# The *Candida albicans* Hwp2 protein is necessary for proper adhesion, biofilm formation and can complement a *Saccharomyces cerevisiae muc1* null strain.

By

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## ABSTRACT


### The *Candida albicans* Hwp2 protein is necessary for proper adhesion, biofilm formation and can complement a *Saccharomyces cerevisiae muc1* null strain.

*Candida albicans* is a fungal pathogen of humans that is responsible for the majority of mucosal and systemic candidiasis. The host-pathogen interaction in *C. albicans* has been the subject of intense investigation as it is the primary step of infection. Hwp2 is a GPI-anchored cell wall protein that was previously shown to be necessary for hyphal and invasive growth on solid media. The purpose of the current study is to further characterize the protein as far as its role in oxidative stress, sensitivity to cell wall disrupting agents, adhesion to human epithelial and endothelial cells, biofilm formation and chitin content. It appears that Hwp2 is necessary for proper oxidative stress tolerance, adhesion and biofilm formation as an *hwp2* null is more susceptible to increasing doses of hydrogen peroxide, unable to adhere efficiently to epithelial and endothelial cell lines, and unable to form biofilms. Furthermore and according to the *Candida* genome database Muc1 is the protein with the closest similarity-albeit slight- to Hwp2 in *Saccharomyces cerevisiae*. By transforming *HWP2* into a *muc1* null strain we were able to complement the lack of pseudohyphal growth of the mutant and confer upon *S. cerevisiae* adhesive properties. We thus show that in this case sequence similarity reflects true orthology.

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## GLOSSARY

5FOA: 5Fluoroorotic acid

PDA: Potato Dextreose Agar

PDB: Potato Dextrose Broth

YNB: Yeast Nitrogenous Base

PCR: Polymerase Chain Reaction

FBS: Fetal Bovine Serum

GPI: Glycosylphosophatidylinositol

S.c: *Saccharomyces cerevisa*

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*Chapter 1*

# INTRODUCTION

## 1.1 *Candida albicans*

The yeast *Candida albicans* is a commensal diploid microorganism found in the gut flora. *C. albicans* usually resides in a benign manner in the host mouth, gut, and vagina without causing disease with the host immune system keeping the virulence nature of the microorganism at bay (Southern et al., 2008). *C. albicans* is the most virulent species of the genus. It is capable of achieving filamentous growth, attachment and invasion of human epithelia and is a causative agent of severe and potentially fatal disseminated disease in the human host (Karkowska-Kuleta et al., 2009). Superficial infections of skin and mucosal membranes by *C. albicans* causing local inflammation and discomfort are common in many human populations. At certain disease states when a patient is immunocompromised, *C. albicans* overcomes host defenses. Physiological states such as diabetes, pregnancy, neutropenia, and immunosuppressive therapy predispose individuals to serious candidiasis encompassing infections that range from superficial, such as oral thrush and vaginitis, to systemic and potentially life-threatening diseases (Viudes et al., 2002). AIDS patients exhibit thrush among early complications (Southern et al., 2008). *C. albicans* is the most common human fungal pathogen which is commonly retrieved from blood cultures. In the United States, mortality rates associated with candidemia and disseminated candidiasis is close to 50% with estimated treatment costs of billions of dollars per year (Viudes et al., 2002).

## 1.2 Dimorphism and switching

Many factors are needed for *C. albicans* to be a successful pathogen. For example *C. albicans* is able to interchange between two main morphologies, blastospores and hyphal cells. "Dimorphism is reversible". Blastospores are round separately floating yeast cells which grow mitotically by budding. The elongated hyphal cells arise from formation and extension of germ tube and adhere to one another then forming filamentous structures (Ten cate et al., 2009). See figure 1 for more details.

*In vitro*, and under favorable growth conditions, *C. albicans* exists as blastospores. However, many environmental cues such as stress conditions, or conditions that mimic the host environment – liquid serum media, temperature of 37°C, glucose starvation, neutral or alkaline pH, and hypoxia – can trigger switching to the filamentous form (Sudbery et al., 2004). When incubated with epithelial cell lines, *C. albicans* exhibit hyphal formation which is necessary for adhesion to and subsequent invasion of the mammalian cells (Zakikhany et al., 2008). *C. albicans* dimorphism ability is crucial for the organism virulence. *In vivo*, the yeast form is necessary for rapid clonal expansion, while the hyphal form allows tissue invasion needed for dissemination (Karkowska-Kuleta et al., 2009). In fact, the filamentous form was isolated from patients suffering from systemic candidosis (Sudbery et al., 2004). Knocking out genes involved in promoting filamentation, notably the transcriptional activators *EFG1* or *CPH1* that activate hyphal genes, renders the *Candida* strains avirulent in a mouse model of disseminated infection. However even though filamentation appears to be necessary for virulence, it is not sufficient. For example, deleting filamentation repressor genes, such as *RFG1* and *TUP1*, results in constitutively filamentous strains that are avirulent (Braun et al., 2001). Thus it appears that the ability to switch between the two forms is essential for successful infection.

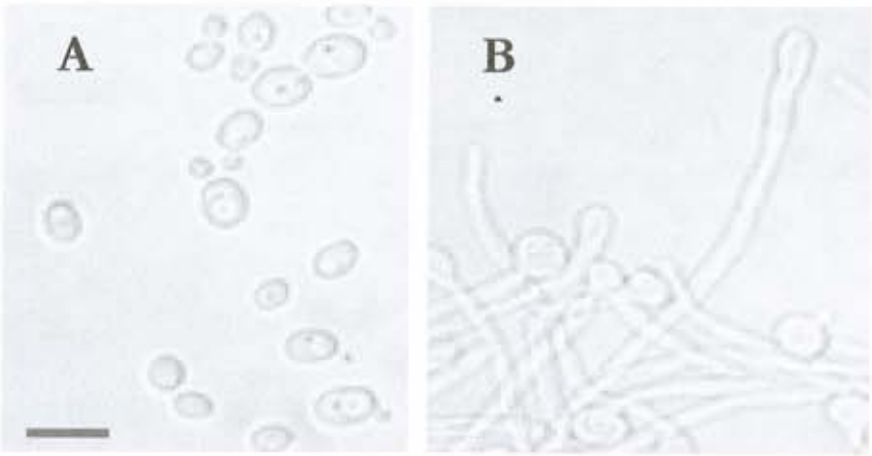


**Figure 1. Dimorphism in *C. albicans*:** A) Free floating blastospore cells; B) Hyphal cells forming filamentous structure (Saville et al., 2003).

Furthermore, *C. albicans* can undergo colony morphology switching. It has the ability to switch between white and opaque colony morphologies with the opaque form composed of large oval shaped flat cells necessary for mating and for evading the immune system whereas the white cells are virulent and antigenic. White to Opaque transition is also an important virulence factor in *C. albicans;* however, it takes place at low frequencies and randomly when compared to dimorphic switching that takes place en masse and in response to specific environmental cues. For example, upregulation of *SAP2* and *EFG1* favors white colony morphology, while *WOR1* (White Opaque Regulator gene), *OPA1* and *SAP3* upregulation favors the opaque forms (Yang 2003). See Fig 2 for more details.

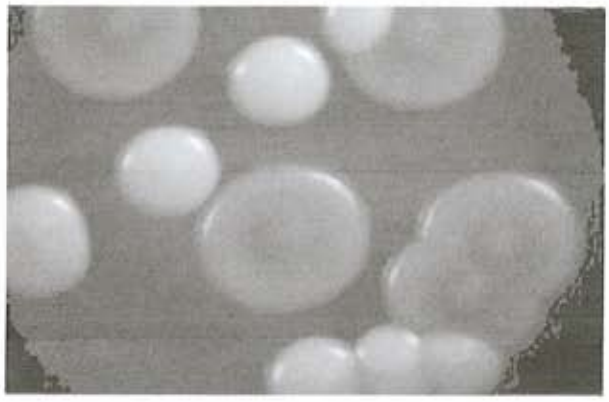

**Fig.2 White Opaque colony morphology.** Note the dark and flat opaque colonies and the creamy smooth white ones. (Slutsky et al., 1987)

## 1.3 Virulence factors

*C. albicans* has evolved a wide set of genes coding for secreted virulence factors such as phospholipases, proteases, lipases, dismutases, adhesins and others. This wide arsenal allows cell wall biosynthesis, self defense against the host, adhesion and invasion, as well as other pathogenic processes (Naglik et al., 2003; Brown et al., 2007). One factor that contributes to the process of virulence in *C. albicans* is secretion of hydrolase enzymes. Besides digesting molecules and acquiring nutrients, these enzymes play a major role in pathogenesis by digesting or distorting host cell membranes, thus contributing to host tissue invasion (Ibrahim et al., 1995). One major form of hydrolytic enzyme is phospholipases (PL) such as *PLA1* and *PLA2* which cleave the ester bonds in glycerol molecules. *PLC* and *PLD* hydrolyze amphipathic phospholipid molecules (Theiss et al., 2006). The major phospholipase in *C. albicans* is phospholipase B. During infection, it is secreted at elevated levels, and it cleaves the ester bonds in glycerophospholipids damaging the host cell membrane (Ghannoum 2000). *PLB5* disruptive mutants were shown to be significantly attenuated in virulence in a mouse model of infection (Theiss et al., 2006). *C. albicans* also secretes several proteases mainly the Secreted Aspartyl Proteases (SAP) family consisting of 10

proteases. Each protease expresses optimal function at a specific pH, with the family covering a wide range of activity from low pH (*SAP3*) to high (*SAP6*). SAP proteins damage host cell membrane thus allows *C. albicans* to adhere to and invade host tissues (Naglik et al., 2003). In order to avoid or overcome the host immune system, *C. albicans* secretes various catalases and dismutases and other virulence factors which help protect the pathogen from Reactive Oxygen Species (ROS) burst generated by the host macrophages (Brown et al., 2007). *PHR1* and *RPH2* help *C. albicans* tolerate neutral and acidic pH, respectively (Yang, 2003).

