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Human, mouse, fly and yeast *GUP1* orthologues in *Candida albicans*.

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Abstract

In *C. albicans* the deletion of *GUP1* causes drastic alterations at both morphologic and physiologic levels, compromising several virulence factors, such as (i) peculiar colony morphology, (ii) filamentous growth defects, (iii) impairment on the ability of cells to adhere, invade and form biofilms, and (iv) resistance to the antifungal agents.

Gup1 is a yeast *O*-acyltransferase with close homologues in higher Eukaryotes, which has been implicated in the regulation of the Hedgehog pathway in mouse. Based on that, a primarily aim for the present work concerned the creation of a strain collection of *C. albicans* Δ*gup1* mutant harboring available *GUP1* homologues from well known model organisms/higher Eukaryotes: yeast *Saccharomyces cerevisiae (ScGUP1),* Fly *Drosophila melanogaster (DmGUP1),* Mouse *Mus musculus (MmGUP1),* and Man *Homo sapiens (HsGUP1)*. These transformed *C. albicans* strains were tested for several virulence factors, such morphology and differentiation, agar adherence and invasion capacities, and resistance to antifungals agents. Another important purpose of this study was to obtain for the first time a detailed characterization of the proteins secreted onto the *C. albicans* extracellular matrix. For that we imitated biofilm formation on plate and compare the wt, Δ*gup1 mutant* and transformants.

Our results suggest an apparent initial development of *hyphae*/pseudoh*yphae* cells in liquid cultures, when the *GUP1* homologues are inserted into Δ*gup1* mutant strain, yet the same strains are unable to give continuity to that differentiation. In agreement, on solid cultures those transformant strains, though displaying a colony morphology closer to wt strain were powerless to differentiate into hyphas. Moreover all the *Ca*Δ*gup1GUP1* homologues showed the same impairment displayed by Δ*gup1*mutant strain, in what regards adherence and agar invasion capacities. Concerning antifungal resistance, it seems that only *DmGUP1* gene complements Δ*gup1* mutation. Additionally, the analysis of Δ*gup1* mutant extracellular matrix protein profile revealed lack of some proteins, presence of other that were not on wt profile and different concentration of some of the common proteins, suggesting that the lack of *Gup1p* triggers *C. albicans* to produce or to excrete distinct proteins. In conclusion, our results open a field of options to future research, mainly regarding proteomics of ECM and its relation to the virulence of *C. albicans.*

Resumo

A deleção do gene *GUP1* em *C. albicans* provoca-lhe alterações drásticas tanto a nível morfológico como fisiológico, comprometendo vários factores de virulência tais como a capacidade de desenvolver hifas, a capacidade de aderir invadir e formar biofilmes, alterações de morfologia nas colónias bem com um aumento da resistência a antifúngicos.

A proteína Gup1p é uma *O*-aciltransferase presente em leveduras e que partilha uma elevada homologia com os eucariotas superiores, tendo sido descrita como essencial na regulação da via Hedgehog, em ratinhos. Com base nestes conhecimentos, foi proposto como 1° objectivo deste trabalho a criação de uma colecção de estirpes de *C. albicans* mutada no gene *GUP1* complementada com vários homólogos do *GUP1* de eucariotas superiores: levedura *Saccharomyces cerevisiae (ScGUP1)*, mosca *Drosophila melanogaster (DmGUP1*), ratinho - *Mus musculus (MmGUP1)*, humano -*Homo sapiens (HsGUP1)*. Nestas novas estirpes foram testados vários factores de virulência: morfologia, capacidade de diferenciação em filamentos, aderência e invasão ao agar, e ainda a resistência a antifúngicos. Um outro objectivo importante deste trabalho consistiu na caracterização detalhada das proteínas secretadas para a matriz extracelular da *C. albicans*. Para tal simulou-se a formação de biofilmes em placas comparando as estirpes wt e mutada no *GUP1*.

Os resultados obtidos mostram que os vários homólogos do *GUP1* quando inseridos na estirpe mutada tiveram a capacidade de iniciar diferenciação em hifas/pseudohifas, em meio líquido. No entanto, não foram aptas para dar continuidade à referida diferenciação. Do mesmo modo, em meio sólido as colónias destas estirpes foram morfologicamente mais semelhantes à wt que ao mutante *GUP1*, sem no entanto apresentarem hifas. Para além disso, nenhum dos transformantes mostrou capacidade de aderir ou invadir o agar. Quando testada a sua resistência a antifúngicos apenas a estirpe complementada com o *DmGUP1* (da mosca) reverteu o fenótipo do mutante *GUP1*. Por fim, a análise das proteínas da matriz extracelular da wt e do mutante *GUP1* revelou perfis proteicos muito distintos, presença de diferentes proteínas e também em diferentes quantidades, o que sugere que a ausência do *GUP1* provoca uma alterada excreção de proteínas.

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Background

1. *Candida* and Candidosis

Since the beginning of the 80s, fungi, mainly *Candida* yeast species have emerged as important causes of serious human infections (Blumberg et al, 2001). This is particularly true among the immuno-compromised patients and those hospitalized with serious sickness (Rees et al, 1998). The rise of infections caused by *Candida* species (candidosis) are largely due to a limited number of sources: increasing number of AIDS seropositives, transplants, novel cancer treatments, and a widespread use of large-spectrum antibiotics and indwelling medical devices (Pfaller et al, 2007). In the early 90s, the introduction of triazoles like fluconazole appeared like a promising option to treat systemic fungal infections (Trick et al, 2002). Still, the incidence of mortality rates associated with this infectious disease remains the same for more than a decade. This has been explained not only by the growing resistance of *Candida* species to the antifungal agents, as to delays in the administration of appropriate antifungal therapy (Pfaller et al, 2007). Moreover, despite the fact that *Candida albicans* is the most common species causing candidosis, approximately 66%, other *non albicans Candida* species have emerged as potential human pathogens: *C. glabrata* (≈12%), *C. parapsilosis* (≈11%), *C. tropicalis* (≈10%) and *C. krusei* (≈1%) (Trick et al, 2002).

C. albicans is a normal commensal of human microflora, residing at the gastrointestinal tract, the oral cavity, the vaginal and urinary environment, yet this organism is also an opportunistic pathogen (Pfaller et al, 2007). This diploid yeast, thought until recently to be asexual (Bennett et al, 2003), causes infections such as denture stomatitis, thrush and urinary tract-infections, but can also provoke more severe systemic infections (Pfaller et al, 2007). The severity of such infections depends on the immune mechanisms of the host that are impaired (Calderone et al, 1997). These can be generally separated in: (i) mucosal infections and (ii) blood stream infections. Mucosal infections, medically called pseudomembranes candidosis are characterized by white spots and affect vaginal, esophageal, oral and gastrointestinal mucosae. Blood stream infections, also known as candidemia, affect individuals who have deficient number of neutrophils *i.e*. immuno-compromised. Candidemia of the internal organs it's called disseminated candidosis, and it is a serious medical condition that leads to mortality rates between 30% and 50% (Sudbery et al, 2011).

The pathogenicity of *C. albicans* is based on several attributes of this species, denominated virulence factors, and on the development of specific strategies that assist in the yeast ability to colonize host tissues, cause disease, and overcome host defences (Naglik et al, 2003). These include (i) the production of certain proteins-secreted hydrolytic enzymes, (ii) the dimorphic transition (morphogenetic conversion from budding yeast into filamentous yeast), the ability to switch between different cell phenotypes commonly named as phenotypic switching, (iii) the production of invasive biomolecules such proteases and phospholiases, which enable *C. albicans* to adhere and invade different inert and biological substrates, (iv) the ability to form biofilms, (iv) and the immunomodulation of the host defense and the antigenic variability (Martinez et at, 1998; Calderone et al, 1997). This complexity, agrees with an opportunistic infectious agent life cycle, and therefore with *C. albicans* considerable genome plasticity (Sudbery et al, 2011). An example is the appearance of an isochromossome V that carries genes involved on the expression of ergosterol pathway enzymes targeted by azole drugs (Selmecki et al, 2006).

1.1 Virulence factors/morphogenesis in *C. albicans*

C. albicans morphogenic transition is defined as the switch between unicellular yeast cells and a filamentous growth form (Calderone et al, 2001). In the yeast form, the cells reproduce by budding-off daughter cells that typically disassociate from the mother cell. In the filamentous or hyphal mode, the cells continually grow at a tip which leads to an elongated tube-like structure – *hyphae* – made of multiple contiguous cells separated by *septae* (Whiteway et al, 2007). In addition this fungus can naturally occur in other morphological forms characteristic of specific cellular functions, namely the *opaque form* - described as characteristic of mating-competent cells (Soll, 2004), and the *chlamydospore form* - characterized by a ticked cell wall typically formed under suboptimal growth conditions (Fabry et al, 2003), and the *pseudohyphal form* consisting of the state between yeast and hyphal form, which coexists with the yeast and hyphal forms during infections (Sudbery et al, 2004).

Fungal invasion has been described to depend on these changes in morphology especially *hyphae* growth, which tip extension can generate significant tip pressures for

tissue penetration (Gow et al, 2002). Different morphological forms confer distinct properties that have advantages at different stages and sites of infection. During the earliest stages of infection, *C. albicans* forms *hyphae*. Once infection is established these are replaced by both yeast cells and pseudo*hyphae* (Gow et al, 2002).

The first studies on *C. albicans* filamentous growth ignored the pseudo*hyphae* state, or tended to consider it as an intermediate stage between yeast and true hyphal growth forms. Sudbery and collaborators work (Sudbery et al, 2004) described a systematic evaluation of these morphological states, having into account the next parameters;

- Cell shape: *hyphae* have no constrictions at the neck of mother cell and have parallel side in all entire length; pseudo-*hyphae* have constrictions at neck of mother cell and the length and width vary enormously.

- Cell cycle: is fundamental for the mode of growth and organization, and is related to the location of the first mitoses within the germ tube (*hyphae*) or across the bud neck (pseudo-*hyphae*).

It has been established also that the environmental conditions that favor *hyphae*, yeast or pseudo*hyphae* growth are different (Odds, 1988). Hyphal growth is promoted at conditions such as: growth at 37°C, presence of serum, neutral pH, high CO_2 concentrations (Sudbery et al, 2011) and presence of N-acetylglucosamine (Cassone et al, 1985). On the other hand, yeast growth is favored by a growth at lower temperatures 30°C (or under) and acidic pH (4.0).

Hyphal development in *C. albicans* is regulated by at least five different pathways: i) the conserved pH signaling pathway, that activates hyphal growth in response to environment pH, (ii) the morphogenetic pathway induced by the transcription factor Cph2p, (iii) the hyphal formation pathway, stimulated by Czf1p, (iv) the basic helix-loop-helix Efg1p transcription factor, involved in the Ras-cAMP signaling pathway that responds to nutrient deprivation and, (v) the mitogen activated protein (MAP) kinase pathway that activates hyphal development in response to nutrient limitation (reviewed by Gow et al, 2002).

Hyphae and pseudo-*hyphae* formation is the ultimate phenotype of polarized growth. In the yeast form, polarized growth is defined by a crescent-shaped polarisome at the tip of the growing bud (**Fig. 1**) (Whiteway et al, 2007). The processes of polarized growth in *C. albicans* yeast form and pseudo-*hyhae* formation in *S. cerevisiae* are similar. It occurs through formation of secretory vesicles that are transported to a specific site of polarized growth, which is marked by cortical markers (Sudbery et al, 2011). These activate Cdc42, a protein involved in the regulation of the cell cycle, resulting in the formation of the polarisome, the exocyst and a septing ring. The septin ring organizes the arrangement of the primary and the secondary *septae* between the mother and daughter cells. The polarisome initiates the formation of actin filaments along which post-Golgi secretory vesicles are delivered to the sites of polarized growth, and where they dock with the exocyst before fusing with the plasma membrane (**Fig. 1**) (Sudbery et al, 2011). In *hyphae* forms, the polarized growth is considerably different. There is a structure rich in secretory vesicles formed at the apex, called Spitzenkörper or the tip body (Sudbery et al, 2011).

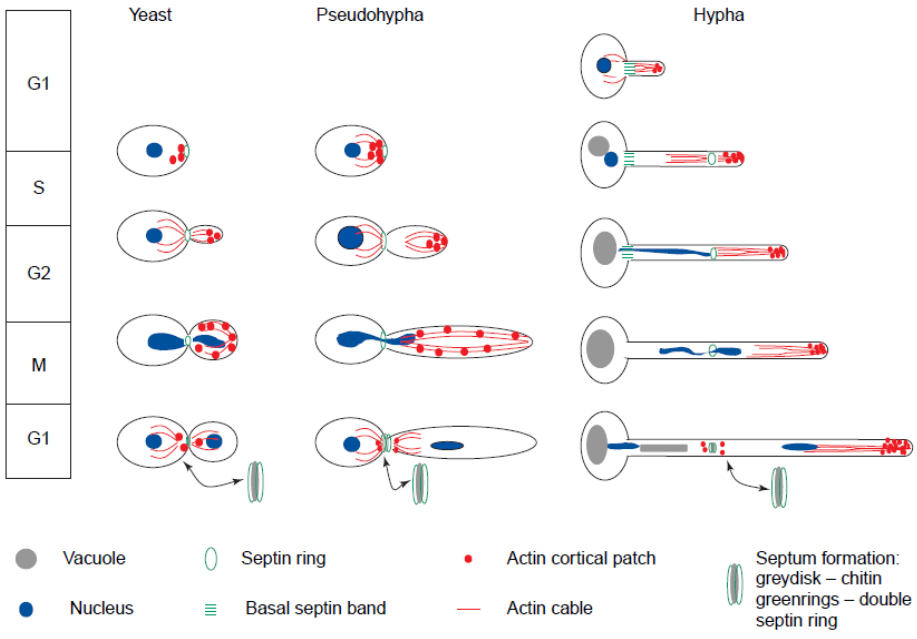

Fig. 1. Cell cycle of yeast and the first cell cycle of *hyphae* and pseudo*hyphae*. Extract from Sudbery et al, 2004.

1.2 *C. albicans* biofilms

The ability to form biofilms it is intimately associated with the ability to cause infection and is considered an important virulence determinant during candidosis. Candidosis are frequently seeded from biofilms developed on medical devices (Nobile et al, 2009). The most common *Candida* associated with biofilm formation is *C. albicans* (Ramage et al, 2005). *C. albicans* can colonize and develop a biofilm on almost any medical device (Uppuluri et al, 2009), including prostheses, implants, several types of catheters, etc. Furthermore, the formation of biofilms in medical devices besides serving as a source for infections, can also cause the failure of the device (Nett et al, 2006).

1.2.1 Formation and features of *C. albicans* biofilms

Biofilm formation is a process in which microorganisms irreversibly attach and grow on a surface producing and secreting polymers that facilitate the attachment and a matrix formation. This corresponds to a change in the microorganism growth rate and gene transcription profile (Donlan, 2001).

