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Identification of new yeast protein phosphatases involved in the regulation of Bax

Tese de Mestrado em Bioquímica Aplicada

Trabalho efetuado sob a orientação da

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E coorientação da

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Identification of new yeast protein phosphatases involved in the regulation of Bax

Abstract

Apoptosis is a genetically controlled cell suicidal program that is highly orchestrated and which contributes to the elimination of unnecessary or damaged cells in multicellular organisms. In this way the organism is capable to maintain the tissue homeostasis. Key regulators of apoptosis include the Bcl-2 family members, which control the permeabilization of mitochondria and the ensuing release of pro-apoptotic factors. Bax is the major pro-apoptotic member of this family. It is mainly cytosolic and remains inactive in proliferating cells, but is translocated to mitochondria and activated in cells undergoing apoptosis, which ultimately leads to permeabilization of the mitochondrial outer membrane. Bax can be regulated through phosphorylation/dephosphorylation by protein kinases and protein phosphatases that integrate different signaling pathways. Currently, the structure of inactive Bax has been determined, but the structures of its active form are still unsolved.

The yeast *Saccharomyces cerevisiae*, has a high degree of conservation of many cellular processes that share fundamental aspects with mammalian. For this reason it has been widely used for the study of the function and regulation of different Bcl-2 family members.

In order to identify novel protein phosphatases involved in the dephosphorylation of Bax, we heterologously expressed human Bax in yeast cells lacking non-essential protein phosphatases and determined whether there were differences in the Bax phosphorylation profile. We found several putative protein phosphatase candidates, such as three yeast protein phosphatases (Pph21p/22p and Pct4p), which have as human orthologs PP2A and WIP1 protein phosphatases, respectively. These two human protein phosphatases have been previously described as involved in the dephosphorylation of Bax, which validates the approach developed to identify new candidate protein phosphatases of Bax. The results will be discussed in terms of the consequences of the regulation of Bax by the protein phosphatases identified.

Identificação de novas fosfatases de proteína em levedura envolvidas na regulação de Bax

Resumo

A apoptose é um programa de suicídio celular altamente orquestrado e sob controlo genético que contribui para a eliminação das células danificadas ou desnecessárias em organismos multicelulares. Deste modo, o organismo é capaz de manter a homeostasia dos tecidos. Existem alguns reguladores-chave da apoptose, como os membros da família Bcl-2, os quais controlam a permeabilização da membrana mitocondrial externa e a consequente libertação de fatores pró-apoptóticos. A proteína Bax é o principal membro pró-apoptótico da família Bcl-2. Esta proteína é principalmente citosólica e permanece inativa nas células em proliferação, sendo translocada para a mitocôndria e ativada em células apoptóticas, levando à permeabilização da membrana mitocondrial. A proteína Bax pode ser regulada através de processos de fosforilação/desfosforilação por cinases e fosfatases de proteína que integram diferentes vias de sinalização. Atualmente, já se conhecem as estruturas da forma inativa de Bax, mas as estruturas da sua forma ativa ainda não foram determinadas.

A levedura *Saccharomyces cerevisiae*, tem um elevado grau de conservação de muitos processos celulares partilhando aspetos fundamentais com os mamíferos. Por este motivo, tem sido amplamente utilizada para o estudo da função e regulação de diferentes membros da família Bcl-2.

A fim de identificar novas fosfatases de proteína envolvidas na desfosforilação de Bax, procedemos à expressão heteróloga de Bax humana em células de levedura deficientes em fosfatases não essenciais a fim de determinar diferenças no perfil de fosforilação de Bax. Encontramos várias fosfatases candidatas, entre elas, três fosfatases de levedura (Pph21p / 22p e Pct4p), que têm como ortólogos humanos, as fosfatases PP2A e WIP1, respetivamente. Estas duas fosfatases de proteína humanas foram previamente descritas como estando envolvidas na desfosforilação de Bax, validando assim a abordagem desenvolvida para identificar novas fosfatases candidatas. Os resultados serão discutidos em termos das consequências da regulação de Bax pelas fosfatases identificadas.

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Abbreviations

3D – Three Dimensional
ACD – Accidental
AIF – Apoptosis Inducing Factor
AKT/PKB - protein kinase B
AMID – Mitochondria-associated Inducer of Death
ANT – Adenosine Nucleotide Translocator
APAF – Apoptosis Protease Activating Factor
Arg – Arginine
ART – Apoptosis Regulator of Targetting
ATP – Adenosine Triphosphate
BI-1 – Bax-inhibitor 1
BOP – BH3-only Protein
BSA - Bovine Serum Albumin
Ca^{2+} - Calcium Ion
CARD – Caspase Recruitment Domain
CFU – Colony-Forming Units
Cit. c – Cytochrome *c*
CK – Creatine Kinase
CsA – Cyclosporine A
CypD – Cyclophilin D
DED – Death Effector Domain
DHE – Dihydroethidium
DISC – Death Inducing Signaling Complex
DNA – Deoxyribonucleic acid
DR – Death Receptors
ER - Endoplasmic Reticulum
FADP – Fas Associated Death Domain Protein
GFP – Green Flourescent Protein
Gly – Glycine
GSK3β – Glycogen Synthase Kinase
H_2O_2 – Hydrogen Peroxide
Hα – α Helix
IMM – Inner Mitochondrial Membrane
IMS – Intermembrane space
JNK – Jun N-Terminal Kinase
LB – Luria-Betani Broth
MAC – Mitochondrial Apoptosis-Induced Channel
MOMP – Mitochondrial Outer Membrane Permeabilization
mPT – Mitochondrial Permeability Transition
mPTP - Mitochondrial Permeability Transition Pore
NADH – Nicotinamide Adenine Dinucleotide
NCCD - The Nomenclature Committee on Cell Death
NMR - Nuclear Magnetic Resonance
O_2^- – Superoxide Anion
OD - Optical Density
OMM – Outer Mitochondrial Membrane
PBR – Peripheral Benzodiazepine Receptor
PCD – Programmed Cell Death
PGK1 – Phosphoglycerate Kinase
PGK1 – Phosphoglycerate Kinase 1
Pi – Phosphate
PKB – Protein Kinase B
PKC – Protein Kinase C
PPM – Protein Phosphatase Mg^{2+}
PPP – Phosphoprotein Phosphate
Pro – Proline
PTP- Protein Tyr Posphatase
PVDF – Polyvinylidene Diflouride
RCD – Regulated Cell Death
ROS – Reactive Oxygen Species
SC – Synthetic Complete
SD – Standard Deviation
Ser – Serine
Thr – Threonine
TNF – Tumor Necrosis Factor
VDAC – Voltage-Dependent Anion Channel
YMUC – Yeast Mitochondrial Unselective Channel
YPD - Yeast Peptone Dextrose

1. INTRODUCTION

1.1 Historical perspective

Although the study of cell death started to be reconsidered and addressed more intensively in the eighties, the interest in it was modest and only a decade later it began to grow exponentially, totaling nowadays more than 80.000 publications (Wyllie, 2010). During the early twentieth century cell death was a subject of interest mainly because of its crucial role in tissue homeostasis or in the development of multicellular organisms or even in some isolated studies that recognized its involvement during metamorphosis and embryogenesis. It was assumed that during the developmental, at least, two forms of regulated cell death were present, one that was activated via the mitochondria and a second that was independent of this organelle. The studies pursued and in 1963/64, it was Lockshin and William that introduced the concept of Programmed Cell Death (PCD) (Lockshin and Zakeri, 2001). In 1973 Schweichel and Merker defined three types of cell death, heterophagy or apoptosis, autophagy and non-lysosomal death, which was not associated with any type of digestion. This distinction was mostly based on the location and on the role of lysosomes (Lockshin and Zakeri, 2001; Baehrecke, 2002). Over the years several attempts have been made to classify cell death subroutines based on cell morphological characteristics.

More recently the Nomenclature Committee on Cell Death (NCCD), was created to unify criteria for the definition of cell death and of different cell death morphologies, while formulating several caveats against the misuse of words and concepts that slow down progress in the area of cell death research (Kroemer *et al*, 2005). The NCCD has formulated several subsequent rounds of recommendations, in Cell Death and Differentiation journal (Kroemer *et al*, 2005, Galluzzi *et al*, 2012 and Galluzzi *et al*, 2014, aiming to define a new systematic classification of cell death based on measurable biochemical features. It was propose that the functional classification of regulated cell death modes should include three major pathways: apoptotic, necrotic and autophagic (Galluzzi *et al*, 2012). More recently, it was considered that cell death could be generally classified as regulated (RCD) or accidental (ACD) in the sense that can be initiated by a genetically encoded machinery, or not. The term PCD should be used to designate cell death scenarios that take place as part of a developmental program or in the context of physiologic adult tissue homeostasis (Galluzzi *et al*, 2014).

1.2 Apoptosis in the context of regulated cell death

The past decade has witnesses a steady accumulation of findings that suggests apoptosis, necrosis and autophagy are often regulated by similar pathways, involving common organelle and sub-cellular sites or sharing effectors and initiator molecules (Nikoletopoulou *et al*, 2013). Basically, necrotic death is typically followed by inflammatory reactions and mechanistically is not associated with activation of caspases (Los *et al*, 2002; Kerr *et al*, 1972). Morphologically necrotic cells are characterized by the swelling of organelles, such as mitochondria and endoplasmic reticulum (ER), the rupture of the plasma membrane and the lysis of the cell, resulting in damage to neighboring cells (Nikoletopoulou *et al*., 2013). The process of autophagy or autophagocytosis (from the Greek for 'self-eating'), first called macroautophagy, is characterized by sequestration of cytoplasmic material like the internal lysis of dysfunctional or unnecessary proteins, organelles, or other sub-cellular components. (Reynolds, 2014), in autophagosomes that are subsequently degraded by lysosomes. The fusion among lysosomes and autophagosomes generates autolysosomes. In turn the acidic lysosomal acid hydrolases will degrade both the autophagosome inner membrane and its luminal content, and this catabolic process marks the completion of the autophagic pathway. But it is not always so linear, and more functional tests are required to investigate the process of autophagy, once an increase in the number of autophagosomes does not necessarily mean that the autophagic route is being induced (Kroemer *et al*, 2009). "Autophagic cell death" is morphologically defined as a type of cellular death that occurs in the absence of chromatin condensation, but accompanied by massive autophagic vacuolization of cytoplasm (Kroemer *et al*, 2009). The NCCD has expressed concern that "the term 'autophagic cell death' is a linguistic invitation to believe that cell death is occurring through autophagy" rather than occurring with autophagy (Reynolds, 2014). Developmental autophagic cell death of *Drosophila* salivary glands following growth arrest and autophagy induction is one example of autophagic cell death with a physiological role in the clearance of unnecessary cells during development (Berry and Baehrecke, 2007).

Apoptosis is a type of regulated cell death, which means that cells follow a sequence of controlled steps towards their own destruction, with morphological characteristics distinct from those found in necrosis and autophagy. The term "apoptosis" was introduced by John Kerr and his coworkers Alastair Currie and Andrew

Wyllie in 1972 to define a new type of death (Kerr *et al*, 1972). Morphologically, apoptosis is characterized by marginal condensation of nuclear chromatin, cytoplasmic retraction, modifications of the cytoskeleton and cytoplasmic membranes, nuclear fragmentation, and blebbing of the plasma membrane. Subsequently, the cell breaks up into membrane-enclosed fragments, termed apoptotic bodies, which are rapidly recognized and engulfed by phagocytosis by neighbouring cells or macrophages, allowing the elimination of damaged or infected cells (Orrenius *et al*, 2011).

In the development context, apoptosis is important, for example in the formation of structure such as fingers and toes of the hand and foot in early life, being responsible for the death of cells from the interdigital membranes. Because of its crucial role in survival of multicellular organism when inappropriately controlled it causes several pathologies leading to a variety of disorders such as degenerative diseases like Alzheimer's disease and Huntington's disease, ischaemic damage, autoimmune disorders and several forms of cancer (Green and Evan, 2002). The execution stage of apoptosis involves the proper function of several enzyme systems activated through elaborate signaling pathways (Vaculova and Zhivotovsky, 2008). Two classical apoptotic pathways called extrinsic and intrinsic have been recognized (Wyllie, 2010).

1.2.1 Main components/regulators of the apoptotic machinery

Pioneering genetic and biochemical studies in mammals and invertebrate models have led to the identification of many apoptotic players from diverse organisms and also have shown that the cell death program has been conserved throughout evolution (Aouacheria *et al*, 2005). The core of apoptotic program consist of three major components: the Bcl-2 family proteins, the Apoptosis Protease Activating Factor-1 (Apaf-1)/CED-4 protein that relays the signal integrated by Bcl-2 family proteins to caspases and the caspases (Adams and Cory, 1998).

1.2.1.1 Caspases

Caspases belong to a family of cysteine proteases that cleave after aspartic acid residues. Caspases are synthesized as inactive proenzymes, called procaspases, which are activated following cleavage at specific aspartate cleavage sites. In the active site, caspases contain a cysteine residue crucial for their proteolytic activity (Cohen, 1997). Caspase activation plays a central role in the execution of apoptosis. Caspases turn off cell-protective mechanisms and activate pathways that lead to cell destruction (Fischer

et al, 2003). On the one hand, it is apparent that cells can survive limited caspase activation and conversely, that inhibiting caspases will often block the morphological manifestations of apoptosis, but cell death proceeds nevertheless (Alam *et al*, 1999). Caspases can be divided into executioners (caspases 3, 6, and 7) and initiators (caspases 2, 8, 9 and 10). Executioner caspases have a small pro-domain, whereas initiator caspases have a long pro-domain. In the case of caspases 8 and 10 they have a Death Effector Domain (DED), and in case of caspase 2 and 9 they have a Caspase Recruitment Domain (CARD) (Budihardjo *et al*, 1999). The two well-studied pathways of caspase activation are the cell surface death receptor pathway and the mitochondria-initiated pathway also known as the extrinsic and intrinsic apoptotic pathways, respectively, as will be discussed below. The following figure presents a diagram classification of the different types of cell death (Figure 1.1).

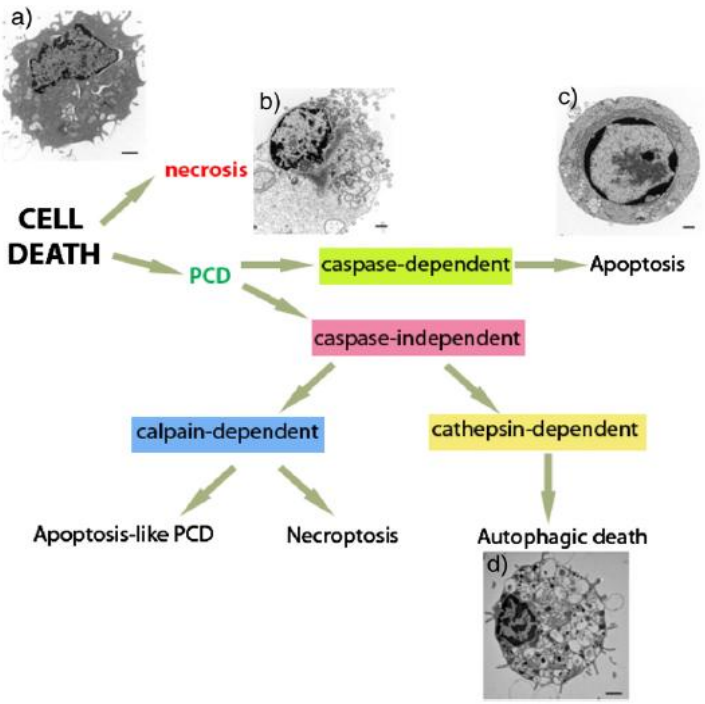

Figure 1.1 - Types of cell death and their morphological hallmarks. Classification diagram of the different types of cell death. PCD: programmed cell death. Morphological features of a) a healthy cell, b) a necrotic cell, c) an apoptotic cell and d) an autophagic cell. [Taken from (Nikoletopoulou *et al*, 2013)].

1.2.1.2 The Bcl-2 family members: structure and function

As referred above, the effector processes responsible for apoptosis are now mostly well known, involving the proteolytic activation of caspases, that provides a biochemical basis for the apoptotic phenotypes, and Bcl-2 family members in response to a wide variety of physiological and injury-induced signals (Vaculova and Zhivotovsky, 2008). The Bcl-2 protein which gave the name to the Bcl-2 family was the first member of this family identified on the basis of its involvement in B-cell malignancies (Zavala *et al*, 1985). Bcl-2 extends cell survival against apoptotic signals induced by a variety of treatments including: growth factor deprivation; ultraviolet and *y*-radiation; heat shock; some cytotoxic lymphokines; calcium ionophores; viral infection and free radicals-promoting agents (Reed, 1994). It was later shown that is oncogenic characteristic stems from its ability to prevent apoptosis rather than promoting proliferation (Tsujimoto, 1998).

It is remarkable that proteins from the same family, constitute an expanding and heterogeneous family divided by their opposite functions into either pro- or anti-apoptotic members, such as anti-apoptotic proteins Bcl-2 or Bcl-xL which tend to prevent the release of apoptogenic molecules from the mitochondria and subsequent caspase activation, while pro-apoptotic proteins, such as Bax and Bak, promote these deleterious events (Aouacheria *et al*, 2005). Other significant fact about this family is the structural homology of the proteins, because they share a common tertiary structure, even though their primary structures are somewhat distant (Petros *et al*, 2001). Since the identification of Bcl-2, other members of the family have been identified on the basis of the primary structure homologies in the so-called "Bcl-2 Homology" domains (BH1 to BH4) that are conserved through the whole animal Kingdom (Lucken-Ardjomande and Martinou, 2005; Aouacheria *et al*, 2005). These proteins can form homo-dimmers and/or hetero-dimmers essentially through the interaction of their BH3 domain (Huang *et al*, 1998 and Sattler *et al*, 1997). The interaction between the BH3-domain of a protein and the BH1/BH2 domain of its partner, is an asymmetric interaction and is one of the bases of the regulation of the apoptotic network (Renault and Manon, 2011). The BH4 domain that corresponds to the first α helix (Hα1) of anti-apoptotic proteins is implicated in the control of their anti-death functions (Huang *et al*, 1998 and Sattler *et al*, 1997). A variety of Bcl-2 family members have been identified in mammalian cells (Adams and Cory, 1998), and were initially divided into three groups according to their

structure and function (Er *et al*, 2006). Although the understanding of activity and function for several Bcl-2 family proteins (e.g., Bcl-2, Bcl-XL, Bid and Bad), has grown rapidly most members of this family have received only an initial characterization (Aouacheria *et al*, 2005). Currently, with new results obtained for a sub-group of this family, the BH-3 only proteins (BOP), Bcl-2 family members are divided into four categories: the anti-apoptotic Bcl-2 proteins (A1, Bcl-2, Bcl-w, Bcl-xL and Mcl-1), Bcl-2 family effector proteins, (Bak and Bax), direct activators BOP (Bid, Bim and Puma) and sensitizers/de-repressors BOP (Bad, Bik, Bmf, Hrk and Noxa) (Chipuk *et al*, 2010). Figure 1.2 shows the homology shared by the Bcl-2 family members. The anti-apoptotic proteins like Bcl-2, Bcl-xL and Bcl-W are characterized by the presence of four Bcl-2 homology domains (BH: BH1, BH2, BH3 and BH4) [Figure 1.2(a)]. The pro-apoptotic proteins, such as Bax, Bak, contain three homology domains BH1, BH2 and BH3 [Figure 1.2(b)] and the BOP only shares one domain [Figure 1.2 (c)].