*C. albicans* has the ability to form biofilm and can adhere to abiotic surfaces (e.g. catheters, intravenous tubing). Biofilms consist of both blastospore and hyphal cells, the hyphal cells are responsible for increased adhesion. Biofilm forming *C. albicans* are 1000x more resistant to antifungal drugs than free floating cells causing serious problems in hospitals where infection spread to patients via the bloodstream. This increased resistance is due to upregulation of efflux pumps that pump out the antifungal agents. In addition, biofilm mass is composed of glycoproteins and polysaccharides. This dense meshwork is thought to limit drugs permeability. β-1,3-glucans of the cell wall are thought to bind drugs and prevent them from entering cell. Biofilm cells secrete more adhesins and proteases than planktonic cells. (Nett et al., 2008).See Fig 3 for more details.

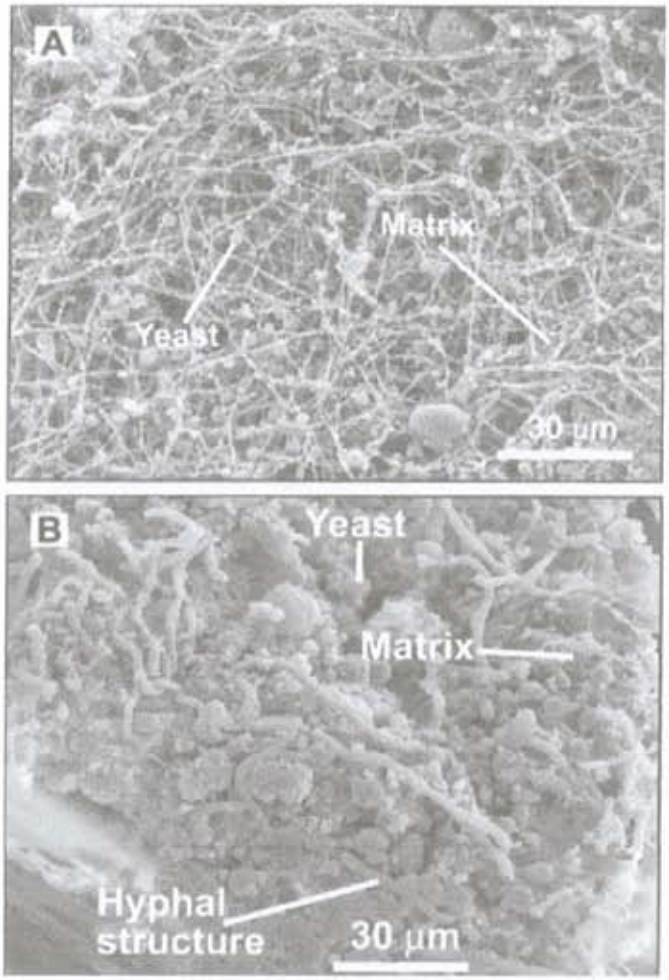


**Fig 3. Biofilm formation**. Biofilm is composed of a mixture of yeast and hyphal cells forming a thick meshwork (Andes et al., 2004).

## 1.4 Cell wall

The cell wall is perhaps the most crucial *C. albicans* structure. The cell wall is responsible for adhesion, the first and most important process that establishes infection. It is also responsible for morphological switching, stress tolerance, antigenicity and other virulence related processes (Masuoka., J 2004). The

structure of the cell wall and its modulable nature is crucial for infection. Hyphal cells for example exhibit a thicker cell wall with increased chitin deposition and with different cell wall proteins being expressed than do yeast cells (Kapteyn et al., 2000). 80-90% of the cell wall composition is carbohydrates mainly beta-glucan, chitin and mannan (Lajean Chaffin et al., 1998). The *C. albicans* cell wall is a bi-layer structure composed of an inner stress tolerant chitin layer bound to mainly β-1,3-glucan and some β-1,6- glucan molecules and an outer layer of mannoproteins. Hydrogen, hydrophobic or covalent linkages are found between the different components (Tronchin et al., 1981). The cell wall proteins (CWP) are divided into two categories. The first is bond to the β-1,6-glucan through a glycophosphatidylinositol (GPI) anchor, while the second type of proteins are directly linked to the β-1,3-glucan and are called Pir proteins (LaJean Chaffin, 2008). See Fig 4 for more details.

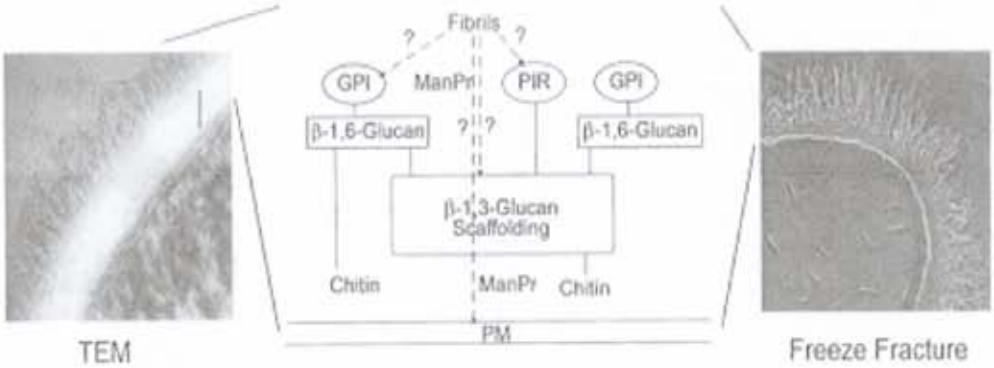


**Figure 4. *C. albicans* cell wall components and organization.** The structural composition of the *C. albicans* cell wall using Transmission Electron Microscopy and Freeze Fracture Electron Microscopy (Masuoka, 2004).

Adhesins are one of the major virulence factors in *C. albicans*. Most characterized adhesins such as Hwp2, Mnt1, Hwp1, Pmt1, and members of

the Agglutinin Like Sequence (Als) protein family attach to the cell wall via a preformed GPI-anchor added postranslationally to the C terminus. Many adhesins studied to date have common structural features, with an N terminal globular Ig like domain, serine threonine rich region containing conserved repeats and a C terminal glycosylated stalk region that extends the active site from the cell wall surface (Otoo et al., 2008). Adherence to host cell is the first step in the process of infection in *C. albicans*. Besides mediating yeast-to-host tissue adherence, ALS proteins can also promote yeast aggregation which has amyloid-like characteristics. As antigenic determinants, deletion of many of these adhesins rendered the mutant cell significantly defective in adhesion and virulence (Yang 2003; Hayek et al. 2009).

Hyphal wall protein 2 (Hwp2) is a 908 aa long putative GPI-anchored protein which is expressed under filamentous conditions. *hwp2* deletion mutants showed lack of invasive growth on solid media and slight attenuation of virulence in a mouse model. This indicates that Hwp2 might be involved in adhesion and invasiveness (Hayek et al. 2009). In fact, BLASting the Hwp2 ORF onto the *C. albicans* database at www.candidagenome.org revealed two proteins with strong sequence similarity: Hwp1 and Rbt1. Both have been characterized and have been found to be involved in adhesion and virulence (Hayek et al, 2009)

## 1.5 *Saccharomyces cerevisiae*

*S. cerevisiae* is a species of budding yeast used in baking and brewing. It is one of the most extensively studied eukaryotic model organism. It has a short life cycle, is easily amenable to modern biochemical and genetic techniques, and shares a wide array of basic cellular processes with higher eukaryotes, including humans. In many cases, elements of these processes are widely interchangeable. This is true in the case of transcriptional regulation, where the RNA Polymerase

II holoenzyme in yeast is very similar to that of humans, and many of its components are functionally interchangeable. (Krogan et al., 2002)

*C. albicans'* distant cousin, *S. cerevisiae* also has a sizable number of adhesins. Given that *S. cerevisiae* is a non-pathogenic yeast, the adhesins do not mediate virulence but flocculation and aggregation. One notable member of this family is Flo11/Muc1, a cell surface bound mucin like protein containing serine threonine rich conserved repeats found to be necessary for blastospore-pseudohyphal switching in addition to flocculation and aggregation. Furthermore Muc1 is necessary for diploid pseudohyphal formation, haploid agar invasion and biofilm formation (Dranginis et al., 2007; Lambrechts et al., 1996) as a null mutant lacks all of the above. According to the *C. albicans* database (www.candidagenome.org), the closest possible *HWP2* orthologue in S. cervisiae is *MUC1* and actually an *hwp2* null shows similar phenotype to a *muc1* null as the strain was afilamentous and hence uninvasive on solid media such as sabouraud and commeal agar (Hayek et al., 2009).

