Universidade do Minho
Escola de Ciências

Yolanda Bedsabé Delgado Silva

Involvement of the gene *CaRLM1* in *Candida albicans* virulence

Abril de 2012

UMinho | 2012 Yolanda Bedsabé Delgado Silva **Involvement of the gene *CaRLM1* in *Candida albicans* virulence**

Universidade do Minho
Escola de Ciências

Yolanda Bedsabé Delgado Silva

Involvement of the gene *CaRLM1* in *Candida albicans* virulence

Tese de Doutoramento em Ciências
Especialidade de Biologia

Trabalho realizado sob a orientação da
Professora Doutora Ana Paula Sampaio
e do
Professor Doutor Rui Oliveira

Abril de 2012

Dissertação apresentada à Escola de Ciências da Universidade do Minho para obtenção do grau de Doutor em Ciências, Área de Conhecimento em Biologia.

Este trabalho foi realizado no Departamento de Biologia da Universidade do Minho, sob a supervisão da Professora Doutora Paula Sampaio e Professor Doutor Rui Oliveira. A sua execução foi financiada pela Bolsa de Doutoramento E07D400922PE, atribuída pelo Programme ALβAN, the European Union Programme of High Level Scholarships for Latin America.

À memória da minha mãe

A ti mãe, que sempre nos acompanham!

LOS HERALDOS NEGROS

Hay golpes en la vida, tan fuertes...Yo no sé.
Golpes como del odio de Dios; como si ante ellos,
la resaca de todo lo sufrido
se empozara en el alma...Yo no sé.

Son pocos; pero son... Abren zanjas oscuras
en el rostro más fiero y en el lomo más fuerte.
Serán tal vez los potros de bárbaros Atilas;
o los heraldos negros que nos manda la Muerte.

Son las caídas hondas de los Cristos del alma,
de alguna fe adorable que el Destino blasfema.
Esos golpes sangrientos son las crepitaciones
de algún pan que en la puerta del horno se nos quema.

Y el hombre... Pobre...pobre! Vuelve los ojos, como
cuando por sobre el hombro nos llama una palmada;
vuelve los ojos locos, y todo lo vivido
se empoza, como charco de culpa, en la mirada.

Hay golpes en la vida, tan fuertes... Yo no sé!

César Vallejo

Acknowledgements/Agradecimentos

Manifesto de maneira muito especial o meu agradecimento à Professora Paula Sampaio e ao Professor Rui Oliveira, orientadores deste trabalho, por todo apoio, dedicação, determinação, constante estímulo, e em particular por todos os ensinamentos transmitidos. O meu mais sincero obrigado, por tudo.

À Professora Célia Pais, pelo seu apoio e valiosas críticas deste trabalho.

I am also very grateful to Professor Joachin Morschhäuser for welcoming me so well in his laboratory in Würzburg Germany. For providing the conditions and all the scientific inputs I ever needed for the development of the work concerning *Candida albicans* construction mutant *RLM1*. I thank Bernardo Ramirez and all the members in the laboratory for treating me as a member of the "Candida Group". I can't express my gratitude for having the chance to work in such a highly regarded group with so high scientific standards.

À Alexandra Correia, Professor Augusto Faustino, Laura Carreto, Sandra Tenreiro, e Professor Manuel Villanova pelos ensinamentos e pela amabilidade com que me receberam no seu laboratório.

A todos os docentes, investigadores e funcionários do Departamento de Biologia, por toda amizade e apoio disponibilizados.

À Raquel, Flavio, Andreia, Célia, Neide, Hanna, Flavia, Rui, João, a todos os meus colegas do laboratório Micro II e do CBMA, pelo auxílio nos reagentes e pela amizade, simpatia e camaradagem. Assim também à Su e Sofi pela companhia em casa.

Aos meus amigos: Divian, Néstor, Omar, Sussy, Marge, Jaime e César pelos e-mail de incentivo, que à distancia sempre me acompanharam (msg)!

A ti Julito pelo amor e por tudo.....amore!!!

A toda minha família, pelas palavras de incentivo que sempre me deram e fizeram sentir acompanhada à distância. Aos meus irmãos Segundo, Maruja, David, Clarissa, Rosario e Ciro, pelo carinho, exemplo e apoio incondicional.

A ti papá, que en tú silencio me comprendes que esté lejos de la familia……te quiero mucho!

Abstract

The cell wall is an essential structure that maintains the viability of fungal cells, conferring their typical morphology and protection. As the most external cellular structure of pathogenic microorganisms, it also carries important antigenic determinants and mediates adhesion to the host tissues, being crucial to initiate colonization and to cause disease. *Saccharomyces cerevisiae* and *Candida albicans* respond to cell wall perturbations by activation of the cell wall integrity (CWI) mitogen-activated protein (MAP) kinase pathway (also known as the PKC pathway). In *S cerevisiae* one of the transcription factors of this signalling cascade is the MADS-box protein Rlm1, of which an orthologue was identified in *C. albicans* based solely in sequence homology. In this thesis we aimed at studying the molecular diversity and functional characterization of the *RLM1* gene as well as its involvement in *C. albicans* cell wall maintenance and virulence.

Candida albicans Rlm1 is a transcription factor that presents a great variability at its C-terminus, conferred by the CAI microsatellite. One hundred twenty-three *C. albicans* isolates were genotyped with CAI microsatellite $(CAA/G)_n$ and 35 alleles were found with repeat units varying from 11 to 49. Interestingly the strains with higher number of (CAA/G) repetitions displayed higher tolerance to cell wall stress agents. These observations suggested that CAI repetitive region confers a high genetic variability to the *RLM1* gene, which is reflected in different strain susceptibilities to different stress conditions, conferring a higher plasticity to *C. albicans* isolates.

To determine if *C. albicans RLM1* is involved in the CWI pathway*,* as described for *S. cerevisiae,* a *rlm1*Δ*/rlm1*Δ mutant was constructed, using the *SAT1*-flipping strategy to avoid the use of auxotrophic markers, and its functional characterization was performed. The wild-type (WT), *mutant* (*rlm1*Δ*/rlm1*Δ*)* and complemented (*rlm1*Δ*/rlm1*Δ*+RLM1)* strains were tested with several cell wall stress agents in parallel with *S.cerevisiae rlm1*Δ mutant and WT strains. *Candida albicans rlm1Δ/rlm1Δ* mutant displayed phenotypes associated to cell wall deficiency such as, hypersensitivity to Congo red, caspofungin and calcofluor white. Upon osmotic stabilization with 1M sorbitol, the caspofungin phenotype was reverted, suggesting cell wall weakening in the mutant. Quantification of cell wall components showed a two-fold increase in chitin and mannans in the *C. albicans rlm1Δ*/*rlm1*Δ mutant in comparison with the WT strain. The *S. cerevisiae rlm1*Δ mutant displayed several phenotypic differences in comparison with the *rlm1Δ*/*rlm1*Δ mutant of *C. albicans*: insensitivity to Congo red and caspofungin, more resistance to calcofluor white, and higher sensitivity to SDS. In agreement with a transcription factor function, we found evidence indicating nuclear localization of the Rlm1-GFP fusion protein. Microarray analysis showed that the absence of a functional *C. albicans RLM1* significantly increased transcription of genes involved in cell adhesion, like *ECE1*, *ALS1*, *ALS3*, *HWP1, RBT1,* and decreased transcription of genes involved in the catabolism of carbohydrates, *DAK2*, *GLK4*, *NHT1* and *TPS1*. The increased transcription of genes involved in cell adhesion correlated well with

adhesion and biofilm assays. These results and the homology with other MADS-box Rlm1 transcription factors strongly suggest that *C. albicans RLM1,* like the *S. cerevisiae* orthologue, is involved in cell wall remodeling. Furthermore, the increase of cell adhesion binding proteins involved in biofilm formation was confirmed in the *rlm1Δ*/*rlm1*Δ mutant in comparison with the WT strain, suggesting that *C. albicans* Rlm1 acts as a negative biofilm regulator.

The involvement of the Rlm1 transcription factor of the human fungal pathogen *C. albicans* in virulence was evaluated in a murine model of disseminated candidiasis. Mice infected with *rlm1Δ/rlm1*Δ mutant cells presented a higher survival time than mice infected with the WT and complemented strains, both presenting higher fungal burden and invasive micelial growth through kidneys in hystopathological analysis. Additionally, in the murine macrophage-like cell line J744A, the TNF-α was lower in response to *rlm1Δ/rlm1*Δ mutant and the cellular toxicity, measured as extracellular lactate dehydrogenase activity, caused by this mutant was significantly lower in comparison with the WT and complemented strains. Finally, qRT-PCR determination showed that the expression of the cell wall-related genes, *CRH11* and *PHR2,* was clearly higher in *rlm1Δ/rlm1*Δ mutant in relation to WT strain, in mRNA of kidney samples after 7 days post-infection. Since these proteins are part important of the cell wall and *in vivo* the yeast cells are under constant cell wall stress by the immune system, it is conceivable that the mutant presents a higher expression of these genes, which may compensate the weakened cell wall. Overall, these results showed that the transcription factor Rlm1 is involved in the stability of the cell wall in the interaction with the host, being important for the virulence of *C. albicans* and invasion of the kidneys during hematogenously disseminated candidiasis.

Altogether, the objective proposted in this thesis were achieved, demonstrating that *RLM1* gene is an important transcription factor involved in *C. albicans* cell wall remodelling, such that the mutant is practically avirulent. Additionally, we also think that this yeast developed a way of increasing the genetic variability of this important gene (through CAI repetitive region), confering a high plasticity to *C. albicans* isolates.

Resumo

A parede celular é uma estrutura essencial responsável pela manutenção da viabilidade das células fúngicas, conferindo-lhes morfologia típica e protecção. Como estrutura celular mais externa de microrganismos patogénicos, contém determinantes antigénicos importantes e medeia a adesão aos tecidos do hospedeiro, sendo essencial para iniciar a colonização e, portanto, causar doença. As células de *Saccharomyces cerevisiae* e *Candida albicans* respondem a perturbações da parede celular através da activação da via "mitogenic activated protein kinase" (MAPK) da integridade da parede celular (CWI), também conhecida como a via da PKC. Um dos factores de transcrição presente nesta cascata de sinalização é a proteína MADS-box Rlm1, cujo ortólogo em *C. albicans* foi identificado apenas por homologia de sequência. A presente tese teve como objectivo o estudo da diversidade molecular e caracterização funcional do gene *RLM1*, bem como estudo da sua participação na manuntenção da parede celular e virulência de *C. albicans*.

A proteína Rlm1 de *C. albicans* é um factor de transcrição que apresenta uma grande variabilidade na sua extremidade terminal C, conferida pelo microssatélite CAI. Cento e vinte e três isolados de *C. albicans* foram genotipados com microssatélite CAI (CAA/G)n e 35 alelos foram encontrados com unidades repetitivas variando entre 11 a 49. È interessante notar que as estirpes com maior número de repetições (CAA/G) apresentaram maior tolerância a agentes de stresse da parede celular. Estas observações sugeriram que a região repetitiva CAI confere uma alta variabilidade genética ao gene *RLM1*, que se reflecte em diferentes susceptibilidades das estirpes a diferentes condições de stresse, conferindo uma elevada plasticidade aos isolados de *C. albicans*.

De forma a determinar se o gene *RLM1* de *C. albicans* está envolvido na via CWI, como descrito para *S. cerevisiae,* foi construído o mutante *rlm1*Δ/*rlm1*Δ utilizando a estratégia *SAT1*-flipping, de modo a evitar o uso de marcadores de auxotrofia. A estirpe selvagem (WT), a estirpe mutante (*rlm1Δ*/*rlm1Δ*) e a complementada (*rlm1Δ/rlm1Δ+RLM1*) foram testadas com vários agentes de stresse da parede celular em paralelo com as estirpes WT e *rlm1Δ* de *S. cerevisiae*. O mutante de *C. albicans rlm1Δ/rlm1Δ* apresentou fenótipos associados à deficiência de parede celular, tais como, a hipersensibilidade ao Congo red, à caspofungina e ao calcofluor white. O fenótipo com caspofungina foi revertido por estabilização osmótica com sorbitol 1M, indicando enfraquecimento da parede celular no mutante. A quantificação de componentes da parede celular mostrou um aumento de duas vezes em quitina e mananos no mutante *rlm1Δ/rlm1Δ* de *C. albicans* em comparação com a estirpe WT. A estipe mutante *rlm1Δ* de *S. cerevisiae* exibiu várias diferenças fenotípicas em comparação com o mutante *rlm1*Δ/*rlm1*Δ de *C. albicans*: insensibilidade ao Congo red e caspofungina, uma maior resistência ao calcofluor white e uma maior sensibilidade ao SDS. De acordo com a função de factor de transcrição, encontramos dados indicando a localização nuclear da proteína de fusão Rlm1-GFP. A análise de "microarrays" demonstrou que a ausência do gene *RLM1* em *C. albicans* induziu um

aumento significativo na transcrição de genes envolvidos na adesão celular, tais como, *ECE1, ALS1, ALS3, HWP1, RBT1* e uma diminuição na transcrição de genes envolvidos no catabolismo de hidratos de carbono, tais como, *DAK2 GLK4, NHT1* e *TPS1.* O aumento na transcrição de genes envolvidos na adesão celular foi confirmados por resultados em ensaios de aderência celular e formação de biofilme. Estes resultados e a homologia com outros factores de transcrição MADS-box do mesmo tipo sugerem fortemente que o gene *RLM1* de *C. albicans,* bem como o seu ortólogo em *S. cerevisiae*, estão envolvidos na remodelação da parede celular. Além disso, observou-se um aumento de proteínas de adesão celular envolvidas na formação de biofilme no mutante *rlm1Δ/rlm1Δ* em comparação com a estirpe WT, sugerindo que o *RLM1* de *C. albicans* actua como um regulador negativo da formação de biofilme.

Por fim o envolvimento do factor de transcrição Rlm1 de *C. albicans* na virulência foi avaliado num modelo murino de candidíase disseminada. Ratinhos infectados com células mutantes *rlm1Δ/rlm1Δ* apresentaram um maior tempo de sobrevivência do que ratinhos infectados com as estirpes WT e complementada, estas últimas apresentando uma maior carga fúngica e crescimento micelial invasivo através dos tecidos na análise histopatológica dos rins. Além disso, na linha celular *murine macrophage-like* J744A, a produção de TNF-α foi menor em resposta ao mutante *rlm1Δ/rlm1Δ* e a toxicidade celular, medida pela actividade extracelular do lactato desidrogenase, foi significativamente mais baixa em comparação com as estirpes WT e complementada. Mais, a quantificação por qRT-PCR demonstrou que a expressão dos genes relacionados com parede celular, *CRH11* e *PHR2,* foi claramente maior no mutante *rlm1Δ/rlm1Δ* em relação à estirpe WT, em mRNA de amostras de rim após 7 dias pós-infecção. Uma vez que estas proteínas são parte importante da parede celular e que *in vivo* as células de levedura estão sob stresse constante pelo sistema imunitário, é plausível que o mutante apresente uma expressão maior de estes genes, podendo compensar o enfraquecimento da parede celular. Em conclusão, estes resultados mostraram que o factor de transcrição Rlm1 está envolvido na estabilidade da parede celular, na interacção com o hospedeiro, sendo importante para a virulência de *C. albicans* e invasão durante a candidíase hematogenicamente disseminada.

No seu conjunto, os objetivos propostos desta tese foram alcançados, demostrando que o gene *RLM1* é um importante factor de transcrição *em C. albicans,* sendo essencial na remodelação da parede celular, de tal modo que o respectivo mutante é praticamente avirulento. Além disso, verificamos tambiém que nesta levadura o gene *RLM1* apresenta uma elevada variabilidade genética (região repetitiva CAI), conferindo desta forma uma maior plasticidade aos isolados de *C. albicans.*

Index

Abbreviations list

AIDS	Acquired Immune Deficiency Syndrome
APS	Ammonium Persulfate
BP	Base Pairs
BSA	Bovine Serum Albumin
BSI	Bloodstream infection
cDNA	Complementary deoxyribonucleic acid
CFG	Caspofungine
CFU	Colny forming unit
CFW	Calcofluor wite
CR	Congo red
CWI	Cell wall integrity
CWP	Cell wall protein
CV	Crystal violet
DAPI	4 6-diamino-2-phenylindol
dH_2O	Deionized H_2O
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immuno Sorbent Assay
FBS	Foetal Bovine Serum
GDP	Glyceraldehyde-3-Phosphate Dehydrogenase
GPI	Glycosyl Phosphatidyl Inositol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
HOG	High osmolarity glycerol
IFN-**γ**	Gamma interferon
IL	Interleukin
LDH	Lactate Dehydrogenase
Lip	Lipases
MADS	Mcm1, Argamous-Deficiens-Serum response factor
MAPK	Mitogen activeted protein kinase
MEF	Myocite Enhancer Factor 2
Nou	Nourseothricin
OD	Optical Density
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PKC	Cell integrity or protein kinase C
PLs	Phospholipases
QSMs	Quorum-sensing molecules

RNA	Ribonucleic Acid
qRT-PCR	Real-Time Reverse-Transcription Polymerase Chain Reaction
SAP	Secreted Aspartic Proteinase
SD	Synthetic dextrose
SDA	Sabouraud dextrose agar, NaCl, CaCl2, egg
SDS	Sodium Dodecyl Sulfate
SBF	Swi4-Swi6 cell cycle Box binding Factor
SRF	Serum Response Factor
SVG	Sterile Vegetative Growth
TE	Tris, EDTA
TEMED	1,2-bis-(dimethylamino)-Ethane
Th	T helper cells
TNF-α	Tumour Necrosis Factor α
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YCB	Yeast carbon base
YNB	Bacto yeast nitrogen base without amino acids
YE	Yeast extract
YEPD	Yeast extract, Peptone and Dextrose
WO	White opaque
WT	Wild type

Chapter 1

General Introduction

1. An overview of fungal infections

Fungi play a critical role impacting nearly all other forms of life in virtually all ecosystems as either beneficial or detrimental. Saprotrophic fungi are important in the environment in the cycling of nutrients through the decomposition of organic material, especially the carbon that is sequestered in wood and other plant tissues. Mutualistic symbiont fungi through relationships with prokaryotes, plant (including algae) and animals have enabled a diversity of other organisms to exploit novel habitats and resources. Indeed, the establishment of mycorrhizal associations may be a key factor that enabled plants to make the transition from aquatic to terrestrial habitats. Other groups are pathogenic and parasitic fungi that attack virtually all groups of organisms such as, bacteria, plants, other fungi, and animals, including humans. The economic impact of such fungi is massive either beneficial by the production of antibiotics or extremely detrimental by the devastating impacts in diseases (Pirozynski and Malloch, 1975; Moss, 1987; Alexopoulos, 1996; Blackwell, 2000).

Human fungal infections have been increasing over the past decades. Fungi are gaining importance with the rising incidence of chronic mycoses, often fatal in immunocompromised patients, decreasing their chance of recovery and seriously hampering their quality of life. These problems are a consequence of the introduction of a variety of therapeutic modalities into clinical medicine (invasive technologies, anticancer drugs and therapy prior to organ transplantation). Additionally, fungal infections increased dramatically with the emergence of acquired immune deficiency syndrome (AIDS) and the widespread use of injectable illicit drugs, such as heroin (Ghannoum and Abu-Elteen, 1990; Edwards, 1991; Zaoutis *et al.*, 2005; Pfaller and Diekema, 2007). Different fungal species are found associated with human diseases. *Candida* spp. and *Cryptococcus* spp. are the yeast species most frequently isolated in clinical practice. *Aspergillus* spp. are the most frequent filamentous fungi (moulds) isolated, but *Fusarium* spp., *Scedosporium* spp., *Penicillium* spp. and Zygomycetes are increasingly seen (Marr *et al.*, 2002; Husain *et al.*, 2003).

Candida albicans is a diploid eukaryotic yeast of the Kingdom Fungi (phylum Ascomycota: subphylum Saccharomycotina: class Saccharomycetes: order Saccharomycetales: family *Saccharomycetaceae*). A distinctive characteristic of *C. albicans* is its ability to grow in three distinct morphologies that include unicellular budding yeast (blastospore), true hyphae, and pseudohyphae (Fig. 1.1). *Candida albicans* also can form chlamydospores, that is to say, asexual spores that develop on pseudohyphal support cells and appear under unfavourable environmental conditions (Kurtz, 1990; Sudbery *et al.*, 2004). This fungus is an opportunistic pathogen, which exists as part of the normal flora of the mucocutanous, gastrointestinal and genitourinary areas in a significant part of the population, where it behaves as a harmless commensal organism (Odds, 1988; Calderone and Fonzi, 2001). However, when the normal host defence mechanisms are impaired, *C. albicans* is capable of causing a range of infections, from superficial thrush to dangerous systemic candidiasis (Larriba *et al.*, 2000; Calderone, 2002).

The diploid *C. albicans* has eight pairs of homologous chromosomes, with a genome size of 14.3 Mb, based on the genome sequencing of SC5314 strain. *Candida albicans* has a genome with a GC content of 33.5%, 6,107 genes, an average gene size of 1,468 bp and an intergenic average size of 858 bp (Magee, 1993; Butler *et al.*, 2009). *Candida albicans* present a non-canonical codon usage, translating the normally leucine-specific codon CUG as serine (Santos and Tuite, 1995). Despite the demonstration that an elaborate mechanism for mating is still operational in *C. albicans* (Magee and Magee, 2000; Johnson, 2003; Kim and Sudbery, 2011), to date, a complete sexual cycle has not been observed. Therefore, *C. albicans* has been reported as presenting parasexual cycle (mating of diploid cells followed by mitosis and chromosome loss instead of meiosis) (Noble and Johnson, 2007; Kim and Sudbery, 2011).

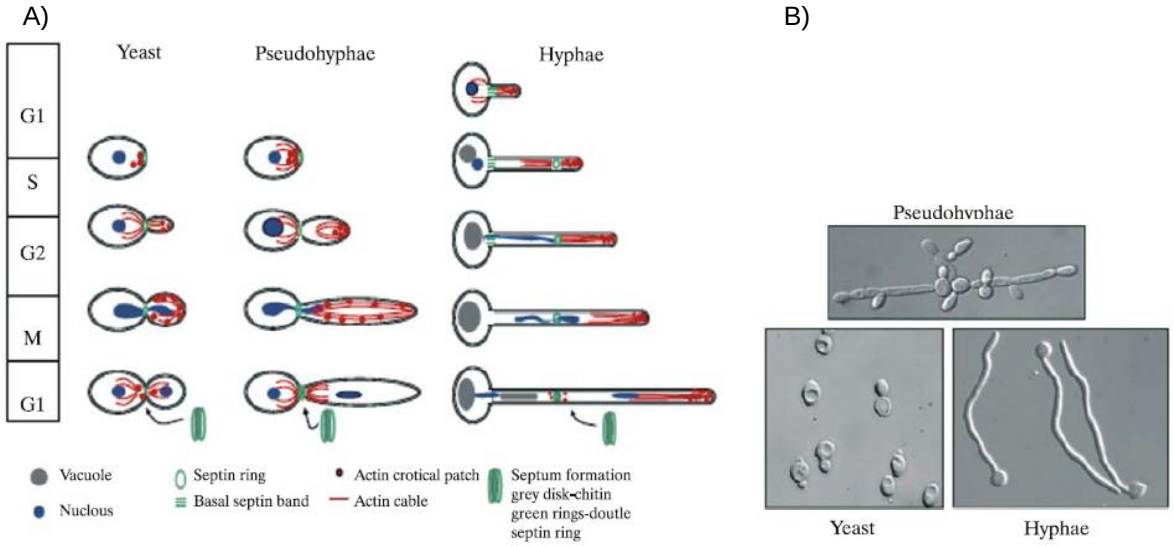

Figure 1.1. Different growth morphologies of *C. albicans* A) Diagrammatic representation of the cell cycle of yeast and of the first cycle after induction of pseudohyphae and hyphae B) Differential interference (DIC) images of cells in hyphae, pseudohyphae, and yeast morphologies. Adapted from Sudbery *et al.*, 2004.

1.1. *Candida albicans* as a pathogenic agent

The genus *Candida* is ubiquitous and approximately 200 species are known, of which nearly 20 have been identified as etiologic agents of infections, but the list of medically important yeasts continues to grow (Calderone, 2002). *Candida albicans* is the most common fungal pathogen, representing a global average of 66% of all *Candida* species isolated in clinical practice, even though other species of *Candida* are increasing in prevalence, such as: *C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei* (Pfaller and Diekema, 2002; Morgan, 2005; Tortorano *et al.*, 2006; Pfaller and Diekema, 2007; Pfaller *et al.*, 2010; Pfaller *et al.*, 2011; Tortorano *et al.*, 2011).

As an opportunistic pathogen *C. albicans* may establish an infection when adaptative and constitutive host defenses are compromised, for example, in haematological disorders, chemotherapy, transplant, diabetes, or patients with AIDS, among others.

1.1.1. Types of candidiasis

Candidiasis can range from superficial disorders to invasive infections, mainly in immunocompromised hosts, and usually are classified into two main groups as follows:

Superficial candidiasis

Candida albicans, as a commensal organism, is not normally a problem however it can cause symptomatic infections of mucosal membranes (Odds, 1988). These infections affect the different systems and have several clinical manifestations, namely thrush, a superficial infection of one or more epithelial surfaces (Schwebke, 1996); oropharyngeal candidiasis (OPC), which occurs in approximately 70% of patients with AIDS; vulvo vaginitis candidiasis, that may affect up to 70% of women (with or without AIDS) at least once in their life time and 20% will experience recurrent disease (Fidel *et al.*, 1999), and cutaneous candidiasis. Recurrent *Candida* infections of mucous membranes, skin and nails can lead to chronic mucocutaneous candidiasis (Ruhnke, 2002).

Invasive Candidiasis

Invasive candidiasis (also known as systemic candidiasis or hematogenously disseminated candidiasis) involves the infection and spread of *Candida* via the bloodstream to multiple organs, such as the brain, kidneys, heart, lungs, liver and spleen (Parker *et al.*, 1976). Invasive candidiasis can be further classified as candidemia or disseminated candidiasis.

- **Candidemia**. Blood stream infections by *C. albicans* are known as candidemia. In normal healthy individuals, adequate protection against such infections is provided by the action of the innate immune system. However, candidemia can develop in patients who have abnormally low numbers of neutrophils as a result of certain blood cancers or immunosuppressant therapy (Kim and Sudbery, 2011). Candidemia represents 10-20% of all candidiasis and is considered as the tip of the iceberg of *Candida* spp. infections (Eggimann *et al.*, 2003).
- **Disseminated candidiasis.** Candidemia may give rise to hematogenous spread of *Candida* to one or multiple organs. In this case, it is referred as acute disseminated candidiasis. Multiple organs are frequently involved, and discrete persistent microabscesses occur in the liver, spleen, and kidneys (Parker *et al.*, 1976; Bodey *et al.*, 1992; Filler, 2002; Hedayati, 2009).

1.1.2. Epidemiology of candidemia

Fungal infections have been increasing over the past decades and today represent a serious health problem even in industrialized countries. *Candida* infections have been documented as the fourth most common hospital-acquired infections, and the second most common cause of death from such infections in the United States (Pfaller *et al.*, 1998). In fact, over the past two decades the incidence of *Candida* bloodstream infections among immunocompromised patients has increased 15 to 20-fold (Perlroth *et al.*, 2007) and is presently the third most common nosocomial infection in the United States (Wisplinghoff *et al.*, 2004; Perlroth *et al.*, 2007). Despite the widespread use of antifungals for prophylaxis and treatment of invasive fungal infections, candidemia and disseminated candidiasis have a high mortality rate of 30–50% (Pfaller *et al.*, 1998; Kibbler *et al.*, 2003).

Although *C. albicans* has been, in the past, the most common causative organism of fungemia and disseminated candidiasis, other *Candida* species are becoming increasingly more prevalent. A worldwide study of bloodstream infections from 1997 to 1999 showed that at least 45% of yeast infections were caused by other *Candida* species than *C. albicans* (Pfaller *et al.*, 2001). The most commonly isolated species, apart from *C. albicans*, are *C. parapsilosis* (20 to 40% of all reported episodes of candidemia), *C. glabrata* (5 to 40%), *C. tropicalis* (10 to 30%), *C. krusei* (10 to 35%), *C. guilliermondii* (2 to 10%) and *C. lusitaniae* (up to 8%) (Sandven, 2000; Krcmery and Barnes, 2002).

Studies of candidemia in Europe reported frequent association of candidemia with patients undergoing surgical interventions, patients with intensive care treatments, and patients suffering from solid tumor and haematological malignancies all of which were deeply immune suppressed. The rates of candidemia in Europe ranged from 0.20 to 0.38 per 1000 admissions and 0.31 to 0.44 per 10,000 patient-days. These values are however lower than the ones reported in the USA, which are 1.5 per 10,000 patient-days (Tortorano *et al.*, 2006). Compared to 20 years ago, a larger proportion of Candida bloodstream infections is presently caused by *C. glabrata* in the United States (Trick *et al.*, 2002) and by *C. parapsilosis* and *C. tropicalis* in European, Canadian and Latin American hospitals (Wingard, 1995; St-Germain *et al.*, 2001; Almirante *et al.*, 2006). In Portugal, the candidemia rate found in acute leukemia patients (6.3%) was similar to rates published in the literature (Ribeiro *et al.*, 1997; Costa-de-Oliveira *et al.*, 2008). A recent epidemiological study in a Portuguese oncology hospital during a 6-year period by Sabino *et al.,* 2010 found that solid tumors and hematological disease were the most common underlying medical conditions. The most frequent species found was *C. albicans* (48.7%), followed by *C. Parapsilosis* (20.2%), *C. Tropicalis* (.8.4%), *C. krusei* (6.7%) and *C. glabrata* (5.0%) but *Saccharomyces cerevisiae* and *Rhodotorula mucilaginosa* were also isolated. *Candida albicans* was more frequently associated with solid tumors of the gastrointestinal and genitourinary tracts and breast, while non-albicans *Candida* species were most frequently recovered from hematological patients. During a six-year survey, they found that in the Portuguese oncology hospital the incidence of candidemia and nosocomial candidemia were 2.02 and 1.49 per 1,000 hospital admissions, respectively, and the mortality rate associated with candidemia was 31.9% (Sabino *et al.*, 2010).

1.1.3. Antifungal therapy

The massive emergence of fungal diseases associated with AIDS in the 1980s and the rising frequency of fatal mycoses associated with increasing use of immunosuppressive medical therapies since the 1970s stimulated research directed towards the discovery of novel antifungal agents. The antifungal drugs available to treat fungal infections frequently cause deleterious side-effects because of the close biological similarity between the fungal cell and the mammalian cell. For that reason, an important goal in antifungal drug discovery is the identification of new suitable fungal targets with no mammalian homologies. All the antifungal agents currently available are classified, according with their specific mode of action.

The azoles act by inhibiting the fungal cytochrome P450-dependent ergosterol biosynthesis, specifically by blocking 14-α sterol demethylase. The primary differences between the azoles (itraconazole, imidazoles, fluconazole and ketoconazole) involve their spectrum of activity, pharmacokinetics, and chemical formulations. Amphotericin B, a polyene, works by binding to ergosterol in the fungal cell membrane, making the membrane more permeable. Despite of the conformational difference between cholesterol and ergosterol, and the major binding affinity of amphotericin B for the latter, this antifungal agent has potential toxicity for mammalian cells, so a variety of reformulated versions of the agent have been introduced in the last years, to overcome amphotericin B nephrotoxicity. Echinocandins (caspofungin, anidulafungin and micafungin) inhibit β-(1,3)-D-glucan synthase, an essential enzyme for the synthesis of the fungal cell wall. Echinocandins have a broad spectrum of activity and tend not to affect human cells due to their targeted mechanism of action (Dupont, 2002; McPheeters, 2005; Perlroth *et al.*, 2007).

Emerging resistance to some antifungals might further limit the options available. For example, reduced susceptibility to fluconazole, one of the preferred drugs in the treatment of fungal infection, is frequent among non-albicans *Candida* spp. The mechanisms of resistance include changes in the cell wall or plasma membrane leading to an impaired uptake of antifungals, efflux pumps that take antifungals outside the cell, overexpression of the antifungal targets, mutations in the antifungal target that decrease its binding ability, activation of alternative pathways that increase the metabolism of the antifungal, sequestration of the antifungal in organelle-like vacuoles, or chromosomal changes that increase the number of copies of the required gene (Eggimann *et al.*, 2003). A 5-year retrospective review published recently by Forrest *et al.*, (2008) showed significant correlations between increased caspofungin usage and an increased incidence of *C. parapsilosis* candidemia and reduction of *C. tropicalis* and *C. glabrata* candidemia. According to Pfaller *et al.*, (2008) *Candida parapsilosis* is innately less susceptible to the echinocandins than many other species of *Candida*, although the vast majority of isolates remain susceptible to all three echinocandins in medical use. Several data suggest that *C. orthopsilosis* and *C. metapsilosis* are more susceptible to echinocandins and amphotericin B but probably less sensitive to fluconazole than *C. parapsilosis, C. metapsilosis* being the less susceptible to this azole (Tavanti *et al.*, 2007; Gomez-Lopez *et al.*, 2008; Lockhart *et al.*, 2008; Szabo *et al.*, 2009; van Asbeck *et al.*, 2009). *Candida glabrata* can be resistant to usual doses of triazoles, but sensitive to higher doses (Eggimann *et al.*, 2003; Tortorano *et al.*, 2006).

1.2. *Candida albicans* virulence factors

The definition of virulence is the ability of a microorganism to cause disease, and is determined by many factors. Virulence describes the degree of pathogenicity, where some strains may be more, or less, virulent than others. Most pathogens, including *Candida* spp, possess a wide range of putative virulence factors and specific mechanisms that assist in their ability to colonize host tissues, causing disease, and overcoming host defences (Romani *et al.*, 2002). The interaction between virulence factors of *C. albicans* and host defense mechanisms plays a central role in determining whether colonization remains harmless or leads to infection of the epithelium and ultimately to systemic

infection. These virulence determinants include adherence to host cells, morphogenesis, biofilm formation, phenotypic switching, secretion of degradative enzymes and quorum sensing (Calderone and Fonzi, 2001; Schaller *et al.*, 2005; Chauhan *et al.*, 2006; Lim *et al.*, 2011).

1.2.1. Adherence

Adherence of *Candida* spp. to host cells and tissues is an initial step that assist colonization or the establishment of infection (Kamai *et al.*, 2002). Adherence involves the interaction between complementary molecules present in both the pathogen and the host surfaces (Gozalbo *et al.*, 1998; Lopez-Ribot *et al.*, 1999). *Candida albicans* is known to bind to different subendothelial extracellular matrix proteins such as fibronectin, laminin, entactin, and collagens, and these proteins are used as possible target molecules in the process of dissemination (Gozalbo *et al.*, 1998; Klotz *et al.*, 2001), penetrating into blood vessel walls and organs in systemic infections (Sundstrom, 1999; Calderone and Fonzi, 2001).

Adhesins may be defined as cell surface biomolecules that promote the adherence of *C. albicans* to host cells or host–cell ligands (Calderone and Fonzi, 2001) such as polysaccharides (Fukazawa and Kagaya, 1997), lipids (Ghannoum *et al.*, 1986), and glycoproteins (Agglutinin-Like Sequence, Als, family or Hwp1) (Gaur and Klotz, 1997; Staab *et al.*, 1999; Hoyer, 2001). The Als protein family is encoded by eight genes, *ALS1*–7, and *ALS9* (Hoyer, 2001), which are differentially expressed depending on the yeast growth conditions. Als proteins were shown to be expressed in vivo in the kidney, spleen, heart, liver and lung following systemic infection in a mouse model, and Als1 was also important for the adherence of *C. albicans* to the oral mucosa during the early stage of the infection (Hoyer *et al.*, 1999; Kamai *et al.*, 2002). *HWP1* encodes a cell-surface adhesin that promotes strong interactions between *C. albicans* and host oral and vaginal epithelial cells. (Staab *et al.*, 1999; Naglik *et al.*, 2006).

1.2.2. Secreted degradative enzymes

The secretion of degradative enzymes during infections may be used as a virulence attribute that promotes degradation of host surfaces allowing adhesion and invasion, destruction of host immune factors, and nutrient acquisition (Hube and Naglik, 2001). The three most significant hydrolytic enzymes produced by *C. albicans* are secreted aspartic proteinases (SAP), phospholipases (PLs), and lipases (Lip) (Mavor *et al.*, 2005).

Proteinases belonging to the *SAP* family are responsible for the proteolytic activity of different *Candida* species (Hube and Naglik, 2001). Proposed functions of SAP during infection include digestion of host proteins on host surface for nitrogen nutrient supply (Backman, 2005), evasion of host defences by degrading immunoglobulins and complement proteins, resisting antimicrobial attack when phagocitosed (Kaminishi *et al.*, 1995), and adherence and degradation of host barriers during invasion (Colina *et al.*, 1996; Morschhauser *et al.*, 1997). The SAP family has ten members (Sap1 to Sap10) and is the most extensively studied group of hydrolytic enzymes and has been linked with the

virulence of the fungus, since its discovery (Staib, 1969; Kwon-Chung *et al.*, 1985). The genes exhibit differential expression profiles under various conditions, in different *in vivo* models and in patients with candidiasis (Naglik *et al.*, 2003; Naglik *et al.*, 2004; Mavor *et al.*, 2005). *In vivo SAP* expression has been mainly studied using the mouse model of systemic infection (Felk *et al.*, 2002; Naglik *et al.*, 2003). In the model of bloodstream disseminated candidiasis, *SAP4* to *SAP6* were the main *SAP* activated genes, while *SAP5* was the main gene activated in a disseminated intraperitoneal infection, both at the initial time of inoculation and during invasion and subsequent dissemination to the kidneys (Staib *et al.*, 2000). *SAP1* to *SAP3* were observed in a later phase of the infectious process (Staib *et al.*, 2000). This pattern of *SAP* gene activation during systemic infections suggested a role for *SAP4* to *SAP6* during the initial phases of invasion and organ colonization and for *SAP1* to *SAP3* in later phases, when *C. albicans* had already established the infection (Staib *et al.*, 2000; Naglik *et al.*, 2003). The contribution of the *SAP* gene family to disease was also confirmed with *sap* mutants, which showed attenuated virulence in a mouse model of systemic infection (Hube *et al.*, 1997; Sanglard *et al.*, 1997). However, Correia *et al.*, (2010) suggested a limited role of Sap1 to Sap6 in *Candida albicans* virulence and host immune response in murine hematogenously disseminated candidiasis, since that no clear difference in the virulence of the different mutants was observed in comparison with the wild-type strain.

Putative roles of microbial extracellular lipases include the digestion of lipids in nutrient acquisition, the adhesion to host cells and tissues, the initiation of inflammatory processes by affecting immune cells, and self-defense by lysing competing microflora (Trofa *et al.*, 2008). Like SAP, lipases are also encoded by a gene family *LIP1* to *LIP10*, and in a similar manner to the *SAP* gene family, the *LIP* genes are differentially expressed at different stages and sites of infection (Mavor *et al.*, 2005). Expression of *LIP5, LIP6, LIP8, and LIP9*, but not the other members was detected in the mouse model of *C. albicans* peritonitis (Hube *et al.*, 2000).

Phospholipases are enzymes capable of hydrolyzing one or more ester linkages in glycerophospholipids. Four PLs have been identified so far in *C. albicans* (PLA, PLB, PLC, and PLD), although only *PLB1* has been shown to be necessary for virulence, in which, Plb1activity was detected at the tips of hyphae during tissue invasion (Ghannoum, 2000). The function of phospholipases during infection is not well understood, although it is believed that they are involved in the adhesion, disruption of host membranes and penetration through host cells (Trofa *et al.*, 2008; van Asbeck *et al.*, 2009).

1.2.3. Morphogenesis

Candida albicans is a polymorphic diploid fungus that can grow either as unicellular budding yeast (blastospores), as hyphae or as pseudohyphae (Lee *et al.*, 1975; Odds, 1988; Calderone and Fonzi, 2001; Romani *et al.*, 2003; Sudbery *et al.*, 2004). The ability to switch rapidly from yeast-to-filamentous growth or vice versa has been often considered to be a critical virulence factor for this fungus (Lo *et al.*, 1997; Mitchell, 1998; Odds *et al.*, 2001; Rooney and Klein, 2002; Nemecek *et al.*,

2006). The yeast form is important for dissemination through the bloodstream (Bendel *et al.*, 2003; Saville *et al.*, 2003), and adheres to endothelial surfaces (Grubb *et al.*, 2009) while the filamentous forms, on the other hand, is more adapted for invasion through the host epithelial tissue (Rooney and Klein, 2002), and also have a higher resistance to phagocytosis (Lorenz *et al.*, 2004). Several environmental factors can induce yeast cells to form hyphae and pseudohyphae (Romani *et al.*, 2003; Sudbery *et al.*, 2004). Hyphal growth is promoted by a variety of environmental conditions such as the temperature of 37°C, the presence of serum, neutral pH, high CO_2 and growth on N-acetylglucosamine. Yeast form growth is favoured by a 30°C growth temperature and acidic pH (pH 4.0), while pseudohyphae are triggered by amino acid starvation, high phosphate concentrations and by growth at pH 6.0 and 35ºC (Sudbery *et al.*, 2004; Whiteway and Oberholzer, 2004; Kim and Sudbery, 2011).

In the last 10 years, the use of gene knockout mutagenesis and transcriptional studies, revealed signalling pathways, transcriptional factors, as well as other regulatory components that collectively drive the yeast-to-hyphal transition (Gow, 2009). The roles of signalling pathways and transcriptional regulators on *C. albicans* morphogenesis have been discussed in many review articles (Brown and Gow, 1999; Ernst, 2000; Liu, 2001; Navarro-Garcia *et al.*, 2001; Liu, 2002; Biswas *et al.*, 2007; Roman *et al.*, 2007; Alonso-Monge *et al.*, 2009; Hogan and Sundstrom, 2009; Wang, 2009; Han *et al.*, 2011).

1.2.4. Phenotypic switching

In addition to the yeast-hyphae transition, *C. albicans* is capable of undergoing a different type of morphological change that has been termed phenotypic switching (Slutsky *et al.*, 1985; Soll *et al.*, 1987; Soll, 1992; Soll, 1997; Soll, 2001). This change involves the spontaneous and reversible generation of different morphological and physiological states, expressing different patterns of pathogenicity-related traits, such as virulence factors, antigens, and resistance to antifungal drugs (Braun and Johnson, 1997; Lo *et al.*, 1997). The white–opaque transition in the WO-1 strain is the most widely studied phenotypic switching and this switch gives rise to distinct cellular forms (Soll, 1997). White-opaque switching contributes to a better adaptation of *C. albicans* to new host niches. While white cells are much more virulent than opaque cells after intravenous infection, opaque cells are better able to infect skin (Kvaal *et al.*, 1997; Kvaal *et al.*, 1999). Moreover, switching has been demonstrated to regulate a number of phenotypic characteristics involved in pathogenesis such as adhesion, expression of cell surface hydrophobicity and proteinases and phospholipases secretion (Antony *et al.*, 2009).

Both processes, dimorphic transition and switching, confer *C. albicans* cells the ability to generate variants which allow a better selective adaptation to changing environmental conditions, and particularly to evade the host immune system (Braun and Johnson, 1997; Lo *et al.*, 1997).

1.2.5. Biofilm formation

Biofilms are universal, complex, interdependent communities of surface-associated microorganisms. *Candida albicans*, is able to form a biofilm when free-living cells, called planktomic cells, rearrange in to a complex structure of yeast and filamentous cells that coexist as an organized community with a complex three-dimensional architecture that is enveloped within an exopolysaccharide matrix, attached to a solid substratum (Chandra *et al.*, 2001; Blankenship and Mitchell, 2006; Nobile and Mitchell, 2006). Biofilms, with their elaborate architecture present a serious medical problem in treating patients with systemic infections because they have a higher resistance to both antifungal drugs and the host immune response (Baillie and Douglas, 1999; Chandra *et al.*, 2001; Ramage *et al.*, 2001; Kuhn *et al.*, 2002; Mishra *et al.*, 2007). The ability of *C. albicans* to switch from the yeast form to the hyphal form, which is one of the major virulence determinants of this fungal species (Lo *et al.*, 1997; Saville *et al.*, 2003), is a key factor for *C. albicans* biofilm development (Baillie and Douglas, 1999; Ramage *et al.*, 2001).

