Role of Tem1 in Signalling Mitotic Exit in the Human Fungal Pathogen *Candida albicans*

Submitted by Stephen William Milne

to the University of Exeter as a thesis for the degree of *Doctor of Philosophy* in Biological Sciences, May 2011.

STEPHEN W. MILNE

Abstract

The human pathogen *Candida albicans* is polymorphic, and its ability to switch growth forms is thought to play an important role in virulence. The primary research aim of this thesis was to understand the role the mitotic exit network plays in *C. albicans* with particular focus on the Tem1 GTPase protein. This aim was split into three specific goals; to study the role of Tem1 through the construction of a regulatable *tem1* mutant, to understand the regulation of Tem1 through localisation and protein interaction studies, and to construct new molecular tools utilising the *NAT1* positive selection marker in order to achieve two previous goals.

In this thesis we demonstrated that *TEM1* is an essential gene in *C. albicans*, and its essential function is signalled through the Cdc15 protein. Surprisingly, Tem1p depleted cells arrested as hyper-polarised filaments containing one or two nuclei and ultimately lost viability. These filaments formed from budding yeast cells, suggestive of a blockage late in the cell cycle. Ultimately the failure of these filaments to undergo cytokinesis was linked to a defect in septin ring dynamics and the formation of actomyosin ring.

To understand the regulation of Tem1 we localised both the Tem1 and Lte1 proteins and found that Tem1 localised to spindle pole bodies in a cell-cycle dependent fashion by recruited at the onset of S phase. In contrast, the Lte1 homolog localised to the daughter cell cortex prior to release into the cytoplasm at the end of the cell cycle. A yeast 2-hybrid analysis of the MEN components demonstrated the potential of Bfa1/Bub2 and Tem1 to form a complex and the ability of Tem1 to homodimerise which may play a role in its self-activation.

In order to carry out various aspects of this work we constructed a fully functional set of cassettes, including the constitutively active *ENO1* promoter, V5-6xHIS epitope tag and various fluorescent protein genes fused to the *NAT1* positive selection marker.

When considered together, these results indicate that Tem1 is required for timely mitotic exit and cytokinesis in *C. albicans*, similar to *S. cerevisiae*, but the final output of the pathway must have diverged.

Acknowledgements

I would firstly like to thank Steve Bates for giving me the opportunity and for skilfully guiding me through the PhD. I am grateful for his patience, encouragement and endless advice. I would also like to thank my second supervisor Steve Aves for his enthusiastic support, advice and eye for detail.

I would also like to thank Jill, Sariqa and all the other past and present members of lab 3.10 and 4.10 for all their words of encouragement and advice during the duration of this project. Derek deserves a special mention for assistance with experiments, vast words of encouragement and for proof reading this thesis.

Thanks to my family for your encouragement throughout my seeming endless education. Also to Debs, thank you for your help with various aspects of this work and for your boundless encouragement and support without which this project would have been even more daunting.

Finally I would like to thank Pete and Martin for all the help and advice for imparting to me some of their vast knowledge of microscopy

Table of contents

Figures and Tables

Chapter 4.

Chapter 5.

