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PROTEIN KINASES AND PHOSPHATASES REGULATING THE YEAST PROTON PUMP

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“Allah loves that whenever any of you does something, he should excel in it.” Prophet Muhammad

TO MY MOM (NAHED HUSSEIN)

Acknowledgment

I love reading, one of my biggest joys ever is when I spend time with a good book or novel, and I believe it was not a coincidence that the book I was carrying with me during my first flight from Egypt to Spain was Alchemist, by Paulo Coelho. Ironically, the main character of the novel was also travelling between Egypt and Spain but he was in the opposite direction of mine. One of the biggest inspirational phrases of this amazing writer was "When I have been truly searching for my treasure, I've discovered things along the way that I never would have seen had I not had the courage to try things that seemed impossible for a shepherd to achieve". The truth is what starts out as a journey to accomplish my aim turned into a discovery of the treasure found within the journey itself. It is all about the journey and what a journey it was! I am so grateful to many people and I hope I won't forget any of them.

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SUMMARY

The plasma membrane H^+-ATPase (Pma1) is essential for yeast growth and is activated by glucose metabolism by an unknown mechanism involving double phosphorylation of a regulatory site at the C-terminus (Ser911 Thr912). In this thesis we have investigated in *Saccharomyces cerevisiae* the role of two protein phosphatases, type 1 Glc7 and type 2A Sit4, and of an essential atypical protein kinase, TORC1, in the activation of Pma1 by glucose. The regulatory site of activated Pma1 can be dephosphorylated "in vitro" by recombinant Glc7 and Sit4, but inhibition "in vivo" of these phosphatases does not activate Pma1. Inhibition of Glc7 by regulated expression of a dominant-negative truncated form (the null mutant is not viable) had no effect on Pma1 activity while deletion of *SIT4* gene decreased both Pma1 activity and double phosphorylation of the regulatory site. Inhibition of TORC1 protein kinase by treatment of yeast cells with the drug rapamycin or by exposure to non-permissive temperature of a temperature-sensitive mutant (*tor1Δ tor2*^{ts}) inhibited Pma1 and decreased double phosphorylation of the regulatory site. We conclude that Sit4 and TORC1 are required for full activation of Pma1 by glucose while Glc7 either does not participate or is redundant with other phosphatases.

RESUMEN

La H^+-ATPasa de la membrana plasmática (Pma1) es esencial para el crecimiento de la levadura y se activa por metabolismo de glucosa por un mecanismo desconocido que lleva consigo la doble fosforilación de un sitio regulador en el extremo C-terminal (Ser911 Thr912). En la presente tesis hemos investigado en *Saccharomyces cerevisiae* la participación de dos proteína fosfatasas, Glc7 de tipo 1 y Sit4 de tipo 2A, y de una proteína kinasa atípica esencial, TORC1, en la activación de Pma1 por glucosa. El sitio regulador de Pma1 en su estado activo puede defosforilarse "in vitro" por Glc7 y Sit4 recombinantes pero la inhibición "in vivo" de estas fosfatasas no activa Pma1. La inhibición de Glc7 mediante la expresión regulada de una forma truncada que actúa como dominante-negativa (el mutante nulo no es viable) no tiene efecto en la actividad de Pma1 mientras que la deleción del gen *SIT4* disminuye tanto la actividad de Pma1 como la doble fosforilación del sitio regulador. Inhibición de la proteína kinasa TORC1 mediante tratamiento de las células de levadura con el fármaco rapamicina o exponiéndolas a temperatura no permisiva en el caso de un mutante termosensible (*tor1Δ tor2*^{ts}) resulta en inhibición de Pma1 y disminución de la doble fosforilación del sitio regulador. Estos resultados indican que Sit4 y TORC1 son necesarias para la máxima activación de Pma1 por glucosa mientras que Glc7 podría no participar o hacerlo de forma redundante con otras fosfatasas.

RESUM

L'H^+-ATPasa de la membrana plasmàtica (Pma1) és essencial per al creixement dels llevats i s'activa gràcies al metabolisme de glucosa per un mecanisme desconegut que porta associat la doble fosforilació d'una regió reguladora a l'extrem C-terminal (Ser911 Thr912). En aquesta tesi hem investigat en *Saccharomyces cerevisiae* la participació de dos proteïnes fosfatases, Glc7 de tipus 1 i Sit4 de tipus 2A, i d'una proteïna quinasa essencial atípica, TORC1, en l'activació de Pma1 per glucosa. La regió reguladora de Pma1, en seu estat activat, pot desfosforar-se "in vitro" per Glc7 i Sit4 recombinants, però la inhibició "in vivo" d'aquestes fosfatases no activa Pma1. La inhibició de Glc7 mitjançant l'expressió regulada d'una forma truncada que actua com a dominant-negativa (el mutant nul no és viable) no té cap efecte en l'activitat de Pma1 mentre que la deleció del gen *SIT4* disminueix tant l'activitat de Pma1 com la doble fosforilació de la regió reguladora. La inhibició de la proteïna quinasa TORC mitjançant un tractament de cèl·lules de llevat amb el fàrmac rapamicina o la seua exposició a temperatures no permissives en el cas d'un mutant termosensible (*tor1Δ tor2*^{ts}) resulta en la inhibició de Pma1 i la disminució de la doble fosforilació de la regió reguladora. Aquests resultats indiquen que Sit4 i TORC1 són necessàries per a l'activació màxima de Pma1 per glucosa, mentre que Glc7 podria no participar o fer-ho d'una forma redundant amb altres fosfatases.

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Abbreviation list:

BSA: Bovine Serum Albumine
DTT: Dithiothreitol
eIF2α: Eukaryotic initiation factor 2α (Sui2)
FW: Fresh weight
GLC7': truncated *GLC7*
GST: glutathione S-transferase
GTED 20: 20% Glycerol, Tris-HCl pH 7.6, EDTA, DTT buffer
IPTG: Isopropyl β-D-1-thiogalactoside
MCS: Multi Cloning Site
MES: 2-(N-morpholino) ethane sulphonic acid
MOPS: 3-(N-morpholino) propane sulphonic acid
OD: optical density (absorbance, extinction)
PCl: Phenol/Chloroform/Isoamyl alcohol (25:24:1)
PK: Protein kinase
Pma1: Plasma membrane H^+-ATPase from *Saccharomyces cerevisiae*
Protein phosphatase: PP
PMSF: Phenylmethylsulfonyl fluoride
RT- PCR: Reverse transcriptase - polymerase chain reaction
S. cerevisiae: Saccharomyces cerevisiae
SCD: Synthetic Complete Dextrose medium
SD: Synthetic Dextrose medium
SDS-PAGE: Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
SDS: Sodium Dodecyl Sulphate
TAE: Tris-acetate-EDTA buffer
TBE: Tris borate EDTA
TBS-T: Tris-buffered saline-Tween 20
TBS: Tris-buffered saline
TCA: Trichloroacetic acid
TE: Tris EDTA buffer
TEMED: Tetramethylethylenediamine
TOR: Target of rapamycin
TORC1: Target of rapamycin complex 1
X-Gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside
YNB: Yeast Nitrogen Base
YPD: Yeast Peptone Dextrose medium
α-pS899: antibody against phosphorylated serine 899
α-pST: antibody against double phosphorylation of serine 911 and threonine 912

INTRODUCTION

INTRODUCTION

Plasma membrane H^+-ATPase (Pma1) is an extremely important enzyme for yeast cell growth and metabolism and on the other hand it is a very expensive one in terms of ATP consumption, which makes its regulation a vitally important subject. Until now complete understanding of Pma1 regulation is not accomplished and the systems that modulate the activity of Pma1 remain largely unknown. Knowing that almost all eukaryotic proteins are regulated by phosphatases and kinases, in this thesis we have tested two major protein phosphatases and one protein kinase of yeast cells for their possible role in regulation of Pma1.

Plasma membrane H^+-ATPase

The yeast plasma membrane H^+-ATPase (in the following "Pma1") is a proton pump that plays a crucial role in the physiology of *Saccharomyces cerevisiae* (in the following "yeast") (Serrano, 1985). It is encoded by *PMA1* gene (YGL008c) and composed of a polypeptide of about 100 kDa (Serrano, 1985; Serrano *et al*., 1986). There is another Pma1 gene, *PMA2,* but it is expressed at very low levels and is not essential for growth (Schlesser *et al*., 1988). As indicated in Figure 1, Pma1 energizes the plasma membrane by generating an electrical membrane potential (positive outside) and a pH gradient (acid outside) to drive active nutrient influx and sodium efflux. It also regulates intracellular (pHi) and extracellular pH for yeast growth and external acidification, respectively (Serrano, 1985 and 1991). Pma1 is a major transport system, accounting for 25-50% of yeast plasma membrane protein (Serrano, 1991) and consuming 15-50% of the ATP produced in yeast cells (Gancedo and Serrano, 1988).

Changes in pHi are of high importance to control the cell growth and proliferation and rates of DNA and RNA synthesis appear to increase with higher pHi within the normal physiological range (Nuccitelli and Deamer, 1982). Also, key glycolytic enzymes are believed to be regulated by pHi, particularly phosphofructokinase (Holyoak *et al*., 1996). Accordingly, the *PMA1* gene is essential for yeast life (Serrano *et al*., 1986) and there is an impressive correlation between the activity of the enzyme and yeast growth (Portillo and Serrano, 1989; Vallejo and Serrano, 1989). Finally, the electrical membrane potential of yeast plasma membranes, negative inside, drives the uptake of toxic cations such as sodium, lithium, aminoglycosides and polyamines and it is set by the relative activities of Pma1 and of the major potassium uptake system Trk1 (see Figure 2 and Goossens *et al.,* 2000).

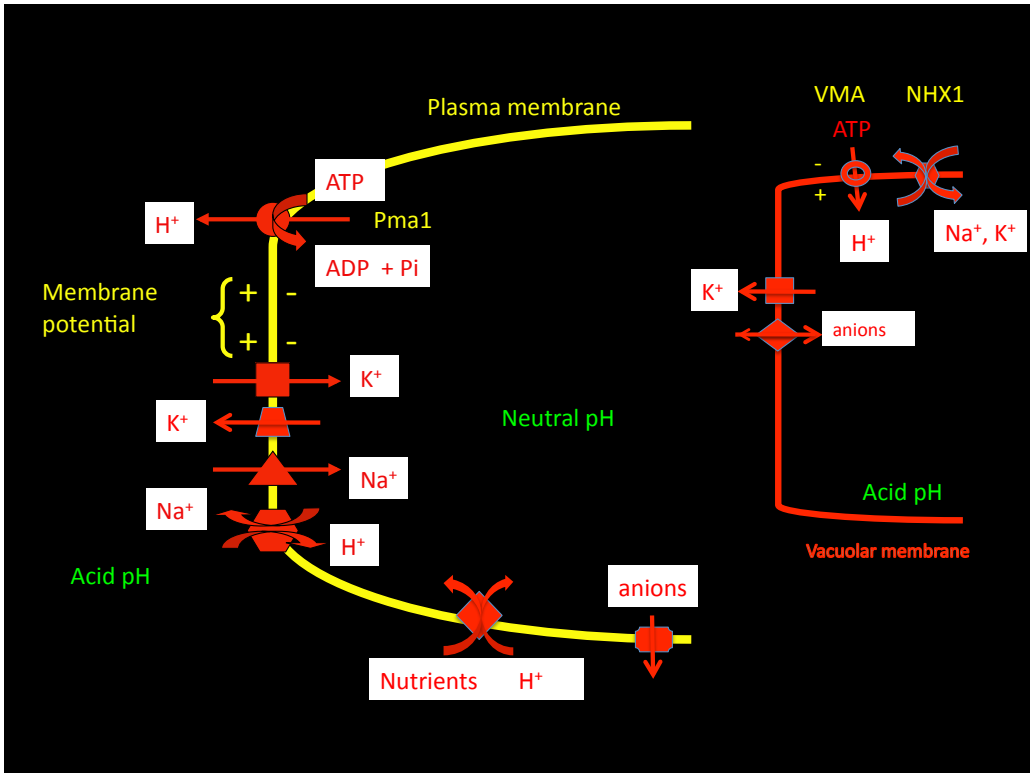


Figure 1. Scheme of ion homeostasis in yeast. The proton pumping H^+-ATPase Pma1 utilizes the energy of ATP hydrolysis to pump protons out of the cell. This generates a pH gradient, acid outside, and a membrane potential, positive outside. Both components of the electrochemical proton gradient drive nutrient uptake and extrusion of sodium and anions. The vacuolar H^+-ATPase (VMA) energizes the vacuolar membrane by generating a pH gradient (acid inside) and a membrane potential (positive inside). Influx and efflux K^+ channels are indicated as well as systems involved in nutrient uptake and sodium and anion efflux.

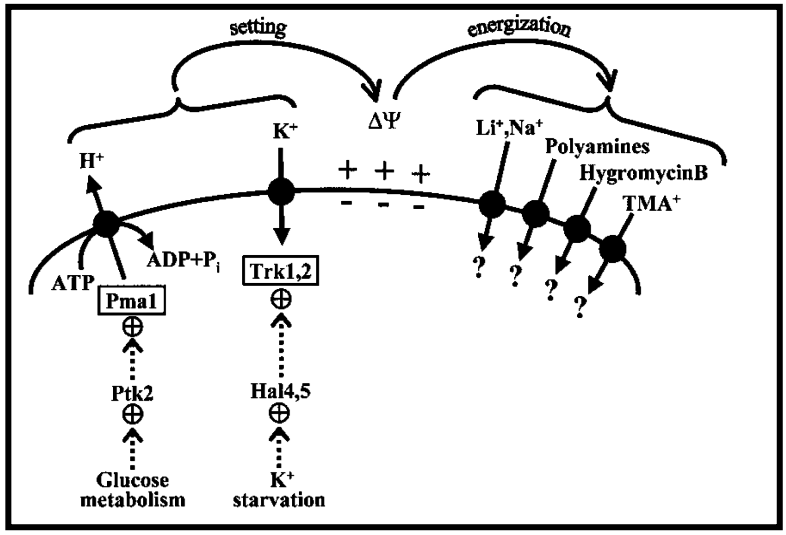


Figure 2. Model for the role of Pma1 and Trk1, 2 on yeast tolerance to toxic cations by modulation of the electrical membrane potential (ΔΨ), which determines the uptake of toxic cations by different voltage-sensitive channels (from Goossens *et al.,* 2000).

From an applied point of view, growth of yeasts in the presence of weak-acid preservatives is a major concern in the food industry. It is well known that the principal inhibitory mode of action of these preservatives is to lower pHi, causing growth arrest and cell death. Despite the presence of the maximum permitted level of preservative and good manufacturing practice, yeast and mold spoilage still happens. Actually, the resistance of the spoilage yeast *Zygosaccharomyces bailii* to such preservatives has become a huge problem to the food industry in products, which rely on low pH and weak acids for preservation. Mechanisms of resistance to weak organic acids seem to involve increased activity of either Pma1 or Trk1 (Holyoak *et al*., 1996). The concerted activity of the two systems modulating pHi is needed for electrical balance during proton efflux (see Figures 1 and 2).

We will make a brief introduction to cellular ion pumps before describing the regulation of Pma1, which constitutes the subject of this thesis.

Types of ion pumps

As indicated in Figure 3, living cells must maintain a set of major ion concentrations in the cytosol for proper operation of cellular systems despite variable external concentrations. In the course of evolution cells developed ion pumps to energize the membranes and maintain intracellular ion homeostasis (Maloney and Wilson, 1985). In addition to pumps energized by light and by red-ox reactions, ion-pumping ATPases of two major kinds (Figure 4) were developed by ancestor cells, more than 2 x 10^9 years ago: the P-ATPases (also called E_1E_2-ATPases) and the rotary ATPases (also called F_oF_1, A_oA_1 or V_oV_1-ATPases). The rotary ATPases are present in bacteria and derived mitochondria and chloroplasts (F_oF_1), archaea (A_oA_1) and vacuolar or lysosomal membranes (V_oV_1). These are very complex reversible enzymes with many subunits (up to 20) and structured into a membrane embedded domain (X_o; involved in transport of H^+ and more rarely of Na^+) and a membrane-protruding domain (X_1; catalyzing ATP hydrolysis or synthesis). The atomic structure and mechanism of these enzymes has been determined and the name of this type of pumps makes reference to their operation as a rotary motor (see the schematic drawing of Figure 5), with a central rotor within a surrounding stator. Rotation of the proton-binding site changes the accessibility of the site to the two sides of the membrane mediated by two half-channels in the transmembrane part of the stator (Muench *et al*, 2011).

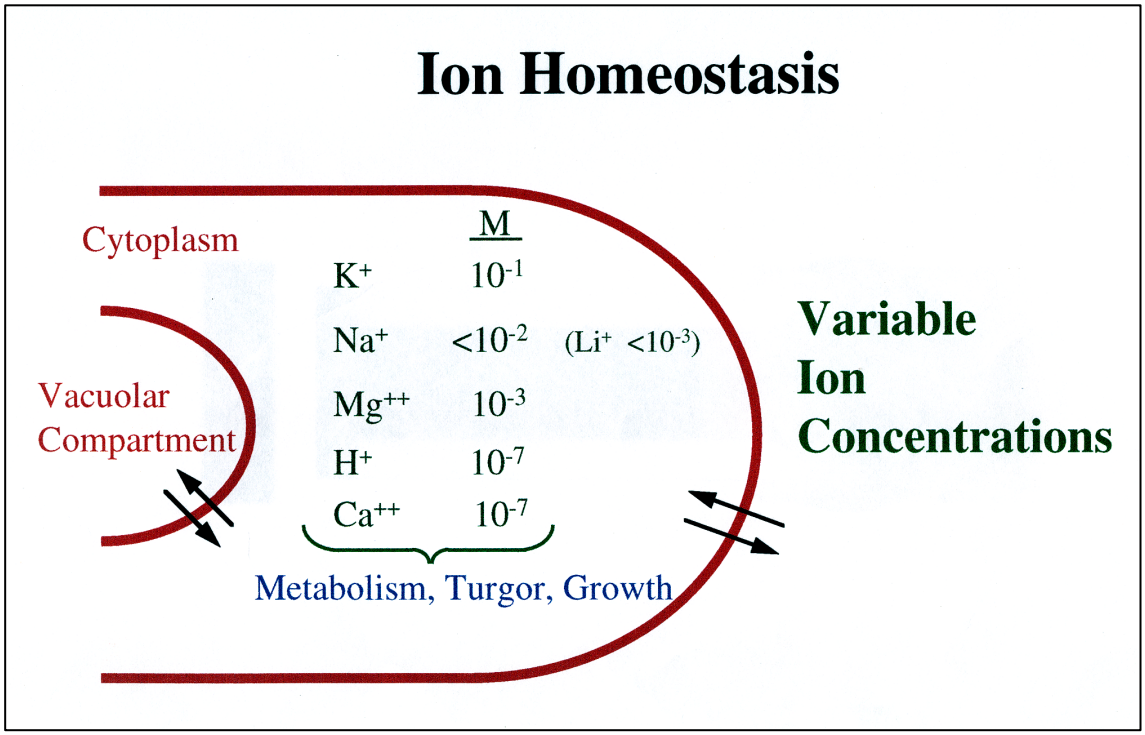


Figure 3. Scheme of cellular ion homeostasis in most living cells. The cytosol must maintain a set of major ion concentrations for the proper operation of cellular machineries despite variable external concentrations. K^+ is the major cation but Mg^{2+} and Ca^{2+} also play important roles. Na^+ and H^+ are toxic at high concentrations.

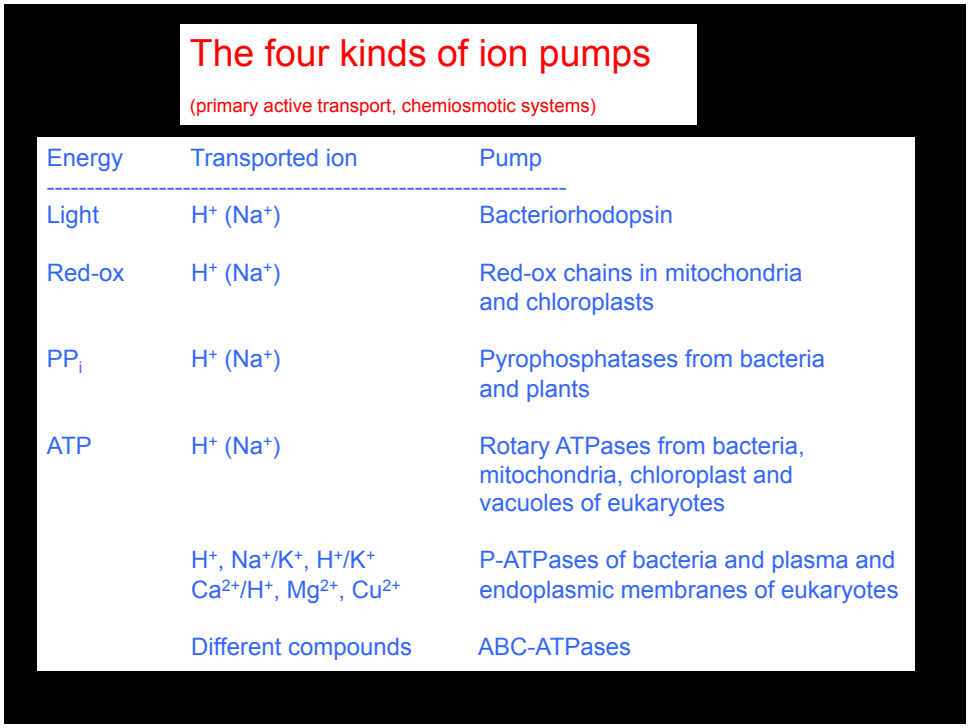


Figure 4. Types of ion pumps in living cells. Within the ATPases the so called ABC are not typical ion pumps and can transport many different molecules.

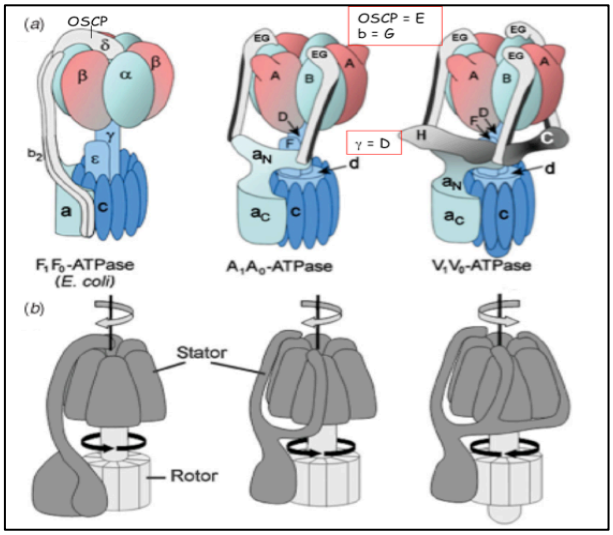


Figure 5. Schematic structure of rotary ion-pumping ATPases (Modified from Muench *et al*., 2011). Part (a) indicates the complex structure of these pumps and part (b) the division into stator and rotor pieces.

P-type ion-pumping ATPases

Pma1 belongs to the P-type ion-pumping ATPases, a versatile family of enzymes made of a single subunit and with different members pumping H^+, Na^+, K^+, Ca^{2+}, Mg^{2+} and Cu^{2+} (Axelsen and Palmgren, 1998; Kühlbrandt, 2004). The family is termed P-type because the mechanism of transport involves direct phosphorylation of a conserved aspartyl residue by ATP followed by hydrolysis of this aspartyl phosphate intermediate as a necessary part of the transport cycle. This has not to be confused with regulatory phosphorylations on serine and threonines by protein kinases acting on regulatory sites. More than 50 members of this class of membrane transport proteins have been sequenced and the family has been divided into five distinct classes based on their sequence, ion specificity and biological occurrence (Axelsen and Palmgren, 1988; Green, 1992). P-type ATPases are present in all species from bacteria to mammals but in fungi and plants the dominant plasma membrane ATPases are P-type proton pumps (H^+-ATPases) while in animal cells a P-type Na^+/K^+-ATPase is the dominant pump (Serrano, 1985). Also, the H^+-ATPases of yeast and other fungi do not contain a glycosylated β-subunit that is found in mammalian Na^+/K^+ and H^+/K^+-ATPases (Lutsenko and Kaplan, 1995). All P-ATPases have a catalytic mechanism based in four conformational states of the enzyme and a model based on the insight of William P. Jencks (1989) is shown in Figure 6. These are "linear" motors whose structure has been determined at the atomic level in some cases (Bublitz *et al*., 2010), including the plant proton pump that is highly homologous to yeast Pma1 (Pedersen *et al*., 2007). A representation of various structures of P-ATPases is shown in Figure 7.

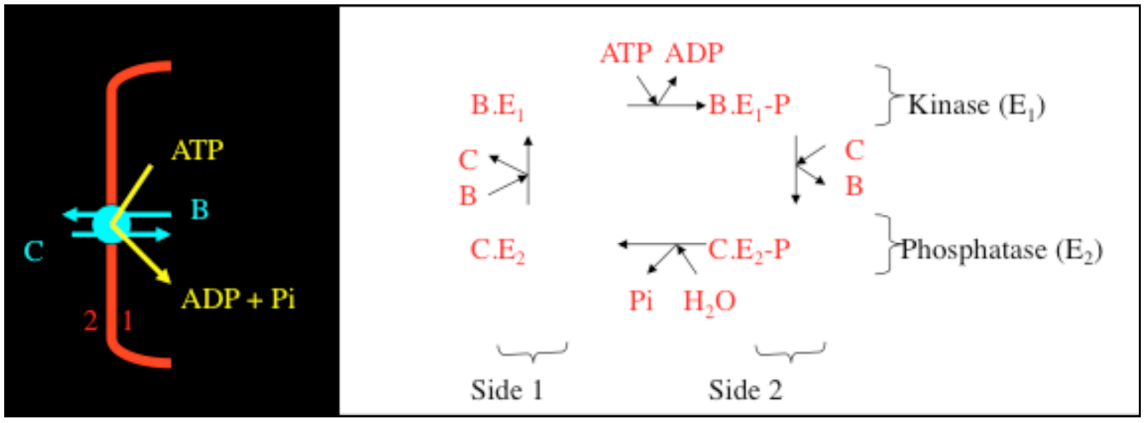


Figure 6. The catalytic cycle of P-ATPases. The cycle for a generic enzyme pumping cation "B" out of the cell and cation "C" into the cells is shown. Yeast Pma1 only pumps protons ($B = H^+$) out. After binding "B" from the cytosolic side (side 1), the enzyme acts as an "auto-kinase" (E_1) and phosphorylates himself at the catalytic site, making a covalent phosphorylated intermediate (aspartyl-phosphate; E-P). This has not to be confused with regulatory phosphorylations on serine and threonines by protein kinases acting on regulatory sites. This auto-phosphorylation triggers a conformational change that shifts the sideness and specificity of the cation binding site, now exposed to side 2 (outside the cell) and with decreased affinity for cation B to exchange "B" by "C". Binding of the latter cation then determines a new catalytic activity as an auto-phosphatase (E_2), releasing the phosphate (P_i) from E-P. This again triggers a conformational change that shifts the sideness and specificity of the cation binding site to side 1 (inside the cell) and exchange of "C" by "B", completing the cycle. After Jencks (1989).

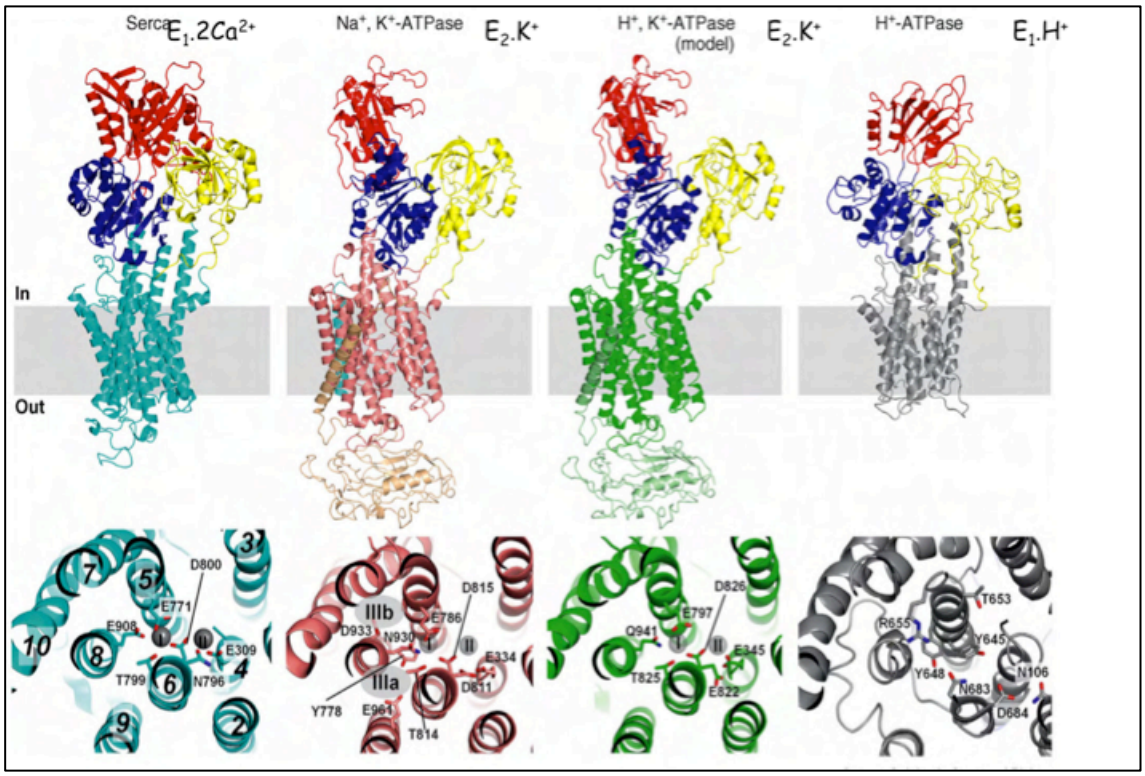


Figure 7. Schematic structure of several P-ATPases in different conformations. Ca^{2+}/H^+-ATPase from sarcoplasmic reticulum (Serca), Na^+/K^+-ATPase and H^+/K^+-ATPase from mammalian plasma membranes and H^+-ATPase from plant plasma membranes. Modified from Bublitz *et al.,* (2010). The upper part represents the overall structure of different P-ATPases by α-helices and β-sheets. The lower part represents the transmembrane helices with the cation binding sites. The accessibility of these sites to the two sides of the membrane and their affinity changes depending on the auto-phosphorylation of the ATPases (see Figure 6).

Regulation of Pma1 activity

The regulation of Pma1 activity was shown for the first time by Serrano (1983), who discovered that incubation of yeast cells with glucose resulted in a reversible several-fold enhancement of the enzyme's activity, a decrease in Km, and an increase in Vmax. Sugars utilized by the glycolytic pathway (glucose, fructose and mannose) were shown to lead to the enhancement of activity. Sugars metabolized through other pathways (galactose), as well as non-metabolized glucose analogs (xylose, 3-O-methylglucose and deoxyglucose), did not result in any enhancement (Serrano, 1983).

Concerning regulation of expression, glucose metabolism, through the Rap1/TUF and Gcr1 transcription factors, increases the expression of the *PMA1* gene together with that of genes of glycolytic enzymes, ribosomal proteins and translation factors, all of them important for fast growth in glucose medium (Capieaux *et al*., 1989; Portillo, 2000). However, this transcriptional regulation of Pma1 is relatively small, less than 2-fold, and regulation occurs mostly at the level of activity, not amount of the enzyme (Portillo, 2000). In other words, regulation of Pma1 is mostly due to posttranslational modification. Pma1 activity is increased by conditions that stimulate yeast growth, such as good carbon sources like glucose (Serrano, 1983) and by stress conditions that can be alleviated by the activity of the enzyme, such as intracellular acidification induced by low external pH (Eraso and Gancedo, 1987), nitrogen starvation (Benito *et al*., 1992) and supraoptimal growth temperature (Viegas *et al*., 1995). In the case of glucose the activation of proton pumping is much greater than the activation of ATP hydrolysis because it increases the number of protons pumped per ATP hydrolyzed (Venema and Palmgren, 1995). The activation by glucose requires metabolism of the sugar and it is independent of the plasma membrane glucose receptors Snf3/Rgt2 and Gpr1 (Belinchón and Gancedo, 2007).

The regulation of Pma1 activity by glucose metabolism and acidification described above depends on a regulatory domain of Pma1 present at the C-terminus of the enzyme (Benito *et al*., 1992). In the scheme of Figure 8 it is shown the domain structure of Pma1 and at the C-terminus is the regulatory domain identified by deletion analysis of the enzyme (Portillo *et al*., 1989; Serrano and Portillo, 1990). The last 21 amino acids of this domain (from Arg898 to Thr918) mediate the activation of the enzyme by glucose metabolism and by acidification.