Biofilm-forming potential is cited as a reason for catheters removal in patients with *Candida*-infected catheters, particularly *C. parapsilosis*, because the ability to grow as a biofilm is directly related to clinically significance of the disease (Kuhn *et al.*, 2002).

1.2.6. Quorum sensing

Quorum sensing is the phenomenon of cell-to-cell communication and signalling in microorganisms. Quorum sensing is often referred to as autoinduction, a process by which individual cells release small diffusible molecules into their environment and these molecules are sensed by all cells in the population (Fuqua *et al.*, 1994; Gray *et al.*, 1994; Hense *et al.*, 2007). The existence of quorum sensing in prokaryotes has been studied extensively, but quorum sensing research in eukaryotes, especially in the medically important diploid fungi *Candida* spp., has gained momentum only recently (Hornby *et al.*, 2001; Chen *et al.*, 2004). Tryptophol and phenylethyl alcohol were the first quorum-sensing molecules (QSMs) identified in *C. albicans* (Lingappa *et al.*, 1969), three additional QSMs have been isolated from *C. albicans*, farnesol, tyrosol and farnesoic acid (Hornby *et al.*, 2001; Oh *et al.*, 2001; Chen *et al.*, 2004; Hornby and Nickerson, 2004). Initially, it was thought that QSMs such as farnesol could be manipulated to treat invasive candidiasis as a fungistatic agent (Hornby *et al.*, 2001), but the discovery that endogenous farnesol actually contributed to *C. albicans* virulence has redirected recent research into understanding QSMs as an important new virulence factor of systemic candidiasis.

1.3. Host immune response

Effective host response against fungal organisms requires the coordinated contribution of both innate and adaptative immunity (Antachopoulos and Roilides, 2005). The initial mechanism of innate immunity against *Candida* infections includes the barrier function of the skin and mucosal epithelial surfaces, the microbial antagonism of the resident flora and the local defence mechanisms (Romani, 2004). Local defences include proteins, such as lactoferrin, beta-defensins, lysozyme, transferrin,

lactoperoxidase, mucins, and secretory immunoglobulin A. Together, these mechanisms contribute to avoid adhesion and growth of *Candida* cells on mucosa surfaces (Steele *et al.*, 2000). If these barriers in the body are damaged by surgery, polytrauma, drug treatment, or spreading from biofilms produced on medical devices, *Candida* cells may enter the bloodstream by direct penetration from epithelial tissues (Mavor *et al.*, 2005). Neutrophils, macrophages and monocytes are fundamentally important antifungal effector cells and constitute the second barrier that *Candida* cells have to overcome. Phagocytes already residing in target organs at the time of infection, attempt to kill or damage fungi. Additional effector cells, including neutrophils and monocytes, are recruited to sites of infection by the action of inflammatory signals, such as cytokines, chemokines and complement components. Hyphae are capable of inducing production of inflammatory cytokines to a higher extent than phagocytosed yeasts (Romani., 2002).

Phagocytic cells recognize and engulf yeast cells via cell-surface pattern recognition receptors. Binding to those receptors activates specialized antifungal effector functions on neutrophils and other phagocytes (Bellocchio *et al.*, 2004). Neutrophils usually lack the machinery required to present antigen to T cells, being their primary function to engulf and kill *Candida* cells (Oehler *et al.*, 1998). At a site of infection, after the resident macrophages, other exudative monocytes are recruited, several hours after neutrophils, which persist for a longer period (van Burik and Magee, 2001). Macrophages possess the machinery required for antigen presentation, being their central role the killing and presentation of pathogen antigens. Whereas neutrophils appear to be able to phagocytose the fungal yeasts, the filamentous forms are not easily phagocytosed. After recognition, *C. albicans* is internalized into the phagosome, which fuses with the lysosome creating the phagolysosome, an organelle with acidic pH and several antimicrobial compounds (Lorenz and Fink, 2002). Degradation of phagocytosed *C. albicans* cells is carried out by oxidative mechanisms, including generation of reactive oxygen and nitrogen intermediates, as nitric oxide, and by non-oxidative mechanisms (Kobayashi *et al.*, 2004). Fungi are then killed or damaged by production and/or release of reactive oxygen intermediates and antimicrobial peptides (Diamond *et al.*, 1980; Mambula *et al.*, 2000). Whether the cells use intracellular or extracellular antifungal mechanisms depends upon the infecting species, morphotype, and route of exposure (Diamond *et al.*, 1978; Schaffner *et al.*, 1982; Kan and Bennett, 1988). Quantitative and qualitative abnormalities of neutrophils and monocytes are associated with systemic candidiasis. Thus, patients with lymphoma, leukaemia, chronic granulomatous disease, and recipients of intensive cancer chemotherapy with resultant neutropenia are at increased risk for disseminated infection.

Complement system is also an important part of the innate resistance to infection (Romani., 2002). *Candida* cells or antibodies directed against mannan antigens activate the complement cascade, leading to destruction of the pathogen directly through the membrane attack complex or by phagocytic cells called by complement fragments that recognize complement opsonized *Candida* cells.

Upon microorganism interaction, phagocytes secrete cytokines and chemokines that polarize the immune response through interaction with T-cells. Thus, macrophages and dendritic cells are the phagocytes with a greater responsibility in presenting antigen and inducing a T-cell mediated response, with cytokines production (Kim *et al.*, 2005). This adaptative immunity is composed by adaptive T helper-cell responses that can be mainly classified as pro-inflammatory (Th1 and Th17 responses) or anti-inflammatory (Th2 response). Dendritic cells that ingest the yeast form induce differentiation of CD4+ T cells toward a Th1 pathway. In contrast, hyphae induce Th2 responses (d'Ostiani *et al.*, 2000). Moreover, development of Th1 or Th2 responses is an important determinant of the host's ability to contain infection. Th1 responses are correlated with protection and their development is influenced by the concerted action of several cytokines such as INF-γ (interferon gama), IL (interleukin)-6, TNF-α (Tumor necrosis factor alfa), IL-12 and IL-17, in the relative absence of Th2 cytokines, such as IL-4, IL-5, IL-10 and IL-13, which inhibit the induction of Th1 responses (Romani., 2002). INF-γ, IL-6, and TNF-α are proinflammatory cytokines readily produced upon interaction of phagocytes with fungal cells and are detected in *Candida* sepsis. The anti-inflammatory responses involve the production of Th2 cytokines that are known to reduce the ability of macrophages to produce nitric oxide (Cenci *et al.*, 1993) and elicit the production of non-opsonizing antibodies and allergic reactions. Regulatory T-cells allow the balance between the two responses, operating from commensalism to infection. Although neutropenia is a major predisposing factor to candidemia, the propensity for persistence of the fungus in infected tissues may be a consequence of cell-mediated immune deregulation with suppression of Th1 and overexpression of Th2 responses (Shoham and Levitz, 2005). Future therapies for invasive fungal infections may include agents that augment antifungal activity of effector cells and alter Th balance.

1.4. Cell wall

1.4.1. Molecular structure

The cell wall is an essential structure that maintains the viability of fungal cells, conferring their typical morphology, osmotic stabilization and protection against external injuries (Klis *et al.*, 2001; Klis *et al.*, 2002; Netea *et al.*, 2008). As the most external cellular structure of pathogenic microorganisms, it also carries important antigenic determinants and mediates adhesion to the host tissues, being therefore crucial to initiate colonization and therefore, cause disease (Calderone and Fonzi, 2001; Sundstrom, 2002). The cell wall is the structure sensed by the host immune cells. As a consequence, it participates in triggering the whole innate and adaptive immune response against the microorganism. Structurally, the yeast cell wall accounts for 15–30% of the cell dry weight and in *C. albicans* the cell wall is made of 80-90% polysaccharides, 6-25% proteins, and in a minor amount of 1-7% lipids (Chaffin *et al.*, 1998). The major polysaccharides of the cell wall are represented by three basic constituents, (i) branched polymers of D-glucose containing β-1,3 and β-1,6 linkages (β-glucans), (ii) unbranched polymers of N-acetyl-D-glucosamine (GlcNAc), containing β-1,4 bonds (chitin), and (iii) polymers of mannose (also called phosphopeptidomannan) covalently associated with proteins (gluco or mannoproteins). β-1,3- and β-1,6-glucans and chitin, represent the inner structural components of

the fungal cell wall to which a protective mannoprotein layer is attached (Fig. 1.2) (Casanova *et al.*, 1992; Klis *et al.*, 2001; Klis *et al.*, 2002; Netea *et al.*, 2008).

The glucan polymer network is composed of β-1,3-glucan linear chains interconnected by means of modules of β-1,6-glucans, accounting for 50–60% of the total cell wall. The enzyme responsible for the synthesis of β-1,3-glucan is the glucan synthase (GS) whose catalytic subunit is encoded by the genes *FKS1* and *FKS2*. The function of β-1,6-glucans is the connection of the network of β-1,3-glucan with some cell wall proteins. β-1,3-glucans are distributed through the cell surface and are covalently linked to some chitin chains, providing a scaffold to which mannoproteins are also covalently attached (Fig. 1.2). Glucans form the structural skeleton of the cell wall and their importance was recognized by the fact that inhibition of glucan synthesis or degradation of its chains confers cell fragility and morphological alterations on *Candida* cells (Lipke and Ovalle, 1998; Kapteyn *et al.*, 1999; Klis *et al.*, 2001; Klis *et al.*, 2002; Gozalbo *et al.*, 2004).

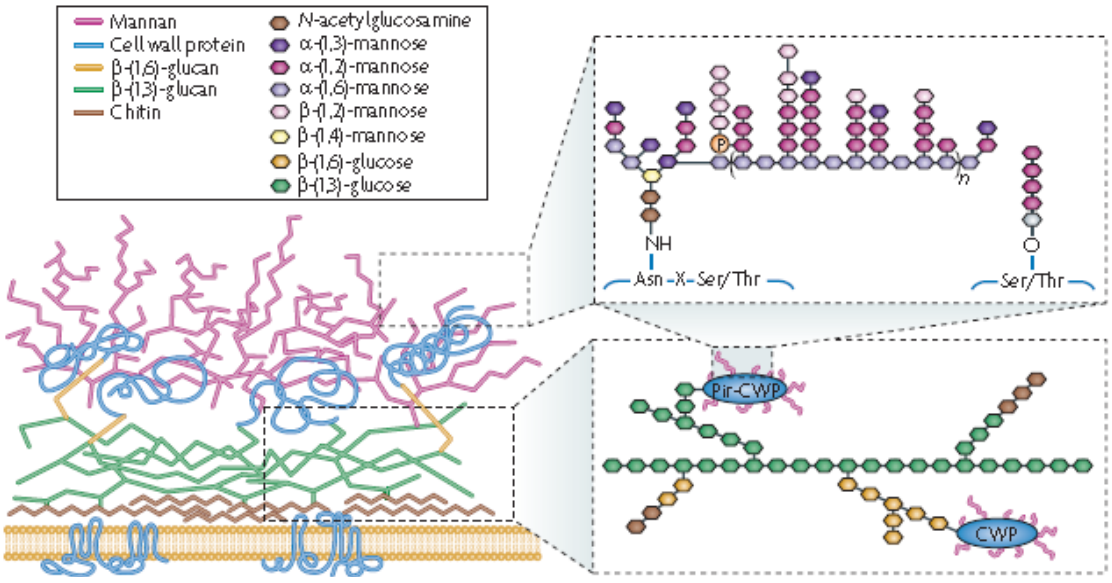

Figure 1.2. The structure of the *Candida albicans* cell wall. The schematic shows the major components of the cell wall and their distributions. β-1,3-glucan and chitin (poly-β-1,4-N-acetylglucosamine) are the main structural components, and these are located towards the inner layer of the cell wall. The outer layers are enriched with cell wall proteins (CWP) that are attached to this skeleton mainly via glycosylphosphatidylinositol remnants to β-1,6-glucan or, to mannoproteins with internal repeat domains (Pir-CWP), via alkali-sensitive linkagers to β-1,3-glucan. The inserts show the structure of glucan and mannan components. Adapted from Netea *et al.*, 2008.

The external outer layer is composed of mannoproteins (35-40% of the total cell wall), also called cell wall proteins (CWPs), which are normally highly glycosylated (either O- or N-glycosylated) with mannose-containing polysaccharides that can account for up to 90% of the mannoproteins molecular mass (Arana 2009). Mannans are largely responsible for determining the porosity of the cell wall and protecting the glucan layer of the action of degradative enzymes. In addition, mannans are the main molecules responsible for the adherence ability, as well as for resistance to a wide range of environmental conditions (Lipke and Ovalle, 1998; Kapteyn *et al.*, 1999; Klis *et al.*, 2002). In *C. albicans*, CWPs frequently contain internal repeats (named Pir-CWPs) that are directly linked to β-1,3-glucan, whereas others contain a glycosylphosphatidylinositol derived-structure (GPI-CWPs) and are

attached to the β-1,6-glucan. Mannans exist as glucoconjugates and were first identified as the major cell surface antigen of *C. albicans,* even before the revelation of their chemical structure (Whiteway and Oberholzer, 2004). Receptors for mannans are found on the surface of macrophages, suggesting their involvement in the phagocytosis of *Candida* cells (Masuoka, 2004).

Chitin is an essential component (1-2% of the total cell wall) found dispersed in the cell wall, in the bud neck, in the primary septum and in bud scars (Cabib and Duran, 2005). This particular localization together with the fact that upon stress the content in chitin can increase considerably (Aguilar-Uscanga and Francois, 2003), suggest that chitin is the material used for cell wall remodelling. Chitin and β-1,3-glucans are mainly responsible for providing the cell wall strength and rigidity, which ultimately determine the morphology of the cell, therefore their synthesis play a fundamental role in maintaining fungal cell integrity during growth and morphogenesis as well as in adaptation to stress (Shaw *et al.*, 1991).

1.4.2. The signalling MAP kinase cascade

Cells have developed mechanisms that sense external stimuli and trigger cellular responses that allow a rapid adaptation to new extracellular conditions. These responses involve changes in gene expression and precise routes that translate the signal from the cell surface to the nucleus, the site of the transcriptional machinery. The sensor proteins located in the plasma membrane are responsible for detecting the signals produced by environmental disturbances. Once a stimulus is detected, the signal is transmitted to cellular targets by different protein kinases. In some cases these kinases are organized in a module, conserved throughout evolution, known as cascade of MAP kinases (*mitogen-activated protein*), which are highly conserved among eukaryotic cells (Kultz and Burg, 1998). The structure comprises three kinases that work by sequential phosphorylation. Upon receiving a stimulatory signal, the MAP kinase kinase kinase (MAPKKK or MEKK) is phosphorylated by different upstream modules, which frequently involve the PAK (p21 activated kinase) family of protein kinases and small GTPases. Phosphorylation of MAPKKK triggers the phosphorylation of the MAP kinase kinase (MAPKK or MEK), which in turn phosphorylates the MAP kinase (MAPK). The signal is finally transferred to downstream effectors, normally a transcription factor or other molecules, which either activate or relieve the repression of the corresponding target genes, modulating gene expression. The expression of the target genes is essential in the adaptive response of the cell to the activating stimulus (Marshall, 1994).

These pathways have been extensively characterized in non-pathogenic model yeasts such as *S. cerevisiae (Gustin et al., 1998)* and *Schizosaccharomyces pombe* (Ikner and Shiozaki, 2005). In *S. cerevisiae,* MAPK pathways have been implicated in at least five different processes: i) the cell integrity mitogen-activated protein (MAP) kinase pathway or protein kinase C (PKC) pathway; ii) the adaptation to stress (osmotic, but also oxidative, acid and heat) mainly occurs through the high osmolarity glycerol (HOG) pathway; iii) the pheromone response pathway or mating and iv) the filamentous growth pathway, that share many members of the central module of MAP kinases; and v)

the sporulation pathway, also called nutrient deprivation, controls the formation of spores when extracellular environmental conditions are adverse. Each of these pathways detects and translates specific signals from different types of stimuli (Posas *et al.*, 1998; Schmidt and Hall, 1998; Heinisch *et al.*, 1999; Millar, 1999; O'Rourke *et al.*, 2002; Perez and Calonge, 2002; Edmunds and Mahadevan, 2004; Schwartz and Madhani, 2004; Levin, 2005).

In the pathogenic yeast *C. albicans*, genetic analysis allowed the identification of different routes, especially by the phenotypic characterization of mutants altered in the Mkc1, Cek1/2 and Hog1 MAP kinases (Fig. 1.3). The cell integrity pathway is mediated by Mkc1, in a manner dependent of the presence of Pkc1, and is primarily involved in the biogenesis of the cell wall (Navarro-Garcia *et al.*, 1995; Diez-Orejas *et al.*, 1997; Navarro-Garcia *et al.*, 1998; Navarro-Garcia *et al.*, 2005). The HOG pathway participates in the response to osmotic stress while the Cek1/2 pathway mediates mating and filamentation (Whiteway *et al.*, 1992; San Jose *et al.*, 1996; Chen *et al.*, 2002; Alonso-Monge *et al.*, 2003; Smith *et al.*, 2004; Arana *et al.*, 2005). Their actual functions are, however, much broader. Mkc1 senses several types of stress, while Hog1 is also responsive to other stress conditions and participates in two morphogenetic programs: filamentation and chlamydospore formation (Alonso-Monge *et al.*, 1999; Calera *et al.*, 2000; Alonso-Monge *et al.*, 2003; Arana *et al.*, 2005). Furthermore, it has been recently shown that Cek1 participates in a putative pathway involved in the construction of the cell wall, which seems to be operative under basal conditions (Chen *et al.*, 2002; Roman *et al.*, 2005).

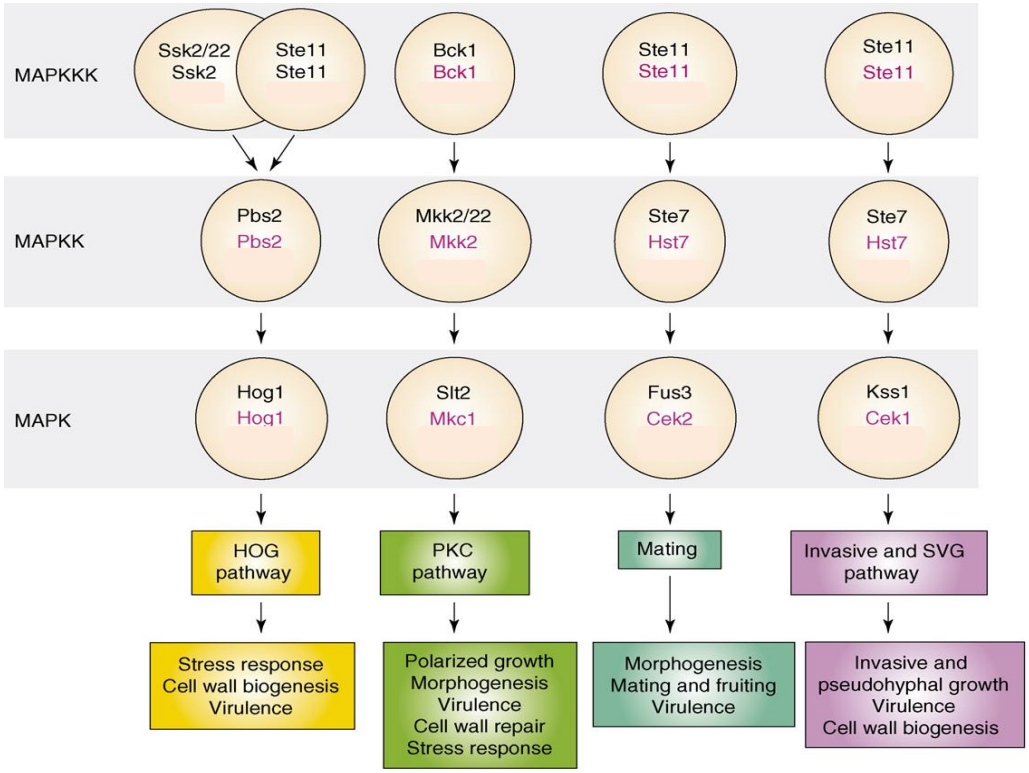

Figure 1.3. MAP kinase pathways in fungi. The central core genes of MAPK pathways in two selected fungal models are shown: *Saccharomyces cerevisiae* (black) and *Candida albicans* (pink). The functions associated to each of them are shown beneath the core genes. Adapted from Román *et al.,* 2007.

1.4.3. Cell Wall integrity (CWI) pathway

Wall remodeling is monitored and regulated mainly by the cell wall integrity (CWI) signaling, which activates a MAP kinase cascade. The cell integrity or Pkc1-mediated pathway responds to perturbations in the cell surface, so the first components of the pathway must be membrane- or cell wall-associated proteins that would function as sensors. This is well understood from studies in the budding yeast *S. cerevisiae*, where the CWI pathway comprises the sensors Mid2 and Wsc1 in the plasma membrane, which, upon cell wall damage, interact with the GDP/GTP exchange factor Rom2, leading to the conversion of the G protein Rho1 to its activated GTP-bound state. Interaction with Rho1 activates the control kinase Pkc1 that phosphorylates the MAPKKK Bck1, which, at least *in vitro*, is phosphorylated in multiple sites (Levin *et al.*, 1994). It is assumed that Bck1 phosphorylates serine and threonine residues of two redundant MAPKK, Mkk1 and Mkk2 (Irie *et al.*, 1993; Kamada *et al.*, 1995; Paravicini *et al.*, 1996), which in turn phosphorylate the MAP kinase. MAPK Slt2 (also called Mpk1) is simultaneously phosphorylated on both threonine and tyrosine conserved residues present in subdomain VIII of the kinase superfamily, a distinctive feature that separates this one from other kinases (Lee *et al.*, 1993; Marshall, 1994).

Mutants defective in elements of CWI pathway display an osmotic-remediable lytic phenotype caused by defective cell wall biogenesis (de Nobel *et al.*, 2000; Levin, 2005). This route, among other functions, is involved in the biosynthesis of the new cell wall when polarized growth is required, as well as in maintenance of the cell wall integrity under stress conditions. This pathway is activated upon heat shock (Kamada *et al.*, 1995), hypoosmotic conditions (Davenport *et al.*, 1995) and treatment with mating pheromone due to cell wall remodelling during pheromone-induced morphogenesis (Buehrer and Errede, 1997). Agents that interfere with cell wall biogenesis such as the chitin antagonist calcofluor white (Ketela *et al.*, 1999), Congo red, caffeine or zymolyase stimulate this pathway (de Nobel *et al.*, 2000; Martin *et al.*, 2000).

In *C. albicans*, Mkc1, the homologue of the *S. cerevisiae* Slt2/Mpk1 MAPK, mediates the CWI pathway. The kinase Mkc1 becomes activated in response to several types of stress (oxidative, osmotic, cell wall damage, calcium ions and temperature) (Navarro-Garcia *et al.*, 2005). Oxidative stress-mediated phosphorylation of Mkc1 is partially dependent on an intact HOG pathway (Arana *et al.*, 2005; Navarro-Garcia *et al.*, 2005). Moreover, Mkc1 becomes activated when cells contact solid surfaces, therefore being related to biofilm formation (Kumamoto, 2005); and it is also activated in the presence of rapamycin, an inhibitor of the Tor kinase (Tsao *et al.*, 2009). Mutants affected in *MKC1* are more sensitive to cell wall-degrading enzymes and antifungals, and display surface alterations when grown under restrictive conditions such as high temperature (Navarro-Garcia *et al.*, 1995; Navarro-Garcia *et al.*, 1998). Not surprisingly, *mkc1* mutants display a reduced virulence in the mouse model of systemic infection (Diez-Orejas *et al.*, 1997).

CWI signaling pathway induces activation of the two known transcription factors: the transcription factor heterodimer complex SBF (Swi4-Swi6 cell cycle Box binding Factor), which is involved in the activation of expression of cell cycle-regulated genes in the G1/S phase, including genes involved in cell wall synthesis, (Nasmyth and Dirick, 1991; Madden *et al.*, 1997; Baetz *et al.*, 2001) and the MADS-box transcription factor Rlm1, which regulates the expression of genes involved in the cell wall integrity pathway (Dodou and Treisman, 1997; Watanabe *et al.*, 1997).

1.4.4. The MADS-box transcription factor Rlm1

The MADS-box proteins have been identified in yeasts, plants, insects, nematodes, lower vertebrates and mammals. These proteins are characterized by a domain that presents DNA binding and dimerization conserved regions named the MADS-box (Schwarz-Sommer *et al.*, 1990) because of the five founding members of the family: Mcm1 (yeast) (Passmore *et al.*, 1989), Arg80 (yeast) (Dubois *et al.*, 1987) or Agamous (plant) (Yanofsky *et al.*, 1990), Deficiens (plant) (Sommer *et al.*, 1990) and SRF (human) (Norman *et al.*, 1988). In animal and fungi, two distinct types of MADS-box genes have been identified, the SRF-like (type I) and the MEF2-like (type II) (Shore and Sharrocks, 1995; Alvarez-Buylla *et al.*, 2000). MADS-box family members generally recognize AT rich consensus sequences, with a highly conserved core of 10 bp, the $CC(A/T)_6GG$ is the binding site of SRF-like proteins known as CArG box (Treisman, 1990), and the $CTA(A/T)_4TAG$ is the binding site of MEF2-like proteins (Pollock and Treisman, 1991). Transcription factors of the MADS box family can act by forming homodimers or heterodimers with other proteins. Their association with different proteins can selectively direct the transcription regulation of various genes. In *S. cerevisiae* four MADS-box proteins have been found: Mcm1 and Arg80 which are related to the SRF human, and Rlm1 and Smp1 which belong to the MEF2-like family (Alvarez-Buylla *et al.*, 2000).

RLM1 was first identified as a gene conferring resistance to lethality of $Mkk1^{S386P}$ overexpression (Watanabe *et al.*, 1995). The Rlm1 protein has three distinct domains: an N-terminal DNA-binding domain related to the MADS-box family of transcriptional regulators that, in *S. cerevisiae*, is known to regulate the expression of genes involved in the cell wall integrity pathway (Watanabe *et al.*, 1995; Dodou and Treisman, 1997). The C-terminal part of the protein contains the transcriptional activation domain and the central part of the protein that is the target for Slt2/Mpk1-dependent phosphorylation (Watanabe *et al.*, 1997). Moreover, the Rlm1 present a repetitive region next to the C-terminal end. Rlm1 is most closely related to mammalian factor MEF2, sharing the same *in vitro* binding specificity ($CTAW_4TAG$) (Dodou and Treisman, 1997). In *S. cerevisiae* Rlm1 is localized in the nucleus irrespective of its activation or phosphorylation status. Rlm1 is regulated through phosphorylation by the protein kinase Slt2p/Mpk1 at two residues within its transcriptional activation domain (Ser 427 and Thr 439), which stimulates its activity (Watanabe *et al.*, 1997; Jung *et al.*, 2002). Genome-wide analysis of gene expression from activation of Slt2/Mpk1 revealed that Rlm1 regulates the expression of at least 25 genes, most of which encode cell wall proteins or have been implicated in cell wall biogenesis in response to cell wall stress (Watanabe *et al.*, 1997; Jung and Levin, 1999). Moreover, it has also been determined that the transcription factor Rlm1 can act as both activator and repressor of

the gene expression (Watanabe *et al.*, 1997; Jung and Levin, 1999). On the other hand, although Rlm1 mediates gene expression regulated by CWI signaling, its deletion does not produce characteristic cell lysis phenotype of CWI pathway mutants. Therefore, it is assumed that there are other transcription factors that mediate the regulation of CWI pathway-dependent cell wall gene expression.

Putative *RLM1* orthologues were identified in *C. albicans*, *Paracoccidioides brasiliensis, Aspergillus niger* and *C. glabrata* (Damveld *et al.*, 2005; Fernandes *et al.*, 2005; Bruno *et al.*, 2006; Miyazaki *et al.*, 2010). An *in silico* analysis of sequence comparison showed that *RLM1* homologe sequences are present in all yeasts belonging to Ascomycota, as well as in yeasts belonging to Basidiomycota and "Zygomycota" (taxonomy still being studied). This observation would indicate that the presence of this gene is important in the maintenance of cell wall integrity in other fungi (Chávez-Galarza, 2009). In *C. albicans* the role of *RLM1* gene is not well established yet but it seems to be required for cell wall integrity, at least under caspofungin and Congo red stress (Bruno *et al.*, 2006).

1.5. Objectives and outline of the thesis

Three main objectives were outlined for this work. The first one was the determination of the influence of the genetic variability of CAI microsatellite, the repetitive region of *RLM1* in *C. albicans,* in cell wall stress resistance. The second one was the evaluation of the *RLM1* role in *C. albicans* and its molecular and functional characterization regarding the remodeling of the cell wall under stress conditions. Finally, the third objective was the investigation of the involvement of *RLM1* in *C. albicans* virulence in the disseminated model of systemic infection. To accomplish these objectives this thesis is organized in five chapters. **Chapter 1** encloses an overview about fungal infection particularly the pathogen *C. albicans* covering, types of candidiasis, epidemiology, an outline on virulence factors and host response, as well as a description of the cell wall integrity pathway. **Chapter 2** regards the correlation of increased number of glutamine repeats in the C-terminus of Rlm1 with the resistance to stress agents. **Chapter 3** presents information concerning the role of *RLM1* in *C. albicans* cell wall integrity and includes the methods to disrupt *RLM1* gene, the phenotypic characterization against cell wall damaging stress agents, the subcellular localization and genome-wide expression regulated by Rlm1 in *C. albicans*. **Chapter 4** presents data and discussion of the role of *RLM1* in *C. albicans* virulence by using the murine model of hematogenously disseminated candidiasis. **Chapter 5** presents the concluding remarks, regarding the results of Chapter 2, 3 and 4 in the context of the initially proposed objectives.

1.6. References

Aguilar-Uscanga, B. and J. M. Francois (2003). "A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation." *Lett Appl Microbiol* 37(3): 268-274.

Alexopoulos, C. J., Mims, C.W., and Blackwell, M (1996). "Introductory Micology." *4th edition. John Wiley & Sons Inc., New York, EE.UU.* .

Almirante, B., D. Rodriguez, M. Cuenca-Estrella, M. Almela, F. Sanchez, J. Ayats, C. Alonso-Tarres, J. L. Rodriguez-Tudela and A. Pahissa (2006). "Epidemiology, risk factors, and prognosis of *Candida parapsilosis* bloodstream infections: case-control population-based surveillance study of patients in Barcelona, Spain, from 2002 to 2003." *J Clin Microbiol* 44(5): 1681-1685.

Alonso-Monge, R., F. Navarro-Garcia, G. Molero, R. Diez-Orejas, M. Gustin, J. Pla, M. Sanchez and C. Nombela (1999). "Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans.*" *J Bacteriol* 181(10): 3058-3068.

Alonso-Monge, R., F. Navarro-Garcia, E. Roman, A. I. Negredo, B. Eisman, C. Nombela and J. Pla (2003). "The Hog1 mitogen-activated protein kinase is essential in the oxidative stress response and chlamydospore formation in *Candida albicans*." *Eukaryot Cell* 2(2): 351-361.

Alonso-Monge, R., E. Roman, D. M. Arana, J. Pla and C. Nombela (2009). "Fungi sensing environmental stress." *Clin Microbiol Infect* 15 Suppl 1: 17-19.

Alvarez-Buylla, E. R., S. Pelaz, S. J. Liljegren, S. E. Gold, C. Burgeff, G. S. Ditta, L. Ribas de Pouplana, L. Martinez-Castilla and M. F. Yanofsky (2000). "An ancestral MADS-box gene duplication occurred before the divergence of plants and animals." *Proc Natl Acad Sci U S A* 97(10): 5328-5333.

Antachopoulos, C. and E. Roilides (2005). "Cytokines and fungal infections." *Br J Haematol* 129(5): 583-596.

Antony, G., V. Saralaya, K. Gopalkrishna Bhat, M. Shalini Shenoy and P. G. Shivananda (2009). "Effect of phenotypic switching on expression of virulence factors by *Candida albicans* causing candidiasis in diabetic patients." *Rev Iberoam Micol* 26(3): 202-205.

Arana, D. M., C. Nombela, R. Alonso-Monge and J. Pla (2005). "The Pbs2 MAP kinase kinase is essential for the oxidative-stress response in the fungal pathogen *Candida albicans*." *Microbiology* 151(Pt 4): 1033-1049.

Backman, D. (2005). "Interaction Studies of Secreted Aspartic Proteases from *Candida albicans*." *Aplication for Drug discovery, in Acta Universitatis Upsaliensis. Faculty of Science and Technology: Uppsala.* 2005.

Baetz, K., J. Moffat, J. Haynes, M. Chang and B. Andrews (2001). "Transcriptional coregulation by the cell integrity mitogen-activated protein kinase Slt2 and the cell cycle regulator Swi4." *Mol Cell Biol* 21(19): 6515-6528.

Baillie, G. S. and L. J. Douglas (1999). "*Candida* biofilms and their susceptibility to antifungal agents." *Methods Enzymol* 310: 644-656.

Bellocchio, S., C. Montagnoli, S. Bozza, R. Gaziano, G. Rossi, S. S. Mambula, A. Vecchi, A. Mantovani, S. M. Levitz and L. Romani (2004). "The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo." *J Immunol* 172(5): 3059-3069.

Bendel, C. M., D. J. Hess, R. M. Garni, M. Henry-Stanley and C. L. Wells (2003). "Comparative virulence of *Candida albicans* yeast and filamentous forms in orally and intravenously inoculated mice." *Crit Care Med* 31(2): 501-507.

Biswas, S., P. Van Dijck and A. Datta (2007). "Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*." *Microbiol Mol Biol Rev* 71(2): 348-376.

Blackwell, M. (2000). "Evolution. Terrestrial life--fungal from the start?" *Science* 289(5486): 1884-1885.

Blankenship, J. R. and A. P. Mitchell (2006). "How to build a biofilm: a fungal perspective." *Curr Opin Microbiol* 9(6): 588-594.

Bodey, G., B. Bueltmann, W. Duguid, D. Gibbs, H. Hanak, M. Hotchi, G. Mall, P. Martino, F. Meunier, S. Milliken and et al. (1992). "Fungal infections in cancer patients: an international autopsy survey." *Eur J Clin Microbiol Infect Dis* 11(2): 99-109.

Braun, B. R. and A. D. Johnson (1997). "Control of filament formation in *Candida albicans* by the transcriptional repressor TUP1." *Science* 277(5322): 105-109.

Brown, A. J. and N. A. Gow (1999). "Regulatory networks controlling *Candida albicans* morphogenesis." *Trends Microbiol* 7(8): 333-338.

Bruno, V. M., S. Kalachikov, R. Subaran, C. J. Nobile, C. Kyratsous and A. P. Mitchell (2006). "Control of the *C. albicans* cell wall damage response by transcriptional regulator Cas5." *PLoS Pathog* 2(3): e21.

Buehrer, B. M. and B. Errede (1997). "Coordination of the mating and cell integrity mitogen-activated protein kinase pathways in *Saccharomyces cerevisiae*." *Mol Cell Biol* 17(11): 6517-6525.

Butler, G., M. D. Rasmussen, et al. (2009). "Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes." *Nature* 459(7247): 657-662.

Cabib, E. and A. Duran (2005). "Synthase III-dependent chitin is bound to different acceptors depending on location on the cell wall of budding yeast." *J Biol Chem* 280(10): 9170-9179.

Calderone, R. (2002). "*Candida* and candidiasis." *ASM Press, washington, DC*.

Calderone, R. A. and W. A. Fonzi (2001). "Virulence factors of *Candida albicans*." *Trends Microbiol* 9(7): 327-335.

Calera, J. A., X. J. Zhao and R. Calderone (2000). "Defective hyphal development and avirulence caused by a deletion of the SSK1 response regulator gene in *Candida albicans*." *Infect Immun* 68(2): 518-525.

Casanova, M., J. L. Lopez-Ribot, J. P. Martinez and R. Sentandreu (1992). "Characterization of cell wall proteins from yeast and mycelial cells of *Candida albicans* by labelling with biotin: comparison with other techniques." *Infect Immun* 60(11): 4898-4906.

Cenci, E., L. Romani, A. Mencacci, R. Spaccapelo, E. Schiaffella, P. Puccetti and F. Bistoni (1993). "Interleukin-4 and interleukin-10 inhibit nitric oxide-dependent macrophage killing of *Candida albicans*." *Eur J Immunol* 23(5): 1034-1038.

Chaffin, W. L., J. L. Lopez-Ribot, M. Casanova, D. Gozalbo and J. P. Martinez (1998). "Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression." *Microbiol Mol Biol Rev* 62(1): 130-180.

Chandra, J., D. M. Kuhn, P. K. Mukherjee, L. L. Hoyer, T. McCormick and M. A. Ghannoum (2001). "Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance." *J Bacteriol* 183(18): 5385-5394.

Chauhan, N., J. P. Latge and R. Calderone (2006). "Signalling and oxidant adaptation in *Candida albicans* and *Aspergillus fumigatus*." *Nat Rev Microbiol* 4(6): 435-444.

Chávez-Galarza, J. D.-S., Y.; Pais, C.; Sampaio, P. (2009). "Preliminary studies of the gene *RLM1* to phylogenetic analysis of the kingdom Fungi" *Journal of Museum of Natural History, Particular University of Antenor Orrego. ARNALDOA* 16(2): 7-22.

Chen, H., M. Fujita, Q. Feng, J. Clardy and G. R. Fink (2004). "Tyrosol is a quorum-sensing molecule in *Candida albicans.*" *Proc Natl Acad Sci U S A* 101(14): 5048-5052.

Chen, J., S. Lane and H. Liu (2002). "A conserved mitogen-activated protein kinase pathway is required for mating in *Candida albicans*." *Mol Microbiol* 46(5): 1335-1344.

Colina, A. R., F. Aumont, N. Deslauriers, P. Belhumeur and L. de Repentigny (1996). "Evidence for degradation of gastrointestinal mucin by *Candida albicans* secretory aspartyl proteinase." *Infect Immun* 64(11): 4514-4519.

Correia, A., U. Lermann, L. Teixeira, F. Cerca, S. Botelho, R. M. da Costa, P. Sampaio, F. Gartner, J. Morschhauser, M. Vilanova and C. Pais (2010). "Limited role of secreted aspartyl proteinases Sap1 to Sap6 in *Candida albicans* virulence and host immune response in murine hematogenously disseminated candidiasis." *Infect Immun* 78(11): 4839-4849.

Costa-de-Oliveira, S., C. Pina-Vaz, D. Mendonca and A. Goncalves Rodrigues (2008). "A first Portuguese epidemiological survey of fungaemia in a university hospital." *Eur J Clin Microbiol Infect Dis* 27(5): 365-374.

d'Ostiani, C. F., G. Del Sero, A. Bacci, C. Montagnoli, A. Spreca, A. Mencacci, P. Ricciardi-Castagnoli and L. Romani (2000). "Dendritic cells discriminate between yeasts and hyphae of the fungus *Candida albicans*. Implications for initiation of T helper cell immunity in vitro and in vivo." *J Exp Med* 191(10): 1661-1674.

Damveld, R. A., M. Arentshorst, A. Franken, P. A. vanKuyk, F. M. Klis, C. A. van den Hondel and A. F. Ram (2005). "The *Aspergillus niger* MADS-box transcription factor RlmA is required for cell wall reinforcement in response to cell wall stress." *Mol Microbiol* 58(1): 305-319.

Davenport, K. R., M. Sohaskey, Y. Kamada, D. E. Levin and M. C. Gustin (1995). "A second osmosensing signal transduction pathway in yeast. Hypotonic shock activates the PKC1 protein kinase-regulated cell integrity pathway." *J Biol Chem* 270(50): 30157-30161.

de Nobel, H., C. Ruiz, H. Martin, W. Morris, S. Brul, M. Molina and F. M. Klis (2000). "Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2-mediated increase in FKS2-lacZ expression, glucanase resistance and thermotolerance." *Microbiology* 146 (Pt 9): 2121-2132.

de Nobel, H., H. van Den Ende and F. M. Klis (2000). "Cell wall maintenance in fungi." *Trends Microbiol* 8(8): 344-345.

Diamond, R. D., R. Krzesicki, B. Epstein and W. Jao (1978). "Damage to hyphal forms of fungi by human leukocytes in vitro. A possible host defense mechanism in aspergillosis and mucormycosis." *Am J Pathol* 91(2): 313-328.

Diamond, R. D., F. Oppenheim, Y. Nakagawa, R. Krzesicki and C. C. Haudenschild (1980). "Properties of a product of *Candida albicans* hyphae and pseudohyphae that inhibits contact between the fungi and human neutrophils in vitro." *J Immunol* 125(6): 2797-2804.

Diez-Orejas, R., G. Molero, F. Navarro-Garcia, J. Pla, C. Nombela and M. Sanchez-Perez (1997). "Reduced virulence of *Candida albicans* MKC1 mutants: a role for mitogen-activated protein kinase in pathogenesis." *Infect Immun* 65(2): 833-837.

Dodou, E. and R. Treisman (1997). "The *Saccharomyces cerevisiae* MADS-box transcription factor Rlm1 is a target for the Mpk1 mitogen-activated protein kinase pathway." *Mol Cell Biol* 17(4): 1848-1859.

Dubois, E., J. Bercy and F. Messenguy (1987). "Characterization of two genes, *ARGRI* and *ARGRIII* required for specific regulation of arginine metabolism in yeast." *Mol Gen Genet* 207(1): 142-148.

Dupont, B. (2002). "Overview of the lipid formulations of amphotericin B." *J Antimicrob Chemother* 49 Suppl 1: 31-36.

Edmunds, J. W. and L. C. Mahadevan (2004). "MAP kinases as structural adaptors and enzymatic activators in transcription complexes." *J Cell Sci* 117(Pt 17): 3715-3723.

Edwards, J. E., Jr. (1991). "Invasive candida infections--evolution of a fungal pathogen." *N Engl J Med* 324(15): 1060-1062.

Eggimann, P., J. Garbino and D. Pittet (2003). "Epidemiology of *Candida* species infections in critically ill non-immunosuppressed patients." *Lancet Infect Dis* 3(11): 685-702.

Ernst, J. F. (2000). "Transcription factors in *Candida albicans* - environmental control of morphogenesis." *Microbiology* 146 (Pt 8): 1763-1774.

Felk, A., M. Kretschmar, A. Albrecht, M. Schaller, S. Beinhauer, T. Nichterlein, D. Sanglard, H. C. Korting, W. Schafer and B. Hube (2002). "*Candida albicans* hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs." *Infect Immun* 70(7): 3689-3700.

Fernandes, L., M. A. Araujo, A. Amaral, V. C. Reis, N. F. Martins and M. S. Felipe (2005). "Cell signaling pathways in *Paracoccidioides brasiliensis*--inferred from comparisons with other fungi." *Genet Mol Res* 4(2): 216-231.

Fidel, P. L., Jr., J. A. Vazquez and J. D. Sobel (1999). "*Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*." *Clin Microbiol Rev* 12(1): 80-96.

Filler, S. C. K. B. J. (2002). "Deep-seated candidal infections." *In Calderone RA, ed. Candida and Candidiasis. Washington DC, USA; ASM Press,* pp: 341-348.

Forrest, G. N., E. Weekes and J. K. Johnson (2008). "Increasing incidence of *Candida parapsilosis* candidemia with caspofungin usage." *J Infect* 56(2): 126-129.

Fukazawa, Y. and K. Kagaya (1997). "Molecular bases of adhesion of *Candida albicans*." *J Med Vet Mycol* 35(2): 87-99.

Fuqua, W. C., S. C. Winans and E. P. Greenberg (1994). "Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators." *J Bacteriol* 176(2): 269-275.

Gaur, N. K. and S. A. Klotz (1997). "Expression, cloning, and characterization of a *Candida albicans* gene, *ALA1*, that confers adherence properties upon *Saccharomyces cerevisiae* for extracellular matrix proteins." *Infect Immun* 65(12): 5289-5294.

Ghannoum, M. A. (2000). "Potential role of phospholipases in virulence and fungal pathogenesis." *Clin Microbiol Rev* 13(1): 122-143, table of contents.