## 1.6 Purpose of the current study

Here, we present our attempt to investigate the role of Hwp2 by assessing whether the lack of invasive growth initially observed in the mutant is prompted by a lack of adhesion to human cells and whether a lack of adhesion is mirrored as a decrease in biofilm formation. Furthermore, we were interested in determining whether the deletion of Hwp2 affects chitin deposition and in turn affect the susceptibility of the mutant to various cell surface disrupting agents, and tolerance to oxidative stress. Finally, homology between Muc1 and Hwp2 has only been determined through sequence similarity and not by functional complementation. We thus attempted a cross species complementation test by transforming the *C. albicans HWP2* into an *S. cerevisiae muc1* null to determine whether complementation of the *muc1* null phenotype was possible. Successful

complementation will be determined on various filamentation inducing media. In addition, the complemented *S. cerevisiae* strain will be assessed for its ability to adhere to human cells mediated by the adhesin Hwp2.

*Chapter 2*

# MATERIALS & METHODS

## 2.1 Strain preparation and culture conditions

The *Candida albicans* wild type strain RM1000 + pABSK2 (*ura3Δ::λimm434/ura3Δ::λimm434his1::hisG/his1::hisG*) (Negredo et al., 1997) histidine and uridine auxotroph, *HWP2/hpw2::URA3* heterozygote, *hwp2:URA3/hwp2::HIS1* null, and a revertant *ura3::HWP2/hwp2::HIS1* strains were used in this study (Hayek et al., 2009).

The *C. albicans* strains were grown on rich potato dextrose agar (PDA) medium (HiMedia India) for routine cultures. PDA plates were supplemented with uridine and histidine in all experiments of spotting, adhesion, biofilm and oxidative stress tolerance. For the biofilm assay, yeast nitrogen base (YNB) synthetic medium (Fluka, Switzerland) (Kaiser et al. 1994) was used and was supplemented with uridine and histidine. Rich potato dextrose broth (PDB) liquid media (Hi Media, India) was used to grow the strains until exponential phase for oxidative stress, cell surface disrupting media and adhesion experiments. Strains were incubated at 30°C under aerobic conditions.

The *muc1* null (ISP15 *muc1*Δ) and wild type *ISP15* (*a, STA2, his3, leu2, ura3, trp1, thr1*) were grown on rich PDA medium for routine cultures. For experiments requiring selective growth, Yeast Nitrogen Base (YNB + Glucose) media supplemented with appropriate amino acids was utilized (Fluka, Switzerland) (Kaiser et al., 1994). For filamentation experiments, Cornmeal agar (HiMedia, India) supplemented with 1% Tween 80 and Synthetic Low Ammonium Dextrose SLAD agar media supplemented with uridine, histidine, leucine, tryptophan and threonine was prepared as previously described (Gimeno et al.,

1992) and were cultured at 30°C under aerobic conditions. Cornmeal and SLAD agar plates were incubated for 4-5 days before microscopic observation. Plate and colony images were captured by the use of an Olympus E330-ADU-1.2X stereomicroscope. Microphotographs were taken by the use of an Olympus CX41 system coupled with a Sony DSC-S40 digital camera.

## 2.2 Human epithelial/endothelial cell culture

Human colon adenocarcinoma epithelial cell line HT-29 ATCC number HTB-38 was utilized in this study for epithelial adhesion experiments. The human cells were cultured in RPMI 1640 medium (Biowhittaker, Belgium) which contains 25mM HEPES and L-glutamine. The medium was supplemented with 10% FBS (Fetal Bovine Serum, Biowhittaker), 100U/ml of penicillin, 100μg/ml of streptomycin, and the culture flasks were incubated at 37°C in 5% $CO_2$. Human endothelial cell line ECV304 originating from human bladder carcinoma with a perfect endothlial phenotype (ATCC number CRL 1998) was utilized in this study for endothelial adhesion experiments. Culture conditions utilized were identical to that of the epithelial adenocarcinoma cell line.

## 2.3 Oxidative stress tolerance

The *C. albicans* wild type, heterozygote and null mutant were assayed for their ability to withstand a potentially lethal dose of hydrogen peroxide. Strains were incubated with 10, 25 and 50mM concentration of hydrogen peroxide for one hour during their exponential growth phase. After incubation, 5μl of a serial dilution were spotted on PDA plates (Pedreño et al., 2007).

## 2.4 Cell surface disrupting agents

The three candida strains were grown until exponential phase and serially diluted. 5μl of the serial dilutions were spotted on PDA plates treated with 0.2 to

0.05% SDS (Sigma-Aldrich, Germany), 100 to 300μg/ml calcofluor white (Sigma-Aldrich, Germany), and 100 to 300μg/ml Congo red (Sigma-Aldrich, Germany). A control plate lacking cell surface disrupting agents was spotted with the exact same volumes of the three strains. The plates were incubated at 30°C for 3-4 days (Plaine et al., 2008).

## 2.5 Adhesion to human epithelial and endothelial cells

Adhesion ability of the strains was assayed against human epithelial cell line HT-29 and human endothelial cell line ECV304 as previously described (Tsuchimori et al., 2000). Briefly, around 100 cells of the three strains were incubated with human cell lines in 6 well microtitre plates for either 90mn or 180mn followed by washing with PBS and overlay with molten PDA agar. Colonies were counted after overnight growth and were compared with a control plated inoculated with the same number of *C. albicans* cells. The results of this experiment are expressed as percentage adhesion of the control plate.

## 2.6 Chitin quantification

Cell wall chitin content was measured for the *Candida* strains according to a modified protocol previously described (Munro et al., 2003; Kapteyn et al., 2000). Briefly, 6M HCl was used to hydrolyze 50mg wet weight purified cell walls at 100°C overnight. After centrifugation the pellet was reconstituted in 1 ml of distilled water. A 0.1 ml aliquot of this sample was added to 0.1 ml of solution A (1.5 N $Na_2CO_3$ in 4% acetylacetone). The mixture was incubated at 100°C for 20 minutes and after cooling to room temperature, 0.7 ml of 96% ethanol was added to the mixture followed by the addition of a 0.1 ml of solution B (1.6 g of *p*-dimethyl-aminobenzaldehyde in 30 ml of concentrated HCl and 30 ml of 96% ethanol). The mixture was incubated for 1 hour at room temperature and absorbance of the samples was measured spectrophotometrically at 520 nm. The

results were plotted against a standard curve generated by the use of known glucosamine standards taken through the same procedure as our samples.

## 2.7 Biofilm formation

The ability of the strains to form biofilms on polystyrene microtiter wells was assayed according the previously described protocol (Peeters et al., 2008). Briefly, $5X10^6$ cells of the wild type, *HWP2/hpw2::URA3* heterozygote, *hwp2:URA3/hwp2::HIS1* null and *ura3::HWP2/hwp2::HIS1* revertant strains were incubated in a flat bottomed microtiter well pretreated with 5% serum overnight. Strains along with an uninoculated control well, were left to incubate at 37°C in a shaking incubator at 75 rpm for 2 hours. Wells were rinsed with PBS to remove non-adherent cells followed by addition of YNB and incubation at 37°C for 48 hours. After incubation, plates were washed again, fixed with methanol and air dried. A 0.2% crystal violet solution was added and left for 20 minutes. Excess crystal violet was washed with distilled water and the crystal violet bound to biofilm cells was released by acetic acid treatment. Absorbance of the released crystal violet was measured spectrophotometrically at 590 nm.

## 2.8 Construction of Sc. *URA3-HWP2*-Sc. *URA3* transformation cassette

The wild type RM1000 strain was used to generate this cassette. Primers utilized contained 100 bp DNA sequences homologous to a 100 bp region upstream and downstream of the *Sc (Saccharomyces cerevisiae) URA3* at their 5' end and *HWP2* sequences at -310bp for the forward primer and at +2792 downstream for the reverse primer at their 3' end (See Table 1 for a list of primers generated). Amplification resulted in a cassette containing the entire *HWP2* ORF and upstream promoter region flanked by regions of homology to *S.c URA*. The cassette was used to transform the *S. cerevisiae ISP15Δmuc1* strain (*a, STA2, his3, leu2, ura3, trp1, thr1, muc1::URA3*) (Lambrechts et al., 1996).

Amplification of the *HWP2-URA3* deletion cassette was done according to the following conditions: A PCR mix containing 50ng of *C. albicans* genomic DNA, 5 µl of 2 mM dNTP mix, 5 µl of 10x long PCR buffer with $MgCl_2$, 1 µl of 60 mM forward and reverse primers, 2 µl of DMSO, 0.5 µl of Long PCR enzyme mix (Fermentas Life sciences, USA), and 35.5µl of distilled nuclease free water. The polymerase chain reaction was performed in a Gene Amp PCR system 9700 (Applied Biosystems) with the following conditions: Initial denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 20 seconds, annealing at 62°C for 30 seconds, elongation at 68°C for 2 minutes; followed by a final extension at 72°C for 10 minutes. The PCR product was stored at 4°C until further usage.

## 2.9 Transformation of cassettes

The *S. cerevisiae muc1* strain was transformed with the Sc.*URA3-HWP2-*Sc.*URA3* using a modified protocol of the alkali yeast cation transformation Kit (Q-Biogene, Germany, Khalaf and Zitomer 2001). Transformants were counter selected for on 0.1% 5Fluoroorotic acid (5FOA) (Sigma-Aldrich, Germany) in YNB media supplemented with 20 µg/ml uridine (Boeke et al., 1984).