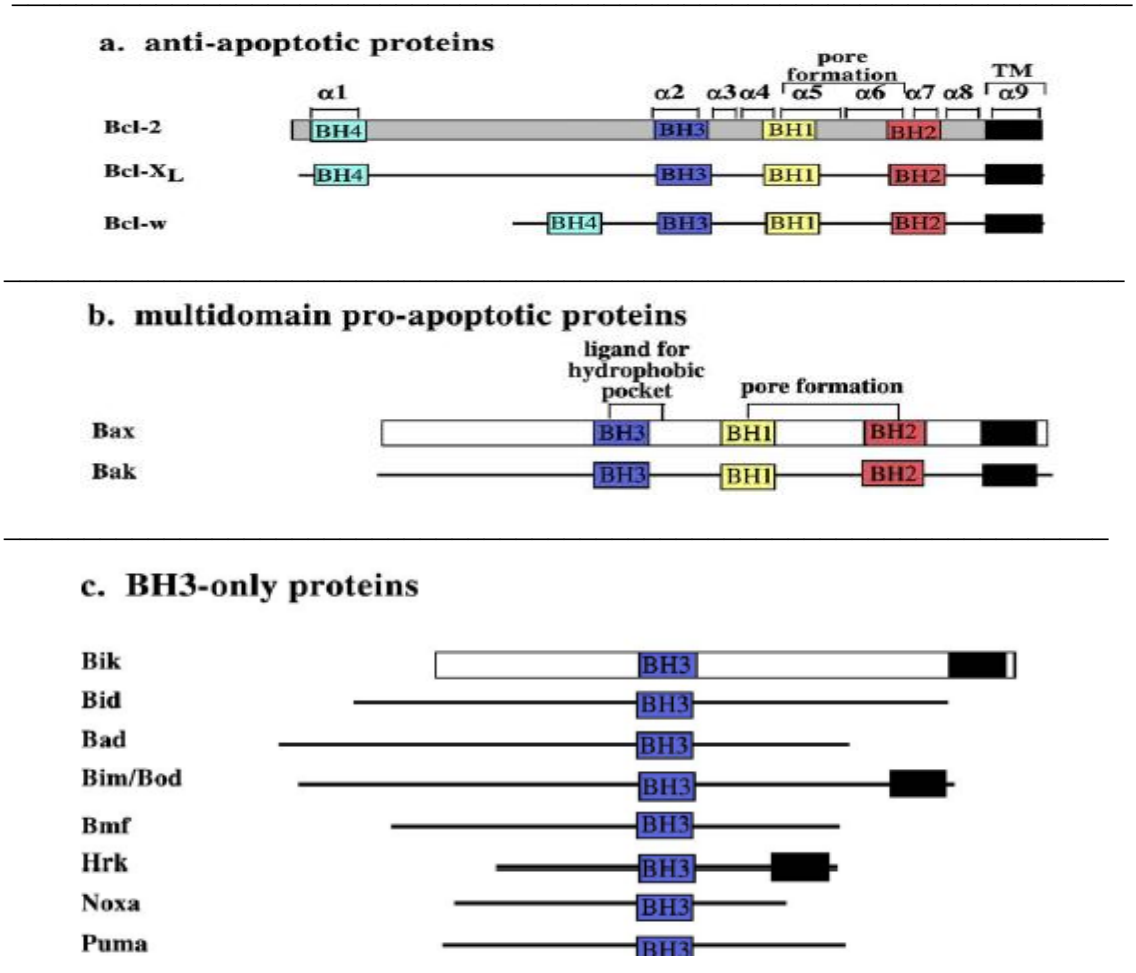

Figure 1.2 - Representation of all the known mammalian Bcl-2 family members. Bcl-2 homology regions 1-4 (BH1-4) are indicated. [Taken from (Er *et al*, 2006)].

Some members of this family are constitutively localized on the outer mitochondrial membrane while other family members can be found in various other sub-cellular locations including the ER, the cytosol and bound to microtubules (Sharpe *et al*, 2004). The control over the subcellular localizations of different members of the Bcl-2 family occurs through heterodimerization, phosphorylation, proteolysis, or interaction with *FKBP38* (FK506-binding protein 8 is a protein that in humans is encoded by the *FKBP8* gene) (Sharpe *et al*, 2004). Most of these proteins contain a C-terminal hydrophobic Hα, which is a potential transmembrane domain involved in their localization to the membranes of organelles such as the mitochondria, the ER and the nucleus, as will be explained later (Er *et al*, 2006). The BOP family members are thought to act as the trigger to initiate apoptosis, and therefore much effort has been devoted to understanding how the cell controls the activity of these lethal proteins (Guscetti *et al*, 2005). BOP members share only one sequence homology domain with the Bcl-2 family (BH3 domain), an amphipathic helix required to interact with other Bcl-2 family members (Huang *et al*, 2000). BOP members induce apoptosis by activating pro-apoptotic proteins like Bax or by inhibiting anti-apoptotic proteins like Bcl-2, so they are crucial intermediates that link specific apoptotic stimuli to the permeabilization of mitochondria (Antonsson *et al*, 2000). Once they reach the mitochondria, they need to cooperate with other mitochondrial proteins to induce the release of apoptogenic proteins (Wang, 2001). Several systems have been described that can keep the protein inactive until the initiation of the apoptotic signal. Examples exist that utilize regulated tethering of BOP to cytoplasm anchors, activation of precursor proteins, or stress-dependent transcriptional induction (Guscetti *et al*, 2005). Conformational changes that lead to the exposure of key domains within the molecule are required for Bax activation and insertion into the outer mitochondrial membrane and then to kill the cell (Gautier *et al*, 2011). Nowadays, with the recent completion of the whole-genome sequencing efforts and progress in bioinformatics a great effort has been made towards the identification of all the members of the family.

1.2.2 Apoptotic pathways

As referred before, two main apoptotic pathways have been identified. The first, known as the extrinsic pathway, is activated when ligands bind to death receptors on the external surface of the plasma membrane. The second, the intrinsic pathway, is activated by various stress signals, such as growth factor withdrawal, DNA damage, or anoikis (cell detachment), and involves the permeabilization of mitochondria, which then release apoptogenic factors that are normally sequestered in the mitochondrial intermembrane space (Lucken-Ardjomande and Martinou, 2005).

1.2.2.1 Extrinsic pathway

The extrinsic apoptosis pathway is involved in activation of death domain-containing receptors, commonly called death receptors (DR), belonging to the tumor necrosis factor (TNF) receptor family located at the plasma membrane, such as TNF-R1 (also called p55 or CD120a), Fas (also called CD95), DR4 and DR5. These receptors share a common activation mechanism, although each of these receptors is activated by its own ligand. When the ligand binds to the receptor its trimerization is induced and this promotes the connection between the cytoplasmic proteins Fas-associated death domain protein (FADD) and procaspase-8 (or procaspase-10) to form a complex known as the death-inducing signaling complex (DISC) which leads to self-activation of caspase 8, activation of effector caspases 3, 6 and 7 and completion of apoptosis (Gómez-Sintes *et al*, 2011; Jope, 2006; Peter and Krammer, 2003) (Figure 1.3).

In certain cell types, the direct activation of downstream caspases by the DISC appears to be sufficient for the execution of Fas-mediated apoptosis because Bcl-2 does not protect against Fas killing in these cell types. However, in other cell systems, Bcl-2 or Bcl-xL were reported to protect against Fas mediated apoptosis (Gewies *et al*, 2000).

1.2.2.2 Intrinsic pathway

Mitochondria have a central role in the induction of apoptosis in the intrinsic pathway which is mediated by mitochondrial outer membrane permeabilization (MOMP). The discovery that mitochondria were able to release apoptogenic factors during apoptosis was a major breakthrough in the understanding of the regulation of this cell death process (Renault and Manon, 2011).

MOMP is mediated by Bcl-2 family members, Bax and Bak, through their interaction and formation of pores in the outer membrane of mitochondria (OMM), such as, the mitochondrial apoptosis-induced channel (MAC), a pore that allows the release of pro-apoptotic proteins (Garrido *et al*, 2006). MOMP is a decisive event in apoptosis not only due to the release of lethal factors, but also because it may lead to cell death by accumulation of lethal Reactive Oxygen Species (ROS), causing the loss of cell homeostasis. Accumulation of ROS along with calcium ion (Ca^{2+}) overload induces mitochondrial permeability transition (mPT) that is associated with opening of the non-selective pathological mitochondrial permeability transition pore (mPTP) at the contact sites between the inner membrane of mitochondria (IMM) and the OMM. Opening of the mPTP is accompanied by loss of the mitochondrial membrane potential and proton gradient across IMM because it allows the passage of solutes and water into the mitochondrial matrix, causing despolarization and osmotic swelling and resulting in the rupture of the OMM and subsequently in the release of proapoptotic proteins that reside in the intermembrane space (Javadov and Kuznetsov, 2013). Though the exact molecular composition of the PTP is not defined it appears to be formed/regulated by the adenine nucleotide translocator (ANT), the voltage-dependent anion channel (VDAC), the peripheral benzodiazepine receptor (PBR), the hexokinase, creatine kinase (CK), and the mitochondrial matrix cyclophilin D (Cyp D) (Garrido *et al*, 2006). Nowadays recent studies suggest that reconstituted dimmers of the F_OF_1 ATP synthase form a channel with properties identical to those of the mitochondrial mega channel (MMC), the electrophysiological equivalent of the PTP (Bernardi, 2013).

VDAC forms a large voltage-gated pore when incorporated into planar lipid bilayers (Shimizu *et al*, 2000). Mitochondrial PT can occur at low and high conductance leading to reversible or irreversible events (Javadov and Kuznetsov, 2013).

In mammalian cells the MOMP occurs in response to various apoptotic stimuli that can cause the release of cytochrome *c* (cyt *c*), a component of the mitochondrial electron transport chain, and other pro-apoptotic proteins like Apoptosis Inducing Factor (AIF) and EndoG, from the intermembrane space of mitochondria into the cytoplasm (Abdelwahid *et al*, 2012). In the cytoplasm, released cyt *c* binds to the APAF, ATP and procaspase 9 to form the apoptosome and leading to activation of caspases which finally execute cell death (Linseman *et al*, 2004; Gómez-Sintes *et al*, 2011) (Figure 1.3). Only caspase-9 binds to the apoptosome in an energy dependent manner and is able to efficiently cleave and activate downstream executioner caspases,

such as caspase 3 and caspase 7 (Rodriguez and Lazebnik, 1999). The Bcl-2 family members have a critical role in determining whether or not the multimeric scaffold/procaspase complex, often termed "apoptosome", can be assembled (Hengartner, 2000).

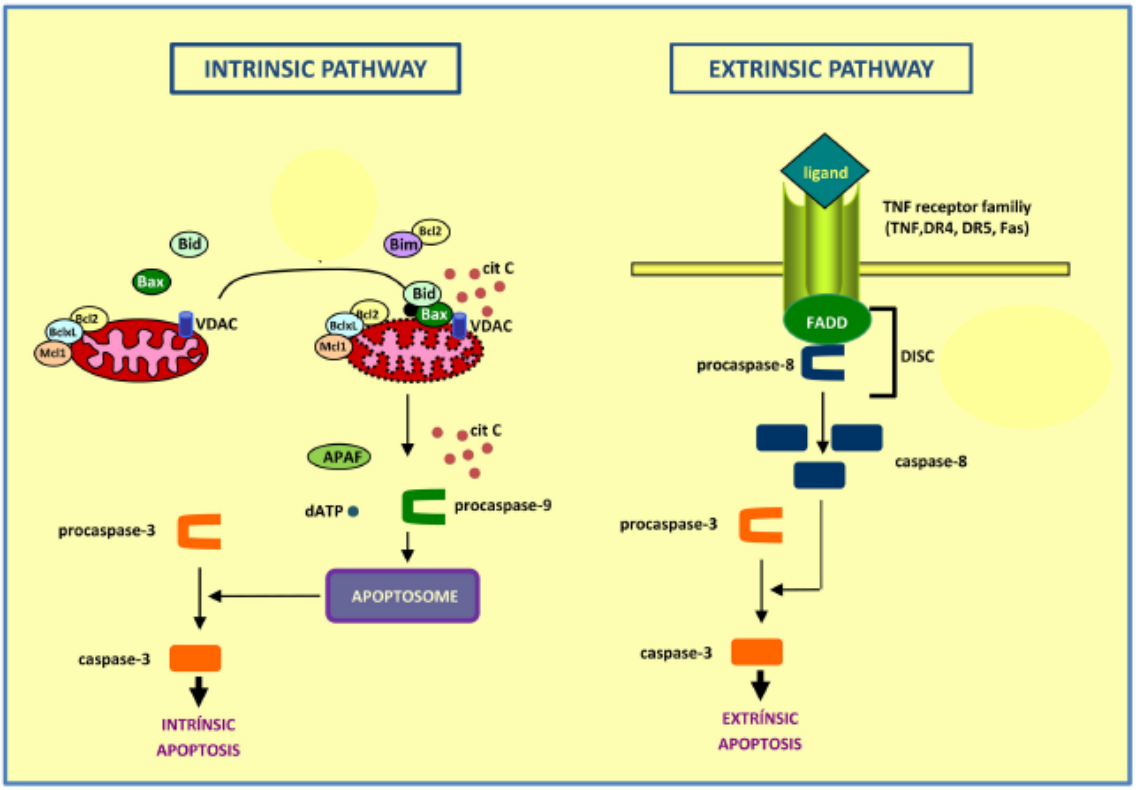

Figure 1.3- Diagram showing the intrinsic (left) and extrinsic (right) apoptotic pathways (Gómez-Sintes *et al*, 2011).

1.3 The Bax Protein

1.3.1 Conformational studies: The C and N-terminal role

In mammalian systems Bax alone or together with other pro-apoptotic members, has proven to be sufficient to induce mitochondrial membrane permeabilization (Shimizu *et al*, 2000). The first evidence that Bcl-2 family members, namely Bax, displayed this ability was obtained with purified proteins reconstituted in liposomes or in planar lipid bilayers. Currently Bax is the best-studied member of this family of proteins. In healthy cells Bax can adopt at least two stable conformational states: i) inactive Bax with cytosolic localization (Annis *et al*, 2005 and Pierre-François Cartron *et al*, 2008) or loosely bound to the OMM, with important functions in mitochondrial

morphogenesis and hardly present in the other membrane fractions such as the ER, remains under a closed conformation, making it poorly able to interact with other partners (Amsel *et al*, 2008; Priault *et al*, 2003; Arokium, *et al*, 2004 and Karbowski *et al*, 2006) ; ii) fully activated Bax with mitochondrial localization (Annis *et al*, 2005 and Pierre-François Cartron *et al*, 2008). The pro-apoptotic function of Bax depends on its ability to translocate, oligomerize and insert into the mitochondrial membrane following stress (Silva *et al*, 2011b). Recent studies propose that Bax is converted into an active conformation capable of being inserted into mitochondria by interaction with BH3 proteins, such as Bim (Walensky and Gavathiotis, 2011). Other authors (Lovell *et al*, 2008) suggests that Bax can only interact with BH3 proteins if both are connected to membranes which implies that mitochondrial targeting of Bax is a prerequisite for its subsequent activation by BH3 binding domain. So Bax targeting to mitochondria turns out to be more complicated than it seems. Furthermore, mitochondrial Bax may be removed back to the cytosol through its interaction with antiapoptotic proteins such as Bcl-xL(Edlich *et al*, 2011).

Even more recent studies show that Bax is in a dynamic equilibrium between the cytosol and mitochondria. In healthy cells, Bax is not exclusively cytosolic, rather, it is distributed between the cytosol and the mitochondria suggesting that Bax exists in equilibrium between these two subcellular compartments, the proportion of each fraction depending on the level of stress and survival signaling (Schellenberg et al., 2013).

It is thought that oligomerization of Bax and/or Bak facilitates the release of proteins from the Mitochondrial Intermembrane Space (IMS) (Sheridan *et al*, 2008) and it is known that Bax and Bak promote mitochondrial fission, while dominant-negative interfering mutant forms of proteins, such as Drp1, which antagonize fission of mitochondria, have also been reported to antagonize both cytochrome *c* release and apoptosis (Sheridan *et al*, 2008). The dependence of Bax-induced cell death on mitochondrial lipid oxidation reinforces the importance of mitochondria physiology in Bax killing effect (Priault *et al*, 2002).

Some proteins of the Bcl-2 family have a hydrophobic C-terminal tail that allows their association with membranes (Schinzel *et al*, 2004). When compared with the full length Bcl-2 and Bcl-xL proteins the C-terminal truncated versions lose their ability to insert into membranes and prevent apoptosis in mammalian cells (Janiak *et al*, 1994). Movemente of N-terminal domain of Bax, leads to Bax translocation to the

mitochondria and insertion into the outer membrane as an alkali-resistant form (Makin *et al*, 2001). This N-terminal domain, designated as Apoptotic Regulation of Targetting (ART), has the ability to lock the proteins under a soluble inactive conformation and its movement is important for mitochondrial translocation of Bax (Goping *et al*, 1998). The ART of human Bax contains 2 Proline(Pro) residues flanking an Arginine (Arg)-Glycine (Gly)-Gly-Gly sequence, in position 8 and 13, and the function of ART has been further investigated by introducing point mutations. For example the replacement of Pro8 and Pro13 by Gly favored the mitochondrial translocation of Bax both in human and in yeast cells, and apoptosis in human cells (Cartron *et al*, 2002). Interactions between ART and residues localized in other domains of Bax are central regulators of ART movements and, subsequently, of Bax mitochondrial translocation (Arokium *et al*, 2004).

The homologous helices in the anti-apoptotic Bcl-2 family members and Bcl-xL are transmembrane anchors and so their complete suppression, prevents the membrane-insertion of these proteins, and only residual loosely-bound Bcl-2 remains attached to mitochondrial and ER membranes (Janiak *et al*, 1994).

Like other proteins of the Bcl-2 family Bax is formed by alpha helices connected by loops. In aqueous solution Bax is composed by 9 α-helices of which the 2 central helices (Hα5 and Hα6) are mostly hydrophobic (Lalier *et al*, 2007). The 19 first residues of Bax, that precede the Hα1, are very mobile, as suggested by Nuclear Magnetic Resonance (NMR) data (Suzuki *et al*, 2000). The Hα1 by itself has been shown to be involved in the translocation of Bax to the OMM (Cartron *et al*, 2003). The BH-3 domain of Bax roughly corresponds to the Hα2 of the protein (Zha *et al*, 1996). Movements between Hα1 and Hα2 are involved in Bax activation, and once ART has been moved Bax can be activated (Renault and Manon, 2011). The helices Hα5 and Hα6 may directly participate to the permeabilization processes induced by Bax. Indeed it was shown that the capacity of the protein to promote membrane permeabilization and to trigger apoptosis is altered in mutant proteins lacking these helices (Valero *et al*, 2011). So, in summary, Hα5, Hα6 and Hα9 helices are likely involved in the interaction of Bax with OMM (Schendel *et al*, 1998). In the three-dimensional (3D) structure of Bax it is clear that, in the native conformation, Hα9 is tightly sequestered in the hydrophobic pocket and cannot insert into membranes in this conformation (Silva *et al*, 2011b). Many cell biology studies made with a fusion Green Fluorescent Protein (GFP)-Bax showed that the absence of Hα9 prevent the ability of the fusion protein to be

translocated to the OMM following an apoptotic signal (Nechushtan *et al*, 1999) (Figure 1.4). Therefore the movement of Hα9 seems to be a crucial event in Bax translocation, not because it might be a membrane anchor, but because this movement unmasks the BH domain and the hydrophobic hairpin Hα5/Hα6, that are crucial for the subsequent events of dimerization and oligomerization (Lalier *et al*, 2007).

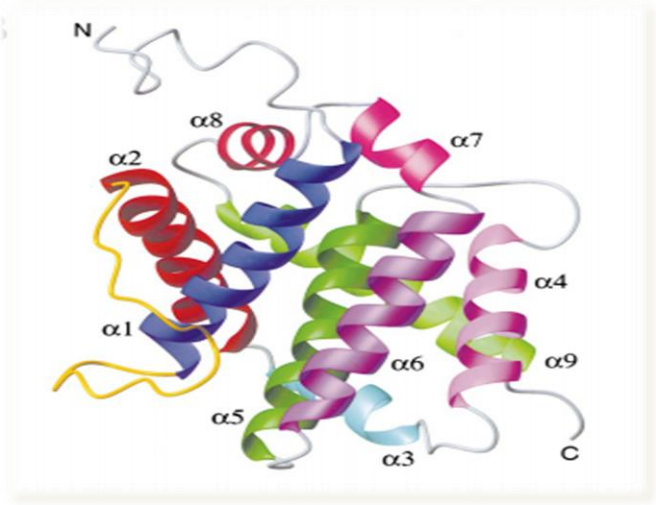

Figure 1.4 – A structure of Bax monomer. All the α-helices were indicated

1.3.2 Regulatory mechanisms by protein interactions and post-translational modifications

A huge importance has been given to the regulation of Bcl-2 family proteins by post-translational modifications, in the very last years. Proteins of the Bcl-2 family can be regulated by interaction with other members of the Bcl-2 protein family or other proteins, or even by post-translational modifications. A key event in regulation of the Bcl-2 family members is the regulated protein-protein interactions (Shimizu *et al*, 2001). Members of this family can interact and form heterodimers blocking the activity of each other (Hanada *et al*, 1995). Inhibiting protein interactions might be a method for pharmacological intervention. A peptide and non-peptide mimetics of the BH3 domain are able to interfere with Bax/Bcl-2 interaction. These BH3 mimetics are small molecule antagonists of the anti-apoptotic Bcl-2 members. They act as a competitive inhibitors of the pro-apoptotic proteins though binding to the hydrophobic cleft of the anti-apoptotic proteins (for a review see Chonghaile and Letai, 2008).

It has already been reported that phosphorylation of different Bax residues modulates its activity (Gardai *et al*, 2004). Phosphorylation is the most common regulation mechanism by post-translational modifications of the members of Bcl-2 protein family and in most cases leads to the loss of the biological function of these proteins (Basu *et al*, 2006). Bax has been identified as a substrate of different kinases that regulate its activity. As mentioned above, Bax is activated when Hα9 is forced to move away from BH3 domain. So, it would be expected that the phophorylation of Ser184 introduced a size constraint that would also help the movement of Hα9. However Bax phosporylation on Ser184 prevents its translocation to the mitochondria, whereas non-phophorylated Bax was mitochondrial (Suzuki *et al*, 2000). Consistently, phosphorylation of Ser184 by protein kinase B (AKT/PKB) promotes cell survival (Gardai *et al*, 2004). The protein kinase Cζ (PKCζ) (Yamaguchi and Wang, 2001 and Xin *et al*, 2007) promotes cell survival that is prevented by dephosphorylation by the protein phosphatase 2A (Xin and Deng, 2006). Apoptosis modulation-associated proteins may also be regulated through phosphorylation by different proteins kinases, such as protein kinase c (PKC) (Saraiva *et al*, 2006). Generally, classical and atypical PKCs appear to be associated with cell survival, whereas novel PKCs are associated with apoptosis stimulation. It was shown that different PKC isoforms modulate the Bcl-xL anti-apoptotic activity differently through interference with the phosphorylated forms of Bcl-xL (Saraiva *et al*, 2006). Akt/PKB also phosphorylates Bad increasing cell survival (Datta *et al*, 1997), Bcl-2 phosphorylation is required for its anti-apoptotic function (Ito *et al*, 1997) and Bcl-xL is phosphorylated and inactivated by the Jun N-terminal kinase (JNK) (Fan et al., 2000).