Abbreviations

ALS	Agglutinin-like sequence
A. nidulans	*Aspergillus nidulans*
APC	Anaphase promoting complex
APC^{Cdc20}	Anaphase promoting complex-Cdc20 complex
APC^{Cdh1}	Anaphase promoting complex-Cdh1 complex
A. thaliana	*Arabidopsis thaliana*
BFP	Blue fluorescent protein
C. albicans	*Candida albicans*
C. glabrata	*Candida glabrata*
cAMP	Cyclic adenosine monophosphate
CAR	Contractile actomyosin ring
CDK	Cyclin-dependant kinase
CFP	Cyan fluorescent protein
CFW	Calcofluor white
ChIP-chip	Chromatin immunoprecipitation
DAPI	4,6-diamidino-2phenylindole-dihydrochloride
DIC	Differential interference contrast microscopy
DNA	Deoxyribonucleic acid
Dox	Doxycycline
DTT	Dithiothreitol
E. coli	*Escherichia coli*
Em	Emission
EML	Extracellular matrix layer
END	Emi1/NuMA/dynein-dynactin
ENO1p	Enolase promoter
Ex	Excitation
FCS	Foetal calf serum
FEAR	Cdc fourteen early anaphase release network
5-FOA	5-Fluoro-orotic acid
GAP	GTPase activating proteins
GDI	Guanosine nucleotide dissociation inhibitor
GEF	Guanosine exchange factor
GFP	Green fluorescent protein
G. mellonella	*Galleria mellonella*
GlcNAc	*N*-acetylglucosamine
GPI	Glycophosphatidylinositol
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HA	Haemagglutinin
HeLa	Human cervical epithelial
HyB	Hygromycin B

LiOAc/ssDNA/PEG	Lithium acetate/single stranded DNA/polyethylene glycol
MBF	*MluI* cell cycle box binding factor
MEN	Mitotic exit network
Mnt	Mannosyltransferase
MPA	Mycophenolic acid
mSPB	Mother-bound spindle pole body
MUAG	4-methylumbelliferyl-*N*-acetyl-D-glucosamine
MTL	Mating type locus
NAT1	Nourseothricin acetyltransferase
N. crassa	*Neurospora crassa*
NLS	Nuclear localisation sequence
Nt	Nucleotide
OMPD	Orotidine-5'-phosphate
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKA	Protein kinase A
Pmt	Protein-O-mannosyltransferase
pNP-GlcNAc	*p*-nitrophenyl-β-*N*-acetylglucosamine
PP2A^{Cdc55}	Protein phosphatase 2A-Cdc55 complex
pYES2.1	Yeast expression system plasmid
RFP	Red fluorescent protein
RHE	Reconstituted human epithelium
RT-PCR	Reverse transcriptase PCR
SAC	Spindle assembly checkpoint
SAP	Secreted aspartic proteinases
SAT1	Streptothricin acetyltransferase
SBF	Swi4/6 cell cycle box binding factor
SC	Synthetic complete media
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
S. pombe	*Schizosaccharomyces pombe*
SPB	Spindle pole bodies
dSPB	Daughter spindle pole body
START	G_1/S checkpoint
TAP	Tandem affinity purification
TBS	Tris buffered saline
TBS-T	TBS plus Tween-20
TBT-T+BSA	TBS-T plus 5 % BSA
TEM	Transmission electron microscopy
TetO	Tet operator sequence
TetR	Tetracycline repressor protein

v/v	Volume/volume
w/v	Weight/volume
Y2H	Yeast 2-hybrid
YFP	Yellow fluorescent protein
YPD	Yeast extract peptone dextrose
YPGal	Yeast extract peptone galactose

Chapter 1. Introduction.

1.1. History of *Candida* species.

Candida infections are not a new issue. The first reported documentation of a thrush infection dates back to the 4th century BC by Hippocrates, but it was not until 1846 that Berg demonstrated thrush was caused by a fungus (Berg, 1846). The name *Candida albicans* was officially accepted for the organism at the Eighth Botanical Congress in 1954. However it has only been from 1990 onwards, when molecular biology approaches became available, that we started making clear progress in understanding this important pathogen.

The *Candida* genus belongs to the saccharomycotina subphylum of the ascomycetes fungi and consists of more than 150 different species. The majority of these species exist as environmental saprophytes with only a few species being human pathogens including *Candida albicans*, *Candida glabrata* and *Candida tropicalis* (Odds, 1998). The *Candida* genus consists of one major group with the exception of *C. krusei* and *C. glabrata*. This major group is characterised by the reading of the CUG codon as serine and not leucine (Santos and Tuite, 1995). Within this diverse group are some species that are haploid such as *C. lusitaniae* while others such as *C. albicans* are found to be diploid.

