Universidade do Minho
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Modulation of Bax by PKCε: An approach to eliminate cancer cells

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Tese de Mestrado em Bioquímica Aplicada

Trabalho efectuado sob a orientação de
Doutora Susana Chaves
Professora Doutora Manuela Côrte-Real

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DECLARAÇÃO

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Soraia Pereira

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Modulation of Bax by PKCε: An approach to eliminate cancer cells

Abstract

Apoptosis is a type of cell death, known as a highly regulated program of cellular suicide; however this process is often deregulated in tumor cells. A mechanism used by tumor cells to evade apoptosis is the alteration of the different members of the Bcl-2 family. These proteins control a key step in the intrinsic apoptotic pathway: permeabilization of the outer mitochondrial membrane and release of several apoptotic factors. The pro-apoptotic members Bax and Bak are essential for this permeabilization, since deletion of either protein compromises the intrinsic apoptotic pathway. These proteins may be regulated by members of the Bcl-2 but also by other proteins, such as the protein kinase C (PKC) family.

The PKC family consists of kinases that regulate a variety of cellular functions. Of these, PKCepsilon (PKCε) is involved in carcinogenesis and has been considered an oncogene. It has been shown that this protein increases the expression of anti-apoptotic members of the Bcl-2 family and inhibits the pro-apoptotic members such as Bax. The interaction between Bax and PKCε in mammalian cells prevents Bax translocation to the outer mitochondrial membrane, leading to a decrease in its activation. Identifying residues required for this interaction is therefore crucial to design a strategy to inhibit it.

In this work, we aimed to identify residues required for the Bax/PKCε interaction. For this purpose, different domains of PKCε cloned in frame with the FLAG epitope in a yeast inducible expression vector were co-expressed with Bax in *Saccharomyces cerevisiae*, in order to evaluate their interaction with Bax. An alternative approach using yeast-two-hybrid was also pursued to verify the interaction between these two proteins. Our ultimate goal is to develop specific modulators to target the PKCε/Bax interaction that could open the door to new clinical opportunities for cancer treatment.

Modulation of Bax by PKCε: An approach to eliminate cancer cells

Resumo

A apoptose é um tipo de morte celular, mais conhecida como um programa de suicídio celular altamente regulado. No entanto, em células tumorais este processo está frequentemente desregulado. Um mecanismo usado pelas células tumorais para escapar à apoptose é a alteração dos níveis de expressão dos diferentes membros da família Bcl-2. Estas proteínas controlam um passo essencial da via apoptótica intrínseca, a permeabilização da membrana mitocondrial externa e a libertação de vários fatores apoptóticos. Os membros pró-apoptóticos Bax e Bak são essenciais para esta permeabilização, pois a deleção de ambas as proteínas compromete a via apoptótica intrínseca. Estas proteínas podem ser reguladas pelos membros da família Bcl-2 mas também por outras proteínas, como as proteínas da família da cinase C PKC).

A família PKC é uma família de cinases que regulam uma grande variedade de funções celulares. Em particular, a PKCepsilon (PKCε) está envolvida na carcinogénese e tem sido considerada um oncogene. Tem sido demonstrado que esta proteína aumenta a expressão dos membros anti-apoptóticos da família Bcl-2 e inibe os membros pró-apoptóticos, como a proteína Bax. A interação entre a PKCε e a proteína Bax em células de mamíferos leva a que haja uma diminuição da ativação da Bax e da sua translocação para a membrana mitocondrial externa, impedindo que a Bax desempenhe a sua função na apoptose. Assim, a identificação dos resíduos responsáveis para esta interação torna-se extremamente importante para que se consiga encontrar um método de a inibir.

Neste trabalho pretendeu-se identificar os resíduos necessários para essa interação. Para este efeito, os diferentes domínios de PKCε clonados como fusões com o epítopo FLAG num vector de expressão indutível em levedura, foram co-expressos com Bax em *Saccharomyces cerevisiae* a fim de avaliar a sua interação com Bax. Uma abordagem alternativa usando a técnica de Yeast-two-hybrid também foi efetuada para verificar a interação entre PKCε e Bax. O objetivo final seria o desenvolvimento de moduladores específicos para inibir a interação PKCε/ Bax de modo a abrir a porta para novas oportunidades clínicas no tratamento de cancro.

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Abbreviations

Ac-CoA- acetyl-coenzyme A
AIF – Apoptosis Inducing Factor
ANT - Adenine Nucleotide Translocator
Apaf-1 - Apoptotic Protease Activating Factor-1
Asn - asparagine
Asp - aspartic acid
ATP - Adenosine Triphosphate
c-FLIP - Cellular-FLICE (FADD-like IL-1β-converting enzyme)-inhibitory Protein
Caspases - Cysteine-dependent Aspartate-specific Proteases
Cyt *c* – Cytochrome *c*
DD - Death Domain
DED - Death Effector Domain
DISC - Death Inducing Signaling Complex
DNA - Deoxyribonucleic Acid
DR - Death Receptors
DTT - Dithiothreitol
Endo G - Endonuclease G
ER - Endoplasmatic Reticulum
FADD - Fas-Associated Death Domain
Glu – glutamic acid
h – hours
H_2O- water
HtrA2/Omi - High Temperature Requirement Protein A2
IAPs - Inhibitors of Apoptosis Proteins
IMM - Inner mitochondrial membrane
MAC - Mitochondrial Apoptosis-induced Channel
Met - methionine
MOMP - Mitochondrial Outer Membrane Permeabilization
NADH - Nicotinamide Adenine Dinucleotide
NADPH - Nicotinamide Adenine Dinucleotide Phosphate
Nat – N-terminal acetyltransferase
min - Minutes
NE – Nuclear envelope
NF-KB - Nuclear factor kappa-light-chain-enhancer of activated B cells
OD – Optical density
OMM - Outer mitochondrial membrane
PCD – Programmed Cell Death
PCR - Polymerase Chain Reaction
PTP - Permeability Transient Pore
RACKs - receptors for activated C kinases
ROS - Reactive Oxygen Species
rpm – Rotations per minute
sec - Seconds
Smac/Diablo - Second mitochondria-derived Activator of Caspases/Direct Inhibitor of Apoptosis Protein (IAP)-Binding Protein With Low Pi
TNF- Tumor Necrosis Factor α
TNF-R - Tumor-Necrosis Factor Receptor
TRADD - TNF-R-Associated Death Domain
TRAIL-R - TNF-related Apoptosis-Inducing Ligand Receptor
VDAC - Voltage-Dependent Anion Channel

Preamble

The work developed in the scope of this thesis includes the study of Bax modulation by PKCε initially planned and presented in chapter 1, and a study of Bax modulation by Nt-acetyltransferase B reported in chapter 2.

Chapter1.
Bax modulation by PKCε

1. General Introduction

1.1. Cell death

The historical development of the concept of cell death originated in 1880 when Weigert and Cohnheim described the appearance of necrotic cell death in tissue by microscopy. In 1885, Flemming described the process chromatolysis (first name of apoptosis) (Majno & Joris, 1995), in which the nucleus of mammalian ovarian follicles broke and disappeared by a process of cell death (Kam & Ferch, 2000). Since the mid 19th century, many observations indicate that cell death plays an important role in the physiological processes of multicellular organisms, particularly during embryogenesis and metamorphosis. The term programmed cell death (PCD) was introduced in 1964 (Majno & Joris, 1995), suggesting that cell death during development is not accidental in nature, but follows a controlled sequence of steps leading to cellular destruction. In turn, the term apoptosis, a form of PCD, was first used in articles by Kerr, Wyllie and Currie in 1972 to describe a morphologically distinct form of cell death, although some features of apoptosis were explicitly described many years before (Elmore, 2007). Apoptosis was later recognized and accepted as an important characteristic mode of PCD with distinct morphologic characteristics from those found in necrosis. Nowadays, the Nomenclature Committee on Cell Death proposes the term regulated cell death (RCD) as a broader term for a genetically controlled death, in contrast to accidental cell death, and reserves the term programmed cell death for RCD instances that occur as part of a developmental program or to preserve physiologic adult tissue homeostasis. The three major pathways of RCD are: apoptotic, necrotic and autophagic (Galluzzi *et al.*, 2015).

1.2. Apoptosis

The term apoptosis is derived from the Greek meaning “dropping off,” and refers to the falling of leaves from trees in autumn. Since apoptosis was described by Kerr et al in the 70´s, it is used in contrast to necrosis, to describe the situation in which a cell actively pursues a course toward death upon receiving certain stimuli (Wong, 2011). This process normally occurs during differentiation, aging, and as a homeostatic mechanism to maintain populations of cells in the tissue (Elmore, 2007).

Apoptosis and necrosis are the two modes of cell death whose molecular mechanisms have been studied in most detail. Although originally thought to be mutually exclusive, recent discoveries show cellular contexts that require a balanced interaction between these two modes of cell death (Nikoletopoulou *et al.*, 2013).

Autophagy is predominantly a cytoprotective process that begins with formation of autophagosomes, double membrane-bound structures surrounding cytoplasmic macromolecules and organelles destined for recycling. Autophagy has been associated with necrosis and apoptosis and plays a crucial role in cell survival and homeostasis. However, it also plays a pro-death function since excessive stress results in autophagic cell death (Nikoletopoulou *et al.*, 2013) (Ouyang *et al.*, 2012).

During the early process of apoptosis, cell shrinkage and pyknosis are the most visible morphological characteristics. With cell shrinkage, the cells are smaller in size, the cytoplasm is dense and the organelles are more tightly packed. Biochemical changes include chromosomal DNA cleavage into internucleosomal fragments, phosphatidylserine externalization and cleavage of a number of intracellular substrates by specific proteolysis (Ouyang *et al.*, 2012) (Figure 1).

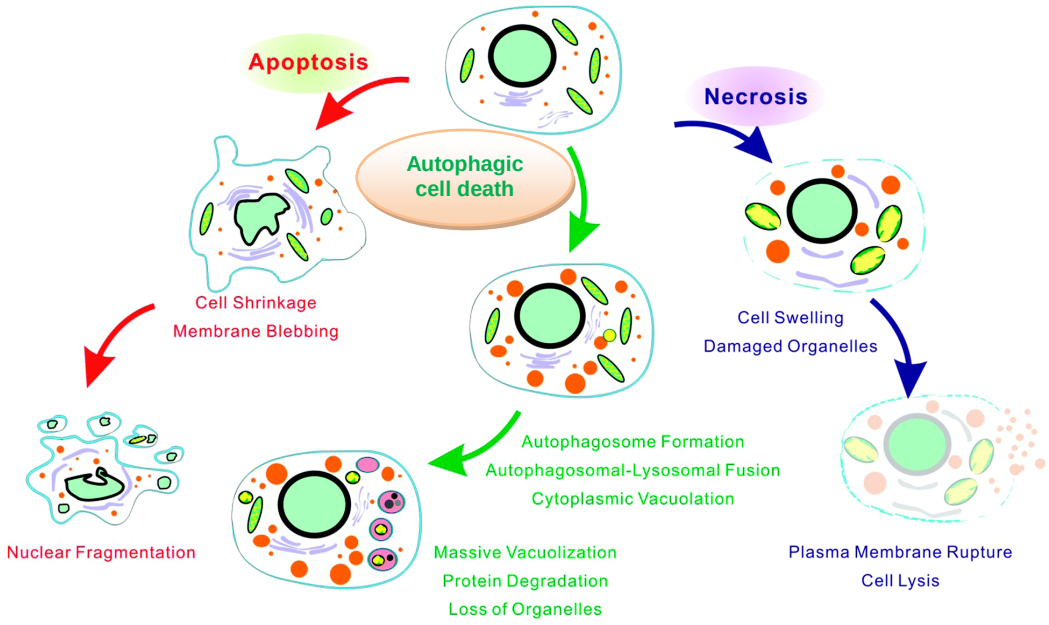


Figure 1. Molecular and morphological events associated with the different types of cell death: apoptosis, autophagic cell death and necrosis (Tan *et al.*, 2014).

1.2.1. Apoptotic pathways

Similarly to other pathways, apoptosis can be initiated by activation of various internal and external factors, such as activation of death receptors at the plasma membrane or through mitochondrial membrane permeabilization.

The two most common apoptotic pathways are usually described as intrinsic (mitochondrial) and extrinsic (death receptor), which eventually can lead to a common

path; the third is less well known, and is executed through the endoplasmic reticulum (Wong, 2011) (Figure 2).

1.2.1.1. Major apoptotic pathways and their regulation

The extrinsic pathway is one of the best characterized apoptotic signaling pathways and is triggered when death ligands bind to a death receptor. Although several death receptors have been described, the best characterized are the tumor necrosis factor TNF 1 receptor (TNFR1) and Fas (CD95); its ligands are referred to as TNF and Fas (FasL), respectively (Wong, 2011). In Fas signaling, FasL binds to Fas, leading to receptor trimerization. Adaptor proteins bind to the cytosolic death domains (DD) of Fas protein (Fas-associated death domain,FADD) via their DDs. In addition, FADD contains a death effector domain (DED), to which the DED of pro-caspase-8 can bind. The complex of Fas, FasL, FADD and pro-caspase-8 is called the DISC. The procaspase-8 molecules are brought into close proximity in the DISC, so that they can transactivate one another. Active caspase-8 then can directly cleave caspase-3 or other executioner caspases, eventually leading to the apoptotic outcome. Its active form, caspase 3, will cleave the inhibitor of caspase-activated deoxyribonuclease, responsible for nuclear apoptosis (Lawen, 2003). DISC signaling can be inhibited by c-FLIP, a dominant negative caspase-8, which leads to the formation of a signaling-inactive DISC.

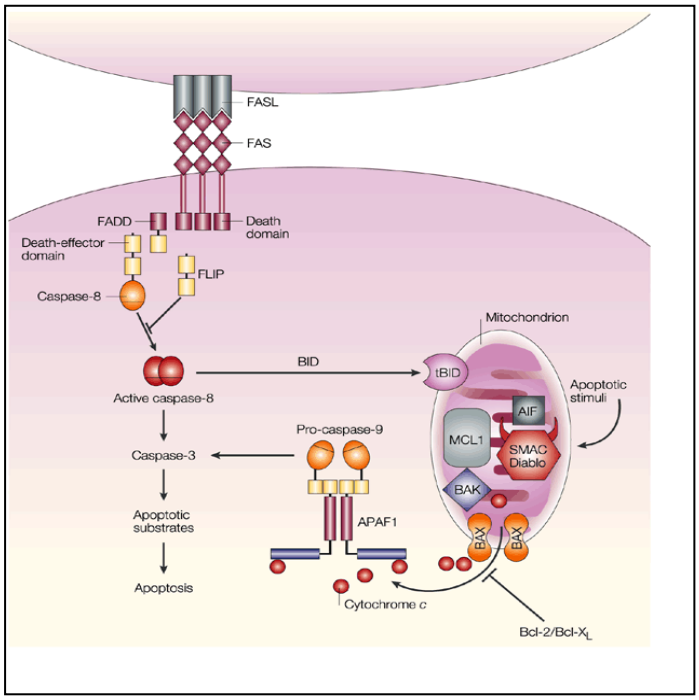


Figure 2. Schematic representation of death receptor (DR) and mitochondrial pathways for the induction of apoptosis (Pope, 2002).

The intrinsic apoptotic pathway may be activated in response to stress conditions, such as hypoxia, oncogene activation, or DNA damage caused by radiation or chemicals (Jia *et al.*, 2012). The apoptotic signal leads to the release of cytochrome *c* (cyt *c*) from the mitochondrial intermembrane space into the cytosol, where it binds to the Apoptotic Protease Activating Factor-1 (Apaf-1).

The mitochondrial pathway of apoptosis begins with permeabilization of the mitochondrial outer membrane (MOMP). The mechanisms through which this occurs remain controversial, thought permeabilization is usually characterized as either permeability transition pore- (PTP) dependent or independent. The exact molecular composition of the PT is still unknown but the main components are the matrix protein cyclophilin D, the inner mitochondrial membrane protein adenine nucleotide translocator (ANT**),** and the outer mitochondrial membrane protein voltage dependent anion channel (VDAC) (Green & Kroemer, 2004).

In response to apoptotic stimuli, pro-apoptotic members of the Bcl-2 protein family (Bax and Bak) become activated and act on the mitochondria to induce the release of cyt *c*. Other pro-apoptotic proteins are also released by the mitochondria, including Smac/Diablo (Second Mitochondrial derived activator of Caspase/Direct IAP-Binding protein with a Low pI), the serine protease Omi/HtrA2, endonuclease G (EndoG) and apoptosis inducing factor (AIF) (Portt *et al.*, 2011).

The cytoplasmic release of cyt *c* will activate caspase 3 by forming a complex known as the apoptosome, which is formed by binding of cyt *c* to the cytoplasmic adapter protein Apaf-1, ATP or dATP, which recruits pro-caspase 9 (cysteine protease); the heptamer formed by these apoptotic factors activates pro-caspase 3. Smac/DIABLO released from the mitochondrial intermembrane space enhances this cascade by binding to IAP (inhibitor of apoptosis protein), which subsequently leads to disruption of the interaction between IAPs and caspase 3, promoting apoptosis (Sukhanova *et al.*, 2012).

1.2.2. The Bcl-2 family members

The Bcl-2 gene was first identified by its involvement in B-cell lymphomas, and encodes a protein of 25-26 kDa. The proteins of the Bcl-2 family do not just regulate apoptosis, they also have alternate functions in other homeostatic pathways, including glucose metabolism, cell cycle checkpoints, DNA damage and regulation of mitochondrial morphology (Danial, 2007).

The Bcl-2 family consists of at least thirty related proteins characterized by the presence of up to four conserved domains called BH (Tzifi *et al.*, 2012). Most members

of the Bcl-2 family contain a transmembrane domain in their C-terminus, which encodes a stretch of hydrophobic amino acids important for targeting to intracellular membranes, closely associated with their ability to regulate apoptosis. Subcellular localization studies confirmed that proteins of the Bcl-2 family are present in the mitochondrial outer membrane (MOM), in the nuclear membrane and in the endoplasmic reticulum (Brown, 1997). The BH4 domain of Bcl-2 comprises 26 amino acids and the structure shows an amphipathic character after interaction with membranes, like antimicrobial peptides (Tzifi *et al.*, 2012).

The Bcl-2 family of proteins is divided into two different sub-classes based on structural and functional characteristics: the anti-apoptotic members such as Bcl-2, Bcl-xL, Bcl-w and MCL-1 contain all four subtypes of BH domains, and promote cell survival by inhibiting the function of pro-apoptotic members. The pro-apoptotic members can be separated into two structurally distinct subfamilies: 1) "multidomain" proteins Bax and Bak, which share three BH regions and lack the BH4 domain. They are structurally similar to the anti-apoptotic proteins; 2) BH3-only proteins, including BNIP3, Nix/Bnip3L, Bid, Noxa, Puma, Bim and Bad, share only the BH3 domain and are structurally diverse (Figure 3) (Gustafsson & Gottlieb, 2007).

