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Candida albicans: Regulation of gene expression - function of CEK1
Candida albicans: Regulace genové exprese - funkce CEK1

Degree paper – Diplomová práce

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Contents

1.1 Abstrakt 4
1.2 Abstract 5
2. Background – Zadání diplomové práce 6
3. Candida albicans: Regulation of gene expression - function of CEK1 7
3.1 Materials and methods 8
3.1.1 Strains 8
3.1.2 Plasmids 9
3.1.3 Culture conditions 14
3.1.4 DNA manipulation and molecular biology techniques 15
3.1.5 Analysis and detection of proteins 15
3.1.6 Protein extract of *C.albicans* 16
3.1.7 Electrophoresis of proteins 16
3.1. 8 Transference and immunodetection 17
3.1.9 Western blotting 17
3.1.10 The Oddysey development system 17
3.1.11 Dot – blot test 18
3.1.12 Hybridisation DNA-DNA non-radioactive (Southern blot) 18
3.1.13 Luciferase Reporter Gene Assay 20
3.2 Introduction 22
3.2.1 Candida *albicans* 22
3.2.2 Sterile vegetative growth (SVG) route in S. cerevisiae 30
3.2.3 Cek 1 pathway 31
3.3 Results 37
3.3.1 CEK 1 gene expression 37
3.3.2 Study of CEK1 function 44
3.4 Discussion 48
4. Conclusion 51
5. Bibliography 52

1.1 Abstrakt

Eva Procházková

Candida *albicans*: Regulace genové exprese - funkce *CEK1*

Diplomová práce

Univerzita Karlova v Praze, Farmaceutická fakulta v Hradci Králové

studijní obor Farmacie

Cíl práce

Tato práce je zaměřená na gen C. *albicans* zvaný *CEK*1, na jeho regulace související s velikosti promotoru a vlivem dráhy sterilního vegetativního růstu na genovou expresi této mitogenem aktivované proteinkinázy.

Metody

K vyhodnocení vysledků bylo použito běžných metod genové manipulace s biologickým materiálem ke konstrukci mutant C. *albicans* a imunoanalýzy s využitím vhodných protilátek k jejich detekci. K posouzení genové exprese byla použita medota luminescenčního měření za použití luciferázy.

Výsledky

Exprese *CEK*1 se zdá být klíčová pro filamentaci kvasinek. Práce tedy spočívá v konstrukci mutant C.*albicans,* které obsahují oblast pro expresi Cek1. Mutanty s delecí různých genů, podílejících se na činnosti MAPK kaskády byly vystaveny stresovým faktorům jako je působení antimykotika tunikamycinu nebo teploty, a byl sledován vliv jednotlivých mutací na expresi *CEK1*.

Závěry

Použité promotory *CEK1* umožňují jeho expresi a zvolené mutace zbůsobují rozdílnosti v intenzite genové exprese v závislosti na čase.

1.2 Abstract

Eva Procházková

Candida *albicans*: Regulation of gene expression - function of *CEK1*

Degree paper

Charles university in Prague, Faculty of Pharmacy Hradci Králové

Farmacie

Background

This work is is aimed on *CEK*1 gene in C. *albicans* and its regulation related to the promotor of this gene and the influence of sterile vegetative growth pathway on gene expression of this mitogen – activated protein kinase.

Methods

Common methods of genetic manipulation with biological material and immunoblot assays with different antibodies were used for constructinon of C.*albicans* mutants and their detection. Luciferase assay, the luminescence method, was used for gene expression measuring.

Results

CEK1 expression seems to be a key element during the filament growth of C. *albicans*, the most invasive form of this organism. This research is based on construction of C.*albicans* mutants containing area of Cek1 expression. Mutants containing deletion of different genes involved in MAPK cascade were exposed to various stress factors like tunicamycine or temperatrure. The effect of those mutations on *CEK1* expression was observed.

Conclusions

Used *CEK1* promotors allow the expression of the gene and the mutations used in the work cause differences in intensity of gene expression according to the time.

2. Background – Zadání diplomové práce

Candida *albicans* commensal organism of human body is nowadays one of the main pathogens causing various infections. According to its ability to transform itself in more organic forms the treatment is complicated. Genome of C. *albicans* is already decoded however the function and regulation are still not well known.

This work is is aimed on *CEK*1 gene, analogue of *FUS*3 gene known from S. cerviciae, and its regulation related to the promotor size of this gene and the influence of sterile vegetative growth pathway on gene expression of this mitogen – activated protein kinase. Regulation of this gene might play very important and perspective role for further biomedical treatment.

Common methods of genetic manipulation with biological material were used for constructin of the mutants of C.*albicans* and immunoblot assays with different antibodies were used for detection of the transformation. Luciferase assay, the luminescence method, was used for gene expression measuring.

Candida *albicans* je dnes jeden z nejčastějších lidských patogenů způsobujících závažné infekce. Schopnost vyskytovat se v několika buněčných formách činí jeho léčbu a eliminaci velmi obtížnou a onemocnění často recidivuje. Genom C.*albicans* je dnes již znám ovšem funkce a regulační mechanism jednotlivých genů jsou zmapovány jen málo.

Tato práce je zaměřená na gen C. *albicans* zvaný *CEK*1, který je analogem *FUS*3 známého u S.*cervisiae*, na jeho regulaci související s velikosti promotoru a vlivem dráhy sterilního vegetativního růstu na genovou expresi této mitogenem aktivované proteinkinázy. Pochopení regulačních mechanismů je prvním krokem k zahájení úspěšné léčy pomocí metod molekulární genetiky a biomedicíny.

Ke konsttrukci mutant C. *albicans* bylo použito běžných metod genové manipulace s biologickým materiálem a imunoanalýzy s využitím vhodných protilátek k jejich detekci. K posouzení genové exprese byla použita medota luminescenčního měření za použití luciferázy.

3. Candida albicans: Regulation of gene expression - function of CEK1

3.1 Materials and methods

3.1.1 Strains

Strains used in this work are listed in the chart below:

Microorganism	*Strain*	*Genotype*	*Relevant genotype*	
E. coli	*DH5αF'*	*K12 Δ(lacZYA-argF)$_{u169}$ supE44 thi1 recA1 endA1 hsdR17 gyrA relA1 (ø80lacZΔM15) F'*		*(Hanahan,D., 1988).*
C. albicans	*SC5314*	*wild type*	*wt*	*(Gillum et al., 1984)*
C. albicans	*CAF-2*	*ura3Δ::imm434/URA3*	*wt*	*(Fonzi and Irwin, 1993)*
C. albicans	*CAI-4*	*ura3Δ::imm434/ura3Δ::imm434*	*ura3*	*(Fonzi and Irwin, 1993)*
C. albicans	CK43B-16	*ura3Δ::imm434/ura3Δ::imm434 cek1Δ::hisG/cek1Δ::hisG*	*cek1 ura3*	*Csank 98*
C. albicans	CDH12	*ura3Δ::imm434/ura3Δ::imm434 hst7Δ::hisG/hst7Δ::hisG*	*hst7 ura3*	Leberer 1996
C. albicans	*REP4*	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG sho1Δ::hisG/sho1Δ::hisG*	*sho1 ura3*	*Román 2005*
C. albicans	REP18	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG msb2Δ::FRT/msb2Δ::FRT*	*msb2 ura3*	*Román 2009*

Table 1: Used strains

3.1.2 Plasmids

Pasmid	Markers	Characteristics	Reference
pDU-L	*Cat CaURA3*	Cbluciferase in the pDARD1 *URA3* plasmid *CbLUC pDARD1 URA3*	This study
pDU8-L	*Cat CaURA3*	0.8 Kb Cek1promoter controls Cb luciferase expression. prCEK108-CbLUC-pDARD1ura3mod	This study
pDU 9-L	*Cat CaURA3*	2.1 Kb Cek1promoter controls Cb luciferase expression Cek1prE-SCbLucpDARD1Umod	This study
pDUM-L	*Cat CaURA3*	Cbluciferase in the pDARD1 URA3 plasmid, with a *Kpn*I restriction site in the 3´ARD1 flanking region *CbLUC pDARD1 URA3 mod*	This study
pDUM8-CM	*Cat CaURA3*	0.8 kb *CEK1* promoter control the expression of Cek1-Myc. CEK1pr0,8-CEK1pDAUmod	This study
pDUM8-CQ	*Cat CaURA3*	0.8 kb CEK1 promoter control the expression of *cek1w*/o poli Q-Myc	This study
pDUM8-CR	*Cat CaURA3*	0.8 kb CEK1 promoter control the expression of *cek1*^{K42R}-Myc.	This study
pDUM9-CM	*Cat CaURA3*	2.1 Kb Cek1promoter controls Cek1-Myc expression	This study
pDUM9-CQ	*Cat CaURA3*	2.1 Kb Cek1promoter controls Cek1w/o poly Q-Myc expression	This study
pDUM9-CR	*Cat CaURA3*	2.1 Kb Cek1promoter controls *cek1*^{K42R}-Myc expression	This study

pNIM1-Cek1-Myc	*Bla CaSAT1*	CEK1-Myc gene under the control of doxycycline inducing promoter	Román unpublished
pNIM1-Cek1 55aa-Myc	*Bla CaSAT1*	55 aa were deleted from the N-terminus of Cek1-Myc and expressed under the doxycycline inducible promoter	Román unpublished
pNIM1-Cek1-K42R	*Bla CaSAT1*	A *cek1*^{K42R}-Myc version of Cek1 under the control of doxycycline inducible promoter	Román unpublished
Table 2: Used plasmids			

Plasmids construction

The pDUL-8 plasmid was obtained by insertion of the 0.8 Kb *Nhe*I-*Sal*I fragment from 0.8KbprCEK1-pGEMT in the *Nhe*I-*Sal*I restriction sites of pDU-L (CbLUC in pDARD1 URA3) (Figure 1).

The plasmid was digested with *Kpn*I and *Sac*I to force homologue recombination into the *ARD1 locus* of *C. albicans*.

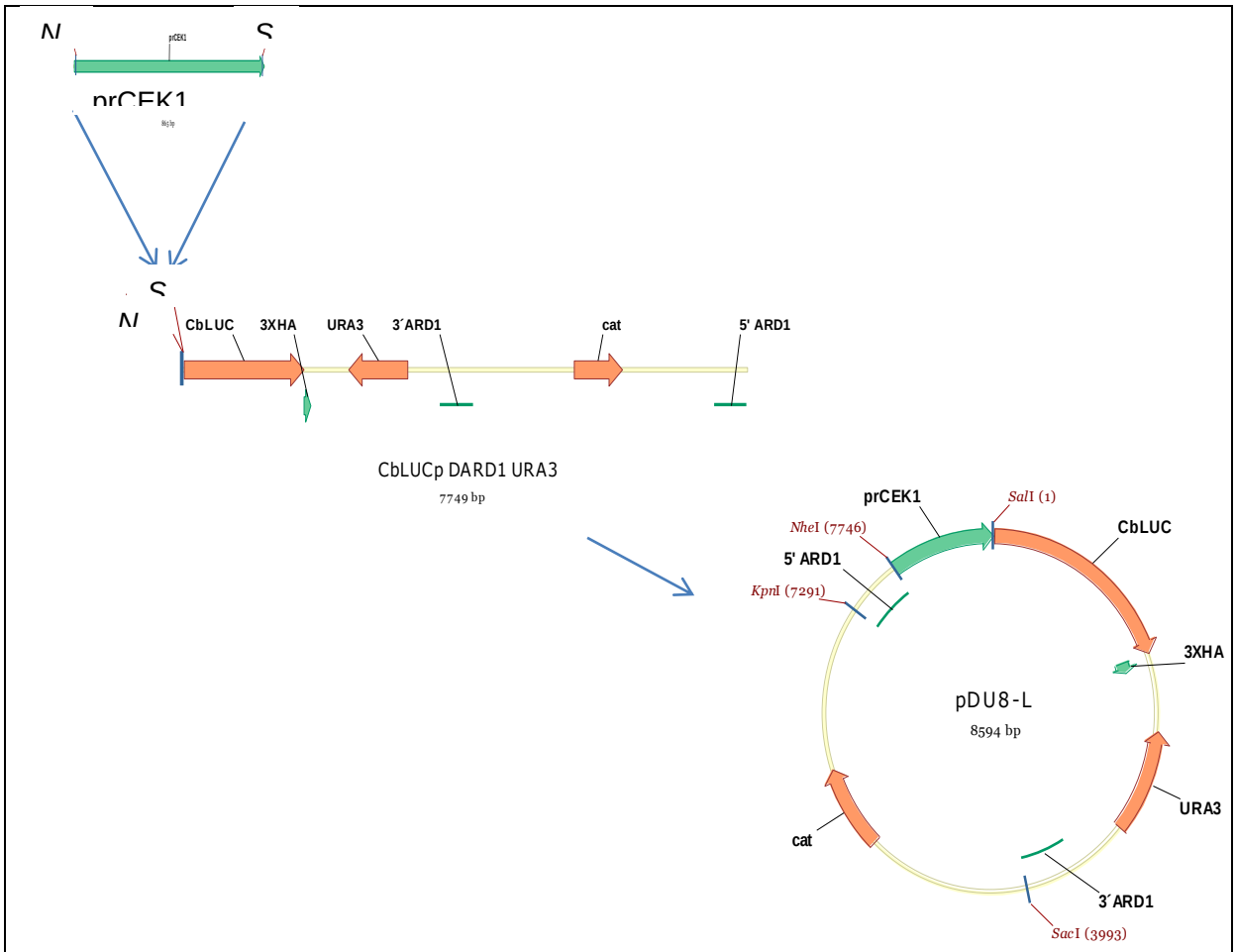


Figure 1: Construction of pDU8-L plasmid

The pDUL-9 plasmid was obtained by insertion of the 2,1 Kb *EcoR*V-*Sal*I fragment from prCEK1-pGEMT in the *Ava*I-*Sal*I restriction sites of pDU-L (CbLUC in pDARD1 URA3) (Figure 1). For this aim, the pDU-L plasmid was cut with the *Ava*I enzyme and cohesive end was filled with klenow enzyme. Afterward, the plasmid was digested with *Sal*I prior ligation. The resulting pDUL-9 plasmid was digested with *Kpn*I and *Sac*I and introduce in the genomic DNA of *C. albicans* to force homologue recombination into the *ARD1 locus.*

