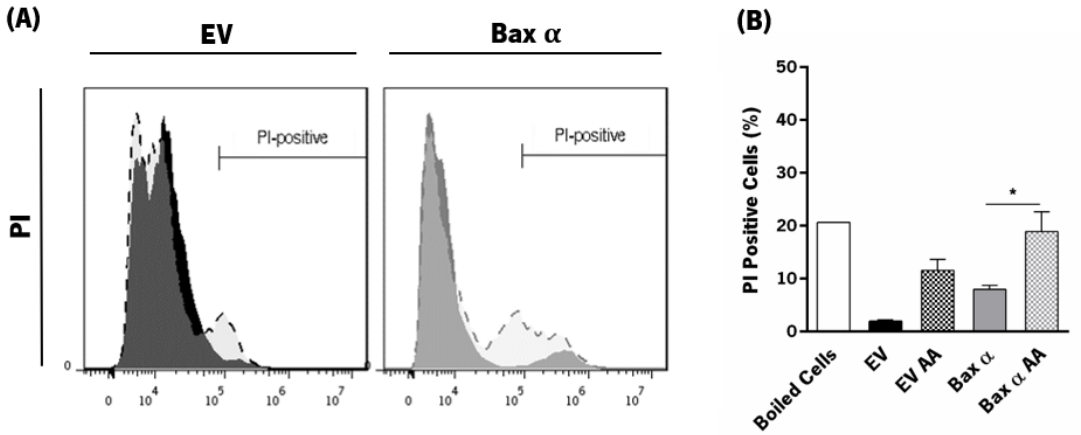

Figure IV.6.1: Sub-lethal concentrations of acetic acid do not induce considerable loss of plasma membrane integrity in yeast cells expressing Bax α. (A) Representative monoparametric histograms of the red fluorescence of PI-stained cells 60 minutes after treatment with acetic acid. Full line histograms correspond to non-treated cells (0 mM AA) and punctuate line histograms correspond to 80 mM acetic acid treatment. **(B)** Percentage of cells exhibiting loss of plasma membrane integrity determined by PI staining of the indicated yeast strains treated and non-treated with 80 mM acetic acid, pH=3, for 60 minutes. Boiled cells were used as a control of loss of plasma membrane integrity. The data displayed are the mean of three independent experiments analysed by one-way ANOVA, where *$p<0.05$.

IV.7. Acetic acid-triggered cell death through Bax α activation is associated with superoxide anion accumulation

In mammalian apoptosis, Bax induces production and accumulation of ROS (Kirkland *et al*. 2002). When using H_2O_2 (Madeo *et al*. 1999) and acetic acid ((Ludovico *et al*. 2002) as exogenous triggers of yeast apoptosis, ROS accumulation was also observed. Moreover, it was confirmed that ROS are mediators rather than by-products of yeast apoptosis (Madeo *et al*. 1999; Giannattasio *et al*. 2005). Thus, we sought to investigate if cell death of Bax α-expressing cells induced by sub-lethal concentrations of acetic acid is associated with ROS accumulation. For this purpose, we assessed the levels of superoxide anion, one of the ROS species that is mainly produced in mitochondria, by flow cytometry using DHE and MitoSOX™ Red. DHE monitors cellular superoxide anion while MitoSOX™ Red detects this species in the mitochondrial compartment. Results indicate that after 60 minutes in medium pH 3.0 (control conditions), cellular superoxide anion accumulation do not change, in contrast to mitochondrial superoxide anion accumulation that increases significantly in Bax α-expressing cells in comparison to control cells (**Figure VIII.3**). However, after 60 minutes treatment, cellular superoxide anion accumulation only significantly increases in Bax α-expressing cells (**Figure IV.7.1A and B**), while mitochondrial superoxide anion accumulation increases significantly independently of Bax expression (**Figure IV.7.1A and C**).

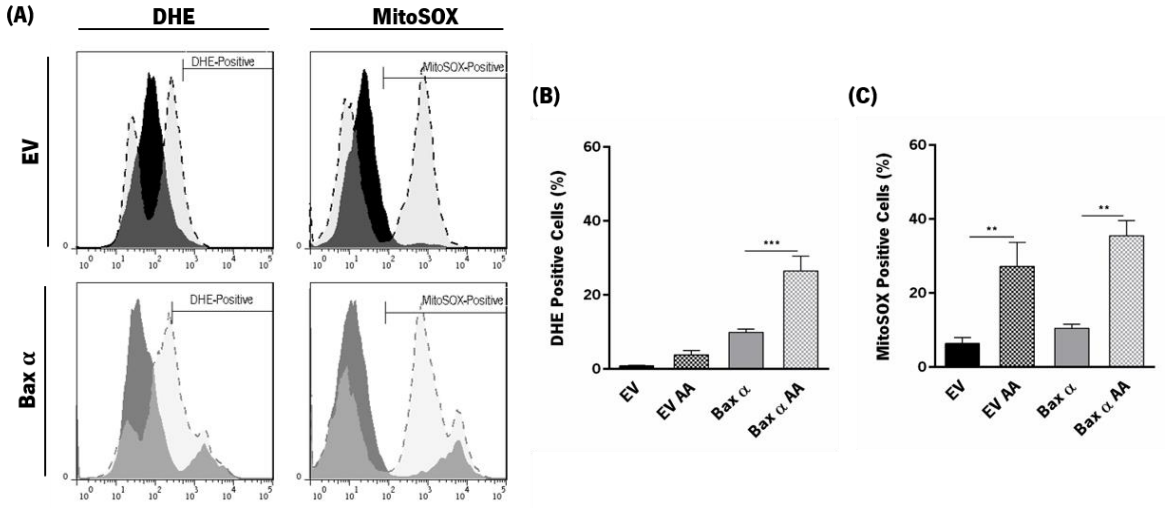

Figure IV.7.1: Sub-lethal concentrations of acetic acid induce mitochondrial superoxide anion accumulation. (A) Representative monoparametric histograms of red fluorescence of cells stained with DHE or MitoSOX™ Red 60 minutes after treatment with 80 mM acetic acid, pH=3. Full line histograms correspond to non-treated cells (0 mM AA) and punctuate line histograms correspond to acetic acid treatment. Quantification of the percentage of **(B)** DHE-positive cells and **(C)** MitoSOX™ Red -positive cells. The values displayed the mean of four independent experiments analysed by one-way ANOVA, where **p<0.01; ***p<0.001.

