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Identificação de cinases reguladoras da VDAC1 mitocondrial das leveduras

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Identificação das cinases reguladoras da VDAC1 mitocondrial das leveduras

Identification of regulatory kinases of the yeast mitochondrial VDAC1

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Paula Gonçalves, Professora do Departamento de Biologia da Universidade de Aveiro e da Doutora Clara Isabel Ferreira Pereira, Investigadora no Instituto de Inovação e Investigação para a Saúde do Porto.

Dedico este trabalho aos meus pais.

o júri

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palavras-chave   Mitocôndrias, Por1p, cinases, fosforilação, sinalização, respiração.

resumo   O canal aniónico dependente de voltagem (VDAC) é um canal tipo poro que atravessa a membrana externa da mitocôndria. O VDAC tem um papel essencial no metabolismo mitocondrial, mediando a troca de metabolitos essenciais para a respiração, produção de energia e crescimento celular. Em mamíferos, a probabilidade de abertura do canal VDAC pode ser regulada por modificações pós-traducionais como a fosforilação. Nas leveduras, o papel da fosforilação na fisiologia da VDAC1/Por1p e as cinases reguladoras são ainda desconhecidos. Deste modo, o objetivo deste estudo incide sobre a identificação de cinases reguladoras do Por1p, e a avaliação do seu papel na função mitocondrial e na fisiologia celular.

Para identificar as cinases reguladoras, mutantes deficientes em 15 cinases (selecionadas de acordo com a função ou localização mitocondrial) foram avaliados em relação ao ponto isoelétrico (pI) do Por1p, através de um 2D SDS-PAGE seguido da imunodeteção do Por1p. Dois mutantes deficientes nas cinases Hog1p e Rim15p, apresentaram uma alteração no pI do Por1p, compatível com a falta de fosforilação, sugerindo que estas cinases poderão ser potenciais reguladores do Por1p. Para reforçar estes resultados, alguns fenótipos foram analisados, tais como o crescimento, consumo de oxigénio, indução de autofagia/mitofagia, resistência ao calor ou stress oxidativo, para avaliar possíveis interações genéticas entre o *POR1* e a cinases identificadas, indicativo de uma relação funcional. Foi observado que a ausência de *HOG1* interfere com o crescimento do *por1Δ* em condições fermentativas, com a indução de mitofagia e resistência ao stress oxidativo. Na ausência de *RIM15*, o crescimento em condições fermentativas, consumo de oxigénio, o fluxo e a indução autofágica e a indução mitofágica são afectados no mutante *por1Δ*. Estes resultados apoiam a hipótese das cinases Hog1p e Rim15p serem reguladoras do Por1p, sendo que mais estudos serão necessários para confirmar se esta esta regulação é directa.

A identificação das primeiras cinases reguladoras do Por1p será um importante avanço no conhecimento da regulação do Por1p/função mitocondrial por vias de sinalização metabólica.

keywords　　　　　　　　Mitochondria, Por1p, protein kinases, phosphorylation, signaling, respiration.

abstract　　　　　　　　The voltage-dependent anion channel (VDAC) is a conserved porin ion channel located on the outer mitochondrial membrane. VDAC plays an essential role in mitochondrial metabolism as it mediates the exchange of metabolites essential for respiration, energy production and cell growth. In mammalian cells VDAC opening probability can be regulated by post-translational modifications such as phosphorylation. In yeast, the role of phosphorylation in VDAC/Por1p physiology and the regulatory kinases are still unknown. Therefore, the focus of the present study is to identify Por1p regulatory kinases, and evaluate its impact on mitochondrial function and cellular physiology.

To identify Por1p regulatory kinases, 15 kinase-deleted mutants (selected based on kinase function or mitochondrial location) were screened for alterations in Por1p isoelectric point (pI), by 2D SDS-PAGE followed by immunoblot using an antibody against Por1p. Two strains among these, lacking Hog1p and Rim15p, exhibited a shift in Por1p pI compatible with lack of phosphorylation, suggesting these kinases may be Por1p regulators. To reinforce these result, several phenotypes such as growth, oxygen consumption, autophagy/mitophagy induction, resistance to heat or oxidative stress, were evaluated to assess genetic interactions between *POR1* and the identified kinases, indicative of a functional relationship. It was observed that absence of *HOG1* interferes with *por1Δ* growth in fermentative conditions, mitophagy induction and oxidative stress resistance. Absence of *RIM15* interferes with *por1Δ* growth in fermentative conditions, mitochondrial oxygen consumption, autophagy flux and induction and mitophagy induction. These results support Hog1p and Rim15p as Por1p regulators, but future work will be needed to confirm them are direct regulators of Por1p phosphorylation.

Identification of the first Por1p regulatory kinases in yeast will be an important advance on the understanding of the regulation of Por1p/mitochondrial function by metabolic signalling pathways.

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Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CFU	Colony forming units
CKI	Casein kinase I
CKII	Casein kinase II
CytC	Cytochrome C
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
FADH$_2$	Flavin adenine dinucleotide reduced
GSK3β	Glycogen synthtase kinase 3 beta
HK	Hexokinase
HOG	High osmolarity glycerol
JNK3	c-Jun N-terminal kinase 3
MAPK	Mitogen-activated protein kinase
MCK1	Meiosis and centromere regulatory kinase 1
MOM	Mitochondrial outer membrane
NADH	Nicotinamide adenine dinucleotide reduced
OCR	Oxygen consumption rate
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDS	Post-diauxic shift
PEG	Polyethylene glycol
pI	Isoelectric point
POR1	Porin 1
POR2	Porin 2
RIM15	Regulator of Ime2
SDS	Sodium dodecyl sulfate
VDAC	Voltage-dependent anion channel
YPD	Yeast peptone dextrose
YPDA	Yeast peptone dextrose agar
YPG	Yeast peptone glycerol

Chapter 1
INTRODUCTION

1. INTRODUCTION

1.1. Mitochondria

Mitochondria are double-membrane organelles present in nearly all eukaryotes (1). It is widely accepted that these organelles are descendants of an endosymbiont protobacteria that became settled in a host cell. Consistently with this hypothesis, mitochondria exhibit their own genome and have the ability to self-replicate (2), but some mitochondrial proteins are encoded by nuclear DNA. Mitochondria are central players in several biological processes, including energy production, stress resistance, lipid metabolism, apoptosis between others.

It is at the mitochondria that cell respiration occurs, an important mechanism in energy production from food and nutrients. During the glycolysis process, pyruvate is converted to acetyl-coA, which in turn participates in the Krebs cycle inside the mitochondria, producing NADH and $FADH_2$, which is then used by enzymes located in the mitochondrial inner membrane to produce adenosine tri-phosphate (ATP) (4). In these reactions, electron transfers occur to promote production of free energy (ATP). This free energy it is then used to pump protons that activate ATP synthase for ATP synthesis (3). This process is known as oxidative phosphorylation and it happens in the matrix of the mitochondria.

In our model organism, the yeast *Saccharomyces cerevisiae*, when glucose is limiting or lacking, the cell enters the respiratory phase, also known as the post-diauxic phase, in which the ethanol produced during fermentation will react with oxygen (consumed during this phase), releasing water, CO_2 and free radical species such as the superoxide radical, O_2^- (5).

Therefore, these activities promote oxidative stress, since the energy production affects redox equilibrium, with the activity of electron transport chain, the release of ATP and the production of free radical species, especially the reactive oxygen species (ROS) (6). Mitochondria shows a variety of factors and proteins that regulate oxidative stress, such as cytochrome c or the c-Jun N-kinase (JNK) that promote redox equilibrium and also some transcription factors such as Rtg1/Rtg2/Rtg3 system, that recruit protein kinases such as Hog1p and regulate the TOR pathway (7) to promote homeostasis.

Mitochondria are also essential for the synthesis of pyrimidines and purines, contribute to the synthesis of heme, regulation of nitrogen balance through the urea

cycle, production of ketones and are key regulators of the apoptotic program in mammals (8).

Since mitochondria are fundamental for cell homeostasis, damages to mitochondria's functions will lead to pathological states and even cell death. To avoid further damages to the cell, mitochondria can be eliminated by autophagy or mitophagy, if the energy cost of maintaining them is superior to their effective metabolism (9,10).

Autophagy is an important process in which the cell ensures homeostasis by eliminating superfluous, damaged or harmful organelles, including mitochondria, and cytoplasmic compounds. This process initially involves the engulfment of compounds to be discarded in a double membrane vesicle and consequent transport to the lysosome or vacuole to be degraded and recycled. Thus, autophagy allows counteracting mostly the lack of nutrients, but also internal and external stress conditions (11,12). Mitochondria can also be selectively eliminated by mitophagy, when an accumulation of dysfunctional mitochondria can lead to a compromised cell growth and even cell death (10). Mitophagy has been described to occur when dysfunctional mitochondria show signs of high energy cost maintenance to the cell (10,12).

There is also emerging evidence of the involvement of mitochondria in multiple other processes such as cytoskeleton organization and microtubule network remodelling (13–15).

Morphologically, mitochondria are dense and double membrane-enclosed organelles, with a mitochondrial outer membrane (MOM) and an inner membrane (MIM), each one with distinct composition and proprieties. The inner membrane houses the respiratory chain and the ATP synthase held in the cristae, which are prolongations of the mitochondrial inner membrane into the matrix. In the MIM there are present the translocases of the inner membrane (TIM). The outer membrane shows a balance between lipids and protein in its constitution, exhibiting, among others, the translocase of the outer membrane (TOM), and the voltage-dependent anion channel (VDAC), as important channels for mitochondrial function (14,16). Their permeability is also different, the outer membrane presenting a higher permeability to ions and macromolecules than the inner membrane (17).

1.2. Voltage-dependent Anion Channel (VDAC)

The existence of channels in the MOM has been evidenced by electrophysiology and electron microscopy during the 1970s, presenting the MOM as the interface between

the cytosol and the mitochondrial spaces. It separates the organelle from its environment and act as selective barrier to the entry and exit of metabolites, water and ions (17,18).

Permeation through the MOM is believed to be based in a MOM protein called VDAC (also known as mitochondrial porin), which forms a voltage-dependent, anion-selective channel (19,20). In mammals, three isoforms of VDAC are described: VDAC1, VDAC2 and VDAC 3 (21,22), with VDAC1 being the major form. In the eukaryote model used in this study, *Saccharomyces cerevisiae*, two isoforms of VDAC are expressed, named Por1p and Por2p. Por1p is the major porin in yeast MOM while Por2p is less expressed and is not essential for the permeability of the mitochondria (23).

VDAC exhibits an essential role in mitochondrial metabolism, participating in the regulation of oxidative phosphorylation, flux of metabolites, intracellular redox status, energy generation and cell growth and survival (Fig. 1).
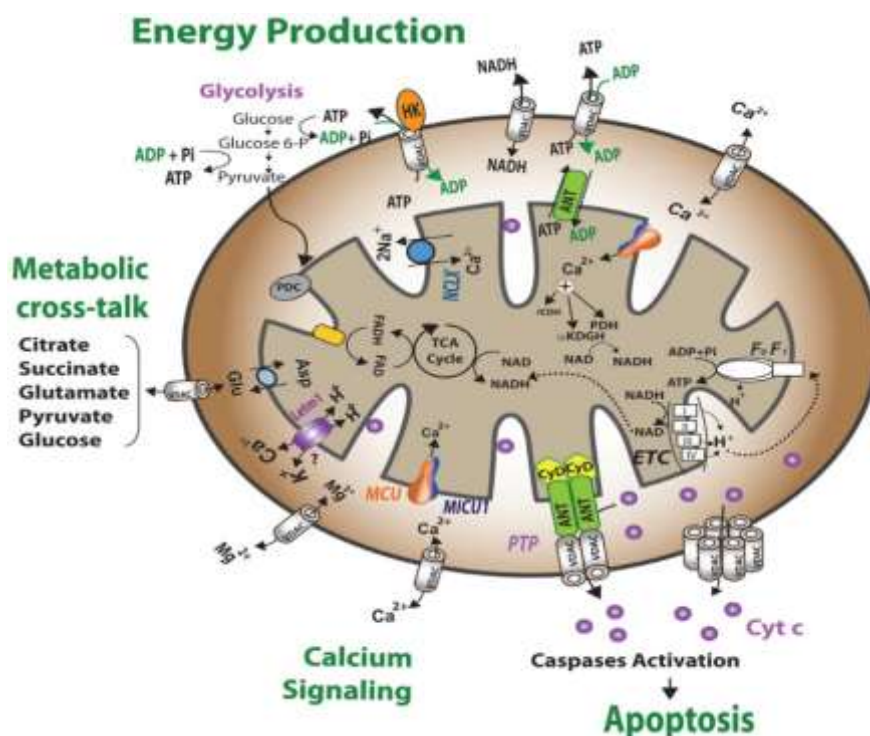
Figure 1: Schematic representation of VDAC1 as a crucial channel for a variety of cell signals. This scheme was reproduced from Shoshan-Barmatz and Mizrachi, 2012 (25).

The prototypal VDAC exhibits one α-helix and 19 β strands that result in a pore with an estimated internal diameter of 2.5 nm (26,27). VDAC depends on the mitochondrial membrane potential to define its conformational state. When the VDAC-pore is at a zero-transmembrane potential, it remains in the open state, enabling the flux of anions, such as ATP/ADP, between the mitochondria and the cytosol. When the

membrane voltage increases beyond 30 mV, the inversion of ion selectivity in VDAC occurs on one side of the channel, by translocation of a region of the VDAC channel, designated as voltage sensor region that encompasses part of the α-helix, towards the outside of the channel, resulting in a reduction of VDAC diameter, compromising the flux of some metabolites, especially ATP and ADP (Fig. 2) (26,28,29).