There are a number of similarities between *Saccharomyces cerevisiae* and *C. albicans*, and the organisms are often compared. However studies have shown there is considerable variation between the two species. These variations included the identification of *C. albicans* as diploid while *S. cerevisiae* is haploid, and the alternative reading of the CUG codon. The former was established using flow microfluorometry to measure the DNA contents of cells, and the latter by sequencing and mass spectrometry of a recombinant protein expressed in *C. albicans* (Olaiya and Sogin, 1979; Santos and Tuite, 1995). Additionally about 40% of all *C. albicans* genes have no homologues in *S. cerevisiae* and genes that are essential in *S. cerevisiae* are often not essential in *C. albicans* and *vice-versa* (Mio *et al*., 1997; Nagahashi *et al*., 1998; Lussier *et al*., 1998; Kelly *et al*., 2000).

1.2. *Candida* pathogenicity.

A number of *Candida* species are constituents of the human commensal micro-flora of the gastrointestinal tract and genitourinary system. It has been demonstrated using oral swabs that 40-70% of healthy individuals carry *C. albicans* as part of their natural flora. *C. albicans* becomes an opportunistic human pathogen if allowed to outgrow other competing organisms of the natural flora e.g. due to the use of antibacterial drugs, or the introduction of *C. albicans* into sterile sites (Pappas *et al*., 2004). Analysis of blood samples taken from candidemia patients has identified *C. albicans* as the predominant human pathogen of the *Candida* genus accounting for 50% of all cases (Pfaller *et al*., 1999). Infections caused *by C. albicans* range from superficial infections in healthy individuals to disseminated blood stream infections (candidemia) in immunocomprimised or severe burns patients (Figure 1; Kao *et al*., 1999). The superficial infections typically occur on mucosal membranes of the mouth and vagina or on the skin, external ear or nails (Jayatilake and Samaranayake, 2010). The number of cases of disseminated infection caused by *Candida* species has risen to the extent that candidemia is now the fourth most common nosocomial infection in the United States and is believed to account for over 10000 deaths and $1 billion in treatment and care costs a year (Beck-Sague and Jarvis, 1993; Miller and Mejicano, 2001; Sudbery *et al*., 2004).

Candida albicans is not the only member of the *Candida* genus that is a human pathogen. At least six other *Candida* species; *C glabrata, C. dubliniensis, C. krusei, C. parapsilosis, C. tropicalis* and *C. lusitaniae*, have been isolated from candidiasis patients (Moran *et al*., Emergence of Non-*Candida albicans Candida* species as pathogens. *Candida* and Candidiasis. Ed. R.A. Calderone. 2002). Worldwide *C. glabrata* is the most common cause of non-*albicans* candidiasis infections. However there are geographical variations such as in Brazil where *C. tropicalis* is the second most common cause of candidiasis infection while in Australia it is *C. parapsilosis* (Colombo *et al*., 2006; Chen *et al*., 2006).

1.3. *Candida* virulence factors.