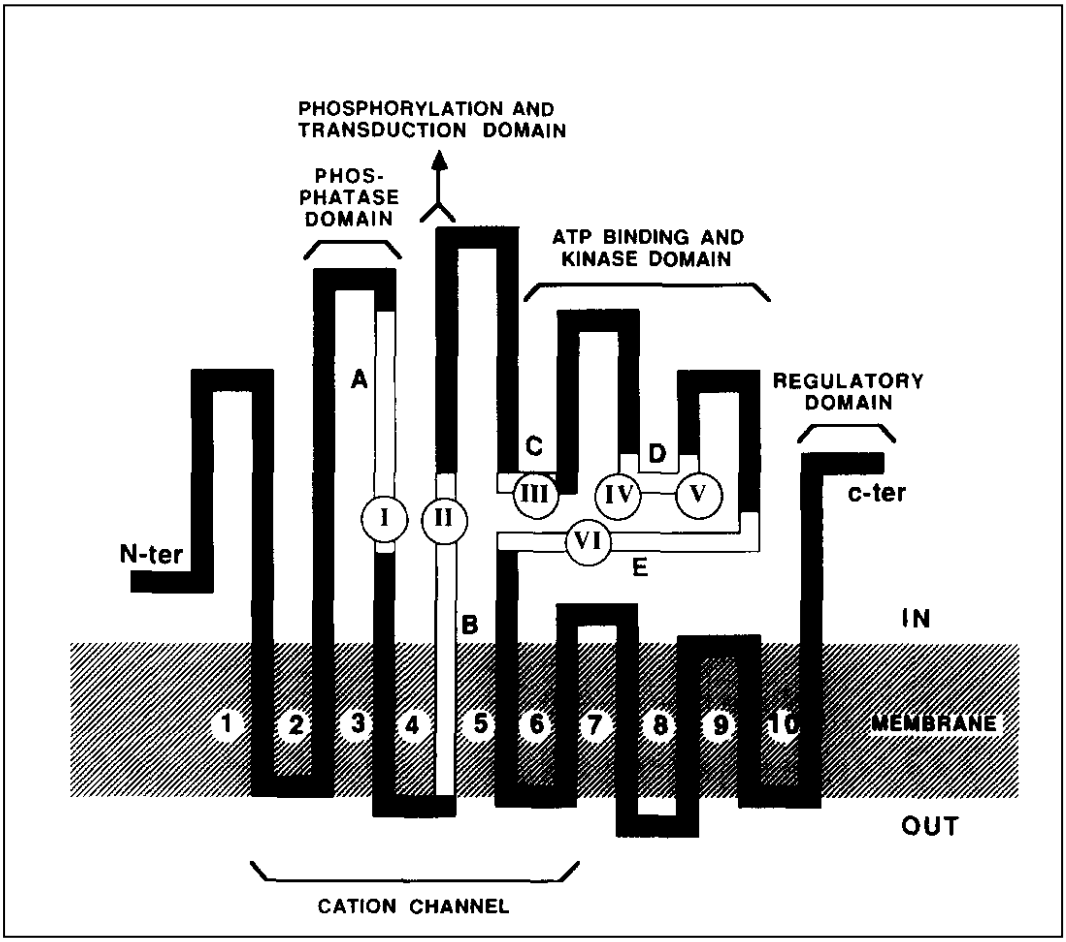


Figure 8. Model for the domain structure of yeast Pma1. The ten transmembrane helices and the conserved motifs I to VI of P-ATPases are indicated. Motif I (TGES) is the active site of the auto-phosphatase domain, motif II (D[K,R]TGT[L,I]T) include the aspartate (D) auto-phosphorylated in the reaction intermediate and motifs III (KGAP), IV (DPPR), V (M[L,I,V]TGD) and VI (GDGXND) are involved in ATP binding for the auto-kinase reaction. From Serrano and Portillo (1990). The regulatory domain is at the C-terminus of the enzyme and is not conserved in different types of P-ATPases. Its conformation is unstableand is not visible in the crystal structures of Figure 7.

The suggested mechanism (Portillo, 2000) is that this regulatory domain in the inactive state blocks the binding to the enzyme of both the transported proton and ATP. Upon some modification induced by glucose metabolism or acidification the regulatory domain is displaced and allows access of both proton and ATP to the enzyme (Figure 9).

In the case of activation by glucose metabolism this modification has been shown to correspond to phosphorylation of two sites: Ser899 and double phosphorylation at Ser911 and Thr912. The sequence of the regulatory domain is shown in Figure 10, with the two phosphorylation sites important for activation by glucose metabolism indicated. Although an inhibitory phosphorylation of Ser507 (within the kinase domain) by casein kinase I (Yck1) has been described, this site has a minor effect on the activation by glucose of Pma1 (Estrada *et al*., 1996). Phosphorylation of Ser899 by protein kinase Ptk2 slightly activates Pma1 by improving the affinity of the enzyme for ATP (Eraso and Portillo, 1994; Goossens *et al*., 2000, Eraso *et al*., 2006). However, the most important regulation of Pma1 by glucose metabolism is mediated by double phosphorylation of Ser911 and Thr912, which greatly affects the maximal rate of the enzyme (Portillo *et al*., 1991; Lecchi *et al*., 2007). As Ser911 is phosphorylated in the absence of glucose (Lecchi *et al*., 2007), the phosphorylation of Thr912 is the crucial modification of Pma1 during activation by glucose metabolism.

At present the protein kinases phosphorylating Pma1 at Thr912 in response to glucose metabolism remains unknown. A scheme of the regulatory pathways triggered by glucose in yeast is shown in Figure 11 (modified from Belinchón and Gancedo, 2007). A major yeast protein kinase activated by glucose, protein kinase A, is not involved in glucose activation of Pma1 (Mazón *et al*., 1989; dos Passos *et al*., 1992). Protein kinase C and Ca^{2+}/calmodulin-dependent protein kinases have been proposed to regulate Pma1 in response to glucose metabolism (Brandao *et al.*, 1994) but a demonstration that these kinases phosphorylate Pma1 remains to be reported.

Campetelli *et al.,* (2005) have reported that in glucose starved cell Pma1 forms a complex with acetylated tubulin that is disrupted when the enzyme is activated by glucose metabolism. This change in interaction of Pma1 with acetylated tubulin as well as the changes in distribution of Pma1 in the membrane surface observed by Permyakov *et al*., (2012) using immunoglod labeling may be structural consequences of the conformational changes triggered by phosphorylation of the regulatory domain of Pma1.

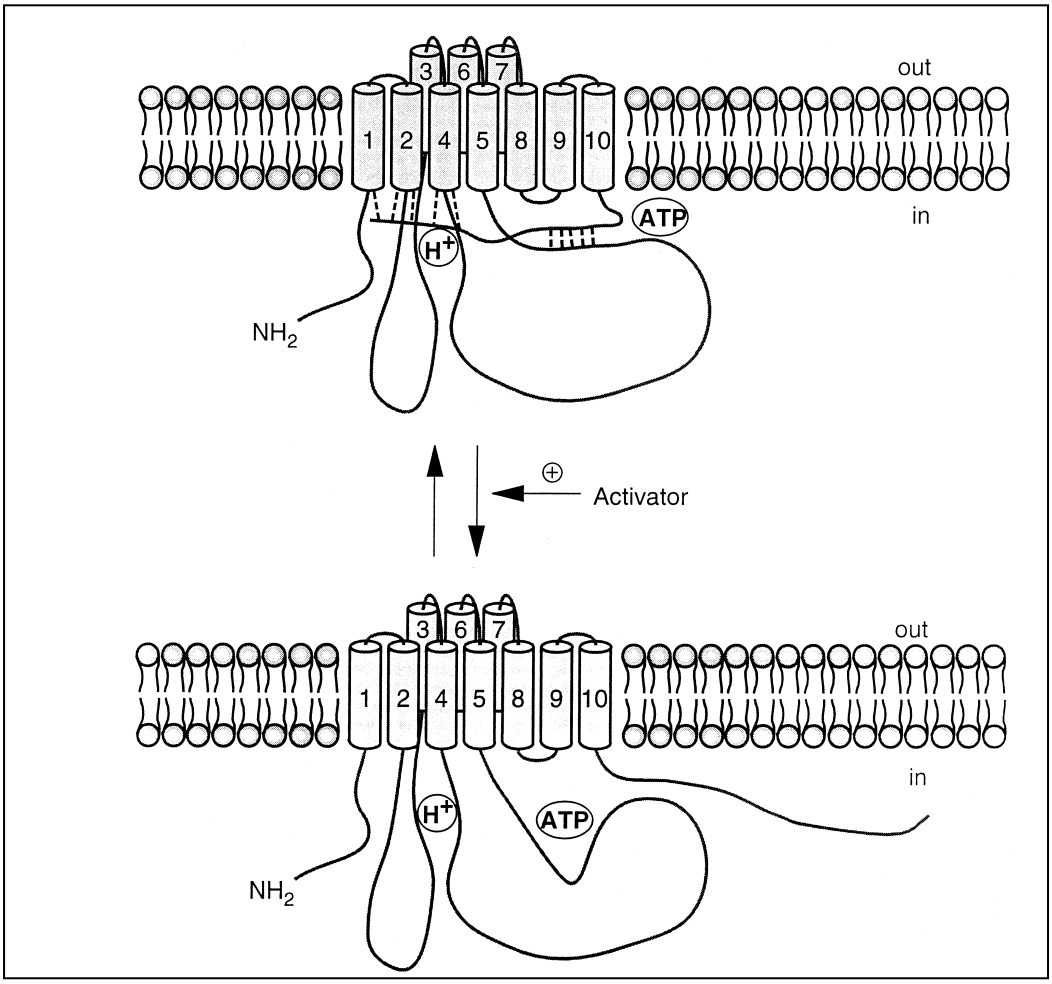


Figure 9. Model for the activation of Pma1. In the low-activity state (upper), the C-terminus would interact with the active site of the enzyme, limiting access of the protons and ATP substrates. The activators would trigger a modification of the C-terminus, releasing the inhibitory interaction and allowing the ATPase to adopt a more active conformation (bottom). From Portillo (2000).

Figure 10. Amino acid sequence of the last 21 amino acids of Pma1. The two activating phosphorylation sites, Ser899 and Ser911 Thr912 are indicated. p: O-PO_3H

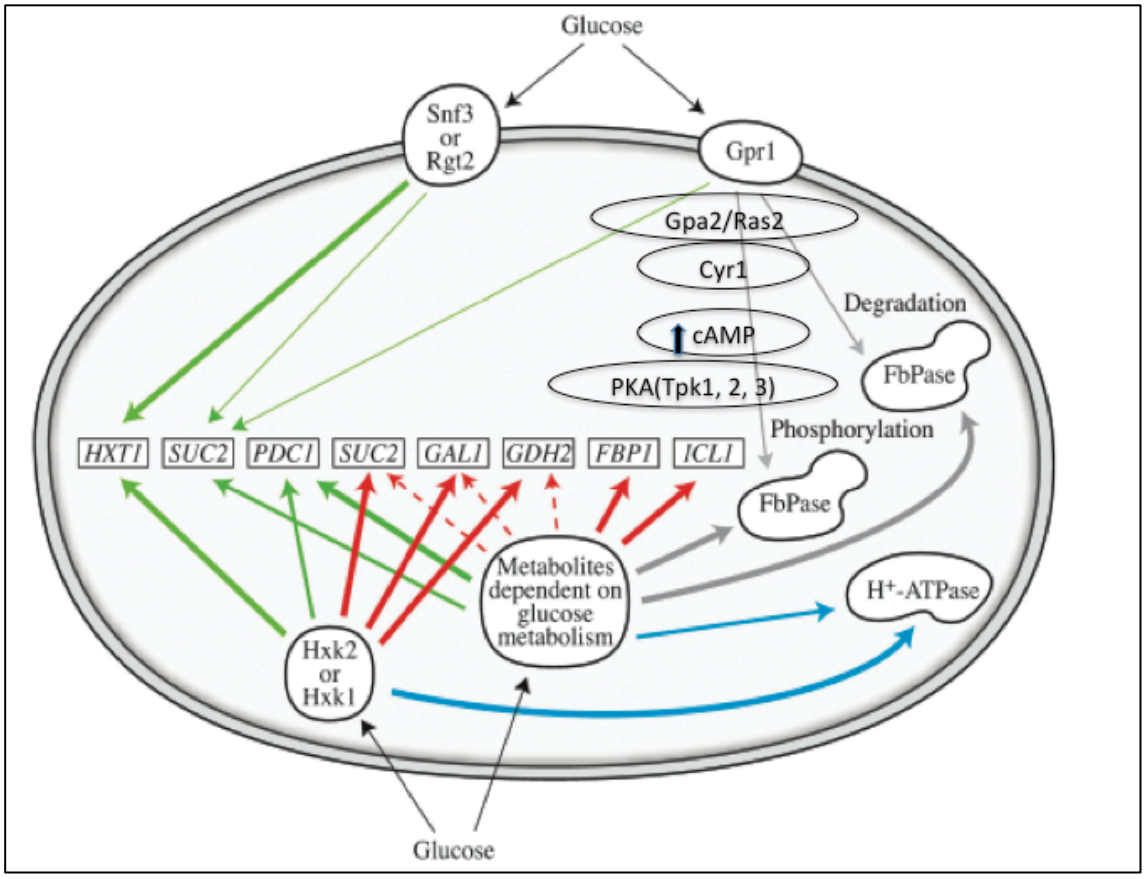


Figure 11. The effects of glucose on different processes in yeast depend on different combinations of early signals. Induction of SUC2 by low glucose depends partially on the glucose sensors, and requires glucose metabolism; in the absence of metabolism, induction requires high glucose and is fully dependent on Gpr1. Induction of HXT1 requires Snf3 or Rgt2, and is partially dependent on Hxk2. Induction of PDC1 is independent of the glucose sensors, has some dependence on Hxk2 or Hxk1, and requires glucose metabolism. Repression of different genes is independent of the glucose sensors; for SUC2, GAL1 and GDH2, Hxk2 or Hxk1 are required, and presumably glucose metabolism; for FBP1 and ICL1, glucose metabolism is necessary, but any glucose-phosphorylating enzyme sustains repression. In the absence of glucose metabolism, phosphorylation of FbPase is impaired, and the effect is reinforced by lack of Gpr1; degradation of FbPase depends on glucose metabolism, and is decreased when Gpr1 is lacking. Activation of H^+-ATPase Pma1 is independent of the glucose sensors, and has a strong dependence on Hxk2 or Hxk1; it is nearly blocked in the absence of glucose metabolism. The interactions between the initial elements of signaling and their targets are indicated by arrows; the width of the arrows is related to the strength of the signal, and putative interactions are indicated by dashed lines. Green color for induction; red, for repression; , blue for activation; grey for inactivation (by phosphorylation or degradation). (Modified from Belinchón and Gancedo, 2007)

Protein kinases and Phosphatases

Regulation of protein activities by phosphorylation is one of the most important regulatory mechanisms in eukaryotic cells (Hanks and Hunter, 1995). The process of phosphorylation and dephosphorylation of a protein serves as an "on-and-off" switch in the regulation of cellular activities. For optimal regulation, protein kinases and phosphatases have to reach a balance in any given cell (Luan, 2003). A large fraction of eukaryotic proteins, about one-third, are controlled by phosphorylation of specific serine, threonine, and/or tyrosine residues (Ceulemans and Bollen, 2004). Adding or removing a phosphate group from a protein often has profound effects on the structure and thereby the functional properties of it. For example, phosphorylation can regulate an enzyme activity by initiation of allosteric conformational changes that may affect the access to its active site (Johnson and O'Reilly, 1996). The interaction among protein partners that must form complexes in order to function is also affected by phosphorylation (Pawson, 1995). Some proteins need to be phosphorylated to be able to target a certain destination site in the cell where they function. Almost all aspects of cell function involve reversible phosphorylation: metabolism, cell cycle progression, ion transport, developmental control, and stress responses. This diversity in cellular functions is reflected by the large number of intracellular proteins subjected to reversible phosphorylation and the large number of protein kinases and phosphatases that catalyze these reactions (Luan, 2003).

There are about a hundred protein tyrosine kinases and the same number of protein tyrosine phosphatases in mammalian cells. In fungi and plants tyrosine phosphorylation is much less important and there are only a few kinases and phosphatases for this modification. Concerning protein serine/threonine (Ser/Thre) phosphorylation, mammalian cells have about 400 protein kinases and 25 protein phosphatases (Plowman *et al*., 1999), and this suggest distinct diversification strategies during evolution (Ceulemans *et al*., 2002). At variance with mammalian cells, the yeast genome encodes and expresses 106 protein phosphatases (Stark, 1996) and 113 protein kinases (Hunter and Plowman, 1997), the number of protein kinases and phosphatases is similar. The true diversity of protein Ser/Thre phosphatases is only seen at the holoenzyme level because of the variety of regulators that can interact with a given catalytic subunit (Ceulemans and Bollen, 2004).

The atypical TOR protein kinase

There are also a few non-conventional, atypical protein kinases. These are enzymes more related by sequence to metabolic kinases that phosphorylate metabolites but behave in cells as kinases that phosphorylate proteins. One of these rare enzymes is TOR (Target Of Rapamycin), an atypical protein kinase sensitive to the drug rapamycin that plays a crucial role in nutrient signaling and growth control. It was discovered in yeast and is homologous to phosphatidylinositol kinases involved in lipid metabolism although it phosphorylates proteins at serine and threonine residues. (Loewith and Hall, 2011). TOR is a highly conserved protein kinase among eukaryotes but *S. cerevisiae* is unusual in having two TOR genes (*TOR1* and *TOR2*) whereas almost all the other eukaryotes, including plants, worms, flies, and mammals, have a single TOR gene (Loewith and Hall, 2011). In yeast Tor1 and Tor2 proteins both have 282 kDa (2470 and 2474 amino acids, respectively) and are 67% identical. They are the founding members of the PIKK family of atypical Ser/Thr-specific kinases related to phosphatidylinositol kinases (Keith and Schreiber, 1995). Disruption of both *TOR1* and *TOR2* genes in yeast leads to growth arrest similar to that caused by rapamycin treatment, suggesting that Tor1 and Tor2 are the ultimate targets of rapamycin, a potent inhibitor of yeast growth (Kunz *et al*., 1993).

Tor1 and Tor2 have a central role in controlling yeast growth as part of two separate signaling branches. They are functionally different despite being structurally similar (Helliwell *et al*., 1994). Disruption of only *TOR1* has little or no effect but disruption of *TOR2* alone causes cells to arrest growth within a few generations and to have a randomized actin cytoskeleton (Schmidt *et al*., 1996). *TOR2* has two essential functions: one function is redundant with *TOR1* and the other function is unique to *TOR2* (Helliwell *et al*., 1998). This is explained because Tor1 and Tor2 are found as part of two essential complexes, TORC1 and TORC2, conserved in eukaryotes (Loewith and Hall, 2011). In yeast TORC1 contains either Tor1 or Tor2 but only Tor2 can form part of TORC2 (Figure 12).

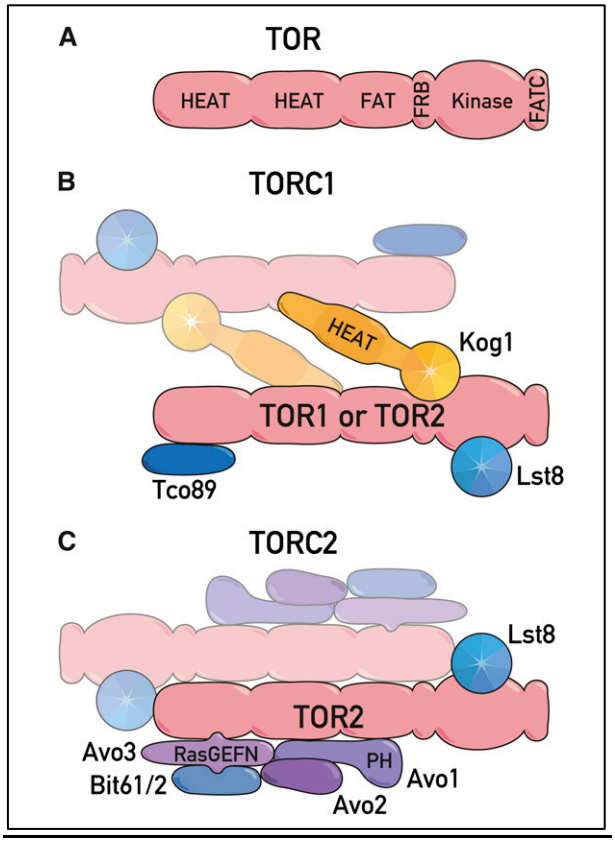


Figure 12. Conserved domain structure of TOR. The N-terminal half of TOR is composed of two blocks of 20 HEAT repeats, 40 aa that form pairs of interacting antiparallel a-helices. The 500-aa FAT (FRAP-ATM- TRRAP) domain contains modified HEAT repeats. Missense mutations in the 100-aa FRB (FKBP12-rapamycin-binding) domain confer complete resistance to rapamycin. The kinase domain phosphorylates Ser/Thr residues in protein substrates, but at the sequence level resembles the catalytic domain of phosphatidylinositol kinases. (B) Composition of TOR complex 1. TORC1 is 2 MDa in size and contains Kog1, Tco89, Lst8, and either Tor1 or Tor2. The HEAT repeats found in Kog1 and the seven-bladed propellers of the WD-40 repeats found in Kog1 and Lst8 are depicted. The binding of Kog1 to TOR is complex, involving multiple domains on each protein. Lst8 binds to the kinase domain of TOR. Each component is likely present in two copies. (C) Composition of TOR complex 2. TORC2 is 2 MDa in size and contains Avo1, Avo2, Avo3, Bit61, and/or its paralog Bit2, Lst8, and Tor2 but not Tor1. The RasGEFN domain of Avo3 and the PH domain of Avo1 are indicated. Each component is likely present in two copies (Loewith and Hall, 2011).

Regulation of and by TORC1

Both TOR complexes have a dual localization, cytoplasmic and membrane-bound, with TORC1 partially bound to the vacuolar membrane and TORC2 partially bound to the plasma membrane (Sturgill *et al*., 2008). TORC1 signaling is responsive to nutrients, which are mainly stored in the yeast vacuole; this makes such localization of TORC1 very logical (Figure 13). The EGO complex resides on the vacuolar/lysosomal membrane and is thought to couple amino acid signals to TORC1. It is an important regulator of TORC1 (Binda *et al*., 2009). Under inappropriate conditions of growth,

cells enters a quiescence (G0) state in which they stop dividing, slow their metabolism, induce the expression of stress-responsive proteins, and accumulate energy stores.

Genetic disruption of TORC1 or rapamycin treatment mimics starvation, (De Virgilio and Loewith, 2006a; Loewith and Hall, 2011). Both starvation and exposure of yeast cells to rapamycin result in a high drop in protein synthesis, induction of autophagy, and exit from the cell cycle and entrance into a quiescent G0 state (Figure 14). The transcriptional response of yeast cells exposed to rapamycin, nutrient starvation, or noxious stressors is similar (Gasch and Werner-Washburne, 2002).

TORC1 does not only respond to extracellular signals, it can sense the intracellular amino acids and also the outputs from distal effectors can regulate TORC1 by feed back mechanism (Loewith and Hall, 2011). Example to this feedback, TORC1 activity stimulates translation initiation (Wullschleger *et al.,* 2006) and inhibiting the translation with cycloheximide leads to high increase in TORC1 activity in response to increased concentration of free amino acids in the cytoplasm (Binda *et al.,* 2009). Another example of the feedback is the regulation of ribosome biogenesis; TORC1 regulates this through two substrates, Sch9 and the transcription factor Sfp1 (Loewith and Hall, 2011).

As shown in Figure 13, one important direct substrate of yeast TORC1 is a protein kinase called Sch9 (Powers, 2007) this substrate gets phosphorylated by TORC1 and becomes active as a result (Urban *et al.,* 2007). TORC1 also regulates type 2A and 2A-related protein phosphatases like Sit4. Zheng and Jiang (2005) showed that when TORC1 is active, Tap42 is phosphorylated and bound tightly to the Sit4 phosphatase. On the other hand, TORC1 inactivation results in Tap42 dephosphorylation and a weakened association with phosphatases which finally results in their activation and/or change in substrate preference (Yan *et al.,* 2006).

As a rule, under appropriate growth conditions, TORC1 is active and its signals promote the accumulation of cellular mass i.e., growth, via regulation of several processes (Loewith and Hall, 2011), a scheme of them is represented in Figure 14.

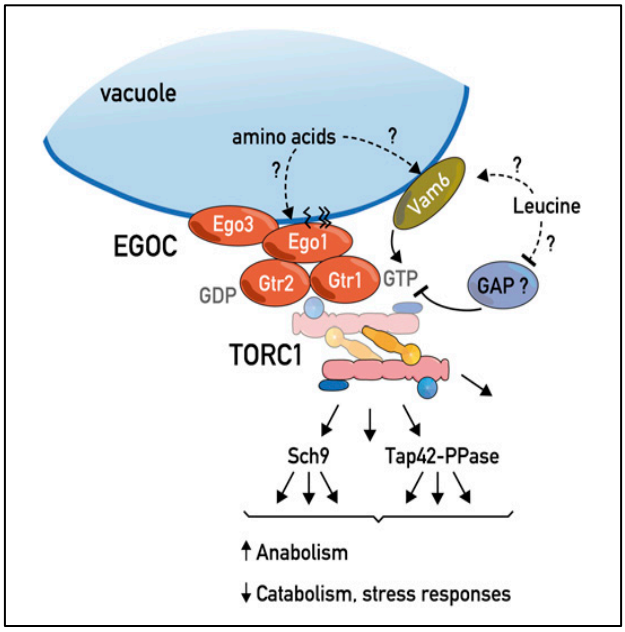


Figure 13. The EGO complex is a major regulator of TORC1. This complex is composed of four proteins (in red), binds to the vacuolar membrane and activates TORC1. Activated TORC1, via its two main effector branches, the AGC kinase Sch9 and the Tap42-PP2A and PP2A-like protein phosphatases, stimulates growth by favoring anabolic processes and by antagonizing catabolic processes and stress-response programs (Loewith and Hall, 2011).

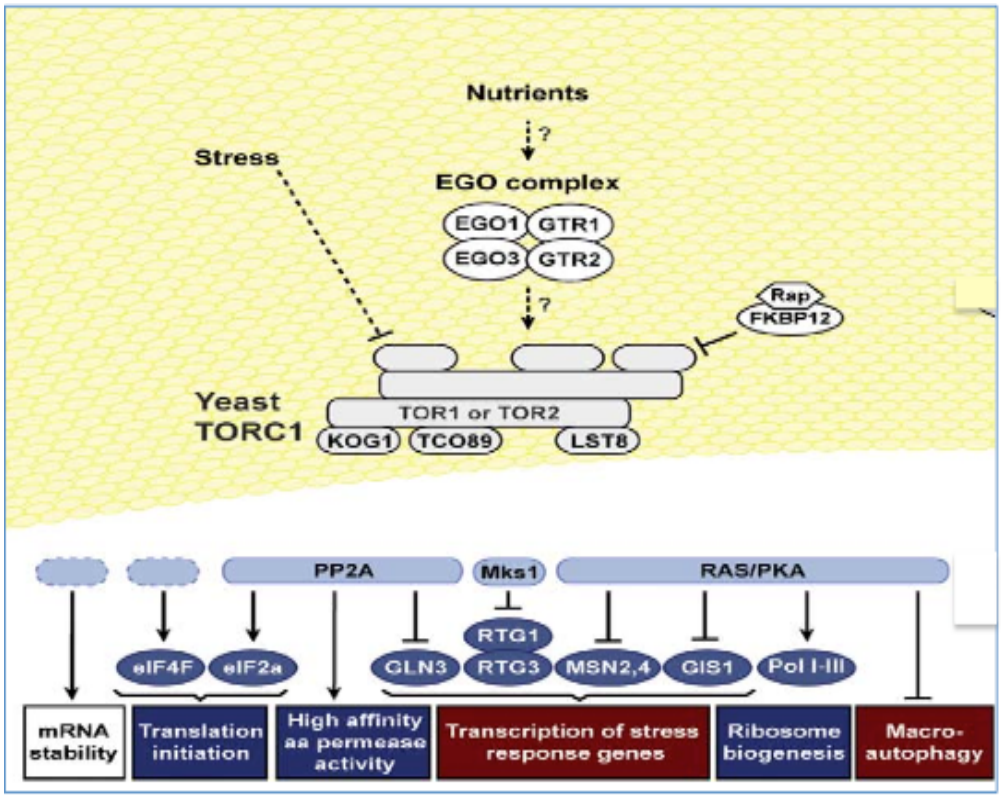


Figure 14. Rapamycin-sensitive yeast TORC1 (TORC1) couples growth to nutrients and stresses that are sensed by signaling pathways that impinge upon TORC1. TORC1-regulated processes that promote growth are boxed in dark blue; TORC1-regulated processes that inhibit growth are boxed in dark red. Arrows and bars denote positive and negative interactions, respectively, and dashed arrows and bars refer to potential interactions (De Virgilio and Loewith, 2006a)

Rapamycin treatment elicits a high drop in protein synthesis by blocking translation initiation (Barbet *et al*., 1996). As indicated in Figure 15 (Ashe *et al*., 2000) this regulation occurs mainly through the translation factor eIF2. Amino acid starvation or rapamycin treatment leads to phosphorylation of the α-subunit of eIF2 (Hinnebusch, 2005). TORC1 signals to eIF2a through both the Sch9 and Tap42-PP2A branches. The only eIF2-α kinase is the conserved Gcn2 protein. Gcn2 is activated when bound to uncharged tRNAs that accumulate when cells are starved for an amino acid. Gcn2 activity is also regulated by phosphorylation on Ser577. This reduces tRNA binding and, consequently, kinase activity. Treating cells with rapamycin elicits a rapid, Tap42-PP2A-dependent dephosphorylation of Ser577 and, consequently, an increase in Gcn2 activity and a reduction in translation (Cherkasova and Hinnebusch, 2003).

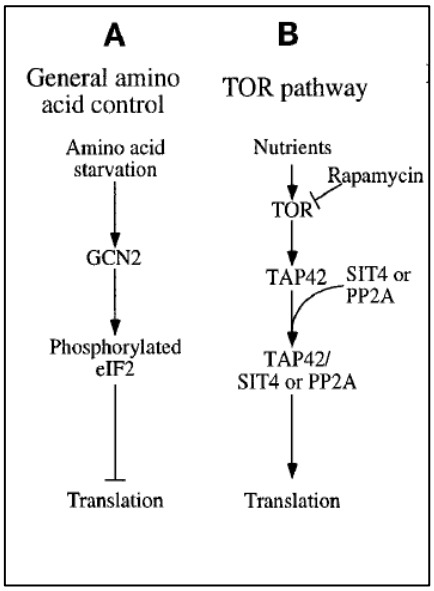


Figure 15. Control of translation in *S. cerevisiae* by the Gcn2 and TORC1 protein kinases (Ashe *et al*., 2000).

In the following I will list some of the many functions of TORC1.

- Ribosome biogenesis: TORC1 plays a central role in regulating ribosome biogenesis at both transcriptional and posttranscriptional levels (Loewith and Hall, 2011). TORC1 also regulates various catalytic steps of ribosome assembly per se (Loewith, 2010).
- Cell cycle/cell size: Cell growth and cell division are two distinct, yet highly linked processes. In yeast, cells start a new round of cell division only after reaching a critical size (Cook and Tyers, 2007). Poor growth conditions reduce the activity of TORC1 and subsequently the activities of Sfp1 and Sch9. Consequently, this would

decrease ribosome biogenesis, which would lower the cell-size threshold required for cell division. In contrast, acute inhibition of TORC1 with high concentrations of rapamycin leads to an arrest in G1 (Barbet *et al.,* 1996) and a paradoxical increase in cell size. The increased cell size is due to swelling of the vacuole as a consequence of increased autophagy.

- TORC1 promotes growth also via inhibiting the stress responses. Activation of stress-responsive pathways is incompatible with rapid growth, and constitutive activation of these pathways results in cell death (Loewith and Hall, 2011).
- Nutrient uptake and intermediary metabolism. In response to the availability of different nutrients yeast cells express different permeases in order to compete with other microorganisms for growth. TORC1 regulates these permeases primarily via Tap42-PP2A (Breitkreutz *et al.,* 2010). Many nutrient and cation permeases have been identified as rapamycin-sensitive phosphoproteins (Huber *et al.,* 2009). TORC1 regulates not only permease localization but also expression (Shamji *et al.,* 2000). One effect on metabolism is through affecting the Gcn4 transcription factor, which gets activated upon amino acid starvation (Hinnebusch, 2005). Rapamycin treatment or amino acid starvation results in a rapid decrease in translation initiation by phosphorylating the α-subunit of eIF2 which results in the repression of bulk translation and transcriptional induction of nearly all genes encoding amino acid biosynthetic enzymes (Loewith and Hall, 2011).
- TORC1 inhibits autophagy. When cells are exposed to starvation conditions they express stress-responsive proteins to help them. The energy and amino acids required for this new synthesis are obtained by inducing autophagy. Autophagy is conserved across eukaryotes (Yang and Klionsky, 2010) and it refers to the different mechanisms by which cytoplasmic material, including proteins and lipids, is translocated to the vacuole and catabolized. The produced amino acids and fatty acids are, respectively, used to synthesize new proteins and to produce ATP. Yeast has two different modes of autophagy: one is microautophagy, in which the cytoplasm is directly transferred into the vacuole via invaginations of the vacuolar membrane. The other mode is macroautophagy, which involves the “de novo” formation of double-membrane vesicles called autophagosomes, which encapsulate cytoplasm and then fuse with the vacuole. The two forms of autophagy are regulated by TORC1 (De Virgilio and Loewith, 2006b).
- TORC1 accelerates aging: Aging is the progressive deterioration of cell, tissue

and organ function. The genetic or chemical inhibition of TORC1 has been demonstrated to increase life span in yeast (Vellai *et al.,* 2003) mostly through the multiple TORC1 effector pathways (Blagosklonny and Hall, 2009). Yeast life span can be assayed in two ways. RLS or replicative life span is defined as a measure of the number of progeny that a single mother cell can produce before senescence. Chronological life span (CLS) is a measure of the length of time a population of yeast cells can remain in stationary phase and keep the ability to restart growth following reinoculation into fresh media. RLS increases upon TORC1 inhibition. TORC1 and Sch9 inhibition and also induction of macroautophagy increases both RLS and CLS (Madeo *et al.,* 2010). Since TORC1, Sch9, and Gcn2 homologs are found in most eukaryotes, the aging pathway appears to be conserved (Kaeberlein and Kennedy, 2011).

Regulation of and by TORC2

The majority of Tor2, almost 90%, is in TORC2 (vs. TORC1) and that is why Tor2 localization studies presumably detect mainly, if not only, TORC2. As described above, TORC2 seems to be located both at the cytoplasm and at the plasma membrane (Sturgill *et al.,* 2008). Localization at the plasma membrane is consistent with the role of TORC2 in controlling the actin cytoskeleton and endocytosis (Loewith and Hall, 2011).

TORC2 is rapamycin insensitive and its regulation is poorly characterized (Cybulski and Hall, 2009). There is a lack of reported evidence to support that TORC2 is controlled by nutrients. Knockout of TORC2 does not induce a starvation-like phenotype, and EGO, the nutrient-sensitive complex, appears not to be affecting TORC2. In mammalian cells, and perhaps also in yeast cells TORC2 is activated by direct association with the ribosome. Such a mechanism ensures that TORC2 is active only in growing cells because ribosomes determine the growth capacity of a cell (Loewith and Hall, 2011).