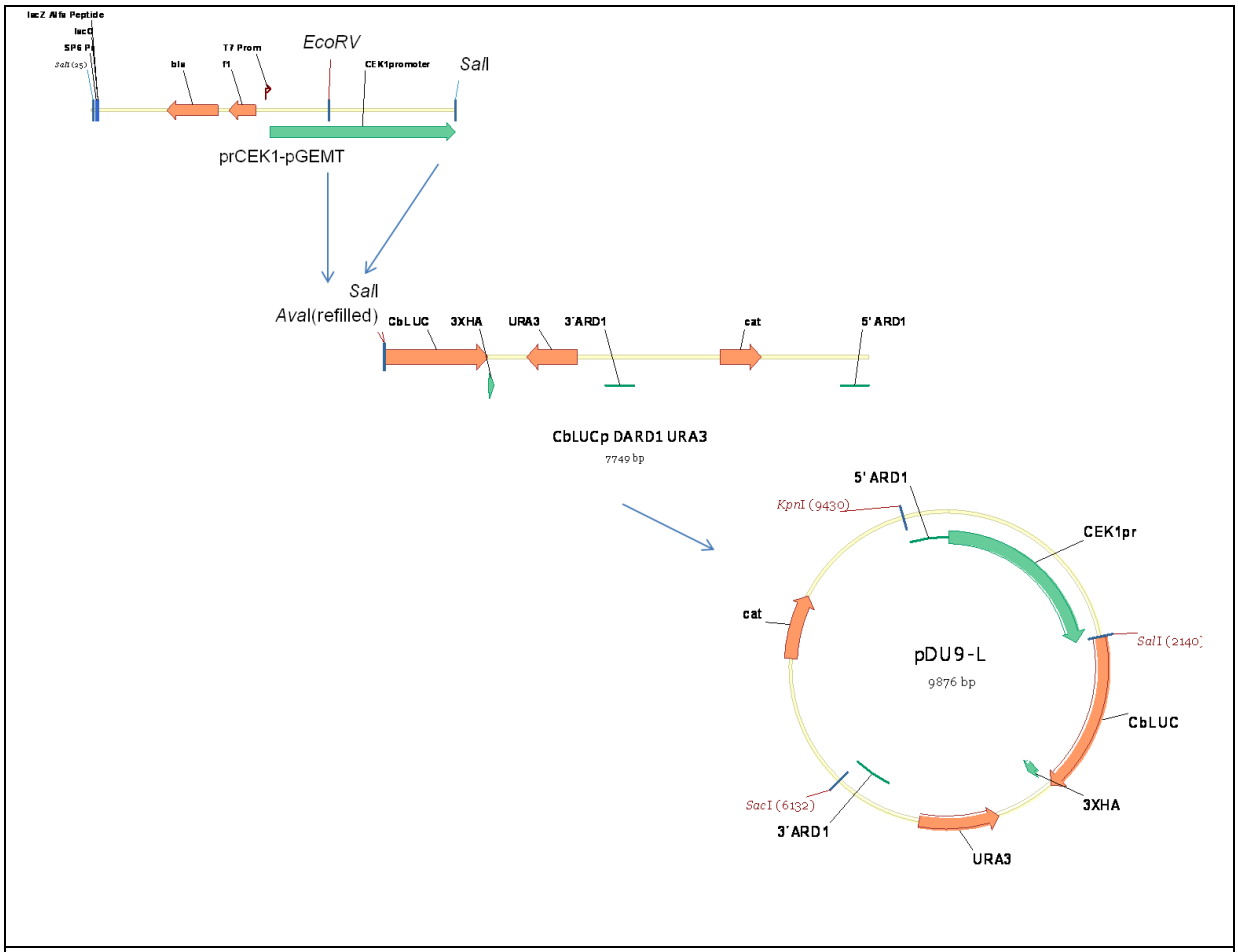


Figure 2: Construction of pDU9-L plasmid

The pDUM8-CM and pDUM9-CM plasmids were obtained by replacement of the *CbLUC* gene from the pDUM8-L and pDUM9-L, with the *CEK1* ORF tagged with Myc1 from the pNIM1-Cek1-Myc (Román unpublish data). The *CEK1*-Myc was digested with *Sal*I-*Bgl*II and inserted into *Sal*I-*Bgl*II sites of pDUM-L-8 and pDUM-L-9 plasmids (figure 3 and 4). The same strategy was pursued with the mutated version of the *CEK1* gene, *cek1*-poli Q Δ-Myc and *cek1*^{K52R}-Myc (Román, unpublish data) obtaining the pDUM8-CQ, pDUM9-CQ, pDUM8-CR and pDUM9-CR.

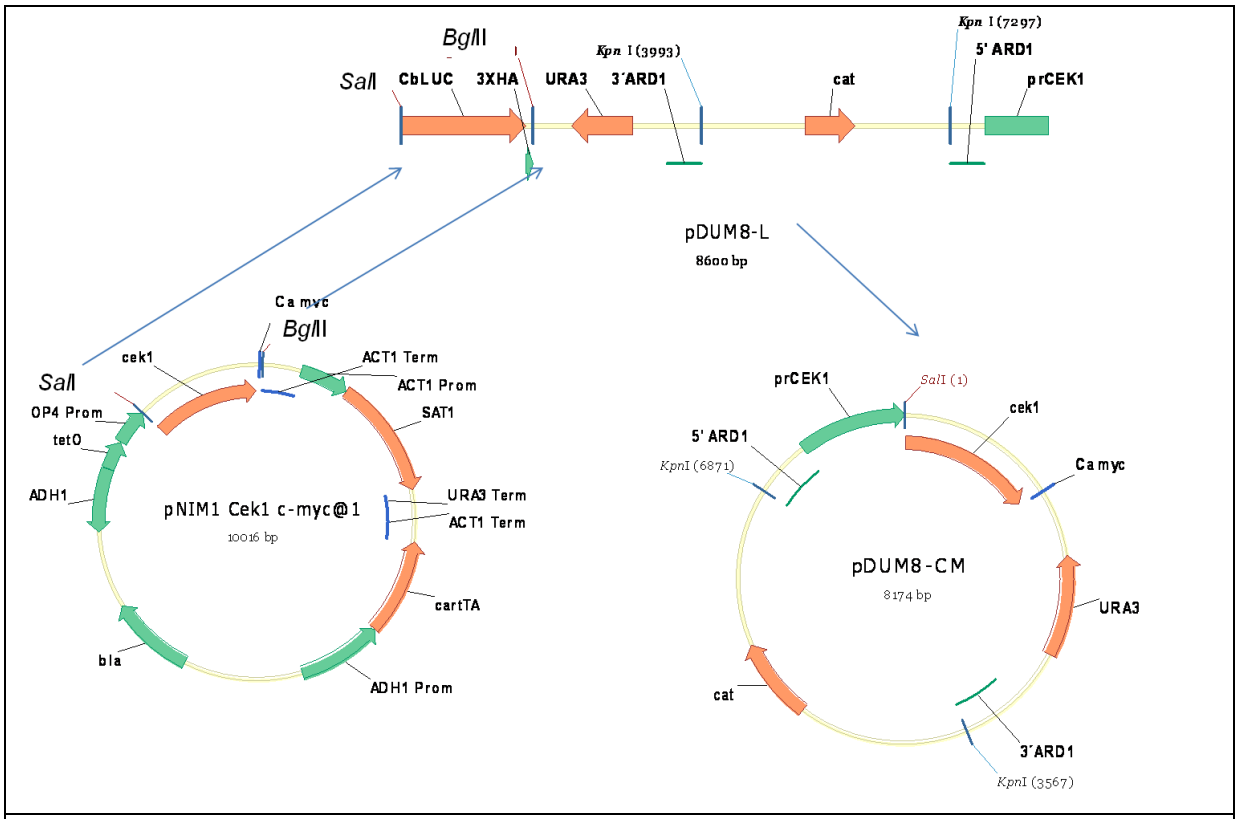


Figure 3: Construction of pDUM8-CM

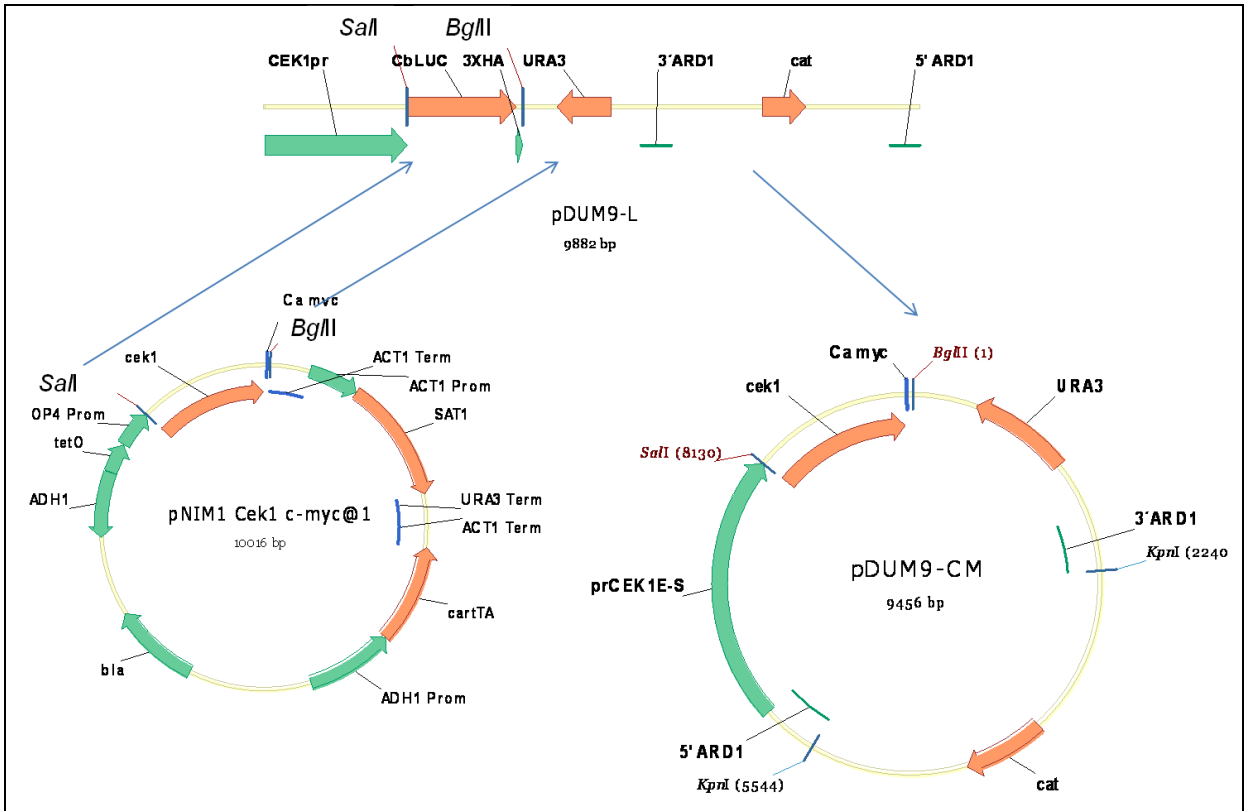


Figure 4: pDUM9-CM construction

3.1.3 Culture conditions

Growth of *E. coli* took place in liquid LB media with chloramphenicol (0,5 μl/ml) at 37°C.

C. albicans was grown in liquid or on solid media of YEPD at 37°C. Cell concentration was quantified spectrometrically at A_{600} and expressed as optical density (O.D.).

Gained transformations were grown on MM without uracil to select those who are containing the marker of *CaURA3*. All the strains are preserved at 4°C, refreshed every 30 days beyond the material conserved in 50% glycerol in -70°C.

The composition and use of the media are described in table 3.

Media	*Composition*	*Use*	**Reference**
LB (Luria-Bertani).	*10 g/l bactotriptone, 5 g/l yeastextract, 5-10 g/l NaCl*	Rich media for growth of bacteria. Supplementation with chloramphenicol (0,5 μg/ml) was used for selecting transformations containing the plasmid construction	(Sherman *et al*., 1986)
YEPD (Yeast Extract Peptone Dextrose)	*20 g/l glucose, 20 g/l peptone, 10 g/l yeast extract*	Common media for growth of yeasts and bacteria	(Sherman *et al*., 1986)
Minimal Media (MM) or Synthetic Dextrose (SD)	*20 g/l glucose, 5 g/l ammonium sulfate, 1.7 g/l basicnitrogen (Difco) and 1.92 g/lsynthetic complete mixture drop-outura-(Formedium).*	Synthetic media for cultivation of yeasts, does not permit the growth of auxotrophic strains	(Sherman *et al*., 1986)

Table 3.