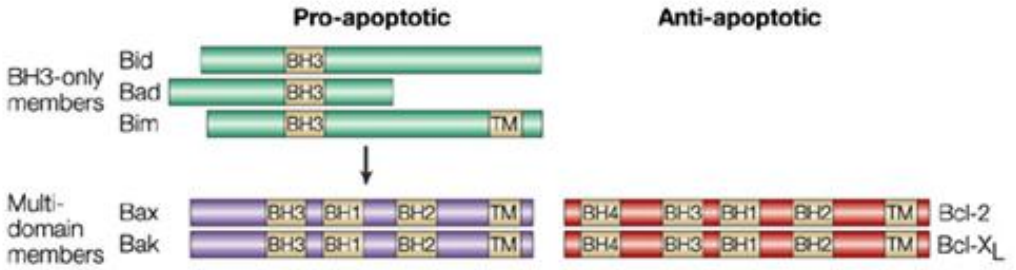


Figure 3. Schematic representation of members of each Bcl-2 sub-family. Bcl-2 homology (BH) and the transmembrane domains (TM) are indicated. Image adapted from (Jesenberger & Jentsch, 2002).

Expression of Bcl-2 or other related anti-apoptotic proteins, including myeloid cell leukemia-1 (Mcl-1), Bcl-XL, Bcl-w and Bcl-2-related protein A1 (Bfl-1), block cell death in response to many stimuli by preventing the activation and homooligomerization of both Bax and Bak. Anti-apoptotic proteins perform their anti-death function by sequestering BH3-only proteins or inactivating Bax and Bak. Cells that survive continuous, permanent death signaling owing to the presence of Bcl-2 are dependent on Bcl-2 for their survival (Figure 4) (Brunelle & Letai, 2009).

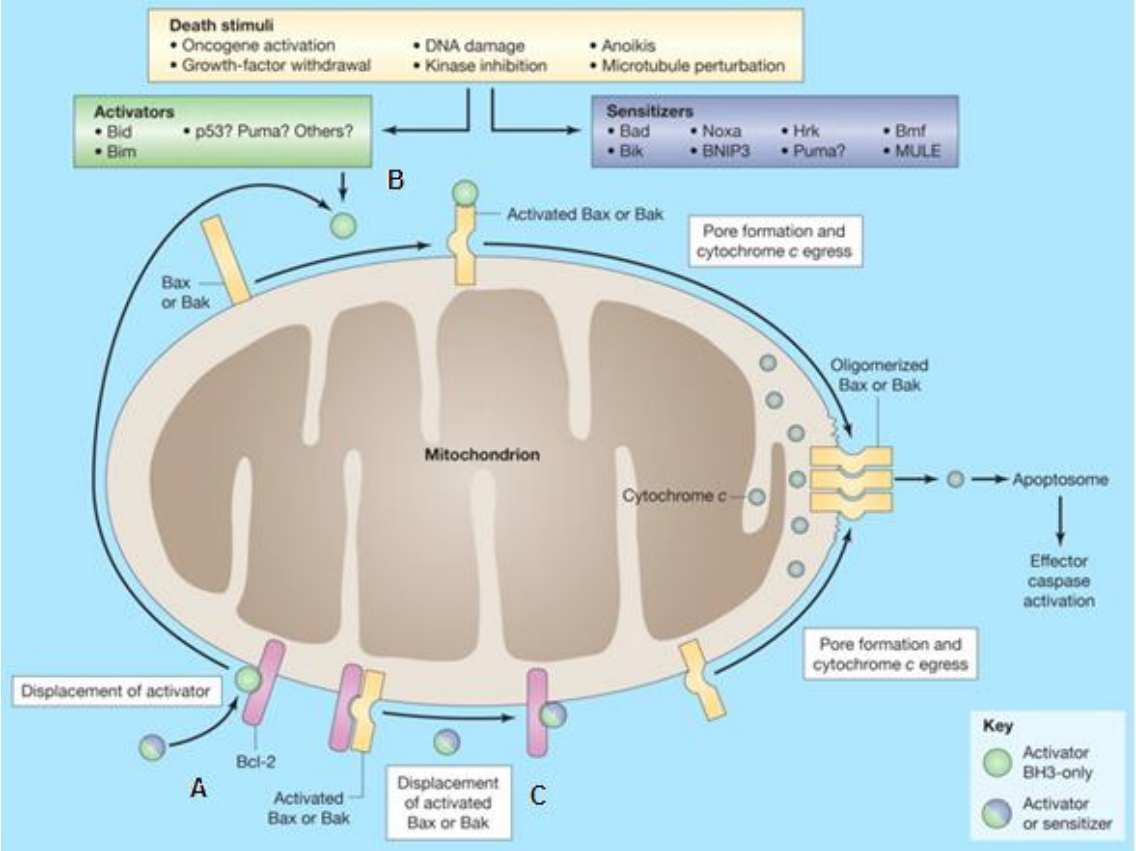


Figure 4. Models of Bcl-2 protein regulation in apoptosis. A: BH3-only proteins bind to and neutralize anti-apoptotic Bcl-2 proteins, allowing Bax/Bak to become activated and initiate apoptosis. B: BH3-only proteins directly activate pro-apoptotic Bax/Bak protein. C: Anti-apoptotic Bcl-2 proteins sequester BH3-only proteins and keep them inactive. Image adapted from (Brunelle & Letai, 2009).

BH3 proteins with high affinity for binding and activating Bax and Bak are designated as “activators,” and those that only bind to antiapoptotic proteins are called “sensitizers.” The activators interact and activate Bax and Bak to promote MOMP while sensitizer proteins compete for binding with the antiapoptotic proteins releasing the activators and promoting MOMP through activation and oligomerization of Bax and Bak (Shamas-Din *et al.*, 2013). The antiapoptotic proteins inhibit MOMP by sequestering the BH3 activators.

1.2.2.1. Bax

Bax resides mainly in the cytosol and translocates to the mitochondria after induction of apoptosis. The mitochondrial form of Bax found in non-apoptotic cells is a monomer of 21 kDa weakly associated with the MOM or soluble in the cytosol. After induction of apoptosis, the monomer progresses to a complex of high molecular weight (96-260 kDa), suggesting that the oligomerization of Bax occurs after insertion in the MOM (Cartron *et al.*, 2008) (Er *et al.*, 2006). A small portion of Bax was also found in the endoplasmic reticulum, although the function of this subpopulation remains undetermined. The Bax protein is composed of nine helices connected by short loops. Three of these helices, namely α5, α6 and α9, are probably involved in the interaction of Bax with the mitochondrial outer membrane. Indeed, the helices 5 and 6 of the Bax hydrophobic hairpin may independently form pores in lipid membranes (Walensky & Gavathiotis, 2011). The α9 helix is hydrophobic, and although it has the necessary characteristics of a transmembrane helix, experimental data suggest that it is not a typical transmembrane anchor of α-helices of the C-terminal of anti-apoptotic proteins of the Bcl-2 family (Renault & Manon, 2011).

The first nineteen residues of Bax are very mobile and data suggests that removal of these residues, leading to initiation of translation at the Met20 residue, results in a protein with a strong ability to be inserted into the MOM. Renault and Manon suggest that the N-terminal domain of Bax has the ability to lock the protein in a soluble and inactive conformation and that its movement is necessary to facilitate mitochondrial translocation of Bax; this domain is called "Apoptotic Regulation of Targeting" (ART). The ART of Bax contains two proline residues at position 8 and 13, and replacement of Pro8 or Pro13 by Gly promotes mitochondrial translocation of Bax in both yeast and human cells and in human cells undergoing apoptosis. This suggests that interactions between ART residues and residues located in other domains of Bax are central regulators of the movements of ART and subsequently the mitochondrial translocation of Bax (Renault & Manon, 2011).

Homo-oligomerization of Bax requires its BH3 death-domain (the α2 helix). Deletion of Bax segments suggests that its α2–α5 helices alone can oligomerize and that this core, together with α9, suffices for MOMP. Recent crosslinking studies suggest that homo-oligomerization of Bax starts when the BH3 domain of one monomer is exposed and engages the canonical binding groove (mainly α3–α5) of another activated monomer, forming “BH3-in-groove” dimers that multimerize by a separate interface (Czabotar *et al.*, 2013).

1.2.2.2. Bax activation and involvement in MOMP

Several models of MOMP have been proposed: nonspecific MOM rupture or the formation of specific channels in the MOM.

One of the proposed models involves the rupture of the MOM after swelling of the mitochondrial matrix and opening of the PTP. The opening of this channel leads to swelling of mitochondria, which causes rupture of the MOM and the massive release of cyt *c*. However, several studies showed that the swelling is not an absolute pre-requisite for apoptosis *in vivo* and release of cyt *c* can occur in the absence of mitochondrial depolarization (Er *et al.*, 2006).

Other studies have examined the ability of Bcl-2 family proteins to render lipid bilayers permeable to proteins. Tsujimoto *et al* reported that Bax interacts with voltage-dependent anion channel (VDAC), an abundant protein in the MOM; moreover, these investigators observed that Bax stimulated the release of cyt *c*, but not of larger proteins, from liposomes reconstituted with VDAC, apparently through a widening of the VDAC pore just large enough to allow efflux of cyt *c* (Kuwana & Newmeyer, 2003).

Kuwana *et al.* showed that Bcl-2-family proteins can themselves permeabilize lipid bilayers, allowing the release of macromolecules considerably larger than cyt *c*. This group also found that the signature mitochondrial lipid cardiolipin was required not merely for the targeting of Bid to mitochondrial membranes, but also for the membrane permeabilizing activity of Bax (Kuwana & Newmeyer, 2003). Similarly to VDAC, Bax and ANT may also form some kind of protein pore. Additionally, since these two proteins play important roles in facilitating the transport of small metabolites and nucleotides across the mitochondrial membrane, binding of Bax may also contribute to the observed blockage of ATP/ADP exchange and the export of creatine phosphate during apoptosis induced by cytokine withdrawal (Wang, 2001).

Further investigations showed that Bax interacts with a component of the translocase of the MOM called TOM22, and that depleting this component from isolated mitochondria and whole cells decreases Bax translocation and the ability to trigger apoptosis. The interaction between Bax and TOM22 is transient, supporting the hypothesis that association of Bax with the TOM complex is the first step in the formation of another structure containing oligomeric Bax and possibly other mitochondrial targets (Renault & Manon, 2011) (Bellot *et al.*, 2007).

In other models, Bax is constitutively active and therefore must be inhibited by anti-apoptotic proteins for the cell to survive. To initiate apoptosis, BH3 proteins displace Bax from the anti-apoptotic proteins to promote Bax-mediated MOMP. Because BH3 proteins selectively interact with a limited spectrum of anti-apoptotic proteins, a

combination of BH3 proteins is required to induce apoptosis in cells expressing multiple anti-apoptotic Bcl-2 family members (Shamas-Din *et al.*, 2013) (Lalier *et al.*, 2007).
Other studies showed that pro-apoptotic Bax can be inactivated by Ku70, a DNA repair protein, and that Bax is regulated by Ku70-dependent deubiquitynation. In the cytoplasm of a normal cell, Bax forms a complex with Ku70, which inactivates Bax function. The release of Ku70 from the complex induces a conformational change in Bax that allows tBid to bind to Bax. The activated Bax is inserted into the MOM, destabilizing it and therefore cells proceed to apoptosis execution (Yonekawa & Akita, 2008).

Cytoplasmic Bax can undergo large but reversible conformational changes after interacting with the MOM, which increase the affinity for BH3 proteins, causing a further conformational change and allowing insertion in the mitochondrial membrane. This interaction with BH3 proteins incorporates features from two models: displacement and direct activation, because the sensitizer BH3 proteins neutralize the dual function of the anti-apoptotic proteins by displacing both the activator BH3 proteins and Bax from the membrane-embedded conformers of the anti-apoptotic proteins. Because it is the activated form of Bax that is bound to the membrane-embedded anti-apoptotic proteins, sensitizer proteins release Bax conformers competent to oligomerize and permeabilize membranes (Shamas-Din *et al.*, 2013).

An alternative possibility is that activated Bax/Bak form pores directly in the MOM. Amphipathic α-helical peptides can permeate membranes via two separate mechanisms, termed barrel-stave or toroidal. In both models, the helices line the pore perpendicularly to the membrane. The barrel-stave model creates a proteic pore devoid of lipids, while a toroidal pore is composed of protein and lipid components, where Bax inserts three amphipathic helices (5, 6, and 9) into the MOM before oligomerization and MOMP. Electrophysiological studies identified a pore that was termed the mitochondrial apoptosis-induced channel (MAC). MAC contains oligomeric Bax or Bak, providing the first indication that these proteins can create a proteic pore (Shamas-Din *et al.*, 2013).

Since it was identified, Bax has been the subject of many biochemical studies to identify its function at the molecular level. It is known that Bax plays a key role in apoptosis acting on mitochondrial permeability, however the mechanism of action is not yet fully known, since there are other interactions between Bax and other proteins with similar functions to the Bcl-2 family, such as the protein kinase C family.

1.2.3 The protein kinase C family

The protein kinase C (PKC) family was discovered in 1977 by Yasutomi Nishizuka, and is composed of protein kinases activated by several different substrates (Toton *et al.*, 2011). This family of serine/threonine kinases plays key roles in the regulation of many cellular processes, including cell cycle, apoptosis, differentiation, angiogenesis, multi-drug resistance and senescence; however, how PKCs regulate these processes *in vivo* is not evident, since all the cells or tissues express multiple isozymes of PKC (Silva *et al.*, 2012) (Marengo *et al.*, 2011). The PKC family contains at least 12 kinases encoded by nine different genes. These can be classified into three different subfamilies based on their structure and cofactors required for its activation: classical or conventional PKCs (cPKCs: α, βI, βII and γ) that require calcium (Ca^{2+}), phosphatidylserine and diacylglycerol (DAG) for activation; novel PKCs (nPKCs: δ,ε,η and θ) that require phosphatidylserine, DAG but not Ca^{2+} and atypical PKCs (aPKCs: ζ e λ/1) which only require phosphatidylserine for their activation (Silva *et al*, 2012.).

All isozymes of this family have a conserved carboxyl-terminal tail that serves as a phosphorylation-dependent anchoring site for key regulatory molecules and a pseudosubstrate sequence that holds PKCε in an inactive state. The V3 region lies between the regulatory domain and the catalytic domain, and is accessible to proteolytic cleavage by activation and conformational changes of PKCs (Fig.5). Cleavage at this site leads to the release of a constitutively active catalytic domain, suggesting that many inhibitory intramolecular interactions occur between these domains (Marengo et al., 2011).

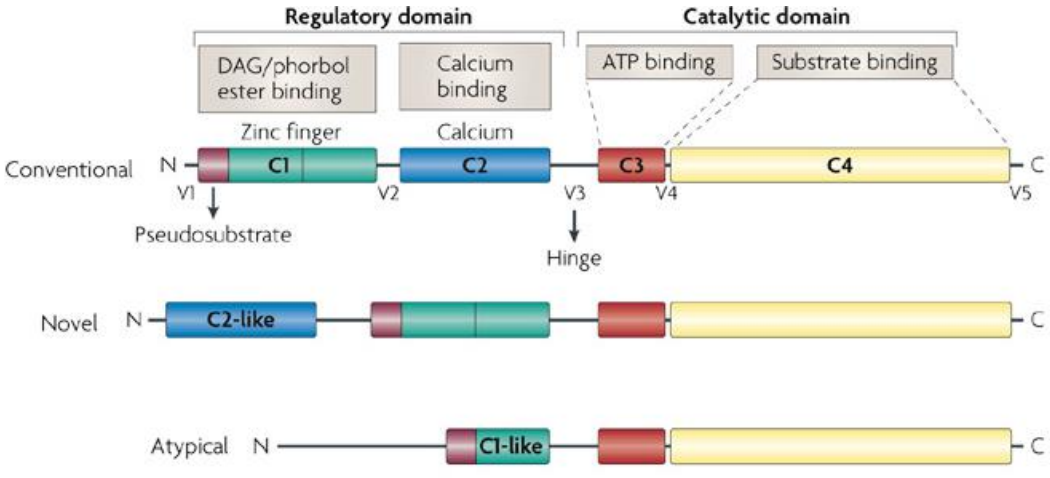


Figure 5. Schematic representation of structure and classification of the family of PKC isozymes (Mackay & Twelves, 2007).

Members of the PKC family of isozymes differ in their structure and mode of activation, but also in their tissue distribution, subcellular localization and substrate specificity. Activation of these isozymes results in changes in their subcellular localization, followed by the translocation of specific anchoring proteins, called receptors of activated C kinase (RACKS) (Mochly-Rosen & Gordon, 1998).

Other anchoring proteins have been suggested as inactivating PKC isozymes, receptors for inactive C-kinase isozymes (RICKS). Mochly-Rosen and colleagues took advantage of isoenzyme-specific interactions between PKCs and RACKS to overcome the lack of specific activators and inhibitors of PKC isozymes. They developed a number of first-generation peptides derived from PKCs or RACKS, based on their interaction sites, which interfere with PKC/RACK protein-protein interactions. By the same principle, peptides that interfere with the PKC/RICK interaction should act as specific agonists of PKCs. The advantages of using these peptides as drugs are their flexibility and fit abilty, since they are naturally selected for a particular protein interaction site, allowing them to interact more effectively and specifically with proteins and interfere with several interaction sites on a protein.

Some of these peptides, such as a PKCε-activator and a PKCδ-inhibitor have been used as pharmacological modulators of PKC activity in animal models of disease and in basic research (Wu-Zhang & Newton, 2013).

1.2.3.1. Regulation of apoptosis by PKC isoforms

The activation of PKCs can induce apoptosis in some cell types, while in other cases prevents it, making it difficult to predict the role of PKCs in the apoptotic process. In general, PKCα, ε, ζ and λ/ι are considered anti-apoptotic, promoting survival and proliferation, while PKCδ is characterized as pro-apoptotic, having a tumor suppressor role (Reyland, 2009).

Conventional PKCs prevent cell shrinkage mediated by FAS since they inhibit the TRAIL death receptor and in turn aggregation of the FAS receptor, recruitment of FADD and DISC formation. In that group, the most studied is the PKCα isoenzyme, which has been associated with the intrinsic pathway due to its ability to mediate expression and phosphorylation of Bcl-2, leading to an increase in the anti-apoptotic function of this protein. PKCα also suppresses drug-induced apoptosis by increasing the promoter activity of the multidrug resistance gene (MDR1), and thus the stability and expression of P-glycoprotein (Lønne, 2010).

The role of PKCβ in apoptosis is contradictory. The gene encoding for this protein creates two isoforms by alternatively splicing, PKCβI and PKCβII, and several studies

have failed to distinguish the contribution of each of these variants to apoptosis. It is believed that PKCβII is involved in the prevention of apoptosis, and expression of the oncogene v-abl causes translocation of PKCβII to the nucleus, preventing apoptosis, and indicating that PKCβII is anti-apoptotic (Gutcher *et al.*, 2003).