## 2.10 DNA extraction

Genomic DNA extraction from the transformed *S. cerevisiae* colonies was conducted according to the yeast DNA extraction protocol described previously (Dib et al., 2008).

## 2.11 Verification of integration by PCR

Insertion of the cassette into the *S. cerevisiae URA3* locus was verified by a pair of primers which hybridized at -50bp and +1680bp of the *HWP2* ORF confirming the presence of *HWP2* in *S. cerevisiae*, and by a primer set which

hybridized at - 100bp and 330bp of the *S.c.URA3* were used, confirming the absence of the *URA3* allele. (See Table1 for a list of primers generated). The *C. albicans URA3* plasmid YCp(33) (Gift from Dr. R. Zitomer) was used as a control template for the presence of *URA3*. The PCR conditions use for these verification experiments were as follows: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 52°C for 50 seconds, extension at 72°C for 2 minutes and a final extension at 72°C for 10'.

**Table 1. PCR primers used in this study.** Forward: F, Reverse: R. Gene sequences were obtained from the *Candida* genome database www.candidgenome.org )

| Primer name | Primer sequence | 5' hybridization site |
|---|---|---|
| Sc*URA3-HWP2*F | 5'ATGTCGAAAGCTACATATAAGGAACGTGCTGCTACTCATCCTAGTCCTGTTGCTGCCAAGCTATTTAATATCATGCACGAAAAGCAAACAAACTTGTGTGCATCAGATCATGCCATACCACTGCC3' | *C. albicans HWP2* at -310 |
| Sc*URA3-HWP2*R | 5'CGGGTAATAACTGATATAATTAAATTGAAGCTCTAATTTGTGAGTTTAGTATACATGCATTTACTATAATACAGTTTTTTAGTTTTGCTGGCCGCATCTTACTAGGTGTATTGTGTTGAAAACCGA3' | *C. albicans HWP2* at +2792 |
| *HWP2* Integration Verification F | 5'CCAATACGACACAAGTCTCCGATA3' | *C. albicans HWP2* at -50 |
| *HWP2* Integration Verification R | 5'AGATGATTCTGAAGTTGATGATCTCACAG 3' | *C. albicans HWP2* at +1680 |
| Sc*URA3 F* | 5'CGCATATGTGGTGTTGAAGAAACATGA3' | *S. cerevisiae URA3* at -100 |
| Sc*URA3 R* | 5'GTATACACCCGCAGAGTACTGCAATT3' | *S. cerevisiae* |

| | | |
|---|---|---|
| | | *URA3* at +330 |

## 2.12 Agarose gel electrophoresis

Following PCR, gel electrophoresis was conducted to determine the size of the PCR products using pharmacia Biotech gel electrophoresis apparatus (GNA-100, Sweden) and 0.8% agarose gels along with a 500 bp DNA marker.

## 2.13 Filamentation on SLAD and cornmeal agar

The *S. cerevisiae HWP2* transformant, *muc1* null, and the *MUC1* wild type strains were plated on SLAD and cornmeal agar media at 30°C for 4 days. Wet mounts were prepared and observed at 1000X magnification.

## 2.14 *Saccharomyces cerevisiae* adhesion assay

The *S. cerevisiae* wild type *ISP15*, *muc1* null *ISP15 muc1*Δ (*muc1::URA3*), and transformant *ISP15* (*muc1*, *ura3*::HWP2) were assayed for their ability to adhere to human epithelial cell line HT-29 as previously described. Strains were platted on cornmeal agar for 4 days to allow for possible pseudohyphal formation prior to incubation with the human cell line.

## 2.15 Statistical analysis

Data was coded and entered into the SPSS 17 program (SPSS Inc., USA) for analysis. Mean and standard deviations were calculated. Analysis of Variance (ANOVA) was carried out with multiple comparison technique to control for alpha inflation. P-value less than 5% was considered significant.

*Chapter 3*

# RESULTS

## 3.1 Oxidative stress response

The *C. albicans* strains were grown until stationary phase and treated with various concentrations of hydrogen peroxide. The homozygous *hpw2/hwp2* mutant strain showed increased sensitivity to hydrogen peroxide when compared to the wild type strain. See Fig 5 for more details.

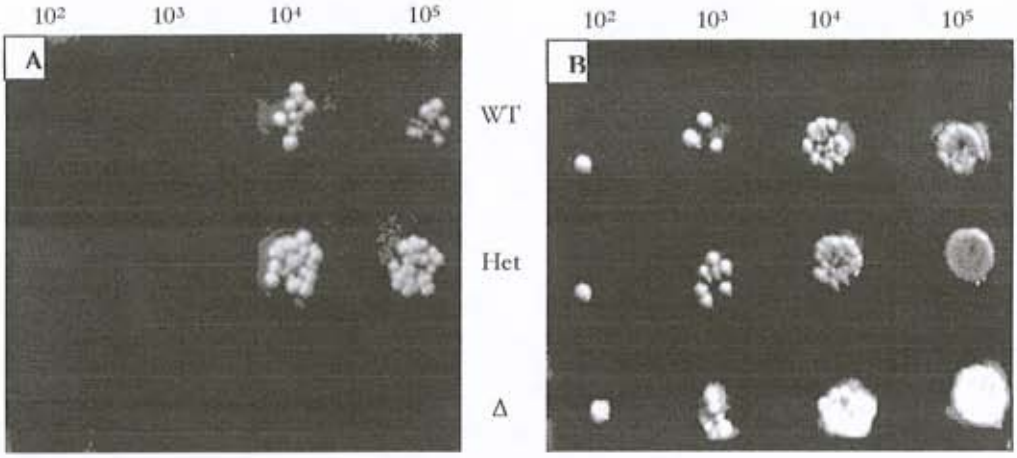


**Figure 5. Oxidative stress response.** The wild type (WT), heterozygote (Het), and *hwp2* null (Δ) mutant were incubated with 50 mM concentration of hydrogen peroxide for one hour. Each dot represent a single colonie. The numbers indicate serial dilutions and number of cells. A) 50 mM concentration of hydrogen peroxide. The mutant clearly shows increase susceptibility to hydrogen peroxide when compared to the wildtype. B) Untreated control plate.

## 3.2 Cell wall disrupting agents

The wild type, *HWP2/hwp2*, and *hwp2/hwp2* strains were spotted on PDA plates containing various cell wall disrupting agents. The mutant strain showed a very slight sensitivity to SDS, when compared to the wild type strain. No discrepancy was seen between the mutant, heterozygote, and wild type strains on plates containing calcofluor white or Congo red. See Fig 6 for more details.

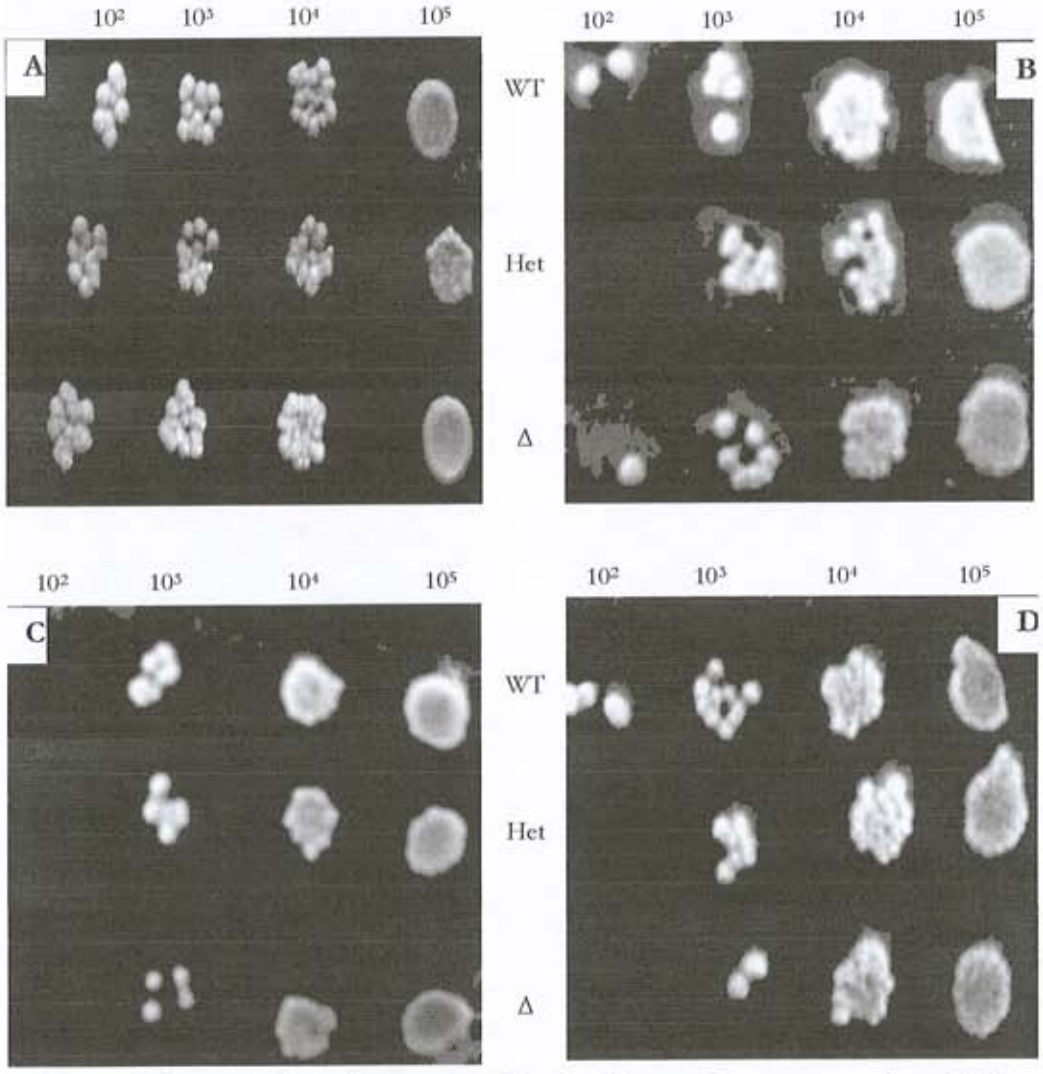


**Figure 6. Cell wall disrupting agents.** The *C. albicans* strains were spotted on PDA plates treated with various concentrations of cell wall disrupting agents. Each dot represent a single colonie. The numbers indicate serial dilutions and number of cells. A)

Untreated control plate. B) 150μg/ml calcofluor white. C) 200μg/ml Congo red. D) 0.03% SDS. Note the lack of any significant difference between the wild type and mutant.