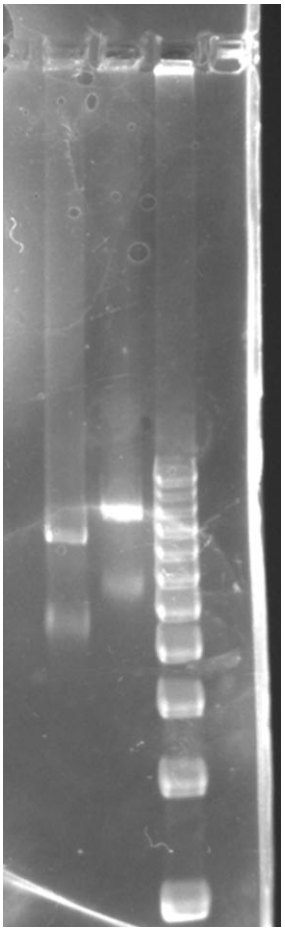
2% weight/volume (w/v) of agar was added to get solid media.

For the dilution of the media was used desionizated water prepared with the system of purification Milipore. The water, as well as the media was sterilized by autoclave during 22 minutes at 2 atm (121ºC).

3.1.4 DNA manipulation and molecular biology techniques

All the techniques of DNA manipulations such as DNA extraction, DNA electrophoresis in agarose gel (figure 5), PCR reaction, etc. used in this work followed previously standardized protocols (Sambrook, Fritsch, & Maniatis, 1989). Some protocols are specified below. DNA was purified from the agarose gel using Geneclean Turbo Kit (MB) following the procedure indicated in the kit.

Figure 5
DNA elecrtophoresis in agarose gel
Cut of plasmid DNA digested with *Sac*I and *BamH*I enzymes proved that we gained two different sizes of promotors



E. coli was transformed with thermic shock described previously by Hanahan (Hanahan, 1988). *C. albicans* was transformed following the protocol described by Köhler and c-worker that combine lithium acetate and electroporation (Köhler, White, & Agabian, 1997).

3.1.5 Analysis and detection of proteins

For immunoblot assays the overnight cultures were diluted in fresh YEPD media, procedures employed for cell collection, lysis, protein extraction, fractionation by SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose membranes have been previously described (Martín, Rodríguez-Pachón, Ruiz, Nombela, & Molina, 2000) and detailed underneath. To equalize the amount of protein loaded, samples were analyzed

measuring the absorbance at 280 nm.

3.1.6 Protein extract of *C.albicans*

Cells growing overnight were taken as stationary phase growth cells and in parallel transfer to new fresh medium. Samples were taken after different times we collected the culture and mixed it with same volume of ice in 40ml Falcon tube. Tubes were then centrifuged 2´ in 4°C and 2400rmp. Obtained cells were washed once with 1ml of cold water and kept frozen at -80°C.

For degradation of the cell wall and obtaining pure proteins we added 200 μl of cold solution lysis (50 mMTrisHCl, 10% glycerol, 1 % Triton X-100, 0.1 % SDS, 150 mMNaCl, 50 mMNaF, 1 mM sodium ortovanadate, 50 mMβ-glycerol phosphate, 5 mM sodium pyrophosphate, 5 mM EDTA pH=8, 1 mM PMSF and inhibitors of tosylphenylalaninecloromethylcetone proteases, tosylchloromethylcetone, leupeptine, antipaine, pepstatine and aprotinine at 25 μg/ml) Shake properly while kept whole time on ice. Then centrifuge at 4 ºC for 10 min and 13.000 rpm. This process was followed by spectrophotometric quantification of the protein extract at 280nm to equalize the amount of samples for electrophoresis. Referring amounts of buffer for electrophoresis (1 ml TrisHCl, 1M pH=6.8, 2 ml glycerol, 4 ml SDS 10 %, 2 mg blue bromofenol and 2 ml H_2O) were tan added to each simple and heated for 10 minutes at 99,4°C.

3.1.7 Electrophoresis of proteins

Electrophoresis was the method to separated proteins according to their molecular weight. Protein extract run over the denaturalized conditions (SDS-PAGE) in 10% polyacrylamide gel. Approximately 150 μg of protein marker (Bio-Rad) is used for comparing the molecular weights. Electrophoresis was proceed in a Bio-Rad Mini Protean II, in the buffer of electrophoresis (buffer 10X: Tris 30.285 g/l, glycerin 144.6 g/l y 10 g/l SDS) under the voltage of 150V.

3.1.8 Transference and immunodetection

For further use of protein is essential to transfer it on nitrocellulose membrane (Towbin, Staehelin, & Gordon, 1979). This operation was proceed in Bio-Rad Mini Trans-Blot device deeped in transference buffer (buffer 10X: 58 g/l Tris-base, 29 g/l glycine y 3.7 g/l SDS) during 1 hour under the 100 V.

3.1.9 Western blotting

After the transference, membranes were blocked with blocking buffer (5% separated milk in TTBS) incubated with Anti-phospho-p44/p42 MAPK (Thr^{202}/Tyr^{204}) antibody (New England Biolabs) to detect dually phosphorylated Mkc1 and Cek1 MAPKs. Primary and secondary antibodies were incubated in presence of 1% separated milk for 2 hours in room temperature or overnight in 4°C. For altering the antibodies the membrane was washed in TTBS to eliminate the rests of previous antibodies, blocking and incubating with new antibodies 1 hour in room temperature. Finally after excess secondary antibody is washed free of the blot, Detection reagent the Hybond ECL kit (Amersham Pharmacia biotech) is added according to manufacturer's conditions.

3.1.10 The Oddysey development system

Alternatively the western-blot was developed by an infrared detection method called Odyssey. This method gives the possibility to detect simultaneously two antibodies with a higher sensitivity. Methods of extracting proteins, electrophoresis and transfer are mentioned previously. Membrane was blocked for 1 hour in blocking buffer (PBS plus 2-5%BSA). Then incubated with primary antibodies (in 6ml blocking buffer, 6ml 0,1% Tween 20). Washed 4 times (PBS+0,1%Tween) before adding rabbit and rat secondary antibodies and incubating them in dark. Follow by washing wit PBS with and then without Tween 20 and scanning on Odyssey device results in bands emitted in green (800nm) and red (680nm).

The antibodies used in this study are listed in table 4

Antibody	*Characteristics*	*Distributor*
Anti-myc (9E10)	*Monoclonal*	*Santa Cruz Biotechnology Inc.*
Anti-CEK	*Polyclonal*	*Roman et al 2005*
Anti-phospho p42/44 MAPK	*Polyclonal*	*Cell signaling*
Table 4.		

3.1.11 Dot – blot test

This technique was performed to detect the Cek1-Myc fusion in *C. albicans* transformants. Tiny dots of purified protein were spilled on nitrocellulose membrane with pipette. Lay out was marked properly and the membrane was dried on the air. Then membrane was processed for immunodetection involving blocking, binding of primary and secondary antibodies and detection previously described by western blot assay.

3.1.12 Hybridisation DNA-DNA non-radioactive (Southern blot)

Extraction of genomic DNA

Culture of *C. albicans* grown in 37°C in YEPD liquid media was put into a tube and centrifuged consequently added 200µl of lysosome solution to break the cell wall followed by alkaline hydrolysis (10% NaOH 2N, 10% SDS 10%, 80%H_2O) and precipitations of all components except plasmid with High salt (sodium acetate). After centrifuge the supernatant is mixed with isopropanol to precipitate under cold conditions. Obtained DNA material is then washed with ethanol 75% and cut with restriction enzyme *EcoR*I during 2 hours at 37 ºC.

Electrophoresis in agarose gel and transferation to nitrocellulose membrane

DNA fragments were separated by electrophoresis in agarose gel (0,8 agar in TAE 1x). DNA was transferred on positively furred nylon membrane (Boerhinger Mannheim). This process includes three steps:

1) depuration (HCl 0,25M) until the genomic material turns in yellow colour.

2) denaturation (0,5N NaOH; 1,5M NaCl) until it turns back to blue

3) neutralization (1M TrisHCl pH 0,8; 1,5M NaC) followed by an hour in transferring solution (20x SSC: 80% H_20; 8,82% sodium citrate; 17,5% NaCl 3M)

Hybridization with specific probe

The Southern-blot was performed following the classical method (Ausubel, Brent, Kingston, Moore, & Seidman, 1993). The membrane was incubated with digoxigenin labelled probe at 65ºC to increase the specificity of the hybridization. After hybridization different blocking and washes steps were done and the results were detected by luminescence.

	Composition
Blocking reagent	0,1% N-lauroyl, 0,02% SDS, 5x SSC
Buffer I	0,1M Tris-HCl, 0,15M NaCl (pH 7,5)
Buffer II	2% pulverized blocking reagent solver in Buffer I
Table 5: Solutions for Southern blot detection	

Synthesis of the labelled probe by PCR

The labelled probe used for the Southern-blot was obtained by PCR using the digoxigenin marked system of Boerhinger Mannheim. The Cb luciferase ORF was amplified using the primers o-CbLucXNSup and o-ACTTermSlw . The same amount of d NTPs and Dig labelling mix dNTPs was added to the reaction mixture. The pDU-L plasmid was used as template and High Fidelity Expand as DNA

polymerase. A 1.6 kb fragment was obtained as PCR product and used as digoxigenin labelled probed.

.

NAME	*Sequence*	*Tªmeltingn*
o-CbLucXNSup	CCGCTCGAGGCTAGCAATAGT CGACGTGAATGGTTAAAAGAG	*Tm 75,4ºC*
o-ACTTermSIW	*GATACTAGTGGAATGAATGGGATG AATCATCAAAC*	*Tm 64,2ºC*

Table 6: Oligonucleotides

3.1.13 Luciferase Reporter Gene Assay

Luciferasse assay is a specific method for real-time monitoring of the temporal progression of gene expression which can help us to understand different interactions in pathogenic organism. Sensitive in vivo imaging technologies can detect low levels of light emitted from luciferase reporters, but not all the existing reporters are optimal for fungal infections.

For the luciferase assay overnight cultures growth in YEPD at 37ºC were diluted in pre-warmed fresh YPED media at 0,2 O.D. Samples were taken at different time points. Alternatively, overnight cultures were refreshed into pre-warmed YEPD liquid medium at 0.2 O.D., incubated for 4 hours and 2,5 µg/ml tunicamycin was added to the cultures. Samples were collected after 1 and 2 hour of incubation.

Cells were washed with water and diluted to 5×10^7 cells/ml in luciferase buffer (15 mM $MgSO_4$; 30 mM sodium citrate pH 2,5). Final volume of each sample came up to 500 µl. 50 µl of the sample were mixed with 50 µl of luciferin solution (1mM

luciferin in luciferase bufferd) and luminescence was measured (in an *OPTOCOMP I*luminometer) during 30 seconds in an integration mode.

Principle:

Click beetle luciferase is expressed under the control of promoter of interest; this luciferase is localized in the cytoplasm of *C. albicans* carrying the construction and cleaves D-luciferin to yield oxyluciferin and light energy.

luciferin + O_2 → oxyluciferin + light

The reaction between luciferin and oxygen is extremely slow until it is catalysed by luciferase mediated by the presence of cofactors such as magnesium ions. Energetic efficiency is very high because majority of energy gained from the reaction is transformed into the light detected in 560nm.

3.2 Introduction

3.2.1 Candida *albicans*

Candida albicans is a microorganism commonly found in the gastrointestinal tract, oral cavity, and genital area as a harmless commensal (Kavanagh, 2007). On the other hand it is an opportunistic fungal pathogen and the most prevalent cause of fungal infections, mostly known as candidiasis. The fungus is present on the skin and mucosal surfaces of many organisms, including humans, acquiring mainly a unicellular yeast-like form, while in infected tissues, different morphologies (yeast, mycelia and even chlamydospores) have been observed (Cole, Seshan, Phaneuf, & Lynn, 1991; Cutler, 1991). These types of morphologies have distinct abilities to adhere, proliferate, invade, or escape phagocytic cells and, therefore, contribute by different degrees to the pathogenesis of the infection (Pla, et al., 2006). When *C. albicans* gains access to the blood stream it disseminates to all major organs including the liver, lungs, spleen, brain, and kidneys (Odds, 1988; Anaiffie, Pinczowfski, & Louria, 1993), which have an associated high mortality rate (Kavanagh, 2007).