The isoenzymes of the novel PKC subfamily PKCδ and PKCε are the best studied. Overexpression of PKCδ stimulates apoptosis in a wide variety of cell types via a mechanism that is not completely understood. When this protein is absent, cells are unable to respond to DNA damage-induced apoptosis, suggesting that PKCδ is required for a response to apoptotic stress (Teicher, 2006). When PKCδ is targeted to the cytosol, mitochondria, or nucleus frequently behaves as pro-apoptotic, while PKCδ targeted to the ER protected against tumor necrosis factor-related apoptosis, ligand-induced apoptosis and etoposide-induced apoptosis. PKCε increases the expression of anti-apoptotic members of the Bcl-2 family and inhibits proapoptotic members of this family, such as Bax and Bad (Sivaprasad *et al.*, 2007). PKCε is the only isoenzyme that is considered an oncogene and regulates cancer cell proliferation through cell signaling by interacting with three major factors RhoA/C, Stat3, and Akt; however, when PKCε is activated it has protective roles in cardiac and brain ischemia, nociception and heat shock response (Jain & Basu, 2014) (Huang *et al.*, 2011).

1.2.3.2. Protein kinase C epsilon

As previously mentioned, PKCε protein belongs to the subfamily of the novel PKCs, whose structure comprises a regulatory N-terminus and a C-terminal catalytic domain with four conserved regions (C1-C4) and a variable region (V3) domain (Figure 6). The variable domain V3 is the region where the PKC isoforms can be cleaved by caspases (Newton, 1995).

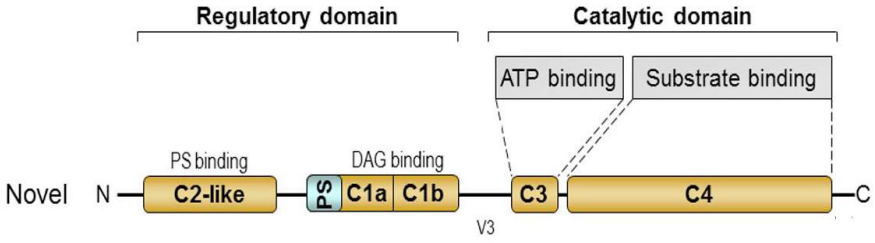


Figure 6. Structural representation of the PKCε protein. Image adapted from Silva *et al.,* 2012

After the discovery of PKCε, there have been many studies on the involvement of this kinase in cell survival and cell death. Though the majority of the studies suggest

that it favors life, some showed that activation of PKCε could contribute to apoptosis. PKCε acts as an oncogene with antiapoptotic effects when overexpressed in cancer cells, and is also oncogenic in colon epithelial cells through interference with Ras signal transduction (Yonekawa & Akita, 2008). Activation of the Ras/Raf/MAP kinase cascade results in the transcription of genes involved in cell proliferation and growth. Stimulation of Ras results in the translocation of Raf-1 from the cytosol to the plasma membrane being activated by a specific set of kinases including PKCε (Toton *et al.*, 2011).The anti-apoptotic ability of PKCε also depends on the increased expression of anti-apoptotic proteins of the Bcl-2 family and the suppression of pro-apoptotic members (Figure 7). Moreover, overexpression of PKCε increases activation of nucleophosmin (NPM), a phosphoprotein capable of inducing carcinogenesis (Gorin & Pan, 2009). PKCε is cleaved by caspases in response to several apoptotic stimuli, including chemotherapeutic agents, starvation and TNF. In one study, cleavage of this protein was inhibited by inhibitor of caspase-3, and PKCε was cleaved by recombinant human caspase-3, suggesting that PKCε is a substrate for this caspase. The results of this study suggest that caspase-7 is the major caspase that cleaves PKCε at the Asp383 site in intact cells (Basu & Sivaprasad, 2007).

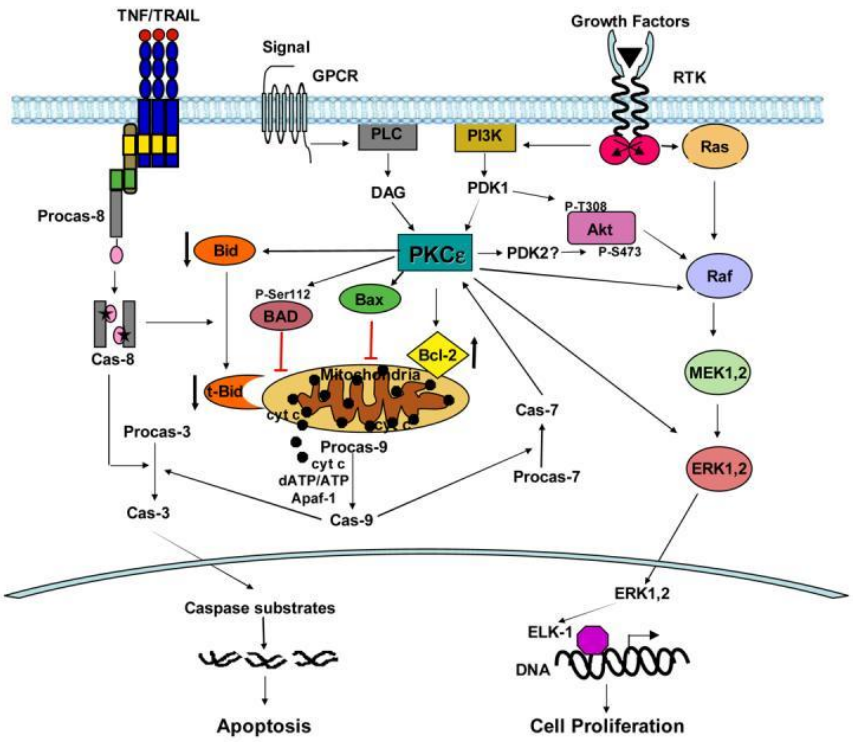


Figure 7. Regulation of cell proliferation and cell death by PKCε (Basu & Sivaprasad, 2007)

The subcellular distribution is an essential feature of activation of PKCε. When PKCε is activated, it inhibits the release of cit *c* from mitochondria, activation of caspases, and suppresses apoptosis. Thus, activation of PKCε in mitochondria is associated with the suppression of cell death and improved cell survival (Nowak *et al.*, 2004). To become responsive to second messengers, PKCε requires the phosphorylation of three conserved sites: Thr-566 in the activation loop, Ser-729 site in the hydrophobic C-terminus, Thr-710 and an autophosphorylation site. The subcellular localization of this kinase will depend in part on the second messenger that is connected to the C1 domain. Thus, PKCε translocates from the plasma and/or membrane to the cytoskeleton in response to DAG and tridecanoic acids, or the Golgi network in response to arachidonic acid and linoleic acid (Akita, 2002).

1.2.3.3. Regulation of Bax by PKCε

The anti-apoptotic properties of PKCε appear to involve the regulation of several members of the Bcl-2 family, increasing the expression of anti-apoptotic members of this family and inhibiting pro-apoptotic members (Silva et al., 2012). Different studies show that PKCε interacts with several proteins, one of which is pro-apoptotic Bax protein, preventing its integration into the mitochondrial membrane. Thus, PKCε allows the survival of various cancer cells by inhibiting Bax conformational rearrangements that are important for its oligomerization, mitochondrial integration and release of cyt *c*, which inhibits cell death. PKCε does not contain a similar BH3 domain, and since the crystal structure of the protein is not entirely known, it is not possible to predict if it has an appropriate interface to directly interact with Bax (McJilton *et al.*, 2003).

The PKCε protein is an important signaling molecule, and understanding how it determines the decision of life or death of a cell will help in understanding the process of carcinogenesis and facilitate the identification of new targets for cancer therapy. In this project, we aim to understand how the interaction between this protein and Bax occurs, so that in the future new drugs to inhibit this interaction and promote cell death can be developed.

1.3. Yeast as a powerful model to study the regulation of Bax

The yeast *Saccharomyces cerevisiae* has already been used as a tool to understand molecular mechanisms of apoptotic pathways, such as the complexities of the function of Bcl-2 family members. The initial observation that the expression of Bax

confers a lethal phenotype in yeast was made during yeast-two-hybrid studies to analyze interactions between Bax and Bcl-2. These results prompted the hypothesis that the mammalian Bcl-2 proteins act on elements of a conserved endogenous yeast machinery to mediate effects on cell viability, and suggest that Bax-mediated cell death in yeast involves a regulated insertion into mitochondrial membranes and mitochondrial dysfunction leading to the release of cyt c and apoptosis, supporting the hypothesis that Bax exerts effects in yeast that are comparable to mammalian cells (Khoury & Greenwood, 2008). Manon and his team suggest that, in yeast cells, Bax induces growth arrest due to defects in the respiratory chain such as a decrease in the amount of the cyt c oxidase (COX) complex and the release of cyt c to the cytosol (Ludovico *et al.*, 2005). Priault *et al* also demonstrated that Bax-c-myc induces a massive release of cyt c in yeast ; the observation that wild-type Bax is unable to induce the cyt c release and yeast cell death, confirm the central role of mitochondria in apoptosis (Priault *et al.*, 2003).

Expression of Bax induces hyperpolarization of mitochondria, production of reactive oxygen species (ROS), cyt c release and mitochondrial network fragmentation reinforcing the importance of mitochondria in Bax-induced death (Silva *et al.*, 2011)

In yeast, there are no known obvious orthologs of Bcl-2 family members; however the core components of the mammalian PTP are highly conserved in eukaryotic organisms. Almost all the studies concerning the role of PTP components in death and mitochondrial permeabilization in yeast have been performed in cells heterologously expressing the pro-apoptotic Bax protein; however, some data suggest that the PTP components do not exhibit a significant role in Bax-induced cell death (Pereira *et al.*, 2008). In fact, was discovered that yeast cells lacking cyt c still die after Bax expression, although at a slower rate, indicating that cyt c release is not essential for Bax-induced cell death (Priault *et al.*, 1999a).

1.4. Aims

The residues of PKCε required for interaction with Bax were never identified. This information is crucial to direct screening of libraries of compounds with affinity for the key residues in the interaction sites to specifically disrupt the PKCε /Bax interaction.

The aim of this study was to identify the PKCε residues required for interaction with Bax, using the following approaches:

1. Assess which domains of PKCε interact with Bax by immunoprecipitation and Yeast-two-hybrid
2. Identify which residues could be involved in this interaction by single substitution of selected residues.

1.5. Materials and Methods

1.5.1. Yeast strains and growth conditions

All strains used in this study are listed in Table I.

For Yeast-two-hybrid (YTH), the PJ69-4a strain was used (Figure 8). This strain has three reporter genes under the control of distinct GAL4 upstream activating sequences (UASs) and TATA boxes (ADE2, HIS3, and lacZ). These promoters yield strong and specific responses to GAL4. As a result, two major classes of false positives are eliminated: those that interact directly with the sequences flanking the GAL4 binding site and those that interact with transcription factors bound to specific TATA boxes.

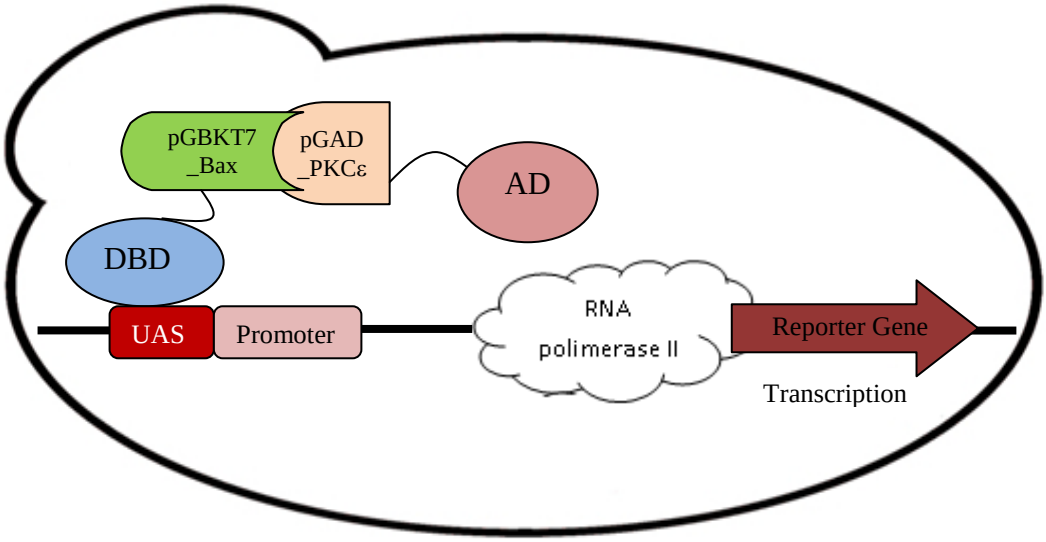


Figure 8. The classical YTH system. The protein of interest is fused to the DNA binding domain (DBD), a construct called bait. The potential interacting protein is fused to the activation domain (AD) and is called prey. The bait binds the upstream activator sequence (UAS) of the promoter. The interaction of bait with prey recruits the AD and thus reconstitutes a functional transcription factor, leading to further recruitment of RNA polymerase II and subsequent transcription of a reporter gene.

Table I. List of strains used in this study.

Strain	Genotype	Reference
XL1 blue	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173* *endA1 supE44 thi-1 recA1* gyrA96 relA1 lac *[F'* proAB lacI^{q}ZΔM15 *Tn*10 *(Tet^{r})]*	Stratagene
W303-1A	MATa, leu2-3, ura3-1, trp1-1, his3-11, ade2-1, can1-100	(Rothstein, 1983)
PJ69-4a	MATa trp1-Δ901 leu2-3,112 901 ura3-52 his3-Δ200 gal4Δ gal8Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ	(James *et al.*, 1996)

W303-1A pGAD_PKCε	W303-1A harboring pGAD_PKCε	This study
XL1 blue pGBKT7_Bax	XL1 blue harboring pGBKT7_Bax	This study
PJ69-4a pGBKT7_Bax pGAD_PKCε	PJ69-4a harboring pGBKT7_Bax and pGAD424_PKCε	This study
PJ69-4a pAS2_Kap120 pGAD_PKCε	PJ69-4a harboring pAS2_Kap120 and pGAD424_PKCε	This study
PJ69-4a pGBKT7 pGAD_Ran	PJ69-4a harboring pGBKT7 and pGAD_Ran	This study
PJ69-4a pGBKT7 pGAD_PKCε	PJ69-4a harboring pGBKT7 and pGAD424_PKCε	This study
PJ69-4a pAS2_Kap120 pGAD_Ran	PJ69-4a harboring pAS2_Kap120 and pGAD_Ran	This study
W303-1A pYES3 pESC-His	W303-1A harboring pYES3 and pESC-His	This study
W303-1A pYES3 pESC-His C1	W303-1A harboring pYES3 and pESC-His C1	This study
W303-1A pYES3 pESC-His C2	W303-1A harboring pYES3 and pESC-His C2	This study
W303-1A pYES3 pESC-His Kin	W303-1A harboring pYES3 and pESC-His Kin	This study
W303-1A pYES3 pESC-His PKCε	W303-1A harboring pYES3 and pESC-His PKCε	This study
W303-1A pYES3_Baxwt pESC-His C1	W303-1A harboring pYES3_Baxwt and pESC-His C1	This study
W303-1A pYES3_Baxwt pESC-His C2	W303-1A harboring pYES3_Baxwt and pESC-His C2	This study
W303-1A pYES3_Baxwt pESC-His Kin	W303-1A harboring pYES3_Baxwt and pESC-His Kin	This study
W303-1A pYES3_Baxwt pESC-His PKCε	W303-1A harboring pYES3_Baxwt and pESC-His PKCε	This study
W303-1A pYES3_Baxwt pESC-His	W303-1A harboring pYES3_Baxwt and pESC-His	This study
W303-1A pYES3 YEplac181	W303-1A harboring pYES3 and YEplac181	This study
W303-1A pYES3 YEplac181_PKCε	W303-1A harboring pYES3 and YEplac181_PKCε	This study
W303-1A pYES3_Baxwt YEplac181	W303-1A harboring pYES3_Baxwt and YEplac181	This study
W303-1A pYES3_Baxwt YEplac181_PKCε	W303-1A harboring pYES3_Baxwt and YEplac181_PKCε	Silva R.

Strains were transformed with the indicated plasmids using the lithium acetate method as described in section 1.5.3. Transformants were selected on Synthetic Complete medium [SC: 0.17% (w/v) Yeast nitrogen base without aminoacids and

ammonium sulfate, 0.5% (w/v) ammonium sulfate, 0.14% (w/v), drop-out mixture lacking histidine, leucine, tryptophan and uracil, 0.008% (w/v) Histidine, 0.04% (w/v) Leucine, 0.008% (w/v) Tryptophan and 0.008% (w/v) Uracil] lacking the appropriate aminoacids plus 2% (w/v) of carbon source and 2% agar. Yeast strains were maintained on solid Yeast extract peptone dextrose (YPD) or Synthetic complete (SC) medium (lacking the appropriate aminoacids), grown at 30°C for 48h-72h, stored at 4°C, and refreshed every 2-3 weeks. Yeast cultures were grown aerobically in SC medium with 2% Glucose or Galactose as a carbon source or anaerobically in SC medium with 2% Lactacte pH 5.5. Strains transformed with plasmids were grown in SC medium lacking the appropriate aminoacids. Cells were incubated at 30°C with orbital shaking (200 rpm) and a liquid/air ratio of 1:5.

1.5.2. Plasmids

All the plasmids and oligonucleotides used in this study are listed in Tables II and III, respectively. The vector used to express Bax was pGBKT7 (Clontech). The pGBKT7 vector expresses proteins fused to amino acids 1–147 of the GAL4 DNA binding domain (DNA-BD). This vector also has other characteristics which make it suitable for use in the YTH system, such as two independent origins of replication in bacteria and yeast, the Kanamycin resistance for selection in *E. coli* and the *TRP1* auxotrophic marker for selection in yeast. It also has a multiple cloning site (MCS) for insertion of the DNA fragment under consideration.

The vector used to clone PKCε was pGAD424 (Clontech). pGAD424 is a shuttle vector that replicates autonomously in both *E. coli* and *S. cerevisiae* and generates a hybrid protein that contains the sequences for the GAL4 activation domain (DNA-AD). It carries Ampicillin resistance for selection in *E. coli* and the *LEU2* auxotrophic marker that allows yeast auxotrophs carrying pGAD424 to grow on limiting synthetic medium lacking Leucine (LEU).

Table II. List of plasmids used in this study.

Plasmid	Description	Reference
pGBKT7	*TRP1*; *KanR*	Clontech
pGBKT7_Bax wt	Bax wt inserted in pGBKT7	This study
pGAD424	*LEU2;AmpR*	Clontech
pGAD424_PKCε	PKCε inserted in pGAD424	This study
pYES3	*TRP1*; *AmpR*	Invitrogen

YEplac181	*LEU2;AmpR*	(Gietz & Sugino, 1988)
YEplac181_PKCε	PKCε inserted in YEplac181	Nigel Goode
pESC-HIS	*HIS3;AmpR*	Agilent
pYES3_Bax wt	Bax wt inserted in pYES3	Silva R.
pESC-HIS_*C1*	*C1* inserted in pESC-HIS	Vieira S.
pESC-HIS_*C2*	*C2* inserted in pESC-HIS	Vieira S.
pESC-HIS_*KIN*	*KIN* inserted in pESC-HIS	Vieira S.
pESC-HIS_PKCε	PKCε inserted in pESC-HIS	Vieira S.
pAS2_Kap120	Kap120 inserted in pAS2	Chaves S.
pGAD_Ran	Ran inserted in pGAD	Chaves S.