Although ATP presents 0.48 nm of diameter, compared to the 0.9 nm of VDAC closed state, the altered charge inside VDAC inhibits its flow. In addition to ATP, the flux of several molecules crucial for the oxidative phosphorylation pathway (NADH, NADPH, succinate, pyruvate, phosphate, etc.) is also inhibited when the VDACs pore is closed, leading to destabilization of the mitochondria, endangering the cell survival (30). VDAC, besides controlling the influx of oxidative phosphorylation substrates, controls the efflux of the ATP produced in mitochondria, avoiding its accumulation in the mitochondrial matrix that could block the oxidative phosphorylation (31).
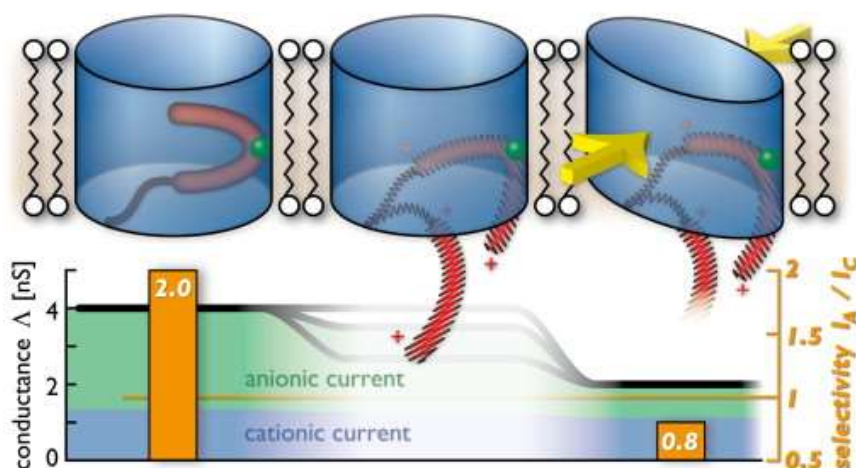
Figure 2: Suggested model of voltage-induced VDAC open/close conformations. The dynamic changes in VDAC, influenced by the variation of the mitochondrial membrane potential, affect the selectivity and conductance of anions and of cations. This scheme was reproduced from Shoshan-Barmatz et al, 2003 (32).

VDAC has also been shown to allow the release of mitochondrially-produced superoxide radicals (O_2^-) to the cytosol, controlling intramitochondrial O_2^- levels. Reactive oxygen species can induce cell damage, but some species have been found to have an important role as intracellular signals (33–35). Hence, VDAC closure provides a simple and flexible mechanism of decreasing release of O_2^- that, although promoting intramitochondrial oxidative stress, spares this stress in the cytosol (36,37).

VDAC also takes part in the redox regulation of expression and activity of several proteins such as those involved in protein import into mitochondria and antioxidant

enzymes. Absence of VDAC in yeast has been shown to affect levels of nuclear transcripts sensitive to changes in the intracellular redox state, including nuclear transcription factors related to stress (e.g., Msn2, Msn4) as well as transcription factors involved in communication between mitochondria and the nucleus (Rtg1, Rtg2) (35,38,39). Thus, in addition to regulating the metabolic and energetic functions in mitochondria, VDAC is involved in the communication between mitochondria and the rest of the cell.

In mammals, VDAC also appears to play an important role in apoptosis induction, with at least three major hypothesis described (Fig. 3): i) the permeability transition pore (PTP) model establishes that opening of the PTP, which contains VDAC and the adenine nucleotide translocator (ANT), increases permeation of the MOM, increasing flux of water and metabolites towards the mitochondria and leading to swelling and rupture; ii) another model focus on VDACs defective closure, which inhibits normal exchange of ATP/ADP leading to swelling and rupture of the mitochondria and later to cell death; iii) the third model proposes VDAC as an anchor for pro-apoptotic proteins such as Bax (40). In yeast, it was shown that cells lacking Por1p are sensitive to apoptotic stimuli, suggesting that Por1p acts as a negative regulator of cell death (41).
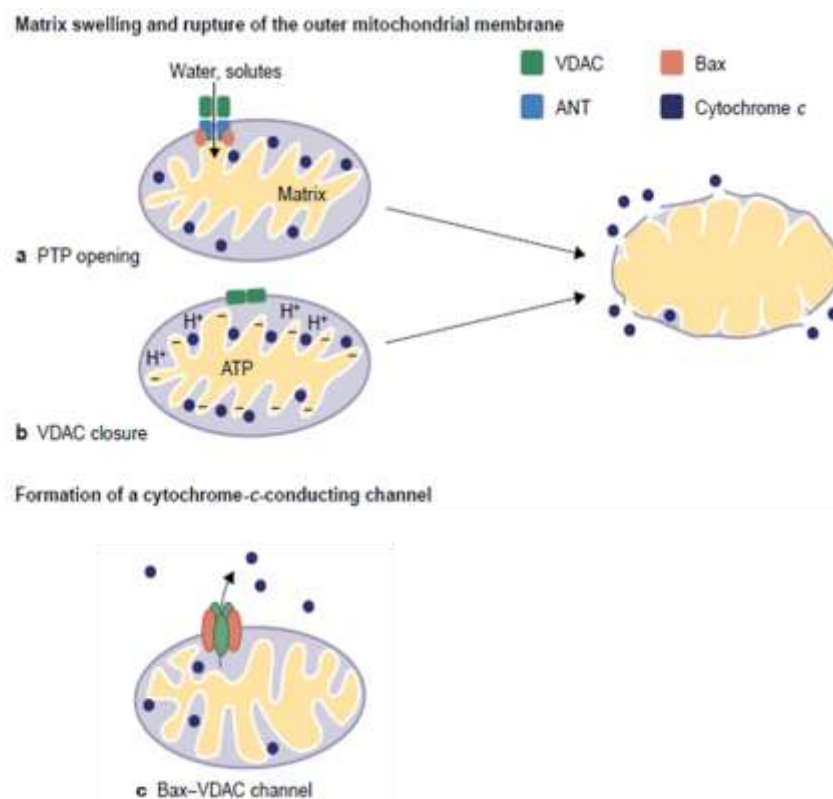

Figure 3: Models for the release of cytochrome c from mitochondria. During the process of apoptosis, cytochrome *c* is released from mitochondria into the cytosol. These models represent

some theories that might lead to cell death involving VDAC. This scheme was reproduced from Martinou et al. 2000 (40).

Because of VDAC vital roles, a dysfunction in VDAC regulation has been associated to the development of some cancers and neurodegenerative diseases, such as Alzheimer's disease (24). Thus, understanding VDAC regulation will help to define strategies to prevent or attenuate the development of such diseases (25,42,43).

1.3. VDAC regulation by post-translational modifications

Various types of post-translational modifications (PTMs) of VDACs have been reported, although their impact on channel function and consequently on mitochondrial function is not well understood. PTMs increase the functional diversity of the proteome by the covalent addition of functional groups to proteins. These modifications include phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation and proteolysis and influence almost all aspects of normal cell biology and pathogenesis (44).

Reversible protein phosphorylation is regulated by kinases and phosphatases and is one of the most common mechanisms of metabolic modulation (44). Protein phosphorylation occurs mainly on serine, threonine and tyrosine residues, and is one of the most important and well-studied post-translational modifications, being commonly known as an on/off mechanism for various cellular processes (45). Phosphorylation can affect protein catalytic activity (it can be activated or deactivated), turnover, subcellular localization or interaction with proteins that have structurally conserved domains that recognize and bind to phosphomotifs. Phosphorylation plays a vital role on the regulation of cell growth, transcription of genes, protein synthesis, metabolism and signaling (46–48) by affecting proteins in several ways (Fig. 4).
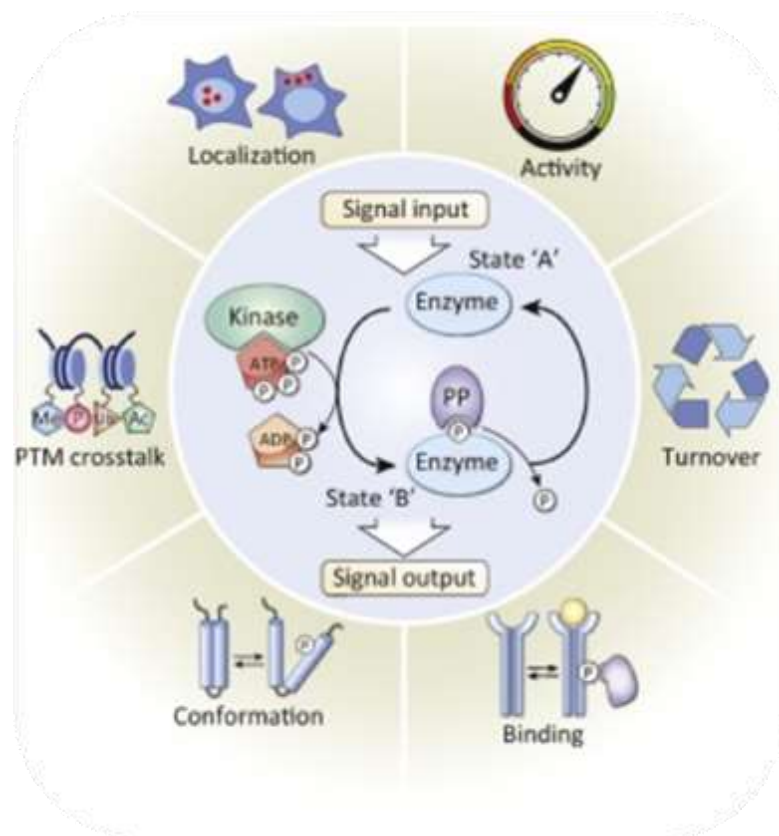
Figure 4: Protein regulation by phosphorylation. Phosphorylation can affect protein activity, subcellular localization, protein turnover and protein interactions, induce conformational changes, and regulate other post-translational modifications. This scheme was reproduced from Humphrey et al. 2015 (49).

According to some reports, phosphorylation of VDAC by cytosolic kinases may be involved in its regulation. To date, a few mammalian kinases have been described as regulating VDAC. Never-in-mitosis A related kinase 1 (Nek1), cAMP-dependent protein kinase A (PKA), protein kinase C (PKC) and glycogen synthase kinase 3 (GSK3β), p38 and c-Jun N-terminal kinase 3 (JNK3) have been reported to regulate VDAC gating (50–52) and binding to other proteins such as hexokinases I and II (HKI and HKII) and tubulin (53–55).

Through co-immunoprecipitation tests and mutagenesis assays, it was demonstrated that these regulatory kinases interact with VDAC, having some of its effects been already described. GSK3β, PKA, Nek1 and PKCs have been reported to phosphorylate VDAC Ser12, Ser103 and Ser193 residues, which are located at crucial sites on VDAC. The phosphorylation of these sites have an impact on VDAC configuration, modulating VDAC conformation state leading to a closed state, therefore, preventing apoptosis (56). Some of these regulatory kinases also promote the binding of tubulin and HK to VDAC (57,58). However, GSK3β also been reported to increase opening of VDAC via Thr51 phosphorylation, leading to sensitization to cell death (58).

GSK3-β showed effects on VDAC binding to both tubulin and HK, being activated when there is mitochondrial dysfunctions, promoting apoptotic activity with the detachment of HK from VDAC, since HK bound to VDAC maintain this channel closed to prevent further apoptotic activity, leading to VDAC open state (59). The phosphorylation of VDAC-Ser193 is known to be affected by Nek1, altering VDAC conformation to a closed state, preventing the leakage of cytochrome c, thus, preventing cell death (56).

Other studies pointed out p38 kinase and JNK3 as potential VDAC1 regulatory kinases. Still not well defined, it is known that p38 affects indirectly VDAC phosphorylation on a tyrosine residue, in response to stress (60). JNK3 is thought to phosphorylate two VDAC1 residues, Ser104 and Ser137, possibly affecting VDAC conformational state, but no regulatory function was attributed yet (61). Other VDAC1 phosphorylated residues and their possible regulatory protein kinases have been reported but their physiological effect have not been determined (62–65) .

Some of the kinases that may regulate mammalian VDAC1 have homologs in yeast, such as GSK3 (Mck1p homologue) (66,67), p38 and JNK3 (Hog1p homologues) (68) and PKA (Tpk1/2/3p homologues) (69). However, although yeast VDAC/Por1p is phosphorylated (Table 1), the role of these modifications and the regulatory kinases are unknown.

Table 1: Por1p residues known to be phosphorylated. Location of the aminoacid in the Por1 protein is indicated. Adapted from Phosphogrid registry (84)

Location	Residue	Sources
2	S	(51,54)
91	T	(87)
100	T	(87)
103	T	(51,53)
109	S	(88)
117	T	(51)

1.4. Hog1p and Rim15p kinases – an overview

1.4.1. Hog1p

Protein kinases mediate signal transduction in eukaryotic cells, being involved in the control of several cellular processes. The yeast Hog1p is a mitogen-activated protein kinase (MAPK) (homolog of mammalian p38 and JNK3) (70) with a central role in the osmorregulatory signal transduction cascade (HOG pathway), which regulates G1 and

G2 cell cycle progression, and cellular ion levels in response to hyperosmotic stress (71–73) (Fig. 5). Loss of Hog1p activity results in reduced growth on high osmolarity media and abnormal cell and budding morphology (71,74), but the constitutive activation of Hog1p has been shown to be lethal (70).

