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**DEPARTAMENTO DE CIÊNCIAS DA VIDA**

FACULDADE DE CIÊNCIAS E TECNOLOGIAS DA UNIVERSIDADE DE  
COIMBRA

*Candida albicans OPY2* gene complementation in *Saccharomyces cerevisiae*.

Carla Cristina Mestre Veríssimo Dias

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# *Candida albicans* *OPY2* gene complementation in *Saccharomyces cerevisiae*

Dissertação apresentada à  
Universidade de Coimbra para cumprimento  
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grau de Mestre em Bioquímica, realizada sob  
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Madrid), da Professora Doutora Rebeca  
Alonso-Monge (Universidad Complutense de  
Madrid) e da Professora Doutora Teresa  
Gonçalves (Universidade de Coimbra).

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# Summary

*Candida albicans* is a commensal fungus that can be virulent when the immune system is compromised. Pathogens have mechanisms that allow them to survive under different kinds of stresses. The MAP kinase pathways are an example of these mechanisms and are vital for adaptation to environmental changes and regulation of several functions such as morphogenesis, cell growth, adaptation to stress and cell wall construction. One of these pathways is the HOG pathway that is responsible for generating an adaptive response to high-osmolarity environments, among others stresses. In *S. cerevisiae* the Opy2 protein was discovered to have a role in this pathway and it is assumed to contribute to the HOG pathway specificity. In this work we studied the role of the *C. albicans* *OPY2* gene when introduced into a *S. cerevisiae* strain in which both the *OPY2* and the *SSK1* genes were deleted and we verified that the *C. albicans* *OPY2* gene partially complements the osmosensitivity of the *opy2 ssk1* mutant and it is not able to activate the HOG1 MAPK in the same mutant under osmotic stress. After different experiment we concluded that the Opy2 plays a different role in *C. albicans* and in *S. cerevisiae*.

**Key Words:** *Candida albicans*, MAPK pathways, osmotic stress, *OPY2* Gene.

*Candida albicans* é um fungo comensal que pode ser virulento quando o sistema imunitário está comprometido. Os microorganismos têm mecanismos que lhes permitem sobreviver quando expostos a diferentes tipos de stress. As vias de sinalização MAPK são um exemplo destes mecanismos e são vitais para a adaptação em casos de mudanças ambientais e para regular diversas funções como morfogénese, crescimento celular, adaptação a stress e construção da parede celular. Uma dessas vias de sinalização é a via de sinalização HOG que é responsável por gerar uma resposta adaptativa a ambientes com alta osmolaridade, entre outros stresses. Em *S. cerevisiae* a proteína Opy2 tem uma função nesta via de sinalização e parece contribuir para a sua especificidade. Neste trabalho estudámos o papel do gene *OPY2* de *C. albicans* quando introduzido numa estirpe de *S. cerevisiae* em que os genes *OPY2* e *SSK1* foram delectados. Verificámos que o gene *OPY2* de *C. albicans* parcialmente complementa a osmosensibilidade do mutante *opy2 ssk1* e que não é capaz de activar a HOG1 MAPK no mesmo mutante sob stress osmótico. Após diversas experiências concluímos que Opy2 tem um papel diferente em *C. albicans* e em *S. cerevisiae*.

**Palavras-Chave:** *Candida albicans*, Vias de sinalização MAPK, stress osmótico, Gene *OPY2*

# **Acknowledgments**

*Para os meus pais e o meu  
irmão, porque sem eles NADA seria  
possível.*



It was not always easy! But it never is....

Fortunately, I had excellent people around me to help me in the not so good moments, in the moments that I just wanted to give up, and mostly, to tell me “Good job!”.

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# Abbreviations

°C – Celsius Degree	CO <sub>2</sub> – Carbon dioxide
% –Percentage	CR – Congo Red
µg – Microgram	DDT – Dichlorodiphenyltrichloroethane
µg/mL – Microgram per milliliter	DMSO – Dimethyl sulfoxide
µL – Microliters	DNA – Deoxyribonucleic acid
µm – Micrometer	dNTP – Deoxyribonucleotide triphosphate
µM – Micromolar	Ds DNA – Double Strain DNA
A <sub>600</sub> – Absorbance at 600 nm	<i>E. coli</i> – <i>Escherichia coli</i>
AcK – Potassium Acetate	EDTA – Ethylenediamine tetraacetic acid
Amp – Ampicilin	g – Grams
Asn – Asparagine	GlcNAc-1-P – <i>N</i> -acetylglucosamine-1-phosphate
ATP – Adenosine-5'-triphosphate	h – Hours
bp – Base pair	H <sub>2</sub> O – Water
BSA – Bovine serum albumin	H <sub>2</sub> O <sub>2</sub> – Hydrogen Peroxide
<i>Ca/C. albicans</i> – <i>Candida albicans</i>	HCl – Chloridric acid
CF – Calcofluor	HL60 – Human Promyelocytic Leukemia Cells
CFU – Colony-forming unit	Hog1-P – Phosphorylated Hog1
Cl <sub>2</sub> Ca.2H <sub>2</sub> O – Calcium (II) Chloride Dihydrate	
CMV – Human cytomegalovirus	

HRP – Horseradish peroxidase	mM – Millimolar
IgG – Immunoglobulin G	MnCl <sub>2</sub> .4H <sub>2</sub> O - Manganese(II) Chloride Tetrahydride
IPTG – Isopropyl β-D-1-thiogalactopyranoside	MOPs – 3-(N-morpholino)propanesulfonic acid
Kb – Kilobase	ms <sup>-1</sup> – Meter per second
kD – KiloDalton	NaCl – Sodium chloride
L – Liters	NaF – Sodium fluoride
LB – Lysogeny broth	NaOH – Sodium hydroxide
M – Molar	nm – Nanometer
MAPK - Mitogen-Activated Protein Kinase	OD – Optical density
MAPKK - Mitogen-Activated Protein Kinase Kinase	P-Hog1 – Hog1 Phosphorylated
MAPKKK - Mitogen-Activated Protein Kinase Kinase Kinase	PAKs – Protein Activated Kinases
MCS – Multiple Cloning Site	PBS – Phosphate buffered saline
MD – Menadione	PCR – Polymerase chain reaction
MgCl <sub>2</sub> – Magnesium chloride	PEG – Polyethylene glycol
min – Minutes	pH – Potential Hydrogen
mL – Milliliters	pI – Isoelectric point
mm – Millimeter	PMSF – Phenylmethylsulfonyl fluoride
	PSA – Ammonium persulfate



PVDF – Polyvinylidene difluoride

RbCl – Rubidium chloride

reint - Reintegrant

RNA – Ribonucleic acid

rpm – Revolutions per minute

RPMI – Royal Park Memorial Institute

s – Seconds

*Sc/S. cerevisiae* – *Sacharomyces cerevisiae*

SD – Synthetic Dextrose

SDS – Sodium dodecyl sulfate

SDS-Page – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Ser – Serine

SO<sub>4</sub>(NH<sub>4</sub>)<sub>2</sub> – Ammonium Sulfate

SVG – Sterile Vegetative Growth

t – Time

TAE – Tris-acetate-EDTA

TBS – Tris-buffered saline

TEMED – Tetramethylethylenediamine

Thr – Threonine

Tm – Melting temperature

Tyr – Tyrosine

U/mL – Unit per milliliter

UDP-GlcNAc – Uridine diphosphate *N*-acetylglucosamine

URA - Uracil

UV – Ultra violet

V – Volts

v/v % – Volume/Volume

w/v % – Mass/Volume

WT – Wild Type

X-gal – 5-bromo-4-chloro-indolyl-β-D-galactopyranoside

YPD – Yeast extract Peptone Dextrose

# Introduction

## **1. *Candida albicans*: a pathogenic fungi**

*Candida albicans* is a very important human fungal pathogen that has the ability to colonize mucosa and skin in about 50% of healthy individuals but without causing disease or danger. In spite of that, it can cause opportunistic infections that can go from superficial to systemic. The last ones are frequently severe, difficult to treat and may lead to the death of the patient (Reviewed by Netea *et al.*, 2008; Reviewed by Alonso-Monge *et al.*, 2009).

Opportunistic fungal infections are a very serious problem to health all around the world, even in countries with a developed health system. A study from the Sepsis Occurrence in Acutely Ill Patients (SOAP) reported that 17% of septic infections appearing in intensive care units in Europe are fungal infections (Reviewed by Tortorano *et al.*, 2011).

Although *C. albicans* normally does not cause disease, when the immune system is compromised, or when the normal microflora balance is disrupted, it becomes an opportunistic pathogen (Calderone *et al.*, 2001; Reviewed by Fernández-Arenas *et al.*, 2009).

In immunocompromised individuals (e.g.: AIDS, cancer chemotherapy, organ or bone marrow transplantation) this microorganism can behave as a pathogen, causing localized or disseminated candidiasis. In addition, hospital-related infections in patients not previously considered at risk (e.g.: patients on an intensive care unit) have become a cause of major health concern (Calderone *et al.*, 2001).

Usually for a microorganism to cause disease it is necessary to reach the host, adhere to its cells, multiply and proliferate when it is attached to the host cells and be able to resist to the host defences (Odds, 2000). Microorganism must be, as well, able to counterattack the several different conditions that can differ in the host as are pH, temperature, nutrient starvation, diverse stresses as oxidative and osmotic, among others. It is essential for the survival of the host to be able to adapt to all these conditions.

*C. albicans* is a polymorphic fungus (Figure 1) that has the ability to modify its morphology from the unicellular (yeast) to mycelial (filamentous) form of growth - this process is called dimorphic transition (Reviewed by Odds, 1988).

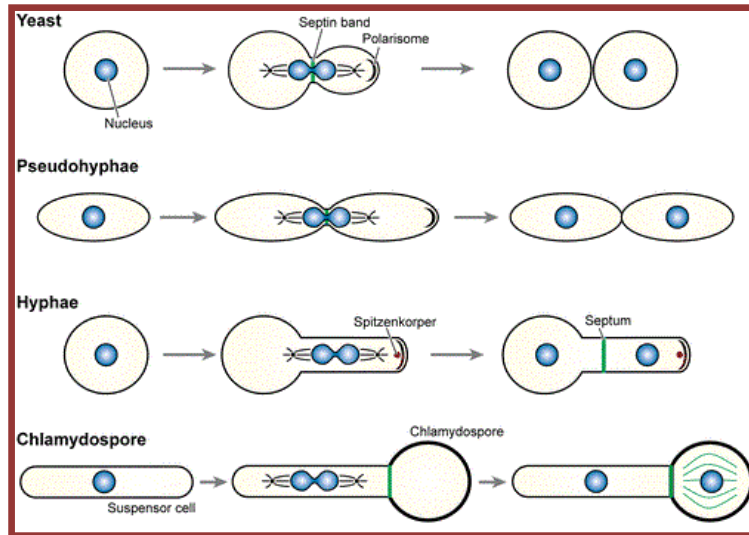


Figure 1 - Polymorphism of *C. albicans*. (Vercruyse, 2010-2011)

This fungus can experience reversible morphogenetic transitions between budding, pseudohyphal and hyphal growth forms (Figure 2) Reviewed by Brown *et al.*, 1999; Reviewed by Sudbery *et al.*, 2004).

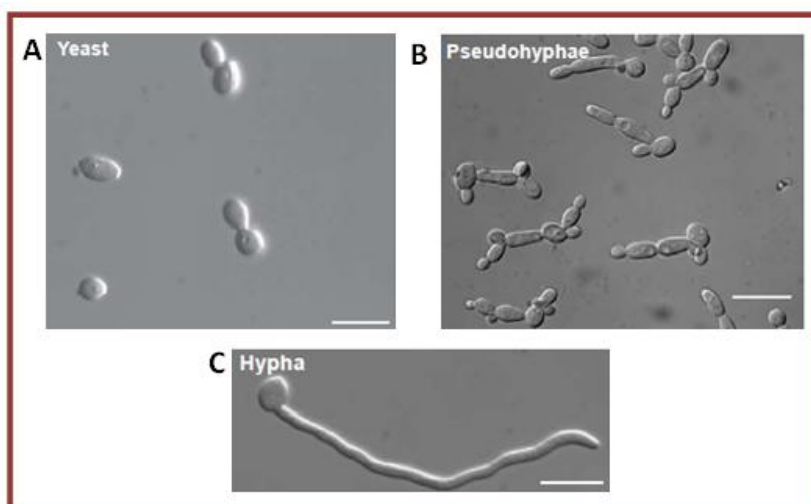


Figure 2 – Yeast (A), pseudohyphal (B) and hyphal (C) morphologies of *C. albicans*. (Sudbery *et al.*, 2004)

Pseudohyphae vary from relatively short to extended cells and, distinctively from hyphae, pseudohyphae have constrictions at their septa. There have not yet been

established the relationship of the development between pseudohyphae and hyphae, but pseudohyphae is described by many as the intermediate between yeast and hyphal cells (Reviewed by Brown *et al.*, 1999).

*C. albicans* is able to switch rapidly from yeast form to filamentous growth under certain conditions. Filamentous growth not only increases the ability to invade the host tissues but also to escape from phagocyte and neutrophil killing (Reviewed by Calderone *et al.*, 2001).

Yeast form is the usual way of propagation in the bloodstream and the one responsible for adherence to endothelial surfaces.

When strains are not capable of changing to the hyphal growth form are typically less pathogenic than the wild type strains, although hyperfilamentous strains have also been reported to be less virulent (Reviewed by Braun *et al.*, 1997; Alonso-Monge *et al.*, 1999).

Formation of hyphae in *C. albicans* is also linked to cell density and quorum sensing. *C. albicans* expresses several molecules for quorum sensing, mainly farnesol and tyrosol, which are responsible for the response to cell density. Farnesol suppresses the formation of filaments and tyrosol stimulates the yeast to hyphae conversion. These molecules also have other functions such as a role in biofilm formation (Hornby *et al.*, 2001; Chen *et al.*, 2004; Alem *et al.*, 2006; Chen *et al.*, 2006).

Another highly studied phenotypic transition is the white-opaque switch (which refers to the morphology of the colonies). These phenotypes contribute to the virulence of *C. albicans* since they are linked to differences in adhesion and affinity properties and antigen expression (Reviewed by Calderone *et al.*, 2001).

Although polymorphism is a very important virulence factor in *C. albicans*, it is not the only one. As was referred before, the fungal cell wall is not present in the

mammalian cells, what makes particles such as the echinocandins target for antifungals.

Different components of the fungal cell wall interact with host receptors, allowing adhesion and invasion of the host tissues and due to this interaction causing immune responses (Reviewed by Vázquez-Torres *et al.*, 1997). Enzymatic proteins such as aspartyl proteases or phospholipases are also considered as virulence factors, which facilitate initial penetration of keratinized cells (Reviewed by Vázquez-Torres *et al.*, 1997).

Fungal infections are difficult to treat due to the fact that mammalian and fungal cells are very similar – both are eukaryotic and this reduces the number of antibiotics that are available to take care of these infections. Due to these facts, the treatment possibilities are limited. The current antifungal treatments have several limitations like the cost, the toxicity, the pharmacokinetic problems (Reviewed by Rentz *et al.*, 1998) and the increasing resistance to the standard antifungal therapies as there are example the azoles (Reviewed by Akins, 2005). The medicines that exist nowadays to treat fungal infections are still few compared to the ones to treat bacterial infections and the ones that exist produce adverse side-effects and toxicity (Reviewed by Román *et al.*, 2007).

Three classes of drugs are available for treating fungal infections: polyens (provokes damage to the cell membrane), azoles (inhibits the synthesis of the ergosterol) and echinocandins (inhibits the glucan formation in the cell wall). These cell wall inhibitors are useful agents in the treatment of fungal infections since mammalian cells do not have a cell wall so the human cells are not targeted with these drugs.

The existent drugs for antifungal treatment are not enough and it is necessary a deeper study of the fungus virulence mechanisms to find new targets for the antifungal treatment.

This microorganism is able to colonize and infect in several microenvironments in the body which includes the bloodstream, superficial sites in the mucosa and the major internal organs, resulting in a systemic disease. (Reviewed by Brown *et al.*, 1999)

Adaptation of the micro-organism to its host is essential cause if it cannot respond to the environmental changes or cope with the host defences presented by the immune system it will not be able to survive, more even, it will probably get eliminated (Reviewed by Arana *et al.*, 2007).

The Mitogen-Activated Protein Kinase pathways play an important role in this process for *C. albicans*.

## **2. Mitogen-Activated Protein Kinase (MAPK) Pathways**

### **2.1 General**

To avoid damage caused by neutrophils/macrophages and by the cells' own metabolism, both long term and short term adaptive mechanisms exist in *C. albicans*.

MAP Kinase pathways are a special type of signal transduction pathways preserved in all eukaryotic cells and that are triggered upon environmental signals (Reviewed by Román *et al.*, 2007; Reviewed by Alonso-Monge *et al.*, 2009; Reviewed by Herrero de Dios *et al.*, 2010).

The complexity in the different organisms differ but the core structure (a cascade of three kinases) is conserved in all of them (Figure 3). These pathways are essential for adaptation to environmental changes and regulate several functions: morphogenesis, cell growth, adaptation to stress and cell wall construction.

In *Saccharomyces cerevisiae*, a non-pathogenic yeast, these pathways are well understood. This yeast has been used as a model for the study of these cascades in other fungi such as *C. albicans*.

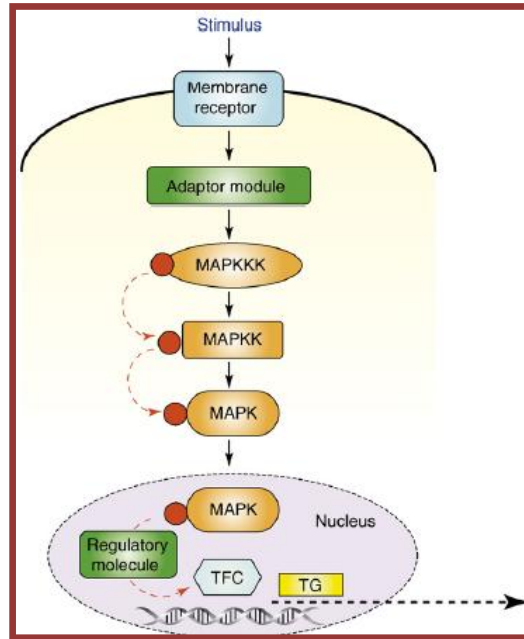


Figure 3 - General representation of the MAPK pathway. Stimuli are sensed by specific sensors or membrane receptors at the level of the membrane and transmitted to the MAPK core. The MAP kinase kinase kinase becomes phosphorylated and phosphorylates the MAP kinase kinase which in turn does so to the MAP kinase. The phosphorylated MAPK translocates to the nucleus where phosphorylates a downstream regulatory protein or a transcription factor. This allows the expression of those genes needed for the specific response that permit adaptation to the stimulus. The pathway is controlled by a feedback mechanism which avoids continuous activation of the proteins. (Román *et al.*, 2007)

As stated before, MAPK cascades have a crucial role in sensing changes in the external and internal environment. It is extremely important for the cells the effectiveness of the generated response because it allows them to survive in a hostile environment.

Three protein kinases are present in the MAPK core: the MAP kinase kinase kinase (MAPKKK), the MAP kinase kinase (MAPKK) and the MAP kinase (MAPK). Transmembrane sensors, PAKs (Protein Activated Kinases), GTPases, scaffold proteins, transcription factors and co-factors also take part in the MAPK signalling cascades (Reviewed by Herrero de Dios *et al.*, 2010).



The three proteins kinases are activated by a sequential phosphorylation on a serine, threonine and/or tyrosine residue. When the MAPK is phosphorylated, it translocates to the nucleus and the transcription factors needed for the response are activated. To avoid non desired crosstalk between the different pathways of the MAPK cascade, as many elements are part of more than one pathway, specific regulations are needed. Phosphatases and scaffold proteins have a critical task doing so. If elements of these pathways are not present, the cells become less virulent. Since these cascades regulate essential functions for fungal life, they are considered as virulence factors (Reviewed by Román *et al.*, 2007).

## **2.2 Mitogen-Activated Protein Kinase (MAPK) Pathways in *C. albicans***

### ***The cell integrity pathway***

The cell integrity pathway or PKC pathway (Figure 4) mediates the cell wall construction. This pathway is the one responsible for the maintenance of a dynamic and stable cell wall able to adapt to the physiologic requests and to the external changes that are produced during the yeast grow.

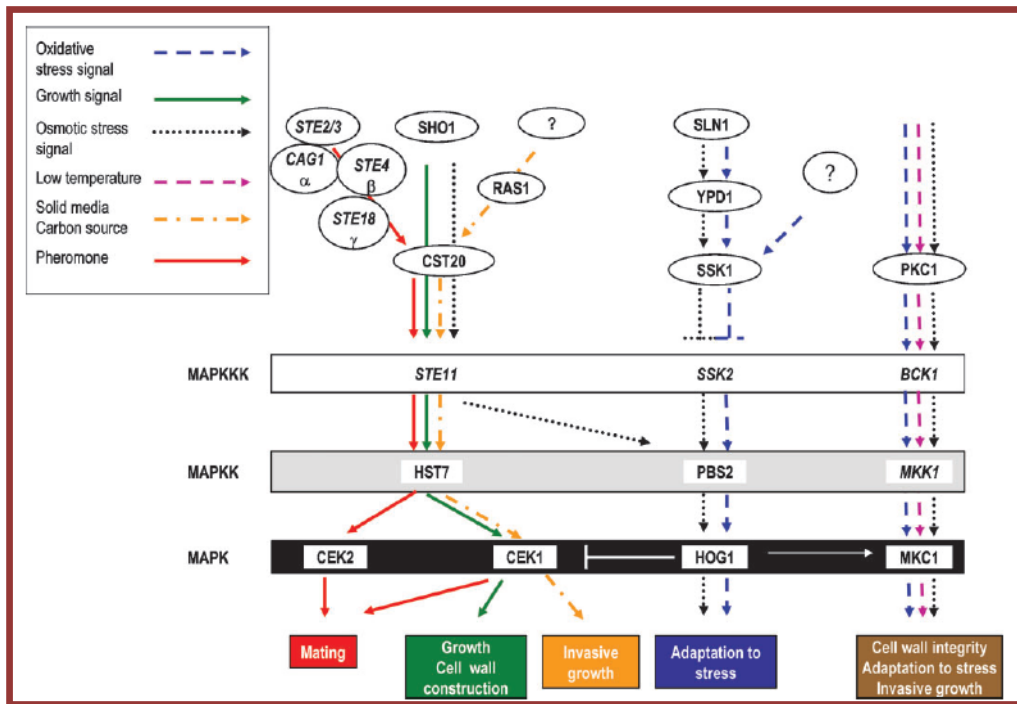


Figure 4 - Model of the MAP kinases pathways in *C. albicans* and the response to different stimuli. (Alonso-Monge *et al.*, 2006)

This pathway is essential in certain ambient conditions like hypotonic shock, nutrient starving and high temperature growing, during morphogenic processes to regulate the biogenesis of the cellular wall and the dynamic of the actin cytoskeleton, and to face certain compounds that interfere with the correct junction of the cellular wall components and their formation which are examples congo red, zymolyase and caffeine. (Davenport *et al.*, 1995; Kamada *et al.*, 1995; Martín *et al.*, 2000).

This pathway participates in the compensatory pathway, a system that is activated to respond to the damage in the cell wall to assure its integrity. It has associated with it an increase of chitin and cell wall proteins, a transition redistribution of the 1,3-β glucan synthase complex and changes in the association of different polymers that are in the cell wall.

In *S. cerevisiae*, the basic kinase module consists of the Bck1 protein (bypass of C protein kinase) MAPKKK, the redundant Mkk1/Mkk2 MAPKKs and the Sit2 MAPK (also called Mpk1), but the elements involved in sensing signals are still not found in *C. albicans*.

In *C. albicans*, the elements involved in sensing the signals are still not found in *C. albicans* but the main core of MAPK has been described – Bck1 MAPKKK, Mkk1 MAPKK and Mkc1 MAPK. Mkc1 is the homologue of the *S. cerevisiae* Slt2/Mpk1 MAPK, and it plays a role in maintaining cellular integrity and cell wall formation (Reviewed by Alonso-Monge *et al.*, 2006).

When the cell wall is compromised, the Mkc1 MAPK is activated and *mkc1* mutants are more sensitive to cell wall degrading enzymes/compounds and antifungals, and this fact proves that the pathway is involved in cell wall construction (Navarro-García *et al.*, 1995; Navarro-García *et al.*, 1998).

The pathway plays a role in the virulence since *mkc1* mutants are less virulent in a murine systemic infection model and is also involved in invasive growth, morphological transition and biofilm formation (Diez-Orejas *et al.*, 1997; Kumamoto, 2005).

Mkc1 is activated upon oxidative stress, changes in osmotic pressure, cell wall damage and a decrease in the growth temperature (Navarro-García *et al.*, 2005). *mkc1* mutants survive less when in contact with leukocytes (Arana *et al.*, 2007).

### ***The Cek1 mediated pathway***

In *S. cerevisiae*, a conserved MAPK pathway mediated by the Fus3/Kss1 MAPKs participates in at least three different processes: mating, invasive growth and vegetative growth. This pathway is essential to maintain the integrity of the cell wall during the vegetative growing (Reviewed by Alonso-Monge *et al.*, 2006).

The first process where this MAPK pathway was found out to participate was the formation of diploid cells from sexually compatible haploid cells  $a$  and  $\alpha$  types (Reviewed by Elion, 2000). The elements of this pathway are the Ste20 MAPKKK, the Ste7 MAPKK, the functionally partially redundant Fus3/Kss1 MAPKs and the scaffold protein Ste5.

When a situation of nitrogen starvation happens, diploid cells undergo a pseudohyphal mode of growing, but haploid cells experience a morphological change to respond to the glucose depletion that results in invasive growth. The two processes are different as their upstream elements differ and the processes are functionally different (Gimeno *et al.*, 1992).

Elements of this route also take part in the cell wall biogenesis the sterile vegetative growth (SVG) pathway. Under basal conditions this pathway is able to promote vegetative growth, and it is essential in mutants deficient in protein mannosylation. This pathway shares elements with the HOG MAPK pathway as the Sho1-Ste20-Ste11 branch drives the specific response either to Ste7-Kss1 or to Pbs2-Hog1 (Posas *et al.*, 1997; Posas *et al.*, 1998b)).

In *C. albicans*, this pathway comprises the Ste 11 MAPKKK, the Hst7 MAPKK and the Cek1 MAPK (Figure 5).

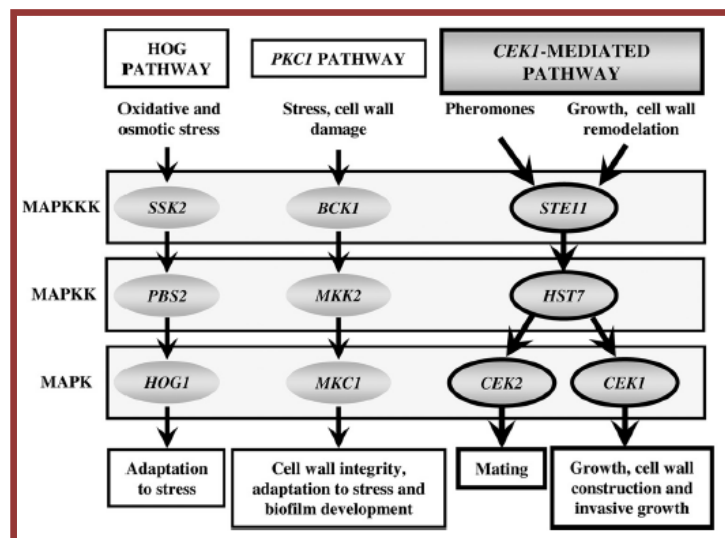


Figure 5 - *Candida albicans*-MAPK signal transduction pathways with the *cek1*-mediated MAPK pathway highlighted. (Galán-Díez *et al.*, 2010)

The upstream elements that have already been described and are responsible for the activation of Cek1 are Sho1 and Msb2. Sho1 is a transmembrane adaptor

protein and Msb2 a transmembrane mucine (Figure X) (Román *et al.*, 2005; Román *et al.*, 2009).

Several reports indicate that this pathway is involved in morphogenesis and filamentation in *C. albicans*. Deletion of *Cek1* results in hyphal formation defects on certain solid media in which the nitrogen source is limiting. Due to this fact, this pathway was initially described as the filamentous growth pathway. Recent studies have shown that this pathway is important in vegetative growth and cell wall construction. It is also involved in virulence since mutants show a reduced virulence in a mouse systemic model of infection (Chen *et al.*, 2002; Román *et al.*, 2005; Alonso-Monge *et al.*, 2006; Román *et al.*, 2009;).

*cek1* mutants are partially defective in mating and both *hst7* and *cek1cek2* mutants are unable to mate. This may presuppose that *Cek1* and *Cek2* have a complementary function in the mating mechanism (Alonso-Monge *et al.*, 2006).

### ***The HOG pathway***

In *S. cerevisiae*, the HOG (high osmotic glycerol) pathway is the one responsible for generating an adaptive response to high-osmolarity environments. Moreover, it plays an important role in the response to mainly osmotic stress (Posas *et al.*, 1998a), although also to oxidative stress and tricarboxylic acids, among others.

In *S. cerevisiae* the HOG pathway has two branches (Figure 6). The first one comprises as upstream elements the *Sln1* two-component protein and the phosphorelay proteins *Ypd1* and *Ssk1* and the *Ssk2* MAPKKKs, the *Pbs2* MAPKK and *Hog1* MAPK. *Sln1* is a sensor histidine kinase, *Ypd1* a phosphotransfer protein and *Ssk1* a response regulator (Posas *et al.*, 1998a); Reviewed by Hohmann, 2002).

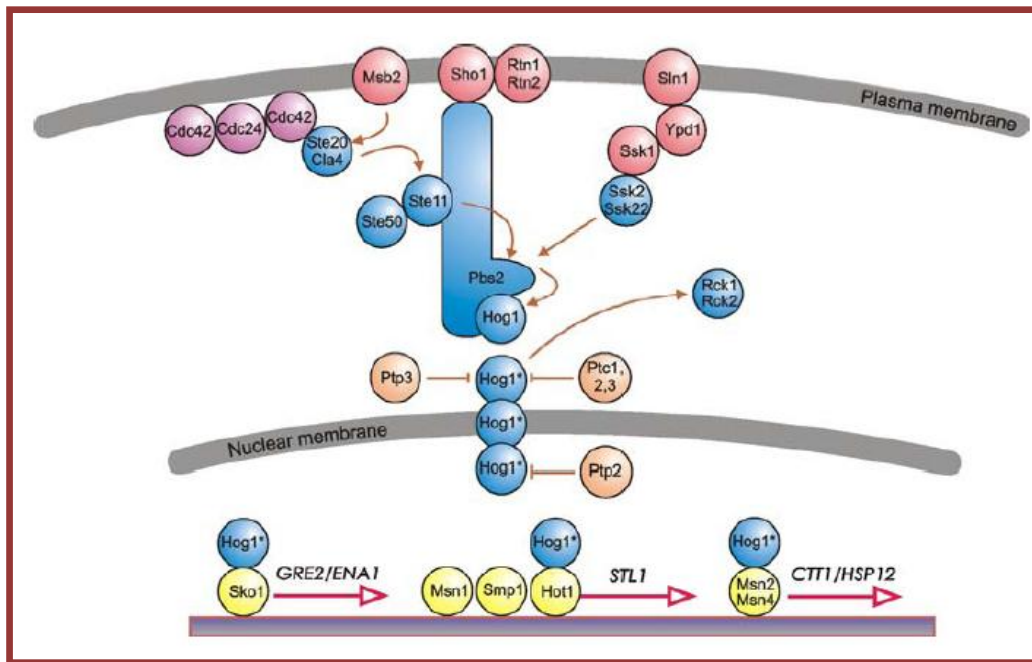


Figure 6 – Scheme of the HOG pathway in *S. cerevisiae*. The osmotic stress is perceived by two distinct branches, the Sho1 and Sln1 branch, both of which activate the MAPKK Pbs2 via distinct MAPKKK. (Krantz *et al.*, 2006)

Sln1 has an extracellular domain, a cytoplasmic domain with histidine kinase activity and a receptor domain. Under isotonic conditions, a His<sup>576</sup> of the kinase domain is phosphorylated, the phosphate is transferred to the Asp<sup>1144</sup> of the receptor domain, then to the His<sup>64</sup> of Ypd1 and, at least, to the Asp<sup>554</sup> of the receptor domain of Ssk1. The phosphorylated Ssk1 blocks further activation of the downstream cascade. This situation is reversed under high osmolarity, where Ssk1 becomes dephosphorylated allowing activation of the Ssk2/Ssk22 functionally redundant proteins (Reviewed by Banuett, 1998).

A second input to this cascade comes from another defined route through the Ste11 MAPKKK, the Ste11-interacting protein Ste50, the Ste20 p21-activated kinase (PAK), the small GTPase Cdc42 and the transmembrane Sho1 adaptor protein. Both signals converge at the level of the MAPKK Pbs2, which is able to activate the Hog1 MAPK by phosphorylation, thus leading to downstream effectors (Reviewed by Banuett, 1998).