The first described and best-characterized TORC2 readout is the actin cytoskeleton (Figure 16). TORC2 also regulates sphingolipid biosynthesis (Powers *et al.,* 2010). Sphingolipids serve as essential structural components in lipid bilayers and as signaling molecules. There is a mutual antagonism between TORC2 and the stress-responsive calcium-calcineurin pathway in yeast (Mulet *et al.*, 2006).

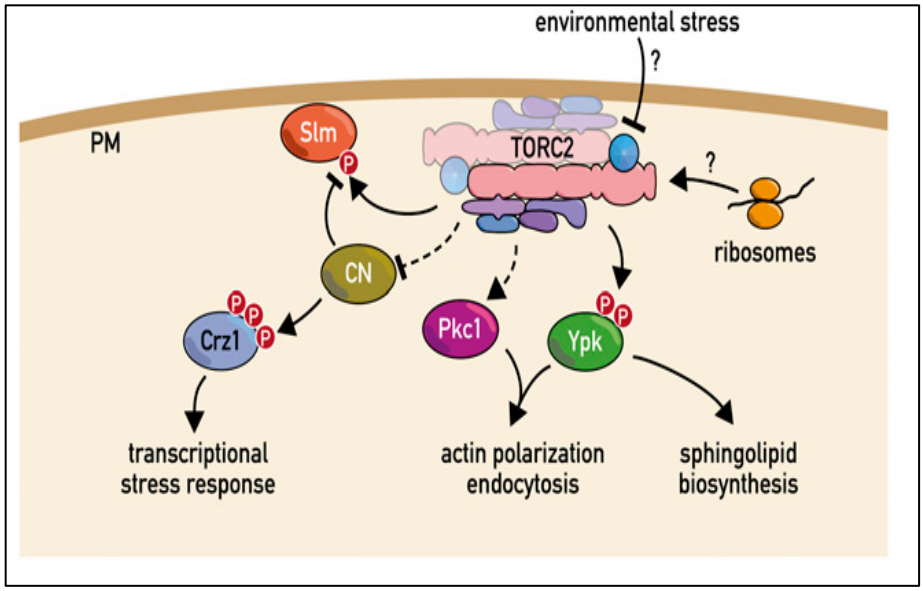


Figure 16. Signaling by TORC2. TORC2 directly phosphorylates the AGC kinase family member Ypk (Ypk1 and 2) and the PH domain containing protein Slm (Slm1 and -2). Downstream effectors include the phosphatase calcineurin, the transcription factor Crz1, and Pkc1. TORC2 controls organization of the actin cytoskeleton, endocytosis, sphingolipid biosynthesis, and stress-related transcription. The effector pathways by which TORC2 controls these processes are incompletely understood (Loewith and Hall, 2011).

Protein phosphatases

In the cellular regulations by phosphorylation protein kinases have been considered as the controlling part, with protein phosphatases (Cohen, 1989) being considered as mere "reset buttons", to put cells back in the resting state when protein kinases are inactivated (Ceulemans and Bollen, 2004). However, the recent discovery of the receptors of the plant hormone abscisic acid and its mechanism of action as inhibitors of protein phosphatases (Santiago *et al*., 2012) has shown that regulation of protein phosphatases can also control phosphorylation signaling cascades. More recently, the plasma membrane H^+-ATPase of the plant *Arabidopsis thaliana* has been reported to be activated by auxin by a mechanism consisting on the inhibition of PP2C-D phosphatases by some SAUR proteins induced by the hormone (Spartz *et al*., 2014).

Typical protein phosphatases catalyze the dephosphorylation of the hydroxyl group on amino acid residues and are classified into two major groups, protein Ser/Thre (Ser/Thr) and protein tyrosine phosphatases. There is a significant structural diversity inside each family generated by the presence of unique regulatory and targeting domains or by the attachment of regulatory subunits to the catalytic subunits. These enzymes have very broad substrate specificities "in vitro" and the function of these regulatory domains or subunits is to modulate their catalytic activity and, most

important, to target the catalytic subunit to a specific subcellular compartment to determine the "in vivo" substrate specificity of protein phosphatases (Cohen, 1989; Luan, 2003).

As indicated in Figure 17, there are two major types of protein Ser/Thr phosphatases, based on their substrate specificity and pharmacological properties: Type 1 (PP1) and Type 2 (PP2). There are two main differences and the first one is the subunit of phosphorylase kinase that is acted on as substrate. PP1 acts on the beta subunit while PP2 dephosphorylates the alpha subunit. The second difference is the effect of nanomolar concentrations of two small peptide inhibitors; inhibitors 1 and 2 inhibit PP1 while PP2 is insensitive to them. PP2 enzymes are further divided according to their dependence on external divalent cations: PP2A, like PP1, does not require them for activity while PP2B and PP2C are activated by Ca^{2+} and Mg^{2+}, respectively. Okadaic acid and calyculin A are considered as potent inhibitors of PP1 and PP2A but are not effective with PP2B and PP2C. PP1 and PP2A sequences are highly conserved among eukaryotes and are about 50% identical (Cohen, 1989; Luan, 2003). The crystal structure shows that some intrinsic metal ions are important for the catalytic reaction such as Mn^{2+} for PP1 (Egloff *et al*., 1995).

More than 90% of protein Ser/Thr phosphatase activity in eukaryotic cells is contributed by PP2A and PP1 enzymes, so they are considered as main regulators of a variety of important cellular processes in yeast and other eukaryotes (Ceulemans and Bollen, 2004; Castermans *et al.*, 2012). PP1s can be considered as a "green" enzyme because it promotes the rational use of energy, a reversal of the cell to a basal and/or energy-conserving state and the recycling of protein factors. When nutrients are abundant PP1s promote the storage of energy in the form of glycogen. PP1s also play a role in the recycling of transcription and splicing factors and the return to basal patterns of protein synthesis; moreover, it has a main role in the recovery from stress but promotes apoptosis when cells cannot be repaired. In addition, PP1 downregulates ion pumps and transporters in different tissues and finally, stimulate exit from mitosis, modulate actin organization and keep cells in the G1or G2 phases of the cell cycle. Finally, in yeast PP1s modulate sporulation and glucose repression (Santangelo, 2006; Stark, 1996 and 2004).

In this respect there are two yeast protein phosphatases that may participate in the regulation of Pma1: the major PP1 Glc7 has been connected with ion homeostasis (Williams-Hart *et al*., 2002), and the PP2A Sit4 controls yeast growth in a similar way

that Pma1, promoting progression through the START or restriction point of the G1 phase of the cell division cycle (Cid *et al*., 1987; Sutton *et al*., 1991; Johnson and Skotheim, 2013; Dechant *et al*., 2014).

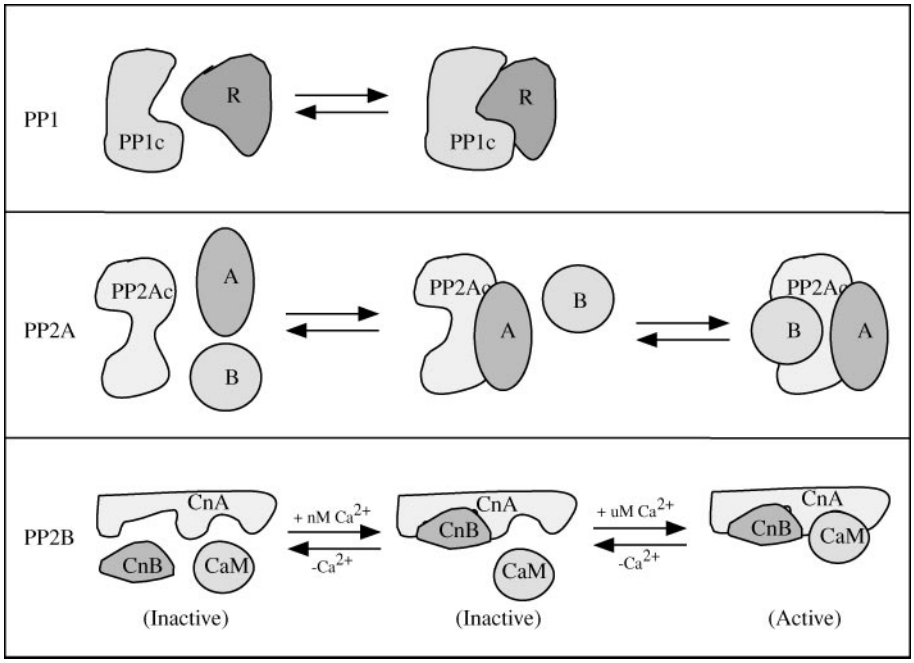


Figure 17. Subunit composition and regulation of PPP family enzymes. PP1 holoenzymes are heterodimers composed of catalytic PP1c and regulatory R subunit. PP2A holoenzymes exist in either heterodimer or heterotrimer form the C-subunit (PP2Ac), and a scaffolding protein, the A subunit. The substrate specificity of PP2A as well as its intracellular localization are defined by an array of distinct groups of B-type regulatory subunits that bind to the AC dimeric core to form a variety of heterotrimeric complexes (Mumby and Walter, 1993). PP2B is often present in a heterodimer form with low activity (catalytic CnA and regulatory CnB) under "resting" levels of calcium. Elevated levels of calcium recruit calmodulin (CaM) into the heterodimer to form highly active trimeric enzymes (Luan, 2003).

Glc7 protein phosphatase

In yeast, Glc7 is a Ser/Thr PP1 that was first discovered upon examination of glycogen synthase activity in a collection of glycogen-deficient mutants, *glc1-glc8*, where the *glc7* mutant strain had the greatest effect. Upon cloning and sequencing it was found that *GLC7* encodes a major PP1 enzyme and the decreased protein phosphatase activity in the *glc7* strain was partly restored by transformation with a plasmid carrying the *GLC7* gene (Feng *et al*., 1991). Glycogen synthase is activated by dephosphorylation (Rothman-Denes and Cabib, 1971) and decreased Glc7 activity impairs the activation of glycogen synthase. The *GLC7* gene has 1,464 bp with a 525-bp intron after codon 59. There is a single copy of *GLC7* in *S. cerevisiae*, its mRNA length is 1.4-kilobase and it increases 4-fold at the end of exponential growth, suggesting that

activation of glycogen synthase is mediated by increased expression of PP1 as cells reach stationary phase (Feng *et al.*, 1991)**.** *GLC7* deletion is lethal yet the essential function is not the regulation of glycogen synthase because disruptions in both known genes encoding this enzyme still produce a viable mutant. More experiments are required to identify targets of the Glc7 phosphatase in *S. cerevisiae* .

Glc7 has an important role in regulating the expression of amino acid biosynthetic genes in yeast cells, through dephosphorylation of eIF-2α (see Figure 15). In *S. cerevisiae*, amino acid starvation, or a defective aminoacyl-tRNA synthetase, leads to increased transcription of more than 30 genes encoding amino acid biosynthetic enzymes, in a mechanism known as "general amino acid control" or "general control of nitrogen metabolism" (GCN). The immediate effector of the general control response is the Gcn4 protein, a transcriptional activator that binds upstream of all the amino acids biosynthetic genes that are subject to the general control. Expression of Gcn4 itself is regulated by amino acid availability; on amino acid starvation Gcn2 stimulates Gcn4 expression by phosphorylating eIF-2α and increasing translation of its mRNA (Hinnebusch, 2005).

Some important regulatory subunits of Glc7 and their functions (between brackets) are: Gip1 (sporulation), Reg1 (glucose repression), Gac1 and Pig1 (glycogen metabolism), Scd5 (actin organization) and Reg2 and Sds22 (cell cycle progression) (Santangelo, 2006). The glycogen accumulation defect of *glc7-1* is due at least in part to the inability of the mutant protein phosphatase to interact with its regulatory subunit Gac1 (Stuart *et al.*, 1994). A short motif present in most known regulatory subunits is very important in the interaction with a hydrophobic groove in PP1 (Egloff *et al*., 1997). The multiple regulatory subunits compete for Glc7 binding in vivo and almost all of them interact with more than one site on the PP1 surface (Wu and Tatchell, 2001). The Glc7 localization pattern dynamically changes throughout the yeast cell cycle due to the effect of the regulatory subunits (Bloecher and Tatchell, 2000).

Sit4 protein phosphatase

Yeast has two PP2A catalytic subunits, Pph21and Pph22 and three PP2A-like catalytic subunits: Sit4, Pph3 and Ppg1. PP2As are involved in a lot of cellular processes, including signal transduction, metabolism, cell cycle progression, gene expression, and protein translation (Stark, 1996 and 2004; Castermans *et al.*, 2012). Sit4 promotes progression through G1, via regulation of G1 cyclin production (Sutton *et al*., 1991). The regulatory subunits of Sit4 include several members of the SAP family (Luke *et al*., 1996) and a novel regulatory subunit termed Tap42 in yeast (Di Como and Arndt, 1996). This regulatory subunit interacts with Sit4 when TORC1 is active and it disassociates from the phosphatase when TORC1 is inhibited (Di Como and Arndt, 1996) (see Figure 13). The interaction of Sit4 with Tap42 is required for the function of Sit4 (Wang *et al*., 2003).

OBJECTIVES

OBJECTIVES

Taking in consideration the background information presented at the introduction, the objectives of the present thesis are as follows:

1- Determine if Glc7, the major PP1 protein phosphatase of yeast and also implicated in ion homeostasis, has a role in the activation of yeast plasma membrane H^+-ATPase (Pma1) by glucose

2- Determine if Sit4, a PP2A protein phosphatase implicated in yeast growth, has a role in the activation of Pma1 by glucose

3- Investigate the participation of the growth-promoting protein kinase TORC1 in the activation of Pma1 by glucose

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Materials

1.1. Strains and culture conditions of bacteria

Strain DH5α of *Escherichia coli, E. coli,* was used for the isolation and propagation of plasmids. Standard methods were used for bacterial culture and manipulation (Sambrook and Russell, 2001). Cells were grown at 37°C in LB (Luria Bertani) medium (0.5% yeast extract, 1% tryptone, 1% NaCl adjusted to pH 7 with NaOH). If solid medium was required 1.5 % agar was added. For plasmid selection, 50 µg/ml ampicillin was added and if applicable: X-Gal (isopropyl β-D-1-thiogalactopyranoside) and IPTG (isopropyl β-D-1-thiogalactopyranoside) were also added to a final concentration of 10 µg/ml.

1.2. Strains and culture conditions of yeast

Strains of *Saccharomyces cerevisiae* used in this work are represented in **Table 1**.

Collection of R. Serrano	Genotype	Reference
RS-58	BWG1-7A *(MATa ade1-100 ura3-52 leu2-3112 his4-519)*	Guarente *et al.*, 1982
	RS-58 /YEp24	G. Hueso
	RS-58 /YEp24-*GLC7'*	G. Hueso
	RS-58 /YEp352	This study
	RS-58 /YEp352-*GLC7'*	This study
RS-132	RS-58 transformed with *LEU2* fragment	R. Serrano
	RS-132 / pUN50	This study
	RS-132 / pUN50-*GLC7'*	This study
	RS-132 / YEp352	This study
	RS-132 / YEp352-*GLC7'*	This study
RS-259	BY4741 *(MATa ura3-Δ0 leu2-Δ0 his3-Δ1 meth15-Δ0)* (derived from S288C)	Euroscarf
RS-119	RS-259 / PCM262	G. Hueso
RS-120	RS-259 / PCM262-*GLC7'-HA3-His6*	G. Hueso
RS-121	RS-259 / PCM262-*GLC7'*	G. Hueso

	RS-259 / pYEX 4T-1	This study
	RS-259 / pYEX 4T-1-*SIT4*	This study
RS-1373	*KT1112 (MATa ura3-52 leu2 his3)*	Stuart *et al*., 1994
RS-1376	RS-1373 *glc7-109*	Williams-Hart *et al*., 2002
RS-1377	RS-1373 *glc7-132*	Williams-Hart *et al*., 2002
RS-1379	RS-1373 *glc7-1*	Stuart *et al*., 1994
RS-624	BY4741 *GCN2^{c}*	Menacho-Marquez *et al.,* 2007
	BY4741 *Sit4Δ*	Euroscarf
SH221	*MATa leu2-3,112 trp1 ura3 rme1 his4 HMLa ade2 his3 HIS4 tor1::HIS3 tor2::ADE2 / YCplac111::tor2-21^{ts}*	Helliwell *et al*., 1998
SH100	*MATa leu2-3,112 trp1 ura3 rme1 his4 HMLa ade2 tor2::ADE2 / YCplac111::TOR2*	Helliwell *et al*., 1998

Yeast cells were cultured and manipulated following standard methods (Sherman, 1991). Temperature was 28°C unless otherwise mentioned, with shaking (180 - 200 rev/min) in the media described below:

Minimal medium (SD)

Synthetic Dextrose: 2% glucose, 0.7% Yeast Nitrogen Base (YNB) without amino acids, 50 mM MES-Tris pH 5.5. Whenever required, amino acids (0.10 mg/ml methionine, 0.10 mg/ml leucine, 0.03 mg/ml histidine) and nitrogen bases (0.03 mg/ml adenine, 0.03 mg/ml uracil) were added.

Minimal complete medium (SCD)

Synthetic Complete Dextrose: 2% glucose, 0.7% YNB, 50 mM MES-Tris pH 6. This medium contains all amino acids and nitrogen bases except when there is a need for plasmid selection, then some of them such as uracil or leucine were removed.

YPD

Rich (complete) medium: 1% Yeast extract, 2% Bacto Peptone, 2% glucose. Agar 2% is added whenever solid media was required.

1.3. Plasmids

All plasmids used in this study are listed in **Table 2:**

Plasmid	Description	Reference
YEp24	Amp^r, 2μ, *URA3*	Botstein *et al.*, 1979
YEp352	Amp^r , 2μ, *URA3*	Hill *et al*., 1986
pUN50	Amp^r , CEN, *URA3*	Elledge and Davis, 1988
pCM262	Amp^r , 2μ, *URA3*	Ariño and Herrero, 2003
pYEX 4T-1	Amp^r	Amrad Biotech (Ward *et al*., 1994)
pGEX-3X	Amp^r	Smith and Johnson, 1988
pCM262-*GLC7'-HA3-His6*		G. Hueso (see methods)
pCM262-*GLC7'*		G. Hueso (see methods)
pGEX-3X-*GLC7*		Rodriguez-Hernandez *et al*., 2012
YEp24-*GLC7'*		Hueso *et al*., 2012
YEp352-*GLC7'*		This study
pUN50- *GLC7'*		This study
pYEX 4T-1-*SIT4*		This study

The restriction maps of several plasmids utilized in the present study are shown in Figures (18-19-21-22)

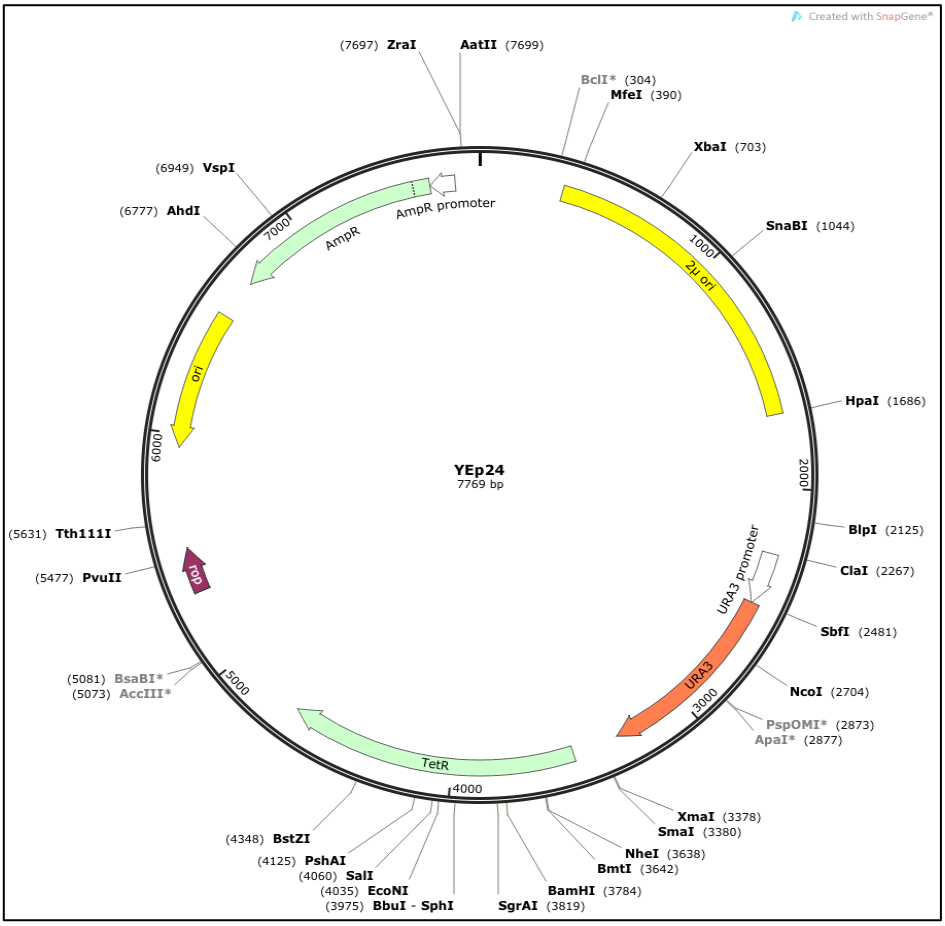


Figure 18. Restriction map of YEp24, an episomal (2µ origin, high-copy number) vector with a *URA3* marker for selection in yeast. Confers ampicillin resistance for selection in *E. coli*. (Botstein *et al.*, 1979). http://www.snapgene.com/resources/plasmid_files/yeast_plasmids/YEp24/

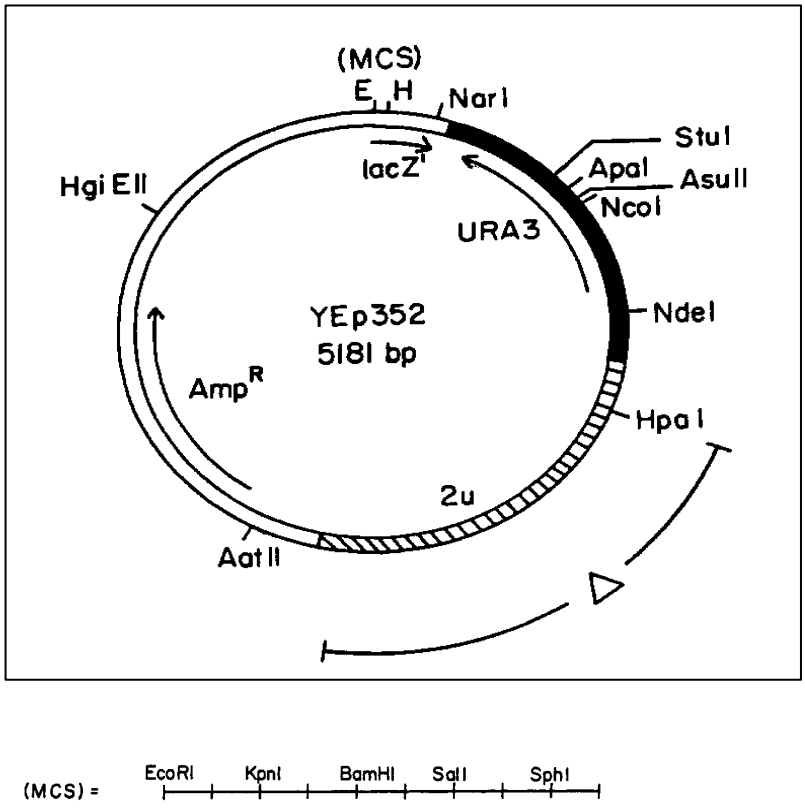


Figure 19. Restriction map of YEp352. An episomal (2µ origin, high-copy number) cloning vector with a *URA3* marker for selection in yeast. Contains the β-lactamase gene that confers ampicillin resistance for selection in *E. coli* (Hill *et al*. 1986).

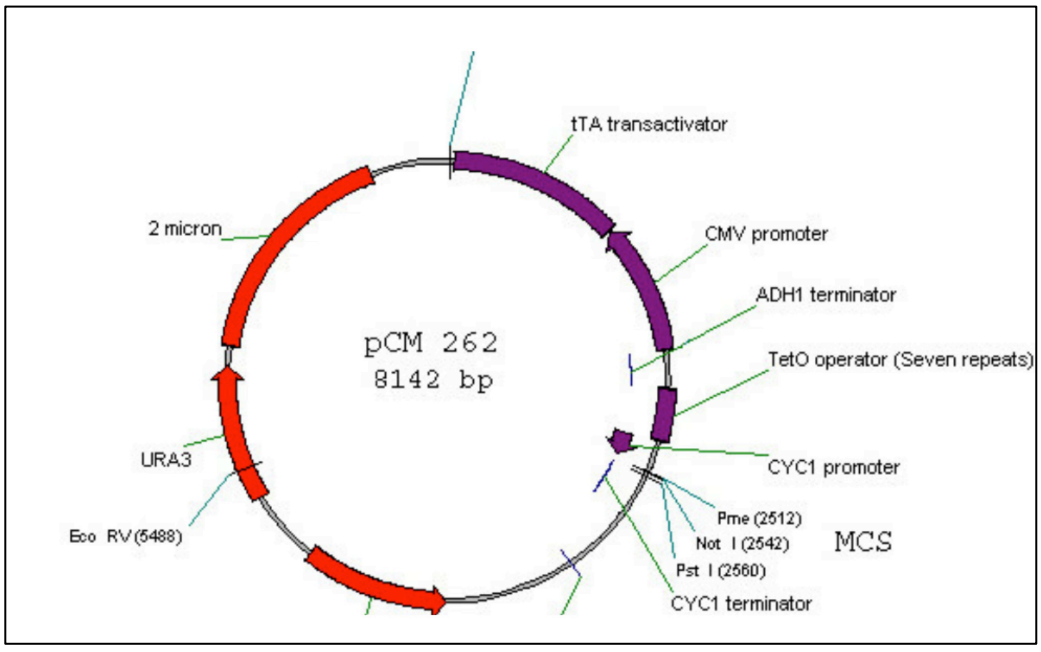


Figure 20. Restriction map of pCM262, an episomal (2µ origin, high-copy number) plasmid used for expression and isolation of recombinant proteins in yeast. It contains a cloning site that allows cloning a gene in reading frame with six histidine and three copies of epitope HA under control of a doxycycline regulated promoter (Ariño and Herrero, 2003).

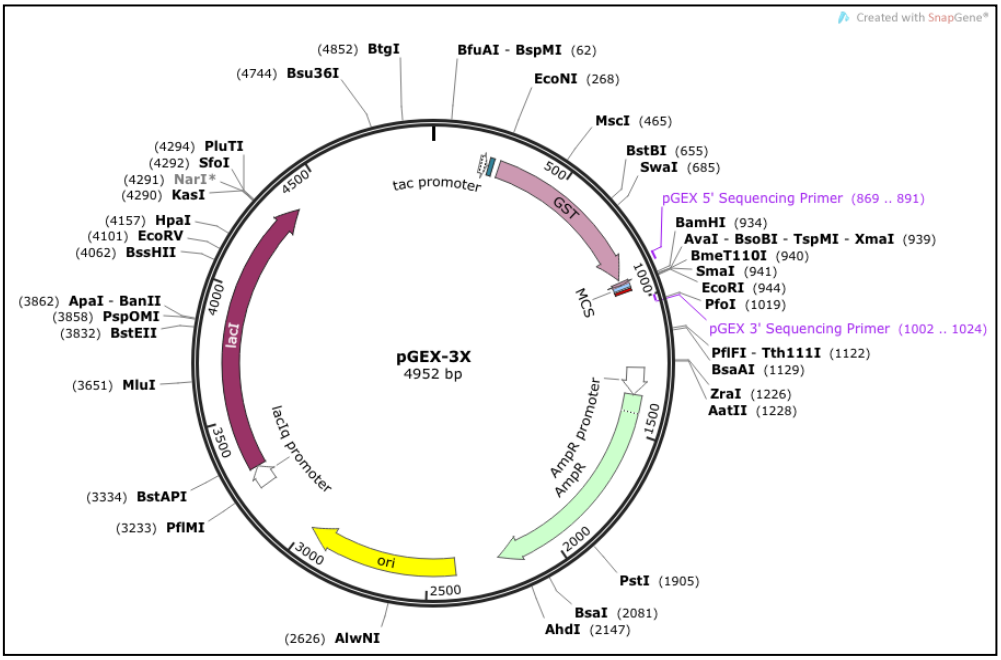


Figure 21. pGEX-3X is a GST-fusion vector for expression in *E. coli* under control of *tac* promoter and with ampicillin resistance for selection (Smith and Johnson, 1988).
http://www.snapgene.com/resources/plasmid__files/pgex_vectors_%28ge_healthcare%29/pGEX-3X/

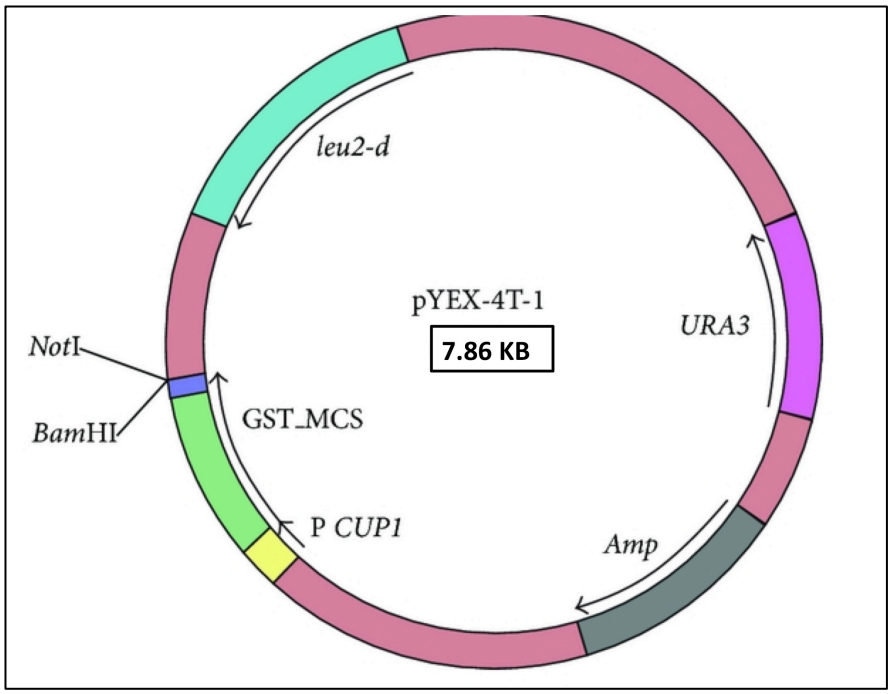


Figure 22. pYEX 4T-1 is a GST-fusion vector for protein expression in yeast under control of the Cu^{2+}-inducible *CUP1* promoter. The GST coding region encodes a protein of approximately 27.5 kDa. It includes the *E. coli* Amp^r gene and the yeast selectable marker *URA3*. It is maintained at high copy number by *leu2-d* (a *LEU2* gene with a truncated, inefficient promoter) during growth selection on media lacking leucine (Ward *et al.*, 1994).
http://apps2.bvl.bund.de/vectorwww/protected/main/vector.do?method=detail&theId=438&d-49653-p=9

2. Isolation of yeast membranes and "in vitro" determination of Pma1 activity

(Modified from Serrano, 1983 and 1988)

2.1. Isolation of yeast membranes

2.1.1. Culture preparation

A single yeast colony was inoculated in 2 ml medium and grown to stationary phase at 30°C with shaking at 200 rev/min. This culture was stored in the cold and used as inoculum. A flask of 250 ml with 50 ml medium was inoculated with the saturated culture (50-500 µl) and incubated at 30°C with shaking at 200 rev/min to reach exponential phase or stationary phase as specified in each experiment.

2.1.2. Membrane isolation

The 50 ml culture (50-100 mg FW cells) was centrifuged during 5 min at 3,000 rev/min in a Nahita 2655 centrifuge and resuspended in 25 ml sterile water, centrifuged again and finally resuspended in 2 ml sterile water. The concentrated and washed cells were incubated for 15 min at 30°C and 1 ml was taken and frozen in liquid nitrogen (Glucose Starved or GS sample). Then 2% final glucose was added to the other 1 ml which was then incubated for 10 min at 30°C and frozen in liquid nitrogen (Glucose Fermenting or GF sample). For cell homogenization, 200 µL of protein extraction buffer 5x (0.25 M Tris- HCl pH 8, 0.3 M KCl, 25 mM EDTA, 10 mM DTT and protease inhibitor cocktail from Roche) was added to 1 ml cell suspension, 1.5-2 ml of glass beads (0.5 mm diameter) were added and vortexed for six cycles of 30 sec vortex and 30 sec incubation in ice. Cell debris was removed by centrifugation of the homogenate 2 min at 3,000 rev/min at 4°C in an Eppendorf R5426 centrifuge and a membrane fraction was obtained from the supernatant by centrifugation 20 min at 13,000 rev/min and 4 °C in the same refrigerated centrifuge as before. The pellet was resuspended with a Dounce-type manual homogenizer in 100 µL of cold GTED 20 solution (20% glycerol, 10 mM Tris HCl pH 7.6, 1 mM EDTA and 1 mM DTT) and diluted with 900 µL cold distilled water to lyse the membranes and wash free of inorganic phosphate. After a final centrifugation of 30 min at 13,000 rev/min at 4°C membranes were resuspended in 150 µL cold GTED 20, divided into several micro tubes and kept at -80ºC.

2.2. Protein determination by the method of Bradford (1976)

In a microtiter plate, duplicates of 0, 2, 4, 6, 8,10 µL of the standard bovine-ɣ-globulin 1 µg/µL were placed in the wells. Triplicates of the membrane preparations (2-10 µL) were also placed. Then 150 µL of the freshly diluted 5X BioRad Protein assay reagent (BioRad) was added to each well, gently mixed and left 25 minutes for the blue color to stabilize. Finally, the absorbance was measured at 595 nm using an iMark™ Microplate Absorbance Reader (BioRad). Protein concentration in the membrane preparations was calculated from the calibration curve.