The Hog1p MAPK also controls a rapid transcriptional response that involves a large number of genes required for cellular adaptation (75). It regulates transcriptional induction by phosphorylating transcriptional factors such as Sko1p, Smp1 Msn2/4 and Hot1p (76–78) and other factors involved in chromatin remodelling and Pol II recruitment (79–81).

Hog1p is also involved in the oxidative stress response, modulating arrest in cell cycle progression and promoting progression of cell cycle when exiting from quiescence (Fig. 5). Most importantly and still not well studied, Hog1p has been described to control the respiratory metabolism (82) since absence of Hog1p resulted in an increase of oxygen consumption, suggesting Hog1p plays a role in the suppression of the respiratory metabolism. Reports have shown that Hog1p is also a specific regulator of mitophagy, participating indirectly in Atg32p phosphorylation and, in a later stage of mitophagy, in the assembly of the mitophagosome (83) and other unknown cytoplasmic substrates (84).
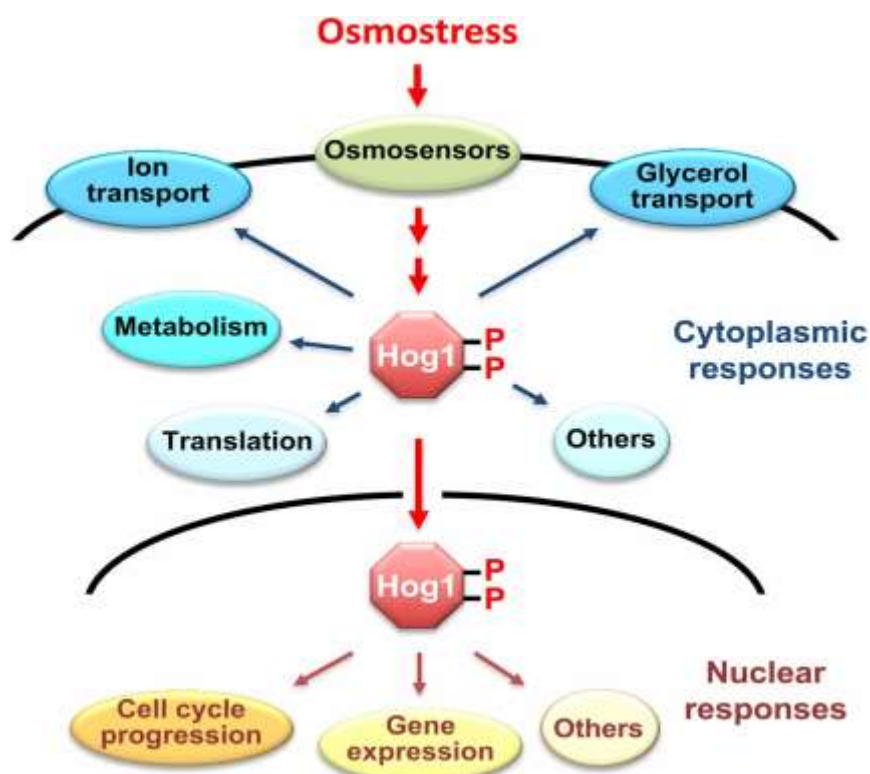
Figure 5: Role of Hog1p in osmostress response in yeast cells. As a response to an increased extracellular osmolarity, Hog1p is activated, stimulating the induction of cytoplasmic and nuclear responses. This scheme was reproduced from Haruo Saito et al. 2012 (85).

1.4.2. Rim15p

Identified as a stimulator of meiotic gene expression, Rim15p is also proposed to integrate signals from various nutrient signalling networks, being one of the most relevant protein kinases in cell proliferation. Regulation of Rim15p activity and localization is dependent of nutrient sensing pathways, such as TOR, Sch9p, PKA and the Pho80-85p kinase pathway. This sensing pathways exhibit sensitivity to nitrogen, sugar and phosphorus status in the cell, promoting cell quiescence when there is lack of these nutrients, showing also sensitivity to various external stresses (86–88), inducing Rim15p activity in both scenarios (Fig. 6). Rim15p is inhibited by TOR1, Sch9p and PKA when nutrients are sensed in the environment. Under nutrient limitation conditions, Rim15p is dephosphorylated and transported to the nucleus, where it regulates Gis1p, a transcription factor that is activated inducing post-diauxic shift (PDS) phase genes, and Msn2/4p that regulate the expression of genes containing stress response elements (STRE) in response to nutrient limitation and stress (oxidative, heat and osmotic) conditions (89). This activity will promote cell quiescence and survival upon lack of nutrients.

A model for Rim15p function has been proposed in which Rim15p coordinates also cell growth (is important for budding index) and the exit of cell cycle to quiescence in response to various sensing pathways (90). Rim15p appears to be relevant for survival and robustness during the stationary phase, in which cells are subjected to prolonged glucose starvation (87,91), promoting non-selective autophagy for ATP recovery and ensuring cell homeostasis (92). Rim15p also regulates oxidative stress responses (90). In stationary-phase cultures, deletion of *RIM15* causes a strongly decreased accumulation of storage carbohydrates and trehalose (89), and reduced thermotolerance and life span (91,93).
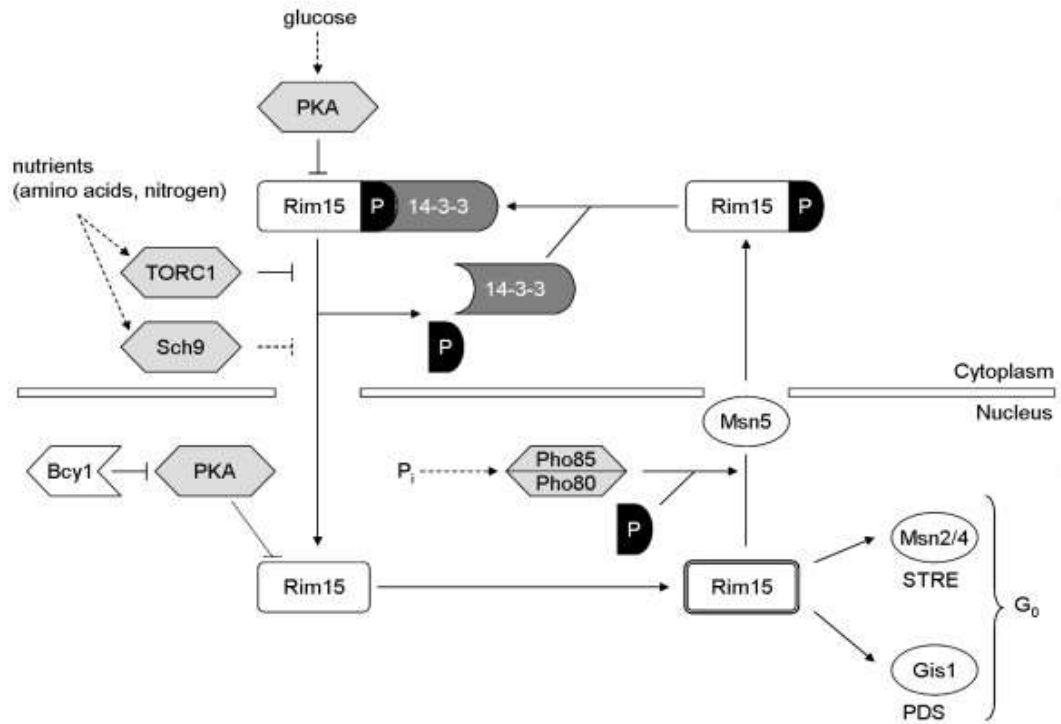
Figure 6: Rim15p activity regulated by nutrient sensitive proteins. Rim15p integrates signals from various nutrient sensing pathways to different effectors. This scheme was reproduced from Swinnen et al. 2006 (87).

Chapter 2

AIMS

2. AIMS

The voltage-dependent anion channel (VDAC) is an outer mitochondrial membrane protein at the interface between mitochondrial and cellular metabolisms. VDAC display two states: an open state that allows the passage of anions (e.g. ATP and NADH); and a closed state, associated with an exchange in channel selectivity allowing the passage of cations. In mammals, VDAC activity and interaction with other proteins can be regulated by post-translational modifications such as phosphorylation. In yeast, the physiological relevance of VDAC1/ Por1p phosphorylation and regulatory kinases have not been characterized.

This project aimed to identify VDAC1/Por1p regulatory kinases, using *S. cerevisiae* as a model organism. The evaluation of VDAC1 phosphorylation was assessed by a 2D-immunoblotting procedure, using mitochondria enriched samples from a pre-selected collection of kinase deleted yeast strains.

The impact of the absence of potential regulatory kinases on VDAC1 physiology was characterized by assessing mitochondrial respiratory activity, directly related with VDAC1 activity in vivo, monitored by measuring oxygen consumption and growth in a respiratory substrate. Additionally, fermentative growth, stress resistance (oxidative and thermotolerance) and induction of autophagy and mitophagy, was evaluated.

Chapter 3

**MATERIALS AND
METHODS**

3. MATERIALS AND METHODS

3. Yeast strains and plasmids

S. cerevisiae strains used in this study are listed in Table 2.

Table 2: Yeast strains used in this work.

Strain	Genotype	Source
BY4741	*Mata, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	EUROSCARF
tpk1Δ	BY4741 *tpk1::KanMx4*	EUROSCARF
tpk2Δ	BY4741 *tpk2::KanMx4*	EUROSCARF
tpk3Δ	BY4741 *tpk3::KanMx4*	EUROSCARF
yck1Δ	BY4741 *yck1::KanMx4*	EUROSCARF
yck2Δ	BY4741 *yck2::KanMx4*	EUROSCARF
cka1Δ	BY4741 *cka1::KanMx4*	EUROSCARF
cka2Δ	BY4741 *cka2::KanMx4*	EUROSCARF
kkq8Δ	BY4741 *kkq8::KanMx4*	EUROSCARF
slt2Δ	BY4741 *slt2::KanMx4*	EUROSCARF
rim15Δ	BY4741 *rim15::KanMx4*	EUROSCARF
hog1Δ	BY4741 *hog1::KanMx4*	EUROSCARF
mck1Δ	BY4741 *mck1::KanMx4*	EUROSCARF
ypk1Δ	BY4741 *ypk1::KanMx4*	EUROSCARF
sak1Δ	BY4741 *sak1::KanMx4*	EUROSCARF
sch9Δ	BY4741 *sch9::KanMx4*	EUROSCARF
por1Δ	BY4741 *por1::HIS3*	This study
por1Δrim15Δ	BY4741 *por1::HIS3 rim15::KanMx4*	This study
por1Δhog1Δ	BY4741 *por1::HIS3 hog1::KanMx4*	This study

3.1. Mutant construction

To generate *por1Δ*::*HIS3* cells, the *KanMX4* cassette in *por1Δ*::*KanMX4* was replaced by *HIS3*, using a deletion fragment containing the heterologous *HIS3* and the flanking regions of *KanMX4*, amplified by polymerase chain reaction (PCR). To delete *POR1* in *hog1Δ* and *rim15Δ* cells, the cassette *HIS3MX6* with the flanking regions of *POR1* was amplified from the BY4741 *por1Δ*::*HIS3MX6* strain using the primers, *POR1*_Amp_Fw (AGTTTAATGGTCAGAATGGGCG) and *POR1*_Amp_Rv (GGAGTTTATCACAATGTTCGAAACC) by colony PCR. The reaction mix contained 1x Reaction Buffer (Thermo Scientific), 1.5 mM MgCl$_2$ (Thermo Scientific), 0.2 mM sense

primer, 0.2 mM antisense primer, 0.2 µM dNTPs (Thermo Scientific), 1 U Taq Polymerase (Thermo Scientific) and the PCR was performed at an annealing temperature of 47ºC. All the PCR products were analysed by nucleic acid electrophoresis (using TAE agarose gel). PCR products were purified with Gel Pure (NZYTech) and used to transform yeast.

The correct replacement of *POR1* by the *HIS3* cassette was confirmed by PCR using a primer for *HIS3* (GAATGCTGGTCGCTATAC) and one upstream *POR1* (CGTCATCTTCTAACACCGTATATG), following the already described procedures, at an annealing temperature of 51ºC. Absence of Por1p was also confirmed by Western-blotting.

For the analysis of autophagy and mitophagy induction in different strains, yeast cells were transformed with plasmids expressing GFP-Atg8 (pRS416, kindly provided by T. Yorimitsu (94)) and OM45-GFP (pKC2, kindly provided by D. Klionsky (95)), respectively, and selected in minimal medium lacking uracil and leucine, respectively.

3.2. Yeast Transformation

For yeast transformation, the polyethylene glycol (PEG)/lithium acetate method was used (96). Cells were grown in 20 mL of YPD medium to an $OD_{600}=0.8$, harvested, washed and incubated with the transformation mix, containing 240 µL of PEG 3350 50% (w/v), 36 µL lithium acetate 1.0 M, 25 µL single stranded DNA (2.0 mg/ml), 300ng DNA and sterile water to a final volume of 360 µL. The cells were incubated at 26ºC for 30 min followed by incubation at 42ºC for 30 min. Lastly, the cells were centrifuged, washed with H_2O and plated on selective medium without histidine. A negative control containing the cells and transformation mix without DNA was used.