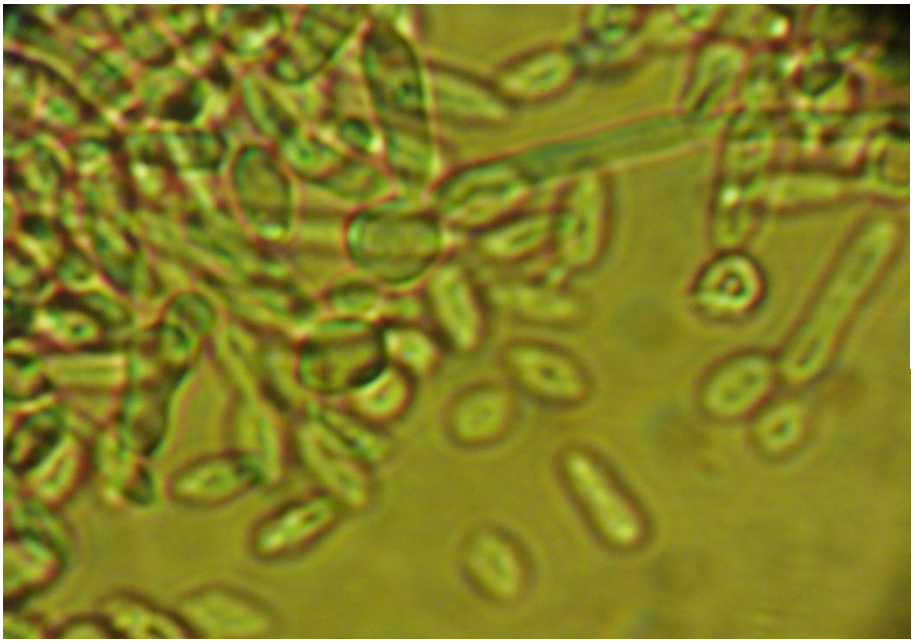


Figure 6

Overnight cultivated culture of C. albicans mutant

Model organism

Common human pathogen became soon in the middle of interest of many scientists. Currently many molecural tools for analysing *C. albicans* have been developed. It is worthwhile to remark some speciall characteristic of *C. albicans*, for example the alternative use of the CUG codon. This CUG codon is normally decoded as leucine, although it is translated as serine in *C. albicans* (Tuite, Santos, & Mick, 1995) and this cause that most standard reporter genes such as *Escherichia coli lacZ*, firefly luciferase or jellyfish GFP are not expressed in a functional form in *C. albican* (Brown, 2006). This problem was circumvented by the development of specialized reporter genes for *C. albicans*, such as Click beeatle luciferase (unpublished data; J. Pla et al.) or *Streptococcus thermophilus lacZ* (Uhl & Johnson, 2001; Srikantha, et al., 1996). Other essential components of the molecular toolbox were developed in the 90s. As Brown mentioned in his publication: “These toolboxes included improved plasmid vectors, ectopic expression vectors and reguladable promoter systems Significantly, robust procedures were established for the generation of *C. albicans* molecular mutants since this yeast is diploid and the sexual cycle is not common in nature. Lately, others disruption methods were improved further for example PCR-based gene disruption methods The latest genome sequence assembly from the Stanford Group represents the diploid genome sequence of *C. albicans.* In addition to the Stanford sequencing website, two main *C. albicans* genome databases have been established. CandidaDB was first released in 2001 and the Candida Genome Database was released in 2004” (Brown, 2006).

Release of the human annotated genome sequence of *C. albicans* strain SC5314 (d'Enfert, et al., 2005; Nantel, 2006) has been very important for understanding the mechanisms copeing in *C. albicans*. Genome sequence data has allowed a number of significant advances, including identification of *C. albicans* genes (including homologues of *Saccharomyces cerevisiae* genes), application of further molecular tools for the fungus, design of DNA microarrays for gene transcription studies, and improved identification of proteins in proteomic analysis (Kavanagh, 2007).

Biological characteristic

C. albicans is a diploid fungus, and it was thought to be asexual (Magee, Koltin, Gorman, & Magee, 1988). However C*.albicans* is indeed able to mate (Magee & Magee, 2000; Hull, Raisner, & Johnson, 2000), and the tetraploid products of these matings can be induced to shed chromosomes, such that diploid recombinants can be isolated (Brown, 2006). Hence, a parasexual cycle has now been defined. However, meiosis has not been demonstrated in *C. albicans*. Genome sequencing has revealed homologues of many *Saccharomyces cerevisiae* genes involved inthe sexual cycle. Mating processes in *S. cerevisiae* and *C. albicans* are phenotypically similar, both controlled by a conserved MAT locus (Hull & Johnson, Identification of a mating type-like locus in the asexual pathogenic yeast Candida albicans, 1999) but the regulatory arrangements that specify the mating types are different. But *C. albicans* appears to lack critical factors which are required for meiosis in budding yeast (Tzung, et al., 2001).

“Diploid *C. albicans* alternates between a yeast form and mycelia and pseudomycelia forms, which demand cell wall remodeling during its life. Cell wall of this fungus is a complex dynamic structure based on a core structure of β-(1,3)-glucan covalently linked to β -(1,6)-glucan and chitin and an outer layer or matrix composed mainly of annose-glycosylated proteins. The fungal cell wall surface represents the interface between the host and the infective pathogen. It is a valuable therapeutic target, as its highly conserved pathogen-associated molecular patterns (PAMPs) are recognized by different pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) and C-type lectins. Recognition by these PRRs mediates microbial uptake and killing as well as antigen presentation and the production of proinflammatory cytokines.” (Galán-Díez, et al., 2010)

Factors of virulence

Genomics has revealed unexpected links between metabolism and virulence in *C. albicans* which were not exposed by pre-genomic experimentation (Brown, 2006). Many *C. albicans* genes, including those encoding metabolic enzymes, have been reported to be required for virulence (Navarro-Garcia, Sanchez, Nombela, & Pla, 2001), for example a failure to adapt to environmental changes may lead to the elimination of the microorganism from the host (Roman, Cottier, Ernst, & Pla, 2009)
Also the ability of the *C. albicans* to resist various stresses is of great importance in its ability to infect and cause disease (Kavanagh, 2007). Stresses influencing *C. albicans* behavior during infection and disease include oxidative and nitrosative stresses and nutrient limitation, which triggers the development of true parallel-sided hyphae as well as pseudohyphae. Hyphal differentiation has been found to be linked to systemic virulence (Leberer, et al., 1997; Lo, et al., 1997) and the ability of *C. albicans* cells to evade macrophages (Lo, et al., 1997). Filamentous forms are also better than yeast forms at invading epithelial cells and agar surfaces in vitro (Csank, et al., 1998). Adhesins are thought to promote adherence to host tissue and colonization (Hoyer, et al., 2001). This may be the result of both the mechanical advantages of hyphal forms in the penetration of solid substrates (Gow, 1994) and the production of hypha specific hydrolytic enzymes such as some of the secreted aspartyl proteinases which also appear to contribute to virulence. Together with lipases may promote invasion, counteract host defenses and provide nutrients (Brown, 2006).

The transfer from the yeast form to the filamentous form of growth can be induced by certain chemicals, a temperature close to 37°C, and a neutral pH (Eisman, et al., 2006). High-frequency switching between different phenotypic forms might help the fungus to evade host defenses (Soll, 2002). These virulence attributes are thought to be required to differing extents during disease establishment and progression (Brown, 2006).

Infection and treatment

C. albicans is the most common fungus causing nosocomial infections remains a major diagnostic and therapeutic challenge to the clinician (Ruhnke, 2006). These infections can occur most usually in immunocompetent and immunocompromised persons.

“Between most common infections cause by *C. albicans* belong soor, the oral mycosis, and some forms of vulvovaginal discomfort. Risk factors associated with oral candidiasis include dry mouth and denture wearing, as well as poorly controlled diabetes mellitus and immunosuppression.” (Kavanagh, 2007)

The treatment is nowadays based on usage of spectrum of antimycotics, including azoles, cyclic lipopeptides echinocandins or pyrimidine analogues (Sobel & Akins, 2009) but we are still fighting with rising resistance to those drugs and also with the additional side effects of those substances. Possibilities of molecular genetic are opening new ways how to deal with those infections, promising less damage for human organism.

Signal transduction pathways mediated by MAP kinases

Extracellular signal-regulated protein kinase (ERK) or mitogen activated protein kinase (MAPK) are pivotal regulatory cascades specialized signal transduction mechanisms which transduce signals from the cell surface to the nucleus. The activation of those kinases require the phosphorylation by upstream kinases. Upon activation, some of this kinase translocate to the nucleus of the stimulated cells, where it phosphorylates nuclear targets (Khokhlatchev, et al., 1998). They are involved in a wide variety of cellular processes such as differentiation, transcription regulation and development also in the stress response to chemicals, the adaptation to environmental changes as well as cellular proliferation and cell wall integrity in higher eukaryotic cells (Kavanagh, 2007). MAPK activity is mainly regulated by dual

phosphorylation that is a ubiquitous and reversible modification that is crucial for the regulation of cellular events (Seet, Dikic, Zhou, & Pawson, 2006; Beltrao, Trinidad, Rogue, Fiedler, & Lim, 2009). Protein kinases phosphorylate (or dephosphorylate) their peptide substrates by specific phosphatases recognizing motifs that consist of a few key residues surrounding the target amino acid, concretely a TXY motif present in the kinase subdomain VIII (Román, et al., 2009).

MAPK pathways are quite conserved structures in every organism (Manjithaya, Jain, Farré, & Subramani, 2010), though, individual kinase–substrate interactions might change quickly. Phosphorylation levels within specific processes are highly conserved characteristic of the gene, hence, evolutionary changes in phosphosite position should be considered as a change of kinase regulation (Beltrao, Trinidad, Rogue, Fiedler, & Lim, 2009). In the light of putative changes in regulation it´s interesting to compare the differences in gene interaction between homologues of *S. cervisiae* and *C. albicans* (figure 9).

Structure and function of MAPK in C. albicans

Mitogen activated protein kinase cascade is homologous to many mammalian systems of the mitogen activated protein kinase (MAPK) family. These pathways generally consist of two or three steps (figure 7), as mentioned at work of Saito et al. (Saito, Posas, Takekawa, 2002), each step involves the activation of a protein kinase, which in turn activates the next enzyme in the system. Typically, each enzyme requires two distinct phosphorylation events in order to become fully active (Thalhauser & Komarova, 2010). In our case phosphorylation of tyrosine and threonine residues activates MAP kinases, but either dual-specificity or monospecificity phosphatases can inactivate them (Csank, et al., 1997).

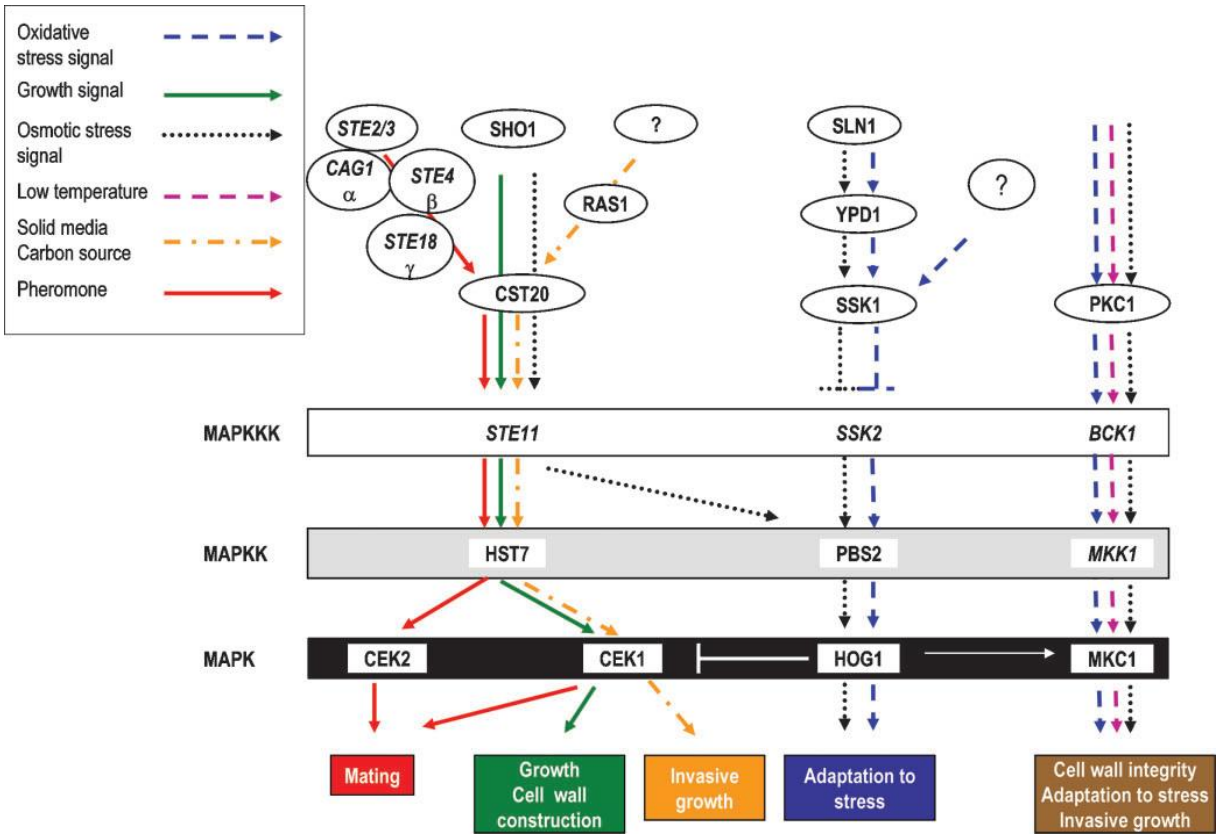


Figure 7

Main elements of the MAPK signal transduction network in C. albicans
(Alonso Monge, Román, Nombela, Pla, 2006)