The progression to a mature biofilm depends of three factors: cell adhesion, extracellular matrix production and transition yeast-to-*hyphae* (Nett et al, 2006). In biofilm formation cell initially adhere to biomaterial surfaces (Li et al, 2003). The characteristics of the *substratum* could affect the rate and extend of the attachment. In general, biofilms develop more quickly in rougher and more hydrophobic materials (Donlan, 2001). The attachment of *Candida* to biomaterials is mediated by nonspecific factors like hydrophobicity and electrostatic forces, and by specific adhesins that recognize ligands in the conditioning films, like serum or salivary proteins (Ramage et al, 2005). A mature biofilm of *Candida* species is composed by a complex three-dimensional structure and displays extensive spatial heterogeneity (Ramage et al, 2005). The biofilm acts like a "filter" to entrap, among others, nutrients and water and facilitate their influx as well as the disposal of waste products throughout the many layers of cells (Ramage et al, 2005). Observations through Scanning Electron Microscopy (SEM) reveal the fully mature *C. albicans* biofilms as a dense network of yeasts, *hyphae*,

pseudo*hyphae*, as well as extracellular polymeric material, the matrix (Ramage et al, 2005; Hawser et al, 1994).

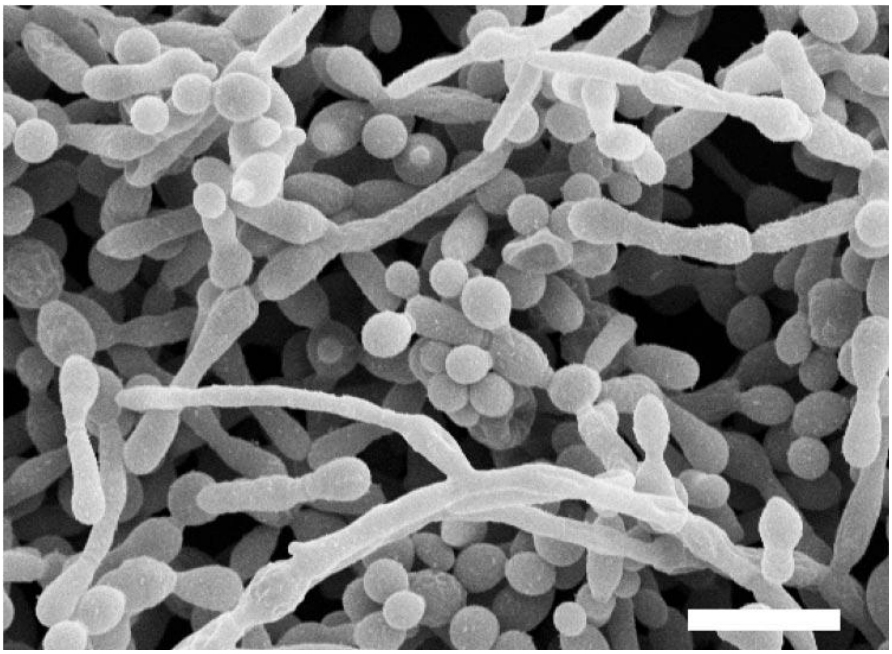

Fig. 2. SEM image of mature *C. albicans* biofilm. Bar corresponds to 10 µm. Extracted from Ramage *et al*, 2005.

1.2.2 Morphogenic conversion in *C. albicans* biofilm

It is widely recognized that morphogenesis plays a pivotal role in the development of *C. albicans* biofilms (Ramage et al, 2005). Baillie and collaborators demonstrated that *hyphae* are essential elements of the architecture and structural integrity of mature biofilm (Baillie et al, 1999). In this study they compare the biofilms produced by two wild-type (wt) strains with those formed by two mutants, incapable of yeast and hyphal growth, respectively. An analysis using SEM revealed that the biofilms of the wt strains consists in two different layers: a basal yeast layer and a thicker hyphal layer. On the other hand, the *hyphae*-mutant produced only the basal layer and the yeast mutant formed only a thicker layer. The biofilms of the yeast mutants were more easily detached that the ones formed by wt strains, which suggested that the basal yeast layer is crucial for the anchoring of the biofilm to the surface (Baillie et al, 1999). More recently our group found that *Cagup1Δ* null mutant strain was not able to form typical biofilm structures, presenting much less *hyphae*/pseudo-*hyphae* comparing to wt

(Ferreira et al, 2010). Still, dimorphism may not be an absolute prerequisite for biofilm formation, since biofilms harboring only yeast cells have been described (Ramage et al, 2010).

Very little is still known about the regulation of biofilm formation, yet, some genetic studies have been made. The regulator Efg1 protein was described as a key factor in the formation and subsequent development of mature *C. albicans* biofilms in a variety of biological and artificial surfaces (Ramage et al, 2002) and Zap1p, a zinc-responsive regulatory protein as a negative regulator of biofilm maturation (Nobile et al, 2009).

C. albicans quorum sensing modulates all stages of biofilm life cycles: attachment, maturation and dispersal (Donlan, 2002; Deveau et al, 2011). Farnesol, a *C. albicans* autoregulatory molecule that regulates the transition between yeast and *hyphae* form, is the best characterized *quorum* sensing molecule, and leads to a reduction on the biofilm sizes (Hornby et al, 2001; Deveau et al, 2011).

1.2.3 Biofilms resistance to antifungals

The increased resistance of biofilm-associated infections to antimicrobial agents is a serious problem for public health (Donlan, 2001). Presently, there are four groups of drugs available to treat fungal infections: (i) the antifungal agents that inhibit macromolecule synthesis (*e.g.* flucytosine), (ii) the ones that impair membrane barrier function (nystatin and amphotericin), (iii) the inhibitors of ergosterol synthesis (azoles derivates such as clotrimazole, ketoconazole or fluconazole) and, (iv) drugs that interact with microtubules (*e.g*. Griseofulvin) (Bossche, 1997).

Hitherto, it looks like that no class of antifungal agent is immune to the development of resistance. Therefore it becomes necessary to consider the development of a systematic program of *in vitro* susceptibility testing, in order to take therapeutic decisions (Pfaller et al, 2007). Several works showed *Candida* biofilms high levels of resistance to antifungal agents and proposed explanations. It has been suggested, for instance that the matrix of extracellular polymeric material would exclude or limit the access of the drug (Al-Fattani et al, 2006). Still, in another study, Al-Fattani and collaborators (Al-Fattani et al, 2006) found that the matrix did not constitute a barrier for 5 clinically used antifungals of different chemical structure, suggesting that the matrix plays a minor role

in drug resistance. In their study, they compared biofilms incubated without agitation (that have little matrix) with biofilms incubated with gentle shaking (produce more matrix material) and these did not show significant differences in susceptibility to any of the drugs tested (Al-Fattani et al, 2006). Other mechanism pointed to have a role on *Candida* biofilms resistance was the nutrient limitation and growth impairment, since mature biofilms are more resistant to antifungals (Ramage et al, 2005).

1.3 Extracellular matrix (ECM)

One of the most important features of biofilms is the extracellular matrix. ECM is a three-dimensional highly hydrated gel-like environment where the microorganims are mainly immobilized (Flemming et al, 2000).

The composition of the matrix varies according to the nature of the organisms present. For instances, exopolysaccharides are the most abundant matrix polymers of bacterial biofilms. These can be attached to carboxyl, sulphate or phosphate groups becoming negatively charged. Nucleic acids, lipids, uronic acid and proteins can also be present but in smaller amounts (Al-Fattani et al, 2006). The ECM of mammalian cells is mainly constituted by a large variety of fibrous proteins (such as glycoproteins and proteoglycans) and glycosaminoglycans, although it varies from tissue to tissue (Mecham, 2011). In terms of functions, mammalian ECM is much more sophisticated and diverse than the ECM of yeasts, being for instance essential on the repair of the tissue (Mecham, 2011). In *C. albicans* ECM were identified structures called “towers”, which are microcolonies of the matrix, separated by water channels that allow an efficient nutrient circulation within the biofilm (Donlan et al, 2002).

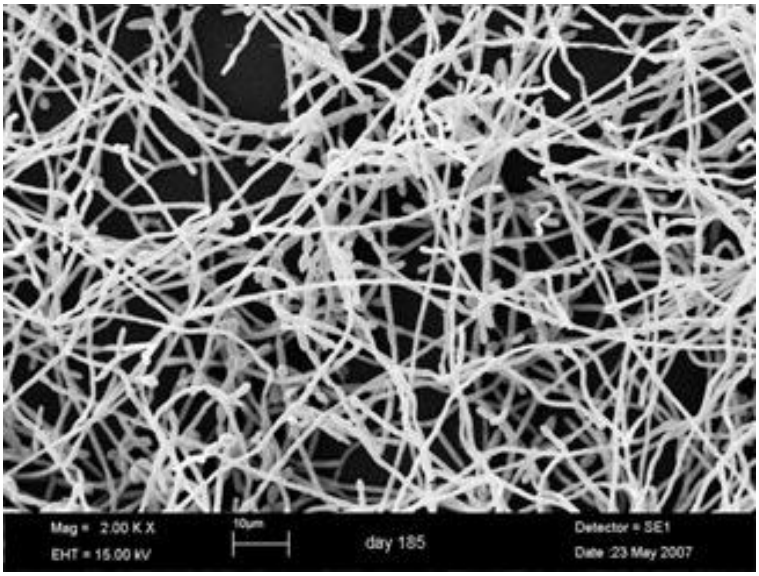

Fig. 3. *C. albicans* forms dense biofilms composed of yeast cells, *hyphae*, and extracellular matrix. Images produced using SEM. (ref:http://hsc.unm.edu/som/medicine/id/msclinic.shtml).

More recently, it was proposed that extracellular DNA is also a component of *C. albicans* ECM and that it may play a crucial role in biofilms structural role, based on the observation that the addition of exogenous DNA increases the biofilm mass and conversely, when treated with DNase the biofilm mass decreases (Martins et al, 2010). Some extracellular polysaccharides and proteins have been reported to be essential components of the matrix (Sutherland, 2001). Also dead cells have been observed in some biofilms, which suggests that cell debris can accumulate in the ECM (Webb et al, 2003). Possibly the extracellular DNA derives from these dead cells. Furthermore, it was recently described that the matrix has a function of cellular protection from surrounding environment, not only by preventing the uptake of drugs, by being a shield for other kinds of stress, but also by acting as a maintainer of the architectural stability of the biofilm (Nobile et al, 2009). Matrix production is described in close link to biofilm formation (Nobile et al, 2009). It is known that the ECM abundance in *C. albicans* can be regulated positively or negatively by glucoamylases and alcohol dehydrogenases, for instance Gca1p, Gca2p and Adh5p which act as positive regulators, while Cshp and Ifd6p act as negative regulators (Nobile et al, 2009). In the study mentioned above, Al-Fattani and collaborators (Al-Fattani et al, 2006) showed that the synthesis of ECM material is dramatically increased during the biofilm development, which is subjected to a liquid flow and shaking, in opposition to minimal ECM synthesis under static incubation conditions. Despite this understanding, still very little is known about its regulation or production mechanisms (Nobile et al, 2009).

2. *GUP1*

GUP1 gene was firstly described in *S. cerevisiae* encoding a multi-membrane-spanning protein essential for glycerol active uptake by proton symport (Holst et al, 2000). This was based on the fact that the deletion of *GUP1* resulted (i) on a slow grow on glycerol as sole carbon and energy source, (ii) on a diminished ability to use extracellular glycerol for rescuing form osmotic stress and (iii) on a defect on active uptake of glycerol and metabolism (Holst et al, 2000).

Soon after, Gup1p was included on the MBOAT (membrane-bound O-acetyl transferases) family (Hoffman, 2000; Neves et al, 2004), and involved in several processes connected with cell structure organization and biogenesis. It was described as having a role in the bipolar site selection, and several proteins sorting (Ny et al, 2001; bonangelino et al, 2002), cytoskeleton polarization (Ny et al, 2001), vacuole morphology (Bonangelino et al, 2002) telomere length maintenance (Askree et al, 2004) and plasma membrane composition evidenced by a reduction in phospholipids and an increase in triacylglycerols (Oelkers et al, 2000).

This pleiotropism suggested the role of this protein in cellular processes to be of a complex nature. Further ahead, *GUP1* was associated to cell wall morphology, synthesis and integrity (Ferreira et al, 2006). This is reinforced by the described *GUP1* genetic interactions with genes associated with chitin and β1,6-glucan synthesis, including *SKT5* (activator of a chitin synthase involved in cytokinesis), *RSV161* and *RSV167* (both involved in actin polarizaton) (Ferreira et al, 2006). *GUP1* gene interferes in lipid metabolism leading to deep alterations on sphingolipid-sterol ordered domains integrity/assembly as well as an abnormal sterol distribution at the level of plasma membrane (Ferreira et al, 2008).

Ergosterol is an important constituent of membranes being target of common antifungals like azoles and polyenes (Pasrija et al, 2005). Several important roles have been attributed to sterol/sphingolipid-rich domains in dynamics processes such as protein sorting during exo- and endocytoses, cell polarity and signaling (Pasrija et al, 2005). Ferreira and Lucas (Ferreira et al, 2008) showed *Scgup1Δ* mutant moderate sensitivity to sphingolipids biosynthesis inhibitors (SBIs), but a high resistance to ergosterol biosynthesis inhibitors (EBIs), including azoles. Following, Gup1p was

directly implicated in GPI anchors remodeling (Bosson et al, 2006). The authors showed that in Δ*gup1* mutants the incorporation of ceramide in the anchors was disturbed, consequently the transport of the GPI protein Gas1p from endoplasmatic reticulum to Golgi was slower and part of it was lost into the medium (Bosson et al, 2006).

All these observations clearly associating ScGup1p with lipids metabolism, with consequences on the resistance to antifungals (Ferreira et al, 2008), as well as with cell wall constitution, morphology and assembly (Ferreira et al, 2006), stress a putative importance for the biology of *C. albicans,* in particular, the switch from commensal to pathogenic nature and the subsequent increased resistance to antifungal drugs.

In the last years, our group started to study *GUP1* gene in *C. albicans*. We have showed that *GUP1* gene clearly has a role in *C. albicans* virulence (Ferreira et al, 2010). The deletion of *GUP1* in *C. albicans* changes ergosterol plasma membrane constitution/distribution, presenting an increased resistance to azoles. More importantly, *CaGup1p* strongly interferes with the capacity of cells to develop *hyphae*, to adhere, to invade and to form biofilms, all of which considered significant virulence factors. Moreover, when treated with macrophages, the mutant *Ca*Δ*gup1* was much easily phagocytosed than wt strain and its survival was clearly impaired (Ferreira et al, 2010).

2.1 *GUP1* homologues

Recently *GUP1* gene attracted a great deal of attention because its mouse homologue was described as a negative regulator of the N-palmilotylation of Sonic hedgehog pathway from higher Eukaryotes (Abe et al, 2008), which controls morphogenesis, differentiation and patterning during embryogenesis, including proliferation and cell fate. This highly stresses the roles of Gup1 in cellular morphology and division control and comes into accordance with the results in yeast suggesting the existence of a putative Hh-like pathway in lower Eukaryotes.

Using the gene and protein databases available, it is possible to identify the existence of *GUP* genes and Gup proteins in many organisms, including *Drosophila melanogaster, Mus musculus* and *Homo sapiens*, which we chose to study.