2.3. Determination of Pma1 activity

Triplicates of 3-10 µg protein in less than 5 µL of yeast membranes were placed in two rows of wells of microtiter plates. The reaction was started in one row by adding preheated (10 min, 30°C) 65 µL of ATPase reaction buffer (50 mM MES-Tris pH 5.7, 5 mM $MgSO_4$, 50 mM KNO_3, 5 mM sodium azide, 0.3 mM ammonium molybdate, 2 mM ATP, the latter added at the moment of use). To the other row, the same preheated buffer but containing 0.15 mM sodium orthovanadate (Na_3VO_4) was added. The plate was incubated (30 min, 30°C). The reaction with terminated by adding 130 µL of phosphate reagent (2% H_2SO4, 0.5% ammonium molybdate, 0.5% SDS, 1% ascorbic acid, the latter added at the moment of use). As standard, duplicates were prepared with 0, 2, 4, 6, 8, 10 and 20 µL of 5 mM inorganic phosphate (Pi) to which ATPase reaction buffer was added followed by phosphate reagent in the same manner as the tested samples. The microplate was left for 25 minutes for the blue color to stabilize and then the absorbance was measured at 750 nm using an iMark™ Microplate Absorbance Reader (BioRad). Pi concentration was calculated from the calibration curve with standard Pi. Pma1 activity was defined as the vanadate-sensitive component of the total ATPase activity and it corresponded to 80-90 % of the latter.

3. Assay for Pma1 activity in membrane preparations of cells treated with PP1 inhibitors

3.1. Treatment of yeast cells with the phosphatase inhibitor

S. cerevisiae strain RS-58 was used for this experiment and it was grown in YPD medium to stationary phase. Cells were washed, concentrated and preincubated in H_2O as

described above and then protein phosphatase 1 inhibitors were added as described below. The stock solutions were: okadaic acid (MW= 805) 0.1 mM in methanol, calyculin A (MW= 805) 0.2 mM in methanol and tungstate (MW= 330) 1 M in water. Harvested and washed cells were divided into 4 portions, 1ml each. Two portions did not receive any inhibitor, and glucose was only added to one of them and processed as described above. To the other two portions, one inhibitor was added (final concentration 0.5 μM okadaic acid, 1 μM calyculin A and 10 mM tungstate) together with glucose to one portion and without glucose to the other one. Then all samples were incubated for 20 min at 30°C and Pma1 activity determined as described above. Okadaic acid was obtained from Kamiya Biomedical Company (Seattle, USA), calyculin A from LC Laboratories (Woburn, MA, USA) and tungstate from Fisher Scientific (Madrid, Spain).

3.2. Growth inhibition test

To detect the effect on yeast growth of every phosphatase inhibitor, an stationary phase culture of RS-58 was used to inoculate 2 ml of YPD (initial OD of about 0.1) and okadaic acid (0, 0.1 and 0.5 μM, calyculin A (0, 2 μM) or methanol (as a control) were added. All tubes were incubated (24 hrs, 200 rev/min, 30°C) and OD was measured at 660 nm.

4. Assay for yeast tolerance to toxic cations

Serial dilutions of stationary phase cultures of the specified strain were spotted (about 3 μL) with either a replicator (Sigma) or by micropipetting on a plate of solid medium with the indicated toxic cations (see Goossens *et al*., 2000). Plates were incubated for 2-3 days at 28°C.

5. Purification and manipulation of nucleic acid

5.1. Isolation of plasmid DNA from *E. coli*

A modified small-scale alkaline lysis method (Birnboim, 1983) was employed. 1.5 ml of overnight culture in LB with amplicillin was pelleted by centrifugation for 1 min at 13,000 rev/min in an Eppendorf D5425 centrifuge. Pellet was resuspended in 200 μL of GTE buffer (50 mM glucose, 25 mM Tris, pH 8, 10 mM EDTA, pH 8). 300 μL of freshly prepared solution with 0.2 N NaOH and 1% SDS was added, mixed by tube inversion and left on ice for 5 min. Then 300 μL of 3.0 M potassium acetate taken to pH 4.8 with acetic acid was added, mixed by inversion and left on ice for 5 min. The tube was centrifuged for

10 min as before and the supernatant transferred to a new tube. RNAse A was added to 20 µg/ml and incubated 20 min at 37°C. A chloroform extraction was made using 2 volumes of organic solvent, mixed by shaking then spinned for 1 min in top speed and the aqueous phase saved to a new tube. DNA was precipitated by adding an equal volume of isopropanol, incubation in ice for 1 hour or more and centrifuged 10 min at top speed. The pellet was washed with 500 µL of 70% etanol, which was then poured after centrifugation for 1 min. Finally the pellet was dried in a vacuum centrifuge and resuspend in 25 µL TE buffer.

5.2. Isolation of yeast genomic DNA

A modified small-scale protoplast method (Winston *et al*., 1983) was employed. Yeast was inoculated in 5 ml YPD and incubated till reaching late exponential phase (OD at 660 nm= 1-2 measured in a DINKO SP8001 spectrophotometer, Barcelona, Spain). Cells were harvested by centrifugation 5 min at 3,000 rev/min (Eppendorf D5425 centrifuge), resuspended in 1 ml SoE solution (0.9 M sorbitol, 0.1 M EDTA, pH 8 with NaOH), centrifuged again and resuspended in 200 µL SoE to which 10 µL of DTT 0.5 M and 25 µL 1% zymolyase 100T (Miles) were added, mixed and incubated 30 min at 37°C to make protoplasts. Then 500 µL of TCES (0.2 M Tris, 0.2 M NaCl, 50 mM EDTA, 2% SDS adjusted to pH 8 with HCl), 2 µL β-mercaptoethanol and 4 µL of 2% protease K were added and incubated 30 min at 65°C. Protoplasts were extracted with 0.7 ml PCI (phenol: chloroform: isoamyl alcohol, 25: 24: 1) followed by centrifugation 5 min at 11000 rev/min. DNA was precipitated from the aqueous phase by adding an equal volume of isopropanol and incubation in ice for 15 min or more. The pellet was washed with 500 µL 70% etanol which was then poured after centrifugation. The pellet was resuspended in 400 µL H_2O with 5 µL RNAase 1% and incubated 30 min at 37°C. Then extraction was made with an equal volume of PCI and centrifugation to take the aqueous phase. DNA was precipitated by adding 1 ml etanol:ammonium acetate 6:1 (6 volumes of absolute etanol+ 1 volume ammonium acetate 7.5 M) and incubation in ice for 15 min or more. After centrifugation during 15 min at 13,000 rev/min, the pellet was washed with 1 ml 70% ethanol which was then poured after centrifugation. The pellet was dried by leaving the tube open in RT for 20 min and resuspended in 30 µL TE by shaking for about 30 min.

5.3. DNA electrophoresis

To detect the integrity of isolated genomic DNA and the result of PCR or restriction endonuclease reactions, we made an electrophoresis of DNA. An agarose gel at 0.7% was prepared in electrophoresis buffer TBE 0.5X (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA pH 8). The electrophoresis buffer and also the gel buffer contained 0.5 µg/ml ethidium bromide. Before injecting the samples into the wells they were mixed with loading buffer 6X (0.25% bromophenol blue, 40 % sucrose, 0.1 M EDTA). Markers used were 1KB or λ DNA digested with *Hind* III (Invitrogen). Bands were detected under UV light of 254 nm wavelength.

To purify DNA fragment from preparative gels a "NucleoSpin DNA purification" kit (Macherey-Nagel) was used.

5.4. Preparation of RNA from *S. cerevisiae*

Steps were basically according to (Li *et al.,* 2009).

5.4.1. Culture preparation

To detect the expression of *GLC7'* in strain RS-121 after doxycycline removal, an exponential phase culture of 15 ml was prepared in medium with doxycycline as described above. Cells were harvested by centrifugation (1 min at 13,000 rev/min), washed with water twice and resuspended in the same volume of medium. Then divided into 4 portions of 5 ml and 2 µg/ml doxycycline final concentration was added to two of them. Two tubes with doxycycline and two tubes without were incubated for 1.5-2 h before RNA isolation.

5.4.2. RNA Isolation

Cells were harvested by centrifugation (1 min at 13,000 rev/min), resuspended in 100 µL RNA extraction buffer (50 mM tris pH 6, 10 mM EDTA, 5% SDS) and incubated (5 min, 65°C). Then 50 µL of K buffer (KCl 0.3 M + 10 mM MES –Tris pH 6) were added to precipitate denatured proteins and SDS, mixed thoroughly and the tube incubated 5 min in ice. After centrifugation (5 min, 13,000 rev/min) the supernatant was transferred to new microtubes and an equal volume of acidic phenol was added and well mixed for phase partition. To remove any remaining proteins, another centrifugation (5 min, 13,000 rev/min) was made and the upper phase was taken, to which 0.1 volume of NaAc 3M pH 5.2 and 2.5 volumes absolute ethanol were added followed by incubation (15 min at -20°C). RNA was precipitated by centrifugation (10 min, 13,000 rev/min), the pellet was

washed with 70 % ethanol which was then removed after centrifugation. The microtube was left open in the air to dry the pellet which was finally resuspended in 20 µL RNAase-free water and stored at -80°C till further processing.

5.5. RNA gel electrophoresis

To test the integrity of the RNA preparation samples were applied to a 1% agarose gel prepared in TAE buffer (40 mM Tris-acetic acid pH 8.0 and 1 mM EDTA). The samples and the ladder standard (0.5-10 kb RNA ladder) were prepared with loading buffer 1.25X [12.5% MAE 10X (0.2 M MOPS, 50 mM Na Acetate, 10 mM EDTA, pH 7), 55% formamide, 20% formaldehyde, 8% glycerol, 0.05% bromophenol blue, 0.001% ethidium bromide] and incubated (10 min, 56°C) before loading into the wells. The electrophoresis was run in TAE buffer.

5.6. Synthesis of cDNA

The extracted RNA was purified with a NucleoSpin RNA kit and checked again for integrity. This purification also eliminated contaminating DNA in the RNA samples by a treatment with DNAase when the RNA is bound to the silica gel plate

The RNA eluted from the silica gel plate (20 µL) was mixed with 6 µL of 5X Reaction Mix and 2 µL of Maxima Enzyme Mix (Thermo Scientific) in a final reaction volume of 30 µL and incubated in two steps, first for 10 min at 25°C then for 30 min at 50°C. Finally the reaction was inactivated by heating at 85°C for 5 minutes.

5.7. PCR

For PCR or semi-quantitative RT-PCR, the following PCR reaction mixture was made:

- 0.5 µL cDNA (see above) or 10 ng DNA template
- 2 µL PCR buffer 10X (with 1.5 mM MgCl2)
- 2 µL dNTPs 10 mM
- 0.5 µL Forward primer 20 µM
- 0.5 µL Reverse primer 20 µM
- 0.5 µL polymerase (Taq) 1U/ µL
- Complete to 20 µL with sterile miliQ H_2O

For reactions that required DNA amplification of high fidelity, the enzyme Phusion High-Fidelity DNA polymerase (New England Biolabs) was applied with all the conditions and PCR program mentioned in the company booklet.

All the PCR reactions were made in Mastercycler personal (Eppendorf), those with Taq received the following PCR program:

- 1 cycle of initial denaturation at 95°C for 5 min
- 25 cycles, for semi-quantitative RT-PCR, or 30-35cycles, for normal PCR, of:
    - Denaturation at 95°C for 30 sec
    - Hybridization for 30 sec at an annealing temperature 2 degrees lower than the Tm of the primers
    - Extension at 72°C for 1 min for 1 KB.
- 1 cycle of final extension at 72°C for 3-5 min
- Hold at 4°C

Table 3: Primers used for PCR reactions. See Figure 23 for explanation.

Name	Sequence (restriction sites underlined)	Product size (kb)
GLC7'c F	CCGGAATTCATTTCTTGTGAGCACAACCTC	1.6
GLC7'c R	CGCGGATCCTTATGGATCTGACCACAATAAGTCA	
GLC7'd, e F	TATGCGGCCGCTATGGACTCACAACCAGTT	1.1
GLC7'd R	ATAAGCGGCCGCCTAGATCTGTTCCATACTATT	
GLC7'd, e F	see above	1.1
GLC7'e R	ATAAGCGGCCGCGATCTGTTCCATACTATT	
GLC7'f F	GAGGGAACCATGAATGTGCTTC	0.23
GLC7'f R	CTGGTGATCCGTCGACCTGCA	
UBC6 F	CGGCAAATACAGGTGATGAAAC	0.11
UBC6 R	TTCAGCGCGTATTCTGTCTTC	
SIT4 F	GCGGAATTCATGGTATCTAGAGGCCCCG	1
SIT4 R	GCGCTCGAGGTTATAAGAAATAGCCGGCTC	

For semi-quantitative RT-PCR, a cDNA from wild type yeast, non-transformed with a *GLC7'* plasmid, was used to check the primer specificity. Other than *GLC7*' primers, *UBC6* primers were applied on the samples cDNA for another PCR reaction mixture as a control. *UBC6* is a house-keeping gene with constant levels of expression. RT-PCR product samples were checked by DNA electrophoresis using 2% agarose gel. The length of the amplified sequence is 227 bp for *GLC7'* and 107 bp for *UBC6.*

5.8. Plasmid construction

GLC7'c (truncated *GLC7* under control of its own promoter) of 1.6 kb was produced by PCR using yeast genomic DNA as a template and primers *GLC7'c* F and *GLC7'c* R, containing sites for *Eco*RI and *Bam*HI, respectively (Table 3). The fragment of 1.6 kb was

inserted into centromeric plasmid pUN50. An episomal version was produced by blunt end ligation with plasmid YEp352 after cutting with *Sma*I.

Starting from the 1.9 kb *GLC7'* fragment isolated by Hueso *et al*. (2012), two 1.1 kb fragments containing the ORF of the truncated gene (*GLC7'd* and *GLC7'e*) were amplified with Phusion High-Fidelity DNA polymerase (New England BioLabs) and primers *GLC7'd, e* F and *GLC7'd* R (for *GLC7'd* gene) and primers *GLC7'd, e* F and *GLC7'e* R (for *GLC7'e* gene). All primers contained *Not*I restriction sites and in the first case the *GLC7'd* ORF contained a stop codon but in the second (*GLC7'e*) there was no stop codon. The amplified fragments were digested with *Not*I and cloned into the *Not*I site of plasmid pCM262, resulting in doxycycline-regulated expression of the simple truncated protein (*GLC7'd*) or expression of a C-terminal in-frame fusion with 3 repetitions of the HA antigen and 6 histidine codons (*GLC7'e*) (Ariño and Herrero, 2003).

SIT4 was produced by PCR using yeast genomic DNA as a template and primers *SIT4*F and *SIT4R*, containing sites for *Eco*RI and *Xho*I, respectively (Table 3). The fragment (≈1 kb) was inserted into plasmid pYEX 4T-1 to be expressed in yeast.

There were many previous trials to express Sit4 in *E. coli* in the soluble fraction but in all of them the protein always was produced in the inclusion bodies. The *SIT4* fragment was inserted into plasmid pGEX-KG. Because of the slight difference in the reading frame of the two plasmids (pGEX-KG and pYEX 4T-1) the forward primer was different: *SIT4* F GCGGAATTCTAATGGTATCTAGAGGCCCCG while the reverse primer was the same. Then several trials were made, using different IPTG concentrations and induction temperature and times (37°C, 0.4 mM IPTG for 17, 30, 40, 50, 60, 90 minutes and 20°C, 0.4 mM IPTG with sorbitol 0, 500 and 333 mM for 17, 30, 45 minutes and 1, 2, 3, 4, 5 hours). We tried also to transform the plasmid to another strain (BL21) by electroporation; again the expressed Sit4 was in the inclusion bodies. The second construct was by cutting *SIT4* from pGEX-KG and putting it directly on pMAL plasmid, then trying to express Sit4 under different conditions (37°C, 0.4 mM IPTG for 0.5, 1, 2 hours; 22°C, 0.4 mM IPTG for 0.5, 1, 2 hours) induction occurred at both temperature tested but never in soluble proteins.

TGATCCTATTACATTATCAATCCTTGCGTTTCAGCTTCCACTAATTTAGATGACTATTTC
TCATCATTTGCGTCATCTTCTAACACCGTATATGATAATATACTAGTAACGTAAATACTA
GTTAGTAGATGATAGTTGATTTTTACTCCAACAAAAGAATTACAGTCAGTGGCTGTTTGC
TGACATTTCATTGCTTCCCTTACAACCTGATTTCCATTCGAGTTGAAAGGTTGAGAAAAA
TTGCAAGACTTCTCTCTCATCTACCTTCCCCTTTTCTTGAAGACAAGGTGAGAGACAATT
TAGTACTAAAGCTTTTGCGGTTGTTATTCAATTGAGGTAGATACCTGGCAAAACATTTCT
TGTGAGCACAACCTCAATTAAAGTTAGACAAGTAGGTGCACTATTGGTTGCTTGTTGGCT
CATCTCGTTGAGGAATGTAATAACTACTTGTTATTAACCTGTTTTTGTGCCATCTATAGT
GGAGAGCTTATTGCAATTTGTTTTTTATTTCTTGACTGCATATATCAGTCTTTGACAGGC
TGCATGGGGATGACAGTTAGAAACTAGCCAAATTACCCCTTATGTAGATAACAATCATTG
CTTATTCGCTCTTCCCCCATTTTTTTTCTTGCTCTTGCTGTTTTTTCTTTTAGCGTTCGT
TTCAAGGAACAAGAGAGGGAAAAAAAAATCAAAAGTAGAAAAGAAGAAGAAAAAAACAAC
GTAACACAAGTTAACACCACAACTGAAAAAAAAAAATAAGAGGTGAACGAACGAGTAACTG
GGGAGAGGAAAGCAGATTACCACAATATACATTCAAATTAAAGAAATGGACTCACAACCA
GTTGACGTTGATAATATCATCGATAGATTATTGGAAGTAAGAGGATCTAAACCTGGTCAA
CAAGTTGATCTAGAAGAAAATGAAATCAGATACTTATGTTCGAAAGCCAGATCTATATTC
ATAAAGCAACCCATTTTACTAGAGTTAGAAGCCCCAATTAAAgtatgttaatgttgaact
tcgcagtcaagagatagaatgcctagagcttcaggtgttttatgtgttctactcctgttg
cggcagtaagatagttacagcagttatttccgtttgactgaaagaatttaccaagcatct
gatgttagcctactcatatgtcgagatagccgagataatgtgtgtgtttagctcttcatt
tttctatcttattaggcatttttttaccaagatttccttatgttttgtatcatcatccgg
ccggcgcctcccatattcagaaaaatccccccttgctcacactaaaaaaagaaggcatttt
ttcgttatgaagaaaacgaatctttttttttttttgagcccgagagaaaggtcggacgaaa
accaagaaatgtggatttgcagaaggcattgggagaaatgaagcgtttttgaCAGCAGTA
ATTGTTCTCTTAAAGTCAACGGAATGAGCTAGATTTAGTGACAAATTCATTTACTAACTT
ATTTTTTTCTATTTTTTTTTTTTTTTAGATATGTGGTGACATTCATGGGCAATACTATGAT
TTACTACGTCTATTTGAGTACGGTGGATTCCCGCCAGAATCTAATTATCTATTTTTGGGT
GATTATGTCGACCGTGGTAAACAATCCTTAGAGACTATTTGTCTATTACTGGCTTACAAA
ATTAAGTATCCAGAAAACTTTTTCATTTTAAGAGGGAACCATGAATGTGCTTCCATTAAT
AGAATTTACGGGTTTTATGATGAATGTAAGAGACGTTATAATATCAAACTTTGGAAAACT
TTCACGGATTGTTTCAATTGTTTACCAATTGCTGCAATTATTGATGAGAAAATCTTCTGT
ATGCATGGTGGTCTCTCACCAGATTTGAATAGTATGGAACAGATCAGAAGGGTGATGAGG
CCAACAGATATTCCCGACGTTGGCTTATTATGTGACTTATTGTGGTCAGATCCA

Figure 23. Nucleotide sequence of chromosome V from coordinates 431670 to 433643, including the truncated *GLC7'* genes of Wek *et al*. (1992; gene *GLC7'a* of 1.6 kb), Hueso *et al*. (2012; gene *GLC7'b* of 1.9 kb) and the one generated in the present work (*GLC7'c*). Small letters correspond to the intron of the gene. *GLC7'a* extends from the *Hin*d III site underlined (AAGCTT) to the end of the sequence (codon CCA for proline 208, underlined). *GLC7'b* extends from the beginning of the sequence to the underlined codon GAA (glutamate 183). *GLC7'c* has 1.6 kb from the underlined ATT (40 nt after the *Hin*d III site) to the end of the sequence (codon CCA for proline 208, underlined). *GLC7'd* is the coding region of *GLC7'b* plus two more codons, stop codon and *Not* I sites at both ends to clone into pCM262 plasmid. *GLC7'e* is the coding region of *GLC7'b* plus two more codons (but no stop codon) to fuse in frame with 3 repetitions of the HA antigen and six histidine codons. It also contains *Not* I sites at both ends to clone into pCM262 plasmid. *GLC7'f* is the 227 bp fragment used to quantify *GLC7'd* expression from the doxycycline-regulated promoter. It extends from the GAG … TTC primer sequence in the *GLC7'* gene (underlined) to the sequence in the pCM262 plasmid just after the *Not* I cloning site (beginning of 3xHA region; Ariño and Herrero, 2003). The complete ORF encodes 312 amino acids. The starting codon ATG is also underlined. See primers at Table 3.

6. Genetic transfer

6.1. Transformation of *E. coli*

The method of Inoue et al. (1990) was used. DH5α competent cells (100 µL) were mixed with 10 µl ligation reaction. The mixture was incubated for 30 minutes in ice, then (1 min, 42˚C) and finally back to ice for 2 minutes. 900 µL of LB medium was added and incubated 50 min at 37°C. Finally the cells were spread on LB agar with ampicillin for selection.

6.2. Transformation of yeast (Gietz, 2014)

6.2.1. Preparation of competent yeast cells

Fresh stationary phase culture was inoculated into YPD broth and incubated till reaching exponential phase. Cells were harvested and resuspended in 0.1M Li Ac-TE (Lithium acetate 0.1 M, 10 mM Tris-HCl pH 7.6, 1 mM EDTA) with 1/10 volume of the culture volume. Again centifuged, resuspended in 1 ml of 0.1 M Li Ac-TE and incubated 30 min at 30°C. Then 0.23 ml of 80% glycerol and 0.1 ml 1% single stranded salmon sperm DNA was added to each 1 ml volume of cells, mixed well then distributed into 100 µL aliquots and stored at -80°C till being used.

6.2.2. Yeast Transformation

The competent yeast cells were mixed with 0.1-10 µg of the specific plasmid, incubated 5-10 min at room temperature and then 600 µL of PEG-Li Ac-TE (40% (w/v) PEG 4,000 in Li Ac-TE). The mixture was incubated (30 min at room temperature, then 10 min, at 42°C), centrifuged for 10 seconds at 13,000 rev/min and cells finally resuspended in 200 µL water and spread on a plate with selective medium. The plate was incubated at 28°C for 2 or more days till the appearance of colonies.

7. Extraction, electrophoresis and detection of proteins

7.1. Extraction of soluble proteins

7.1.1. Method of boiling with Laemmli buffer

Cells from 5 ml of exponential phase culture were harvested, washed and put under the conditions of study and harvested by centrifugation during 5 min at 3,000 rev/min. The pellet was resuspended in 100 µL of 1X Laemmli sample buffer (5X: 7.5 % SDS, 0.1 M DTT, 10 mM EDTA, 30% sucrose, 0.25 mg/ml bromophenol blue, 0.3 M Tris taken to pH

6.8 with HCl), then heated 5 min at 100°C. Finally, the suspension was centrifuged during 2 min at 13,000 rev/min and the supernatant kept at -20°C until being utilized.

7.1.2. Method of NaOH (von der Haar, 2007)

Cells were harvested from 5 ml exponential phase culture as described above. Lysis buffer (0.1 M NaOH, 0.05 M EDTA, 2% β-mercaptoethanol and 2% SDS) was freshly prepared and heated at 90°C for 5 minutes before used (200 µL) to resuspend the cells. The resulting suspension was incubated 10 min at 90°C, then the lysate was brought to neutral pH by mixing well with 5 µL of 4 M acetic acid, and incubated 10 min at 90°C to increase solubilization. 50 µL loading buffer (0.25 M Tris-HCl, pH 6.8, 50% glycerol, 0.05% Bromophenol blue) was well mixed and finally the suspension was centrifuged for (1 min, 13,000 rev/min, 4°C). Denatured proteins in the supernatant were kept at -20°C until being utilized.

7.1.3. Method of TCA (Grassl *et al*., 2009)

Equal volume of 20% trichloroaceticacetic acid (TCA) was added to exponential phase culture, mixed well then kept in ice for 30 minutes before being centrifuged (8 min, 3,000 rev/min). The pellet was resuspended in 1 ml cold absolute ethanol and left in ice for 5 minutes then centrifuged (5 min, 12,000 rev/min, 4°C) and this washing step was done twice. The pellet was dried by leaving the microtube open for 20 minutes at RT or by vacuum centrifugation and was then resuspend in 1X Laemmli (200 µL) and heated 5 min at 100°C to solublize proteins. Finally, the suspension was centrifuged (2 min, 13,000 rev/min, 4°C) to remove debris and the supernatant kept at -20°C until being utilized.

7.2. Extraction of Pma1

The above methods cannot be utilized to extract Pma1 because it aggregates by boiling in Laemmli sample buffer. Instead, in the case of Pma1 the extraction of a crude membrane fraction must be made in the presence of protease inhibitors as described above and then samples treated with Laemmli sample buffer by incubation during 15 min at 42 ºC.

7.3. Electrophoresis and detection techniques of proteins

7.3.1. Electrophoresis of proteins

The denatured proteins were separated by electrophoresis in SDS-polyacrylamide gels (SDS-PAGE) using Mini-Protean 3 (BioRad). The gel consisted of two layers above each other. The upper layer (stacking gel) consisted of (3% acrylamide: bisacrylamide 30:0.8, 0.1 % SDS, 125 mM Tris-HCl pH 6.8) and the lower layer (resolving gel) consisted of (8 or 10 % acrylamide: bisacrylamide 30:0.8, 0.1 % SDS, 325 mM, Tris-HCl pH 8.8), ammonium persulphate and tetraethylmethylenediamine (TEMED) were added to both gels. Acrylamide concentration in the resolving gel was according to the protein size, 8% gel was used for Pma1 and 10% was used for eIF2α, GST-Glc7 and GST-Sit4 detection. The gel can be prepared for either 0.75 or 1 mm thickness tray. Electrophoresis buffer used was 0.19 M Glycine, 0.1 % SDS, pH to 8.3 with Tris base. 25 A current fixed all the time was applied for separation of proteins of one gel.

7.3.2. Protein detection with Coomassie Brilliant Blue R-250

After protein separation on SDS-PAGE, the gels were incubated for 15 minutes in Coomassie (0.05 % Coomassie Brilliant Blue R-250, 50 % methanol, 10 % acetic acid). The gel was then destained using a destaining solution (10% methanol, 10% acetic acid) for 1 hour up to overnight.

7.3.3. Membrane transfer

After being separated on the gel, proteins were transferred to nitrocellulose membrane or to PVDF membrane. This process was carried out in Mini-Protean 3 (BioRad) in the presence of Towbin buffer (14.4 g/L glycine, 2 ml 10% SDS, pH to 8.3 with Tris base, 20% Methanol) at 100 V for 1 hr at 4°C.

7.3.4. Direct Blue staining of the membrane

In some experiment the membrane with transferred proteins was incubated for 10 minutes in 0.008%, direct blue [(Direct Blue 71, 0.1 % in H_2O diluted with washing solution (250 ml H_2O, 200 ml absolute ethanol, 50 ml glacial acetic acid)]. Stained membrane was left in the washing solution for 10 min and after scanning it was destained by incubating in destaining solution (175 ml H_2O, 250 ml absolute ethanol, 75 ml 1 M $NaHCO_3$) for 5 minutes. Finally the membrane was neutralized with TBS-T (0.1 % Tween

20, 150 mM NaCl, 20 mM Tris-HCl pH 7.6) for 30 minutes then could be used to continue western procedures.

7.3.5. Immunodetection of the proteins transferred to the membrane

After the proteins were transferred to the membrane, the membrane was blocked by 1-5% skimmed milk in TBS-T for 1 hr with agitation. In case of applying antibodies against the phosphorylated regulatory domain of Pma1, skimmed milk was substituted with bovine serum albumin (BSA) 1% for antibody α-pST and 5% for antibody α-pS899 also in this latter case, α-pS899, the blocking time was much shorter only 5-15 minutes. The blocked membrane was incubated (overnight, 4°C or 1 hr, RT) with the primary antibody prepared in the same blocking solution to the dilution mentioned in Table 4. The membrane was washed three times for 10 minutes each with TBS-T then it was incubated with the specific secondary antibody (α –rabbit Ig-HRP) prepared in the same blocking solution to the dilution mentioned in Table 4 for 30 minutes at room temperature, followed by three washings for 10 minutes each with TBS-T. Finally the detection was done using enhanced chemiluminescence (ECL Plus Western Blotting Detection System, Amersham Biosciences) and the images were taken by the imaging system [LAS-3000 (FUJI)] or by radioactive method. When the band intensity needed to be quantified, Image Gauge software was used. In cases where it was necessary to reuse the membrane, it was incubated for 30 minutes with agitation in stripping solution (5 ml of 1 M glycine, 1 ml of 10% SDS, 19 ml H_2O, pH adjusted to 2.5 with 12 M HCl), washed once, blocked and then reincubated with any desired antibody, except α- pS899, in the same way as described above. α-Kar2 was used in combination with α-pST or α-pS899 as a control.

All antibodies used are listed in **Table 4**

Primary antibody	Dilution	Secondary antibody	Dilution	Source
α-PeIF2α	1:2000	α –rabbit Ig-HRP	1:5000	Cell Signaling Technology
α-eIF2α	1:500	α –rabbit Ig-HRP	1:8000	Tom Dever
α-pST	1:2000	α –rabbit Ig-HRP	1:5000	F. Portillo
α-pS899	1:100	α –rabbit Ig-HRP	1:5000	F. Portillo
α-Pma1	1:10000	α –rabbit Ig-HRP	1:5000	R. Serrano
α-GST	1:3000	α –rabbit Ig-HRP	1:5000	Santa Cruz biotechnology
α-Kar2	1:3000	α –rabbit Ig-HRP	1:5000	F. Portillo

8. Recombinant protein expression and purification

8.1. GST-Glc7 protein expression and purification from *E. coli* (García-Gimeno *et al*., 2003)

Saturated culture of *E. coli* containing pGEX-3x-*GLC7* (García-Gimeno *et al*., 2003; provided by Dr. Jose R. Murguía) and also of *E. coli* containing pGEX-3x, as a negative control were used to inoculate 50 ml of LB-Ampicillin (50 μg/ml) medium with 0.5 mM $MnCl_2$ and it was incubated 3 hours, 200 rev/min at 37°C (OD 0.6 - 0.8). Expression was induced by adding 0.1-0.2 mM IPTG and incubation overnight at 25°C. The culture was centrifuged (5 min, 3000 rev/min, RT) and the pellet was resuspended in 3 ml of sonication buffer (50 mM Tris-HCl pH 7.6, 0.2 mM EGTA, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 2 mM DTT, 2 mM PMSF, 2 mM $MnCl_2$). A complete mini protease inhibitor mixture 1 tablet [Roche Applied Science] was added for 7 ml. The cells were broken by sonication, three cycles for 10 seconds each at maximum intensity with 30 seconds pause and centrifuged (20 min, 15,000 rev/min, 4°C).

For purification of the soluble proteins obtained, all the supernatant was added to the washed magnetic glutathione beads (Pierce™ Glutathione Magnetic Beads) and incubated (1 hr, 4°C) with rotation, then beads were harvested, washed three times with the same buffer and resuspended in GTED 20 (100 μL) with 2 mM $MnCl_2$.

8.2. GST-Sit4 protein expression and purification from yeast

8.2.1. Check the expression of Sit4 in Soluble fraction

S. cerevisiae competent cell of strain (BY4741) was transformed with pYEX 4T-1-*SIT4*, and also pYEX 4T-1 (negative control). Transformants were cultured on plates of SD supplemented with histidine, methionine and leucine. At this stage leucine was added to the culture because if not the growth will be extremely slow. Saturated cultures of 5 different transformant colonies and one colony transformed with pYEX 4T-1 were prepared in SD supplemented with histidine and methionine, the second culture lacked leucine to increase the plasmid number and consequently the protein production. Transformants with pYEX 4T-1-*SIT4*) took 3 days to reach stationary phase while the negative control transformants took only 1 day. After that, fresh cultures of the same composition were inoculated and incubated to reach exponential phase. Expression was induced by adding 0.5 mM $CuSO_4$ to each flask and incubating for 2 hours. The cultures

were centrifuged (5 min, 3000 rev/min, RT) and The pellet was resuspended in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% glycerol, 0.1% Triton X-100, 2 mM DTT, 0.5 mM PMSF, 2 mM $MnCl_2$, complete protease inhibitor mixture (Roche). Cells were broken by the aid of sterile glass beads (0.5 mm diameter) for 3-3.5 minutes by vortexing in 30 sec. cycles separated by ice incubation. Cell debris were removed by centrifuging the homogenate (2 min, 3,000 rev/min, 4°C), soluble proteins were separated from insoluble ones by another centrifugation (20 min, 13,000 rev/min, 4°C) and taking the supernatant. Proteins were denatured by Laemmli method, and checked with antibody α-GST.