Table III. List of oligonucleotides used in this study.

Number	Name	Oligonucleotide sequence
1	pAS2-1_BaxFw	5´-AAAGACAGTTGACTGTATCGCCGGAATTCATGGACGGT TCCGGTGAACAA-3´
2	pAS2-1_BaxRv	5´- ATTCGCCCGGAATTAGCTTGGCTGCAGTCAACCCATCT TCTTCCAGATGG-3´
3	pGAD_PKCFw	5´-ACCCAAAAAAAGAGATCGAATTCATGGTAGTGTTCAAT GGCCTTCTTAAG-3´
4	pGAD_PKCRv	5´-ATCTACGATTCATAGATCTCTGCAGTTAGGGCATCAGG TCTTCACCAAA-3´
5	pAS2_seq_F	5´-TCATCGGAAGAGAGTAGTAACAAAGG-3´
6	M13Rv	5´-TCCTGTGTGAAATTGTTATCCGCT-3´
7	pGAD_seq_F	5´-CACTACAGGGATGTTTAATACCACTAC-3´
8	pGAD_seq_R	5´-GTTCACTTCAACTGTGCATCGT-3´

pGBKT7_Bax was constructed by standard ligation (Figure 9). Briefly, the Bax gene was amplified by PCR using pYES2_Baxwt (URA) as a template and the PCR product was purified using a DNA Clean & Concentrator™ -5 Kit (Zymo Research) according to manufacturer's instructions. The pGBKT7 vector and purified Bax were digested with *EcoR*I and *Pst*I and the digestion product was purified using a DNA Clean & Concentrator™ -5 Kit (Zymo Research) in order to remove the endonucleases. Ligation was performed using 1μl of vector DNA, 10μl of insert DNA, 2μl of 10X Ligase Buffer, 1μl of T4 DNA ligase and H_2O to a total volume of 20μl, and the reaction was incubated at 4ºC overnight. 10μl of reaction were transformed into *E. coli* XL1 Blue, and cells plated on Luria Bertani medium [LB; 1% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 1% (w/v) NaCl and 2% (w/v) Agar] supplemented with 100 μg/mL Kanamicin. The plasmid was then extracted from *E. coli* using the GenElute Plasmid Miniprep kit (Sigma-Aldrich) and correct integration of the insert was confirmed by restriction analysis, PCR using oligonucleotides 5 and 6, and sequencing.

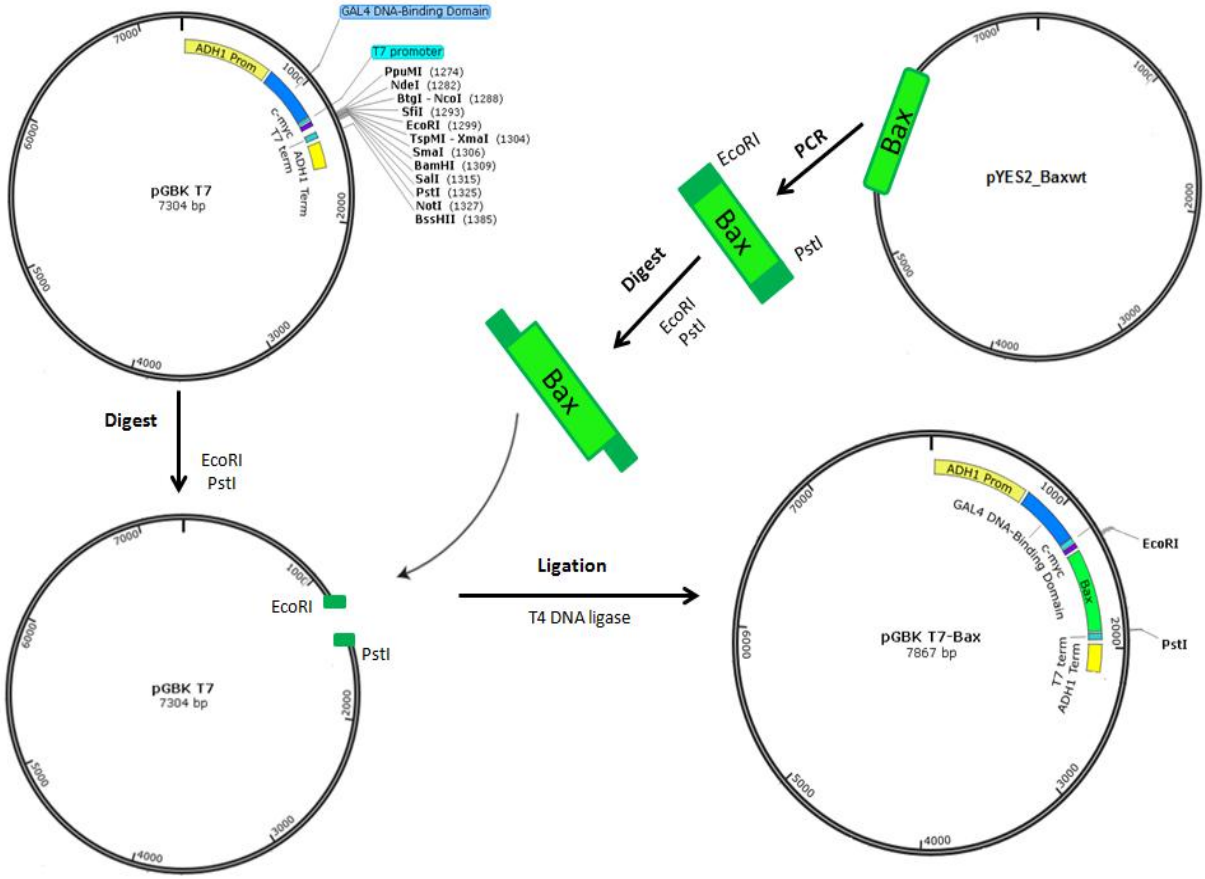


Figure 9. Schematic representation of the methodology used to construct pGBKT7-Bax.

The pGAD-PKCε clone was constructed in W303-1A by homologous recombination (Figure 10). The gene encoding the protein was amplified by Polymerase Chain Reaction (PCR) using the oligonucleotides listed in Table III (numbers 3-4), and co-transformed in yeast with cut pGAD424 through the lithium acetate method (described in section 1.5.3). After growth on SC medium (lacking the appropriate aminoacids) at 30°C for 48h, genomic DNA was extracted from putative positive clones and amplified in *E. coli* XL1 Blue by transformation using standard procedures and selection on LB supplemented with 100 µg/mL Ampicillin. The plasmid was then extracted from *E. coli* using the GenElute Plasmid Miniprep kit (Sigma-Aldrich) and correct integration of the insert was confirmed by restriction analysis and PCR using oligonucleotides 7/8 and by sequencing.

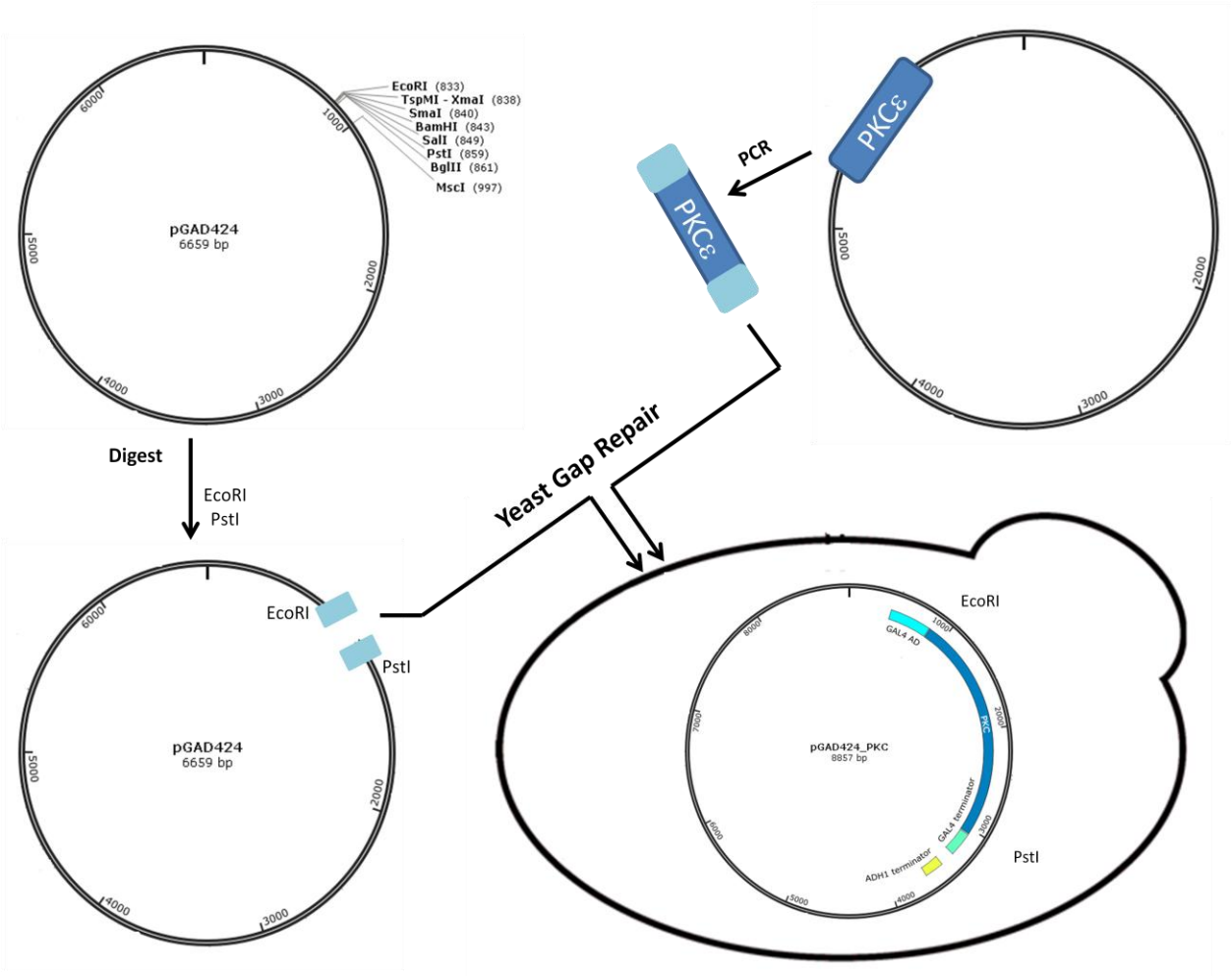


Figure 10. Schematic representation of the methodology used to construct pGAD424-PKCε.

1.5.3. Yeast transformation by the lithium acetate method

All transformations in yeast strains were performed using the lithium acetate method. 240µl of 50% (w/v) Polyethylene Glycol (PEG), 36µl of 1 M LiAc, 40µl of 2 mg/ml salmon sperm DNA (ssDNA) and 36µl (water + plasmid DNA) were mixed, vortexed, and 50µl of competent yeast cells were added. Then, the mix was incubated at 30ºC for 20 minutes, heat shocked for 30 minutes at 42°C and cooled on ice.

Cells were pelleted by centrifugation at 5000 rpm for 3 minutes, and the pellet was resuspended in 100uL of water. The total cell suspension (100µL) was plated on appropriate selection medium consisting of SC medium supplemented with all essential aminoacids, except for the aminoacids of selectable markers on each plasmid and incubated at 30°C until the appearance of colonies.

1.5.4. Growth curves

Yeast strains W303-1A pYES3 pESC-His, W303-1A pYES3 pESC-His C1, W303-1A pYES3 pESC-His C2, W303-1A pYES3 pESC-His Kin, W303-1A pYES3 pESC-His PKCε, W303-1A pYES3_Baxwt pESC-His C1, W303-1A pYES3_Baxwt pESC-His C2, W303-1A pYES3_Baxwt pESC-His Kin, W303-1A pYES3_Baxwt pESC-His PKCε, W303-1A pYES3_Baxwt pESC-His, W303-1A pYES3 YEplac181, W303-1A pYES3 YEplac181_PKCε, W303-1A pYES_Baxwt YEplac181 and W303-1A pYES_Baxwt YEplac181_PKCε were first grown in SC medium with 2% of glucose lacking the appropriate aminoacids. Cells were then transferred to SC medium with 2% of Lactacte, pH 5.5, and grown to an O.D (640nm) of 0.3-0.5 (exponential phase). Then, 2% of galactose was added to induce PKCε and Bax expression. The growth of yeast strains was assessed at different times. All incubations were performed at 30°C, 200 r.p.m.

1.5.5. Analysis of protein expression

1.5.5.1. Preparation of protein extracts

For detection of protein expression by Western blot of total cellular extracts, 5mL of cell culture were grown in SC medium with 2% of glucose and were then transferred to SC medium with 2% of galactose for 16h. Cells were harvested at an O.D (640nm) of 1, and resuspended in 1ml of H_2O. After centrifugation at 5000 rpm for 3 min, the pellet was suspended in 500μl of H_2O and 50μl of a mixture of 3.5% β-mercaptoethanol in 2M NaOH was added. After 15 min incubation on ice, proteins were precipitated with 50μl of 50% (w/v) trichloroacetic acid (TCA) for 15 min on ice. Following centrifugation at 10000 g for 10 min, the pellet was solubilized in 1x Laemmli Buffer (63mM Tris-HCl pH 6.8; 10% glycerol; 0,0005% bromophenol blue; 2% SDS and 0.1M DTT) and the samples were boiled at 100ºC for 5 min in order to denature proteins.

1.5.5.2. SDS gel electrophoresis/Western blot

Total cellular extracts were separated electrophoretically in a 15% SDS polyacrylamide gel at 25 mA and transferred to a Hybond-P Polyvinylidene Difluoride Membrane (PVDF) (Hybond-ECL, GE Healthcare) at 60 mA for 1 h and 30 min. Membranes were blocked for 1h in PBS-T [80 mM Na2HPO4, 20 mM NaH_2PO_4 and 100 mM NaCl with 0.05% (v/v) Tween-20] containing 5% (w/v) non-fat dry milk, washed in PBS-T and then cut into strips and incubated with the primary antibodies: mouse

monoclonal anti-yeast phosphoglycerate kinase (PGK1) antibody (1:5000, Molecular Probes), rabbit polyclonal anti-human Bax (BAX) antibody (1:5000, Sigma), mouse anti-FLAG antibody (Sigma) and rabbit polyclonal anti-human PKCε (PKCε) antibody (1:100, Santa Cruz Biotechnology). Then, membranes were incubated with IgG-peroxidase secondary antibodies against mouse or rabbit (1:5000; Sigma Aldrich). Pgk1p was used as a loading control. Immunodetection of bands was revealed by chemiluminescence (Chemidoc XRS, BioRad).

1.5.6. Immunoprecipitation

Imunoprecipitation was performed using protein G-coupled Dynabeads (Invitrogen). Cells were ressuspended in resuspension buffer (10 mM Tris–malate, 0.6 M Mannitol, 1 mM EGTA, pH 6.7) supplemented with a mixture of protease inhibitors (0.4 µl/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride and phosphatase inhibitors (PhosSTOP phosphatase inhibitor cocktail tablets; Roche Applied Science)). Cells were broken mechanically by vortexing with glass beads, after which 10× IP buffer (1x IP buffer: 50mM Tris-HCl, pH7.5; 100mM NaCl; 2mM EDTA) was added to the cell lysate to a final concentration of 1x, and incubated at 4 °C during 1 h. After incubation with IP buffer, 2 µg of monoclonal anti-FLAG antibody (Sigma) was added, and the lysate incubated overnight at 4 °C. Protein G-coupled Dynabeads (Invitrogen) were added and the extracts incubated for 6 h. Washing was performed with 1x IP buffer and elution was performed with Laemmli sample buffer, followed by denaturing at 65ºC for 30 min.

1.5.7. Yeast-two-hybrid

To verify the interaction between Bax and PKCε proteins, pGBKT7_Bax and pGAD_PKCε vectors were transformed into strain PJ69-4a, as well as various negative controls: pAS2_Kap120-pGAD424_PKCε, pGBKT7-pGAD_Ran and pGBKT7-pGAD424_PKCε and a positive control tested before: pAS2_Kap120-pGAD_Ran.

The cells were plated on appropriate selection medium supplemented with all essential aminoacids, except tryptophan and Leucine (SD -TRP-LEU), and incubated at 30°C until the appearance of colonies.

In order to assess whether the proteins studied are independently capable of activating transcription of the reporter genes or interact with each other, isolated colonies of the yeast strain PJ69-4a pre-transformed with the plasmids mentioned

above were plated on SD -TRP-LEU-HIS and SD -ADE-TRP-LEU). These colonies were also tested for the expression of β-galactosidase (lacZ gene product) by X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) filter-assay as described in (Mockli & Auerbach, 2004).

1.6. Results

1.6.1. Previous Results

Before this study, the DNA sequence coding for PKCε was cloned in the pESC-HIS plasmid, in frame with the FLAG epitope, and transformed into a W303-1A strain expressing Bax. Then, Bax was immunoprecipitated from extracts from this strain with the Bax-2D2 antibody, bound proteins run on an SDS-PAGE gel, transferred to PVDF and probed with an anti-PKCε antibody in order to verify whether PKCε and Bax interact in yeast (Figure 11A, B).