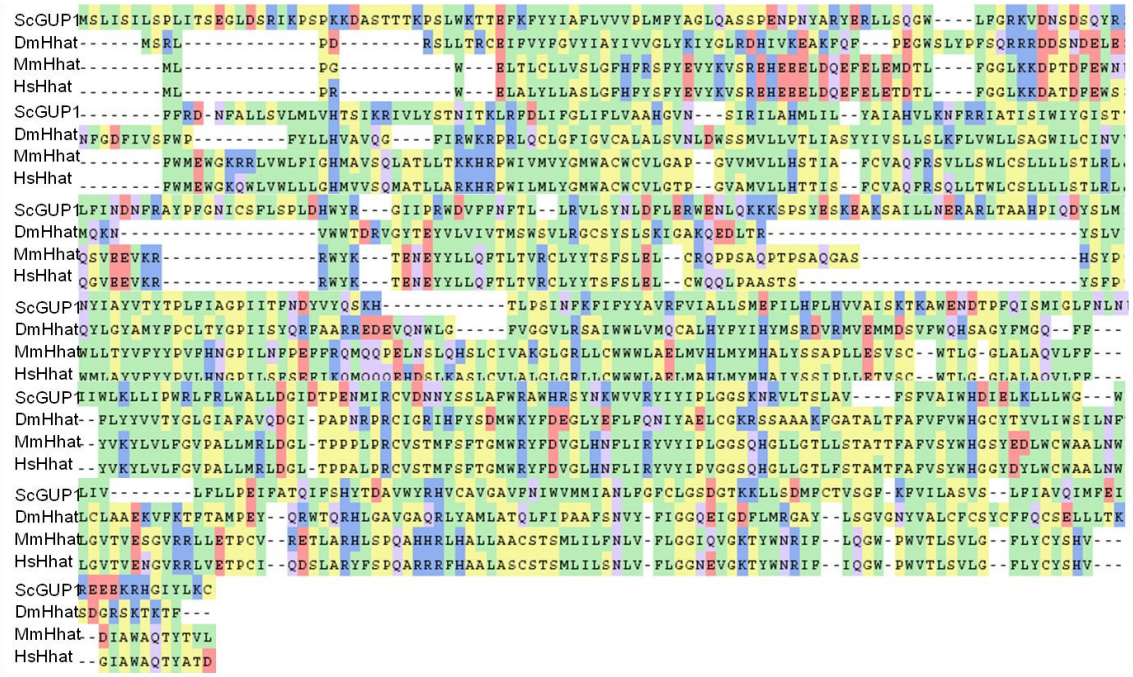

Fig.4. *GUP1* homologues alignment based on sequence homology (NCBI Data Base Designation).

Mammalian *GUP1* is described on NCBI gene database as a homolog of *S. cerevisiae GUP1* (Abe et al, 2008), and is consider to be a member of the membrane-bound *O*-acyltransferase (Hoffman, 2000). *MmGUP1* is also highly homologous to mammalian hedgehog acyltransferase, Skn, and importantly, the subcellular localization of *MmGUP1* has been shown to be indistinguishable from the mouse Skn by green fluorescent protein analysis. As mentioned above it was identified as a negative regulator of Hh pathway (Abe et al, 2008).

DmGUP1 is known as Rasp. The literature concerning this protein is practically inexistent. There is a study performed by Miccheli and co-authors, based on molecular alignment analysis, which describes Rasp as a multipass transmembrane protein that has homology to a family of membrane bound *O*-acyl transferases (Hoffman, 2000). They suggested that Rasp acylation is necessary to activate Hedgehog protein, an essential

component of Hedgehog pathway (Miccheli et al, 2002). An *in silico* analysis, revealed that the Rasp ORF (CG11495) used on the above mentioned work (Hoffman, 2000) presents a high homology with *ScGUP1* gene (this study, **Fig. 4**).

As for *DmGUP1*, there is very little information on Human *GUP1*. In fact, no studies with this gene have been made or published in the literature. Human *GUP1* genomic sequence, available at NCBI database presents a high homology with *MmGUP1* (this study, **Fig. 4**).

2.2 HedgeHog pathway in mammalians

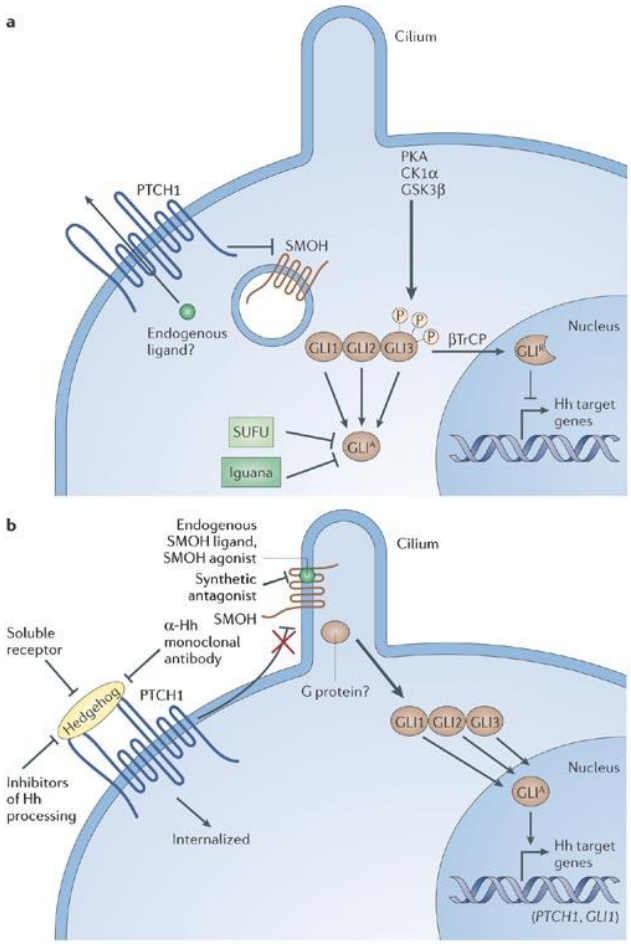

Fig. 5. Classical hedgehog pathway that leads to cells morphogenesis and proliferation (http://www.nature.com/nrd/journal/v5/n12/fig_tab/nrd2086_F1.html).

The glycoprotein Sonic Hedgehog (SHH) is essential for the development of several tissues during embryogenesis (Britto et al, 2000) and is also involved in organogenesis (Abe et al, 2008). SHH has additional functions in cell survival and cell proliferation (Ahlgren et al, 2002). Recent pieces of evidence suggest a major role for the activation of Sonic Hedgehog (SHH) signaling pathway in tumor development (Mao et al, 2009a; Mao et al, 2009b). Namely, SHH was involved in pathogenesis of neuroblastoma due to its high expression level in this pathology cells lines. Additionally, SHH inhibition induced apoptosis and stopped proliferation, abolishing the tumorgenecity of neuroblastoma (Mao et al, 2009a).

Aims

Aims

Gup1 is a yeast *O*-acyltransferase with close homologues in higher Eukaryotes (Hoffman, 2000; Neves et al, 2004) which has been implicated in the regulation of the Hedgehog pathway in mouse (Abe et al, 2008).

In *C. albicans* the deletion of *GUP1* causes drastic alterations both at morphologic and physiologic levels (Ferreira et al, 2010), such as (i) changes in the constitution and distribution of ergosterol in the plasma membrane, (ii) resistance to antifungals (azoles), (iii) aberrant colony morphology, (iv) impairment on the capacity of cells to develop *hyphae*, and (v) defects on the ability of cells to adhere, invade and form biofilms.

Furthermore, when a copy of *CaGUP1* gene is re-introduced to Δ*gup1* mutant these phenotypes are reversed, indicating that the above phenotypes are directly connected to *Gup1p* function or its absence (Ferreira et al, 2010).

Based on that, it was proposed as aim for the present work, to create and study several strains of *C. albicans* Δ*gup1* mutant harboring available *GUP1* homologues from well known model organisms/higher Eukaryotes:

- Yeast - *Saccharomyces cerevisiae (ScGUP1)*
- Fly - *Drosophila melanogaster (DmGUP1)*
- Mouse - *Mus musculus (MmGUP1)*
- Man - *Homo sapiens (HsGUP1)*

These newly transformed *C. albicans* strains were tested for several virulence factors, such as: (i) morphology and differentiation; (ii) adherence and invasion capacities; and (iii) resistance to antifungals agents.

Another important purpose of this study was to obtain a first characterization of the proteins secreted onto the *C. albicans* extracellular matrix, for which it was chosen to mimic biofilm formation on plate and compare the wt, Δ*gup1 mutant* and transformants for that matter.

Material and Methods

1. Strains & growth conditions

In this study *C. albicans* strains used were: wild type (wt) BWP17 (*ura3*Δ*::*λ*imm434/ura3*Δ*::*λ*imm434his1::hisG/his1::hisGarg4 ::hisG/arg4::hisG*) (Wilson et al, 1999), several clones of homozygous *C. albicans* Δ*gup1/* Δ*gup1* (isogenic to BWP17 but *gup1::URA3-dpl200/gup1::ARG4*) named as Δ*gup1* mutant (Kayingo et al, 2009) and Δ*gup1* mutant complemented with *GUP1* homologues from *S. cerevisiae (ScGUP1), D. melanogaster (DmGUP1), Mus musculus* (*MmGUP1*) and *H. sapiens (HsGUP1*), generally referred below as Ca*Δgup1GUP1* homologues.

Yeast strains were batch-grown on rich medium (YPD - yeast extract (1% w/v), peptone (2% w/v), glucose (2%, w/v)), minimal medium (YNBD – yeast nitrogen base (0.67% w/v), glucose (2% w/v)) or Spider´s medium (Nutrient broth (1% w/v), manitol (1% w/v), K_2HPO_4 (0.2% w/v)), at 30ºC (YPD; YNBD) or 37ºC (YPD; Spider), with orbital shaking, at 200 rpm. Solid cultures media were supplemented with 2% agar (YPD, YNBD) or 1.5% agar (Spider) and identically incubated at 30 and 37°C. Transformants were grown at 30ºC on YNBD supplemented with 200 µg/mL geneticin (G418), until colonies were observed. Strains were kept at 4ºC on solid media.

Escherichia coli XL1Blue strain (*end*A1 *gyr*A96 (nalR) *thi*-1 *rec*A1 *rel*A1 *lac gln*V44 F'[::*Tn*10 *pro*AB+ *lac*Iq Δ(*lac*Z)M15] *hsd*R17(rK- mK+) was used for DNA propagation. Bacterial cells were grown in Luria-Bertani (LB) medium (yeast extract (0.5%, w/v); tryptone (1%, w/v); NaCl (1%, w/v)) at 37ºC in an orbital shaker. Strains were maintained in LB medium supplemented with 2% agar, previously grown overnight at 37ºC and kept at 4ºC. Bacterial transformants were grown overnight on LB medium with ampicilin (100 mg/ml) at 37ºC, until colonies were observed.

2. DNA manipulation

A pair of plasmids, p413GPD and p423GPD, were used in this work to clone *GUP1* homologues (**Fig. 7**). Both are expression plasmids containing the strong GPD promoter and the selective marker histidine (Mumberg et al, 1995), but differ in replicative strength. p413 is centromeric and p423 harbours the 2µ element, which result in one copy per cell or in 10-30 copies per cell, respectively (Mumberg et al, 1995). The plasmid pRS42K was also used in this work (Taxis et al, 2006) (**Fig. 7**). This vector derives from pRS plasmid series and is an episomal shuttle plasmid used to easy genetic manipulation in yeast. It contains selective markers both to bacteria (ampicilin, kanamicin) and yeast (geneticin).

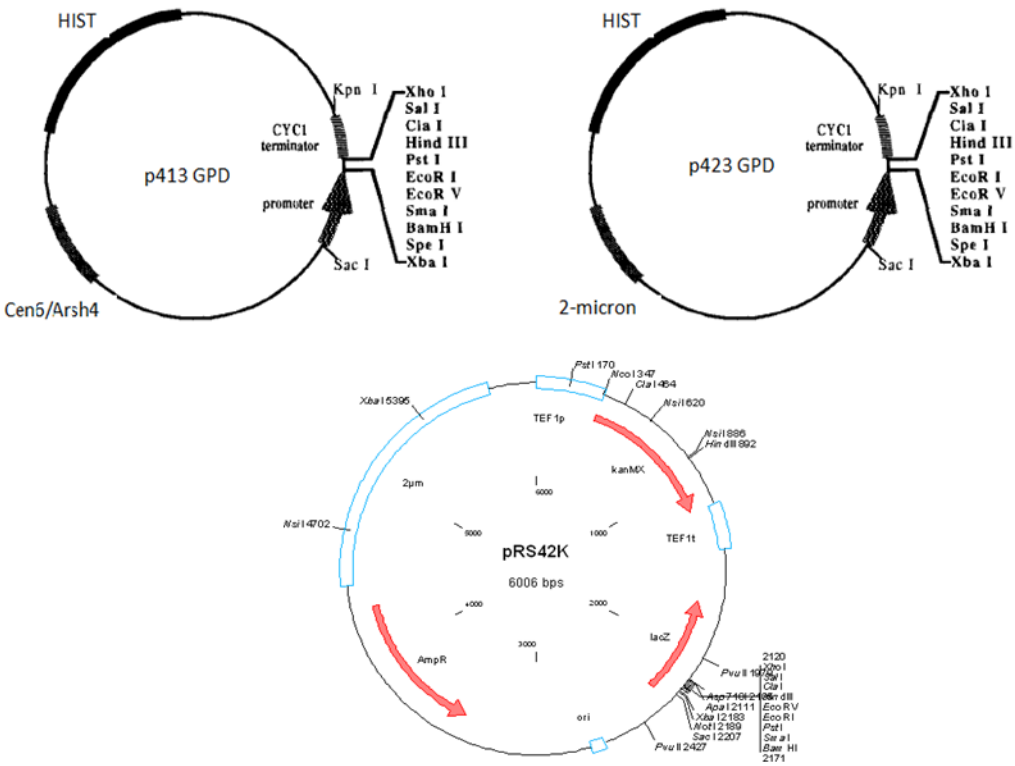

Fig. 7. Schematic representation of p413GPD, p423GPD and pRS42K plasmids.

2.1 Transformation of *C. albicans*Δ*gup1* mutant strain

The referred vectors, p413GPD, p423GPD and pRS42K were used to clone the several *GUP1* homologues, *ScGUP1*, *DmGUP1*, *MmGUP1* and *HsGUP1* in *C. albicans*. These constructions were previously propagated in *E. coli XL1Blue* strain.

2.1.1 Transformation in *E. coli XL1Blue*

E. coli XL1Blue transformation was made following standard protocols and using prepared competent *E. coli XL1Blue* cells (Ausubel et al, 1996). The transformation selection was performed based on the autotrophic mark ampicillin present in both p4X3GPD and pRS42K plasmids. The procedure is next described:

E. coli XL1Blue competent cells preparation was made using the Simple and Efficient Method (Inoue et al, 1990). The method basically consists on inducing membrane alterations that make cells more permeable to macromolecules, in the present case DNA on plasmids, which crosses the membrane more easily.

To transform *E. coli XL1Blue*, a volume of 5 µl plasmid DNA (≈50 µg/µl) was added to 200 µl of competent cells. These were maintained on ice for 40 minutes, subjected to a heat shock by incubation at 42ºC for 2 minutes and then incubated on ice for 2 minutes. In order to allow cells to recover, 500 µl of LB medium was added and the cell suspension further incubated at 37ºC for 1 hour. A volume of 100 µl of this suspension was then platted on solid LB medium supplemented with 100 µg/ml of ampicillin. Cells were allowed to grow overnight at 37ºC.