Ghannoum, M. A. and K. H. Abu-Elteen (1990). "Pathogenicity determinants of *Candida*." *Mycoses* 33(6): 265-282.

Ghannoum, M. A., G. R. Burns, K. A. Elteen and S. S. Radwan (1986). "Experimental evidence for the role of lipids in adherence of *Candida* spp. to human buccal epithelial cells." *Infect Immun* 54(1): 189-193.

Gomez-Lopez, A., A. Alastruey-Izquierdo, D. Rodriguez, B. Almirante, A. Pahissa, J. L. Rodriguez-Tudela and M. Cuenca-Estrella (2008). "Prevalence and susceptibility profile of *Candida metapsilosis* and *Candida orthopsilosis*: results from population-based surveillance of candidemia in Spain." *Antimicrob Agents Chemother* 52(4): 1506-1509.

Gow, N. A. (2009). "Fungal morphogenesis: some like it hot." *Curr Biol* 19(8): R333-334.

Gozalbo, D., I. Gil-Navarro, I. Azorin, J. Renau-Piqueras, J. P. Martinez and M. L. Gil (1998). "The cell wall-associated glyceraldehyde-3-phosphate dehydrogenase of *Candida albicans* is also a fibronectin and laminin binding protein." *Infect Immun* 66(5): 2052-2059.

Gozalbo, D., P. Roig, E. Villamon and M. L. Gil (2004). "*Candida* and candidiasis: the cell wall as a potential molecular target for antifungal therapy." *Curr Drug Targets Infect Disord* 4(2): 117-135.

Gray, K. M., L. Passador, B. H. Iglewski and E. P. Greenberg (1994). "Interchangeability and specificity of components from the quorum-sensing regulatory systems of Vibrio fischeri and *Pseudomonas aeruginosa.*" *J Bacteriol* 176(10): 3076-3080.

Grubb, S. E., C. Murdoch, P. E. Sudbery, S. P. Saville, J. L. Lopez-Ribot and M. H. Thornhill (2009). "Adhesion of *Candida albicans* to endothelial cells under physiological conditions of flow." *Infect Immun* 77(9): 3872-3878.

Gustin, M. C., J. Albertyn, M. Alexander and K. Davenport (1998). "MAP kinase pathways in the yeast *Saccharomyces cerevisiae*." *Microbiol Mol Biol Rev* 62(4): 1264-1300.

Han, T. L., R. D. Cannon and S. G. Villas-Boas (2011). "The metabolic basis of *Candida albicans* morphogenesis and quorum sensing." *Fungal Genet Biol* 48(8): 747-763.

Hedayati, T., Choi J (2009). "Candidiasis." *http://emedicine.medscape.com/article/781215-overview. 12th December, 2009*.

Heinisch, J. J., A. Lorberg, H. P. Schmitz and J. J. Jacoby (1999). "The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in *Saccharomyces cerevisiae*." *Mol Microbiol* 32(4): 671-680.

Hense, B. A., C. Kuttler, J. Muller, M. Rothballer, A. Hartmann and J. U. Kreft (2007). "Does efficiency sensing unify diffusion and quorum sensing?" *Nat Rev Microbiol* 5(3): 230-239.

Hogan, D. A. and P. Sundstrom (2009). "The Ras/cAMP/PKA signaling pathway and virulence in *Candida albicans*." *Future Microbiol* 4(10): 1263-1270.

Hornby, J. M., E. C. Jensen, A. D. Lisec, J. J. Tasto, B. Jahnke, R. Shoemaker, P. Dussault and K. W. Nickerson (2001). "Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol." *Appl Environ Microbiol* 67(7): 2982-2992.

Hornby, J. M. and K. W. Nickerson (2004). "Enhanced production of farnesol by *Candida albicans* treated with four azoles." *Antimicrob Agents Chemother* 48(6): 2305-2307.

Hoyer, L. L. (2001). "The *ALS* gene family of *Candida albicans*." *Trends Microbiol* 9(4): 176-180.

Hoyer, L. L., J. Clevenger, J. E. Hecht, E. J. Ehrhart and F. M. Poulet (1999). "Detection of Als proteins on the cell wall of *Candida albicans* in murine tissues." *Infect Immun* 67(8): 4251-4255.

Hube, B. and J. Naglik (2001). "*Candida albicans* proteinases: resolving the mystery of a gene family." *Microbiology* 147(Pt 8): 1997-2005.

Hube, B., D. Sanglard, F. C. Odds, D. Hess, M. Monod, W. Schafer, A. J. Brown and N. A. Gow (1997). "Disruption of each of the secreted aspartyl proteinase genes *SAP1*, *SAP2*, and *SAP3* of *Candida albicans* attenuates virulence." *Infect Immun* 65(9): 3529-3538.

Hube, B., F. Stehr, M. Bossenz, A. Mazur, M. Kretschmar and W. Schafer (2000). "Secreted lipases of *Candida albicans*: cloning, characterisation and expression analysis of a new gene family with at least ten members." *Arch Microbiol* 174(5): 362-374.

Husain, S., B. D. Alexander, P. Munoz, R. K. Avery, S. Houston, T. Pruett, R. Jacobs, E. A. Dominguez, J. G. Tollemar, K. Baumgarten, C. M. Yu, M. M. Wagener, P. Linden, S. Kusne and N. Singh (2003). "Opportunistic mycelial fungal infections in organ transplant recipients: emerging importance of non-Aspergillus mycelial fungi." *Clin Infect Dis* 37(2): 221-229.

Ikner, A. and K. Shiozaki (2005). "Yeast signaling pathways in the oxidative stress response." *Mutat Res* 569(1-2): 13-27.

Irie, K., M. Takase, K. S. Lee, D. E. Levin, H. Araki, K. Matsumoto and Y. Oshima (1993). "*MKK1* and *MKK2*, which encode *Saccharomyces cerevisiae* mitogen-activated protein kinase-kinase homologs, function in the pathway mediated by protein kinase C." *Mol Cell Biol* 13(5): 3076-3083.

Johnson, A. (2003). "The biology of mating in *Candida albicans*." *Nat Rev Microbiol* 1(2): 106-116.

Jung, U. S. and D. E. Levin (1999). "Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway." *Mol Microbiol* 34(5): 1049-1057.

Jung, U. S., A. K. Sobering, M. J. Romeo and D. E. Levin (2002). "Regulation of the yeast Rlm1 transcription factor by the Mpk1 cell wall integrity MAP kinase." *Mol Microbiol* 46(3): 781-789.

Kamada, Y., U. S. Jung, J. Piotrowski and D. E. Levin (1995). "The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response." *Genes Dev* 9(13): 1559-1571.

Kamai, Y., M. Kubota, T. Hosokawa, T. Fukuoka and S. G. Filler (2002). "Contribution of *Candida albicans ALS1* to the pathogenesis of experimental oropharyngeal candidiasis." *Infect Immun* 70(9): 5256-5258.

Kaminishi, H., H. Miyaguchi, T. Tamaki, N. Suenaga, M. Hisamatsu, I. Mihashi, H. Matsumoto, H. Maeda and Y. Hagihara (1995). "Degradation of humoral host defense by *Candida albicans* proteinase." *Infect Immun* 63(3): 984-988.

Kan, V. L. and J. E. Bennett (1988). "Lectin-like attachment sites on murine pulmonary alveolar macrophages bind *Aspergillus fumigatus* conidia." *J Infect Dis* 158(2): 407-414.

Kapteyn, J. C., H. Van Den Ende and F. M. Klis (1999). "The contribution of cell wall proteins to the organization of the yeast cell wall." *Biochim Biophys Acta* 1426(2): 373-383.

Ketela, T., R. Green and H. Bussey (1999). "*Saccharomyces cerevisiae* mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway." *J Bacteriol* 181(11): 3330-3340.

Kibbler, C. C., S. Seaton, R. A. Barnes, W. R. Gransden, R. E. Holliman, E. M. Johnson, J. D. Perry, D. J. Sullivan and J. A. Wilson (2003). "Management and outcome of bloodstream infections due to *Candida* species in England and Wales." *J Hosp Infect* 54(1): 18-24.

Kim, H. S., E. H. Choi, J. Khan, E. Roilides, A. Francesconi, M. Kasai, T. Sein, R. L. Schaufele, K. Sakurai, C. G. Son, B. T. Greer, S. Chanock, C. A. Lyman and T. J. Walsh (2005). "Expression of genes encoding innate host defense molecules in normal human monocytes in response to *Candida albicans*." *Infect Immun* 73(6): 3714-3724.

Kim, J. and P. Sudbery (2011). "*Candida albicans*, a major human fungal pathogen." *J Microbiol* 49(2): 171-177.

Klis, F. M., P. de Groot and K. Hellingwerf (2001). "Molecular organization of the cell wall of Candida albicans." *Med Mycol* 39 Suppl 1: 1-8.

Klis, F. M., P. Mol, K. Hellingwerf and S. Brul (2002). "Dynamics of cell wall structure in *Saccharomyces cerevisiae*." *FEMS Microbiol Rev* 26(3): 239-256.

Klotz, S. A., M. L. Pendrak and R. C. Hein (2001). "Antibodies to alpha5beta1 and alpha(v)beta3 integrins react with *Candida albicans* alcohol dehydrogenase." *Microbiology* 147(Pt 11): 3159-3164.

Kobayashi, S. D., J. M. Voyich, K. R. Braughton, A. R. Whitney, W. M. Nauseef, H. L. Malech and F. R. DeLeo (2004). "Gene expression profiling provides insight into the pathophysiology of chronic granulomatous disease." *J Immunol* 172(1): 636-643.

Krcmery, V. and A. J. Barnes (2002). "Non-albicans *Candida* spp. causing fungaemia: pathogenicity and antifungal resistance." *J Hosp Infect* 50(4): 243-260.

Kuhn, D. M., J. Chandra, P. K. Mukherjee and M. A. Ghannoum (2002). "Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces." *Infect Immun* 70(2): 878-888.

Kultz, D. and M. Burg (1998). "Evolution of osmotic stress signaling via MAP kinase cascades." *J Exp Biol* 201(Pt 22): 3015-3021.

Kumamoto, C. A. (2005). "A contact-activated kinase signals *Candida albicans* invasive growth and biofilm development." *Proc Natl Acad Sci U S A* 102(15): 5576-5581.

Kurtz, M. B., Kelly, M.R., and Kirsch, D.R. (1990). "Overview of *Candida* physiology, pathogenicity, and new anticandidal agents." *In D. R. Kirsch, R. Kelly, and M. B. Kurtz (eds.), The genetics of Candida. CRC Press, Florida.*: 1-20.

Kvaal, C., S. A. Lachke, T. Srikantha, K. Daniels, J. McCoy and D. R. Soll (1999). "Misexpression of the opaque-phase-specific gene *PEP1* (*SAP1*) in the white phase of *Candida albicans* confers increased virulence in a mouse model of cutaneous infection." *Infect Immun* 67(12): 6652-6662.

Kvaal, C. A., T. Srikantha and D. R. Soll (1997). "Misexpression of the white-phase-specific gene *WH11* in the opaque phase of *Candida albicans* affects switching and virulence." *Infect Immun* 65(11): 4468-4475.

Kwon-Chung, K. J., D. Lehman, C. Good and P. T. Magee (1985). "Genetic evidence for role of extracellular proteinase in virulence of *Candida albicans*." *Infect Immun* 49(3): 571-575.

Larriba, G., J. J. Rubio Coque, A. Ciudad and E. Andaluz (2000). "*Candida albicans* molecular biology reaches its maturity." *Int Microbiol* 3(4): 247-252.

Lee, K. L., H. R. Buckley and C. C. Campbell (1975). "An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans*." *Sabouraudia* 13(2): 148-153.

Lee, K. S., K. Irie, Y. Gotoh, Y. Watanabe, H. Araki, E. Nishida, K. Matsumoto and D. E. Levin (1993). "A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C." *Mol Cell Biol* 13(5): 3067-3075.

Lenardon, M. D., I. Lesiak, C. A. Munro and N. A. Gow (2009). "Dissection of the *Candida albicans* class I chitin synthase promoters." *Mol Genet Genomics* 281(4): 459-471.

Levin, D. E. (2005). "Cell wall integrity signaling in *Saccharomyces cerevisiae*." *Microbiol Mol Biol Rev* 69(2): 262-291.

Levin, D. E., B. Bowers, C. Y. Chen, Y. Kamada and M. Watanabe (1994). "Dissecting the protein kinase C/MAP kinase signalling pathway of *Saccharomyces cerevisiae*." *Cell Mol Biol Res* 40(3): 229-239.

Lim, C. S., R. Rosli, H. F. Seow and P. P. Chong (2011). "*Candida* and invasive candidiasis: back to basics." *Eur J Clin Microbiol Infect Dis*.

Lingappa, B. T., M. Prasad, Y. Lingappa, D. F. Hunt and K. Biemann (1969). "Phenethyl alcohol and tryptophol: autoantibiotics produced by the fungus *Candida albicans*." *Science* 163(863): 192-194.

Lipke, P. N. and R. Ovalle (1998). "Cell wall architecture in yeast: new structure and new challenges." *J Bacteriol* 180(15): 3735-3740.

Liu, H. (2001). "Transcriptional control of dimorphism in *Candida albicans*." *Curr Opin Microbiol* 4(6): 728-735.

Liu, H. (2002). "Co-regulation of pathogenesis with dimorphism and phenotypic switching in *Candida albicans*, a commensal and a pathogen." *Int J Med Microbiol* 292(5-6): 299-311.

Lo, H. J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti and G. R. Fink (1997). "Nonfilamentous *C. albicans* mutants are avirulent." *Cell* 90(5): 939-949.

Lockhart, S. R., S. A. Messer, M. A. Pfaller and D. J. Diekema (2008). "Geographic distribution and antifungal susceptibility of the newly described species *Candida orthopsilosis* and *Candida metapsilosis* in comparison to the closely related species *Candida parapsilosis*." *J Clin Microbiol* 46(8): 2659-2664.

Lopez-Ribot, J. L., J. Bikandi, R. S. San Millan and W. L. Chaffin (1999). "Interactions between *Candida albicans* and the human extracellular matrix component tenascin-C." *Mol Cell Biol Res Commun* 2(1): 58-63.

Lorenz, M. C., J. A. Bender and G. R. Fink (2004). "Transcriptional response of *Candida albicans* upon internalization by macrophages." *Eukaryot Cell* 3(5): 1076-1087.

Lorenz, M. C. and G. R. Fink (2002). "Life and death in a macrophage: role of the glyoxylate cycle in virulence." *Eukaryot Cell* 1(5): 657-662.

Madden, K., Y. J. Sheu, K. Baetz, B. Andrews and M. Snyder (1997). "SBF cell cycle regulator as a target of the yeast PKC-MAP kinase pathway." *Science* 275(5307): 1781-1784.

Magee, B. B. and P. T. Magee (2000). "Induction of mating in *Candida albicans* by construction of MTLa and MTLalpha strains." *Science* 289(5477): 310-313.

Magee, P. T. (1993). "Variations in chromosome size and organization in *Candida albicans* and *Candida stellatoidea*." *Trends Microbiol* 1(9): 338-342.

Mambula, S. S., E. R. Simons, R. Hastey, M. E. Selsted and S. M. Levitz (2000). "Human neutrophil-mediated nonoxidative antifungal activity against *Cryptococcus neoformans*." *Infect Immun* 68(11): 6257-6264.

Marr, K. A., R. A. Carter, F. Crippa, A. Wald and L. Corey (2002). "Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients." *Clin Infect Dis* 34(7): 909-917.

Marshall, C. J. (1994). "MAP kinase kinase kinase, MAP kinase kinase and MAP kinase." *Curr Opin Genet Dev* 4(1): 82-89.

Martin, H., J. M. Rodriguez-Pachon, C. Ruiz, C. Nombela and M. Molina (2000). "Regulatory mechanisms for modulation of signaling through the cell integrity Slt2-mediated pathway in *Saccharomyces cerevisiae*." *J Biol Chem* 275(2): 1511-1519.

Masuoka, J. (2004). "Surface glycans of *Candida albicans* and other pathogenic fungi: physiological roles, clinical uses, and experimental challenges." *Clin Microbiol Rev* 17(2): 281-310.

Mavor, A. L., S. Thewes and B. Hube (2005). "Systemic fungal infections caused by *Candida* species: epidemiology, infection process and virulence attributes." *Curr Drug Targets* 6(8): 863-874.

McPheeters, L. (2005). "Combination Antifungal Therapy: When Is Two Better Than One? ." *Drug Therapy Topics* 34: 13-18.

Millar, J. B. (1999). "Stress-activated MAP kinase (mitogen-activated protein kinase) pathways of budding and fission yeasts." *Biochem Soc Symp* 64: 49-62.

Mishra, N. N., T. Prasad, N. Sharma, A. Payasi, R. Prasad, D. K. Gupta and R. Singh (2007). "Pathogenicity and drug resistance in *Candida albicans* and other yeast species. A review." *Acta Microbiol Immunol Hung* 54(3): 201-235.

Mitchell, A. P. (1998). "Dimorphism and virulence in *Candida albicans*." *Curr Opin Microbiol* 1(6): 687-692.

Miyazaki, T., T. Inamine, S. Yamauchi, Y. Nagayoshi, T. Saijo, K. Izumikawa, M. Seki, H. Kakeya, Y. Yamamoto, K. Yanagihara, Y. Miyazaki and S. Kohno (2010). "Role of the Slt2 mitogen-activated protein kinase pathway in cell wall integrity and virulence in *Candida glabrata*." *FEMS Yeast Res* 10(3): 343-352.

Morgan, J. (2005). "Global trends in candidemia: review of reports from 1995-2005." *Curr Infect Dis Rep* 7(6): 429-439.

Morschhauser, J., R. Virkola, T. K. Korhonen and J. Hacker (1997). "Degradation of human subendothelial extracellular matrix by proteinase-secreting *Candida albicans*." *FEMS Microbiol Lett* 153(2): 349-355.

Moss, M. O. (1987). "Fungal biotechnology roundup." *Mycologist* 21: 55-58.

Naglik, J., A. Albrecht, O. Bader and B. Hube (2004). "*Candida albicans* proteinases and host/pathogen interactions." *Cell Microbiol* 6(10): 915-926.

Naglik, J. R., F. Fostira, J. Ruprai, J. F. Staab, S. J. Challacombe and P. Sundstrom (2006). "*Candida albicans HWP1* gene expression and host antibody responses in colonization and disease." *J Med Microbiol* 55(Pt 10): 1323-1327.

Naglik, J. R., S. J. Challacombe and B. Hube (2003). "*Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis." *Microbiol Mol Biol Rev* 67(3): 400-428, table of contents.

Nasmyth, K. and L. Dirick (1991). "The role of *SWI4* and *SWI6* in the activity of G1 cyclins in yeast." *Cell* 66(5): 995-1013.

Navarro-Garcia, F., R. Alonso-Monge, H. Rico, J. Pla, R. Sentandreu and C. Nombela (1998). "A role for the MAP kinase gene *MKC1* in cell wall construction and morphological transitions in *Candida albicans*." *Microbiology* 144 (Pt 2): 411-424.

Navarro-Garcia, F., B. Eisman, S. M. Fiuza, C. Nombela and J. Pla (2005). "The MAP kinase Mkc1p is activated under different stress conditions in *Candida albicans*." *Microbiology* 151(Pt 8): 2737-2749.

Navarro-Garcia, F., M. Sanchez, C. Nombela and J. Pla (2001). "Virulence genes in the pathogenic yeast *Candida albicans*." *FEMS Microbiol Rev* 25(2): 245-268.

Navarro-Garcia, F., M. Sanchez, J. Pla and C. Nombela (1995). "Functional characterization of the *MKC1* gene of *Candida albicans*, which encodes a mitogen-activated protein kinase homolog related to cell integrity." *Mol Cell Biol* 15(4): 2197-2206.

Nemecek, J. C., M. Wuthrich and B. S. Klein (2006). "Global control of dimorphism and virulence in fungi." *Science* 312(5773): 583-588.

Netea, M. G., G. D. Brown, B. J. Kullberg and N. A. Gow (2008). "An integrated model of the recognition of *Candida albicans* by the innate immune system." *Nat Rev Microbiol* 6(1): 67-78.

Nobile, C. J. and A. P. Mitchell (2006). "Genetics and genomics of *Candida albicans* biofilm formation." *Cell Microbiol* 8(9): 1382-1391.

Noble, S. M. and A. D. Johnson (2007). "Genetics of *Candida albicans*, a diploid human fungal pathogen." *Annu Rev Genet* 41: 193-211.

Norman, C., M. Runswick, R. Pollock and R. Treisman (1988). "Isolation and properties of cDNA clones encoding *SRF*, a transcription factor that binds to the c-fos serum response element." *Cell* 55(6): 989-1003.

O'Rourke, S. M., I. Herskowitz and E. K. O'Shea (2002). "Yeast go the whole *HOG* for the hyperosmotic response." *Trends Genet* 18(8): 405-412.

Odds, F. C. (1988). "Candida and candidosis: a review and bibliography." *3rd edn. Balliere Tindall, London.*

Odds, F. C., N. A. Gow and A. J. Brown (2001). "Fungal virulence studies come of age." *Genome Biol* 2(3): REVIEWS1009.

Oehler, L., O. Majdic, W. F. Pickl, J. Stockl, E. Riedl, J. Drach, K. Rappersberger, K. Geissler and W. Knapp (1998). "Neutrophil granulocyte-committed cells can be driven to acquire dendritic cell characteristics." *J Exp Med* 187(7): 1019-1028.

Oh, K. B., H. Miyazawa, T. Naito and H. Matsuoka (2001). "Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in *Candida albicans*." *Proc Natl Acad Sci U S A* 98(8): 4664-4668.

Paravicini, G., A. Mendoza, B. Antonsson, M. Cooper, C. Losberger and M. A. Payton (1996). "The *Candida albicans PKC1* gene encodes a protein kinase C homolog necessary for cellular integrity but not dimorphism." *Yeast* 12(8): 741-756.

Parker, J. C., Jr., J. J. McCloskey and K. A. Knauer (1976). "Pathobiologic features of human candidiasis. A common deep mycosis of the brain, heart and kidney in the altered host." *Am J Clin Pathol* 65(6): 991-1000.

Passmore, S., R. Elble and B. K. Tye (1989). "A protein involved in minichromosome maintenance in yeast binds a transcriptional enhancer conserved in eukaryotes." *Genes Dev* 3(7): 921-935.

Perez, P. and T. M. Calonge (2002). "Yeast protein kinase C." *J Biochem* 132(4): 513-517.

Perlroth, J., B. Choi and B. Spellberg (2007). "Nosocomial fungal infections: epidemiology, diagnosis, and treatment." *Med Mycol* 45(4): 321-346.

Pfaller, M. A., M. Castanheira, S. A. Messer, G. J. Moet and R. N. Jones (2010). "Variation in *Candida* spp. distribution and antifungal resistance rates among bloodstream infection isolates by patient age: report from the SENTRY Antimicrobial Surveillance Program (2008-2009)." *Diagn Microbiol Infect Dis* 68(3): 278-283.

Pfaller, M. A. and D. J. Diekema (2002). "Role of sentinel surveillance of candidemia: trends in species distribution and antifungal susceptibility." *J Clin Microbiol* 40(10): 3551-3557.

Pfaller, M. A. and D. J. Diekema (2007). "Epidemiology of invasive candidiasis: a persistent public health problem." *Clin Microbiol Rev* 20(1): 133-163.

Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, K. P. Ng, A. Colombo, J. Finquelievich, R. Barnes and J. Wadula (2008). "Geographic and temporal trends in isolation and antifungal susceptibility of *Candida parapsilosis:* a global assessment from the ARTEMIS DISK Antifungal Surveillance Program, 2001 to 2005." *J Clin Microbiol* 46(3): 842-849.

Pfaller, M. A., D. J. Diekema, R. N. Jones, H. S. Sader, A. C. Fluit, R. J. Hollis and S. A. Messer (2001). "International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and in vitro susceptibilities to fluconazole, ravuconazole, and voriconazole of isolates collected from 1997 through 1999 in the SENTRY antimicrobial surveillance program." *J Clin Microbiol* 39(9): 3254-3259.

Pfaller, M. A., R. N. Jones, S. A. Messer, M. B. Edmond and R. P. Wenzel (1998). "National surveillance of nosocomial blood stream infection due to *Candida albicans*: frequency of occurrence and antifungal susceptibility in the SCOPE Program." *Diagn Microbiol Infect Dis* 31(1): 327-332.

Pfaller, M. A., G. J. Moet, S. A. Messer, R. N. Jones and M. Castanheira (2011). "Candida bloodstream infections: comparison of species distributions and antifungal resistance patterns in community-onset and nosocomial isolates in the SENTRY Antimicrobial Surveillance Program, 2008-2009." *Antimicrob Agents Chemother* 55(2): 561-566.

Pirozynski, K. A. and D. W. Malloch (1975). "The origin of land plants: a matter of mycotrophism." *Biosystems* 6(3): 153-164.

Pollock, R. and R. Treisman (1991). "Human SRF-related proteins: DNA-binding properties and potential regulatory targets." *Genes Dev* 5(12A): 2327-2341.

Posas, F., M. Takekawa and H. Saito (1998). "Signal transduction by MAP kinase cascades in budding yeast." *Curr Opin Microbiol* 1(2): 175-182.

Ramage, G., K. Vandewalle, B. L. Wickes and J. L. Lopez-Ribot (2001). "Characteristics of biofilm formation by *Candida albicans*." *Rev Iberoam Micol* 18(4): 163-170.

Ribeiro, P., A. B. Sousa, O. Nunes, F. Aveiro, J. P. Fernandes and J. Gouveia (1997). "Candidemia in acute leukemia patients." *Support Care Cancer* 5(3): 249-251.

Roman, E., D. M. Arana, C. Nombela, R. Alonso-Monge and J. Pla (2007). "MAP kinase pathways as regulators of fungal virulence." *Trends Microbiol* 15(4): 181-190.

Roman, E., C. Nombela and J. Pla (2005). "The Sho1 adaptor protein links oxidative stress to morphogenesis and cell wall biosynthesis in the fungal pathogen *Candida albicans*." *Mol Cell Biol* 25(23): 10611-10627.

Romani, L. (2004). "Immunity to fungal infections." *Nat Rev Immunol* 4(1): 1-23.

Romani, L., F. Bistoni and P. Puccetti (2002). "Fungi, dendritic cells and receptors: a host perspective of fungal virulence." *Trends Microbiol* 10(11): 508-514.

Romani, L., F. Bistoni and P. Puccetti (2003). "Adaptation of *Candida albicans* to the host environment: the role of morphogenesis in virulence and survival in mammalian hosts." *Curr Opin Microbiol* 6(4): 338-343.

Romani. (2002). "Immunology of invasive candidiasis." *In Calderone RA, ed. Candida and Candidiasis. Washington DC, USA; ASM Press* pp: 223-242.

Rooney, P. J. and B. S. Klein (2002). "Linking fungal morphogenesis with virulence." *Cell Microbiol* 4(3): 127-137.

Ruhnke, M. (2002). "Skin and mucous membrane infections." *In R. A. Calderone (ed.), Candida and Candidiasis. American Society for Microbiology, Washington, D.C.* pp: 307-325.

Sabino, R., C. Verissimo, J. Brandao, C. Alves, H. Parada, L. Rosado, E. Paixao, Z. Videira, T. Tendeiro, P. Sampaio and C. Pais (2010). "Epidemiology of candidemia in oncology patients: a 6-year survey in a Portuguese central hospital." *Med Mycol* 48(2): 346-354.

Sampaio, P., L. Gusmao, C. Alves, C. Pina-Vaz, A. Amorim and C. Pais (2003). "Highly polymorphic microsatellite for identification of *Candida albicans* strains." *J Clin Microbiol* 41(2): 552-557.

San Jose, C., R. A. Monge, R. Perez-Diaz, J. Pla and C. Nombela (1996). "The mitogen-activated protein kinase homolog *HOG1* gene controls glycerol accumulation in the pathogenic fungus *Candida albicans*." *J Bacteriol* 178(19): 5850-5852.

Sandven, P. (2000). "Epidemiology of candidemia." *Rev Iberoam Micol* 17(3): 73-81.

Sanglard, D., B. Hube, M. Monod, F. C. Odds and N. A. Gow (1997). "A triple deletion of the secreted aspartyl proteinase genes *SAP4, SAP5,* and *SAP6* of *Candida albicans* causes attenuated virulence." *Infect Immun* 65(9): 3539-3546.

Santos, M. A. and M. F. Tuite (1995). "The CUG codon is decoded in vivo as serine and not leucine in *Candida albicans*." *Nucleic Acids Res* 23(9): 1481-1486.

Saville, S. P., A. L. Lazzell, C. Monteagudo and J. L. Lopez-Ribot (2003). "Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection." *Eukaryot Cell* 2(5): 1053-1060.

Schaffner, A., H. Douglas and A. Braude (1982). "Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to *Aspergillus*. Observations on these two lines of defense in vivo and in vitro with human and mouse phagocytes." *J Clin Invest* 69(3): 617-631.

Schaller, M., C. Borelli, H. C. Korting and B. Hube (2005). "Hydrolytic enzymes as virulence factors of *Candida albicans.*" *Mycoses* 48(6): 365-377.

Schmidt, A. and M. N. Hall (1998). "Signaling to the actin cytoskeleton." *Annu Rev Cell Dev Biol* 14: 305-338.

Schwartz, M. A. and H. D. Madhani (2004). "Principles of MAP kinase signaling specificity in *Saccharomyces cerevisiae*." *Annu Rev Genet* 38: 725-748.

Schwarz-Sommer, Z., P. Huijser, W. Nacken, H. Saedler and H. Sommer (1990). "Genetic Control of Flower Development by Homeotic Genes in *Antirrhinum majus*." *Science* 250(4983): 931-936.

Schwebke, J., and Hillier, S.L. (1996). "Sexually-transmitted diseases and sexual health." *In DJ Weatherall, JGG Ledingham and DA Warrell (eds). Oxford Textbook of Medicine,3rd ed. Oxford: Oxford University Press.* pp: 3343-3372.

Shaw, J. A., P. C. Mol, B. Bowers, S. J. Silverman, M. H. Valdivieso, A. Duran and E. Cabib (1991). "The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle." *J Cell Biol* 114(1): 111-123.

Shoham, S. and S. M. Levitz (2005). "The immune response to fungal infections." *Br J Haematol* 129(5): 569-582.

Shore, P. and A. D. Sharrocks (1995). "The MADS-box family of transcription factors." *Eur J Biochem* 229(1): 1-13.

Slutsky, B., J. Buffo and D. R. Soll (1985). "High-frequency switching of colony morphology in *Candida albicans*." *Science* 230(4726): 666-669.

Smith, D. A., S. Nicholls, B. A. Morgan, A. J. Brown and J. Quinn (2004). "A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*." *Mol Biol Cell* 15(9): 4179-4190.

Soll, D. R. (1992). "High-frequency switching in *Candida albicans*." *Clin Microbiol Rev* 5(2): 183-203.

Soll, D. R. (1997). "Gene regulation during high-frequency switching in *Candida albicans*." *Microbiology* 143 (Pt 2): 279-288.

Soll, D. R. (2001). "Phenotypic switching" *In R. Calderone (ed.), Candida and Candidiasis. American Society for Microbiology, Washington, D. C.* pp: 123-142.

Soll, D. R., C. J. Langtimm, J. McDowell, J. Hicks and R. Galask (1987). "High-frequency switching in *Candida* strains isolated from vaginitis patients." *J Clin Microbiol* 25(9): 1611-1622.

Sommer, H., J. P. Beltran, P. Huijser, H. Pape, W. E. Lonnig, H. Saedler and Z. Schwarz-Sommer (1990). "Deficiens, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*: the protein shows homology to transcription factors." *EMBO J* 9(3): 605-613.

St-Germain, G., M. Laverdiere, R. Pelletier, A. M. Bourgault, M. Libman, C. Lemieux and G. Noel (2001). "Prevalence and antifungal susceptibility of 442 *Candida* isolates from blood and other normally sterile sites: results of a 2-year (1996 to 1998) multicenter surveillance study in Quebec, Canada." *J Clin Microbiol* 39(3): 949-953.

Staab, J. F., S. D. Bradway, P. L. Fidel and P. Sundstrom (1999). "Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1." *Science* 283(5407): 1535-1538.

Staib, F. (1969). "Proteolysis and pathogenicity of *Candida albicans* strains." *Mycopathol Mycol Appl* 37(4): 345-348.

Staib, P., M. Kretschmar, T. Nichterlein, H. Hof and J. Morschhauser (2000). "Differential activation of a *Candida albicans* virulence gene family during infection." *Proc Natl Acad Sci U S A* 97(11): 6102-6107.

Steele, C., J. Leigh, R. Swoboda and P. L. Fidel, Jr. (2000). "Growth inhibition of *Candida* by human oral epithelial cells." *J Infect Dis* 182(5): 1479-1485.

Sudbery, P., N. Gow and J. Berman (2004). "The distinct morphogenic states of *Candida albicans*." *Trends Microbiol* 12(7): 317-324.

Sundstrom, P. (1999). "Adhesins in *Candida albicans*." *Curr Opin Microbiol* 2(4): 353-357.

Sundstrom, P. (2002). "Adhesion in *Candida* spp." *Cell Microbiol* 4(8): 461-469.

Szabo, Z., J. Szilagyi, A. Tavanti, G. Kardos, F. Rozgonyi, S. Bayegan and L. Majoros (2009). "In vitro efficacy of 5 antifungal agents against *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida* metapsilosis as determined by time-kill methodology." *Diagn Microbiol Infect Dis* 64(3): 283-288.

Tavanti, A., L. A. Hensgens, E. Ghelardi, M. Campa and S. Senesi (2007). "Genotyping of *Candida orthopsilosis* clinical isolates by amplification fragment length polymorphism reveals genetic diversity among independent isolates and strain maintenance within patients." *J Clin Microbiol* 45(5): 1455-1462.

Tortorano, A. M., G. Dho, A. Prigitano, G. Breda, A. Grancini, V. Emmi, C. Cavanna, G. Marino, S. Morero, C. Ossi, G. Delvecchio, M. Passera, V. Cusumano, A. David, G. Bonaccorso, A. Corona, M. Favaro, C. Vismara, M. G. Garau, S. Falchi and M. R. Tejada (2011). "Invasive fungal infections in the intensive care unit: a multicentre, prospective, observational study in Italy (2006-2008)." *Mycoses*.

Tortorano, A. M., C. Kibbler, J. Peman, H. Bernhardt, L. Klingspor and R. Grillot (2006). "Candidaemia in Europe: epidemiology and resistance." *Int J Antimicrob Agents* 27(5): 359-366.

Treisman, R. (1990). "The SRE: a growth factor responsive transcriptional regulator." *Semin Cancer Biol* 1(1): 47-58.

Trick, W. E., S. K. Fridkin, J. R. Edwards, R. A. Hajjeh and R. P. Gaynes (2002). "Secular trend of hospital-acquired candidemia among intensive care unit patients in the United States during 1989-1999." *Clin Infect Dis* 35(5): 627-630.

Trofa, D., A. Gacser and J. D. Nosanchuk (2008). "*Candida parapsilosis*, an emerging fungal pathogen." *Clin Microbiol Rev* 21(4): 606-625.

Tsao, C. C., Y. T. Chen and C. Y. Lan (2009). "A small G protein Rhb1 and a GTPase-activating protein Tsc2 involved in nitrogen starvation-induced morphogenesis and cell wall integrity of *Candida albicans*." *Fungal Genet Biol* 46(2): 126-136.

van Asbeck, E. C., K. V. Clemons and D. A. Stevens (2009). "*Candida parapsilosis*: a review of its epidemiology, pathogenesis, clinical aspects, typing and antimicrobial susceptibility." *Crit Rev Microbiol* 35(4): 283-309.

van Burik, J. A. and P. T. Magee (2001). "Aspects of fungal pathogenesis in humans." *Annu Rev Microbiol* 55: 743-772.

Wang, Y. (2009). "CDKs and the yeast-hyphal decision." *Curr Opin Microbiol* 12(6): 644-649.

Watanabe, Y., K. Irie and K. Matsumoto (1995). "Yeast *RLM1* encodes a serum response factor-like protein that may function downstream of the Mpk1 (Slt2) mitogen-activated protein kinase pathway." *Mol Cell Biol* 15(10): 5740-5749.

Watanabe, Y., G. Takaesu, M. Hagiwara, K. Irie and K. Matsumoto (1997). "Characterization of a serum response factor-like protein in *Saccharomyces cerevisiae*, Rlm1, which has transcriptional activity regulated by the Mpk1 (Slt2) mitogen-activated protein kinase pathway." *Mol Cell Biol* 17(5): 2615-2623.

Whiteway, M., D. Dignard and D. Y. Thomas (1992). "Dominant negative selection of heterologous genes: isolation of *Candida albicans* genes that interfere with *Saccharomyces cerevisiae* mating factor-induced cell cycle arrest." *Proc Natl Acad Sci U S A* 89(20): 9410-9414.

Whiteway, M. and U. Oberholzer (2004). "*Candida* morphogenesis and host-pathogen interactions." *Curr Opin Microbiol* 7(4): 350-357.

Wingard, J. R. (1995). "Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients." *Clin Infect Dis* 20(1): 115-125.

Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel and M. B. Edmond (2004). "Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study." *Clin Infect Dis* 39(3): 309-317.

Yanofsky, M. F., H. Ma, J. L. Bowman, G. N. Drews, K. A. Feldmann and E. M. Meyerowitz (1990). "The protein encoded by the *Arabidopsis* homeotic gene agamous resembles transcription factors." *Nature* 346(6279): 35-39.

Zaoutis, T. E., J. Argon, J. Chu, J. A. Berlin, T. J. Walsh and C. Feudtner (2005). "The epidemiology and attributable outcomes of candidemia in adults and children hospitalized in the United States: a propensity analysis." *Clin Infect Dis* 41(9): 1232-1239.

Chapter 2

Increased number of glutamine repeats in the C-terminal of *Candida albicans* Rlm1 enhances the resistance to stress agents

This chapter comprises parts from the following publication:

Sampaio, P., E. Nogueira, A Loureiro, Y Delgado-Silva, A. Correia and C. Pais (2009). Increased number of glutamine repeats in the C-terminal of *Candida albicans* Rlm1 enhances the resistance to stress agents. *Antonie van Leeuwenhoek* 96:395–404.

Abstract

The highly polymorphic microsatellite CAI described for *Candida albicans* genotyping was found to be located within the *RLM1* gene which codes for a transcription factor from the MADS box family that, in *Saccharomyces cerevisiae*, is known to regulate the expression of genes involved in the cell wall integrity pathway. The aim of this work was to study CAI genetic variability in a wide group of *C. albicans* isolates and determine the response of genetic variants to cell wall damaging stress agents. One hundred twenty-three *C. albicans* isolates were genotyped with CAI microsatellite $(CAA/G)_n$, and 35 alleles were found with repeat units varying from 11 to 49. Alleles with less than 29 repetitions were the most frequent, while the longer ones were underrepresented and had a more complex internal structure. Combinations of *RLM1* alleles generated 66 different genotypes. Significant differences ($P < 0.05$) in the susceptibility patterns to menadione, hydrogen peroxide, SDS, acetic acid, and CFW, stress agents affecting cell integrity, were found between strains harbouring alleles ranging from 17 to 28 repetitions and strains with longer alleles, suggesting that an increased number of repetitive units in the *C. albicans RLM1* gene could be related to stress response.

Keywords: Allelic frequency - *Candida albicans* - *RLM1* gene - Stress resistance.

Introduction

Candida albicans is part of the human commensal flora with carriage rates in healthy individuals of around 50% in the gastrointestinal tract, and between 10 and 20% in the oral cavity, anorectal tract, and vagina. However, in immunocompromised patients this species is one of the most important opportunistic fungal pathogen, being responsible for both superficial and systemic infections (Pelroth *et al.,* 2007). Molecular epidemiology studies showed that *C. albicans* isolates exhibit a high level of genetic diversity. Microsatellite length polymorphism and multilocus sequence typing have been used to discriminate *C. albicans* strains and to detect small genetic changes or micro variations that may be indicative of adaptability processes (Sampaio *et al*., 2005; Bougnoux *et al.,* 2006; Garcia-Hermoso *et al*., *2007*). One of the most polymorphic microsatellites described in *C. albicans* was designated as CAI, which, together with other loci in a multiplex system, proved to be an excellent tool for strain discrimination and for the detection of microevolutionary events (Sampaio *et al*., 2003, 2005). It was reported that sequential isolates from patients with recurrent infections presented changes at CAI locus, suggesting a possible role of this locus in strain adaptation to stress in the host environment. Furthermore, this repetitive region was found to be located within the *RLM1* gene which codes for a transcription factor from the MADS (Mcm1p-Agamous-Deficiens-Serum response factor) box family that, in *S. cerevisiae*, is known to regulate the expression of genes involved in the cell wall integrity pathway (Watanabe *et al.,* 1995; Dodou and Treisman 1997). In *C. albicans* the role of *RLM1* gene is not established yet but it also seems to be required for cell wall integrity, at least under caspofungin and Congo red stress (Bruno *et al.,* 2006). It is likely that the presence of a repetitive region confers genetic variability to *RLM1* gene and may contribute to the adaptation of *C. albicans* strains if some genetic variations of this gene confer a better response to stresses affecting the cell integrity, like antifungal agents, oxidative stress, or others. Thus, the objectives of the present work were, (1) to determine the extent of genetic variability of CAI microsatellite, the repetitive region of *RLM1*, by studying 123 *C. albicans* clinical isolates from different geographic and anatomic origins; and (2) to investigate if the genetic variants behave differently regarding cell damaging stress agents.

Materials and methods

Yeast strains

A total of 123 *C. albicans* unrelated clinical isolates with different geographical origins and collected from distinct body locations were used in this study (Table 2.1). Reference strains WO-1 and PYCC 3436 (ATCC 18804) were also included. Stock cultures were maintained on Sabouraud glucose agar medium at 4°C and cryopreserved in 30% glycerol (w/w) at −80°C.

Table 2.1. *Candida albicans* isolates studied, their origins and CAI genotypes.