Since mitochondria are the powerhouse of the cell, these organelles regulate important metabolic pathways and participate in the decision between life and death. Acetic acid has been shown to trigger a RCD in yeast associated with mitochondrial dysfunctions including reduction in $\Delta\Psi_m$, mitochondrial fragmentation and mitochondrial degradation (Pereira *et al.* 2010) that are also hallmarks of mammalian apoptosis. However, these findings were obtained using lethal concentrations of acetic acid. Therefore, we next assessed if treatment with sub-lethal concentrations of acetic acid would lead to the same phenotype in yeast cells expressing Bax α. The MitoTracker Green FM probe stains mitochondria regardless of its membrane potential and has been used to monitor changes in mitochondrial mass by flow cytometry. Thus, possible alterations in mitochondrial mass stemming from exposure of cells expressing Bax α for 16 hours to 80 mM acetic acid treatment for 60 minutes were assessed with that probe. Though, 60 minutes after exposure to pH 3.0 medium, cell suspensions were quite heterogeneous with three subpopulations, the population exhibiting lower green mean fluorescence intensity was the predominant one. Expression of Bax α did not alter this distribution, in contrast to exposure to acetic acid. Indeed, acetic acid treatment clearly led to a decrease of the subpopulation with lower green mean fluorescence intensity associated with an increase of the other

two, suggesting an accumulation of mitochondrial mass (**Figure VIII.4**). Indeed, the populations with higher green mean fluorescence intensity increased after acetic acid treatment independently of Bax expression (**Figure IV.7.2**).

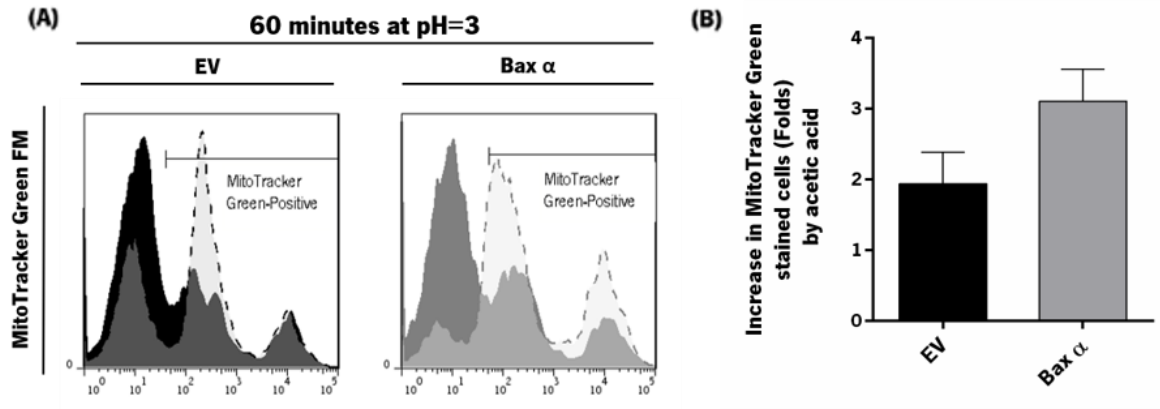

Figure IV.7.2: Sub-lethal concentrations of acetic acid increase mitochondrial mass of yeast cells independently of Bax α expression. (A) Representative monoparametric histograms of the fluorescence of MitoTracker Green FM stained control and Bax α-expressing cells 60 minutes after treatment with acetic acid. Full line histograms correspond to non-treated cells (0 mM AA) and punctuate line histograms correspond to 80 mM acetic acid treatment, pH 3.0. **(B)** Alterations (folds) of gated MitoTracker Green FM stained cells following acetic acid treatment. The values displayed correspond to the mean of three independent experiments analysed by student *t*test, where **p≤0.01.

Taken together, our results suggest that the activation of Bax by 80 mM acetic acid neither result in a significant increase in mitochondrial ROS levels compared with control cells nor in an increase in mitochondrial mass. Nevertheless, other effects at the mitochondria level may occur.

IV.8. Acetic acid-induced Bax **α**-dependent cell death is associated with cytochrome *c* release

As sub-lethal concentration of acetic acid (80 mM, pH 3) induced cell death in Bax α-expressing cells through a regulated process, we questioned if under these conditions Bax translocates to mitochondria and causes cytochrome *c* release, as in mammalian cells upon apoptosis induction. To evaluate cytochrome *c* release, mitochondrial and cytosolic fractions were isolated from Bax α-expressing cells treated (80 mM, pH 3.0) and non-treated with acetic acid. The release of cytochrome *c* was then assessed by western blot by qualitatively comparing the intensity of the bands in the different fractions. The results indicate that there is a higher amount of cytochrome *c* in the cytosol of cells expressing Bax α exposed to acetic acid comparatively to non-treated cells, suggesting that cytochrome *c* was released from mitochondria, consistent with an active conformation of Bax (**Figure IV.8.1**).

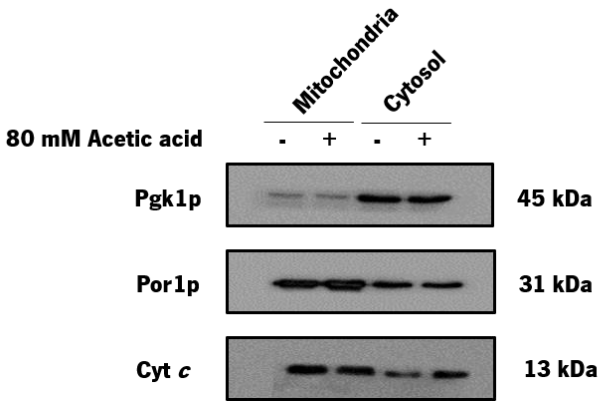

Figure IV.8.1: Sub-lethal concentrations of acetic acid induce cytochrome *c* release in yeast cells expressing Bax α. Immunodetection of cytochrome *c* in yeast cells expressing Bax α 16 hours after expression and 60 minutes after treatment with 80 mM acetic acid, pH3.0. Pgk1p and Por1p were used as loading controls for cytosol and mitochondrial fractions, respectively.