2.1.2 Plasmid isolation from *E. coli XL1Blue* cells

In order to isolate plasmidic DNA in large scale, several colonies of *E. coli XL1Blue* transformed with *GUP1* homologues were inoculated in 5ml of LB-amp liquid medium and grown overnight at 37ºC. The plasmid extraction was performed with *GeneElute*^{TM} *Plasmid Miniprep Kit* [Sigma], following the protocol of the manufacturer.

2.1.3 Enzymatic digestion of plasmidic DNA

To extract the several *GUP1* homologues from the different plasmids, appropriate pairs of restriction enzymes were used (**Table 1**).

Table 1. Restriction enzymes used to extract *GUP1* homologues.

***GUP1* origin**	**Restriction enzymes**
ScGUP1	*Hind III* (1.5 µl); *Xho I* (0.75 µl)
DmGUP1	*Bam HI* (1.5 µl); *Xho I* (0.75 µl)
MmGUP1	*Xba I* (1.5 µl); *Xho I* (0.75 µl)
HsGUP1	*Spe I* (0.5 µl) e *Xho I* (2 µl)

The restriction enzymes *Kpn I* and *Sac I* were also used to clone these *GUP1* genes into pRS42K, already harboring the GPD promoter and the terminator CYC1 (Danielly, master thesis, 2008).

Plasmids linearization was obtained by the following reaction mixture:

- 8 µl of vector,
- X µl of enzyme A,
- Y µl of enzyme B,
- 2 µl of buffer,
- Z µl ultrapure autoclaved water,

As can be seen in **Table 1**, the volumes of the different enzyme combination are distinct. The ratio of volumes was calculated using the *Fermentas double digestion* tool (http://www.fermentas.com/en/tools/doubledigest). Ultrapure water was added to complete a final reaction volume of 20 µl. The mix was incubated for 2.5 to 3 hours at 37ºC.

2.1.4 Extraction of DNA fragments of agarose gel electrophoresis

In order to separate, identify and purify DNA fragments, 1% agarose gel electrophoresis was performed. The DNA was visualized by UV illumination (245nm) at *Eagle Eye II* (*Stratagene*), previously stained with Gel RED (*Biotium*) dye. The molecular weight marker was Lambda DNA/Eco471 [Fermentas]. The electrophoresis ran for approximately 1 hour at 70 volts. The DNA fragments of interest were extracted with *QIAquick Gel Extraction Kit* (QUIAGEN) system following the manufacture recommendations.

2.1.5 Ligation reaction

Prior to ligation reaction the plasmid was treated with the enzyme *Shrimp Alkaline Phosphatase* (SAP) in order to remove phosphate 5' ends of the vectors. This elimination prevents the auto-ligation of the vector and reduces the frequency of plasmid circularization during the ligation reaction (Sambrook et al, 1989). For that, the vector was incubated with SAP enzyme for 10 minutes at 37ºC. The mix was further incubated 15 minutes at 65ºC in order to a complete inactivation of the enzyme.

The ligation reaction was performed using the following conditions:

- 1 µl ligation buffer 10x,
- 1 µl plasmid,
- 7 µl of purified digestion product,
- 1 µl of T4 DNA ligase,
- 10 µl ultrapure water to achieve final volume of 20 µl,

The reaction mixture was incubated overnight at 4ºC. For DNA propagation, the ligation product was used to transform *E. coli XL1Blue* competent cells, as described above.

2.1.6 Yeast transformation

C. albicans Δ*gup1* mutant strain was transformed following a protocol adapted from Gietz R.D. and collaborators, 1995 and Walther A. and collaborators, 2003. The procedure is next described:

- Grow of Δ*gup1* mutant strain in ≈5ml of YPD, overnight at 30ºC until stationary phase,
- Ressuspend the cells on fresh YPD to OD_{600}= 0.3,
- Incubate for 4-5h at 30ºC,
- Harvest the cells by centrifugation 5 min at 5000 rpm. Wash the pellet once with water. Carefully remove the remaining water,
- Ressuspend the pellet in 1.5ml of LiAc/TE solution* (0.5 ml 10xTE; 0.5 ml 10x LiAc; 4 ml water),
- Set up transformation tubes as follows:

  . 1-5 µl DNA

  . 10 µl ssDNA (DNA carrier)

  . 80 µl cells

  . 600 µl PEG4000/LiAc solution* (2.5g PEG4000, 0.5 ml 10x LiAc, 4.5 ml water)

- Vortex briefly, and incubate overnight at 30ºC,
- Heat shock for 15 minutes at 44ºC,
- Spin down cells at 13000 rpm for 15 seconds and pour off PEG;
- Spin down again and tip off remaining PEG,
- Ressuspend the pellet in 100-200 µl water and plate the suspension on selective plates (YNB w/o aminoacids supplemented with geneticin (200 µg/ml));

- Incubate for 24-48h at 30ºC.;

*Both solutions were sterilized by filtration.

3. Virulence assays

3.1 Morphology and hyphal formation

In order to observe different colony morphology/differentiation, the cultures were diluted and inoculated in both liquid and solid medium, under hyphal non-inducing conditions (YPD at 30ºC) and under hyphal-inducing conditions (Spider medium, YPD at 37º and also YPD supplemented with 10% Fetal Bovine Serum (FBS) at 37º C).

Solid media: Cultures were allowed to grow for 2-3 weeks. During this period the colonies were several times observed at light microscopy to inspect hyphal development.

Liquid media: To discriminate between *hyphae* and pseudo-*hyphae*, young cultures were diluted to 1×10^7 cells/ml (OD_{600}=1), and incubated in the presence of FBS 10% (Fetal Bovine Serum) at 37ºC. At several time points a sample was collected and cells were stained with CFW (Calcofluor White) by adding to 3 ml of cell suspension 300 µl of CFW (300 µg/ml). Samples were incubated at room temperature for 5 min, after which 5 µl of the suspension was placed on microscopic slide, visualized by light microscopy (LM) and photographed. CFW dye binds primarily to chitin, but also in less extent to glucans, staining cell wall and septa of the cells (Ferreira et al, 2010).

Microscopy assessments were done in a Leica Microsystems DM-5000B epifluorescence microscope, with appropriate filter settings. Images were acquired by a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

3.2 Adherence to agar and invasion capacities

Equal volumes of young cultures of each strain were diluted to 1×10^7 cells/ml (OD_{600}=1), and 1 ml of cells suspension was spotted into YPD and Spider medium agar

plates. Cultures were allowed to grow at 37°C for 10 days. The cells on the surface were then removed by washing under running water (Guo et al, 2000; Hube et al, 2001) and visualized by LM.

3.3 Resistance to antifungal drugs

To attest *Ca*Δ*gup1GUP1* homologues sensibility/resistance to antifungal drugs, YPD-grown cultures were used for drop tests, containing approximately 1×10^7 cells/ml (OD_{600}=1) as described before (Ferreira et al, 2008; Ferreira et al, 2010). Six-fold serial dilutions were made, and 5 µl of each suspension was applied on selective media. Results were scored after 3-5 days of incubation at 30°C. YPD medium was complemented as follow: clotrimazole (68.8 µg/ml), ketoconazole (86.1 µg/ml), fluconazole (30.6 µg/ml). All chemicals were obtained at the highest available grade from Sigma Aldrich (Ferreira et al, 2010).

4. Proteins secreted to extracellular matrix (ECM)

The extraction of proteins secreted to the extracellular matrix (ECM) was performed using an optimized protocol develop by our group (Joana Carvalho, unpublished results):

In order to obtain a biofilm-like structure and ensure relevant quantities of secretome, 1 ml of cells (OD_{600}= 1) were spread on different mediums (YPD, Spider and YNB without aminoacids). The overlays were allowed to grow 7 days at 30°C and 37°C. The biomass of at least 5 plates (90mm∅) of each strain was scraped and immersed in PBS buffer containing a cocktail of proteases inhibitors (PMSF 0.2 µg/ml; Aprotinin 0.32 µg/ml; Pepstatin 1µg/ml; Leupeptin 1µg/ml) in which cells were washed for 10 minutes on a roller under constant rotation, at 4°C. The suspension was then spun down for 5 minutes at 5000 rpm. The supernatant was collected and stored at 4ºC for further protein analysis.

To obtain a maximum protein recovery, proteins were precipitated overnight with a mix of TCA (Trichloroacetic acid) and DTT (Dithiothreitol) at 4°C. DTT has a protein protector effect that increases the yield of proteins recovery. A crucial step to perform

efficient protein extraction is the protein solubilization. Two alternative buffers were tested, urea and a modification of Laemmli buffer consisting on the withdraw of the bromophenol blue and the addition of urea (6M of final concentration), to previous Laemmli buffer (Joana C., unpublished results). This last one was chosen according to ressuspension efficiency determined by electrophoretic profile, as well as because it is compatible with the utilization of Bradford protein quantification method. To enhance the performance of the protein precipitation, two further steps of vortex and sonication were performed.

SDS-PAGE was made in 9% of polyacrylamide in order to allow the use of a broad range of molecular weight marker. Electrophoretic run was performed during 1 hour at 140 volts. The gel was stained with $AgNO_3$. A formaldehyde fixation step, which enhances the staining sensitivity but precludes any further use of the stained bands, was introduced to the staining process.

Results

1.Strains harboring *GUP1* homologues

The cloning of several of the *GUP1* homologues (*ScGUP1*; *DmGUP1*; *MmGUP1* and *HsGUP1*) was initially performed into a pair of p4X3 series plasmids, with different expression strength (Mumberg et al, 1995). These plasmids have different origins of replication, a centromeric (p413) and a 2µ (p423) that vary in the number of copies produced (1 and 10-30 copies per cell respectively) (Mumberg et al, 1995). Yet, both have a strong promoter (GPD) and also share the same terminator (CYC1).

In general, the strategy applied to clone the *GUP1* homologues consisted in extracting the gene from the original plasmid (all *GUP1* homologues were obtained already cloned in several plasmids) and cloned them into p4X3GPD plasmids. A restriction map was performed for the genomic DNA coding sequence of each *GUP1* homologue in order to select the appropriate restriction enzymes to be used on the cloning steps, considering both the plasmidic Multi-Cloning-Site (MCS) and the restriction analysis. Each construction, p4X3GPD-*GUP1* homologues, was then transformed in *E. coli XL1Blue* in order to obtain a larger stock of DNA. The *E. coli XL1Blue* transformants were selected in LB medium supplemented with 100 µg/ml of ampicilin. The positive clones, those who carry the *GUP1* gene fragment, were verified through electrophoresis of the enzymatic digestion confirming the expected bands size. These positive clones were then frozen and stored at -80°C in glycerol stocks.

To otain the *ScGUP1-GFP* chimera a previous pYES2-*GUP1-GFP* construction available at the collaborators Franscesco Grieco and Gianluca Bleeve (Istituto di Scienze Delle Produzioni Alimentari del Consiglio Nazionale Delle Ricerche (ISPA), Lecce, Italy) was used. The *in silico* restriction map was studied, covering the chimera *GUP1-GFP* and not only *ScGUP1* gene (**Fig. 8**). *Hind III* and *Xho I* enzymes were chosen to extract the fragment form pYES2 and to clone it in p4X3GPD, once these were included simultaneously into the MCS (Multiple Cloning Site) of both plasmids and do not cut the chimera *ScGUP1-GFP* (**Fig. 8**).

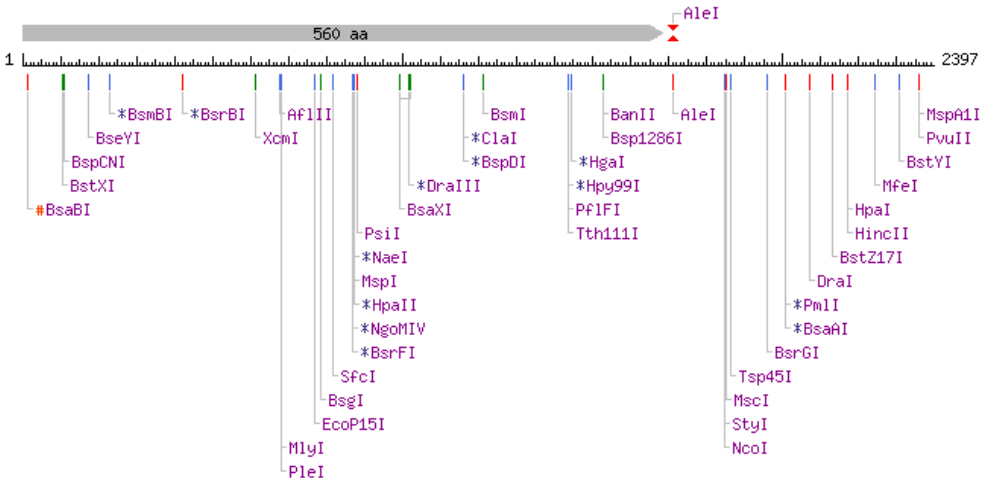

Fig.8. Restriction map of *GUP1-GFP* chimera, obtained with Nebcutter V2.0 program [New England Biolabs Inc.].

The *XL1Blue* clones were digested with *Hind III* and *Xho I* enzymes and the resulting product analyzed by electrophoresis (**Fig. 9**). The positive clones were confirmed by the presence of the two expected bands: a band of approximately 6000 bp corresponding to the empty plasmid (5876 bp) and of a band of approximately 2500 bp corresponding to the chimera *ScGUP1+GFP* (1683 bp of *GUP1* plus 717 bp of *GFP*).

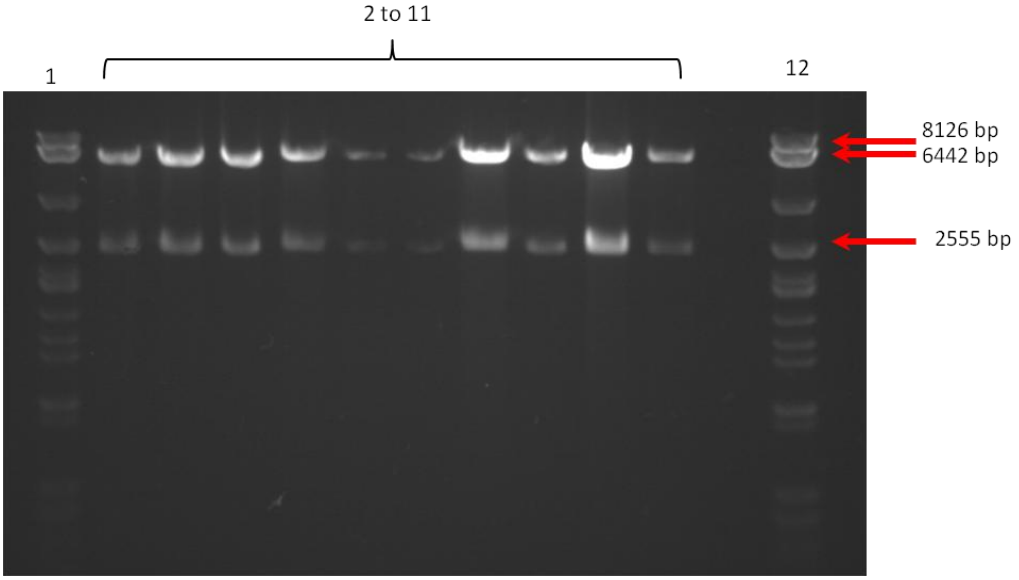

Fig. 9. Electrophoretic analysis of clones containing p4X3GPD-*ScGUP1-GFP* construction. Molecular weight marker Lambda DNA/Eco471, Marker 13 [Fermentas] – lanes 1 and 12; Product of the double digestion with *Hind III* and *Xho I* of the clones harboring p413GPD-*ScGUP1-GFP* - lanes 2 to 11.