8.2.2. Soluble protein fraction purification

For purification of the soluble proteins obtained, all the supernatant was added to washed glutathione Sepharose 4B (GE Healthcare, Sweden) and incubated (1 hr-overnight, 4°C) with rotation then slurry was harvested (5 min, 2,000 rev/min, 4°C), washed three times with the same buffer and resuspended in 100 µL GTED 20 (see section 2.1.2) with 2 mM $MnCl_2$

9. "In vitro" dephosphorylation of Pma1 by Glc7 or Sit4

The purified protein fused to glutathione beads or slurry was directly distributed into 4 microtubes (20 µL each sample)

	Zero time	30 min	1 hr	2 hrs
Bead or slurry - GST	1	2	3	4
Bead GST – Glc7 or slurry GST- Sit4	5	6	7	8

GTED 20 was discarded after putting the microtubes in magnetic rack, or harvesting the slurry by centrifugation then each one received 20 µl of a mixture of (GTED 20 + 2 mM $MnCl_2$ + protease inhibitor (1x) + 4 µg activated Pma1) followed by incubation at 30°C with rotation. Directly after the specific time of incubation, reaction was stopped by protein denaturation with 20 µL of 2x mixture of (Laemmli, protease inhibitor, PMSF) added to each microtube, well mixed then incubated (10 min, 42°C). Samples were injected in polyacrylamide gel (2 µg total membrane protein per lane).

A portion of recombinant Glc7 and its control (GST) was kept without any further treatment, extracted with Laemmli to check the protein expression by staining the gel with Coomassie.

10. Measurement of pH changes of yeast suspensions induced by glucose (Serrano, 1980)

A stationary phase culture is used to inoculate 15 ml fresh culture (YPD or SD supplied with required amino acids in case of plasmid transformants) in 100 ml flask and incubated till reaching stationary phase. Cells were harvested, washed and resuspended in sterile H_2O and incubated 2 hours at 200 rev/min and 30°C for starvation. Cells were harvested and resuspended in 2 ml of 100 mM KCl and 10 mM glycylglycine, adjusted to pH 4 with 1 M HCl. Calibration pulses of 100 nmol HCl were added at the beginning and at the end of the experiment. Proton pumping of the cells was started by addition of 50 μmoles of glucose. A pH meter GLP22 (Crison) was used to record pH.

11. Investigation of the effect of inhibiting PP1 by regulated expression of Glc7' on Pma1 activity

11.1. Determination of the best doxycycline concentration to control gene expression of *GLC7'*

SD medium was supplemented with leucine, histidine, methionine and 10 μg/ml doxycycline to inhibit expression. *S. cerevisiae* strains RS-119, RS–120, RS-121 were tested for the best concentration of doxycycline to control *GLC7*' expression. Stationary phase cultures were prepared as described in section 2.1.1. These cells were washed and resuspended in sterile distilled water then served as inocula in fresh broth containing different doxycycline concentrations and the growth was determined by measuring OD at 660 nm after 18 and 22 hours.

11.2. Doxycycline removal and Pma1 assay

Stationary phase cultures of strains RS-121 and RS-120 were prepared in the same medium described in 4.1 but doxycycline concentration was only 2 μg/ml. They were used to inoculate fresh medium and incubated till reaching exponential phase. A portion of the culture, 20 ml, was separated and labeled zero time. Cells were harvested, resuspended in 0.8 ml (fresh broth with 2 μg/ml doxycycline) and incubated for 10 minutes. Doxycycline was removed from the rest of the cells by harvesting and washing twice with water as described in section 2.1.2. Cells was resuspended in SD medium without doxycycline, then distribute into three 100-ml flasks, 20 ml each. Each flask was incubated for a specific time

interval then cells were harvested, resuspended in 0.8 ml SD medium without doxycycline and incubated for 10 minutes. After the incubation period they were put in liquid N_2 or immediately homogenized as described in section 2.1.2. Protein concentration was determined and ATPase activity was assayed as described in sections 2.2 and 2. 3.

11.3. Investigation of inhibition of Glc7 by expression of Glc7' under doxycycline promoter by detecting the phosphorylation level of eIF2*α*

Exponential phase culture of strains RS-121 was obtained just as described in section 11.2, and washed as described in section 2.1.2. Cells were resuspended in SD medium without doxycycline and a zero control with doxycycline was made. Both test and the control were incubated for 1.5 hr. After the incubation time, protein was extracted with Laemmli method (section 7.1.1) and eIF2α phosphorylation levels were detected using the procedures described in section 7.

12. Dephosphorylation rate experiment

Culture preparation was carried out as described in 2.1.1. The tested strain was RS-132 transformed with YEp24, YEp24-*GLC7'*, YEp352, and YEp352-*GLC7'* and the medium was SD supplemented with adenine and histidine. Also were tested the Sit4 null mutant and its wild type RS-259 and they were grown in YPD. Cells were grown to late exponential phase. Membrane isolation was according to the method described in section 2.1.2 except that after incubation with 2% glucose for 10 minutes and taking a sample, glucose was quickly removed by centrifugation (2 min, 3,000 rev/min) and cells resuspended in the same volume of H_2O. This was followed by incubation for 10 and 20 minutes without glucose. Then the procedures of section 2. 2. were followed to determine protein concentration. Extracted Pma1 samples were denatured and subjected to electrophoresis and immunodetection as described in section 7.

13. Rapamycin effect on Pma1 activity

Culture preparation was carried out as described in section 2.1.1. The tested strain was RS-132 and grown in YPD till exponential phase. A sample, 25 ml, was taken as zero time and to the rest of the culture 500 nM rapamycin prepared in DMSO was added, and the culture incubated at 28°C and 200 rev/min. Equal volume samples were taken after 2, 3 and 5 hours incubation. The membrane was isolated from the samples by the same procedures described in section 2.1.2 except that at the step of incubation before glucose,

the resuspended pellet of all the samples (except the zero time one) received 10 μM rapamycin, approximately 25 times increased concentration to compensate for the 25 times increased concentration of cells. The Pma1 activity was assayed as described in section 2. The phosphorylation level of Pma1 in these samples was compared using Laemmli method to denature the protein as described in section 7 and applying antibodies α-ST911,2 and anti total Pma1 as control.

14. TORC1 and TORC2 thermosensitive mutant

The strains used for this experiment were SH221 *(tor1Δ tor2^{ts})* and its control SH100. First the two strains were checked for the phenotype by growing three different colonies of each strain on YPD plate and incubating at 37°C and RT. Second the growth difference was also checked in liquid medium by inoculating fresh YPD with stationary phase culture, starting OD at 660 nm = 0.2, and they were incubated at 37°C and 24°C. Growth was monitored by measuring the OD at 660 nm after 3, 6 and 24 hours.

To determine Pma1 activity "in vitro" same procedures described in section 2 were followed. Stationary phase cultures of strains SH100 and SH221 were used to inoculate fresh YPD, initial OD at 660 nm = 0.2, and both were incubated at 37°C for 6 hours. The phosphorylation level of Pma1 in these samples was compared as described in section 7 by applying α-pST. As control α-Pma1 was also applied on stripped membrane and the gel was stained with Coomassie.

15. Protein phosphatase assay (Garcia-Gimeno *et al*., 2003 and Silberman *et al*., 1984)

The reaction buffer was (50 mM Tris-HCl, pH 7.5, 0.1mM EGTA, 2 mM $MnCl_2$, and 1 mM DTT). *p*-nitrophenylphosphate disodium salt hexahydrate (PNPP) (Roche, Germany) (Mol.Wt = 371.1 g) stock solution was prepared (40 mM in phosphatase buffer) and kept at -80. The experiment was carried out by mixing 20 μL of the sample (purified GST-Glc7) or negative control (purified GST) with 180 μL of PNPP 40 mM and incubating the mixture for 10-20 min at 30 °C. The reaction was stopped by adding 1ml 0.5 M EDTA. The absorbance was measured at 405 nm using Microplate Reader. *p*-nitrophenol concentration was calculated from the calibration curve with standard *p*-nitrophenol.

RESULTS

RESULTS

Typical chemical inhibitors of protein phosphatases do not affect Pma1 activity

In order to test the role of the yeast major type 1 protein phosphatase Glc7 on the activation of Pma1 by glucose metabolism, we have first tested chemical inhibition of protein phosphatases during the activation process. If Glc7 were involved in dephosphorylation of the activating site of Pma1, addition of chemical inhibitors of the protein phosphatase could increase phosphorylation and activate the enzyme. Okadaic acid and calyculin A are cell-permeable inhibitors of animal type 1 and 2A protein phosphatases that have been shown to increase protein phosphorylation in intact animal cells (Shenolikar, 1994; Haystead *et al*., 1989). However, these inhibitors were never used in yeast, where only tungstate, a non-specific phosphatase inhibitor, has been utilized (Rodriguez-Hernandez *et al*., 2012). *GLC7* is an essential gene in yeast (Stark, 1996) and therefore any inhibitor of the encoded phosphatase should inhibit yeast growth. However, at the tested concentrations of 0.5 μM okadaic acid and 10 mM tungstate no inhibition of growth was observed and also no inhibition of Pma1 activity in cells with glucose metabolism (Figure 24 A and B). In the case of calyculin A growth was clearly inhibited but still no effect on Pma1 activity was observed (Figure 24 C and D). As the concentration of okadaic acid was much higher than those utilized with animal cells (0.01 μM; Shenolikar, 1994; Haystead *et al*., 1989) and as Glc7 is highly homologous to animal type 1 protein phosphatases (Stark, 1996), it seems that okadaic acid is not permeable in yeast and we cannot reach conclusions with this inhibitor about the participation of Glc7 on Pma1 regulation. In the case of calyculin A, however, the inhibitor is permeable because it inhibits growth but still, Pma1 activity is not affected. There is evidence in another yeast strain that tungstate, at the tested concentration of 10 mM, increases phosphorylation of the Glc7 substrate eIF2α, although it only produced a slight inhibition of growth (Rodriguez-Hernandez *et al*., 2012). Therefore we may conclude from the data with calyculin A and tungstate that probably Glc7 has only a minor role in regulation of Pma1 activity, perhaps because it affects only the Km or because other protein phosphatases are redundant with it.

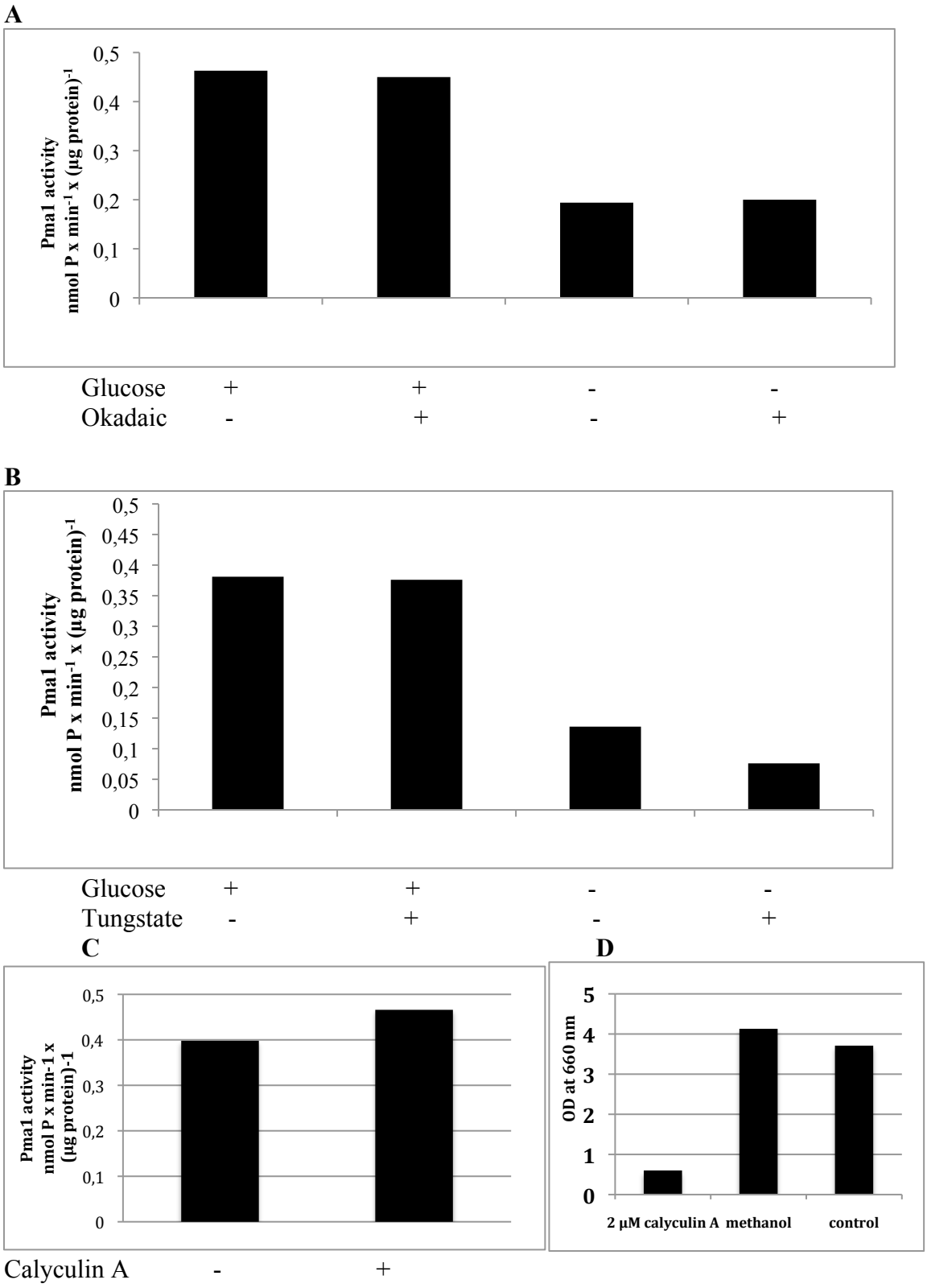


Figure 24. Specific activity of Pma1 in the total membrane fraction from cells treated or not with okadaic acid (A), tungstate (B) or calyculin A (C) and growth inhibition by calyculin A (D).

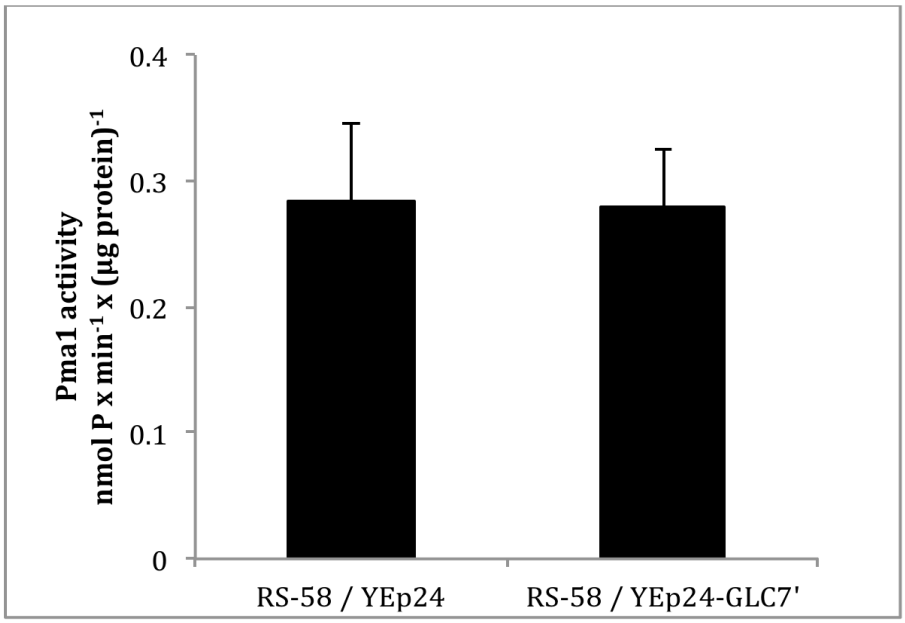
S. cerevisiae strain RS-58 was grown to first stationary phase and harvested, washed and resuspended with water and incubated for 15 minutes with glucose and okadaic acid (0.5 µM) or tungstate (10 mM) or calyculin A (2 µM) as indicated. After isolation of total membranes, Pma1 specific activity was assayed as described in Materials and Methods.

Expression of a dominant-negative form of *GLC7* does not affect Pma1 activity

Another strategy to inhibit Glc7 "in vivo" is to express a truncated gene, *GLC7'*, encoding the first 208 amino acids of Glc7 (312 in total) and which has a dominant-negative effect by inhibiting wild type Glc7 (Wek *et al*., 1992; Rodriguez-Hernandez *et al*., 2012). We have utilized several constructions made by G. Hueso in our laboratory with another truncated form encoding the first 183 amino acids of Glc7 (YEp24-*GLC7'*; Hueso et al., 2012). In addition we have made a novel construction with the original deletion of Wek *et al*. (1992) encoding the first 208 amino acids (YEp352-*GLC7'* and pUN50-*GLC7'*).

In the first place we tested *GLC7'* expressed from its own promoter in single copy (centromeric) and in multicopy (2μ) plasmids and there was no change in Pma1 activity in cells with glucose metabolism either in isolated membranes (ATP hydrolysis, Figure 25) or in whole cells (proton extrusion, Figure 26). Under these conditions it has been observed increased phosphorylation of a "bona-fide" substrate of Glc7, the translation initiation factor eIF2α encoded by the *SUI2* gene in a partially defective mutant of *GCN2*, encoding the involved kinase (Wek *et al*., 1992; Rodriguez-Hernandez *et al*., 2012).

A



B

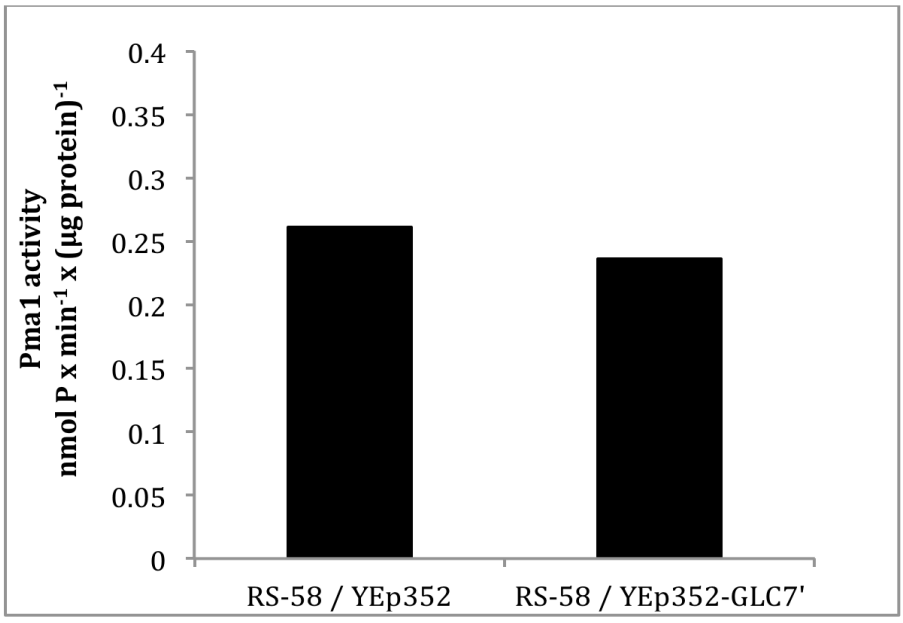


Figure 25. "In vitro" Pma1 activity of yeast transformed with *GLC7'*

S. cerevisiae (A) Strain RS-58 transformed with YEp24-*GLC7'* compared to its control RS-58 transformed with YEp24. (B) Strain RS-58 transformed with YEp352-*GLC7*' compared to its control RS-58 transformed with YEp352. Cultures were first grown in SD medium supplemented with leucine, histidine and adenine. Stationary phase cultures were used as inoculums in SD broth till reaching late exponential phase then cells were harvested and resuspended with H_2O and processed as indicated in Materials and Methods to determine Pma1 specific activity. The experiment (A) was repeated three times and error bars correspond to standard error. Experiment (B) was repeated twice with similar results and data presented are corresponding to a typical experiment.

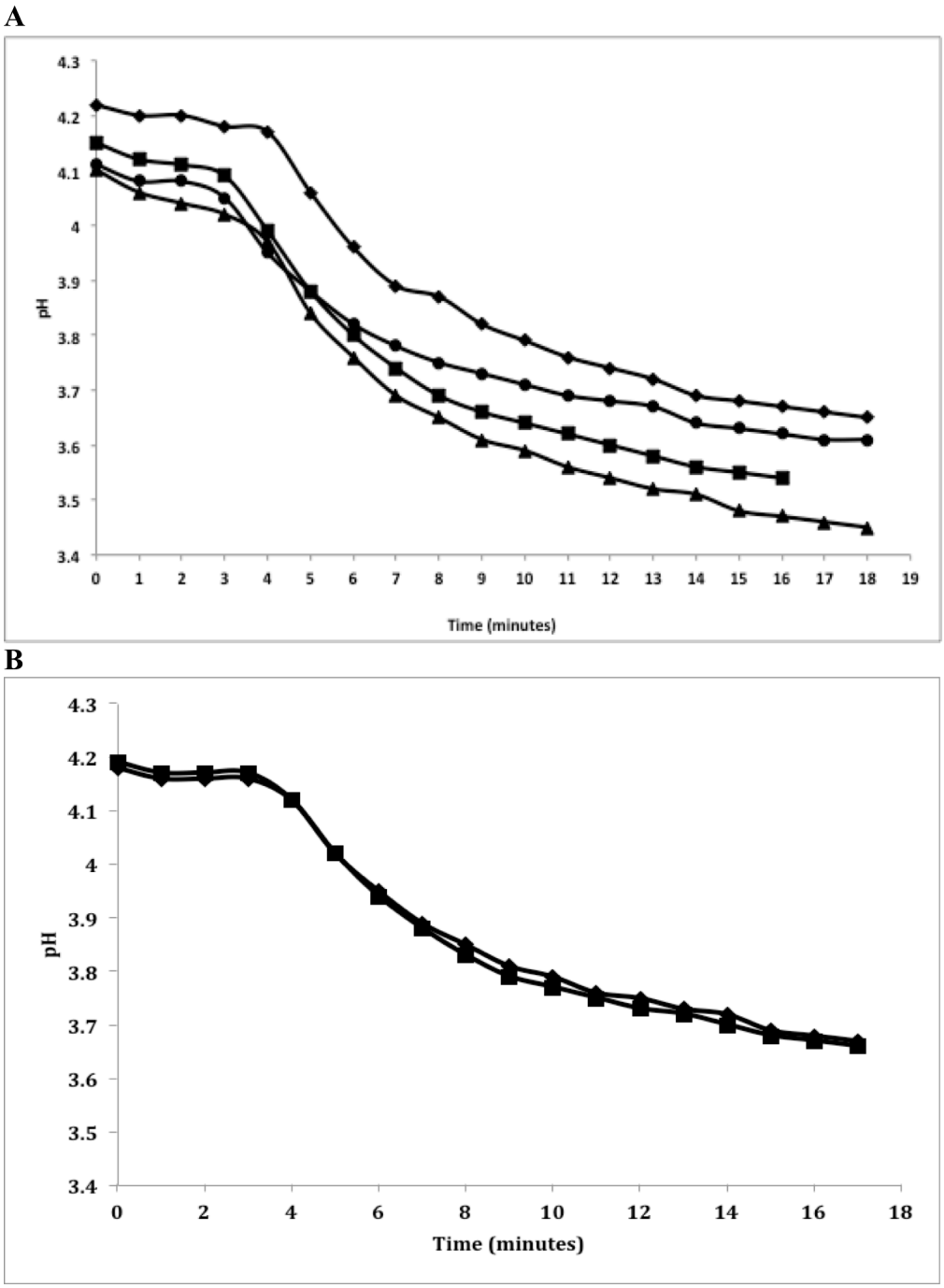


Figure 26. pH changes of yeast suspension induced by glucose

S. cerevisiae (A) Strain RS-58 / YEp24-*GLC7*' (•) compared to its control RS-58 / YEp24 (⬥) and strain RS-58 / YEp352-*GLC7'* (■) compared to its control RS-58 / YEp352 (▲). (B) Strain RS-132 / pUN50-*GLC7'* (■) in comparison to its control RS-132 / pUN50 (◆). Specific mg of each strain was suspended in 2 ml of (100 mM KCl and 10 mM glycylglycine adjusted to pH 4 with HCl) and the pH was recorded as described in Materials and Methods. At the times indicated in materials and method glucose (50 µmol) and calibration pulses of 100 nmol HCl were added. The maximal rate of proton pumping was (A) RS-58 / YEp24= 24.5, RS-58 / YEp24-*GLC7'*= 23.8, RS-58 / YEp352= 23.8, RS-58 / YEp352-*GLC7'* =22.7 (B) RS-132 / pUN50= 8.1, RS-132 / pUN50-*GLC7'*=8.07 nmol × min^{-1}× mg $yeast^{-1}$.The experiments were made once.

Expression of *GLC7'* under control of a doxycycline-regulated promoter

It has been described in yeast that when genes expressed from multicopy plasmid are deleterious for yeast growth the copy number of the plasmid is greatly reduced (Eraso *et al.*, 1987). As Glc7 activity is essential for yeast growth, too much inhibition by *GLC7'* in multicopy plasmid may have selected cells with low copy number and small inhibition. Accordingly we have utilized a system where the expression of *GLC7'* is under control of a doxycycline-regulated promoter (Ariño and Herrero, 2003).

As indicated in Figures 27, 28 and 29, when the expression of *GLC7'* is induced by removal of doxycycline the yeast growth is inhibited with two different constructs (with and without HA3-His6 at the C-terminus), suggesting that Glc7 is inhibited by the truncated protein acting as a dominant-negative of an essential enzyme. We used different concentrations of doxycycline as shown in the Figures (27 and 28) in order to determine the best concentration to be used to permit the cells to grow by inhibiting the gene expression and also avoiding toxic effects. The concentration selected was 2 μg/ml and it was used in the following experiments.

Then we checked if Pma1 activity is affected by expression of *GLC7'* and, as indicated in Figure 30, no change in Pma1 activity was observed with the two different constructs inhibiting yeast growth.

When the results showed such a lack of effect of Glc7' on Pma1 activity, there was a slight doubt that maybe *GLC7'* was not expressed under the experimental conditions. So in order to confirm expression of Glc7' we carried out a semiquantitative RT-PCR from strain BY4741 transformed with PCM262-*GLC7'* before and after doxycycline removal. As indicated in the Figure 31 the gene did get expressed after 1.5 hours incubation of the cells in absence of doxycycline. These data are in agreement with our previous conclusion that Glc7 protein phosphatase is minorly involved or it is not the only phosphatase involved in regulation of Pma1 activity.

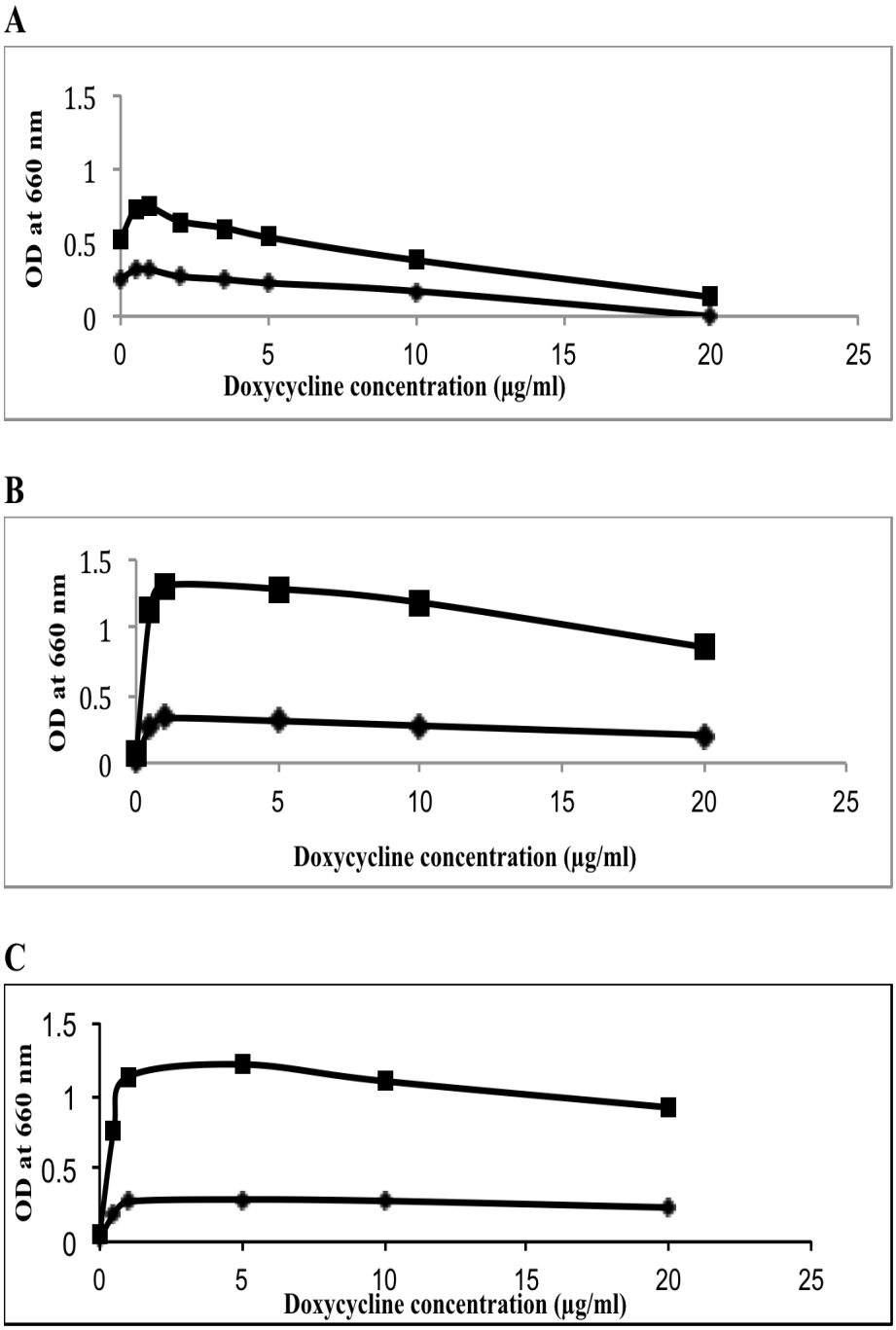
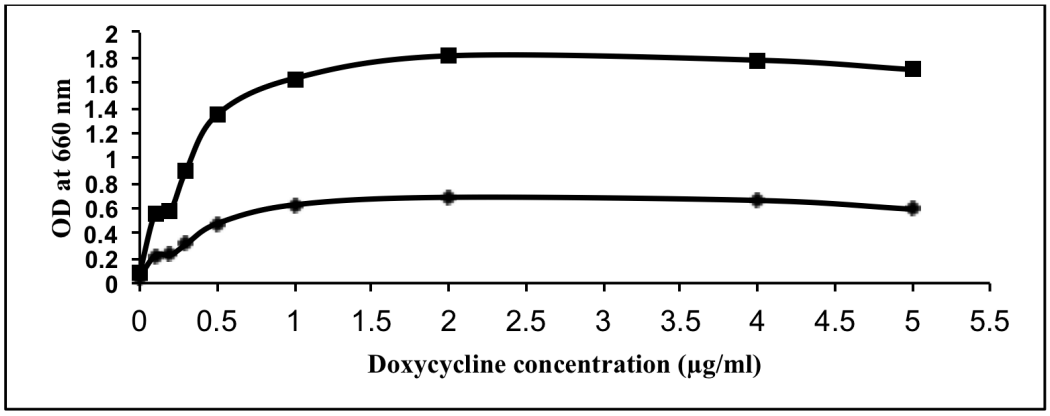


Figure 27. Effect of different doxycycline concentrations (0-20 µg/ ml) on growth of *S. cerevisiae* strains derived from BY4741 by transformation with different plasmids. (A) strain RS-119 (transformed with PCM262) (B) strain RS-120 (transformed with PCM262-*GLC7*'-HA3-His6) (C) strain RS-121(transformed with PCM262- *GLC7'*).

S. cerevisiae cells were grown in SD medium supplemented with leucine, histidine, methionine and doxycycline 10 µg/ ml. Stationary phase cultures were washed twice and resuspended with sterile distilled water and used as inoculums in fresh medium of the same complosition with different doxycycline concentrations. Growth was then determined by measuring optical density (OD) after incubation at 30°C with shaking 200 rev/min for 18 (◆) and 22 (■) hours. The experiment was repeated twice with similar results and data presented are corresponding to a typical experiment.

A



B

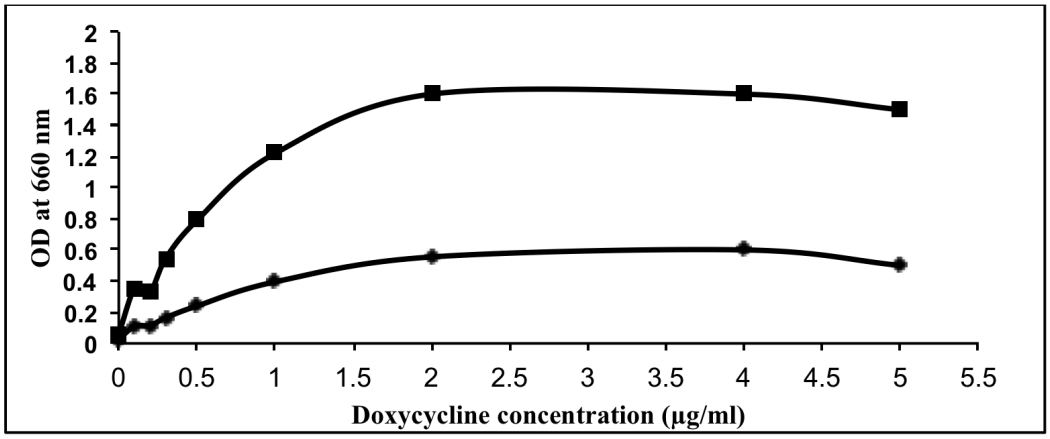


Figure 28. Effect of different doxycycline concentrations (0-5 µg/ ml) on growth of *S. cerevisiae* strains derived from BY4741 by transformation with different plasmids. (A) strain RS-120 (transformed with PCM262-*GLC7*'-HA3-His6) (B) strain RS-121 (transformed with PCM262- *GLC7'*).

Conditions and symbols as in **Figure 27** but with lower range of doxycycline concentrations. The experiment was made once.