Strain	Country of origin	Isolated from	CAI genotype	Strain	Country of origin	Isolated from	CAI genotype
ATCC-18804	Uruguay	Skin lesion	17/23	31C	Portugal	VE	21/25
WO1	EUA	–	16/27	36C	Portugal	VE	11/18
3	Portugal	VE	17/23	39C	Portugal	VE	20/28
7	Portugal	VE	30/30	45C	Portugal	VE	11/18
8	Portugal	VE	30/32	46C	Portugal	VE	27/27
11	Portugal	VE	27/45	49C	Portugal	VE	21/26
12	Portugal	VE	18/25	52C	Portugal	VE	22/34
17	Portugal	VE	21/21	53C	Portugal	VE	27/47
18	Portugal	VE	20/29	55C	Portugal	VE	27/42
20	Portugal	VE	26/26	57C	Portugal	VE	11/28
22	Portugal	VE	23/27	58C	Portugal	VE	28/47
27	Portugal	VE	22/23	60C	Portugal	VE	21/21
29	Portugal	VE	20/20	62C	Portugal	VE	28/47
31	Portugal	VE	21/26	64C	Portugal	VE	20/28
35	Portugal	VE	25/25	69C	Portugal	VE	27/42
37	Portugal	VE	18/27	70C	Portugal	VE	21/25
39	Portugal	VE	21/22	72C	Portugal	VE	21/24
41	Portugal	VE	23/27	140A	Portugal	Blood	34/34
45	Portugal	VE	25/25	CIPO46	Portugal	Feces	30/42
H58	Portugal	Blood	22/22	CIPOX	Portugal	Blood	11/11
H37	Portugal	RT	25/27	IPOL13	Portugal	Blood	40/40
1M	Portugal	Urine	21/25	IPOL15	Portugal	Blood	35/44
5M	Portugal	RT	13/32	IPOL33	Portugal	VE	32/46
8M	Portugal	Urine	21/25	IPOL35	Portugal	VE	38/48
10M	Portugal	RT	17/17	FC27	USA	–	28/49
13M	Portugal	Urine	21/25	122.072	Switzerland	–	21/21
26M	Portugal	RT	20/28	180.004	USA	–	18/24
33M	Portugal	VE	23/24	FC29	USA	–	21/26
35M	Portugal	RT	24/27	FC8	USA	–	20/31
39M	Portugal	RT	18/18	152.082	USA	–	11/20
41M	Portugal	Urine	21/22	178.042	USA	–	21/26
49M	Portugal	Urine	36/36	FC12	USA	–	27/27
52M	Portugal	Urine	21/21	157.039	Brazil	–	26/34
61M	Portugal	RT	17/21	FC11	USA	–	27/45
62M	Portugal	RT	28/28	152.085	Canada	–	18/18
63M	Portugal	Urine	25/27	175.006	Spain	–	21/25
64M	Portugal	RT	22/22	180.012	USA	–	26/34
88M	Portugal	Urine	20/28	FC23	USA	–	24/29
69M	Portugal	RT	21/25	FC6	USA	–	17/27
74M	Portugal	Urine	20/28	122.095	Germany	–	26/32
77M	Portugal	RT	18/18	176.079	USA	–	25/25
79M	Portugal	RT	18/34	FC15	USA	–	27/41
82M	Portugal	Urine	18/47	175.010	Belgium	–	21/25
90M	Portugal	RT	24/24	175.031	Italy	–	11/21
91M	Portugal	RT	21/27	122.077	United kingdom	–	18/25
1C	Portugal	VE	17/21	FC19	USA	–	21/25
2C	Portugal	VE	26/33	FC20	USA	–	25/25
3C	Portugal	VE	20/37	FC17	USA	–	29/33
5C	Portugal	VE	18/18	175.071	Italy	–	18/25
6C	Portugal	VE	21/26	157.096	Portugal	–	21/26
7C	Portugal	VE	25/26	180.021	Italy	–	25/25
9C	Portugal	VE	24/26	FC13	USA	–	27/27
10C	Portugal	VE	27/49	FC7	USA	–	21/26
11C	Portugal	VE	21/25	A1587	Angola	VE	15/15
13C	Portugal	VE	20/27	A1598	Angola	VE	15/15
14C	Portugal	VE	39/46	A1605	Angola	VE	15/15
16C	Portugal	VE	21/26	A1618	Angola	VE	15/15
19C	Portugal	VE	27/27	A1634	Angola	VE	15/15
24C	Portugal	VE	17/21	A8450	Angola	VE	15/15
27C	Portugal	VE	16/27	M11	Madagascar	VE	15/15
				M1615	Madagascar	VE	15/15
				M8627	Madagascar	VE	15/15

VE vaginal exudate, *RT* respiractory tract, – unknown

Microsatellite amplification and fragment analysis

DNA extraction and PCR reactions were performed as previously described (Sampaio *et al.,* 2005). PCR products were run on an ABI 310 Genetic Analyser (Applied Biosystems) together with the GeneScan-500 (TAMRA) size standard (Applied Biosystems). Fragment sizes were determined automatically using the GeneScan 3.7 analysis software. Alleles have been designated by the number of trinucleotide repeats.

DNA sequence analysis

Amplified DNA fragments were separated by electrophoresis in polyacrylamide gels (T6C5) in denaturing conditions and DNA fragments visualized by a silver staining method (Budowle *et al.,* 1991). Fragment bands were cut individually from the gel, eluted in 250µl of TE buffer, frozen and thawed three times, reamplified, and purified with Microspin S-300 HR columns (Pharmacia). The purified products were submitted to dideoxy cycle sequencing reaction using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Sequence analysis was performed on an ABI 310 Genetic Analyzer using the Sequencing 3.7 Analysis software. Direct sequencing was performed on both DNA strands for all the fragments analyzed.

Susceptibility assays

Candida albicans cells were grown overnight in YEPD medium (0.5% w/v yeast extract, 1% w/v peptone and 2% w/v glucose) at 30ºC and 160 rpm. $2x10^7$ cells were overlayed on YEPD agar plates prepared with and without 200µg/ml Congo red (CR). Six millimeter diameter paper discs imbibed in 10µl of either 99.8% (v/v) acetic acid (Merck), 0.5 M menadione (Sigma), 0.5 M SDS (BDH Chemicals), 35% (v/v) hydrogen peroxide (Merck) and 10mg/ml calcofluor white (CFW) were loaded over the *C. albicans* cells. Halos of growth inhibition were measured after 24h of incubation at 37ºC. The results presented are the mean of three independent experiments. Statistical analysis was performed with SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Location of CAI microsatellite within *Candida albicans RLM1* gene

A search in *C. albicans* genome revealed that CAI microsatellite is located within a gene coding for a transcription factor from the MADS box protein family, designated by *RLM1*. A comparison of DNA and protein homology between *C. albicans RLM1* and the *S. cerevisiae* ortholog was performed by using the program T-COFFEE Version 5.05. *Saccharomyces cerevisiae RLM1* gene and protein sequences were obtained from SGD (*Saccharomyces* Genome Database). The DNA sequence homology is very low, (nucleotide identity of 47%) as well as the protein identity (29%). However, some characteristic features of this family are present, namely the MADS box region at the 5′-terminus, responsible for the DNA binding, and a repetitive region at the 3′-terminus (Fig. 2.1).

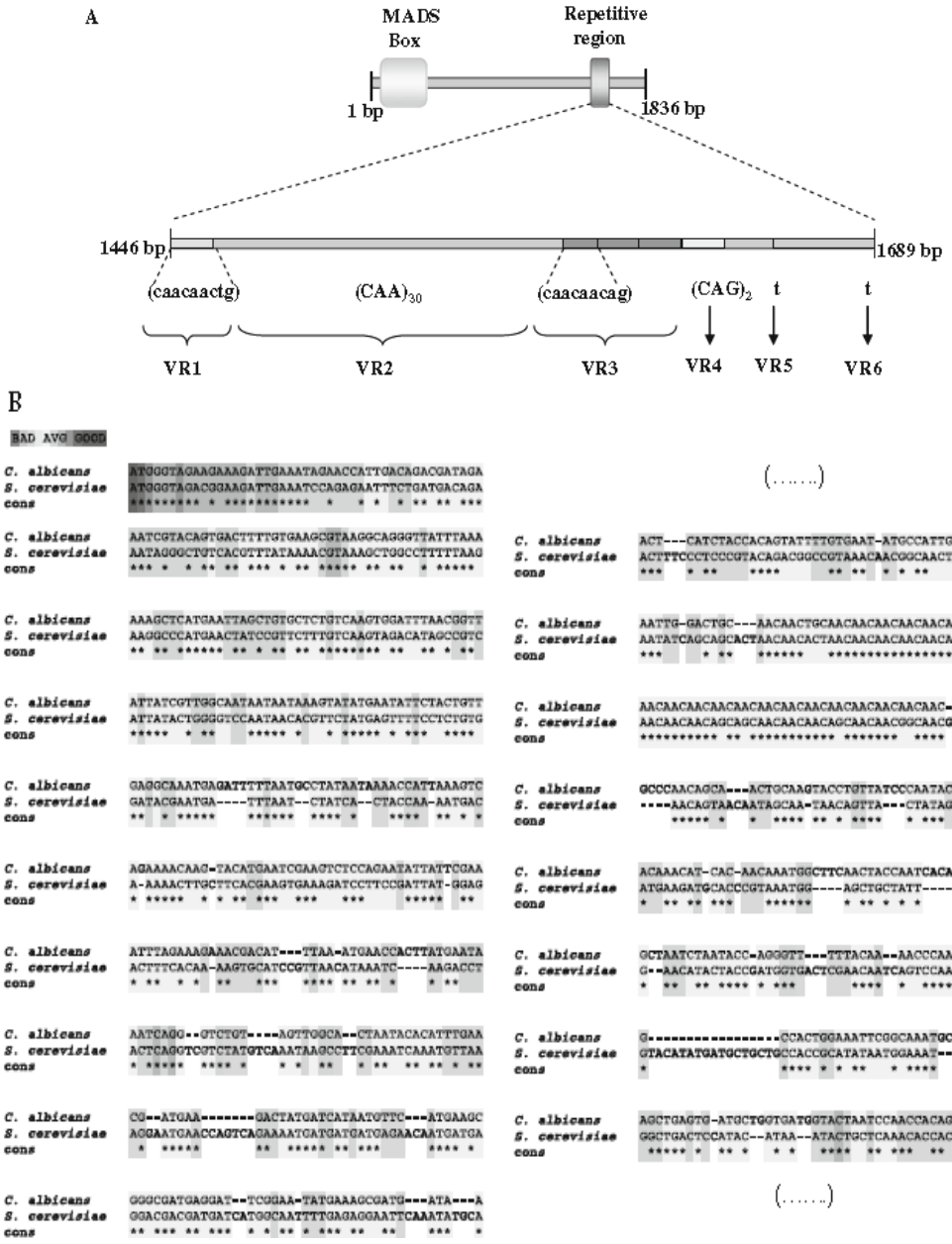

Figure 2.1. A) Schematic representation of *C. albicans RLM1* gene. The scheme is based on the sequence of strain SC5314. The repetitive CAI region is highlighted with the correspondent variable regions (VRs) for allele 45. B) DNA sequence alignment of *C. albicans* and *S. cerevisiae RLM1* gene showing the MADS box and the repetitive region, the zones with higher homology.

The variability within the 3′-terminus of *RLM1* gene was assessed by amplification of the CAI microsatellite, which constitutes the main repetitive region of this gene. CAI allele length variation was analysed by sequencing all the different PCR fragments obtained. This analysis showed that the *RLM1* repeat region had a complex structure, with trinucleotide and nanonucleotide regions varying simultaneously (Table 2.2). Independently of the complexity of the variable regions the repeat unit present, CAA or CAG, codes only for glutamines, resulting a protein with a stretch of 11–49 glutamines in its C-terminus. These variable regions (VRs) are indicated in Fig. 2.1A and Table 2.2.

Table 2.2. Sequence alignment of *C. albicans RLM1* alleles, showing the defined variable regions, VR1 to VR6

Alleles : Sequence							Group
	VR1	VR2	VR3	VR4	VR5	VR6	
11 to 15	:CTGcaacaactg$(CAA)_n$(caacaacag)			$(CAG)_4$----------c----------g			I
16 to 29	:CTGcaacaactg$(CAA)_n$$(caacaacag)_2$			$(CAG)_2$----------c/t----------t/g			II
30 to 49	:CTGcaacaactg$(CAA)_n$$(caacaacag)_2CAA(caacaacag)_3$$(CAG)_2$----------t----------t						III

Lowercase letters indicate the nanonucleotide variable motifs

Allelic and genotypic frequencies

One hundred and twenty-one *C. albicans* independent clinical isolates and two reference strains collected from different geographic origins and anatomical sites were genotyped using the CAI microsatellite. A total of 35 different alleles were obtained with repeat units varying from 11 to 49 (Fig. 2.2). Alleles presenting 11–28 repeat units represented 82% of all alleles typed, being the most frequently observed the ones with 21, 25 and 27 repetitions. The remaining alleles corresponded to only 18%, and were clearly underrepresented. In these isolates 66 different genotypes were observed from which 44 were detected only once (Table 2.1). All strains from Angola and Madagascar presented the genotype 15/15. This genotype constitutes a curious case since allele 15 was observed exclusively in this particular group of strains. No association between allele length and geographic origin or body location of the isolates was observed except for allele 15 that was exclusively identified in isolates from Angola and Madagascar.

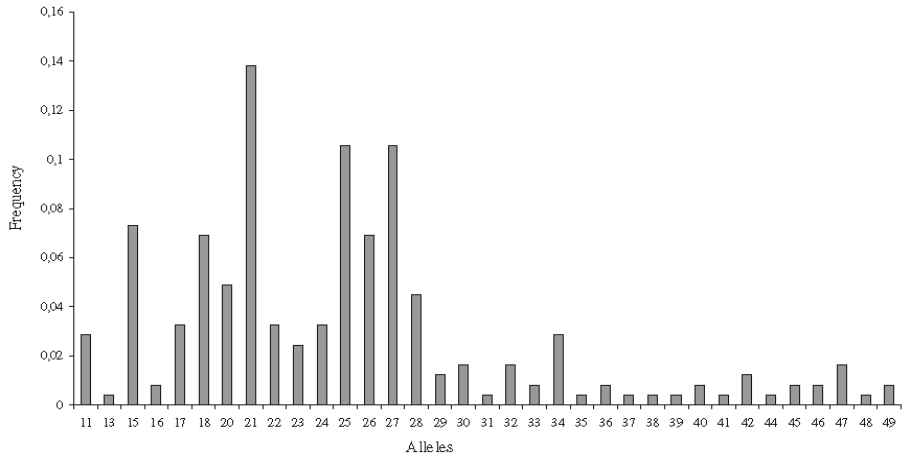

Figure 2.2. *RLM1* allele frequencies based on CAI microsatellite analysis.

Susceptibility to stress agents

In order to investigate whether the variability found at the *RLM1* locus could be correlated with susceptibility we tested three groups of strains formed according to the length and complexity of *RLM1* alleles. One group representative of genotypes presenting alleles with less than 16 repetitions (group I), one including strains with alleles from 17 to 28 repetitions (group II), and a third with strains presenting genotypes composed of longer alleles, more than 28 repetitions (group III). Only strains presenting the two alleles within the same allelic group were tested in this analysis. The susceptibility to menadione, hydrogen peroxide, SDS, acetic acid, and CFW was tested in ten strains of each group. The stress agents were used alone or combined with CR, an additional cell wall damaging agent.

The results (Fig. 2.3) obtained when testing the stress agents alone showed that strains within group I were significantly more susceptible than strains from the other groups, except the case of CFW. Comparing strains from groups II and III no significant differences were observed in the susceptibility to all the stress agents (Fig. 2.3).

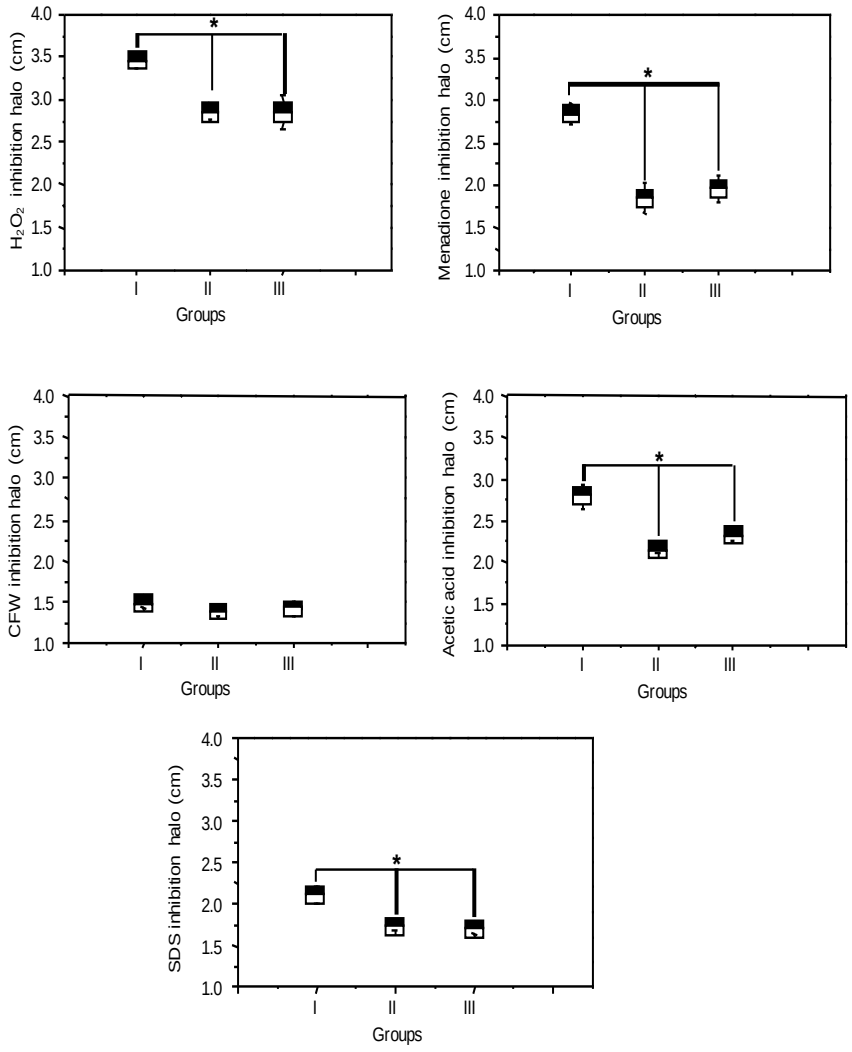

Figure 2.3. Susceptibility of *C. albicans* strains presenting alleles within groups I, II and III to menadione, acetic acid, hydrogen peroxide, SDS and Calcofluor White (CFW) at 37ºC. Susceptibility was determined measuring the inhibition halo (cm) and results are shown in *Box plots*. Standard error bars * $P < 0.05$. The values displayed are the mean of three independent experiments.

On the contrary, in the presence of CR the susceptibility of strains from group II to the stresses tested was significantly enhanced ($P > 0.05$), while no significant differences were observed in the growth inhibition halos of strains from group III (Figs. 2.4 and 2.5). Curiously, in the presence of CR no growth was observed in strains from group I, indicating a hypersensitivity to the combination of the different stresses with CR (Fig. 2.5).

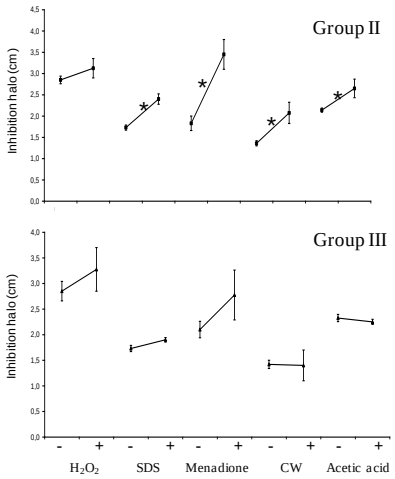

Figure 2.4. Susceptibility of *C. albicans* strains within groups II and III to hydrogen peroxide, SDS, menadione, Calcofluor White (CFW) and acetic acid at 37°C in the absence (−) and in the presence (+) of Congo red (CR). Susceptibility was determined by measuring the inhibition halo (cm). Standard error bars * $P < 0.05$. The values displayed are means of three independent experiments.

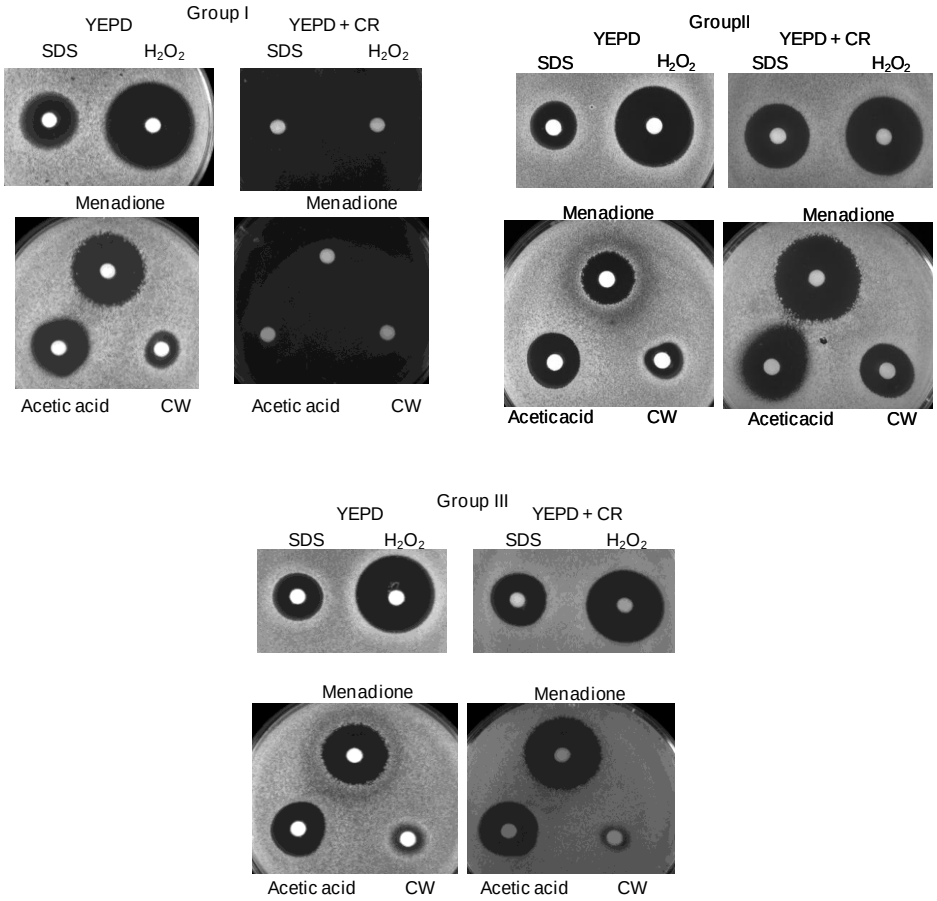

Figure 2.5. Representative pictures of the susceptibility of *C. albicans* strains within groups I, II, and III to hydrogen peroxide, SDS, menadione, Calcofluor White (CFW) and acetic acid in the presence and absence of Congo red (CR). Diameter of the blank paper disk is 0.6 cm.

Discussion

Candida albicans is an opportunistic fungus responsible for serious infections and the identification of strains with a particularly well-adapted genotype is of great interest to understand local epidemiology, pathogenicity and host interactions (Odds 1988; Pujol *et al.,* 1993). The microsatellite used in this study is located inside *RLM1*, a gene from the MADS box family. The MADS box genes encode a family of eukaryotic transcriptional regulators involved in diverse and important biological functions, controlling gene expression and cell development (Messenguy and Dubois 2003). In *S. cerevisiae*, *RLM1* is the major effector of the cell wall integrity pathway, responsible for the regulation of genes required in the repair of damages of the cell wall. Since the composition of the cell wall of both *C. albicans* and *S. cerevisiae* is very similar the role of *C. albicans RLM1* in the maintenance of the cell wall integrity is expectedly conserved. A previous work reported that *RLM1* is required for normal growth of *C. albicans* at least in the presence of caspofungin and CR, but surprisingly although the apparent conserved biological function, very few caspofungin-inducible *RLM1* dependent genes were identified (Bruno *et al*., 2006). We found that *C. albicans RLM1* presents a great variability at its 3′-terminus. The allelic frequencies of the 35 different alleles did not follow the typical Gaussian distribution, since alleles with more than 28 repetitions were clearly underrepresented. These longer alleles presented a complex internal structure with 3–5 CAG units interrupting the CAA simple tract due to the incorporation of nanonucleotide tracts among the trinucleotide repeats (see Table 2.2). As a consequence, these alleles are likely to present a lower mutational rate. In fact, it has been described that the incorporation of variants in the simple repetitive tract of a microsatellite greatly contributes to its stabilization, lowering its mutational rate (Petes *et al.,* 1997). Although the longer alleles had lower frequencies they represented the majority of the alleles found among the strains studied, suggesting that they may have a positive role in strain adaptation. In fact, results from the susceptibility tests showed that strains possessing longer alleles were significantly more resistant to the tested stress agents indicating that, at least in vitro, the longer alleles may confer advantages to the strains. *Candida albicans* is a diploid species and the majority of the strains presenting long alleles are heterozygotic, with the other allele within group II (i.e., 27/49). This suggests that the diploid genome of *C. albicans* allows to create further variability and adaptation by differential evolution of even the two alleles of a single gene as reported previously for *ALS3*, *ALS7* and *SAP2* (Staib *et al.,* 2002; Zhang *et al.,* 2003; Oh *et al.,* 2005). It is noteworthy to refer also the susceptibility of Group I strains to almost all stresses tested, particularly in the presence of CR. All strains included in this group were from Angola and Madagascar and belonged to the so-called atypical *C. albicans* group that has been proposed to constitute a new species, *C. africana* (Tietz *et al.*, 2001). Our results support the view that these strains present different characteristics not only in their hypersensitivity but also in the fact that they were all homozygous to allele 15 that possessed a unique internal structure.

Candida albicans Rlm1 is a combinatory transcription factor that presents a great variability at its C-terminus, conferred by the CAI microsatellite. The C-terminus of proteins from the MADS box family is necessary for dimerization and required for transcriptional activation (Messenguy and Dubois 2003). It is known that their regulatory specificity depends on accessory factors and in many cases the cofactor

with which the protein interacts specifies which genes are regulated, when they are regulated and if these genes are transcriptionally activated or repressed (Shore and Sharrocks 1995). This characteristic may account for the different susceptibility pattern observed among strains from the three groups of alleles, if the interaction of accessory factors with Rml1 is somehow correlated with the C-terminus length, conditioning the transcriptional activity.

The functional importance of the C-terminal domain of MADS-box genes has already been demonstrated in other organisms. The loss-of-function observed in alleles that carried mutations in the C-terminus or the dominant-negative phenotypes that were generated by overexpressing MADS box genes lacking the C-terminus in plants and humans are some of the examples (Vandenbussche *et al.,* 2003; Wang *et al.,* 2003).

In conclusion, we demonstrated that the CAI repetitive region confers a high genetic variability to *RLM1* gene, which is reflected in strain susceptibility to different stress conditions. Although the role of *RLM1* gene in *C. albicans* is not well established yet our observations suggest that an increased number of repetitive units in the *C. albicans RLM1* gene enhances the resistance to stress agents. Additional work is under development by using isogenic strains in order to confirm the phenotypes seen and further investigate whether *RLM1* gene variability may be related to *C. albicans* pathogenicity.

Acknowledgments

We thank Claude Pujol and Timothy Lott for providing part of the strains used in this study. We are also indebted to Adelaide Alves (Hospital de S. Marcos, Braga) and Cidália Pina-Vaz (Hospital de S. João, Porto) for providing the clinical isolates. Magda Graça is gratefully acknowledged for operating the nucleic acid sequencer. This research was supported by Fundação para a Ciência e Tecnologia (FCT), Portugal, through a multi-year contract with Centro de Biologia da Universidade do Minho.

References

Bougnoux, M. E., D. Diogo, N. François, B. Sendid, S. Veirmeire, J.F. Colombel, C. Bouchier, H. van Kruiningen, C. d'Enfert and D. Poulain (2006). "Multilocus sequence typing reveals intrafamilial transmission and microevolutions of *Candida albicans* isolates from the human digestive tract." *J Clin Microbiol* 44:1810–1820.

Bruno, V. M., S. Kalachikov, R. Subaran, C. J. Nobile, C. Kyratsous and A. P.Mitchell (2006). "Control of the *C. albicans* cell wall damage response by transcriptional regulator Cas5." *PLoS Pathog* 2(3):e21.

Budowle, B., R. Chakraborty, A. Giusti, M. A. J. Eisenberg and R. C. Allen (1991). "Analysis of the VNRT *locus D1S80* by the PCR followed by high resolution PAGE." *Am J Hum Genet* 48:137–144.

Dodou, E. and R. Treisman (1997). "The *Saccharomyces cerevisiae* MADS-box transcription factor Rlm1 is a target for the Mpk1 mitogen-activated protein kinase pathway." *Mol Cell Biol* 17:1848–1859.

Garcia-Hermoso, D., O. Cabaret, G. Lecellier, M. Desnos-Ollivier, D. Hoinard, D. Raoux, J. M. Costa, F. Dromer and S. Bretagne (2007). "Comparison of microsatellite length polymorphism and multilocus sequence typing for DNA-based typing of *Candida albicans*." *J Clin Microbiol* 45:3958–3963.

Messenguy, F. and E. Dubois (2003). "Role of MADS box proteins and their cofactors in combinatorial control of gene expression and cell development." *Gene Rev* 316:1–21.

Odds, F.C. (1988). "Candida and candidosis." *Bailliere Tindall, London*, pp 1–6, 60–230

Oh, S.H., G. Cheng, J. A. Nuessen, R. Jajko, K. M. Yeater, X. Zhao, C. Pujol, D. R. Soll and L. Hoyer (2005). "Functional specificity of *Candida albicans* Als3p proteins and clade specificity of *ALS3* alleles discriminated by the number of copies of the tandem repeat sequence in the central domain." *Microbiology* 151:673–681.

Pelroth, J., B. Choi and B. Spellberg (2007). "Nosocomial fungal infections: epidemiology, diagnosis, and treatment." *Med Mycol* 45:321–346.

Petes, T. D., P. W. Greenwell and M. Dominska (1997). "Stabilization of microsatellite sequences by variant repeats in the yeast *Saccharomyces cerevisiae*." *Genetics* 146:491–498.

Pujol, C., J. Reynes, F. Renaud, M. Raymond, M. Tibayrenc, F. J. Ayala, F. Janbon, M. Mallie and J-M Bastide (1993). "The yeast *Candida albicans* has a clonal mode of reproduction in a population of infected human immunodeficiency virus-positive patients." *Proc Natl Acad Sci USA* 90:9456–9459.

Sampaio, P., L. Gusmão, C. Alves, C. Pina-Vaz, A. Amorim and C. Pais (2003). "Highly polymorphic microsatellite for identification of *Candida albicans* strains." *J Clin Microbiol* 41:552–557.

Sampaio, P., L. Gusmão, A. Correia, C. Alves, A. G. Rodrigues, C. Pina-Vaz, A. Amorim and C. Pais (2005). "New microsatellite multiplex PCR for *Candida albicans* strain typing reveals microevolutionary changes." *J Clin Microbiol* 43:3869–3876.

Shore, P. and A. Sharrocks (1995). "The MADS-box family of transcription factors". *Eur J Biochem* 229:1–13.

Staib, P., M. Kretschmar, T. Nichterlein, H. Hof and J. Morschhauser (2002). "Host versus in vitro signals and intrastrain allelic differences in the expression of a *Candida albicans* virulence gene." *Mol Microbiol* 44:1351–1366.

Tietz, H. J., M. Hopp, A. Schmalreck, W. Sterry and V. Czaika (2001). "*Candida africana* sp. nov., a new human pathogen or a variant of *Candida albicans*?" *Mycoses* 44:437–445.

Vandenbussche, M., G. Theissen, Y. Van de Peer and T. Gerats (2003). "Structural diversification and neo-functionalization during floral MADS-box gene evolution by C-terminal frameshift mutations." *Nucleic Acids Res* 31:4401–4409.

Wang, L., C. Fan, S. E. Topol, E. J. Topol and Q. Wang (2003). "Mutation of MEF2A in an inherited disorder with features of coronary artery disease." *Science* 302:1578–1581.

Watanabe, Y., K. Irie and K. Matsumoto (1995). "Yeast *RLM1* encodes a serum response factor-like protein that may function downstream of the Mpk1 (Slt2) mitogen-activated protein kinase pathway." *Mol Cell Biol* 15:5740–5749.

Zhang, N., A. L. Harrex, B. R. Holland, L. E. Fenton, R. D. Cannon and J. Schmid (2003). "Sixty alleles of the *ALS7* open reading frame in *Candida albicans*: *ALS7* is a hypermutable contingency locus." *Genome Res* 13:2005–2017.

Chapter 3

Candida albicans transcription factor Rlm1 is involved in cell wall remodelling and behaves as a negative biofilm regulator

This chapter comprises parts from the following publication:

Delgado-Silva, Y., B. Ramírez, L. Carreto, S. Silva, J. Chávez-Galarza, C. Vaz, C. Carneiro, J. Morchhäuser, C. Pais, R. Oliveira and P. Sampaio. 2012. *Candida albicans* transcription factor Rlm1 is involved in cell wall remodelling and behaves as a negative biofilm regulator. *Submitted manuscript*.

Abstract

Saccharomyces cerevisiae and *Candida albicans* respond to cell wall perturbations by activation of the cell wall integrity (CWI) mitogen-activated protein (MAP) kinase pathway. One of the transcription factors of this signalling cascade is the MADS-box protein Rlm1, which function has been characterized mainly in *S. cerevisiae*. In this work we studied the involvement of the *C. albicans* orthologue, also named Rlm1, in cell wall remodelling.

Candida albicans rlm1Δ/*rlm1*Δ mutant was tested with several cell wall-perturbing agents in parallel with *S. cerevisiae rlm1*Δ mutant. Results showed that *C. albicans* mutant was hypersensitive to Congo red, caspofungin and calcofluor white. Caspofungin phenotype was reverted in the presence of 1M of sorbitol, indicating that *C. albicans RLM1*, like the *S. cerevisiae* orthologue, is important for the maintenance of cell wall integrity. Quantification of cell wall components showed a two-fold increase in chitin and mannans in the *C. albicans rlm1Δ*/*rlm1*Δ strain in comparison with the WT. In agreement with a transcription factor function, we found evidence indicating nuclear localization of Rlm1-GFP fusion protein. Microarray analysis showed that the absence of a functional *C. albicans RLM1* significantly up regulated genes involved in cell adhesion, like *ECE1*, *ALS1*, *ALS3*, *HWP1, RBT1,* and down regulated genes involved in the catabolism of carbohydrates, *DAK2*, *GLK4*, *NHT1* and *TPS1*. Accordingly, transcription of *GAL4*, coding for a transcription factor involved in the control of glycolytic enzymes, was also up regulated. These results suggest that deletion of *RLM1* induces a rearrangement in metabolic pathways allowing the use of alternative carbon sources. The up-regulation of genes involved in cell adhesion correlated with adhesion and biofilm assays in which an increase in the amount of adhering cells and biofilm biomass was observed in the mutant strain in comparison with the WT.

This study suggests that, in the absence of *RLM1 C. albicans* activates a “compensatory mechanism” involving the increase in cell wall chitin and mannoproteins, changing the metabolism of the mutant to cope with it, as observed for *S. cerevisiae*. Furthermore, the increase of cell adhesion binding proteins involved in biofilm formation and the confirmation that the *rlm1Δ*/*rlm1*Δ mutant biofilm biomass is much higher, suggests that Rlm1 acts as a negative biofilm regulator, a function that has not been detected for *S. cerevisiae*.

Introduction

The yeast cell wall is an essential cellular structure in the osmotic stabilization, protection against mechanical damage, maintenance of cell shape, adhesion and invasive growth (Klis *et al.*, 2006). Polysaccharides, β-1,3- and β-1,6-glucan and chitin, represent the inner structural components of the fungal cell wall to which a protective mannoprotein layer is attached. Mannoproteins may account to nearly half of the cell wall mass and may be non-covalently bound, covalently bound and GPI-modified and covalently linked to the β-1,3-glucan network. The polysaccharide β-1,3-glucan comprises the inner layer, highly elastic, that serves as a scaffold for the protective outer layer of mannoproteins (Lipke and Ovalle, 1998). Chitin is found dispersed in the cell wall, in the bud neck, in the primary septum and in bud scars (Cabib and Duran, 2005). This particular localization together with the fact that upon stress, the content in chitin can increase considerably (Aguilar-Uscanga and Francois, 2003), suggest that chitin is the material used in cell wall remodelling. Chitin and β-glucans provide the strength and rigidity to the cell wall, which ultimately determine the morphology of the cell, therefore their synthesis play a fundamental role in maintaining fungal cell integrity during growth and morphogenesis and in stress adaptation (Shaw *et al.*, 1991).

The cell wall is a dynamic structure since it changes with alterations of the surrounding growth conditions (Garcia *et al.*, 2004) and is remodeled as the cell increases in size and during morphogenetic processes such as mating, sporulation, or pseudohyphal growth. Upon cell wall damage, cells activate the cell wall integrity (CWI) mitogen-activated protein (MAP) kinase pathway (also known as the PKC pathway) so that the cell wall is repaired and cell integrity maintained. This response involves several processes (Garcia *et al.*, 2004): (i) the balance between cell wall polysaccharides is modified, as indicated by hyper-accumulation of chitin; (ii) the type of association between β-glucan, mannoproteins, and chitin is changed; (iii) an increase of cell wall proteins occurs; and (iv) the β-1,3-glucan synthase complex is transiently redistributed throughout the cell (Garcia *et al.*, 2004; Martinez-Lopez *et al.*, 2004). The response to cell wall damage is well understood from studies with the budding yeast *S. cerevisiae*, where the CWI pathway comprises the sensors Mid2 and Wsc1 in the plasma membrane, which, upon cell wall damage, interact with the GDP/GTP exchange factor Rom2, leading to the conversion of the G protein Rho1 to its activated GTP-bound state. Interaction with Rho1 activates the control kinase Pkc1 that phosphorylates Bck1, the first component of the MAP kinase cascade three-component module. Sequential phosphorylations of the components of the MAP kinase cascade activate downstream kinases: the redundant Mkk1 and Mkk2 and the final kinase Slt2. The targets of the CWI pathway are the transcription factor heterodimer complex SBF (composed by Swi4 and Swi6), and the MADS-box transcription factor Rlm1 (Reinoso-Martin *et al.*, 2003; Levin, 2005). The SBF transcription factor (Sw4-Swi6 cell cycle Box binding Factor) is involved in the activation of expression of cell cycle-regulated genes in the G1/S phase, including genes involved in cell wall synthesis (Nasmyth and Dirick, 1991), and the MADS (Mcm1p-Agamous-Deficiens-Serum Response Factor) box transcription factor Rlm1 (Schwarz-Sommer *et al.*, 1990) activates genes involved in cell wall reinforcement and remodeling in response to cell wall stress (Jung and Levin, 1999; Garcia *et al.*, 2004).

The *C. albicans* CWI counterpart is the Mkc1 signaling pathway (Navarro-Garcia *et al.*, 1995; Navarro-Garcia *et al.*, 1998; Navarro-Garcia *et al.*, 2005) which is important for virulence since the disruption mutant in *MKC1*, the *SLT2* homolog, is less virulent than the wild-type (Diez-Orejas *et al.*, 1997). The Pkc1 and Mkc1 components of this pathway were identified based on sequence and functional homologies with *S. cerevisiae* CWI pathway components (Navarro-Garcia *et al.*, 1995; Paravicini *et al.*, 1996), while other components with sequence homology in *C. albicans* remain to be characterized. Targets of Mkc1 have not yet been identified, however the presence of sequence homologues of *S. cerevisiae SWI4*, *SWI6* and *RLM1* in the *C. albicans* genome suggests that these could act as final effectors of the signaling cascade. Accordingly, *C. albicans* mutant in the homologue of *RLM1* displays higher sensitivity to caspofungin and Congo red (Bruno *et al.*, 2006). This is the only study including the characterization of *RLM1* in *C. albicans* and results indicated that *RLM1* does not appear to be involved in the cell wall remodelling after caspofungin treatment. In this study our aim is to extend the characterization the *C. albicans RLM1* in a new set of *rlm1* mutants from the prototrophic wild-type model strain SC5314 constructed using the *SAT1*-flipping strategy (Reuss *et al.*, 2004). This study was made in parallel with the *rlm1*Δ mutant in the *S. cerevisiae* orthologue in order to assess parallelism between sequence homology and function.

Materials and Methods

Strains and growth conditions

Candida albicans and *S. cerevisiae* strains used in this study are listed in table 3.1. All strains were stored as frozen stocks with 15% w/v glycerol at -80ºC and cultured on yeast extract-peptone-dextrose (YPD) agar plates (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose and 2% w/v agar) at 30°C. For routine growth, YPD liquid medium, lacking agar, was used. Selection of *C. albicans* nourseothricin-resistant (Nou^R) transformants was performed on YPD agar plates containing 200µg/ml nourseothricin (Werner Bioagents, Jena, Germany). To obtain nourseothricin-sensitive (Nou^S) derivatives in which the *SAT1* flipper was excised by FLP-mediated recombination, transformants were grown overnight on YCB–BSA–YE medium (2.34% w/v yeast carbon base, 0.4% w/v bovine serum albumin, 0.2% w/v yeast extract, pH 4.0) without selective pressure to induce the *SAP2* promoter controlling *caFLP* expression. One hundred to two hundred cells were then spread on YPD plates containing 10µg/ml nourseothricin and grown for 2 days at 30ºC. Nou^S clones were identified by their small colony size and confirmed by re-streaking on YPD plates containing 200µg/ml nourseothricin as described previously (Reuss *et al.*, 2004). For integration of GFP for gene fusion constructions we used strain CAI4 (*ura3Δ::imm434/ura3Δ::imm434*) as a host. This strain was grown on YPD agar plates supplemented with uridine (50µg/ml) while strains carrying the *RLM1*-GFP fusion genetic construct were grown on solid or liquid synthetic complete medium without uridine (SC-uridine; 0.67% w/v yeast nitrogen base without amino acids (DIFCO), supplemented with dropout mix without uridine) with 2% w/v glucose. Liquid cultures (lacking agar) were incubated at 30ºC, 200 revolutions per min (rpm) and growth was monitored by optical density at 640nm (OD_{640}).

Table 3.1. Strains used in this study.

Strain	Parental strain	Relevant characteristics or genotype[a]	Source or reference
C. albicans			
SC5314		Wild-type *C. albicans* model strain	Gillum *et al.* (1984)
SCRLM1M1A	SC5314	*rlm1*Δ::*SAT1-FLIP*/*RLM1*	This study
SCRLM1M1B	SC5314	*RLM1*/*rlm1*Δ::*SAT1-FLIP*	This study
SCRLM1M2A	SCRLM1M1A	*rlm1*Δ::*FRT*/*RLM1*	This study
SCRLM1M2B	SCRLM1M1B	*RLM1*/*rlm1*Δ::*FRT*	This study
SCRLM1M3A	SCRLM1M2A	*rlm1*Δ::*FRT*/*rlm1*Δ::*SAT1-FLIP*	This study
SCRLM1M3B	SCRLM1M2B	*rlm1*Δ::*SAT1-FLIP*/*rlm1*Δ::*FRT*	This study
SCRLM1M4A	SCRLM1M3A	*rlm1*Δ::*FRT*/*rlm1*Δ::*FRT*	This study
SCRLM1M4B	SCRLM1M3B	*rlm1*Δ::*FRT*/*rlm1*Δ::*FRT*	This study
SCRLM1K1A	SCRLM1M4A	*rlm1*Δ::*FRT*/*RLM1-SAT1-FLP*	This study
SCRLM1K1B	SCRLM1M4B	*RLM1-SAT1-FLP*/*rlm1*Δ::*FRT*	This study
SCRLM1K2A	SCRLM1K1A	*rlm1*Δ::*FRT*/*RLM1-FRT*	This study
SCRLM1K2B	SCRLM1K1B	*RLM1-FRT*/ *rlm1*Δ::*FRT*	This study
124a		Wild-type *C. albicans clinical isolate*	Sampaio *et al.* (2010)
124aRLM1M1A	124a	*rlm1*Δ_::*SAT1-FLIP*/*RLM1*	This study
124aRLM1M1B	124a	*RLM1*/*rlm1*Δ::*SAT1-FLIP*	This study
124aRLM1M2A	124aRLM1M1A	*rlm1*Δ_::*FRT*/*RLM1*	This study
124aRLM1M2B	124aRLM1M1B	*RLM1*/*rlm1*Δ::*FRT*	This study
124aRLM1M3A	124aRLM1M2A	*rlm1*Δ_::*FRT*/*rlm1*Δ_::*SAT1-FLIP*	This study
124aRLM1M3B	124aRLM1M2B	*rlm1*Δ::*SAT1-FLIP*/*rlm1*Δ::*FRT*	This study
124aRLM1M4A	124aRLM1M3A	*rlm1*Δ_::*FRT*/*rlm1*Δ_::*FRT*	This study
124aRLM1M4B	124aRLM1M3B	*rlm1*Δ::*FRT*/*rlm1*Δ::*FRT*	This study
124aRLM1K1A	124aRLM1M4A	*rlm1*Δ::*FRT*/*RLM1-SAT1-FLP*	This study
124aRLM1K1B	124aRLM1M4B	*RLM1*_::*SAT1-FLIP*/*rlm1*Δ_::FRT	This study
124aRLM1K2A	124aRLM1K1A	*rlm1*Δ::*FRT*/*RLM1-FRT*	This study
124aRLM1K2B	124aRLM1K1B	*RLM1-FRT*/ *rlm1*Δ::*FRT*	This study
CAI4	SC5314	*ura3Δ::imm434/ura3Δ::imm434*	Fonzi and Irwin (1993)
CAI4-RLM1-GFP	CAI4	*RLM1/RLM1-GFP-URA*	This study
S. cerevisiae			
BY4741		MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachmann *et al.* (1998)
rlm1	BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YPL089c::kanMX4*	EUROSCARF

[a] *SAT1-FLIP* denotes the *SAT1* flipper cassette.