DmGUP1 was obtained from the same source as *ScGUP1-GFP* and cloned into p426GPD plasmid. After the restriction analysis, *Bam HI* and *Xho I* enzymes were chosen to extract *DmGUP1* from p426GPD and clone into p4X3GPD plasmids (**Fig. 10**).

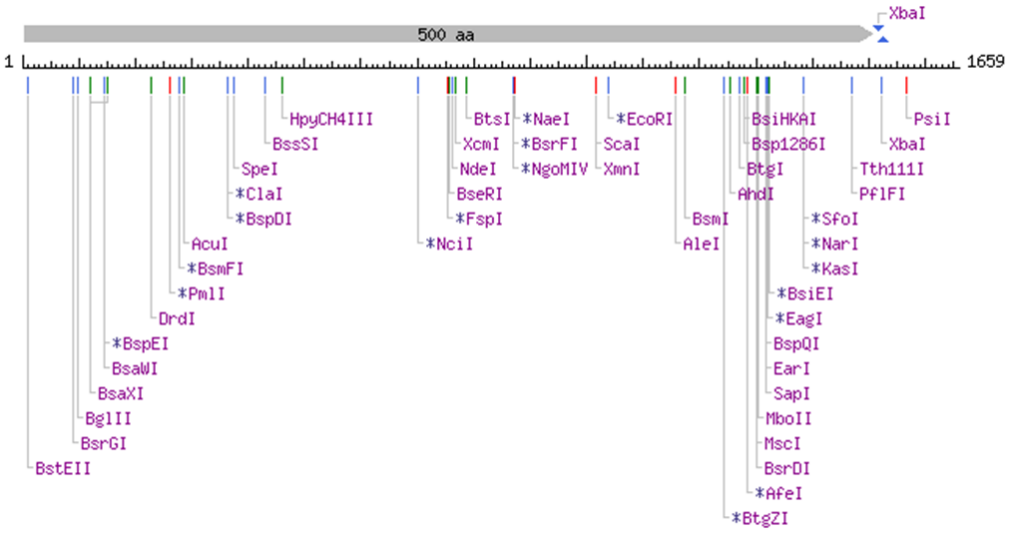

Fig. 10. Restriction map of *DmGUP1* gene, obtain with NEBcutter V2.0 program [New England Biolabs Inc.].

Again to verify the presence of *DmGUP1* gene on the *XL1Blue* transformants, the plasmidic DNA was extracted and double digested with *Bam HI* and *Xho I* enzymes.

The gel electrophoresis of the double digestion confirmed the presence of *DmGUP1* gene with approximately 1600bp (**Fig. 11** -lanes 2 and 3- green arrow). Empty p413GPD plasmid (lane 2 – blues arrow) and p423GPD plasmid (lane 3 – black arrow) have approximately 5900 bp and 6700 bp, respectively.

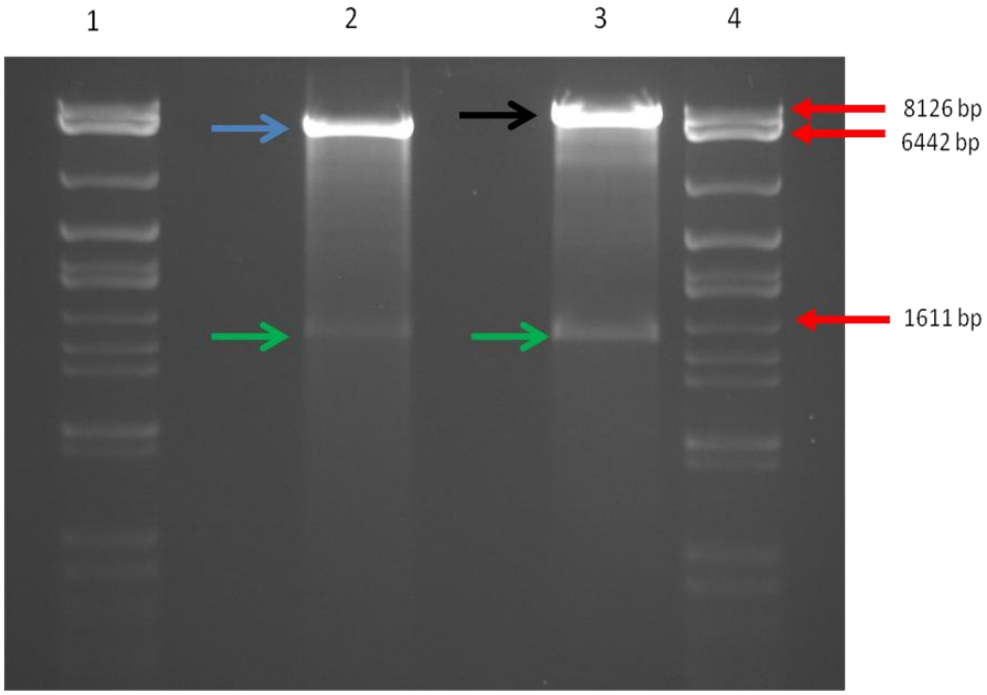

Fig. 11. Electroforetic analysis of double digestion of p4X3-*DmGUP1*. Molecular marker Lambda DNA/Eco471 Marker, 13 [Fermentas] - lanes 1 and 4; Double digestion with *Bam HI* and *Xho I* of p4X3GPD -*DmGUP1* clones - lanes 2 and 3.

MmGUP1 gene was gently provided by Abe et al (Abe et al, 2008) into a p426GPD plasmid. As before, a restriction analysis was made and *Xho I* and *Xba I* enzymes chosen for the extraction of the fragment and the cloning into p413GPD and p423GPD plasmids (**Fig. 12**).

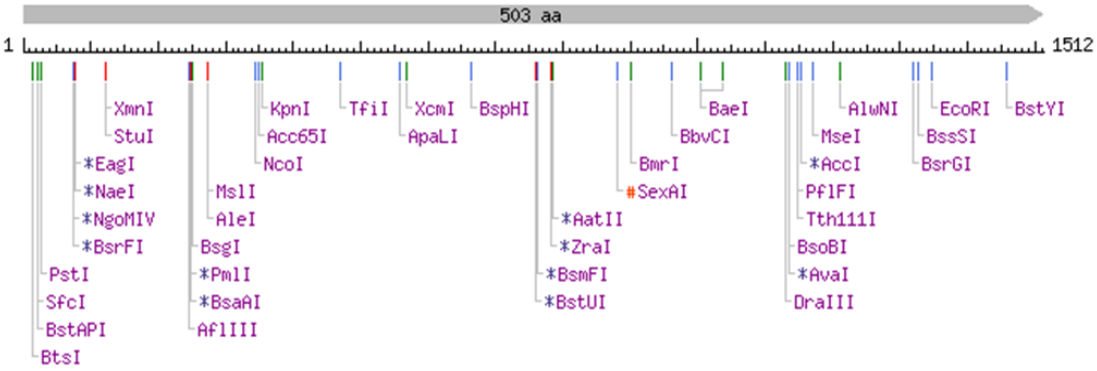

Fig. 12. Restriction map of *MmGUP1* gene, obtain with NEBcutter V2.0 program [New England Biolabs Inc.].

Three *XL1Blue* transformants were double digested with the same enzymes and analyzed by electrophoresis (**Fig. 13**). Two clones displayed two bands, one of *MmGUP1* gene (≈1500bp) and the other corresponding to the empty p413GPD (≈5900bp) (**Fig. 13**. lanes 3 and 4). On the other hand, in the clone of lane 2 only the presence of empty plasmid was visualized, meaning that the *MmGUP1* gene was not incorporated into this clone. Therefore, only clones 3 and 4 were considered positive.

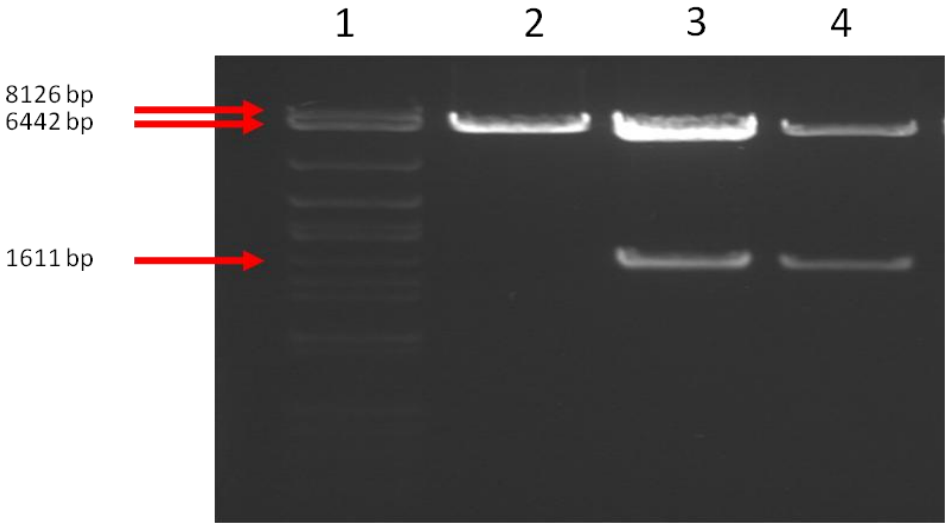

Fig. 13. Electrophoretic analysis of the double digestion of p413-*MmGUP1*. Molecular Marker - lane 1; Double digestion with *Xba I* and *Xho I* of p413-*MmGUP1* clones- lanes 2, 3 and 4;

A turning point on this work arose when we tried to clone Δ*gup1* mutant strain with the p4X3GPD constructions harboring the *GUP1* homologues (from *ScGUP1*; *DmGUP1*; *MmGUP1*). Despite of the several attempts and protocols used, Ca*Δgup1GUP1* homologues clones were never achieved. We hypothesized that this difficulty could be related with the aminoacid selection of the p4X3GPD plasmids. Apparently the marker gene, histidine, was not stringent enough as selection force, preventing us to obtain positive clones. These complications have been described by other researchers (Danielly, master thesis, 20008; Pacheco, PhD thesis, 2008).

We decided to clone all *GUP1* homologues into a new plasmid, the pRS42K, an integrative episomal plasmid, whose selective force arises as more reliable since is an antibiotic (Taxis et al, 2006). However, this vector is not an expression plasmid, since it lacks the promoter and terminator (Taxis et al, 2006). The strategy thus to extract from the p4X3GPD plasmids constructions previously build, the fragment containing the GPD promoter, the *GUP1* homologues and the CYC1 terminator and insert the whole

fragment into pRS42K plasmid, thereby arming pRS42K of a promoter and terminator (**Fig. 14**). The restriction enzymes *Kpn I* and *Sac I* were used to extract the fragments containing *ScGUP1* and *DmGUP1* homologues from p413 plasmids and to clone into pRS42K.

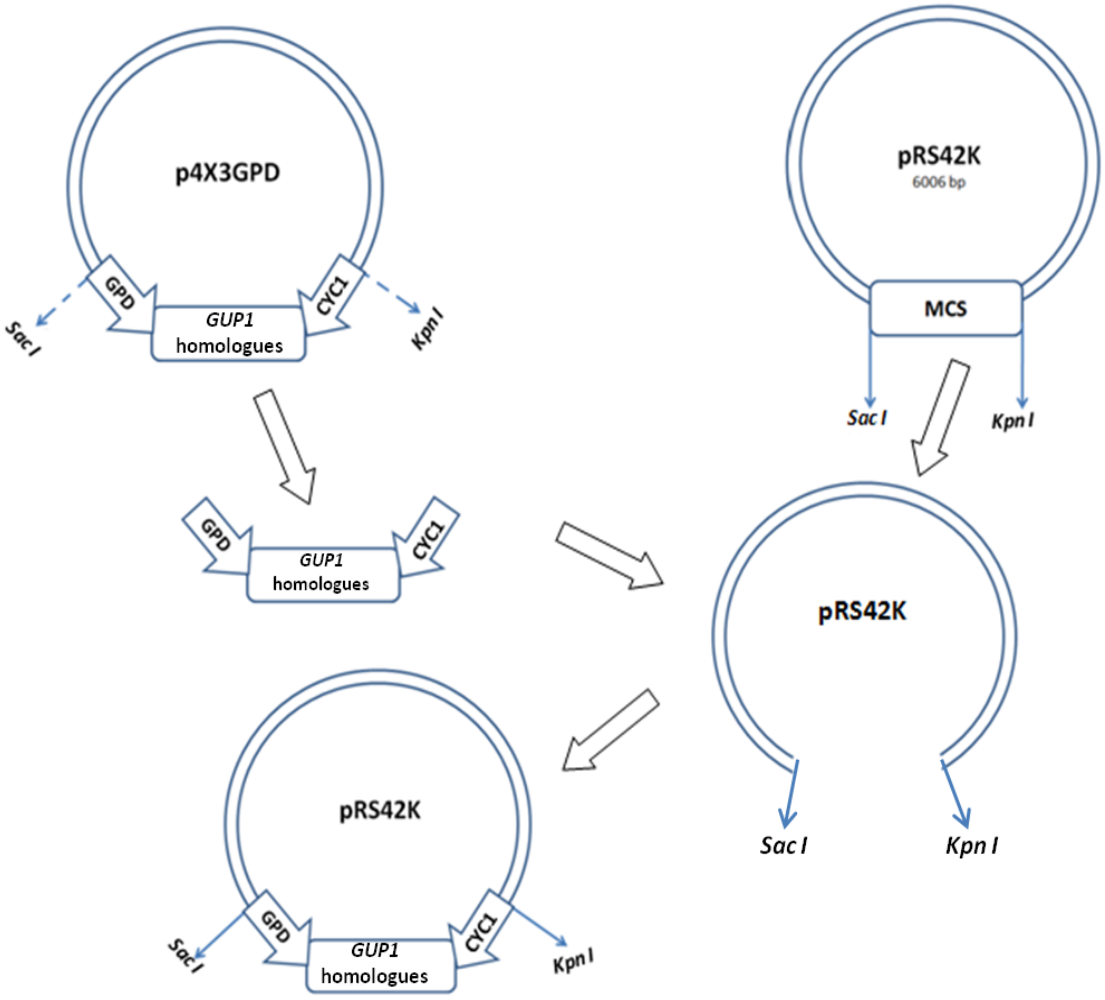

Fig. 14. Schematic representation of the cloning strategy used to insert the fragment GPD-*GUP1*homologues- CYC1 into pRS42K plasmid.

The constructions obtained were transformed into *E. coli XL1 Blue* strain for DNA propagation. The confirmation of positive clones was achieved as before, by extracting the plasmid from *E. coli XL1 Blue* clones and subjecting these to enzyme digestion according to each *GUP1* homologue and subsequent gel electrophoresis analysis:

The pRS42K-*ScGUP1* clones were digested with *Kpn I* (**Fig. 15**). The resulting linearized plasmid of a positive clone should give a band with 9373 bp corresponding to pRS42K-GPD-*ScGUP1*-*GFP*-CYC1 full size (6006bp of pRS42K plus 3367 of the fragment GPD-*ScGUP1*-*GFP*-CYC1) (**Fig. 15-** lanes 2 and 4).