Others residues can also be involved in regulation of Bax by phosphorylation; two examples are the phosphorylation on Ser163 that can be another possible target by glycogen synthase kinase-3β (GSK-3β), and the phosphorylation Thr167 by JNK and p38 kinase, leading to Bax activation and cell death. GSK-3β is a crucial activator of cell death in numerous models of neuronal apoptosis (Linseman *et al*, 2004; Silva *et al*, 2011b). Arokium *et al*, 2007 identified a new putative phosphorylation site at Ser60, which is located in a consensus target sequence for PKA. These phosphorylations results allowed the study of the complex phoshorylation/dephosphorylation events that regulates Bcl-2 protein family activity by heterologous expression of different kinases and protein phosphatases.

As it is well known, proteins phosphatases are enzymes whose function is to remove the phosphate group from a substrate, though a dephosphorylation mechanism while kinases have an opposite function, and phosphorylate the substrates through an ATP consuming reaction. Protein phosphatases can be classified according to their specificity regarding the aminoacid residue they dephosphorylate (table below).

Class	Example	Substrate	Reference
Histidine protein phosphatase	**PHP**	**Phospho-Histidine**	**(Moorhead, 2007)**
Dual specificity protein phosphatases	**VHR**	**Phosphotyrosine/-serine/-threonine**	**(Camps *et al*, 2000)**
Serine-/threonine-pecific protein phosphatases	**PP2C**	**Phosphoserine/-threonine**	**(Moorhead, 2007)**
Lipid protein phosphatase	**PTEN**	**Phosphatidyl-Inositol-3,4,5-Triphosphate**	**(Maehama *et al*, 2004)**
Tyrosine-specific protein phosphatases	**PTP1B**	**Phosphotyrosine**	**(Zhang, 2002)**

Although protein phosphatases can be divided based on their substrate specificity they can also be divided into four main groups based on their catalytic function, structure and sequence. So according to this classification the major classes of protein phosphatases are the protein phosphatase (PPP) family, corresponding to PP1, PP2A, PP2B, PP4, PP5, PP6 and PP7, the protein phosphatase Mg^{2+} (PPM) family, corresponding to PP2C, the PTP, and the aspartate-based protein phosphatase (Barford, 1996).

1.4 Contributions of the yeast model to study the function of proteins of the Bcl-2 family

Several studies have examined the response of yeast to the heterologous expression of proteins of the Bcl-2 family, in order to address basic questions on their mechanisms of action. The idea of using yeast as an alternative system for the elucidation of molecular aspects of the function of these apoptotic regulators arose accidentally (Pereira *et al,* 2008), even before yeast was a well established unicellular eukaryotic model to study cell death. Indeed for many years, apoptosis was assumed to be restricted to multicellular organisms. However, it is now established that several stimuli can induce in unicellular organisms some of typical apoptotic markers that are

observed in multicellular organisms. Through this concept initially generated a big controversy due to the difficulty to find an advantage of a cell suicide in an unicellular organism additional evidence showed that apoptotic-like cell death can greatly benefit a population of unicellular organisms, such as yeast, for instance by eliminating virus-infected and damaged cells or by releasing nutrient sources for the fittest individuals (Büttner *et al*, 2006 and Rego *et al*, 2012). So far several yeast orthologs of crucial apoptotic regulators have been discovered (for a revision see Carmona-Gutierrez *et al*, 2010). For instance, a caspase-like protein termed Yca1p has been identified, which revealed to be involved in programmed cell death induced by acetic acid, hydrogen peroxide ($H_2O_{2)}$ and ageing. Absence of Yca1p reduces cell death whereas its overexpression enhances apoptotic-like death of the cells (Frank Madeo *et al*, 2002 and Fahrenkrog *et al*, 2004). It was also recognized in yeast a mitochondria-mediated apoptosis pathway in response to a variety of stimuli, similar to the mammalian intrinsic apoptotic pathway, involving cytochrome *c* release from mitochondria to the cytosol (Ludovico *et al*, 2002; Pereira et al, 2008; Giannattasio *et al*, 2008). The use of yeast mutants defective in genes encoding mitochondrial proteins enabled to identify several other mitochondrial regulators/mediators of apoptosis in this organism (Figure 1.5). The proteins identified are components of the electron transport chain, of the IMM or of OMM. While most of the yeast mitochondrial proteins recognized as implicated in apoptosis have their mammalian counterparts also involved in apoptosis, a few were only identified in yeast (e.g. Ysp1p and Ysp2p).

An ortologue of Apoptosis Inducing Factor (AIF), YNR074C was identified in yeast that displays sequence similarity to AIF and AIF-homologous mitochondrion-associated inducer of death (AMID) and regulates apoptosis in a similar way to that AIF in mammalian cells. However, the inner membrane mitochondrial Nicotinamide Adenine Dinucleotide (NADH) dehydrogenase (Ndi1p), which is the first component of the electron transport chain in yeast, was found to be the closest yeast orthologue to mammalian AMID proteins (Li *et al*, 2006). Nuc1p, an ortolog of another classical apoptotic regulator, EndoG has also been classified and characterized (Büttner *et al*, 2007).

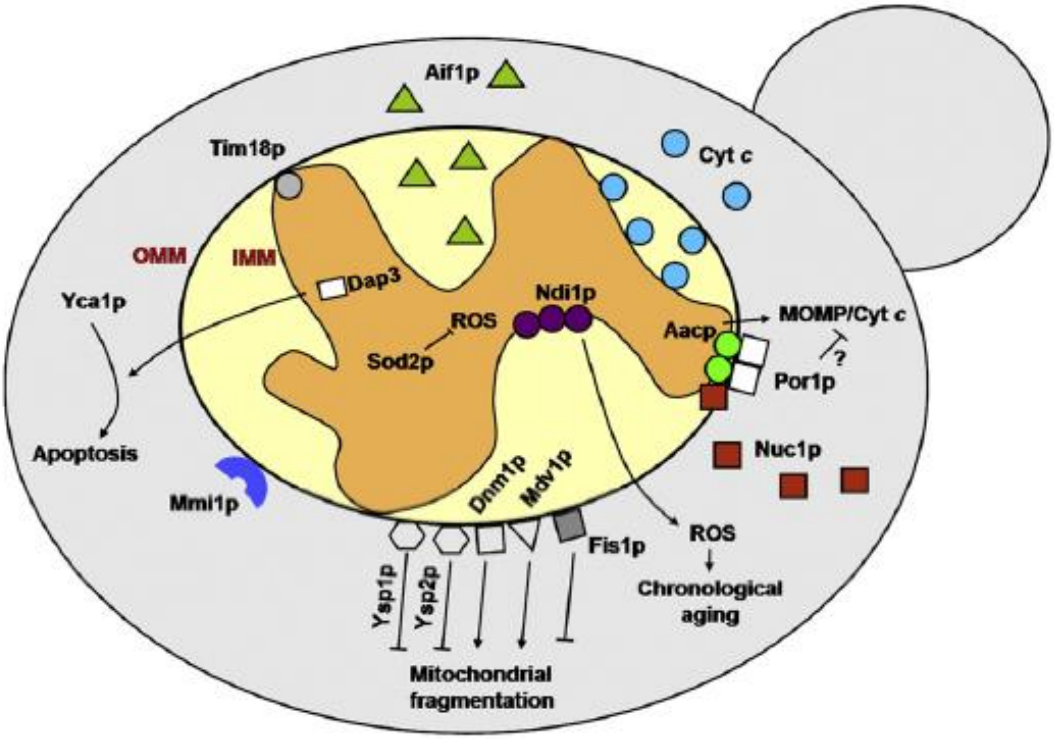

Figure 1.5- Mitochondrial proteins involved in the regulation of yeast apoptotic cell death. (Taken from *(Pereira et al*, 2008).

Some of those yeast mitochondrial proteins, like their mammalian akin, are translocated from the mitochondria to the cytosol or to the nucleus (Aif1p and Nuc1p), others are proposed to be involved in mitochondrial permeabilization and release of the former proteins (Aac1p, Aac2p and Aac3p, and Por1p) or in the fission/fusion of mitochondria (Dnm1p, Mdv1/Net2, Fis1p, Ysp1p and Ysp2p) (Pereira *et al*, 2008). All these findings firmly established that yeast and metazoan apoptosis have in essence the same cellular program (Li *et al*, 2006).

Until recently it was thought that yeast lacks obvious homologues of the Bcl-2 protein family members, and it has therefore been used as an "in vivo" system to study several of these apoptotic regulators without the interference of other family members. But in the last decade Büttner *et al*, 2011 report that the yeast genome encodes a BH3 domain-containing protein (Ybh3p) which interacts with Bcl-xL and shares functional characteristics with the pro-apoptotic members of the mammalian Bcl-2 family. Ybh3p is capable of translocating to mitochondria and regulate the mitochondrial apoptotic pathway in a phylogenetically conserved manner. This study has also proved that the overexpression of Ybh3p enhances stimulus-induced apoptosis, while its knockout

reduces cell death. Death mediated by Ybh3p was accompanied by a burst of ROS. The deletion of *YBH3* protects against cell death induced by H_2O_2, acetic acid, mammalian Bax expression and ageing. Ybh3p was first described as a member of the family of Bax-inhibitor 1 (BI-1) proteins, which operate as antiapoptotic proteins in the endoplasmic reticulum of all phyla, including animals, plants and yeast, and contributes to the development of cancer. BI-1 inhibits Bax induced apoptosis and appears to regulate the concentration of Ca^{2+} in the ER and the cytosol, in mammalian cells. Plant BI-1 form is able to suppress mammalian Bax induced cell death in yeast (Cebulski *et al*, 2011).

1.4.1 Advantages of the yeast model system

Because of the conservation of many cellular processes in yeast and of its simple manipulation and genetic tractability, this organism, among the different cell models exploited to comprehend several mammalian cellular pathways and processes, emerged as a powerful tool and a model of choice to answer important biological questions. Specifically, the easy manipulation of its mitochondria led to an increased interest in using this cell model to unveil unknown features of the mammalian intrinsic apoptotic pathway and its regulation by members of the Bcl-2 family (Silva *et al*, 2011a).

It was demonstrated that Bcl-2 family members when expressed in yeast are likely to act upon highly conserved mitochondrial components that correspond directly to their apoptotic substrates in mammalian cells, generating similar, if not identical, biochemical and physiological responses (Guscetti *et al*, 2005). It was described that expression of Bax in *S. cerevisiae* causes growth arrest and induces cell death with similar characteristics to those observed in apoptotic metazoan cells (Ligr *et al*, 1998). However, another work showed the occurrence of autophagic features in yeast cells expressing Bax, including increased accumulation of Atg8p and activation of the targeting-deficient mutant of the vacuolar alkaline protein phosphatase. Inactivation of autophagy slightly accelerated Bax-induced cell death showing a protective role for this process (Kissová *et al*, 2006).

Bax was also shown to induce cell death in other yeast species namely in *Schizosaccharomyces pombe*, *Pichia pastoris* and *Candida albicans* (Ink *et al*, 1997 and Greenhalf *et al*, 1996). The budding yeast *S. cerevisiae* has proved particularly useful for studying Bax-induced mitochondrial changes, because Bax is able to induce the release of cyt *c* from mitochondria (Manon *et al*, 1997) and induces apoptotic

mitochondrial changes in isolated yeast mitochondria like mammalian mitochondria (Shimizu *et al*, 1999). Bax in its native conformation does not induce cell death in yeast (Silva *et al*, 2011b). In native Bax, the position of its C-terminal and N-terminal allows Bax to keep a cytosolic stable conformation. When expressed in yeast, human full-length Bax is not localized in mitochondria, but the addition of a *c-myc* tag at the C-terminal end, or the replacement of a part of Hα9 helix by a random sequence are sufficient to promote the mitochondrial localization (Priault *et al*, 2003). The conformational change of its N-terminal end is associated to its translocation, but this is not sufficient to promote the full activation and insertion of the protein (Arokium *et al*, 2004). So to be activated, Bax needs to suffer dramatic conformational alterations in order to insert into the mitochondria membrane. This can be achieved by deletions of portions of the protein, single substitutions or addition of an epitope, and allowed to show that the Hα1 is necessary for Bax translocation to mitochondria and contains an addressing signal to the mitochondria, as referred above.

Ionic interactions between some residues of the protein are stabilized by the position of Bax C-terminal and N-terminal conformation and its disruption can lead to Bax activation (Silva *et al*, 2011a). Subsequent work towards identifying the genetic determinants of Bax-mediated cell death have indicated a significant role for the oxidative phosphorylation machinery and respiratory status in Bax-mediated yeast apoptosis (Khoury and Greenwood, 2008).

Some authors (Arokium *et al*, 2007) also showed that human Bax α is phosphorylated in yeast cells and mutation of possible phosphorylation serine sites in the protein enhances the ability of Bax α to insert to the mitochondria and to induce cyt *c* release. For example, the phosphorylation of Ser163 was shown to stimulate Bax-dependent apoptosis, and when expressed in yeast, as compared to Bax wild-type, the phosphomimetic mutant Ser163 to Asp did not exhibit any significant increase of activity (Arokium *et al*, 2007).

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2. OBJECTIVES

Objectives

Although it is known that specific residues of Bax are phosphorylated and can regulate its insertion into mitochondria, phosphorylated Bax has yet to be found in mitochondrial membranes. These observations indicate that, kinases and protein phosphatases are involved in Bax regulation. Though some mechanisms and phosphorytable residues have already been identified, the sequence analysis of this protein showed the presence of other amino acid residues that are potential targets of this modification.

So this project aimed to identify new protein phosphatases involved in regulation of Bax by dephosphorylation and to understand whether this modification influences the different steps of Bax activation and ultimately apoptosis.

The work plan proposed focused on:

- The identification of protein phosphatases involved in Bax regulation
- The identification of new Bax residues crucial for its regulation through dephosphorylation.

These approaches took advantage of heterologous expression of human Bax in the yeast S. *cerevisiae*.

Since protein phosphatases are potential drug targets, we expected our results may could lead to the proposal of new therapeutic strategies against diseases involving apoptotic dysfunctions.

3. MATERIALS AND METHODS

3.1 Yeast strains and plasmids

The *Saccharomyces cerevisiae* BY4741 strain (MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0), and the respective deletion mutants in the protein phosphatase genes listed below (Table 3.1) from Euroscarf collection, were used in this study. In total we studied 23 protein phosphatases that have mammalian orthologues.

Table 3.1- Yeast protein phosphatase, encoded by non-essential genes and respective human orthologues.

Yeast non-essential protein phosphatase genes	ORF	Description	Human orthologue genes
LPT1	YPR073C	**Protein phosphotyrosine protein phosphatase** of unknown cellular role; activated by adenine	ACP1
MIH1	YMR036C	**Protein tyrosine protein phosphatase** involved in cell cycle control; regulates the phosphorylation state of Cdc28p	CDC25A; CDC25B; CDC25C
PTC1	YDL006W	**Type 2C protein phosphatase (PP2C)**; dephosphorylates Hog1p, inactivating osmosensing MAPK cascade; involved in Fus3p activation during pheromone response	PPM1K
PTC2	YER089C	**Type 2C protein phosphatase (PP2C)**; dephosphorylates Hog1p to limit maximal osmostress induced kinase activity; dephosphorylates Ire1p to downregulate the unfolded protein response; dephosphorylates Cdc28p; inactivates the DNA damage checkpoint	PPM1A; PPM1B; PPM1E; PPM1F; PPM1N
PTC3	YBL056W	**Type 2C protein phosphatase (PP2C)**; dephosphorylates Hog1p (see also Ptc2p) to limit maximal kinase activity induced by osmotic stress; dephosphorylates T169 phosphorylated Cdc28p (see also Ptc2p); role in DNA damage checkpoint inactivation	
PTC4	YBR125C	**Cytoplasmic type 2C protein phosphatase (PP2C)**; identified as a high-copy number suppressor of cnb1 mpk1 synthetic lethality	PPM1D (WIP1); PPM1G
PTC5	YOR090C	**Mitochondrial type 2C protein phosphatase (PP2C)**; involved in regulation of pyruvate dehydrogenase activity by dephosphorylating the serine 133 of the Pda1p subunit; acts in concert with kinases Pkp1p and Pkp2p and phosphatase Ptc6	PDP1; PDP2
PTC7	YHR076W	**Type 2C protein phosphatase (PP2C)**; alternatively spliced to create two mRNA isoforms; protein from spliced form localizes to the mitochondria while the one from the unspliced form is localized to the nuclear envelope	PPTC7
PTP1	YDL230W	**Phosphotyrosine-specific protein phosphatase**; dephosphorylates a broad range of substrates in vivo, including Fpr3p; localized to the cytoplasm and the mitochondria; proposed to be a negative regulator of filamentation	PTPN6; PTPRQ; PTPRO; PTPN11
YHV1	YIR026C	**Protein phosphatase**; involved in vegetative growth at low temperatures, sporulation, and glycogen accumulation; mutants are defective in 60S ribosome assembly; member of the dual-specificity family of protein phosphatases	DUSP12; DUSP15; DUSP19; DUSP22
CNA1	YLR433C	**Calcineurin A**; **one isoform (the other is Cmp2p) of the catalytic subunit of calcineurin, a Ca++/calmodulin-regulated protein phosphatase** which regulates Crz1p (a stress-response transcription factor).	PPP3CA; PPP3CB; PPP3CC
CMP2	YML057W		
	YKL190W	**Calcineurin B; the regulatory subunit of calcineurin, a**	PPP3R1;

CNB1		**Ca++/calmodulin-regulated type 2B protein phosphatase** which regulates Crz1p (a stress-response transcription factor	PPP3R2
SIT4	YDL047W	**Type 2A-related serine-threonine protein phosphatase**; functions in the G1/S transition of the mitotic cycle; regulator of COPII coat dephosphorylation; required for ER to Golgi traffic; interacts with Hrr25p kinase; cytoplasmic and nuclear protein that modulates functions mediated by Pkc1p including cell wall and actin cytoskeleton organization	PPP6C
PPH21	YDL134C	**Catalytic subunit of protein phosphatase 2A (PP2A)**; functionally redundant with Pph22p; methylated at C terminus; forms alternate complexes with several regulatory subunits; involved in signal transduction and regulation of mitosis; forms nuclear foci upon DNA replication stress	PPP2CA; PPP2CB; PPP4C
PPH22	YDL188C		
PPT1	YGR123C	**Protein serine/threonine protein phosphatase**; regulates Hsp90 chaperone by affecting its ATPase and cochaperone binding activities; has similarity to human phosphatase PP5; present in both the nucleus and cytoplasm; expressed during logarithmic growth	PPP5C; PPEF1; PPEF2
SER2	YGR208W	**Phosphoserine protein phosphatase** of the phosphoglycerate pathway; involved in serine and glycine biosynthesis, expression is regulated by the available nitrogen source	PSPH
NEM1	YHR004C	**Probable catalytic subunit of Nem1p-Spo7p protein phosphatase holoenzyme**; regulates nuclear growth by controlling phospholipid biosynthesis, required for normal nuclear envelope morphology and sporulation	CTDNEP1
TDP3	YAL016W	**Regulatory subunit A of the heterotrimeric protein phosphatase 2A (PP2A)**, which also contains regulatory subunit Cdc55p and either catalytic subunit Pph21p or Pph22p; required for cell morphogenesis and transcription by RNA polymerase III	PPP2R1A; PPP2R1B
CDC55	YGL190C	**Non-essential regulatory subunit B of protein phosphatase 2A (PP2A)**	PPP2R2A; PPP2R2B; PPP2R2C; PPP2R2D
PSY2	YNL201C	**Subunit of protein phosphatase PP4 complex**; active complex is composed of catalytic subunit Pph3p and Psy2p, with Psy4p apparently providing additional substrate specificity in some cases	SMEK1; SMEK2
RTS1	YOR014W	**B-type regulatory subunit of protein phosphatase 2A (PP2A)**	PPP2R5A; PPP2R5B; PPP2R5C; PPP2R5D; PPP2R5E

To express the different Bax forms we used the PYES2 plasmid that has the URA3 gene as a selective marker. The plasmids used on this study are listed in Table 3.2.

Table 3.2- List of plasmids used in this study.

Plasmid	Description	Source
PYES2 Ø	URA3; AmpR	Invitrogen
PYES2 Bax alpha	Bax alfa inserted in PYES2	This study
PYES2 Bax P168A	Bax P168A inserted in PYES2	This study
PCM189 Bax *c-myc*	Bax *c-myc* inserted in PCM189	This study

3.2 Transformation of yeast mutants, with the plasmids expressing the forms of Bax and growth conditions

The strains (protein phosphatases deleted mutants and wild type mentioned above) were grown overnight in *Yeast Peptone Dextrose* (YPD) medium (1% of yeast extract, 1% of peptone and 2% of glucose) and, in the following day they were diluted to 0.2, and incubated again until reaching mid-exponential phase (optical density $(OD)_{640nm}$ = 0.8). Transformation of *S.cerevisiae* cells with the above mentioned plasmids was done by the lithium acetate general protocol (Ito *et al*, 1983). The cells were ressuspended in 200µL of lithium acetate at 0.1M and incubated at 4ºC overnight. Simultaneously, *E. Coli* cells transformed with the different plasmids were grown in *Luria-Betani broth* (LB) medium (0.5% of Yeast extract, 1% of NaCl and 1% of tryptone, pH 7.5) with 5 µl of ampicillin at 37 ºC, 200rpm. The extraction of the plasmid, containing the isoforms of Bax, was done by Miniprep kit (GenElute Plasmid Miniprep kit, Sigma-Aldrich) according to manufacturer's instruction.