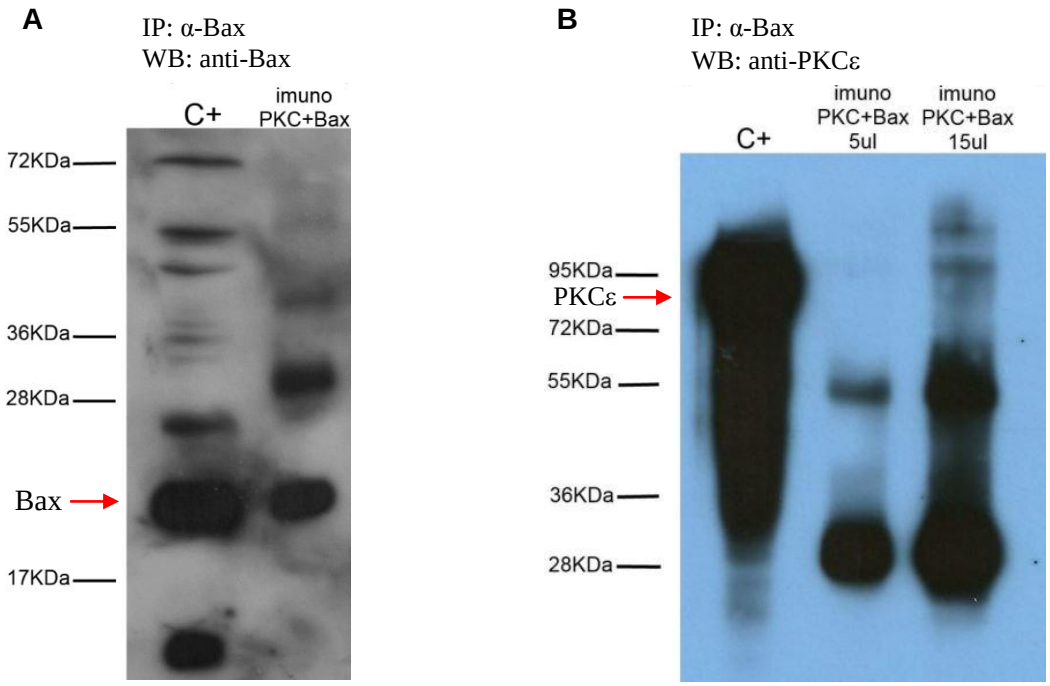


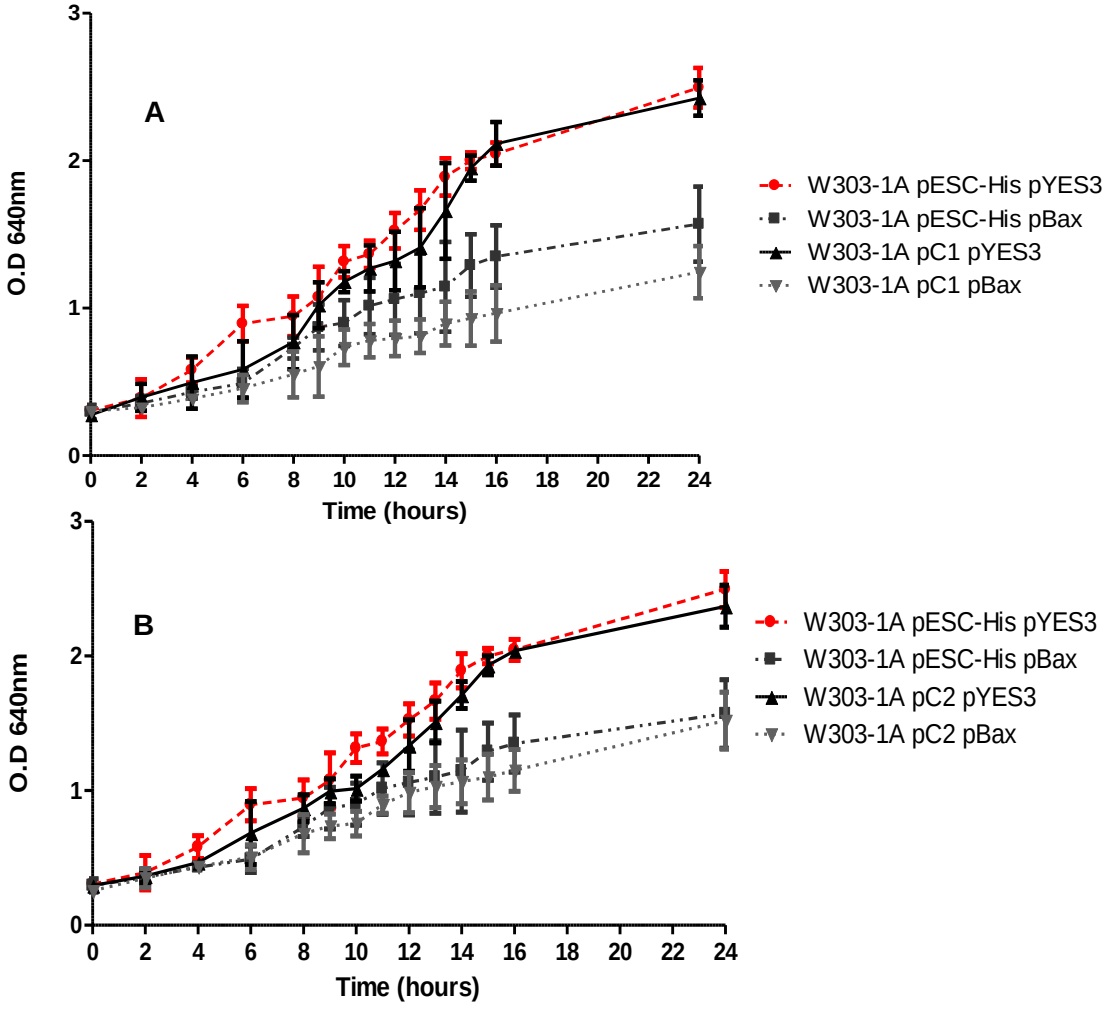
Figure 11. Immunoprecipitation of Bax-interacting proteins. Lysates from cells expressing Bax and PKCε were immunoprecipitated with Bax antibody (2D2). Bound proteins were eluted with loading buffer, denaturated at 65ºC for 30min, run on SDS-PAGE and transferred to PVDF. Western blots were performed with anti-Bax antibody (A) and anti-PKCε antibody (B). C+ WB:anti-Bax was W303-1A pYES3-Baxwt and C+ WB:anti-PKCε was W303-1A YEplac181-PKCε pYES3-Baxwt in order to verify expression of Bax and PKCε, respectively.

However, PKCε was not detected in the immunoprecipitated extract, and therefore an interaction between full length PKCε and Bax could not be demonstrated in yeast.

1.6.2. Effect of expressing Bax and different PKCε fragments on cell growth

Yeast has been considered an important tool to discover the different cellular targets of several proteins. Moreover, the preservation of functional characteristics of mammalian PKCs in yeast led to the identification and characterization of the mode of action of several PKC modulators in yeast cells expressing individual PKC isforms (Sprowl *et al.*, 2007). It had previously been shown that expression of PKCε reverted the slow growth phenotype of yeast cell expressing Bax, indicating PKCε could function as an inhibitor of Bax, presumably by protein-protein interaction (R. Silva, personal communication). This could be a transient or weak interaction, and therefore not detected in the immunoprecipitation studies. We therefore sough to determine whether one or more domains of PKCε had the same effect of the growth of Bax-expressing cells.

In this study, full length and individual domains of PKCε (C1, C2, Kinase) cloned in pESC-His (S. Vieira) were transformed in the W303-1A yeast strain expressing Bax. We next sough to identify which of these domains could be involved in the interaction with Bax by determining if they caused a reversal of the slow growth phenotype of cells expressing Bax (Figure 12 A, B, C, D and E).



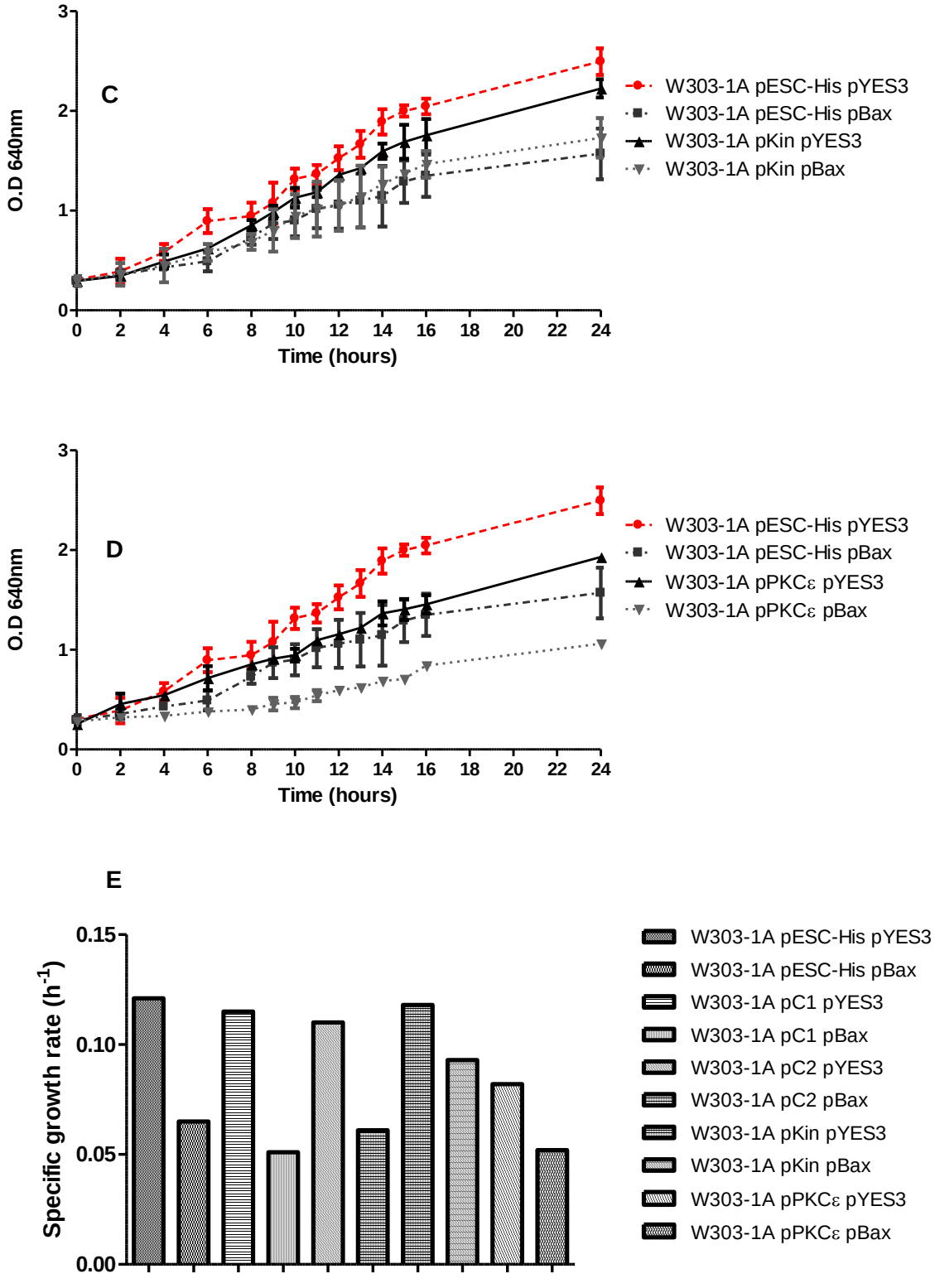


Figure 12. Growth curves of the *S. cerevisiae* strain W303-1A expressing the indicated proteins induced by galactose at T0. **(A)-** Growth curves for yeast strain W303-1A carrying C1 domain (pC1) and empty vector of Bax (pYES3) (•); pC1 plus pBax (■); empty vector of PKCε (pESC-His) plus pYES3 (▲) and pESC-His plus pBax(▼). **(B)-** Yeast strain W303-1A carrying C2 domain (pC2) and pYES3 (•); pC2 plus pBax (■); pESC-His plus pYES3 (▲) and pESC-His plus pBax(▼). **(C)-** Yeast strain W303-1A carrying Kinase domain (pKin) and pYES3 (•); pKin plus pBax (■); pESC-His plus pYES3 (▲) and pESC-His plus

pBax(▼). **(D)-** Yeast strain W303-1A carrying PKCε protein (pPKCε) and pYES3 (•); pPKCε plus pBax (■); pESC-His plus pYES3 (▲) and pESC-His plus pBax(▼). **(E)-** Specific growth rates (h^{-1}) of W303 cells expressing the indicated proteins. Empty plasmids used as a control. These data result from the mean of three independent experiments. Specific growth rate was calculated using the formula $\mu = \frac{\ln X - \ln X_0}{t - t_0}$

Analyzing the data obtained, it appears that there is no reversal in the slow growth phenotype of Bax-expressing cells by PKCε protein. Only expression of the PKCε-Kinase domain partially reverts the slow growth phenotype of Bax-expressing cells. These results are not in agreement with previous data from the lab (R. Silva, unpublished data). We therefore decided to study the growth phenotype of the W303-1A strain expressing Bax and PKCε from the same plasmids used in the previous study, *i.e.* YEplac181 instead of pESC-HIS to express PKCε (Figure 13 A and B). In this case, expression of PKCε is able to partially revert the slow growth phenotype of the W303-1A strain expressing only Bax.

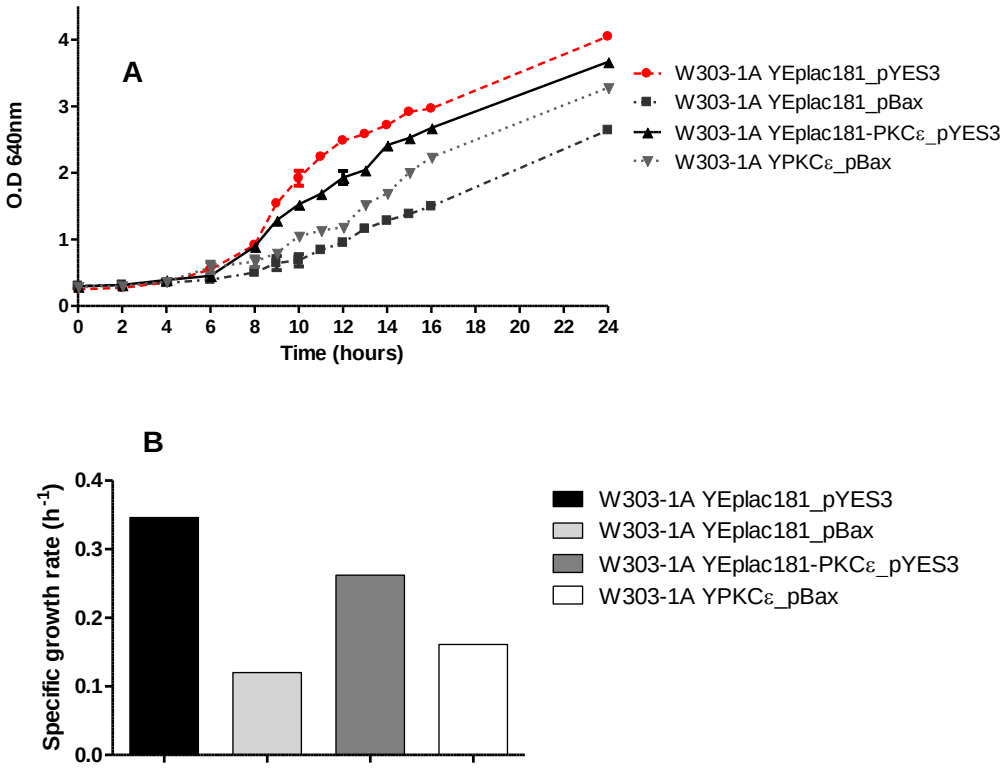


Figure 13. Growth curves of the *S. cerevisiae* strain W303-1A expressing the indicated proteins induced by galactose at T0. **(A)-** Growth curves for yeast strain W303-1A carrying PKCε protein (YPKCε) and empty vector of Bax (pYES3) (•); YPKCε plus pBax (■); empty vector of PKCε (YEplac181) plus pYES3 (▲) and YEplac181 plus pBax (▼). **(B)-** Specific growth rates (h^{-1}) of W303 cells expressing the

indicated proteins. Empty plasmids were used as a growth control. These data result from the mean of three independent experiments. Specific growth rates was calculated using the formula $\mu = \frac{\ln X - \ln X_0}{t - t_0}$

Previous studies by Silva et al. (2012) found that PKCε leads to Bax dephosphorylation and inhibits translocation of Bax into mitochondria. They hypothesized that an interaction between these two proteins in yeast would probably prevent Bax phosphorylation by endogenous yeast kinases by blocking their access to Bax, and would also retain Bax in the cytosol, which would be in agreement with the data from the growth curves.

In order to understand why expression of PKCε from different plasmids led to opposing results, we determined if its levels differed. Western blot of whole cell extracts from strains W303-1A pESC-HIS PKCε-FLAG pYES3-Bax and W303-1A YEplac181-PKCε pYES3-Bax shows that the strain expressing FLAG-tagged PKCε from pESC-HIS has much higher levels of PKCε than the strain expressing untagged PKCε from YEplac 181 (Figure 14).

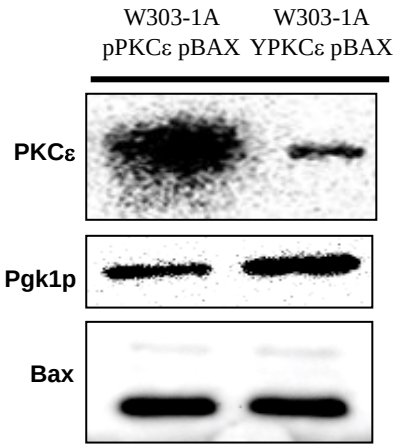


Figure 14. Western Blot of total cellular extracts of *S. cerevisiae* strains W303-1A pPKCε pBax and W303-1A YPKCε pBax. Anti-PKCε and anti-Bax antibodies were used to detect expressing of PKCε and Bax, respectively. Anti-PGK was used as a control.

Expression of Bax appears to be the same in both strains, so a possible explanation for the growth curve differences is that the increased expression of PKCε protein causes increased toxicity and results in a slow growth phenotype, masking any positive effects on cell growth from its presumed interaction with Bax.

1.6.3. Analysis of the interaction between the Kinase domain of PKCε and Bax by immunoprecipitation

Since the PKCε-Kinase domain partially reverts the slow growth phenotype of Bax-expressing cells, immunoprecipitation was performed to detect whether there is an interaction between these 2 proteins (Figure 15).

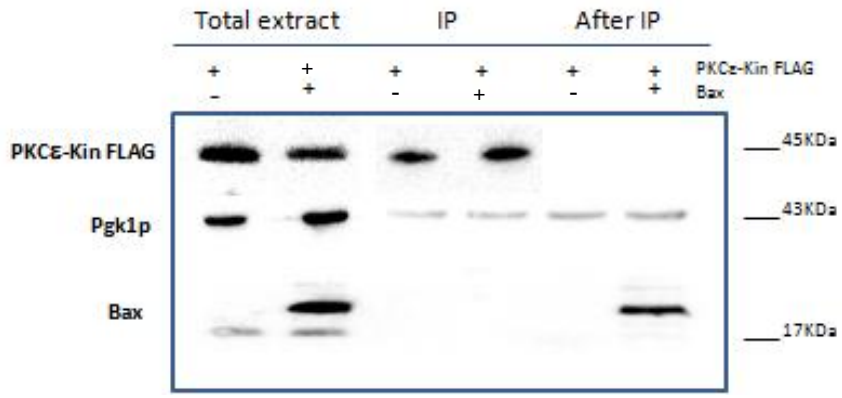


Figure 15. Co-Immunoprecipitation (IP) between PKCε-Kin Flag and Bax. IP of the Kinase domain was performed with an anti-FLAG antibody and Bax and Kin proteins detected with an anti-Bax or anti-FLAG antibody by Western Blot.

As shown in Figure 15, Bax was only detected in total extracts and in the unbound proteins (after the IP with an anti-FLAG antibody). Therefore, we could not detect an interaction between this domain and Bax by IP. This can indicate that the interaction between the kinase domain and Bax may be indirect, weak or transient; hence it was not possible to verify an interaction by this technique.

1.6.4. Analysis of the interaction between PKCε and Bax by Yeast-two-hybrid

The protein interactions play a crucial role in almost of biological processes, from DNA replication, transcription, polyadenylation, splicing, transport or translation. Currently, it is estimated that more than 80% of the proteins are in active protein complexes (Berggard *et al.*, 2007). The yeast two hybrid system (YTH), is one of the most used methods to identify interactions between proteins, mainly because it is simple to implement and is a good approximation of the *in vivo* environment of higher eukaryotes (Van Criekinge & Beyaert, 1999).