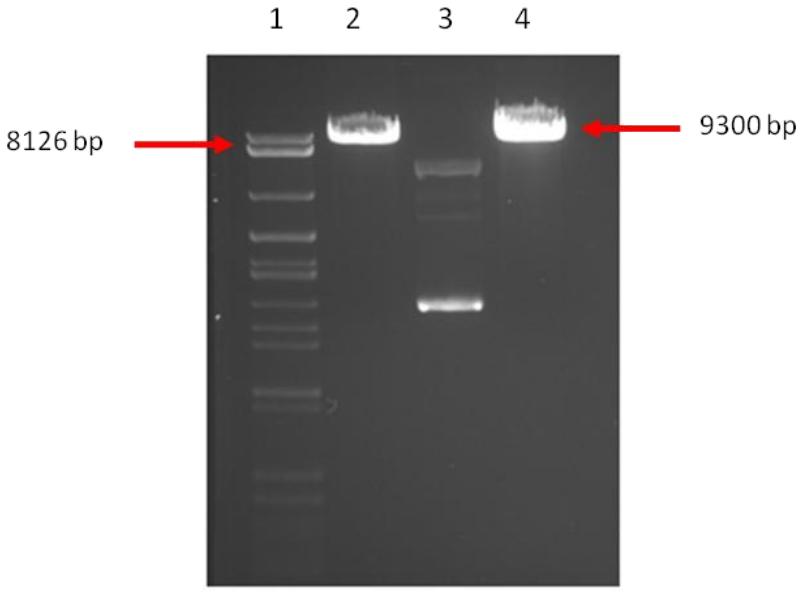

Fig. 15. Electroforetic analysis of pRS42K-GPD-*ScGUP1*-*GFP*-CYC1 digested with *Kpn* I. Molecular weight marker - Lane 1; pRS42K–GPD–*ScGUP1*-*GFP*-CYC1 clones - lanes 2, 3 and 4;

The pRS42K *DmGUP1* clones were double digested with *Kpn I* and *Sac I* (**Fig. 16**). We expected two bands for the digestion product of positive clones: one corresponding to the empty pRS42K (≈6006 bp) and another corresponding to the fragment GPD-*DmGUP1*-CYC1 (~2500 bp) (**Fig.16 –** lanes 2, 4 and 5).

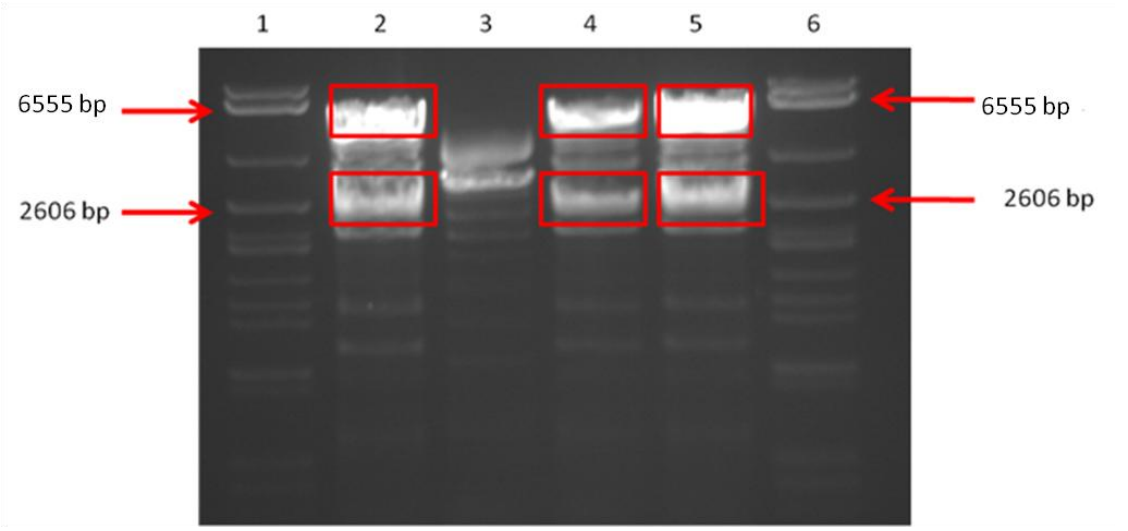

Fig. 16. Electrophoretic analysis of ligation product pRS42K-GPD-*DmGUP1*-CYC1 digested with *Kpn I* and *Sac I.* Molecular weight marker - Lane 1 and 6; pRS42K-GPD-*DmGUP1*-CYC1 clones - Lanes 2, 3, 4, and 5.

Concerning the *HsGUP1* homologue a different strategy was taken to clone it into pRS42K. This gene was available in our lab cloned into p426GPD plasmid (Fábio Faria-Oliveira, unpublished results), originating from a cDNA form ORFeome Collection from Dana Farber Cancer Institute, Boston, USA). p426GPD plasmid belongs to the same series of plasmids of p4X3 (Mumberg et al, 1995) and therefore has similar MCS. The restriction enzymes, *Kpn I* and *Sac I*, used before, have restriction sites inside the *HsGUP1* sequence and therefore couldn't be used. Instead we used another pRS42K plasmid construction, containing already this promoter and this terminator pRS42K-GPD-*DhXYLH*-CYC1 (Danielly, master thesis, 2008). *DhXYLH* is the genomic sequence from the yeast *Debaryomices Hansenii* xylose symporter (Danielly, master thesis, 2008). The sequence was removed from pRS42K, using *Spe I* and *Xho I* restriction enzymes. The same enzymes were used to extract *HsGUP1* gene from p426GPD plasmid and to clone it into the pRS42K (**Fig. 17**).

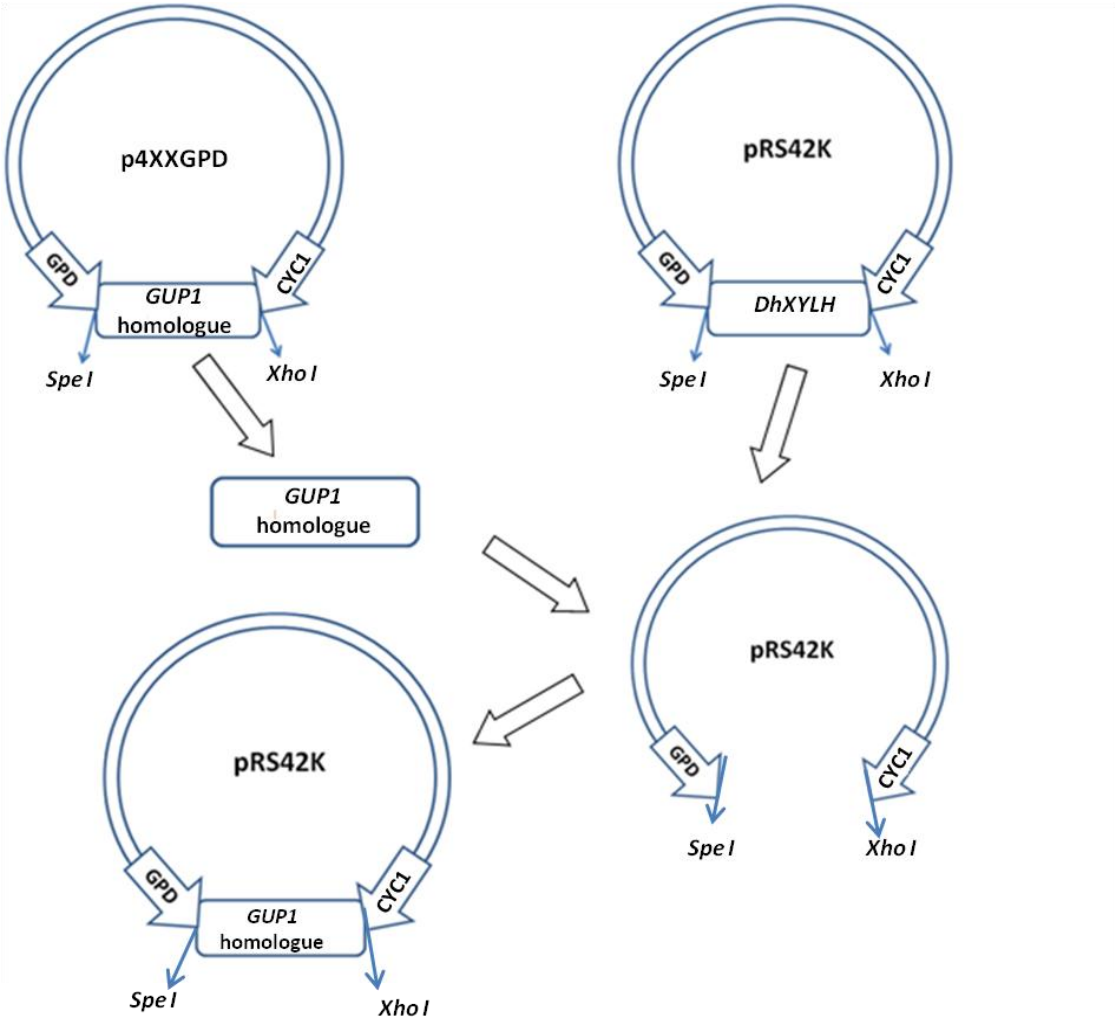

Fig. 17. Schematic representation of the cloning strategy used to insert *HsGUP1* and *MmGUP1* into pRS42K plasmid containing a promoter and a terminator.

The construction was used to transform *E. coli XL1Blue* strain. The clones pRS42K *HsGUP1* were digested again with the same enzymes to confirm *HsGUP* insertion (**Fig. 18**). The two expected bands were obtained: one of approximately 6000bp corresponding to linearized pRS42K containing the promoter and the terminator, and another band of 1500bp, corresponding to the size of *HsGUP1* gene. Two positive clones were obtained following with this approach (**Fig. 18**. lanes 2 and 3).

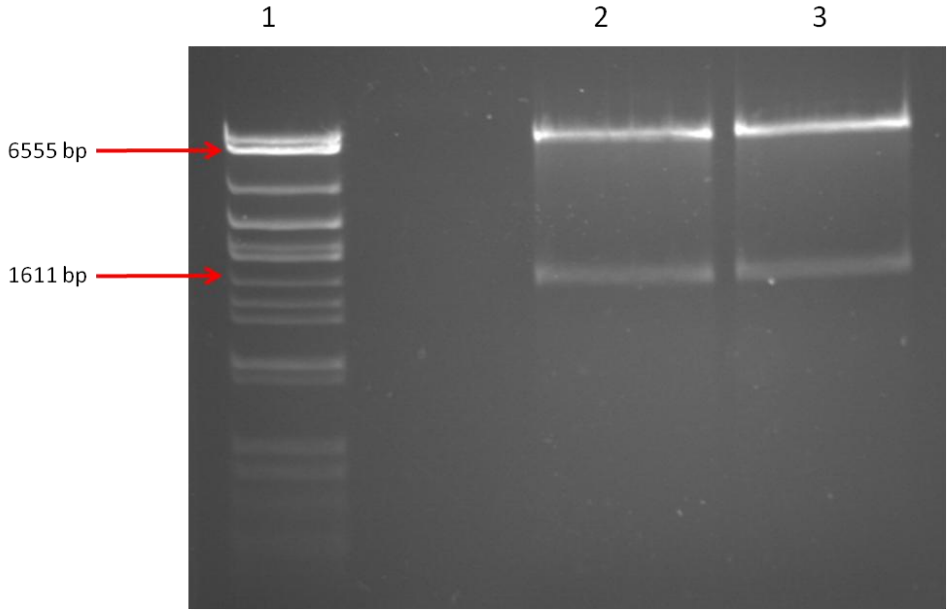

Fig. 18. Electroforetic analysis of pRS42K-GPD-*HsGUP1*-CYC1 digested with *Spe I* and Xho *I*. Molecular weight marker - lane 1; pRS42K-GPD-*HsGUP1*-CYC1 clones - lanes 2 and 3;

To clone *MmGUP1* we used the same approach as for *HsGUP1*, meaning that the fragment *MmGUP1* was removed from p426GPD plasmids (Fabio Faria-Oliveira-unpublished results) with *Spe I* and *Xho I* enzymes and cloned into empty pRS42K plasmid containing GPD promotor and CYC1 terminator obtained before (**Fig. 17**). The construction was used to transform *E. coli XL1Blue* strain and the insertion of *MmGUP1* gene was verified as before. Despite of several attempts, none of the clones obtained with this construction harbored the *MmGUP1* gene.

2. Virulence assays with the *C. albicans* complemented strains

C. albicans Δ*gup1* mutant strain was transformed with the pRS42K constructions harboring *GUP1* homologues (from *ScGUP1*, *DmGUP1* and *HsGUP1*) as described in Material and Methods section **2.1.6.** Yeast transformants were selected through its resistance to geneticin and tested for virulence related phenotypes.

2.1 Colony morphology and hyphal formation capacity

The colony morphology and capacity of cells to develop *hyphae* of the several *Ca*Δ*gup1GUP1*homologues were tested in both solid and liquid media.

Solid media: wt, Δ*gup1* mutant and *Ca*Δ*gup1GUP1* homologues strains were grown for 2 weeks on YPD agar plates, at 30°C (non-*hyphae*-inducing conditions) and on YPD and Spider medium at 37°C (*hyphae*-inducing conditions), before colony morphology inspection. As described before, Δ*gup1* mutant exhibit different colony morphology when compare with wt (Ferreira et al, 2010). On the other hand, the colonies of CaΔ*gup1GUP1* homologues strains didn't display the spaghetti/flower kind morphology characteristic of Δ*gup1* mutant and in that sense, were closer to the wt colony morphology (Ferreira et al, 2010). Yet, neither of the CaΔ*gup1GUP1* homologues presents *hyphae* differentiation and wt strain has that capacity (Ferreira et al, 2010). Thus, in solid media the *GUP1* homologues were not able to revert Δ*gup1* mutant defect to develop *hyphae*, although all affected colony morphology (not shown).

Liquid media: Time-course of *hyphae* formation induced by FBS (fetal bovine serum) in liquid medium was checked. Calcofluorwhite (CFW) binds mainly to chitin (Herth et al, 1980), one of the cell wall compounds, allowing us to see the cell wall, the budding scars and the septas (Hoch et al, 2005). Contrarily to Δ*gup1* mutant, the *Ca*Δ*gup1GUP1*homologues harboring the different homologues *ScGUP1*, *DmGUP1* and *HsGUP1* were able to differentiate into pseudo-*hyphae*/*hyphae* cells, very soon after induction (5 min.) as shown in **Fig. 19**. This, however, stabilizes/stops after 30 minutes of incubation with FBS (**Fig. 19**). Our statement is based on the the observation that after the refered period is not possible to see alterations on the size of *hyphae*/pseudo-*hyphae* cells. Furthermore, the stained wall indicate that the filamentous cells developed by these strains are more likely to correspond to pseudo-*hyphae* rather than true *hyphae*, since i) first septum is close to the mother neck, ii) present constrictions at the septa junctions and iii) the side walls are not parallel (**Fig. 19 -** white arrows). This is in clear contrast to wt cells, which form true *hyphae*, once the first septum of these filamentous cells is distant from the mother neck and the other septa do not present constrictions (**Fig. 19 -** red arrows). It is further noteworthy that *Ca*Δ*gup1GUP1*homologues cells were significantly smaller than wt.