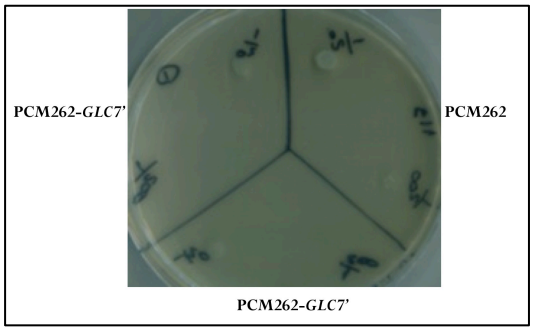


Figure 29. Inhibition of growth of cells expressing *GLC7'* on solid medium in absence of doxycycline.

S. cerevisiae strain RS-119 (strain BY4741 transformed with PCM262) and strain RS-121 (strain BY4741 transformed with PCM262-*GLC7'*) were grown in SD medium supplemented with leucine, histidine, methionine and doxycycline (2 µg/ml) till stationary phase, harvested, washed and resuspended with sterile distilled water same volume . Two dilutions (1/20 and 1/200) were made and 3 µL of each dilution were spotted on a plate of the same medium. The experiment was repeated twice with similar results.

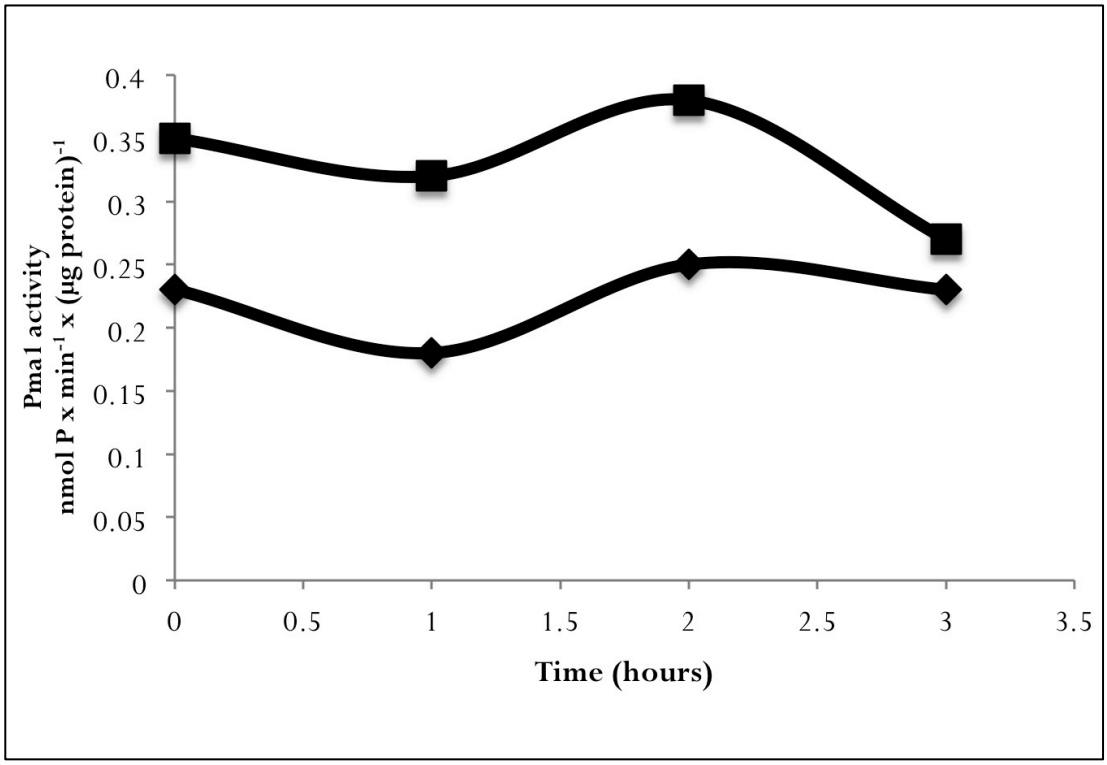


Figure 30. Lack of effect of doxycycline removal on Pma1 activity of strains RS-120 (strain BY4741 transformed with PCM262-*GLC7*'-HA3-His6) and RS-121 (strain BY4741 transformed with PCM262- *GLC7*').

S. cerevisiae cultures were first grown in SD medium supplemented with leucine, histidine, methionine and doxycycline (2 µg/ml). Stationary phase cultures of strains RS-120 (■) and RS-121(◆) were used as inoculums in SD fresh broth with the same composition till reaching exponential phase then cells were harvested, washed and resuspended with the same medium without doxycycline. At indicated times, samples were processed as indicated in Materials and Methods to determine Pma1 specific activity. The experiment with strain RS-120 was repeated twice, and with strain RS-121 for 5 times and measured once in galactose medium and the repetitions gave similar results and data presented are corresponding to a typical experiment.

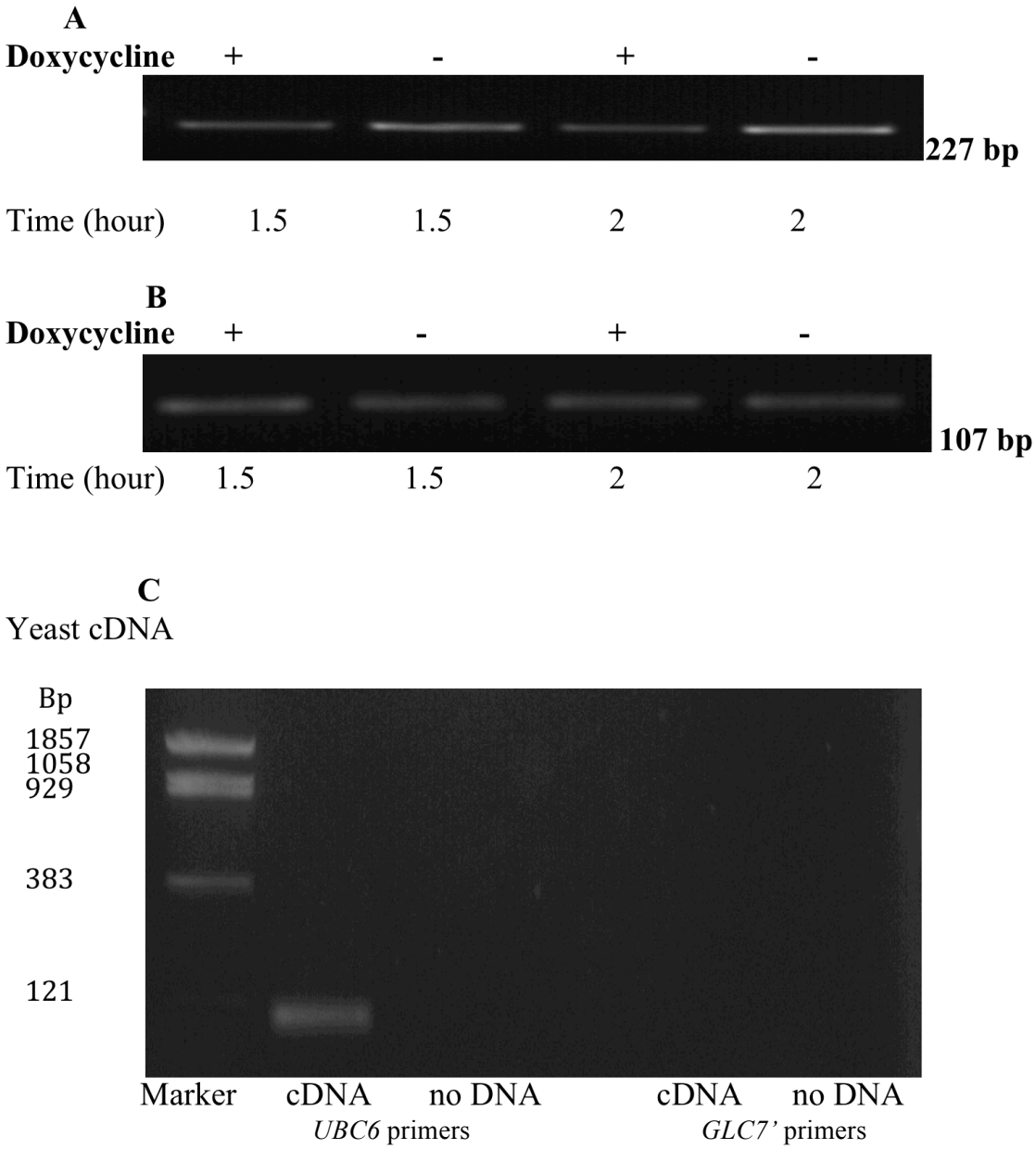


Figure 31. Semi quantitative RT-PCR analysis of expression of *GLC7'* in RS-121 (strain BY4741 transformed with PCM262-*GLC7*') before and after removal of doxycycline.

(A) Semi quantitative RT-PCR with *GLC7'* primers of samples taken after 1.5 and 2 hours incubation of exponential phase cultures in presence (+) or in absence (-) of doxycycline (2 µg/ml). The amplified *GLC7'* fragment has 227 bp. (B) Control experiment using UBC6 primers on all the samples to check that the expression of genes other than *GLC7'* is not affected. (C) Control experiment, *GLC7'* primers are specific because no product is amplified in a sample from yeast not transformed with a *GLC7'* plasmid. One of the primers corresponds to a flanking sequence of the plasmid. On the other hand *UBC6* primers result in amplification. The used marker is pBR322-*Bst*NI. The experiment was made once.

Expression of a dominant-negative form of *GLC7* does not affect dephosphorylation of Pma1 after removal of glucose

One further strategy to investigate if protein phosphatase Glc7 participates in the regulation of Pma1 has been to determine if inhibition of Glc7 by expression of a truncated dominant-negative form of the enzyme affects the dephosphorylation of the regulatory domain of Pma1 after removal of glucose from the yeast medium. If Glc7 dephosphorylates this regulatory domain its inhibition should result in slower dephosphorylation after removal of glucose from the medium.

Recently, the group of Francisco Portillo at Madrid has developed specific antibodies against the two phosphorylated sites in the regulatory domain of Pma1 modified by glucose metabolism (Mazón *et al.,* 2015). Antibody α-pS899 specifically recognizes phosphorylated Ser899, which slightly contributes to Pma1 activation by increasing the affinity of the enzyme for ATP. Antibody α-pST specifically recognizes the double phosphorylation on Ser911 and Thr912 that is the major regulatory event by increasing the maximal activity of the enzyme. By using these two antibodies we could show that expression of the dominant-negative form of Glc7 has no effect on the rate of dephosphorylation of Pma1 after removal of glucose from the medium of the cells (Figure 32). In the same figure it is clear that the phosphorylation level of Pma1 increases after glucose addition for 10 minutes and also shows the decreased phosphorylation on glucose removal.

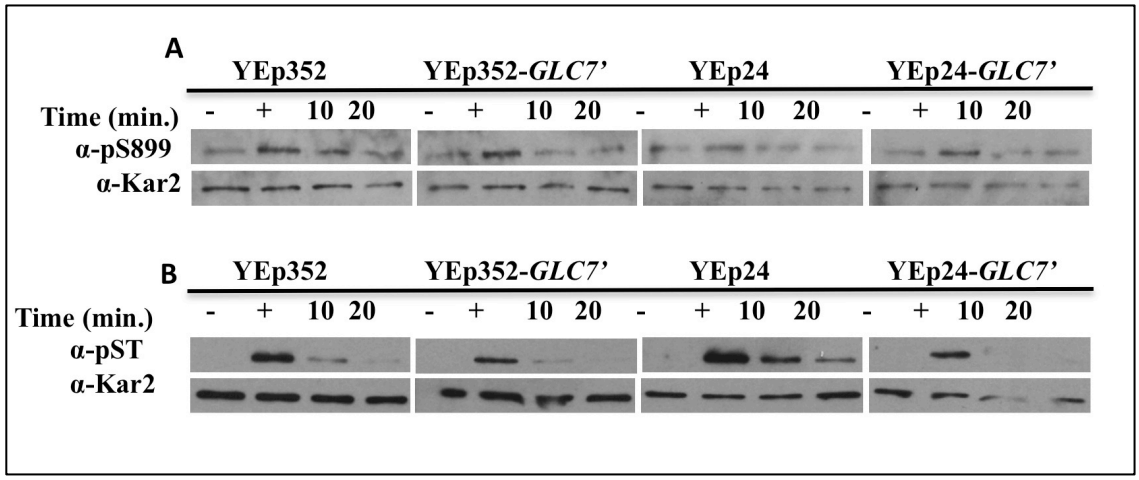


Figure 32. Effect of *GLC7'* on the "in vivo" dephosphorylation rate of Pma1 phosphorylation site Ser899 (A) and Ser 911 Thr 912(B)

S. cerevisiae strain RS-132 transformed with YEp352-*GLC7'* compared to its control RS-132 transformed with YEp352, also RS-132 transformed with YEp24-*GLC7*' compared to its control (RS-132 transformed with YEp24). Late exponential phase cultures were harvested, washed and resuspended with sterile distilled water and incubated for 15 minutes (-), then incubated with glucose for 10 minutes (+), glucose was removed by centrifugation then cells were incubated in water for 10 and 20 minutes as indicated. The levels of phosphorylated serine 899 (pS899) (A) and double phosphorylation at serine 911 and threonine 912 (pST) (B) were determined by western blotting as described in Materials and Methods. α-Kar2 was used as control of loading. The bands recognized by the antibodies represent Pma1 of 100 KD and Kar2 of 75 KD. The experiment was repeated twice with similar results.

Phenotype of *GLC7'* transformants with hygromycin B and acetic acid

Any activation of Pma1 by increased phosphorylation of the regulatory sites should manifest by increased tolerance to acetic acid and decreased tolerance to toxic cations such as hygromycin B (Hueso *et al.,* 2012). Yeast cells with a more active Pma1 should maintain a more neutral intracellular pH after treatment with acetic acid because of increased capability for proton extrusion. Also, these cells should have a hyperpolarized membrane potential and therefore a decreased tolerance to toxic cations such as hygromycin B whose uptake is driven by membrane potential (Perlin *et al*., 1989; Goossens *et al*., 2000). As indicated in Figure 33 *GLC7'* expressing cells have a slightly less tolerance to hygromycin but this phenotype is not very significant. On the other hand no phenotype of tolerance to acetic acid could be observed (Figure 34). Therefore again we conclude that Glc7 is minorly involved (or it is not the only phosphatase involved) in regulation of Pma1 activity.

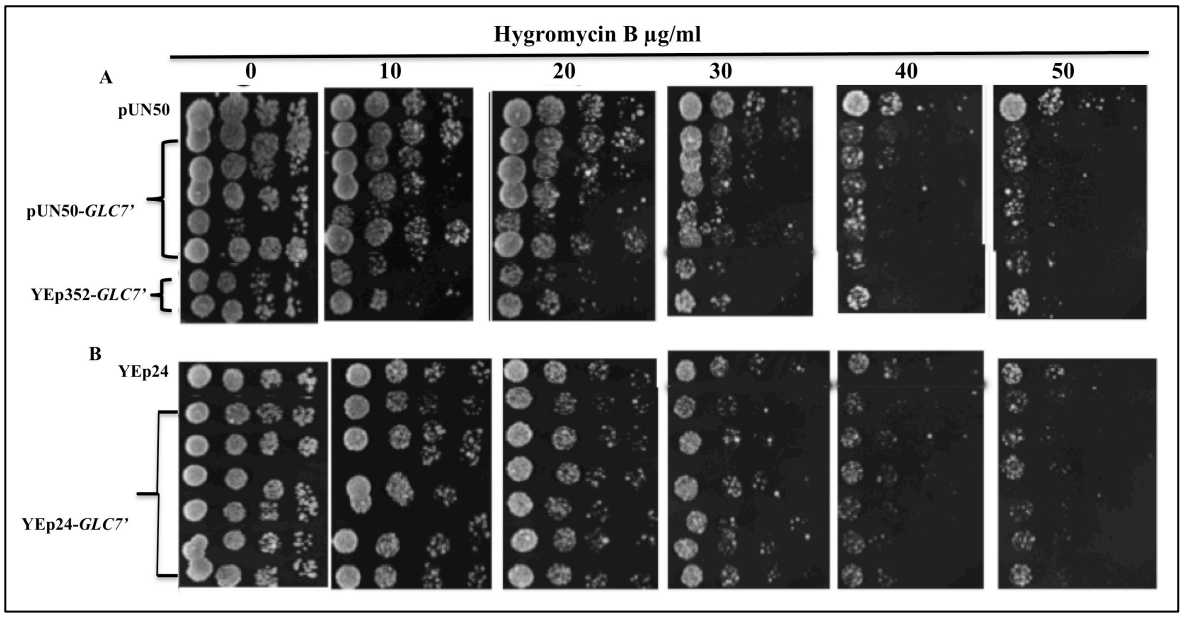


Figure 33. Effect of *GLC7*' on hygromycin B resistance.

S. cerevisiae (A) Strain RS-132 transformed with pUN50, pUN50-*GLC7'*, YEp352-*GLC7'*. (B) Strain RS-132 transformed with YEp24, YEp24-*GLC7'*. Serial dilutions of stationary phase cultures of these strains prepared in SD, were incubated on YPD media for 2–3 days at 30°C. The experiment was made once with 5 different colonies of RS-132 / pUN50-*GLC7'* and 6 different colonies of RS-132 / YEp24-*GLC7'* and 2 colonies of RS-132 / YEp352-*GLC7'*.

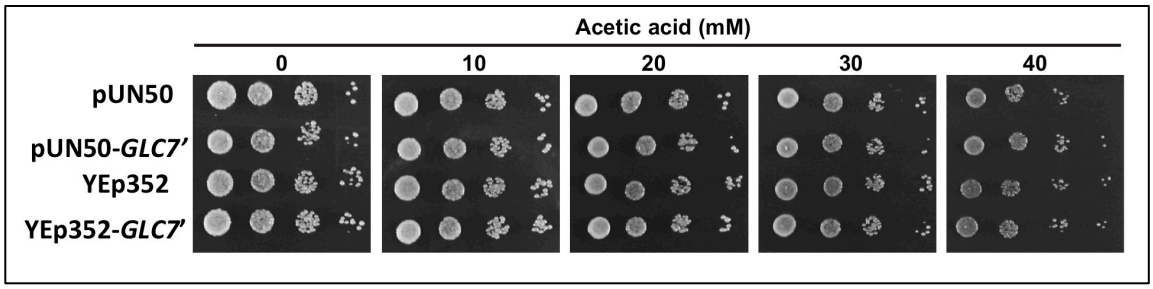


Figure 34. Effect of *GLC7'* on acetic acid resistance.

S. cerevisiae strain RS-132 transformed with pUN50 , pUN50-*GLC7'*, YEp352, YEp352-*GLC7*'. Serial dilutions of stationary phase cultures of these strains prepared in SD, were incubated on YPD media of pH 3.5 , for 2–3 days at 30°C. The experiment was repeated twice with similar results.

Mutants in *GLC7* exhibit altered ion homeostasis

Another approach to investigate the role of Glc7 in Pma1 activity has been to determine this activity in mutants of the *GLC7* gene previously identified in the laboratory of Kelly Tatchell (Baker *et al.,* 1997; Williams *et al*., 2002). Protein phosphatases exhibit little substrate specificity "in vitro" while the specificity "in vivo" is dictated by regulatory subunits that target the phosphatase catalytic subunit to its phosphorylated protein substrate

(Cohen, 1989; Stark, 2004). One of the functions of Glc7 is to promote glycogen synthesis by dephosphorylating and activating glycogen synthase 2 (Gsy2) as cells approach stationary phase. The *glc7-1* mutant is defective in interacting with the required regulatory subunit for this function, Gac1, and accumulates little glycogen (Stark, 2004). The *glc7-109* and *glc7-132* mutants were isolated by directed mutagenesis of clusters of charged residues that could mediate interactions with regulatory subunits (Baker *et al.,* 1997). The *glc7-109* mutant exhibits increased glycogen accumulation while the *glc7-132* mutant resembled the *glc7-1* mutant in accumulating less glycogen. Interestingly, the *glc7-109* mutant, but not the other two, is more sensitive to toxic cations such as Na^+, Li^+ and hygromycin B and has a greater membrane potential (Williams-Hart *et al*., 2002). The activity of Pma1, however, was not affected.

We have reinvestigated the sensitivity of these mutants to acetic acid and toxic cations (Figure 35). The results indicate that *glc7-1* and *glc7-132* are more tolerant than wild type to acetic acid, *glc7-109* is more sensitive than wild type to acetic acid and the three mutants are more sensitive than wild type to lithium and hygromycin B. These phenotypes are difficult to explain in terms of changes of Pma1 activity. Determinations "in vitro" and "in vivo" (Figure 36) indicate that the *glc7-1* mutant exhibits a slight increase in Pma1 activity with respect to wild type both "in vivo" and "in vitro" while the other two mutants had no change in the "in vitro" assay, while *glc7-132* has increased rate of proton pumping from cells.

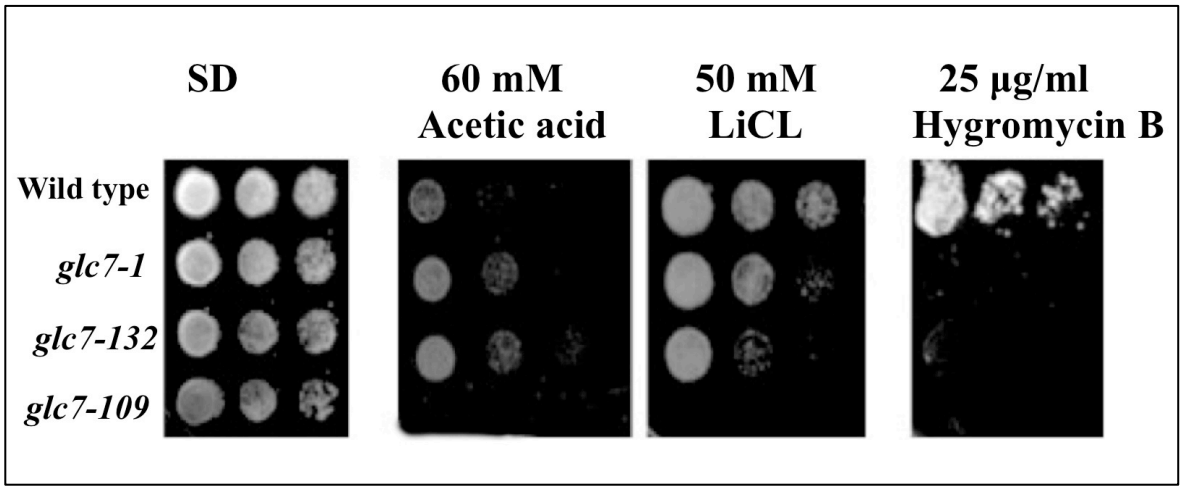
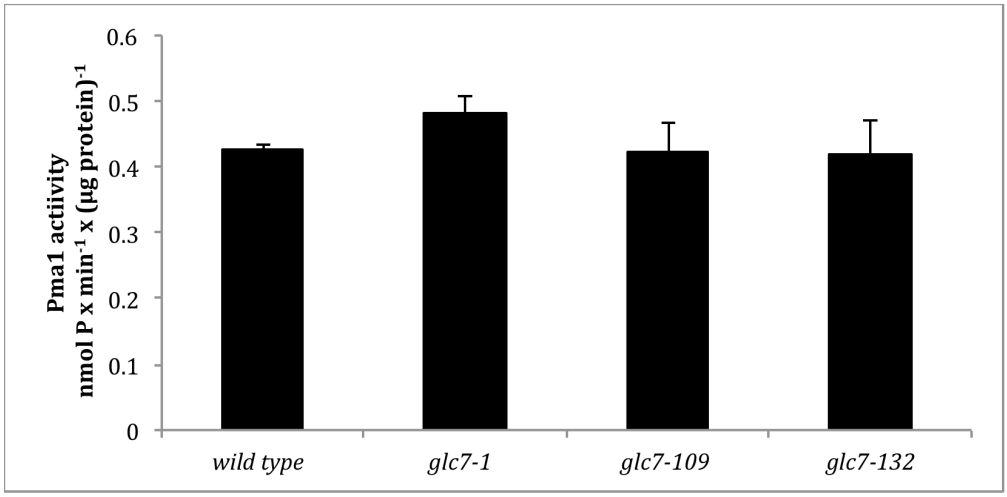


Figure 35. The growth phenotypes of 3 *glc7* mutants.

S. cerevisiae WT (strain KT1112) and mutants, *glc7-1*, *glc7-132* and *glc7-109* serial dilutions of stationary phase cultures were incubated on the indicated agar medium for 2–3 days at 30°C. The experiment was repeated twice with similar results.

A



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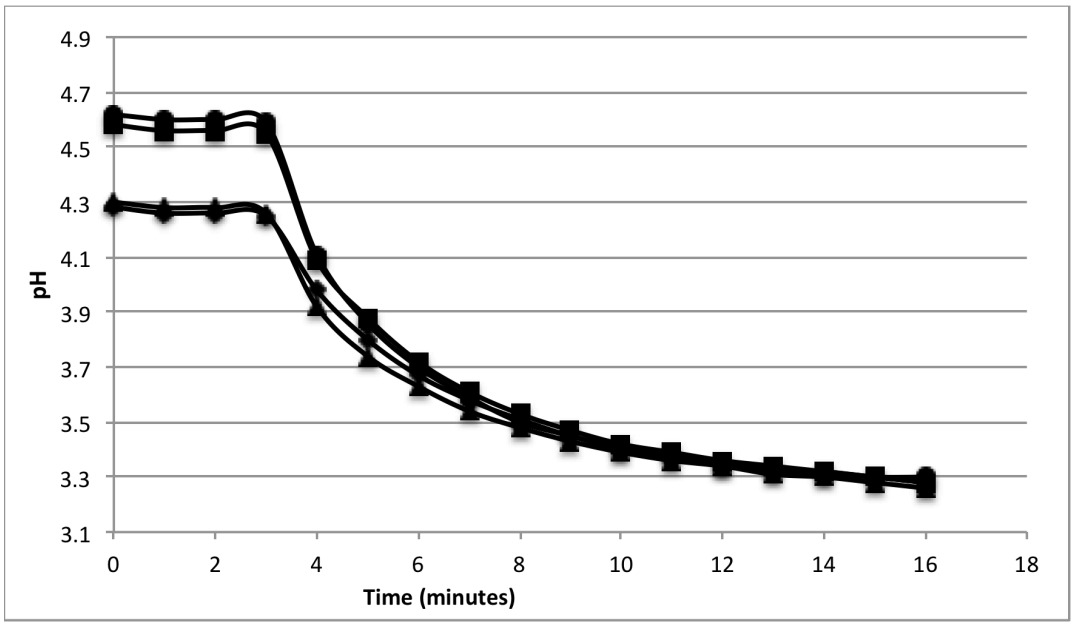


Figure 36. Pma1 specific activity of 3 *GLC7* mutants. (A) "In vitro" activity (B) pH changes of yeast suspension induced by glucose.

(A) *S. cerevisiae* stationary phase cultures were used as inoculums in YPD broth till reaching stationary phase and then cells were harvested and resuspended with H_2O then processed as indicated in Materials and Methods to determine Pma1 specific activity. (B) Wild type (strain KT1112) (◆), *glc7-1* (■), *glc7-109* (▲) and *glc7-132* (●) specific mg of each strain was resuspended in 2 ml of (100 mM KCl and 10 mM glycylglycine adjusted to pH 4 with HCl) and the pH was recorded as described in Materials and Methods. At the times indicated in Materials and Methods, glucose (50 µmol) and calibration pulses of 100 nmol HCl were added. The maximal rate of proton pumping was (A) wild type= 20.03, *glc7-1*= 27.2, *glc7*-109= 21.1, *glc7-132* =32.5 nmol × min^{-1}× mg $yeast^{-1}$. The experiment (A) was repeated three times and error bars correspond to standard error and experiment (B) was made once.

Effect of expression of a dominant-negative form of *GLC7* and of glucose metabolism on phosphorylation of eIF2α

The translation factor eIF2α is a known substrate of Glc7 and is phosphorylated by protein kinase Gcn2 in response to amino acid starvation (Wek *et al.* 1992). We have tested the effect of glucose metabolism and of expression of the truncated dominant-negative form of Glc7 on the phosphorylation level of eIF2α. As indicated in Figures 37 and 38, glucose metabolism greatly increases eIF2α phosphorylation, as it also does in the case of Pma1 phosphorylation at Ser911 Thr912 (Mazón *et al.,* 2015). However, in a mutant with constitutive activity of Gcn2 (Menacho-Marquez *et al*., 2007) eIF2α phosphorylation was high in the absence and presence of glucose. GCN2 is not known to be activated by glucose metabolism and therefore it could be that glucose inhibits Glc7.

Figure 37 also shows the results of a comparison that we made between three different methods of protein extraction for SDS-PAGE. It was reported by Wright *et al.* (1989) that TCA method is the best and that it produces much higher protein amount. Our results indicated that the two other methods tested (Laemmli and alkaline lysis) produce almost equal amounts of protein like the tedious, more time consuming than TCA method that results in slightly more protein extraction.

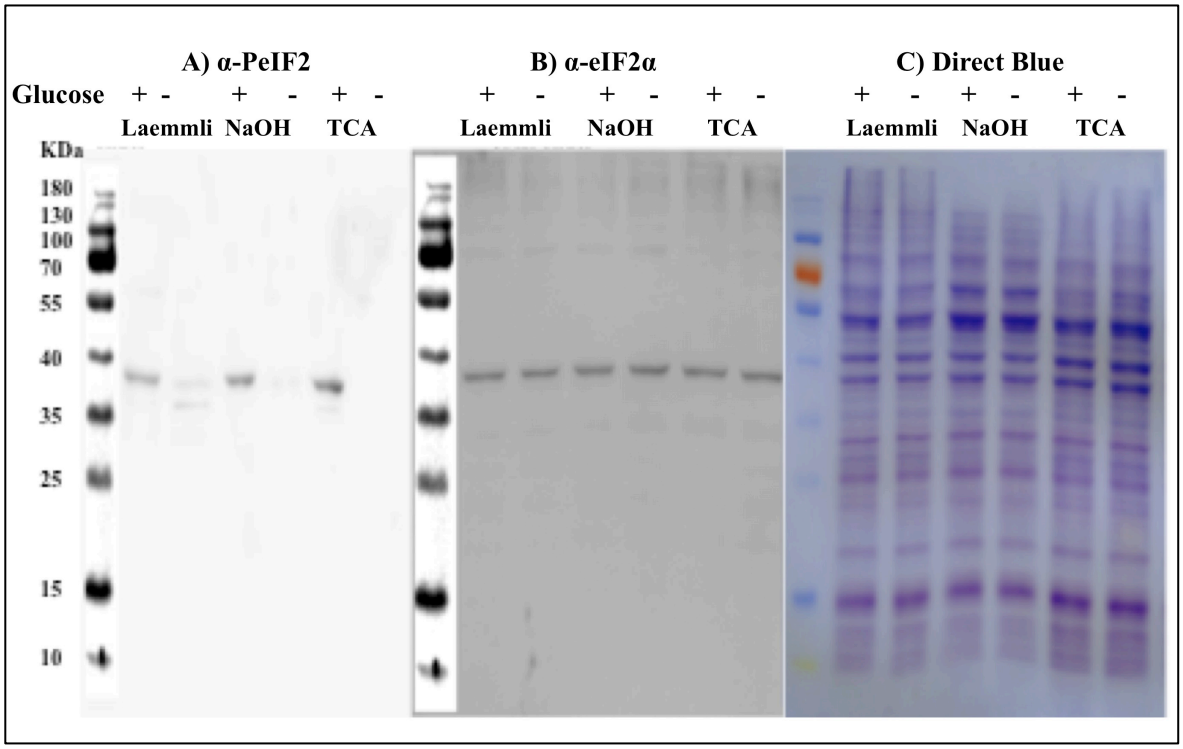


Figure 37. Comparison between three methods of protein extraction.
(A) Western blotting with antibodies against P-eIF2α (B) Western blotting with antibodies against total eIF2α (C) Direct blue stained membrane.

S. cerevisiae strain RS-259 stationary phase cultures were used as inoculums in YPD broth till reaching early exponential phase then cells were harvested, washed and resuspended with H_2O then distributed into 6 samples, glucose was added to only 3 of them and incubated all for 10 minutes. Extraction was made by : harvesting the cells then boiling with hot Laemmli 1X for 10 minutes at 100°C or hot NaOH or precipitated by TCA as mentioned in details in Materials and Methods. Standard protein markers (PageRuler Prestained Protein Ladder, from Thermo Scientific), with masses indicated in kDa. The experiment was repeated twice with similar results.