3.3. Growth Conditions

Yeast cells were grown at 26ºC in a gyratory shaker at 140 rpm, with a ratio of flask volume/medium of 5:1, to logarithmic phase (log) ($OD_{600}=1$) or to PDS phase ($OD_{600}=6-10$). The growth media used were yeast peptone dextrose [YPD, 1% (wt/vol) yeast extract (Conda Pronadisa), 2% (wt/vol) bacteropeptone (LabM) and 2% (wt/vol) D-glucose (Fisher Scientific), yeast peptone glycerol [YPG, containing 2% (wt/vol) glycerol (NZYtech) instead of glucose] and synthetic complete [SC medium; containing drop-out, 2% (wt/vol) glucose and 0.67% (wt/vol) yeast nitrogen base without amino acids (BD Biosciences)], being supplemented with appropriate amino acids or nucleotides (0.04% (wt/vol) leucine, 0.008% (wt/vol) histidine, 0.008% (wt/vol) tryptophan and 0.008% (wt/vol) uracil). For solid medium 2% (wt/vol) agar (Conda Pronadisa) was added.

3.4. Mitochondrial Isolation

Mitochondrial extracts were prepared based on a previous method (97). Briefly, yeast cells grown to the PDS phase in YPD medium were harvested at 4700 rpm for 10 min and washed with dH_2O before suspension in digestion buffer [2 M sorbitol, 1 M sodium-potassium phosphate pH 7.5, 0.5 M EDTA, 1% (vol/vol) 2-mercaptoethanol] at a concentration of 10 g cells (wet weight) to 30 mL digestion buffer to 50 mg of zymolyase. Cells were then incubated at 37ºC until most of cells have been converted to spheroplasts. The spheroplasts were washed with 1.2M sorbitol and suspended in suspension buffer (0,5 M Tris, pH 7.5, 1 mM EDTA) and lysed using a Douce homogenizer. Afterwards the suspensions were transferred to 2 ml microtubes and subjected to 3 cycles of low-speed/high speed centrifugation (30 min at 2700 rpm; 2 cycles of 15 min at 13300 rpm). The mitochondrial pellet was stored at -80ºC. Protein quantification of the mitochondrial extracts was performed using the Lowry method.

3.5. 2D-gel electrophoresis and immunoblot analysis

3.5.1. Isoelectric focusing

To separate proteins by isoelectric point (pI), 35 µg of mitochondrial proteins were suspended in a rehydration buffer [2M Urea, 2M thiourea, 1% Triton X-100 (vol/vol), 1% CHAPS (vol/vol), 0.4% DTT (wt/vol), 0.5% Pharmalyte (vol/vol), 2% ASB (wt/vol), Bromophenol Blue), incubated for 30 min at room temperature with shacking and used to rehydrate an immobiline drystrip (Immobiline™ DryStrip pH6-11, 7cm, GE Healthcare) overnight. Focusing (IEF BioRad Protean) of the strips, covered with mineral oil (Sigma), was performed, following the described settings: 1. Step and Hold, 300V, 30 min, 0.2 kVh; 2. Gradient, 1000V, 30 min, 0.3 kVh; 3. Gradient, 5000 V, 1h20min, 4.0 kVh; 4. Step and Hold, 5000 V, 25 min, 2.0 kVh.

3.5.2. Immunoblotting

After focusing, the strips were washed with dH_2O, reduced with 100 mM DTT in equilibrium buffer (6 M urea, 2 % sodium dodecyl sulphate (SDS), 0.1 mM EDTA, 0.01 % bromophenol blue, 50 mM Tris pH 6.8, 30 % glycerol, 100 ml H_2O final vol) for 30 min

at room temperature, and alkylated with 260 mM iodoacetamide in equilibrium buffer for 30 min at room temperature.

Then, the strips were washed with ddH$_2$O, and proteins were separated in a 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. After blocking with 5% low fat dry milk in TTBS (20 mM Tris, 140 mM NaCl, 0.05% (v/v) Tween-20 pH 7.6), membranes were probed overnight with the primary antibodies anti-por1 (1:6000, Invitrogen A6449) or anti-cytC (1:6000), washed and then incubated for 2h with the correspondent secondary antibody conjugated to horseradish peroxidase, anti-mouse (1:6000, Santa Cruz sc-2005) or anti-rabbit IgG light chain (1:7000, Sigma 9169).

To probe for phosphorylated proteins, membrane stripping was performed as described in the Abcam protocol. The membrane was washed with TTBS and incubated in a stripping buffer [62.5 mM Tris-HCl pH 6.8, 2% (wt/vol) SDS, 100 mM 2-mercaptoethanol] for 30 min at 50ºC. After that, the membrane was thoroughly washed, blocked with 5% bovine serum albumin in TTBS, and probed overnight with the primary antibody anti-phospho S/T/Y (1:3000, Abcam), followed by a 2h incubation with the correspondent secondary anti-mouse (1:3000, Invitrogen G21040). Immunodetection was performed by chemiluminescence, using a kit from GE Healthcare (RPN2109).

3.6. Hydrogen peroxide and heat sensitivity

For the analysis of hydrogen peroxide (H$_2$O$_2$) resistance, yeast cells were grown in YPD to PDS phase and treated with 100 mM H$_2$O$_2$ (Merck) for 1h. Cells viability was determined by standard dilution plate counts on YPD medium containing 2% (wt/vol) agar. Colonies were counted after growth at 26ºC for 3 days, and viability expressed as the percentage of the colony-forming units (CFUs) of treated cells versus non-treated with H$_2$O$_2$.

For the analysis of heat sensitivity, the same number of cells of each strain was plated in ten-fold serial dilutions into YPD medium containing 2% (wt/vol) agar 2%), and the plates were incubated at either 26ºC or 37ºC.

3.7. Oxygen consumption rate and growth in respiratory substrates

For the analysis of growth in respiratory substrate, the same number of cells of each strain was plated in ten-fold serial dilutions into two solid agar and glycerol plates.

The plates were composed of YPD or YPG medium containing 2% (wt/vol) agar, and the plates were incubated at 26ºC.

Oxygen consumption rate (OCR) was measured in whole cells (1×10^8 in PBS buffer) grown to an $OD_{600}=1$ and $OD_{600}=11$, using a Clark oxygen electrode. Cells were collected, ressuspended in 1 ml PBS buffer (137 mM NaCl pH 7.4, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.46 mM KH_2PO_4) and transferred to the water-jacketed microcell, magnetically stirred, at 26ºC. The OCR was measured for 2 min using an Oxygraph system (Hansatech) and data analysed with Oxyg32 V2.25 software (Hansatech).

3.8. Analysis of autophagy and mitophagy induction

Yeast cells were harvested by centrifugation for 3 min at 4,000 r.p.m. (4ºC), and ressuspended in 50 mM sodium potassium phosphate buffer (pH 7.4) containing protease inhibitors (Complete, Mini, EDTA free Protease Cocktail Inhibitor Tablets; Boehringer Mannhein) and 1% Triton X-100 (Sigma). Total protein extracts were obtained by mechanical disruption through vigorous shaking of the cell suspension in the presence of zirconium beads for 5 min. Short pulses of 1 min were applied followed by 1 min incubation on ice. Cell debris was removed by centrifugation at 13,000 r.p.m. for 12 min and protein content was determined by the Lowry method, using bovine serum albumin as a standard.

For the immunoblot analysis, 80 µg of the proteins were mixed with Laemmli buffer containing 1% 2-mercaptoethanol and heated at 95ºC for 5 min. The electrophoresis was performed on a 15% and 12.5% polyacrylamide gel, for GFP-ATG8 and OM45-GFP respectively. Immunoblotting was performed as described above, using mouse anti-green fluorescent protein (GFP) (1:4000, Roche) as primary antibody and anti-mouse (1:5000, Invitrogen G21040) as secondary antibody.

Chapter 4

**RESULTS AND
DISCUSSION**

4. RESULTS AND DISCUSSION

4.1. Identification of phosphorylated forms of Por1p

Since the aim of this project was to determine which kinases regulate the yeast Por1p by phosphorylation, it was first necessary to implement a screening assay. It was previously reported that phosphorylation leads to Por1p isoforms with distinct isoelectrical points (pI) (98).To confirm this result, mitochondria were isolated from cells grown to post-diauxic shift (PDS) phase – to induce respiratory phase conditions – and proteins were separated by 2D-gel electrophoresis followed by immunoblotting (2D-immunobloting) using an antibody against Por1p or cytochrome c (as a control). We observed Por1p exhibits three major forms at pI of approximately 8.5 (spot 1), 7.9 (spot 2) and 7.3 (spot 3) and one minor form with a pI of approximately 9 (Fig. 7). To determine if the growth phase affects Por1p pI forms, similar studies were performed using cells grown to log, PDS and stationary phase. Since the results showed no variation of the spots (Supplemental Figure S1), the next studies were performed at PDS phase as Por1p function is more relevant during this growth phase.

Proteins may be subjected to modifications that change the pI both to the acidic pole (e.g., ubiquitination, phosphorylation, acetylation) or to the basic pole (ex sumoylation). To identify Por1p phosphorylated forms, the membrane was reprobed with an anti-phospho-serine, -tyrosine and -threonine (anti-phospho S/T/Y) antibody. It was observed (Fig. 7) that the three main forms of Por1p (1-3) are phosphorylated. As such, alterations in the pI of Por1p were assessed by 2D-immunoblotting to screen for potential Por1p regulatory kinases.
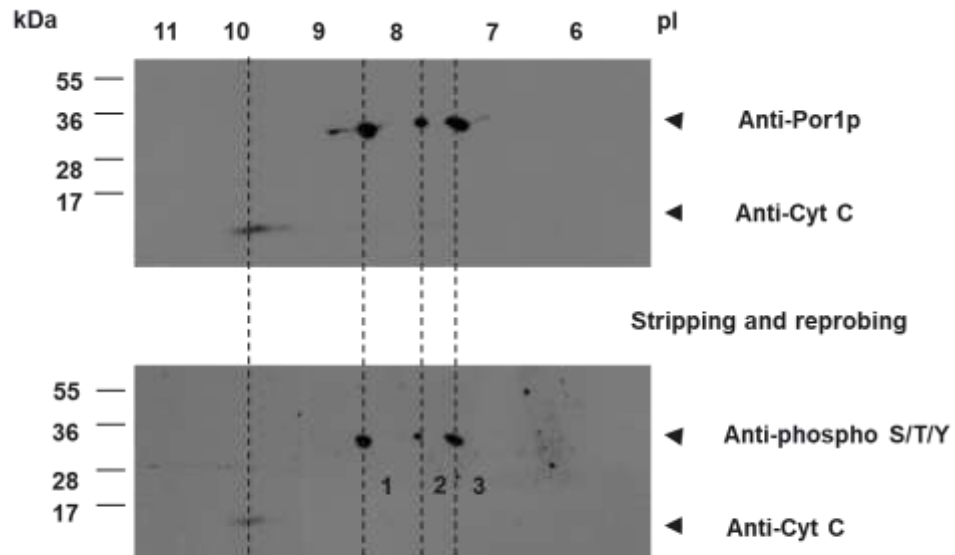
Figure 7: Analysis of Por1p phosphorylation: WT (BY4741) cells were grown to PDS phase in YPD medium. Protein phosphorylation was analysed by 2D-immunoblotting, using antibodies raised against Por1p, Cytochrome C (Cyt C; internal control for the first dimension). or anti-phospho S/T/Y. The 3 main Por1p forms were detected as phosphorylated and numbered 1-3 from the highest to the lowest pI.

4.2. Screening for kinases with a potential role in Por1p phosphorylation

Kinases evaluated as potential Por1p regulators were selected based on their mitochondrial localization (70,99,100) and their role in the regulation of mitochondrial function. This screening was based on the premise that, in the absence of a potential Por1p regulatory kinase, Por1p pI is altered (shift to the basic pole) due to the disappearance of the kinase-mediated phosphorylation. As such, 15 kinase-deleted mutants were tested and mitochondria isolated from cells grown to PDS phase (OD$_{600}$~14) samples analysed by 2D-immunobloting (Table 3).

Some kinases selected to this work, namely the catalytic subunits (Tpk1/2/3) of protein kinase A, Mck1p (homolog of mammalian GSK3β) and Yck1/Yck2 (homologues to CKI), have homologues that were previously implicated in mammalian VDAC regulation (62). Yet, in this work, no alteration in Por1p pI was observed in the absence of this kinases/catalytic subunits. Yet, for PKA and for CKI, because the tested mutants may have redundant functions it would be needed to assess Por1p phosphorylation in triple and double mutants, respectively, to exclude this kinases as Por1p regulators. Also, since we performed these studies in PDS phase, PKA might have not intervened, since it is mostly active during exponential phase (88). From the tested kinase-deleted

mutants, only *hog1Δ* and *rim15Δ* cells showed a shift in Por1p pI compatible with a lack of phosphorylation (shift to the acidic pole) (Table 3; Fig 8). Comparing with wild type cells, *hog1Δ* cells showed a shift in Por1p pI of spots 1 and 2, and *rim15Δ* cells showed a shift in Por1p pI of spot 1 (Fig. 8). For the remaining kinase-deleted strains, there were no differences in Por1p pI (Supplemental Fig. S2).