The transformation requires: 250µL LiAc / PEG (225µL PEG (50%) + 25µL LiAc (1M), 5µL salmon sperm ssDNA (must be boiled) and 5µL DNA (100-200ng DNA). Then the solution was vortex to mix well and 50mL of cells in 0.1 M LiAc were added and vortex again. After, the mixture was incubated 30 minutes at 30 ºC, 200 rpm, followed by 30 minutes in a bath at 42 ºC and then 60 minutes in the ice. When this step was completed cells were plated to confirm the transformation. For such the suspension was centrifuged at maximum speed (14500rpm), ressuspend in 200µL of sterile H_2O and plating 200µL in a plate with Synthetic Complete (SC) medium [SC containing 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, 0.008% (w/v) histidine, 0.04% (w/v) leucine, lacking the appropriate aminoacids and 2% agar].

Yeast strains were maintained on solid SC medium (lacking the appropriate amino acids), grown at 30°C for 48 h, stored at 4°C and refreshed every week. Yeast cultures were grown aerobically in SC medium with 2% glucose or galactose as a carbon source. Strains transformed with plasmids were grown in the same medium lacking the appropriate amino acids. Cells were incubated at 30°C with orbital shaking (200 rpm) and a liquid/air ratio of 1:5.

3.3 Induction conditions for the different Bax forms

Strains harboring the plasmid Bax *c-myc* were grown overnight in SC glucose2% lacking uracil and supplemented with doxycycline (10 µg/ml) to repress Bax *c*-myc expression. Cells were centrifuged, washed three times with sterilized water and ressuspended in the same medium without doxycycline, to induce Bax *c*-myc expression with 14h of incubation.

For the strains with the Bax alpha and Bax P168A plasmids we use two different kinds of inductions. In the first, strains with the two plasmid were grown overnight in SC glucose 2% lacking uracil. The following day the cells cultures were diluted to 0.2, and incubated again in SC galactose 2% lacking uracil to induce Bax expression. After 14h of incubation the cells were again normalized to OD (by the lower OD, calculate to collect the same amount of cells). In the second, strains with the two plasmid were grown overnight in SC glucose 2% lacking uracil. The following day the cells were normalized to OD (by the lower OD, calculate to collect the same amount of cells) in SC lactate 0.5% lacking uracil and ethanol 0.5%. On the third day, galactose was added to the medium to a final concentration of 0.5%, to induce Bax for 4h.

3.4 Protein sample preparation

After 14h of incubation the strains with different forms of Bax (Bax alpha, P168A and *c-myc*) (which have different types of Bax induction aforementioned) has the same procedure for the preparation of the protein sample.

Approximately $2x10^6$ cells (1ml; OD=2) were harvested, ressuspended in 0.5 mL of water, and added to 50µL of mixture of 3.5% β-mercaptoethanol in 2M NaOH in a 1.5mL microcentrifuge tube and incubated on ice for 15 minutes. Then the proteins were precipitated by adding 50µL of TCA 50% and incubating for 15minutes on ice. After, the cells were centrifuged for 10 minutes at 10000 g. The supernatant was aspirated and the pellet re-solubilized in 16 µL of 5% SDS plus 16 µL of Laemnli buffer

(2% β-mercaptoethanol, 2% SDS, 0.1 M tris-HCl, pH 8.8, 20%glycerol, 0.02% bromophenol blue). 3 µl of tris 1M pH 9.5 were then added (to adjust the pH, the solution was half purple). The samples were boiled in the thermoblock 5 min at 100ºC. Then centrifuge and store the pellet at -20 º C.

3.5 SDS gel electrophoresis/Western blot

Proteins were separated electrophoretically on a 12.5% SDS polyacrylamide gel at 25mA and transferred to a Hybond-P Polyvinylidene Difluoride Membrane (PVDF) (Hybond-ECL, GE Healthcare) at 60mA for 1h:30 minutes. Membranes were blocked for at least 1h at room temperature in PBS-T [PBS with 0.05% (v/v) Tween-20] containing 5% (w/v) non-fat dry milk. After, the membranes were washed in PBS-T and then cut in strips and incubated for 2h with the primary antibody (rabbit polyclonal anti-human Bax (BAX) antibody (1:5000, Sigma) and mouse monoclonal anti-yeast phosphoglycerate kinase (PGK1) antibody (1:5000, Molecular Probes)), if we want to do the all experience in the same day or, alternatively, put overnight at 4ºC. Then the membranes were washed for 3 times, 5 minutes with PBS-T. After this, the membranes were incubated with the secondary antibody for 1h, at room temperature; against mouse or rabbit IgG-peroxidase (1:10000; Sigma Aldrich) and washed 6 times, 10 minutes with PBS-T. Immunodetection of bands was revealed by chemiluminescence (Immobilon, Millipore).

3.6 Viability assays in strains displaying changes in Bax phosphorylation

Mutant strains that exhibit an altered phosphorylation levels or pattern were assessed by colony-forming colony units (CFU). Strains were grown by the two induction methods described above. When galactose is added corresponds to time zero (t_0) and after induction times of 4h or 14h corresponds to (t_4) and (t_{14}) respectively. Cell viability was measured as CFU on YPD medium after 2 days of growth at 30ºC. The percentage of survival for the different time-points was calculated by the formula: number of colonies in time X min (TX)/ number of colonies in T_0 x 100.

3.7 Flow cytometric assays

All the flow cytometric 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 Forward Scatter (FS) x Side Scatter (SS). Thirty thousand cells per sample were analyzed. The obtained data were further analyzed by WinMDI 2.8 software.

Strains were grown by the second type of induction method described above. When galactose is added corresponds to time zero (t_0) and after induction time of 4h corresponds to (t_4).

3.7.1 Mitochondrial membrane potential

The cells were normalized to OD_{640nm} = 0.6 washed with 500 μL of PBS1x [80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl)] and incubated with 4 μg/mL Mito Tracker Green and 10 μg/mL Mito Tracker Red (Molecular Probes, Eugene, U.S.A.) for 30 min at 37ºC in the dark. Mito Tracker Green and Red measure respectively the mitochondrial mass and the mitochondrial membrane potential. Cells with red fluorescent [FL-4 channel (660/700) nm] and cells with green fluorescent [FL-1 channel (505/545) nm] were considered.

3.7.2 ROS

Intracellular superoxide anion was detected by flow cytometry using Dihydroethidium (DHE). For DHE staining, cells were normalized to OD_{640nm} = 0.6, harvested by centrifugation, ressuspended in 500 μL PBS1x [80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl)] and incubated with 1.25 μg/mL DHE (Molecular Probes, Eugene, U.S.A.) for 40 min in the dark at RT. Cells with red fluorescent FL-4 channel (660/700 nm)] were considered to contain superoxide anion.

3.8 Assessment of Bax content of isolated mitochondria and post mitochondrial fractions by *Western blotting*.

3.8.1 Preparation of spheroplasts

Cells were grown overnight in SC glucose 2% medium without appropriate aminoacids, on the next day the cells were normalized and incubated with SC lactate 0.5% lacking uracil + ethanol 0.5%. For the induction of Bax, cells were grown until late exponential phase (OD_{640} = 0.8) and the culture was divided in two: one culture, without induction, was used as a negative control and the other was treated with galactose 0.5% (for Bax induction) during 4h. Cells were collected and incubated in resuspension buffer [2M Sorbitol, 1M sodium phosphate (Na_3PO_4), 0.5M EDTA] in a concentration of 10g of cells per 15mL of resuspension buffer. β-mercaptoethanol (0.5mL per each 50mL) and zymoliase (100mg/mL) were also added. Digestion of cell wall was performed during 30 up to a maximum of 55min for treated and untreated cells. Spheroplasts were obtained by incubation of 50mL of 1.2M Sorbitol buffer, then centrifuge at 6000 rpm, 5min, discard supernatant and resuspended with 10 mL of 1.2M Sorbitol buffer and re-centrifuge.

3.8.2 Preparation of mitochondrial and cytosolic fractions

Spheroplasts were washed with lysis buffer [0.5M sorbitol, 20 mM Tris, pH 7.5 and 1 M EDTA]. A Dounce homogenizer was used to homogenize the cell suspension to preserve the outer mitochondrial membrane integrity, controlling cell lysis on the microscope. Then the homogenate was centrifuge at 2500 rpm for 10 min to remove cell debris and nuclei. The supernatant was centrifuged at 15000 rpm for 10 min, if the pellet is already small centrifuge at 14000 rpm for 15 min to lower the mitochondria and the cytosolic fraction will match to supernatant. Mitochondria were then ressuspended in lysis buffer.

3.8.3 SDS gel electrophoresis/*Western blotting*

To estimated the protein concentration of the fractions we use the Bradford method using bovine serum albumin (BSA) as a standard as described on Bradford, 1976. Then 50µg of protein of the subcellular fractions were separated by electrophoresis on a 12.5% SDS polyacrylamide gel at 25mA and transferred to a PVDF membrane (Hybond-ECL, GE Healthcare) at 60 mA for 1h:30 min. Membranes were blocked at least for 1 h in PBS-T [PBS with 0.05% (v/v) Tween-20] and then 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 (1:2000, custom-made by Millegen), rabbit polyclonal anti-human Bax (Bax) antibody (1:5000, Sigma). Then the membranes were washed for 3 times, 5 min. each, with PBS-T. After the membranes were incubated with the secondary antibody for 1 h, against mouse or rabbit IgG-peroxidase (1:10000; Sigma Aldrich). Por1p were used as control of the mitochondrial fraction and Pgk1p as control of the cytosolic fraction. Immunodetection of bands were revealed by chemiluminescence (Immobilon, Millipore).

3.9 Reproducibility and statistical analysis

The results obtained are represented by mean and standard deviation (SD) values of at least three independent experiments. Statistical analyses were carried out using GraphPad Prism Software v5.00 (GraphPad Software, California, USA). P-values lower than 0.05 were considered to represent a significant difference.

4. RESULTS AND DISCUSSION

4.1 Screen of yeast protein phosphatase mutants involved in Bax phosphorylation profile

In this study we aimed to identify novel protein phosphatases involved on the dephosphorylation of Bax. To this end we heterologously expressed human Bax in yeast cells lacking non-essential protein phosphatases. Yeast cells were transformed with plasmids expressing Bax alpha (mainly cytosolic and inactive form), Bax P168A (a mitochondrial and active form) or Bax *c-myc* (an even more toxic form of active Bax, with mitochondrial localization) (Figure 4.1).

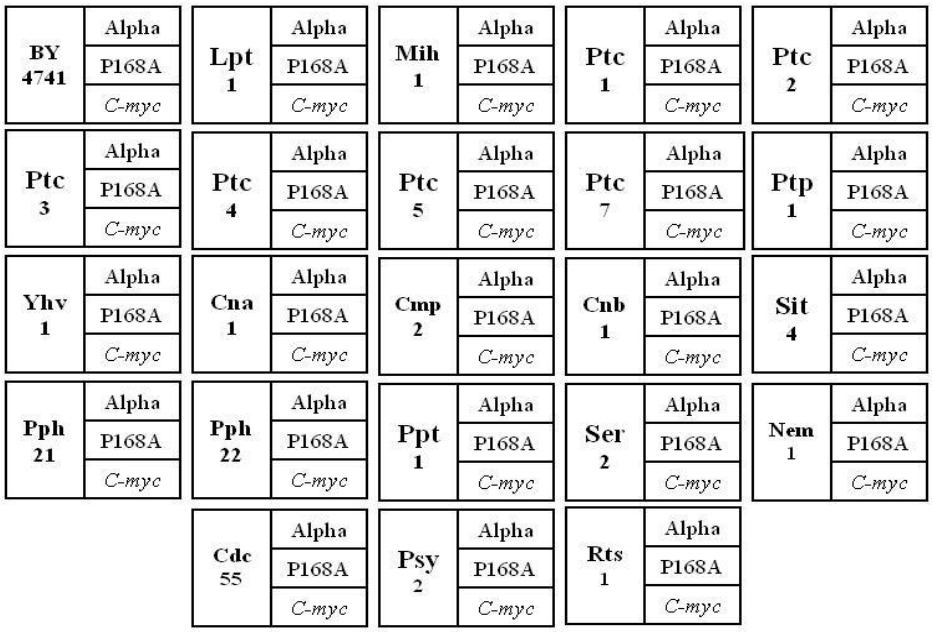

Figure 4.1 – *S. cerevisiae* mutant strains from the Euroscarf collection lacking non-essential protein phosphatases that have mammalian orthologs, and which were used to express the three forms of Bax (Bax alpha, Bax P168A and Bax *c-myc*).

All the yeast transformed strains constructed were confirmed for the expression of Bax, and compared regarding the profile of Bax phosphorylation (Figure 4.2, Figure 4.3, Figure 4.4 and Figure 4.5). Although the same set of protein phosphatases mutant strains expressing Bax *c-myc* was constructed, we decided to focus only on the mutants expressing Bax alpha and Bax P168A.

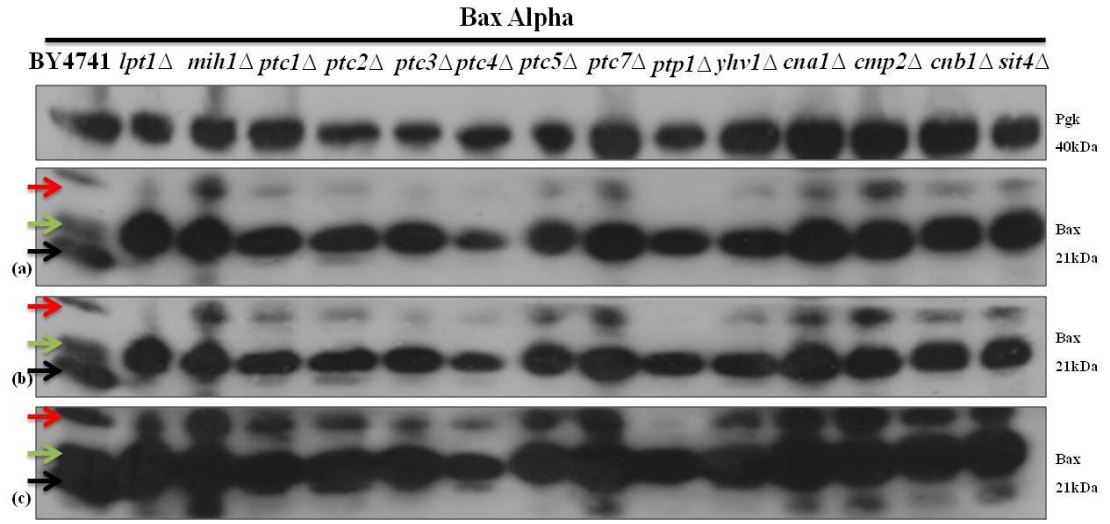

Figure 4.2 - Immunodetection of Bax in *S. cerevisiae* BY4741 and in the different mutant cells lacking non-essential protein phosphatases expressing Bax alpha. Pgk1p was used as a loading control. Panels correspond to (a) 5 sec of exposure, (b) 15 sec of exposure and (c) 30 sec of exposure. Three distinct band were identified by three arrows and the meaning of which is explained below.

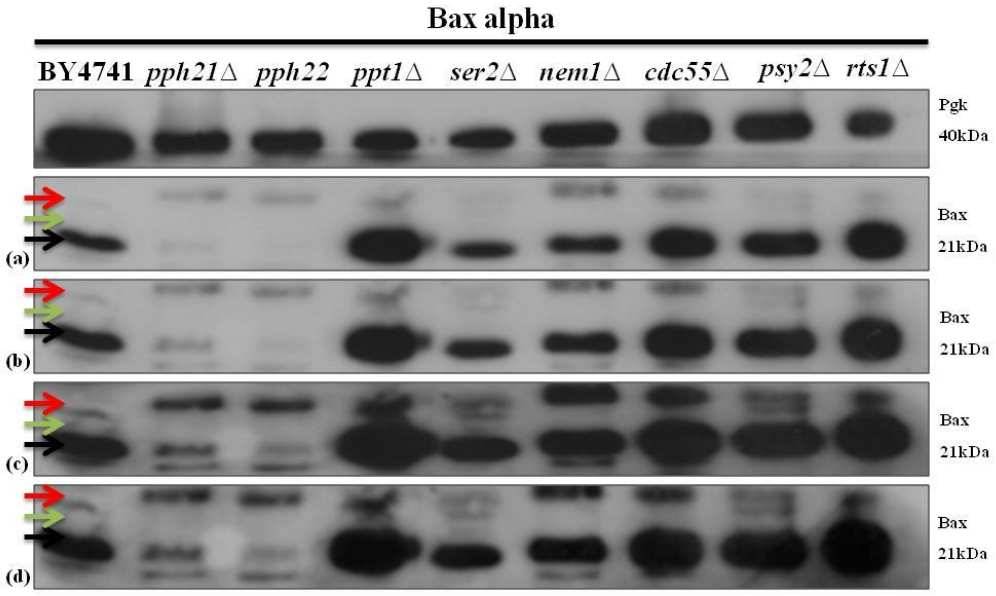

Figure 4.3 - Immunodetection of Bax in *S. cerevisiae* BY4741 and in the different mutant cells lacking non-essential protein phosphatases expressing Bax alpha. Pgk1p was used as a loading control. Panels correspond to (a) 5 sec of exposure, (b) 15 sec of exposure, (c) 30 sec of exposure and (d) 60 sec of exposure. Three distinct band were identified by three arrows and the meaning of which is explained below.

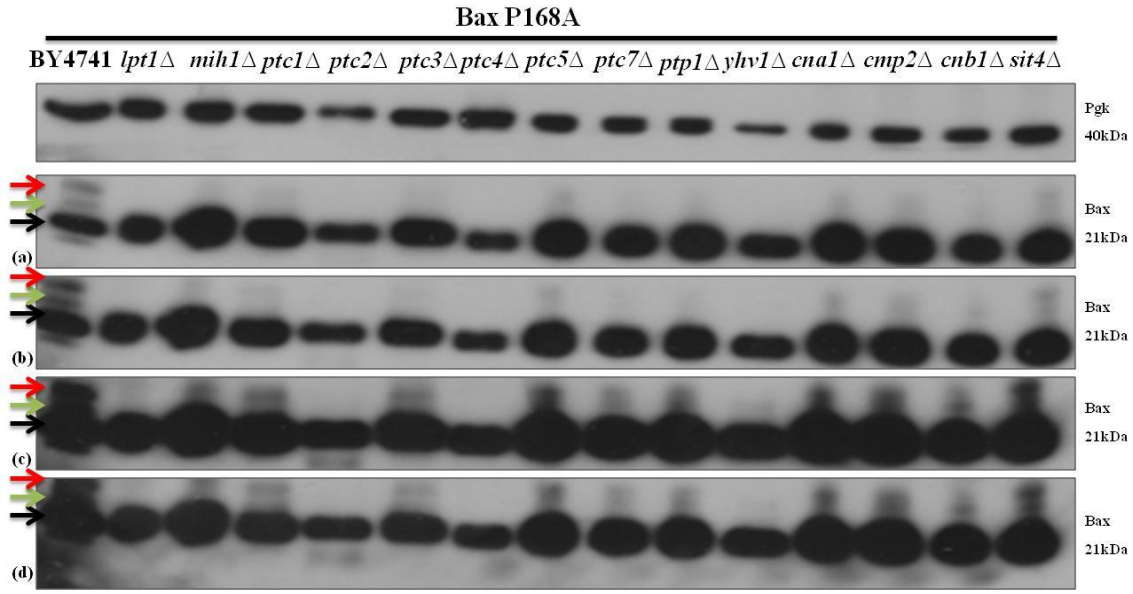

Figure 4.4 - Immunodetection of Bax in *S. cerevisiae* BY4741 and in the different mutant cells lacking non-essential protein phosphatases expressing Bax P168A. Pgk1p was used as a loading control. Panels correspond to (a) 5 sec of exposure, (b) 15 sec of exposure, (c) 30 sec of exposure and (d) 60 sec of exposure. Three distinct band were identified by three arrows and the meaning of which is explained below.