In *C. albicans*, the HOG pathway is involved in at least three different processes: response/ adaptation to stress, morphogenesis and cell wall formation (Reviewed by Alonso-Monge *et al.*, 2006). Unlike *S. cerevisiae*, the HOG pathway in *C. albicans* displays only the homolog to Ssk1 signaling branch (Cheetham *et al.*, 2011)

Mutants of the *hog1* and *pbs2* genes are sensitive to osmotic stresses. When sodium chloride is added, Hog1 is phosphorylated in a Pbs2-dependent manner and it translocates to cell nucleus. To counteract cell dehydration when Hog1 is activated, glycerol is accumulated. Other compatible solutes can be accumulated instead of glycerol (e.g. arabinitol) (Alonso-Monge *et al.*, 2003; Arana *et al.*, 2005; Reviewed by Alonso-Monge *et al.*, 2006).

The HOG pathway has a role in the resistance to oxidative stress. Upon oxidative stress the HOG pathway is activated through a mechanism involving Ssk1 and Ssk2 MAPKKK and mutants are hypersensitive to both oxidative and nitrosative stress (Chauhan *et al.*, 2003). Additionally, *hog1* and *psb2* mutants are susceptible to oxidants like hydrogen peroxide, potassium superoxide, among others and these mutants show reduced viability in systemic mouse model of infection presumably due to the host control of the fungal infection which is mediated by oxidative stress and they are easily killed by macrophages in vitro (Alonso-Monge *et al.*, 2003; Arana *et al.*, 2005; Alonso-Monge *et al.*, 2006; Arana *et al.*, 2007).

### **3. The OPY2 protein**

As it was stated before, in *S. cerevisiae*, in the HOG pathway, there are two upstream osmosensing branches both containing membrane proteins – one the Sln1 and the other the Sho1 – and both share a redundant function in the activation of a common downstream MAPK cascade consisting of the Pbs2 MAPKK and the Hog1 MAPK. (Ekiel *et al.*, 2009). To activate the HOG pathway by the Sln1 branch two functionally redundant MAPKKKs are involved – Ssk2 and Ssk22, and their activator Ssk1. in the SHO1 branch, Ste11 MAPKKK is the one that is able to activate the pathway (Ekiel *et al.*, 2009).

Although the mechanisms of how the MAP kinase cascade transmits the signals to activate the HOG pathway are well studied, how the upstream branches are able to detect and act against osmotic stress has not been well described (Ekiel *et al.*, 2009).

Studies were made to detect synthetic osmosensitivity by the deletion of genes when the SLN1 branch was absent, and it was seen that the protein Opy2 was an essential component in the Sho1/Ste11/Ste50 part of the HOG pathway (Ekiel *et al.*, 2009).

In *S. cerevisiae*, Opy2 is an integral membrane protein, located at the level of the plasma membrane, with 360 amino acids. It has a single 93-115-aa transmembrane domain associated with the plasma membrane and interacts with Ste50 through its cytoplasmic C-terminal region (Figure 7) (Wu *et al.*, 2006; Ekiel *et al.*, 2009).

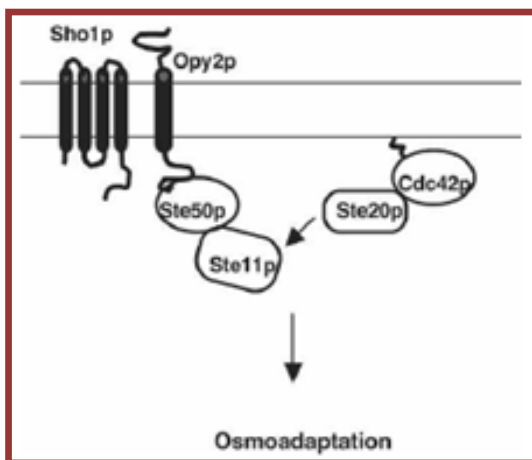


Figure 7 – Model of Opy2 as a component of the HOG pathway. In the Figure we can see a schematic representation of Opy2 with a single transmembrane domain that is shown as a cylinder. (Adapted from Wu *et al.*, 2006)

Genetically, Opy2 performs its function between the level of the Sho1 and the osmosensors Msb2 and Hkr1, and the level of the Ste50/Ste11 complex (Wu *et al.*, 2006; Tatebayashi *et al.*, 2007; Ekiel *et al.*, 2009).

The cytosolic tail of Opy2 interacts with Ste50 that interacts with Ste11. Through these protein–protein interactions, Opy2 acts together with Sho1 to present Ste11 to the plasma membrane, where it is phosphorylated by Ste20 under the control



of Cdc42, itself isoprenylated and attached to the plasma membrane. Under these conditions, the Ste50/Ste11 complex that interacts with Opy2 is activated, and this may contribute to the HOG pathway specificity (Wu *et al.*, 2006).

During activation of the HOG pathway, Opy2 function has a plasma membrane attachment site for the adaptor protein Ste50 (Wu *et al.*, 2006; Ekiel *et al.*, 2009). Ste50 is not only involved in the HOG pathway, but it also modulated two other MAPK pathways in *S. cerevisiae* – the pheromone response and pseudohyphal growth control pathways (Figure 8).

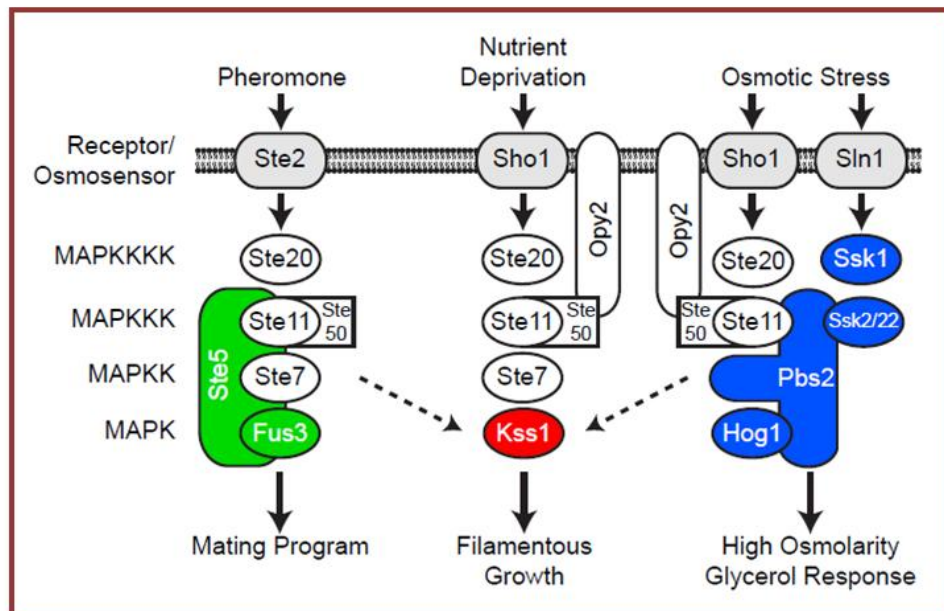


Figure 8 – Regulation of three different yeast MAPK pathways that respond to different stimuli and regulate distinct biological processes by the MAPKKK Ste11 and the adapter Ste50. (Cappell *et al.*, 2011).

The main function of the Ste50 to activate these different MAPK pathways is in the plasma membrane localization of the Ste11 MAPKKK. The Ste50 is able to associate with the Ste11 MAPKKK through the interaction of its RA (Ras association) domain with Opy2 (Wu *et al.*, 2006) or with other membrane-anchored proteins, such as the small GTPase Cdc42 (Ekiel *et al.*, 2009).

Opy2 is, indeed, necessary, in *S. cerevisiae*, for Ste50/Ste11 activation in response to high osmolarity stress (Wu *et al.*, 2006). It interacts through its C-terminal fragment with the RA-like domain of Ste50. (Wu *et al.*, 2006).

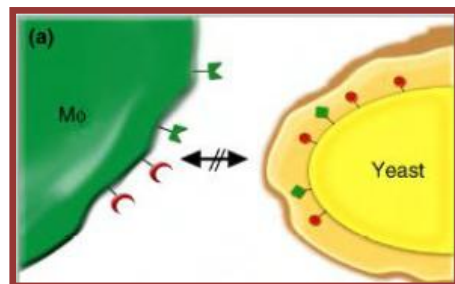
There are significant differences between *S. cerevisiae* and *C. albicans* signaling pathways. In *C. albicans* the branch in which Sho1, Msb2 and Ssk2 are implicated belongs to the SVG pathways. Although, it has been reported that only the Ssk1, Sln1, Ste11 branch is involved in signaling to the MAPK Hog1 (Cheetham *et al.*, 2011) there are some evidences that suggests an implication of the SVG pathway in the response to osmotic stress.

In an attempt to analyze this role, we propose the study of the Opy2 protein in *C. albicans*.

#### 4. Surviving in the host

The response of the immune system to *C. albicans* is not a simple process. The first structure recognized by the mammalian host cells is the cell wall. A protective layer of glycoproteins such as mannoproteins masks immunostimulatory pathogen-associated molecular patterns (PAMPs) and these proteins are the responsible for the start of an immune response (Figure 9) (Medzhitov *et al.*, 2002; Reviewed by Seider *et al.*, 2010).

Figure 9 – The protective layer of mannoproteins in *C. albicans*. (Seider *et al.*, 2010)



To fight against the invading microorganisms, all immune system plays an important role. Keratinocytes, neutrophils, macrophages, eosinophils and basophils are the first line of the host immune system against mucosal *C. albicans* infections. Neutrophils, monocytes, and monocyte-derived macrophages, as well as committed

tissue macrophages are the key effector cells in host defence against deeply invasive candidiasis (Reviewed by Blanco *et al.*, 2008).

In order to fight the infection and to recruit enough immune cells to the site of infection, it is important that chemotaxis takes place. There are several different chemotactic factors – chemokines that are produced by leukocytes, epithelial cells and many other cells, cytokines and peptides derived from the activation of the complement system (Reviewed by Blanco *et al.*, 2008). The Pathogen Associated Molecular Patterns (PAMPs) present in the pathogen activate the chemotactic factors, and the PAMPs are recognized by pattern recognition receptors (PRRs) such as Toll-like receptors present in several cells of the immune system (Reviewed by Roeder *et al.*, 2004).

When the pathogens are able to cross the physical barriers and the infection reaches the tissues, the immune system reacts in two ways – first it is activated a non-specific immune system and afterwards a specific immune response.

In the non-specific immune system, phagocytic cells (neutrophils, monocytes and macrophages) are the primary effector cells in *C. albicans* infection but also eosinophils and basophils may play a role (Reviewed by Blanco *et al.*, 2008).

After this first response, the adaptive one is activated. This is responsible for a specific immune response with the activation of specific phagocytic cells through a cell mediated immunity via a Th1 response and the formation of antibodies through an antibody mediated immunity via a Th2 response (Reviewed by Blanco *et al.*, 2008).

The immune system works by exposing invading microorganisms to oxidative stress by reactive oxygen species (ROS) which are produced by macrophages and neutrophils.

Neutrophils have a large number of antimicrobial compounds to accomplish their function. In case of inflammation, neutrophils are the first immune cells that are recruited from the bloodstream (Reviewed by Urban *et al.*, 2006).

Upon contact, neutrophils engulf the microbes into a phagocytic vacuole, called a phagosome. Subsequently, intracellular granules fuse with the phagosome and discharge their contents to form a phagolysosome (Figure 10) (Reviewed by Urban *et al.*, 2006).

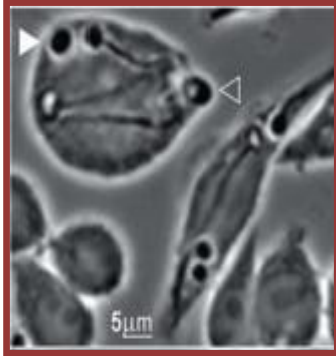
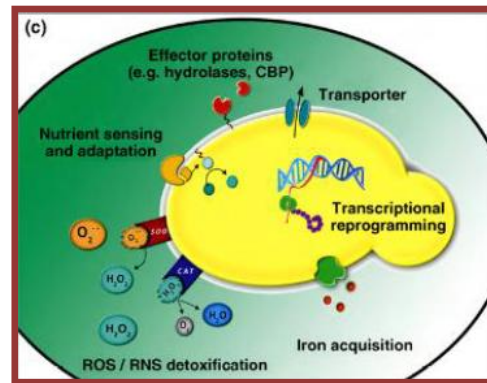


Figure 10 – Phagocytosis of a *C. albicans* wild-type strain. Close arrowheads point at ingested fungal cells, while open arrowheads point at non-ingested fungal cells. (Fernández-Arenas *et al.*, 2009)

In the phagolysosomes, the microorganisms are killed by a mixture of non-oxidative and oxidative mechanisms. There are three types of oxygen-independent effectors and they are stored in different granule subsets of the neutrophil. The specific granules (or secondary granules) and the gelatinase granules (or tertiary granules) are the first ones to be discharged. The importance of these granules is that they contain a combination of antimicrobial proteins such as lactoferrin, lipocalin, lysozyme and gelatinase as well as metalloproteases that are important for tissue breakdown. This makes easy for neutrophil to migrate and for them to act. After this step, there are discharged the azurophilic granules (or primary granules). These granules are peroxidase-positive and contain small antimicrobial peptides,  $\alpha$ -defensins, seprocidins and antibiotic proteases (Reviewed by Urban *et al.*, 2006).

A non mitochondrial generation of reactive oxygen species (ROS) is implicated in the oxygen-dependent mechanism and this is known as the respiratory burst (Reviewed by Suzuki *et al.*, 2011). During this procedure, different kinds of ROS are produced (Figure 11).

Figure 11 – The respiratory burst produced within the phagosome to counteract antimicrobial activities. (Seider *et al.*, 2010)



To study the interaction between the cells of immune system and invading microorganisms, tests with the HL60 cell line might be performed.

### ***The HL60 cell line***

The HL60 cell line (Figure 12) was firstly originated from a female patient with acute myeloid leukaemia. The culture of her peripheral blood leukocytes in conditioned medium resulted in the development of a growth-factor-independent immortal cell line with distinct myeloid characteristics (Birnie, 1988; Reviewed by Flec *et al.*, 2005).

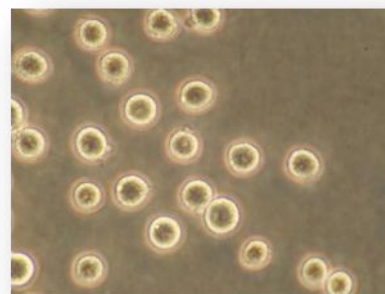


Figure 12 – HL60 cells.

The sublines of HL60 display a myeloblastic/promyelocytic morphology: large, blast-like cells with characteristic large, rounded nuclei containing 2-4 distinct nucleoli, and a basophilic cytoplasm with azurophilic granules. The cultures of HL60 cells comprise 90-95% of cells with this morphology; the remaining cells display morphologies resembling those of more mature myeloid cells (mainly myelocytes, with

some neutrophils and monocytes). The majority of the cells carry a variety of cell surface antigens characteristic of immature myeloid cells as shown by their reaction with an extensive panel of monoclonal antibodies. Many histochemical markers characteristics of myeloid lineage cells are also present, as well as receptors for insulin, transferrin and complement (Birnie, 1988).

Cytogenetic analysis of HL60 cells shows the occurrence of many karyotypic abnormalities, including monosomy, trisomy and tetrasomy, and a variety of chromosomal translocations (Birnie, 1988).

It is possible to induce differentiation of the HL60 cells. Polar-planar compounds such as dimethyl sulphoxide (DMSO), 1,25-dihydroxyvitamin D<sub>3</sub>, phorbol esters and sodium butyrate induce monocyte/macrophage differentiation. After a 7 to 10 day DMSO treatment, HL60 cells acquired a neutrophilic phenotype (Jacob *et al.*, 2002; Reviewed by Fleck *et al.*, 2005).

The course of the differentiation induced by any of these agents is accompanied by a large number of changes in the cells and is easily monitored by morphological, histochemical and immunological criteria. Thus, incubation with DMSO or retinoic acid leads, over a period of 5 days, to a progressive decrease in the size of HL60 cells and condensation of nuclear material with the appearance of kidney-shaped nuclei characteristic of the myelocyte and, later, lobed nuclei characteristic of banded and segmented neutrophils. The nuclear/cytoplasmic ratio decreases, and the cytoplasm becomes more diffuse. In parallel with these changes there occur marked changes in histochemistry, including decreased myeloperoxidase activity and the appearance of cells capable of reducing nitroblue tetrazolium (a granulocyte marker) (Birnie, 1988).

Histochemical changes that occur include a decrease in myeloperoxidase activity and the appearance of non-specific esterase activity (a monocyte/macrophage marker) in all adherent cells. In addition, markedly elevated levels of lysozyme can be detected in the medium within 16-24h after initiation of differentiation. Changes in cell

surface antigens characteristic of maturation along the monocyte/macrophage pathway also occur (Birnie, 1988).

Due to the possibility to differentiate the HL60 cells to have a myelomonocytic cell line, this cell line is ideal to study their interaction with different strains of *C. albicans* cells and the effect that the production of ROS might have in the survival of mutant strains.

# Objectives



*Candida albicans* is a commensal fungus that can be virulent when the immune system is compromised. One of the ways that the immune system has to fight against invading microorganisms is to expose them to oxidative stress by producing ROS, for example. Pathogens have developed different mechanisms in order to survive under different stresses such as the MAP kinase pathways.

These pathways are essential for adaptation to environmental changes and regulate several functions: morphogenesis, cell growth, adaptation to stress and cell wall construction.

The main core structure of these MAPK kinase pathways is already known but the way that the upstream elements of the different branches notice and act against the different stresses are not known yet.

In *S. cerevisiae*, the protein Opy2 was identified as a membrane anchor that can interact with the Ste50p/Ste11p module in the activation of the HOG pathway. When this gene was deleted, the pathway would go to a second branch able to respond in cases of high osmolarity – the *SSK1* branch. When both *OPY2* and *SSK1* genes were deleted, no response in the HOG pathway was detected.

Even though there are differences between *S. cerevisiae* and *C. albicans* signaling pathways, in this work we propose to study the role of *C. albicans* *OPY2* gene when introduced into a *S. cerevisiae* strain in which both the *OPY2* and the *SSK1* genes were deleted. It is also our purpose to study if *C. albicans* gene is able to complement the osmosensitivity and the response to osmotic stress in *S. cerevisiae*.

Moreover, as the HOG pathway has a role in the response to other stresses, we test if the *CaOPY2* is able to improve the growth of *opy2 ssk1* mutants under oxidative and cell wall stresses.

Another part of this work aims to investigate if the adaptive response to tunicamycin and zymolyase stresses requires the activation of several pathways and by this the activation of other MAPK's.

We propose to study the Opy2 protein in *C. albicans* and assess if this protein undergoes post-translational modifications that might be identical or not in *S. cerevisiae* and *C. albicans*.

# **Materials and Methods**

## 1. Strains and cultures

All strains were maintained at 4°C and were refreshed every month in YPD or SD minimal medium plates from preserved material (from the previous plates used or 50% glycerol (v/v %) stored at -80°C).

For the experiments the cultures were grown at 30°C overnight with gentle shaking (200 rpm) to collect stationary phase cells. To collect cells in their exponential phase, these cultures were diluted in pre warmed medium to an OD of  $A_{600}=0.2$  and grown until they reached an OD of  $A_{600}=1$  to 2.

The absorbance was measured in a DU®640 Spectrophotometer (Beckman Coulter Inc., USA) at a wavelength of 600nm. For this, appropriate dilutions were made so that the optical density was measured in the range where the relation between the measured absorbance and the number of cells is linear.

All the wild type and mutant strains used for the experiments are listed in Table 1 together with the information about the *E. coli* strain used for transformation.

Table 1 – Different strains used during this work.

<b>Microorganism</b>	<b>Strain</b>	<b>Genotype</b>	<b>Name in text and figures</b>	<b>Reference</b>
<i>E. coli</i>	DH5αF'	<i>K12Δ(lacZYA-argF)u169 supE44 thi1 recA1 endA1 hsdR17 gyrA relA1 (ø80lacZΔM15) F'</i>	DH5α	(Hanahan, 1988.)
<i>S. cerevisiae</i>	BY4741	<i>MATa his3 1 leu2 0 met15 0 ura3 0</i>	WT	EUROSCARF
<i>S. cerevisiae</i>	YCW1380	<i>MATa opy2::Kan<sup>R</sup> ssk1::Nat<sup>R</sup> ura3 his3 leu2</i>	<i>opy2 ssk1</i>	(Wu et al., 2006)
<i>S. cerevisiae</i>	BY4741 <i>opy2</i> (Y05494)	<i>MATa opy2::KanMX4</i>  ( <i>BY4741 Mat a his3 1 leu2 0 met15 0 ura3 0 YPR075c::kanMX4</i> )	<i>opy2</i>	EUROSCARF
<i>S. cerevisiae</i>	BY4741	<i>MATa ssk1::kanMX4</i>	<i>ssk1</i>	EUROSCARF

	<i>ssk1</i>			
<i>S. cerevisiae</i>	CD1-1	<i>MATa his3 1 leu2 0 met15 0 ura3 0:: TetO pCMV-URA3</i>	WT pCM189	<i>This work</i>
<i>S. cerevisiae</i>	CD2-4	<i>MATa his3 1 leu2 0 met15 0 ura3 0:: TetO pCMV-CaOPY2 myc-URA3</i>	WT CaOPY2-Myc pCM189	<i>This work</i>
<i>S. cerevisiae</i>	CD3-2	<i>MATa his3 1 leu2 0 met15 0 ura3 0:: TetO pCMV-ScOPY2 myc-URA3</i>	WT ScOPY2-Myc pCM189	<i>This work</i>
<i>S. cerevisiae</i>	CD8-1	<i>MATa opy2::Kan<sup>R</sup> ssk1 ::Nat<sup>R</sup> ura3 his3 leu2:: TetO pCMV-URA3</i>	<i>opy2 ssk1</i> pCM189	<i>This work</i>
<i>S. cerevisiae</i>	CD9-1	<i>MATa opy2Δ::Kan<sup>R</sup> ssk1Δ::Nat<sup>R</sup> ura3 his3 leu2:: TetO pCMV-CaOPY2 myc-URA3</i>	<i>opy2 ssk1</i> CaOPY2-Myc pCM189	<i>This work</i>
<i>S. cerevisiae</i>	CD10-23	<i>MATa opy2Δ::Kan<sup>R</sup> ssk1Δ::Nat<sup>R</sup> ura3 his3 leu2:: TetO pCMV-ScOPY2 myc-URA3</i>	<i>opy2 ssk1</i> ScOPY2-Myc pCM189	<i>This work</i>
<i>S. cerevisiae</i>	CD4-1	<i>BY4741 ssk1::KanMX4 :: TetO pCMV-URA3</i>	<i>ssk1</i> pCM189	<i>This work</i>
<i>S. cerevisiae</i>	CD5-2	<i>BY4741 opy2::KanMX4 :: TetO pCMV-URA3</i>	<i>opy2</i> pCM189	<i>This work</i>
<i>S. cerevisiae</i>	CD6-1	<i>BY4741 opy2::KanMX4 :: TetO pCMV-CaOPY2 myc-URA3</i>	<i>opy2</i> CaOPY2-Myc pCM189	<i>This work</i>
<i>S. cerevisiae</i>	CD7-2	<i>BY4741 isogenic, opy2::KanMX4 :: TetO pCMV-ScOPY2 myc-URA3</i>	<i>opy2</i> ScOPY2-Myc pCM189	<i>This work</i>
<i>C. albicans</i>	CAF2	<i>ura3Δ::imm434/ura3Δ::imm434-URA3</i>	CAF2	(Fonzi et al., 1993)
<i>C. albicans</i>	CHO4-1	CAF2 <i>opy2::FRT/opy2::FRT</i>	<i>opy2</i>	(Herrero de Dios, unpublished data)
<i>C. albicans</i>	CHO39	CAF2 <i>opy2::FRT/opy2::FRT</i> <i>ARD1/ard1:: pOPY2::pOPY2-OPY2-URA3</i>	OPY2 reint	(Herrero de Dios, unpublished data)
<i>C. albicans</i>	REP29F-1	CAF2 <i>ssk1::hisG/ssk1:: hisG msb2::FRT/ msb2:: FRT sho1::hisG/sho1::hisG-URA3-hisG</i>	<i>ssk1 sho1 msb2</i>	(Román et al., 2009)
<i>C. albicans</i>	CHO31-1	CAF2 <i>ssk1::hisG/ssk1::hisG msb2::FTR/msb2::FRT</i>	<i>ssk1 sho1 msb2 opy2</i>	(Herrero de Dios,

		<i>sho1::hisG/sho1::hisG-URA3-hisG</i> <i>opy2::FRT/opy2::FRT</i>		<i>unpublished data</i> )
<i>C. albicans</i>	CHOM-16	CAF2  <i>opy2::FRT/opy2::FRT</i> <i>ADH1/adh1:: tTA pTET-OPY2-myc-SAT1</i>	<i>CaOPY2-Myc</i>	(Herrero de Dios, <i>unpublished data</i> )

## 2. Media

Yeast and bacteria were grown in both liquid and solid media (containing 2% w/v agar). All media were autoclaved at 121°C during 20 minutes before use.

The media used during this work are listed in Table 2.

Table 2 - Composition of the different media used during this work.

<b>Abbreviation</b>	<b>Name</b>	<b>Composition</b>	<b>Characteristic</b>	<b>Organism</b>	<b>Reference</b>
YPD	Yeast extract Peptone Dextrose	1% yeast extract, 2% pepton, 2% glucose	Rich	<i>S. cerevisiae</i> <i>C. albicans</i>	(Sherman, F., 2002)
SD	Synthetic Dextrose	2% glucose, 0.17% nitrogen base for yeast with amino acids except uracil (URA), 0.5% $\text{SO}_4(\text{NH}_4)_2$	Minimal	<i>S. cerevisiae</i> <i>C. albicans</i>	(Sherman, F., 2002)
LB	Lysogeny broth	1% tryptone, 0.5% yeast extract, 0.5% NaCl	Rich	<i>E. coli</i>	(Sherman, F., 2002)

## 3. Plasmids

The plasmids used in this work are resumed in Table 3.

Table 3 – Plasmids used in this work.

<b>Plasmids</b>	<b>Characteristics</b>	<b>Reference</b>
pNIM1-OPY2-Myc	<i>CaOPY2</i> gene merged with a Myc-tag under the control of a tetracycline inducible promoter	(Herrero de Dios, unpublished data)
pGEM-T	Commercial to subclone PCR products	Promega
<i>CaOPY2</i> -Myc-pGEM-T	<i>CaOPY2</i> gene merged with a Myc-tag in pGEM-T	<i>This work</i>
pCM189	Expression plasmid in <i>S. cerevisiae</i> under the control of a tetracycline repressive promoter	(Gari et al., 1997)
<i>CaOPY2</i> -Myc-pCM189	<i>CaOPY2</i> gene merged with a Myc-tag in pCM189	<i>This work</i>
<i>ScOPY2</i> -Myc-pCM189	<i>ScOPY2</i> gene merged with a Myc-tag in pCM189	<i>This work</i>

#### 4. DNA Manipulation

All techniques used for manipulating DNA such as isolation of plasmid DNA from *E. coli*, DNA electrophoresis on agarose gels, determination of purity and concentration of the DNA, among others were executed by standard procedures (Sambrook *et al.*, 1989). *E. coli* was transformed following the protocol described by Hanahan (1988).

The representations of DNA sequences, vector and plasmids in this work, as well as the calculation of theoretic molecular weights from the proteins used, were obtain by the Vector NTI Advance<sup>®</sup> Software, by Life Technologies.

**a) PCR amplification of the CaOPY2-Myc gene**

For the PCR amplification, template DNA of the *CaOPY2-Myc* gene was amplified on a *Eppendorf Mastercycler DNA Engine Thermal Cycler PCR* with the forward primer o-CaOpy2Bup (CCGGATCCAATCAATCATGCCAATACCCAGATCG), and the reverse primer o-CaOpy2Plw (GGCTGCAGTTACAAGTCTTCTTCAGAAATCAATTTTTTGTTTC), using standard PCR conditions: 5 min at 95°C, followed by 40 cycles of 20s at 95°C, 20s at 55-60°C and 20s at 72°C with a final extension of 5 min at 72°C.

The following compounds were required: pNIM1-*CaOPY2-Myc* as DNA template, Thermostable Taq DNA polymerase (Roche, Switzerland) with proofreading activity, dNTP mix (Roche, Switzerland), Buffer 10x (with MgCl<sub>2</sub>), forward and reverse primers and deionized water, on a total volume of 50µL.

The PCR product was subsequently separated in a 0.8% agarose gel.

**b) DNA Gel Electrophoresis**

The amplified PCR product and all the diverse DNA samples used in this work were resolved by electrophoresis in a 0.8% agarose gel in 1x TAE buffer (4.84g Tris-base, 1.142mL glacial acetic acid, 2mL EDTA 0.5M pH 8 and H<sub>2</sub>O till prefacing 1 L) at 100V for 80 min using a Bio-Rad electrophoresis apparatus (USA).

Depending on the resolution that is wanted for the separation of the more or less heavy fragments, the percentage of agarose in the gel can be changed.

In each DNA Gel Electrophoresis it is added a 1Kb Plus DNA Ladder marker (Invitrogen, USA) (Figure 15) as a reference to help us estimate the size of our DNA molecules (40µL *Ladder* enzyme, 20µL 10x buffer and 140µL H<sub>2</sub>O Millipore in a final volume of 250µL).



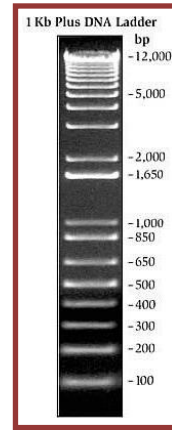


Figure 15 - 1Kb Plus DNA Ladder marker used in the DNA Gel Electrophoresis to distinguish the different sizes of the samples loaded.

To each sample, before they are loaded in the gel, it is mixed the loading buffer. The loading buffer consists on glycerol mixed with bromophenol blue and H<sub>2</sub>O (Lab made, Tris-EDTA and bromophenol blue) and it is added to the samples in the proportion of 1:1. This dye is important in two ways: first it helps to weigh down the DNA so that it can sink into the bottom of the wells and not float in the buffer solution; secondly, it moves more quickly than the actual DNA so it is an indicator to when to turn off the power on the electrophoresis chamber.

The gels were stained using GelRed (a substitute of ethidium bromide since it has a higher sensibility and it has a low risk to human health) (10 $\mu$ L of GelRed (GelRed Biotium Inc., USA) in 100mL of NaCl 0.1M) during 30 min. After visualizing the gels in a MiniLumi Gel-Documentation System (DNR Bio-imaging systems, Israel), the images were saved as a TIFF file to posterior analyze.

### **c) *Elution of DNA bands***

During this work it was necessary to isolate specific DNA bands from the agarose gels for it use in subsequent procedures.

For this, a DNA Gel Electrophoresis as explained before is performed with some differences: the TAE buffer should not have been used before; the agarose must be more pure that the one normally used with a lower melting temperature; and the

stained of the gel must be done in a new solution of GelRed. It is essential that the exposure to UV light is as minimal as possible to prevent DNA damage.

After having the desired DNA band, the DNA is purified using the GENE CLEAN® *Turbo* Kit (Qbiogene, Inc., Carlsbad, California).

**d) *Rapid Isolation of DNA from Agarose Gels***

To perform the isolation of DNA from the bands extracted from the agarose gels, the gel slice was placed in a 1.5mL eppendorf vial. For each 0.1g of gel slice, 100µL of the GENE CLEAN® *Turbo* Salt Solution was added and mixed. The mixture was incubated at 55°C for 5 min to melt the gel. The tube was inverted several times to mix.

The DNA/Salt solution is added to a GENE CLEAN® *Turbo* Cartridge placed inside a cap-less Catch Tube and it is centrifuged for 5s until all liquid has passed through the filter. The Catch Tube must be empty as much as needed.

500µL of the prepared GENE CLEAN® *Turbo* Wash Solution are added to the filter and it is centrifuged again for 5s. The Catch Tube must be, again, empty as much as needed. To remove residual Wash Solution, the GENE CLEAN® *Turbo* Cartridge is centrifuged for an additional 4 min.

The cap of a new, clean Catch Tube is removed and the GENE CLEAN® *Turbo* Cartridge which contains the bound DNA is inserted.

30µL of H<sub>2</sub>O milli-Q is added onto a GLASSMILK®-embedded membrane and incubated at room temperature for 5 min and centrifuged for 1 min to transfer eluted DNA to GENE CLEAN® *Turbo* Catch Tube. The GENE CLEAN® *Turbo* Cartridge is discarded and the Catch Tube is capped.

### ***e) DNA Ligation***

Joining linear DNA fragments together with covalent bonds is called ligation. More specifically, DNA ligation involves creating a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another. The enzyme used to ligate DNA fragments is T4 DNA ligase. This enzyme can join DNA fragments with cohesive ends or fragments with blunt ends, although higher concentrations of the enzyme are usually recommended for this last purpose. The optimal incubation temperature for T4 DNA ligase is 16°C but it works properly at room temperature.

In this work we performed this ligation in some steps of the procedure in order to ligate the insert with the vector. For this reaction we mixed: insert, vector, Buffer 2x (this buffer contains ATP) and T4 DNA ligase (Roche, Switzerland) on a final volume of 15µL. The ligation reactions were performed at room temperature during 2 hours.

Before proceeding to the ligation of the insert into the vector, an electrophoresis is done with equal volume of DNA from both the vector and the insert. The intensity of the bands provides an estimative of the quantity of DNA to use from each sample.