IV.9. Human PKCε adds to Bax α killing activity in acetic acid-induced Bax α-dependent cell death

After finding conditions of expression and activation of Bax α in yeast, we sought to investigate the regulation of this pro-apoptotic protein through interaction with a protein kinase other than Rim11p. Our group has previously investigated the role of the PKC family in Bax regulation, since these serine/threonine kinases were shown to play an important role in cell survival and apoptosis as well as in Bax regulation (Silva *et al.* 2011). It has been reported that independent expression of Bax α or PKCε in yeast does not induce cell death, and that expression of either protein in yeast cells treated with lethal concentrations of acetic acid increase their killing activity (Saraiva *et al.* 2006; Pereira *et al.* 2015). In prostate cancer cells, an interaction between PKCε and Bax was observed, and resulted in inhibition of Bax activation and, consequently, inhibition of its killing activity (McJilton *et al.* 2003). We therefore hypothesized that co-expression of PKCε with Bax α could result in protection from cell death induced by acetic acid. To confirm this hypothesis, PKCε was co-expressed with Bax α and the effect in cell viability analysed by CFU counting. Our results show that cell viability was not affected in yeast cells expressing Bax α and PKCε (**Figure IV.9.1A**). Next, we evaluated if this isoform has any effect on acetic acid-induced cell death of cells expressing Bax α. Results indicate that sub-lethal concentrations of acetic acid increase cell death in cells expressing Bax α, as shown above, but not in control cells

(**Figure IV.9.1B**). As a matter of fact, cells co-expressing Bax α and PKCε are more sensitive to acetic acid than cells expressing Bax α alone.

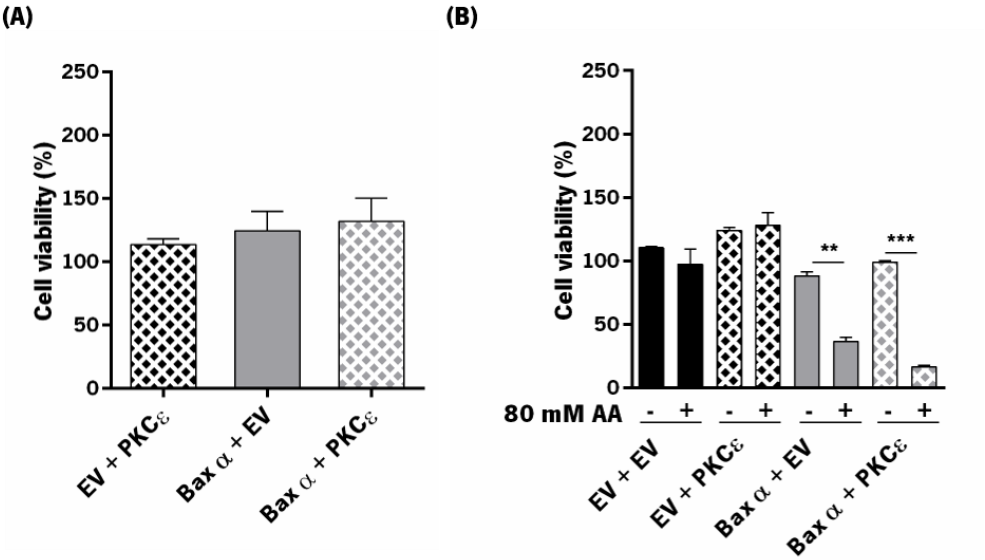

Figure IV.9.1: Co-expression of PKCε and Bax α adds to acetic acid-induced cell death of Bax α expressing cells. (A) Cell viability of yeast cells co-expressing PKCε and Bax α, 16 hours after expression induction. The results displayed are normalized to T0 of expression and to control cells (transformed with both EVs). **(B)** Cell viability of yeast cells co-expressing PKCε and Bax α, 16 hours after protein´s expression, treated with 80 mM acetic acid for 60 minutes. Data are normalized T0 of acetic acid treatment and are the mean of three independent experiments analysed by one-way ANOVA, where **p<0.01; *** p<0.001.

IV.10. Bcl-x_L rescues cells from acetic acid-induced cell death in yeast cells expressing Bax **α**

We next aimed to study Bax α regulation by interaction with one of its family members, which are known regulators of Bax function. Indeed, Bax interaction with its anti-apoptotic relative Bcl-x_L has already been confirmed not only in mammalian but also in yeast cells (Renault *et al.* 2015). This interaction restrains Bax activation and appears to regulate Bax localization, as co-expression of Bcl-x_L with Bax favours Bax translocation to mitochondria and, additionally, Bcl-x_L participates in Bax retrotranslocation to the cytosol (Renault *et al.* 2015; Renault *et al.* 2017). We therefore sought to decipher if Bcl-x_L is able to rescue cells from cell death induced by exposure of Bax α-expressing cells to acetic acid and modulate its localization, mimicking the phenotype observed in other backgrounds. For this purpose, we heterologously co-expressed Bax α with Bcl-x_L or with a truncated version, Bcl-x_LΔC,

which does not contain the C-terminal transmembrane domain and is unable to promote Bax retrotranslocation (Renault *et al.* 2015). After 16 hours of expression, no significant differences were found in cell survival (**Figure IV.10.1A**). However, cell viability decreased in cells expressing Bax α alone or co-expressing Bax α with Bcl-x_L or its truncated version 60 minutes after 80 mM acetic acid treatment (**Figure IV.10.1B**). Nonetheless, co-expressing Bax α with full-length Bcl-x_L significantly increased cell survival in comparison with the strain expressing Bax α alone, suggesting that Bcl-x_L protects cells from cell death caused by activation of Bax α by acetic acid. As expected, since Bcl-x_LΔC is not able to retrotranslocate Bax from the mitochondria to the cytosol, its effect on cell death was not significantly different from the strain expressing only Bax α.