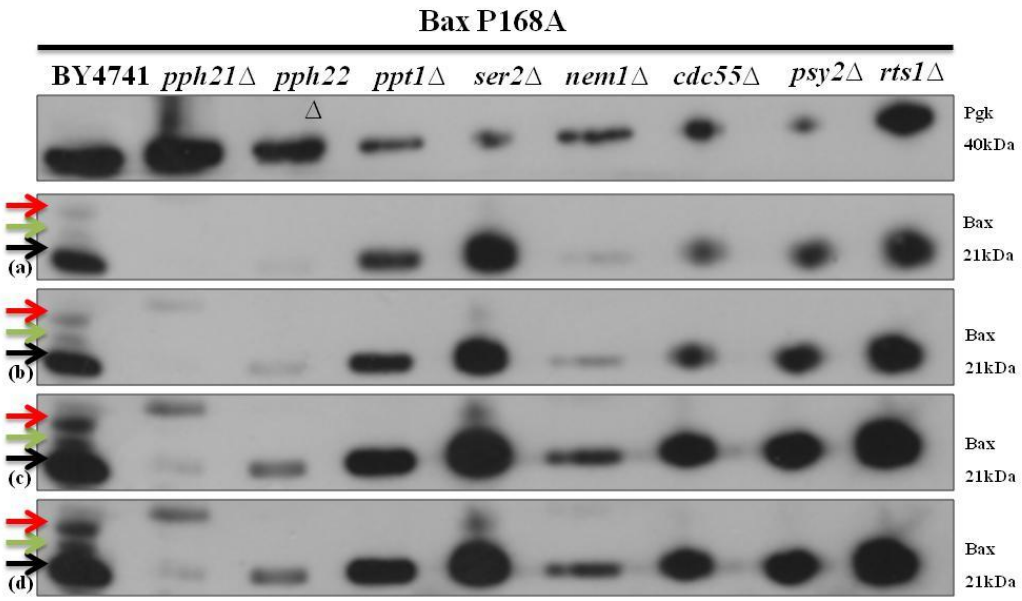

Figure 4.5- Immunodetection of Bax in *S. cerevisiae* BY4741 and in the different mutant cells lacking non-essential protein phosphatases expressing Bax P168A. Pgk1p was used as a loading control. Panels correspond to (a) 5 sec of exposure, (b) 15 sec of exposure, (c) 30 sec of exposure and (d) 60 sec of exposure. Three distinct band were identified by three arrows and the meaning of which is explained below.

Analysis of these figures shows that when Bax alpha or Bax P168A are expressed in yeast they are phosphorylated by endogenous protein kinases, as previously reported (Arokium *et al*, 2007 and Rui Silva, 2006). Three characteristics bands were observed on the samples from the control BY4741 strains expressing Bax alpha or Bax P168A namely: a lower band ( ), which corresponds to Bax protein; a band on the top ( ) which represents a nonspecific band as it is present in the extract of cells transformed with the empty vector (not shown), and finally, a band from the middle ( ), which may be representative of phosphorylated Bax. In some cases another band below Bax was visualised.

The Bax phosphorylation profiles in the protein phosphatase mutants were compared with those on BY4741 expressing Bax alpha or Bax P168A. Since protein phosphatases remove phosphate groups, the absence of a specific protein phosphatase that acts on Bax will lead to an increase of the Bax phosphorylation level. However, it is conceivable that if the protein phosphatase in question inactivates a kinase involved in Bax phosphorylation, it can have the same effect. So, in the case a protein phosphatase mutant exhibits a decrease in Bax phosphorylation level it may indicate that the protein phosphatase activates a protein kinase involved in Bax phosphorylation.

Differences in the total amount of Pgk1p, used as a loading control, as well as problems in the running of the samples made the comparison of the phosphorylation profiles between the wild type and mutant strains difficult. To better support the selection of the mutants we tried different exposure times for each mutant, and analysed different western blots (see Annex A.1, A.2, A.3 and A.4). Taking these aspects into account and being aware of some subjectivity we selected the following potential protein phosphatase candidates: *ptc1*Δ, *ptc2*Δ, *ptc3*Δ, *ptc4*Δ, *ptc5*Δ, *ptp1*Δ, *yhv1*Δ, *cnb1*Δ, *pph21*Δ, *pph22*Δ, *nem1*Δ and *cdc55*Δ for Bax alpha; and *ptc2*Δ, *ptc4*Δ, *pph21*Δ, *pph22*Δ and *nem1*Δ for Bax P168A (Figure 4.6). Considering the screening performed, from the twenty two protein phosphatases analysed fourteen and five were identified as potentially involved in phosphoregulation of Bax alpha and Bax P168A, respectively (Figure 4.6).

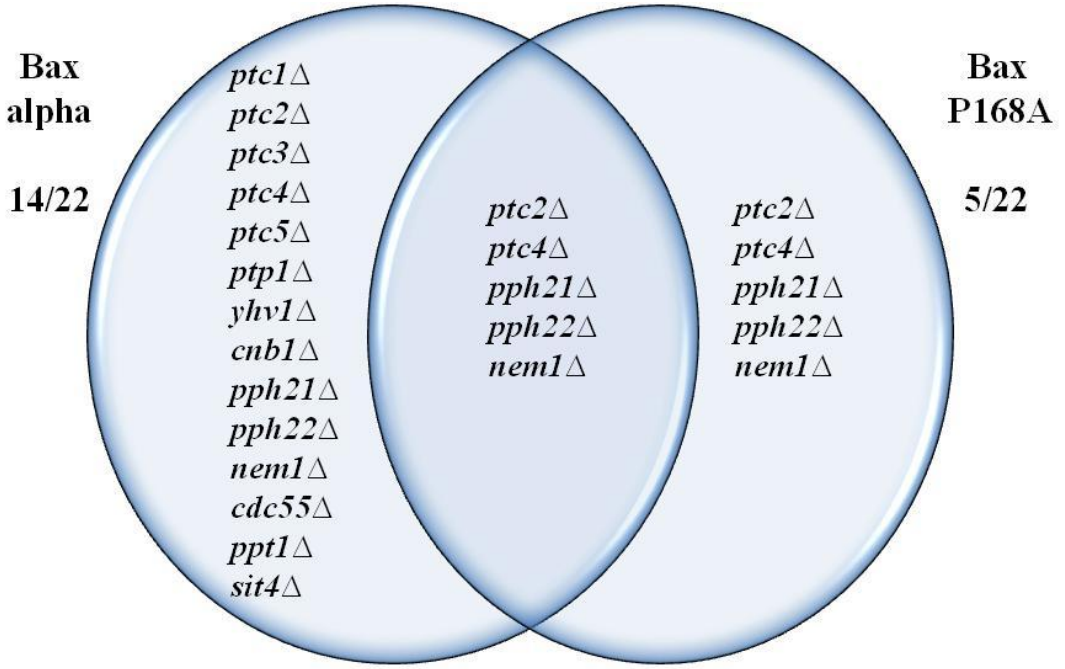

Figure 4.6 - Putative protein phosphatases identified in the screening of the different mutant cells lacking non-essential protein phosphatases expressing Bax alpha or Bax P168A.

Among the protein phosphatases selected we found two yeast protein phosphatases (Pph21p/22p and Ptc4p), orthologs of human phosphatases PP2A and WIP1, respectively and which were previously described as involved on the phosphorylation of Bax (Xin and Deng, 2006 and Song *et al*, 2013).

We also found other new putative yeast protein phosphatase candidates, not previously implicated in Bax regulation. To further validate the approach developed to identify novel yeast protein phosphatases involved in the regulation of Bax, we assessed the consequences on alterations of Bax alpha and Bax P168A phosphorylation profile on its activity, as assessed by Bax-induced cell death and the ensuing mitochondrial dysfunctions.

4.2 Effect of the protein phosphatases Ptc4p, Pph21p and Pph22p on Bax-dependent cell death

Cell viability of the protein phosphatase mutant strains expressing Bax was assessed by CFU. As we referred above we chose the Pph21p/Pph22p and Ptc4p, orthologues of two protein phosphatases previously described as involved in regulation of Bax to assess the role on Bax-dependent cell death. For this purpose we used yeast cells deleted in the genes encoding these protein phosphatases and expressing Bax alpha and Bax P168A and compared cell survival with cells transformed with the PYES2 empty vector (Ø), to ascertain whether the mutant strains behaved differently from the BY4741 under the conditions tested in response to Bax expression.

As expected expression of Bax alpha in the BY4741 wild type background had no significant effect on cell survival, 104±6.2% [Figure 4.7 (a)] and 98.5±1.6% [Figure 4.7 (b)], as compared to the control strain transformed with the empty vector, 114±10.6% [Figure 4.7 (a)] and 99.1±0.4% [Figure 4.7 (b)] for both induction conditions. Regarding the mutant *ptc4Δ* Ø we can observe that though it already displays a lower survival without Bax expression, it leads to a slight but not significant decrease of the cell survival after 14h of induction of Bax alpha expression in 2% galactose medium (71.7±21.6% compared to 73.7±5.8%) [Figure 4.7 (a)]. These results indicate that absence of *ptc4Δ* does not sensitize the cells for Bax alpha expression. However, when we analyse the results obtained for the other induction condition tested [Figure 4.7 (b)] some differences become evident. Indeed *ptc4*Δ Ø strain (97.3±5.5%) had no longer a lower survival compared to BY4741 Ø and Bax expression in the absence of Ptc4p had a significant effect on survival causing more death (67.6±14.1%). Comparison of the ratio between the percentages of survival of the strain expressing Bax alpha and the strain transformed with the empty vector, both for the wild type BY4741 and the mutant strain, indicates that the absence of this phophatase sensitizes the cells for expression of Bax alpha [Figure 4.7 (b)].

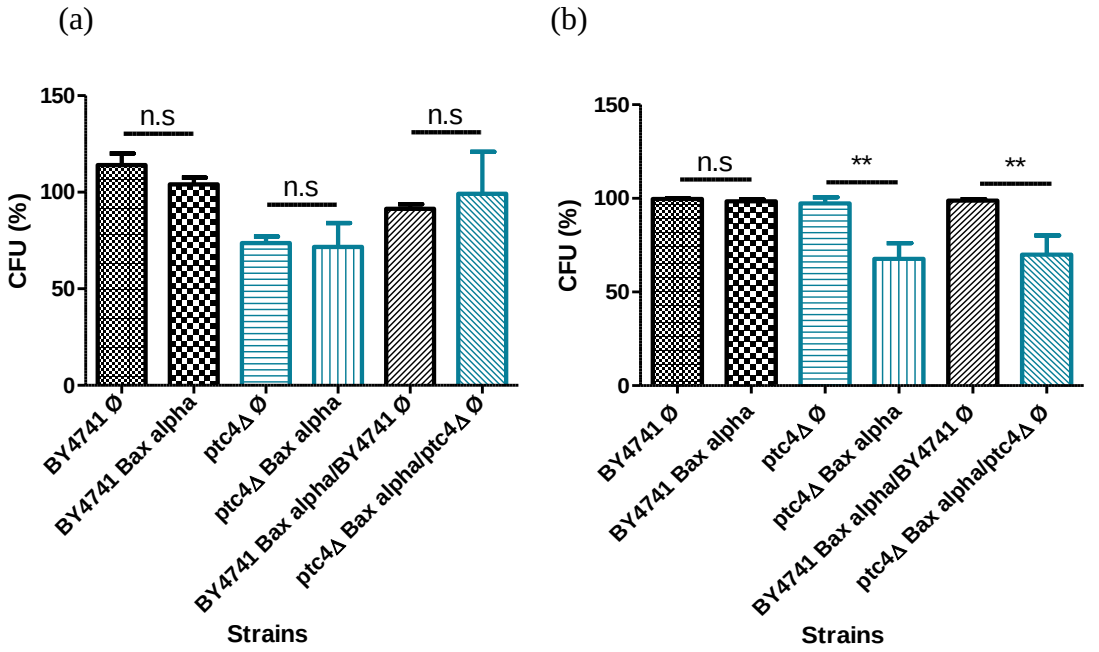

Figure 4.7 - Survival of *S. cerevisiae* BY4741 and mutant cells lacking Ptc4p protein phosphatase after (a) 14h of induction of Bax alpha expression in 2% galactose medium in cells pre-grown in glucose and (b) 4h of induction of Bax alpha expression in 0.5% of galactose in cells pre-grown in lactate+ethanol. Cell viability was determined by CFU counts. The percentual values of cell survival were calculated considering 100% at t_0. For each bar, mean ± S.E.M. of at least three experiments is represented $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, compared to the empty vector. One-way ANOVA was used to compare cell survival of BY74741 and protein phosphatase mutant cells expressing Bax alpha. The ratio between the percentages of survival of the BY4741 and mutant strain expressing Bax alpha and the respective strains only transformed with the empty vector was also calculated.

The expression of active Bax P168A, as expected, led to a more pronounced decrease in viability, compared to Bax alpha, once it is a more toxic form of Bax. The decrease on cell survival for the *ptc4*Δ was more visible after 4h induction (61.3±8.8%) [Figure 4.8 (b)], than after 14h (94.0±7.9%) [Figure 4.8(a)]. The strain *ptc4*Δ expressing Bax P168A behaved similarly under both induction conditions. When the values were normalised for the control strain *ptc4*Δ Ø, the ratios were not statistically different for both induction conditions.

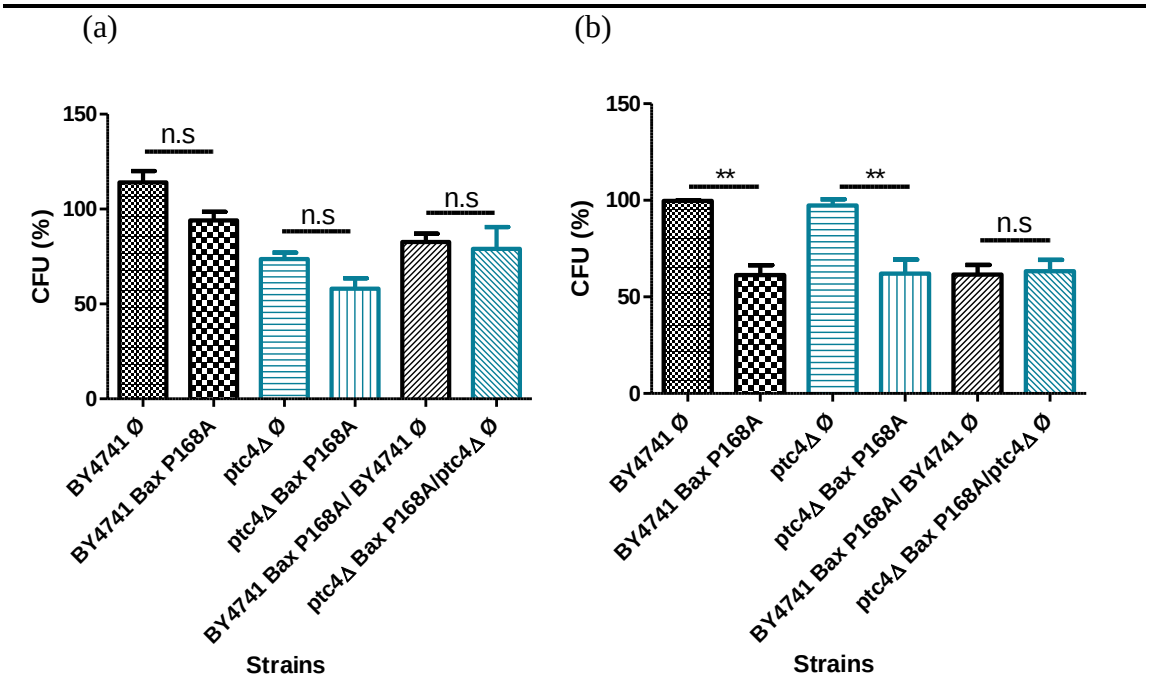

Figure 4.8 - Survival of *S. cerevisiae* BY4741 and mutant cells lacking Ptc4p protein phosphatase after (a) 14h of induction of Bax P168A expression in 2% galactose medium in cells pre-grown in glucose and (b) 4h of induction of Bax P168A expression in 0.5% of galactose in cells pre-grown in lactate+ethanol. Cell viability was determined by CFU counts. The percentual values of cell survival were calculated considering 100% at t_0. For each bar, mean ± S.E.M. of at least three experiments is represented $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, compared to the empty vector. One-way ANOVA was used to compare cell survival of BY74741 and protein phosphatase mutant cells expressing Bax P168A. The ratio between the percentages of survival of the BY4741 and mutant strain expressing Bax P168A and the strains only transformed with the empty vector was also calculated.

Concerning the strain *pph21*Δ we found that the survival of *pph21*Δ Ø strain was very similar to the BY4741 Ø strain, and that Bax alpha induction in *pph21*Δ strain caused an accentuated decrease in cell survival (41.7±14.0% and 45.3±10.0%) independently of the induction conditions [Figure 4.9 (a) and (b)]. When the values were normalised the ratios were statistically different indicating that the absence of this protein phosphatase render the cells sensitive to Bax alpha expression.

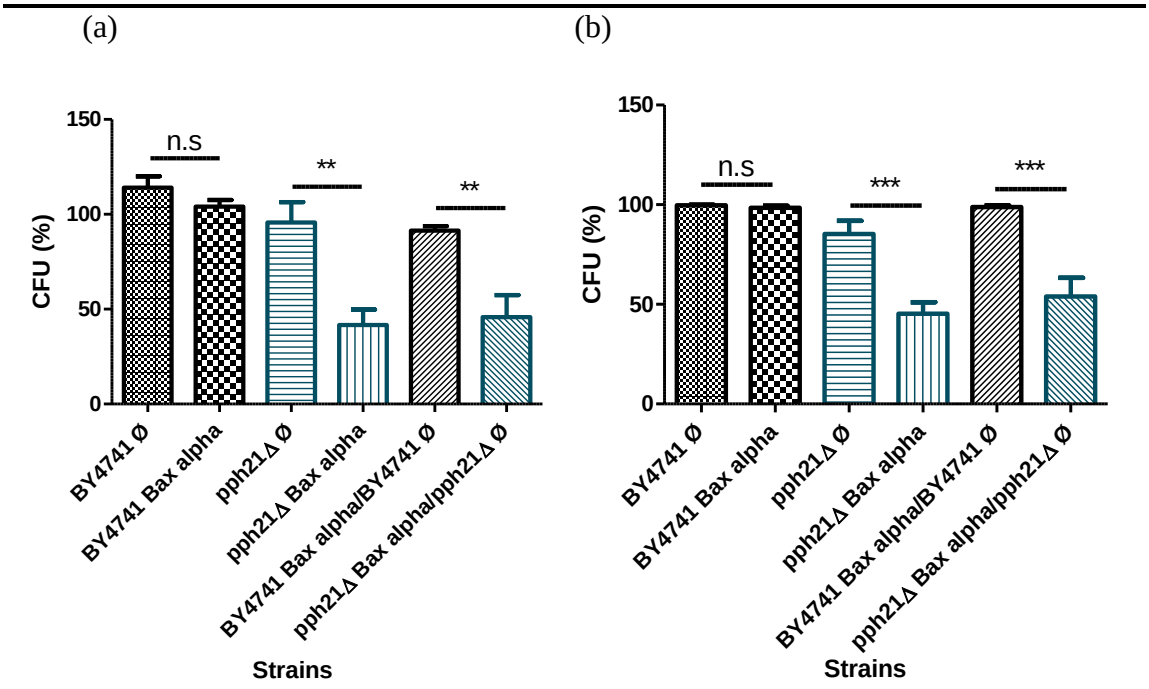

Figure 4.9 - Survival of *S. cerevisiae* BY4741 and mutant cells lacking Pph21p protein phosphatase after (a) 14h of induction of Bax alpha expression in 2% galactose medium in cells pre-grown in glucose and (b) 4h of induction of Bax alpha expression in 0.5% of galactose in cells pre-grown in lactate+ethanol. Cell viability was determined by CFU counts. The percentual values of cell survival were calculated considering 100% at t_0. For each bar, mean ± S.E.M. of at least three experiments is represented $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, compared to the empty vector. One-way ANOVA was used to compare cell survival of BY74741 and protein phosphatase mutant cells expressing Bax alpha. The ratio between the percentages of survival of the BY4741 and mutant strain expressing Bax alpha and the strains only transformed with the empty vector was also calculated.

In the strain *pph21*Δ, *e*xpression of Bax P168A causes less death than expression of Bax alpha. When we compare, Figure 4.10 (a) with Figure 4.10 (b), the strain *pph21*Δ Bax P168A has a higher decrease of cell viability with the induction of 4h (70.7±7.0%) than for the 14h induction (93.7±15.9%). When the values were normalised for the control, the ratios were not statistically different in either conditions. So it appears that though not statistically significant Pph21p is required for Bax P168A-induced cell death.