Before the ligation, it is essential to previously digest the plasmid with the appropriate restriction enzymes, to obtain an opened vector.

### ***f) Restriction enzymes digestion***

During this work it was necessary to use restriction enzymes to cut the DNA plasmid to obtain inserts or vectors, according to what was need to proceed with the experiments. For this, it was necessary: plasmid DNA, Buffer 10x (specific for the restriction enzyme), restriction enzyme (Roche, Switzerland), H<sub>2</sub>O milli-Q in a final volume of 100µL. The reaction is left for 2 hours, at 37°C. The time of the reaction can be expanded to make sure that the digestion is totally done.

The buffer used in each reaction depends on the enzymes used and it should, if two enzymes are used, be adequate for the both – this allows a 100% of cut efficiency. When this is not possible, first the fragment is digested with one enzyme; than the DNA is precipitated as a way of recovering it and then the precipitated DNA is digested with the second enzyme.

In this work there were used the following restriction enzymes: *PstI*, *BamHI*, *NotI* and *HindIII* (Roche, Switzerland) to obtain regulated expression plasmids, sequences to posterior integration in a vector or to prove the correct obtainment of the different plasmids (Figure 16).

<b>PstI</b>	<b>BamHI</b>	<b>NotI</b>	<b>HindIII</b>
<b>Recognition Site:</b>	<b>Recognition Site:</b>	<b>Recognition Site:</b>	<b>Recognition Site:</b>
5'...GGT <b>N</b> ACC...3'	5'...GGAT <b>C</b> CC...3'	5'...GCGG <b>C</b> CGC...3'	5'...A <b>A</b> GGCTT...3'
3'...CCANT <b>G</b> G...5'	3'...CCTA <b>G</b> G...5'	3'...CGCC <b>G</b> CG...5'	3'...TTCGA <b>A</b> ...5'

Figure 16 - Recognition Sites for the Restriction enzymes *PstI*, *BamHI*, *NotI* and *HindIII*

After the digestion, samples are subjected to a DNA Gel Electrophoresis to confirm that, in fact, the DNA was digested. The plasmid DNA is then precipitated.

#### **g) DNA ethanol precipitation**

Ethanol precipitation is a method used to purify and/or concentrate DNA from aqueous solutions.

To precipitate DNA, first it should be added 1/10 of the volume of the solution of Sodium Acetate (3M, pH 5.2). 2 volumes of cold 100% ethanol are added and left at least 20 min at -20°C. The mixture is centrifuged at 13000rpm for 10 min and after the supernatant be discarded, 1mL 75% ethanol is added to the pellet. After another centrifugation of 10 min, the supernatant is discarded and the pellet is dried at 37°C. The pellet is then resuspended in 30µL of milli-Q H<sub>2</sub>O.

#### ***h) Plasmid DNA Isolation (Mini-Prep)***

Isolation of plasmids DNA by this method, often referred to as Mini-Prep, yields fairly clean DNA quickly and easily.

For the extraction of the plasmid DNA, overnight cultures were grown on SD minimal medium with shaking. After being subjected to centrifugation during 5 min at 10000rpm, the pellet was resuspended in 200µL of lysozyme solution (1.8% glucose, 2.5% 1M Tris HCl pH 8, 2% 0,5M EDTA pH 8 and egg lysozyme (Sigma, USA)) to break the peptidoglycans of the cell wall, 400µL of a bacteria lysis buffer (10% SDS detergent and 10% NaOH 2M and milli-Q H<sub>2</sub>O) and 300µL of a High-Salt solution (3M). The treated samples were centrifuged for 10 min at 10000rpm, and to the supernatant was added isopropanol (0.6% of the volume of the supernatant). The eppendorfs vials were then placed at -20°C during 20/30 min. After centrifugation of the samples during 15 min, ethanol was added to the pellet and centrifuged again during 10 min. The pellet was then dried at 37°C and resuspended in 30µL of H<sub>2</sub>O milli-Q. The samples were then separated in a 0.8% DNA Gel Electrophoresis as explained before.

#### ***i) Hanahan Transformation of E. coli***

Bacterial transformation is the process by which bacterial cells take up naked DNA molecules. If the foreign DNA has an origin of replication recognized by the DNA polymerases of the host cell, the bacteria will replicate the foreign DNA along with its own DNA.

When transformation is coupled with antibiotic selection techniques, bacteria can be induced to uptake certain DNA molecules, and those bacteria can be selected for that incorporation. Bacteria which are able to uptake DNA are called "competent" and are made so by treatment with calcium chloride in the early log phase of growth.

The bacterial cell membrane is permeable to chloride ions, but is non-permeable to calcium ions. As the chloride ions enter the cell, water molecules

accompany the charged particle. This influx of water causes the cells to swell and this is necessary for the uptake of the DNA. The exact mechanism of this uptake is unknown. It is known, however, that the calcium chloride treatment has to be followed by heat. When *E. coli* are subjected to 42°C heat, a set of genes are expressed which help the bacteria in surviving at such temperatures. These set of genes are called “the heat shock genes”.

Competent *E. coli* cells (previously treated with 0.15% RbCl, 0.8% Ca<sub>2</sub>Cl<sub>2</sub>.H<sub>2</sub>O and 15% glycerol, pH 6.8) are stored at -80°C. The bacterial cells and the plasmid DNA to introduce in the cells have to be kept cold throughout the transformation. For 100µL of competent cells it is usually added 2µL of plasmid DNA (but this depends mostly on the concentration of our DNA). The mix is then kept on ice for 20 min followed by a heat shock at 42°C during 2 min.

After the heat shock, the transformed cells were left on ice for 5 min and then incubated in LB medium with ampicillin for 30 min under soft shaking (800rpm) at 37°C (Thermomixer compact, Eppendorf, Germany). This step is important so that the cells that received the plasmid DNA are able to express the proteins which are responsible for antibiotic resistance (β-lactamase for ampicillin resistance) which makes it possible to select the clones carrying the plasmid.

Cells were plated on LB solid medium containing ampicillin (100µg/mL) and were grown overnight at 37°C. Colonies that were able to grow in this selective medium were chosen to isolate the plasmid they were carrying and cultured in 2mL LB liquid medium containing ampicillin.

#### **j) Preparation of *E. coli* Competent Cells**

First it is necessary to prepare the pre-inoculum from the DH5α *E. coli* cells in a test tube with LB medium, at 37°C with gentle shaking, overnight.

In an Erlenmeyer flask with 100mL of LB medium, the previous grown pre-inoculum is inoculated and the flask is left growing at 37°C, with gentle shaking till a OD of 0.4 at 550nm.

When the culture reaches the wanted OD, the 100mL are transferred to a 500mL flask previously on ice and left it there for 10 min. The culture is centrifuged for 10 min at 5000rpm, the supernatant discharged and it is added 1/3 of the initial volume of cold RF<sub>1</sub> solution in the pellet (Table 4). The culture is kept on ice for 30 min with the RF<sub>1</sub> solution.

After 30 min, the cultures are centrifuged for another 10 min at 5000rpm, the supernatant is discharged and in the pellet it is added 1/10 of the initial volume of RF<sub>2</sub> solution (Table 4).

Aliquots of 200µL each are performed and kept on dry ice with ethanol. Once all the aliquots are done, they are saved at -80°C.

With 100mL of LB medium it is obtained about 40 aliquots of *E. coli* competent cells.

Table 4 – Solutions used to prepare *E. coli* competent cells for 200mL of initial LB medium.

RF <sub>1</sub> (75mL)	RF <sub>2</sub> (20mL)
100mM RbCl	10mM MOPs pH=6.8
50mM MnCl <sub>2</sub> .4H <sub>2</sub> O	10mM RbCl
30mM AcK	75mM Cl <sub>2</sub> Ca.2H <sub>2</sub> O
10mM Cl <sub>2</sub> Ca.2H <sub>2</sub> O	
15% Glycerol	15% Glycerol
Ajust pH=5.8 with acetic acid 0.2N	Ajust pH=6.8 with NaOH

## 5. One-step transformation of *S. cerevisiae*

To introduce heterologous DNA into *S. cerevisiae*, the one-step transformation protocol is used.

Cultures of the yeast strains (WT BY4741, *opy2 ssk1*, *opy2* and *ssk1*) were grown at 30°C in SD minimal medium, overnight. The cells were then subjected to centrifugation, the pellet resuspended with One Step Buffer (0.2N lithium acetate, 40% PEG and 0.77μL β-mercaptoetanol) and vortexed.

0.6μg of the plasmid DNA was added to the mixture, vortex and then maintained for 10 min at room temperature. 50μg of single-stranded carrier DNA previously denatured for 5 min at 99°C (to reduce membrane binding to our plasmid) (Salmon Sperm DNA, Invitrogen) was added to the mixture, vortex again and maintained for 5 more min at room temperature.

After incubation at 45°C for 30 min, suspensions are then plated directly onto SD minimal medium plates and incubated at 30°C for 2 days.

## 6. Protein Extraction

To extract the proteins from the different mutants, cultures of yeast cells were grown at 30°C, overnight, in SD minimal medium.

Proteins were extracted by beat beating in the following way: the cells were collected in eppendorf vials by gentle centrifugation (13000rpm, 5 min) and the pellet resuspended in 150μL of lysis buffer (3mL proteins buffer (50mM Tris HCl (pH 8), 10% glycerol, 1% triton X-100, 0.1% SDS, 150mM NaCl, 50mM NaF, 1mM orthovanadate, 50mM β-glycerol phosphate, 5mM sodium pyrophosphate, 5mM EDTA (pH8)), 300μL protein inhibitor cocktail mix (Roche, Switzerland) and 150μL PMSF, and 200μL of 0.5mm glass beads were added to the mix.



To extract the proteins by breaking the cells mechanically with the glass beads, one cycle of bead beating was run through with a Fast prep cell breaker (MP Biomedicals, USA) at  $5.5 \text{ ms}^{-1}$  for 30s with a minute of cooling step on ice afterwards (one time is enough when working with *S. cerevisiae*).

The resulting supernatants were subjected to low speed centrifugation (3000rpm) to remove beads and high speed centrifugation to remove cell debris (13000rpm).

A 1/500 dilution with each protein is performed (998 $\mu\text{L}$  of  $\text{H}_2\text{O}$  with 2 $\mu\text{L}$  of the sample in a final volume of 1mL) and the OD is measure in a quartz cuvette at 280nm.

The protein concentration is then adjusted with Protein Loading Buffer (Tris HCl 0.1M at pH 6.8, 20% glycerol, 4% SDS, bromophenol and DDT) to obtain a concentration of 20 $\mu\text{g}/\text{mL}$  in 100 $\mu\text{L}$ .

Before the samples are loaded in the gel, they have to be boiled for 10 min at 99°C and centrifuged during 5 min at 13000rpm.

Protein samples should be kept at -80°C and the loading samples at -20°C.

## **7. Western Blot analysis**

Western blotting identifies, with specific antibodies, proteins that have been separated from one another according to their size by gel electrophoresis. The blot is a membrane, almost always of nitrocellulose or PVDF. The gel is placed next to the membrane and an application of an electrical current induces the proteins in the gel to move to the membrane where they adhere. The membrane is then a replica of the gel's protein pattern, and is subsequently stained with antibodies specific to the target protein.

In this work, the previously extracted proteins were subjected to electrophoresis through 10% SDS-polyacrylamide gel (Running Gel Solution (for 1 gel:

1.52mL H<sub>2</sub>O; 1.36mL Acrylamide; 1.4mL 1,5M Tris (pH 8.8); 40 μL 10% SDS; 40μL 10% PSA; 1.6μL TEMED); Stacking Gel Solution (for 1 gel: 1.15mL H<sub>2</sub>O; 150μL Acrylamide; 190μL 1M Tris (pH 6.8); 15 μL 10% SDS; 15μL 10% PSA; 1.5μL TEMED)).

5μL of each loading samples are loaded in the gel to have a concentration of the loaded sample of 10μg/mL.

A protein ladder (Precision Plus Protein™ All Blue Standards #161-0373, BioRad, USA) was added to each gel to allow distinction of the different molecular weights (Figure 17).

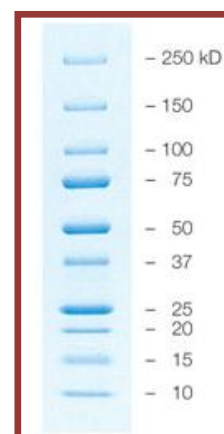


Figure 17 - Precision Plus Protein™ All Blue Standards, BioRad, USA.

The separation of the proteins was performed in cuvettes Mini-Protean II Cell (Bio-Rad, USA), in Electrophoresis buffer 1X (Tris-base 30.285g; Glicine 144.6g; SDS 10g and deionized water till 1L) during one hour at 150V.

The proteins were then transferred to a nitrocellulose membrane by electroblotting in 1X Transfer buffer (Tris-base 50g; Glicine 29g; SDS 3.7g and deionized water till 1L). An Amersham Hybond ECL Nitrocellulose Membrane (GE Life Sciences, UK) was placed onto the polyacrylamide gel and this was placed between filter paper and sponges in a Mini Trans-Blot Transfer Cell (Bio-Rad, USA). A constant voltage of 100V was applied for one hour to transfer the proteins from the gel to the membrane.

The membrane was then blocked in a 5% milk PBS or TBS/Tween 20 solution (PBS from BD Biosciences, USA, Tween 20 from Duchefa, Netherlands, TBS 10x (pH=7.6) – Tris-base 24.2g; NaCl 80g; H<sub>2</sub>O till 1L) to avoid non-specific antibody binding.

After being probed with the primary antibody (1:2500 dilution) in 1% milk PBS or TBS/Tween 20 during 2 hours at room temperature, the membrane was washed 3 times with PBS or TBS/Tween 20 and the secondary antibody in 1% milk PBS or TBS/Tween 20 was added to the membrane (1:5000 dilution) during 1 hour at room temperature. After the second antibody was removed, the membrane was washed again 3 times with PBS or TBS/Tween 20.

During this work two methods of revealing membranes were used – Fluorescent detection and Chemiluminescence.

For the analysis by Fluorescent detection the membranes were scanned in an Odyssey scanner (Li-cor Biosciences, USA) that detects infrared fluorescence and analyzed in the Odyssey V3.0 programme.

When chemiluminescence is used, the procedure has more steps, as follows:

First, in an eppendorf vial, Western Blotting detection reagents 1 and 2 are mixed in a ratio of 1:1 (250 $\mu$ L of the mix are added per membrane) (GE Healthcare UK Limited, UK). The mixed reagent should be protected from light either by wrapping in aluminum foil or storing in a dark place, if it is not used immediately. To have higher signal, 2 $\mu$ L of hydrogen peroxide (PRS Panreac Quimica SA, Barcelona, Spain) can be added to the mix.

What happens with these reagents is the following:

When energy in the form of light is released from a substance due a chemical reaction, the process is called chemiluminescence. Luminol is one of the most widely used chemiluminescent reagent and its oxidation by peroxide results in creation of an excited state product called 3-aminophthalate. This product decays to a lower energy state by releasing photons of light (Figure 18).

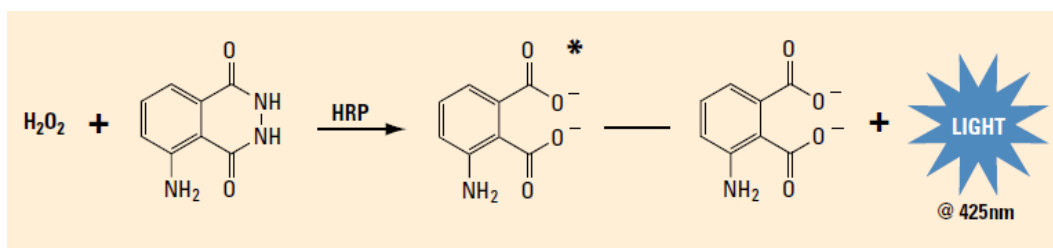


Figure 18 – Luminol is oxidized in the presence of HRP and hydrogen peroxide to form an excited state product (3-aminophthalate). The 3-aminophthalate emits light at 425nm as it decays to the ground state. (Western Blotting Handbook and Troubleshooting Tools, 2010)

Horseradish peroxidase (HRP) is a 40kD protein that catalyzes the oxidation of substrates by hydrogen peroxide, resulting in a colored or fluorescent product or in the release of light as a byproduct.

After adding the reagents to the membrane, the excess wash buffer from the washed membranes should be drained and the membrane should be placed with the protein side up on a sheet of plastic wrap in a X-ray film cassette (Figure 19). It must be ensured that there is not any detection reagent free in the cassette – the film must not get wet.



Figure 19 – Example of a X-ray film cassette.

Then, the mixed detection reagent is added onto the membrane and the excess of detection reagent should be drained off. The X-ray film cassette is closed and taken to a dark room using red safe lights.

In this room, the cassette is open and a sheet of autoradiography film (Amersham GE Life Sciences, UK) is placed on top of the membrane. The cassette is closed and the film is exposed from 60s to minutes depending on the antibody used. The film should not be moved while it is being exposed.

The first film is then removed and replaced with a second sheet of unexposed film.

The first piece of film is developed immediately, and on the basis of its appearance it is estimated how long to continue to expose the second piece of film. Second exposures can vary from 1 min to 1 hour.

To develop the films, initially they are placed in a Developer Solution (X-OMAT EX II Developer and Replenisher, Kodak), then washed in water, followed by placing the film in a Fixer Solution (RP X-OMAT LO Fixer and Replenisher, Kodak) and to finish the films are washed with water and dried.

In this work several antibodies were used in different procedures to detect different proteins. To detect the proteins that had attached a Myc-tag, a primary antibody anti-myc (Millipore, USA) was used followed by a second antibody IRDye 800CW Goat anti-mouse (Li-cor Biosciences, USA) when the Western Blot was revealed with the Odyssey scanner and by the  $\alpha$ -Mouse-HRP antibody when they were revealed by chemiluminescence.

To detect the Hog1 protein the primary antibody used was the anti-Hog (Santa Cruz Biotechnology, USA) and to detect the activated Hog1 Kinase two different anti-phospho-p38 MAPK were used, one from Cell Signaling Technology Inc., USA and, another from Santa Cruz Biotechnology. For both these detections, the secondary antibody used was the IRDye 800CW Goat anti-rabbit (Li-cor Biosciences, USA) when the membranes were revealed with the Odyssey scanner. When chemiluminescence was applied, the  $\alpha$ -Rabbit-HRP antibody was used.

An antibody to detect phosphorylation of Mkc1 and Cek1 in *C. albicans* and the phosphorylation of Slt2, Kss1 and Fus3 in *S. cerevisiae* was used too – the anti-phospho-p44/42 MAPK (Thr202/Tyr204) from Cell Signaling Technology I. The same secondary antibody as with the Hog1 protein was used for these detections.

The antibodies used in this work are resumed in Table 5.

Table 5 – Antibodies used for detection of proteins in the Western blot analysis.

<b>Antibody</b>	<b>Features</b>	<b>Reference</b>
anti-Hog1 (y-215): sc-9079	Primary antibody, detecting the Hog1 protein, rabbit polyclonal IgG	(Santa Cruz Biotechnology, USA)
anti-phospho-p38 MAPK (Thr180/Tyr182)	Primary antibody, detecting phosphorylated Hog1, rabbit monoclonal IgG	(Cell-Signalling, USA)
anti-phospho-p38 MAP Kinase (Thr180/Tyr182)	Primary antibody, detecting phosphorylated Hog1, rabbit polyclonal	(Cell-Signalling, USA)
anti-phospho-p38 MAP Kinase (Thr180/Tyr182)-R: sc-17852-R	Primary antibody, detecting phosphorylated Hog1, rabbit polyclonal	(Santa Cruz Biotechnology, USA)
anti-phospho-p44/42 MAPK (Thr202/Tyr204)	Primary antibody detecting dually phosphorylated Mkc1 and Cek1 rabbit IgG monoclonar	(Cell-Signalling Technology I., USA)
Anti-Myc Tag, clone 4A6	Primary antibody detecting the Myc Tag, mouse monoclonal IgG1	(Millipore, USA)
IRDye 800CW Goat anti-mouse	Secondary antibody	(Li-cor Biosciences, USA)
IRDye 800CW Goat anti-rabbit	Secondary antibody	(Li-cor Biosciences, USA)
$\alpha$ -Rabbit-HRP	Secondary antibody	(Amersham, England)
$\alpha$ -Mouse-HRP	Secondary antibody	(Amersham, England)

#### **a. Dot Blot Assay**

Dot blot is an immunological technique based on Western blotting (without separating the protein on SDS-Page) to detect the protein with antibodies in a sample like tissue lysate or in a mixture of proteins. Similar to Western blot, the technique involves spotting of antigens or proteins on to the nitrocellulose membrane and then the remaining protein-binding sites are blocked by treating the membrane with blocking reagent (5% skimmed milk). The spotted membrane is then treated with specific primary antibody followed by washing to remove the unbound and nonspecifically bound antibody. Then a secondary antibody is added. The analysis is performed as in a Western Blot.

To detect the presence of the Myc epitope in the extracted proteins, Dot Blot Assays were developed. 2 $\mu$ L of each samples of the proteins to test were blotted in an Amersham Hybond nitrocellulose membrane (GE Life Sciences, USA). To each blot, both negative and positive controls were added. Blocking of the membrane was performed during 30 min using a solution of 5% milk PBS/Tween 20. Immunolocalisation of the Myc epitope was performed using the primary antibody anti-myc (Millipore, USA) in 1% milk PBS/Tween 20 during 2 hours at room temperature. The membrane was washed 3 times with PBS/Tween 20 (1mL Tween in 1L PBS) and the secondary antibody IRDye 800CW Goat anti-mouse (Li-cor Biosciences, USA) in 1% milk PBS/Tween 20 was added to the membrane during 1 hour at room temperature. After the second antibody was removed, the membrane was again washed 3 times with PBS/Tween 20.

The blot was scanned in an Odyssey scanner (Li-cor Biosciences, USA) and analyzed in the Odyssey V3.0 programme.

#### **b. Coomassie Staining**

To calculate the volume of samples to load in a SDS-Page and have equal concentration of each sample, a SDS-Page as previously explained can be run for 30 min and the gel stained with Coomassie dye. This offers one of the simplest protein staining technique, although not the most sensitive.

After the gel been removed from the electrophoresis chamber, it is placed in enough 0.5% Coomassie Blue G-250 (prepared in 50% methanol/ 10% acetic acid) to cover the gel and it is stained for about 15 min. The stain is then removed and the gel is destained with a solution of 40% methanol/ 10% acetic acid, replacing the solution every 10-20 min until faint bands are observed.

According to the different intensity that the different samples appear with, the volume of the samples to load in the Western Blot is calculated.

**c. *Stripping and reprobing membranes***

One of the major advantages offered by chemiluminescent detection is the ability to strip reagents from a blot and then reprobe it. This is possible because the product detected is light rather than a colored precipitate on the membrane.

A blot may be stripped and reprobed several times to visualize other proteins or to optimize detection of a protein without the need for multiple gels and transfers.

The key to this process is to use conditions that cause the release of antibody from the antigen without removing a significant amount of antigen from the membrane. The membranes should be submerged in stripping buffer (7.5g Glicine pH 2.2; 0.5mL NP-40 (5mL 10%); 1% SDS (100mL 10%); H<sub>2</sub>O till 1L) and left with agitation for one hour at room temperature. The membrane is then washed 3 times, approximately 10 min each, with TBS/Tween 20 at room temperature. Next, the membrane is blocked in a 5% milk TBS/Tween 20 solution for 1 hour at room temperature.

Both incubation and detection protocol are the same as explained before. After each immunodetection membranes should be stored in a refrigerator.

## **8. Activation of the HOG pathway**

The activation of the HOG pathway by osmotic shock was studied as follows:

First, cultures of the different mutants to be tested were grown at 30°C in SD minimal medium, overnight. For each culture, an Erlenmeyer flask with SD minimal medium was prepared with a fixed volume – for each point that was pretended to study, 20mL of SD minimal medium was added, and left at 30°C, overnight.

In the following morning, the OD was measured at 600nm in a dilution of 1:20 (50µL of cells in 950µL H<sub>2</sub>O). The cultures were then diluted to obtain, in the pre warmed medium, 0.2 of OD and the cultures were left growing for 5 hours.



After 5 hours, when cultures should be in an OD around 1 (exponential phase), and 20mL of the cultures were removed to a falcon and to the remaining volume was added NaCl (PRS Panreac Quimica SA, Barcelona, Spain) - final concentrations differed during the different attempts of the experiment. The cultures were left growing for specific times and when it ended, the volume was removed to another falcon. The falcons without and with the cell have to be leave on ice during all these procedures to stop to the reaction when the reaction time ends.

A scheme of the first experiment performed is shown in Figure 20.

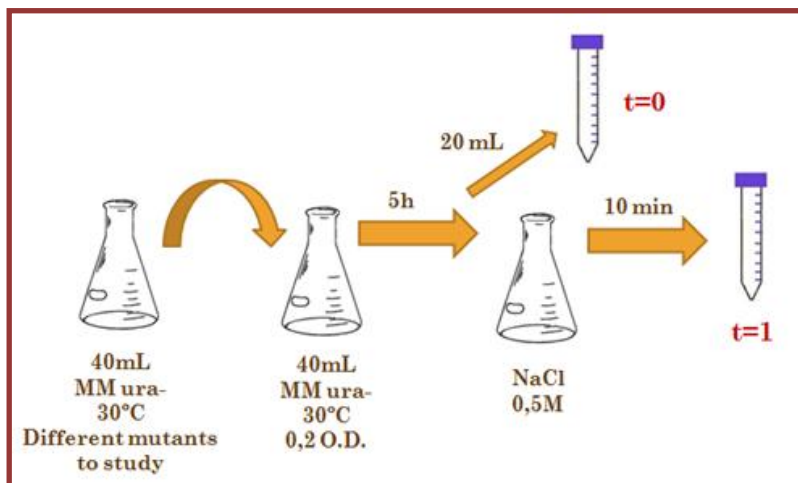


Figure 20 – Scheme of the procedures done to see activation of the HOG pathway adding NaCl to the different cultures.

Cells were then centrifuged during 5 min at 3000rpm in an Eppendorf Centrifuge 5810R. Supernatant is removed and the pellets were two times washed with cold deionized H<sub>2</sub>O. The proteins are then extracted as explained before and a Western Blot with the different samples is performed afterwards.

## 9. Inhibition of the *OPY2* N-glycosylation by tunicamycin

To see differences in the glycosylation of *CaOPY2* in *C. albicans* and the *ScOPY2* in *S. cerevisiae*, another experiment was performed with the use of tunicamycin to block the synthesis of all N-linked glycoproteins (N-glycans).

For this, the following procedures were performed:

The different cultures to test were grown in SD minimal medium, overnight, at 37°C for *C. albicans* strains and at 30°C for *S. cerevisiae* strains.

For each culture, an Erlenmeyer flask with SD minimal medium was prepared with a fixed volume – for each point that was pretended to study, 15mL of SD minimal medium was added, and left at the correspondent temperature, overnight.

In the following afternoon, the OD was measured at 600nm in a dilution of 1:20 (50µL of cells in 950µL H<sub>2</sub>O). The cultures were then diluted to obtain, in the pre warmed medium, an OD that allowed having in the next morning the cultures with an OD around 1.

In the following morning, the OD was measured to confirm that the cells were in the wanted OD and, if so, 10mL of the initial cultures were removed to a falcon and the remaining volume was separated into two Erlenmeyer flasks – to one of them, 1.5µg/mL of tunicamycin was added and the other one remaining the same. The cultures were left growing for 6 hours and 20mL samples were removed at 2 hours, 4 hours and 6 hours. Two tests were performed with different mutants and in different times of reaction, cells were removed.

A scheme of the 2 experiments performed with tunicamycin is shown in Figure 21.

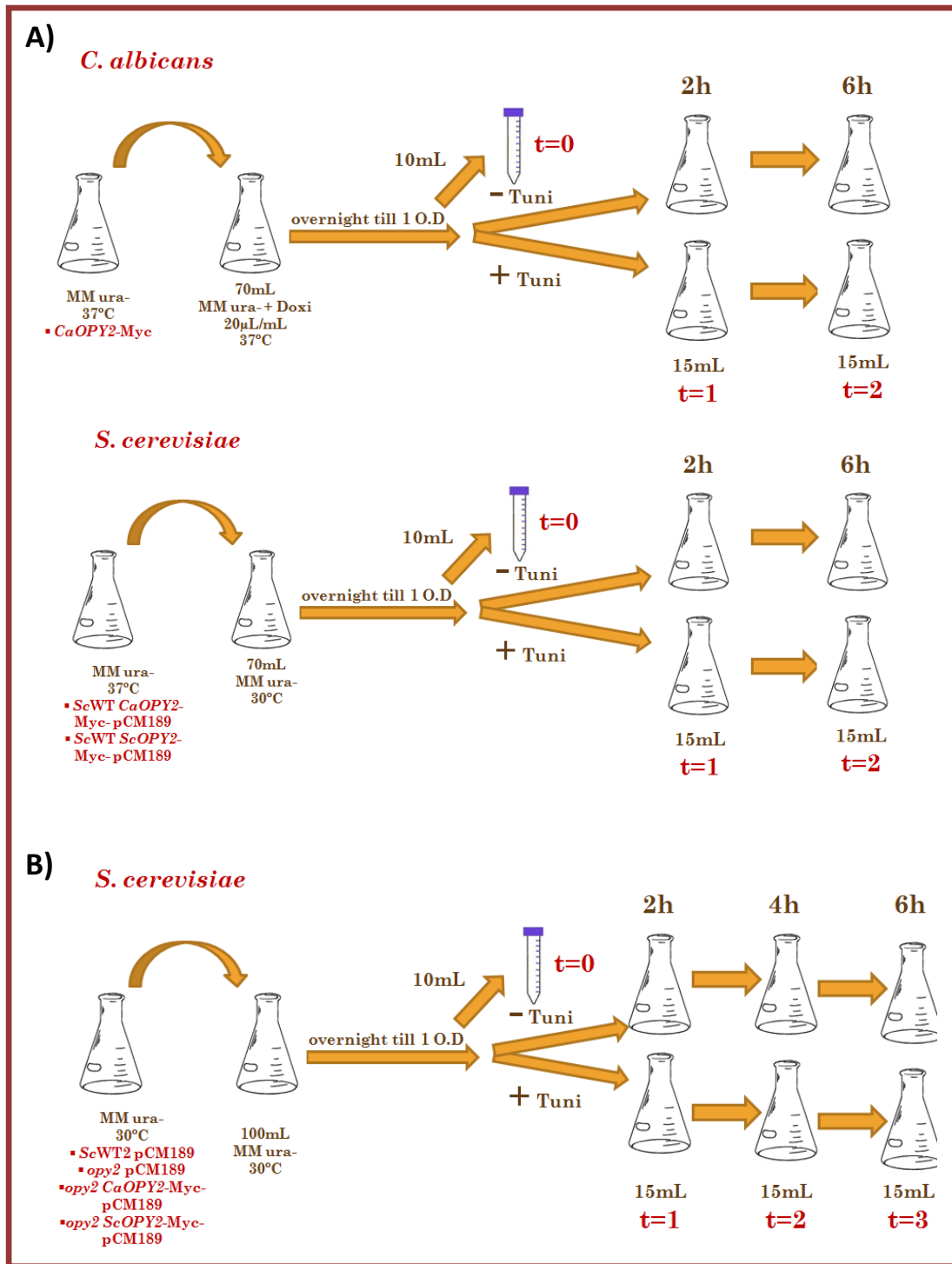


Figure 21 – Scheme of the procedures performed in two experiments to test the effect of 1,5µg/mL tunicamycin in different mutants.

Cells were then centrifuged during 5 min at 3000rpm, the supernatant is removed and the proteins are then extracted as explained before and Western Blots with the different samples are performed afterwards.

## 10. Adaptation to zymolyase-mediated cell wall damage

To test the response generated upon cell wall stressing conditions, cells were exposed to zymolyase in an experiment similar to the one previously performed with tunicamycin but in this case, 0.4U/mL of zymolyase (100T from *Arthrobacter luteus*, MP Biomedicals, Aurora, OH) was added to the cultures of the mutants to test (ScWT2, *opy2* pCM189, *opy2 CaOPY2-Myc-pCM189* and *opy2 ScOPY2-Myc-pCM189*) (Figure 22).

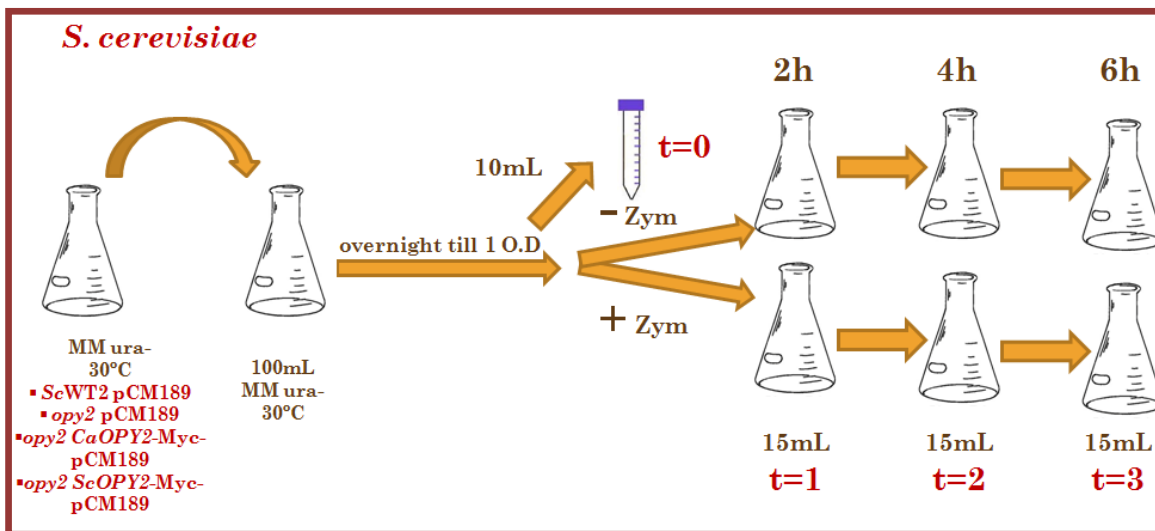


Figure 22 – Scheme of the procedures done to see adaptation to zymolyase-mediated cell wall damage adding 0.4U/mL of zymolyase to the different cultures.