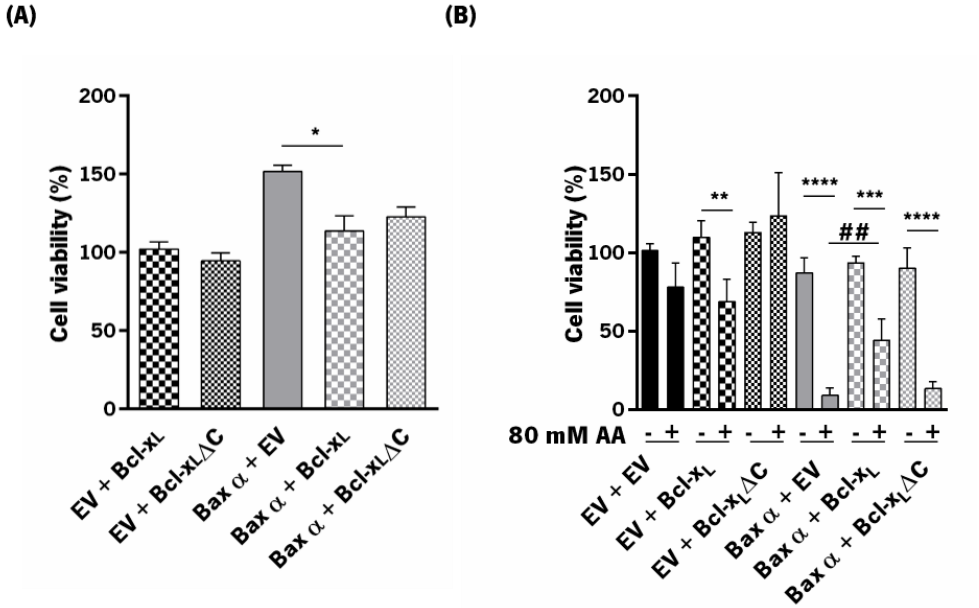

Figure IV.10.1: Expression of Bcl-x_L, but not its truncated form Bcl-x_LΔC, protects Bax α-expressing cells from acetic acid-induced death. (A) Cell viability assessed by CFU counting of yeast cells co-expressing Bax α and Bcl-x_L or Bcl-x_LΔC, 16 hours after induction of expression. The values displayed are normalized to T0 of expression induction and to control cells (transformed with both EVs). **(B)** Cell viability of yeast cells co-expressing Bax α and Bcl-x_L or Bcl-x_LΔC, 16 hours after protein´s expression, treated with 80 mM acetic acid for 60 minutes, pH 3.0. Data are normalized to T0 of acetic acid treatment and are the mean of three independent experiments analysed by one-way ANOVA, where *p<0.05; **p<0.01; ## p<0.01; ***p<0.001; **** p<0.0001.

Chapter V

DISCUSSION AND FUTURE PERSPECTIVES

Given the important role of the pro-apoptotic protein Bax in cell death, its potential as a therapeutic target in mis-regulated cell death-associated diseases arose quickly. In fact, its mode of action and regulation has been extensively studied, but multiple aspects remain elusive, as the model systems used are complex. Here, we took advantage of the genetically tractable yeast *S. cerevisiae,* whose genome lacks genes coding obvious homologues of the human Bcl-2 family, to study Bax independently of the apoptotic network. To achieve our goal, we heterologously expressed native Bax α in *S. cerevisiae* under the control of a *GAL10* promoter, a system already well characterized, and optimized the conditions for expression. The highest Bax α expression level was achieved 16 hours after induction with 1% galactose, which caused a significant reduction in the specific growth rate. However, this reduction did not reflect net growth of the Bax α-expressing cell population as concurrence of growth and death. Indeed, viability of Bax α-expressing cells 16 hours and 24 hours after induction was not affected, which is consistent with an inactive protein conformation (Gallenne *et al*. 2009). Bax-mediated growth inhibition may be due to impairment in cell cycle progression, as described for another genetic background (Bounhar *et al*. 2006). In fact, apart from growth inhibition and cell death, it was observed that expression of Bax containing a N-terminal HA tag (which mainly localizes at mitochondrial contact sites between MOM and inner mitochondrial membrane) causes growth arrest (Greenhalf *et al*. 1996; Gross *et al*. 2000) and blockage in the S-phase of the cell cycle (Bounhar *et al*. 2006). Growth inhibition occurs independently of mitochondrial function, though it can be reverted by the anti-apoptotic Bcl-x_L (Gross *et al*. 2000). Another study showed that wild-type mouse Bax cDNA-mediated cell death in yeast *S. cerevisiae* is reverted in a Bcl-2 protein family-independent way by the human prion protein (PrP). PrP also counteracts the S-phase arrest, yet it is not able to revert growth inhibition because cells are retained in the G_2/M phase (Bounhar *et al*. 2006). These deleterious effects of Bax heterologous expression are extended to other yeast species (Khoury and Greenwood 2008). In *Pichia pastoris*, Bax (mouse *BAX* cDNA) expression induced cell death and growth inhibition, as well as chromatin condensation, typical of mammalian apoptosis (Martinet *et al*. 1999). The same phenotype was detected in the pathogenic yeast *Candida albicans* expressing a synthetic codon-optimized *BAX* gene (De Smet *et al*. 2004). Whereas in *S. cerevisiae* N-terminal tagged Bax killing activity depends on oxidative phosphorylation (Gross *et al*. 2000), in *Kluyveromyces lactis* it does not. In fact, *K. lactis* mitochondrial mutants are even more sensitive to Bax expression (Šabova *et al*. 2002). In *Schizosaccharomyces pombe*, in addition to Bax, Bak expression also affected cell cycle progression. Whereas Bak expression results in defects in cell cycle progression at G_1-phase, and remaining cells are

unable to complete M-phase, Bax expression allows cells to enter S-phase, even though with less efficiency, but results in problems after M-phase (Torgler *et al*. 1997). Torgler and colleagues suggested that in *S. pombe* Bak and Bax differ only in the severity of the phenotype; Bak expression is lethal, whereas Bax expression causes a decrease in population growth, without causing cell death. Altogether, these reports demonstrate that the Bax mode of action is conserved from lower to higher eukaryotes, which reinforces the exploitation of these simpler single cell organisms as powerful model systems to further highlight Bax function. Further studies on the effect of Bax α on cell cycle progression will allow assessing whether it is involved in the growth inhibitory effect. Similarly to *S. pombe*, under our experimental conditions, Bax α does not induce cell death of *S. cerevisiae*, mimicking what happens in healthy mammalian cells. Thus, we also sought to characterize the phenotype of an active form of Bax, Bax *c*-myc, as a positive control of Bax α activation and subsequent cell death, under the same conditions.

As previously reported, we also found that expression of the mitochondrial and active form Bax *c*-myc caused a significant reduction in yeast survival (Priault *et al*. 1999). Although Bax α and Bax *c*-myc expression affect cell viability differently, both do not impair metabolic activity as assessed by the ability to process FUN-1 in the vacuole and to cleave FDA in an esterase-dependent way. While maintenance of metabolic activity in Bax α correlates with maintenance of cell viability, it might appear unexpected that only 25% of cells expressing Bax *c*-myc survive, but almost 100% keep their metabolic activity. Although non-viable cells can keep their metabolic activity, the observed discrepancy may be explained by the different experimental times used. Indeed, while clonogenic assays evaluate the ability of cells to proliferate when transferred to a rich medium for 2-3 days 16 hous after Bax expression, the metabolic activity is assessed just 16 hours after Bax expression. Thus, the assessment of metabolic activity should be performed under the experimental conditions used in clonogenic assays for correct interpretation on the effect of Bax on metabolic activity and its relation with cell viability. Cytotoxicity is often associated with an increase in superoxide anion accumulation, as previously observed (Silva *et al*. 2011). Bax α-expressing cells exhibit an accumulation of superoxide anion though at a lower level than those expressing Bax *c*-myc, which may in part explain the different outcomes in cell viability. Interestingly, expression of a DNA fragment coding for the murine Bax gene in *K. lactis* also results in cell death associated with ROS accumulation. Notwithstanding, cell death is reverted by co-expression with Bcl-x_L but maintaining ROS accumulation, indicating that an oxidative burst is not required for *K.*

lactis death (Šabova *et al.* 2002). Nevertheless, the results regarding metabolic activity and ROS accumulation correspond to a single experiment, thus further confirmation is required.