Table 3: Evaluation of Por1p pI shift by 2D-immunoblotting in indicated kinase-deleted strains.

| Kinase Name	Mutant	pI shift
cAMP-dependent protein kinase type 1	*tpk1Δ*	No
cAMP-dependent protein kinase type 2	*tpk2Δ*	No
cAMP-dependent protein kinase type 3	*tpk3Δ*	No
Casein kinase I homolog 1	*yck1Δ*	No
Casein kinase I homolog 2	*yck2Δ*	No
Casein kinase II subunit alpha 1	*cka1Δ*	No
Casein kinase II subunit alpha 2	*cka1Δ*	No
Probable serine/threonine-protein kinase KKQ8	*kkq8Δ*	No
Mitogen-activated protein kinase SLT2/MPK1	*slt2Δ*	No
Serine/threonine-protein kinase RIM15	*rim15Δ*	Yes
High osmolarity glycerol response protein 1	*hog1Δ*	Yes
Meiosis and centromere regulatory kinase 1	*mck1Δ*	No
Yeast protein kinase 1	*ypk1Δ*	No
Sucrose nonfermentating protein-activating kinase 1	*sak1Δ*	No
Serine/threonine-protein kinase SCH9	*sch9Δ*	No
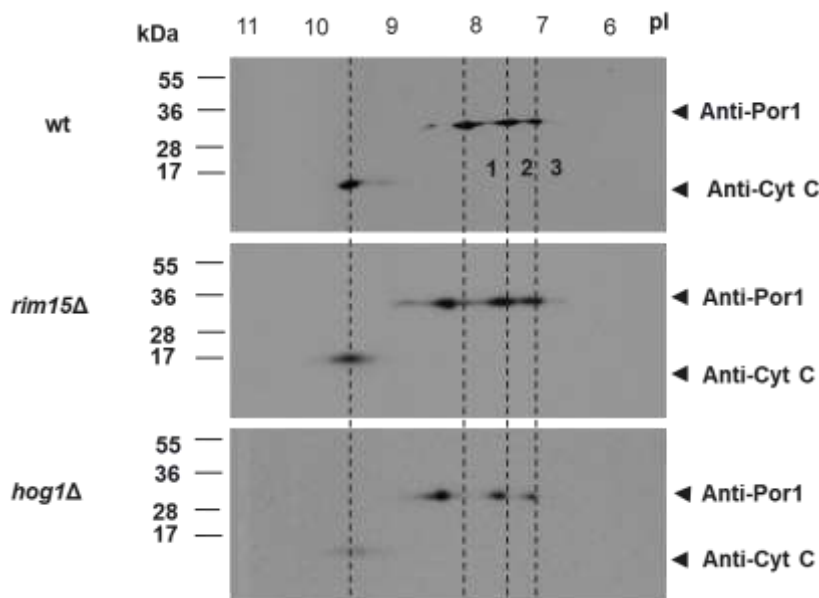
Figure 8: Modification of Por1p pI in isolated mitochondria from *rim15Δ* and *hog1Δ* mutants: wt, *rim15Δ* and *hog1Δ* mutants were grown to PDS phase, in YPD medium. Evaluation of Por1p pI was performed by 2D-immunoblotting.

To confirm that the Por1p pI alteration in *rim15Δ* and *hog1Δ* yeast cells was due to absence of phosphorylation and to identify the Por1p phosphorylated residues, mitochondrial fractions from wt and *rim15Δ* and *hog1Δ* cells were analysed by mass spectrometry LC-MS analysis, at the Proteomics Facility CBM-SO (Madrid). In wt cells, it was found that Por1p was phosphorylated in two aminoacid residues, T91 and T103, which were already described as phosphorylated sites (101). However, these phosphosites were also found in both *rim15Δ* and *hog1Δ* yeast cells, indicating that these residues are not regulated by these kinases. Additional mass spectrometry LC-MS analysis should be performed for the identification of additional residues that are phosphorylated in wt but not in the kinase-deleted mutants, which will indicate the possible regulated sites by these kinases.

4.3. Por1p and kinases Rim15p and Hog1p: Genetic interaction

4.3.1 Growth curve analysis

Some mutations in two genes produce a phenotype that is unexpected in comparison with the mutation's individual effect. These are divided in two types of interactions: negative interactions refer to a more severe fitness defect than expected and positive interactions refer to double mutants with a less severe fitness defect than expected (102). Because a genetic interaction, defined as a deviation from the expected phenotype when combining genetic mutations, can reveal functional relationships between genes, double mutant strains were constructed, namely *por1Δrim15Δ* and *por1Δhog1Δ* mutants, and several phenotypes (related with Por1p or Rim15/Hog1p function) were tested.

One of the phenotypes evaluated was cell growth in liquid glucose-medium. No significant growth defects were observed for *por1Δ*, *hog1Δ* and *rim15Δ* single mutants compared with wt, while the double mutant strains exhibited a significant growth defect (Fig. 9A). The growth defect was more evident at log phase (Fig. 9B). In the log phase, a significant growth defect was also observed for *por1Δ* mutant strain, which reflects the importance of Por1p for cellular growth, since its deletion has been shown to delay growth (23,103). These results show that absence of Hog1p and Rim15p worsen the

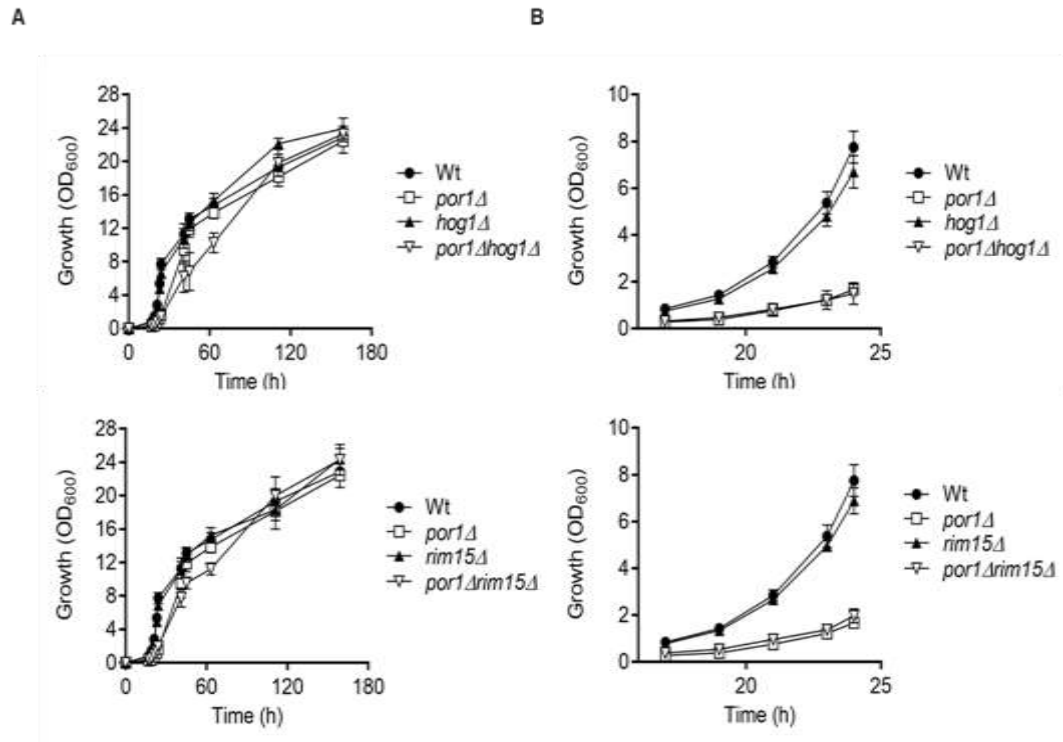growth defects of *por1Δ* at the respiratory phase indicating a negative interaction regarding this phenotype.

Figure 9: Analysis of cell growth. Growth of wt, *por1Δ*, *hog1Δ*, *rim15Δ*, *por1Δhog1Δ* and *por1Δrim15Δ* strains was monitored by OD_{600} measurements over time: (A) total growth untill early stationary phase; (B) log phase; Values are the mean ± SD (n=3). (A) $p < 0.001$ (wt vs *por1Δhog1Δ*), $p < 0.01$ (wt vs *por1Δrim15Δ*) (B) $p < 0.0001$ (wt vs *por1Δ*) $p < 0.0001$ (wt vs *por1Δrim15Δ*), $p < 0.0001$ (wt vs *por1Δhog1Δ*), two-way ANOVA.

4.3.2 Mitochondrial function: mitochondrial respiration and growth in a respiratory substrate

Regulation of Por1p opening can affect mitochondrial respiration, since Por1p is the major mediator of the flux of metabolites between the cytosol and the mitochondrial space, such as NADH and ADP essential for mitochondrial respiration (104,105). To evaluate if potential alterations in Por1p phosphorylation due to deletion of *RIM15* or *HOG1* affects mitochondrial function, mitochondrial respiratory capacity was evaluated both by analysing the growth of the strains in respiratory media and by measuring the mitochondria respiration. To evaluate the growth of the strains in respiratory media, the same number of cells was plated in serial dilutions in solid plates containing either glucose as a carbon source (control) or glycerol, a strictly respiratory medium.
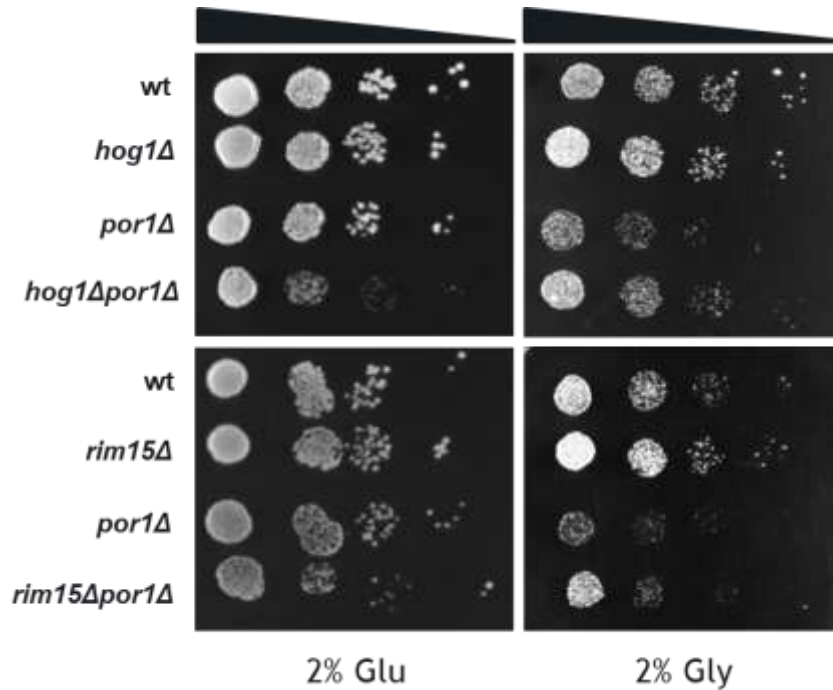
Figure 10: Growth assessment in strictly respiratory (glycerol) media. wt yeast (BY4741), the mutant strains *hog1Δ, rim15Δ* and *por1Δ*), or double mutants (*por1Δrim15Δ, por1Δhog1Δ*) were spotted in a ten-fold dilution series, on glycerol (Gly) and glucose (Glu)-based solid medium as control.

In glucose conditions, *por1Δ*, *hog1Δ* and *rim15Δ* mutant strains showed similar growth in comparison with wild type (Fig. 10). However, both *por1Δhog1Δ* and *por1Δrim15Δ* double mutants showed a decrease in growth, indicating a possible relationship between Hog1p and Rim15p and Por1p.

In respiratory conditions, it was observed that both *hog1Δ* and *rim15Δ* mutants showed an improved growth (Fig. 10). Since these kinases are described to have inhibitory activity towards respiration (70,106), it was expected to observe such increased growth. The *por1Δ* mutant strain showed a decreased growth in comparison with wt, as expected, since lack of Por1p reduces mitochondrial outer membrane (MOM) permeability to respiratory substrates (107). In the absence of Por1p, both *hog1Δ* and *rim15Δ* mutants exhibit comparable growth to wt in glycerol, indicating this effect is independent of Por1p.