Budding yeast contain at least four distinct MAPK cascades that transduce a variety of intracellular signals: mating-pheromone response, pseudohyphal/invasive growth, cell wall integrity, and high osmolarity adaptation. Although each MAPK cascade contains a conserved set of three protein kinases, the upstream activation mechanisms for these cascades are diverse (Saito, Posas, Takekawa, 2002)

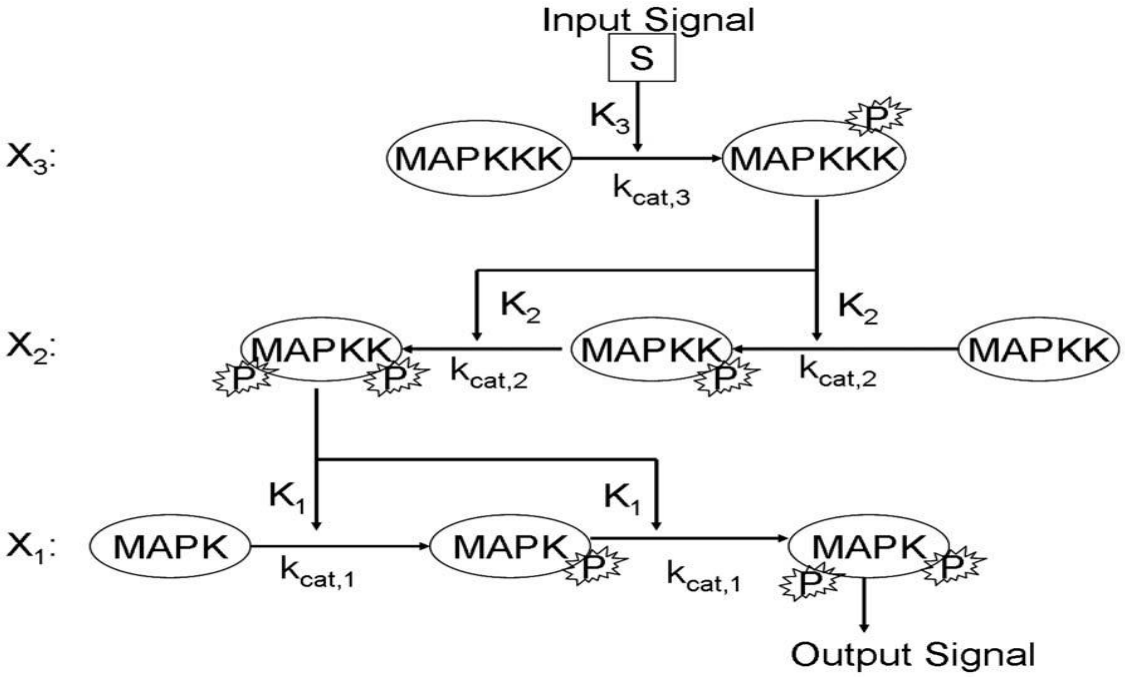


Figure 8. Cartoon representation of the canonical MAPK signaling pathway. (Thalhauser & Komarova, 2010)

Four MAPKs have been identified in *C. albicans*. Components of MAP kinase cascade can trigger the transition from the budding yeast form to a more invasive filamentous form (Csank, et al., 1997). In addition, the MAP kinase pathway plays a crucial role in cell wall integrity and mediation of the mating response triggered by pheromone (Stynen, Dijck, & Tournu, 2010). Pathways involved in filamentation are partially mediated by MAP kinases and include the Cek1-mediated pathway and the HOG pathway where the HOG pathway represses the activation of the CEK-mediated pathway. The High Osmolarity Glycerol (HOG) pathway is mediated by the Hog1 MAPK and enables adaptation to both osmotic and oxidative stress (Román, et al., 2009). Another MAP kinase pathway involved in morphogenesis, hypha formation, and virulence has been characterized through the isolation of the Cek1 MAP kinase (Román, Nombela, & Pla, 2005). The Cek1 pathway involves genes *CST20*, *HST7*, and *CPH1* that are homologues of *S. cerevisiae* genes encoding a mitogen-activated protein (MAP) kinase kinase kinase kinase (STE20), a MAP kinase kinase (*STE7*), and a transcription factor (*STE12*) (Csank, et al., 1997). The *C. albicans* Ste11 is considered to be the MAPKKK that activates Hst7

(Stynen, Dijck, & Tournu, 2010). Mutants in these genes present defects in hyphal development to a different degree on certain media and have a reduced virulence in animal models (Eisman, et al., 2006). In *C. albicans*, all these genes function in glucose-independent in vitro hyphal formation on solid surfaces (Csank, et al., 1997).

The cell integrity pathway is mediated by the Mkc1 MAPK (Navarro-García, Sánchez, Pla, & Nombela, 1995) the homolog of the *S. cerevisiae* Slt2/Mpk1 MAP kinase, plays a role in cell wall construction, the response to certain stress conditions (Navarro-García, Eisman, Fiuza, Nombela, & Pla, 2005) and biofilm formation. The other MAPK present in *C. albicans*, Cek2 participates in mating (Chen, Chen, Lane, & Liu, 2002) since double mutant *cek1 cek2* is unable to mate.

The MAP kinases Cek2 and Cek1 both interacted with Cph1 and they are believed to activate this transcription factor (Csank, et al., 1997).

3.2.2 Sterile vegetative growth (SVG) route in S. cerevisiae

In *S. cerevisiae*, Kss1 participates in the SVG pathway, which is involved in cell wall biogenesis (Eisman, et al., 2006) and we can observe some similarity in behavior of *C. albicans*. In the yeast model organism *S.cerevisiae* the SVG pathway functions, in part, to promote cell wall integrity in parallel with the protein kinase C pathway. During vegetative growth, the SVG pathway is inhibited by the mating MAPK Fus3 (Lee & Elion, 1999). „In response to mating pheromone, the MAPK Fus3p is activated on tyrosine and threonine residues by the MAPK kinase (MAPKK or MEK) Ste7p. Ste7p is activated by the serine/threonine MAPKK kinase (MAPKKK) Ste11p, which may itself be activated by the serine/threonine MAPKKK kinase (MAPKKKK) Ste20p. All of these MAPK cascade components, with the replacement of Fus3p with the homologous MAPK Kss1p, are also involved in activating the Ste12p transcription factor to drive filamentous growth in response to starvation” (Csank, et al., 1998).
“*S. cerevisiae* diploids cells, when exposed to severe nitrogen limitation, start to grow as chains of attached elongated cells which invade solid surfaces, rather than

growing as individual budding cells. In response to nutrient starvation, *S. cerevisiae* haploid cells also start to grow invasively, but in a more random direction than diploid cells. Similarily, nutrient starvation is a potential environmental signal for *C. albicans* in different microenvironments including the spleen and liver, but serum is the best inducer of the true parallel-sided hyphal form. In both *S. cerevisiae* and *C. albicans*, environmental changes induce filamentous growth through at least two parallel signal transduction pathways. Cells with homozygous deletions of both of two independently regulated putative transcriptional activators (called Ste12p and Phd1p in S. cerevisiae and Cph1p and Efg1p in *C. albicans*) are locked in the yeast phase and cannot grow filamentously.“ (Csank, et al., 1998)

The same MAPK module Ste11, Ste7 and Fus3 acts in the pheromone response or mating pathway. Commonly the system responds to a mating factor secreted by a nearby cell of opposite type. The factor binds to and activates a G-protein coupled receptor, which in turn activates a heterotrimeric G protein, which is responsible for activating the kinase cascade (Thalhauser & Komarova, 2010). In the yeast system (*S. cervisiae*), G protein activation leads to the activation of a MAPKKK, Ste11 (Bardwell, 2004). Ste11 phosphorylates two serine or threonine residues at conserved positions in the activation loop of its target MAPKK Ste7, which is a dual-specificity (serine/threonine and tyrosine) protein kinase (Chen & Thorner, 2007). Ste7 has two possible target MAPKs, Fus3 and Kss1 (Bardwell, 2004) While most of the MAPK cascade components are shared for the different development pathways, process-specific factors help to guide the specificity of response (Madhani & Fink, 1997).

3.2.3 Cek 1 pathway

Cek1p belongs to the ERK family of MAP kinases and is closely related to the *S. cerevisiae* kinases Kss1 and Fus3 and it encodes kinase, that was first identified in a screen of *S. cerevisiae* (Whiteway, Dignard, & Thomas, 1992) by its ability to interfere with the cell cycle. Although CEK1 overexpression does not reestablish mating in a *fus3 kss1* strain, disruption of the kinase domain of *CEK1* blocks its ability to interfere with pheromone arrest. It was later shown to be an important mediator of filamentous growth in this microorganism (Csank, et al., 1998) and within

a cascade that contributes in cooperation with cAMP in the morphological switch characteristic of this pathogenic fungus (D'Souza & Heitman, 2001)
Deletion of *CEK1* results in defects in hyphal formation and reduced virulence in certain animal models (Román, et al., 2009) because the cells are defective in shifting from a unicellular budding colonial growth mode to an agar-invasive hyphal growth mode when nutrients become limiting on solid medium with mannitol as a carbon source or on glucose when nitrogen is severely limited. Reduced virulence is also evidenced when the *MKC1* or the *HOG1* MAPK genes are deleted (Alonso-Monge, et al., 1999) although they repress the morphogenesis and Cek1 cause its induction. In addition to this role in dimorphism and virulence, the Cek1-mediated pathway also participates in cell wall biogenesis as *cek1* – and *hst7* and *sho1* mutants as well – are sensitive to compounds that interfere with its construction (Eisman, et al., 2006).

Another experiment demonstrates involvement of the Cek1 MAPK in masking β-glucan within the *Candida* cell wall. Disruption of *CEK1*-mediated MAPK pathway causes enhanced cell wall beta-glucan exposure, triggering immune responses more efficiently than the wild type (Galán-Díez, et al., 2010).

Cek1 become phosphorylated when cells resume growth in a fresh medium. Cek1 phosphorylation requires both a low cell density and a fresh medium suggesting the implication of QS molecules in the activation of this MAPK (Román, et al., 2009).
If we consider signal transduction pathways as sensing mechanisms, it is appropriate to assume that some of them could be under quorum-sensing (QS) regulation. Although QS is frequent in bacterial systems (Miller & Bassler, 2001), it is less documented in the eukaryotic world. In *C. albicans*, farnesol and tyrosol (Chen, Fujita, Feng, Clardy, & Fink, 2004) have been identified as QS molecules. Those molecules behave in an opposite manner (Chen et al., 2004). Farnesol is actively released by the cells during growth, accumulates in stationary-phase cells where inhibits filament formation (Hornby et al., 2001) and biofilm formation (Ramage et al., 2002), so this compound regulates the dimorphic transition in this fungus. In addition, it also represses Cek1 activation and on the other hand it induces the activation of Mkc1, the MAPK of the cell integrity pathway. The role of farnesol in Cek1

phosphorylation is independent of the Chk1 histidine kinase, a putative QS sensor, as revealed by genetic analysis (Román, et al., 2009).

Others stimuli that trigger Cek1 phosphorylation are an increase of temperature or cell wall disturbing agents such as tunicamicyn or congo red. Interestingly, the transcription of Cek1 protein also increased with some of the signals that cause its phosphorylation. “Among the factors that more critically influence Cek1 transcription, temperature is the most important. Physiological (37 °C) and higher (42 °C) temperatures induce Cek1 mRNA transcription as well as an increased amount of Cek1, a situation that may reflect its importance in cellular physiology and its adaptation to commensalism“ (Román, Nombela, & Pla, 2005).

The HOG pathway is important in controlling the specificity of the activation signal to the Cek1 cascade: osmotic stress can activate Cek1 in the absence of *HOG1* (Román, Nombela, & Pla, 2005) and a deficient HOG pathway results in enhanced basal Cek1 phosphorylation levels (Navarro-García, Eisman, Fiuza, Nombela, & Pla, 2005) and a hyperfilamentous phenotype in *hog1* cells (Alonso-Monge, et al., 1999). In an inverse manner, transient activation of the HOG pathway by farnesol could repress Cek1 phosphorylation.

In *S. cerevisiae*, unphosphorylated (active) Ssk1p is selectively degraded by the Ubc7p-dependent ubiquitin–proteasome system to downregulate the HOG pathway after the completion of the osmotic adaptation (Sato, Kawahara, Toh-e, & Maeda, 2003). In a similar manner, *C. albicans* may degrade Cek1 once it ensures its function.

“Cek1 levels – but neither Mkc1 nor Hog1 – are regulated not only posttranslationally by phosphorylation but also by proteolytic degradation through the ubiquitin proteasome pathway as revealed by the use of a conditional lethal protein degradation mutant, *pre1*. Obtained data indicate that Cek1 synthesis is not triggered only under defined conditions, but that Cek1 levels are also regulated by protein degradation, suggesting a short half-life and increased protein turnover for this protein. Cek1 MAPK can be considered as a short-lived protein and a target of the proteasome degradation machinery.” (Román, et al., 2009)

Sho1 and its role in activation of Cek1

“Sho1 is essential for the activation of the Cek1 MAP kinase under different conditions that require active cell growth and cell wall remodeling, such as the resumption of growth upon exit from the stationary phase. Blockage in cellular growth results in a failure to detect phosphorylated Cek1. Cek1 is activated in certain HOG pathway mutants to compensate for their defects in cell wall architecture. Deletion of *SHO1* in an *ssk1* and a *hog1* background suppresses the Congo red resistance phenotype as well as Cek1 basal activation in these mutants. Cek1 is also activated in response to the transfer from the stationary phase to the exponential mode of growth and Sho1 controls this process. The activation is evident as early as 15 min after dilution of the culture is dependent on the growth inhibition caused by protein synthesis inhibition, and takes place in mutants of the HOG pathway were Cek1 is hyperactive. In fact, biochemical analysis has revealed that Cek1 is phosphorylated via the transmembrane protein Sho1 and the MAPKK Hst7” (Román, Nombela, & Pla, 2005) upon growth resumption from the stationary phase.