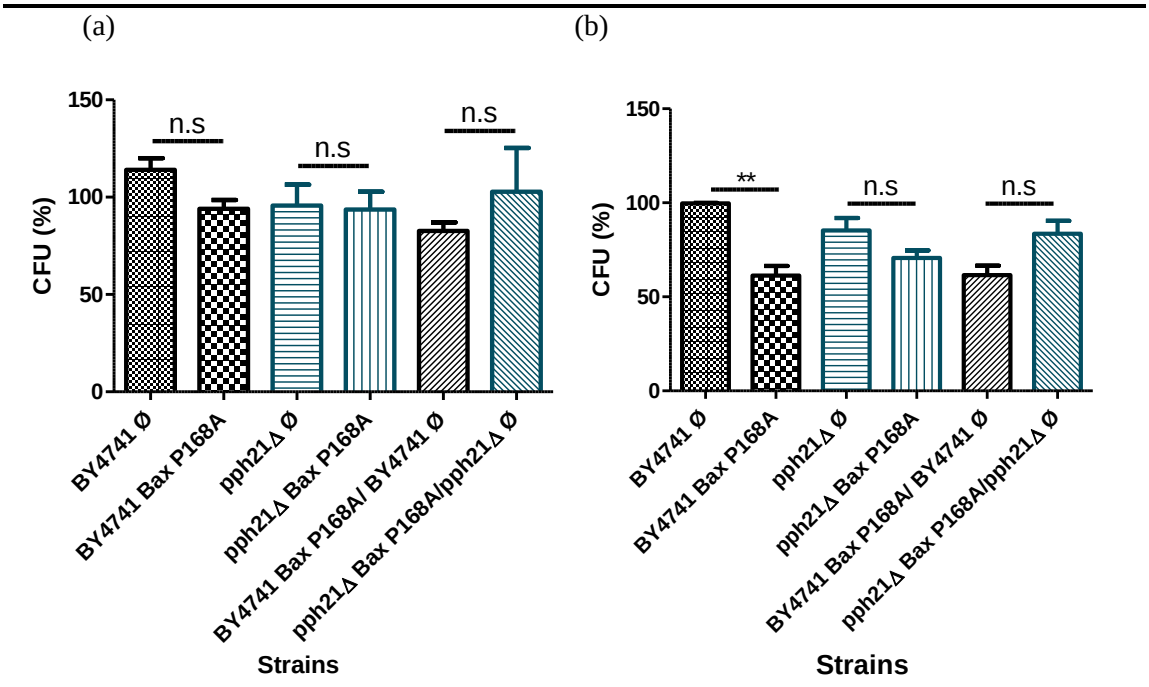

Figure 4.10 - Survival of *S. cerevisiae* BY4741 and mutant cells lacking Pph21p protein phosphatase after (a) 14h of induction of Bax P168A expression in 2% galactose medium in cells pre-grown in glucose and (b) 4h of induction of Bax P168A expression in 0.5% of galactose in cells pre-grown in lactate+ethanol. Cell viability was determined by CFU counts. The percentual values of cell survival were calculated considering 100% at t_0. For each bar, mean ± S.E.M. of at least three experiments is represented $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, compared to the empty vector. One-way ANOVA was used to compare cell survival of BY74741 and protein phosphatase mutant cells expressing Bax P168A. The ratio between the percentages of survival of the BY4741 and mutant strain expressing Bax P168A and the strains only transformed with the empty vector was also.

Regarding the mutant *pph22*Δ the expression of Bax alpha has opposite effects accordingly to induction conditions [Figure 4.11 (a) and (b)]. Absence of Pph22p causes a decrease and an increase of loss cell survival to 69.3±9.3% and 130.6±4.1%, after 14h (2% galactose) and 4h (0.5% galactose) of Bax alpha expression, respectively. When these values were normalised for the control the ratios were statistically different indicating that the altered sensitivity of *pph22*Δ to Bax alpha expression depends on the derepression level. So we may say that the absence of this protein phosphatase render the cells sensitive to Bax alpha expression.

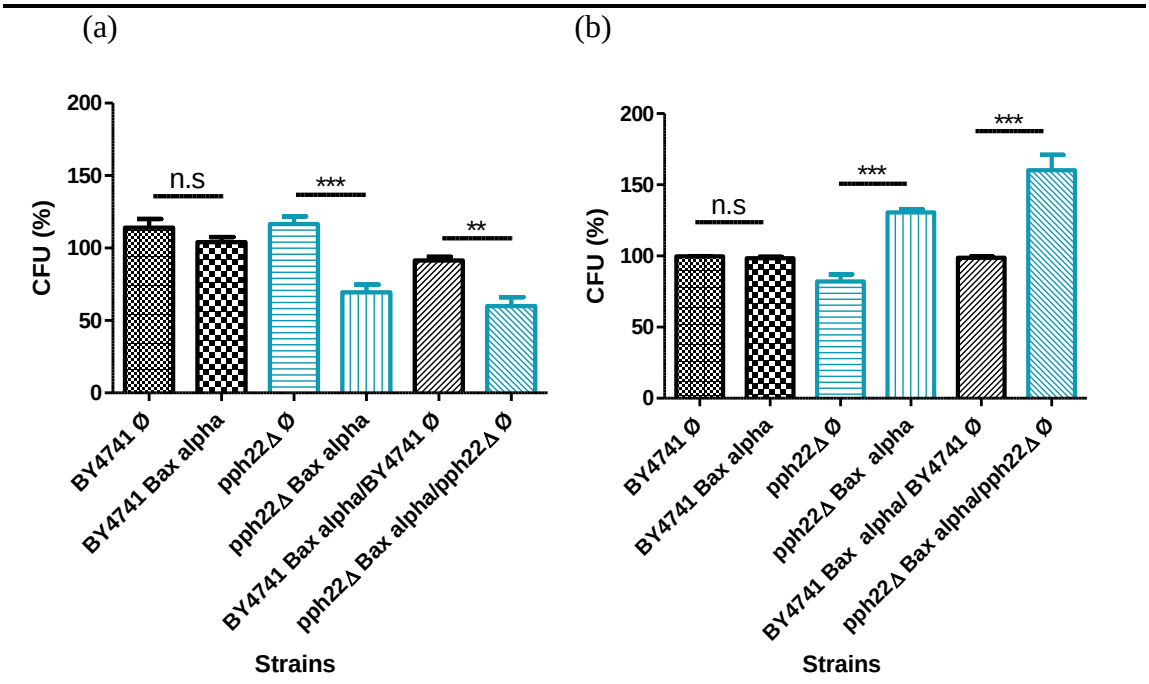

Figure 4.11 - Survival of *S. cerevisiae* BY4741 and mutant cells lacking Pph22p protein phosphatase after (a) 14h of induction of Bax alpha expression in 2% galactose medium in cells pre-grown in glucose and (b) 4h of induction of Bax alpha expression in 0.5% of galactose in cells pre-grown in lactate+ethanol. Cell viability was determined by CFU counts. The percentual values of cell survival were calculated considering 100% at t_0. For each bar, mean ± S.E.M. of at least three experiments is represented $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, compared to the empty vector. One-way ANOVA was used to compare cell survival of BY74741 and protein phosphatase mutant cells expressing Bax alpha. The ratio between the percentages of survival of the BY4741 and mutant strain expressing Bax alpha and the strains only transformed with the empty vector was also calculated

Still for the same mutant but with expression of Bax P168A, we may say that, as observed for *pph22*Δ, the strain *pph22*Δ Bax P168A had a more pronounced decrease in cell viability, compared to *pph22*Δ Bax alpha, in spite of being a more toxic form of Bax. This decrease in toxicity was more evident after 4h induction (88.7±7.1%) [Figure 4.12 (b)]. The strains *pph22*Δ Ø/P168A ratios were statistically different on the induction with 0.5% galactose [Figure 4.12 (b)].

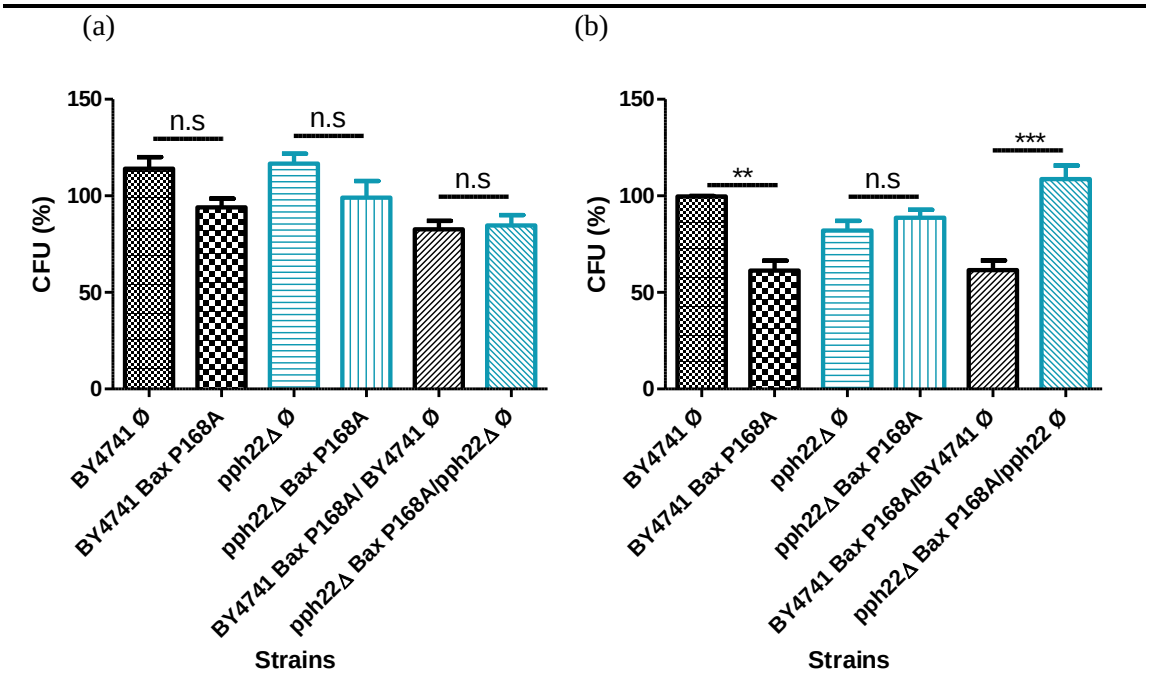

Figure 4.12- Survival of *S. cerevisiae* BY4741 and mutant cells lacking Pph22p protein phosphatase after (a) 14h of induction of Bax P168A expression in 2% galactose medium in cells pre-grown in glucose and (b) 4h of induction of Bax P168A expression in 0.5% of galactose in cells pre-grown in lactate+ethanol. Cell viability was determined by CFU counts. The percentual values of cell survival were calculated considering 100% at t_0. For each bar, mean ± S.E.M. of at least three experiments is represented $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, compared to the empty vector. One-way ANOVA was used to compare cell survival of BY74741 and protein phosphatase mutant cells expressing Bax P168A. The ratio between the percentages of survival of the BY4741 and mutant strain expressing Bax P168A and the strains only transformed with the empty vector was also.

The protein phosphatase mutant phenotypes expressing Bax alpha or Bax P168A obtained for the two induction conditions tested are systematised in Table 4.1. The phenotypes associates with Bax alpha expression in the absence of protein phosphatases Ptc4p, Pph21p and Pph22p are more prominent when mitochondrial respiration is less repressed (0.5% galactose, in cells pre-grown in lactate+ethanol). This same relationship is observed for Bax P168A expression, excepting in the case of *ptc4*Δ. Notably, the absence of Pph21p causes opposite phenotypes in Bax alpha-induced. Indeed while it appears to be required to keep Bax alpha inactive, it is required for BaxP168A-induced cell death.

Table 4.1 - Yeast putative protein phosphatases involved in phosphoregulation of Bax alpha and Bax P168A under different conditions of Bax induction. (n.s.) means that the effect of Bax expression on cell viability is not statistically different from that on the wild type strain and (**) means $p < 0.05$; ** $p \leq 0.01$and (***) means $p \leq 0.01$; *** $p \leq 0.001$.

Strain	2% Galatose (14h) in cells pre-grown in glucose	0.5% Galactose (4h) in cells pre-grown in lactate+ethanol
***ptc4*Δ Bax alpha**	**n.s.**	**decreased survival****
***ptc4*Δ Bax P168A**	**enhanced survival ****	**n.s.**
***pph21*Δ Bax alpha**	**decreased survival ****	**decreased survival *****
***pph21*Δ Bax P168A**	**n.s.**	**n.s.**
***pph22*Δ Bax alpha**	**decreased survival ****	**enhanced survival *****
***pph22*Δ Bax P168A**	**n.s.**	**enhanced survival *****

The results altogether indicate that Bax expression after 4h induction in 0.5% lactate and 0.5% galactose seems to be more adequate to find mutant phenotypes. So we select this condition of Bax of induction in the following assays.

4.3 Effect of protein phosphatases on Bax-induced mitochondrial dysfunctions ($\Delta\psi_m$)

Different yeast death scenarios have been associated with mitochondrial dysfunctions namely, ROS accumulation, mitochondrial membrane potential changes and cytochrome *c* release (Pereira *et al*, 2008). Therefore, the survival phenotypes of the different protein phosphatase mutants upon expression of Bax may be associated with such events. So we monitored the accumulation of superoxide anion (O_2^-) and changes in mitochondrial membrane potential as well as in mitochondrial mass by staining with dihydroethidium (DHE), MitoTracker Red and MitoTracker Green, respectively. DHE is a non fluorescent probe able to penetrate into the living cells through the membrane and which has the capacity to intercalate double stranded DNA following is oxidation to ethydium. MitoTracker Red and MitoTracker Green probes must be used always together to normalize the mitochondrial mass value with the potential value. Only then we are can analyse properly whether there is hyperpolarization or depolarization of the mitochondrial membrane.

- *ptc4*Δ mutant expressing Bax alpha

As expected, expression of Bax alpha in BY4741 strain did not cause a statistical change in ROS accumulation compared with the control strain (BY4741Ø) [Figure 4.13 (a)]. The mutant strain *ptc4*Δ showed a similar behaviour and when the values were normalised for the control strain (*ptc4*ΔØ) and compared with the ratio obtained for the BY4741 strain they were not statistically different [Figure 4.13 (a)]. Likewise, the expression of Bax alpha in BY4741 and in *ptc4*Δ mutant did not caused significant changes in $\Delta\Psi_m$ [Figure 4.13 (b)].

(a) (b)

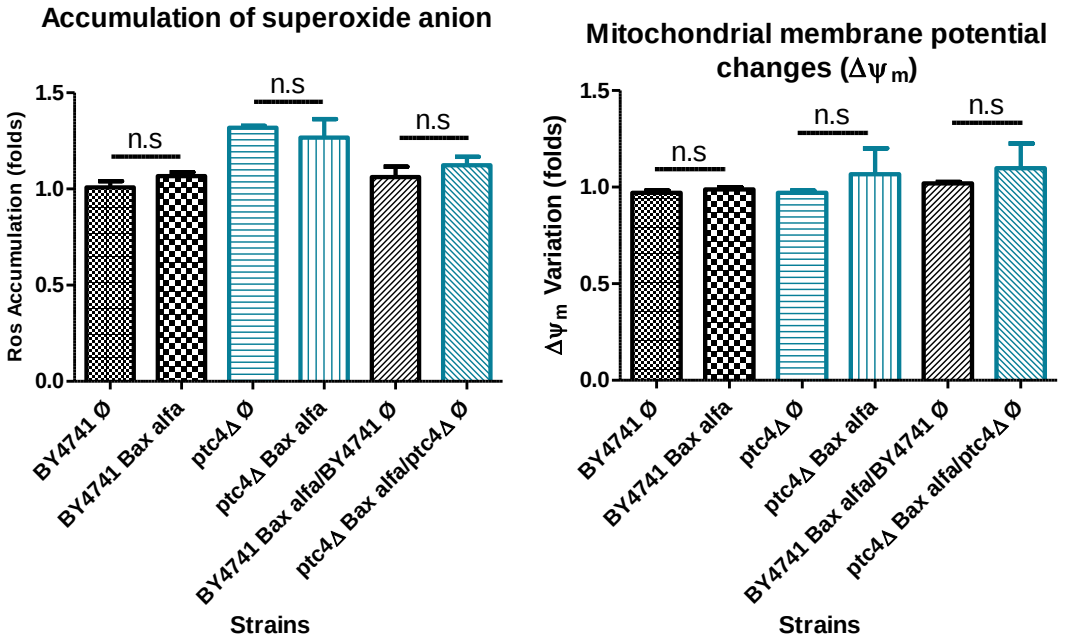

Figure 4.13 –Levels of superoxide anion accumulation (a) and changes in mitochondrial membrane potential (b) in *S. cerevisiae* BY4741 and mutant cells lacking Ptc4p protein phosphatase expressing Bax alpha after 4h of Bax induction in 0.5% of galactose in cells pre-grown in lactate+ethanol. Values are mean ± S.E.M of at least three independent experiments (b). Values significantly different from BY4741: * P<0.05, ** P<0.01 and *** P<0.001, One-way ANOVA was used.

- *ptc4*Δ mutant expressing Bax P168A

While as expected, expression of Bax P168A in BY4741 strain caused a statistical increase in ROS accumulation and an increase in $\Delta\Psi_m$ compared with the control strain (BY4741Ø), in *ptc4*Δ mutant strain though there was no significant increase in ROS accumulation a significant decrease in $\Delta\Psi_m$ was observed [Figure 4.14 (a) and (b)]. These results indicate that Bax P168A- induced depolarization depends on Ptc4p.

(a) (b)

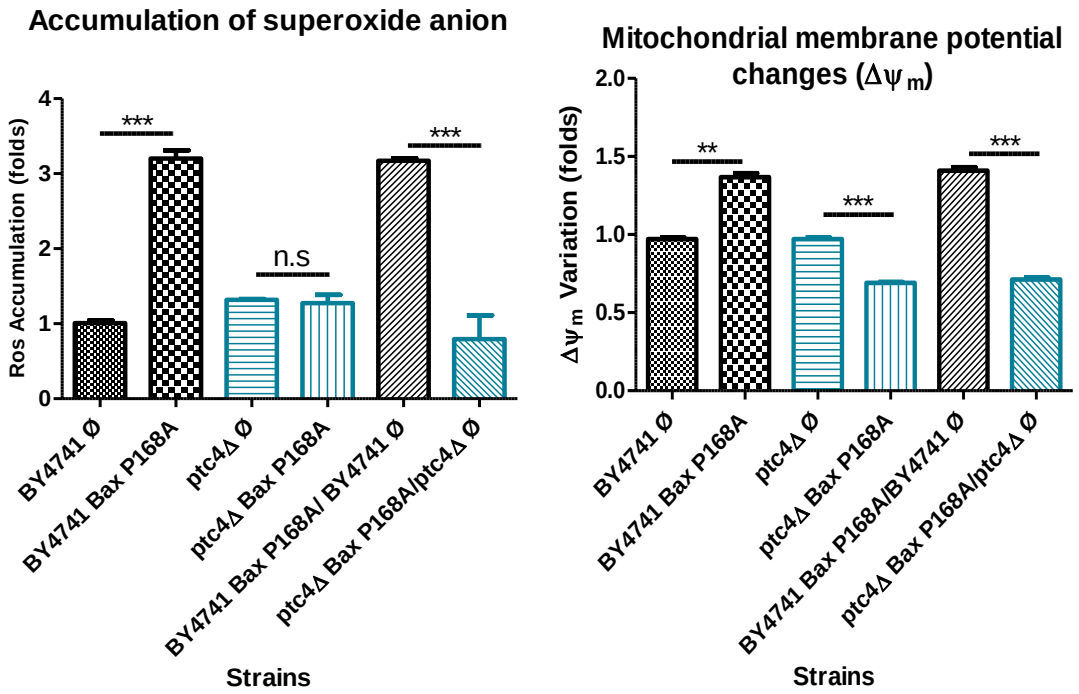

Figure 4.14 – Levels of superoxide anion accumulation (a) and changes in mitochondrial membrane potential (b) in *S. cerevisiae* BY4741 and mutant cells lacking *ptc4*Δ protein phosphatase expressing Bax P168Aafter 4h of Bax induction in 0.5% of galactose in cells pre-grown in lactate+ethanol. Values are mean ± S.E.M of at least three independent experiments (b). Values significantly different from BY4741: * P<0.05, ** P<0.01 and *** P<0.001, One- way ANOVA was used.

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- *pph21*Δ mutant expressing Bax alpha

Expression of Bax alpha in *pph21*Δ mutant strain caused a significant increase in ROS accumulation and a significant decrease in $\Delta\Psi_m$ [Figure 4.15 (a) and (b)]. These results indicate that Pph21p can prevent Bax alpha - induced ROS accumulation and depolarization.

(a) (b)

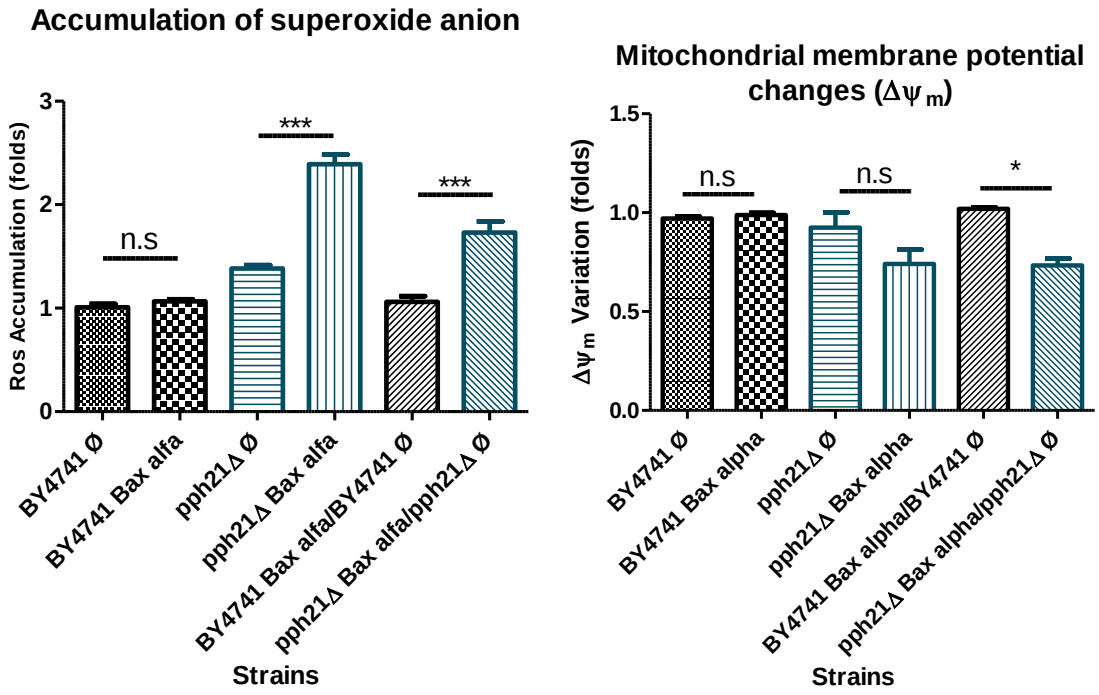

Figure 4.15 – Levels of superoxide anion accumulation (a) and changes in mitochondrial membrane potential (b) in *S. cerevisiae* BY4741 and mutant cells lacking Pph21p protein phosphatase expressing Bax alpha after 4h of Bax induction in 0.5% of galactose and 0.5% lactate in cells pre-grown in lactate+ethanol. Values are mean ± S.E.M of at least three independent experiments (b). Values significantly different from BY4741: * P<0.05, ** P<0.01 and *** P<0.001, One- way ANOVA was used.