### 3.3 Adhesion to human epithelial and endothelial cells

The three *C. albicans* strains were incubated with human epithelial (Ht-29) and endothelial (ECV304) cell lines. After 90' (data not shown) and 180' minute incubation, the mutant strain showed a significant 75% decrease in ability to adhere to human epithelial cells when compared to the wild type strain (p-value <0.001). The null mutant also showed a significant 68% decrease in ability to adhere to human endothelial cells. See figures 7 and 8 for more details.

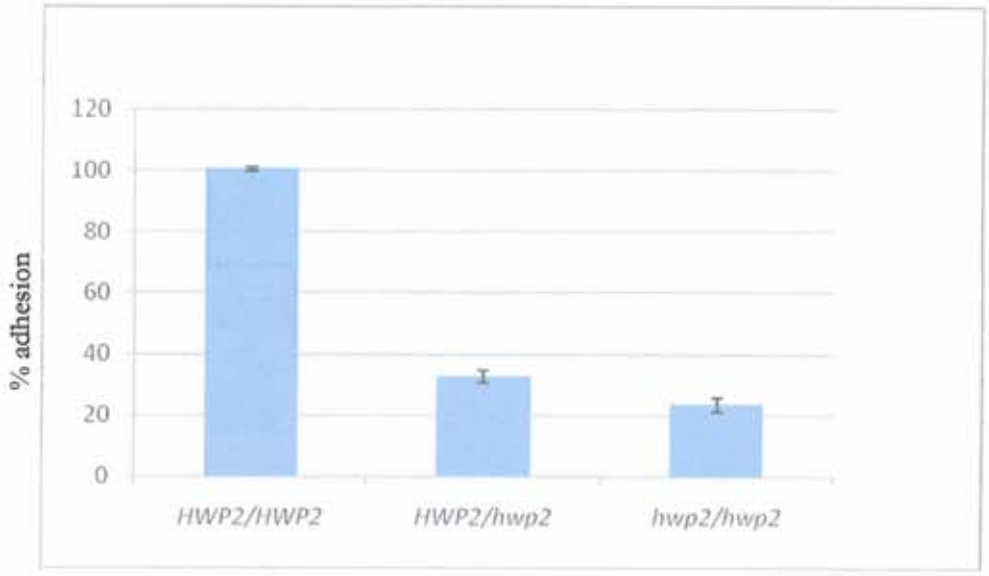


**Figure 7. Adhesion assay to epithelial cells.** Around 100 *C. albicans* cells were added to human epithelial cells in 6-well microtiter plates. After 180' non adherant cells were washed away. Results are expressed as percent of total cells added. Compared to the wildtype, the mutant clearly shows a dramatic and significant decrease (75%) in its ability

to adhere to human epithelial cells (p-value <0.001). This indicates a defect in adhesion in the mutant strain.

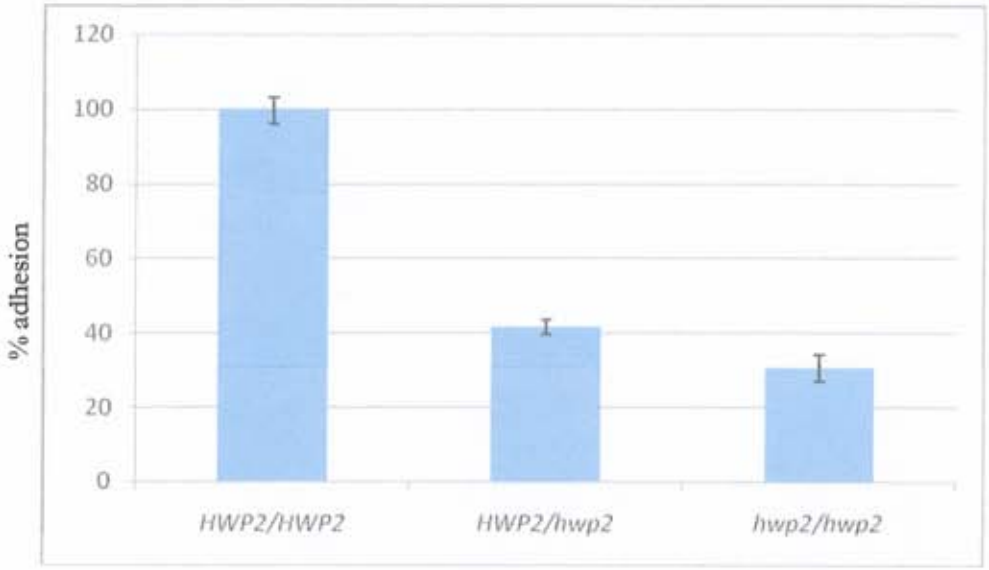


**Figure 8. Adhesion to human endothelial cells.** Figure shows the percentage adhesion of cells relative to a control well. Note the statistically significant (p less than 0.001) decrease in adhesion of the *hwp2* null mutant strain compared to the wild type.

## 3.4 Chitin content

The amount of cell wall chitin was determined by acid hydrolysis for all the *C. albicans* strains. A slight difference was observed between the wild type and the mutant strains (15% less chitin in the mutant). See Fig 9 for more details.

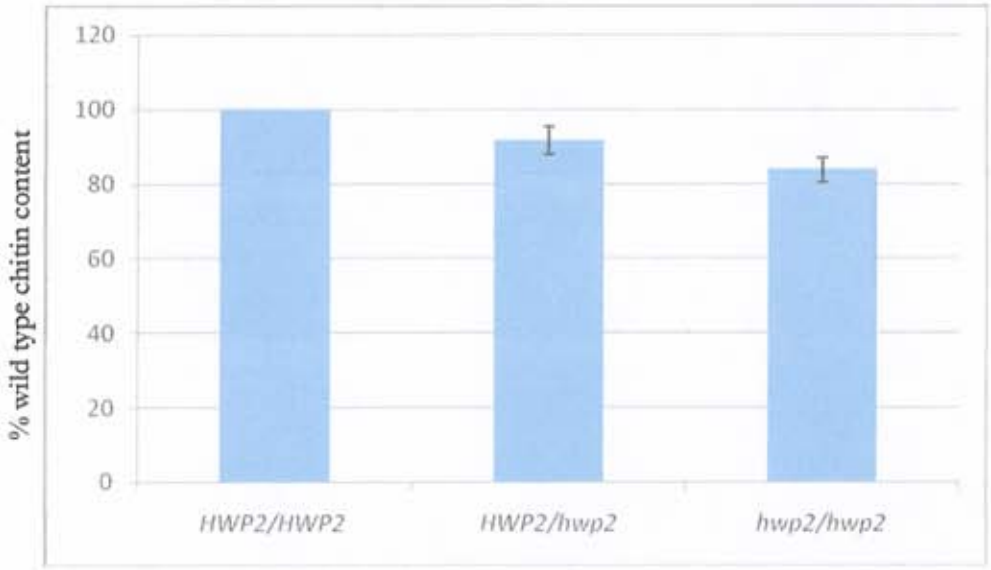


**Figure 9. Chitin content.** The amount of cell wall chitin was determined spectrophotometrically following acid hydrolysis. Data represents percent chitin content as compared to the wild type. Note the slight decrease in chitin content in the mutant strain.

### 3.5 Biofilm formation

The wild type, heterozygote, null mutant and revertant strains were assayed for ability to form a biofilm on polystyrene plastic wells. The mutant strain showed a 30% decrease in biofilm mass (p-value = 0.008) when compared to the wild type, while the revertant exhibited an intermediate phenotype. See Fig 10 for more details.