## 11. Serial dilution spotting assay

This method for spotting yeast cells allows seeing cell growth. With serial dilutions, it is possible to estimate the concentration till when the yeast can grow. It can be added to the medium several different compounds to see if the cell growing is different.

The different strains were grown, overnight, in SD minimal medium at 30°C. The OD was measured at 600nm in a dilution of 1:20 (50µL of cells in 950µL H<sub>2</sub>O). Dilutions with SD minimal medium were performed to obtain, in a total of 1mL, the OD of 0.8.

In a 96-well plate (Cellstar greiner bio-one), 100 $\mu$ L of the previous dilution was placed in the first column. To the following 5 columns (columns 2, 3, 4, 5, 6), 90 $\mu$ L of SD minimal medium was added and serial dilutions of 10 $\mu$ L from the first to the following column were done. In the last column, 10 $\mu$ L were discarded. This will allow to have a dilution serie of  $10^0$ ,  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  as can be shown in the Figure 23.

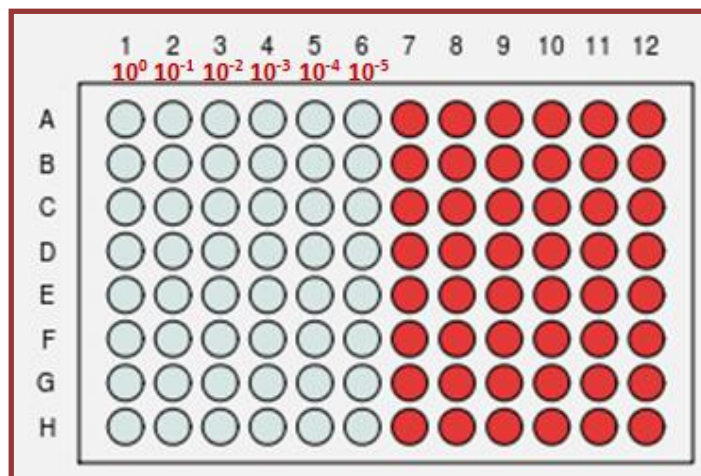


Figure 23 – Scheme of the 96-well plate used to do the dilutions for the Spot Assay.

The dilutions are dropped onto appropriate plates using a VP 407 Multi-Blot Replication (V&P Scientific, Inc, USA) (Figure 24), creating a grid of descending cell concentrations. Plates of YPD and YPD with the different compounds tested were used and duplicates were made.

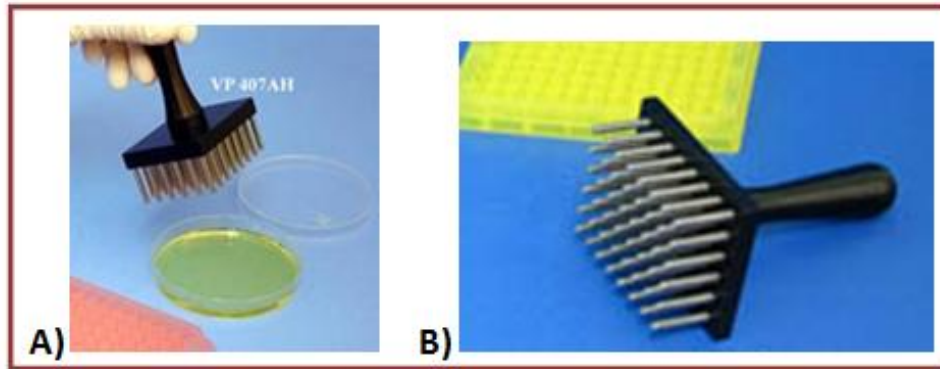


Figure 24 – A) and B) VP 407 Multi-Blot Replication used to perform the Spot Assays (V&P Scientific, Inc, USA).

The plates have to be completely dried or drops will run into each other. The plates were then incubated one group at 30°C and another at 37°C.

Scanners of the plates were made after 2 or 3 days of incubation.

During this work Spots Assays were performed in YPD plates with different compounds – NaCl (PRS Panreac Quimica SA, Barcelona, Spain), Sorbitol (Duchefa Biochemie BV, Netherlands), Congo Red (Merck), Menadione (Sigma, USA), Calcofluor (Sigma, USA) and Hydrogen peroxide (PRS Panreac Quimica SA, Barcelona, Spain). Different concentrations of the different compounds were used.

## 12. *OPY2* viability test with HL60

The HL60 cell line was obtained from ATCC (American Type Culture Collection) and was maintained in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), glutamine (Gibco) and 1% streptomycin/penicillin (Gibco) at 37°C in 5% CO<sub>2</sub> using 50mL tissue culture flasks (IWAKI brand, Japan).

HL60 cells were differentiated by incubation in presence of DMSO (Merck) 1.3% (v/v %) for 4 days. For harvesting, cells were centrifuged at 1000rpm, the supernatant removed and the pellet re-suspended in RPMI at the required final concentration. The

cell population was counted by trypan blue dye exclusion (Sigma, USA) with a hemocytometer (BLAUBRAND®, Germany) using an optical microscope.

The different yeasts strains to test were grown in YPD, overnight, at 37°C. 1mL of the culture was transferred to an eppendorf vial, centrifuged at 13000rpm and the pellet washed twice with PBS.

Killing assays were performed in 24-well tissue culture dishes (Cellstar greiner bio-one). HL60 cells and yeast strains were suspended in RPMI 1640 medium at a cell ratio of 1:40 and 1:20 (yeasts:HL60 cells) as is explained in Figure 25. The cultures were incubated at 37°C in 5% CO<sub>2</sub> for 2 hours.



Figure 25 – Scheme of the 24-well tissue culture dish with the different mutants and the different dilutions performed.

After the 2 hours of incubation, the cultures are left for one hour with PBS under gentle agitation. Because *C. albicans* cells attach to the plastic of the dishes, it is important to scrape very well the wells, especially the ones who have only *C. albicans* cells of the different strains.

Both 10<sup>-1</sup>, 10<sup>-2</sup> and 10<sup>-3</sup> dilutions of each different culture were performed and 100µL from each dilution and from the controls of the cells before incubation with

HL60 were spread into YPD plates with the help of glass beads. Determinations of CFU were performed after incubation for 48 hours at 37°C (Figure 26).

To analyze and compare the obtained data, graphs in Microsoft Office Excel were performed.

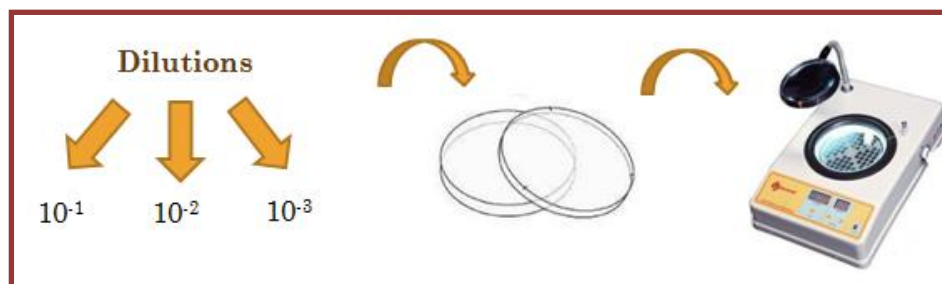


Figure 26 – Scheme of the steps to at last obtain the colony-forming units of each plate spread.

### 13. Microscopy

For analysis of the different phenotypes of the different mutants (*ScWT* pCM189, *opy2 ssk1* pCM189, *opy2 ssk1 CaOPY2-Myc*-pCM189 and *opy2 ssk1 ScOPY2-Myc*-pCM189), cells were grown overnight in YPD and in YPD 1M NaCl and then analyzed by microscopy.

The cells were visualized with a Nikon TE2000 fluorescence inverted microscope equipped with CCD (Melville, NY). Digital images were acquired with an Orca C4742-95-12ER camera (Hamamatsu, Bridgewater, NJ) and processed with the Aquacosmos Imaging Systems software.



# Results

## 1- PCR amplification of the CaOPY2-Myc gene

The *CaOPY2-Myc* gene was amplified using the pNIM1-*OPY2-Myc* plasmid as template (Figure 27) (Herrero de Dios, unpublished data) by PCR. The Myc-tag allows to detect the presence of the protein with the use of an antibody against the Myc epitope.

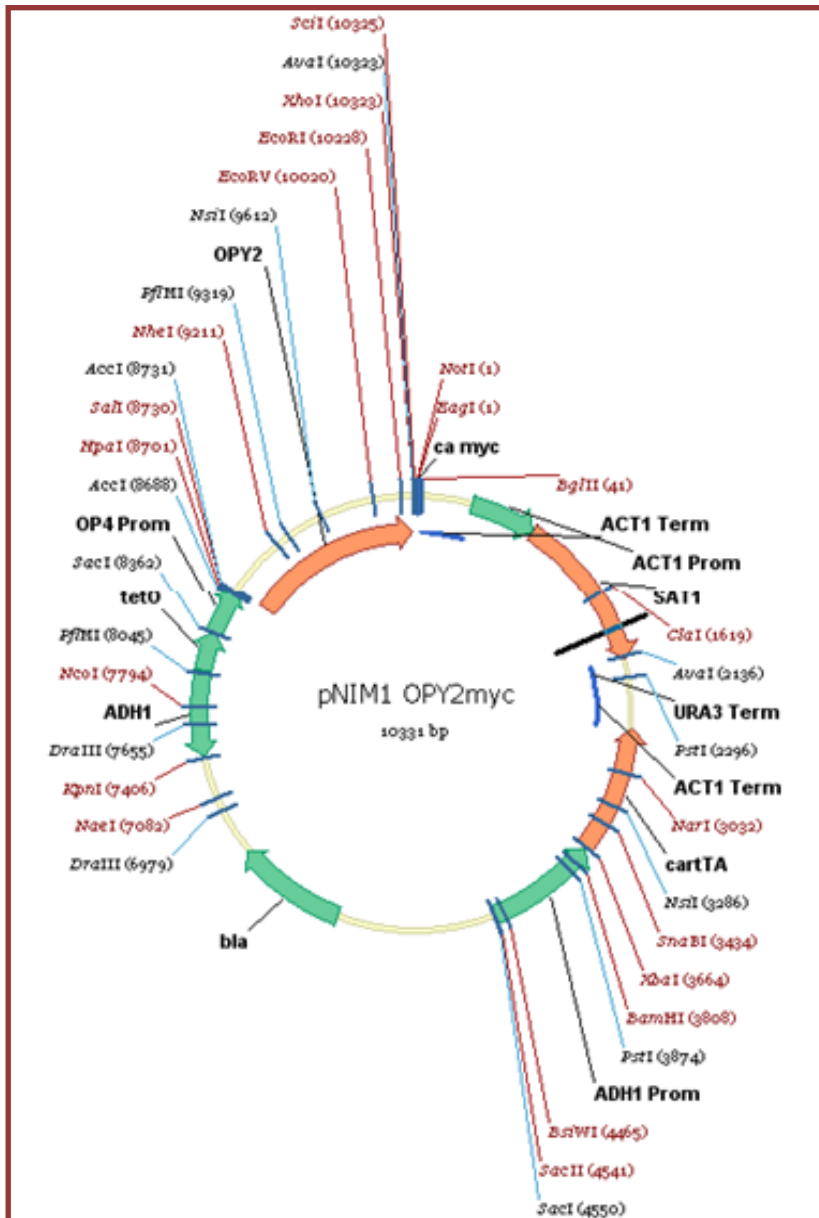


Figure 27 - Structure of the pNIM1-*OPY2-Myc* plasmid.

The *CaOPY2*-Myc is 1652 bp long (Figure 28). The Myc-tag is attached to the end of the gene between restriction sites to *NotI* and *PstI*. In the beginning of the *CaOPY2* there is a restriction site to *BamHI*.

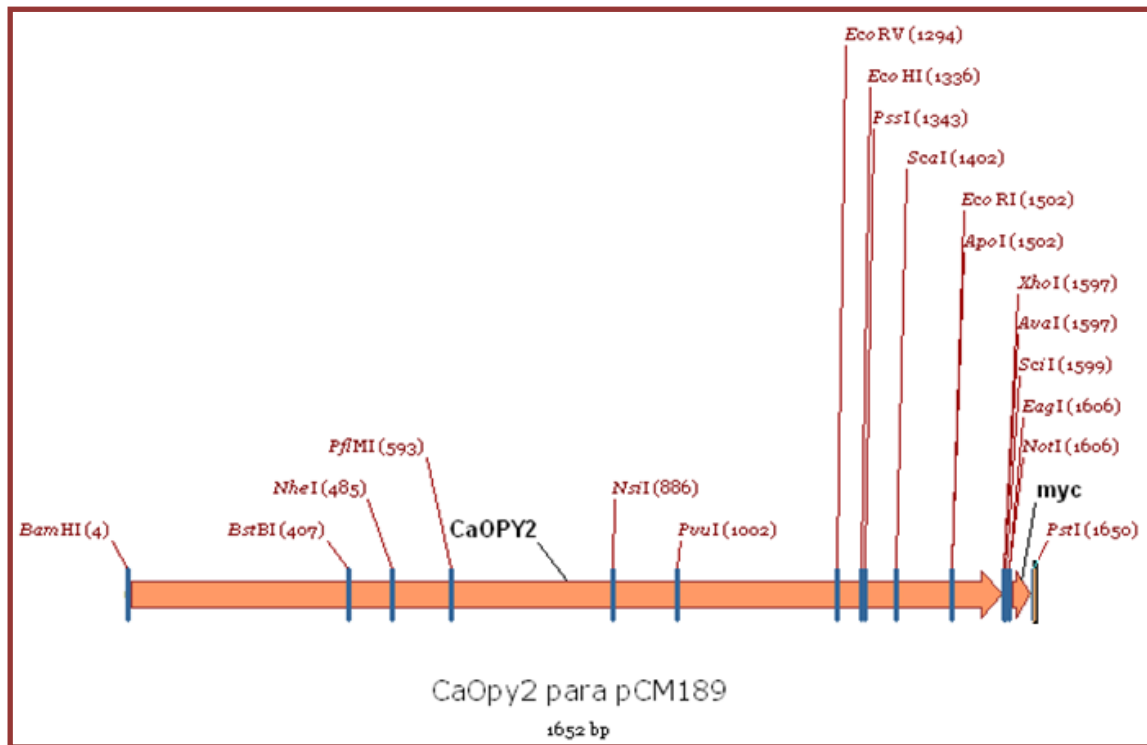


Figure 28 – *CaOPY2*-Myc PCR amplified gene with 1652bp.

To verify if the gene was correctly amplified, a DNA Gel Electrophoresis was performed (Figure 29). As seen in Figure 29, the PCR amplification product appears at the same level of the 1650bp band meaning that the gene was correctly amplified.

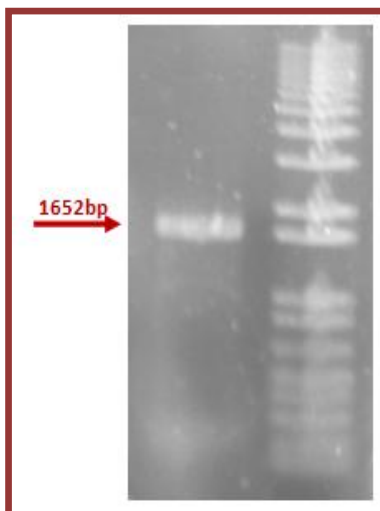


Figure 29 – PCR amplification product of the *CaOPY2*-Myc with 1652bp. 5µL of the sample were loaded in a DNA Gel Electrophoresis and the gel revealed by GelRed staining.

## 2- Construction of the CaOPY2-Myc-pGEM-T

pGEM-T vectors are linearized vectors with a single 3'-terminal thymidine at both ends what provides a compatible overhang for PCR products generated by certain thermostable polymerases. *Taq* polymerase is an example of this thermostable polymerases because amplifies DNA products that have adenine overhangs at their 3' ends. This enables ligation between the PCR product and the vector.

pGEM-T vector (Figure 30) has 3000 bp which includes a *lacZ* start codon, a *lac* operator, a T7 RNA polymerase promoter, a T7 RNA polymerase transcription initiation site, a resistance gene to ampicillin and multiple cloning regions with different restriction sites.

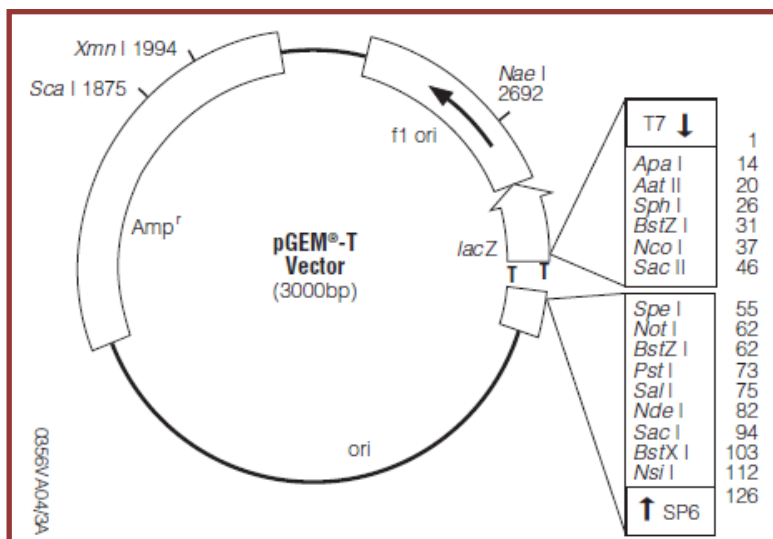


Figure 30 – Structure of the pGEM-T Vector.

After the preparation of the two molecules to be combined, we can initiate the process of ligation. Since the vector has the 3' terminal with thymidines and the insert the 3' terminal with adenines, the extremities are compatible and the impairment between complementary bases by hydrogen bonds is possible. It is necessary to promote a phosphodiester bond between the two nucleotides of the extremities of both molecules to ligate and for this a DNA ligase is used. This enzyme catalyzes the bond between the phosphate of the carbon 5' and the hydroxyl group of the carbon 3', in a reaction that needs ATP.

To clone our insert (*CaOPY2-Myc*) into the vector, T4 DNA Ligase was used at room temperature during 2 hours. In this step it can happen that the cloning vector recirculates without integrating any DNA molecule or that two vector molecules ligate to themselves without integrating the DNA pretended.

The next step is to introduce the molecules of the recombinant DNA in a host cell to reproduce the plasmid DNA introduced. This process is called Transformation. In the end of the process cells are plated in a medium where was added an antibiotic to which the plasmid has a resistance gene, X-gal and IPTG. IPTG induces production of the functional galactosidase which cleaves X-Gal (a compound analog to lactose) and results in a blue colored metabolite. When the insert is introduced in the vector, the *lacZ* gene region is disrupted, so the colonies that have introduced the plasmid are the white ones.

The ligation reaction was introduced in *E. coli* competent cells and the cells were plated in LB Amp Xgal IPTG. Several white colonies were selected and grown separately in LB Amp, at 37°C overnight to isolate single plasmids and analyse them in order to identify the expected plasmid *CaOPY2-Myc-pGEM-T*.

### **3- Obtention of *CaOPY2-Myc-pCM189***

To obtain *CaOPY2-Myc* to afterwards introduce into an expression vector, the *CaOPY2-Myc-pGEM-T* plasmid was digested with *Bam*HI and *Pst*I. Since in the beginning of the *CaOPY2-Myc* sequence there is a *Bam*HI restriction site and in the end extremity a *Pst*I restriction site, after the digestion of the *CaOPY2-Myc-pGEM-T* plasmid with *Bam*HI and *Pst*I will be obtained two bands: the band that corresponds to the pGEM-T vector with 3000bp and the *CaOPY2-Myc* gene with 1652bp (Figure 31).

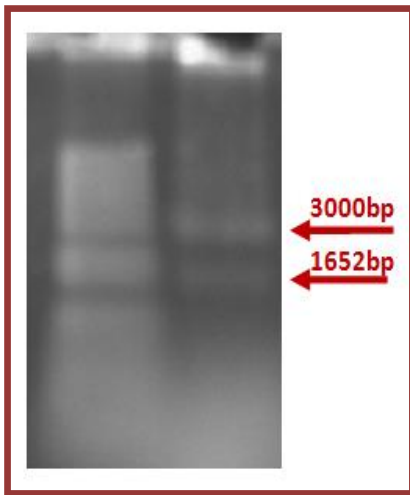


Figure 31 – DNA Gel Electrophoresis of the *CaOPY2*-Myc-pGEM-T digestion with *Bam*HI and *Pst*I with two bands – one with 3000bp and the one corresponding to the *CaOPY2*-Myc gene with 1652bp. 2μL of the sample were loaded in the DNA Gel Electrophoresis and the gel revealed by GelRed staining.

As both enzymes digest in different buffers (*Bam*HI in Buffer B and *Pst*I in Buffer H), the two digestions cannot be made at the same time. First, the plasmid is digested with *Bam*HI at 37°C for 2 hours and this opens the plasmid. The DNA is then precipitated to eliminate both the first enzyme and the buffer used and another digestion follows, this time with *Pst*I.

To express our gene afterwards, an expression vector is used. An expression vector is generally a plasmid used to introduce a specific gene into a target cell. Once the expression vector is inside the cell, the protein that is encoded by the gene is produced by the cells' machinery. The goal is then to produce large amounts of messenger RNA, and consequently proteins.

pCM189 is a tetracycline-regulatable expression vector for *S. cerevisiae* (Figure 32). This means that the genes are expressed in the absence of tetracycline but not in the presence of the antibiotic or analogs.

Since the goal is to introduce the final plasmids into *S. cerevisiae*, this vector is appropriated to introduce the insert.

The pCM189 vector has 8374bp with a CMV promoter, a pMB1 replication origin, an ampicillin resistance gene, a URA3 marker gene, a TetO operator (with Seven repeats) and multiple cloning regions with different restriction sites. The multiple cloning site (MCS) has restriction sites for *Bam*HI and *Pst*I in its extremities.

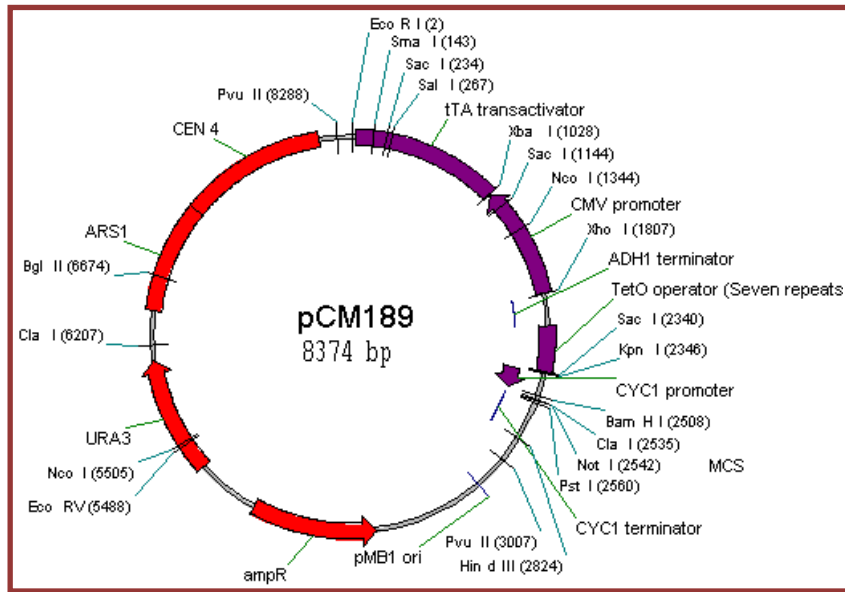


Figure 32 – Structure of the expression Vector pCM189. (ATCC website, 2012)

To extract pCM189, an *E. coli* strain with this plasmid was grown in LBamp, at 37°C overnight and it was extracted using the Mini-Prep protocol.

To introduce the *CaOPY2*-Myc gene into the pCM189 vector, it was necessary to digest the vector with the same enzymes as the insert. The vector was digested with both *Bam*HI and *Pst*I with the same methodology used for the *CaOPY2*-Myc-pGEM-T plasmid (Figure 33 – A). The digestion of the pCM189 vector was confirmed with a DNA Gel Electrophoresis (Figure 33 – B).

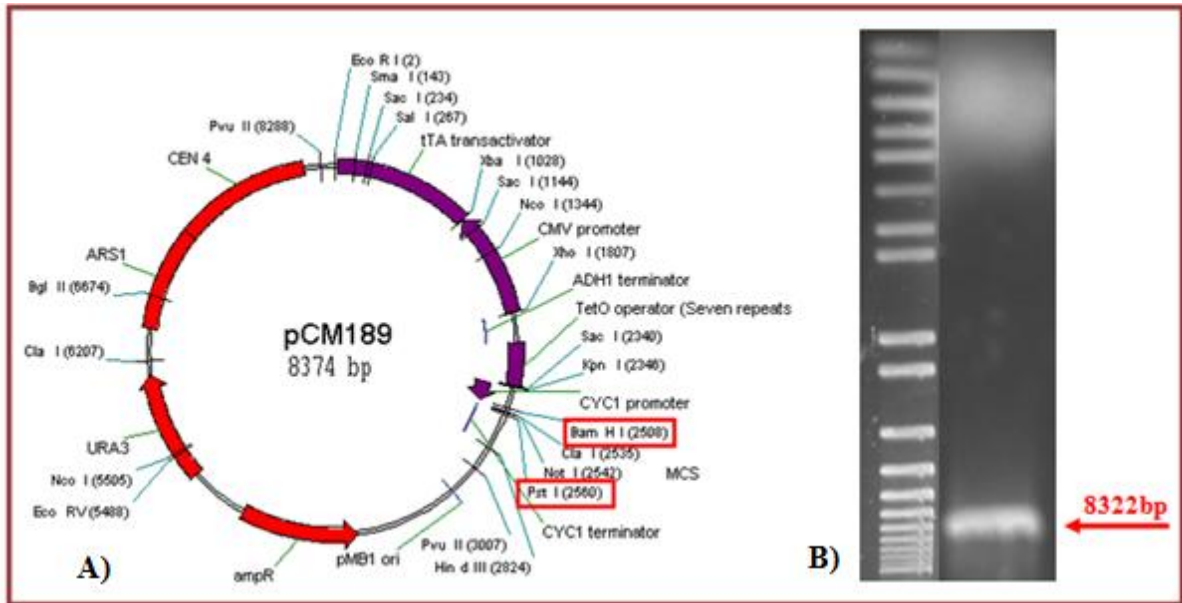


Figure 33 – A) Restriction Sites for *Bam*HI and *Pst*I in the pCM189 vector. B) DNA Gel Electrophoresis of the pCM189 digestion with *Bam*HI and *Pst*I appearing a very intense band with 8322bp corresponding to the open vector. 5 $\mu$ L of the sample were loaded in the DNA Gel Electrophoresis and the gel revealed by GelRed staining.

With this digestion two bands should be obtained – one with 8322 bp that is the one pretended corresponding to the main vector, and another around 50 bp that, because of its small size, don't appear in the gel using this concentration of agarose and the marker used.

To obtain the desired bands of the vector and the insert, a DNA Gel Electrophoresis is performed loading all the volume of both samples. The bands are then eluted and the DNA isolated from the agarose gel. In Figure 34 it is possible to see the DNA Gel Electrophoresis after being eluted the band corresponding to the pCM189 with 8322bp and the one of the *CaOPY2*-Myc insert with 1652bp.



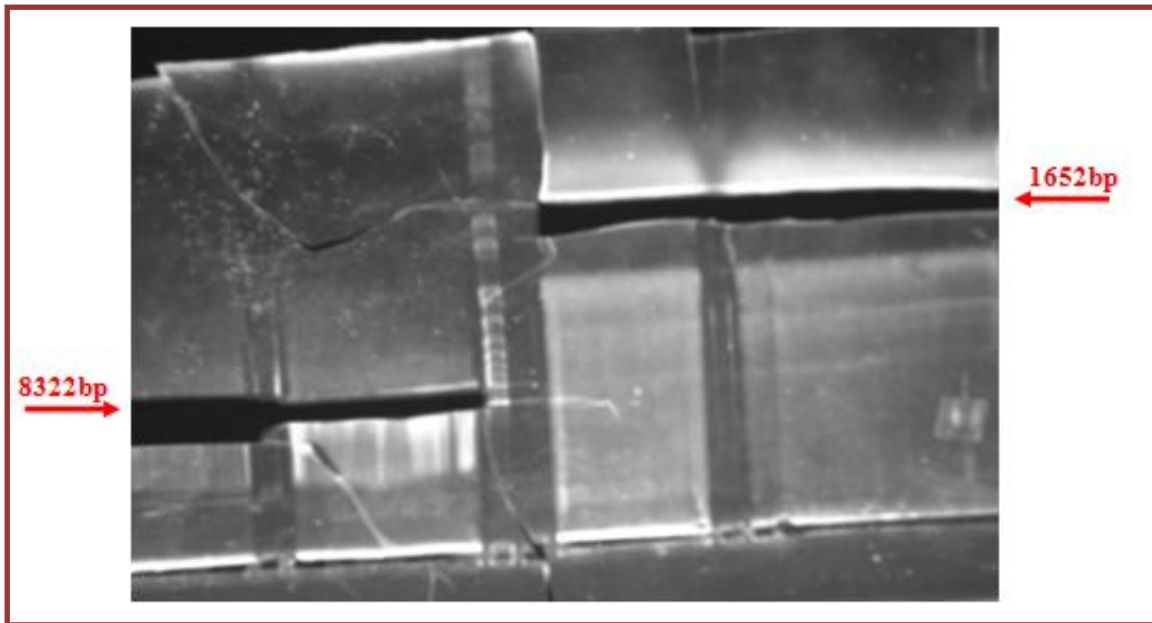


Figure 34 – DNA Gel Electrophoresis from where were eluted two bands: one with 8322bp corresponding to the pCM189 and another with 1652bp from the *CaOPY2*-Myc insert. All volume of the samples were loaded in the DNA Gel Electrophoresis and the gel revealed by GelRed staining.

After the purification of the samples, a ligation to clone the insert *CaOPY2*-Myc into the digested pCM189 expression vector follows. To estimate the quantity of DNA to use from each sample, a DNA Gel Electrophoresis with equal volume of both DNA samples was previously performed (Figure 35).

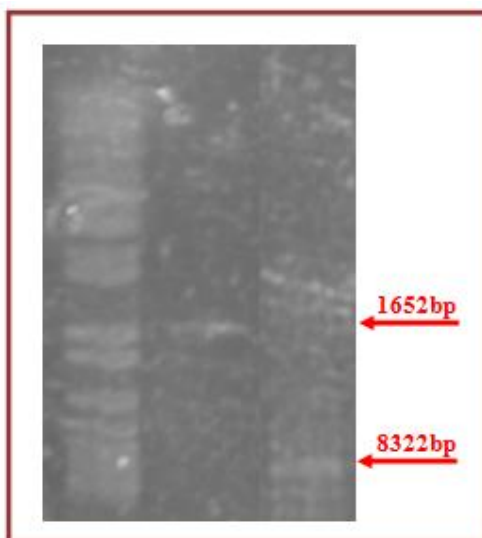


Figure 35 – DNA Gel Electrophoresis to estimate the volume of each sample to use in the ligation: in the first column appears a band with 1652bp corresponding to the *CaOPY2*-Myc and in the second column the band from the vector with 8322bp. 2 $\mu$ L of the samples were loaded in the DNA Gel Electrophoresis and the gel revealed by GelRed staining.

With this DNA Gel Electrophoresis it was seen that the vector band is more intense than the one from the insert so in the ligation it is necessary to use more volume of the insert than the vector.

T4 DNA Ligase is once again the enzyme used, at room temperature, during 2 hours.

The ligation mixture was introduced in *E. coli* competent cells and the cells were plated in LBamp. Several colonies were selected and grown separately in LBamp, at 37°C overnight.

#### 4- CaOPY2-Myc-pCM189 Isolation

Single plasmids were extracted from the colonies using the Mini-Prep protocol and were subjected to digestion with *Bam*HI and *Pst*I to confirm which ones were *CaOPY2-Myc-pCM189* (Figure 36). With this enzyme the plasmid should be digested in two places and two bands should appear in the DNA Gel Electrophoresis – one with 1652 bp and 8374 bp.