The genes encoding the proteins Bax and PKCε were amplified by PCR and then cloned into the vectors pGBKT7 and pGAD424, respectively. Correct insertion of the

Bax and PKCε genes into the plasmids was confirmed by restriction analysis and PCR (Figure 16A and B).

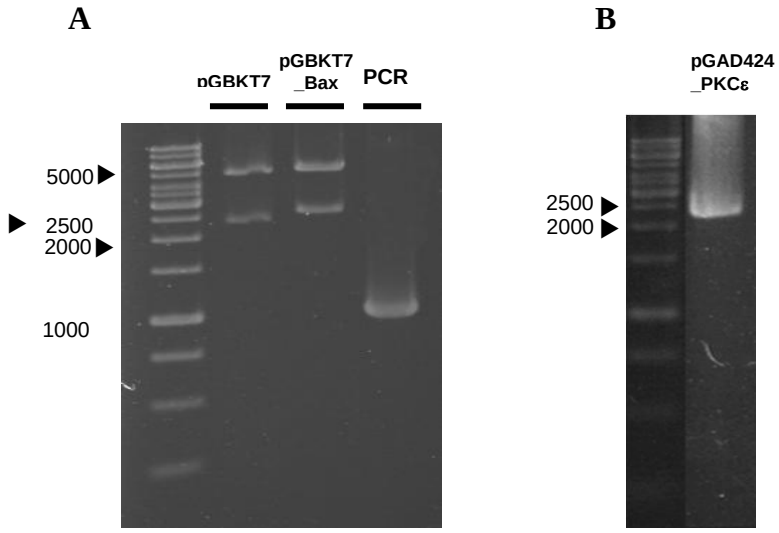


Figure 16. Confirmation gel of Bax and PKCε gene obtained by PCR using oligonucleotides 1, 2, 3 and 4, respectively (see Table III) and digestion of plasmids. **(A)-** Confirmation of the correct insertion of Bax in the pGBKT7 plasmid by digestion the empty plasmid (pGBKT7) and one Bax clone (pGBKT7_Bax) using *EcoR*V and *Sal*I enzymes, and confirmation by PCR using oligonucleotides 5 and 6. **(B)-** Confirmation of the correct insertion of PKCε in the pGAD424 plasmid by PCR using oligonucleotides 7 and 8.

After confirming the correct insertion of genes, the plasmids were sequenced to confirm that there was no mutation that could affect the function of each protein. We then determined whether there is an interaction between them by YTH, as mentioned in the 1.5.7. section.

As described before, in addition to cells expressing pGBKT7_Bax and pGAD_PKCε, we used a previously tested positive control, and several negative controls. Cells were plated on SC-TRP-LEU, after which they replicated onto agar medium without adenine or without histidine, and lifted to filter paper for the X-gal assay (reporter genes) (Figure 17 A and B). As seen in Figure 17A and B, only the positive control used in this assay grows in both media, and turned blue in the X-Gal assay before the negative controls (not shown). Thus, we conclude that PKCε and Bax do not interact in the yeast nucleus, or that the fusion tags prevent these proteins from interacting.

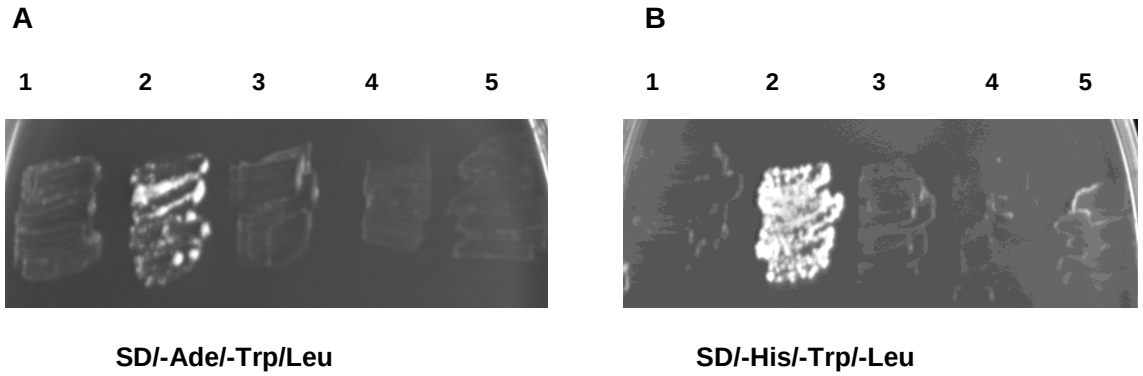


Figure 17. Test self-activating protein reporter genes. Activation of the reporter genes HIS3 and ADE2 was evaluated by the ability of tranformants to grow on plates lacking Adenine (A) or Histidine (B). **1-** pGBKT7_Bax pGAD_PKCε; **2-** pAS_Kap120 pGAD_Ran; **3-** pAS_Kap120 pGAD_PKCε; **4-** pGBKT7 pGAD_Ran; **5-** pGBKT7 pGAD_PKCε to grow on media lacking adenine (B) and histidine (C) (-Ade and -His).

1.7. Discussion and Future perspectives

Several studies have reported that Bax and PKC can interact, leading to higher survival of cells exposed to apoptotic stimuli. McJilton *et al.* showed that PKCε selectively interacts with Bax in the lymph node of prostate carcinoma and suppresses the apoptotic effects of phorbol 12-myristate 13-acetate (PMA). They hypothesized that this interaction can promote cell survival of prostate carcinoma and block PMA-induced apoptosis (McJilton *et al.,* 2003). These results suggested that an interaction between Bax and PKCε inhibits Bax conformational rearrangements that are important for Bax oligomerization, mitochondrial integration, and cyt *c* release. Lu *et al.* (2007) demonstrated in MCF-7 breast cancer cells that overexpression of PKCε inhibited Bax dimerization and mitochondrial translocation in response to TNF. They showed that Bax is required for TNF-mediated cell death in MCF-7 cells and that overexpression of PKCε protected MCF-7 cells from TNF-induced mitochondria.

In this study, yeast was used to verify the interaction between PKCε and Bax, in order to later uncover how to modulate it. Yeast was chosen for this study because it has been a powerful tool to uncover the molecular aspects of several biological processes, including the apoptotic cascade involving mitochondria and several aspects of Bax function. It is particularly relevant that this organism does possess obvious homologues of the mammalian Bcl-2 family proteins, so it becomes easier to study them individually without the interference from other mammalian apoptotic modulators. In addition, the preservation in yeast of the molecular and biochemical functions of mammalian PKCs allowed identifying and characterizing some aspects about the mode of action of PKCε. This favors the use of this simpler model system to identify some of the functions of this family (Silva, 2012).

We first performed an *in vivo* assay based on growth of yeast cells expressing Bax and PKCε or its domains in order to assess which domains of PKCε potentially interact with Bax, reversing the slow growth phenotype of Bax-expressing cells. The results obtained (Figure 12) demonstrated that the PKCε-Kinase domain partially reverts the slow growth phenotype of Bax expressing cells, but so far no interaction was detected *in vitro* by co-immunoprecipitation (Figure 15). However, PKCε-FLAG did not revert the slow growth phenotype of Bax-expressing cells, in agreement with previous results of S. Vieira indicating that no interaction between these proteins was detected *in vitro*, but contrasting those obtained by Silva (unpublished data), who showed that PKCε partially reverts the slow growth of Bax expressing cells (Figure 13). The difference between these studies was the vector in which PKCε was cloned. In this

study, we used the pESC-His vector, a yeast episomal plasmid designed for expression and functional analysis of eukaryotic genes in the yeast *S. cerevisiae*. This vector contains the *GAL1* and *GAL10* promoters in opposite orientation and one or two cloned genes can be introduced into a yeast host strain. A sequence for the FLAG epitope (DYKDDDDK) is located in the multiple cloning site (MCS) downstream of the GAL10 promoter. In the previous study, the vector YEplac181 was used. The main difference between these two vectors is the tag (FLAG epitope). As indicated by the Figure 14, PKCε presents higher levels of expression from the pESC-HIS vector than from vector YEplac181.This may be due to a high percentage of galactose (2%) and the induction time (16h) used. In the future a lower percentage of galactose in a shorter time should be used to induce expression of PKCε-FLAG comparable to that of untagged PKCε from the YEPlac181 vector.

The next step was to use the YTH technique to verify the interaction between the two proteins, since it can detect weak or transient interactions that occur *in vivo*. The YTH system offers several advantages over other biochemical methods, including cost, simplicity and sensitivity. Still there are some problems that have been associated with it. It is assumed that the main disadvantage of the system is the high number of false positives in screens, though they can be identified in subsequent steps with proper controls (von Mering *et al*., 2002). It is also possible there are false negatives, because certain protein interactions are not reproducible in a YTH environment (Stellberger *et al.*, 2010).

In this study, we could not determine that PKCε and Bax do interact in yeast, since the strain expressing these proteins did not grow on plates without His and Ade (Figure 17). This could indicate these proteins do not interact, that the tags used with this technique interfere with the interaction, or that they do not interact in the nucleus. Indeed, McJilton et al. demonstrated that PKCε is associated with numerous proteins and the interaction between PKCε and Bax probably is indirect and mediated by one or several of the intermediate proteins, which may not be present in the nuclear environment. A technical alternative to standard YTH would be the split-ubiquitin two hybrid system, which can detect interactions between proteins in the cytosolic environment, but is technically very challenging.

In order to verify an indirect interaction between Bax and PKCε, it would also be interesting to use an active form of Bax and isolate the mitochondrial and cytosolic fraction of yeast cells to verify the location of Bax and release of cyt *c*. Using this

approach, if Bax and PKCε interact in yeast, Bax cannot oligomerize and be inserted in the outer mitochondrial membrane, remaining into the cytosol, and there will be no release of cyt *c*.

McJilton *et al.* (2003) shows through co-immunoprecipitation assays using a specific cancer cell line that PKCε binds to Bax, and Bax binds to PKCε in LNCaP cells. Therefore, this cell line could be used in future studies to scan for interactions between PKC domains and Bax.

Chapter 2.
Regulation of Bax activation by Nt-acetyltransferase B

2. Introduction

2.1. Nt-acetyltransferases

Post-translational modifications, including phosphorylation, acetylation and others, influence protein function and localization (Yasuda *et al.*, 2013) (Helbig *et al.*, 2010). Protein acetylation is an important modification, significant in several cellular processes and cancer development. One of the most common modifications of proteins in mammals is N-α-acetylation of the amino terminus during *de novo* protein synthesis by N-acetyltransferases (NATs), and is found in 50–70% of yeast proteins and 80–90% of human proteins (Ferrandez-Ayela *et al.*, 2013). Although for majority of proteins its exact biological role has remained enigmatic, in a small number of proteins Nt-acetyation was linked to modulation of various features such as localization, stability and protein-protein interactions. It is therefore considered one of the most important post-translational modifications.

NATs transfer an acetyl group from acetyl-coenzyme A (Ac-CoA) to the N-terminus of the first amino acid residue of a protein (Figure 18). The acetylated amino acid is the initial methionine or the second residue after methionine is cleaved off by methionine aminopeptidase. Several specific molecular functions for Nt-acetylation have been uncovered, such as regulation of protein degradation, prevention of protein translocation from the cytosol to the ER, mediation of protein complex formation and membrane attachment of small GTPases involved in organelle trafficking (Starheim *et al*., 2012). Some studies showed that the catalytic subunits of NATs can also catalyze N-ε-acetylation, which is the acetylation of e-lysine groups on internal sites of proteins, suggesting that they have functions besides the co-translational Nt-acetyltransferase activity (Kalvik & Arnesen, 2013).

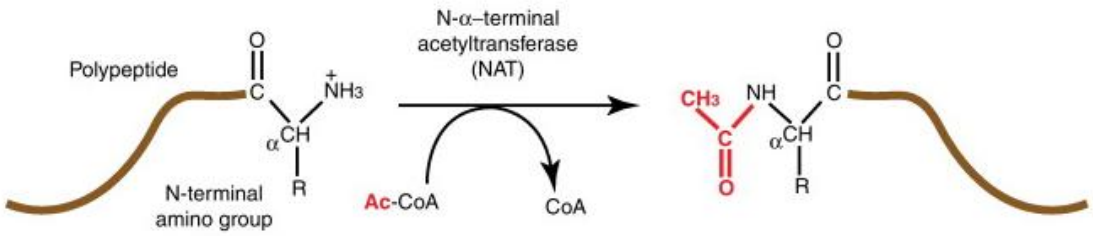


Figure 18. Scheme of Nt-acetylation. A Nat acetylates the N-terminal α-amino group of a protein by transferring an acetyl moiety (Ac) from Ac-CoA (Starheim *et al.*, 2012).

Nt-acetylation is carried out by protein complexes (NatA, NatB, NatC, NatD and NatE) that perform most Nt-acetylations in eukaryotes. Each complex consists of a catalytic and a varying number of auxiliary subunits, and their function appears to be highly conserved across species (Figure 19) (Helbig *et al.*, 2010). Human NatA, NatB and NatC were reported to function in translation initiation and in other events in the cell, while NatD is a specific nt-acetyltransferase of histone H4 and H2A (Yasuda *et al.*, 2013). Higher eukaryotic organisms express another type of Nat, NatF. Human NatF shows very distinct substrate specificity and contributes to about 10% of the total protein Nt-acetylation in the cell (Van Damme *et al.*, 2011).

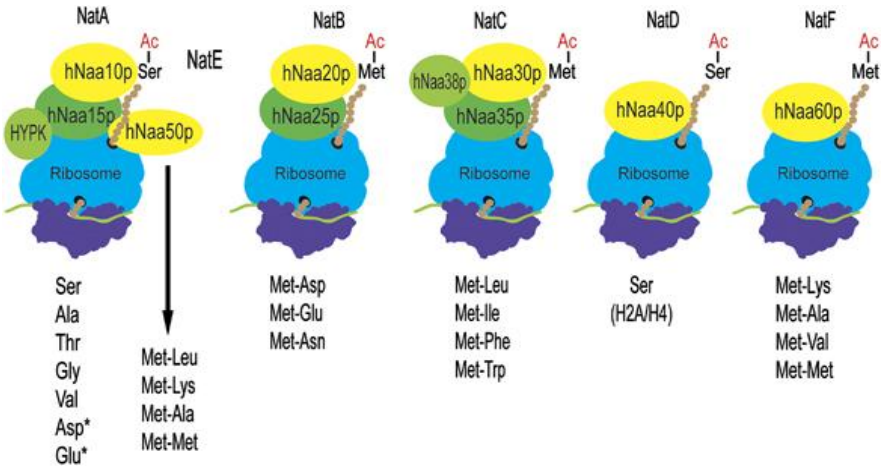


Figure 19. The eukaryotic NATS and substrate specificity. The NATs are found on the ribosome in both humans and yeast. In humans, Naa10p also acetylates β- and γ-actin post-translationally. NatF is only present in higher eukaryotes (Kalvik & Arnesen, 2013).

Nt-acetylation is considered an irreversible process that mainly occurs on the ribosome during protein synthesis (Foyn *et al.*, 2013).Nt-acetylation appears to be essential for life in higher eukaryotes: a mutation in the human Nt-acetyltransferase A, more specifically in the Naa10 gene, was shown to be the cause of Ogden syndrome, resulting in males that are underdeveloped and die at infancy.

Several distinct NATs have been identified in yeast: NatA, NatB, NatC, NatD and NatE which are composed of catalytic and auxiliary subunits. NatD only consist of a catalytic subunit, Nat4p. The deletion of NatA leads to defects in sporulation, slow growth and failure to enter the G0 phase. NatB-deleted strains exhibit decreased utilization of non-fermentable carbon sources, inability to form functional actin filaments, defects in mitochondrial and vacuolar inheritance, increased sensitivity to

anti-mitotic drugs and susceptibility to a number of DNA damaging agents (Kamita *et al.*, 2011).

2.1.1. Nt-acetyltransferase B

In eukaryotes and yeast, multiple protein complexes are capable of performing N-terminal acetylation; one of the most well characterized NATs is the NatB complex. NatB subunits are associated with ribosomes in both humans and yeast, and transfer an acetyl group to the N-terminus of target proteins. However, a large portion of the NatB subunits in humans are present in a non-ribosomal form (Starheim *et al.*, 2012). Yeast and human NatB are composed of the catalytic subunit Naa20p (Nat3p) and the auxiliary subunit Naa25p (Mdm20p), and acetylate the N-terminal methionine residue of substrates with the N-terminal sequence Met-Glu-, Met-Asp- and Met-Asn (Polevoda *et al.*, 2003).

In both humans and yeast, the Naa20 catalytic subunit of NatB localizes to both the cytoplasm and the nucleus, while the Naa25 auxiliary subunit is only present in the cytoplasm. Both subunits of yNatB are found either in the ribosome-bound or non-ribosome-bound form, indicating that these proteins can interact with ribosomes but also may have other functions (Starheim *et al.*, 2008). In yeast, NatB is one of the major NATs and it is responsible for about 15% of all protein Nt-acetylation (Van Damme *et al.*, 2012). Several substrates have been reported for yNatB, including tropomyosin-1 (Tpm1), the 20S proteosomal subunit and the stress-induced carboxypeptidase inhibitor Tfs1p (Van Damme *et al.*, 2012). The *naa20Δ* and *naa25Δ* mutants display several phenotypes that can be linked to the loss of acetylation of the yNatB substrates actin and tropomyosin (Starheim *et al.*, 2008), since Nt-acetylation of tropomyosin is required for high affinity binding of tropomyosin to actin. Previous studies demonstrated that a strain lacking Naa20 has reduced growth rate, reaches lower population densities, and exhibits abnormal morphology, including multiple buds and increased cell size (Caesar *et al.*, 2006). Helbig *et al*. also showed that the effect in mitochondrial inheritance observed when in the absence of Nat3 could be attributed to the loss of the N-acetyl group of Ugo1p, a protein located in the mitochondrial outer membrane where it is required for mitochondrial fusion (Helbig *et al.*, 2010).

Since the yeast and human NatB substrate specificity is largely conserved (Van Damme *et al.*, 2012), yeast has been a great advantage as a model organism to study Nt-acetylation.

2.1.2. Regulation of Apoptosis by Nt- acetylation

Apoptosis and Nt-acetylation have previously been associated. A point mutation in Naa10p, the catalytic subunit of NatA, results in the reduction of acetylation levels by NatA and causes the lethal X-linked disorder in infancy (Rope et al., 2011). Arnesen and coworkers also showed that depletion of this Nat induces p53-dependent apoptosis and p53-independent growth inhibition (Gromyko *et al.*, 2010). Yi and coworkers demonstrated that Nt-acetylation promotes apoptosis and proposed that Ac-CoA might serve as a signaling molecule that couples apoptotic sensitivity to metabolism by regulating protein Nα-acetylation (Yi *et al.*, 2011). They showed that protein Nt-acetylation levels are sensitive to alterations in metabolism and to Bcl-xL expression, since it leads to reduced levels of Ac-CoA and hypoacetylation of protein N-termini through a Bax/Bak-independent mechanism. This study suggests that regulation of Ac-CoA and protein Nt-acetylation may provide a Bax/Bak-independent mechanism for Bcl-xL to regulate apoptotic sensitivity (Figure 20) (Yi *et al.*, 2011). They also suggest that Bcl-xL might block citrate export from the mitochondria by interacting with the voltage-dependent anion channel (VDAC) and that Bcl-xL also inhibits cell proliferation, which might indirectly affect acetyl-CoA levels by reducing TCA cycle activity (Andersen & Kornbluth, 2011).The same study also showed that reduced levels of Ac-CoA mediated by Bcl-xL do not affect N-ε-acetylation of histones H3 and H4.