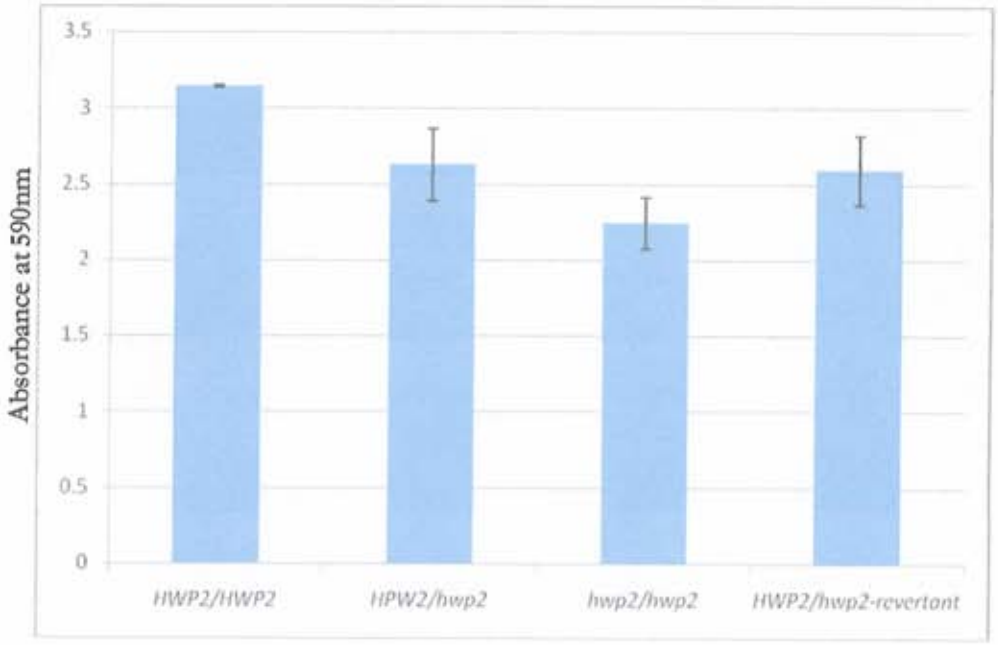


**Figure 10. Biofilm formation.** Biofilm formation on polystyrene plastic microtiter plates was performed as described in the materials and methods. Note the decrease in crystal violet absorbance at 590nm in the mutant strain as opposed to the wild type. The revertant *HWP2/hwp2* strain exhibited an intermediate phenotype.

## 3.6 Cross species complementation

The *C. albicans HWP2* ORF from -310bp to +2792bp was transformed into the *S. cerevisiae muc1* null strain at the *URA3* locus. The successful transformants were counter selected for on YNB plates containing 5FOA. See Fig 11 for more details.

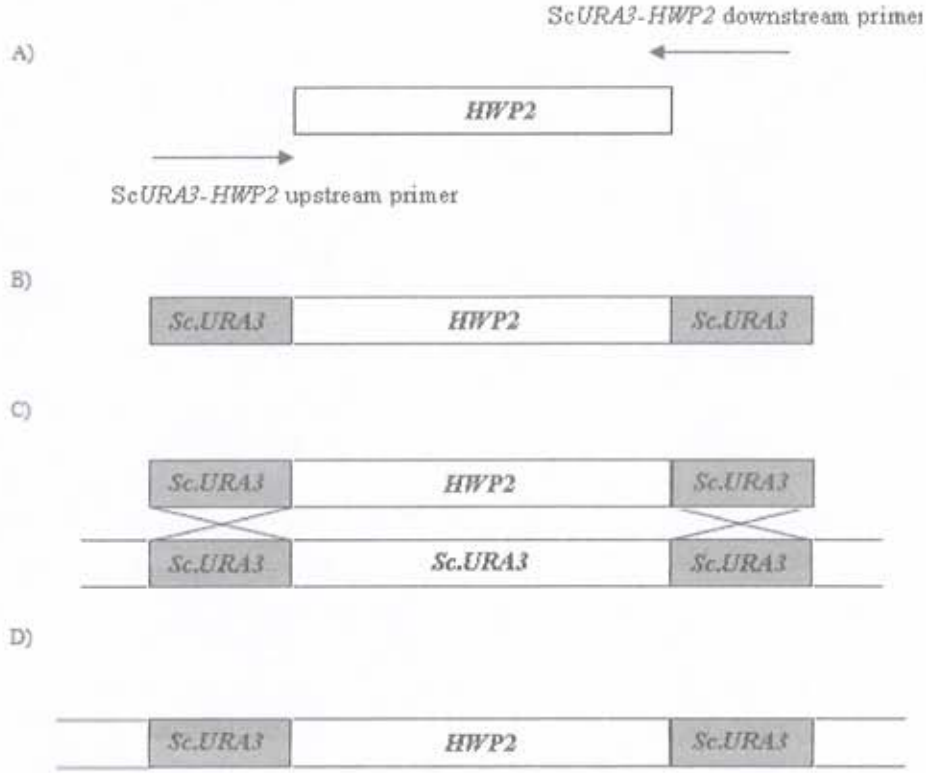


**Figure 11. Transformation strategy.** A) Generation of Sc.*URA3-HWP2*-Sc.*URA3* cassette via PCR on *C. albicans* genomic DNA. B) Sc.*URA3-HWP2*-Sc.*URA3* cassette. C) Homologous recombination event at the *S. cerevisiae URA3* locus. D) *HWP2* transformed into the *S. cerevisiae muc1* null strain.

Verification of integration was achieved by generating a *HWP2* fragment upon amplification with *HWP2* internal primers, and lack of a PCR product upon amplification with *URA3* primers. An S. cerevisiae*URA3* plasmid (YCp(33)) was utilized as a control. Successful integration of *HWP2* displaced the *S. cerevisiae URA3* allele. See Fig 12 for more details.

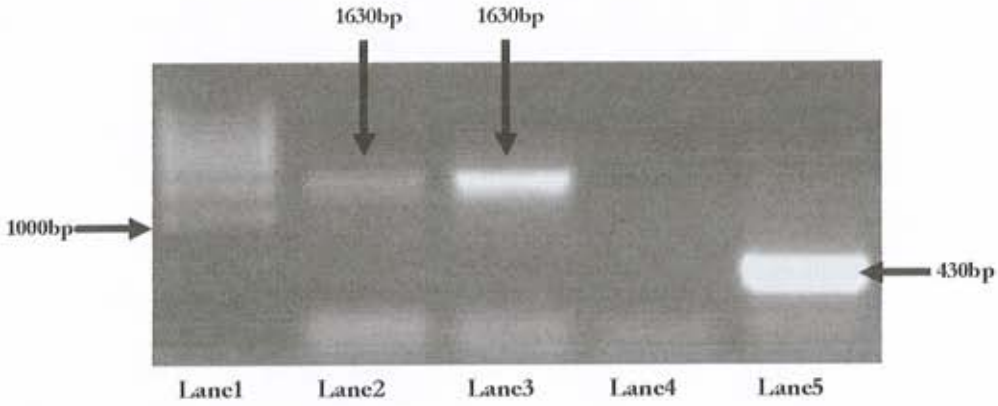


**Figure 12. *HWP2* internal verification.** Lane 1: 500bp ladder. PCR, band present at 1630bp (lane2) indicating the presence of the *HWP2* ORF in the *S. cerevisiae muc1* null strain as a result of successful transformation. Band at 1630bp using *HWP2* internal primers as a control in the *C. albicans* RM1000 parental strain (lane3). *URA3* verification PCR; no product (lane4) denoting the absence of *URA3* in the *ISP15Δmuc1* strain and thus successful integration of *HWP2* at the correct locus. Lane5: Control PCR product indicating the presence of *URA3* fragment at 430bp in the *URA3* plasmid YCp(33).

## 3.7 Filamentation on SLAD and cornmeal agar

The *S. cerevisiae HWP2* transformant, *muc1* null, and the *MUC1* wild type strains were plated on SLAD and cornmeal agar media at 30°C for 4 days. Wet mounts were prepared and observed at 1000X magnification. Compared to the wild type and null strain, the transformant exhibited extensive pseudohyphal growth on SLAD media, and heavy pseudohyphal penetrance on cornmeal agar. See figures 13 and 14 for more details.