Once we optimised and recapitulated in yeast the biochemical features of Bax in healthy mammalian cells, we pursued our study towards the further understanding of Bax function/regulation. Our idea was to explore the use of an exogenous stimulus to activate native Bax α, mimicking Bax activation in mammalian cells, in addition to the use of mutants deficient in putative regulators of Bax as an alternative to the exploitation of artificial active Bax mutants. Previous works from our group (Mendes 2017) suggested that Bax α could be activated through inactivation of the yeast orthologue of GSK3β, Rim11p, implying that it could play a role in Bax α regulation. Our results do not sustain this hypothesis since *rim11*Δ cells expressing Bax α did not display alterations in cell survival. This indicates that, if playing a role in Bax α activation, abrogation of Rim11p *per se* is not sufficient. One possible explanation for this inconsistency can be the use of different strains and growth and expression induction conditions. Indeed, the previous studies were performed using a strain kindly provided by Joris Winderickx, while herein the one used was constructed from the wild-type kindly provided by Stéphen Manon. Another explanation can be the different growth and expression conditions. Indeed, the previous results were obtained after just 4 hours of Bax α expression induced with 2% galactose (Mendes 2017), instead of 16 hours of Bax α expression induced by 1% galactose, as used herein. The same conditions should therefore be used with the newly constructed yeast strain in order to confirm the phenotype of *rim11Δ* cells expressing Bax α. Mendes also detected Bax α in the mitochondria and the release of cytochrome *c* 4 hours after induction of Bax α expression. Curiously, both strains displayed the same survival 3 hours after Bax α expression (Mendes 2017). When growth was evaluated along 32 hours, a reduction in biomass at the stationary phase was observed, but no significant decrease in the specific growth rate of *rim11*Δ EV and *rim11*Δ Bax α was detected. Nevertheless, the absence of Bax killing activity obtained herein is in accordance with the suppression of Bax activation, and its associated translocation to mitochondria by inhibition of GSK3β in mammalian cells undergoing apoptosis (Linseman *et al.* 2004; Zhang *et al.* 2010). Therefore, lack of Bax α activation in the absence of Rim11p as observed herein would be expected. Nevertheless, the effect of Rim11p abrogation in yeast cells should be exploited under conditions of Bax α activation, such as exposure to acetic acid, to mimic apoptotic mammalian cells. Moreover, Linseman and co-authors identified the Bax residue S163 as a target of phosphorylation by GSK3β, needed for induction of

apoptosis (Linseman *et al.* 2004). However, when testing for the involvement of this residue in Bax α activation in yeast, Arokium *et al.* (2007) found that its substitution with a negatively charged residue (phosphomimetic mutant) does not activate Bax α significantly. Thus, the authors combined the phosphomimetic mutant with the inactive double mutant P168A (which allows the movement of α-helix 9 and confers an active conformation) and S60A (which restricts α-helix 1 movement, inactivating the protein), and found that the triple mutant had increased capacity to translocate to mitochondria and to release cytochrome *c* in comparison with the single P168A mutant (Arokium *et al.* 2007). When exploring S60 as another possible target of phosphorylation, its substitution for a phosphomimetic residue induced Bax α translocation to mitochondria without stimulating the release of cytochrome *c*, consistent with a conformation that is not fully activated (Arokium *et al.* 2007). Thus, as it happens with S163, the phosphorylation of S60 *per se* is not sufficient to activate Bax α. Nonetheless, it is interesting to hypothesize that the combination of both substitutions may originate a fully active protein.

Another simpler and more obvious way to activate Bax is by using an exogenous trigger. The stress and death-inducing agent acetic acid is a regular by-product of the alcoholic fermentation carried out by *S. cerevisiae* (Du Toit and Lambrechts 2002; Vilela-Moura *et al.* 2011). At a pH below its pKa (pKa=4.76), acetic acid is mainly present in its undissociated form (CH_3COOH), so it can enter the cell, where it dissociates into protons (H^+) and acetate (CH_3COO^-) and, depending on the pH gradient across the plasma membrane, it can lead to intracellular acidification and acetate accumulation, affecting metabolic activity (Pampulha and Loureiro-Dias 1989; Casal *et al.* 1996). While above certain concentrations acetic acid is detrimental for fermentation performance (Alexandre and Charpentier 1998; Vilela-Moura *et al.* 2011), its cytotoxicity can be exploited as a food preservative (J. *et al.* 2012; Giannattasio *et al.* 2013). Moreover, short-chain fatty acids (SCFA), including acetic acid, have been explored in the therapy and prevention against some diseases, and have been shown to induce differentiation, growth arrest and apoptotic cell death in colorectal cancer cells (Jan *et al.* 2002; Lan *et al.* 2007; Marques *et al.* 2013). In yeast, mammalian apoptotic-like features have also been reported in response to lethal doses of this monocarboxylic acid, including chromatin condensation, exposure of phosphatidylserine, DNA breakdown, cytochrome *c* release, decrease in cytochrome *c* oxidase activity, ROS accumulation and loss of $\Delta\Psi_m$ (Ludovico *et al.* 2001; Ludovico *et al.* 2002). It has long been demonstrated that acetic acid-induced cell death is mitochondria-dependent, and is inhibited in the presence of cycloheximide (Ludovico *et al.* 2001; Ludovico *et al.* 2002). Altogether, these data support the hypothesis that acetic acid could act as an exogenous trigger of Bax when expressed in yeast.