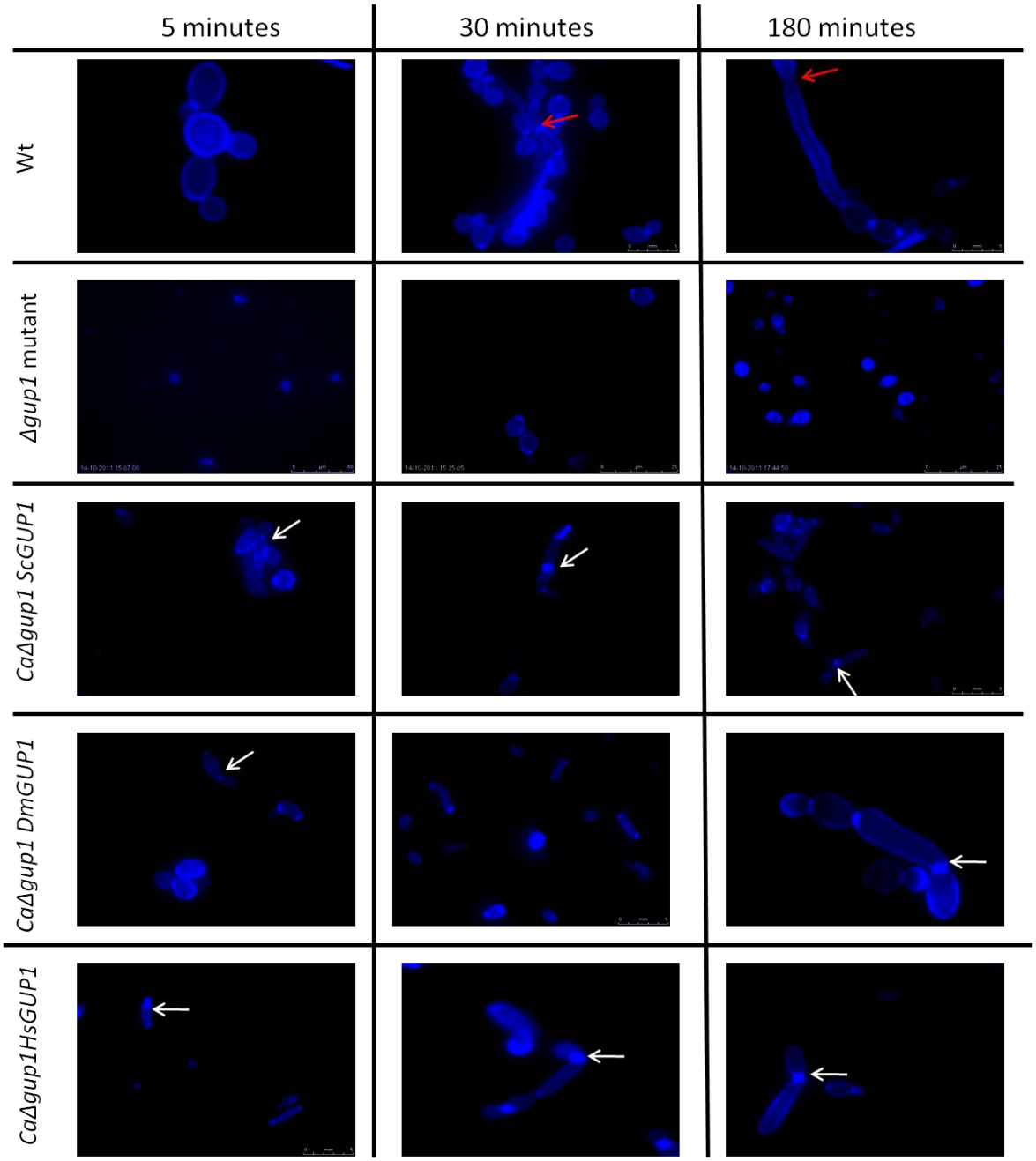

Fig. 19. Time-course pseudo-*hyphae*/*hyphae* formation in wt, Δ*gup1* mutant and *Ca*Δ*gup1GUP1*homologues, in liquid *hyphae* inducing medium (FBS). Cells were stained with CFW and observed by LM with the adquated filters.

2.2 Adherence to agar and invasion capacities

In order to evaluate the *Ca*Δ*gup1GUP1*homologues strains capacity to adhere and to invade agar, cells were grown for ten days on both YPD and Spider agar plates. Ended

this period the plates were washed under running water. In a previous publication it was described how the deletion of *GUP1* interferes in the capacity of the strain to both adhere and invade these surfaces (Ferreira et al, 2010). All the transformants revealed the same lack of adherence capacity/level to YPD and Spider plates, since they were washed out as easy as Δ*gup1* mutant from the agar surface.

Replicas of these plates were further inspected for agar invasion as described in Materials and Methods- section **3.2**. In a previous work we showed that Δ*gup1* mutant,was deficient on the ability to invade these surfaces (Ferreira et al, 2010). Here, we observed that the complementation of the Δ*gup1* mutant with the mentioned homologues did not reverted this phenotype (not shown). In fact, none of the *Ca*Δ*gup1GUP1*homologue strains were able to invaded the agar, at all the conditions tested.

2.3 Resistance to antifungals

The O-acyltransferase *Gup1p* acts on lipids metabolism affecting the assembly and integrity of the plasma membrane, as well as the sphingolipids-sterol order domains (Ferreira et al, 2008). An association between altered lipid-ordered domains and antifungal resistance has been described before as influencing the susceptibility to antifungal drugs (Mukhopadhyay et al, 2004; Ferreira et al, 2008). Regarding *Ca*Δ*gup1GUP1*homologues strains, the two harboring the *ScGUP1* and the *HsGUP1* genes seem to be more resistant to these antifungals than the wt strain, showing a similar behavior to Δ*gup1* mutant strain (**Fig. 20,** 3th and 5th columns). However the strain complemented with *DmGUP1* gene was as sensitive to these drugs as wt strain (**Fig. 20**. 4th colunm), differing from the other complemented strains.

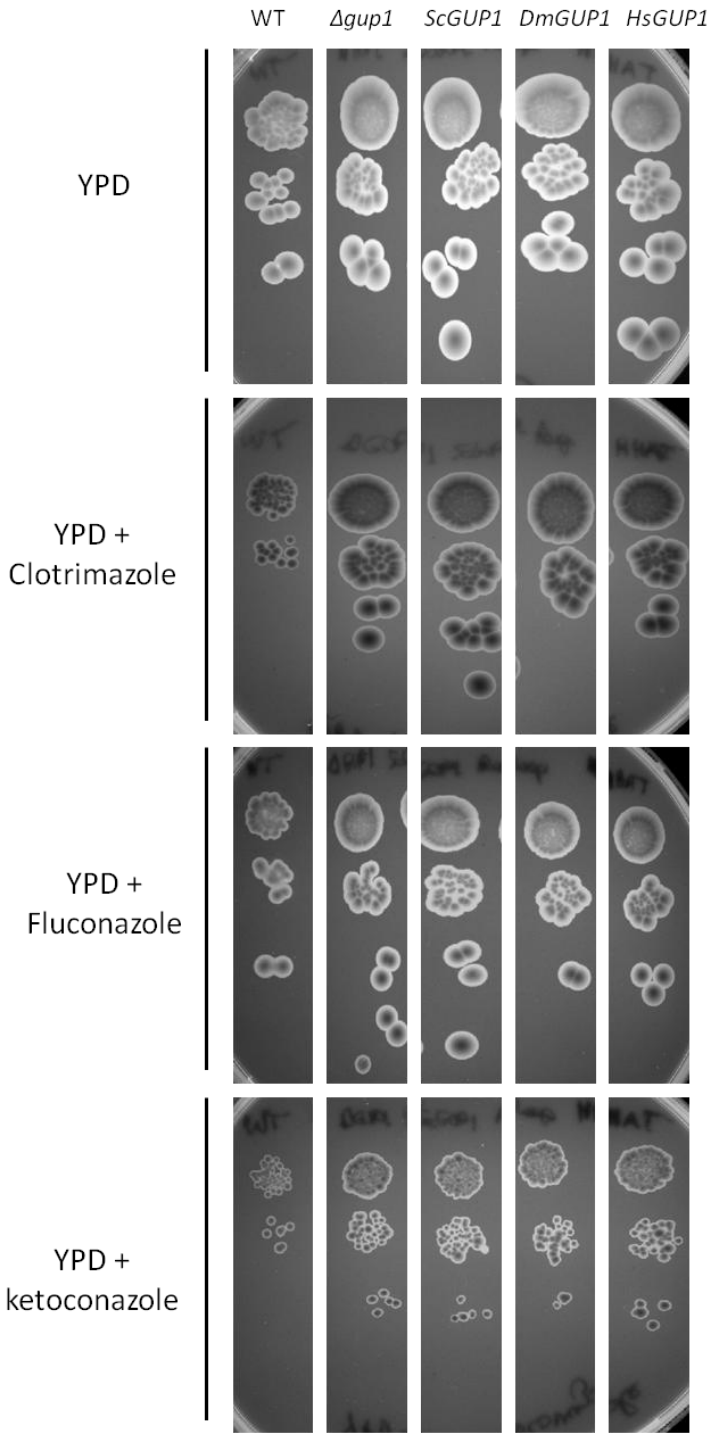

Fig. 20. *C. albicans* wt, Δ*gup1* mutant and *Ca*Δ*gup1GUP1*homologues strains sensitivity to different antifungals. All strains were treated as described in Material and Methods section **1.3.3**.

3. Extracellular matrix profiles

The *C. albicans* ability to invade tissues and form biofilms is related to the formation of an ECM yet uncharacterized in detail (Douglas, 2003). Similarly to what happens in higher Eukaryotes, it is likely that the constituents of the matrix influence and relation to the morphological switch or, more broadly, control the multicellular aggregates morphology. Moreover, the *GUP1* gene from mammals was implicated in the regulation of the morphogenic Hedgehog pathway (Abe et al, 2008). This prompted us to attempt a first approach to the *C. albicans* ECM using the present constructions. The analysis of ECM proteins from *C. albicans* was performed using a novel methodology developed to efficiently extract the yeast ECM (Joana Carvalho, unpublished results).

We started to compare the ECM proteins of wt and Δ*gup1* mutant strains grown on ideal conditions (YPD at 30°C). For that, 5 plates (with 1 ml of culture per plate) of each strain were grown during 5 days, in order to obtain a mature biofilm-like structure. This was collected, the protein and sugar fractions separated and the proteins precipitated as described in Materials and Methods- section **4**. The SDS revealed that different buffers ressuspend different proteins, as is mirroned by the different protein profiles (**Fig. 21-** green arrows) and modified Laemmli appears as the best ressuspension buffer since the bands are better defined and the protein pattern is more easily visualized (**Fig. 21-** lane 3 and 6). Furthermore, regarding the comparison of the protein profile of Δ*gup1* mutant strain with wt strain, the differences in the protein pattern are notorious, independently of the ressuspension buffer used. This suggests that Δ*gup1* mutant excrete different proteins to the extracellular matrix of those excreted by wt, but also excrete proteins in distinct quantities than wt (**Fig. 21**. red circles). This is not unexpected since it was also observed in S. cerevisiae liquid cultures (Ferreira et al, 2006).

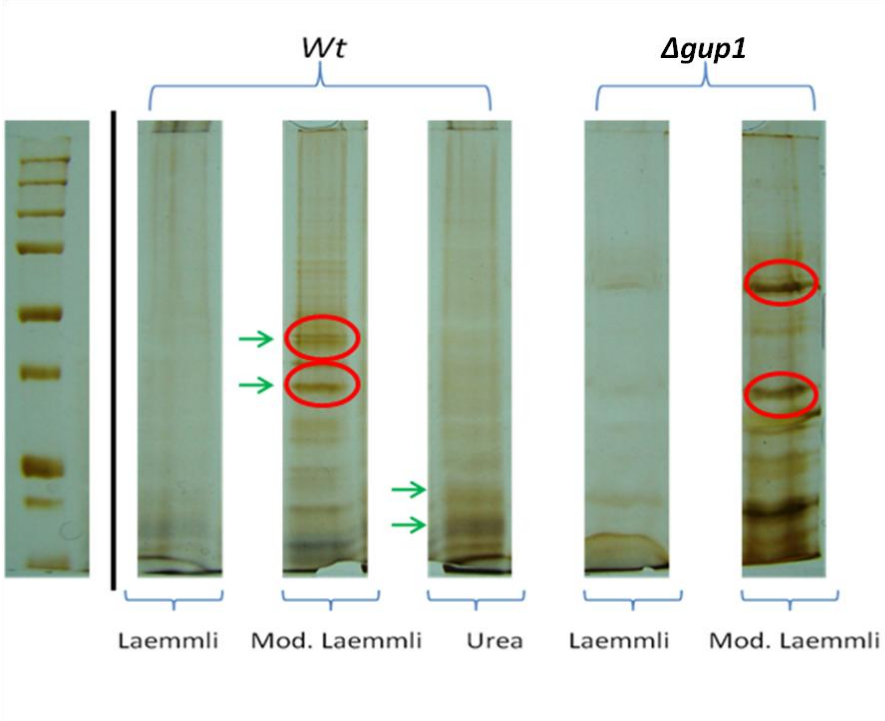

Fig. 21. SDS gel of the ECM proteins of wt and Δ*gup1* mutant grown on YPD 30º C**.**

Next we analyzed the ECM protein profiles of the same strains grown in different conditions: YPD at 37°C, Spider medium at 37°C and YNB without aminoacids at 30°C. These conditions were chosen to observe possible differences in the protein profiles when the strains are submitted to *hyphae* induction conditions (YPD at 37°C and Spider medium at 37°C) and to minimal conditions (YNB without aminoacids at 30°C).

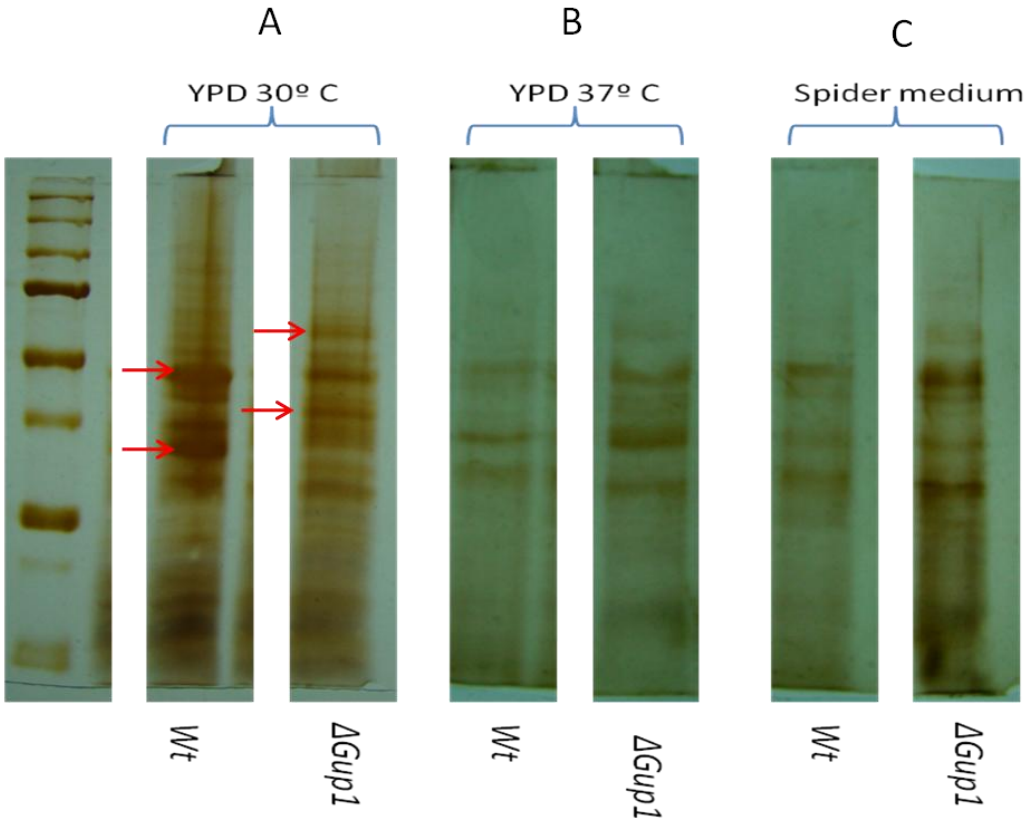

Fig. 22. SDS gel of the ECM proteins wt and Δ*gup1* mutant grown on YPD at 30º C (column A); at 37º C (column B) and on Spider medium at 37º C (column C); All proteins were ressuspended in modified Laemmli buffer.