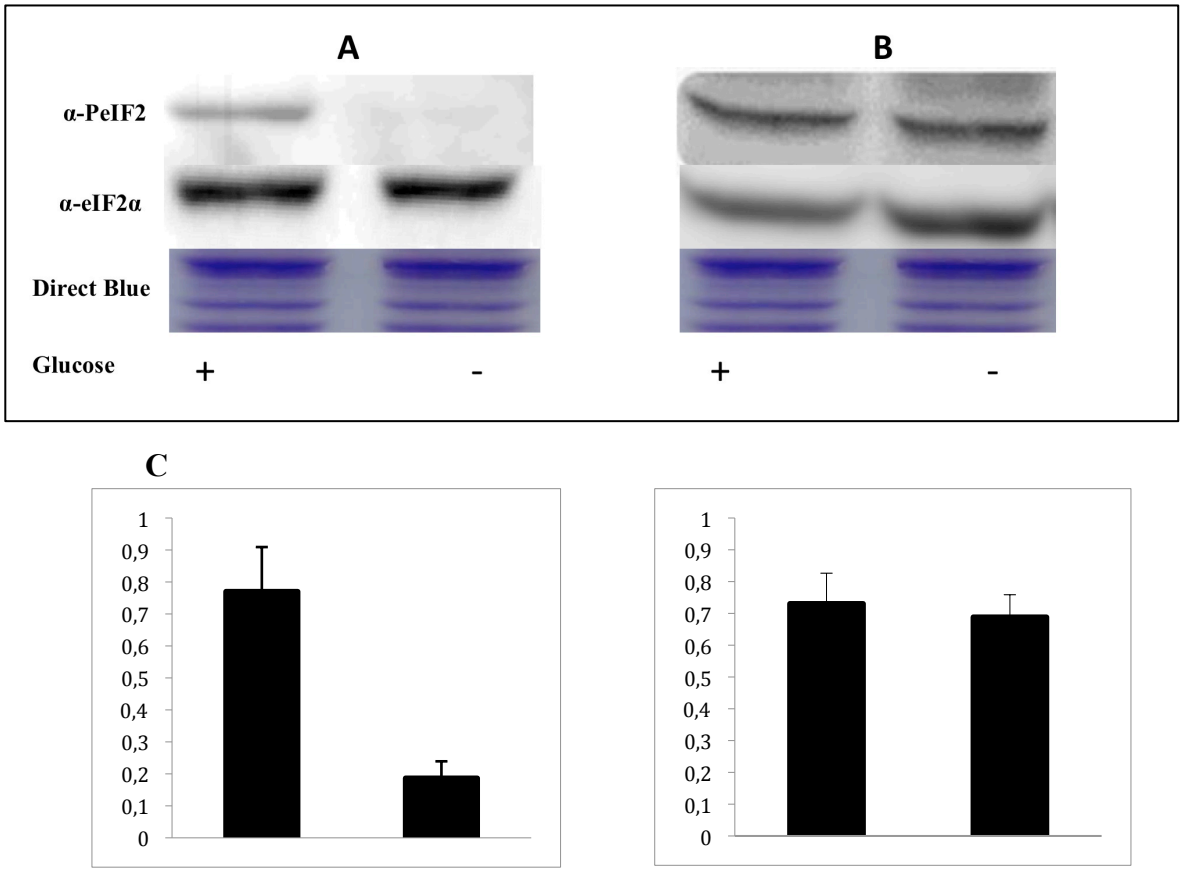


Figure 38. Glucose effect on phosphorylation level of eIF2*α* in (A) wild type yeast (BY4741) and (B) *GCN*2^c mutant

Western blotting with antibodies against PeIF2α and, as control of loading: antibody against total eIF2α and Direct blue stained membrane. *S. cerevisiae* strain RS-259 and *GCN2*^{c} mutant stationary phase cultures were used as inoculums in YPD broth till reaching early exponential phase then cells were harvested, washed and resuspended with H_2O then distributed into (5 ml) samples, 2% glucose was added or not and all were incubated for 10 min. Extraction was made by Laemmli as described in materials and method. The experiment was repeated three times with similar results. The band intensity was quantified by Image Gauge software then relative band intensity (P-eIF2α/total eIF2α) was calculated and the average value was represented in (C) with arbitrary units of the Y-axis and error bars correspond to standard error.

In order to check if inhibition of Glc7 by Glc7' increases phosphorylation of eIF2α, we expressed Glc7' under control of doxycycline and, as indicated in Figure 39, inhibition of Glc7 by expression of the truncated Glc7' had no effect on eIF2α phosphorylation. Therefore, even in a system where the Glc7 phosphatase is known to regulate the phosphorylation level of a protein substrate, inhibition of Glc7 by the truncated dominant-negative form does not increase phosphorylation.

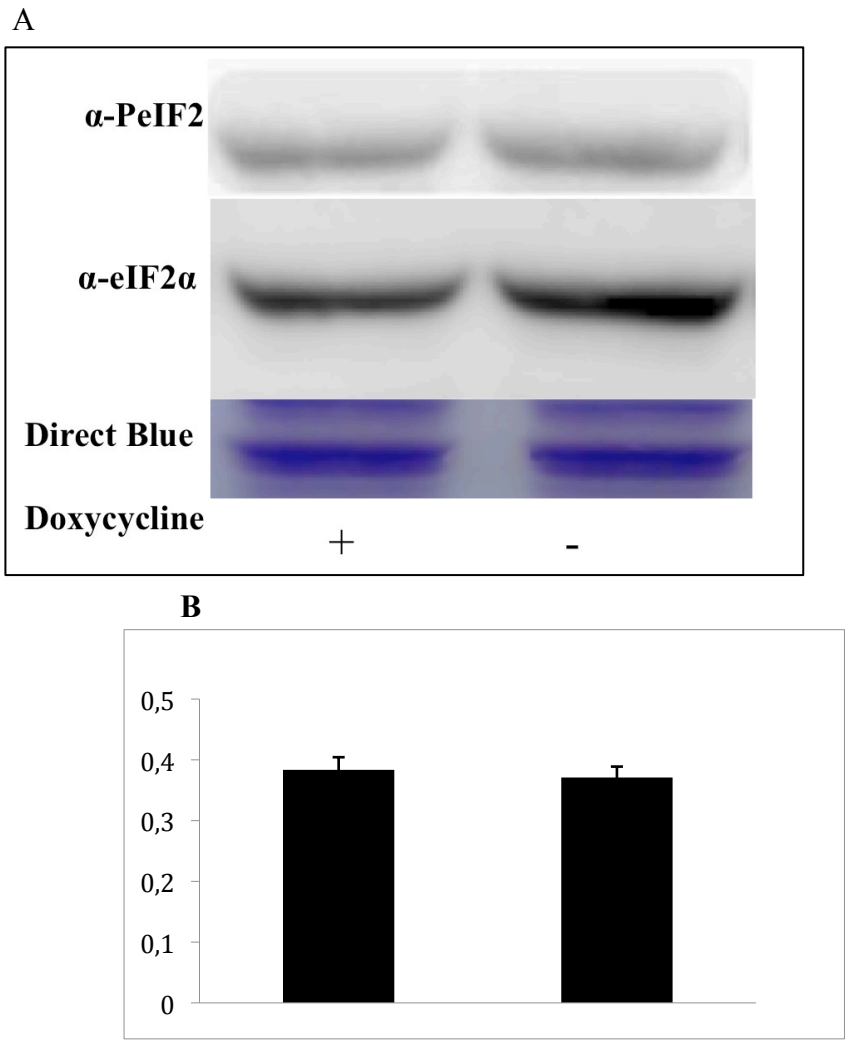


Figure 39. Inducing expression of *GLC7*' has no effect on phosphorylation level of eIF2α.

(A)Western blotting with antibodies against PeIF2α and as control of loading: antibody against total eIF2α and Direct blue stained membrane. *S. cerevisiae* strain RS-121 was grown in SD medium supplemented with leucine, histidine and methionine and doxycycline (2 μg/ml) till reaching exponential phase then doxycycline was removed and samples incubated for one hour and a half with or without doxycycline. True zero control with doxycycline was made. The experiment was repeated three times with similar results. The band intensity was quantified by Image Gauge software then relative band intensity (P-eIF2α/total eIF2α) was calculated and the average value was represented in (b) with arbitrary units of the Y-axis and error bars correspond to standard error.

Effect of *sit4* null mutation on the activity of Pma1

sit4 null mutant is viable, so we used it in order to test the effect of Sit4, which is type 2A protein phosphatase, on Pma1 activity. We tested the rate of dephosphorylation of activated Pma1 of *sit4* null mutant in comparison to the wild type. Results in Figure 40 showed that the dephosphorylation rate of the mutant was slower, yet the level of phosphorylation after glucose addition was less than in the wild type.

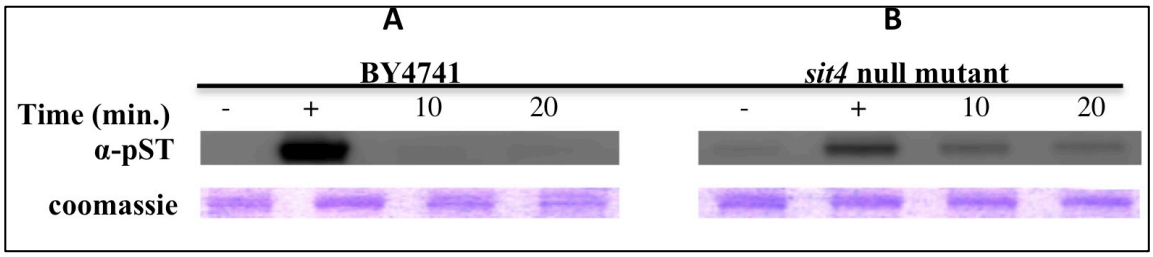


Figure 40. Dephosphorylation rate of Pma1 (A) wild type (strain BY4741) and (B) *sit4* null mutant.

S. cerevisiae late exponential phase cultures were harvested, washed and resuspended with sterile distilled water then incubated for 15 minutes (-), then glucose was added and cells were incubated for 10 minutes (+), glucose was removed by centrifugation then cells were incubated in water for 10 and 20 minutes as indicated. The levels of double phosphorylation at serine 911 and threonine 912 (pST) were determined as described in Materials and Methods. Coomassie stained gel is presented as control of loading. The bands recognized by the antibody and stained by Coomassie represent Pma1 of 100 KD. The experiment was repeated twice with similar results.

In another approach to test the effect of Sit4 on Pma1 activity we measured the "in vitro" activity of Pma1 of the same samples used in dephosphorylation rate experiment, of both the *sit4* null mutant and the wild type. As indicated in Figure 41 the wild type showed higher Pma1 activity after glucose activation than that of the null *sit4* mutant.

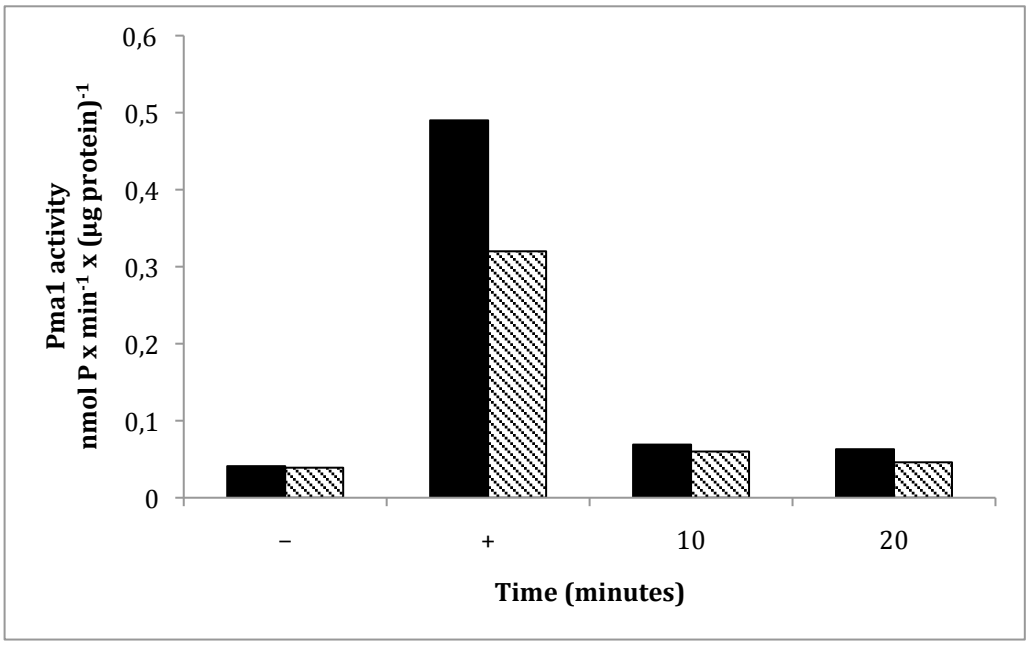


Figure 41. Effect of *sit4* null mutation on specific activity of Pma1.

S. cerevisiae strain RS-259 (■) and null *sit4* mutant (▨) late exponential phase cultures were harvested, washed and resuspended with sterile distilled water then incubated for 15 minutes (-), then incubated with glucose for 10 minutes (+), glucose was removed by centrifugation then cells were incubated in water for 10 and 20 minutes as indicated. The crude Pma1 was extracted and its specific activity was determined as described in materials and method. The experiment was repeated twice with similar results and data presented are corresponding to a typical experiment.

The *sit4* null mutant also showed lower Pma1 activity when the pH changes of yeast suspension induced by glucose were measured, the maximal rate of proton pumping of the wild type was almost twice that of the null mutant as shown in Figure 42. All these results together suggest that Sit4 is having a stimulatory effect on Pma1 activity.

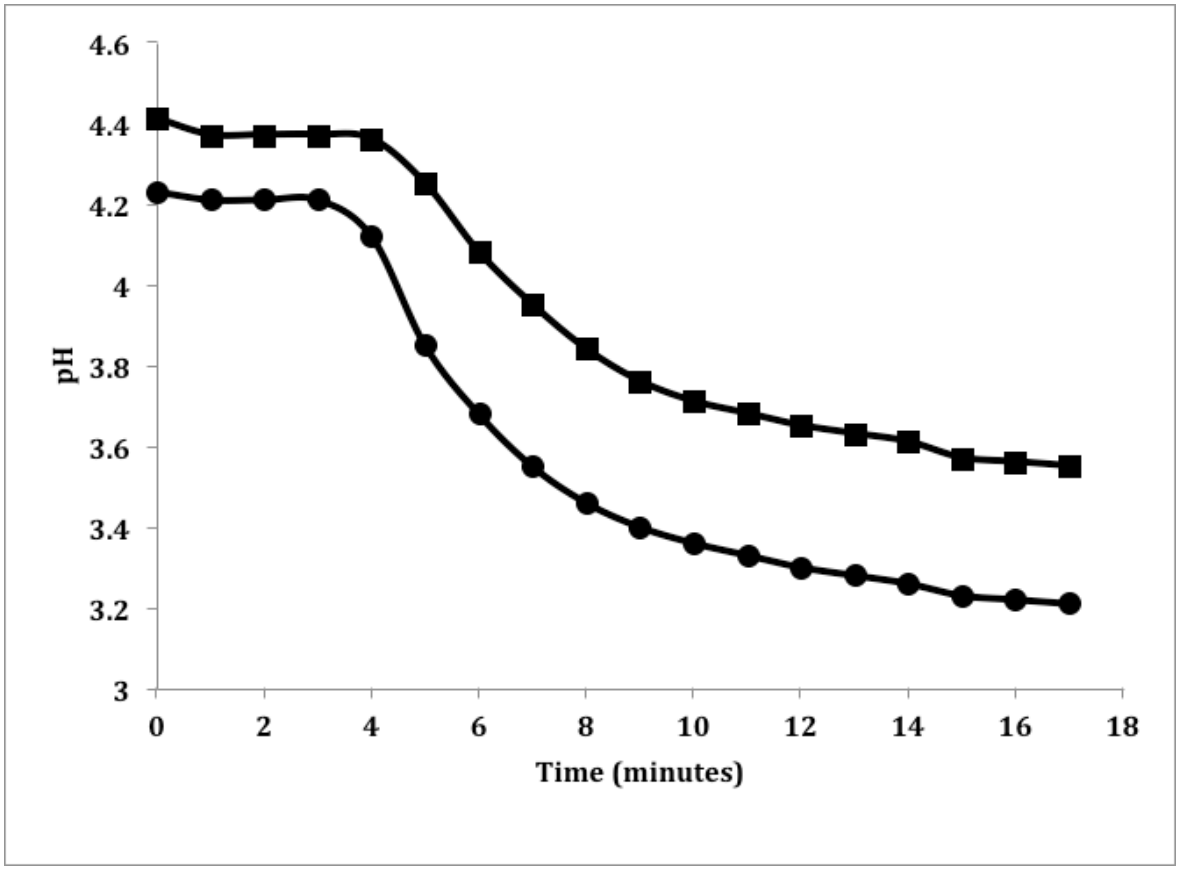


Figure 42. pH changes of yeast suspension induced by glucose.

S. cerevisiae strain RS-259 (•) and null *sit4* mutant (■) . Specific mg of each strain was suspended in 2 ml of (100 mM KCl and10 mM glycylglycine adjusted to pH 4 with HCl) and the pH was recorded as described in Materials and Methods. At the times indicated in materials and method glucose (50μmol) and calibration pulses of 100 nmol HCl were added. The maximal rate of proton pumping was RS-259= 13.7, null *sit4* =7.2 nmol × min^{-1}× mg $yeast^{-1}$. The experiment was made once.

We tested the effect of LiCl and hygromycin B on growth of *sit4* null mutant. In Figure 43, we can see that null *sit4* mutant is showing less growth in presence of LiCL and hygromycin than the wild type, yet the growth of the null mutant is already much lower in absence of any toxic cation, on YPD, as an effect of the null mutation on growth which makes it confusing to get a clear judgement on the mutant resistance.

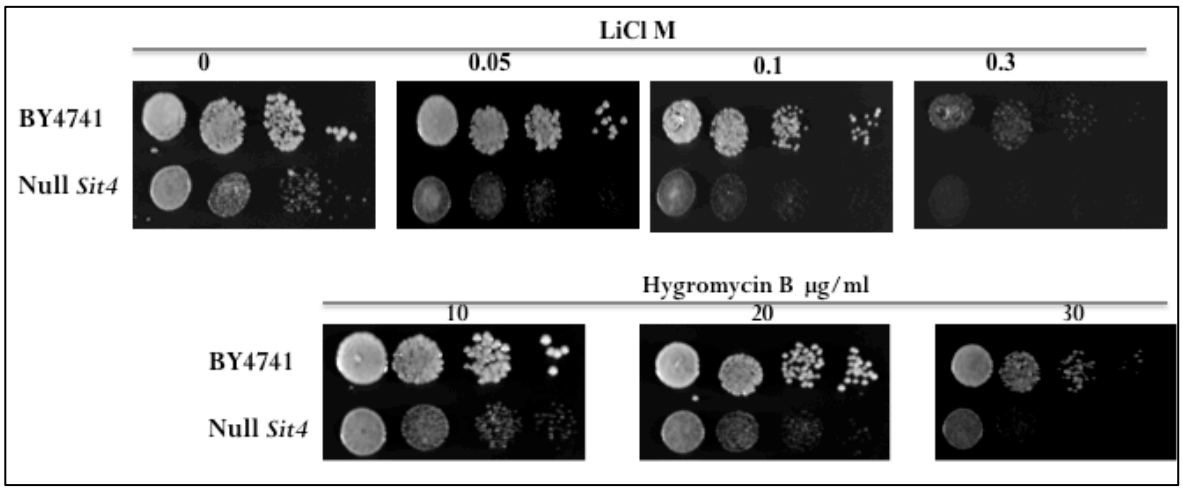


Figure 43. The growth phenotypes of *sit4* null mutant in comparison to its wild type.

S. cerevisiae strains RS-259 (WT) and *sit4* null mutant serial dilutions of stationary phase cultures were incubated on the indicated agar medium for 2–3 days at 30°C. The experiment was made once.

"In vitro" dephosphorylation of Pma1 by Glc7

We have tested if the Glc7 phosphatase could dephosphorylate or not Pma1 "in vitro". In order to do that we used a construction of Glc7 fused to GST (pGEX-3X-*GLC7*) to express the protein in *E. coli* then we purified it with glutathione beads. The presence of purified protein in the soluble fraction after inducing the expression with IPTG was confirmed by staining the membrane with Coomassie, Figure 44.

To test the "in vitro" interaction between Glc7 and Pma1, the purified Glc7 was incubated with activated Pma1 for different time intervals. Figure 45A showed that the level of the double phosphorylation at serine 911 threonine 912 of Pma1 decreased after being incubated with Glc7. A control experiment was conducted simultaneously (Pma1 incubated with purified GST extracted from cells harboring pGEX-3X induced with IPTG) and as shown in Figure 45B the phosphorylation level of Pma1 at ST911, 2 in this control experiment was constant at different time intervals of incubation, which confirms that Glc7 "in vitro" dephosphorylates Pma1.

"In vitro" dephosphorylation of Pma1 by Sit4

In order to test the ability of Sit4 to dephosphorylate Pma1 "in vitro" we used a pYEX 4T-1-*SIT4* construct, which can express the protein in the soluble fraction in yeast. The expression was confirmed with anti-GST antibody after expression induction with $CuSO_4$, Figure 46. The purified Sit4 was incubated with activated Pma1 for different time intervals. Figure 47A shows that Sit4 can dephosphorylate Pma1 "in vitro" as the phosphorylation level at serine 911 threonine 912 decreased after incubation with Sit4, starting from 30 minutes incubation and decreasing more with increased incubation time. A control experiment was conducted simultaneously (Pma1 incubated with purified GST extracted from cells harboring pYEX 4T-1 induced with $CuSO_4$) and as shown in Figure 47B the phosphorylation level of Pma1 at serine 911 threonine 912 in this control experiment was not affected.

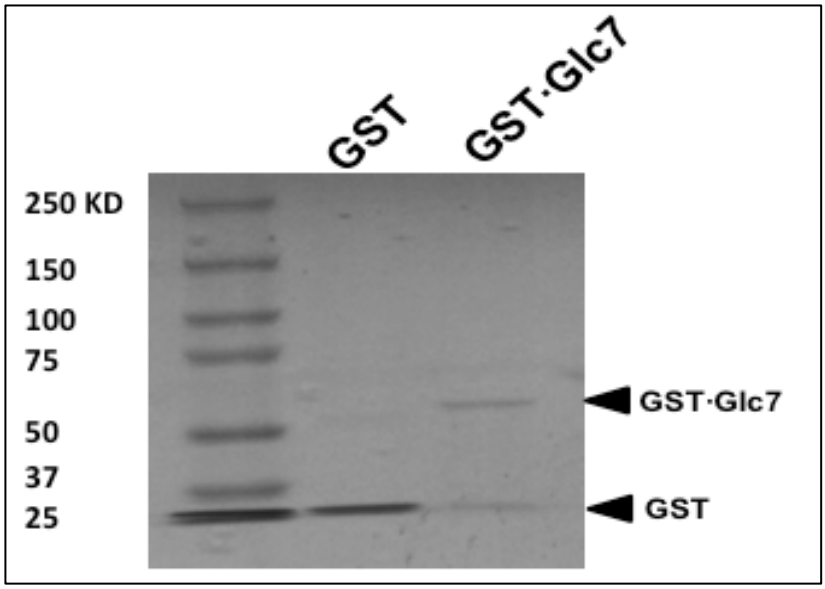


Figure 44. Expression of recombinant Glc7 protein from pGEX-3X vector in *Escherichia coli*.

RS-405 strain (*E. coli* transformed with pGEX-3X-*GLC7*) and a control strain (*E. coli* transformed with pGEX-3X). The cells were grown to exponential phase at 37°C and then induced with IPTG (see"Materials and Methods"). The cells were harvested, sonicated and the soluble materials were purified with Pierce™ Glutathione Magnetic Beads, then subjected to SDS-PAGE and stained for protein with Coomassie. ***Lane** 1* marker (Precision Plus Protein Dual Color Standards) from BioRad; ***lane** 2,* a fraction from the control cells (harboring pGEX-3X) induced with 0.1- 0.2 mM IPTG; ***Lane 3*** a fraction from cells harboring pGEX-3X-*GLC7* induced with 0.1- 0.2 mM IPTG. The upper arrow refers to GST-Glc7 in correct position Glc7 (35.9) + GST (26) = 61 KD. The experiment was made once.

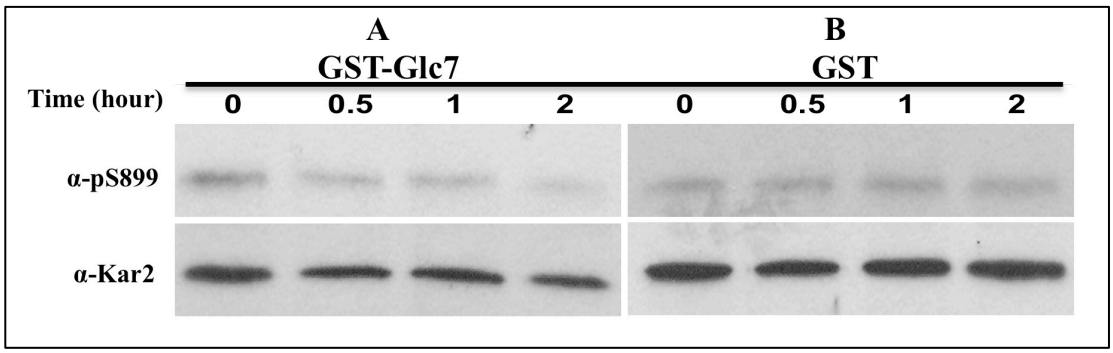


Figure 45. Purified Glc7 shows "in vitro" effect on activated Pma1.

E. coli (A) RS-405 strain (transformed with pGEX-3X-*GLC7)* and (B) a control, strain *E. coli* DH5α transformed with pGEX-3X. The cells were grown to exponential phase at 37°C and then induced with IPTG (see "Materials and Methods"). The cells were harvested, sonicated and the soluble materials were purified with Pierce™ Glutathione Magnetic Beads. The purified soluble protein was incubated with activated Pma1 at 30°C for the specified times then membrane proteins were analyzed by Western blotting with antibodies against phosphorylated Ser899 (pS899) and α-Kar2 as control of loading, as described in materials and method. The bands recognized by the antibodies represent Pma1 of 100 KD and Kar2 of 75 KD. The experiment was made once.

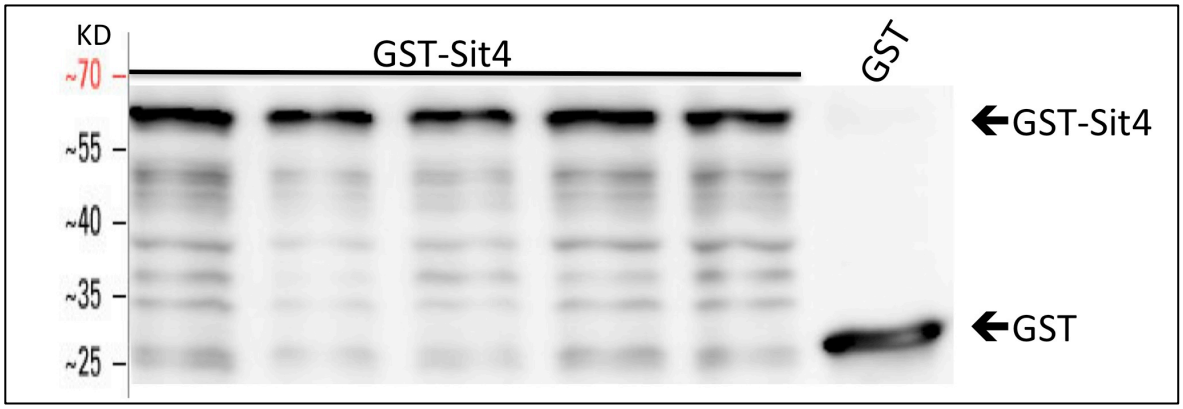


Figure 46. Expression of recombinant Sit4 protein from the pYEX 4T-1 vector in *Saccharomyces cerevisiae*.

S. cerevisiae strain (RS-259 transformed with pYEX 4T-1-*SIT4*) and a control strain (RS-259 transformed with pYEX 4T-1). The cells were grown to exponential phase at 28°C and then induced with $CuSO_4$ 0.5 mM (see "Materials and Methods"). The cells were harvested and broken with the aid of glass beads in specific lysis buffer then soluble material membrane proteins were analyzed by Western blotting with antibodies against GST. ***Lanes*** 1 to 5, fractions from cells harboring pYEX 4T-1-*SIT4* after induction with $CuSO_4$ 0.5 mM ; ***lane*** 6, a fraction from the control cells (harboring pYEX 4T-1) induced with $CuSO_4$ 0.5 mM. The upper arrow refers to GST-Sit4 in correct position Sit4 (35.9) + GST (26) = 61 KD. Standard protein markers (PageRuler Prestained Protein Ladder, from Thermo Scientific), with masses indicated in kDa was used. The experiment was made once.

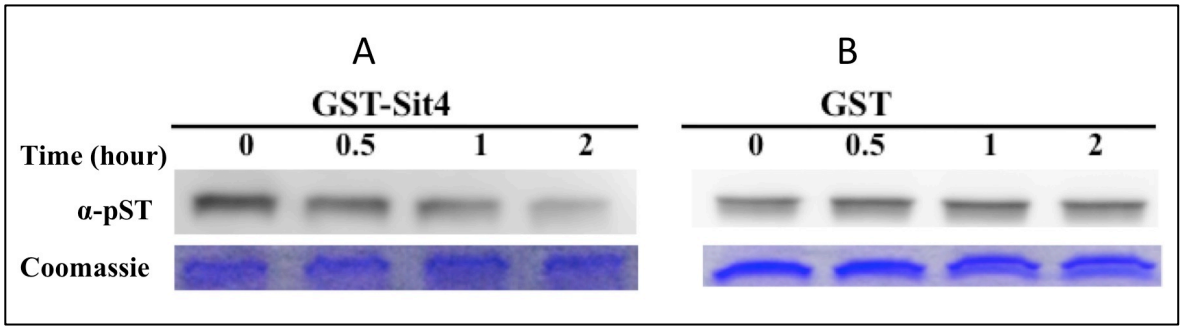


Figure 47. Purified Sit4 shows "in vitro" effect on activated Pma1.

S. cerevisiae strain (A) RS-259 transformed with pYEX 4T-1-*SIT4* and (B) a control, strain RS-259 transformed with pYEX 4T-1. The cells were grown to exponential phase at 28°C and then induced with $CuSO_4$ 0.5 mM (see "Materials and Methods"). The cells were harvested and broken with the aid of glass beads in specific lysis buffer. The soluble materials were purified with (Glutathione sepharose ^{TM} 4B (GE Healthcare) Sweden). The purified soluble protein was incubated with activated Pma1 at 30°C for the specified times then membrane proteins were analyzed by Western blotting with antibodies against the double phosphorylation at serine 911 and threonine 912 (pST). Coomassie stained membrane is presented as control of loading, procedures are described in materials and method. The bands recognized by the antibody and stained by Coomassie represent Pma1 of 100 KD. The experiment was repeated three times with similar results.

TOR kinase is a positive regulator of Pma1 activity

The idea now was to take another direction and test the effect of one of the most important kinases of eukaryotic cells, TOR, on Pma1 activity. Rapamycin is known to inhibit TORC1 so our first approach was to test the effect of rapamycin on Pma1 activity. In order to do that we first had to determine which concentration of rapamycin will give good inhibition of TORC1, and as the latter is necessary for growth, the effect of rapamycin was measured by OD of growing cultures. Figure 48 shows that higher concentrations of rapamycin are inhibiting TORC1, as represented by inhibition of growth.

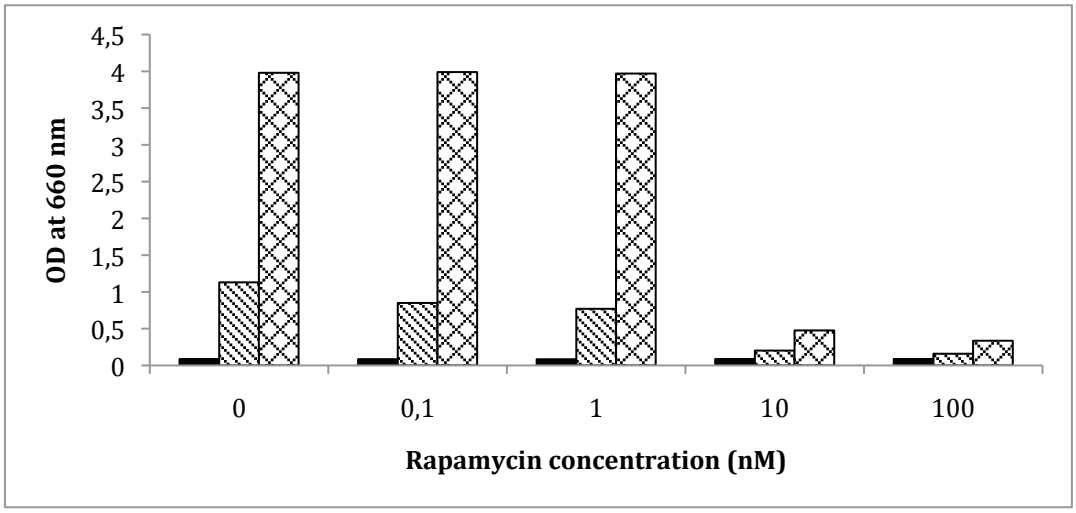


Figure 48. Effect of rapamycin different concentrations on TOR Kinase shown in terms of yeast growth inhibition

S. cerevisiae strain RS-132 was inoculated in YPD with initial OD at 660 nm = 0.1 with the specified rapamycin concentration (0, 0.1, 1, 10, 100 nM) and incubated (30°C, 200 rev/min). Growth was detected by measuring OD at 660 nm at zero time (■), again after 8 hours (▨) and finally after 24 hours (▩). The experiment was made once.

The next step was to treat the cells with rapamycin and this was made at two stages: in the growth medium (0.5 μM) and during treatment of cells with glucose (10 μM). The concentration of this drug had to be increased in order to keep the ratio of rapamycin to cells in the procedures of the experiment. In the experiment of Figure 48 rapamycin was added to cells at an OD = 0.1, while for Pma1 activity the culture was at about OD = 2 and after concentrating cells for incubation with glucose OD was about 40.

When the activity of Pma1 was measured "in vitro" after incubation with rapamycin for different time intervals, it showed a high decrease completed in two hours (Figure 49A). The same decrease was confirmed when the phosphorylation level of these samples was measured using α-pST (Figure 49B). Total Pma1 protein on the other hand did not show any change of amount when tested with the specific antibody, which means that the effect on activity is not explained by decreased amount of the enzyme.

Still in the same direction, testing the effect of TOR kinase on Pma1 activity, the second approach was to measure Pma1 activity in a strain that is TORC1 and TORC2 thermosensitive, SH221 *(tor1Δ tor2^{ts})* in comparison to its control strain (SH100) (Helliwell *et al.,* 1998). First to confirm the phenotype, Figure 50A shows the different growth of the thermosensitive and control strain at non-permissive temperature (37°C) on YPDA: while the control strain could grow normally, the *tor1Δ tor2^{ts}* mutant could not grow. The same difference is confirmed in terms of OD when the strains were grown in broth (Figure 50B). Both strains grew equally at room temperature (Figure 50C).

After finding good difference in growth between the two strains after 6 hours incubation at 37°C, this time interval was used in the following experiment. Figure 51A shows the decreases of Pma1 "in vitro" activity in SH221 strain in comparison to its control strain (SH100) at non-permissive temperature. Such a decrease was confirmed by measuring the phosphorylation level of the same samples using α-pST (Figure 51B). These results confirm that TOR is a positive regulator of Pma1.