- *pph21*Δ mutant expressing Bax P168A

Expression of Bax P168A in *pph21*Δ mutant strain caused an increase in ROS accumulation, associated with a significant increase in $\Delta\Psi_m$ when compared with the *pph21*Δ*Ø* strain [Figure 4.16 (a) and (b)]. The comparison of the normalised values for BY4741 and *pph21*Δ mutant strains revealed that ROS does not induced accumulation, but induce hyperpolarization.

(a) (b)

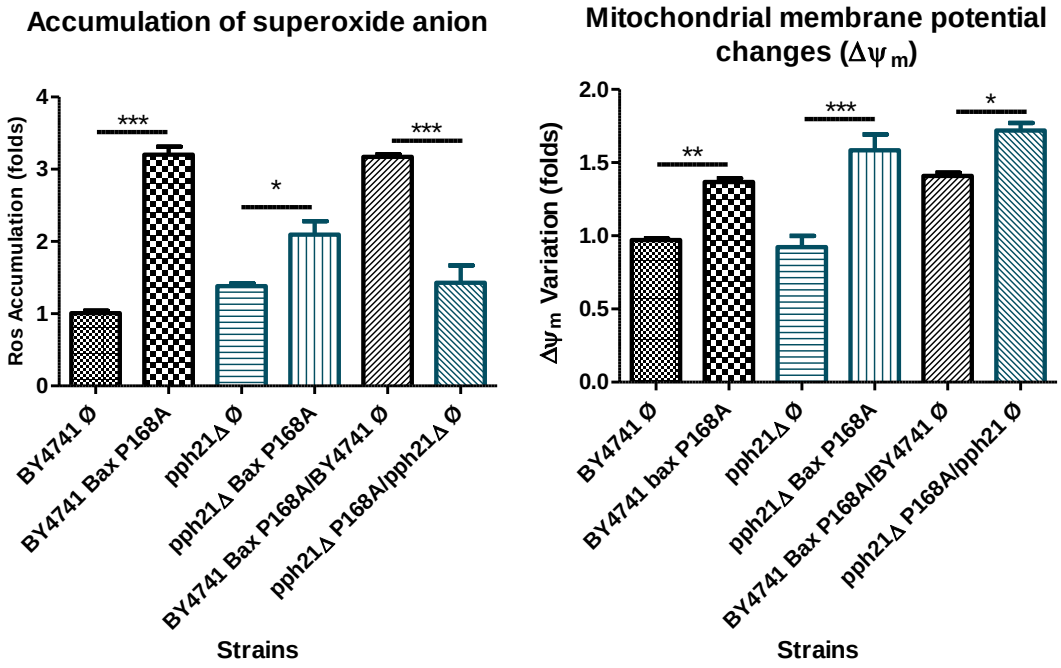

Figure 4.16 – Levels of superoxide anion accumulation (a) and changes in mitochondrial membrane potential (b) in *S. cerevisiae* BY4741 and mutant cells lacking Pph21p protein phosphatase expressing Bax P168Aafter 4h of Bax induction in 0.5% of galactose in cells pre-grown in lactate+ethanol. Values are mean ± S.E.M of at least three independent experiments (b). Values significantly different from BY4741: * P<0.05, ** P<0.01 and *** P<0.001, One- way ANOVA was used.

- *pph22*Δ mutant expressing Bax alpha

Expression of Bax alpha in *pph22*Δ mutant strain caused a significant decrease in ROS accumulation and no changes in $\Delta\Psi_m$ [Figure 4.17 (a) and (b)].

(a) (b)

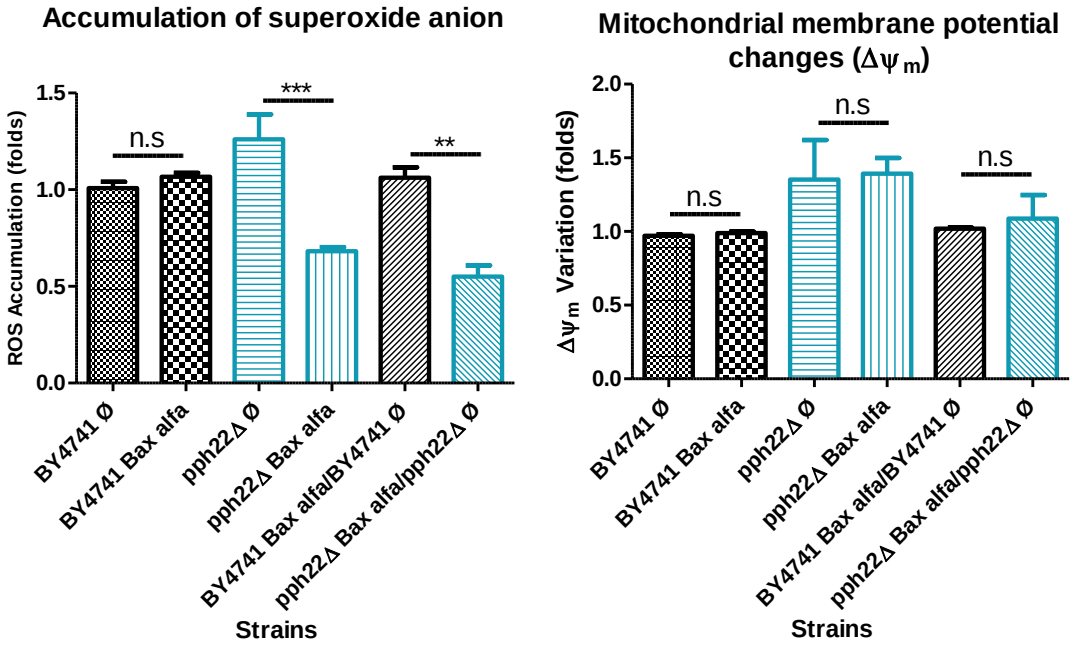

Figure 4.17 – Levels of superoxide anion accumulation (a) and changes in mitochondrial membrane potential (b) in *S. cerevisiae* BY4741 and mutant cells lacking Pph22p protein phosphatase expressing Bax alpha after 4h of Bax induction in 0.5% of galactose in cells pre-grown in lactate+ethanol. Values are mean ± S.E.M of at least three independent experiments (b). Values significantly different from BY4741: * P<0.05, ** P<0.01 and *** P<0.001, One- way ANOVA was used.

- *pph22*Δ mutant expressing Bax P168A

Expression of Bax P168A in *pph22*Δ mutant strain was neither associated with an increase in ROS accumulation nor with significant changes in the mitochondrial membrane potential compared with the BY4741Φ strain [Figure 4.18 (a) and (b)]. The comparison of the normalised values for BY4741 and *pph22*Δ mutant strains revealed that Bax P168A –induced ROS accumulation and hyperpolarization depends on Pph22p.

(a) (b)

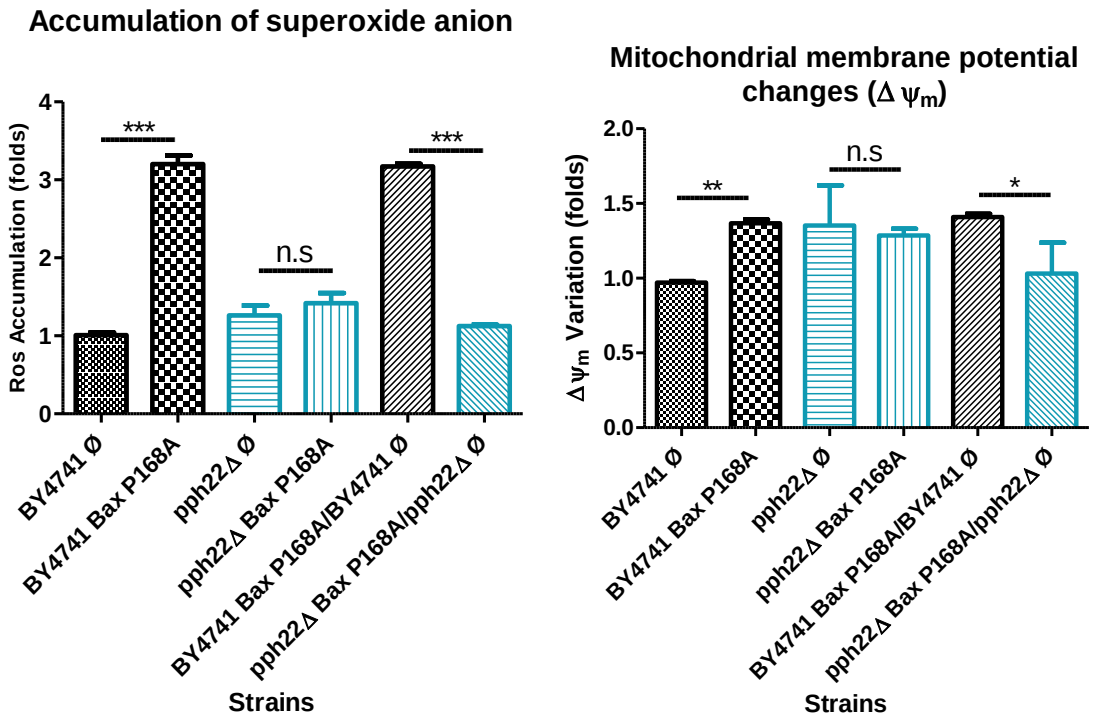

Figure 4.18 - Levels of superoxide anion accumulation (a) and changes in mitochondrial membrane potencial (b) in *S.cerevisiae* BY4741 and mutant cells lacking *Pph22p*Δ protein phosphatase expressing Bax P168Aafter 4h of Bax induction in 0.5% of galactose in cells pre-grown in lactate+ethanol. Values are mean ± S.E.M of at least three independent experiments (b).Values significantly different from BY4741: * P<0.05, ** P<0.01 and *** P<0.001, One- way ANOVA was used.

As aforementioned expression of an active form of Bax in yeast causes the translocation of cyt *c* from the mitochondria to the cytosol (Garrido *et al*, 2006). Therefore, to further assess whether the selected protein phosphatases regulate Bax alpha and Bax P168A activity we monitored the release of cyt *c* in the three protein phosphatase mutants upon Bax expression. We also evaluated their role in Bax cellular localization by monitoring the levels of Bax in both the cytosolic and mitochondrial fractions. These two sub-cellular fractions were isolated by differential centrifugation. Pgk1p and mitochondrial porin (Por1p) levels were used as loading control of cytosolic and mitochondrial fractions, respectively. *Western blot* analysis was then performed in order to detect the levels of cyt *c* in the two fractions as well as the levels of Bax protein (Figure 4.19 and Figure 4.20).

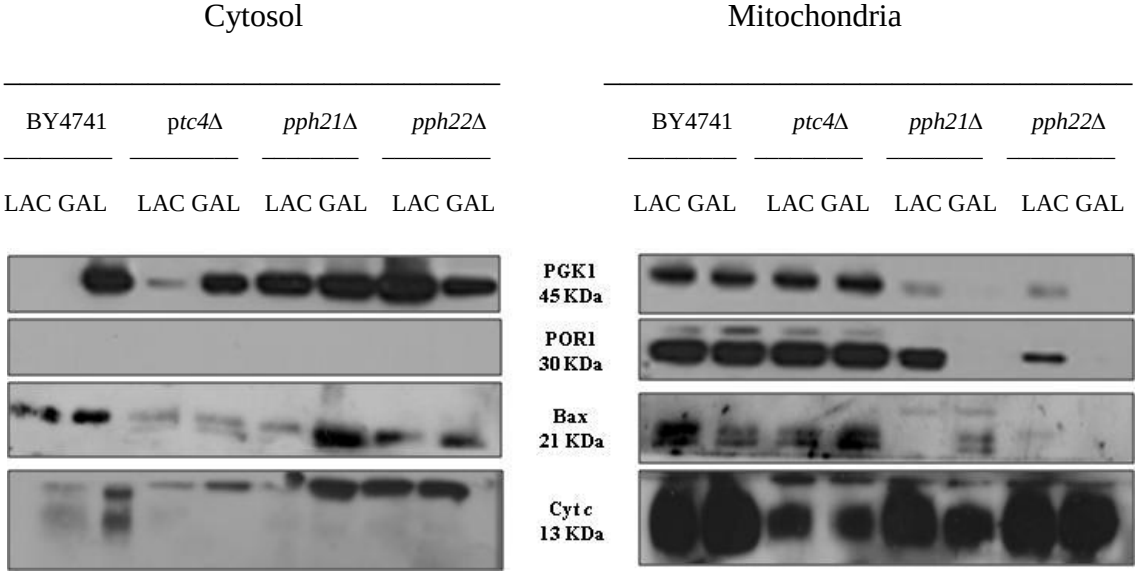

Figure 4.19 – Western blot analysis of cytochrome *c* in *S. cerevisiae* BY4741 and protein phosphatase mutant cells expressing Bax alpha before (LAC) and after (GAL) induction of Bax expression after 4h in 0.5% of galactose, in cells pre-grown in lactate+ethanol, in both mitochondrial and cytosolic fractions. Pgk1p and mitochondrial porin (Por1p) levels were used as loading control of cytosolic and mitochondrial fractions, respectively. A representative experiment is shown of at least two independent experiments with similar results.

Western blot analysis revealed that, under normal non-inducing conditions, cyt *c* is exclusively localized in the mitochondria for all strains.

As expected induction of expression of the inactive Bax alpha in the BY4741 strain did not resulted in cyt *c* release. The bands visualized in the gel correspond to

unspecific bands (cytosol fractions). As to the mitochondrial fraction there was a slight contamination with the cytosol.

Regarding the content of Bax, induction with 0.5% galactose (in cells pre-grown in lactate+ethanol) did not result in a clear increase in the amount of Bax alpha, excepting for the *pph21*Δ strain. Also, detection of Bax was associated with two bands in all strains, likely the unphosporylated Bax and the putative phosphorylated form.

Expression of Bax alpha in the *ptc4*Δ strain appears to lead to a considerably higher and likely lower amount of Bax in the mitochondria and cytosol, respectively. This was apparently associated with a decrease of mitochondrial cyt *c* but not to an increase in cytoslic cyt *c*. On the other hand, expression of Bax alpha in *pph21*Δ and *pph22*Δ strains upon expression of Bax alpha do not cause decrease in cyt *c* mitochondrial content, and consequently in its detection in the cytosol. However, while for *pph21*Δ strain Bax alpha appears to be more retained in the cytosol no differences were observed for *pph22*Δ strain.

The possible role of the three protein phosphatases on the activity of Bax P168A was also assessed by monitoring the release of cyt *c* (Figure 4.20).

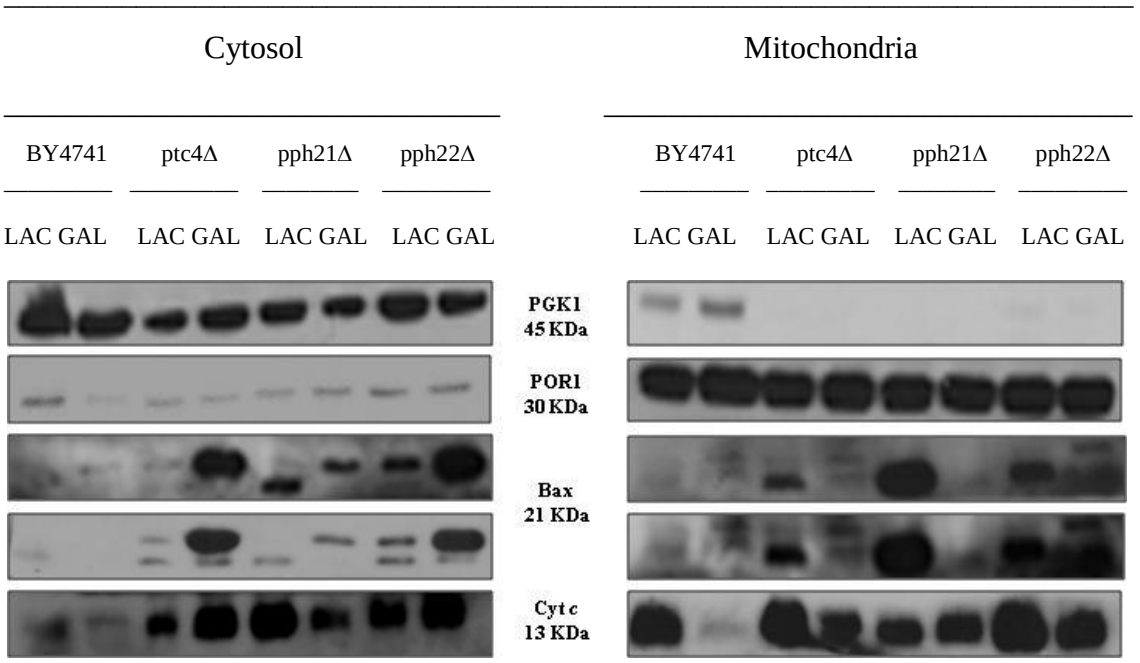

Figure 4.20 - Western blot analysis of cytochrome *c* in *S. cerevisiae* strains BY4741 and protein phosphatase mutant cells expressing Bax P168A before (LAC) and after (GAL) induction of Bax expression after 4h in 0.5% of galactose in cells pre-grown in lactate+ethanol, in both mitochondrial and cytosolic fractions (regarding the content of Bax, we analysed a duplicate of the same extracts). Pgk1p and mitochondrial porin (Por1p) levels were used as loading control of cytosolic and mitochondrial fractions, respectively. A representative experiment is shown of at least two independent experiments with similar results.

The cellular distribution of this form of Bax in the absence of these protein phosphatases was also assessed to relate with the localization of cyt *c*. The expression of the active mitochondrial form Bax P168A in the BY4741 strain led to the release of cyt *c* consistent with mitochondrial localization of this form of Bax (the signal is a little weak).

Bax P168A besides at the mitochondrial fraction was also detected in the cytosol probably due to mitochondrial contamination of the cytosolic fraction as revealed by the presence of Por1p. Regarding the content of Bax we analysed a duplicate of the same extracts.

*ptc4*Δ and *pph22*Δ mutant strains upon expression of Bax P168A displayed a decrease of the cyt *c* mitochondrial content and consequently an increase of cytosolic content of cyt *c*. However, at least for *ptc4*Δ mutant, this was associated with a decrease in mitochondrial Bax P168A content, probably due to mitochondrial contamination of the cytosolic fraction as revealed by the presence of Por1p.

Table 4.2 summarizes the phenotypes of the protein phosphatase mutants studied in what regards Bax alpha- and Bax-P168A-induced cell death and -induced mitochondrial dysfunctions.

Table 4.2 - Comparative table of the phenotypes of the protein phosphatase mutants studied in what regards Bax-alpha- and Bax-P168A-induced cell death and mitochondrial dysfunctions. Non statistically different (n.s.). Comparatively with the control strain. (-) no release and (+) release of cytochrome *c*.