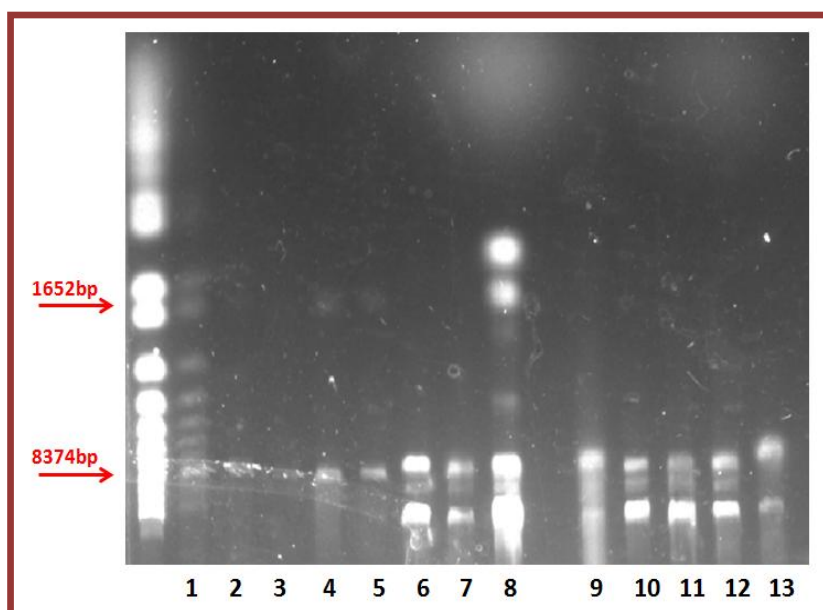


Figure 36 - DNA Gel Electrophoresis of the confirmation with *Bam*HI and *Pst*I of the plasmids extracted from the *E. coli* cultures by Mini-Prep Protocol. 5µL of the sample were loaded in the DNA Gel Electrophoresis and the gel revealed by GelRed staining.

The plasmids 4 and 5 were considered correct due to the fact that the two bands with 1652 bp and 8374 bp appear. The same did not happen with the other samples, so they were excluded from the work.

The DNA from the plasmid 4 was analyzed in a DNA Gel Electrophoresis (Figure 37 – A). The band corresponding to the extracted DNA appeared near the 10000bp band, what was consistent with the size of the expected *CaOPY2-Myc-pCM189* plasmid (Figure 37 – B).

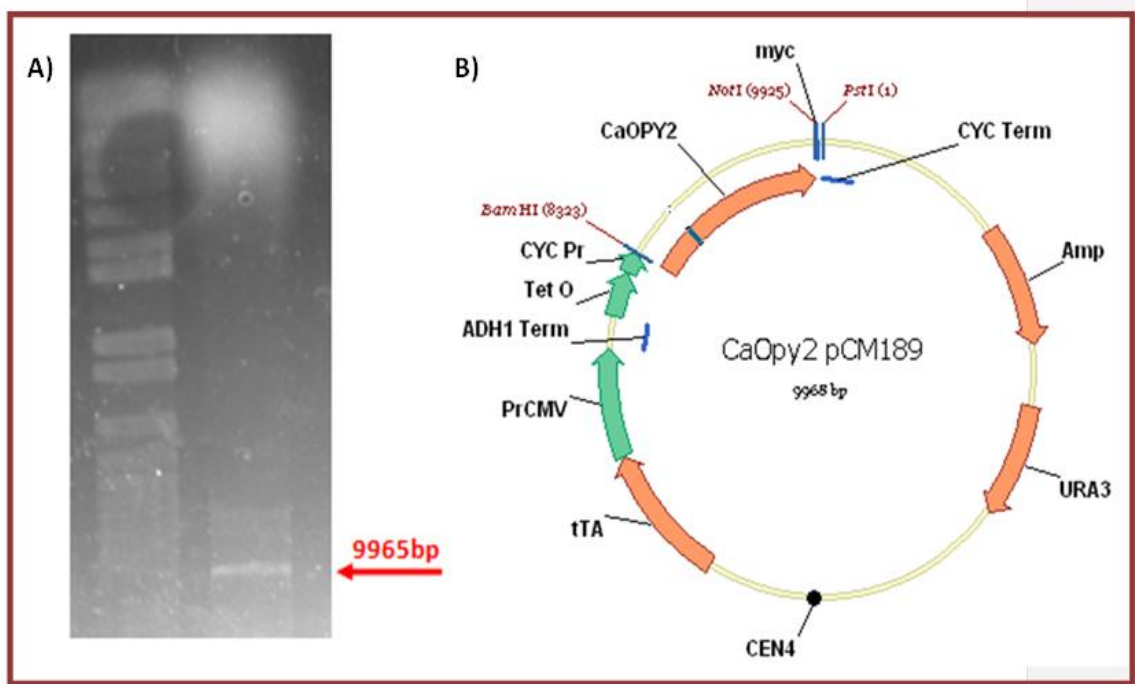


Figure 37 – A) DNA Gel Electrophoresis of the *CaOPY2-Myc-pCM189* with 9965bp extracted from the *E. coli* cultures by Mini-Prep Protocol. 5 $\mu$ L of the sample were loaded in the DNA Gel Electrophoresis and the gel revealed by GelRed staining. B) Structure of the plasmid *CaOPY2-Myc-pCM189*.

### **5- Construction of *ScOPY2-Myc-pCM189* plasmid**

The *CaOPY2-Myc-pCM189* plasmid was used to obtain a similar construction carrying the homolog *OPY2* gene from *S. cerevisiae* to be afterwards compared with the *C. albicans* one.

The *CaOPY2*-Myc-pCM189 plasmid was digested with *Bam*HI and *Not*I to obtain the pCM189-Myc (Figure 38).

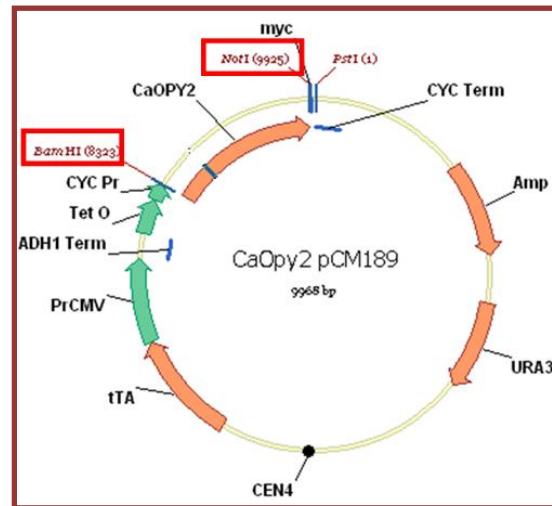


Figure 38 - Digestion sites of *Bam*HI and *Not*I in the *CaOPY2*-Myc-pCM189 plasmid.

Once again, because both enzymes cut in different buffers (*Bam*HI in Buffer B and *Not*I in Buffer H), the digestions have to be done separately, with a step of DNA precipitation in between. The first digestion with *Bam*HI opens the plasmid that continues with 9965bp (Figure 39 - A), and the second digestion with *Not*I separates the pCM189-Myc from *CaOPY2*, appearing two bands on the DNA Gel Electrophoresis - one band with 1602bp correspond to *CaOPY2* and another of 8363bp from the pCM189-Myc (Figure 39 - B).

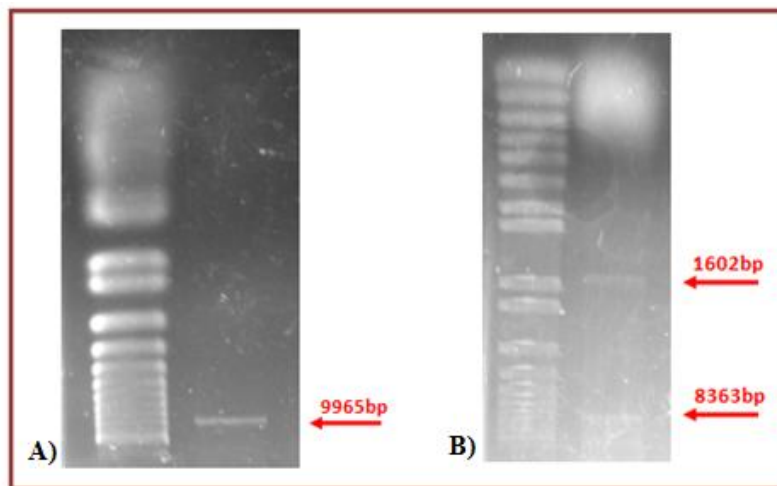


Figure 39 – A) DNA Gel Electrophoresis of the *CaOPY2*-Myc-pCM189 after digestion with *Bam*HI with 9965bp. B) DNA Gel Electrophoresis of the *CaOPY2*-Myc-pCM189 after digestion with *Bam*HI and *Not*I with two bands – one of 1602bp corresponding to *CaOPY2* and another of 8363bp from the pCM189-Myc. 2 $\mu$ L of both samples were loaded in the DNA Gel Electrophoresis and the gel revealed by GelRed staining.

To obtain the pCM189-Myc band, a DNA Gel Electrophoresis is performed with all the volume obtained from the previous digestion. After elution of the bands with 8363bp (Figure 40), the DNA is isolated from the agarose gel.

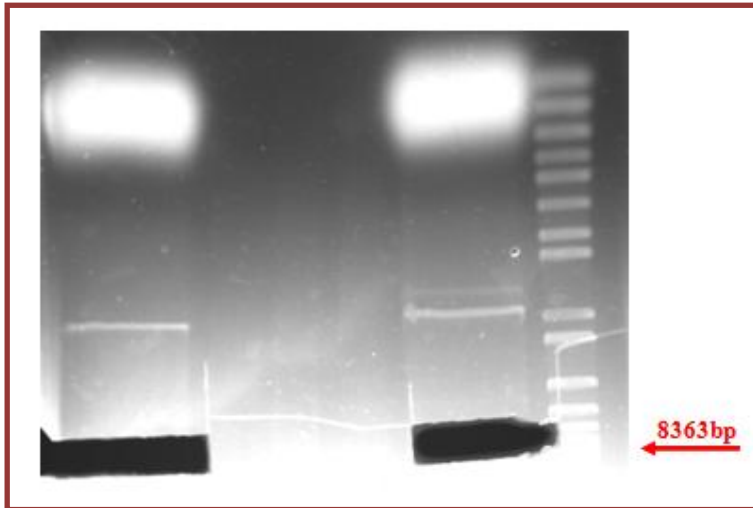


Figure 40 – DNA Gel Electrophoresis of the eluted bands with 8363bp corresponding to pCM189-Myc. All volume of the sample were loaded in the DNA Gel Electrophoresis and the gel revealed by GelRed staining.

The *ScOPY2* gene had already been introduced into a pGEM-T vector. The subsequent step to introduce it into the pCM189-Myc vector was to digest it with the same enzymes as previously was the vector for the ligation been possible.

As can be seen in the Figure 41, the *ScOPY2* gene has in the extremities restriction sites for *Bam*HI and *Not*I, which are the same as the pCM189-Myc was digested with.

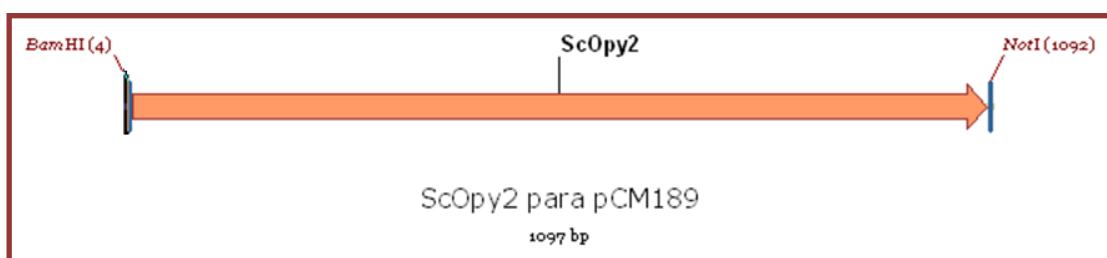


Figure 41 – Structure of the *ScOPY2* gene with 1097bp, and the respective restriction sites of *Bam*HI and *Not*I.

To obtain *ScOPY2*, the *ScOPY2*-pGEM-T plasmid was digested with *Bam*HI and *Not*I, and, once again, the digestions had to be done separately, with a step of DNA precipitation in between.

To obtain the *ScOPY2* band (Figure 42), a DNA Gel Electrophoresis was performed with all the volume from the *ScOPY2*-pGEM-T digestion with *Bam*HI and *Not*I. Once the elution of the bands had been performed, the DNA was isolated from the agarose gel.

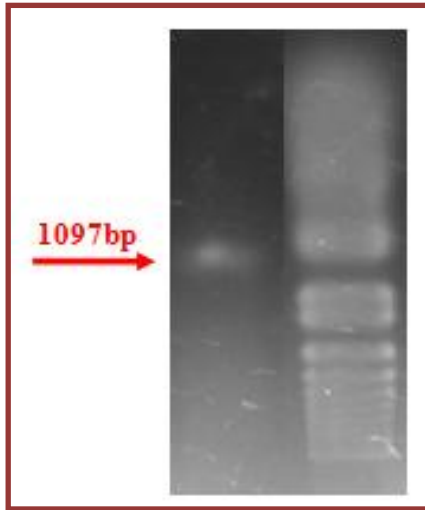


Figure 42 – DNA Gel Electrophoresis of the *ScOPY2* gene with 1097bp. 2 $\mu$ L of the sample were loaded in this DNA Gel Electrophoresis and the gel revealed by GelRed staining.

After the *ScOPY2* gene had been purified from the agarose gel, the insert *ScOPY2* is cloned into the pCM189-Myc vector. Once again, to estimate the quantity of DNA to use from each sample, a DNA Gel Electrophoresis with equal volume of both DNA samples was previously performed (Figure 43).

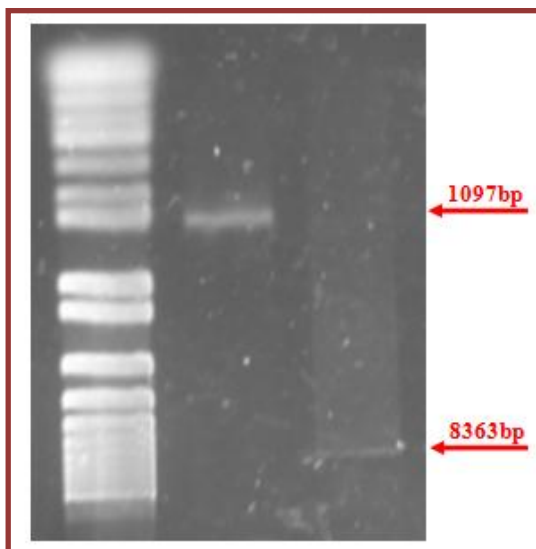


Figure 43 – DNA Gel Electrophoresis to estimate the volume of each sample to use in the ligation: in the first column appears a band with 1097bp corresponding to the *ScOPY2*-Myc and in the second column the band from the vector attached with the Myc-tag with 8363bp. 2 $\mu$ L of the samples were loaded in the DNA Gel Electrophoresis and the gel revealed by GelRed staining.

From this DNA Gel Electrophoresis it is possible to perceive that the insert band is more intense than the one from the vector so in the ligation it is necessary to use more volume from the vector.

T4 DNA Ligase is used at room temperature during 2 hours and the plasmid obtained is *ScOPY2-Myc-pCM189* with 9454bp (Figure 44).

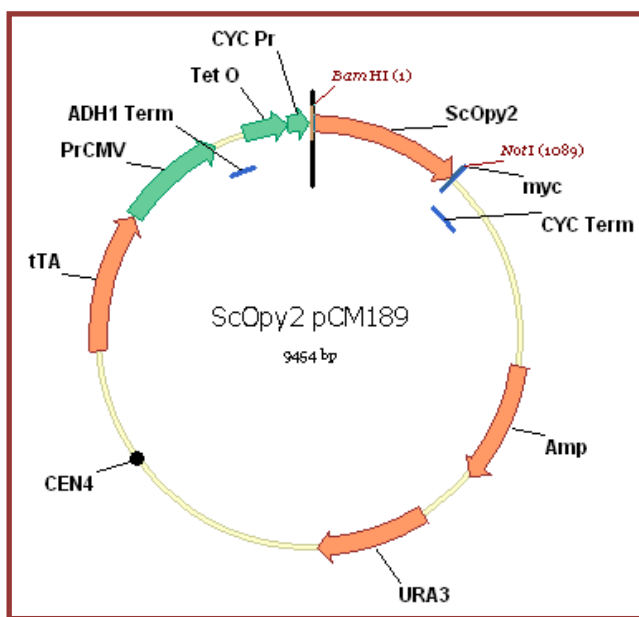


Figure 44 – Structure of the *ScOPY2-Myc-pCM189* plasmid.

The ligation mixture was introduced in *E. coli* competent cells and after the transformation process the cells were plated in LBamp. Several colonies were selected and grown separately in LBamp, at 37°C overnight.

The plasmids were extracted using the Mini-Prep protocol from the previously grown colonies and to confirm if the plasmids were *ScOPY2-Myc-pCM189*, they were subjected to digestion with *HindIII* (Figure 45).

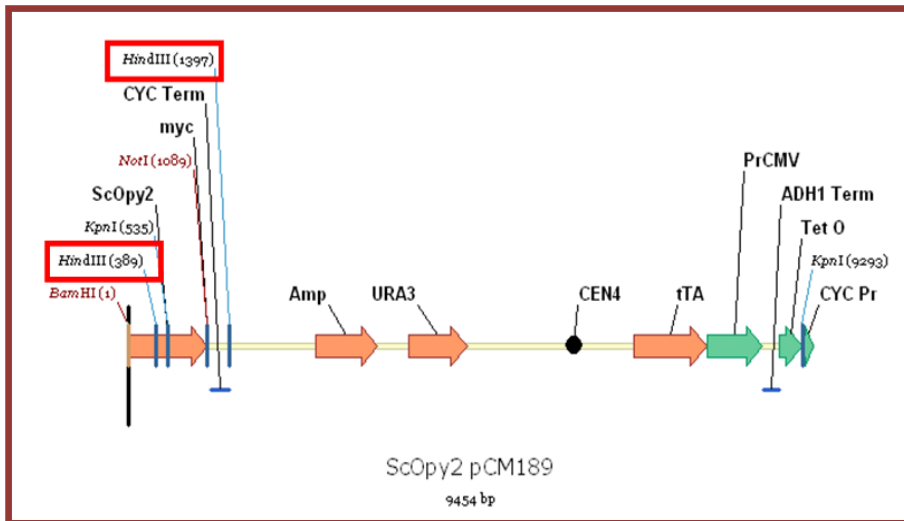


Figure 45 - Restriction Sites for *HindIII* in the *ScOPY2-Myc-pCM189* plasmid.

This enzyme digests the plasmid in two places and two bands should be obtained in the DNA Gel Electrophoresis – one with 1000 bp and another with 8000 bp (Figure 46).

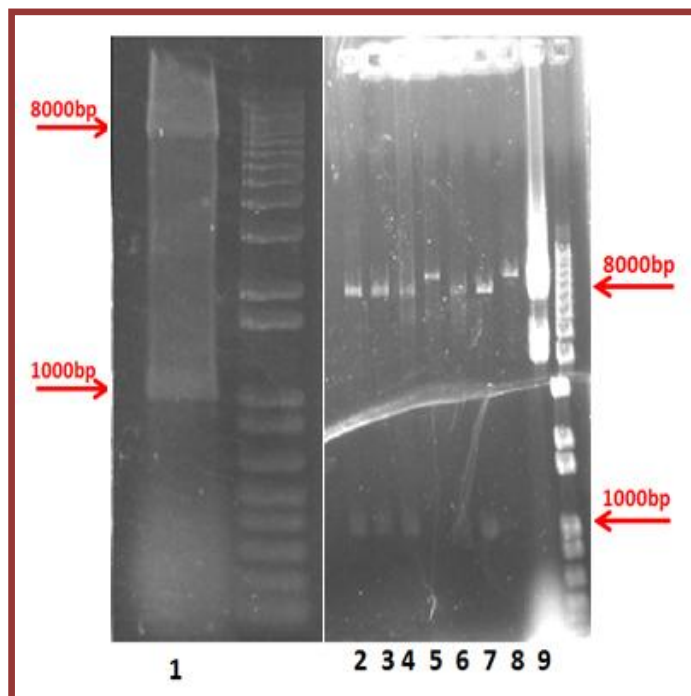


Figure 46 – Distinct DNA Gel Electrophoresis of the plasmids extracted from different *E. coli* cultures by the Mini-Prep protocol and digested with *HindIII* to select the *ScOPY2-Myc-pCM189* plasmid. 5 $\mu$ L of each samples were loaded in the DNA Gel Electrophoresis and the gel revealed by GelRed staining.

The plasmids 1, 4, 5, 7, 8, 9 were considered *ScOPY2-Myc-pCM189* because two bands with 1000 bp and 8000 bp were detected in the DNA Gel Electrophoresis.

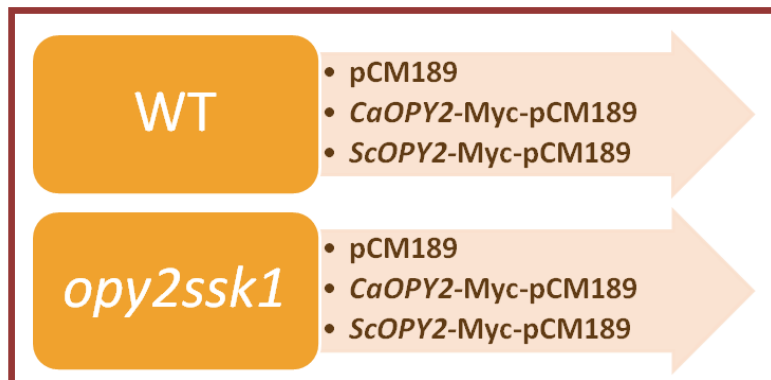


## 6 – Introduction of the genetic constructions into *S. cerevisiae*

Transformation is an important technique in which exogenous DNA is introduced into a cell, resulting in genetic modification. For successful fungal transformation, exogenous DNA must pass through the cell wall and plasma membrane and be delivered in the cytosol to reach the nucleus. The mechanism underlying transformation has not been clarified completely even in *S. cerevisiae*.

Firstly in this work, two strains of *S. cerevisiae* were transformed by the One-step transformation method with pCM189, *CaOPY2-Myc-pCM189* and *ScOPY2-Myc-pCM189*. The strains used were a wild type strain, BY4741, and a mutant strain which lacked of the *SSK1* and the *OPY2* genes (Table 6). The introduction in the wild-type strain of the *OPY2* let us only see the overexpression of the gene as the strain has its own *OPY2*.

Table 6 – Scheme of the plasmids introduced in the *S. cerevisiae* WT strain and in the *opy2 ssk1* strain.



The transformed suspensions were plated into SD minimal medium plates and incubated at 30°C for 2 days. Here, SD minimal medium is used to select the cells carrying the plasmid. After 2 days of incubation, different colonies of each transformation were striated in SD minimal medium plates.

## ***7- Verification of the transformation***

To confirm that the plasmids were correctly introduced into de *S. cerevisiae* cells a dot-blot assay was performed. To proceed with the experiments it was necessary to extract the proteins from the transformed cultures. Cultures of the yeast cells were grown at 30°C, overnight, in SD minimal medium to avoid the plasmid curation and the proteins were extracted as explained in Methodology.

Once the proteins were extracted, a Dot Blot Assay was performed with 2µL of each sample extracted before. With this assay, the objective was to detect the presence of the Myc epitope to assure that the extracted proteins had the correct construction and to choose the ones that gave the higher signal. An antibody against the Myc-tag was used and both positive and negative controls were added in the assay – the positive control was a sample existent in the laboratory that guaranteed had the Myc-tag, and the negative control a sample that was absence of the Myc-tag.

Here it was expected that all clones with the pCM189 of both the WT and the *opy2 ssk1* mutant were negative because this construction does not have the Myc-tag attached, and that all the samples with the *CaOPY2-Myc* and the *ScOPY2-Myc* would be positive to the Myc-tag.

In Figure 47 A) and B) can be seen the result of the Dot Blot Assay.

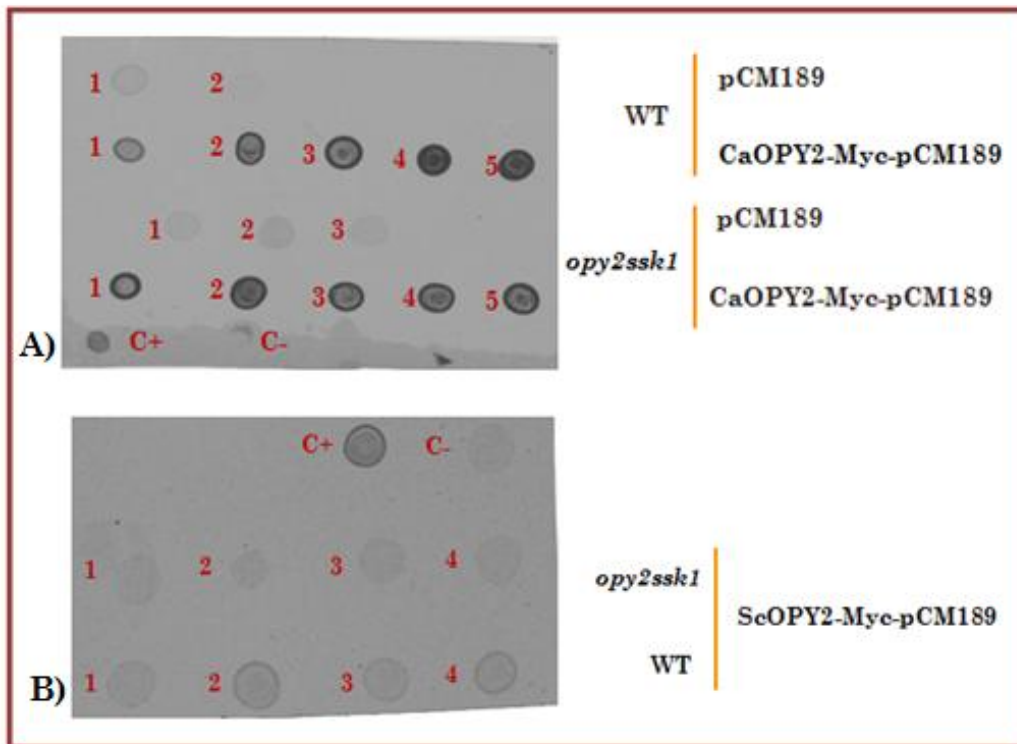


Figure 47 – Dot Blot Assays. A) Proteins from different clones of both *S. cerevisiae* WT strain and the *opy2 ssk1* strain with pCM189 and *CaOPY2-Myc-pCM189*. B) Proteins from different clones of the *ScOPY2-Myc-pCM189* in the *S. cerevisiae* WT strain and in the *opy2 ssk1* strain. 2 $\mu$ L of each sample were blotted in a nitrocellulose membrane, immunoblotting of the Myc epitope was performed using a primary antibody anti-myc and a secondary antibody anti-mouse and the membrane was revealed using an Odyssey scanner.

After analyzing the first Dot Blot, it was possible to conclude that both clones 1 and 2 of the WT pCM189 were not positive for the Myc-tag as expected; all 5 clones of the WT *CaOPY2-Myc-pCM189* were as much or more intense as the positive control; both clones 1, 2 and 3 of the *opy2 ssk1* pCM189 were negative for the Myc-tag as was predictable and all 5 clones of the *opy2 ssk1 CaOPY2-Myc-pCM189* were positive for the Myc-tag.

In the second Dot Blot none of the samples of the *ScOPY2-Myc-pCM189* from the *opy2 ssk1* strain were positive and the ones from the wild type strain were less

intense than the positive control, but more intense than the negative one, so it was necessary to confirm if they were positive or not in a Western Blot Assay.

To reduce the number of samples of each mutant to the proceeding studies, only the following mutants were chosen:

- WT strain: clone 1 and 2 with the pCM189 (from now on referred to as pCM189 WT1 and pCM189 WT2) and clone 4 and 5 from the *CaOPY2*-Myc-pCM189 (from now on referred to as *CaOPY2*-Myc-pCM189 WT4 and *CaOPY2*-Myc-pCM189 WT5);
- *opy2 ssk1* strain: clone 1 and 2 with the pCM189 (from now on referred to as *opy2 ssk1* pCM189 1 and *opy2 ssk1* pCM189 2) and clone 1 and 2 with the *CaOPY2*-Myc-pCM189 (from now on referred to as *opy2 ssk1 CaOPY2*-Myc-pCM189 1 and *ssk1 opy2 ssk1 CaOPY2*-Myc-pCM189 2).

A first Western Blot was then performed to visualize the proteins and in which molecular weight they appear (Figure 8). An anti-Myc antibody was used to detect the Myc-tag present in the proteins. The previous chosen clones of the WT strain were loaded in the gel as well as 4 different clones of the *ScOPY2*-Myc-pCM189 of both the WT and the *opy2 ssk1* strain.

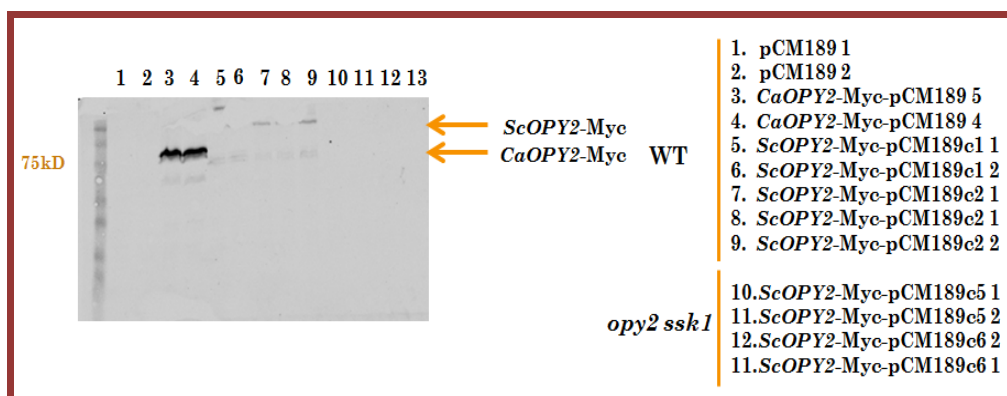


Figure 48 – Western Blot Assay. 10µL from a 20µg/mL protein sample from the samples referred in the image were loaded in the gel, the immunoblotting of the Myc epitope was performed using a primary antibody anti-Myc and a secondary antibody anti-mouse and the membrane was revealed using an Odyssey scanner.

After analyzing the Western Blot, it was possible to notice that in none of the WT clones with only pCM189, the Opy2 protein was detected.

Relatively to the WT samples of the *CaOPY2-Myc-pCM189*, the protein was detected in two bands: one, very intense, around the 75 kD band and another with a lower molecular weight. It was considered that the intense band was our protein, but a doubt persisted of why it was appearing in this molecular weight since the theoretic molecular weight of the protein was 58.65 kD and it should appear lower in the gel.

In the protein samples from the different *opy2 ssk1 ScOPY2-Myc-pCM189* clones, the Myc tag was detected, but in two bands: an intense one in the beginning of the gel and another, with lower intensity, and a molecular weight lower than 75 kD. It was expected that the *ScOpy2* would have a different molecular weight from the *C. albicans* one. Since the theoretic molecular weight of the *ScOPY2-Myc* protein is 40.354 kD, the band was appearing, once again, in a molecular weight higher than the expected one.

Another Western Blot was performed and this time only samples from the mutant strain were loaded (Figure 49).

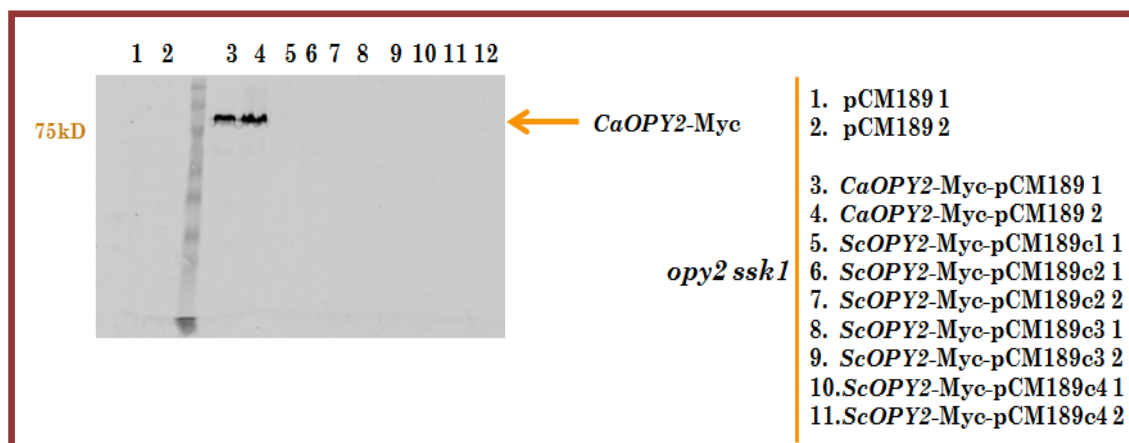


Figure 49 – Western Blot Assay. 10µL from a 20µg/mL protein sample from the samples referred in the image were loaded in the gel, the immunoblotting of the Myc epitope was performed using a primary antibody anti-Myc and a secondary antibody anti-mouse and the membrane was revealed using an Odyssey scanner.

As can be seen in the Figure 49, in the *opy2 ssk1 CaOPY2-Myc-pCM189* samples the *CaOPY2-Myc* protein was detected in both samples with a molecular weight around 75 kD, as was seen previously with the same protein in the WT strain. As before, the protein appears with a molecular weight higher than the expected.