Plasmids construction

For the deletion of *RLM1*, ca. 0.5 kb of upstream and downstream flanking sequences were amplified from the genomic DNA of strain SC5314 with the primers pairs RLM1-1/RLM1-2 and RLM1-3/RLM1-4, respectively (Table 2), and the SacI/SacII- and XhoI/ApaI-digested PCR products were cloned on both sides of the *SAT1*-flipper cassette of pSFS5 (Sasse *et al.*, 2011) to generate pRLM1M1. For reintroduction of *RLM1* into *rlm1* mutants, the *RLM1* coding region and ca. 0.5 kb of upstream and ca. 1.0 Kb downstream sequences were amplified with the primers RLM1-1 and RLM1-compl (Table 2). The PCR product was digested with SacI and SacII and used to replace the *RLM1* upstream region in pRLM1M1, resulting in pRLM1K1A.

To chromosomally tag Rlm1 with GFP, *RLM1*-specific sequences were added to the universal primer sequences described previously (Gerami-Nejad *et al.*, 2001) to generate the oligonucleotide RLM1-GFP*fwd* and RLM1-GFP*rev* (Table 2). These primers were used in combination with the template pGFP-*URA3* to generate the RLM1-GFP cassettes by PCR (Gerami-Nejad *et al.*, 2001). The RLM1-GFP cassettes were transformed into CAI4 cells to create the *C. albicans* strain CAI4-RLM1-GFP (Table 3.1). Correct integration at the *RLM1* locus was confirmed by diagnostic PCR, using RLM1-CAI primer, which anneals to the target gene locus upstream the altered region and RLM1-GFP primer, which anneals within the transformation module (Table 3.2).

Table 3.2. Primers used in this study.

Primer	Sequence[a,b]
RLM1-1	5´-TAGCTGAGCTCAAACCAATATAGTGA-3´
RLM1-2	5´-CGTCTGTCCGCGGTTCTATTTCAATC-3´
RLM1-3	5´-TTCAACTCGAGCAGATGTCAATAAC-3´
RLM1-4	5´-AGTAGGGCCCTAGCCCTAAGTCACG-3´
RLM1-compl	5´-TGTCACCGCGGATAAACTAGTCCTTCAT-3´
RLM1-GFP*fwd*	5´-TCCAACCACAGCAGGAAGTTCAAGTTCAGCAGATGTCAATAACACCAACAATGGACCTAATAAAAA TACA**GGTGGTGGTTCTAAAGGTGAAGAATTATT**-3´
RLM1-GFP*rev*	5´-TCAAAAACAAGGTGACTCTTTATGCTTGTTCTTTCTTTCTATTCACTTGTGAAAATCATGCACAATC AAA**TCTAGAAGGACCACCTTTGATTG**-3´
RLM1-CAI	5´-ATGCCATTGAGTGGAATTGG-3´
RLM1-GFP	5´-TACCGTAAGTAGCATCACCT 3´

[a] Restriction sites introduced into primers are underlined.
[b] The sequence to GFP plasmid templates is shown bold.
The gene-specific sequences included in the primers used in this study were approximately 70 nucleotides in length.

Candida albicans transformation

Candida albicans strains were transformed by electroporation (Kohler *et al.*, 1997) with gel-purified SacI-ApaI fragments from pRLM1M1 used to delete the *RLM1* gene, in strains SC5314 and 124a. The SacI-ApaI fragment from pRLM1K1A was used to reintroduce a functional *RLM1* copy into *rlm1* mutants. Nourseothricin-resistant transformants were selected on YPD agar plates containing 200µg/ml nourseothricin as described previously (Reuss *et al.*, 2004). The correct genomic integration of all constructs was confirmed by Southern hybridization with gene-specific probes.

Southern hybridization

Genomic DNA from *C. albicans* strains was isolated as described previously (Millon *et al.*, 1994). Ten mg of DNA were digested with EcoRI, separated in a 1% w/v agarose gel and, after ethidium bromide staining, transferred by vacuum blotting onto a nylon membrane and fixed by UV crosslinking. The gel-purified SacI-SacII *RLM1* upstream fragment and XhoI-ApaI *RLM1* downstream fragment from pRLM1M1 were used as probes. Southern hybridization with enhanced chemiluminescence-labeled probes was performed with the Amersham ECL™ Direct Nucleic Acid Labelling and Detection System (GE Healthcare, Braunschweig, Germany) according to the instructions of the manufacturer.

Susceptibility assays

Cultures were incubated overnight in liquid YPD medium at 30ºC, 200rpm, and diluted to OD_{640}=1 with fresh medium. Drop tests were performed by spotting 5µl of the serially diluted cell suspension onto 20% YPD (0.4% w/v peptone, 0.4% w/v glucose, 0.2% w/v yeast extract and 2% w/v agar) and YPD plates supplemented with the following compounds: 70µg/ml calcofluor white (CFW), 100µg/ml Congo red (CR), 30ng/ml caspofungin (CFG), 10mM caffeine, 0.035% (w/v) SDS, 1.5M NaCl or 2M sorbitol. Plates were incubated 48h at 30ºC before observation. Starvation sensitivity on solid media was assayed using a nitrogen starvation medium (2% w/v dextrose, 0.17% w/v yeast nitrogen base without amino acids and ammonium sulfate) (Toda *et al.*, 1985). Carbon source utilization was checked by addition (2% v/v glycerol) to YP medium (YPD devoid of dextrose. Diffusion test assays were performed using YPD agar plates with an overlay of $2x10^7$ cells. Paper discs (6 mm diameter), imbibed with 10µl of 0.5M menadione or 35% v/v hydrogen peroxide, were loaded on the agar plates and halos of growth inhibition were measured after 24h incubation at 30ºC.

Filamentation tests

Candida albicans cells were grown for 24h on modified Lee medium (0.5% w/v $(NH_4)_2SO_4$, 0.02% w/v $MgSO_4.7H_2O$, 0.25% w/v K_2HPO_4, 0.5% w/v NaCl, 1.25% w/v D-galactose, 0.05% w/v L-alanine, 0.13% w/v L-leucine, 0.1% w/v L-lysine, 0.01% w/v L-methionine, 0.007% w/v L-ornithine, 0.05% w/v L-proline, 0.05% w/v L-threonine and 0.0001% w/v biotin, pH 5.0) at 26ºC and 150rpm in order to maintain cells in the yeast morphology. Filamentation was induced by plating approximately 30 colony-forming units (CFUs) on Spider medium (1% w/v nutrient broth, 1% w/v mannitol, 0.2% w/v K_2HPO_4, 1.35% w/v Bacto Agar, pH 7.2) or YPD supplemented with 10% v/v fetal bovine serum (FBS) medium. Plates were incubated at 37ºC for 72h and photographed.

Sensitivity to zymolyase and quantification of cell wall components

Sensitivity to zymolyase was tested following the method described previously (van der Vaart *et al.*, 1995). Briefly, cultures at mid-logarithmic growth phase were adjusted to OD_{640}=0.5 in tris buffer (100mM tris-Cl, 1mM dithiothreitol, pH 7.4), containing 5U zymolyase 20T and incubated at 30ºC, 200rpm. Sensitivity of strains to zymolyase was determined by the decrease of OD_{640} of the cell suspension as a result of cell lysis along time. The amounts of the different cell wall sugar polymers was quantified in cells exponentially grown on YPD liquid medium at 30ºC by HPLC, following chemical hydrolysis with concentrated sulphuric acid, as previously described (Francois, 2006).

Fluorescence microscopy and immunofluorescence

Cultures of cells bearing the *RLM1*-GFP fusion construct were grown for 18h at 30ºC, 200rpm, in SC-uridine with 5µg/ml CR and aliquots were collected for nuclei staining with 1µg of 4',6'-diamino-2-phenylindol (DAPI; Sigma, St. Louis, MO) per ml of cell suspension. Control cultures were grown on medium lacking CR. Cells were visualized with a Leica-DM 5000B fluorescence microscope. For immunofluorescence cultures of cells bearing the *RLM1*-GFP fusion construct were grown as previously and stationary phase cells were harvested by centrifugation at 5,000rpm, 4ºC, and fixed with 4 % w/v paraformaldehyde for 2h. After wash with PBS 3 times (5min each), cells were permeabilized with 0.2% w/v Triton X-100 in PBS for 15min. After blocking non-specific binding sites by incubation with 0.5% w/v BSA in PBS for 10min, cells were incubated with the primary antibody for 1h at room temperature (mouse anti-GFP antibody diluted 1:100 in PBS, Santa Cruz, CA) followed by washing with PBS and incubation with the secondary antibody for 30min at room temperature (anti-mouse IgG_{2a} Alexa-Flour 488, 1:500 in PBS, Molecular Probes, Inc., OR; USA). One µg/ml of DAPI (Sigma, St. Louis, MO) was used to stain nuclei and the immunolabeling was visualized with a Zeiss Axiovert 200M fluorescence microscope.

Microarray analysis

RNA isolation and sample labeling

Candida albicans yeast cells from the wild-type SC5314 and mutant SCRLM1M4 (*rlm1ΔIrlm1*Δ) strain were grown in YPD liquid medium overnight at 30ºC. Each overnight culture was used to inoculate 20ml of YPD to an initial OD_{640}=0.4, and incubated at 30ºC for an additional 4h period at 200 rpm. The

cells were then harvested and immediately stored at -80ºC. RNA extraction was performed by using the hot acidic phenol method (Ausubel, 1993). cDNA synthesis and labelling were carried out as described elsewhere (van de Peppel *et al.*, 2003). Briefly, cDNA was synthesized from 40µg of total RNA in the presence of 2-aminoallyl-dUTP. Samples were purified using Microcon-30 (Millipore) columns prior to coupling to NHS ester activated Cyanine 3 (Cy3) and Cy5 fluorofores. Before hybridization, free dyes were removed using Chromaspin-30 (Clontech) columns and the efficiency of cDNA synthesis and dye incorporation was measured by spectrophotometry (NanoDrop). All samples had a degree of labelling (labelled nucleotides per 100 nucleotides) of around 5.0 ± 1.5.

DNA microarrays

Samples were hybridized onto customized Agilent 44K microarrays with probes designed for the *C. albicans* (Assembly 21) genome sequence. The microarray design was developed by the group of Prof. Geraldine Buttler at the School of Biomolecular and Biomedical Science, Conway Institute, University College, Dublin, and was made available upon request (http://www.ucd.ie/biochem/gb/Lab/). The hybridizations were conducted following Agilent Technologies recommendations. Two independent microarray hybridizations for the comparison of SC5314 and SCRLM1M4 strains were carried out using dye-swap labeling. Microarray images were obtained at a 5µm resolution using the Agilent G2565AA scanner. Fluorescence intensity was measured using the Agilent Feature Extraction Software (version 10.5.1.1) and signal and background quantitation was performed according to protocol Agilent recommendations (GE2_105_Dec08). Local background subtraction, Lowess normalization and averaging of replicate probes were performed using BRB Array Tools 3.8.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html). The processed signal was annotated using the data downloaded from the Candida Genome Database (http://www.candidagenome.org/).

Statistical analysis and functional annotation of the data

The log2 intensity ratios were used for identification of differentially expressed genes, using the one class t-test implemented in TM4 Microarray SoftwareSuite (MeV) v4.6.1 and a *P*-value cut-off of 0.05. Only genes with a fold variation above 2 were considered for discussion. The web based tool Genecodis2 (http://genecodis.dacya.ucm.es/) was used for functional enrichment analysis of the differentially expressed genes. *P*-values were calculated using the hypergeometric distribution and were corrected using the simulation-based approach (Carmona-Saez *et al.*, 2007). Enrichment results were filtered using a corrected *P*-value cut-off of 0.05.

Adhesion and biofilm formation

Adhesion and biofilm formation ability was assessed through quantification of total biomass by crystal violet (CV) staining (Stepanovic *et al.*, 2000; Silva *et al.*, 2009) and by determining the number of cultivable cells (Silva *et al.*, 2010). For this, standardized cell suspensions (1ml containing $1x10^7$ cells/ml in YPD) were placed into selected wells on 12 polystyrene plates (Orange, Braine-l`Alleud, Belgium) and incubated at 37ºC, 120 rpm. Adhesion ability was measured after 2h of incubation and

biofilm formation ability was inspected after 24h and 48h. Regarding the 48h sample, an extra step was performed, at 24h, in which 500µl of YPD medium was removed and an equal volume of fresh YPD was added. After the defined times of incubation, the medium was aspirated and non-adherent cells removed by washing the wells with ultra-pure water. Regarding total biomass quantification, first the biofilms were fixed with 1ml of methanol, which was removed after 15min of contact. The plates were allowed to dry at room temperature, and 1ml of CV (1% v/v) was added to each well and incubated for 5min. The wells were then gently washed with ultra-pure water and 1ml of acetic acid (33% v/v) was added to release and dissolve the dye. The absorbance of the obtained solution was read in triplicate in a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 570nm. Results were presented as absorbance/area of the wells (abs/cm^2). Regarding the number of cultivable biofilm cells, the biofilms were resuspended in 1ml phosphate buffer saline (PBS pH 7, 0.1M) by repeated pipetting (complete removal of the biofilm was confirmed by subsequent CV staining), and vigorously vortexed for 5min to disrupt the matrix. Serial decimal dilutions (in PBS) were plated onto YPD agar plates and incubated for 24h at 37ºC. Results are presented as the total number of CFUs enumerated (Log/cm^2). Experiments were repeated in three independent assays and the results were compared using a one-way analysis of variance (ANOVA) by applying Levene´s test of homogeneity of variance and the Tukey multiple-comparions test, using SPSS software (SPSS Inc., Chicago, IL). All tests were performed with a confidence level of 95%.

Phylogenetic analysis

Retrieval of sequences

In order to compare the similarity level between orthologous genes for *CaRLM1* a search in several fungal genomes was performed by using the algorithm BLASTP and BLOSUM62 matrix. Species and database used were: *C. albicans* (Candida Database), Broad Institute (*Aspergillus nidulans, Magnaporthe grisea*), Genolevures (*Candida glabrata, Kluyveromyces lactis*), Saccharomyces Database (*S. cerevisiae*), NCBI (*Aspergillus niger*). Moreover, *MCM1*, *SMP1* and *ARG80* genes, also belonging to MADS-box transcription factor family and present in *S. cerevisiae*, were recovered in Saccharomyces database for a further searching of orthologous genes for fungus above mentioned.

Alignment and phylogenetic inference

The amino acid sequences of each gene were aligned by using MUSCLE (Edgar, 2004) and manually edited in MEGA (Tamura *et al.*, 2007). The phylogenetic topology was inferred by Maximum Likelihood in PHYML 3.0 (Guindon *et al.*, 2010), with a nodal support estimated by 100 bootstrap analyses. The best-fit protein evolutionary model for Maximum Likelihood was JTT, with value gamma, amino acid frequencies and presence of invariable sites, which was obtained with PROTTEST 2.1 (Abascal *et al.*, 2005).

Results

Construction of the *C. albicans rlm1* mutant

To determine the role of *Rlm1* in *C. albicans, rlm1*Δ mutants were constructed from the prototrophic wild-type model strains SC5314 and 124a, using the *SAT1*-flipping strategy (Reuss *et al.*, 2004) to avoid the use of auxotrophic markers. For each *RLM1* allele we generated two independent heterozygous mutants (strains SCRLM1M2A and B) in which one of the *RLM1* alleles was deleted (Table 3.1; Fig. 3.1, lanes 3 and 7). A second round of gene deletion resulted in the homozygous *rlm1* mutants SCRLM1M4A and B (Table 3.1; Fig. 3.1, lanes 5 and 9). Two complemented strains (SCRLM1K2A and B), after reintegration of *RLM1* ORF, were generated (Table 3.1). After each round of insertion and FLP-mediated excision of the *SAT1* flipper cassette the resulting strains were analyzed by Southern hybridization to confirm their specific excision and to exclude, as far as possible, undesired recombination events involving the previously inactivated loci (Fig. 3.1). The absence and reintegration of the target genes from the genome of the mutants was also confirmed by PCR amplification (not shown). The same strategy was applied to strain 124a (Table 3.1 and Fig. 3.1).

Before the phenotyping tests, the constructed strains from SC5314 and 124a, were assessed for growth in YPD liquid medium, both at 30ºC and 37ºC, and in filament inducing media. Results showed that, in both genetic backgrounds, the growth rate of *C. albicans rlm1Δ/rlm1Δ* mutant was unaffected in YPD at both temperatures and that strains did not lose its ability to filament in solid media (Fig. 3.2A and 3.2B).

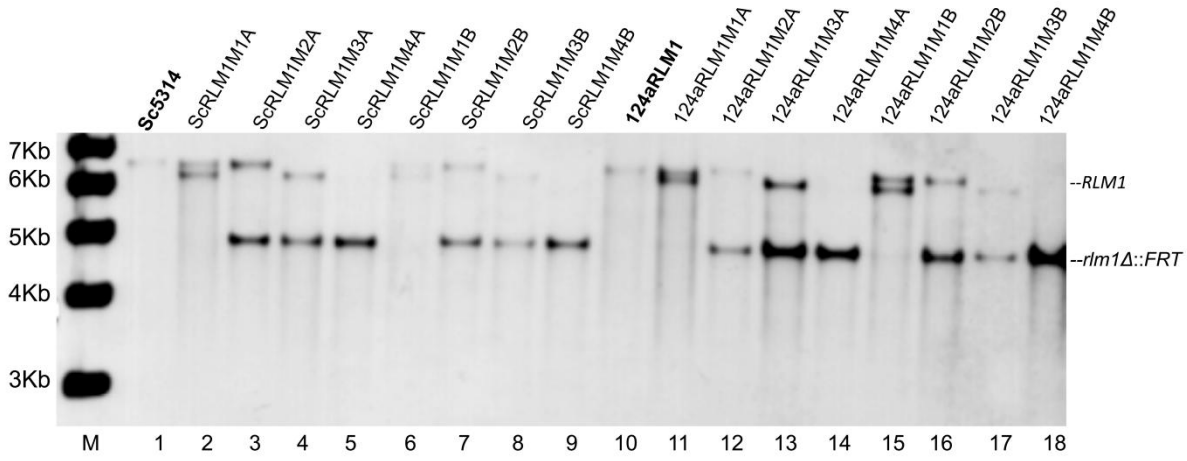

Figure 3.1. Southern hybridization of EcoRI-digested genomic DNA from wild-type strains SC5314, 124a (lane 1 and 10 respectively), heterozygous (lanes 3,7,12 and 16) and homozygous (lanes 5,9,14 and 18) *rlm1* mutants, with the *RLM1*-specific probe (XhoI-ApaI *RLM1* downstream fragment from pRLM1M1). The sizes (in kb) of the 1-kb ladder (lane M), which was also labeled, are shown on the left side of the blot. The positions of the original wild-type *RLM1* alleles and the inactivated *rlm1*Δ alleles are shown on the right side of the blot.

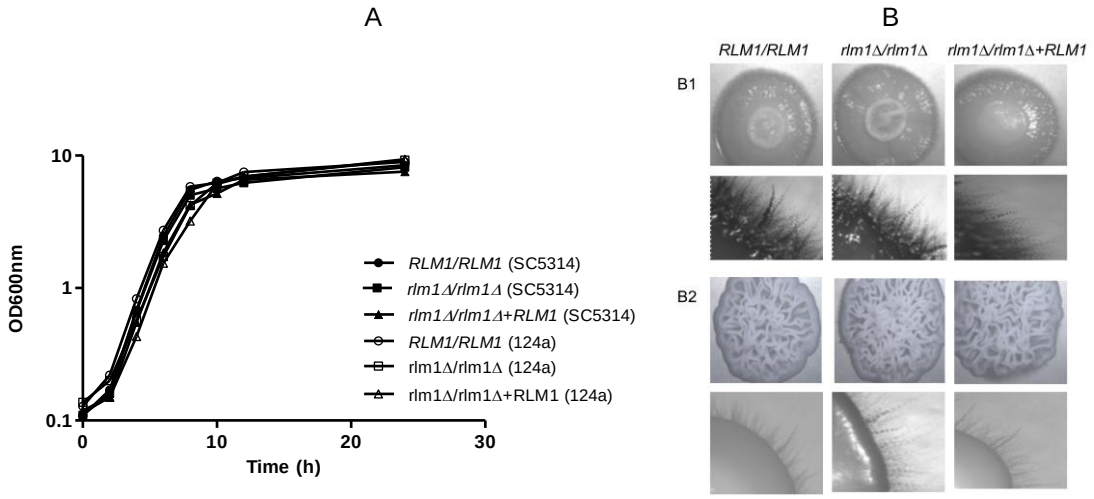

Figure 3.2. (A) Growth curve of *C. albicans* SC5314 (wt; circles), homozygous mutant SCRLM1M4A (*rlm1Δ/rlm1Δ*; squares), complemented SCRLM1K2A (*rlm1Δ/rlm1Δ+RLM1*; triangles), 124a (wt; open circles), homozygous mutant 124aRLM1M4A (*rlm1Δ/rlm1Δ*; open squares) and complemented 124aRLM1K2A (*rlm1Δ/rlm1Δ+RLM1*; open triangles) strains in YPD liquid medium at 30ºC and, (B) morphology on Spider medium (B1) and YPD containing 10% serum (B2) after 6 days of incubation at 30ºC. Photographs of the colony edge were taken by phase-contrast microscopy at 20X magnification.

Response of *rlm1* mutants to agents that affect cell integrity

To determine if *C. albicans* Rlm1 is involved in the CWI pathway, as described for *S. cerevisiae,* we compared the sensitivity of the constructed mutant strains against a range of cell wall-perturbing agents as well as to agents known to be associated with altered cell walls. As depicted in Fig. 3.3, the absence of a functional *RLM1* in *C. albicans* results in hypersensitivity to Congo red (CR) and Calcofluor White (CFW) for both genetic backgrounds. On the contrary, the *S. cerevisiae rlm1Δ* mutant displayed slightly higher resistance to CFW and unaffected growth in the presence of CR (Fig. 3.3).

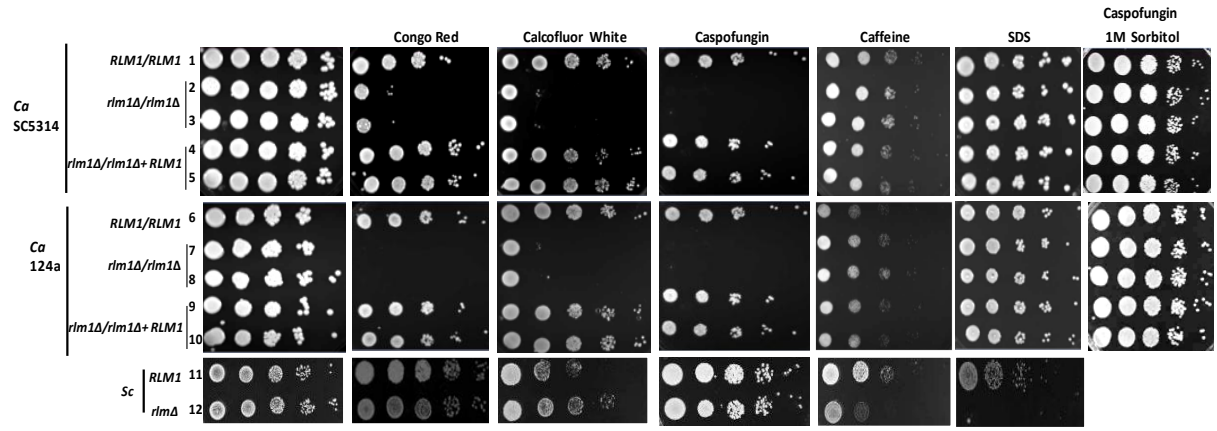

Figure 3.3. Sensitivity of *C.albicans* and *S. cerevisiae* strains against agents that affect cell integrity. Serial 10-fold dilutions of YPD overnight cultures were spotted on YPD plates without or with 100µg/ml Congo red, 70µg/ml calcofluor white, 30ng/ml caspofungin, 10mM caffeine, 0.035% SDS and 1M sorbitol with 30ng/ml caspofungin. After incubation for 2 days at 30ºC plates were photographed. *Candida albicans* strains obtained from SC5314 (1) (*RLM1*/*RLM1*) were two independently constructed homozygous mutant strains (*rlm1Δ/rlm1Δ*) SCRLM1M4A (2) and SCRLM1M4B (3), and two independently constructed complemented strains (*rlm1Δ/rlm1Δ+RLM1*) SCRLM1K2A (4) and SCRLM1K2B (5). *Candida albicans* strains obtained from 124a (6) (*RLM1*/*RLM1*) were two independently constructed homozygous mutant strains (*rlm1Δ/rlm1Δ*) 124aRLM1M4A (7) and 124aRLM1M4B (8) and two independently constructed complemented strains (*rlm1Δ/rlm1Δ+RLM1*) 124aRLM1K2A (9) and 124aRLM1K2B (10). *Saccharomyces cerevisiae* strains were wild-type BY4741 and the derived *rlm1Δ* mutant.

The SC5314- and 124a-derived *rlm1Δ/rlm1Δ* mutants from *C. albicans* were also more sensitive than the complemented and parental strains to the presence of Caspofungin (CFG). Curiously, *S. cerevisiae rlm1Δ* mutant showed no sensitivity to CFG under the same conditions as *C. albicans* (Fig. 3.3). The hypersensitivity of the *C. albicans* mutant to CFG was reverted with the osmotic protection by 1M sorbitol (Fig. 3.3). In the tests with caffeine and SDS, both genetic backgrounds of *C. albicans rlm1Δ/rlm1Δ* mutant were equally resistant to caffeine and unaffected by SDS, while for *S. cerevisiae rlm1Δ* these compounds caused decreased growth. Together, these results suggest that *C. albicans RLM1* is involved in cell wall biogenesis but its function in this process seems to be different from its *S. cerevisiae* orthologue.

Effect of osmolarity and oxidative stress on *rlm1* mutant strains

Cell wall-deficient yeast cells become more sensitive to osmotic stress due to a decreased physical support of the plasma membrane by the cell wall when changes in cellular volume compensate for the osmotic gradient. Upon high osmolarity, the CWI signalling pathway becomes activated, leading to phosphorylation of the MAPK Mkc1 (Navarro-Garcia *et al.*, 2005), the orthologue of Slt2 that in *S. cerevisiae* activates Rlm1. Therefore, we investigated the involvement of *C. albicans RLM1* in response to high and low osmolarity conditions (Fig. 3.4). Strains were tested for growth on hyper-osmotic media by incorporation of 1.5M NaCl and 2M sorbitol, as well as on hypo-osmotic medium, by diluting five-fold the components of YPD medium (20% YPD; Davenport *et al.*, 1995). *Candida albicans* strains displayed the same growth under all conditions, unlike *S. cerevisiae,* in which *rlm1*Δ mutant displayed higher sensitivity to hyperosmotic stress imposed by NaCl. Hypo-osmolarity did not affect growth of the mutant strains relatively to the parental strain in both species (Fig. 3.4). These results suggest that in the absence of Rlm1, and without any other stress, the cell wall is sufficiently strong to support the hypo and hyper-osmotic stresses imposed.

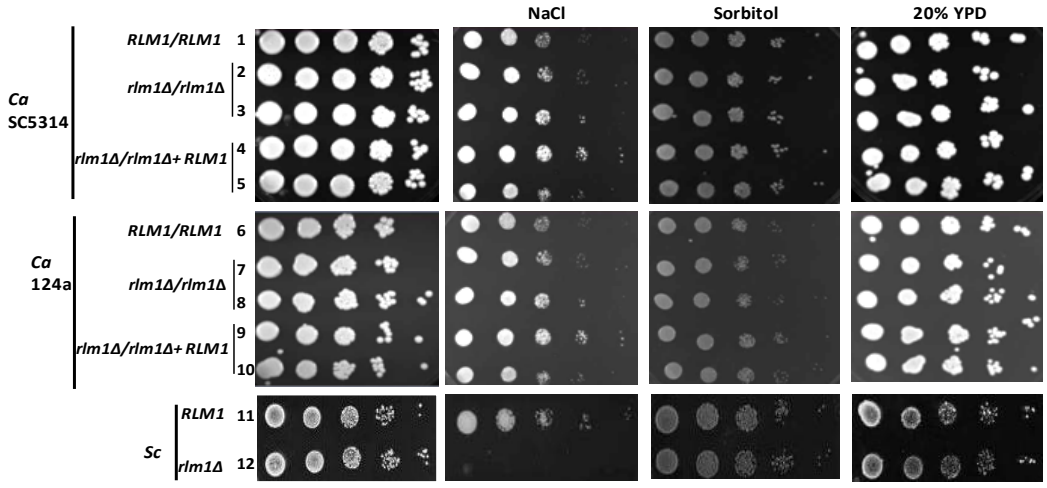

Figure 3.4. Sensitivity of *C.albicans* and *S. cerevisiae* strains to osmotic stress. Serial 10-fold dilutions of YPD overnight cultures of the strains were spotted on YPD plates without or with 1.5M NaCl, 2M sorbitol and 20% YPD and were incubated for 2 days at 30ºC. *Candida albicans* strains obtained from SC5314 (1) (*RLM1*/*RLM1*) were two independently constructed homozygous mutant strains (*rlm1*Δ*/rlm1*Δ) SCRLM1M4A (2) and SCRLM1M4B (3), and two independently constructed complemented strains (*rlm1*Δ*/rlm1*Δ+*RLM1*) SCRLM1K2A (4) and SCRLM1K2B (5). *Candida albicans* strains obtained from 124a (6) (*RLM1*/*RLM1*) were two independently constructed homozygous mutant strains (*rlm1*Δ*/rlm1*Δ) 124aRLM1M4A (7) and 124aRLM1M4B (8) and two independently constructed complemented strains (*rlm1*Δ*/rlm1*Δ+*RLM1*) 124aRLM1K2A (9) and 124aRLM1K2B (10). *Saccharomyces cerevisiae* strains were wild-type BY4741 and the derived *rlm1*Δ mutant.

In a previous study a connection between oxidative stress and the CWI pathway has been reported (Vilella *et al.*, 2005). Moreover, it has been described that the transcription of *S. cerevisiae RLM1* is activated upon treatment with hydrogen peroxide (Staleva *et al.*, 2004). To explore the involvement of *RLM1* (from *S. cerevisiae* and *C. albicans*) in oxidative stress response, strain growth was tested in the presence of the superoxide-generating agents, menadione, and hydrogen peroxide (Staleva *et al.*, 2004; Navarro-Garcia *et al.*, 2005; Vilella *et al.*, 2005; Westwater *et al.*, 2005), in diffusion test assays. For both species, mutants affected in *RLM1* were insensitive to the presence of 0.5M menadione and 35% hydrogen peroxide (Fig. 3.5).

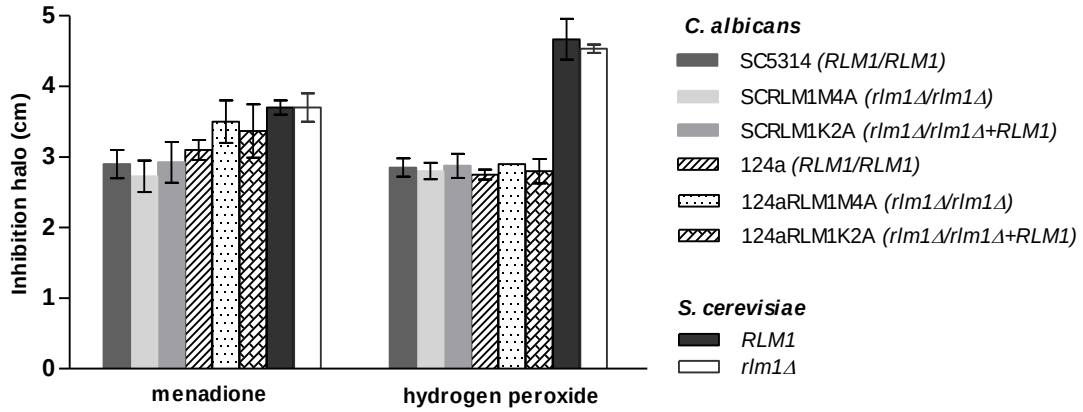

Figure 3.5. Sensitivity to oxidative stress of wild-type strains SC5314 and 124a (*RLM1*/*RLM1*), mutants SCRLM1M4A and 124aRLM1M4A (*rlm1Δ*/rlm1Δ), and complemented strains SCRLM1K2A and 124aRLM1K2A (*rlm1Δ/rlm1Δ+RLM1*) of *C. albicans*, and the haploid parental BY4741 (*RLM1*) and mutant (*rlm1Δ*) strains of *S. cerevisiae*. Diffusion test assays were performed using YPD agar plates with an overlay of $2x10^7$ cells. Paper discs (6 mm diameter), imbibed with 10µl of 0.5M menadione or 35% v/v hydrogen peroxide, were loaded on the agar plates and halos of growth inhibition were measured after 24h incubation at 30ºC.

Response of *rlm*1 mutants to temperature, glycerol and nitrogen starvation

In *S. cerevisiae,* deletion of several genes involved in the cell wall integrity pathway confer typical phenotypes, such as failure to grow at elevated temperatures in the absence of an osmostabilizer, failure to grow on glycerol medium, and sensitivity to nitrogen starvation (Lee and Levin, 1992; Irie *et al.*, 1993; Lee *et al.*, 1993; Martin *et al.*, 1993; Costigan and Snyder, 1994). However, for the *S. cerevisiae rlm1Δ* mutant the only phenotype in common with the other PKC1 pathway mutants is caffeine sensitivity. Thus, in this study we also tested *C. albicans rlm1Δ/rlm1Δ* mutant susceptibility to these parameters. Since our previous results showed no difference neither between phenotypes of SC5314 and 124a strains nor between phenotypes of the independent clones A and B, the following tests were performed only with clones A from wild-tipe SC5314 (SCRLM1M4A and SCRLM1K2A). Results showed that *C. albicans* mutant strains were able to grow on glycerol, resist nitrogen starvation (Fig. 3.6A), and had reduced thermotolerance (Fig. 3.6B). Curiously, regarding thermotolerance, the complemented and mutant strains behaved similarly, suggesting that gene dosage seems to be important for this phenotype. Overall, these results indicate that the function of *RLM1* in *C. albicans* and *S. cerevisiae* differ in the response to temperature and caffeine. The phenotype on glycerol is not clear since our results indicate that *S. cerevisiae rlm1Δ* is sensitive while the literature indicates that it grows normally.

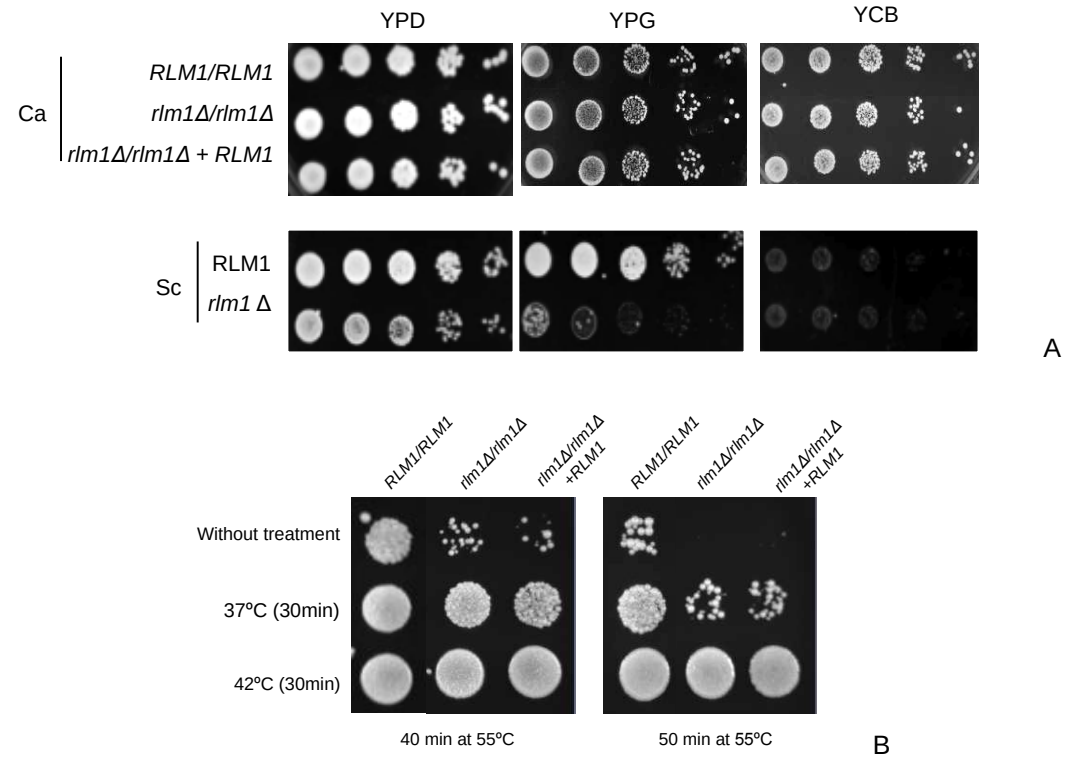

Figure 3.6. A) Ability to grow on glicerol (YPG) and sensitivity to nitrogen starvation (YCB) of wild-type strain SC5314 (*RLM1*/*RLM1*), homozygous SCRLM1M4A (*rlm1Δ*/rlm1Δ) *rlm1* mutant, and complemented strain SCRLM1K2A (*rlm1Δ/rlm1Δ+RLM1*) of *C. albicans* and the haploid parental BY4741 (*RLM1*) and mutant rlm1 (*rlm1Δ*) strains of *S. cerevisiae*. Serial 10-fold dilutions of YPD overnight cultures of the strains were spotted on YPD, YPG and YCB plates and incubated for 2 days at 30ºC. B) Thermal shock and thermotolerance of *C. albicans rlm1Δ/rlm1Δ* mutant. Exponentially grown cells at 30ºC were spotted on YPD plates and incubated at different temperatures for 2 days or subjected to a pre-treatment at 37ºC or 42ºC for 30 minutes, then spotted on YPD, shocked at 55ºC for 40 and 50 min before incubation at 30ºC for 2 days.

Sensitivity to zymolyase and quantification of cell wall components

Our results with agents affecting the cell wall integrity suggest the involvement of *RLM1* in the cell wall remodeling therefore, we investigated the sensitivity of *rlm1Δ/rlm1Δ* mutant cells against the β-1,3-glucan degrading enzyme zymolyase (Fig. 3.7). Deletion of *RLM1* slightly increased resistance to the enzyme in both species. Moreover, after 30 min of incubation around 50% of *S. cerevisiae* cells were lysed, whereas for *C. albicans* the percentage was higher than 75%. It has been previously reported that the decrease in susceptibility to zymolyase could be due to rearrangements in the amount of glucan and/or mannoprotein in the cell wall (Navarro-Garcia *et al.*, 1995; van der Vaart *et al.*, 1995). Thus, we quantified the amounts of the different cell wall sugar polymers in cells exponentially growing in YPD liquid medium.

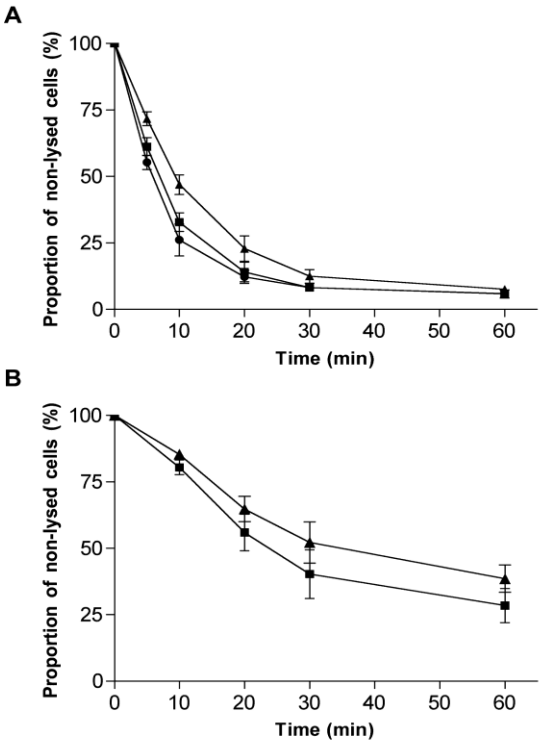

Figure 3.7. Zymolyase sensitivitiy of yeast strains assessed by decrease of optical density along time. Cells of (A) wild-type strain SC5314 (*RLM1*/*RLM1; squares*), homozygous mutant SCRLM1M4A (*rlm1Δ/rlm1Δ*; triangles) and complemented strain SCRLM1K2A (*rlm1Δ/rlm1Δ+RLM1*; circles) of *C. albicans*; and (B) wild-type BY4741 (*RLM1*; squares) and mutant rlm1 (*rlm1Δ*; triangles) strains of *S. cerevisiae* were harvested at the exponential growth phase and were incubated with 5U zymolyase 20T at 30ºC, 200rpm. Decrease of optical density of cell suspensions was monitored along time at 640nm as indication of cell lysis upon cell wall digestion by zymolyase.

As observed in Table 3.3, deletion of *RLM1* in *C. albicans* resulted in an increase of about 2-fold in the amount of chitin and mannans and of only 1.3 in glucans, in comparison with the parental strain. These results indicate that the content of the different cell wall components changed in *C. albicans rlm1Δ/rlm1Δ* mutant strain mostly regarding chitin and mannan content, which correlates with the hypersensitivity with CFW and the low sensitivity to zymolyase. The thicker outer layer of mannans in the *C. albicans* mutant may impede zymolyase the access to glucans but not CFG, making the mutant hypersensitive to CFG and only slightly sensitive to zymolyase.

Table 3.3. Polysaccharides cell wall composition of *C. albicans rlm1* mutant, wild-type and complemented strains.

	Chitin (% of the wall mass)	**Mannan** (µg/mg wall mass)	**Glucan** (µg/mg wall mass)
SC5314	17.8+1.2	71.34+3.3	189.67+3.6
SCRLM1M4A (rlm1Δ/rlm1Δ)	38.9+3.9	137.33+3.6	254.92+1.9
SCRLM1K2A (Δrlm1Δ/rlm1+RLM1Δ)	13.46+3.6	63.23+4.1	165.61+0.6

Subcellular localization of Rlm1P-GFP

To assess the cellular localization of Rlm1 in CAI4 *C. albicans* strain, we inserted the GFP-encoding gene in an in-frame fusion with *RLM1*, leading to the production of a fusion protein with GFP in the C-terminal of Rlm1. Two independent transformants with Rlm1-GFP fusion were grown to stationary phase on SC-uridine culture with CR (5µg/ml) and then assessed by fluorescence microscopy. Green fluorescence was present mainly in the nucleus (identified by DAPI staining), appearing also in the cytoplasm as granules (Fig. 3.8A). In order to increase sensitivity, the fusion protein was detected by indirect immunfluoresce. Detection of the Rlm1-GFP fusion protein with mouse anti-GFP by the anti-mouse IgG_{2a} Alexa-Flour 488 secondary antibody revealed the same pattern of subcellular localization (Fig. 3.8B). The nuclear localization is in accordance with a transcription factor function and the presence of cytoplasmic granules suggests that Rlm1 might be recruited to the nucleus when activated, as previously observed for *S. cerevisiae* (Jung *et al.*, 2002).