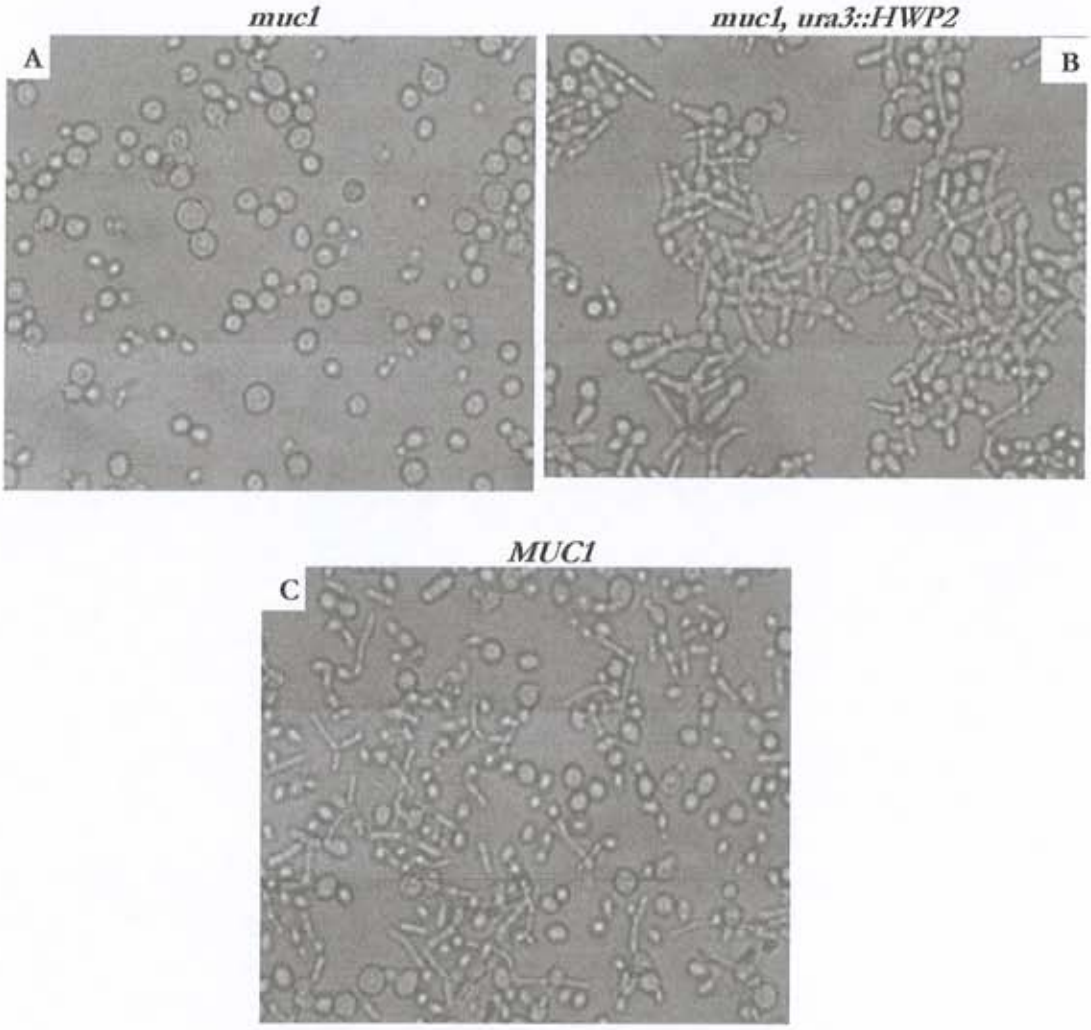


**Figure 13. Filamentation on SLAD agar.** The *S. cerevisiae muc1* null strain (A), *HWP2* transformant (B), and the parental *MUC1* wild type strain (C) were plated on SLAD at 30°C. Wet mounts were prepared and observed at 1000X magnification. Note the network of filaments in the wild type and transformant strain and the lack of filamentation in the null mutant.

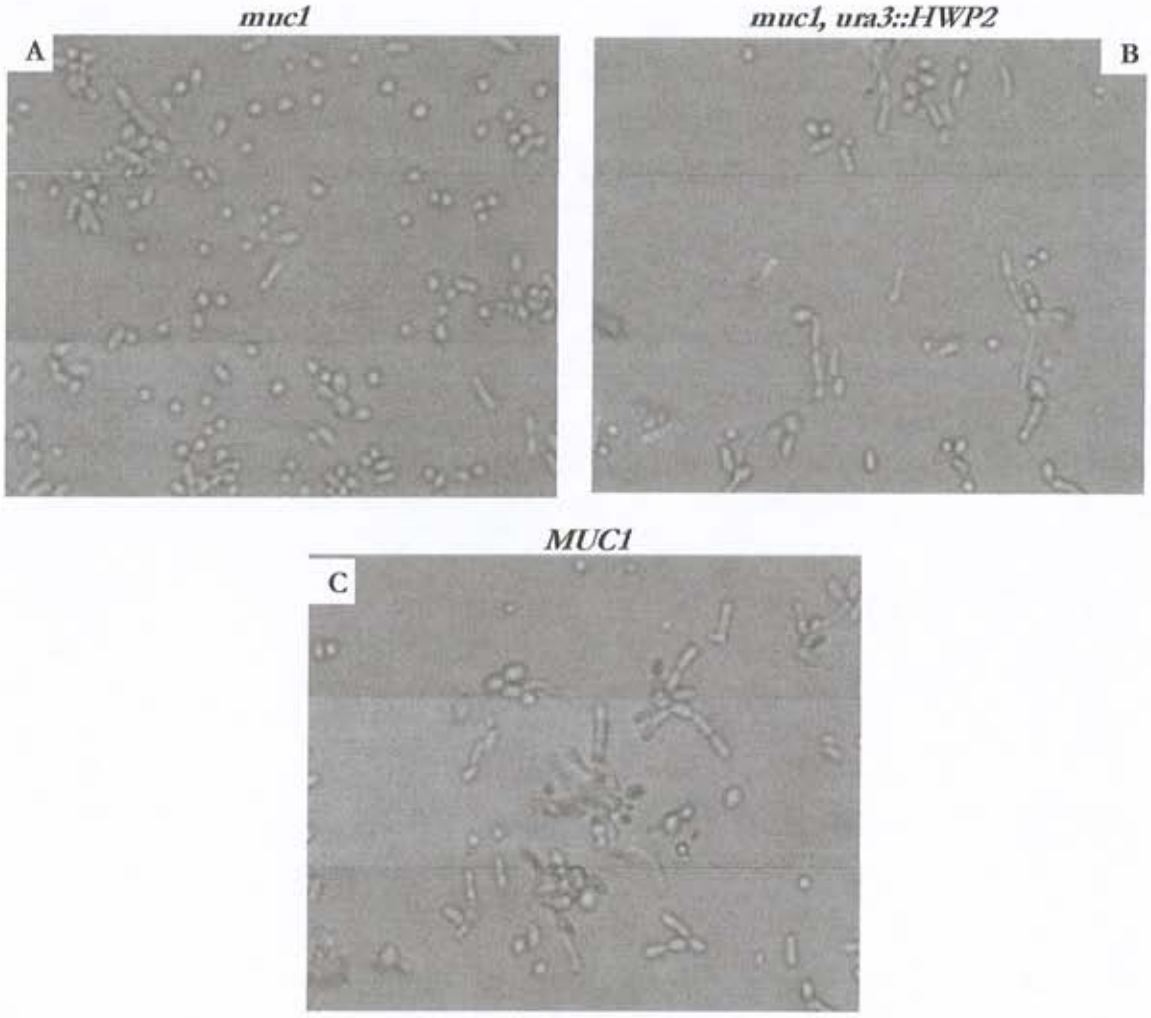


**Figure 14. Filamentation on cornmeal agar.** *S. cerevisiae* strains were plated cornmeal agar media at 30°C for 4 days. (A) shows the *muc1* null mutant lacking hyphal growth. Note the heavy pseudohyphal growth in (B) and (C) transformant and wild type respectively. Cells were photographed at 1000X magnification.

## 3.8 *S. cerevisiae* adhesion to human epithelial cells

The *S. cerevisiae* strains were incubated with human epithelial (Ht-29) cells. As expected the *muc1* Δ mutant strain showed no adhesion ability. The *MUC1*

wild type strain showed slight adhesion (perhaps due to adhesins present on pseudohyphae formed upon SLAD media incubation) but which was not statistically significant (p=0.27). Interestingly, the transformant *muc1*, *ura3::HWP2* strain showed a significant increase in ability to adhere to human epithelial cells a 2X increase versus the wildtype *MUC1*,( p=0.029), and more than a 5X increase (p=0.006) vs. the *muc1*⊿. The *C. albicans* wild type strain was used as a control for the experiment. See Fig 15.

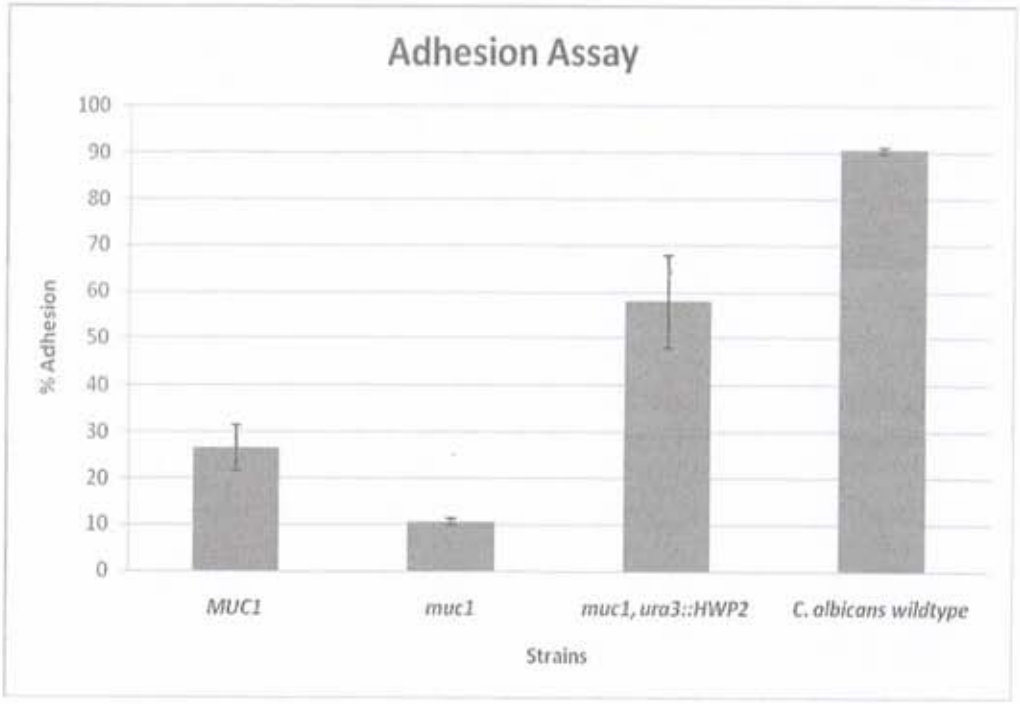


**Figure 15. *S. cerevisiae* adhesion assay.** Adhesion abilities of the three *S. cerevisiae* strains in addition to the wild type *C. albicans* strain to human epithelial cells after 180' incubation was determined. Note the significant increase in adhesion capability of the HWP2 transformed strain.