The ECM protein profile of Δ*gup1* mutant was again very distinct from the one displayed by wt (**Fig. 22**. red arrows). However, probably the most significant result was that under induction *hyphae* conditions the protein profiles displayed a considerable reduced quantity of proteins, independently of the strain used (**Fig. 22**. columns B and C).

Discussion

I

S. cerevisiae Gup1p is an acyltransferase involved in lipids metabolism/rafts stability and on the resistance to antifungals (Ferreira et al, 2008), as well as in the cell wall constitution, morphology and assembly (Ferreira et al, 2006). These are important features to be considered when regarding both *C. albicans* switch from commensal to pathogen and its increased resistance to antifungal drugs. Moreover, in 2008, Abe and collaborators (Abe et al, 2008) described that mammalian *GUP1* as a negative regulator of N-terminal palmitoylation of Sonic hedgehog of Hedgehog pathway, which among other functions is involved in morphogenesis in mammalian cells (Abe et al, 2008). In a previous work, our group has shown that *C. albicans* virulence and drug-resistance requires Gup1p (Ferreira et al, 2010). Our assumptions were based on the following observations. First, *gup1Δ* mutant strain was resistant to common antifungals. Second, *CaGUP1* deletion incited an aberrant evenly ergosterol distribution at the level of plasma membrane. Third, the ability to switch from yeast-form to *hyphae*-growth required *CaGUP1*. Fourth, a distinct growth orientation elicited by the deletion of Ca*GUP1* leaded to colonies with remarkable distinct/aberrant morphology *i.e.* a flower, spaghetti, irregular and wrinkled shape. Fifth, *gup1Δ* mutant strain adherence and invasion abilities were strongly reduced. Sixth, biofilm formation, another important indicator of *C. albicans* virulence, was strongly impaired by the deletion of *CaGUP1*. Finally, the introduction of the *GUP1* gene copy into the *gup1Δ* mutant strain was able to revert all these phenotypes, symptomatic of the *GUP1* gene accountability.

All taken, and considering the fact that *MmGUP1* gene was able to complemented the *hypha* morphogenetic defects on solid media of *gup1Δ* null mutant (Ferreira et al, 2010), we anticipated that Gup1p might be part of a yeast morphogenic pathway, parallel to the mammalian Hedgehog. Several approaches are being developed by our group to unveil and characterize this pathway, both in *S. cerevisiae* and *C. albicans*. The present thesis focused on *C. albicans* approach. For that, we clone several *GUP1* homologues from lower to higher eukaryotes (*S. cerevisiae*, *D. melanogaster* and *H. sapiens*) and evaluated the level of complementation of each homologue concerning some of the virulence factors affected by *GUP1* deletion, namely *hyphae* development, adherence and agar invasion but also regarding antifungals resistance.

II

The initial approach to clone *GUP1* homologues was performed using p4X3GPD plasmids series (Mumberg et al, 1995). p413GPD and p423GPD are considered useful tools for cloning procedures in yeast. The only distinction between them is the different expression strength, p423GPD ensures high expression, while p413GD only produces a copy of the recombinant protein per cell (Mumberg et al, 1995). Although the constructions p4X3GDP-*GUP1* homologues were efficiently cloned in *E. coli*, when transformed into *C. albicans* no clones were achieved regardless of the genomic sequence cloned. We were not able to obtain colonies from these transformations. Instead an undifferentiated amount of biomass (background) was attained on the selective media. This suggests the lost of the insertion and therefore, a high level of instability of these constructions. Although heterologous expression of plasma membrane proteins are traditionally considered very tricky, and consequently the structural instability of the expression vector may result into the lost or rearrangement of the plasmid, we first considered that the main reason could be associated with a weak (not stringent enough) selective force of the aminoacid marker, histidine. This problem has been described before by other groups (Danielly, master thesis, 2008; Pacheco, PhD thesis, 2008). For instance, a plasmid from the same series of p4X3GPD, the p416GPD, was utilized to express the gene *HSP12* in *S. cerevisiae* revealing genetic instability. The same happened on the heterologous expression of *LGT1* in *Torulaspora delbrueckii* (Pacheco, PhD thesis, 2008) and on the heterologous expression of *D. hansennii* xilose symporter *DhXYLH* in *S. cerevisiae* (Danielly, master thesis, 2008).

GUP1 homologues were then cloned into a cloning vector, pRS42K, along with the GPD promoter and CYC1 terminator. This plasmid has a strong selection marker, the antibiotic geneticin (G418). Furthermore, it has been described to enhance the flexibility of genetic manipulations and gene expression in yeast (Taxis et al, 2006), increasing the possibilities of the gene be expressed in larger quantities (Futcher et al, 1983). Using this plasmid the cloning of *ScGUP1, DmGUP1* and *HsGUP1* in *C. albicans* Δ*gup1* mutant strain was successful.

III

The ability to switch rapidly between yeast-to-*hyphae* forms of growth is a defining characteristic of *C. albicans* cells. Nevertheless, each form of growth provides critical functions required for pathogenicity/virulence. Namely, *hyphae* form is thought to facilitate host tissues invasion and escape from phagocytic destruction (Whiteway et al, 2007; Sudbery et al, 2004; Slutsky et al, 1985). According to previous works, Δ*gup1* mutant is not able to develop *hyphae* or pseudo-*hyphae* in solid media, and presents a great delay in doing so in liquid media (Ferreira et al, 2010). Moreover, Δ*gup1* mutant displays distinct colony morphology (spaghetti/flower shape) (Ferreira et al, 2010). The insertion of *GUP1* homologues into Δ*gup1* mutant reverted this morphology pattern, turning colonies smooth and round. Yet, this reversion was not complete, since *Ca*Δ*gup1* transformants were not able to develop the *hyphae* or pseudo-*hyphae* characteristic of wt strain in any tested media (YPD and Spider at 37ºC) (not shown). This suggests the possible existence of some steps during the process of differentiation and that these homologues are only able to complement some of those stages, leading to an intermediated morphology between the sphagetti-like form of Δ*gup1* mutant and the filamentous one of wt. On the other hand, macroscopic morphology may not entirely depend on cellular differentiation/*hyphae* formation, and be under the control of other signaling *via*, which *GUP1* homologues were not able to complement.

In agreement, *Ca*Δ*gup1GUP1*homologues transformant strains when submitted to filament development induction with FBS in liquid cultures were only capable of differentiate into *hyphae* and pseudo-*hyphae* during the first moments of induction. In fact, these strains form filamentous cells as soon as induction is applied (at a minutes scale), whereas Δ*gup1* mutant has a big delay to start developing *hyphae* and pseudo-*hyphae* (Ferreira et al, 2010). After 30 min of incubation with FBS, all the transformants stop the *hyphae* elongation/growth, being for that reason all the *hyphae* cells composed only by two or at most three cells. This is quite interesting since, although Δ*gup1* mutant requires 2.5 h more to start the filamentous development than wt, after 4 or 5 h, the length of Δ*gup1* mutant filamentous cells are quite similar to wt ones (Ferreira et al, 2010). On the other hand, *C. albicans* invasiveness requires that cells differentiate into *hyphae* and/or pseudo-*hyphae* (Sudbery et al, 2004). Accordingly, none of the transformant strains were able to invade the agar. It seems that the signaling for starting filamentous development is complemented by *ScGUP1*, *DmGUP1* and *HsGup1* genes, but the following steps are not. Discover the events that are behind this interruption constitute a stimulating/interesting future project.

Other crucial features on *C. albicans* pathogenicity are the adherence and biofilm formation abilities. In this work we did not evaluate the biofilm formation aptitude of the transformant strains, yet we did assess the adhesion of those strains to YPD and Spider medium. Δ*gup1* mutant has a reduced potential to adhere the agar, which is mirrored by the easiness with which this strain is washed out from the agar (Ferreira et al, 2010). The introduction of the *GUP1* homologues into *gup1Δ* mutant did not cause any amend to its adherence ability, which lead us to extrapolate that possibly also the biofilm formation skill will most probably be defective on those strains.

IV

Modifications on the membrane lipid composition (phospholipids and ergosterol), on its fluidity and asymmetry can be important determinants of yeast cells susceptibility to antifungal drugs (Pasrija et al, 2005). Both *S. cerevisiae* and *C. albicans* Δ*gup1* mutants present an altered sterol distribution across the plasma membrane, accompanied by an increased resistance to antifungal agents such fluconazole, ketoconazole and clotrimazole (Ferreira et al, 2008; Ferreira et al, 2010). These antifungals belong to the class of the azoles and are commonly used to treat *Candida* infections. The pathways involved in *C. albicans* lipid bionsynthesis are not well documented, yet in *S. cerevisiae* it is known that these drugs operate on the biosynthesis of ergosterol at the C-14 demmethylation stage (Song et al, 2007), causing a depletion on ergosterol and accumulation of lanosterol. In our study the *Ca*Δ*gup1GUP1* homologues with *ScGUP1* and *HsGUP1* had a growth behavior very similar to Δ*gup1* mutant, meaning that these strains were as resistant to EBIs as the mutant. This suggests that the resistance to antifungals phenotype of Δ*gup1* mutant was not complemented by *ScGUP1* and *HsGUP1* homologues. On the other hand, *DmGUP1* gene seems to revert Δ*gup1* mutant phenotype, showing a similar susceptibility to EBIs as wt strain. The observation of filipin stained sterols at the plasma membrane of these transformant strains would be of great interest. We would expect an even sterol distribution characteristic of *Δgup1* mutant for the strains harboring *ScGUP1* and *HsGUP1* and a normal punctuated plasma membrane sterol distribution for the strain carrying *DmGUP1* gene.

V

The characterization *C. albicans* ECM proteins is of enormous significance concerning the discovery and identification of the main players of cell-cell communication (Joana Carvalho, unpublished results). While some few studies were published, namely on *C. albicans* ECM that connects the cells within the biofilm structures (Ramage et al, 2005; Mukherjee et al, 2004; Martins et al, 2010; Sutherland, 2001; Al-Fattani et al, 2006), and on wild *S. cerevesiae* strains colonies (Stovicek et al, 2010; Váchová et al, 2011**),** this subject has been largely neglected**.** Thomas and co-workers (Thomas et al, 2006) paper was one of the very few that addressed the identification of *C. albicans* ECM proteins in relation with virulence. These authors performed the proteomic comparative analysis of cell surface-associated proteins and secreted components (among other subcellular fractions) of both liquid and biofilm *C. albicans* cultures. They found some differences that could be associated with the role of biofilm on the *Candida* infections.

Bearing in mind the fact that the *GUP1* homologues from higher Eukaryotes interfere in the morfogenic Hedgehog pathway that controls and is controlled through the ECM components, and in view of all the results above, we considered pertinent to explore the protein composition of the *C. albicans* Δ*gup1* mutant and the above-mentioned transformants, eager to find differences on the yeast ECM protein profiles. In fact, the ECM protein profile of Δ*gup1* mutant was clearly distinct in all conditions tested. The Δ*gup1* mutant SDS-PAGE profile not only showed proteins that were not apparent on the wt profile, but also some of the common proteins were present at different concentrations. Interestingly, when either strain was cultivated at high temperatures (37°C), the ECM proteins profiles exhibited considerably less proteins.

If we bear in mind the phenotypes of Δ*gup1* mutant in *S. cerevisiae*, a possible rationalization for such events could be the instability that the absence of Gup1p causes on the cell wall and membrane composition, assembly, and stability (Ferreira et al, 2006; Ferreira et al, 2008) but also on the protein endocytosis and exocytosis processes (Bosson et al, 2006; Bonangelino et al, 2002). However, one can speculate that some of the proteins excreted by Δ*gup1* mutant present on our profiles may be associated to the adherence, biofilm formation and *hyphae* development deficiencies of this strain, as well as with its increased resistance to the antifungal drugs. The future identification and characterization of the interesting proteins present on those profiles will definitely provide important information in this regard.

Final comments

Considering all the results obtained in this work, we may conclude that *ScGUP1*, *DmGUP1* and *HsGUP1* only partially complement Ca∆*gup1* mutant defects. In what concerns antifungal resistance, only *DmGUP1* which is actually more similar to Gup2 (unpublished results from Fábio faria-Oliveira), was able to revert ∆*gup1* mutant phenotype, showing a similar susceptibility to EBIs as wt strain. Regarding *hyphae* differentiation on liquid media, all the *GUP1* homologues were able to start developing filamentous cells, but unable to give continuity to that differentiation. In agreement, on solid cultures, although displaying a colony morphology closer to wt strain, the transformants were unable to differentiate into *hyphae*, exception made of *MmGUP1* that complemented *hyphae* differentiation defect of ∆*gup1* mutant in solid media (Ferreira et al, 2010). This strain was nevertheless not further tested. It is well known that heterologous expression of proteins despite of the system used, *E. coli*, yeast or even mammalian or plant cells, results in lower expression levels than on the original cells (Grisshammer et al, 1995; Sarramegna et al, 2003). This is even more critical if the protein is a plasma membrane resident (Hamacher et al, 2002; Vilalba et al, 1992), as is the case of Gup1p. Further studies are required to conclude about *GUP1* homologues complementation efficiency of ∆*gup1* mutant phenotypes in relation to expressed protein amounts, and consequently infer on their common functions.

Future work

Future work

Our results, although preliminary open a field of options for future research, mainly regarding proteomics of ECM and its relation to the virulence of *C. albicans*. To consolidate our observations, namely concerning the *hyphae*/pseudo-*hyphae* development and the resistance to antifungals agents of these *GUP1* homologues, further assays are needed:

1. Test new clones of these transformants to attest if the results obtained were due to different expression levels of the proteins.

2. Test the strains with the empty plasmid to confirm that the events are in fact owed only to *GUP1* homologues insertion.

3. Clone *MmGUP1* in the same plasmid (pRS42k) and test it regarding: (i) adherence and agar invasion; (ii) hyphal formation ability in liquid cultures; and (iii) resistance to antifungals;

4. Complete the virulence assays namely study the ergosterol distribution and the biofilm formation ability of these transformant strains.

5. Analyze the protein profile of wt, Δ*gup1* mutant as well as of the transformant strains in 2D-electrophoresis to further identification of key proteins present in the strains.

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