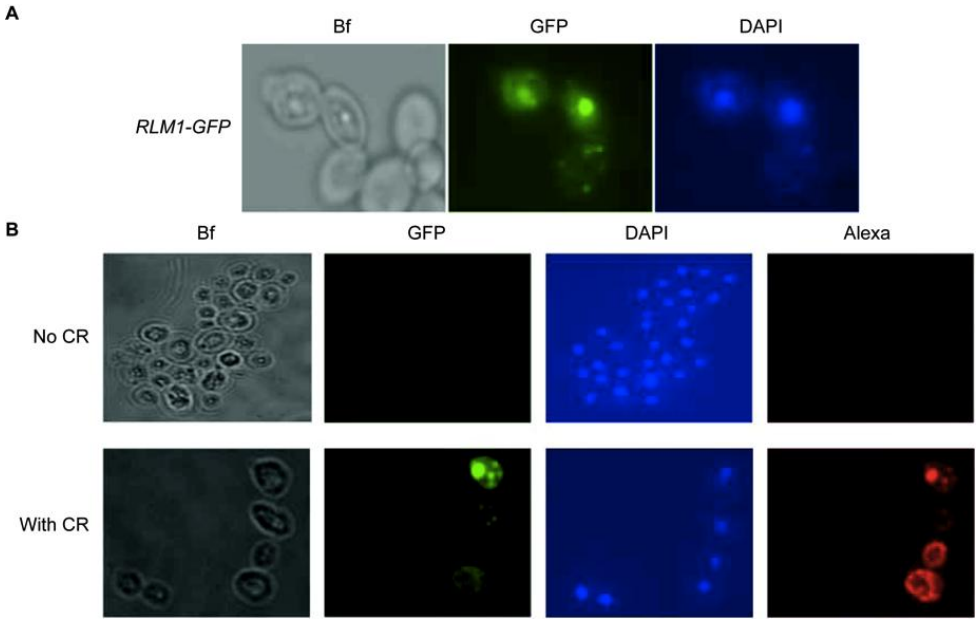

Figure 3.8. Subcellular localization of the Rlm1-GFP fusion protein in *C. albicans*. A) Cells of strain CAI4 bearing a chromosomally integrated *RLM1*-GFP cassette were grown to stationary phase in SC-uridine culture with 5µgCR/ml and then visualized by microscopy in bright field and for GFP and DAPI fluorescences. B) Cells from the same strain were grown under the conditions described above and fixed (in control experiments CR was omitted). Indirect immunodetection was performed using mouse anti-GFP antibody and anti-mouse IgG_{2a} Alexa-Flour 488–conjugated secondary antibody. Nuclear DNA was stained with DAPI. Bf: bright field microscopy.

Global gene expression profile

In order to identify genes whose expression was affected by deletion of *C. albicans RLM1* we performed gene expression profiling analyses on SC5314 and SCRLM1M4A, its *Δrlm1/rlm1Δ* mutant. Taking into consideration that microarray studies with *S. cerevisiae rlm1*Δ mutant (Becerra *et al.*, 2011) were performed in YPD exponential growth phase, the present gene expression analysis was performed under the same conditions. Other conditions, such as elevated temperature or the action of drugs can also activate this pathway but we have chosen growth on YPD to avoid activating general heat shock response or other general responses.

After filtering, the entire data set resulted in a total of 772 statistically significant differentially regulated ORFs (*P*-value <0.05). From those, the transcript profiles of genes differentially expressed by a factor of 2-fold were analyzed by function. The full data set was deposited in the ArrayExpress database from the European Bioinformatics Institute (www.ebi.ac.uk/arrayexpress) with the accession number E-MEXP-3247.

Deletion of *RLM1* in *C. albicans* changed the mRNA level of 101 genes with regulation ratios >2.0 (up-regulated and down-regulated; Appendix 1), which is far above the 20 genes identified for *S. cerevisiae rlm1Δ* mutant grown under the same conditions (Jung and Levin, 1999; Becerra *et al.*, 2011). Curiously, our data showed that more genes (63.4%) have elevated expression than reduced expression, which is exactly the opposite of what was observed for *S. cerevisiae* by Becerra *et al.* (2011) but similar to Jung and Levin (1999) results. Gene annotation and classification with the GO (Gene Ontology) terms for *C. albicans* was performed in Candida Database. The majority of the up-regulated genes (53.1%) have no known function. The remaining genes (corrected *P*-value <0.05) are involved in cell adhesion related to biofilm formation (*P*=0.019) and polyamine transport (*P*=0.037). Regarding down-regulated genes, 48.6% have unknown function, the ones that appeared with significant expression encode proteins involved in catalytic activity (43.2%), mainly oxidoreductase activity (10.8%, *P*= 0.026).

Table 3.4 highlights the genes with known function identified in this study. Genes with higher expression in the mutant than in the wild-type strain correspond to genes that are activated due to lack of Rlm1 and may be involved in a “compensatory mechanism” response (Lagorce *et al.*, 2003). In this study we observed that genes with the highest up-regulation encode for proteins involved in the cell wall organization and biofilm formation, *ALS1*, *ALS3*, *HWP1*, *ECE1* and *RBT1*, with *ECE1* showing an increase of around 76 fold. *PGA25* that encodes for a GPI-anchored protein member of the PGA family displayed also up-regulation in this study. Members of this family are frequently identified in studies involving induction of cell wall stress (Bruno *et al.*, 2006) and cell wall regeneration (Castillo *et al.*, 2006). Although we observed a two-fold increase in the cell wall chitin content of the mutant strain, in which the CHS enzyme family is responsible for the chitin synthesis, only *CHS7* was up-regulated in the mutant strain. However, it has been described that Chs7 is essential for Chs3 activity, the major chitin synthase of *C. albicans* cells (Sanz *et al.*, 2005). This result suggests that Chs7, directly or indirectly, enhance Chs3 activity resulting in the increase in cell wall chitin content observed in this study. Additionally, genes involved in the adaptation to osmotic stress were also up regulated *GCV2*, *CIT1*, *ENA21* as well as *HGT10*, which codes for a glycerol permease. This observation also indicates that the absence of Rlm1 can active genes osmotic stress response, reinforcing the role of *RLM1* in the cell wall maintenance.

Curiously, among the up-regulated genes identified with transporter activity, *AGP2* and *OPT6* are involved in the uptake of amino acids and oligopeptides, respectively. These, together with the action of secreted proteases (*SAP6*), which was also up-regulated, can contribute to the intake of

oligopeptides and amino acids. The up-regulation of *PUT2* and *GCV2*, whose products are involved in amino acid degradation, may suggest the utilization of alternative carbon or energy sources by *C. albicans* mutant cells. Other genes that could be involved in the interconnection of the pathways required to metabolize nonfermentable carbon sources, i.e. involved in the gluconeogenesis, the glyoxylate cycle, and beta-oxidation, are *CIT1*, coding for citrate synthase, *ACS1* an acetyl-CoA synthetase, and *SOU1* a sorbose redutase, which are also up-regulated. Interestingly, *DAK2*, *GLK4*, *GPD1*, *NTH1* and *TPS1*, which products are involved in carbohydrate catabolism appeared as down-regulated (Table 3.4). This observation agrees with the fact that carbohydrates are necessary as building blocks for cell wall remodeling. Gal4, one of the transcription factors known to be involved in the control of glycolytic enzymes in pathogenic species (Askew *et al.*, 2009), was also up-regulated in our analysis. These observations suggest that sugar metabolism is under tight control of Rlm1 and metabolic pathways for the utilization of alternative carbon and energy sources may be activated in the mutant cells.

Table 3.4. Selected Rlm1p regulated genes.

ORF	Gene name	*S.cerevisiae* ortholog	Function / Discription	Ratio mutant/ wild-type	Rlm1 binding sequence (location upstream gene sequence)
orf19.1228	*HAP2*	*HAP2*	Unknown / CCAAT-binding factor involved in low-iron response	3,0	TAWWWWTAGM (-919R)
orf19.1321	*HWP1*	--	Protein binding / Hyphal cell wall protein involved in host defense	37,4	TAWWWWTAGM (-938R)
orf19.1327	*RBT1*	--	Unknown / Cell wall protein with similarity to Hwp1	10,4	-
orf19.1569	*UTP22*	*UTP22*	Unknown/ Putative U3 snoRNP protein involved in rRNA processing	2,6	-
orf19.1743	*ACS1*	*ACS1*	Ligase activity / Putative acetyl-CoA synthetase	2,3	-
orf19.1816	*ALS3*	*SAG1*	Protein binding / Adhesin from the ALS family; role in epithelial adhesion, endothelial invasiveness	14,1	CTAWWWWTAG (-0 F;-10R) TAWWWWTAGM (-9 F;-1R)
orf19.2444	*CHS7*	*CHS7*	Protein binding / Protein required for wild-type chitin synthase III activity	2,7	-
orf19.2606	*HDA1*	*HDA1*	Hydrolase activity / Histone deacetylase	2,8	-
orf19.2896	*SOU1*	*SPS19*	Oxidoreductase activity / Enzyme involved in utilization of L-sorbose	6,3	-
orf19.3265	*TRM1*	*TRM1*	Transferase activity / Protein described as an N2,N2-dimethylguanine tRNA methyltransferase	2,8	-
orf19.3374	*ECE1*	--	Unknown / Hyphal-specific cell wall protein	76,0	TAWWWWTAGM (-145R)
orf19.3548.1	*WH11*	*HSP12*	Unknown / Protein expressed specifically in white phase yeast-form cells	5,4	TAWWWWTAGM (-96F;-781R)
orf19.385	*GCV2*	*GCV2*	Oxidoreductase activity / Glycine decarboxylase P subunit	3,7	TAWWWWTAGM (-213F)
orf19.3974	*PUT2*	*PUT2*	Oxidoreductase activity / delta-1-pyrroline-5-carboxylate dehydrogenase	2,2	-
orf19.3981	*MAL31*	*MAL31*	Transporter activity / Putative high-affinity maltose transporte	2,8	CTAWWWWTAG (-665F;-655R) TAWWWWTAGM (-566R;-897R)
orf19.4093	*PES1*	*NOP7*	Unknown / Pescadillo homolog required for filament-to-yeast switching	3,2	-
orf19.4211	*FET3*	*FET3*	Oxidoreductase activity / Multicopper oxidas	3,1	CTAWWWWTAG (-784F;-774R) TAWWWWTAGM (-5R)
orf19.4393	*CIT1*	*CIT1*	Transferase activity / Protein described as citrate synthase	3,9	-
orf19.4551	*CTN1*	*YAT1*	Transferase activity / Predicted carnitine acetyl transferase	4,9	TAWWWWTAGM (-799R)
orf19.4655	*OPT6*	*OPT2*	Transporter activity / Putative oligopeptide transporter	2,3	TAWWWWTAGM (-834F)
orf19.4679	*AGP2*	*AGP2*	Transporter activity / Protein described as an amino acid permease	3,8	-
orf19.473	*TPO4*	*TPO4*	Transporter activity / Putative sperimidine transporter	2,4	TAWWWWTAGM (-532R)

ORF	Gene name	*S.cerevisiae* ortholog	Function / Discription	Ratio mutant/ wild-type	Rlm1 binding sequence (location upstream gene sequence)
orf19.4815	*YTM1*	*YTM1*	Unknown / Protein similar to *S. cerevisiae* Ytm1, involved in biogenesis of the large ribosomal subunit	2,1	-
orf19.5071	*NRP1*	*NRP1*	Unknown / Nucleic acid binding	2,1	-
orf19.5110	*OPY2*	*OPY2*	Unknown / *S. cerevisiae* ortholog has role in osmosensory signaling pathway, cell cycle arrest	2,6	TAWWWWTAGM (-457F)
orf19.5170	*ENA21*	*ENA2*	Unknown / Similar to *S. cerevisiae* sodium transporters	2,5	-
orf19.5338	*GAL4*	*GAL4*	Transcription regulator activity / Transcription factor involved in control of glycolysis	2,3	-
orf19.5542	*SAP6*	*BAR1*	Hydrolase activity / Secreted aspartyl proteinase	3,2	-
orf19.5595	*SHE3*	--	RNA binding / mRNA-binding protein that localizes specific mRNAs to daughter yeast-form cells and to hyphal tips	2,3	TAWWWWTAGM (-546R)
orf19.5741	*ALS1*	*SAG1*	Peptide binding / Adhesin; ALS family of cell-surface glycoprotein	6,2	TAWWWWTAGM (-853R)
orf19.5753	*HGT10*	*STL1*	Transporter activity / Glycerol permease involved in glycerol uptake induced by osmotic stress, during cell wall regeneration	13,0	TAWWWWTAGM (-204F;-976R)
orf19.6078	*POL93*	*YIL080W*	Unknown / nucleic acid binding	7,7	-
orf19.6139	*FRE7*	*FRE3*	Unknown / Protein similar to ferric reductase Fre10	3,2	-
orf19.6169	*ATO1*	*ATO2*	Unknown / Putative fungal-specific transmembrane protein	4,6	-
orf19.6336	*PGA25*	--	Unknown / Putative GPI-anchored protein	8,0	TAWWWWTAGM (-316F)
orf19.651	*LYP1*	*LYP1*	Unknown / Putative permease, animo acid transmembrane transporter	2,4	-
orf19.6514	*CUP9*	*CUP9*	Unknown / sequence specific DNA binding	2,1	-
orf19.6577	*FLU1*	*TPO1*	Transporter activity / Multidrug efflux pump of the plasma membrane	2,2	TAWWWWTAGM (-840F)
orf19.6948	*CCC1*	*CCC1*	Transporter activity / Putative manganese transporter	2,5	TAWWWWTAGM (-209R)
orf19.918	*CDR11*	*PDR5*	Transporter activity / Putative transporter of PDR subfamily of ABC family	2,1	-
orf19.2525	*LYS12*	*LYS12*	Oxidoreductase activity / mitochondrial homoisocitrate dehydrogenase	-20,1	-
orf19.1868	*RNR22*	*RNR2*	Unknown / ribonucleoside diphosphate reductase	-3,4	-
orf19.7600	*FDH3*	*SFA1*	Oxidoreductase activity / Putative protein of glycine catabolism	-2,9	-
orf19.866	*RAD32*	*RAD30*	Nucleotidyl transferase activity / Protein similar to *S. cerevisiae* protein with role in nucleotide excision repair	-2,9	-
orf19.3749	*IFC3*	*OPT2*	Transporter activity / Oligopeptide transporter	-2,7	-
orf19.2770.1	*SOD1*	*SOD1*	Oxidoreductase activity / Cytosolic copper- and zinc-containing superoxide dismutase	-2,6	-
orf19.1756	*GPD1*	*GPD1*	Oxidoreductase activity / Glycerol-3-phosphate dehydrogenase (enzyme of glycerol biosynthesis)	-2,5	-
orf19.5248	*MSO1*	--	Unknown / Unknown	-2,5	-
orf19.4777	*DAK2*	*DAK2*	Unknown / Dihydroxyacetone kinase	-2,4	-
orf19.4664	*NAT4*	*NAT4*	Transferase activity / Histone acetyltransferase	-2,4	-
orf19.5025	*MET3*	*MET3*	Transferase activity / ATP sulfurlyase of sulfate assimilation	-2,4	-
orf19.6116	*GLK4*	*GLK1*	Hexokinase activity / Glucokinase	-2,3	TAWWWWTAGM (-9R)
orf19.2341	*HNT1*	*HNT1*	Hydrolase activity / Protein kinase C inhibitor-I	-2,3	-
orf19.6640	*TPS1*	*TPS1*	Transferase activity / Trehalose-6-phosphate synthase	-2,3	-
orf19.5228	*RIB3*	*RIB3*	Lyase activity / 3,4-Dihydroxy-2-butanone 4-phosphate synthase	-2,3	TAWWWWTAGM (-756R)
orf19.5001	*CUP2*	*HAA1*	Unknown/ Protein required for normal resistance to copper	-2,2	TAWWWWTAGM (-593R)
orf19.7479	*NTH1*	*NTH1*	Hydrolase activity / Neutral trehalase	-2,1	-
orf19.5000	*CYB2*	*CYB2*	Oxidoredutase activity / Cytochrome b2 precursor protein	-2,1	-
orf19.3359	*ARP8*	*ARP8*	Hydrolase activity / Chromatin-remodeling enzyme complex protein	-2,1	-

Adhesion and biofilm formation

Since the proteins that showed higher up-regulation in the *rlm1Δ/rlm1Δ* mutant are involved in adhesion and biofilm formation, *C. albicans* strains were tested regarding their ability to adhere to a polystyrene surface (within 2h) and to form biofilm after 24h and 48h (Fig. 3.9A and 3.9B). *Candida albicans rlm1Δ/rlm1Δ* showed a higher ability to adhere to polystyrene surface, presenting a higher number of cells compared to the WT strain (P<0.05). However, no differences in biomass production were observed in the adhesion process (Fig. 3.9A, 2h). Regarding biofilm formation, a clear difference (P<0.05) in the total number of CFUs as well as total biomass formation was observed between the mutant and the WT strain. Although the number of CFUs did not change between 24h and 48h, the total biomass still increased in both strains with the mutant showing significant (P<0.05) higher amount of total biomass than the WT. The complemented strain, SCRLM1K2A, showed once more the common phenotype of partial complementation, particularly regarding CFUs counting. The amount of total biomass produced at 48h by the complemented strain was similar to the WT, but no significant difference (P>0.05) was observed in comparison with the mutant strain. No phenotypic differences between the WT and mutant cells were observed when grown in biofilm (Fig. 3.9C). Together, these results indicate that Rlm1 acts as a negative regulator of in vitro biofilm formation.

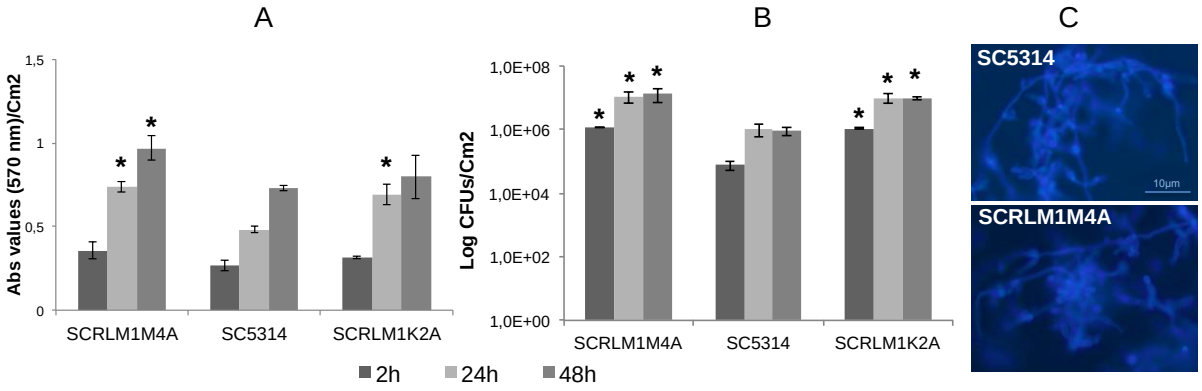

Figure 3.9. *In vitro* adhesion and biofilm formation. *Candida albicans* cells from SC5314 (*RLM1*/*RLM1*), SCRLM1M4A (*rlm1Δ*/*rlm1Δ*) and complemented strain SCRLM1K2A (*rlm1Δ*/*rlm1Δ*+*RLM1*) were allowed to adhere (2h) and to form biofilm (24h and 48h) in polystyrene. Total biomass (A) was then assed by crystal violet staining and viable cells (B) by CFUs counting. (C) Morphology of SC5314 and SCRLM1M4A cells after 24h biofilm growth stained with CFW and visualized under the fluorescence microscope. The symbol * indicates that measurements were significantly different (P<0.005) from the WT strain.

***Candida albicans* genome contains two MADS-box transcription factors while *S. cerevisiae* has four**

Our search in several databases indicated the presence of two MADS-box proteins in *C.albicans* (Rlm1 and Mcm1), and in other fungi belonging to subphylum Saccharomycotina (*Kluyveromyces lactis, C. glabrata, S. cerevisiae*) and Pezizomycotina (*Aspergillus nidulans, Aspergillus niger, Magnaporte grisea*). However, *S. cerevisiae* and *C. glabrata* presented two other MADS-box proteins, Arg80 and Smp1 besides Rlm1 and Mcm1. These additional MADS-box proteins could have arise as consequence of the genome duplication process, since this event has been reported to happened after divergence of *K.lactis* and *S.cerevisiae* (Wolfe and Shields, 1997; Kellis *et al.*, 2004).

In order to evaluate the proximity of *C. albicans* Rlm1 with the other MADS-box transcription factors from closely related species, protein sequence alignment and phylogenetic analysis for the selected species was performed (Fig. 3.10).

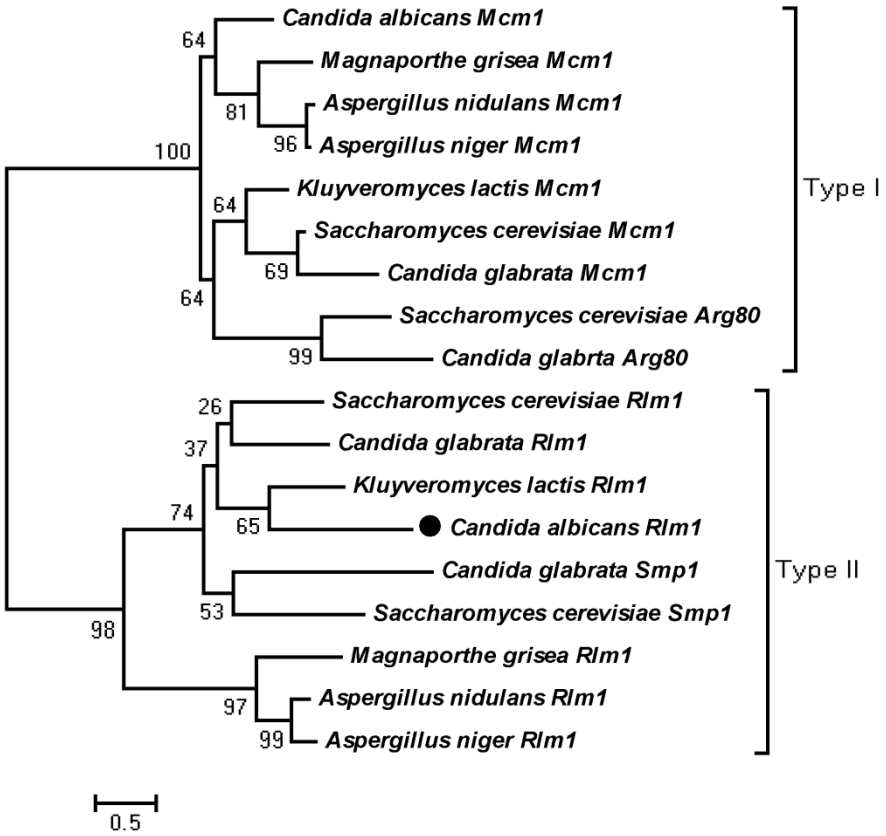

Figure 3.10. Phylogenetic relationship of MADS-box type I and type II proteins from selected fungal species. This phylogeny was obtained by maximum likelihood with a nodal support estimated by 1000 bootstrap analysis.

This analyses clearly showed that fungal MADS-box protein are grouped in two groups, according to the position of MADS-box within protein sequence (type I or type II) (Alvarez-Buylla *et al.*, 2000). The yeast MADS-box sequences present low similarity with their filamentous fungi orthologues, clustering in distinct subgroups, except for *C. albicans* Mcm1 that grouped within the filamentous fungi cluster. *Candida albicans* Rlm1 grouped within the yeast type II cluster, closer to *K. lactis* Rlm1 orthologue. In view of this result we believe that Rlm1 from *C. albicans* would behave similarly to its *K. lactis* and *S. cerevisiae* orthologues rather than to the filamentous fungi.

Discussion

Candida albicans RLM1 is required for cell wall remodeling

In *S. cerevisiae* the signaling pathway responsible for the CWI is the Slt2 MAP kinase pathway, in which the transcription factor Rlm1 plays a key role in the regulation of genes involved in the maintenance of integrity and cell wall biosynthesis (Dodou and Treisman, 1997; Watanabe *et al.*, 1997). In *C. albicans* this role is accomplished through the functionally and structurally homologous Mkc1 MAP kinase pathway (Navarro-Garcia *et al.*, 1995), but the involvement of Rlm1 in CWI maintenance has not been completely clarified (Bruno *et al.*, 2006).

The analysis of *C. albicans rlm1Δ/rlm1Δ* mutant in the presence of compounds that affect the cell wall, showed a hypersensitivity to CFW, CFG and CR. The hypersensitivity to CFW has been reported to be associated with an increase in the cell wall chitin content, after activation of the so-called "compensatory mechanism" in response to the weakening of the cell wall (Popolo and Vai, 1999; Selvaggini *et al.*, 2004; Sanz *et al.*, 2005). In this study, *C. albicans rlm1Δ/rlm1Δ* mutant strain showed a 2-fold increase in the cell wall chitin content in comparison with the wild-type strain. In the microarray analysis, we did not find an over-expression of the genes directly responsible for chitin synthesis, such as *CHS1*, *CHS2*, *CHS3*, or *CHS8* (Mio *et al.*, 1996; Munro *et al.*, 2003; Castillo *et al.*, 2006) but we found a 2.7 fold over-expression of *CHS7* (Chs7p) which is required for wild-type Chs3 activity, the major chitin synthase of *C. albicans* cells (Sanz *et al.*, 2005). This observation suggests that the increase in cell wall chitin content observed in this analysis, that is responsible for the CFW hypersensitivity, could be due to Chs7 enhancing, directly or indirectly, Chs3 activity.

The hypersensitivity to CR, which interferes with the assembly of β-1,3-glucan, and to CFG, an inhibitor of β-1,3-glucan synthase, may suggest an alteration in the composition of the glucans in the cell wall of *C. albicans rlm1Δ/rlm1Δ* mutant. However, no significant differences in resistance to zymolyase or in the total amount of cell wall glucans was observed in the mutant strain in comparison with the wild-type. Changes in the association between the polysaccharides, particularly the amount of mannoproteins, have also been described in response to cell wall weakening (Lagorce *et al.*, 2003). Probably this is the case for *C. albicans rlm1Δ/rlm1Δ* since the total amount of glucans was not significantly altered but a two-fold increase in the cell wall mannans was observed in the mutant in comparison with the wild-type, suggesting an increase in the amount of mannoproteins in the cell wall surface. The thicker outer layer of mannans in *C. albicans* mutant may make difficult the access of zymolyase to glucans but not CFG, rendering the mutant hypersensitive to CFG and slightly sensitive to zymolyase. Microarray analysis also pointed towards this hypothesis, showing a significant increase of transcripts of cell wall proteins. The genes with the highest up-regulation in the mutant are *ALS1*, *ALS3*, *HWP1*, *RBT1* and *ECE1,* which are directly involved in the cell wall organization. These results, together with the osmoremediation observed with CFG, indicates that *C. albicans RLM1* is involved in cell wall remodelling. This regulatory role is further evidenced by the nuclear and cytoplasmic granular localization of Rlm1-GFP fusion protein observed in this study, suggesting that Rlm1 might be recruited to the nucleus when activated. This pattern of cellular localization agrees with data obtained

in *S. cerevisiae* (Jung *et al.*, 2002) and further supports a functional homology between *C. albicans* and *S. cerevisiae* Rlm1.

Role of *RLM1* in response to cell wall damage is different in *S. cerevisiae* and *C. albicans*

Candida albicans Rlm1 is a putative transcription factor from the MADS-Box family whose function has been attributed mainly based on the knowledge from the *S. cerevisiae* orthologue (Reinoso-Martin *et al.*, 2003; Levin, 2005). This is supported by the fact that 74% of *C. albicans* genes are homologous to genes in *S. cerevisiae* (Jones *et al.*, 2004). The homology between *RLM1* from *S. cerevisiae* and *C. albicans* and phenotypes associated with cell wall defects of the correspondent null mutants, strongly suggest that both genes are functionally homologues (Bruno *et al.*, 2006).

Deletions of several *S. cerevisiae* genes involved in the cell wall integrity pathway such as *BCK1*, *MKK1, MKK2* or *SLT2/MPK1* confer typical phenotypes such as, failure to grow at elevated temperatures in the absence of an osmostabilizer; sensitivity to caffeine in the medium; failure to grow on glycerol medium; and sensitivity to nitrogen starvation (Lee and Levin, 1992; Irie *et al.*, 1993; Lee *et al.*, 1993; Martin *et al.*, 1993; Costigan and Snyder, 1994). However, unlike PKC pathway mutants, the *S. cerevisiae rlm1Δ* mutant appears to be able to grow normally at elevated temperatures (38ºC), grows on glycerol medium and is not sensitive to nitrogen starvation. In *C. albicans*, the only gene involved in the cell wall integrity pathway that has been extensively characterized is *MKC*1 (*SLT2* in *S. cerevisiae*) (Navarro-Garcia *et al.*, 1995; Navarro-Garcia *et al.*, 1998). *Candida albicans mkc1Δ/mkc1Δ* deficient cells also showed temperature sensitivity, reduced thermotolerance and caffeine sensitivity. But, contrary to its *S. cerevisiae* orthologue, *C. albicans mkc1Δ/mkc1Δ* Ura^+ mutant cells survived nitrogen starvation and were also able to grow on glycerol medium. In this study, *C. albicans rlm1Δ/rlm1Δ* mutant showed reduced thermotolerance, no sensitivity to caffeine, ability to grow on glycerol and resistance to nitrogen starvation. Recently, the *rlm1Δ* as well as the *slt2Δ* mutants from *C. glabrata* have been studied and results showed that they present temperature sensitivity in the absence of an osmostabilizer (Miyazaki *et al.*, 2010). We may infer from these results that the function of *RLM1* in *C. albicans* and *S. cerevisiae* differ in response to temperature and caffeine. Additionally, the *S. cerevisiae rlm1*Δ mutant was not affected by the presence of CR and CFG, was more resistant to CFW, and unlike *C. albicans* mutant*, S. cerevisiae rlm1*Δ was more sensitive to SDS. The whole-genome duplication event, may explain the fact that *S. cerevisiae* has four MADS-Box transcriptions factor while *C. albicans* only two, which suggests that some cellular functions in *C. albicans* may remain in a transcription factor while in *S. cerevisiae* may be performed by the duplicated copy.

***RLM1*-responsive genes**

In this study, we analyzed gene expression of *C. albicans rlm1Δ/rlm1Δ* mutant in cells growing in YPD, rather than under any cell wall stress condition such as in previous works (Bruno *et al.*, 2006). Analysis of genes with expression ratio mutant/wild-type showed a significant (corrected *P*-value < 0.05) up-regulation of genes involved in cell adhesion related to biofilm formation (*P*=0.019) and

polyamine transport (*P*=0.037). Additionally, the genes that revealed the highest up-regulation were *ALS1*, *ALS3*, *HWP1*, *RBT1* and *ECE1,* which are involved in the cell wall organization. Another important observation was that genes involved in the metabolism of carbohydrates such as *DAK2*, *GLK4*, *GPD1*, *NTH1* and *TPS1* were down-regulated. The products of these genes are involved in the several pathways that control the utilization of glucose, thus we believe that the metabolism of *C. albicans* cells without a functional *RLM1* could be rearranged so that glucose is deviated from utilization as energy source to utilization as building blocks for cell wall. In fact, the microarray analysis identified *AGP2*, *OPT6* and *SAP6* as up-regulated genes, which together contribute to the intake of oligopeptides and amino acids, suggesting the utilization of alternative carbon/energy sources. Curiously, *GAL4* was identified has an up-regulated gene in this study, which indicates that regulation of glycolytic enzymes could be occurring in *C. albicans rlm1*Δ/*rlm1*Δ mutant. Additionally, it has been reported that fungal species lacking *GCR1*/2 homologs, such as *C. albicans*, have an enrichment of the Gal4 motif in the promoter regions of glycolytic genes (Askew *et al.*, 2009).

These observations showed that *C. albicans* mutant appears to rearrange the metabolic pathways so that glucose is deviated from utilization as energy source, being more available for use as cell wall building blocks, as well as increase cell wall protein involved in adhesion. As a consequence the association between β-glucan, mannoproteins, and chitin would certainly change. Additionally, we identified several genes involved in stress response, particularly genes involved in the adaptation to osmotic stress and that are regulated by Hog1 such as *GCV2*, *ECE1*, *CIT1*, *ENA21* and predominantly *HGT10*, which codes for a glycerol permease with an up-regulation of more than ten fold.

As discussed above, we concluded that *RLM1* is important for the cell wall biogenesis. Furthermore, a novel role for *RLM1* that was not described for *S. cerevisiae* was also detected. We observed that the absence of *RLM1* significantly activated genes involved in cell adhesion and biofilm formation. Blankenship *et al.* (2010) had already observed novel functions in *C. albicans* conserved PKs compared to their orthologs in *S. cerevisiae* and *Schizosaccharomyces pombe* when studying cell wall remodeling, which included functions related to filamentation and biofilm formation. Despite the fact that our results do not point to a direct involvement of *RLM1* in *C. albicans* filamentation an increase in proteins involved in adhesion and biofilm formation was clearly observed. Nobile *et al.* (2009) described Zap1/Csr1 as a negative regulator of biofilm formation and in a previous study Nobile and Mitchell (2006) identified Bcr1 as a transcription factor that governs biofilm formation in an *in vitro* catheter model. In this study, Rlm1 behaved as a negative regulator of biofilm in an *in vitro* polysteryne biofilm model, and the *rlm1*Δ mutant strain presented overexpression of *ALS1*, *ALS3*, *HWP1*, *RBT1* and *ECE1,* which includes the same Bcr1 targets (*ALS1*, *ECE1*, and *HWP1*), suggesting that *BCR1* activates genes directly involved in biofilm formation while *RLM1* regulates negatively the same set of genes. Overall, with this study we were able to demonstrate the role of *C. albicans RLM1* in cell wall remodeling and to show that, besides this important role, *RLM1* presents extended functions in negatively regulating biofilm formation.

Acknowledgments

We thank Cheryl Gale, Fonzi and EUROSCARF for the pGFP-URA3; the strain CAI4; and BY4741 and YPL089c strains from *S. cerevisiae,* respectively. We thank Sandra Tenreiro and Inês Mendes for help with the immunofluorescence and operating the HPLC, respectively. This study was supported by FEDER through POFC-COMPETE and by national funds from FCT through the project PEst-C/BIA/UI4050/201. Yolanda Delgado was supported by PhD fellowships from Nº E07D400922PE, The European Union Programme of High Level Scholarships for Latin America, ALβAN.

Rererences

Abascal, F., R. Zardoya and D. Posada (2005). "ProtTest: selection of best-fit models of protein evolution." *Bioinformatics* 21(9): 2104-2105.

Aguilar-Uscanga, B. and J. M. Francois (2003). "A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation." *Lett Appl Microbiol* 37(3): 268-274.

Alvarez-Buylla, E. R., S. Pelaz, S. J. Liljegren, S. E. Gold, C. Burgeff, G. S. Ditta, L. Ribas de Pouplana, L. Martinez-Castilla and M. F. Yanofsky (2000). "An ancestral MADS-box gene duplication occurred before the divergence of plants and animals." *Proc Natl Acad Sci U S A* 97(10): 5328-5333.

Askew, C., A. Sellam, E. Epp, H. Hogues, A. Mullick, A. Nantel and M. Whiteway (2009). "Transcriptional regulation of carbohydrate metabolism in the human pathogen *Candida albicans*." *PLoS Pathog* 5(10): e1000612.

Ausubel, F. M. e. (1993). "Current protocols in molecular biology." *2nd ed. Wiley, New York, NY*.

Becerra, M., L. J. Lombardia, M. Lamas-Maceiras, E. Canto, E. Rodriguez-Belmonte and M. E. Cerdan (2011). "Comparative transcriptome analysis of yeast strains carrying slt2, rlm1, and pop2 deletions." *Genome* 54(2): 99-109.

Blankenship, J. R., S. Fanning, J. J. Hamaker and A. P. Mitchell (2010). "An extensive circuitry for cell wall regulation in *Candida albicans*." *PLoS Pathog* 6(2): e1000752.

Brachmann, C. B., A. Davies, G. J. Cost, E. Caputo, J. Li, P. Hieter and J. D. Boeke (1998). "Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications." *Yeast* 14(2): 115-132.

Bruno, V. M., S. Kalachikov, R. Subaran, C. J. Nobile, C. Kyratsous and A. P. Mitchell (2006). "Control of the *C. albicans* cell wall damage response by transcriptional regulator Cas5." *PLoS Pathog* 2(3): e21.

Cabib, E. and A. Duran (2005). "Synthase III-dependent chitin is bound to different acceptors depending on location on the cell wall of budding yeast." *J Biol Chem* 280(10): 9170-9179.

Carmona-Saez, P., M. Chagoyen, F. Tirado, J. M. Carazo and A. Pascual-Montano (2007). "GENECODIS: a web-based tool for finding significant concurrent annotations in gene lists." *Genome Biol* 8(1): R3.

Castillo, L., A. I. Martinez, A. Garcera, J. Garcia-Martinez, J. Ruiz-Herrera, E. Valentin and R. Sentandreu (2006). "Genomic response programs of *Candida albicans* following protoplasting and regeneration." *Fungal Genet Biol* 43(2): 124-134.

Costigan, C. and M. Snyder (1994). "SLK1, a yeast homolog of MAP kinase activators, has a RAS/cAMP-independent role in nutrient sensing." *Mol Gen Genet* 243(3): 286-296.

Davenport, K. R., M. Sohaskey, Y. Kamada, D. E. Levin and M. C. Gustin (1995). "A second osmosensing signal transduction pathway in yeast. Hypotonic shock activates the PKC1 protein kinase-regulated cell integrity pathway." *J Biol Chem* 270(50): 30157-30161.

Diez-Orejas, R., G. Molero, F. Navarro-Garcia, J. Pla, C. Nombela and M. Sanchez-Perez (1997). "Reduced virulence of *Candida albicans MKC1* mutants: a role for mitogen-activated protein kinase in pathogenesis." *Infect Immun* 65(2): 833-837.

Dodou, E. and R. Treisman (1997). "The *Saccharomyces cerev*isiae MADS-box transcription factor Rlm1 is a target for the Mpk1 mitogen-activated protein kinase pathway." *Mol Cell Biol* 17(4): 1848-1859.

Edgar, R. C. (2004). "MUSCLE: multiple sequence alignment with high accuracy and high throughput." *Nucleic Acids Res* 32(5): 1792-1797.

Fonzi, W. A. and M. Y. Irwin (1993). "Isogenic strain construction and gene mapping in *Candida albicans.*" *Genetics* 134(3): 717-728.

Francois, J. M. (2006). "A simple method for quantitative determination of polysaccharides in fungal cell walls." *Nat Protoc* 1(6): 2995-3000.

Garcia, R., C. Bermejo, C. Grau, R. Perez, J. M. Rodriguez-Pena, J. Francois, C. Nombela and J. Arroyo (2004). "The global transcriptional response to transient cell wall damage in *Saccharomyces cerevisiae* and its regulation by the cell integrity signaling pathway." *J Biol Chem* 279(15): 15183-15195.

Gerami-Nejad, M., J. Berman and C. A. Gale (2001). "Cassettes for PCR-mediated construction of green, yellow, and cyan fluorescent protein fusions in *Candida albicans*." *Yeast* 18(9): 859-864.

Gillum, A. M., E. Y. Tsay and D. R. Kirsch (1984). "Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae* ura3 and E. coli pyrF mutations." *Mol Gen Genet* 198(1): 179-182.

Guindon, S., J. F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk and O. Gascuel (2010). "New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0." *Syst Biol* 59(3): 307-321.

Irie, K., M. Takase, K. S. Lee, D. E. Levin, H. Araki, K. Matsumoto and Y. Oshima (1993). "*MKK1* and *MKK2*, which encode *Saccharomyces cerevisiae* mitogen-activated protein kinase-kinase homologs, function in the pathway mediated by protein kinase C." *Mol Cell Biol* 13(5): 3076-3083.

Jones, T., N. A. Federspiel, H. Chibana, J. Dungan, S. Kalman, B. B. Magee, G. Newport, Y. R. Thorstenson, N. Agabian, P. T. Magee, R. W. Davis and S. Scherer (2004). "The diploid genome sequence of *Candida albicans*." *Proc Natl Acad Sci U S A* 101(19): 7329-7334.

Jung, U. S. and D. E. Levin (1999). "Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway." *Mol Microbiol* 34(5): 1049-1057.

Jung, U. S., A. K. Sobering, M. J. Romeo and D. E. Levin (2002). "Regulation of the yeast Rlm1 transcription factor by the Mpk1 cell wall integrity MAP kinase." *Mol Microbiol* 46(3): 781-789.

Kellis, M., B. W. Birren and E. S. Lander (2004). "Proof and evolutionary analysis of ancient genome duplication in the yeast *Saccharomyces cerevisiae*." *Nature* 428(6983): 617-624.

Klis, F. M., A. Boorsma and P. W. De Groot (2006). "Cell wall construction in *Saccharomyces cerevisiae*." *Yeast* 23(3): 185-202.

Kohler, G. A., T. C. White and N. Agabian (1997). "Overexpression of a cloned IMP dehydrogenase gene of *Candida albicans* confers resistance to the specific inhibitor mycophenolic acid." *J Bacteriol* 179(7): 2331-2338.

Lagorce, A., N. C. Hauser, D. Labourdette, C. Rodriguez, H. Martin-Yken, J. Arroyo, J. D. Hoheisel and J. Francois (2003). "Genome-wide analysis of the response to cell wall mutations in the yeast *Saccharomyces cerevisiae*." *J Biol Chem* 278(22): 20345-20357.

Lee, K. S., K. Irie, Y. Gotoh, Y. Watanabe, H. Araki, E. Nishida, K. Matsumoto and D. E. Levin (1993). "A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C." *Mol Cell Biol* 13(5): 3067-3075.

Lee, K. S. and D. E. Levin (1992). "Dominant mutations in a gene encoding a putative protein kinase (*BCK1*) bypass the requirement for a *Saccharomyces cerevisiae* protein kinase C homolog." *Mol Cell Biol* 12(1): 172-182.

Levin, D. E. (2005). "Cell wall integrity signaling in *Saccharomyces cerevisiae*." *Microbiol Mol Biol Rev* 69(2): 262-291.

Lipke, P. N. and R. Ovalle (1998). "Cell wall architecture in yeast: new structure and new challenges." *J Bacteriol* 180(15): 3735-3740.

Martin, H., J. Arroyo, M. Sanchez, M. Molina and C. Nombela (1993). "Activity of the yeast MAP kinase homologue Slt2 is critically required for cell integrity at 37 degrees C." *Mol Gen Genet* 241(1-2): 177-184.

Martinez-Lopez, R., L. Monteoliva, R. Diez-Orejas, C. Nombela and C. Gil (2004). "The GPI-anchored protein CaEcm33p is required for cell wall integrity, morphogenesis and virulence in *Candida albicans*." *Microbiology* 150(Pt 10): 3341-3354.

Millon, L., A. Manteaux, G. Reboux, C. Drobacheff, M. Monod, T. Barale and Y. Michel-Briand (1994). "Fluconazole-resistant recurrent oral candidiasis in human immunodeficiency virus-positive patients: persistence of *Candida albicans* strains with the same genotype." *J Clin Microbiol* 32(4): 1115-1118.

Mio, T., T. Yabe, M. Sudoh, Y. Satoh, T. Nakajima, M. Arisawa and H. Yamada-Okabe (1996). "Role of three chitin synthase genes in the growth of *Candida albicans*." *J Bacteriol* 178(8): 2416-2419.

Miyazaki, T., T. Inamine, S. Yamauchi, Y. Nagayoshi, T. Saijo, K. Izumikawa, M. Seki, H. Kakeya, Y. Yamamoto, K. Yanagihara, Y. Miyazaki and S. Kohno (2010). "Role of the Slt2 mitogen-activated protein kinase pathway in cell wall integrity and virulence in *Candida glabrata*." *FEMS Yeast Res* 10(3): 343-352.

Munro, C. A., R. K. Whitton, H. B. Hughes, M. Rella, S. Selvaggini and N. A. Gow (2003). "*CHS8*-a fourth chitin synthase gene of *Candida albicans* contributes to in vitro chitin synthase activity, but is dispensable for growth." *Fungal Genet Biol* 40(2): 146-158.

Nasmyth, K. and L. Dirick (1991). "The role of *SWI4* and *SWI6* in the activity of G1 cyclins in yeast." *Cell* 66(5): 995-1013.

Navarro-Garcia, F., R. Alonso-Monge, H. Rico, J. Pla, R. Sentandreu and C. Nombela (1998). "A role for the MAP kinase gene *MKC1* in cell wall construction and morphological transitions in *Candida albicans*." *Microbiology* 144 (Pt 2): 411-424.

Navarro-Garcia, F., B. Eisman, S. M. Fiuza, C. Nombela and J. Pla (2005). "The MAP kinase Mkc1p is activated under different stress conditions in *Candida albicans*." *Microbiology* 151(Pt 8): 2737-2749.