About the *opy2 ssk1 ScOPY2-Myc-pCM189* samples, in none of them the *ScOPY2-Myc* protein was detected. Again, it was thought that the transformation was not successful, with a deficient introduction of the plasmid into the *S. cerevisiae* strain.

### ***8- Introduction of the genetic constructions into S. cerevisiae opy2 ssk1 mutant***

Since we could never detect the *ScOPY2-Myc* protein introduced in the *opy2 ssk1* mutant, another transformation of the *ScOPY2-Myc-pCM189* plasmid into this strain was performed. Once again, the transformed suspension was plated into a SD minimal medium plate and incubated at 30°C for 2 days and after the 2 days of incubation, different colonies of each transformation were striated in SD minimal medium plates.

Another Dot Blot Assay was performed to see if, this time, the Myc-tag was detected. For this, *ScOPY2-Myc* proteins were extracted from different transformed cultures. Once more, cultures of the yeast cells were grown at 30°C, overnight, in SD minimal medium and the proteins were extracted as explained in Methodology.

A Dot Blot assay against the Myc epitope was performed with the previous extracted proteins and the results in Figure 50 were obtained.

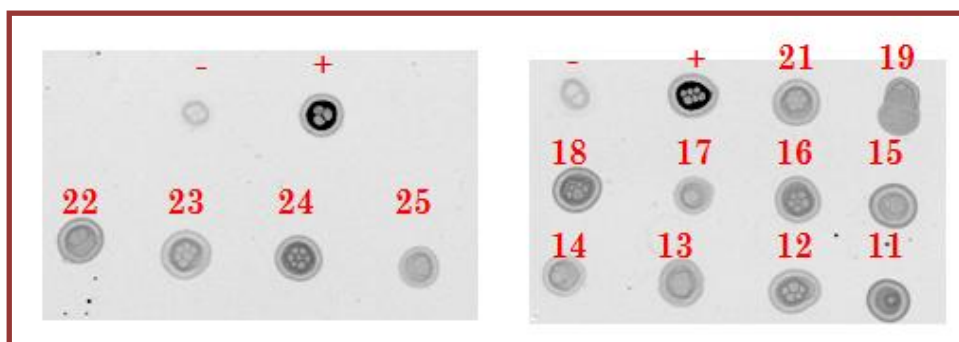


Figure 50 – Dot Blot Assay with the proteins from different clones of the new transformation of the *opy2 ssk1* strain with the *ScOPY2-Myc-pCM189*. 2 $\mu$ L of each sample were blotted in a nitrocellulose membrane, immunoblotting of the Myc epitope was performed using a primary antibody anti-Myc and a secondary antibody anti-mouse and the membrane was revealed using an Odyssey scanner.

Even though the intensities of the Myc signals of the samples compared with the positive control were not as intense, they were more intense than the negative control. Clones 11, 18, 21, 23 and 25 were chosen to follow to work with and see if we could detect the protein in a Western Blot assay.

The previously 5 samples chosen of the *opy2 ssk1 ScOPY2-Myc-pCM189* and the 4 samples of the WT *ScOPY2-Myc-pCM189* were loaded in a SDS-Page.

Before the samples were loaded in the gel, they were boiled to denature the proteins – this is important to perform because both secondary and tertiary structures should be destroyed for the SDS-Page be in real conditions of denaturation.

To test if the heat was interfering with the samples and, for this reason, the *opy2 ssk1 ScOPY2-Myc* bands were not appearing in the previous gels, both boiled and not boiled samples were loaded in different gels and the results are showed in Figure 51.

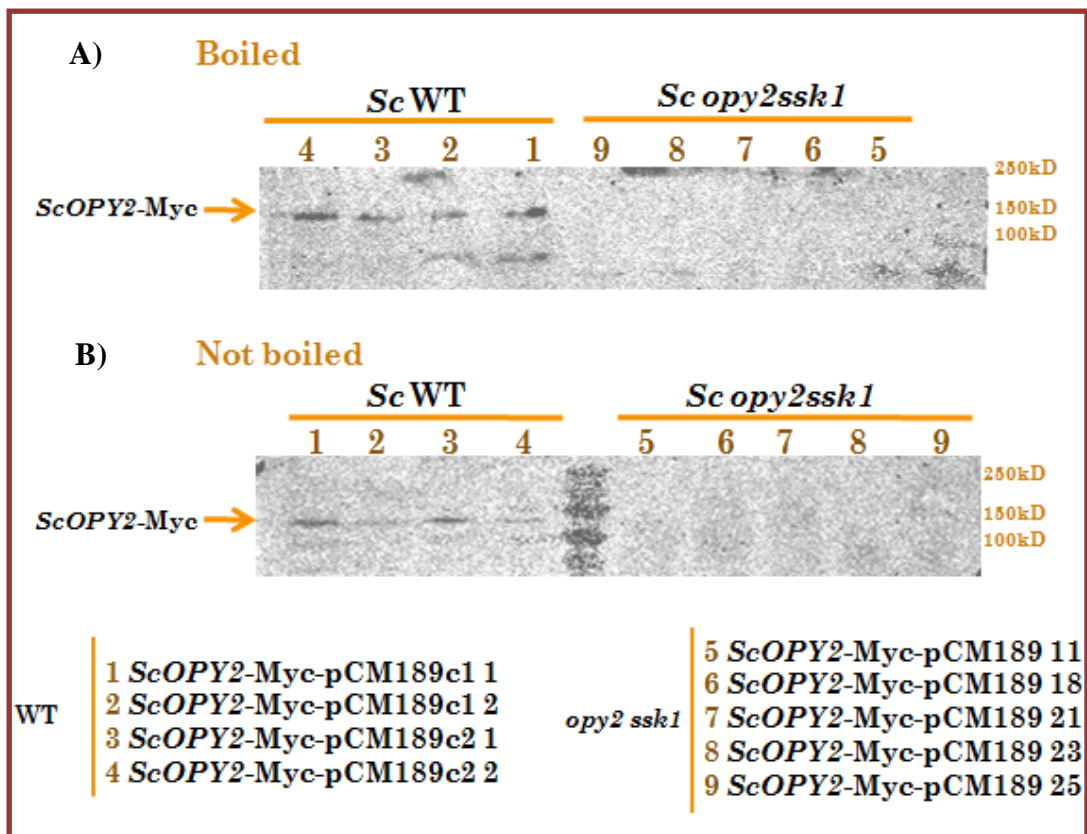


Figure 51 – Western Blot Assays. 10µL from a boiled - A) and not boiled - B) 20µg/mL protein sample from the samples referred in the image were loaded in the gel, the immunoblotting of the Myc epitope was performed using a primary antibody anti-Myc and a secondary antibody anti-mouse and the membrane was revealed using an Odyssey scanner.

After analyzing the Western Blots, it was seen that, once again, only the *ScOPY2-Myc* from the WT strain was detected in the electrophoresis around the 150 kD, once again in a molecular weight higher than it should appear.

Because the *Opy2* is a transmembrane protein, the absence of detection of this protein in the mutant strain could be due to a limitation in the protein extraction procedure – this method could not be the most effective to extract transmembrane proteins and, because of that, the protein was not detected. The problem was not in the transformation because the strains were growing in SD minimal medium, only possible because the cells were loaded with the plasmid and the marker gene (*URA3*) is expressed. So, although the myc tag was not detected we followed the experimental work with these transformants.



## 9 – Study of the OPY2 in response to osmotic stress

### 9.1 – The CaOPY2 gene partially complements the osmosensitivity of the *opy2 ssk1* mutant

In order to study the role of the *CaOPY2* gene Spots Assays were performed in YPD and in YPD with 0.5M NaCl and 0.8M Sorbitol. These substances are added to the plates to test the degree of sensitivity of the different mutants to high salt concentrations. As it has already been explained, the Opy2 is an essential component of the SHO1 branch of the HOG pathway. This pathway is the one responsible for generating an adaptive response to high-osmolarity environments.

The spots were incubated at 30°C and at 37°C to test if the osmotolerance is different at different temperatures. The results can be seen in the Figure 52.

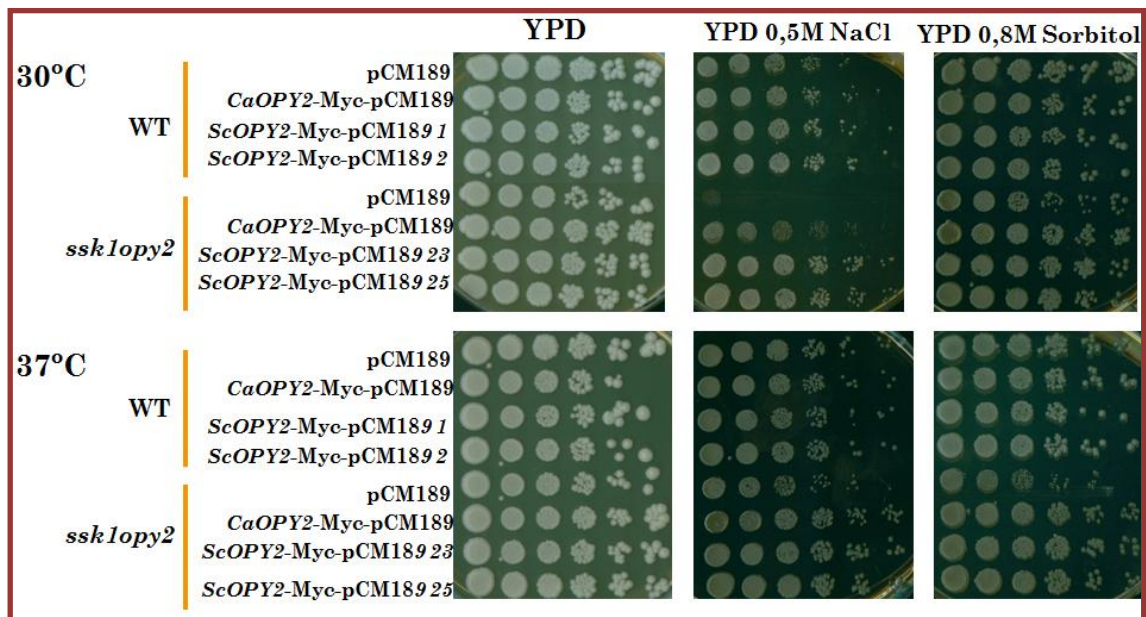


Figure 52 – Susceptibility to different kind of stresses. Equal number of exponentially growing cells was shifted in YPD medium and 10-fold serial dilutions were done. Five microliters were spotted on YPD plates supplemented with the compounds indicated in the upper part. Plates were incubated at 30°C and 37°C for 32h.

After performing the Spots Assays it was possible to realize that the growth of the mutant with only the vector was, as expected, poor in the presence of elevated concentrations of NaCl and Sorbitol.

When with the *CaOPY2*, it was possible to see a recovery in the response to osmotic stress. Even though the response did not match that of the parent wild-type strain, it was possible to conclude that the *C. albicans OPY2* is able to partially complement the osmosensitivity of the *opy2 ssk1 S. cerevisiae* mutant.

Even though we could not detect the *opy2 ssk1 ScOPY2-Myc* protein in either of the Western Blot performed, both mutants improved the growth onto high salt conditions. In conclusion, *ScOPY2* complemented the osmosensitivity in the *opy2 ssk1* mutant.

At 37°C the mutant carrying the empty vector grew better than at 30°C. So it was possible to conclude that at this temperature, the mutants became osmotolerant.

The results were confirmed in YPD 1M Sorbitol with the same results obtained.

### **9.2 – *CaOPY2* partially complements the aberrant cell morphology of *opy2 ssk1* mutant under osmotic stress**

To analyze the different phenotypes of the mutants when grown in medium with and without 0.5M NaCl, the strains were grown overnight in the different conditions and pictures were taken after 24 hours of incubation at 30°C. The pictures obtained are in Figure 53.

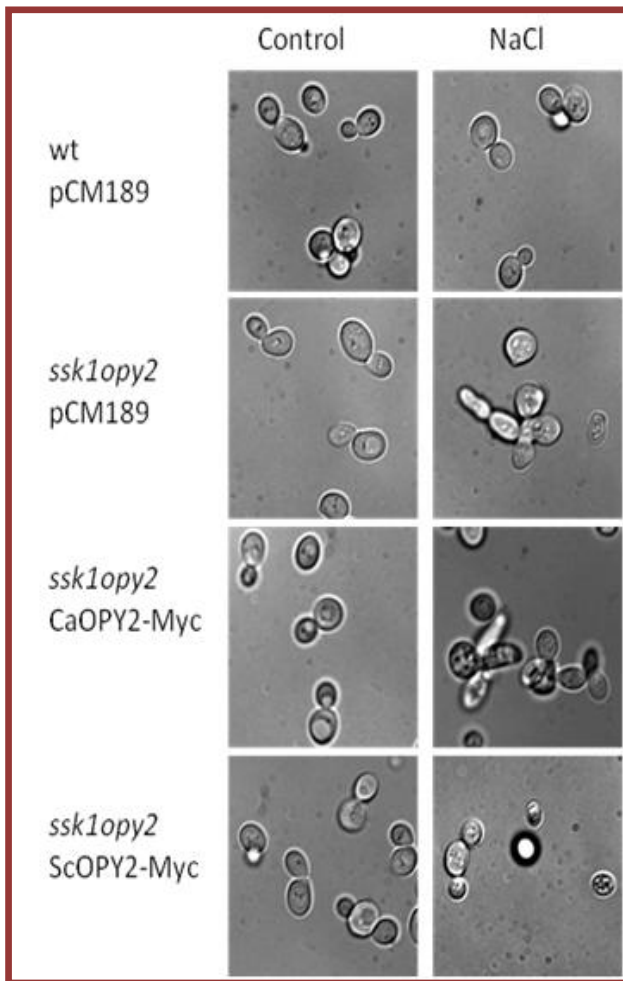


Figure 53 - Effect of NaCl on the phenotypes of the different mutants. Cells were grown overnight in YPD and in YPD 0.5M at 30°C. Pictures were taken after 24h of incubation.

The normal *S. cerevisiae* cells are round to ovoid, with 5 to 10µm in diameter and they divide by an asymmetric process called budding. As can be seen in the control of ScWT pCM189 normal cells are seen, with the expected morphology. When the same cells are grown in a medium with NaCl, the cells swell.

The mutant *opy2 ssk1* pCM189 cells, when grown in 0.5M NaCl, lose the normal morphology and become rounded or elongated.

When the *ScOPY2* is introduced in this strain, the cells should recover the ability to respond to the osmotic stress and have a phenotype similar to the one of the ScWT. As can be seen in the Figure 53, the cells have a similar morphology as the one of the ScWT pCM189 in 0.5M NaCl.

In the case of the *opy2 ssk1 CaOPY2*, the cells when grown in 0.5M NaCl not all the cells are able to restore the morphology.

### **9.3 – *CaOPY2* is not able to activate the HOG1 MAPK in the *opy2 ssk1* mutant**

As it was already explained, the HOG pathway is the one responsible for generating a response in situations of high osmolarity environments, among other stresses.

When this pathway is activated by both branches, the signal converge at the level of the Psb2 MAPKK that activates the Hog1 MAPK by phosphorylation and it is able to translocate to the nucleus where phosphorylates downstream effectors.

Hog1 is a native protein so it should be detected in all samples but the phosphorylated form only is detected in situations with high osmolarity when the pathway is activated.

The ScHog1p has 48.97 kD so both forms of the protein should appear around the 50 kD band in a immune-detection assay.

To test if our mutants were able to activate this pathway, several experiments adding NaCl to the medium were performed. Due to the fact that in most of the Western Blot resulting from these experiments, clear results were not obtained, these will be omitted from this work.

Firstly, to found out which were the best conditions to see activation of the HOG pathway with our mutants, an experiment was performed with only the WT pCM189 strain and with 0.9M NaCl and samples were taken at 0, 5, 10, 15 and 20 minutes. The goal of this test was to determine the time-period needed to see activation of the Hog1-P.

To test if the result was coherent, two different p-38 antibodies were tested in the same membrane at different times, with a process of stripping.

The result can be seen in Figure 54.

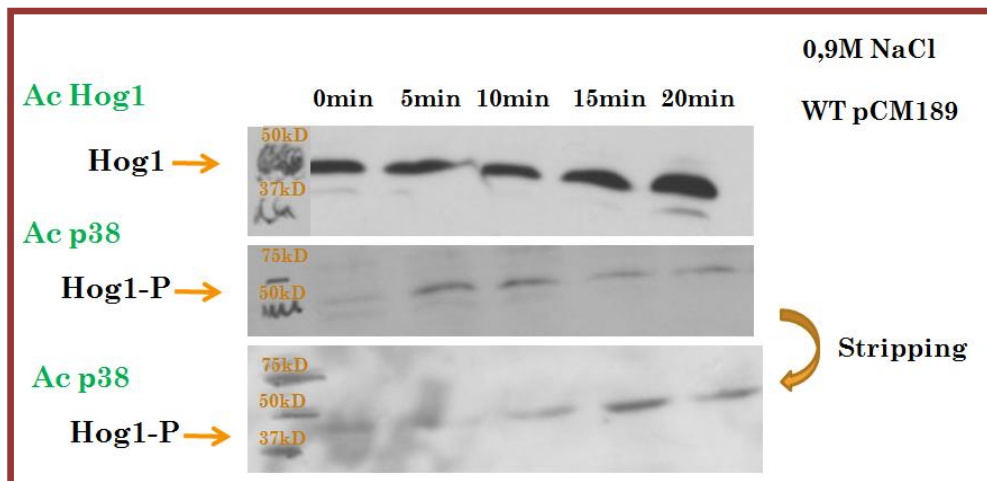


Figure 54 – Western Blot Assay. 10 $\mu$ L from a 20 $\mu$ g/mL protein sample from the samples referred in the image were loaded in the gel, the immunoblotting was performed using the primary antibodies anti-Hog1 and anti-phospho-p38 MAPK (monoclonal, Cell-Signalling, USA) and a secondary antibody anti-rabbit and the membrane was revealed using chemiluminescence. The second membrane was stripped and the membrane immunoblocked with another primary antibody for the anti-phospho-p38 MAP Kinase, this time a polyclonal, Cell-Signalling, USA.

Activation was seen but when different antibodies were used, activation was detected at different times. With an anti-phospho-p38 MAPK (monoclonal, Cell-Signalling, USA), the activation was detected at 5 minutes and with an anti-phospho-p38 MAPK (polyclonal, Cell-Signalling, USA) at 15 minutes.

Due to the fact that it was pretended to observe if *CaOpy2* is able to activate the *S. cerevisiae* Hog1, an experiment with 0.9M NaCl was performed with all the mutant strains, using samples taken at time-intervals where activation was seen in the previous experiment.

A third anti-phospho-p38 MAP Kinase antibody (polyclonal, Santa Cruz Biotechnology, USA) was used to immunodetect the P-Hog1 as well as an anti-phospho-p44/42 MAPK antibody to see if MAPK from other pathways (Slf2, Kss1 and Fus3) were activated. In Figure 55 can be seen the Western Blot with samples from the WT

*CaOPY2-Myc-pCM189* 1 and 2 and from the WT *ScOPY2-Myc-pCM189* 1 and 2. In Figure 56 can be seen the comparison between the WT *CaOPY2-Myc-pCM189* 1 and the samples from the mutants *opy2 ssk1 pcM189*, *opy2 ssk1 CaOPY2-Myc-pCM189* and *opy2 ssk1 ScOPY2-Myc-pCM189* 23 and 25.

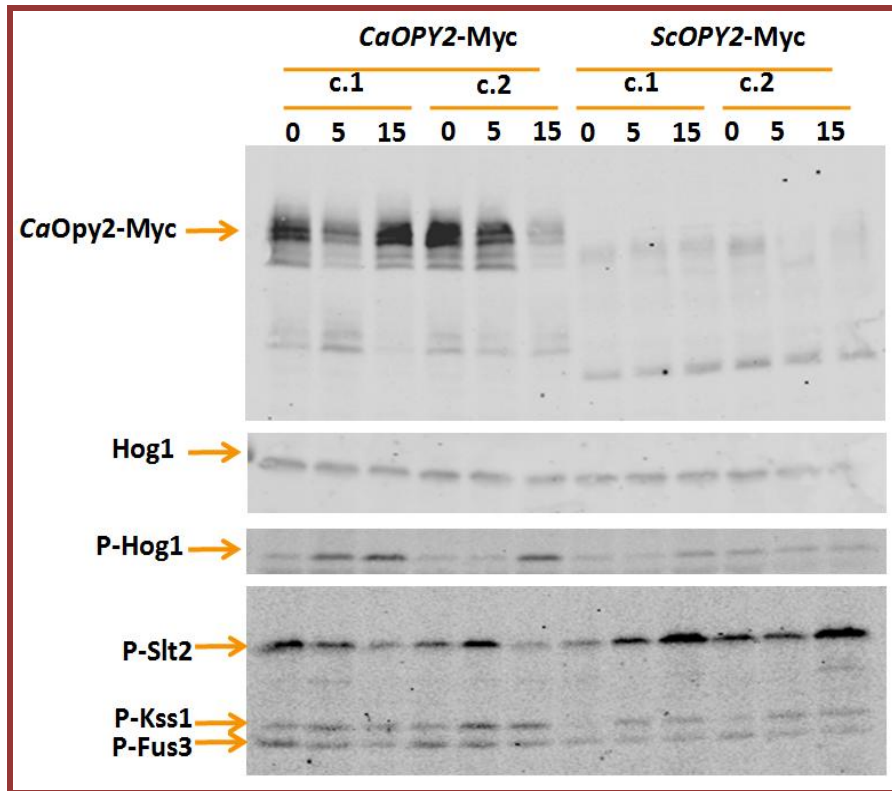


Figure 55 – Western Blot Assay. 10 $\mu$ L from a 20 $\mu$ g/mL protein sample from the samples referred in the image were loaded in the gel, the immunoblotting was performed using the primary antibodies anti-myc, anti-Hog1, anti-phospho-p38 MAPK (polyclonal, Santa Cruz Biotechnology, USA) and anti-phospho-p44/42 MAPK and secondary antibodies anti-mouse and anti-rabbit and the membrane was revealed using an Odyssey scanner.

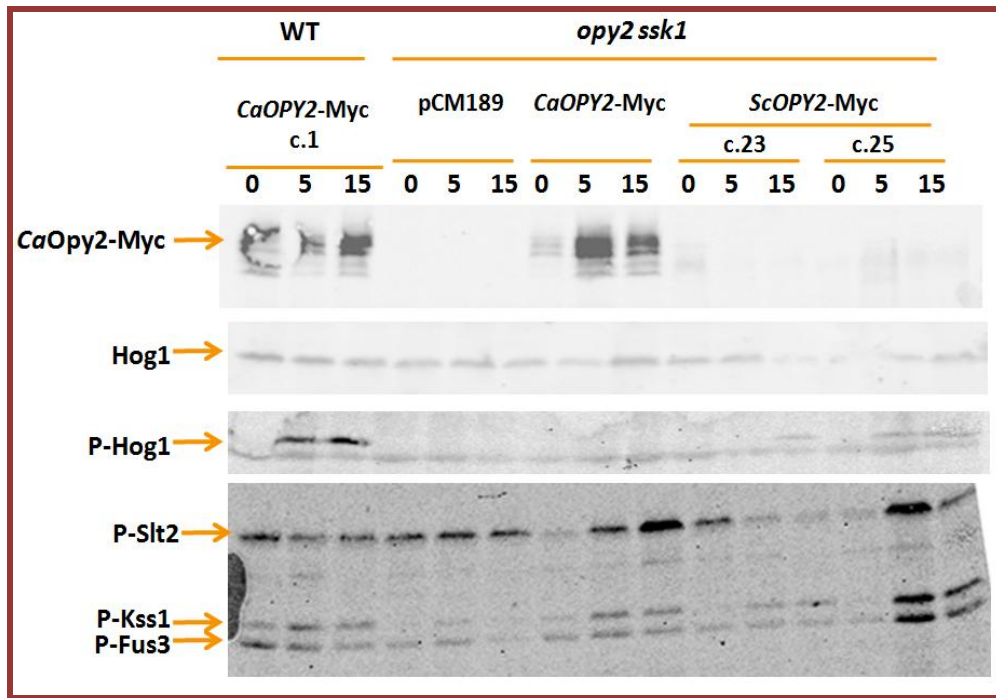


Figure 56 – Western Blot Assay. 10 $\mu$ L from a 20 $\mu$ g/mL protein sample from the samples referred in the image were loaded in the gel, the immunoblotting was performed using the primary antibodies anti-myc, anti-Hog1, anti-phospho-p38 MAPK (polyclonal, Santa Cruz Biotechnology, USA) and anti-phospho-p44/42 MAPK and secondary antibodies anti-mouse and anti-rabbit and the membrane was revealed using an Odyssey scanner.

In the first Western Blot, it is possible to see the detection of the *CaOpy2-Myc*, but once again, the *ScOpy2-Myc* was not detected. Both clones of the WT *CaOPY2-Myc*-pCM189 are able to activate the P-Hog1, the clone 1 at 5 minutes and clone 2 at 15 minutes. About the WT *ScOPY2-Myc*-pCM189, clone 1 is able to activate the P-Hog1, but not the clone 2.

Both WT *ScOPY2-Myc*-pCM189 clones are able to activate Slt2 and Kss1 and slightly deactivate Fus3 as well as the clone 2 of the WT *CaOPY2-Myc*-pCM189.

In the *opy2 ssk1* mutant, activation of the P-Hog1 with both the pCM189 and *CaOPY2-Myc*-pCM189 is not seen. *CaOPY2-Myc* is not able to activate Hog1.

In both mutant clones of the *ScOPY2-Myc*-pCM189, activation of Hog1 is detected – in clone 23 at 15 minutes and in clone 25 at 5 minutes.

Relatively to the activation of other MAPK's in the mutant strains, with *CaOPY2*-Myc Slt2 is activated as well as Kss1. With *ScOPY2*-Myc, in clone 25 there is activation of Slt2, Kss1 and Fus3. The differences seen on the activation of Slt2 in both mutants might be because of differences of protein content.

### 10- Study of the *OPY2* in response to oxidative stress

The mechanism by which the HOG pathway is activated by oxidative stress is dependent on the Ssk1 branch and not by the Sho1 adaptor protein branch. This means that in mutants who lack the *SSK1* gene, there is no response to oxidative stress (Alonso-Monge *et al.*, 2006).

As menadione and hydrogen peroxide are both oxidants, they were tested to see if there were any differences in the growing of the different mutants.

The results are presented in Figure 57.

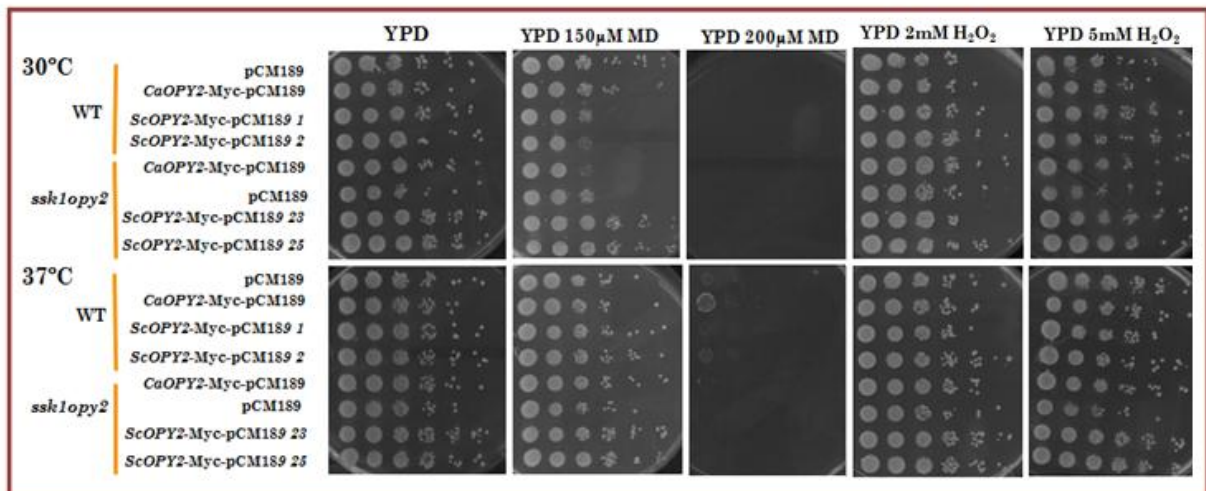


Figure 57 – Susceptibility to oxidative stress by menadione (MD) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Equal number of exponentially growing cells was shifted in YPD medium and 10-fold serial dilutions were done. Five microliters were spotted on YPD plates supplemented with the compounds indicated in the upper part. Plates were incubated at 30°C and 37°C for 32h.



No significant differences were detected between the different strains at 30°C on both oxidants tested. So, it was concluded that the mutants are not sensitive to oxidative stress. Regardless, an unexpected observation was done in this spot assay: at 37°C, with a concentration of 200 µM menadione, only the wild-type strain showed any growth.

In order to verify the previous observed results, the spot assay with Menadione was repeated several times, and it can be seen in Figure 58.

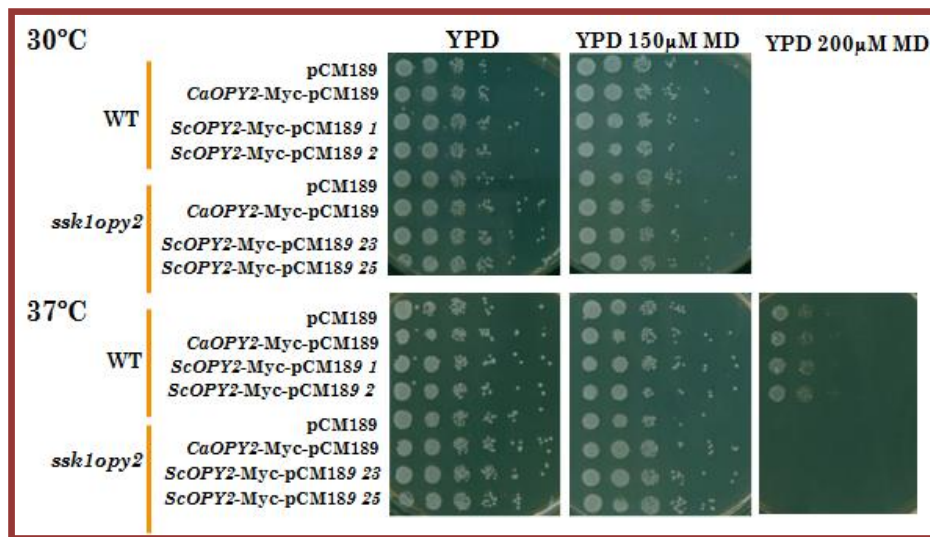


Figure 58 – Susceptibility to menadione stress. Equal number of exponentially growing cells was shifted in YPD medium and 10-fold serial dilutions were done. Five microliters were spotted on YPD plates supplemented with menadione in the concentration indicated in the upper part. Plates were incubated at 30°C and 37°C for 32h.

Again, only the WT strain cells were able to grow under the stress with 200µM of menadione. One possible explanation is that this strain has the *SSK1* gene and then, it is able to respond to this kind of stress. After establishing the end-points of the concentration of menadione in which the strains are able to grow, the *opy2 ssk1* mutants are unable to grow because they lack *SSK1*, and *OPY2* is not able to respond when exposed to oxidative stress.

It is concluded that *CaOPY2* is not able to improve the growth under an oxidative stress.

### ***11- Study of the OPY2 in cell wall biogenesis***

Due to the fact that the HOG pathway is also involved in cell wall biosynthesis, two compounds that are able to interact with the cell wall were tested in Spot Assays: Congo Red and Calcofluor.

Congo red is a dye which interacts with numerous polysaccharides, exhibiting a particularly high affinity for  $\beta$ -Glucans. Through hydrogen bonds, Congo red binds to nascent glucan chains and, as a consequence of this interaction, the glucan chains are prevented from crystallizing to form microfibrils (Nodet *et al.*, 1990).

Calcofluor is a fluorochrome that exhibits antifungal activity and a high affinity for yeast cell wall chitin.

Susceptibility to these compounds correlates with Cek1 activation. In *ssk1* mutants this susceptibility is diminished because in this situation, phosphorylation of Cek1 is higher. The HOG pathway represses the Cek1 pathway and this process helps in the construction of the cell wall (Alonso-Monge *et al.*, 2006).

The result of these Spot Assays is represented in Figure 59. After analyzing the Spot Assays with Calcofluor, it was observed that no significant differences were seen between the different mutants at the different concentrations tested. About the ones with Congo Red, it was seen that only the *opy2 ssk1* mutants were able to grow at both 30°C and 37°C in the different concentrations. These results were intriguing because the effect seen could be from the *CaOPY2* introduced or from the mutant itself that lacked the *ssk1*.