Hst7 is responsible for Cek1 phosphorylation

„Candida *albicans* was thought to be asexual until the discovery of the MTL loci homologous to the mating type (MAT) loci in Saccharomyces cerevisiae led to the demonstration that mating is possible. By analysing disruptions of three of the genes in the MAPK pathway which is involved in filamentous growth in both S. cerevisiae and C. *albicans* and is known to control pheromone response in the former fungus was found that in HST7 and CPH1 mutantsmating was blocked“ (Magee, Legrand, Alarco, Raymond, & Magee, 2002). As far as activation of Cek1 completely disappeare in *hst7* mutants its quite clear that that the Hst7 MAP kinase kinase is required to phosphorylate the Cek1 MAP kinase (Eisman, et al., 2006).

Superficial signaling mucinMsb2

In S. cervisiae Msb2 is a cellsurface glycoprotein which functions at the head of the filamentous growth MAPK pathway (Cullen, et al., 2004). The pathway is regulated by this signaling mucin that mediates signaling through the RHO guanine nucleotide triphosphatase (GTPase) (Vadaie, Dionne, Akajagbor, Nickerson, & Krysan, 2008). Msb2 is localized to polarized sites on the cell surface and interacts with Cdc42 and with the osmosensor for the high osmolarity glycerol response (HOG) pathway (Cullen, et al., 2004). Protein is processed in its extracellular domain by the aspartyl protease Yps1p, and release of the extracellular domain is required for filamentous growth pathway activation (Vadaie, Dionne, Akajagbor, Nickerson, & Krysan, 2008). Induction of *MSB2* expression depends on starvation and is regulated by RAS protein (Chavel, Dionne, Birkaya, Joshi, & Cullen, 2010). Interestingly, we cen observe hyperactivation of the filamentous growth pathway Msb2p lacking the mucin homology domain (Cullen, et al., 2004), which is connected to a cytoplasmic domain that regulates RAS/RHO GTPases and their effector MAPK pathways (Carraway, Funes, Workman, & Sweeney, 2007). Activated versions of Msb2p were dependent on Sho1p for MAPK signaling which indicate that MAPK signaling is initiated by Msb2p and propagated through Sho1p (Vadaie, Dionne, Akajagbor, Nickerson, & Krysan, 2008). In *C. albicans* Mbs2 protein is required for Cek1p phosphorylation in response to cell wall stress (Roman, Cottier, Ernst, & Pla, 2009)

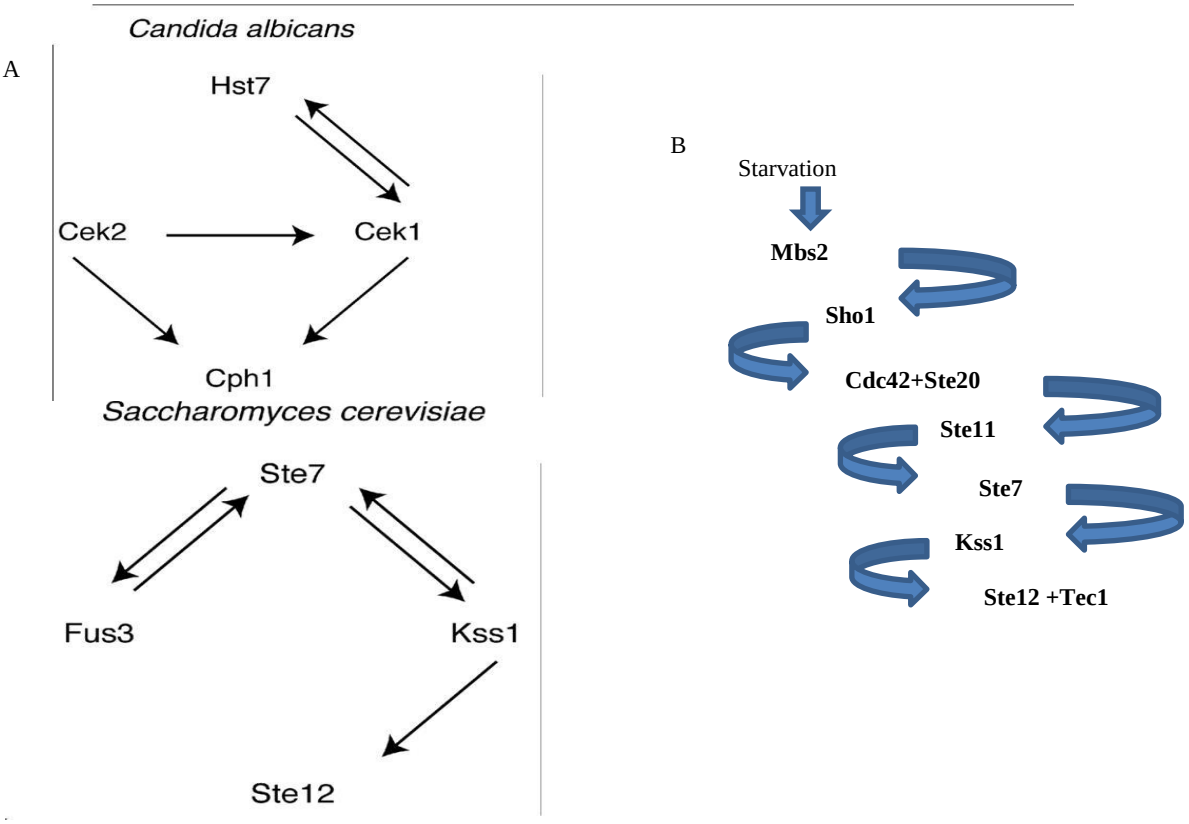


Figure 9

A

Differences in relations between MAPKs in C. albicans and S. cervisiae (Stynen, Dijck, & Tournu, 2010)

B

Stress activated cascade of S. cervisiae

3.3 Results

As presented in the background part *CEK* 1 MAPK is an important gene for adaptation and invasivity of *C. albicans* and participates in the process of filamentation (Román, et al., 2009). This route is also involved in cell wall biogenesis, as either *cek1* mutants or mutants defective in their phosphorylation are sensitive to compounds such as Congo red, calcofluor white, caspofungin, or zymolyase (Román, Nombela, & Pla, 2005).

Remarkably the amount of *CEK1* protein as well as the mRNA transcript increased with the temperature (Román, et al., 2009) and in the presence of tunicamycin (Roman, Cottier, Ernst, & Pla, 2009). In the other hand Cek1 is degraded by the proteasome system. These facts suggest that the amount of Cek1 protein is tightly regulated, thus expression of *CEK*1 responds to different environmental conditions.

In the present work we aimed to analyze the extracellular signals that induce the expression of *CEK*1 and, in parallel if the expression of *CEK*1 under its own promoter is important for the function of this MAPK.

3.3.1 CEK 1 gene expression

Integration of the Cb luciferase as reporter gene under the control of CEK1 promoter

As far as the exact size of *CEK1* promoter which is essential for its expression is not yet confirmed and in the genome of *C. albicans* we can find unusually wide area forgoing *CEK* 1 ORF we proposed to analyze this promoter. We rationed that this long sequence plays a role in the regulation of the expression and that different signaling molecules influence the expression of the gene.

Promoter	Molecular weight
CEK1pr0,8	849bp
CEK1prE-S	2149bp

Table 7: Used promoters

In order to study the role of the promoter we constructed two plasmids carrying the Click beetle luciferase under the control of *CEK1* promoter. The reporter system used for *C. albicans* constructed by members of our group was using luciferase obtained from the click beetle insect and modified with codons containing amino acids commonly occurring in *C. albicans*. Two sizes of this promoter were analyzed (table 7) to identified differences in the Cek1 expression. These plasmids called pDU8-L and pDU9-L with different sizes of CEK1 promoter were introduced into mutant *C. albicans* strains (table 1) with deletion of different genes of SVG pathway.

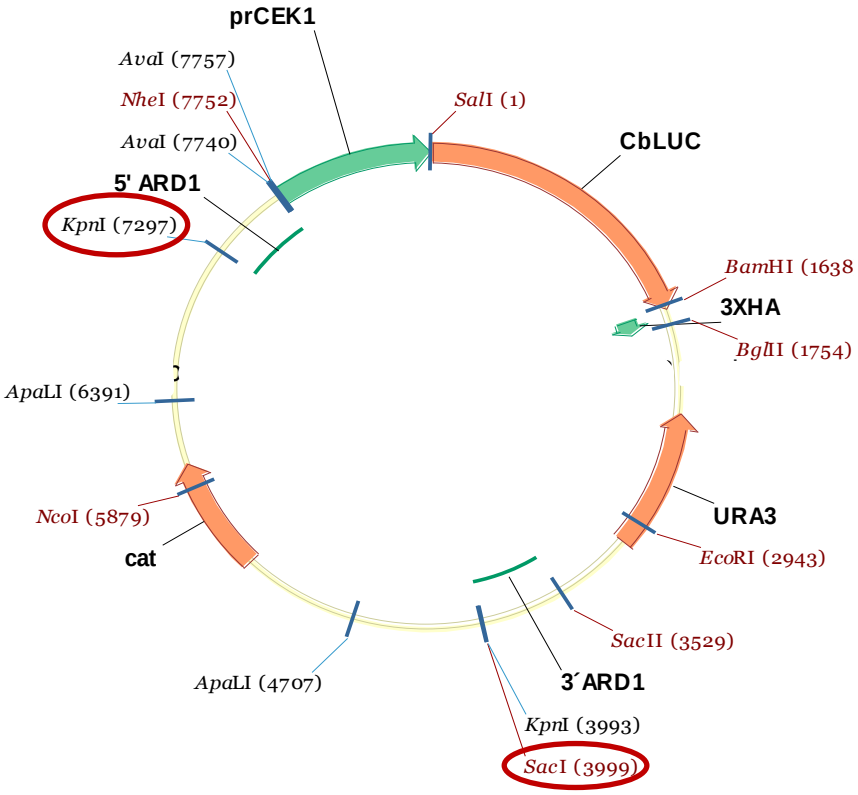


Figure 10

Plasmid construction containing especially constructed CbLUC area and *ARD* 1 locus.. It was digested with KpnI and SacI enzymes in order to integrate the construction in the *ARD1 locus* of *C.albicans* stains.

We confirmed the correct integration by Southern blot. We used as external probe the 5´ sequence of the *ARD* 1 to confirm the correct position of the construction and the Cb luciferase sequence as internal probe to confirm that only one copy was integrated in the *C. albicans* genome.

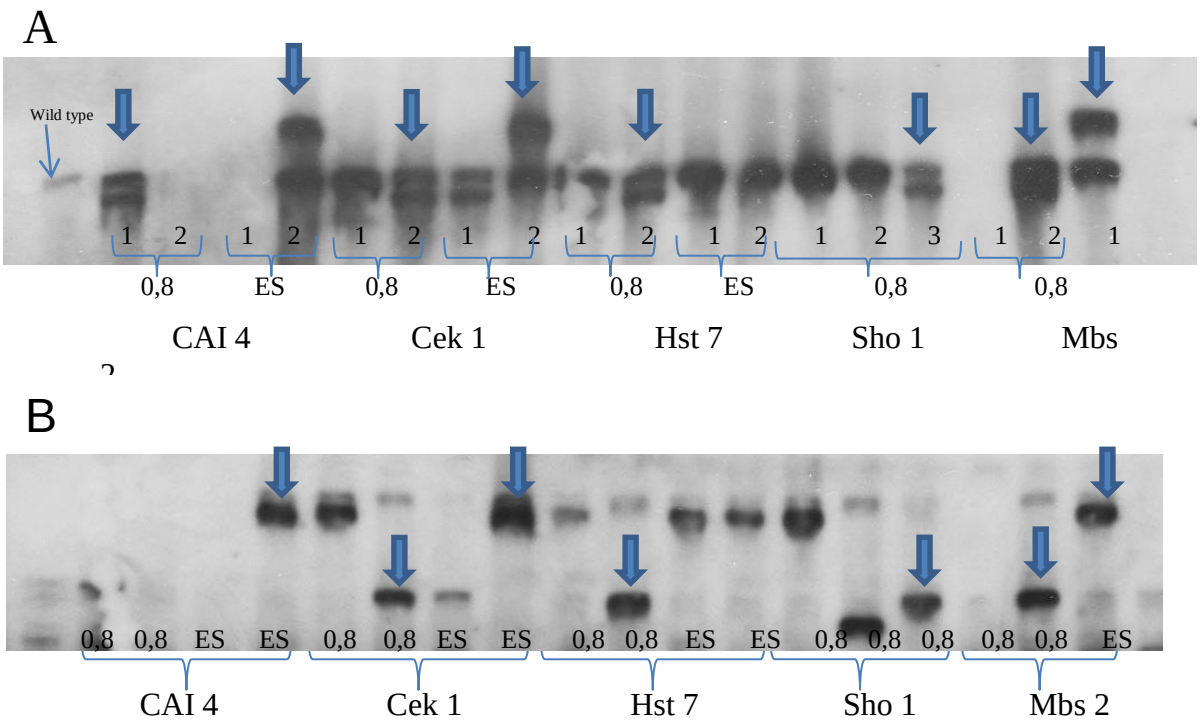


Figure 11

Southern blots with ARD1 [A] and Clickbeetle luciferase [B] probe proved that our construction was transformed corect in mutants marked above with blue arrows
(CAI 4: short promoter (0,8) clone 1, long promoter (ES) clone 2; c*ek* 1: 0,8 clone 2, ES clone 2; *hst* 7: 0,8 clone 2; s*ho* 1: 0,8 clone 3; m*bs* 2: 0,8 clone 2, ES clone 1)
DNA was digested with *EcoR*I, so the size of expected bands with bound antibodies was 7,4 Kb for short promoter and 4,08 Kb for long promoter. *ARD 1 locus* in wild type is recognisible as a band in 6,1 Kb.