Indeed, Pereira *et al*. (2015) observed that acetic acid triggered cell death of Bax α-expressing cells. Herein, we show that under these conditions cell death is associated with maintenance of plasma membrane integrity, which suggest an apoptotic regulated death process, though additional apoptotic markers should be monitored. Moreover, this was accompanied by an increase in mitochondrial superoxide anion levels. However, Bax α-expressing cells only exhibit a slight increase in DHE-positive cells than control cells exposed to the same sub-lethal concentration of acetic acid, suggesting that death is due to Bax α activation. Mitochondria are also involved in yeast cell death induced by lethal doses of acetic acid, shown by the use of different mutant strains lacking mitochondrial DNA, mitochondrial ATPase and a gene encoding a heme lyase, which displayed higher resistance to acetic acid-induced cell death than the wild-type strain (Ludovico *et al*. 2002). Furthermore, another mitochondria dependent-apoptotic death feature of yeast cells expressing Bax *c*-myc (Kiššová *et al*. 2006) or upon exposure to lethal doses of acetic acid (Fannjiang *et al*. 2004; Pereira *et al*. 2010) is the fragmentation and degradation of this organelle. As a matter of fact, mitochondria degradation has been observed in both yeast and colorectal cancer cells after treatment with lethal doses of acetic acid and acetate, respectively (Pereira *et al*, 2010; Oliveira *et al*. 2015). However, counter intuitively, mitochondrial degradation was associated with a higher mitochondrial content in comparison with acetic acid/acetate untreated cells. In fact, exposure of colorectal cancer cells to higher acetate concentrations resulted in decreased levels of Beclin-1 and Atg5p, two proteins required for autophagosome formation (Oliveira *et al*. 2015), which indicate inhibition of autophagy. Similarly, exposure of yeast to lethal doses of acetic acid inhibits autophagy as monitored by the decrease in alkaline phosphatase activity and in the amount of the autophagic Atg8p (Pereira *et al*. 2010). However, in both yeast and colorectal cancer cells, mitochondrial degradation occurs through an autophagy-independent process mediated by the vacuolar/lysosomal Pep4p or Cathepsin D, respectively (Pereira *et al*. 2010; Oliveira *et al*. 2015). Somehow contradictory to the results here obtained is the autophagy activation assessed by accumulation of the autophagic protein Atg8p in cells expressing the active form Bax *c*-myc (Kiššová *et al*. 2006). Altogether, our and other's data led us to suppose that under conditions of Bax α activation, autophagy should also be activated and hence lead to a decrease in mitochondrial content. However, we found that acetic acid treatment increased yeast mitochondrial mass independently of Bax α expression. This increase suggests that sub-lethal doses of acetic acid counteract autophagy activation by Bax α. Nonetheless further experiments should be performed to confirm this hypothesis.

Another common feature of yeast cells undergoing acetic acid-induced cell death (Ludovico *et al*. 2002) and Bax activation (Manon *et al*. 1997) is the release of cytochrome *c* from the mitochondria to the cytosol. We found that acetic acid exogenously triggers Bax activation with subsequent release of cytochrome *c*, since the amount of cytochrome *c* in the cytosol is higher for Bax α treated than non-treated cells, consistent with the viability results obtained. In addition, the extent of cytochrome *c* release should be quantified by redox spectrophotometry, and controls not expressing Bax α included. Moreover, Bax α mitochondrial localization should be assessed to confirm whether it is Bax α activation by acetic acid that leads to cytochrome *c* release. It has been shown by immunostaining that Bax translocates from the cytosol to the mitochondria of colorectal cancer cells undergoing apoptosis induced by SCFA (Lan *et al*. (2007)). Altogether, these results suggest for the first time that sub-lethal concentrations of acetic acid act as an exogenous trigger of Bax in yeast cells resulting in cytochrome *c* release. This finding allows us to mimic what happens in human cells, without the need to use non-natural mutants of Bax, such as phosphomimetic or non-phosphorylatable, or mitochondrial tagged versions. Thus, we next sought to explore how Bax is regulated through different proteins.

PKC is a family of serine/threonine kinases that plays crucial roles in physiology and pathology, participating in proliferation, cell cycle, cell death, invasiveness, drug resistance, cell growth and angiogenesis (Griner and Kazanietz 2007). This family contains 10 isoforms, and their involvement in cell death has been studied in our laboratory for a long time, since they seemed to be involved in the regulation of Bcl-2 family members (Gubina *et al*. 1998; Silva *et al*. 2012). Here, we focused on the PKCε isoform, which is considered an oncogene with anti-apoptotic features (Griner and Kazanietz 2007). We studied the role of this kinase in Bax α regulation, after Bax activation by acetic acid. The heterologous expression of the mammalian PKCε in yeast did not impair cell viability, as previously reported (Saraiva *et al*. 2006). It had also been described that cells expressing PKCε display a higher sensitivity to apoptotic conditions induced by lethal doses of acetic acid (Saraiva *et al*. 2006). In this work, we used sub-lethal doses of acetic acid, and this decrease in cell survival was therefore not observed. Consistently with their independent phenotype, the co-expression of PKCε and Bax α under non-apoptotic conditions did not impairs cell survival. However, when Bax α was activated by acetic acid, we found that yeast cells co-expressing both proteins showed an even lower percentage of cell survival rather than those expressing Bax α alone. These results indicate that PKCε does not play a protective role in yeast cells expressing active Bax α and, in fact, adds to Bax α killing activity.

Nevertheless, to further sustain a PKCε pro-apoptotic role under the used conditions, mitochondrial fractionation should be performed to assess Bax localization and evaluate if PKCε co-expression is associated with an increase in Bax translocation. In addition, co-expression of a catalytic-dead PKCε would allow assessing whether the observed enhanced killing effect and pro-apoptotic role of PKCε depends on its catalytic activity. The results mentioned above suggest a possible interaction between PKCε and Bax α in yeast *S. cerevisiae*. Although previous works in our Lab using co-immunoprecipitation or yeast two-hybrid did not detect this interaction (Pereira S. 2015), several studies have reported an interaction between these two proteins, which culminated in a higher survival of the cells, due to abrogation of Bax translocation (McJilton *et al*. 2003; Lu *et al*. 2007). We should study a possible interaction between Bax α and PKCε by co-immunoprecipitation or yeast two hybrid, which allows the detection of weak and transient interactions, but under our work conditions. Indeed, the previous experiments were performed using steady-state cells.

PKCε also regulates the activity of Akt, another kinase reported to modulate Bax. Overexpression of PKCε increases Akt phosphorylation and activity, abrogating cell death. However, PKCε cannot control Akt levels, and is not able to protect Akt-depleted cells from apoptosis (Lu *et al*. 2006). This anti-apoptotic role of PKCε was previously observed in haematopoietic cells through an increase in the expression of anti-apoptotic Bcl-2 proteins (Gubina *et al*. 1998). As a matter of fact, PKCε overexpression was shown to increase the levels of Bcl-2 while decreasing the levels of the pro-apoptotic Bid (Sivaprasad *et al*. 2007). These data corroborate that the anti-apoptotic role of PKCε is mediated by the protective role of Bcl-2 and Akt. Nonetheless, since yeast is depleted of these proteins, PKCε *per se* may not have been able to prevent cell death of Bax α expressing cells treated with acetic acid, and a different, pro-apoptotic role, could be more evident.