Navarro-Garcia, F., M. Sanchez, J. Pla and C. Nombela (1995). "Functional characterization of the *MKC1* gene of *Candida albicans*, which encodes a mitogen-activated protein kinase homolog related to cell integrity." *Mol Cell Biol* 15(4): 2197-2206.

Nobile, C. J. and A. P. Mitchell (2006). "Genetics and genomics of *Candida albicans* biofilm formation." *Cell Microbiol* 8(9): 1382-1391.

Nobile, C. J., J. E. Nett, A. D. Hernday, O. R. Homann, J. S. Deneault, A. Nantel, D. R. Andes, A. D. Johnson and A. P. Mitchell (2009). "Biofilm matrix regulation by *Candida albicans* Zap1." *PLoS Biol* 7(6): e1000133.

Paravicini, G., A. Mendoza, B. Antonsson, M. Cooper, C. Losberger and M. A. Payton (1996). "The *Candida albicans PKC1* gene encodes a protein kinase C homolog necessary for cellular integrity but not dimorphism." *Yeast* 12(8): 741-756.

Popolo, L. and M. Vai (1999). "The Gas1 glycoprotein, a putative wall polymer cross-linker." *Biochim Biophys Acta* 1426(2): 385-400.

Reinoso-Martin, C., C. Schuller, M. Schuetzer-Muehlbauer and K. Kuchler (2003). "The yeast protein kinase C cell integrity pathway mediates tolerance to the antifungal drug caspofungin through activation of Slt2p mitogen-activated protein kinase signaling." *Eukaryot Cell* 2(6): 1200-1210.

Reuss, O., A. Vik, R. Kolter and J. Morschhauser (2004). "The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*." *Gene* 341: 119-127.

Sampaio, P., M. Santos, A. Correia, F. E. Amaral, J. Chavez-Galarza, S. Costa-de-Oliveira, A. G. Castro, J. Pedrosa and C. Pais (2010). "Virulence attenuation of *Candida albicans* genetic variants isolated from a patient with a recurrent bloodstream infection." *PLoS One* 5(4): e10155.

Sanz, M., L. Carrano, C. Jimenez, G. Candiani, J. A. Trilla, A. Duran and C. Roncero (2005). "*Candida albicans* strains deficient in *CHS7*, a key regulator of chitin synthase III, exhibit morphogenetic alterations and attenuated virulence." *Microbiology* 151(Pt 8): 2623-2636.

Sasse, C., R. Schillig, F. Dierolf, M. Weyler, S. Schneider, S. Mogavero, P. D. Rogers and J. Morschhauser (2011). "The transcription factor Ndt80 does not contribute to Mrr1-, Tac1-, and Upc2-mediated fluconazole resistance in *Candida albicans*." *PLoS One* 6(9): e25623.

Schwarz-Sommer, Z., P. Huijser, W. Nacken, H. Saedler and H. Sommer (1990). "Genetic Control of Flower Development by Homeotic Genes in Antirrhinum majus." *Science* 250(4983): 931-936.

Selvaggini, S., C. A. Munro, S. Paschoud, D. Sanglard and N. A. Gow (2004). "Independent regulation of chitin synthase and chitinase activity in *Candida albicans* and *Saccharomyces cerevisiae*." *Microbiology* 150(Pt 4): 921-928.

Shaw, J. A., P. C. Mol, B. Bowers, S. J. Silverman, M. H. Valdivieso, A. Duran and E. Cabib (1991). "The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle." *J Cell Biol* 114(1): 111-123.

Silva, S., M. Henriques, A. Martins, R. Oliveira, D. Williams and J. Azeredo (2009). "Biofilms of non-Candida albicans Candida species: quantification, structure and matrix composition." *Med Mycol* 47(7): 681-689.

Silva, S., M. Henriques, R. Oliveira, D. Williams and J. Azeredo (2010). "In vitro biofilm activity of non-Candida albicans Candida species." *Curr Microbiol* 61(6): 534-540.

Staleva, L., A. Hall and S. J. Orlow (2004). "Oxidative stress activates FUS1 and RLM1 transcription in the yeast *Saccharomyces cerevisiae* in an oxidant-dependent Manner." *Mol Biol Cell* 15(12): 5574-5582.

Stepanovic, S., D. Vukovic, I. Dakic, B. Savic and M. Svabic-Vlahovic (2000). "A modified microtiter-plate test for quantification of staphylococcal biofilm formation." *J Microbiol Methods* 40(2): 175-179.

Tamura, K., J. Dudley, M. Nei and S. Kumar (2007). "MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0." *Mol Biol Evol* 24(8): 1596-1599.

Toda, T., I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broek, S. Cameron, J. Broach, K. Matsumoto and M. Wigler (1985). "In yeast, RAS proteins are controlling elements of adenylate cyclase." *Cell* 40(1): 27-36.

van de Peppel, J., P. Kemmeren, H. van Bakel, M. Radonjic, D. van Leenen and F. C. Holstege (2003). "Monitoring global messenger RNA changes in externally controlled microarray experiments." *EMBO Rep* 4(4): 387-393.

van der Vaart, J. M., L. H. Caro, J. W. Chapman, F. M. Klis and C. T. Verrips (1995). "Identification of three mannoproteins in the cell wall of *Saccharomyces cerevisiae*." *J Bacteriol* 177(11): 3104-3110.

Vilella, F., E. Herrero, J. Torres and M. A. de la Torre-Ruiz (2005). "Pkc1 and the upstream elements of the cell integrity pathway in *Saccharomyces cerevisiae*, Rom2 and Mtl1, are required for cellular responses to oxidative stress." *J Biol Chem* 280(10): 9149-9159.

Watanabe, Y., G. Takaesu, M. Hagiwara, K. Irie and K. Matsumoto (1997). "Characterization of a serum response factor-like protein in *Saccharomyces cerevisiae*, Rlm1, which has transcriptional activity regulated by the Mpk1 (Slt2) mitogen-activated protein kinase pathway." *Mol Cell Biol* 17(5): 2615-2623.

Westwater, C., E. Balish and D. A. Schofield (2005). "*Candida albicans*-conditioned medium protects yeast cells from oxidative stress: a possible link between quorum sensing and oxidative stress resistance." *Eukaryot Cell* 4(10): 1654-1661.

Wolfe, K. H. and D. C. Shields (1997). "Molecular evidence for an ancient duplication of the entire yeast genome." *Nature* 387(6634): 708-713.

Chapter 4

Decreased virulence of *Candida albicans CaRLM1* mutant in a murine model of hematogenously disseminated candidiasis

This chapter comprises parts from the following publication:

Delgado-Silva Y., A. Correia, A. Faustino, C. Pais and P. Sampaio. 2012. Decreased virulence of *Candida albicans CaRLM1* mutant in a murine model of hematogenously disseminated candidiasis. *Submitted manuscript.*

Abstract

Candida albicans, as an opportunistic pathogen, is able to adapt its growth to a range of environmental changes, by modulation of expression of many genes in a coordinated manner. Many of the mitogen-activated protein (MAP) kinase pathway components are important for virulence and morphological transitions. For this reason the knowledge of the role of the transcriptional factors under the control of the MAP kinase pathways should help us to better understand the interaction between *C. albicans* and the host. The transcription factor Rlm1 has been shown to be involved in cell wall biogenesis. In this study the involvement of Rlm1 in the virulence of the human fungal pathogen *Candida albicans* was evaluated in a murine model of hematogenously disseminated candidiasis. The mutant *rlm1*Δ/*rlm1*Δ strain was found to be less virulent than the wild-type (WT) and complemented strains, as displayed by the different survival time of mice, the lower kidney fungal burdens, and the histological observations. Additionally, in the murine macrophage-like cell line J744A, the TNF-α was lower in response to *rlm1*Δ/*rlm1*Δ mutant and the cellular toxicity (LDH) caused by this mutant was significantly lower in comparison with the WT and complemented strains. On the other hand, qRT-PCR determination showed that the expression of the cell wall related genes, *CRH11* and *PHR2,* was clearly higher in *rlm1*Δ/*rlm1*Δ mutant in relation to WT strain, in mRNA of kidney samples after 7 days post-infection. Since these proteins are part important of the cell wall and *in vivo* the yeast cells are under constant cell wall damage by the immune system it is not a surprease that the mutant present a higher expression of these genes, which may compensate the weakened cell wall. In relation to participation of the Rlm1 in the expression, we observed an increase of transcription of *MKC1* in WT and *rlm1*Δ/*rlm1*Δ mutant strains, suggesting that Rlm1 does not regulate this gene. Overall, these results showed that the transcription factor Rlm1 that is involved in the stability of the cell wall is important in the interaction with the host, being essential for the virulence of *C. albicans* and invasiveness of the kidneys during hematogenously disseminated candidiasis.

INTRODUCTION

The ubiquitous *Candida albicans* is an opportunistic commensal, which exists as part of the normal flora of the mucocutanous, genitourinary, and gastrointestinal area in healthy humans, without causing disease (Calderone, 2002). The fungus colonizes the mucosal membranes of 30–60% of humans (Odds, 1988); however, when the normal host defense mechanisms are impaired (for example, in patients who are undergoing chemotherapy for malignancies, receiving immunosuppressants after an organ transplant, or patients with AIDS), *C. albicans* is capable of causing a range of infections, from superficial thrush to life-theatening systemic candidiasis, making it the most prevalent human fungal pathogen (Larriba *et al.*, 2000).

Host defense against systemic candidiasis relies mainly on the ingestion and elimination of *C. albicans* by cells of the innate immune system, in particular macrophages, monocytes and neutrophils (Romani, 2000; Richardson and Rautemaa, 2009). Activation of leukocytes by *C. albicans*, triggers the release of pro-inflammatory cytokines (Th1 and Th17 responses), such as IFN-γ, TNF-α, IL-1β, IL-6, and IL-17 that in turn activate phagocyte effector functions that promote the elimination of the invading yeast (Djeu, 1990; Netea *et al.*, 1999; Huang *et al.*, 2004). In contrast, anti-inflammatory cytokines (Th2 response) such as IL-4 and IL-10 have immunosuppressive effects. Thus, the balance between pro- and anti-inflammatory cytokines is decisive in determining whether the host defense system is surpassed or able to eliminate the fungal pathogens (Cenci *et al.*, 1995; Netea *et al.*, 2006; Rozell *et al.*, 2006). Although the status of the host immune system is the major factor balancing the transition from commensalism to pathogenicity (Pirofski and Casadevall, 2009), *C. albicans* expresses several virulence attributes that contribute for its successful behavior, both as a commensal colonizer and as a pathogen (Biswas *et al.*, 2007). One of its major virulence traits is the ability to reversibly switch from unicellular budding cells to filamentous forms and the yeast uses this attribute during an infection that, not only provides invasion of tissues, but also allows escape from intracellular phagocyte death by inducing hyphal growth inside the phagosome, resulting in the destruction of the macrophage (Braun and Johnson, 1997; Kobayashi and Cutler, 1998; Calderone *et al.*, 2000; Mansour and Levitz, 2002; Lorenz *et al.*, 2004; Whiteway and Oberholzer, 2004). During these interactions *C. albicans* establishes contact with host cells through its cell wall, which is the outermost fungal structure. The cell wall of *C. albicans* is an essential structure that maintains cell morphology, providing protection from external pressure, aiding in colonization, host pathogenesis, as well as in immune-recognition and immune-avoidance (Klis *et al.*, 2002; Netea *et al.*, 2008). As the most external cellular structure of pathogenic microorganisms, it also carries important antigenic determinants and mediates adhesion to the host tissues, being therefore crucial in triggering and orchestrating the whole innate and adaptive immune response against the microorganism (Calderone and Fonzi, 2001). The cell wall is a multilayered structure composed by β-glucans (*β*1,3- and *β*1,6-glucan), chitin and mannoproteins (also called cell wall proteins - CWPs). Chitin and β-glucans provide the strength and rigidity to the cell wall, which ultimately determine the morphology of the cell, therefore their synthesis play a fundamental role in maintaining fungal cell integrity during growth, morphogenesis and in stress adaptation (Shaw *et al.*, 1991; Munro and Gow, 2001; Klis *et al.*, 2002; Roncero, 2002; Arana *et al.*, 2009).

In *C. albicans*, four different MAP kinase pathways have been identified by genetic analysis, and phenotypic characterization of mutants (Monge *et al.*, 2006). The cell wall integrity pathway is mediated by the Mkc1 (the homologue of the *Saccharomyces cerevisiae* Pkc1-Slt2/Mpk1), in a manner dependent on the presence of *PKC1* and it seems to be important for virulence since the disruption mutant in *MKC1*, is less virulent than the wild-type (Diez-Orejas *et al.*, 1997). Targets of Mkc1 have not yet been identified in *C. albicans*; however the presence of sequence homologues of the *S. cerevisiae* genes encoding Mkc1 transcription factors targets, *SWI4, SWI6* and *RLM1,* in *C. albicans* genome suggests that these could act as final effectors of the pathogenic yeast signalling cascade. The *RLM1* codes for a transcription factor from the MADS (Mcm1p-Agamous-Deficiens-Serum Response Factor) box family that, in *S. cerevisiae,* is known to regulate the expression of genes involved in the cell wall integrity pathway (Schwarz-Sommer *et al.*, 1990; Watanabe *et al.*, 1995; Dodou and Treisman, 1997; Jung and Levin, 1999; Garcia *et al.*, 2004; Levin, 2005).

In a previous work, both copies of the *RLM1* gene of *C. albicans* were deleted using the *SAT1*-flipping strategy and phenotypic analysis of the resulting *rlm1*Δ/*rlm1*Δ mutant was performed (this study, Chapter 3). Results showed that *C. albicans RLM1* is important in cell wall remodeling, as well as in regulating biofilm formation (this study, Chapter 3). The present study was undertaken to address the importance of the *RLM1* gene in *C. albicans* virulence, using the murine model of hematogenously disseminated infection.

MATERIALS AND METHODS

Mice.

Female BALB/c mice, 8 weeks old, were purchased from Charles River (Barcelona, Spain) and kept under specific-pathogen-free conditions at the Animal Facility of the Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal. All procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123), the 86/609/EEC directive, and Portuguese rules (DL 129/92).

Candida albicans culture conditions

The *C. albicans* strains used in this study are listed in Table 4.1. All strains were maintained as frozen stocks in 30% glycerol at -80°C. Yeast growth was tested in synthetic glucose minimal (2% w/v glucose, 0.67% w/v Bacto yeast nitrogen base without amino acids) and complex yeast-peptone-dextrose (YPD, 2% w/v glucose, 1% w/v yeast extract, 2% w/v Bacto peptone) liquid media at 30°C and 37°C in a shaking incubator for 24 h. Growth was measured at 60-min intervals, and the generation time calculated for all strains.

Table 4.1. *Candida albicans* strains used in this study.

Strain	Genotype	Reference
SC5314	Prototrophic wild-type strain	Gillum et al. (1984)
SCRLM1M4A	*rlm1*Δ::*FRT*/*rlm1*Δ::*FRT*	This study (Chapter 3)
SCRLM1K2A	*rlm1*Δ::*FRT*/*RLM1-FRT*	This study (Chapter 3)

To test filamentation, *C. albicans* cells were first grown for 24h on modified Lee medium (0.5% w/v $(NH_4)_2SO_4$, 0.02% w/v $MgSO_4.7H_2O$, 0.25% w/v K_2HPO_4, 0.5% w/v NaCl, 1.25% w/v D-galactose, 0.05% w/v L-alanine, 0.13% w/v L-leucine, 0.1% w/v L-lysine, 0.01% w/v L-methionine, 0.007% w/v L-ornithine, 0.05% w/v L-proline, 0.05% w/v L-threonine and 0.0001% w/v biotin, pH 5.0) at 26ºC and 150rpm in order to maintain cells in the yeast morphology and then filamentation was induced by plating approximately 30 colony-forming units (CFU) in Spider medium (1% w/v nutrient broth, 1% w/v mannitol, 0.2% w/v K_2HPO_4, 1.35% w/v Bacto Agar, pH 7.2) or YPD medium supplemented with 10% (v/v) fetal bovine serum (FBS). Plates were incubated at 37ºC for 72h and photographed.

To prepare the inocula for mice infection *C. albicans* strains were grown in a shaking incubator for 14h at 30°C in Winge medium (0.2% w/v glucose, 0.3% w/v yeast extract), then yeast cells were harvested, washed twice with sterile, nonpyrogenic phosphate buffered saline (PBS), counted in a hemocytometer, and resuspended at $2.5x10^6$ cells/ml. Inocula were confirmed by CFU counts on YPD agar after 48h at 37°C.

***Candida albicans* hematogenously disseminated infections**

Mice (n=8/group) were injected intravenously (i.v.) in the lateral tail vein with $5x10^5$ *C. albicans* yeast cells in 0.2 ml PBS. To evaluate the progress of hematogenously disseminated candidiasis, mice were weighed and monitored twice per day. Moribund mice were humanely terminated, and their deaths were recorded as occurring on the following day. To analyze organ fungal burden, histology and gene expression by quantitative (real-time) reverse-transcription PCR (qRT-PCR), groups of mice (n=4/group) were infected with the same inocula, $5x10^5$ yeast cells, and sacrificed 2 and 7 days postinfection. Control mice were injected i.v. with PBS. After infection, kidneys were aseptically removed, weighed, homogenized, and quantitatively cultured on YPD agar at 37ºC. Values were expressed as log CFU per gram of tissue. Alternatively, the other kidney, the liver and spleen were fixed in 10% phosphate buffered formaldehyde, followed by periodic acid-Schiff (PAS) reagent staining and counterstaining of the paraffin-embedded tissues with hematoxylin in order to evaluate both fungal morphology and composition and distribution of inflammatory infiltrates.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from kidney suspensions, homogenized in PBS, recovered from mice infected with *C. albicans* wild-type SC5314 or mutant SCRLM1M4A (*rlm1*Δ/*rlm1*Δ) strains. The suspensions were centrifuged at 1,500 x *g* at 4ºC for 10 min; pellets were washed twice with ice-cold RNase-free water, and frozen in liquid nitrogen. Total RNA was also obtained from *C. albicans* SC5314 and SCRLM1M4A cells incubated overnight in YPD at 30 ºC, and stored at -80 ºC. RNA extraction was performed using the hot acidic phenol method (Ausubel, 1993).

Quantitative RT-PCR assays and data normalisations were performed according to Correia et al. (2010). Briefly, total RNA from kidneys after infection and YPD stationary phase, was incubated with DNase I, for 15 min at room temperature to eliminate genomic DNA contamination. DNase I was

inactivated according to the manufacturer's instructions. The Superscript III Platinum two-step qRT-PCR kit with SYBR green (Invitrogen) was used to generate first-strand cDNA from each DNase I-treated RNA sample, as follows: 10 min at 65°C, 60 min at 37ºC, and 10 min at 65ºC. Quantitative PCR (qPCR) was performed with Platinum SYBR green qPCR SuperMix-UDG (Invitrogen). Three microliters of each cDNA sample were added to a 25 µl PCR mixture containing 12.5 µl Platinum SYBR green qPCR SuperMix-UDG, 0.5 µl of 10 µM specific forward and reverse primers (Table 4.2), and 8.5 µl RNase-free water. Each reaction was performed in a Corbett Rotor-Gene 6000 instrument (Qiagen). Thermocycling conditions for *CRH11, PGA52, PHR2, RCT1, MKC1* and *ACT1* quantification were 2 min at 50ºC (UDG incubation) and 5 min at 95ºC, followed by 40 cycles of 95ºC for 15 s, 60ºC for 30 s, and 72ºC for 30 s. The specificity of each primer pair was verified by the presence of a single melting temperature peak. The calibration and efficiency of all primers were assessed in titration experiments using *C. albicans* SC5314 genomic DNA (500ng to 5pg) in serial dilutions. A negative control (water) and a four-point curve of SC5314 genomic DNA were included in each run. *CRH11, PGA52, PHR2, RCT1* and *MKC1* gene expression was normalized to the housekeeping gene *ACT1* and analyzed using the standard curve method. Data are presented as ratio of normalized mRNA levels of *rlm1*Δ/rlm1Δ mutant / wild-type. Each experimental condition was performed in duplicate.

Table 4.2. Primers used in this study.

Gene	Primer (5´-3´)		Amplicon length (bp)
	Forward	Reverse	
CRH11	TCTGCATCTGTTTCCACTGC	ACAAAGCCAAAAAGGGAGGT	209
PGA52	AGAAGCGACATTCCTGCCTA	CAGATCTCCAGCACGAACAA	196
PHR2	AATGCTAACGGAACCACAGG	CGGTTCTGACGGTACCAGAT	232
RCT1	TGGTGTTGCTGGTGGTTATG	CCACCATATTGTTGCTCGTG	165
MKC1	ATGGGTCCAAAAAGGTTCC	TTATGGCCCCTGAAGAACTG	163
ACT1	TGCTGAACGTATGCAAAAGG	TGAACAATGGATGGACCAGA	186

Determination of lactate dehydrogenase activity and cytokine measurement

The murine macrophage-like cell line J774A (American Type Culture Center number TIB 67), was routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v heat-inactivated fetal calf serum (FBS), 1% w/v glutamine, 1% w/v sodium pyruvate, and 1% w/v HEPES in 5% v/v CO_2 at 37ºC in cell-culture flasks. After confluent growth, macrophage cells were washed and recovered. Viable cells were determined by Trypan blue (Sigma-Aldrich) exclusion, counted in the hemocytometer, and resuspended in DMEM to a final concentration of $5x10^5$ cell/ml. *Candida albicans* SC5314, SCRLM1M4A and SCRLM1K2A cells were cultivated in Winge medium, and resuspended at $1.25x10^6$ cell/ml.

The release of lactate dehydrogenase (LDH) from cells into the surrounding medium was monitored as a measure of J774A macrophage cell membrane damage. The amount of LDH released in the medium from macrophages cultures (negative control) and from macrophages co-incubated with *C.*

albicans suspension previously preparared ($1.25x10^6$ cell/ml) was measured after 12h of co-incubation using the Cytotoxicity Detection Kit_{PLUS} (LDH) (Roche Diagnostics Corporation, Indianapolis, USA), according to the manufacturer's instructions. This colorimetric assay is based on indirect reduction of tetrazolium salt when LDH is present in the cell culture supernatant. The percentage of cytotoxicity caused by the different *Candida* strains was calculated using the following formula: % cytotoxicity = [(experimental value – low control) / (High control– low control] x 100.

Production of TNF-α by macrophages co-incubated with *C. albicans* SC5314, SCRLM1M4A and SCRLM1K2A strains and bacterial lipopolysacharide, LPS, (5ng/µl, positive control) was quantified with the ELISA ReadySETGo Kit (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. The macrophage incubation medium used for TNF-α quantification was the same used in the LDH analysis. Determinations of LDH and TNF-α were carried out in triplicate, and the results are expressed as the mean of these results.

Secreted aspartic proteinase and phospholipases

The secretion of aspartic proteinases (SAPs) and phospholipases (PLs) by *C. albicans* strains was determined as previously described (Al-Abeid *et al.*, 2004). Briefly, 10µl of yeast suspension ($1.25x10^6$ cell/ml) were spotted onto YCB-BSA plates (1.17% w/v yeast carbon base, Difco, Sparks, USA; 0.01% w/v yeast extract; 2% agar w/v, pH 5.0; containing 0.2% w/v bovine serum albumin) for proteinase determination, and onto sabouraud dextrose agar (SDA) plates (6.5% w/v SDA, 5.84% w/v NaCl, 0.55% w/v $CaCl_2$, 2% w/v agar) supplemented with 8% v/v egg yolk emulsion, for phospholipase determination. For SAPs assays, after 5 days of incubation at 37ºC plates were stained with an amido-black water/alcohol solution (4.0% v/v methanol, 10.0% v/v acetic acid, 0.1% w/v amido black) and destained with the same solution without amido-black. The unstained zones around each colony were measured and the Pz values calculated using the following formula: Pz = (Colony diameter / Halo diameter). Production of PLs was determined as the precipitation zone around each colony and mesured after incubation at 37ºC for 72h using the same Pz formula. Pz values close to zero indicate maximum production of SAPs and LPs whereas values close to 1 indicate low enzymatic activity. Positive results were considered when Pz value was ≤0.5. Each assay was carried out in triplicate, and the results are expressed as the mean of these results.

Statistical analysis

Unless otherwise stated, results shown are from one representative experiment from three independent experiments. The statistical significance of results was determined by the unpaired Student *t* test, and survival data were analyzed with the log-rank test by using GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA). Results were considered statistically significant with *P* values below 0.05.

RESULTS

Influence of Rlm1 on *C. albicans* virulence in a murine model of hematogenously disseminated infection

To determine whether *RLM1* is required for virulence, we investigated the ability of the *rlm1*Δ/rlm1 mutant strain to establish an infection in the murine model of haematogenously disseminated candidiasis. Before performing the virulence studies, we determined the generation time for SC5314, SCRLM1M4A and SCRLM1K2A strains in synthetic defined and complex media at 30ºC and 37ºC and the ability to form hyphae in serum media as well as to secrete proteinases and phospholipases. No yeast growth defects were observed under the conditions tested, all strains presented similar generation times (Fig. 4.1A); and the filamentous growth under the hypha-inducing conditions analyzed was also similar between strains (Fig. 4.1B). Additionally, no significant differences were observed in the Pz values calculated for secreted aspartic proteinase and phospholipase production in the three strains (Fig. 4.1C and 4.1D).

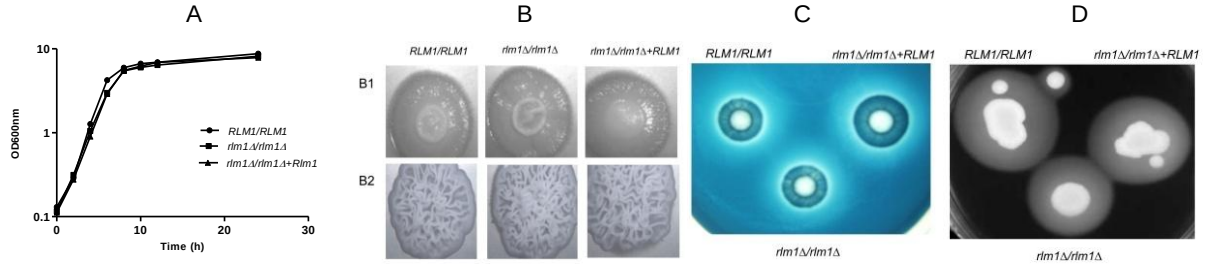

Figure 4.1. Phenotypic characterization of *C. albicans* wild-type SC5314 *(RLM1/RLM1)*, mutant SCRLM1M4A (*rlm1Δ*/*rlm1Δ*) and complemented SCRLM1K2A (*rlm1Δ/rlm1Δ+RLM1*) strains. A) Growth curve at 37ºC, B) morphology on Spider medium (B1) and YPD containing 10% of FBS (B2) after 6 days of incubation at 30ºC. C) secretion of proteinase on YCB-BSA containing 0.2% of BSA and D) fosfolipase activity on SDA supplemented with 8% egg yolk emulsion, after incubation at 37ºC.

BALB/c mice were i.v. injected with $5x10^5$ cells of *C. albicans* wild-type (SC5314), *rlm1*Δ/*rlm1*Δ mutant (SCRLM1M4A) or complemented (SCRLM1K2A) strains. All mice injected with WT succumbed to candidal infection within 24 days (Fig. 4.2). In contrast, at the end of the experimental period (70 days), 75% of the mice infected with the mutant strain survived the infection. The median survival time of SCRLM1M4A-infected mice was undefined whereas, for WT-infected mice, the median survival time was 20 days. Significant differences in survival time were observed between mice infected with SCRLM1M4A strain and SC5314 (*P<0.000*1 by log-rank test) (Fig. 4.2). When *RLM1* gene was reintroduced into SCRLM1M4A strain to form SCRLM1K2A, the virulence of the *C. albicans* organism was partially recovered. Mice infected with SCRLM1K2A had a median survival of 41 days, which was much higher than mice infected with WT (*P*=0.1132) but significantly shorter than that of mice infected with SCRLM1M4A (*P*=0.0463) (Fig. 4.2). These results indicate that the *RLM1* gene is important for the virulence of *C. albicans* and that introduction of one copy of *RLM1* gene did not restore completly the virulence phenotype observed in the WT strain.

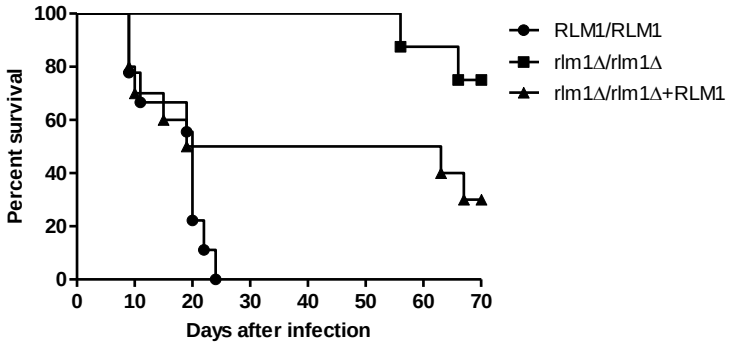

Figure 4.2. Influence of *RLM1* in *C. albicans* virulence in a murine model of hematogenously disseminated candidiasis. Female BALB/c mice were i.v. injected with 5 X 10^5 cells of *C. albicans* wild-tipe SC5314, mutants SCRLM1M4A (*rlm1Δ*/*rlm1Δ*) or complemented SCRLM1K2A (*rlm1Δ/rlm1Δ+RLM1*) strain. Each strain was injected into eight mice per group, per experiment, and survival was monitored twice daily for 70 days. Results are representative of two independent experiments.

The evaluation of the fungal ability to invade the kidneys has been frequently used to measure the virulence of *C. albicans* strains (MacCallum and Odds, 2005; Sampaio *et al.*, 2010). In this study all strains tested produced a similar level of infection in the kidneys after two days of infection (Fig. 4.3). However, after 7 days of infection, the numbers of *C. albicans* CFU in the Kidneys of mice infected with SCRLM1M4A (*rlm1*Δ/*rlm1*Δ) strain were significantly lower than those of mice infected with the SC5314 (*RLM1/RLM1*) and SCRLM1K2A (*rlm1Δ/rlm1Δ+RLM1*) strain (*P*=0.0345 for SC5314 versus SCRLM1M4A strain and *P*=0.0440 SCRLM1K2A versus SCRLM1M4A strain) (Fig. 4.3).

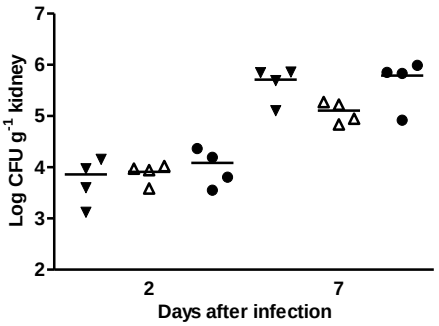

Figure 4.3. Kidney fungal burden of BALB/c mice 2 and 7 days after i.v. injection with 5 x10^5 cells of wild-type SC5314 (▲), mutant SC5314M4A (*rlm1*Δ/*rlm1*Δ) (Δ) and complemented SC5314K2A (*rlm1*Δ/*rlm1*Δ+*RLM1)* (●) *C. albicans* strains. Each symbol represents an individual mouse, and horizontal bars are means of CFU numbers for each group.

The ability of WT, *rlm1Δ/rlm1*Δ mutant and complemented strains to infect and injure several organs were assessed by histopathological analysis of the kidneys, spleen and liver, 2 and 7 days after infection. Intralesional PAS-positive organisms both in yeast and hyphal morphology, septated and branched, were detected 2 days after *C. albicans* i.v. infection with strains SC5314 and SCRLM1K2A (Fig. 4.4). Concomitantly, these kidneys showed moderate multifocal renal medullary interstitial neutrophilic infiltration. In the kidneys of the mice infected with the *rlm1Δ/rlm1*Δ mutant strain, SCRLM1M4A, the fungi appeared as mixture of ovoid and hyphal cells with a more restricted infiltration in comparison with kidneys infection with the other strains. At the later time point tested, 7 days after infection, analysis of WT-infected and complemented-infected mice showed severe, focally extensive to coalescing, renal medullary interstitial neutrophilic infiltration surrounding numerous PAS-

positive organisms. These organisms were present mainly as septated, branched hyphal structures, which escaped the medulla and invaded the pelvis region (Fig. 4.4). In contrast, in kidneys of mice infected with the *rlm1*Δ/*rlm1*Δ mutant strain, a clear reduction of yeast cells was observed and the remainding hyphal structures were present mainly at the pelvis region, surrounded by neutrophilic infiltration, which is in agreement with CFU results (Fig. 4.4). Invasion of spleen and liver was not consistently seen in all strains (data not shown). These differences regarding organ distribution are in accordance with the known higher ability of *C. albicans* to colonize kidneys after mouse systemic infection (Tavares *et al.*, 2003; Correia *et al.*, 2010; Sampaio *et al.*, 2010).

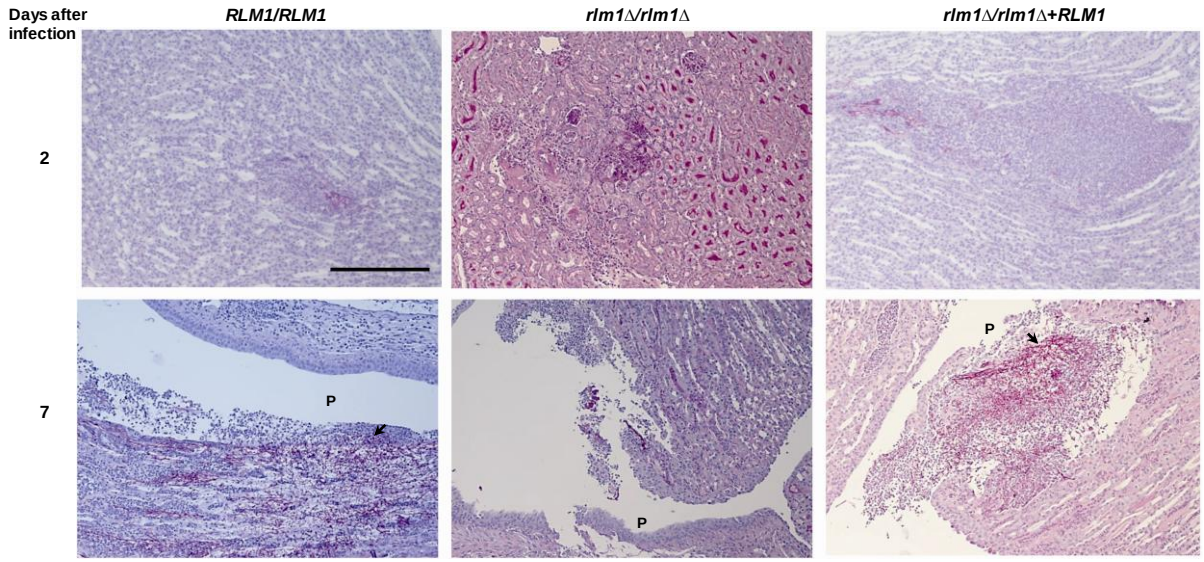

Figura 4.4. Representative photomicrographs of PAS-stained paraffin sections of kidneys recovered from BALB/c mice infected with $5x10^5$ cells of wild-type SC5314, mutant SCRLM1M4A (*rlm1*Δ/*rlm1*Δ) and complemented SCRLM1K2A (*rlm1*Δ/*rlm1*Δ+*RLM1*) *C. albicans* strains at 2 and 7 days post-i.v. injection. Arrows show hyphae invading the pelvis region. P - renal pelvis. Magnification of photographs: 100X. Bar: 100 µm for all photos.

Decreased *in vitro* production of TNF-α and cell damage of macrophages in response to *C. albicans rlm1*Δ/*rlm1*Δ cells

To determine the effect of the disruption of *RLM1* on the immune response elicited by *C. albicans*, the proinflamatory cytokine TNF-α was measured in murine macrophage-like cell line J774A challenged for 12h with *C. albicans* yeast cells from WT, mutant or complemented strain. Results (Fig. 4.5A) showed that TNF-α levels produced by macrophages were significantly lower in response to *rlm1*Δ/*rlm1*Δ mutant cells in comparation with WT (*P*=0.0001). On the other hand, the complemented strain, although inducing a higher amount TNF-α than the mutant strain, did not reached the WT levels. As expected, a strong proinflammatory response was observed with the positive control (*E. Coli* LPS), whereas no detectable levels of TNF-α were found in non-stimulated macrophages.. Furthermore, to quantify the cell damage caused by the WT, *rlm1*Δ/*rlm1*Δ mutant and complemented *C. albicans* strains, we determined the LDH released from macrophages after 12h of co-incubation. As depicted in Fig. 4.5B, the cell damage caused by *rlm1*Δ/*rlm1*Δ mutant was significantly lower in comparison with the WT and complemented strains, which presented similar cell damage as the WT. These results indicate that the deletion of *RLM1* has a significant impact in the immune response elicited by the murine macrophage-like cell line J774A.

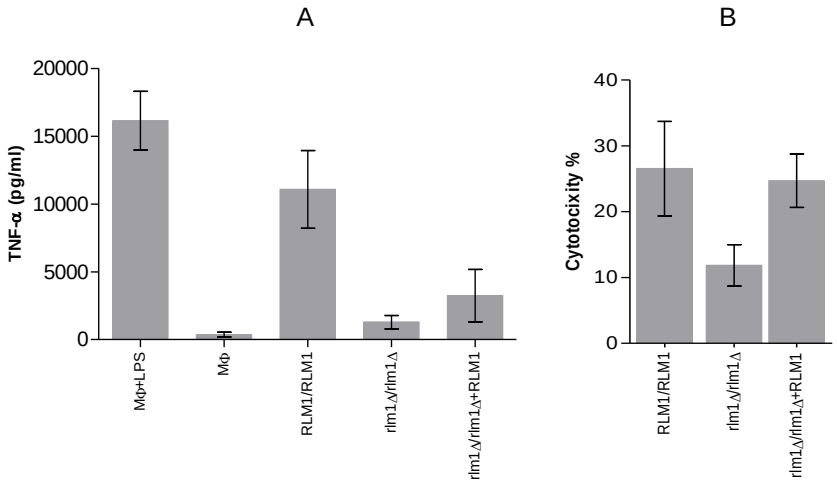

Figure. 4.5. *In vitro* production of TNF-α and cellular damage (LDH released) caused by *C. albicans* wild-type SC5314, mutant SCRLM1M4 (*rlm1Δ/rlm1Δ)* and complemented (*rlm1Δ/rlm1Δ+RLM1*) strains. The murine macrophage-like cell line J774A was co-incubated with the indicated *C. albicans* strains or *E. coli* LPS. Concentration of TNF-α (A) and LDH (B) were mesuared in the culture supernatant after 12h of co-incubation. MΦ - macrophages.

Quantitative RT-PCR

In order to evaluate the role of important cell wall related genes, *CRH11, PGA52, PHR2, RCT1* and *MKC1,* during the infection process, as well as the participation of the transcription factor Rlm1 in their expression, qRT-PCR was performed. These genes were selected from the list of genes in the cell wall compensatory cluster identified for *S. cerevisiae* (Lagorce et al., 2003). In *C. albicans* Crh11*,* Pga52 *and* Phr2 are glycosylphosphatidylinositol (GPI)-anchored cell surface proteins (Fonzi, 1999; De Groot *et al.*, 2003; Pardini *et al.*, 2006). Rct1 is an outer transmembrane protein produced in exponential and stationary growth phase culture (Kusch *et al.*, 2008) and Mkc1 is a protein kinase of the cell wall integrity pathway (Navarro-Garcia *et al.*, 1995).

The expression of the genes encoding these proteins in the WT SC5314 and in the mutant SCRLM1M4A (*rlm1Δ/rlm1Δ*) strains was evaluated by qRT-PCR in kidney samples after 2 and 7 days of infection (*in vivo*) and in YPD-cultivated cells in the stationary phase of grown (*in vitro*). Only the results for the latter time point analyzed in the kidneys are presented, since after 2 days of infection, either the fungal burden was insufficient or the gene expression was too low to obtain reproducible results. Results of qRT-PCR are representative of two independent experiments (Table 4.3 and Fig. 4.6).

Table. 4.3. Expression level of cell wall related genes.

		CRH11 (pg/µl)	***PGA52*** (pg/µl)	***PHR2*** (pg/µl)	***RCT1*** (pg/µl)	***MKC1*** (pg/µl)
"in vivo"	*RLM1/RLM1*	0.0086	0.0019	0.0083	0.0685	1.8624
	rlm1Δ/rlm1Δ	0.0871	0.0010	0.0600	0.0204	2.3703
"in vitro"	*RLM1/RLM1*	0.2210	1.2313	0.2628	1.7166	0.0430
	rlm1Δ/rlm1Δ	0.5059	3.7398	0.1878	1.2114	0.0445

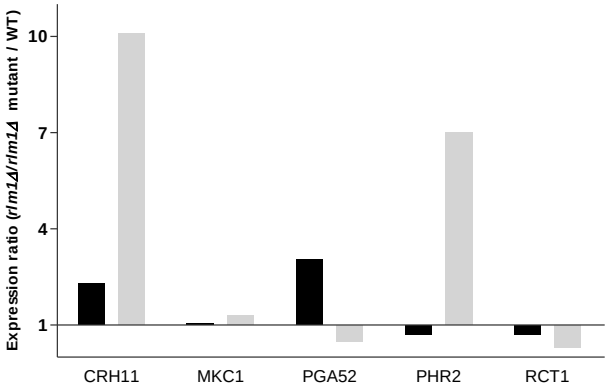

Figure 4.6. Ratio of expression levels of *CRH11, PGA52, PHR2, RCT1* and *MKC1* in *C. albicans* wild-type SC5314 and mutant SC5314M4A (*rlm1Δ/rlm1Δ)* strains by qRT-PCR from kidney homogenates 7 days after i.v. injection with $5x10^5$ cells (■) (*in vivo)* and YPD stationary phase cells (■) (*in vitro)*. Results are presented as ratio of normalized mRNA levels of *rlm1Δ/rlm1Δ* mutant / wild-type.

MKC1, the putative activator of Rlm1, was the gene that presented higher expression *in vivo*, while *in vitro* the genes with the highest expression were *PGA52* and *RCT1* (Table 4.3). Considering the differences in *in vivo* gene expression, in the *rlm1Δ/rlm1Δ* mutant in relation to WT, we observed that the genes with the highest differences in induction were *CRH11* approximately 10x and *PHR2* approximately 7x. On the other hand, in *in vitro* the mostly altered were *CRH11* and *PGA52* that presented 2x and 3x higher expression, respectively, in the *rlm1Δ/rlm1Δ* mutant in relation to WT. The *RCT1* gene presented reduced expression in the mutant in both conditions. Regarding *MKC1*, results do not indicate differences in its expression level in the *rlm1Δ/rlm1Δ* mutant cells in relation to the WT, in both experimental conditions (Fig. 4.6).

DISCUSSION

Candida albicans, as an opportunistic pathogen, is able to adapt its growth to a range of environmental changes, by modulation of expression of many genes in a coordinated manner. Many of the mitogen-activated protein (MAP) kinase pathway components are important for virulence and morphological transitions (Ernst, 2000; Monge *et al.*, 2006; Biswas *et al.*, 2007). For this reason the knowledge of the role of the transcriptional factors under the control of the MAP kinase pathways should help us to better understand the interaction between *C. albicans* and the host. To the best of our knowledge, the influence of the *RLM1* gene in *C. albicans* virulence has not been characterized yet. Therefore, in this study, the virulence of the *rlm1*Δ/*rlm1*Δ mutant was examined *in vivo* and *in vitro* and compared with the WT and the complemented strains. In previous works, phenotypic analysis in *rlm1*Δ/*rlm1*Δ mutants and WT showed that the role of *C. albicans RLM1* is important in cell wall remodeling, and in regulating biofilm formation (Bruno *et al.*, 2006; this study, Chapter 3).