Resume of growth induces CEK1 expression

Adaptation to stress is an essential mechanism for every living cell to ensure its survival under nonoptimal conditions. In this sense importance of the Cek1- mediated pathway which is involved in morphogenesis and hyphal formation (Whiteway, Dignard, & Thomas, 1992) (Whiteway, Transcriptional control of cell type and morphogenesis in Candida albicans, 2000) is quite clear. Biochemical analysis has revealed that Cek1 is phosphorylated via the transmembrane protein Sho1 and the MAPKK Hst7 (Román, Nombela, & Pla, 2005) upon growth resumption which means that Sho1 protein plays relevant role in the activation of the Cek1 MAP kinase in C.albicans (Román, et al., 2009) and defects in protein glycosylation cause its constitutive and *SHO1*-dependent activation. Also the HOG pathway is important in controlling the specificity of the activation signal to the Cek1 cascade because osmotic stress can activate Cek1 in the absence of *HOG1* (Román et al., 2005).

Light emission was detected in whole set of mutants carrying the short and long promoter. We focused on short promoter regulation for further experiments since a similar light signal was detected.
The cultures growth overnight at 37ºC in YPD were transferred to fresh pre-warmed medium and samples were taken after 1 and 2 hour of incubation in the same conditions (YPD at 37ºC). The expression of *CEK1* was quantified using a luminometer. All mutant strains as well as wild type strain were able to induce the expression of the gene reporter (Figure 12). Some differences were detected in the level of induction and the length of the induction.

To further characterize these differences similar experiments were performed taken samples at half an hour, 1, 2, and 4 h after refreshing the cultures (figure 13). The Cek1 expression reached the peak after 30 min or 1 hour of incubation going down gradually. No significant differences were detected among strains. We can conclude that Cek1 expression is induced when cultures are transferred to fresh pre-warmed medium and that this induction is not dependent on upstream elements of the SVG pathway.

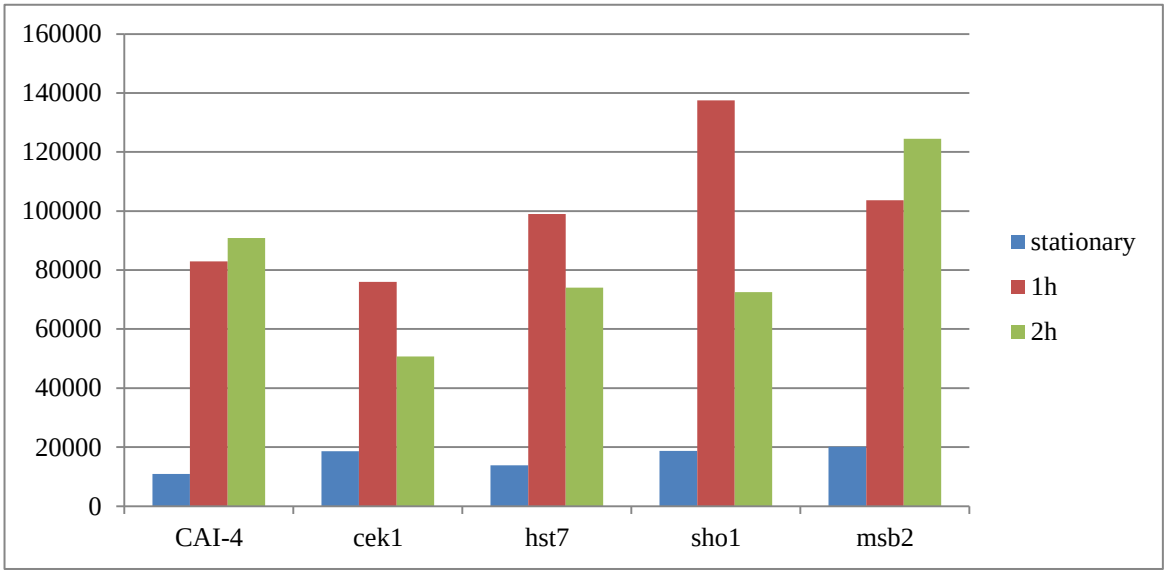


Figure 12

Absolute values of emited signal from cells with CEK1 0,8 promoter obtained from luciferase assay. Cultures were cultivaded overnight in 37°C in YPED medium then refreshed in prewarmed YPED medium and cultivated at 37°C. *CEK*1 expression of the samples taken after the cells entered the exponential phase of growth (blue column) and then after 1 and 2 hours of exponential growth was measures in luminometer. We can notice huge expression in *sho1* mutant which is then rapidly decreasing and slightly increasing expression in wild type and *mbs2* mutant.

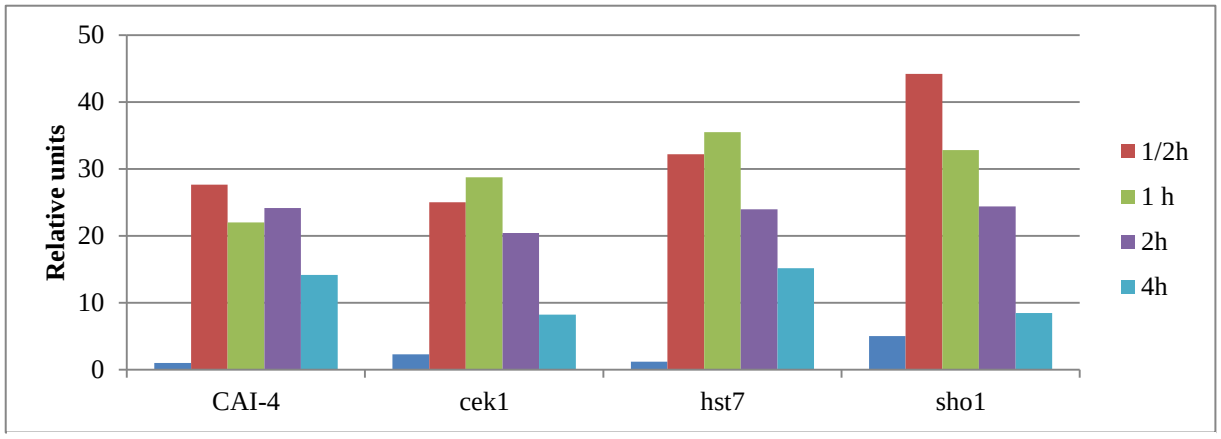


Figure 13

Relative values of *CEK*1 expression measured by Cb Luciferase activity declare decreasing expression after 4 hours of exponential growth and low relative increase of *CEK*1 expression after delection of *cek*1 and *sho*1.

As mentioned above for constructing our mutants we had available two different sizes of *CEK*1 promoter (table 1) and it was not clear whether their length is playing some role in the regulation of gene expression. We analyzed the expression of both constructions in wild type CAI 4 and *msb2* mutant. Similar results were observed in both strains and both promoter sizes (figure 14). The results declared that the size of the promoter is in both cases sufficient to induce gene expression. Nevertheless the existence of enhancers and silencers in the sequence of longer promoter are expected, further experiments are required.

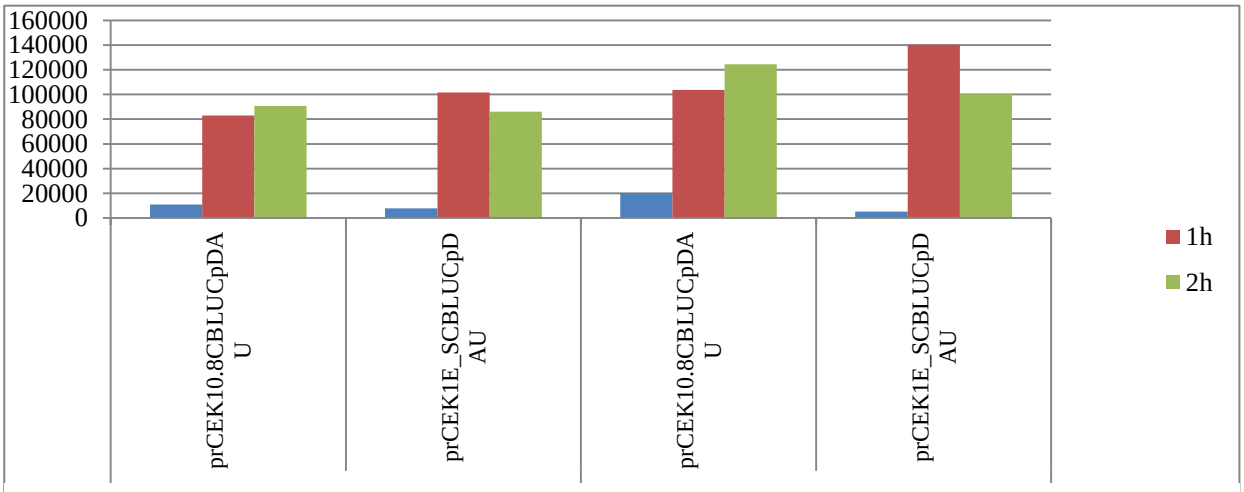


Figure 14

Mutants with different promoter size gave in luciferase assay different expression levels. Short promoter increased the expression even after two hours an in opposite in both cases both long promoter mutants show lower expression in declared time according to stationary phase (blue column).

Tunicamycin as CEK1 expression inducer

Tunicamycin is a drug that blocks with glycosylation in *S. cerevisiae* (Kuo & Lampen, 1974) and therefore causes cell wall damage. It also induces phosphorylation of Cek1 MAPK in *C. albicans* and increased the mRNA *CEK1* transcript (Cantero, et al., 2007; Román, et al., 2009).

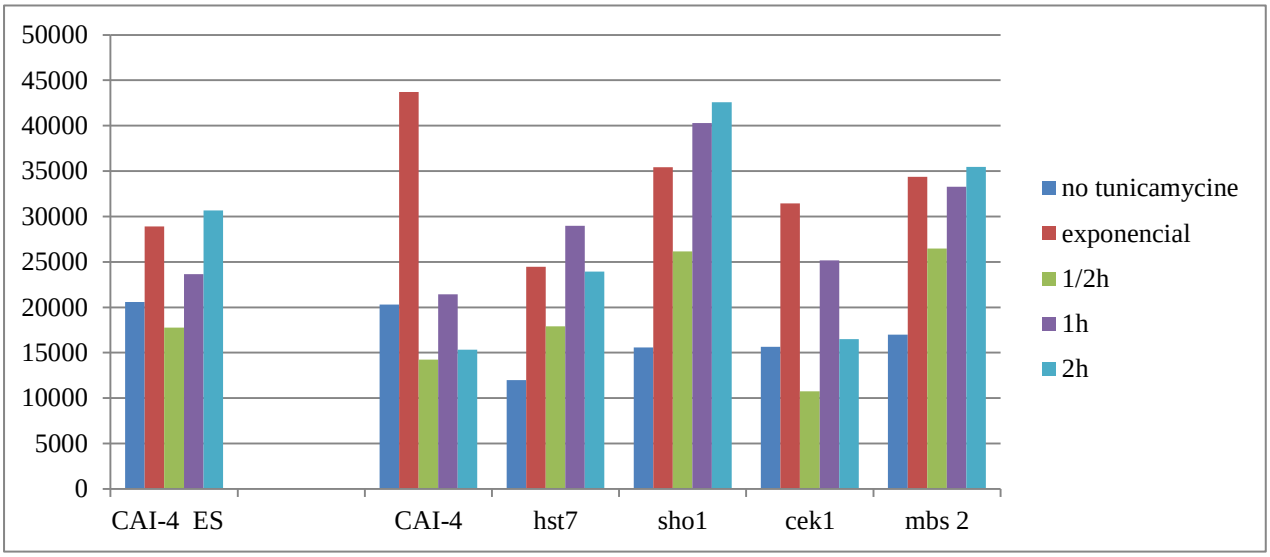


Figure 15

Mutants with different promoter size gave in luciferase assay different expression levels. Short promoter increased the expression even after two hours an in opposite in both cases both long promoter mutants show lower expression in declared time.