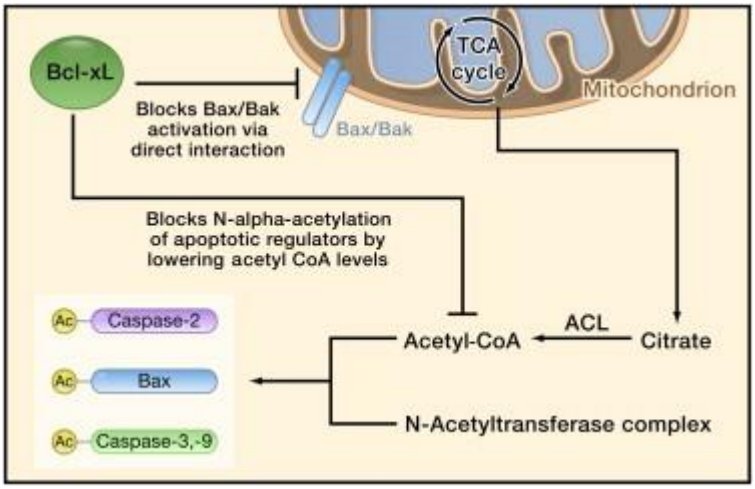


Figure 20. Regulation of Apoptosis by Bcl-xL. Bcl-xL lowers the levels of acetyl-CoA (possibly due to an indirect effect upstream of acetyl-CoA), resulting in decreased Nt-acetylation of apoptotic mediators such as caspase-2, -3, -9 and Bax. In the case of caspase-2, Nt-acetylation is critical for its activation (Andersen & Kornbluth, 2011).

NATs have not only been associated with apoptosis but also have a role in cancer. hNaa10 in particular has been proposed as both an oncoprotein and a tumour

suppressor. It is overexpressed in different types of cancers such as colorectal, lung, and breast cancer, and studies have proposed various molecular explanations for the role of Naa10 as an oncoprotein (Ren *et al.*, 2008). hNaa10 can exert its effects as a major NAT, but also through N-ε-acetylation, directly and/or by recruiting interaction partners with lysine acetyltransferase activity (Kalvik & Arnesen, 2013). It has also been shown that deletion of hNatB subunits inhibits cell growth and proliferation, and disturbs cell cycle progression. The differences in phenotypes resulting from deletions of subunits of hNatB could indicate that one or both may have individual functions in addition to those of the hNaa20/hNaa25 complex (Starheim *et al.*, 2009). Moreover, deletion of hNatC subunits leads to reduced cell proliferation and apoptosis. Cells without the catalytic subunit hNaa30 have a stronger phenotype than those with knockdown of auxiliary subunits hNaa35 and hNaa38 (Figure 21). These phenotypes suggest that hNatC is required for normal cell growth and survival and that all three subunits are needed for hNatC activity (Starheim *et al.*, 2009). However, more studies are needed in order to understand the function and regulation of Nats and which mechanisms mediate their oncogenic and tumor suppressor properties.

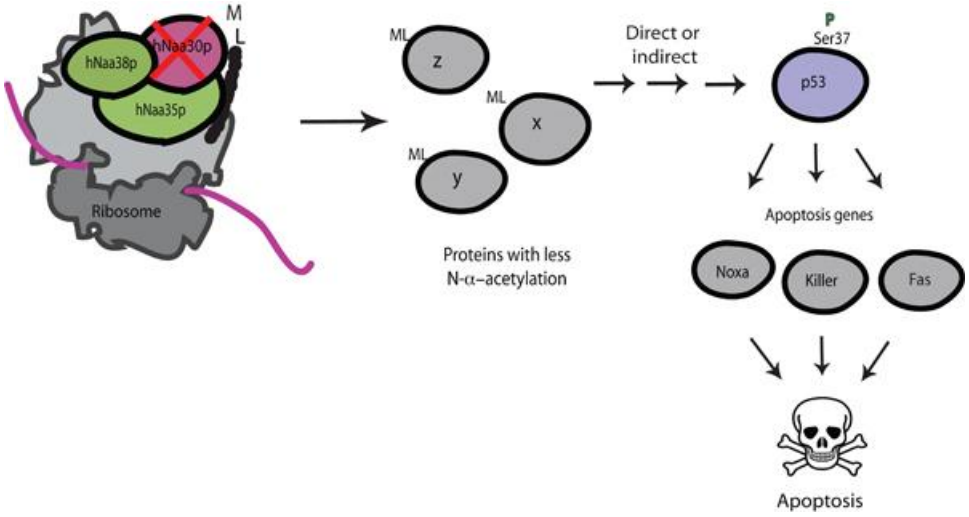


Figure 21. *hNaa30Δ* of the hNatC complex leads to p53-dependent apoptosis and lead to the reduced acetylation of hNaa30p substrates. As a direct or indirect effect of this, p53 is stabilized and phosphorylated on Serine37, and subsequently, transcription of p53 downstream proapoptotic genes NOXA, KILLER and FAS is activated, resulting in apoptosis (Starheim *et al.*, 2009).

Given these data from the literature, a previous study in our laboratory used yeast cells in order to verify whether Bax is acetylated by NatB, affecting its function and role in apoptosis. In fact, each Nat acetylates a subset of protein N-termini roughly

defined by the N-terminal amino acid sequence and Bax contains in its terminal sequence a specific substrate of NatB (Figure 22A, B).

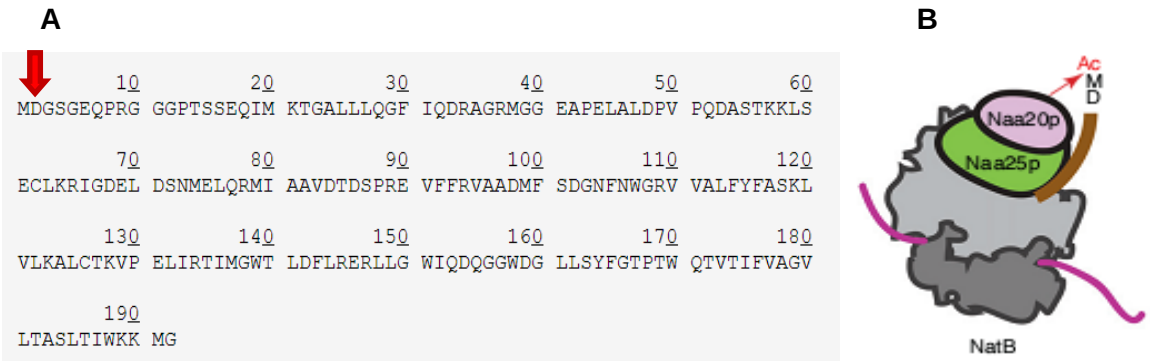


Figure 22. Substrate specificity of NatB. Baxα isoform protein sequence (A) is a putative substrate of NatB (B).

In this previous study, *NAT3* was replaced by the KanMX4 gene cassette in the W303-1A strain, through homologous recombination and the positive colony was transformed with the plasmids pYES3/CT and pYES3/CT Baxα (Bax wt). After confirmation of the clones, viability assays were performed in order to verify if expression of Bax in *nat3*Δ cells affects cell survival (Figure 23). The results suggest that expression of Baxα in the deleted strain seems to induce cell death.

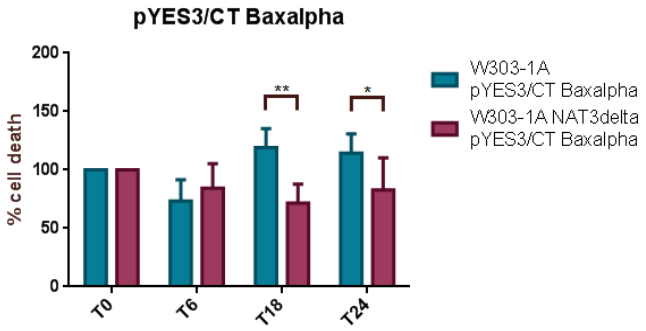


Figure 23. Effect of Baxα in *nat3*Δ yeast cells. Cell viability was measured as a percentage of colony forming units (c.f.u.) at different times on YPD medium after induced by galactose at T0. Vieira S., unpublished data.

The isolation of cytosolic and mitochondrial fractions of all strains was also performed to ascertain whether the cellular localization of Bax was affected (Figure 24).

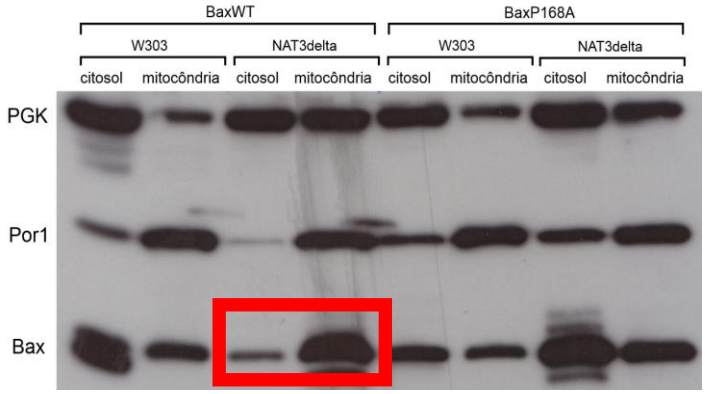


Figure 24. Baxα. Bax translocates to mitochondria of wt and *nat3*Δ cells expressing Baxα. The ability of Bax to induce mitochondria membrane permeabilization might be affected in the absence of Nt-acetylation, since mitochondria from *nat3*Δ retains the most fraction of Bax (Vieira S., unpublished data).

As can be seen in the figure 24, Bax is translocated to mitochondria in both strains expressing Baxα being more evident this translocation in the *nat3*Δ strain.

2.2. Aims

N-terminal acetylation is a common modification, occurring in the great majority of eukaryotic proteins. Is has been thought for a long time that Nt-acetylation protects proteins from degradation, but the role of Nt-acetylation is still unclear. It is known that the regulation of Bax depends on the interaction with different Bcl-2 family proteins and post-translational modifications such as phosphorylation events, which control the conformational changes required for Bax activity. Yet, less is known about the contribution of other Bax post-translational modifications to cell death. In this work, we took advantage of the yeast model system to address this issue, studying acetylation of Bax by hNatB using the following approaches:

1- Assess if expression of hNatB reverts the slow growth phenotype and complements the morphological defects of yeast *nat3*Δ cells;
2- Assess if expression of hNatB affects the survival and mitochondrial dysfunction (ROS) of wt and *nat3*Δ cells expressing Baxα;
3- Verify if expression of hNatB affects Bax localization and cyt *c* releasing activity.

2.3. Materials and Methods

2.3.1. Yeast strains and growth conditions

All strains of *Saccharomyces cerevisiae* used in this study are listed in Table IV. The *nat3*Δ pYES3-Baxα strain was constructed previously by S. Vieira (unpublished).

Strains were transformed with the indicated plasmids using the lithium acetate method as described before. Transformants were selected on Synthetic Complete (SC) medium lacking the appropriate aminoacids plus 2% (w/v) of carbon source and 2% agar. Yeast strains were maintained on solid YPD or SC medium (lacking the appropriate aminoacids), grown at 30 °C for 48h-72h, stored at 4°C, and refreshed every 2-3 weeks. Yeast cultures were grown aerobically in SC medium with 2% Glucose or Galactose as a carbon source or in SC medium with 0.5% Lactacte pH 5.5 supplemented with 0.5% ethanol. Strains transformed with plasmids were grown in SC medium lacking the appropriate aminoacids. Cells were incubated at 30 °C with orbital shaking (200 rpm) and a liquid/air ratio of 1:5.

Table IV. List of *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
W303-1A pYES3-Baxα	W303-1A harboring pYES3-Baxα	Silva R.
*nat3*Δ pYES3-Baxα	*nat3*Δ harboring pYES3-Baxα	Vieira S.
W303-1A pYES3-Baxα pAB3461	W303-1A harboring pYES3-Baxα and pAB3461	This study
*nat3*Δ pYES3-Baxα pAB3461	*nat3*Δ harboring pYES3-Baxα and pAB3461	This study
W303-1A pYES3-Baxα pAB3473-NatB	W303-1A harboring pYES3-Baxα and pAB3473-NatB	This study
*nat3*Δ pYES3-Baxα pAB3473-NatB	*nat3*Δ harboring pYES3-Baxα and pAB3473-NatB	This study

2.3.2. Plasmids

All the plasmids used in this work are listed in Table V.

Table V. List of the plasmids used in this work.

Plasmid	Description	Reference
pAB3461	*URA3;AmpR*	Van Damme *et al.*, 2012
pAB3473-NatB	NatB inserted in pAB3473	Van Damme *et al.*, 2012

2.3.3. Growth curves and viability assay

Yeast strains W303-1A pYES3-Baxα pAB3461, W303-1A pYES3-Baxα pAB3473-NatB, *nat3*Δ pYES3-Baxα pAB3461 and *nat3*Δ pYES3-Baxα pAB3473-NatB were first grown in SC medium with 2% of glucose lacking the appropriate aminoacids.

Cells were then transferred to SC medium with 0.5% of Lactacte pH 5.5 supplemented with 0.5% ethanol and grown to an O.D at 640nm of 0.3-0.5 (exponential phase). Then, 2% of galactose was added to induce Bax expression. The growth of yeast strains was assessed at different times. All incubations were performed at 30°C, 200 r.p.m.

For viability assay, samples were harvested at the indicated times at an O.D. at 640 nm of 0.1 and cell viability was measured as a percentage of colony forming units (c.f.u.) on YPD medium. Statistical analyses were performed using GraphPad Prism Software v5.00 (GraphPad Software, California, USA). P-values lower than 0.05 were assumed to represent a significant difference by Anova.

2.3.4. Accumulation of ROS (Flow Cytometry)

Intracellular superoxide anion was detected by flow cytometry using dihydroethidium (DHE) as a probe. For DHE staining, cells were harvested by centrifugation, resuspended in 500µL PBS and incubated with 5µg/mL DHE for 30 min in the dark. Cells with red fluorescence [FL-3 channel (488/620 nm)] were considered to accumulate superoxide anion.

All flow cytometry assays were performed in an Epics® XL™ (Beckman Coulter) flow cytometer, equipped with an argon-ion laser emitting a 488-nm beam at 15mW. The population of cells with high homogeneity and frequency was gated in a histogram of Side Scatter (SS) x Forward Scatter (FS). Twenty thousand cells per sample were analyzed. The resulting data were analyzed with WinMDI 2.8 software.

2.3.5. Cell morphology and chitin visualization

Since the *nat3*Δ strain shows an abnormal morphology, microscopy was used to verify the morphology and cell wall chitin of each yeast strain. Chitin assembly was assessed by CFW (Calcofluor-white) staining. Cultures were diluted to $1x10^7$ cells/ml and 20µl of CFW (100 µg/ml) added to 1ml of cell suspensions. Samples were incubated at room temperature for 5min and 5µl of each suspension placed on a glass slide for microscopic visualization (Leica Microsystems DM-5000B).

2.3.6. Mitochondrial and cytosolic fractionation

Four hundred milliliters of cells were grown under the same conditions used for growth curves, and harvested at the end of exponential phase (OD_{640}=1.4-1.6) after 14h of galactose induction. Cells were resuspended in Suspension Buffer [60% (v/v) 2 M Sorbitol, 6% (v/v) 1 M Sodium phosphate (pH 7.5) and 2% (v/v) 0.5 M EDTA] and

then digested with 10 mg Zymolyase 20T (ImmunO, MP Biomedicals) to obtain spheroplasts, washed twice with 1.2 M sorbitol, and suspended in Lysis Buffer [0.5 M Sorbitol, 20 mM Tris/HCl (pH 7.5) and 1 mM EDTA]. Spheroplasts were lysed with a few strokes in a glass Dounce homogenizer (tight fitting piston) with care to avoid mitochondrial lysis. Homogenates were centrifuged at 2500 rpm for 10 min and the supernatant then centrifuged at 15000 rpm for 15 min. The supernatant constitutes the cytosolic fraction and was centrifuged two more times at 15000 rpm for 15min to eliminate contamination with mitochondria. The pellet, containing the mitochondrial fraction, was centrifuged two more times and suspended in Lysis Buffer. Both fractions were frozen in liquid nitrogen and stored at -80ºC. Estimation of the protein concentration of the fractions was determined by the Bradford method using BSA as a standard (Bradford, 1976).

2.3.6.1. SDS gel electrophoresis/Western Blot

Mitochondrial and cytosolic fractions were separated electrophoretically on a 15% SDS polyacrylamide gel at 20 mA and transferred to a PVDF membrane as described above. Membranes were cut into strips and incubated with the primary antibodies: mouse monoclonal anti-yeast phosphoglycerate kinase (PGK1) antibody (1:5000, Molecular Probes), mouse monoclonal anti-yeast porin (POR1) antibody (1:5000, Molecular Probes), rabbit polyclonal anti-yeast cytochrome *c* (CYC1) antibody (1:2500, custom-made by Millegen), rabbit polyclonal anti-human Bax (BAX) antibody (1:5000, Sigma). Membranes were incubated with secondary antibodies against mouse or rabbit IgG-peroxidase (1:10000; Sigma Aldrich). Pgk1p and Por1p were used as controls for cytosolic and mitochondrial fractions, respectively. Immunodetection of bands was revealed by chemiluminescence (Immobilon, Millipore).

2.4. Results

2.4.1. Expression of human NatB reverts the slow growth and morphology defects of yeast *nat3*Δ cells

Deletion of yNAA20 (nat3) leads to a reduced growth rate in yeast. Moreover, *Naa20*Δ (*nat3*Δ) cells showed reduced mating, abnormal morphology and cytoskeleton function, defects in mitochondrial division and vacuolar segregation. These defects are attributed to the absence of Nt-acetylation in two essential cytoskeleton proteins, actin and tropomyosin, substrates of yNatB (Van Damme *et al.*, 2012), and the inability of cells to form functional actin cables (Starheim *et al.*, 2012) (Caesar *et al.*, 2006). *nat3*Δ yeast cells are also temperature sensitive for growth and fail to transport mitochondria into newly formed buds (Hermann *et al.*, 1997).

In this work, we first determined if hNatB reverts the slow growth of yeast *nat3*Δ (Figure 25) in cells expressing Baxα.