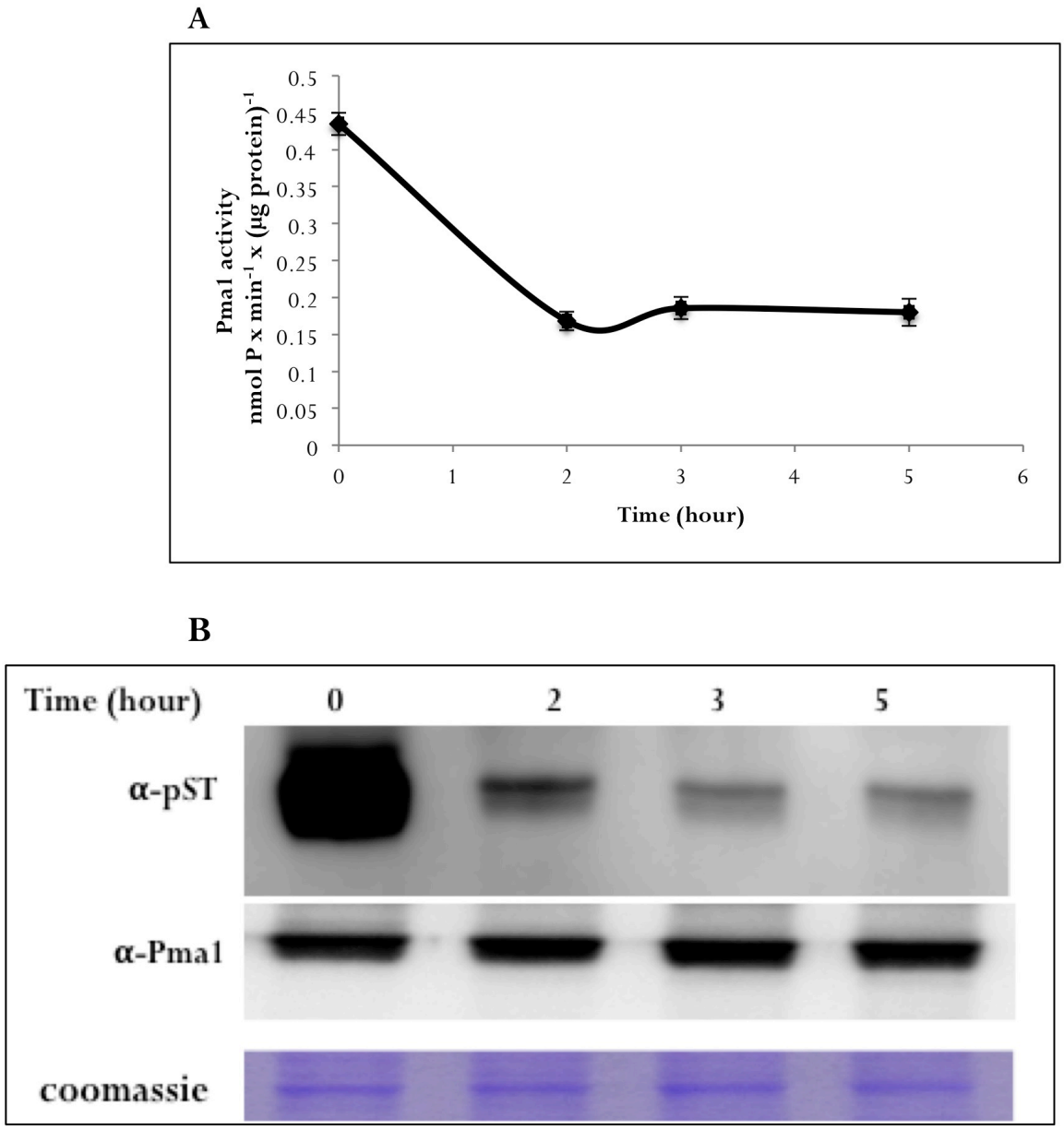
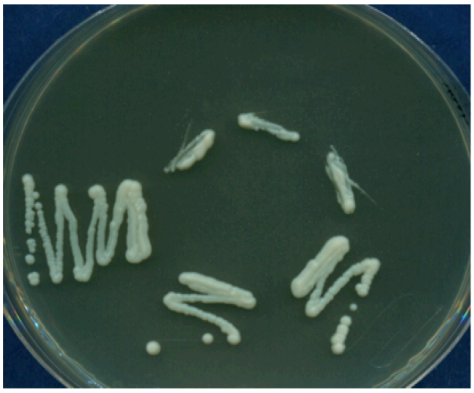


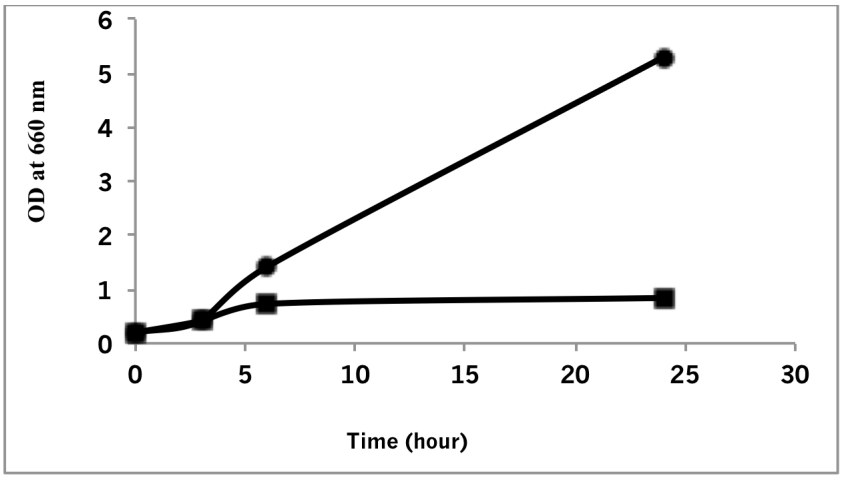
Figure 49. Rapamycin decreases Pma1 activity. (A) "in vitro" Pma1 specific activity of cells treated with rapamycin and (B) phosphorylation level of Pma1 of the same samples.

S. cerevisiae strain RS-132 exponential phase cultures were incubated with rapamycin 500 nM at 30°C, 200 rev/min for the indicated time intervals. At each time interval cells were harvested, washed resuspended with H_2O and rapamycin was added to final concentration of 10 µM and mixed well by vortex. The suspension was proceeded to extract the Pma1. (A) Pma1 specific activity (◆) was assayed "in vitro" as described in materials and method. (B) The same extracted membrane proteins were analyzed by western blotting with antibodies against the double phosphorylation at serine 911 and threonine 912 (pST) and against total Pma1. Coomassie stained membrane is presented as control, procedures are described in Materials and Methods. The bands recognized by the antibody and stained by Coomassie represent Pma1 of 100 KD. The experiment (A) was repeated three times and and error bars correspond to standard error and experiment (B) was repeated twice with similar results.

A



B



C

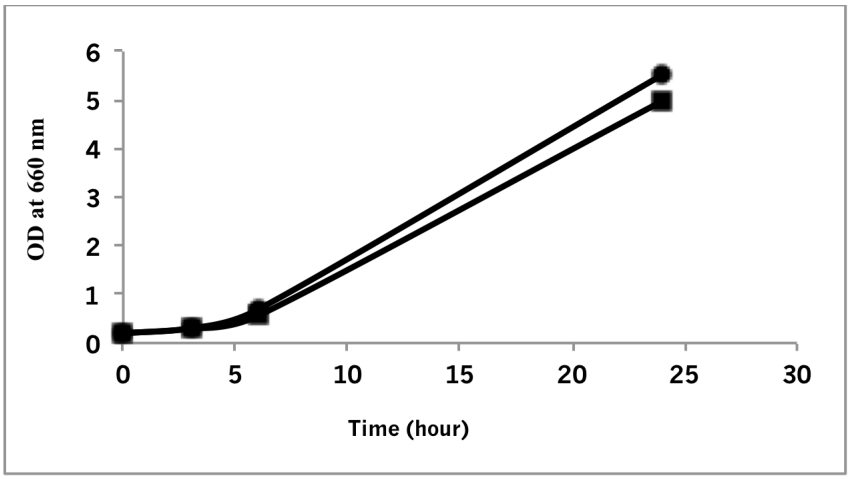
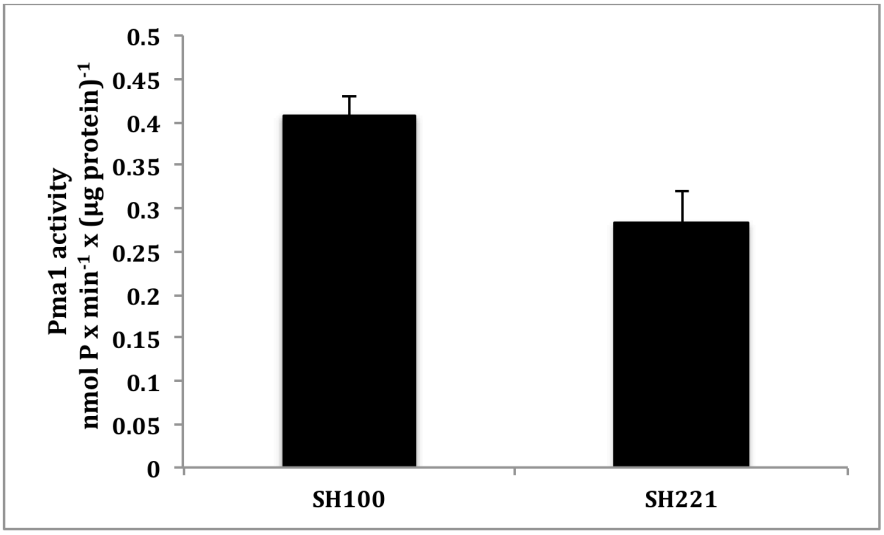


Figure 50. Growth difference between *tor1Δ tor2^{ts}* and its control strain at 37°C and at room temperature.

S. cerevisiae strain SH221 (■) and its control strain SH100 (•) were cultured on YPDA plate (A), three different colonies of each strain and incubated at 37°C for two days. stationary phase culture of one transformant from each strain was inoculated in fresh YPD broth with starting OD of 0.2 then incubated at 37°C (B) or at room temperature (C) and growth was determined by measuring OD at different time intervals. The experiments were made once.

A



B

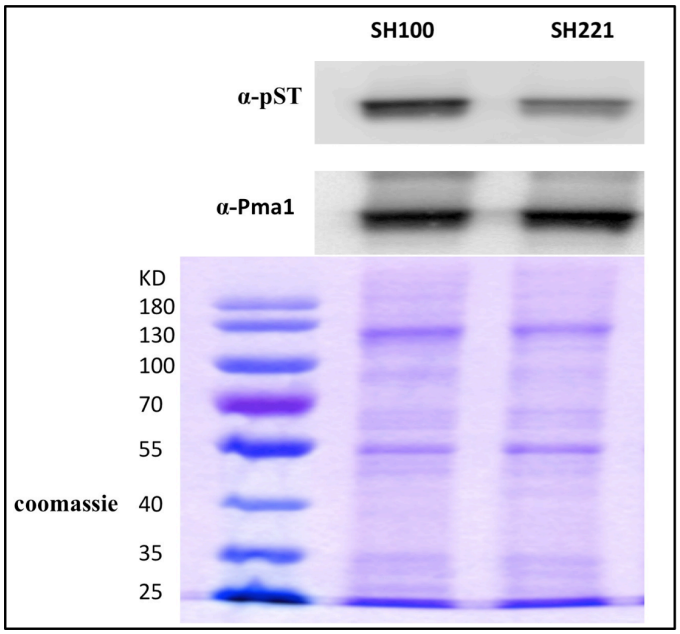


Figure 51. Decrease of Pma1 activity of *tor1*Δ *tor2*^{ts} strain when grown at non-permissive temperature.

S. cerevisiae strain SH221 and its control strain SH100, stationary phase cultures were inoculated in fresh YPD broth with starting OD of 0.2 and incubated at 37°C for 6 hours. cells were harvested and resuspended with H_2O and processed to extract the Pma1. (A) specific activity of the extracted Pma1 was assayed "in vitro". (B) The same extracted Pma1 samples were analyzed by western blotting with antibodies against the double phosphorylation at serine 911 threonine 912 (pST) and also against total Pma1. Coomassie stained membrane is presented as control. Procedures are described in materials and method. Standard protein markers (PageRuler Prestained Protein Ladder, from Thermo Scientific), with masses indicated in kDa was used. Both experiments (A) and (B) were repeated three times with similar results and error bars in (A) correspond to standard error.

DISCUSSION

DISCUSSION

Lack of effect of typical chemical inhibitors of protein phosphatases on Pma1 activity

Okadaic acid and calyculin A are potent inhibitors of type 1 protein phosphatases (PP1) in all mammalian cells because they are permeable. In yeast Glc7 is the most important PP1, it is essential for growth and it has been connected to ion homeostasis. However, when these inhibitors, and also tungstate, were added to a yeast culture there was no effect on Pma1 activity (Figure 24). As no inhibition of growth was observed in case of treatment with okadaic acid and as Glc7 is an essential enzyme, it is likely that this inhibitor has poor uptake in yeast. In case of calyculin A there was a significant growth inhibition, shown in Figure 24D. Pma1 activity of cells treated with calyculin A was slightly higher than in non-treated cells. Yet, we cannot conclude that Glc7 inhibition activates Pma1 because the observed increase is very small (about 10%) and the experiments were only done once as the results were disappointing. Both okadaic acid and calyculin A are hydrophobic compounds and their uptake by cells is affected by many things like the partitioning to cell membrane and also from the membrane to the cytoplasm and they might have needed much longer time in order to be well absorbed as stated by Swingle *et al*. (2007). Also the different compounds do not necessarily diffuse into cells at the same rate, especially if the compounds are added at different concentrations and finally, because organic anion efflux pumps may use these compounds as substrates (Swingle *et al*., 2009). On the other hand, tungstate is water-soluble and at the concentration we used in the experiment (10 mM) it is reported to give a very high inhibition of Glc7 activity (Rodriguez-Hernandez *et al.,* 2012). These considerations indicate that chemical inhibitors of Glc7 have no effect on Pma1 activity and that other methods should be used to reach definitive conclusions.

Effect of expression of a dominant-negative form of *GLC7* on Pma1 activity

As Glc7 is an essential enzyme, a null mutant could not be tested. Truncated Glc7 (Glc7') is reported to have a dominant negative effect, partially inhibiting wild type Glc7, (Wek *et al*., 1992). Constitutive expression of Glc7' driven by its genomic promoter had no effect on Pma1 activity "in vitro" using a multicopy plasmid (Figure 25). The same lack of effect of expressing Glc7' on Pma1 activity was noticed when the activity was measured "in vivo", when the *GLC7'* gene was expressed from multicopy or centromeric plasmids (Figure 26). We also tried to measure the effect of *GLC7'* using

a slightly different method because we thought the kinase effect on Pma1 may be so high under glucose addition that it masks any possible effect of the phosphatase. We removed the glucose after 10 minutes activation to measure the dephosphorylation rate with antibody against phosphorylated Ser899 and also against the double phosphorylation at Ser911 and Thr912 (Mazón *et al.,* 2015), and again even with this indirect approach there was no effect on Pma1: the dephosphorylation rate after removal of glucose in the cells transformed with *GLC7'* was the same as in those transformed with empty plasmid (Figure 32).

When these transformants were tested for resistance to hygromycin B they showed a little more sensitivity than wild type (Figure 33). In the same experiment, we noticed that putting *GLC7'* on high copy number plasmid does not produce higher effect than putting it on centromeric plasmid. On the other hand, when those transformants were tested for acetic acid resistance no change of tolerance was observed with respect to the control strain (the same strain transformed with empty plasmid) (Figure 34). An increase in Pma1 activity should have resulted in improved tolerance to intracellular acidification induced by acetic acid. Because Pma1 activity was not affected (measured either "in vivo" or "in vitro") the small effect on hygromycin tolerance could be due to an effect of Glc7' on potassium transporters (Trk1, Trk2) that are regulated by Hal4 and Hal5 kinases according to Mulet *et al*. (1999). When these genes are disrupted this leads to greater cation sensitivity and increase the membrane potential. Another explanation could be that Glc7 directly affects the channels involved in hygromycin transport.

Expression of *GLC7'* under control of a doxycycline-regulated promoter

As the continuous expression of Glc7' may be deleterious to cells and selections of non-expressing mutants could have occurred, a truncated form of Glc7 under control of a doxycycline-regulated promoter was used. When Glc7' was conditionally expressed growth was inhibited (Figure 27, 28, 29) because Glc7 is a vital protein for cell growth and with such high expression of a dominant negative gene cells cannot grow. Cells were allowed to grow in the presence of a selected concentration of doxycycline until exponential phase and then were incubated in a medium free from doxycycline to induce Glc7' expression. The activity of Pma1 was assayed "in vitro" after different time intervals and there was no effect on Pma1 activity in the two constructs used (with and without HA3-His6 at the C-terminus; Figure 30). The

expression of the *GLC7'* gene, suggested by the observed growth inhibition, was confirmed by semi quantitative RT-PCR and as shown in Figure 31. These results confirm the previous results with constitutive expression of *GLC7'* and suggest that inhibition of Glc7 has a minor effect on Pma1 activity probably because it is involved in the dephosphorylation of S899 which is related to Pma1 Km (Mazón *et al.,* 2015).

Mutants in *GLC7* exhibit altered ion homeostasis

Some Glc7 mutants were tested for Pma1 activity and the results showed that *glc7-1* and *glc7-132*, reported to have decreased glycogen accumulation and less Glc7 activity, both had a higher rate of proton extrusion from cells (higher "in vivo" Pma1 activity) (Figure 36B), higher tolerance to acetic acid and lower tolerance to hygromycin B and LiCl (Figure 35). This may suggest that these *glc7* mutations increase Pma1 activity but when the activity was assayed "in vitro" in isolated membranes only *glc7-1* exhibited slightly higher Pma1 activity while *glc7-132* did not show any increase (Figure 36A). On the other hand a third mutant, *glc7-109*, reported to have a dominant, hyperglycogen defect and that was expected to have a lower Pma1 activity and consequently higher sensitivity to acetic acid, it did show the latter effect but when the activity of Pma1 was assayed it was almost equal to the wild type, both "in vitro" and "in vivo". Also, *glc7-109* was expected to have lower sensitivity to hygromycin and LiCl, yet it acted in an opposite direction showing higher sensitivity to both of them. Some of these phenotypes of *glc7-109* were already tested by Williams-Hart T *et al.* (2002) and are in agreement with our results. So if we tried to make some conclusions about such results with phenotypes of tolerance to toxic cations and acetic acid going in unexpected directions, it seems that the mechanism is not clear and it could be that *glc7* mutations affected the transport system of these cations and acetic acid. When the Pma1 activity was assayed "in vitro", it was equal to wild type (*glc7-132* and *glc7-109*) yet there were effects on acetic acid and toxic cation tolerance. It could be that the effect of these Glc7 mutations is not on Pma1 but for example on potassium transporters (see above). Williams-Hart *et al*. (2002) state that Glc7 itself might be regulating ion homeostasis through directly controlling ion transport and/or plasma membrane potential. However, in the same publication, when the ability of the *glc7-109* mutant to grow on low K concentration conditions was tested, it grew equally to the wild type, suggesting that the increased sensitivity to LiCl and Hygromycin B is not due to a defect in K^+ transporter. As this mutant is clearly hyperpolarized, a more

direct determination of K^+ transport should be made. In any case, these authors also stated that they could not find a direct involvement of Glc7 in Pma1 regulation. So we believe our results are in the same direction, where Glc7 does not affect Pma1 activity yet it affects ion sensitivity, which could be explained by the hypothesis that Glc7 activates either K^+ transport or an unknown mechanism of transport that affects ion homeostasis. Another explanation could be that because Glc7 is involved in dephosphorylation of S899 on glucose starvation (Mazón *et al.,* 2015) which only affects the Pma1 Km, so the final effect on Pma1 activity is minor and is more apparent in terms of ion tolerance than "in vitro" assay with high ATP concentration. To further complicate this issue, the "in vivo" assay of Pma1 activity by proton extrusion from cells is not only dependent on Pma1 activity but also influenced by K^+ transport for electrical balance (Serrano, 1980; Bissoli *et al*., 2012).

Because the regulatory subunits are responsible for Glc7 specificity (Luan 2003), it may be that a mutation of *GLC7* affects its binding to certain regulatory subunits responsible for the enzyme binding to glycogen synthase but that such a mutation will have no effect on the enzyme activity on other substrates. Therefore it is very complicated to explain the glycogen defect and the different acetic acid and toxic cation sensitivity in the mutants.

A plausible role for protein phosphatases in the activation of Pma1 by glucose metabolism

As discussed at the Introduction, protein phosphatases may be more than mere reset buttons of important phosphorylated proteins. The steady-state level of phosphorylation of important regulated proteins such as Pma1 could depend not only on the activity of the protein kinases but also on the relative activities of the kinases and phosphatases acting on the regulatory domain of the enzyme. The fact that these enzymes have not been identified by loss of function approaches suggests that they may be redundant or encode essential functions. As we have discarded the essential *GLC7* gene, a plausible mechanism for glucose activation could be that, in addition to activation of some protein kinases, phosphorylated metabolites derived from glucose cause a general inhibition of protein phosphatases. Although protein phosphatases have little specificity "in vitro" and are inhibited by many phosphorylated compounds, the situation "in vivo" is uncertain because the accessory subunits of the phosphatases put them in close proximity to their protein substrates. In any case, glucose induces in yeast

cells a dramatic increase in phosphorylated metabolites, reaching total values around 10 mM (Gancedo and Gancedo, 1973) and therefore it is not unlikely that many protein phosphatases could be inhibited "in vivo" and contribute to activation of Pma1. This could explain the mysterious regulatory pathway of glucose requiring metabolism of the sugar (Belinchón and Gancedo, 2007).

Effect of expression of a dominant-negative form of *GLC7* and of glucose metabolism on phosphorylation of eIF2α

The effect of the possible inhibition of Glc7 by glucose metabolites was investigated "in vivo" by measuring the phosphorylation level of eIF2*α* with specific antibody in wild type and in the *GCN2*^{c} mutant, before and after addition of glucose. Figures 37 and 38 showed that glucose starvation led to decreased phosphorylation of eIF2α in the wild type. These results differ from those of Castelli *et al*. (2011), who found that glucose starvation does not affect the levels of phosphorylated eIF2α. The experimental conditions, however, were different from ours because they worked with cells in rich YPD medium and then removed the glucose but kept all the other components of media while we resuspended cells in water with or without glucose. It would be interesting to determine what component of medium explains the differences in the results.

The decrease of eIF2α phosphorylation level in the wild type means that glucose starvation leads to either an increase of phosphatase activity or a decrease of the kinase's one. Then checking the effect on a *GCN2*^{c} mutant that has a constitutive hyperactivity of the kinase, we found out that there was no change of the phosphorylation level of eIF2α upon glucose starvation (Figure 38). This may suggest that the effect of glucose on eIF2α phosphorylation is mainly due to activation of Gcn2 because when it was kept constant as in the *GCN2*^{c} mutant there was no change of the phosphorylation level upon removal of glucose. However, the *GCN2*^{c} mutant has a constitutive hyperactivity and this high effect might have masked the increase of Glc7 activity under glucose starvation conditions. Therefore we believe that the hypothesis that glucose metabolism inhibits "in vivo" the activity of protein phosphatases such as Glc7 deserves further investigation.

In the strain expressing Glc7' under doxycycline promoter there was no difference of the phosphorylation level of eIF2*α* before and after inducing the expression (Figure 39). The *GLC7'* expression was confirmed by semiquantitative RT-

PCR under the same conditions as mentioned before (Figure 31). However, in this experiment glucose was present and this may have already inhibited Glc7, making further inhibition by Glc7' undetectable. Also, the experiment was done in minimal medium where Gcn2 is hyperactivated by the lack of amino acids. According to Wek *et al*. (1992) Glc7' produces increased level of eIF2α phosphorylation in the *gcn2-507* mutant, with reduced activity of Gcn2. It maybe that an effect of Glc7'on eIF2α phosphorylation could only be seen in a defective mutant with decreased activity of Gcn2.

Also in the same Figure 37 we have tested the different protein extraction methods in order to choose the best one for all the following experiments of western blotting and the results showed that the amount of protein produced by three different types of protein extraction (Laemmli, alkaline lysis and TCA) was so close unlike what was mentioned by (Wright *et al*., 1989). So we settled to the most convenient one, which is Laemmli method.

Effect of *sit4* null mutation on the activity of Pma1

Another important yeast phosphatase is Sit4, a major PP2A phosphatase. The *sit4* null mutant is viable so we used it to investigate the effect of Sit4 on Pma1. The phosphorylation level of Pma1 by using specific antibody against the double phosphorylation of SerT911 Thr912 (that is the major regulatory event by increasing the maximal activity of the enzyme, see Mazón *et al.,* 2015) was less than that of the control strain after glucose activation (Figure 40). On the other hand, the mutant showed a decreased Pma1 activity "in vitro" and "in vivo" (Figures 41 and 42, respectively). These results together indicate that Sit4 is a positive regulator of Pma1 phosphorylation and activity.

The effect of LiCl and hygromycin on the *sit4* mutant was not clear (Figure 43). The growth of this mutant is much slower than that of control strain in normal conditions and therefore the effect of *sit4* null mutation on tolerance to toxic cations was difficult to ascertain.

"In vitro" and "in vivo" effects of Glc7 and Sit4 on Pma1

It was possible to express active GST-Glc7 in the soluble fraction from *E. coli* (Figure 44). On the other hand, we made many trials to express GST-Sit4 in *E. coli* using several plasmids and growth conditions but we could never recover it in the

soluble fraction. Finally we could produce soluble and active GST-Sit4 by expression in yeast (Figure 46). GST-Glc7 and GST-Sit4 were purified, incubated with glucose-activated Pma1 and finally the level of phosphorylation of Pma1 was checked with specific antibodies. Recombinant Glc7 dephosphorylated phospho-Ser899 (Figure 45) as suggested by Mazón *et al*. (2015). On the other hand, recombinant Sit4 dephosphorylated the major activation site of Pma1 (double phosphorylation at Ser911 Thr912; Figure 47).

The "in vitro" activity of protein phosphatases is not very specific because they depend "in vivo" on regulatory subunits to target them to the different specific substrates (Luan, 2003). Therefore these experiments do not demonstrate that Glc7 and Sit4 act on Pma1 "in vivo". Actually, our experiments on inhibition of Glc7 "in vivo" failed to demonstrate a participation of this phosphatase on the regulation of Pma1. It cannot be discarded, however, that the activating site of Pma1 was dephosphorylated by several redundant phosphatases and that Glc7 could be one of them.

On the other hand, the effect of the null mutation of Sit4 on Pma1 activity ("in vitro", "in vivo" and phosphorylation level at the regulatory site, Ser911 Thr912) suggests that Sit4 promotes "in vivo" the phosphorylation of the activating site of Pma1 instead of decreasing it as expected from a phosphatase. Therefore, instead of having a direct effect on Pma1 by dephosphorylation, Sit4 must act "in vivo" by an indirect mechanism to activate Pma1 by promoting the phosphorylation of its activating site. The nature of this mechanism is presently unknown but it could be related to the growth-promoting effect of Sit4. It can be speculated that Sit4 could dephosphorylate and activate a protein kinase acting on Pma1 or dephosphorylate and inactivate protein phosphatases acting on Pma1.

TORC1 kinase is a positive regulator of Pma1 activity

The participation of TORC1 protein kinase in the regulation of Pma1 was investigated by two different mechanisms: using the specific TORC1 inhibitor (rapamycin) and increasing the temperature in a thermosensitive mutant of the TOR system.

Rapamycin treatment inhibited growth of yeast cells (Figure 48) because TORC1 is necessary for cell growth (Loewith and Hall, 2011). Pma1 activity showed a high decrease upon incubation with rapamycin, (Figure 49A) and there was a decrease on the phosphorylation level of the activation domain of Pma1 (Ser911 Thr912; Figure

49B). This indicates that the TOR kinase is necessary for maximal activation of Pma1 by glucose. Such an effect could either be direct, TORC1 phosphorylating Pma1, or indirect by activating another kinase or inhibiting a phosphatase with negative effect on Pma1. The effect could also be a consequence of the inhibition of growth by rapamycin. Rapamycin treatment leads to inhibition of TORC1 and as a result to a G0 growth arrest within one generation (Barbet *et al*., 1996). However, growth arrest by glucose deprivation does not affect the activation of Pma1 upon glucose addition (Serrano, 1983). Another possibility could be a decrease in Pma1 amount. Rapamycin treatment causes high drop in protein synthesis by blocking translation initiation (Barbet *et al*., 1996). Such an explanation could not be used to justify the decrease in Pma1 activity in terms of decreased protein synthesis because according to Benito *et al*., (1990) the half life of Pma1 under our experimental conditions (presence of glucose and exponential phase culture) is more than 20 hours while the decrease in activity in our experiment was detected after just 2 hours, so clearly this is not the explanation. Accordingly, we did not observe a decrease in Pma1 protein levels measured with antibodies against the whole enzyme during the rapamycin treatment (Figure 49B).

Another way to inhibit TORC1 is by using a non-permissive temperature with a thermosensitive mutant of this essential protein kinase. Mutant SH221 (*tor1 tor2-21^{ts}*) is temperature sensitive for both TOR complexes and functions (activation of translation initiation of TORC1 and the organization of the actin cytoskeleton of TORC2), although only TORC1 is sensitive to rapamycin (Helliwell *et al.,* 1998). Its growth was normal at room temperature but defective at 37ºC as expected from inhibition of TOR (Figure 50). Accordingly, incubation at the non-permissive temperature reduced the activity of Pma1 and the phosphorylation of Ser911 Thr912 in this mutant with no effect on the control strain (Figure 51).

According to Helliwell *et al.* (1998) loss of *TOR1* and *TOR2* functions causes arrest in the early G1 phase of the cell cycle, like rapamycin treatment does (Barbet *et al*., 1996) and the arrested cells show characteristics of starved cells in stationary phase (G0), glycogen accumulation and a decrease in protein synthesis. The block in protein synthesis happens at the level of initiation and is the cause of the cell cycle arrest. TOR2 overlaps with TOR1 and mediates protein synthesis and cell cycle progression through the G1 phase of the cell cycle in response to nutrients (Barbet *et al*., 1996).

We have observed that in order to detect the effect of TOR on Pma1 activity cells must be in exponential phase, because at the beginning we tried treatment of

rapamycin on cells in stationary phase and this did not lead to change of Pma1 activity, probably because TORC1 activity is already decreased in this phase due to lack of nutrients (Galdieri *et al.,* 2010).

Another practical point refers to the fact that although 10-100 nM rapamycin was enough to inhibit growth of inoculated cultures, in grown cultures with much higher cell density, these concentrations of rapamycin had no effect on Pma1 activity (Figure 48). We then considered that when working with hydrophobic inhibitors such as rapamycin there should be an increase in inhibitor concentration when the cell concentration is increased. With cells in late exponential phase and during the glucose activation step before cell homogenization the concentration of yeast is much higher than in inoculated cultures at the beginning of growth. When rapamycin was added to the cells at higher concentration (10 μM) keeping the rapamycin to cells ratio, the effect on Pma1 activity was noticed.

The decrease of Pma1 activity after rapamycin treatment was higher than that obtained when TOR was inhibited in the thermosensitive mutant SH221 by incubation at the non-permissive temperature (activity decreased to 45% and 65% of control, respectively). This could mean that the effect on Pma1 of the high concentrations of rapamycin employed may have additional targets other than TORC1 or that the non-permissive temperature does not fully inhibits TORC1.

Because Sit4 increased Pma1 activity and rapamycin decreased it, and knowing that TORC1 inactivation results in Tap42 weakened association with phosphatases (Yan *et al.,* 2006), so probably Sit4 needs the binding with Tap42 in its pathway to activate Pma1.

Our finding that both TORC1 and Sit4 are required for full activation of Pma1 is in agreement with the finding that Sit4 acts downstream of TORC1 for many important physiological functions, including nutrient sensing for growth control (Rhode *et al*., 2004; Loewith and Hall, 2011), a function also contributed by Pma1, activated by glucose and determining growth rate (Serrano, 1983: Portillo and Serrano 1989).

Recently it has been reported that Pma1 activation increases intracellular pH and this activates TORC1 (Dechant *et al*., 2014). Together with our results this suggest that there is cross activation between TORC1 and Pma1, the two cellular systems responding to nutrients. TORC1 is mostly activated by amino acids while Pma1 is mostly activated by sugar metabolism and therefore it makes sense that there is cross activation to integrate both kinds of nutrient signals.

CONCLUSIONS

CONCLUSIONS

The objectives of the present thesis were:

1- Determine if Glc7, the major PP1 protein phosphatase of yeast and also implicated in ion homeostasis, has a role in the activation of yeast plasma membrane H^+-ATPase (Pma1) by glucose

2- Determine if Sit4, a PP2A protein phosphatase implicated in yeast growth, has a role in the activation of Pma1 by glucose

3- Investigate the participation of the growth-promoting protein kinase TORC1 in the activation of Pma1 by glucose

And the conclusions are:

1- Inhibition "in vivo" of Glc7 results in neither increased activity of Pma1 nor increased phosphorylation of the major regulatory site (Ser911 Thr912) in the presence of glucose metabolism. However, in the *glc7-1* and *glc7-132* mutants acetic acid tolerance and Pma1 activity are increased. Glc7 may be involved in regulation of Pma1 but with only a minor effect and other protein phosphatases are redundant with it.

2- Glc7 itself might be regulating ion homeostasis through directly controlling ion transport and/or plasma membrane potential or an unknown mechanism of transport that affects ion homeostasis.

3- Protein phosphatase Sit4 can dephosphorylate "in vitro" the regulatory site of the glucose-activated form of Pma1. However, deletion of the *SIT4* gene results in decreased phosphorylation of the regulatory site and decreased activity of Pma1 in the presence of glucose metabolism. Therefore Sit4 activates "in vivo" Pma1 by an indirect mechanism instead of inactivating it by direct dephosphorylation. This activation may contribute to the activation of growth and proliferation by this phosphatase

4- Inhibition of protein kinase TORC1 results in decreased phosphorylation of the regulatory site and decreased activity of Pma1 in the presence of glucose metabolism. Activation of Pma1 by TORC1 maybe direct or through contribution to the activation of growth and proliferation by this kinase

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