*Chapter 4*

# DISCUSSION

*C. albicans* cell wall proteins and notably GPI anchor proteins have been the subject of intense investigation. As antigenic determinants, these proteins were shown to be involved in virulence and filamentation, thus becoming possible targets for novel antifungal drugs. Hwp2 is a putative GPI-anchored protein. It was previously shown that an *hwp2* null mutant had a significant deficiency in filamentation on solid media, and a slight reduction in virulence in a mouse model (Hayek et al 2009). In this work we decided to further characterize Hwp2 by addressing the possible role it plays in virulence and adhesion.

## 4.1 Hwp2's role in cell wall integrity

Mutations in cell wall proteins usually lead to a brittle and shaky cell wall phenotype. With their cell wall integrity affected, these mutants show increased susceptibility to drugs, oxidative challenge and other stress conditions (Plaine et al., 2008; Dib et al., 2008).

During infection, several cell wall proteins help protect the *C. albicans* from Reactive Oxygen Species (ROS) burst generated by the host macrophages (Brown et al., 2007). We tried to mimic the macrophage harsh ambiance by spotting the wild type, heterozygote and null mutant strains on PDA plates containing varying concentrations of hydrogen peroxide. When compared to the wild type, *hwp2*Δ mutant strain resistance to hydrogen peroxide decreased dramatically. These data show that Hwp2 is necessary for proper oxidative stress response. In *C. albicans*, a family of superoxide dismutases (Sod's) is responsible for counteracting the effects of oxidative stress. Sod5 in particular was shown to be necessary for such a response (Fradin et al., 2005). Since Sod5 is also a GPI

anchored cell surface protein, the deletion of *hwp2* might have slightly altered the cell wall architecture preventing normal functioning of Sod5; thus providing an explanation for the above mentioned phenotype.

On the other hand, our *hwp2* null mutant did not exhibit a defect regarding cell wall integrity and brittleness. In fact no increase in susceptibility was recorded upon exposure to agents such as SDS, Congo red or calcofluor white. SDS is a cell membrane solubilizing agent; while Congo red and calcofluor prevent glucan and chitin microfibril assembly (Herth, 1980). Recently a correlation was found between increased vulnerability to these cell wall disrupting agents and a decrease in chitin content and vice versa (Plaine et al., 2008). We thus assessed the chitin content of our *hwp2* deletion strain and found no major decrease compared to the wild type. This indicates chitin synthesis and deposition was not significantly impaired in the mutant explaining the lack of susceptibility to the above-mentioned agents. Overall the rigidity and integrity of the cell wall does not seem to be affected. This should not come as a surprise since Hwp2 as a β-1,6-glucan linked adhesin is not an integral cell wall component as is the case with β-1,3-glucan linked integral proteins. Deletion of such β-1,3-glucan linked proteins such as Pir1 dramatically affect cell wall integrity mirrored through decreased resistance to cell surface disrupting agents (Martinez et al., 2004).

## 4.2 Defect in adhesion and biofilm formation

The most striking defect observed in our mutant is the dramatic decrease in adhesion ability and biofilm formation. Our tests showed that, compared to the wild type strain, the *hwp2* null mutant adhered 75% less to human epithelial cells and 68% to human endothelial cell. This phenotype resulted from a significant defect in adhesion and not just a simple delay since even incubating for 3 hours did not rectify the defect.

Furthermore, since adhesion to plastic is a probable precursor to biofilm formation, we assayed our null mutant's ability to form biofilm on polystyrene microtiter wells. The mutant strain showed a 30% decrease in biofilm mass; this result was predictable since a defect in adhesion usually leads to a defect in biofilm formation.

Our observed phenotype is similar to that of many characterized GPI anchored adhesins such as Eap1 that has been shown to be necessary for proper cell-cell adhesion and adhesion to polystyrene surfaces (Li and Palecek, 2003; Li et al., 2007). Another notable adhesin family is the Als family. Depletion of Als2 has bee shown to dramatically affect adhesion and result in abnormal levels of biofilm formation (Hoyer, 2001).

It is worthwhile noting that the above mentioned genes have been shown to be enriched in beta branched aliphatic amino acid repeats of isoleucine , valine, and threonine that have been shown both bionformatically and biochemically to be necessary for aggregation into amyloid like fibers, a precursor step to adhesion (Otoo et al., 2008; Ramsook CB et al., 2010). Members of this family of repeats that also include Ece1, Rbt1, Hwp1 are exclusively cell wall proteins. Null mutants of these proteins show defects in adhesion, invasive growth and filamentation adding further evidence of the role such repeats play in aggregation and adhesion (Yang 2003).

**4.3 Cross species complementation**

According to the *Candida* database (www.candidagenome.com), the *S. cerevisiae* Muc1/Flo11 is the protein with the closest sequence similarity to Hwp2. Overall homology between the two proteins however is very weak and is limited to the serine-threonine rich repeat regions and the I,V,T sequences mentioned above. A *muc1* null has been shown to be afilamentous and uninvasive on nitrogen limiting media and cornmeal agar (Lambrechts et al., 1996), a phenotype

similar to an *hwp2* null strain. We thus decided to determine whether the sequence similarity reflects true orthology and functional identity by cross species complementation. The reason for doing so is the fact that *C. albicans* as a pathogen is notorious for transcriptional rewiring, whereby a protein is recruited to a different function than its orthologue in other species. Notable examples include the Gal4 transcriptional activator which is involved in regulating the *GAL* gene family in *S. cerevisiae* but not in *C. albicans* (Martchenko et al., 2007). Rfg1 in *C. albicans* shows high sequence similarity to Rox1 of *S. cerevisae*, however Rfg1 is a repressor of filamentation while Rox1 is a repressor of hypoxic genes (Khalaf and Zitomer 2001).

We thus generated a Sc. *URA3-HWP2*-Sc.*URA3* cassette containing the entire *HWP2* ORF with its own promoter flanked by a 100bp DNA sequence that is homologous to the Sc. *URA3* ORF in the *muc1* null strain As such, via homologous recombination after transformation, the entire *HWP2* ORF with its own promoter region integrated at the *URA3* locus. Successful integrants were counter selected for on 5Fluoroorotic acid (5FOA) that eliminates all uridine prototrophs (Boeke et al., 1984). By PCR amplification, we then verified the insertion of *HWP2* into *S. cerevisiae*, and consequently the absence of the *S.c.URA3* allele. The above-mentioned transformant, alongside the wild type and *muc1* null strains were grown in nitrogen limiting media and on cornmeal agar, conditions that trigger pseudohyphal formation in *S. cerevisiae*. On both media the *muc1* null mutant grew only as round yeast cell. On the other hand, the transformant *muc1,ura3::HWP2* strains showed a significant increase in filamentation with many pseudohyphal extensions observed. Such data suggests that Hwp2 can complement the *muc1* null phenotype and is thus a functional orthologue of Muc1.

We then decided to determine whether Hwp2 can impart adhesive abilities onto *S. cerevisiae*. As a non-pathogen *S. cerevisiae* lacks the ability to significantly adhere to human cells. We thus harvested cells grown on cornmeal agar and assayed for adhesion to human epithelial cells. Interestingly we found out that the *HWP2* transformant displayed a dramatic increase in adhesion, 5X more than the *muc1* null, and 2X more than the wild type *MUC1* strain. Increased *S. cerevisiae* adherence by transformation of a *C. albicans* gene has been shown previously for other adhesins such as Als1 (Fu et al., 1998). Furthermore transformation of the *C. albicans* Als5 imparted increased aggregation to *S. cerevisiae* cells (Ramsook CB et al., 2010).

*Chapter 5*

# CONCLUSION

In this study, we further characterized Hwp2. As a β-1,6-glucan attached GPI-anchored adhesin, the protein is not an integral protein embedded within the cell wall; instead, its major portion protrudes outwards where it plays its role in interaction with the environment. As such the lack of *HWP2* expression in the null strain did not affect processes that require cell wall integrity, such as resistance to cell wall disrupting agents. However as an adhesin, the most severe mutant phenotype was observed as a defect in adhesion to human cells and a subsequent decrease in biofilm mass.

Furthermore, we have shown that Hwp2 can complement the lack of pseudohyphal growth of an S. cerevisiae *muc1* null strain, and can impart adhesive properties to the non-adhesive yeast *S. cerevisiae*. As such, the sequence similarity between the proteins tends to reflect true orthology.

It is important to note however, that expression of *HWP2* in the *S. cerevisiae* transformant was not verified by RT-PCR, Northern or Western blotting. Such an experiment needs to be conducted in the very near future to prove that the phenotype observed is indeed due to *HWP2* transcription and not due to a secondary mutation in *S. cerevisiae* that suppressed the lack of filamentation phenotype of the *muc1* null strain. If such assays were to show that *HWP2* is not expressed than this raises an interesting point as to what secondary mutation confers such a phenotype. By transforming a DNA library into the mutant strain one can screen for colonies that have lost the ability to filament in a *muc1* null background. Plasmid miniprep isolation and sequencing should reveal the said suppressor gene.

*Chapter 6*

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