We further sought to explore if Bcl-x_L reverts the activation of Bax α by acetic acid. This anti-apoptotic protein is known as one of the main partners of Bax, playing a protective role in cell survival (Manon *et al*. 1997; Renault *et al*. 2017). As the last task of this thesis, we aimed to study the regulation of acetic acid-activated Bax α by Bcl-x_L and the mutant form Bcl-x_LΔC. In the presence of lethal doses of acetic acid, expression of Bcl-x_L in yeast cells increases cell survival, accordingly to its pro-survival role (Saraiva *et al*. 2006). Notwithstanding, in this work we used sub-lethal concentrations, thus acetic acid does not lead to any detrimental effect in cell survival of control cells, contrary to what was detected for Bax α-expressing or co-expressing strains. Acetic acid activates Bax α and thereafter

its killing activity, which is partly reverted by co-expressing Bax α with Bcl-x_L. but not with Bcl-x_LΔC These results were expected and supported the known involvement of Bcl-x_L and of its truncated version in Bax localization, as well as by the ability of Bcl-x_L to increase the mitochondrial content of Bax, yet in an inactive conformation (Renault *et al*. 2015). Moreover, full-length Bcl-x_L was shown to modulate Bax translocation to mitochondria and its retrotranslocation to the cytosol (Edlich *et al*. 2011; Renault *et al*. 2017), while Bcl-x_LΔC is able to modulate Bax location, but owing to its inability to promote Bax retrotranslocation, it induces Bax mitochondrial localization to a higher extent than the unmodified protein (Todt *et al*. 2013; Renault *et al*. 2015). This truncated protein is therefore not able to impair Bax cytotoxic effect as Bcl-x_L (Todt *et al*. 2013; Renault et al. 2015). Hence, we should next determine Bax α localization and the extent of cytochrome *c* release by immunodetection in mitochondrial and cytosolic fractions under co-expression conditions of Bax and Bcl-x_L or Bcl-x_LΔC. Cytochrome *c* content should also be quantified by redox spectrophotometry to complement the cytochrome *c* release by western-blot analysis.

Chapter VI

FINAL REMARKS

In this thesis, we exploited yeast as a cell model to further understand the mechanisms underlying Bax activation and regulation through protein modifications and interaction with other proteins. We showed for the first time that, in contrast to cells lacking the yeast orthologue of the human GSK3β, sub-lethal doses of acetic acid trigger Bax α activation and subsequently cell death. Cell death was associated with maintenance of plasma membrane integrity and mitochondrial mass, superoxide anion accumulation and release of cytochrome *c* to the cytosol. This Bax α killing phenotype was partially reverted by co-expression with Bcl-x_L, but not with its truncated version, Bcl-x_LΔC, or PKCε. We therefore recapitulated in yeast the activation of Bax α by an exogenous stimulus, mimicking what happens in apoptotic mammalian cells. We believe this finding will provide new clues for the understanding of Bax function and regulation, as an alternative to the use of non-natural phosphomimetics or non-phosphorytable mutants or of mitochondrial tagged versions of Bax. Above all, this thesis supports the use of yeast as a valuable tool to support novel therapeutic strategies targeting Bax.

Chapter VII

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Chapter VIII

SUPPLEMENTAL MATERIAL

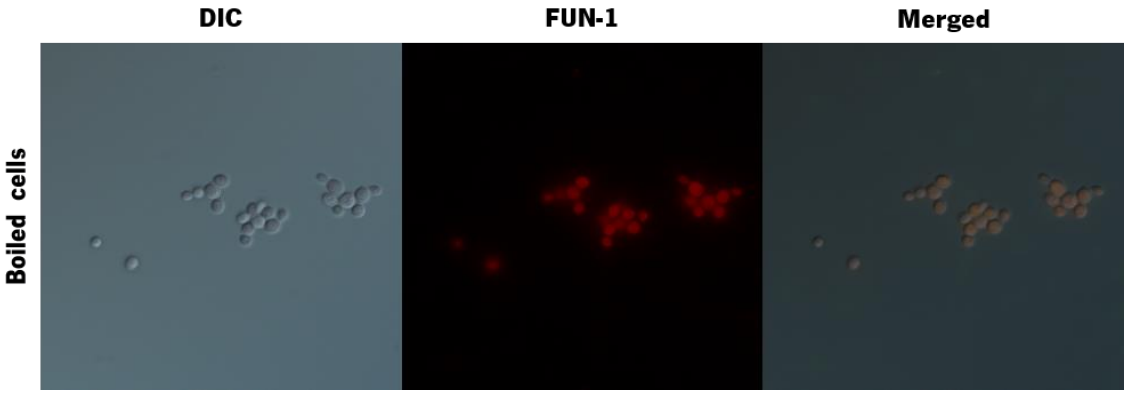

Figure VIII.1: Boiled yeast cells are not able to process FUN-1 into CIVS. Fluorescence microscopy images of boiled cells, for 10 minutes, stained with 4 μM FUN-1 for 30 minutes at 30 °C, in the dark.

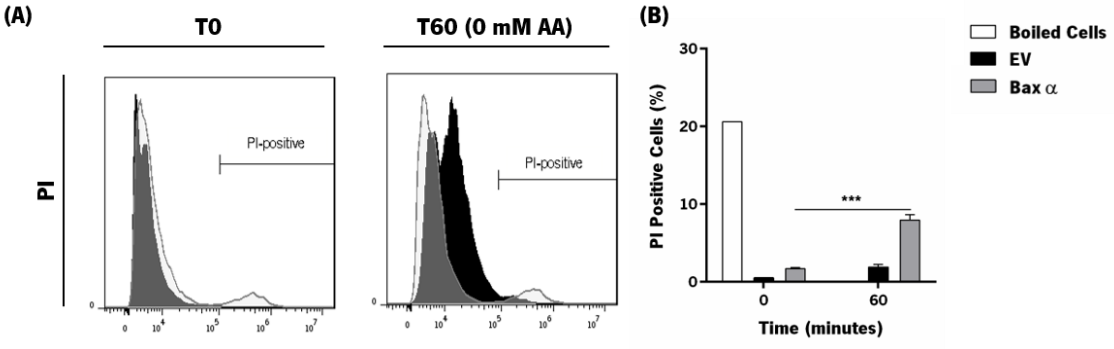

Figure VIII.2: Yeast cells suspensions expressing Bax α display a residual subpopulation exhibiting loss of plasma membrane integrity. (A) Representative monoparametric histograms of the red fluorescence intensity of PI-stained cells 60 minutes after transfer to medium without acetic acid, pH 3.0. Black histograms correspond to control cells and grey histograms correspond to Bax α-expressing cells. **(B)** Quantification of the PI-positive cells shown in (A). Boiled cells were used as a control of loss of plasma membrane integrity. The values displayed are the mean of three independent experiments analysed by one-way ANOVA, where ***p<0.001.