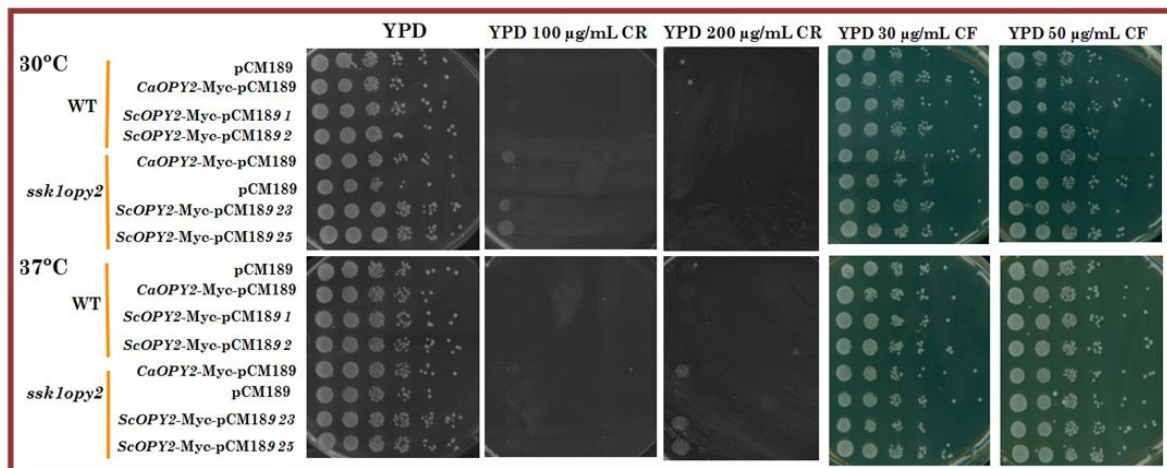


Figure 59 – Susceptibility to different kind of stresses. Equal number of exponentially growing cells was shifted in YPD medium and 10-fold serial dilutions were done. Five microliters were spotted on YPD plates supplemented with the compounds indicated in the upper part. Plates were incubated at 30°C and 37°C for 32h.

To confirm the result obtained with Congo Red, another Spot Assay with lower concentrations of Congo Red – 50µg/mL, 75µg/mL and 100µg/mL was performed (Figure 60).

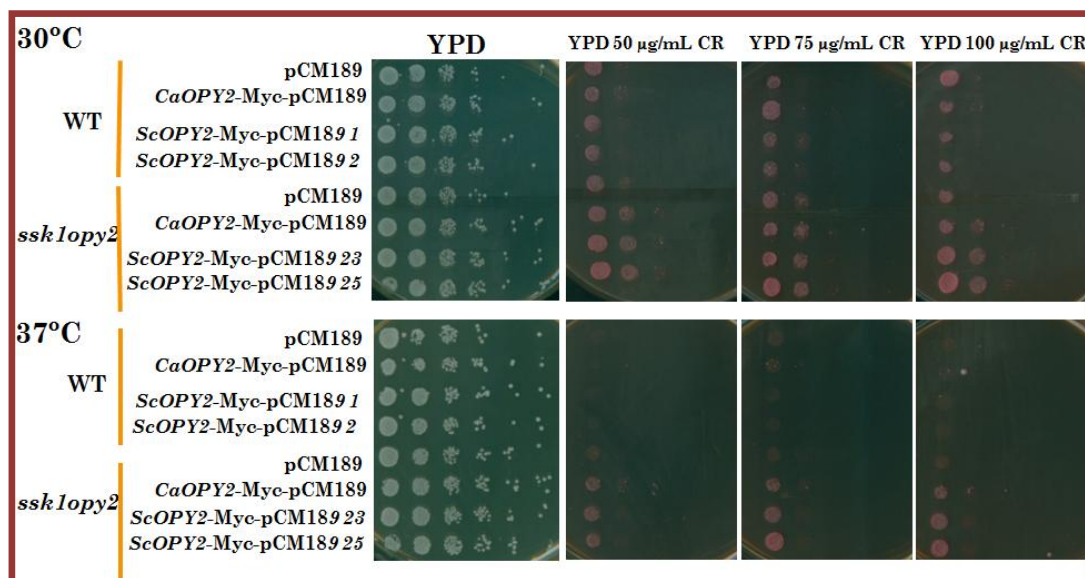


Figure 60 – Susceptibility to Congo Red stress. Equal number of exponentially growing cells was shifted in YPD medium and 10-fold serial dilutions were done. Five microliters were spotted on YPD plates supplemented with Congo Red in the concentration indicated in the upper part. Plates were incubated at 30°C and 37°C for 32h.

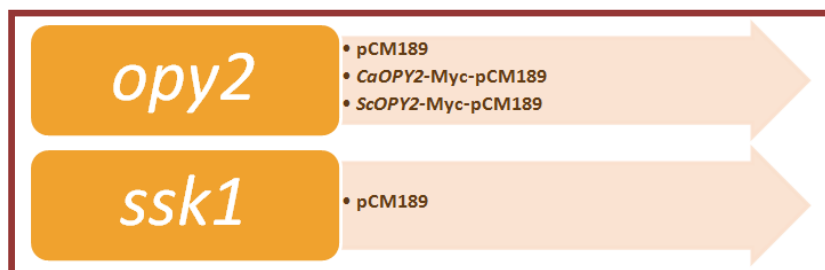
Once again, it was realized that the mutant strains grow better than the wild type strains but it was impossible to know if this effect in the double mutant was due to the introduced *CaOPY2* or the absence of *SSK1* without studying the simple mutants.

To try to answer this question, it was decided to transform *S. cerevisiae* again but this time in a *ssk1* mutant and in an *opy2* mutant.

### **12 – Introduction of the genetic constructions into *S. cerevisiae* *ssk1* and *opy2* mutants**

Two different strains of *S. cerevisiae* were transformed by the One-step transformation method with pCM189, *CaOPY2*-Myc-pCM189 and *ScOPY2*-Myc-pCM189. The *opy2* mutant strain was transformed with all the three plasmids and the *ssk1* mutant strain only with the pCM189 plasmid because this strain has its own intrinsic *OPY2*, so insert the *OPY2* gene in this strain only would allow us to see the overexpression of the gene (Table 7).

Table 7 – Scheme of the plasmids introduced in the *S. cerevisiae* WT strain, in the *opy2* strain and in the *ssk1* strain.



Before the transformation itself, a DNA Gel Electrophoresis was performed to calculate the volume of each plasmid to use in each transformation. Depending on the quantity of DNA seen in the gel the volumes used from each plasmid was different.

The transformed suspensions were plated in SD minimal medium plates and incubated at 30°C for 2 days. After the 2 days of incubation, different colonies of each transformation are striated in SD minimal medium plates.

In order to confirm the introduction of the plasmids into the *S. cerevisiae* strains a Dot Blot Assay was performed to detect the Myc-tag in the proteins extracted from different colonies of diverse transformants.

All the samples from *ssk1* pCM189 and *opy2* pCM189 should be negative since this construction is absent for the Myc-tag and all the samples from *opy2 CaOPY2-Myc-pCM189* and *opy2 ScOPY2-Myc-pCM189*, if the plasmid would have been well introduced, should have a positive signal. The result of the Dot Blot Assay can be seen in Figure 61.



Figure 61 – Dot Blot Assays. Proteins from different clones of both *S. cerevisiae* *opy2* strain and *ssk1* strain with pCM189, *CaOPY2-Myc-pCM189* and *ScOPY2-Myc-pCM189*. 2µL of each sample were blotted in a nitrocellulose membrane, immunoblotting of the Myc epitope was performed using a primary antibody anti-myc and a secondary antibody anti-mouse and the membrane was revealed using an Odyssey scanner.

Only two transformants had a positive signal for the Myc-tag – the *opy2 CaOPY2-Myc-pCM189* 1 and the *opy2 ScOPY2-Myc-pCM189* 2.

Once again, because the Opy2p is a transmembrane protein, when extracting the proteins it is necessary to remove them from the membranes. The method used to extract the proteins is not the most effective for this separation and the mutants may carry the plasmid, but, because the protein was not efficiently extracted, it may not be positive for the Myc-tag due to this fact.

Even if not all these clones were positive in the Dot Blot, clones 1 and 2 from each different mutant were loaded in a Western Blot.

To test if the previously chosen samples were positive for the Myc-tag, a Western Blot was performed with clones 1 and 2 of each different mutant.

As in the Dot Blot, samples with only pCM189 should be negative (*ssk1* pCM189 and *opy2* pCM189) and samples with both *CaOPY2-Myc-pCM189* and *ScOPY2-Myc-pCM189* of the *opy2* strain should be positive.

The result can be seen in Figure 62.

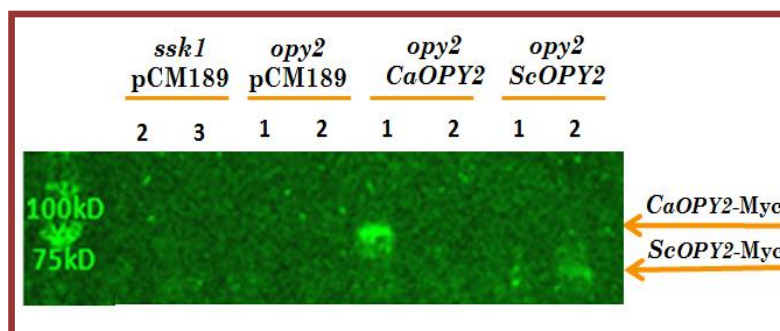


Figure 62 – Western Blot Assay. 10µL from a 20µg/mL protein sample from the samples referred in the image were loaded in the gel, the immunoblotting of the Myc epitope was performed using a primary antibody anti-myc and a secondary antibody anti-mouse and the membrane was revealed using an Odyssey scanner.

Even though the signal was not very intense, it was possible to see a band in the *opy2 CaOPY2-Myc-pCM189 1* around the 75 kD and a band appearing in the *opy2 ScOPY2-Myc-pCM189 2* a little bit above the band of the 75 kD. These molecular weights are consistent with the results obtained with the samples from the WT and the *opy2 ssk1* strains obtain in the section 14 of this work (14 - *The CaOpy2 protein must suffer post-translational modifications*).

As happened in the Dot Blot, in none of the clone 2 of the *opy2 CaOPY2-Myc-pCM189* and the clone 1 of the *opy2 ScOPY2-Myc-pCM189* appeared the protein band.

### ***13 – The ssk1 and opy2 mutants were not sensitive to osmotic, oxidative or cell wall stresses***

To test the different susceptibilities of the single mutants to different compounds, Spot Assays were performed.

Firstly, the strains were tested to visualize their ability to grow under osmotic stress in the presence of 1M Sorbitol. The result is shown in Figure 63.

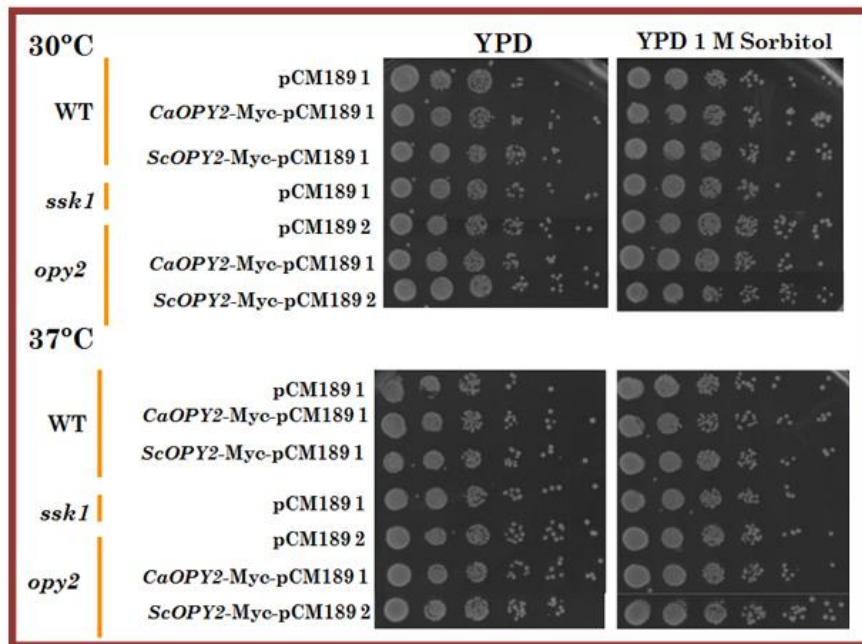


Figure 63 – Susceptibility to Sorbitol stress. Equal number of exponentially growing cells was shifted in YPD medium and 10-fold serial dilutions were done. Five microliters were spotted on YPD plates supplemented with the concentration indicated in the upper part. Plates were incubated at 30°C and 37°C for 32h.

As can be seen, none of the mutants is sensitive to osmotic stress. Even though these strains have genes that participate in the response to osmotic stress, the deletion of only one of the branches did not block the response to osmotic stress. The obtained result was the expected.

Next, to test the respond of the mutants under oxidative stress, Hydrogen Peroxide and Menadione were tested and the result is shown in Figure 64.



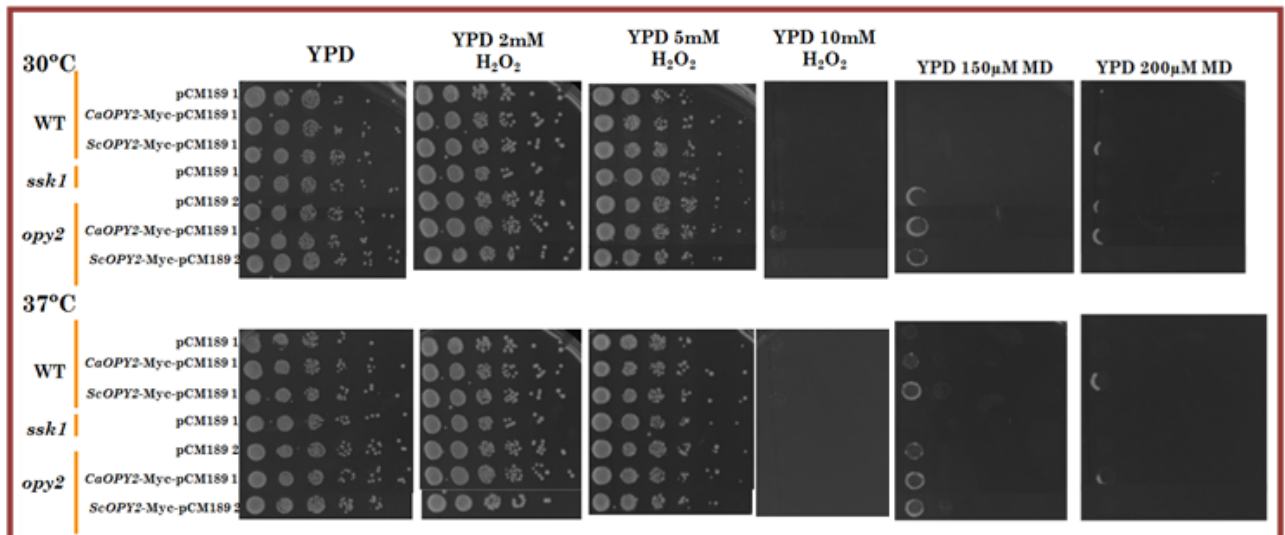


Figure 64 – Susceptibility to different kind of stresses. Equal number of exponentially growing cells was shifted in YPD medium and 10-fold serial dilutions were done. Five microliters were spotted on YPD plates supplemented with the compounds indicated in the upper part. Plates were incubated at 30°C and 37°C for 32h.

Because the branch with the *Ssk1* is the one specialized in sensing/transmitting oxidative stress-mediated signals, it was normal that the *ssk1* pCM189 had no response in a situation of oxidative stress, as was seen in this Assay.

No significant differences were observed between the wild type and the *opy2* mutant.

For the reason that was important to confirm if the previously Congo Red susceptibility results obtained with the double mutant were due to the *ssk1* deletion or from the introduction of the *CaOPY2*, the mutants were as well tested in different concentrations of Congo Red. To test if Calcofluor could have any effect, Spot Assays with this compound were performed too. The results can be seen in Figure 65.

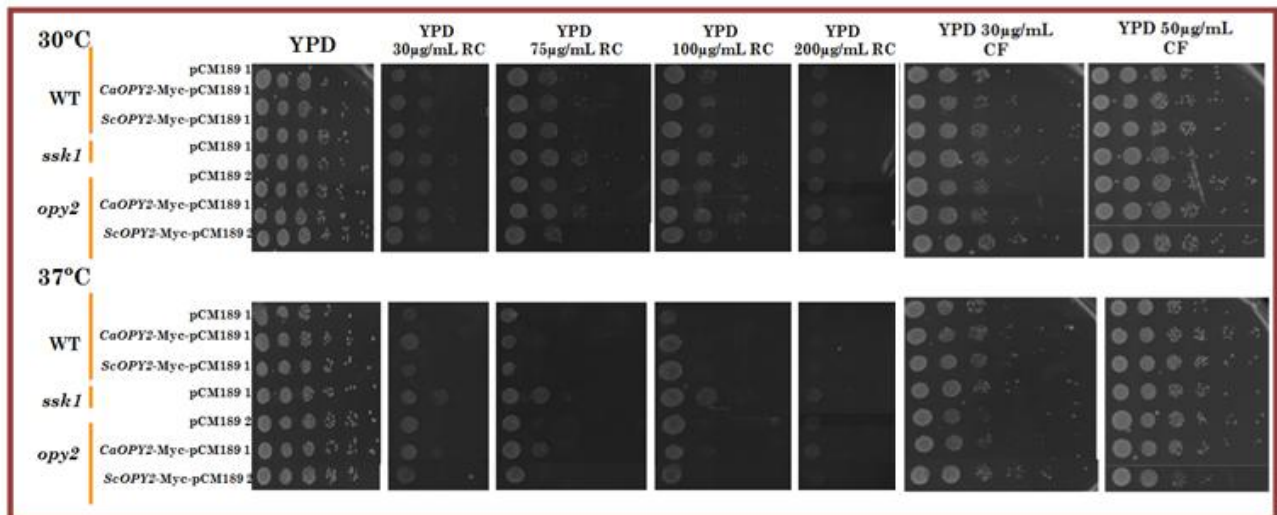


Figure 65 – Susceptibility to different kind of stresses. Equal number of exponentially growing cells was shifted in YPD medium and 10-fold serial dilutions were done. Five microliters were spotted on YPD plates supplemented with the compounds indicated in the upper part. Plates were incubated at 30°C and 37°C for 32h.

With Calcofluor no significant differences were seen between the different mutants at the different concentrations tested.

About the susceptibility to Congo Red, the *ssk1* mutant grow slightly better than the rest of the mutants.

Concerning the effect of the *CaOPY2-Myc-pCM189* in the *opy2* strain, compared to the one with the vector, and the ones of the wild type strain, it was possible to detect an slightly improvement of the growth when the *CaOPY2* is introduced. *CaOPY2* might have a role in cell wall biosynthesis.

#### **14- The *CaOpy2* protein must suffer post-translational modifications**

After the DNA has been transcribed into RNA and translated into proteins the proteins undergo posttranslational modifications. One of these posttranslational modifications is enzymatic glycosylation. Glycosylation is the covalent attachment of an oligosaccharide chain to the protein backbone and is considered to be a very

common protein modification. This modification of proteins through enzymatic glycosylation is an event that reaches beyond the genome and is controlled by factors that differ greatly among cell types and species.

Two major types of glycosylation, referred to as N- and O- linked glycosylation, can be distinguished. N-glycans are attached to Asn residues of the peptide backbone while O-glycans are connected to Ser or Thr residues. Only in recent years, it has been acknowledged that glycosylation of proteins modulates various processes such as subcellular localization, protein quality control, cell-cell recognition and cell-matrix binding events.

If *CaOPY2-Myc* has the same molecular weight in both *C. albicans* and *S. cerevisiae*, it might mean that in both organisms the glycosylation is performed in the same way. This was important to study because more tools are known in *S. cerevisiae*, and thereby the study of *CaOPY2-Myc* would be simplified.

It was important, as well, to see if the *ScOPY2-Myc* and the *CaOPY2-Myc* had different molecular weights.

For this, several Western Blots were performed.

Firstly, to test if the *CaOPY2-Myc* in *S. cerevisiae* had a different molecular weight from the *ScOPY2-Myc* in *S. cerevisiae*, a Western Blot was performed with samples of *CaOPY2-Myc* in both the WT and *ssk1 opy2 ssk1 S. cerevisiae* strains and samples of the *ScOPY2-Myc* of the WT *S. cerevisiae* strain. The Western Blot obtained is represented in Figure 66.

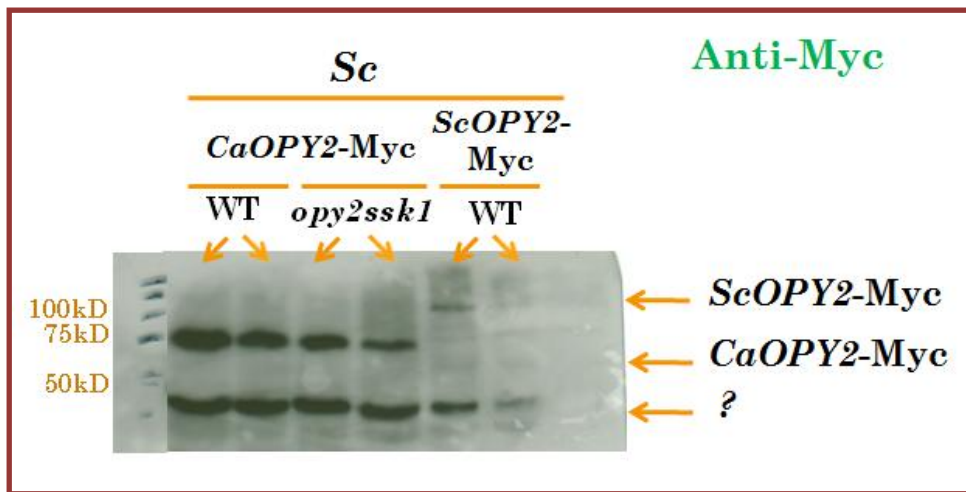


Figure 66 – Western Blot Assay. The samples were loaded in the following order: 10 $\mu$ L of a 20 $\mu$ g/mL protein sample of the WT *CaOPY2-Myc*-pCM189 4 and 5; *opy2 ssk1 CaOPY2-Myc*-pCM189 1 and 2 and the WT *ScOPY2-Myc*-pCM189 1 and 2. The immunoblotting of the Myc epitope was performed using a primary antibody anti-myc and a secondary antibody anti-mouse and the membrane was revealed using the chemiluminescence method.

After analysis of the Western Blot it was possible to see that in the *CaOPY2-Myc* of both WT and *opy2 ssk1* of *S. cerevisiae* two bands were appearing, one around the 75 kD and another below 50 kD. The theoretic molecular weight of *CaOPY2-Myc* is 58.65 kD so even though two bands were appearing with the *CaOPY2-Myc* in *S. cerevisiae*, none of them were appearing in the theoretic molecular weight.

Concerning the *ScOPY2-Myc* two bands were, once again, appearing, one at the same molecular weight as the lower band appearing with the *CaOPY2-Myc* in *S. cerevisiae* and another band a little bit above 100 kD. The theoretic molecular weight of the *ScOPY2-Myc* is 40.354kD so the band was appearing too high compared to the theoretic molecular weight. In Yang *et al.* (2009) *ScOPY2* was already seen in a molecular weight between the 91 and the 114 kD so it could be possible that the band around the 100 kD was the *ScOPY2-Myc*.

Because the band lower the 50 kD was appearing in all the samples it was thought that it could be an unspecific band so another Western Blot with the same samples as before and a ScWT pCM189 that has no Myc-tag attached that would allow

us to see if any band would appear and, if so, it had to be unspecific. This time the Western Blot was revealed with the Odyssey scanner (Figure 67).

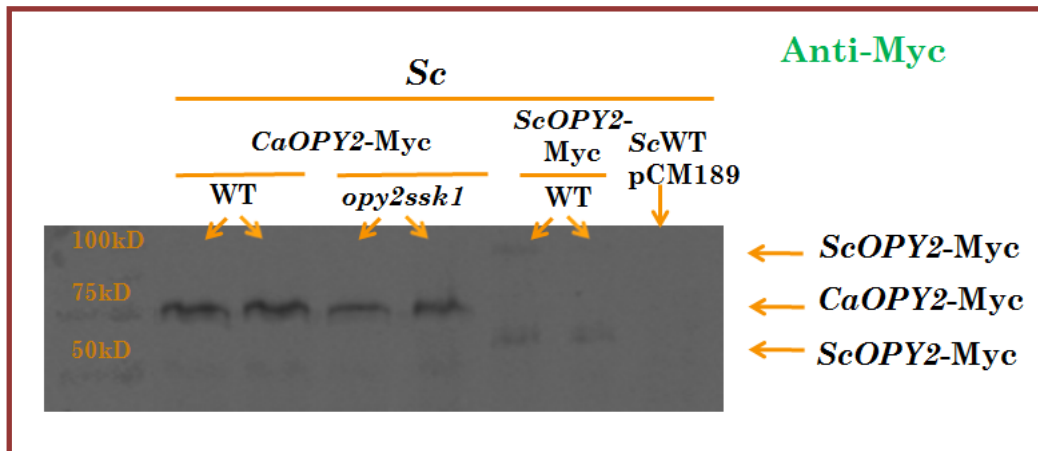


Figure 67 – Western Blot Assay. The samples were loaded in the following order: 5µL from a 20µg/mL protein sample of the WT *CaOPY2-Myc*-pCM189 4 and 5; *opy2 ssk1 CaOPY2-Myc*-pCM189 1 and 2; the WT *ScOPY2-Myc*-pCM189 1 and 2 and the ScWT pCM189. The immunoblotting of the Myc epitope was performed using a primary antibody anti-myc and a secondary antibody anti-mouse and the membrane was revealed using an Odyssey scanner.

In this Western Blot it was possible to see that the previous band that was appearing in all the samples did not appear in this one, as well as in the ScWT pCM189 sample, so it was confirmed that that band was unspecific.

Once again *CaOPY2-Myc* and *ScOPY2-Myc* were appearing in different molecular weights, what was expected. *CaOPY2-Myc* was appearing around 75 kD and in the case of the *ScOPY2-Myc* samples, one band was appearing around 100 kD as in the Western Blot before, but another band that did not appeared in the previous Western Blot was appearing around 63 kD.

From this it was concluded that if the samples are boiled during more time, the intensity of the band that appeared before around the 100 kD is reduced and the band around 63 kD becomes more intense. It is believed that this protein forms dimers and in order to separate them it is necessary to boil the samples for at least 10 minutes.

To compare the *CaOPY2*-Myc expressed in *C. albicans* from the one that was expressed in *S. cerevisiae*, a Western Blot was performed and it is shown in Figure 68.

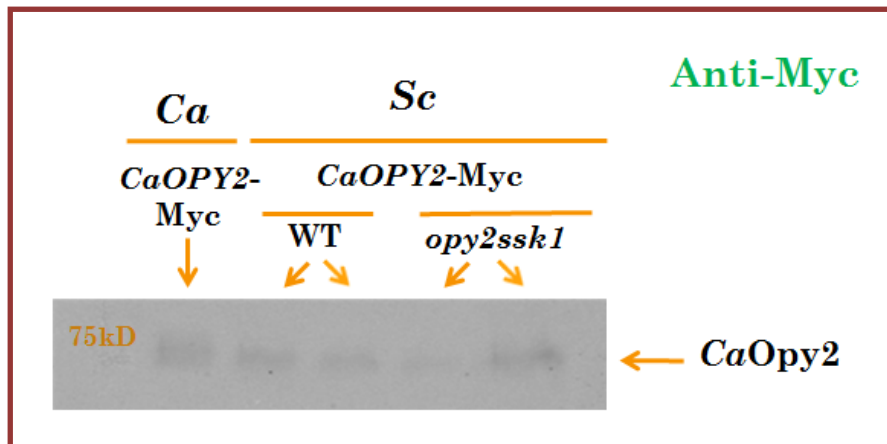


Figure 68 – Western Blot Assay. The samples were loaded in the following order: 4µL from a 20µg/mL protein sample of a *CaOPY2*-Myc-pCM189 expressed in *C. albicans*, and 5µL from the following samples of the WT *CaOPY2*-Myc-pCM189 4 and 5 and the *opy2 ssk1* *CaOPY2*-Myc-pCM189 1 and 2. The immunoblotting of the Myc epitope was performed using a primary antibody anti-myc and a secondary antibody anti-mouse and the membrane was revealed using an Odyssey scanner.

As we can see in this last Western Blot, all samples of *CaOpy2p* from both organisms appear at 75 kD, so it could be possible that both microorganisms glycosylate in the same way this protein.

Although the main way of glycosylation of the *OPY2* gene is O- glycosylation in *S. cerevisiae* (Yang *et al.*, 2009), previous experiments suggest that in *C. albicans* this is not the case since *CaOpy2* did not display differences in its electrophoretical pattern when it was expressed in mutants defective in the enzymes implicated in the O-glycosilation. An experiment, as explained in Methodology, was performed with tunicamycin to block the synthesis of all N-linked glycoproteins (N-glycans) to distinguish if differences in the glycosylation of *CaOPY2* in both microorganisms and of the *ScOPY2* were perceived.

### 15 – Inhibition of the OPY2 N-glycosylation by tunicamycin

N-glycans of the proteins from *C. albicans* are essential for the integrity of the cell wall and for the interactions between the fungus and the host. N-glycosylation can be altered by several products as is the example of tunicamycin.

Tunicamycin is an antibiotic that inhibits N-glycosylation by blocking the transfer of N-acetylglucosamine-1-phosphate (GlcNAc-1-P) from UDP GlcNAc to dolichol-P, decreasing the formation of dolichol-PP-GlcNAc (Almeida *et al.*, 2011).

To see if differences could be seen in the *CaOPY2-Myc* in *C. albicans* and in the *ScWT CaOPY2-Myc-pCM189* and in the *ScWT ScOPY2-Myc-pCM189* when the N-glycosylation was inhibited and only the O-glycosylation was active, an experiment as explained in Methodology was performed and the result can be seen in Figure 69.

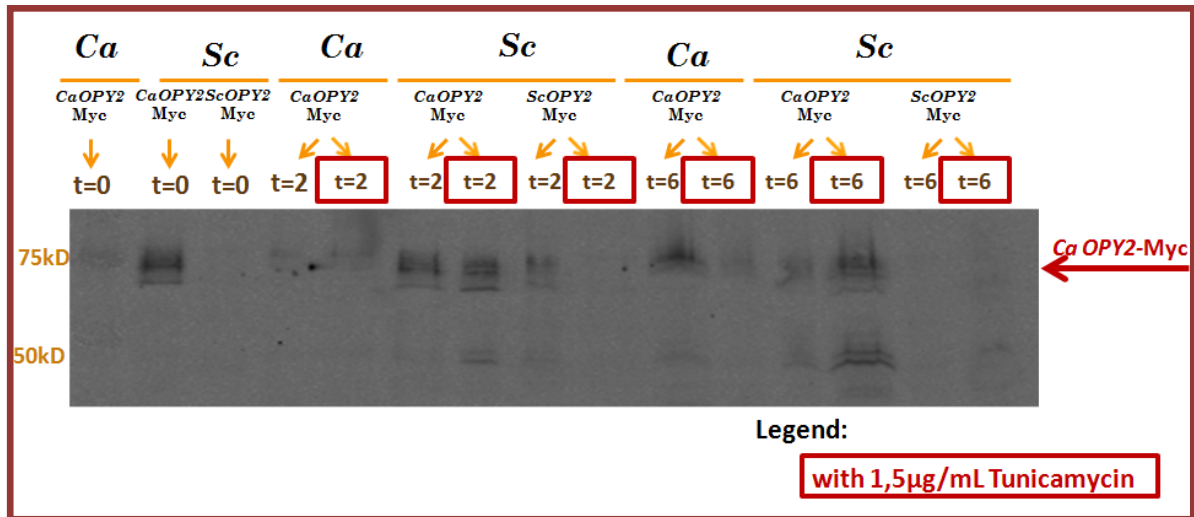


Figure 69 – Western Blot Assay. 5µL from a 20µg/mL protein sample of the samples treated with or without tunicamycin (final concentration of 1.5 µg/ml) referred in the image were loaded in the gel, the immunoblotting of the Myc epitope was performed using a primary antibody anti-myc and a secondary antibody anti-mouse and the membrane was revealed using an Odyssey scanner.

The plasmid that was introduced in the *C. albicans* strain, unlike what happens with the plasmids introduced in the *S. cerevisiae* strains, required Doxycycline to induce the gene expression. At time 0, it was not possible yet to see the *CaOpy2-Myc*, because it was necessary more time to express the *CaOpy2-Myc* protein. At time 2 a

slightly band can be seen, but at time 6 the expression was high enough to detect a clear band.

About the *CaOpy2-Myc* in *S. cerevisiae*, it is possible to see that when tunicamycin is introduced in the medium, the pattern of the band is different. There is an increase in the trail and the size of the bands what means that, in fact, the N-glycosylation is being blocked and that when only the O-glycosylation is active, the resultant protein is distinct. In the samples with tunicamycin, a band around the 50 kD also appears that might be result of degradation.

Comparing the *CaOpy2-Myc* in *C. albicans* from the one in *S. cerevisiae*, it is possible to see differences in the pattern of the bands. In the *CaOpy2-Myc* in *C. albicans*, the 75 kD band is smaller compared to the ones from the *S. cerevisiae*, and the band of the 50 kD almost don't appear. It is possible to conclude that the glycosylation of the *CaOPY2-Myc* is different in the different organisms.

Relatively to the *ScOpy2-Myc*, it is not possible to compare the differences of the glycosylation because the bands are too faint.