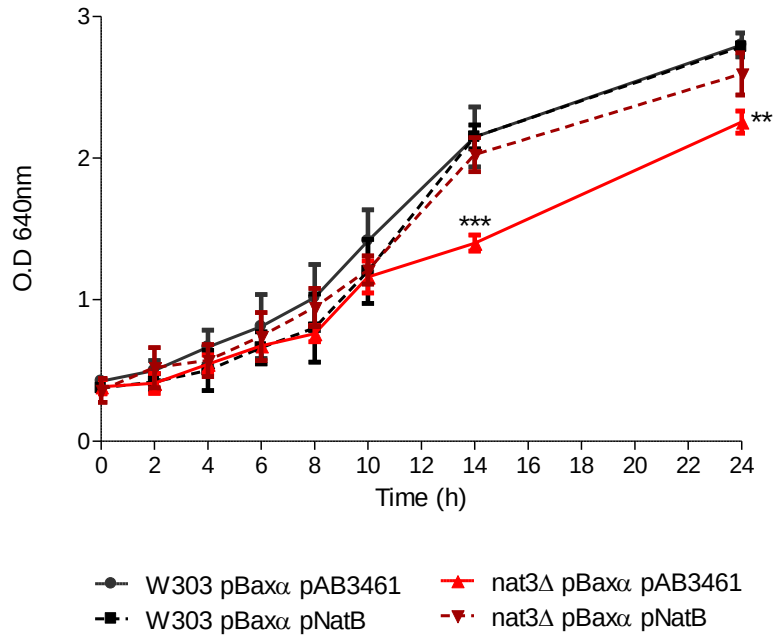


Figure 25. Effect of Baxα and hNatB co-expression in yeast *nat3*Δ growth. Growth curves of *S. cerevisiae* strain W303-1A expressing **Baxα and hNatB** along induction started by galactose at T0. These data result from the mean of three independent experiments. Values significantly different from W303 pBaxα pAB3461: ** P<0.01 and *** P<0.001, 2way Anova Test.

As shown in Figure 25, hNatB reverts the growth defect of *nat3*Δ cells, and had no effect in the growth of wt strain (W303 pBax). Yeast strains were also incubated with CFW and cell wall chitin was observed by fluorescence microscopy (Figure 26 A, B, C and D).

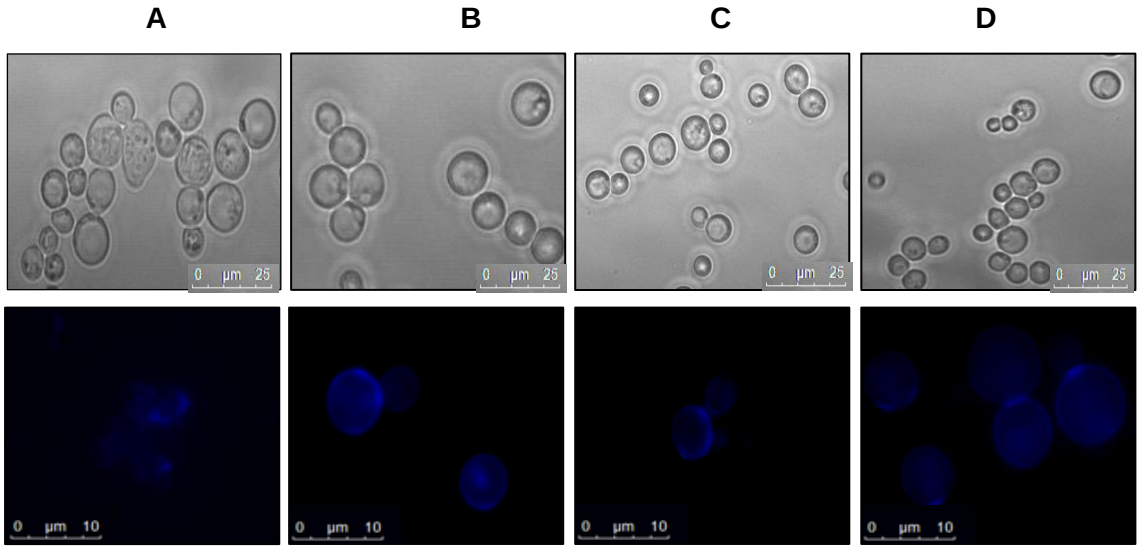


Figure 26. Morphology and chitin visualization of the yeast strains *nat3*Δ Baxα (A) and *nat3*Δ Baxα expressing hNatB (B) as well as strains wt Baxα (C) and wt Baxα expressing hNatB (D) used as a control.

Similarly to other studies, we observed that *nat3*Δ cells are abnormally large and heterogeneous in size, exhibit defects in chitin localization, and accumulate small vesicles (A). However, in the presence of hNatB, *nat3*Δ yeast cells show morphology similar to that observed in the wt strain (B, C) though their size remains larger than the wild type. These data suggest that hNatB (B) can complement the function performed by yeast Nat3 that is required for normal function of the actin cytoskeleton, since loss of interaction between actin and tropomyosin in *nat3*Δ causes dramatic changes in cell physiology (Caesar & Blomberg, 2004). These results are in agreement with the literature, since hNatB was shown to be active in yeast and partially rescue *yNatB*Δ phenotypes (Van Damme *et al.*, 2012).

2.4.2. Expression of hNatB enhances the survival of wt and *nat3*Δ cells expressing Baxα and protects cells from oxidative stress

To ascertain the role of hNatB in cell survival of yeast *nat3*Δ cells expressing Baxα, cell survival of the aforementioned strains was measured as a percentage of colony forming units (c.f.u.) (Figure 27).

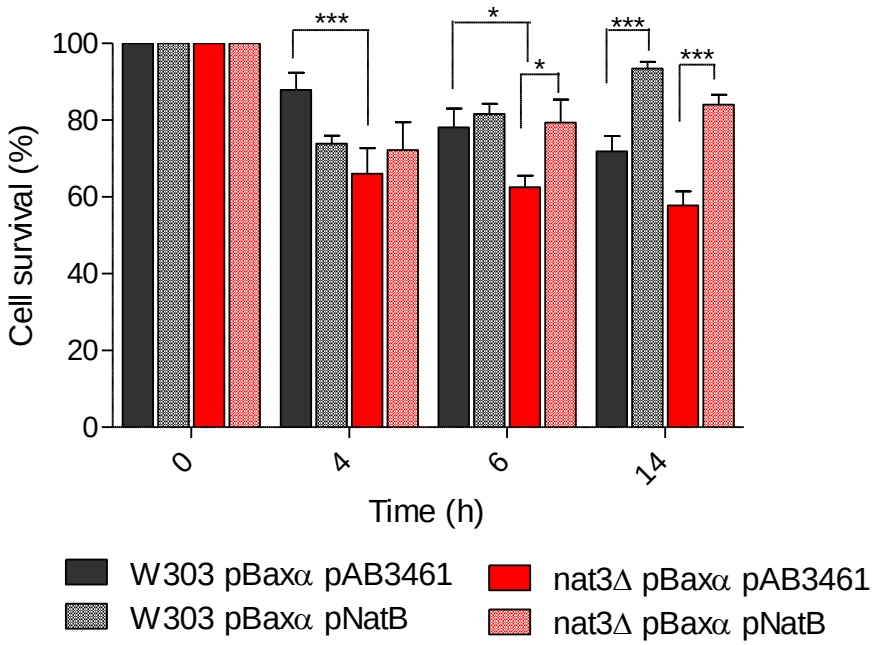
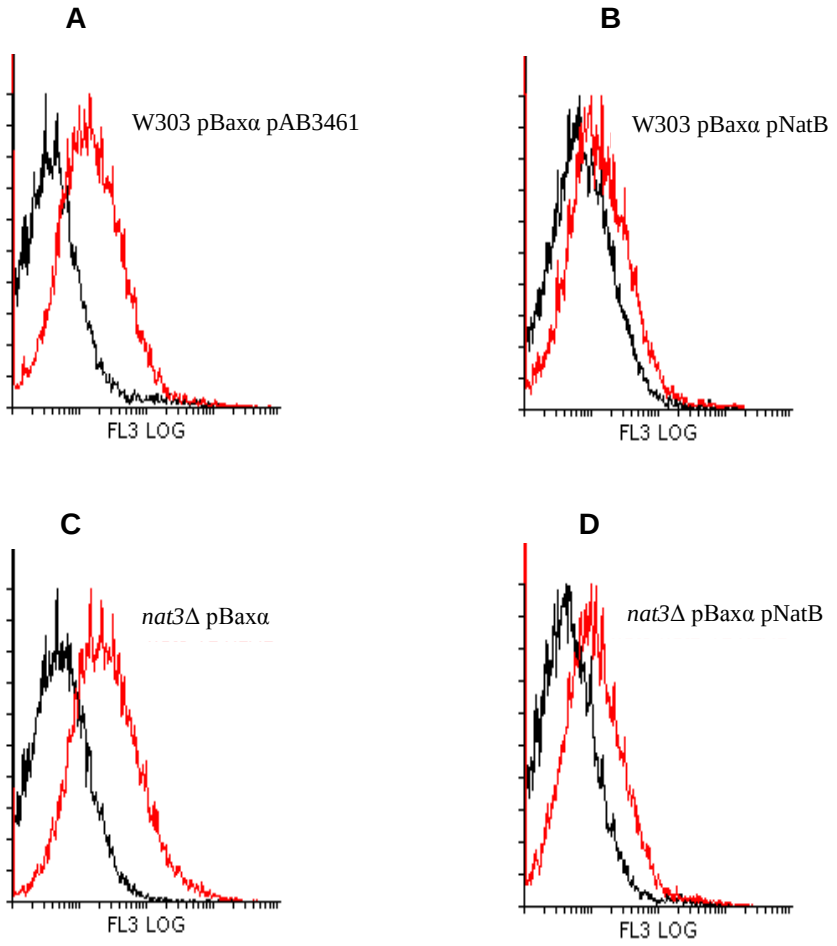


Figure 27. Effect of Baxα and hNatB co-expression in yeast. Cell viability was measured as a percentage of colony forming units (c.f.u.) at different times on YPD medium after induced by galactose at T0. These data result from the mean of four independent experiments. * P<0.05 and *** P<0.001, 2way Anova Test.

After shorter periods of expression of human Baxα, but not after 14h of expression, yeast cells lacking Nat3p have decreased cell survival compared to the wt strain. At the 14h time point, the *nat3*Δ strain expressing hNatB shows cell survival values similar to the wt strain carrying hNatB, indicating that for longer expression times hNatB promotes survival in *nat3*Δ cells as well as in the wt strain.

Expression of Bax leads to mitochondrial dysfunction in yeast cells (Khoury & Greenwood, 2008). In order to verify if Nat3p plays a role in this phenotype, DHE staining was used to detected intracellular superoxide anion accumulation in all strains. In this experiment the role of hNatB and Baxα in oxidative stress was also observed in yeast *nat3*Δ cells (Figure 28 A, B, C, D and E).



E

Strains	W303 pBax pAB3461		W303 pBax pNatB		*nat3*Δ pBax		*nat3*Δ pBax pNatB	
Time	0	14	0	14	0	14	0	14
Mean fluorescence intensity (MnX)	0,444	4,98	0,582	2,22	0,651	5,68	0,546	2,84

Figure 28. ROS accumulations in wt (A) and *nat3*Δ (C) cells expressing Baxα or co-expressing Baxα and hNatB (B and D, respectively). Cells were analysed by flow cytometry using DHE staining. Samples were collected before, T0 (black histogram), and after 14h of protein induction (red histogram). (**E)-** Mean fluorescence intensity over time for each strain. This result is representative of three independent experiments.

The results obtained by flow cytometry show that there is an accumulation of ROS in *ynat3*Δ and wt strains when Baxα is present, which is partially rescued by expression of hNatB. This suggests that hNatB protects cells from Bax-induced oxidative stress in both strains.

2.4.3. Localization of Baxα and cytochrome *c* releasing activity in *nat3*Δ cells expressing hNatB

Bax activation involves subcellular translocation and dimerization. In normal cells, Bax is monomeric and found either in the cytosol or loosely linked to membranes. After a death stimulus, Bax translocates to the mitochondria where it becomes an integral membrane protein (Gross *et al.*, 1999). It is already established that post-translational modifications are important for Bax function. For instance, phosphorylation can affect the function of Bcl-2 or Bax by altering the ability of these proteins to form heterodimers, affecting cell cycle events, regulating cell proliferation and programmed cell death (Delivoria-Papadopoulos & Mishra, 2010). In yeast, expression of Bax releases cytochrome *c* to the cytosol and this is prevented by co-expression of anti-apoptotic proteins. It was described that expression of Bcl-2 alone is able to protect yeast cells against death suggesting that at a part of the death pathway may be shared between yeast and higher eukaryotes (Priault *et al.*, 1999b).

One hypothesis of this study was to verify if Bax is acetylated by NatB and activated. To test this, the cytosolic and mitochondrial fractions of Baxα-expressing cells were isolated and the localization of Baxα and the release of cyt *c* assessed (Figure 29).

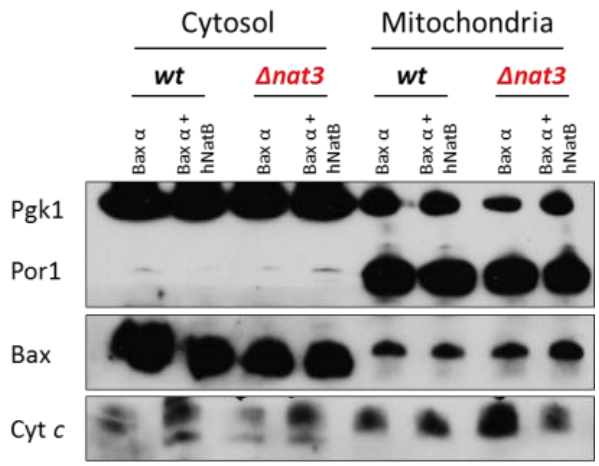


Figure 29. Effect of Nt-acetylation by hNatB on Baxα activity. Bax is translocated to mitochondria of wt and *nat3*Δ cells. The ability of Bax to induce mitochondria membrane permeabilization might be affected in the absence of Nt-acetylation, since mitochondria from *nat3*Δ cells retain most of their cyt *c.*

The localization of Baxα seems to be identical in wt and *Δnat3* cells, and located preferentially in the cytosol; however, small portions are found in mitochondria. These results are in contrast with those obtained previously in the lab, with growth conditions in SC medium with 2% lactate and with the difference of not having the empty vector of

hNatB, but may reflect that Bax is translocated to mitochondria with different kinetics in wt and *Δnat3* cells, but the end-point is similar. In agreement, the *nat3*Δ strain did not show increased release of cyt *c*. This result suggests that Nat3 may affect the kinetics of Bax addressing to the mitochondria, but that it does not affect Bax-induced cyt *c* release. It was also apparent that wt and *nat3*Δ strains carrying hNatB had slightly higher release of cyt *c*. While this was associated with a higher decrease of cyt *c* mitochondrial content for *nat3*Δ the same was not observed for the wild type. Whether it is a specific effect or there was slight contamination of the cytosolic fraction with mitochondrial contents remains to be determined.

2.5. Discussion and Future Perspectives

The majority of cytosolic proteins in eukaryotes contain a covalently linked acetyl moiety at their N terminus. Nt-acetylation has recently been extensively studied, since it affects the stability and function of several proteins. This modification occurs on the α-amino group of a protein during its synthesis by a conserved family of N-terminal acetyltransferases, whose specificity is determined by the amino acid at the mature N terminus of the substrate (Holmes *et al.*, 2014). This study aimed to verify if Baxα is acetylated by hNatB in normal yeast cells and *nat3*Δ cells but also if hNatB complements the absense of Nat3p.

At first, an *in vivo* assay based on growth of yeast cells expressing Baxα and hNatB in *nat3*Δ and wt strain was performed to assess if hNatB complements the slow growth of *ynat3*Δ cells. The results of this assay showed that hNatB partially complements the growth of *ynat3*Δ cells and no differences were found in the wt strain, suggesting that hNatB does not affect growth in this strain (Figure 25). The Nat3p deletion phenotype in yeast is very clear showing abnormal cell morphology. Another aim of this study was to determine whether hNatB was able to reverse this phenotype. Our results show that the *nat3*Δ strain has an abnormal morphology that is reversed by the presence of hNatB, thus resembling the cells of the wt strain (Figure 26). This suggests that hNatB can complement the functions performed by yeast Nat3 that is required for acetylation of actin and tropomyosin that contributes to normal function of actin cytoskeleton.

In order to complete these results, cell survival assays were performed to understand the role of hNatB in cell survival in yeast cells expressing Baxα and in *nat3*Δ cells. The results obtained through c.f.u. counts reveal that expression of Baxα in *nat3*Δ cells seems to induce cell death that is reversed by expression of hNatB. Expression of hNatB in yeast cells partially restores the survival of wt and *Δnat*3 cells expressing Baxα for longer expression times (Figure 27). These results suggest that absence of Baxα Nt-acetylation affects yeast survival.

Since previous studies have shown that Bax induces mitochondrial hyperpolarization and production of ROS (Priault *et al.,* 2003) and deletion of Nat3p also associated with a decrease in the function of the actin resulting in a defective active cables formation (Polevoda et *al.,* 2003), likely linked to an increased in production of ROS, we assessed if hNatB had a role in the oxidative stress caused by expressing Baxα and Nat3p deletion in yeast cells.

The flow cytometry results obtained by DHE staining indicated that there is an accumulation of ROS in *nat3*Δ and wt strains when Baxα is present which is partially rescued by hNatB, suggesting that hNatB had a protective role in oxidative stress in wt and *nat3*Δ strains (Figure 28). This result is in agreement with previous results obtained in our lab by Vieira S., which showed an evident increased in ROS production in *nat3*Δ strain (unpublished data). In the future, it would be interesting to optimize the Mitotracker Red and Mitotracker Green staining to determine if there are also changes in mitochondrial membrane potential, as well as assess if there are alterations in mitochondrial fragmentation/degradation.

To ascertain whether acetylation of Baxα by hNatB affect Bax localization and activity, cytosolic and mitochondrial fractions from wild type and *nat3*Δ cells were made analyzed by western blot. Baxα is located preferentially in the cytosol however, small portions are found in mitochondria of wt and *nat3*Δ cells, but the location was identical in all strains. These results together with those obtained by Vieira S. suggest that though Nt-acetylation does not affect the ability of Bax to insert in the outer mitochondrial membrane, it may alter the kinetics of Bax mitochondrial addressing/insertion. Moreover, it also appears that in the absence of hNatB and yeast Nat3p, expression of Bax does not trigger release of cyt *c* (Figure 29) Indeed, mitochondria from *Δnat3* cells retain most of its cyt *c* which suggests that the ability of Bax to induce mitochondria membrane permeabilization might be unaffected in the absence of Nt-acetylation. On the other hand, expression of hNatB appears to induce cyt *c* release both in the wt as in the *nat3*Δ strain. However, it is necessary to determine whether this is a specific effect or there was a slight contamination of the cytosolic fraction with mitochondrial contents. To discard this possibility would require monitoring the integrity of the inner mitochondrial membrane with the citrate synthase assay. Washing cytosolic and mitochondrial fractions to eliminate contaminations and repeating the extraction would be necessary to draw definitive conclusions. Expression of hNatB should also be checked with an anti-HA antibody. Finally, it will also be necessary to confirm if Baxα is indeed Nt-terminally acetylated in *nat3*Δ cells expressing hNatB by mass spectroscopy.

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