Because respiratory activity correlates with VDAC1 opening *in vivo*, oxygen consumption rate (OCR) in whole cells grown to log and PDS phases was assessed. The OCR during log phase of single and double mutants was not significantly different from wt (Fig. 11A). OCR was evaluated at this early growth phase, in which cells are still

fermenting because we were expecting a catabolite de-repression and thus an increase in OCR for the *HOG1*-deleted mutant, as described (108), but that was not verified in our working conditions. Though there is a tendency for a higher OCR, it was not statistically significant.
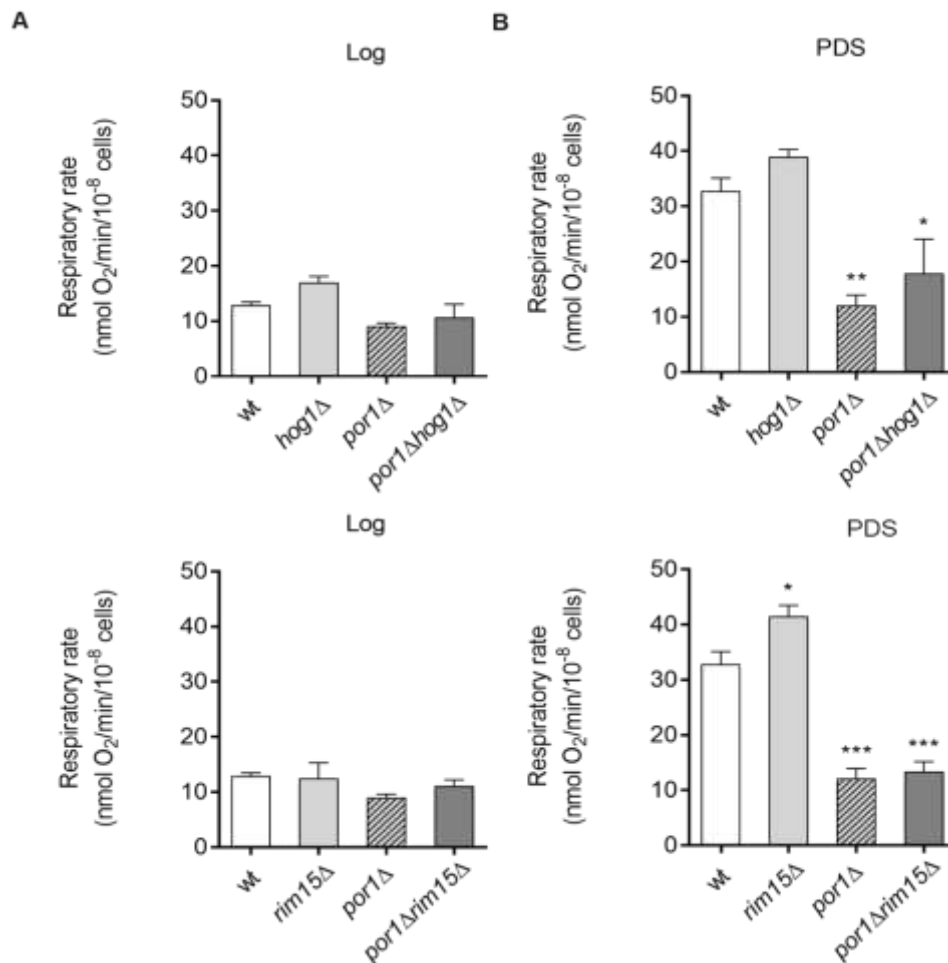

Figure 11: Determination of oxygen consumption rate: Oxygen consumption rate was measured in BY4741(wt), *por1Δ*, *rim15Δ*, *hog1Δ*, *por1Δrim15Δ* and *por1Δhog1Δ* strains grown to (A) at mid-log and (B) post-diauxic phase. Values are mean ± SD (n=3); **$p < 0.01$ (wt vs *por1Δ*) *$p < 0.1$ (wt vs *hog1Δ*), *$p < 0.1$ (wt vs *por1Δhog1Δ*), *$p < 0.1$ (wt vs *rim15Δ*), ****$p < 0.001$ (Wt vs *por1Δrim15Δ*).

In PDS phase, OCR increased comparing to log phase for the wt strain, but not for *por1Δ* cells, as expected, due to the role of Por1p in mitochondrial respiration (109). We observed a similar increase of OCR in *hog1Δ* mutant, indicating Hog1p does not play a significant role on mitochondrial respiration. Because Por1p is important for OCR, it also suggests Hog1p does not play a significant role in possible Por1p opening. For *rim15Δ*, there was a significant increase in OCR at PDS phase, comparing to the wt, as expected, since Rim15p is reported to play an important role in the suppression of

mitochondrial respiration (110). However, OCR in *por1Δrim15Δ* cells was similar to *por1Δ* single mutants, indicating that the increase of OCR in *RIM15* deleted cells is Por1p-dependent and, therefore, Rim15p may play a role in regulating Por1p channel activity (Fig. 11B).

4.3.3 Autophagy and mitophagy induction

Because mitochondrial function and both Rim15p and Hog1p play a role in autophagy and mitophagy regulation (84,11,12,111), autophagy induction during stationary phase was also analysed since autophagy regulation is crucial for maintaining normal cell functions and also promoting cell survivability (112,113).

ATG8 has been identified as one of the autophagic genes at the base of the autophagosome assembly, from the pre-autophagosome formation until its delivery to the vacuole (114). Atg8p is an ubiquitin-like protein that is part of the autophagosomal structure, being cleaved by Atg4p, essential for the biogenesis of the autophagosome. Atg8p is also present during the course of the whole autophagic process, designated for autophagy flux, or the process that leads from the autophagosome formation and maturation, the autolysome formation and later degradation (115). Thus, autophagy can be assessed in cells expressing a fusion of GFP-Atg8. This autophagosome, that contains the cargo, is transported to the vacuole (yeast lysosome), where it suffers degradation by vacuolar hydrolases. In the end, the resulting products of degradation are released back to the cytosol for recycling. GFP is fused in the N-terminal of Atg8p and is resistant to the vacuolar degradation, unlike Atg8p, as such, when the autophagosome is delivered to the vacuole it will result in free GFP in the vacuolar lumen and detection of free GFP can be used as a measure of autophagy flux (115,116). It is known that autophagy can be regulated at the transcriptional level through *ATG8* induction, which can be monitored by quantifying total GFP (GFP-Atg8 + free GFP), and at the flux level, assessed by monitoring Atg8p degradation levels (ratio of free GFP over total GFP).

Using this assay, we observed an induction of autophagy in wt cells grown to stationary phase (Fig. 12A; quantification in 12B-C), as expected. In the *por1Δ* mutant, it was observed a strong suppression of autophagy induction (but not flux) (Fig 12). According to literature (117), impaired mitochondrial function may result in low autophagy induction and flux, which might explain the results in *por1Δ* strain (Fig. 12B-C). The *rim15Δ* mutant strain also showed low levels (suppression) of autophagy induction (Fig.12B). This result is consistent with reports stating that Rim15p upregulates

autophagy in starvation conditions (92,11), which in turn promotes degradation and recycling of cytoplasmic components for ATP recovery and cell survival. The autophagic flux also decreased in *rim15Δ* mutants, although not leading to suppression (Fig. 12C). This might be due to the regulation exerted by Rim15p in the process of autophagy flux, where its role is still not well defined (94,11). The double mutant strain *por1Δrim15Δ* showed a recovery in both autophagy induction and flux (Fig. 12B-C), suggesting that the presence of both Por1p and Rim15p is important for normal regulation of autophagy and there might be a compensatory mechanism in the absence of these two proteins. The *hog1Δ* mutant strain showed low levels of Atg8p, indicating defects in autophagy induction. Hog1p has been described as participating in the phosphorylation and stabilization of Atg8p, collaborating in the formation of autophagosomes (118,119), hence, being a crucial protein kinase in the regulation of autophagy. Our results corroborate this fact (Fig. 12B). In contrast, the autophagic flux was not affected in the *hog1Δ* mutant strain, suggesting that Hog1p is not relevant for the degradation process (Fig. 12C). The *por1Δhog1Δ* mutant strain showed a severe reduction in autophagic induction, accumulating the negative interaction of the absence of both Por1p and Hog1p. In contrast, the autophagic flux was similar to that of both *por1Δ* and *hog1Δ* mutant strains, indicating that both mutants may be reducing the autophagyc flucx by affecting the same process.
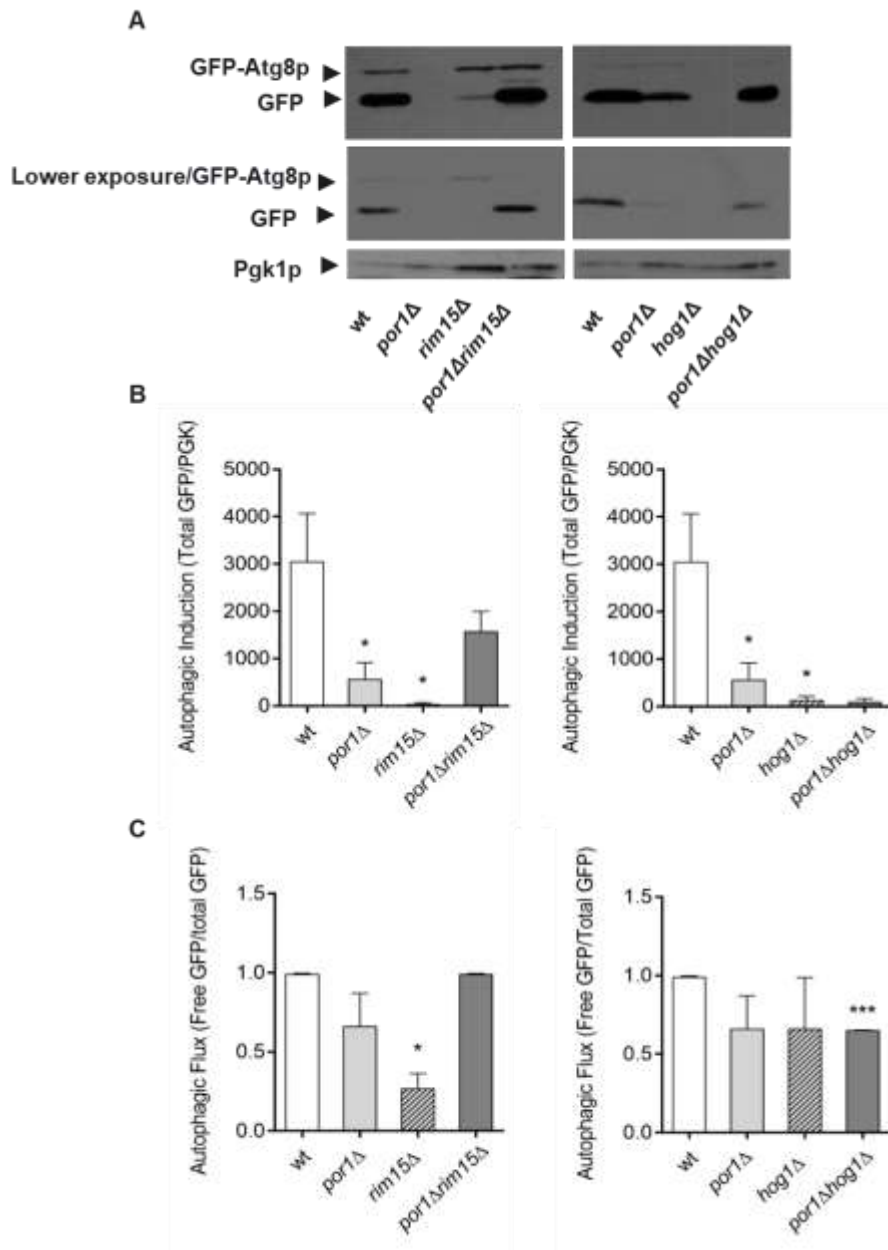
Fig.12: Analysis of autophagy induction and flux: (A) GFP-Atg8 immunoblots of cells grown in YPD, harvested 3 days after PDS phase. (B) The autophagy induction was calculated by the ratio between the sum of free GFP and GFP-Atg8p signals and Pgk1 (loading control). Values are mean ± SD (n=3); *$p < 0.1$ (wt vs *por1Δ*) *$p < 0.1$ (wt vs *rim15Δ*) *$p < 0.1$ (wt vs *hog1Δ*), one-way ANOVA. (C) The autophagic flux was calculated as the ratio between the free GFP signal and the sum of free GFP and GFP-Atg8p signals. Values are mean ± SD (n=3) *$p < 0.1$ (wt vs *rim15Δ*) ****$p < 0.001$ (wt vs *hog1Δ*), one-way ANOVA.

Accumulation of dysfunctional mitochondria can lead to a compromised cell growth and even cell death (10). An effective way to control this accumulation and ensure quality control over mitochondria is mitophagy, a type of selective autophagy, where severely damaged mitochondria are eliminated.

Mitophagy was analysed using an assay similar to the used for autophagy, except that a mitochondrial outer membrane protein (OM45) (12) fused to GFP was used as a reporter of mitochondrial degradation, and mitophagy induction was estimated by measuring the ratio of free GFP (reflecting degraded OM45) over total GFP.