#### **16 - Activation of Kss1 and Mkc1 MAPK induced by N-glycosylation defect with tunicamycin.**

To study if different pathways were activated by a defect induced by tunicamycin, with the samples of the previous experiment with the *C. albicans* strain and the *ScWT* strains with tunicamycin as well as with new samples using the *opy2* strain, Western Blots were carried out and the immune detection was performed with an anti-phospho-p44/42 MAPK antibody. This antibody is able to detect the *S. cerevisiae* Fus3/Kss1 MAPKs and its Cek1 homologue of *C. albicans* from the Cek1-mediated pathway and the *S. cerevisiae* Slt2 MAPK and its homologue Mkc1 of *C. albicans* from the cell integrity pathway. To each different samples two Western Blots are performed – one with the anti-phospho-p44/42 MAPK antibody and another with the anti-Hog1 antibody to work as control of protein. Important to refer again



that the Hog1 from the different organisms are different proteins so they have distinct molecular weights.

Another important aspect is that, in *S. cerevisiae*, the Kss1 MAPK is the one mainly responsible to be activated by glycosylation defects. In case of a treatment with tunicamycin, Kss1 is activated but not Fus3. Moreover, Mpk1 is also activated after a treatment with tunicamycin, but its phosphorylation occurs very slowly and it is independent of Kss1/Fus3 (Yang *et al.*, 2009).

Firstly, to compare the pattern of activation of the *Ca*OPY2-Myc from the *C. albicans* strain with the *Sc*WT *Ca*OPY2-Myc-pCM189 and the *Sc*WT *Sc*OPY2-Myc-pCM189 a treatment with tunicamycin was performed and samples were removed at 0, 2 and 6 hours. The Western Blot obtained is showed in Figure 70.

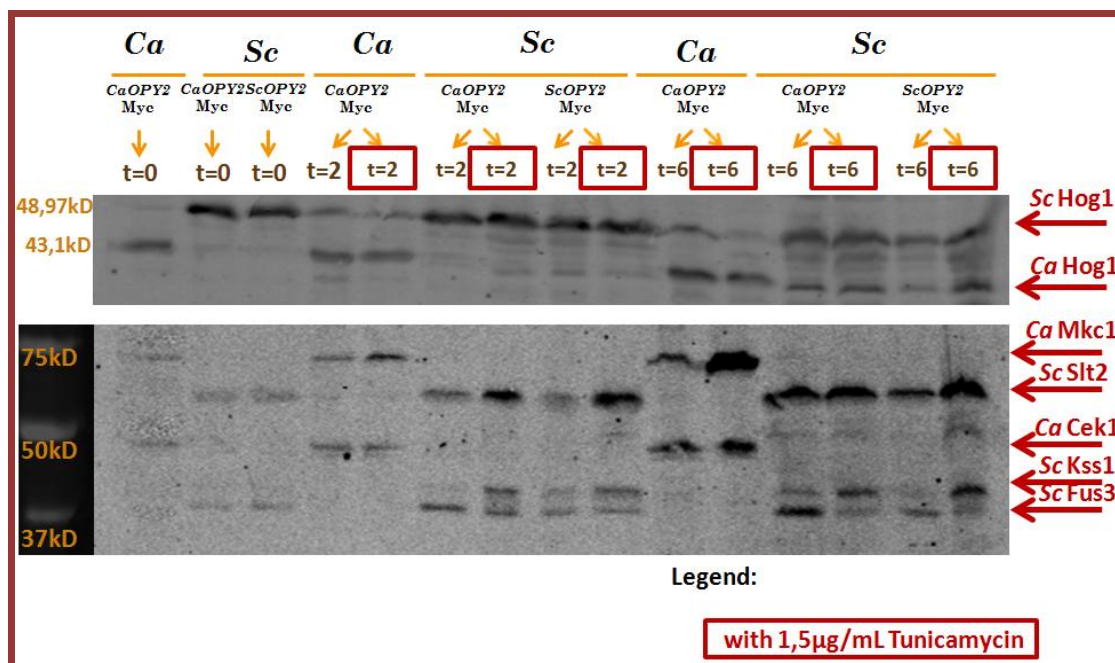


Figure 70 – Western Blot Assay. 5µL from a 20µg/mL protein sample of the samples treated with or without tunicamycin (final concentration of 1.5µg/ml) referred in the image were loaded in the gel, the immunoblotting was performed in the first Western Blot with a primary antibody anti-Hog1 and in the second Western Blot with a primary antibody anti-phospho-p44/42 MAPK (Thr202/Tyr204) and a secondary antibody anti-rabbit in both Western Blots. The membranes were revealed using an Odyssey scanner and the concentrations were equiparated by a Coomassie Blue Staining Assay.

In this Western Blot it is possible to see activation of the Kss1 MAPK at 2 and 6 hours but not of the Fus3 MAPK in the *S. cerevisiae* strains and a slightly activation of the Cek1 MAPK in the *C. albicans* strain at 6 hours.

It is possible, as well, to see activation of the *CaMkc1* and the *ScSlit2* at 2 and 6 hours.

With these results it is possible to conclude that both *S. cerevisiae* and *C. albicans*, when exposed to a tunicamycin stress, activates both Cek1/Kss1-mediated pathway and the cell integrity pathway.

Due to the fact that activation was seen in the *ScWT* strains, the same experiment was performed with the *opy2* strains with pCM189, *CaOPY2-Myc-pCM189* and the *ScOPY2-Myc-pCM189*. With this test it is wanted to study if *CaOPY2* is able to activate these pathways in the mutant strain and the relevance of *Opy2* in the signaling to Kss1.

Firstly, two Western Blots were performed to see in which time activation in the *ScWT* pCM189 and the *opy2* pCM189 strains could be seen. With these first results, another Western Blot would be performed with the samples of all strains of the correspondent time.

The result of the first Western Blots is showed in Figure 71.

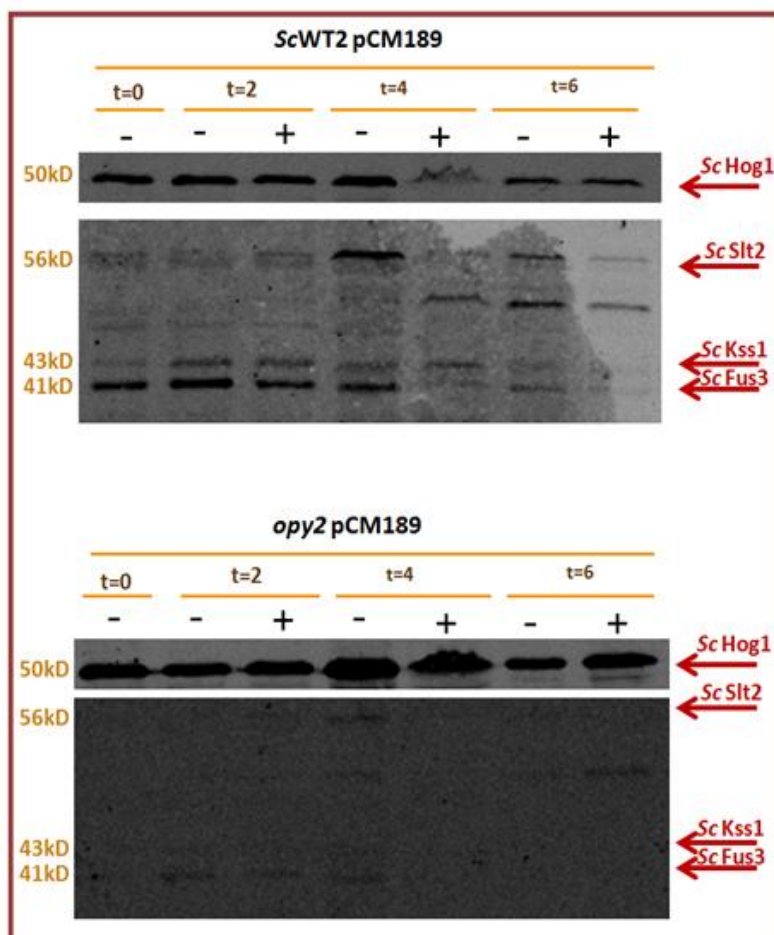


Figure 71 – Western Blot Assay. 5 $\mu$ L from a 20 $\mu$ g/mL protein sample of the samples treated with or without tunicamycin (final concentration of 1.5  $\mu$ g/ml) referred in the image were loaded in the gel, the immunoblotting was performed in the first Western Blot with a primary antibody anti-Hog1 and in the second Western Blot with a primary antibody anti-phospho-p44/42 MAPK (Thr202/Tyr204) and a secondary antibody anti-rabbit in both Western Blots. The membranes were revealed using an Odyssey scanner.

After analyzing these Western Blots, a clear activation of Kss1 is not detected, but it is seen a deactivation of Fus3 over time in both WT and mutant strains. In the previous Western Blot a clear activation of Slt2 was observed, but in both these Western Blots, that is not detected. Although activation of the actually Slt2 is not seen, Slt2 activation might be appreciated in this westerns by the increasing of its degradation band that is clearly detected at 4 hours of treatment. This band is the one appearing under the Slt2 one.

To observe the behavior of the *opy2* mutant strain with the *CaOPY2-Myc* and the *ScOPY2-Myc*, Western Blots were performed with samples from both 2 and 4 hours. The results are shown in Figure 72.

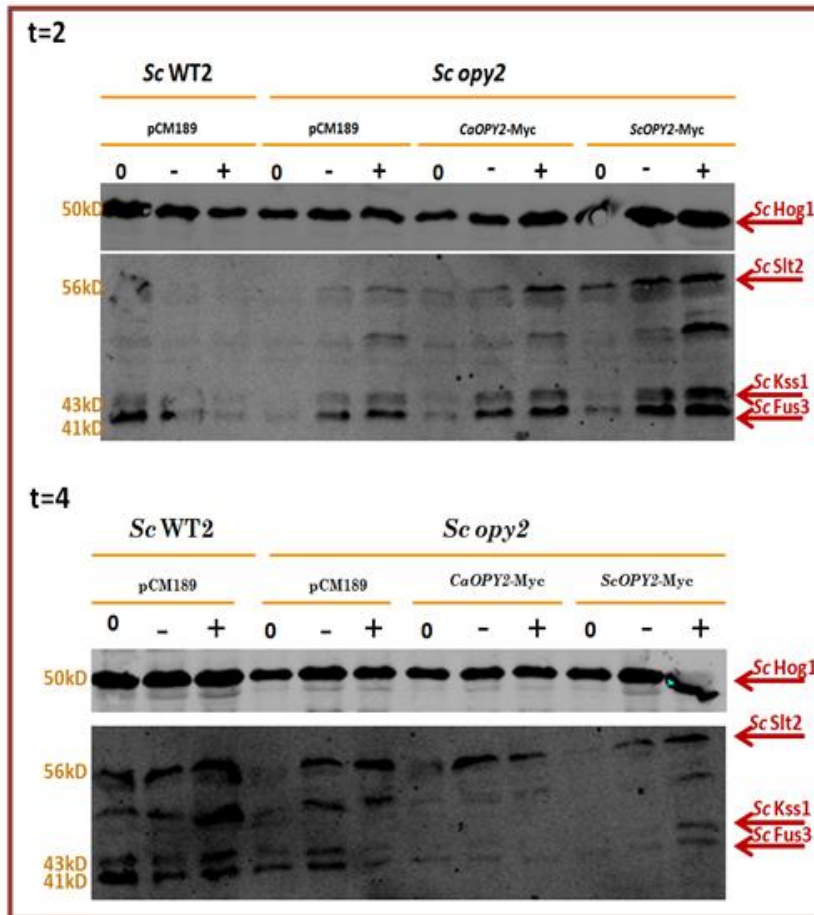


Figure 72 – Western Blot Assay. 5 $\mu$ L from a 20 $\mu$ g/mL protein sample of the samples treated with or without tunicamycin at 2 and 4 hours (final concentration of 1.5 $\mu$ g/ml) referred in the image were loaded in the gel, the immunoblotting was performed in the first Western Blot with a primary antibody anti-Hog1 and in the second Western Blot with a primary antibody anti-phospho-p44/42 MAPK (Thr202/Tyr204) and a secondary antibody anti-rabbit in both Western Blots. The membranes were revealed using an Odyssey scanner.

In these Western Blots it is possible to visualize that in the *opy2 CaOPY2-Myc*, activation of Slt2 is seen at 2 hours, but not of Kss1. At 4 hours, there isn't activation of neither Slt2 nor Kss1. Here we can see that the activation of Kss1 is not dependent of tunicamycin, but the dilution in which the *opy2* mutant is able to phosphorylate Kss1.

From this result it is possible to conclude that *opy2* is not essential for the activation of Kss1, in contrary of what happens in *C. albicans*.

In the *opy2 ScOPY2-Myc*, activation of Slt2 and Kss1 is seen at 2 hours as well as at 4 hours.

### **17 - Adaptation to zymolyase-mediated Cell Wall Damage**

When the cells are exposed to a cell wall damage caused by zymolyase ( $\beta$ -1,3 glucanase), a cell wall integrity response is activated, and this response requires both the HOG and Slt2-mediated pathways. Zymolyase is able to activate both Hog1 and Slt2 MAPKs and in these circumstances, the activation of the Slt2 MAPK is dependent of the Sho1 branch of the HOG pathway (Bermejo *et al.*, 2008).

Recent studies have showed that the regulation of transcriptional responses to cell wall damage mainly depends on the MAPK Slt2. As was seen in the recent work of Bermejo, C. *et al.*, 2008, a rapid increase of the levels of the Slt2-P happens after 15 minutes of the treatment with zymolyase, having a peak of the Slt2 phosphorylation after 1 to 2 hours of treatment, but it remains detectable after 6 hours of the treatment.

To test if the *opy2* mutants with pCM189, *CaOPY2-Myc*-pCM189 and *ScOPY2-Myc*-pCM189 could activate the Slt2 MAPK, the WT pCM189 and the different mutant strains were subject to a treatment with 0.4U/mL of zymolyase and samples removed at 0, 2, 4 and 6 hours.

As in the experiment with tunicamycin, firstly two Western Blots were performed to see in which time activation in the control strains (*ScWT2* pCM189 and *opy2* pCM189) could be detected. According to these first results, Western Blot with the *opy2* mutant with *CaOPY2-Myc* and *ScOPY2-Myc* samples from the time where activation was seen would be performed.

The result of the Western Blots with ScWT2 pCM189 and *opy2* pCM189 is showed in Figure 73.

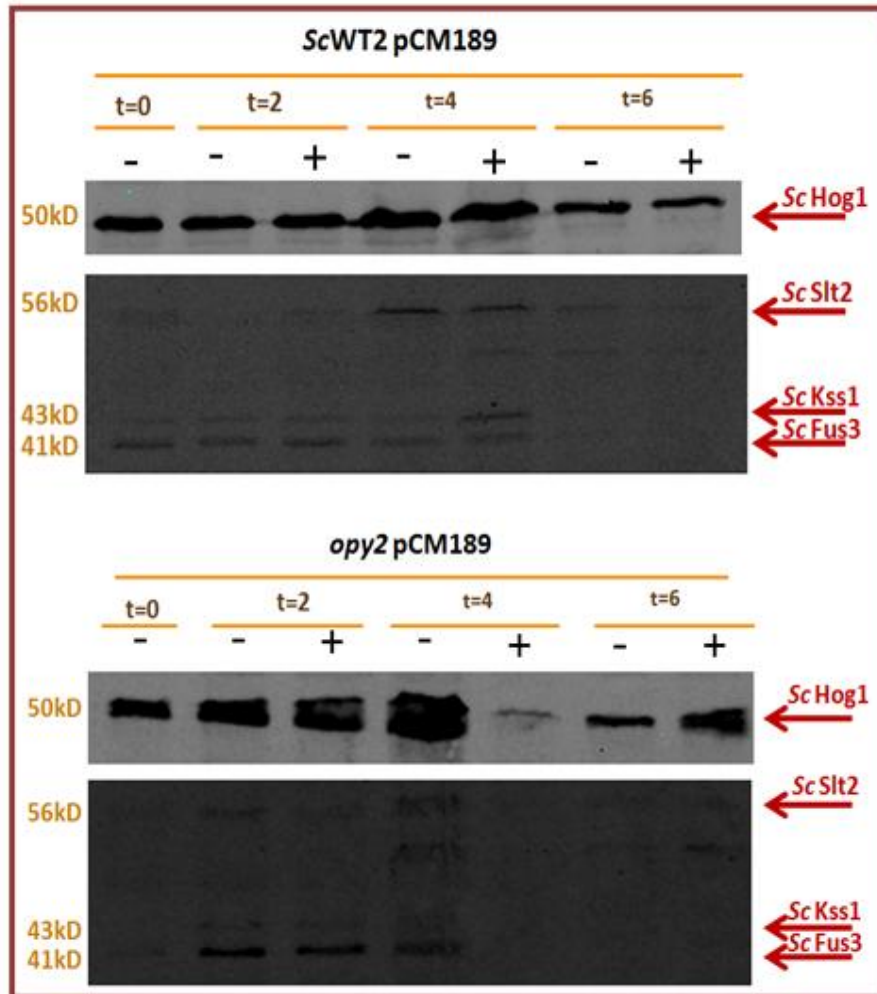


Figure 73 – Western Blot Assay. 5 $\mu$ L from a 20 $\mu$ g/mL protein sample of the samples treated with or without zymolyase (final concentration of 0.4U/mL) referred in the image were loaded in the gel, the immunoblotting was performed in the first Western Blot with a primary antibody anti-Hog1 and in the second Western Blot with a primary antibody anti-phospho-p44/42 MAPK (Thr202/Tyr204) and a secondary antibody anti-rabbit in both Western Blots. The membranes were revealed using an Odyssey scanner.

A clear activation of Kss1 was observed after 4 hours of treatment in the wild type strain. This activation was not detected in the *opy2* mutant.

Following Western Blots were performed with the samples from 2 and 4 hours and the results can be seen in Figure 74.

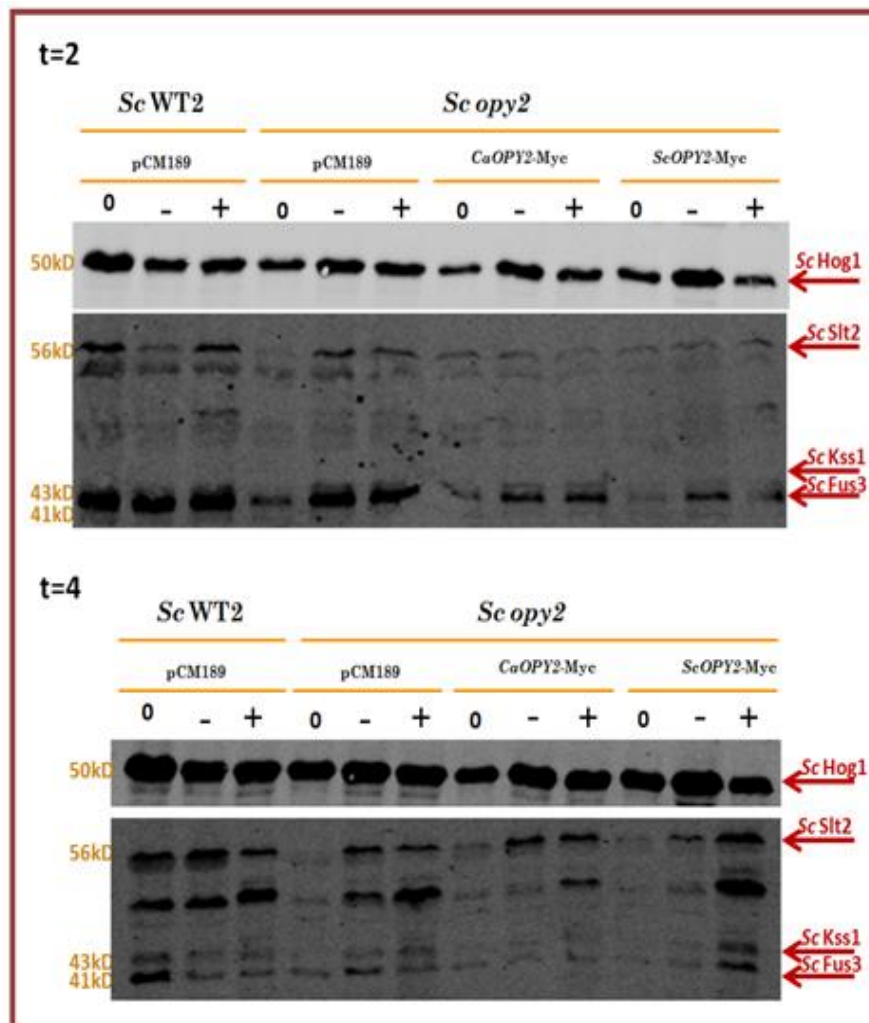


Figure 74 – Western Blot Assay. 5 $\mu$ L from a 20 $\mu$ g/mL protein sample of the samples treated with or without zymolyase (final concentration of 0.4U/mL) referred in the image were loaded in the gel, the immunoblotting was performed in the first Western Blot with a primary antibody anti-Hog1 and in the second Western Blot with a primary antibody anti-phospho-p44/42 MAPK (Thr202/Tyr204) and a secondary antibody anti-rabbit in both Western Blots. The membranes were revealed using an Odyssey scanner.

At time 2, it is only possible to see activation of Slt2 in the ScWT pCM189. However, at 4 hours, activation of Slt2, Kss1 and Fus3 in *opy2* ScOPY2-Myc is seen, but not on any other mutant strain, including the *opy2* CaOPY2-Myc. Even though it is not

possible to see activation at 4 hours in the actually SlT2 band, a smaller band appears under the one of SlT2 that increases with treatment, and the protein bands of the mutant treated with zymolyase appear to be more intense than the one without this treatment.

### **18 – OPY2 viability test with HL60**

To test if different *C. albicans* *OPY2* mutants had different susceptibilities to HL60, viability tests were performed. With this experiment it was wanted to see how the deletion of *OPY2* would affect the behavior of different *opy2* mutants interacting with HL60 cells.

HL60 cell line is a myelomonocytic cell line and it is appropriate to study the granulocyte responses against *C. albicans*. (Arana *et al.*, 2007)

Five strains were tested: a CAF2 strain, used as control, an *opy2* mutant strain, an *OPY2* reintegrant strain in which the *OPY2* gene was deleted and introduced again in a plasmid, a triple mutant of the *ssk1 sho1 msb2* genes and a quadruple mutant of the *ssk1 sho1 msb2 opy2* genes.

The ratios 1:40 and 1:20 were tested (1 yeast for 40 or 20 HL60 cells). The co-cultures were left during 2 hours for infection in an atmosphere of 5% CO<sub>2</sub> at 37°C.

Two independent experiments were performed and the viability of CAF2, *opy2*, *OPY2* reint, *ssk1 sho1 msb2* and *ssk1 sho1 msb2 opy2* mutants is shown in Figure 75. The results are represented with two distinct controls – the yeasts counted before incubation with HL60 and the yeasts after 2 hours of incubation in the same conditions as the ones in the presence of HL60.



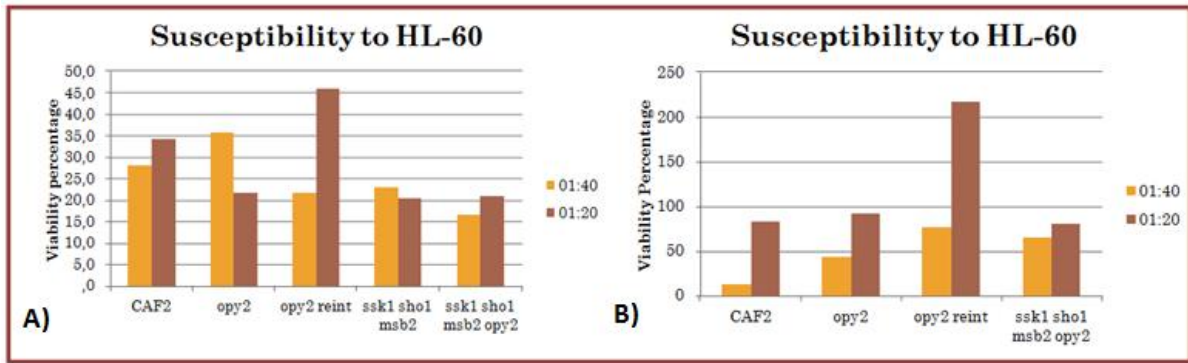


Figure 75 - Viability of *C. albicans* in the presence of HL60. A) Viability of *C. albicans* wild type (CAF2), *opy2*, *OPY2* reint, *ssk1 sho1 msb2* and *ssk1 sho1 msb2 opy2* strains in the presence of HL60 at both 1:40 and 1:20 cell ratios. Viability is calculated as the ratio of colony-forming units (CFU) from the phagocyte–yeasts co-cultures versus those containing only yeasts before incubation with HL60. B) Viability of *C. albicans* wild type (CAF2), *opy2*, *OPY2* reint, *ssk1 sho1 msb2* and *ssk1 sho1 msb2 opy2* strains in the presence of HL60 in a similar experiment to (A). In this case viability is calculated as the ratio of colony-forming units (CFU) from the phagocyte–yeasts co-cultures versus those containing only yeasts after 2 hours of incubation. Data represent the averages for two independent experiments.

*C. albicans* cells attach to plastic, and because these experiments were performed in 24-well tissue culture dishes that are made of plastic, it was hard to remove the totality of the *Candida* cells from the plates. This leads to errors in the final calculation.

It was expected that CAF2 and *OPY2* reint would have the same behavior because in the last one, the *OPY2* gene was deleted and then reintroduced in its own plasmid, so it should behave as in the wild type. In neither of the representation of the results it is possible to see a similarity in the viability percentage of these two strains.

It was expected, as well, that the *opy2* mutant should be more sensitive to the action of the HL60 cells, as the triple and the quadruple mutant. As can be seen in the Figure, it is not clear a difference between the triple and quadruple mutants and the viability of the *opy2* mutant is very elevated in both graphics, so no conclusions can be taken from these tests.

Another expected conclusion was that in the 1:40 ratio, the viability should be lower than with the 1:20 ratio due to the fact that there are more HL60 to one yeast. As can be seen in the graphic A) that does not happen with all the mutants.

To have more clear conclusions, this experiment should be repeated, if possible in eppendorf vials to try to reduce the attachment of the *C. albicans* cells using a vortex machine.

# Discussion

The mechanisms of how the MAPKs cascades transmit the signals in *C. albicans* are mostly known, although how the upstream elements of the branches are able to detect and act against different kinds of stresses are not well understood yet.

Here we studied the Opy2 protein with a role in the HOG pathway, and that was first discovered in *S. cerevisiae*. This pathway is responsible for generating an adaptive response to high-osmolarity environments but has, as well, a role in response to several external stresses such as heavy metals, thermal shock and oxidative stress.

Wu *et al.* (2006) stated that Opy2 in *S. cerevisiae* interacts with Ste50 that in turn interacts with Ste11. This interaction allow the Opy2 together with Sho1 to present Ste11 to the plasma membrane, where is phosphorylated by Ste20. This activates the Ste50/Ste11 complex which interacts with Opy2 and this is believed to contribute to the HOG pathway specificity. Nonetheless, the knowledge about the *C. albicans* Opy2 is scarce.

To study more about the *CaOPY2* gene, a plasmid with this gene attached to a Myc tag was introduced into a *S. cerevisiae* strain which lacked the *SSK1* and the *OPY2* genes. With none of these genes, the response of the HOG pathway to osmotic stress is interrupted in both branches of the pathway. The introduction of the *CaOPY2* had the purpose of studying if, in this strain, that is not able to generate a response when exposed to an osmotic stress, *CaOPY2* was able to complement its osmosensitivity.

In this work we provide evidence that the *CaOPY2* gene partially complements the osmosensitivity of the *opy2 ssk1* mutant. Our data shows that the *CaOPY2* when introduced in the *S. cerevisiae opy2 ssk1* mutant is not able to match the growing of the parent wild-type strain and the one of the mutant strain where the *ScOPY2* is introduced.

When the mutant strains are grown in medium with 0.5M NaCl, *CaOPY2* is only able to partially complement the aberrant cell morphology of *opy2 ssk1* mutant. It is clear that even though some of the cells are able to restore the normal morphology, not all the cells are able to do so.

When experiments were performed to test if the *CaOPY2* was able to activate the Hog1 MAPK in the *opy2 ssk1* mutant under osmotic stress, we observed that the Opy2 from *C. albicans* is not able to activate the Hog1 MAPK, in contrary of the wild type or the mutant with the *ScOPY2*. All these observations allowed us to conclude that *CaOpy2* transmembrane protein does not play a role in signaling to HOG pathway in response to osmotic stress unlike what has been previously described in *S. cerevisiae*.

Since the HOG pathway has a role in the response to other stresses, we tested if the *CaOPY2* is able to improve the growth of the *opy2 ssk1* mutants under oxidative and cell wall stresses. *CaOPY2* was not able to improve the growth of the *opy2 ssk1* mutant under the presence of menadione and hydrogen peroxide. However, we saw that in this genetic background, the deletion of *SSK1* does not mean an increase of the sensibility to oxidative stress. When the strains were stressed with Congo red, it was seen that the *opy2 ssk1* mutant strains were able to grow better than the wild type ones. In *C. albicans* the susceptibility to these compounds is connected with Cek1 activation, when Cek1 phosphorylation is higher, the susceptibility is diminished as is the case of *ssk1* mutants (Navarro-García *et al.*, 2005). Our observation suggested the existence of a similar mechanism in *S. cerevisiae* since the expression of either *ScOpy2* or *CaOpy2* increased the growth on Congo red supplemented plates.

We showed that *CaOpy2* undergoes post-translational modifications that differ in *S. cerevisiae* and in *C. albicans*. Even though *CaOpy2* expressed in *C. albicans* and *S. cerevisiae* were appearing at the same molecular weight, when the O- glycosylation, the main way of glycosylation of Opy2 in *S. cerevisiae*, was blocked by tunicamycin, differences were seen in the pattern of the bands obtained (Yang *et al.*, 2009).

As many adaptive responses to distinct stresses require the activation of several pathways, the activation of other MAPK's was study under tunicamycin and zymolyase stresses.

When glycosylation defects are provoked by tunicamycin, we saw that in the *C. albicans* and in the *ScWT* strains both Cek1/Kss1-mediated pathway and the cell integrity pathway are activated. As in *S. cerevisiae*, in case of a treatment with tunicamycin Kss1 is activated but not Fus3, as well as SlT2 that is also activated but its phosphorylation. This result is in agreement with the previously reported by Yang and co-workers. In this paper they describe that SlT2 phosphorylation occurs very slowly and it is independent of Kss1/Fus3 (Yang *et al.*, 2009). In the *opy2* mutant strains, with *CaOPY2* we saw activation of SlT2 at 2 hours, but not of Kss1 and at 4 hours, we could not detect activation of neither SlT2 nor Kss1. By contrast with the *ScOPY2-Myc* in the *opy2* mutant both Kss1-mediated and the cell integrity pathways are activated. So, some differences in the kinetic of MAPKs phosphorylation can be detected when the *C. albicans* or the *S.cerevisiae* protein is expressed. Nevertheless, the relevance of this phosphorylation on the physiology of the cell remains unclear and deeper analyses are required.

When we exposed the different strains to a cell wall damage provoked by zymolyase, we detected activation of SlT2 in the wild type after 2 h of treatment while it was needed to incubate for 4 h to detect the SlT2 phosphorylation in the *opy2* mutant carrying the plasmids under study. When a cell wall integrity response is sensed, both the HOG and SLT2 pathways are activated, but the main MAPK activated is SlT2, which has a peak of the SlT2 phosphorylation after 1 to 2 hours of treatment (Bermejo, C. *et al.*, 2008). Bermejo and his co-workers reported that the SlT2 phosphorylation in response to zymolyase was dependent on Opy2. In our hand this was not the case since SlT2 phosphorylation was detected in the *opy2* mutant carrying the pCM189 as well as *CaOPY2-Myc-pCM189* and *ScOPY2-Myc-pCM189* after 4 h under the presence of zymolyase. Nevertheless, the conditions of the experiments were different since Bermejo and his co-workers performed the experiments at 24°C and we incubate the *S. cerevisiae* strains at 30°C. We can conclude that Opy2 is not essential to phosphorylate neither Kss1 nor Fus3 under the conditions tested. This fact is opposite to what happen in *C. albicans* where the lack of Opy2 caused no Cek1 phosphorylation upon cell wall disturbing agents. The results generated in this work link to previous observations in our lab evidenced that *CaOpy2* plays its role in the

Cek1-mediated pathways while ScOpy2 exert its function in the HOG pathway. In summary, the paralogous transmembrane proteins Opy2 play different roles in *C. albicans* and *S. cerevisiae*.

# Conclusions



The main conclusions obtained with the development of this work are described afterward:

- 1- *CaOPY2* partially complements the osmosensitivity of the *opy2 ssk1* mutant.
- 2- *CaOPY2* partially complements the aberrant cell morphology of *opy2 ssk1* mutant under osmotic stress.
- 3- *CaOPY2* is not able to activate the HOG1 MAPK in the *opy2 ssk1* mutant under osmotic stress.
- 4- *CaOPY2* is not able to improve the growth of the *opy2 ssk1* mutant under an oxidative stress.
- 5- *CaOPY2* might have a role in cell wall biosynthesis.
- 6- *CaOPY2* is not sensitive to osmotic, oxidative or cell wall stresses in the *ssk1* and the *opy2* mutants.
- 7- *CaOpy2* suffers post-translational modifications that are different in *C. albicans* and *S. cerevisiae*.
- 8- *Opy2* plays a different role in *C. albicans* and in *S. cerevisiae*.

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