In this study, an increased median survival time was consistently observed for mice infected with the mutant lacking both alleles of the *RLM1* gene, when compared with the median survival time observed in animals infected with the WT SC5314 and complemented strains. When another parameter associated with *C. albicans* virulence was analyzed, the CFU in the kidneys, differences among the different mouse groups were clearly observed at 7 days post-infection, where *rlm1*Δ/*rlm1*Δ mutant presented a lower fungal burden. Curiously, despite the quantification of a reduced number of *C. albicans rlm1*Δ/*rlm1*Δ mutant cells in the kidney by CFUs, it was very difficult to observe them in the histopathology analysis. This seems to indicate a reduced ability of the *rlm1*Δ/*rlm1*Δ mutant to invade deeply into the kidneys. In contrast, mice infected with the WT and complemented strains showed extensive invasion into kidneys, with mycelial growth extending throughout the tissue. Addicionaly, a reduced number of inflammatory cells surrounding the *C. albicans rlm1*Δ/*rlm1*Δ mutant cells were also observed. This paucity of inflammatory cells could be due to the low number of PAS-positive organisms invading the kidneys or to a diminished induced host response.

The host response to infection by *C. albicans* is associated with the release of proinflamatory cytokines such as TNF-α (Mencacci *et al.*, 1998). The comparative analysis of the interaction of the WT, *rlm1*Δ/*rlm1*Δ mutant and complemented *C. albicans* strains with J774 macrophages cell line showed that at 12h of co-incubation the levels of TNF-α were much higher in response to WT and complemented strains in comparison with *rlm1*Δ/*rlm1*Δ cells. These observations are in agreement with our previous histopathology results of kidney sections; revealing heavy infiltration of leucocytes around *C*. *albicans* cells, in mice infected with WT or complemented strains and few inflammatory cells around the *rlm1*Δ/*rlm1*Δ mutant cells. Thus, we belive that the difficulty in observing *C. albicans rlm1*Δ/*rlm1*Δ mutant cells in the histopathology of kidney and the lower kidney fungal burden may be mainly due to the lower ability of *C. albicans* mutant cells to resist host deffense mechanisms. These observations are in concordance with previous reports, indicating that pro-inflammatory cytokines are important for antifungal effector functions, particularly during the early phase of the inflammatory response (Huang *et al.*, 2004; Romani, 2004; MacCallum and Odds, 2005). This may be the reason

why at 2 days post infection dispite the lower resitence to host defense mechanisms no significant differences in the kidney fungal burden were observed. Furthermore, a significant lower cellular toxicity (measured as extracellular LDH) was caused by the *rlm1*Δ/*rlm1*Δ mutant cells in comparison with the wild-type and complemented strains. These results correlate well with the histopathological observations, in which cellular kidney tissue destruction is observed in mice infected with WT or complemented strains.

We observed that some phenotypes were not totally complemented with the introduction of only one copy of the *RLM1* gene. It was clearly observed that the complemented strain had a survival curve in between the WT and mutant strain (41 days), and the induction of TNF-α was significantly lower than the observed with WT. This intermediate behaviour was also observed in other studies with *C. albicans* mutants such as *rad52* (Chauhan *et al.*, 2005) where gene dosage is important for the WT phenotype.

Since *RLM1* is involved in the cell wall maintenance, several genes that were considered important for the cell wall formation and integrity, such as *CRH11, PGA52, PHR2, RCT1* and *MKC1*, were evaluated by qRT-PCR in kidney samples after 7 days post-infection (*in vivo*) and in YPD stationary phase growth cells (*in vitro*). Our results indicated that *in vivo* gene expression of *CRH11* and *PHR2* was cleary higher in *rlm1*Δ/*rlm1*Δ mutant in relation to WT, while *in vitro* the higher expression in *rlm1*Δ/*rlm1*Δ mutant in relation to WT were *CHR11* and *PGA52*. Since these proteins are part important of the cell wall and *in vivo* the yeast cells are under constant cell wall damage by the immune system it is not a surprease that the mutant present a higher expression of these genes, which may compensate the weakened cell wall (Fonzi, 1999; Pardini *et al.*, 2006). The higher expression of these genes in the mutant strins suggest that either Rlm1 acts as a negative regulator of these genes, or other transcription factors may be involved in the expression of these genes, so that the absence of the Rlm1 is compensated. It is known that in *S. cerevisiae,* the heterodimer complex Swi4/Swi6 transcription factors have been described to also participate in the remodelling and maintenance of cell wall (Igual *et al.*, 1996; Kim *et al.*, 2008). Furthermore, *C. albicans* presents *SWI4* and *SWI6* orthologues (Nikolaou *et al.*, 2009), for this we could suggest a similar role for these orthologues in the activation of genes encoding cell wall proteins.

The reduced expression of *RCT1* in *rlm1*Δ/*rlm1*Δ mutant in relation to WT in both conditions, *in vivo* and *in vitro,* suggests that Rlm1 could be involved in the expression of this gene. It has been reported that Rct1 is present in exponential and stationary growth phase culture, which is in agreement with our *in vitro* results (Kusch *et al.*, 2008). The induction observed in the *PGA52 gene in vitro,* in cells grown on YPD, is in accordance with the YPD microarrays assays that also showed an increase expression in the mutant cells (this study Table 3.4), however in *in vivo* experiments this gene presented reduced expression in the *rlm1*Δ/*rlm1*Δ mutant. The reason for this diferent behaviour, *in vivo* and *in vivo*, could not be ascertained.

Studies with *S. cerevisiae* using LexA as a reporter gene showed that the ability of Rlm1 protein to regulate transcription is dependent on the activation of the Pkc1-Slt2/Mpk1 pathway (Watanabe *et al.*, 1997). In *C. albicans MKC1,* the *SLT2* homolog, encodes a protein kinase of the cell wall integrity pathway (Navarro-Garcia *et al.*, 1995) that is important for virulence, since the *mkc1*Δ*/mkc1*Δ mutant is less virulent than the wild-type (Diez-Orejas *et al.*, 1997). The much higher expression of *MKC1 in vivo* (1,862 pg/µl in the WT) when compared with *in vitro* experiments (0,043 pg/µl in the WT)*,* agrees with the previous studies that showed its importance in the maintenance of the cell wall under constant damage by the immune system. However, since *MKC1* expression is similar in WT and *rlm1*Δ/*rlm1*Δ mutant strains in both conditions, *in vivo* and *in vitro*, we conclude that Rlm1 does not regulate *MKC1*. Overall, these results showed that the transcription factor Rlm1 is involved in the stability of the cell wall in the interaction with the host, being important for the virulence of *C. albicans* and invasion of the kidneys during hematogenously disseminated candidiasis.

Acknowledgments

Yolanda Delgado was supported by PhD fellowships from Nº E07D400922PE, The European Union Programme of High Level Scholarships for Latin America, ALβAN.

References

Al-Abeid, H. M., K. H. Abu-Elteen, A. Z. Elkarmi and M. A. Hamad (2004). "Isolation and characterization of *Candida* spp. in Jordanian cancer patients: prevalence, pathogenic determinants, and antifungal sensitivity." *Jpn J Infect Dis* 57(6): 279-284.

Arana, D. M., D. Prieto, E. Roman, C. Nombela, R. Alonso-Monge and J. Pla (2009). "The role of the cell wall in fungal pathogenesis." *Microb Biotechnol* 2(3): 308-320.

Ausubel, F. M. e. (1993). "Current protocols in molecular biology." *2nd ed. Wiley, New York, NY*.

Biswas, S., P. Van Dijck and A. Datta (2007). "Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*." *Microbiol Mol Biol Rev* 71(2): 348-376.

Braun, B. R. and A. D. Johnson (1997). "Control of filament formation in *Candida albicans* by the transcriptional repressor TUP1." *Science* 277(5322): 105-109.

Bruno, V. M., S. Kalachikov, R. Subaran, C. J. Nobile, C. Kyratsous and A. P. Mitchell (2006). "Control of the *C. albicans* cell wall damage response by transcriptional regulator Cas5." *PLoS Pathog* 2(3): e21.

Calderone, R. (2002). "Candida and candidiasis." *ASM Press, washington, DC*.

Calderone, R., S. Suzuki, R. Cannon, T. Cho, D. Boyd, J. Calera, H. Chibana, D. Herman, A. Holmes, H. W. Jeng, H. Kaminishi, T. Matsumoto, T. Mikami, J. M. O'Sullivan, M. Sudoh, M. Suzuki, Y. Nakashima, T. Tanaka, G. R. Tompkins and T. Watanabe (2000). "*Candida albicans:* adherence, signaling and virulence." *Med Mycol* 38 Suppl 1: 125-137.

Calderone, R. A. and W. A. Fonzi (2001). "Virulence factors of *Candida albicans.*" *Trends Microbiol* 9(7): 327-335.

Cenci, E., A. Mencacci, R. Spaccapelo, L. Tonnetti, P. Mosci, K. H. Enssle, P. Puccetti, L. Romani and F. Bistoni (1995). "T helper cell type 1 (Th1)- and Th2-like responses are present in mice with gastric candidiasis but protective immunity is associated with Th1 development." *J Infect Dis* 171(5): 1279-1288.

Chauhan, N., T. Ciudad, A. Rodriguez-Alejandre, G. Larriba, R. Calderone and E. Andaluz (2005). "Virulence and karyotype analyses of *rad52* mutants of *Candida albicans*: regeneration of a truncated chromosome of a reintegrant strain (*rad52*/*RAD52*) in the host." *Infect Immun* 73(12): 8069-8078.

Correia, A., U. Lermann, L. Teixeira, F. Cerca, S. Botelho, R. M. da Costa, P. Sampaio, F. Gartner, J. Morschhauser, M. Vilanova and C. Pais (2010). "Limited role of secreted aspartyl proteinases Sap1 to Sap6 in *Candida albicans* virulence and host immune response in murine hematogenously disseminated candidiasis." *Infect Immun* 78(11): 4839-4849.

De Groot, P. W., K. J. Hellingwerf and F. M. Klis (2003). "Genome-wide identification of fungal GPI proteins." *Yeast* 20(9): 781-796.

Diez-Orejas, R., G. Molero, F. Navarro-Garcia, J. Pla, C. Nombela and M. Sanchez-Perez (1997). "Reduced virulence of *Candida albicans MKC1* mutants: a role for mitogen-activated protein kinase in pathogenesis." *Infect Immun* 65(2): 833-837.

Djeu, J. Y. (1990). "Role of tumor necrosis factor and colony-stimulating factors in phagocyte function against *Candida albicans*." *Diagn Microbiol Infect Dis* 13(5): 383-386.

Dodou, E. and R. Treisman (1997). "The *Saccharomyces cerevisiae* MADS-box transcription factor Rlm1 is a target for the Mpk1 mitogen-activated protein kinase pathway." *Mol Cell Biol* 17(4): 1848-1859.

Ernst, J. F. (2000). "Transcription factors in *Candida albicans* - environmental control of morphogenesis." *Microbiology* 146 (Pt 8): 1763-1774.

Fonzi, W. A. (1999). "*PHR1* and *PHR2* of *Candida albicans* encode putative glycosidases required for proper cross-linking of beta-1,3- and beta-1,6-glucans." *J Bacteriol* 181(22): 7070-7079.

Garcia, R., C. Bermejo, C. Grau, R. Perez, J. M. Rodriguez-Pena, J. Francois, C. Nombela and J. Arroyo (2004). "The global transcriptional response to transient cell wall damage in *Saccharomyces cerevisiae* and its regulation by the cell integrity signaling pathway." *J Biol Chem* 279(15): 15183-15195.

Huang, W., L. Na, P. L. Fidel and P. Schwarzenberger (2004). "Requirement of interleukin-17A for systemic anti-*Candida albicans* host defense in mice." *J Infect Dis* 190(3): 624-631.

Igual, J. C., A. L. Johnson and L. H. Johnston (1996). "Coordinated regulation of gene expression by the cell cycle transcription factor Swi4 and the protein kinase C MAP kinase pathway for yeast cell integrity." *EMBO J* 15(18): 5001-5013.

Jung, U. S. and D. E. Levin (1999). "Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway." *Mol Microbiol* 34(5): 1049-1057.

Kim, K. Y., A. W. Truman and D. E. Levin (2008). "Yeast Mpk1 mitogen-activated protein kinase activates transcription through Swi4/Swi6 by a noncatalytic mechanism that requires upstream signal." *Mol Cell Biol* 28(8): 2579-2589.

Klis, F. M., P. Mol, K. Hellingwerf and S. Brul (2002). "Dynamics of cell wall structure in *Saccharomyces cerevisiae*." *FEMS Microbiol Rev* 26(3): 239-256.

Kobayashi, S. D. and J. E. Cutler (1998). "*Candida albicans* hyphal formation and virulence: is there a clearly defined role?" *Trends Microbiol* 6(3): 92-94.

Kusch, H., S. Engelmann, R. Bode, D. Albrecht, J. Morschhauser and M. Hecker (2008). "A proteomic view of *Candida albicans* yeast cell metabolism in exponential and stationary growth phases." *Int J Med Microbiol* 298(3-4): 291-318.

Lagorce, A., N. C. Hauser, D. Labourdette, C. Rodriguez, H. Martin-Yken, J. Arroyo, J. D. Hoheisel and J. Francois (2003). "Genome-wide analysis of the response to cell wall mutations in the yeast *Saccharomyces cerevisiae*." *J Biol Chem* 278(22): 20345-20357.

Larriba, G., J. J. Rubio Coque, A. Ciudad and E. Andaluz (2000). "*Candida albicans* molecular biology reaches its maturity." *Int Microbiol* 3(4): 247-252.

Levin, D. E. (2005). "Cell wall integrity signaling in *Saccharomyces cerevisiae*." *Microbiol Mol Biol Rev* 69(2): 262-291.

Lorenz, M. C., J. A. Bender and G. R. Fink (2004). "Transcriptional response of *Candida albicans* upon internalization by macrophages." *Eukaryot Cell* 3(5): 1076-1087.

MacCallum, D. M. and F. C. Odds (2005). "Temporal events in the intravenous challenge model for experimental *Candida albicans* infections in female mice." *Mycoses* 48(3): 151-161.

Mansour, M. K. and S. M. Levitz (2002). "Interactions of fungi with phagocytes." *Curr Opin Microbiol* 5(4): 359-365.

Mencacci, A., E. Cenci, G. Del Sero, C. Fe d'Ostiani, P. Mosci, C. Montagnoli, A. Bacci, F. Bistoni, V. F. Quesniaux, B. Ryffel and L. Romani (1998). "Defective co-stimulation and impaired Th1 development in tumor necrosis factor/lymphotoxin-alpha double-deficient mice infected with *Candida albicans*." *Int Immunol* 10(1): 37-48.

Monge, R. A., E. Roman, C. Nombela and J. Pla (2006). "The MAP kinase signal transduction network in *Candida albicans.*" *Microbiology* 152(Pt 4): 905-912.

Munro, C. A. and N. A. Gow (2001). "Chitin synthesis in human pathogenic fungi." *Med Mycol* 39 Suppl 1: 41-53.

Navarro-Garcia, F., M. Sanchez, J. Pla and C. Nombela (1995). "Functional characterization of the *MKC1* gene of *Candida albicans,* which encodes a mitogen-activated protein kinase homolog related to cell integrity." *Mol Cell Biol* 15(4): 2197-2206.

Netea, M. G., G. D. Brown, B. J. Kullberg and N. A. Gow (2008). "An integrated model of the recognition of *Candida albicans* by the innate immune system." *Nat Rev Microbiol* 6(1): 67-78.

Netea, M. G., G. Ferwerda, C. A. van der Graaf, J. W. Van der Meer and B. J. Kullberg (2006). "Recognition of fungal pathogens by toll-like receptors." *Curr Pharm Des* 12(32): 4195-4201.

Netea, M. G., L. J. van Tits, J. H. Curfs, F. Amiot, J. F. Meis, J. W. van der Meer and B. J. Kullberg (1999). "Increased susceptibility of TNF-alpha lymphotoxin-alpha double knockout mice to systemic candidiasis through impaired recruitment of neutrophils and phagocytosis of *Candida albicans.*" *J Immunol* 163(3): 1498-1505.

Nikolaou, E., I. Agrafioti, M. Stumpf, J. Quinn, I. Stansfield and A. J. Brown (2009). "Phylogenetic diversity of stress signalling pathways in fungi." *BMC Evol Biol* 9: 44.

Odds, F. C. (1988). "Candida and candidosis: a review and bibliography." *3rd edn. Balliere Tindall, London.*

Pardini, G., P. W. De Groot, A. T. Coste, M. Karababa, F. M. Klis, C. G. de Koster and D. Sanglard (2006). "The *CRH* family coding for cell wall glycosylphosphatidylinositol proteins with a predicted transglycosidase domain affects cell wall organization and virulence of *Candida albicans.*" *J Biol Chem* 281(52): 40399-40411.

Pirofski, L. A. and A. Casadevall (2009). "Rethinking T cell immunity in oropharyngeal candidiasis." *J Exp Med* 206(2): 269-273.

Richardson, M. and R. Rautemaa (2009). "How the host fights against Candida infections." *Front Biosci* 14: 4363-4375.

Romani, L. (2000). "Innate and adaptive immunity in *Candida albicans* infections and saprophytism." *J Leukoc Biol* 68(2): 175-179.

Romani, L. (2004). "Immunity to fungal infections." *Nat Rev Immunol* 4(1): 1-23.

Roncero, C. (2002). "The genetic complexity of chitin synthesis in fungi." *Curr Genet* 41(6): 367-378.

Rozell, B., P. O. Ljungdahl and P. Martinez (2006). "Host-pathogen interactions and the pathological consequences of acute systemic *Candida albicans* infections in mice." *Curr Drug Targets* 7(4): 483-494.

Sampaio, P., M. Santos, A. Correia, F. E. Amaral, J. Chavez-Galarza, S. Costa-de-Oliveira, A. G. Castro, J. Pedrosa and C. Pais (2010). "Virulence attenuation of *Candida albicans* genetic variants isolated from a patient with a recurrent bloodstream infection." *PLoS One* 5(4): e10155.

Schwarz-Sommer, Z., P. Huijser, W. Nacken, H. Saedler and H. Sommer (1990). "Genetic Control of Flower Development by Homeotic Genes in Antirrhinum majus." *Science* 250(4983): 931-936.

Shaw, J. A., P. C. Mol, B. Bowers, S. J. Silverman, M. H. Valdivieso, A. Duran and E. Cabib (1991). "The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle." *J Cell Biol* 114(1): 111-123.

Tavares, D., P. Ferreira and M. Arala-Chaves (2003). "Increased resistance in BALB/c mice to reinfection with *Candida albicans* is due to immunoneutralization of a virulence-associated immunomodulatory protein." *Microbiology* 149(Pt 2): 333-339.

Watanabe, Y., K. Irie and K. Matsumoto (1995). "Yeast *RLM1* encodes a serum response factor-like protein that may function downstream of the Mpk1 (Slt2) mitogen-activated protein kinase pathway." *Mol Cell Biol* 15(10): 5740-5749.

Watanabe, Y., G. Takaesu, M. Hagiwara, K. Irie and K. Matsumoto (1997). "Characterization of a serum response factor-like protein in *Saccharomyces cerevisiae,* Rlm1, which has transcriptional activity regulated by the Mpk1 (Slt2) mitogen-activated protein kinase pathway." *Mol Cell Biol* 17(5): 2615-2623.

Whiteway, M. and U. Oberholzer (2004). "*Candida* morphogenesis and host-pathogen interactions." *Curr Opin Microbiol* 7(4): 350-357.

Chapter 5

Concluding remarks and future perspectives

Concluding remarks

Candida albicans is mainly an opportunistic pathogen, whose incidence is increasing in immunocompromised patients. The cell wall, as the most external cellular component, plays a crucial role in the interaction with host cells mediating processes that are essential during infection, hence the importance of carrying out studies about cell wall molecules that participate in the virulence of this yeast. Cell wall has been characterized in the model organism *S. cerevisiae*, with which *C. albicans* shares many orthologous proteins, including Rlm1.

Rlm1 belongs to the type II MADS-box proteins family characterized by a genetically variable C-terminus due to the presence of a repetitive region of different sizes. Our results in Chapter 2 indicate that variability is due to a trinucleotide microsatellite encoding for glutamine, in which 35 alleles were indentified with repetitive units from 11 to 49. Another important point of this work is that strains with higher repetitions displayed more tolerance to cell wall stress agents. This characteristic may account for the different susceptibility pattern observed among strains. This diference could be explained if the interaction of accessory factors with Rml1 is somehow related with the C-terminus length, hence it is likely that polymorphisms in the microsatellite constrain the transcriptional activity.

In view of these results, in Chapter 3 it was proposed to evaluate the funcionality of the *RLM1* gene by using an approach based on mutant construction by the *SAT1*-flipping strategy. *Candida albicans rlm1Δ/rlm1Δ* mutant, the complemented and WT strains were exposed to several cell wall stress agents and compared with *S.cerevisiae rlm1Δ* mutant and WT strains. *Candida albicans rlm1Δ/rlm1Δ* mutant displayed phenotypes associated to cell wall deficiency such as hypersensitivity to CR, CFW and CFG. Caspofungin phenotype was reverted in the presence of 1M of sorbitol. These results and the homology with MADS-box transcription factors suggest an involvement in a regulatory role of cell wall remodeling. This regulatory role is further evidenced by the nuclear localization of Rlm1-GFP fusion protein observed in this study. We have also observed cytoplasmic granular fluorescence, suggesting that Rlm1 might be present in the cytoplasm as well. A possible recruitment of Rlm1 to the nucleus upon activation requires further confirmation. The *S. cerevisiae rlm1Δ* mutant displayed several phenotypic differences: insensitivity to CR and CFG, more resistance to CFW, and more sensitivity to SDS. These results indicate that the regulatory role of *C. albicans RLM1* presents differences when compared with *S. cerevisiae RLM1*. In accordance with a regulatory role, gene expression in the mutant lacking *RLM1* significantly activated genes involved in cell adhesion like *ECE1*, *ALS1*, *ALS3*, *HWP1 and RBT1,* and decreased transcription of genes involved in the catabolism of carbohydrates, *DAK2*, *GLK4*, *NHT1* and *TPS1*. The increased transcription of genes involved in cell adhesion was confirmed by adhesion and biofilm assays, suggesting that Rlm1 acts as a negative biofilm regulator.

In Chapter 4, we present results of *in vivo* and *in vitro* assays with mutant and wild-type strains of *C. albicans* to investigate the importance of the *RLM1* gene in virulence. Mice infected with the mutant strain presented a higher survival time than mice infected with the WT strain, these latter presenting

higher fungal burden and invasive micelial growth through tissues in hystopathological analysis of kidneys. In addition, decreased production of TNF-α, and lower cellular toxicity was observed in the absence of a functional Rlm1, suggesting that *RLM1* has a significant impact on *C. albicans* virulence. Finally, qRT-PCR analysis showed that the expression of the cell wall related genes, *CRH11* and *PHR2,* was clearly higher in the *rlm1Δ/rlm1Δ* mutant in relation to the WT strain, in mRNA of kidney samples after 7 days post-infection. Since these proteins are part of the cell wall and *in vivo* the yeast cells are under constant cell wall stress by the immune system it is conceivable that the mutant presents a higher expression of these genes, which may compensate the weakened cell wall. Overall, these results showed that the transcription factor Rlm1 is involved in the stability of the cell wall in the interaction with the host, being important for the virulence of *C. albicans* and invasion of the kidneys during hematogenously disseminated candidiasis.

Future perspectives

The studies performed in this work allowed to achieve the main objectives proposed for this thesis. In spite of the advances obtained in this work, regarding molecular and functional characterization *RLM1* gene, new questions have emerged that should be covered in the future.

Our studies regarding *RLM1* CAI microsatellites have demonstrated that this gene presents more than 35 alleles identified and classified into three different groups (I, II and III). We have shown that an increased number of repetitive units in the *C. albicans RLM1* correlate with resistance to stress agents. In addition, we showed that the *C. albicans RLM1* is involved in cell wall remodeling and is importance for the virulence. As the strain used (SC5314), presents both alleles belonging to group III, the construction of derived strain with *RLM1* alleles from groups I and II would complement the studies of the influence of alleles on resistance against cell wall stressing agents, virulence and on the interaction with the host immune system.

Previous studies with *S. cerevisiae* using LexA as a reporter gene showed that the ability of the Rlm1 protein to regulate transcription is dependent on the activation of the Pkc1-Slt2/Mpk1 pathway (Watanabe *et al.*, 1997; Jung *et al.*, 2002). As *C. albicans* possesses homologous of all kinases of this signaling pathway it would be important to investigate whether a similar interaction between Rlm1 and Mkc1 (the *C. albicans* Slt2/Mpk1 orthologue). The same strategy could be used with a strain bearing a construct to direct the expression of a hybrid protein in which the MADS box of *CaRLM1* is replaced with the DNA binding region of the bacterial LexA repressor (LexARlm1ΔN). The ability of the LexA-Rlm1ΔN protein to activate transcription would be monitored by using a *lacZ* reporter gene containing *lexA* DNA binding sites in its promoter. To show that Rlm1 is dependent on the CWI pathway, cells with or without cell wall stress conditions can be assayed, the activity of β-galactosidase being a measure of Rlm1 as transcription factor. Alternatively, interaction between Rlm1 and Mkc1 can be investigated by Western analysis with phosphorylated Rlm1-specific antibodies to show the phosphorylation state in cells under cell wall stress, using a wild type strain and a *mkc1Δ/mkc1Δ* mutant strain.

In agreement with a transcription factor function, we found evidence indicating nuclear localization of the Rlm1-GFP fusion protein. We have also observed cytoplasmic granular fluorescence, suggesting that Rlm1 might be present in the cytoplasm as well. It would be interesting to explore a possible recruitment of Rlm1 to the nucleus upon activation by cell wall stress agents such as calcofluor white and casporfungin. This investigation can be performed by immunofluorescence in stressed and non-stressed cells and by Western blot with GFP-specific antibodies with the cytoplasmic and nuclear fractions of similar samples.

Since that the microarray analysis showed that the absence of a functional *C. albicans RLM1* significantly decreased the transcription of genes involved in the catabolism of carbohydrates and increased genes involved in biofilm formation, it would be interesting to confirm these interactions with a different approach on selected genes. An approach based on the LexA-β-galactosidase reporter system can also be used; however Northern blot analysis or real time RT-PCR can contribute to reliable and fast results.

Since that the thermal shock phenotypes (Fig. 3.6B), survival time of infected mice (Fig. 4.2) and TNF-α levels production (Fig. 4.5A) were not totally complemented with the introduction of only one copy of the *RLM1* gene, the double complemented strain is under construction for further analysis to confirm the important of gene dosage for the reversion to the WT phenotype. As mentioned above, complemented strains can be constructed with different *RLM1* alleles, so that a collection of strains can be created with all combinations of alleles for studies of stress resistance and interaction with the host. In this way the importance of Rlm1 as a virulence factor can be further demonstrated which will open the perspective to new strategies of antifungal therapies targeting kinases of the CWI pathway and, in particular, the transcription factor Rlm1.

References

Jung, U. S., A. K. Sobering, M. J. Romeo and D. E. Levin (2002). "Regulation of the yeast Rlm1 transcription factor by the Mpk1 cell wall integrity MAP kinase." *Mol Microbiol* 46(3): 781-789.

Watanabe, Y., G. Takaesu, M. Hagiwara, K. Irie and K. Matsumoto (1997). "Characterization of a serum response factor-like protein in *Saccharomyces cerevisiae*, Rlm1, which has transcriptional activity regulated by the Mpk1 (Slt2) mitogen-activated protein kinase pathway." *Mol Cell Biol* 17(5): 2615-2623.

Appendix 1

Appendix 1. Rlm1 up-regulated and down-regulated genes, with ratios above 2.0.

Gene name	Description	YPD-Fold regulation
ECE1	Protein of unknown function; hyphal-specific expression increases with extent of elongation of the cell; regulated by Rfg1, Nrg1, Tup1, Cph1, Efg1, Hog1, farnesol, phagocytosis; may contribute to biofilm formation	68,9
HWP1	Hyphal cell wall protein; covalently crosslinked to epithelial cells by host transglutaminase; opaque- and a-specific, alpha-factor induced; at MTLa side of conjugation tube; assessment of virulence role complicated by URA3 effects	37,4
--	Predicted ORF in Assemblies 19, 20 and 21	24,3
--	Predicted ORF in Assemblies 19, 20 and 21; Plc1-regulated	17,0
ALS3	Adhesin; ALS family; role in epithelial adhesion, endothelial invasiveness; allelic variation in adhesiveness; immunoprotective in mice; promotes biofilm formation via binding to the SspB adhesin of S. gordonii	14,1
HGT10	Glycerol permease involved in glycerol uptake; member of the major facilitator superfamily; induced by osmotic stress, at low glucose in rich media, during cell wall regeneration; 12 membrane spans	13,0
--	Predicted ORF in retrotransposon Tca8 with similarity to the Gag region encoding nucleocapsid-like protein; transcription is downregulated in response to ciclopirox olamine; filament induced; regulated by Rfg1, Tup1; overlaps orf19.6078.1	12,4
RBT1	Cell wall protein with similarity to Hwp1, required for virulence; predicted to be cell-wall attached and glycosylated; Tup1 repressed; serum, hyphal and alkaline induced; farnesol, alpha factor induced; Rfg1-, Rim101-regulated	10,4
PGA25	Putative GPI-anchored protein of unknown function; fluconazole-downregulated	8,0
POL93	Predicted ORF in retrotransposon Tca8 with similarity to the Pol region of retrotransposons encoding reverse transcriptase, protease and integrase; downregulated in response to ciclopirox olamine; induced upon biofilm formation	7,7
SOU1	Enzyme involved in utilization of L-sorbose; has sorbitol dehydrogenase, fructose reductase, and sorbose reductase activities; has NAD-binding site motif; transcriptional regulation affected by chromosome 5 copy number	6,3
ALS1	Adhesin; ALS family of cell-surface glycoproteins; adhesion, virulence roles; immunoprotective; in band at hyphal base; amyloid domain; biofilm-induced; Rfg1, Ssk1, growth-regulated; strain background affects expression	6,1
WH11	Cytoplasmic protein expressed specifically in white phase yeast-form cells; expression in opaque cells increases virulence and frequency of opaque-to-white switching; null mutant shows wild-type switching; similar to *S. cerevisiae* Hsp12	5,4
--	Predicted ORF in Assemblies 19, 20 and 21	5,1
CTN1	Predicted carnitine acetyl transferase; required for growth on nonfermentable carbon sources but not for hyphal growth or virulence in mouse systemic model; transcription induced in macrophage; macrophage/pseudohyphal-repressed after 16h	4,9
--	Predicted ORF in Assemblies 19, 20 and 21; induced by Rgt1	4,6
--	Predicted ORF in Assemblies 19, 20 and 21; induced in core caspofungin response; increased expression observed in ssr1 homozygous null mutant; induced by nitric oxide in yhb1 mutant; protein detected by mass spec in stationary phase culture	3,8
CIT1	Protein described as citrate synthase; soluble protein in hyphae; biofilm induced; expression greater in high iron; upregulated upon phagocytosis; Hog1-downregulated; regulated by Efg1 under yeast-form but not hyphal growth conditions	3,8
GCV2	Putative protein of glycine catabolism; downregulated by Efg1; Hog1-induced; upregulated by Rim101 at acid pH; transcription is activated in the presence of elevated CO2; protein detected by mass spec in stationary phase cultures	3,7
--	Predicted ORF in Assemblies 19, 20 and 21	3,5
AGP2	Protein described as an amino acid permease; hyphal downregulated; regulated upon white-opaque switching; induced in core caspofungin response, during cell wall regeneration, or by flucytosine; fungal-specific (no human or murine homolog)	3,3
--	Predicted membrane transporter, member of the vesicular neurotransmitter (VNT) family, major facilitator superfamily (MFS); downregulated in core caspofungin response	3,3
PES1	Pescadillo homolog required for filament-to-yeast switching; mutation confers hypersensitivity to 5-fluorocytosine (5-FC), 5-fluorouracil (5-FU), and tubercidin (7-deazaadenosine)	3,2
--	Predicted ORF in Assemblies 19, 20 and 21	3,2

Gene name	Description	YPD-Fold regulation
SAP6	Secreted aspartyl proteinase; expressed during hyphal growth, oral carriage, infection; sap4 sap5 sap6 triple mutant is defective in protein utilization as N source; assessment of virulence role complicated by URA3 effects	3,2
FRE7	Protein similar to ferric reductase Fre10; transcription is downregulated in response to treatment with ciclopirox olamine or 17-beta-estradiol; transcriptionally induced by alkaline conditions or interaction with macrophage	3,2
FET3	Multicopper oxidase; required for growth under low-iron conditions; null mutant shows decreased prostaglandin E2 production; functional homolog of *S. cerevisiae* Fet3; ketoconazole, caspofungin, amphotericin B repressed	3,1
--	Putative guanyl nucleotide exchange factor with Sec7 domain, required for normal filamentous growth; transcriptionally regulated upon yeast-hyphal switch; filament induced; regulated by Nrg1, Tup1, Mob2; mRNA binds to She3	3,0
HAP2	CCAAT-binding factor regulates low-iron (chelation) induction of FRP1 transcription, and under these conditions CBF comprises Hap43 and probably Hap2 and Hap3; possibly an essential gene, disruptants not obtained by UAU1 method	3,0
--	Predicted ORF in Assemblies 19, 20 and 21; possibly spurious ORF (Annotation Working Group prediction); transcriptionally activated by Mnl1 under weak acid stress	2,9
TRM1	Protein described as an N2,N2-dimethylguanine tRNA methyltransferase; induced upon adherence to polystyrene	2,8
MAL31	Putative protein of unknown function, transcription is upregulated in clinical isolates from HIV+ patients with oral candidiasis; alkaline upregulated	2,8
HDA1	Histone deacetylase; conserved deacetylation motif; regulates white-to-opaque switching frequency but not opaque-to-white switching; greater expression in white cells than opaque cells; inhibited by trichostatin-A; inducer of filamentation	2,8
--	Predicted ORF in Assemblies 19, 20 and 21	2,7
CHS7	Protein required for wild-type chitin synthase III activity; similar to (but not functional homolog of) *S. cerevisiae* Chs7, which effects ER export of Chs3; induced in homozygous cyr1 null mutant hyphae and ras1 yeast-form cells	2,7
--	Predicted ORF in Assemblies 19, 20 and 21	2,6
UTP22	Predicted ORF in Assemblies 19, 20 and 21; decreased expression observed in an ssr1 homozygous null mutant; decreased expression in response to prostaglandins; heterozygous null mutant exhibits resistance to parnafungin	2,6
--	Predicted ORF in Assemblies 19, 20 and 21	2,6
--	Predicted ORF in Assemblies 19, 20 and 21; upregulation correlates with clinical development of fluconazole resistance	2,6
--	Predicted ORF in Assemblies 19, 20 and 21; increased transcription is observed in an azole-resistant strain that overexpresses MDR1; transcriptionally activated by Mnl1 under weak acid stress	2,6
OPY2	Predicted ORF in Assemblies 19, 20 and 21	2,6
--	Predicted ORF in Assemblies 19, 20 and 21	2,5
CCC1	Putative manganese transporter, required for normal filamentous growth; mRNA binds to She3 and is localized to hyphal tips; repressed by nitric oxide and alkaline pH; shows colony morphology-related regulation by Ssn6	2,5
ENA21	Predicted ORF similar to *S. cerevisiae* sodium transporters Ena1 and Ena5; Gcn4-regulated; flucytosine, amphotericin B, or ketoconazole-induced; osmotic stress-induced; overlaps orf19.5170.1, which is annotated as a blocked reading frame	2,5
--	Predicted ORF in Assemblies 19, 20 and 21; transcription detected in high-resolution tiling array experiments	2,5
--	Predicted ORF in Assemblies 19, 20 and 21; transcription detected in high-resolution tiling array experiments	2,4
LYP1	Predicted ORF in Assemblies 19, 20 and 21; amphotericin B induced; flucytosine repressed; possibly an essential gene, disruptants not obtained by UAU1 method	2,4
--	Predicted ORF in Assemblies 19, 20 and 21	2,4
TPO4	Putative sperimidine transporter; fungal-specific (no human or murine homolog)	2,4
--	ORF Predicted by Annotation Working Group	2,4
ACS1	Putative acetyl-CoA synthetase, similar to *S. cerevisiae* Acs1; upregulated in the presence of human neutrophils; fluconazole-downregulated; regulated by Nrg1 and Mig1; shows colony morphology-related gene regulation by Ssn6	2,3

Gene name	Description	YPD-Fold regulation
OPT6	Putative oligopeptide transporter; fungal-specific (no human or murine homolog); expression of OPT6, OPT7, or OPT8 does not suppress defect of mutant lacking Opt1, Opt2, and Opt3; alleles are nonidentical	2,3
--	Predicted ORF in Assemblies 19, 20 and 21	2,3
GAL4	Transcription factor with zinc cluster DNA-binding motif involved in control of glycolysis; ortholog of *S. cerevisiae* Gal4, but not involved in the regulation of galactose utilization genes; caspofungin repressed	2,3
SHE3	mRNA-binding protein that localizes specific mRNAs to daughter yeast-form cells and to hyphal tips; required for normal filamentous growth and host epithelial cell damage; ortholog of *S. cerevisiae* She3 but set of target mRNAs differs	2,3
PUT2	Putative delta-1-pyrroline-5-carboxylate dehydrogenase; alkaline upregulated; protein detected by mass spec in exponential and stationary phase cultures	2,2
FLU1	Multidrug efflux pump of the plasma membrane; MDR family member of the MFS (major facilitator superfamily) of transporters; preferred substrate may be mycophenolic acid; fungal-specific (no human or murine homolog)	2,2
--	Predicted ORF in Assemblies 19, 20 and 21	2,2
CUP9	Protein of unknown function, upregulated in clinical isolates from HIV+ patients with oral candidiasis; transcription reduced upon yeast-hyphal switch; ketoconazole-induced; Plc1-regulated; shows colony morphology-related Ssn6 regulation	2,1
YTM1	Protein similar to *S. cerevisiae* Ytm1, which is involved in biogenesis of the large ribosomal subunit; transposon mutation affects filamentous growth; protein level decreased in stationary phase cultures	2,1
NRP1	Predicted ORF in Assemblies 19, 20 and 21; fungal-specific (no human or murine homolog)	2,1
--	Predicted ORF in Assemblies 19, 20 and 21	2,1
CDR11	Putative transporter of PDR subfamily of ABC family; Gcn4-regulated; upregulated by Rim101 at pH 8	2,1
ARP8	Predicted ORF in Assemblies 19, 20 and 21; mutation confers hypersensitivity to toxic ergosterol analog, and to amphotericin B	-2,1
CYB2	Protein described as precursor protein of cytochrome b2; transcriptionally regulated by iron; expression greater in high iron; alkaline downregulated; shows colony morphology-related gene regulation by Ssn6	-2,1
--	Predicted ORF in Assemblies 19, 20 and 21	-2,1
NTH1	Neutral trehalase; hyphal induction in homozygous null mutant is delayed but not reduced overall; not required for virulence in mouse systemic infection; homodimeric enzyme; possible regulatory cAMP-dependent phosphorylation at S10 and S213	-2,1
RSN1	Protein induced during the mating process	-2,2
--	Predicted ORF in Assemblies 19, 20 and 21	-2,2
--	Predicted ORF in Assemblies 19, 20 and 21	-2,2
IFI3	Predicted ORF in Assemblies 19, 20 and 21; constitutive expression independent of MTL or white-opaque status	-2,2
TPS1	Trehalose-6-phosphate synthase; role in hyphal growth and virulence in mouse systemic infection; upregulated in presence of human neutrophils; macrophage/pseudohyphal-repressed after 16h; detected by mass spec in stationary phase cultures	-2,2
CUP2	Protein required for normal resistance to copper; similar to *S. cerevisiae* Cup2, which is a copper-binding transcription factor that activates transcription of metallothionein genes	-2,2
RIB3	3,4-Dihydroxy-2-butanone 4-phosphate synthase; homodimeric enzyme of riboflavin biosynthesis; converts ribulose 5-phosphate to L-3,4-dihydroxy-2-butanone 4-phosphate; transcription regulated on yeast-hyphal switch, macrophage interaction	-2,3
--	Predicted ORF in Assemblies 19, 20 and 21	-2,3
HNT1	Predicted ORF in Assemblies 19, 20 and 21; protein level decreased in stationary phase cultures	-2,3
--	Predicted ORF in Assemblies 19, 20 and 21; transcription is repressed in response to alpha pheromone in SpiderM medium	-2,3
GLK4	Protein described as a glucokinase; decreased expression in hyphae compared to yeast-form cells	-2,3
MET3	Putative ATP sulfurlyase of sulfate assimilation; repressed by Met or Cys, Sfu1, or in fluconazole-resistant isolate; strongly induced on biofilm formation, even in presence of Met and Cys; Hog1-, caspofungin-, possibly adherence-induced	-2,4

Gene name	Description	YPD-Fold regulation
NAT4	Putative histone acetyltransferase, involved in regulation of white-opaque switching; predicted ORF in Assemblies 19, 20 and 21	-2,4
--	Predicted ORF in Assemblies 19, 20 and 21; increased transcription is observed upon benomyl treatment	-2,4
DAK2	Protein described as similar to dihydroxyacetone kinase; transcription is decreased upon yeast-hyphal switch; fluconazole-induced; caspofungin repressed; protein detected by mass spec in stationary phase cultures	-2,4
--	Predicted ORF in Assemblies 19, 20 and 21; possibly spurious ORF (Annotation Working Group prediction)	-2,4
MSO1	Predicted ORF in Assemblies 19, 20 and 21; expression is down-regulated during pseudohyphal growth in the presence of lysed macrophages	-2,5
--	Transcriptionally regulated by iron; expression greater in high iron; downregulated in core caspofungin response; ketoconazole-repressed; shows colony morphology-related gene regulation by Ssn6; possibly subject to Kex2 processing	-2,5
GPD1	Protein similar to *S. cerevisiae* glycerol-3-phosphate dehydrogenase (enzyme of glycerol biosynthesis); biofilm-induced expression; regulated by Efg1; regulated by Tsa1, Tsa1B under H2O2 stress conditions	-2,5
--	Predicted ORF in Assemblies 19, 20 and 21; induced during planktonic growth	-2,5
--	Predicted ORF in Assemblies 19, 20 and 21; Hog1-downregulated	-2,6
SOD1	Cytosolic copper- and zinc-containing superoxide dismutase, involved in protection from oxidative stress and required for full virulence; alkaline upregulated by Rim101; upregulated in the presence of human blood	-2,6
--	Predicted ORF in Assemblies 19, 20 and 21; shows colony morphology-related gene regulation by Ssn6	-2,6
--	Predicted ORF in Assemblies 19, 20 and 21; possibly an essential gene, disruptants not obtained by UAU1 method	-2,6
IFC3	Oligopeptide transporter; transcriptionally induced upon phagocytosis by macrophage; induced by BSA or peptides; fluconazole-induced; upregulated by Rim101 at pH 8; virulence-group-correlated expression; no human or murine homolog	-2,7
--	Predicted ORF in Assemblies 19, 20 and 21; induced in core caspofungin response; increased expression observed in an ssr1 homozygous null mutant; induced by nitric oxide in yhb1 mutant	-2,7
RAD32	Protein similar to *S. cerevisiae* protein with role in nucleotide excision repair; down-regulation associated with azole resistance	-2,9
FDH3	Putative protein of glycine catabolism; downregulated by Efg1 under yeast-form but not hyphal growth conditions; transcriptionally activated by Mnl1 under weak acid stress	-2,9
--	Predicted ORF in Assemblies 19, 20 and 21; induced upon adherence to polystyrene; oxidative stress-induced via Cap1	-3,1
RNR22	Protein described as ribonucleoside diphosphate reductase; shows colony morphology-related gene regulation by Ssn6; RNA abundance regulated by tyrosol and cell density	-3,4
--	Transcription is positively regulated by Sfu1	-6,5
--	Predicted ORF in Assemblies 19, 20 and 21; fluconazole-induced; filament induced; Hog1-induced; regulated by Nrg1, Tup1; increased expression in response to prostaglandins	-7,2
LYS12	Protein described as mitochondrial homoisocitrate dehydrogenase; clade-associated gene expression; protein level decreased in stationary phase cultures	-20,1