In order to verify that this drug induced the expression of *CEK1* we quantified the light emission of our gene reporter upon addition of tunicamycin and others cell wall disturbing agents such as Congo red. Cultures growth overnight in YPD at 37ºC were transferred to fresh medium to 0,2 OD and then incubated since they reached 1 O.D. thus, they were exponentially growing. Tunicamycin was added to a final concentration of 2,5 µg/ml and Congo red to 50 µg/ml. Congo red did not increased Cek1 expression at all even the light emission was significantly decreased

suggesting that either the stress was too high or Congo red reduce the expression of the MAPK: Different experiments were performed to analyzed the effect of tunicamycin in *CEK1* expression but results were not conclusive. Figure 15 shows sample of the experiment performed, an increase of the signal was detected just after the addition of tunicamycin but this level decreased after half an hour reaching a new peak after two hours of incubation. This not-expected pattern of regulation requires further analyses to verify the results observed.

3.3.2 Study of CEK1 function

Apart to the study of the regulation of Cek1 we aimed to analyze the function of this MAPK. Previous experiments suggested that protein level is important for a correct function since overexpression of the gene (under the control of tetracycline induce promoter, data no shown) did not complement the sensitivity to cell wall disturbing agents of a *cek1* mutant. Taken advantage of plasmids carrying two size of *CEK1* promoter (called pDU8-L and pDU9-L) the luciferase gene was changed by *CEK*1 ORF fused to Myc a tag that allow us to detect it easily by Western-blot.

In addition, *CEK1* mutated versions were introduced in similar plasmids. Deletion of first 55 amino acids of *CEK1* gene leaded to a protein lacking the poly glutamine (poly Q) fragment. This poly-Q is specific for *C. albicans*, it is not present in *S. cerevisiae* and its function remains unknown. The substitution of a lysine (K) for an arginine (R) in position 42 disables the phosphorylation of the MAP kinase and inactivates further action dependent of MAPK pathway in S*. cerevisiae*. A similar mutation of *CEK*1 was integrated in our plamids.

Mutant CEK1pr0,8	**Mutant CEK1prE-S**
prCEK10.8CEk1Myc	prCEK1E-SCEK1Myc
prCEK10.8CEk1Myc R	prCEK1E-SCEK1Myc R
prCEK10.8CEk1Myc w/o poly Q	prCEK1E-SCEK1Myc w/o poly Q

Table 8: Used mutations

Detecting the mutants

Once that the constructions were obtained and checked, we tried to integrate them in a *cek1* mutant in order to analyze the relevance of its own promoter and, on the other hand, the role played by the poly-Q tale and the non-phosphorylated version.

For further testing of Cek1 function was necessary to verify our constructions were transformed correctly. The techniques of protein extraction and immunodetection were used to confirm the integration of the plasmids.

Firstly, we used anti phospho p44/p42 antibody recognizing dually phosphorylated (Thr^{202}/Tyr^{204}) molecules of MAPK such as Mkc1 and Cek1 in *C. albicans*. Different clones obtained after transfom *cek1* mutant were tested. Stationary growing culture and after 1h after refreshing in fresh medium were taken and processed for Western-blot analyses. The MAPK phosphorylation pattern was not as expected since any Cek1 phosphorylation was detected and Mkc1 appeared to be phosphorylated in exponentially growing cultures. When the same blot was incubated with antibodies that recognize the Myc tag, no signal was observed suggesting that the different version of Cek1-Myc were not integrated.

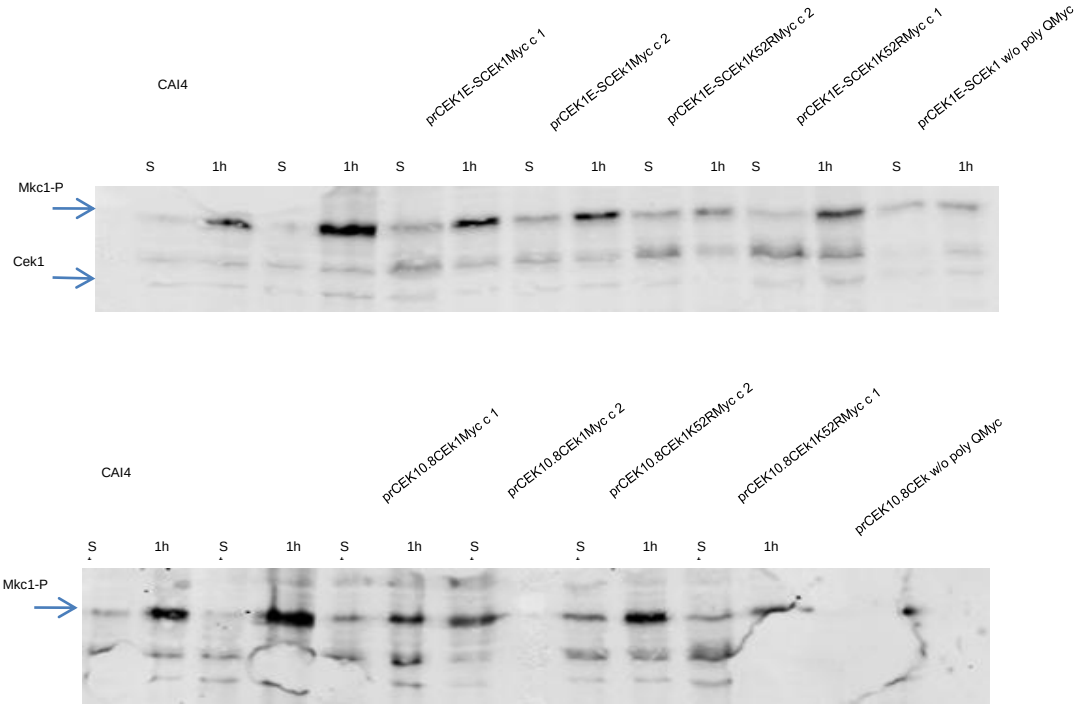


Figure 16

Oddysey with phospho p42/p44 rabbit antibodies emiting green signal at 800nm. Phosphorylation of Mck 1 in 75 kb was detected in cells after 1 hour of exponential growth, thou it was expected in stacionary phase

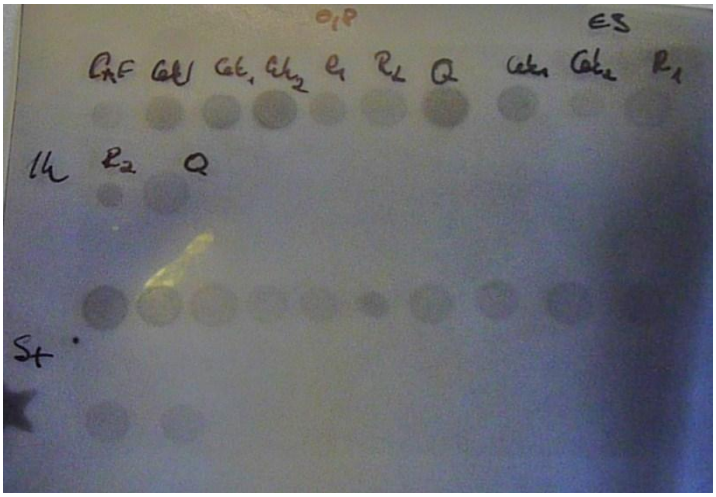


Figure 17

Dot test with *anti myc* antibodies should prove presence of *myc* tag in the mutants. CAF and *cek*1 wild type were used as negative controle of *myc* expression in stationary phase and after cultivating 1 hour in exponential phase of growth. Signal from the mutatnts was weak in most of them.

To ensure that the existence or not the tagged Cek1 version a dot-blot was performed with all the transformants obtained in the transformation. Dot test was used as easy and quick method to detect *Myc* tag in our mutants. None of the

transformats displayed a significant signal in the immunodetection assays. Taken together these results indicate that the transformation was not succeeded and the clones did not carry the expected construction. The lack of time did not allow me to repeat the transformation.

3.4 Discussion

Dimorphism of Candida albicanns is provoking us in every sence. Even this oranism is dimorphic within its relation to humans, once harmless comensal once harmful pathogen. In this work we are at the beginning of reaserch focused on the way which leads from pathogenic fungus to innocuous yeast.

Organisms sense and adapt to environmental conditions. C. albicans depending on extracellular signal can behave as comesal or pathogenic yeast. Signal transduction pathways are key mechanisms by which eukaryotic cells sense environmental changes in order to respond and adapt. Among signal transduction routes it is worth to remark the MAP kinase mediated pathway. These pathways are implicated in relevant physiological processes such as the response to different kind of stresses or growth.

In C. albicans the SVG pathway is activated when external conditions are advantageous to growth. Cek1 is the MAPK laying at the end of the MAPK module and unlike others MAPK the amount of protein depend on extracellular signal. The result showed in this work reinforces previous observation, the amount of Cek1 protein increases significantly when cells are shifted from stationary phase culture to fresh warmed medium. This increase of Cek1 protein concur whit its phosphorylation and therefore with its activation and requirement for the cell. The augment of Cek1 protein is due, at least in part, to an increase of CEK1 expression as we have quantified using the Click beetle luciferase as gene reporter. This up regulation was similar in upstream mutants in the SVG pathway suggesting that the expression of this MAPK is not depend on the elements of the pathway.

From previous experiments (Román, Nombela, & Pla, 2005) we know that Cek1 became phosphorylated as early as 15 min after dilution in YEPD rich medium, reaching a maximum at 1 to 2 h and the signal decreased again after 4 h of growth and completely disappeared after 8 h. This postulate is corresponding with presented data and among others we show that in the background of Cek1 and Sho1 the expression of the gene is decreasing noticeably faster than in wild type or Hst7.

Except the CAI 4 the other mutants (figure 13) reach maximal expression and then more than less continuously loosing on power.

My measurements shown interesting results. Thou the OD of all cultures were always synchronized for A_{600}=0,2 the expression in the beginning of exponential phase in CAI4 and Hst7 was always lower than by the other mutants but during the time reached adequate levels of expression correlating with the rank of the other mutants. In addition, relative increase of value during the determinate time period is even higher than in sho1 or cek1 mutants (figure 13), which at least in the case of Hst7 and Cek1 corresponds to conclusion of Csank (Csank, et al., 1998) that in contrast to disruptions of the Hst7 gene, disruption of the Cek1 gene affects the growth of serum-induced mycelial colonies.

Another important question approached in this work is the size of *CEK1* promoter or the existence of regulatory sequences. Two size of the promoter have been analyzed (0,8 and 1,6 Kb) and in both cases were sufficient to induce gene expression. Remarkably, the length of the sequence lacking ORFs just before the *CEK1* ORF is about 3 Kb. This fact suggests that important regulatory signal must be sited in this genome region. Our studies have been performed integrating the construction in the *ARD1 locus*, thus we have analyzed the *CEK1* expression ectopically and no differences were detected. The integration of the same constructions in *CEK1 locus* can be an alternative to study the expression of this gene. Nevertheless the existence of enhancers and silencers in the sequence of longer promoter are expected, further experiments are required.

As we know, tunicamycin shows a potent inhibitory effect on biofilm (Pierce, Thomas, & López-Ribot, 2008). It affects the formation of hyphae by C. albicans (Vespa & Lebecq, The morphology of Candida albicans in two different Earle base media in the presence of tunicamycin., 1996) and filamentation plays a pivotal role in biofilm formation (Baillie & Douglas, 1999). Concentrations of 4 μM still exerted a general inhibitory effect on fungal growth and filamentation and concentrations of 2 and 1 μM still led to reduction of metabolic activity but the drug did not display any noticeable effects on cell growth and morphology (Pierce, Thomas, & López-Ribot, 2008).

When tunicamycin was added to stationary phase cultures failed to initiate either yeast bud or germ tube formation (Chaffin, 1985) so the drug also prevents

development of germ tubes and development of mycelia from germ tubes (Vespa, Lebecq, & Simonetti, 1993). When tunicamycin was added to growing cells, growth was inhibited but not immediately. Addition of the drug to exponential phase yeast cultures resulted in an approximately 45% increase in cell number before cell division ceased and yeast accumulated in both budded and unbudded stages of the cell cycle (Chaffin, 1985).

Second part of my results focused on *CEK*1 function was not satisfactory but mutants with possibly key mutations in *CEK*1 are ponentially highly usable. We propose interesting results of experiments with different antimycotic agents which may lead to decreasing the dosages of commonly used medication, copeing with resistance or finding new substances for treatmento of candidiasis.

Nowadays our science is on quite high level and allows us to make genomic maps of many organisms inclidung the human genome. The challange upcoming from this event is to get to know more about the genes we can detect. In most of the case we are now in the point where we know the primary input – the gene and more or less the final cause of it. The challange is to describe whats happening on the way between the begining and the end. That should be the next step for scientist followed with putting the separete processes in consequences with orther functions in the organism. All those pathways and their mediators work within some mechanisms. Mechanism is the main goal that can help us to understand the diseases and the ways of their treatment.

4. Conclusion

1. 0.8 Kb upstream of the ORF of *CEK1* gene are enough to induce the expression of the gene under the conditions analyzed.
2. No significant differences were observed on gene expression using two different size of *CEK1* promoter.
3. The luciferase of click beetle adapted to its expression in *C. albicans* can be used us gene reported.
4. Cek1 expression is induced when cultures growth in stationary phase are shifted to fresh warmed medium. This induction is transient since it decreased after 4 hours of incubation.
5. Cek1 expression is not dependent on upstream elements of the SVG pathway.

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