It was observed an induction of mitophagy in wt cells grown to stationary phase (Fig. 13), as expected. In the *por1Δ* mutant strain, though not significantly, an increased induction of mitophagy was observed (Fig. 13). This increase may suggest that the lack of Por1p compromises the normal mitochondrial function, since ATP/ADP flow is affected, although there may be compensatory proteins that promote cell survival (Fig. 13). The *rim15Δ* mutant strain showed similar level of mitophagy induction when compared to wt (Fig. 13). Rim15p is indeed important for non-selective autophagy regulation but there are no reports implicating Rim15p role in mitophagy, although it is known that, indirectly, it may promote mitophagy induction factors (120). The *por1Δrim15Δ* double mutant showed a major decrease in mitophagy induction (Fig.13), compared both to wt and *por1Δ* mutant strain, indicating Rim15p is involved in *por1Δ* mitophagy induction. In *hog1Δ* mutants, it was expected a low level of mitophagy induction, since some reports state that Hog1p is an important mitophagy regulator. The absence of Hog1p leads to a suppression of CK2-dependent Atg32p phosphorylation, which is required for mitophagy induction (84,12). The double mutant *por1Δhog1Δ* also showed a low level of mitophagy induction, compared to both wt and *por1Δ* mutant cells, which might indicate, as with *por1Δrim15Δ* mutant strain, a negative interaction for this phenotype (Fig. 13). These results suggest that *por1Δ*-induced mitophagy is dependent of the presence of Rim15p and Hog1p, though this effect may be direct or not.
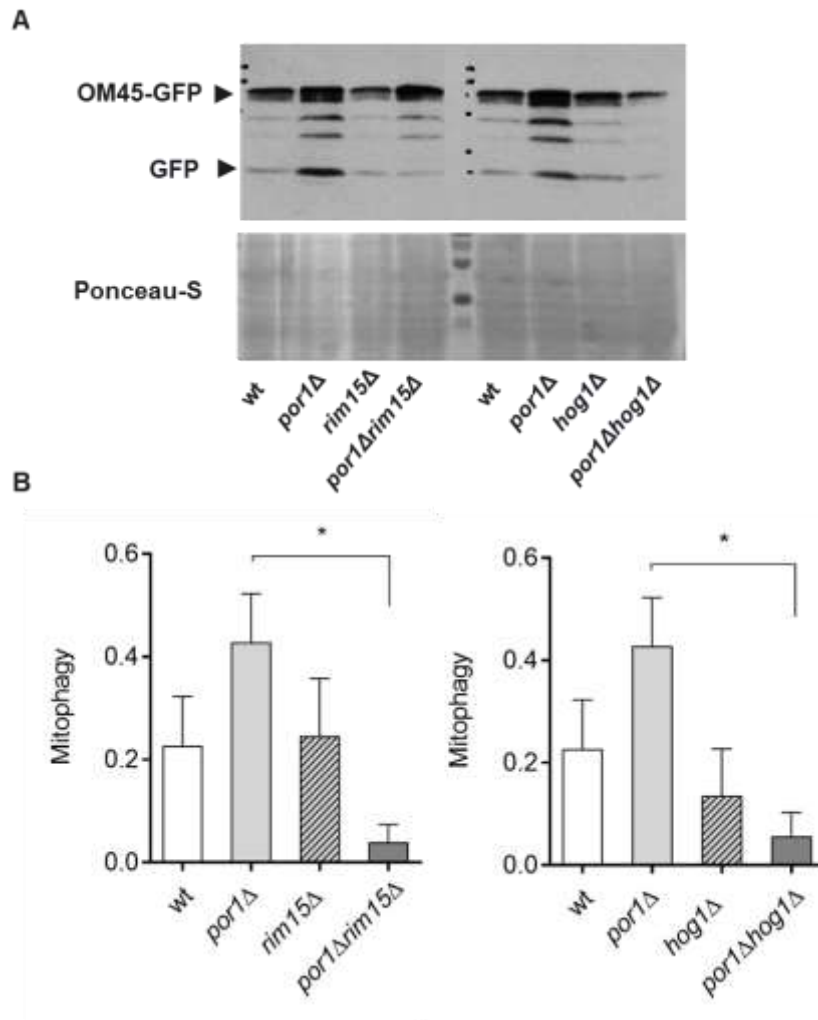
Fig.13: Analysis of mitophagy induction: (A) OM45-GFP immunoblots of cells grown in YPD, harvested 3 days post-PDS phase. (B) The mitophagy induction was calculated by the ratio between the free GFP and the sum of free GFP and OM45-GFP signals (Free GFP/total GFP), using a Ponceau stained membrane as a loading control. Values are the mean ± SD (n=3)), $p < 0.1$ (*por1Δ* vs *por1Δrim15Δ*), one-way ANOVA.

4.3.4 Stress resistance

Because Por1p affects heat sensitivity (121), the response of the mutants under study to elevated temperatures was evaluated by growing the cells in YPD solid media at 37ºC. It was observed that cells lacking Por1p showed a strong growth defect at 37ºC (Fig. 14), corroborating the described role of Por1p as an important protein in thermoresistance. Since loss of Por1p leads to a decrease of MOM permeability, the low exchange of metabolites compromise mitochondrial functions leading to an increased

stress sensitivity. Though Rim15p and Hog1p are important regulators of cellular responses to osmotic stress, oxidative stress or others (110), our results suggest that these kinases do not exert an important role at 37ºC, since both *hog1Δ* and *rim15Δ* mutants showed no differences in growth. The double mutants *por1Δhog1Δ* and *por1Δrim15Δ* exhibited a phenotype similar to *por1Δ* single mutants, indicating no interaction between these proteins regarding growth at 37ºC.
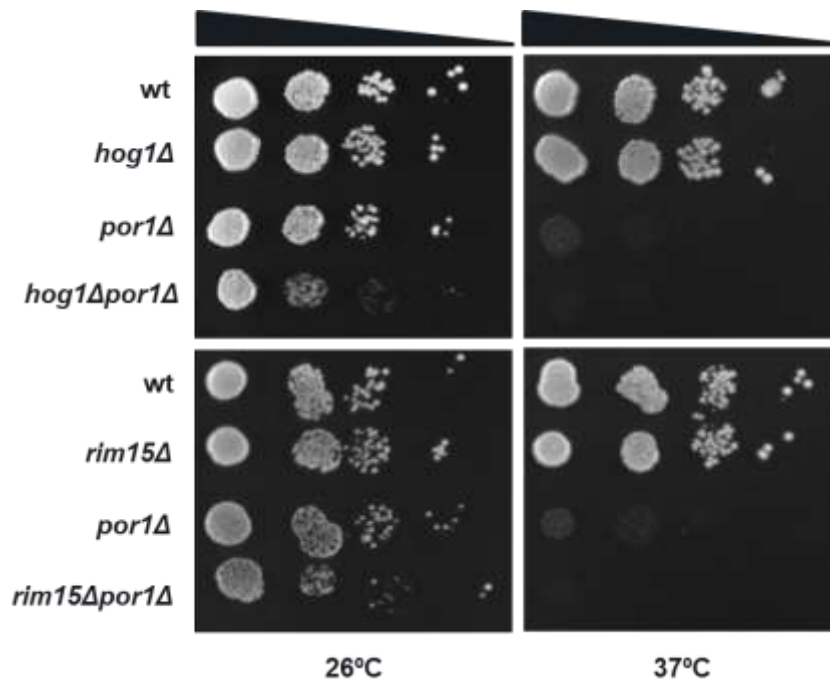

Figure 14: Growth assessment with heat shock: wt yeast (BY4741), the kinase-deleted strains (*hog1Δ, rim15Δ*) and *por1Δ* mutant strain, and both double mutants (*por1Δrim15Δ, por1Δhog1Δ*) were spotted in a ten-fold dilution series, on glucose-based medium and incubated at the indicated temperatures: room temperature (26ºC) and 37ºC.

The strains were also assayed regarding their resistance to H_2O_2, an oxidative stress inducer. The mutant *por1Δ* was sensitive to H_2O_2 comparing with wt (Fig. 15), as reported before (41). The *hog1Δ* mutant also exhibited a decreased resistance to H_2O_2, (Fig. 15) that was not increased by the deletion of *POR1*, suggesting these proteins may be in the same pathway concerning stress resistance. *RIM15*-deleted mutant also showed high sensitivity to H_2O_2, indicating an important role for this kinase in stress resistance (Fig.15). This sensitivity was increased by the additional deletion of *POR1* suggesting Rim15p and Por1p may be in parallel pathways concerning stress resistance.
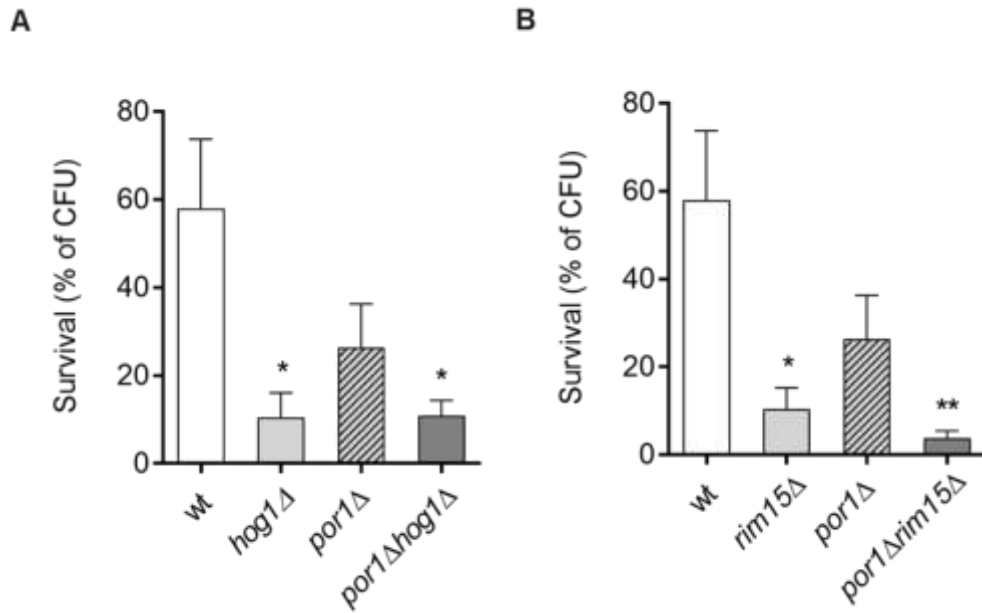
Figure 15: Survival of wt and mutant strains to H_2O_2 treatment: Cells were grown to PDS phase and treated with H_2O_2 (100 mM, 1h) and viability assessed by CFU count. Values are mean ± SD (n=3). * $p < 0.05$ (wt vs *hog1Δ*; wt vs *rim15Δ*; wt vs *por1Δhog1Δ*), ** $p < 0.01$ (wt vs *por1Δrim15Δ*), one-way ANOVA.

Chapter 5

**CONCLUSIONS AND FUTURE
PERSPECTIVES**

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The voltage-dependent anion channel (VDAC) is a mitochondrial protein with an essential role in the mitochondrial metabolism. In mammalian cells, it is known that VDAC can be regulated by post-translational modifications such as phosphorylation and some regulatory kinases have been identified. Yet, in yeast though VDAC/Por1p is reported as phosphorylated, the role of these modifications and its regulatory kinases are unknown. As such, this dissertation aimed to identify Por1p regulatory kinases. For that, 15 kinase-deleted mutants (selected based on kinase function or mitochondrial location) were screened for alterations in Por1p pI by 2D immunoblot. Two Ser/Thr protein kinases, Rim15p and Hog1p, were identified to cause alterations in Por1p pI when deleted. The assessment of genetic interactions between these kinases and Por1p support that a functional relationship may exist between these proteins. It was observed that deletion of *HOG1* interferes with *por1Δ* growth in fermentative conditions, mitophagy induction and oxidative stress resistance, whereas deletion of *RIM15* interferes with *por1Δ* growth in fermentative conditions, mitochondrial oxygen consumption, autophagy flux and induction and mitophagy induction.

Taking in account that both kinases affect Por1p pI and they exhibit genetic interaction with Por1p, our data suggests that these kinases may be Por1p regulators. If the identified kinase directly regulates Por1p, they likely phosphorylate different Por1p residues because some of the phenotypes affected are different. While in mammals VDAC phosphorylation has been associated mainly to apoptosis induction, the distinct phenotypes affected by lack of VDAC1/Por1p in yeast suggest regulation of this channel may impact additional cellular processes, such as autophagy and mitophagy induction, also critical for cell survival. Rim15p has no close homologue in mammals, but Hog1p has two homologues in mammals (p38/JNK) that have been implicated in VDAC1 regulation, suggesting VDAC1 regulation by Hog1p may be conserved.

To confirm that Hog1p and Rim15p directly interact and phosphorylate Por1p, co-immunoprecipitation and *in vitro* kinase assays should be performed. In future work, Por1p MS/MS analysis should be repeated, to identify the Por1p phosphorylated residues by the kinases Hog1p and Rim15p. With that knowledge, it will be possible to study the physiological role of Por1p phosphorylation by mutating the individual residues into nonphosphorylatable (alanine) or phosphomimetic (aspartic acid) versions. Through the evaluation of mitochondrial function, membrane potential, communication cell-mitochondria and even apoptotic activity will also be crucial to better understand the level of Por1p regulation possibly promoted by these potential regulatory kinases.

Por1p plays a central role in mitochondrial metabolism affecting crucial processes as proliferation, and oxidative stress resistance. Because VDAC is conserved, identifying its regulatory kinases can provide new pharmaceutical targets for treatment of human diseases such as cancer and Alzheimer's disease in which VDAC1 activity has been implicated (43,52,53).

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**SUPPLEMENTARY
MATERIAL**

7. SUPPLEMENTARY MATERIAL
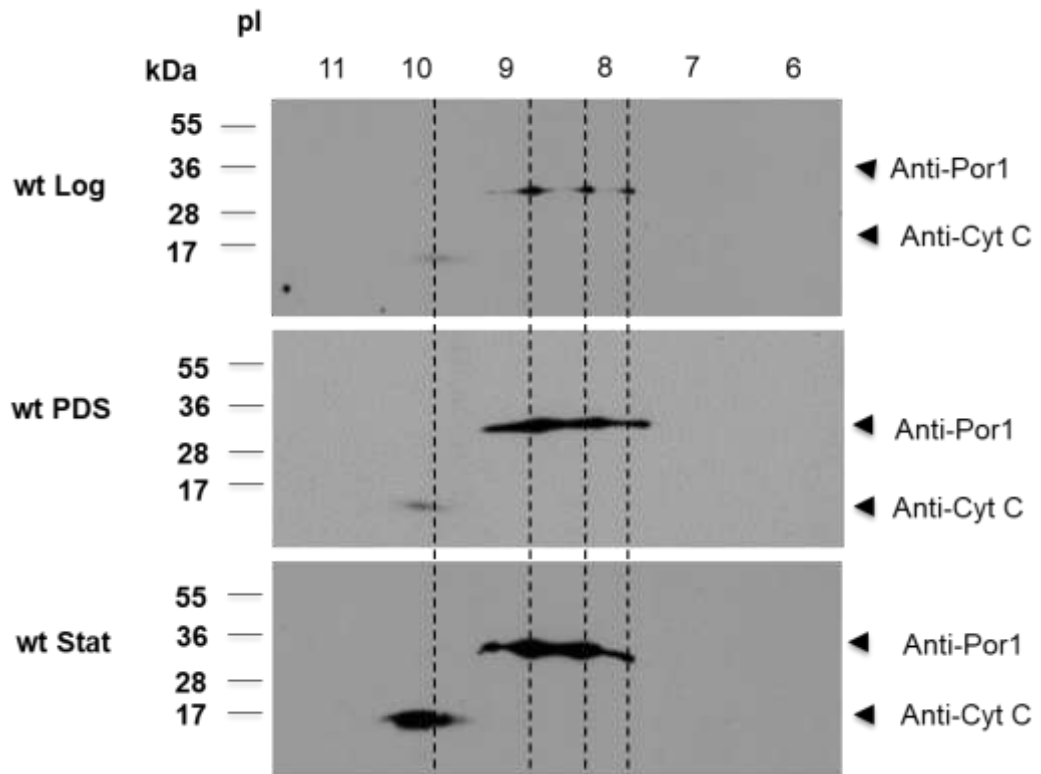
## Analysis of Por1p pI in cells at different growth phases

Figure S1: Analysis of Por1p pI in cells at different growth phases. Mitochondria were isolated from wt cultures grown until log, PDS and stationary phase (OD_{600}= 0.2; OD_{600}= 9.83; OD_{600}= 16.41). Analysis of Por1p pI was performed by 2D-imunoblotting, with antibodies anti-Por1p and anti-cyt C, as internal control of the 1st dimension.