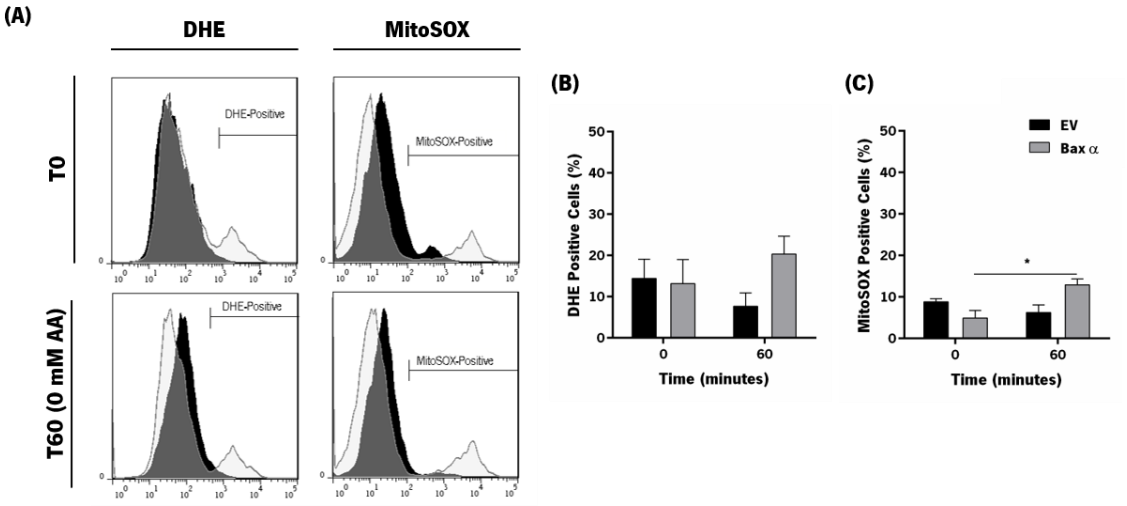

Figure VIII.3: Yeast cells expressing Bax α accumulate mitochondrial superoxide anion. (A) Representative monoparametric histograms of red fluorescence of DHE- or MitoSOX™ Red-stained cells, 60 minutes after transfer to pH 3.0 medium. Black histograms correspond to control cells and grey histograms correspond to Bax α-expressing cells. **(B)** Quantification of the DHE-positive cells and **(C)** MitoSOX™ Red-positive cells shown in (A). The values displayed are the mean of four independent experiments analysed by one-way ANOVA, where *p<0.05.

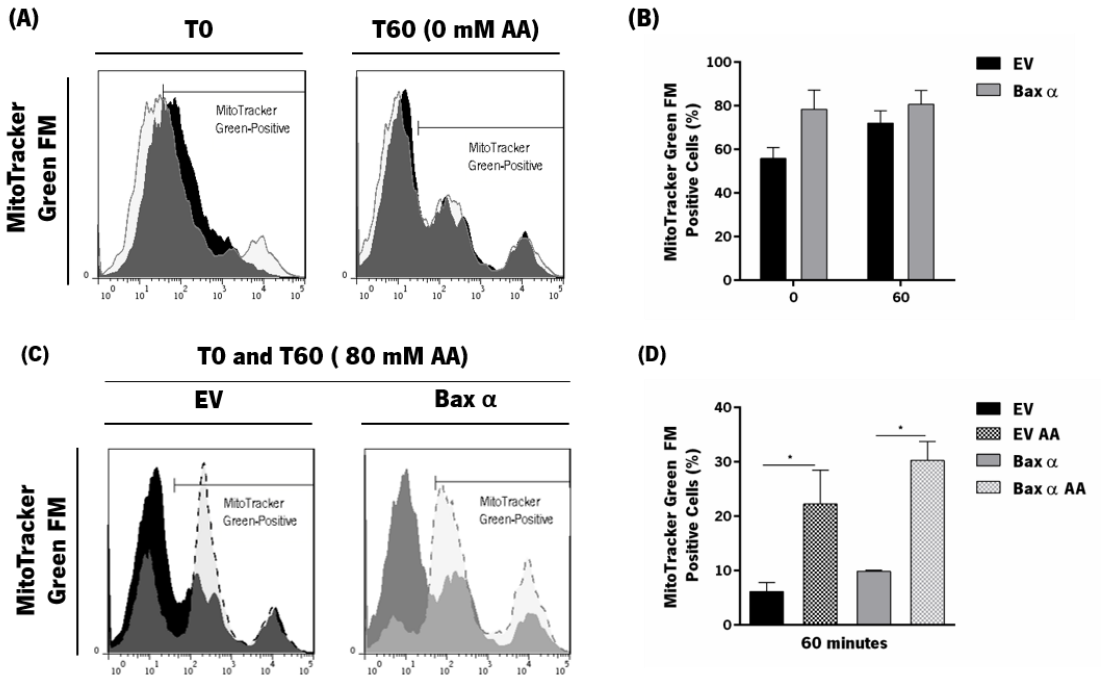

Figure VIII.4: Yeast cells expressing Bax α as well as control cells exhibit mitochondrial mass increase after treatment with sub-lethal concentrations of acetic acid. (A) Representative monoparametric histograms of MitoTracker Green FM-stained cells fluorescence intensity at T0 and 60 minutes after transfer to medium without acetic acid, pH 3.0. Black histograms correspond to control cells and grey histograms correspond to Bax α-expressing cells. Cursors are positioned to the right side of autofluorescence. **(B)** Quantification of the gated MitoTracker Green FM-positive cells shown in (A). The values displayed correspond to the mean of three independent experiments analysed by one-way ANOVA. **(C)** Representative monoparametric histograms of MitoTracker Green FM-stained cells fluorescence intensity 60 minutes after exposure to 0 mM or 80 mM acetic acid, pH 3.0. Full line histograms correspond to non-treated cells (0 mM AA) and punctuated line histograms correspond to acetic acid treated cells (80 mM) pH 3.0. **(D)** Quantification of the gated MitoTracker Green FM-positive cells shown in (C). The values displayed are the mean of three independent experiments analysed by one-way ANOVA, where *p<0.05.