Strains	Survival phenotype (CFU)	ROS Accumulation (folds)	$\Delta\Psi_m$ Membrane Potential (folds)	Release of Cyt *c*
BY4741 Bax alpha	**98.5% Does not induce death**	**1.07 Without accumulation**	**0.98 Unchanged**	**-**
BY4741 Bax P168A	**61.3% Decreased survival ****	**3.20 Enhanced ROS *****	**1.37 Enhanced $\Delta\Psi_{m}$ ****	**+**
***Ptc4p*Δ Bax alpha**	**67.6% Decreased survival ****	**1.27 n.s**	**1.07 n.s.**	**-**
***Ptc4p*Δ Bax P168A**	**62% n.s**	**1.28 Enhanced ROS *****	**0.69 Decreased $\Delta\Psi_{m}$ *****	**+**
***Pph21p*Δ Bax alpha**	**45.33% Decreased survival*****	**1.73 Enhanced ROS *****	**0.71 Decreased $\Delta\Psi_{m}$ ***	**-**
***Pph21p*Δ Bax P168A**	**70.7% n.s**	**1.96 Enhanced ROS *****	**1.58 Enhanced $\Delta\Psi_{m}$ ***	**-**
***Pph22p*Δ Bax alpha**	**130.6% Enhaced survival *****	**0.68 Decreased ROS ****	**1.07 n.s**	**-**
***Pph22p*Δ Bax P168A**	**88.7% Enhaced survival *****	**1.42 Enhanced ROS *****	**1.14 Decreased $\Delta\Psi_{m}$ ****	**+**

5. CONCLUSIONS AND FUTURE PERSPECTIVES

RCD is crucial to numberless biological processes, and understanding its role is continuously increasing. This sustained progress is very important since RCD is involved in numerous human diseases, and targeting these mechanisms will have therapeutic benefit (Galluzzi *et al*, 2014). Therefore, the investigation of the different mechanisms that regulate cell death either through apoptosis, autophagy, necrosis or others will be a significant endeavor (Walsh, 2014). Apoptosis is a genetically programmed mechanism and an evolutionary conserved type of cell death crucial for promoting the organized demise of cells through normal tissue homeostasis and development. Deregulation of apoptosis may contribute to several pathologies or diseases, like neurodegenerative disorders, cancer, and even autoimmune diseases associated with an extreme loss of cells, uncontrolled cell proliferation, and malfunction in the elimination of the abnormal cells, respectively (Fischer and Schulze-osthoff, 2005 and Walsh, 2014). The Bcl-2 protein family consists of different members with opposite functions, either promoting or inhibiting cell death (Lee *et al*, 2014). The Bcl-2 family member Bax is an essential mediator of apoptosis which following a death stimulus adopts a conformation allowing it to oligomerize and insert into mitochondrial outer membrane (Lindsten *et al*, 2000 and Wei *et al*, 2001). Bax protein can be regulated by post-translational modification, the most common being phosphorylation/ dephosphorylation (Basu, 2006; Gardai *et al*, 2004; Linseman *et al*, 2004). So far only few protein kinases and protein phosphatases as well as critical protein residues have been identified as involved in the regulation of Bax with consequences on its activity (for a review see Arokium *et al*, 2007; Linseman *et al*, 2004 and Gardai *et al*, 2004b). However, taking into account the structure of Bax it is conceivable that other residues may be phosphorylated/dephosphorylated by protein kinases/phosphatases not yet identified and that may be relevant in Bax phosphoregulation.

In this study, we used yeast to find new protein phosphatases involved in Bax mediated cell death that ultimately may provide new strategies to modulate its function. This model system was selected because the apoptotic core machinery is conserved in yeast to a degree that makes it a suitable model organism to approach pending questions on human apoptosis and its deregulation in the context of neoplasia, neurodegenerative diseases, and aging (Carmona-Gutierrez *et al*, 2010). Moreover, this model system does not possess obvious homologues of the mammalian Bcl-2 family proteins, which allows studying them individually without the interference from other mammalian apoptotic modulators.

We first expressed human Bax (alpha and P168A) in yeast cells lacking non-essential protein phosphatases and then we determine the profile of Bax phosphorylation by *wb* analysis to detect phenotype alterations. The results obtained from this screening identified different mutant strains displaying an altered Bax phosphorylation profile including *ptc1*Δ, *ptc2*Δ, *ptc3*Δ, *ptc4*Δ, *ptc5*Δ, *ptp1*Δ, *yhv1*Δ, *cnb1*Δ, *pph21*Δ, *pph22*Δ, *nem1*Δ and *cdc55*Δ for Bax alpha; and *ptc2*Δ, *ptc4*Δ, *pph21*Δ, *pph22*Δ and *nem1*Δ for Bax P168A. However, it was not possible to confirm with a λ-phosphatase assay that changes in migrating bands with different mobility result from changes in Bax phosphorylation level. It has been reported that protein phosphatases have different substrate specificity and, as such some may be more adequate than others to dephosphorylate Bax. Therefore, other phosphatases should be tested to optimize this assay and confirm potential changes in the Bax phosphorylation profile due to the absence of protein phosphatases.

Next we decided to focus on the yeast mutants deficient in *pph21*Δ*/22*Δ and *ptc4*Δ, orthologs of human PP2A and WIP1 phosphatases, respectively, that have been previously described as involved on the phosphorylation of Bax. This would allow optimizing the approaches used to characterize the changes in Bax activity as a consequence of deficiency in each of the unknown protein phosphatases identified in the screening with the mutants.

Previous studies reported that specific protein phosphatases are implicated in both extrinsic and intrinsic apoptotic pathways (Figure 5.1). Indeed, PP2A besides affecting directly the MAPKs and AKT pathways, also acts on members of the Bcl-2 protein family (Sun and Wang, 2012). PP2A was found to dephosphorylate Bax and regulate Bax translocation to mitochondria (Xin and Deng, 2006), though the residue involved has not yet been identified. The authors also showed that overexpression of PP2A/C resulted in the Bax dephosphorylation and promoted apoptotic cell death, and using specific knockdown of PP2A/C expression by RNAi increased Bax phosphorylation and enhanced cell survival. The phosphatase 2A belonging to the PPP family is considered the major protein serine/threonine phosphatase which participates in many signaling pathways in mammalian cells (Xin and Deng, 2006). It is a heterotrimer consisting of a 36-kDa catalytic subunit (PP2A/C), a 65-kDa structural A subunit (PP2A/A), and a variable regulatory subunit (PP2A/B, which can vary in size from 50 to 130 kDa). The distinct B subunits can recruit PP2A/C to distinct subcellular locations and then define a specific substrate target determining the substrate specificity of PP2A (Cegielska *et al*,

1994; Estelle *et al*, 1995 and Mccright *et al*, 1996) while the AC catalytic complex alone has phosphatase activity, and it can interact with at least three families of regulatory subunits B, B' and B'' (Cegielska *et al*, 1994). The A and C subunits are evolutionary conserved and ubiquitously expressed (Mayer-jaekel *et al*, 1992).

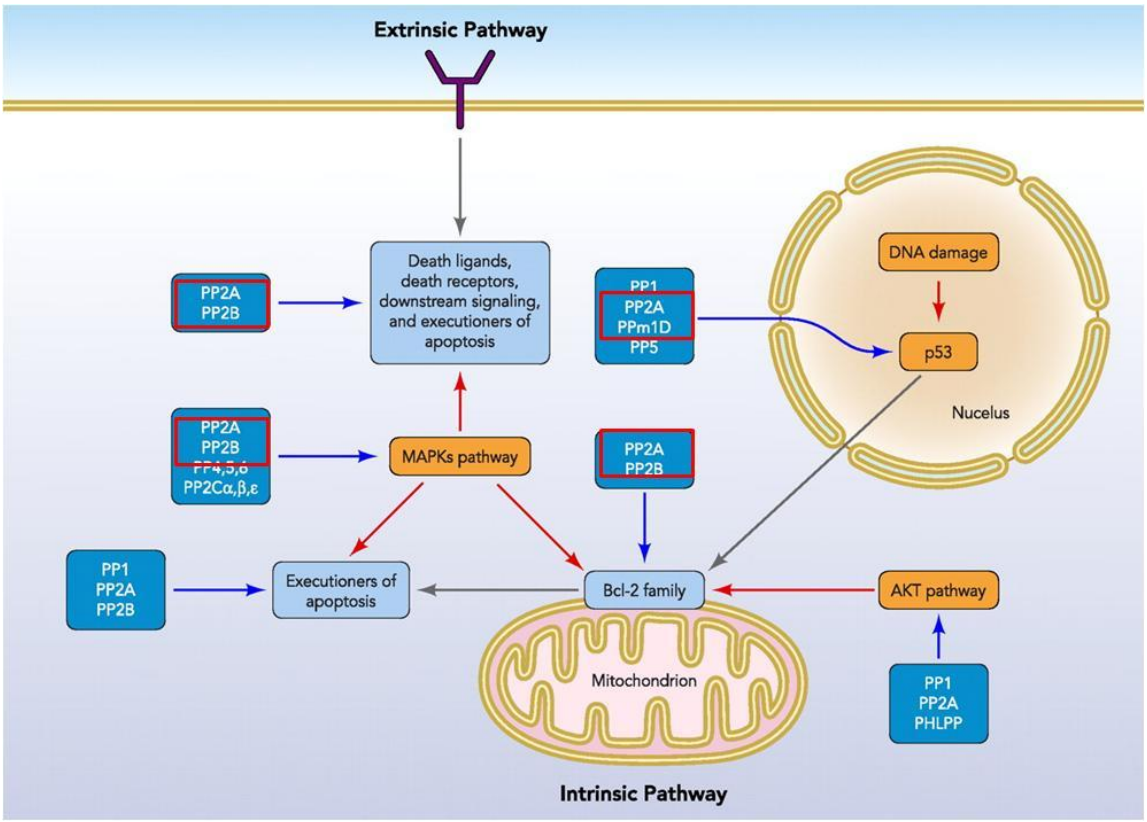

Figure 5.1 – Protein Ser/Thr protein phosphatases implicated in both intrinsic and extrinsic apoptotic pathways. The protein phosphatases are listed in dark blue boxes. Blue arrows indicate direct targets of protein phosphatases, and red arrows indicate indirect targets via MAP kinases and AKT and p53 pathways. Red boxes mark the protein phosphatases studied (Adapted from Sun and Wang, 2012).

It was also shown that PPm1D/Wip1, a p-53 inducible serine/threonine phosphatase that switches off DNA damage checkpoint responses by the dephosphorylation of certain proteins (Song *et al*, 2013), targets directly p53 in the nucleus, removing a phosphate group from Ser15 (Figure 5.2). WIP1 also interacts with and dephosphorylates Bax to suppress Bax-mediated apoptosis in response to γ–irradiation in prostate cancer cells (Song *et al*, 2013). We anticipate that the expression in yeast of non-phosphorylatable or phosphomimetic variants of Bax will contribute to the identification of new Bax residue(s) that are dephosphorylated by Pph21p/22p as well as additional residues that may be targeted by Ptc4p. These data may then be

further validated by heterologously expressing the Bax mutants in a Bax knockout cell line and assessment of the consequences on Bax activity.

STPs	Target	Phosphorylation Residue
PP1	AKT	Thr450
	Caspase-9	Thr125
	Cofilin	
PP1cγ1	JNK1	
	p53	
PP2A	Caspase-9	Thr125
	Cofilin	Ser3
	Bcl-2	Ser70
	Bax	
	Bim	
	AKT	Ser473/Thr308
	AKT	Ser473
	p53	Ser46
	ERK	
	p38 MAPK	Thr-180/Tyr-182
	Caspase-3	Ser150
	p38 MAPK	Thr-180/Tyr-182
	ASK-1	Ser976
PP2A/α4	MEK3	Thr193
	p53	Ser18
	Jun	Ser63
PP2A/PR55	c-SRC	Ser12
PP2A/B56γ	CREB	Ser-108/111/114 and Ser-121
PP2A/B56γ	ATF1	Ser-47/50/51
PP2A/PR65β	DAPK	Ser308
PP2B/Calcineurin	Caspase-3	
PHLPP isoforms	AKT isoforms	Ser473/Thr308
PPm1D/Wip	p53	Ser15

Figure 5.2 - List of protein Ser/Thr protein phosphatases (STPs) involved in cell death regulation and their reported targets (Adapted from Sun and Wang, 2012).

As referred before it was demonstrated that Bax, when expressed in yeast acts on mitochondria generating similar mitochondrial dysfunctions as found in mammalian cells.

To ascertain the effect of Pph21p/22p and Ptc4p on Bax activity, we evaluate changes in mitochondrial function by monitoring ROS accumulation, mitochondrial potential changes ($\Delta\Psi_m$), and release of cyt *c* from mitochondria to the cytosol in single mutant strains lacking these protein phophatases. Since Bax alpha has almost no effect on cell viability while Bax P168A induces cell death, we also performed CFUs assays to assess whether these protein phosphatases affect Bax function (Table 4.2). Moreover taking into account that Bax translocation is necessary but not sufficient for Bax activation, the expression of Bax alpha and BaxP168A allowed assessing the effect of protein phosphatases in these two sequential steps. We found that expression of the

inactive form of Bax in cells lacking Ptc4p induced cell death. However this apparent activation of Bax was not associated with mitochondrial dysfunctions, since the absence of this protein phosphatase does not causes an increase in accumulation of superoxide anion, changes in $\Delta\Psi_m$ or release of cyt *c*. This suggests that Ptc4p acts as a negative regulator of Bax. The *pph21*Δ mutant is also sensitive to the expression of Bax alpha associated with a significant increase in the accumulation of superoxide anion and changes in $\Delta\Psi_m$. However, the mutant decreased survival is not linked to cyt *c* release. In contrast Pph22p appears to enhance Bax activity since its deficiency causes a reduction in the accumulation of superoxide anion though this is not related to change in $\Delta\Psi_m$ and release of cyt *c* in comparison with the wild type strain.

The role of the three protein phosphatases on Bax function was also assessed by their effect on cell death induced by the BaxP168A active form. The absence of Ptc4p does not change BaxP168A-induced cell death associated with the release of cyt *c*, though it is associated with a reduced mitochondrial dysfunction. On the other hand, deficiency of Pph21p inhibits the release of cyt *c* and the accumulation of superoxide anion suggesting that this protein phosphatase is directly or indirectly involved in the regulation of BaxP168A activity. The use of the 6A7 monoclonal Bax antibody would help to confirm this hypothesis. Pph22p, the other PP2A complex subunit studied, appears to have a role in the modulation of BaxP168A activity since, though in its absence there is still cyt *c* release, this is associated with an enhanced survival consistently with a decrease in mitochondrial dysfunction.

The comparison of the protein phosphatase deficient mutant phenotypes expressing Bax alpha or BaxP168A allows to infer on their individual role in the modulation of a cytosolic inactive or mitochondrial active form of Bax (Table 4.2). Ptc4p, Pph21p and Pph22p seem to be required to maintain Bax alpha inactive. Assessment of mitochondrial Bax insertion (by western-blotting after chaotropic treatment) and Bax conformation (by comparing the efficiency of immunoprecipitating Bax with 2D2 and 6A7 monoclonal antibodies recognizing total Bax and activated Bax, respectively) could allow confirming this hypothesis. Regarding the regulation of the BaxP168A by the three protein phosphatases, the results suggest that Ptc4p is not involved in its modulation since its absence does not affect cell death and cyt *c* release. Pph21p also does not seem to interfere with BaxP168A-induced cell death but unexpectedly it reverts cyt *c* release, suggesting that loss of cell survival does not depend on cyt *c* release. Monitoring accumulation of Atg8p, activation of the targeting-

deficient mutant of the vacuolar alkaline protein phosphatase, or GFP-Atg8p processing would allow to confirm whether these cells are dying through an autophagic death as described by Kissová *et al*, 2006. It would also be interesting to confirm the activity of BaxP168A with 6A7 Bax antibody when expressed in the *pph21*Δ mutant. Regarding Pph22p it does not seem to be required for BaxP168A activity as assessed by cyt *c* release. However, this is in disagreement with the decrease in BaxP168A-induced cell death. Further isolation should be done to discard whether the presence of cyt *c* in the cytosolic fraction is just due to a mitochondrial contamination or as consequence of cyt *c* release.

In all cell fractionation assays performed the effect of the protein phosphatases in the distribution of Bax alpha and BaxP168A between the cytosolic and mitochondrial fraction was also determined with the aim to relate with the changes in Bax activity, as assessed by cyt *c* release. However, the results obtained both for the inactive and active forms of Bax are not conclusive and are difficult to interpret. Indeed, the absence of Ptc4p appears to promote Bax alpha addressing to mitochondria associated with a decrease in mitochondrial cyt *c* that is not related with an increase in the level of cytosolic cyt *c*. Therefore, further assays are required to clarify if Ptc4p modulates Bax alpha addressing and activity. On the other hand, it appears that Pph21p as well as Pph22p have no role in modulation of Bax alpha function. Regarding modulation of BaxP168A it seems that Ptc4p, Pph21p and Pph22p appear to be required for mitochondrial Bax addressing. However, while for Ptc4p and Pph22p this addressing of BaxP168A is not associated with Bax activation, it is for Pph21p. As referred above assessment of Bax activity with the 6A7 antibody will allow confirming this hypothesis.

Altogether the results obtained with the yeast orthologs of PP2A (Pph22p) and WIP1 (Ptc4p) mimic the previously reported activation and inactivation of Bax by these protein phosphatases in mammalian cells linked to an enhancement and inhibition of apoptosis. Moreover, the results obtained with BaxP168A suggest that these two protein phosphatases regulate Bax addressing but are not involved in its activation. These data reinforce that the approach developed is adequate to find new protein phosphatase candidates, which directly or indirectly target Bax and regulate its function. Finally it will also enhance our understanding of the mechanisms underlying Bax phosphoregulation to develop novel therapeutic strategies against diseases involving apoptosis dysfunctions, especially in cancer.

6. REFERENCES

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7. ANNEX

A.1 - *Western Blotting*

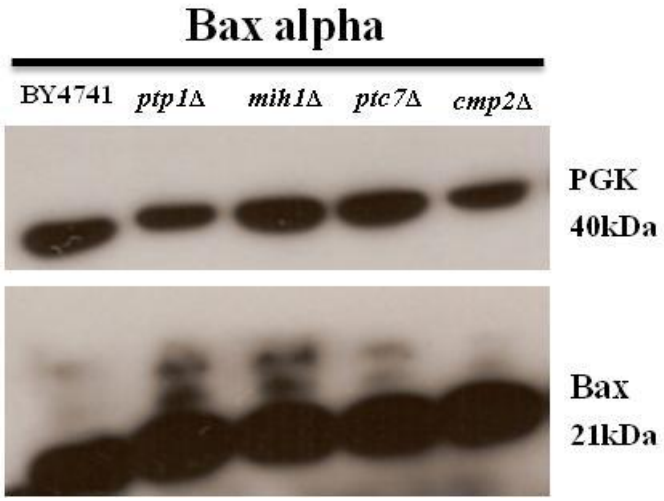
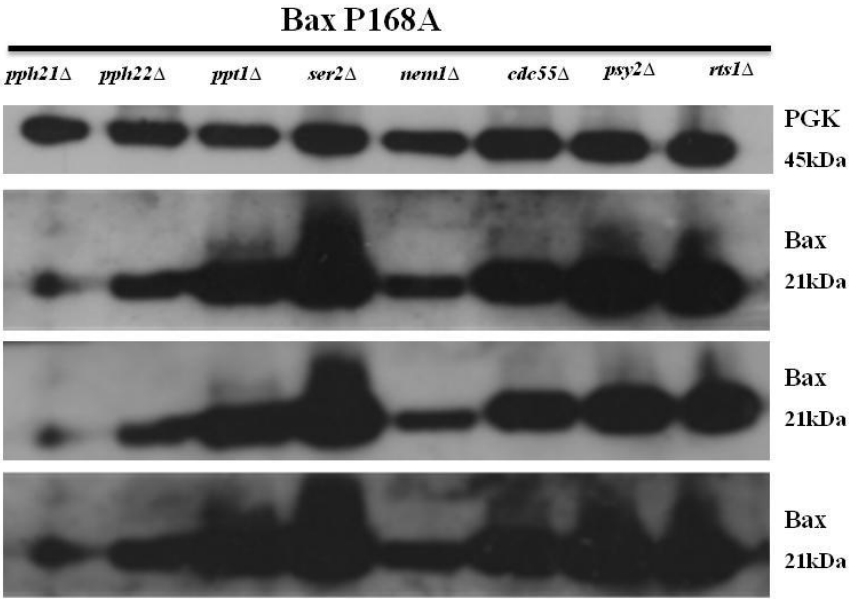
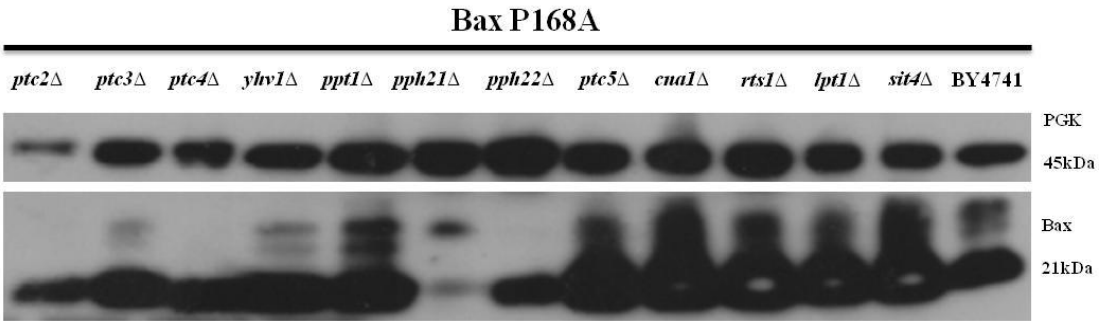


Figure A.1 - Immunodetection of Bax in S. cerevisiae BY4741 and different mutant cells lacking non-essential protein phosphatases expressing Bax alpha. PGK1 was used as a loading control.

A.2 - *Western Blotting*



A.3 - *Western Blotting*



A.4 - *Western Blotting*

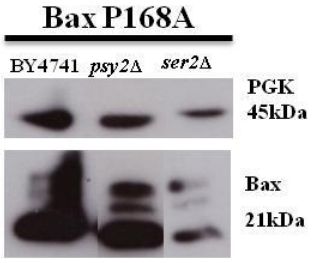


Figure A.3, A.4 and A.5- Immunodetection of Bax in S. cerevisiae BY4741 and different mutant cells lacking non-essential protein phosphatases expressing Bax P168A .PGK1 was used as a loading control.