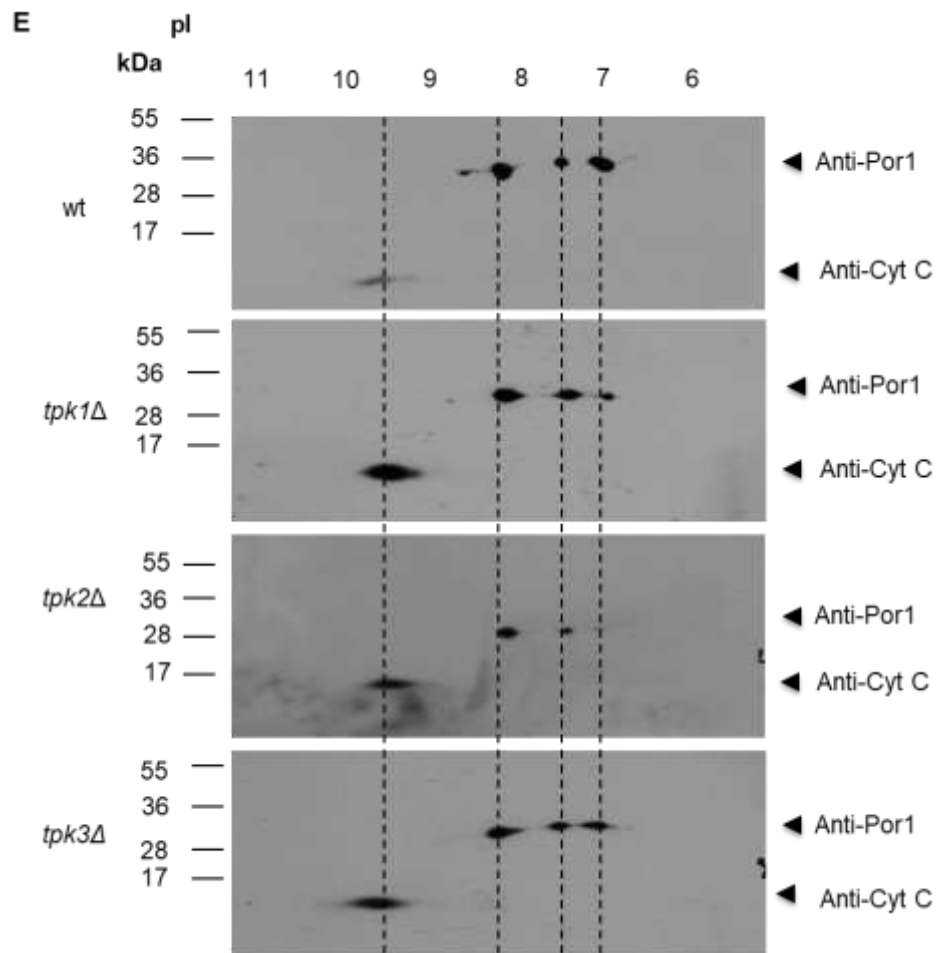**Analysis of Por1p pI in different kinase-deleted strains**
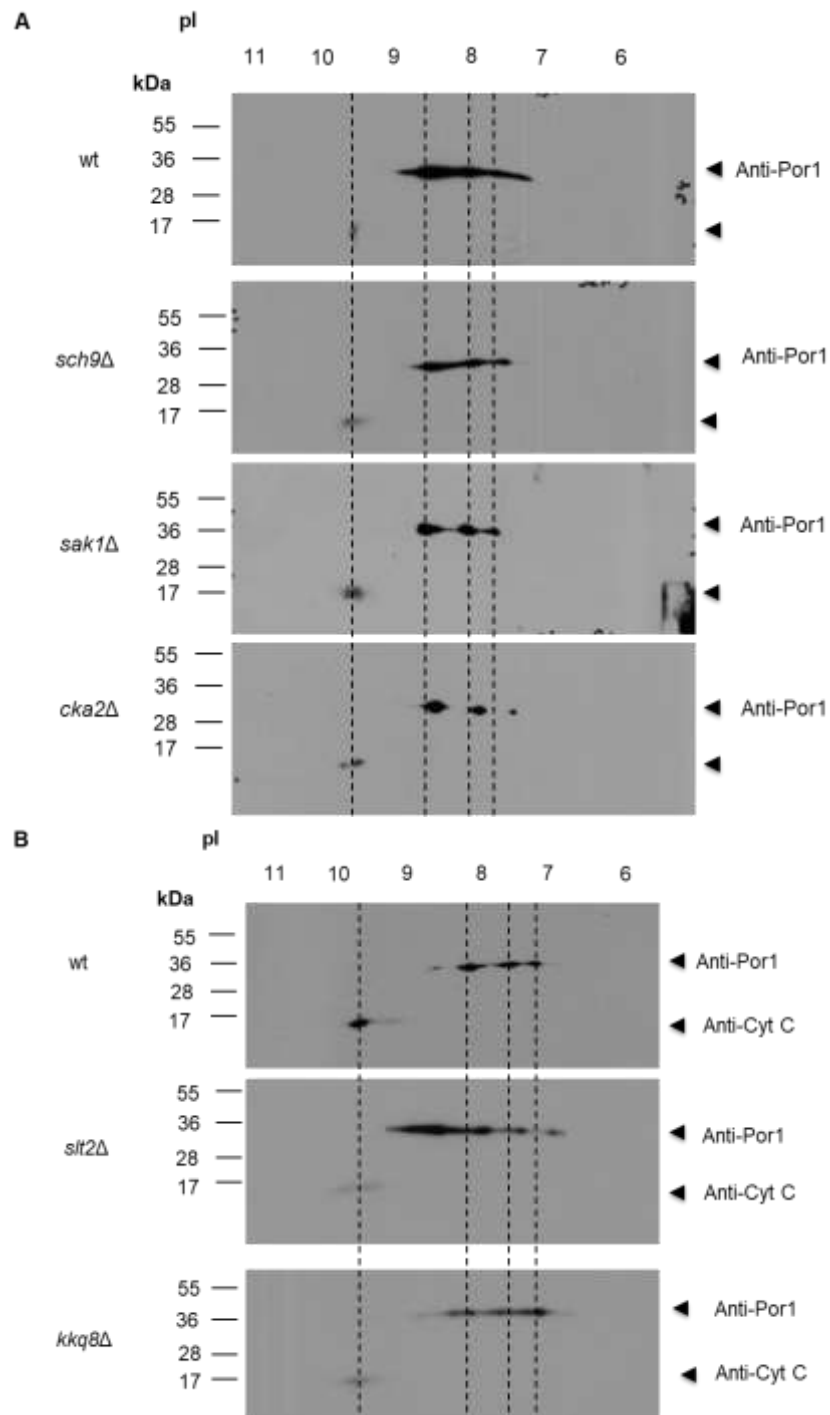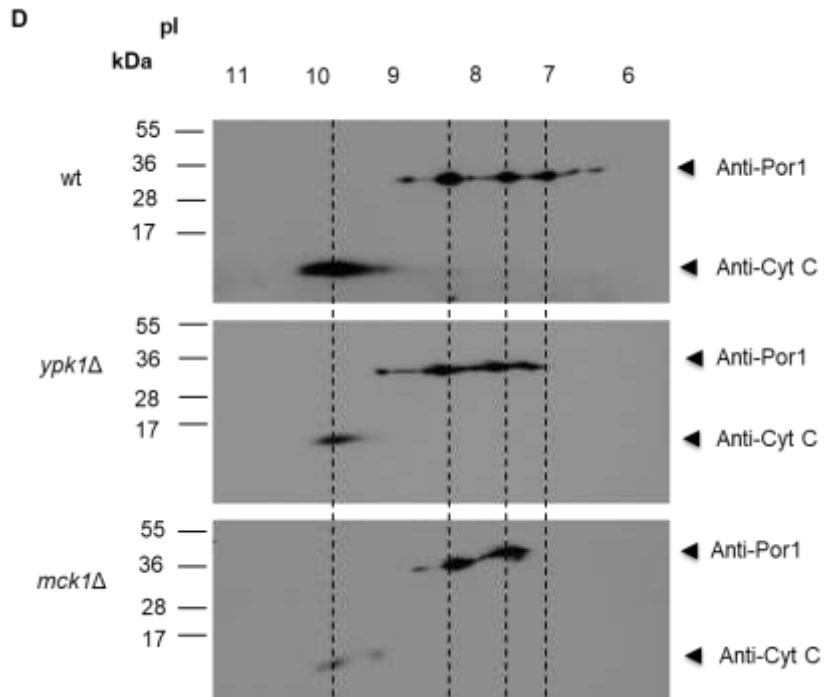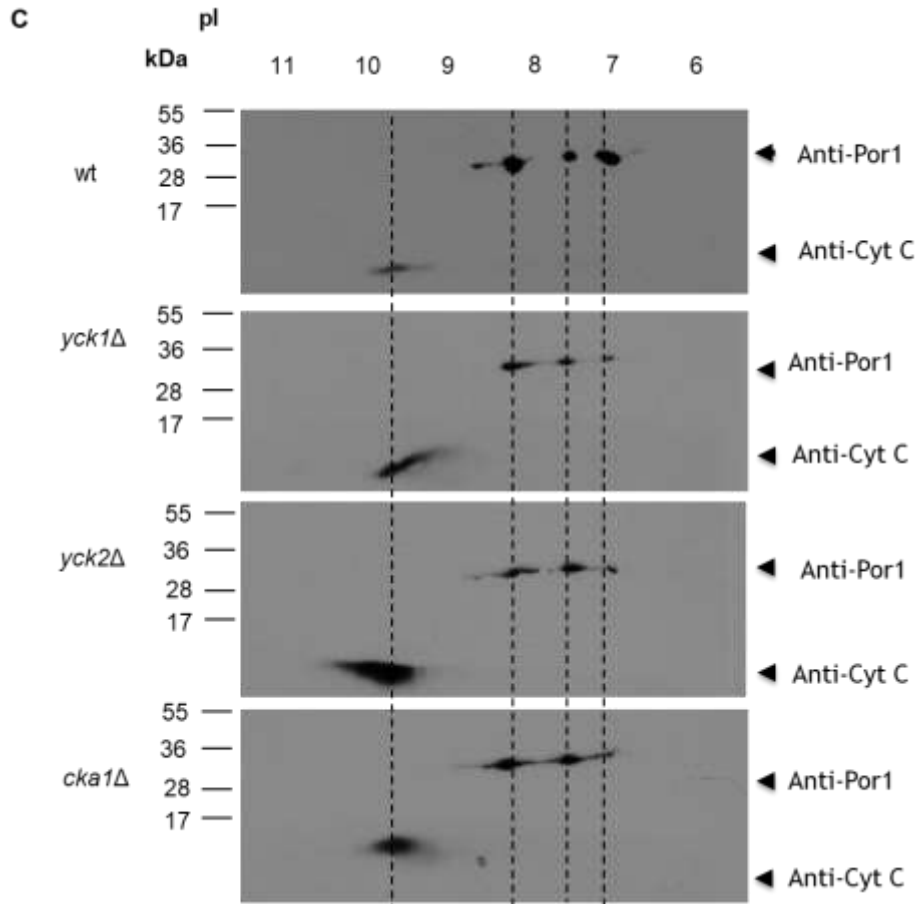
Figure S2: Mitochondria from PDS grown cells were isolated from wt and selected kinase-deleted strains. Analysis of Por1p pI was performed by 2D-imunoblotting, with antibodies anti-Por1p and anti-cyt C, as internal control, in selected kinase-deleted strains: (A) *sch9Δ*, *sak1Δ* and *cka2Δ* mutant strains (B) *slt2Δ* and *kkq8Δ* mutant strains (C) *yck1Δ*, *yck2Δ* and *cka1Δ* mutant strains (D) *ypk1Δ* and *mck1Δ* mutant strains (E) *tpk1Δ*, *tpk2Δ* and *tpk3Δ* mutant strains compared with wt harvested simultaneously.