The mechanisms that allow *C. albicans* to switch from being a non-harmful commensal to a pathogen have long been debated. Unlike many bacterial pathogens *C. albicans*
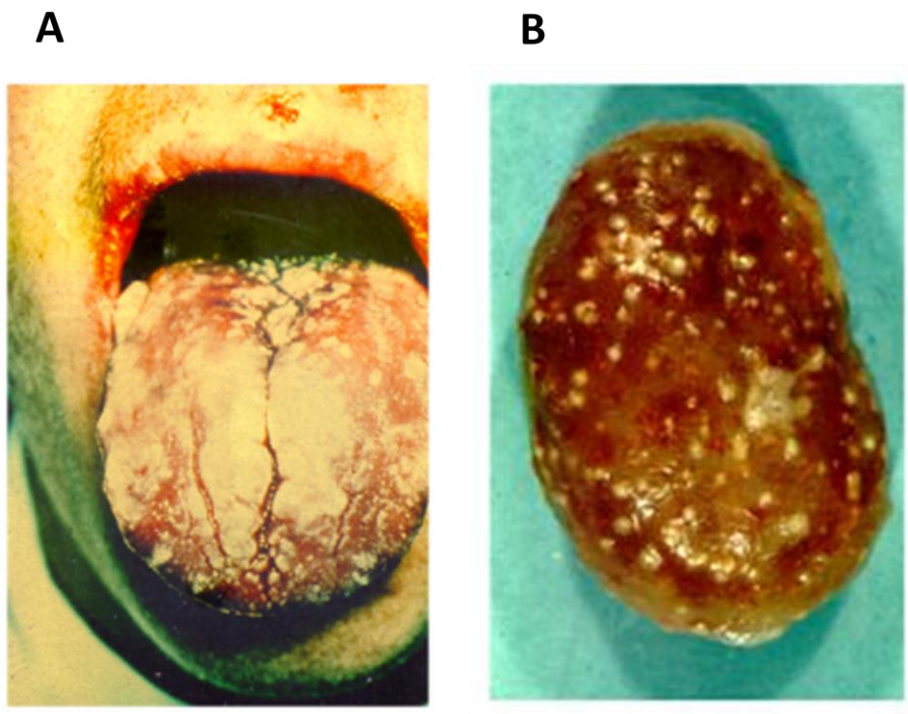
Figure 1. *Candida* infections.   A) *C. albicans* infection of the membranous regions of an individual's mouth. B) Systemic *C. albicans* infection of a rabbit kidney. (Photos kindly provided by Professor Frank Odds).

does not possess one dominant virulence factor, such as the tetanus toxin of *Clostridium tetani*, but requires a number of contributing factors in order to be pathogenic. For example *C. albicans* is known to react very quickly to external conditions to form hyphae and to express various virulence factors. A four stage infection model has been proposed for *C. albicans* infections (Figure 2; Naglik *et al.*, 2003). The model starts with the initial colonisation of an epithelial surface by the cells. This colonisation is assisted by various virulence factors involved in adhesion to different host surfaces. Stage two, the superficial infection stage, degradation or manipulation of host epithelial cells by enzymes and mechanical force generated by *C. albicans* hyphae help invasion of epithelial cells. Such infections typically afflict healthy immune-competent individuals (Figure 1A). The last two stages of the model represent more serious life threatening infections and include deep-seated and disseminated infections. These stages include tissue penetration, dissemination around the body and immune evasion (Figure 1B). A number of different virulence factors are involved in each stage of infection. These various virulence factors and the roles they play in virulence are discussed in more detail in the subsequent sections.

Many potential virulence factors have been suggested, that are believed to be involved in the pathogenicity of *C. albicans*. Recently three studies have been published which call into doubt the merits of some of these virulence factors (Lermann and Morschhaüser, 2008; Correia *et al.*, 2010; Noble *et al.*, 2010). These studies suggest that many attenuated mutant strains were wrongfully identified and that the virulence defect was actually caused by use of the *URA3* gene as the selection marker in gene disruption and the failure to restore *URA3* to its native locus. Virulence defects of strains lacking the *ura3* gene were first observed in 1991 during a study of virulence characteristics of auxotrophic marker mutants using an immunocompromised mouse virulence model (Kirsch and Whitney, 1991). Additional studies utilising both systemic candidiasis murine virulence models and enzyme activity assays have found that strains with variable levels of the enzyme encoded by the *URA3* gene, OMPD, exhibit virulence defects (Kirsch and Whitney, 1991; Laurenson and Rine, 1992; Cole *et al.*, 1995; Bain *et al.*, 2001; Cheng *et al.*, 2003; Staab and Sundstrom, 2003; Brand *et al.*, 2004). These virulence defects can be avoided by integrating a copy of the *URA3* gene into its native locus or into the RPS10 (Brand *et al.*, 2004). Despite this evidence the
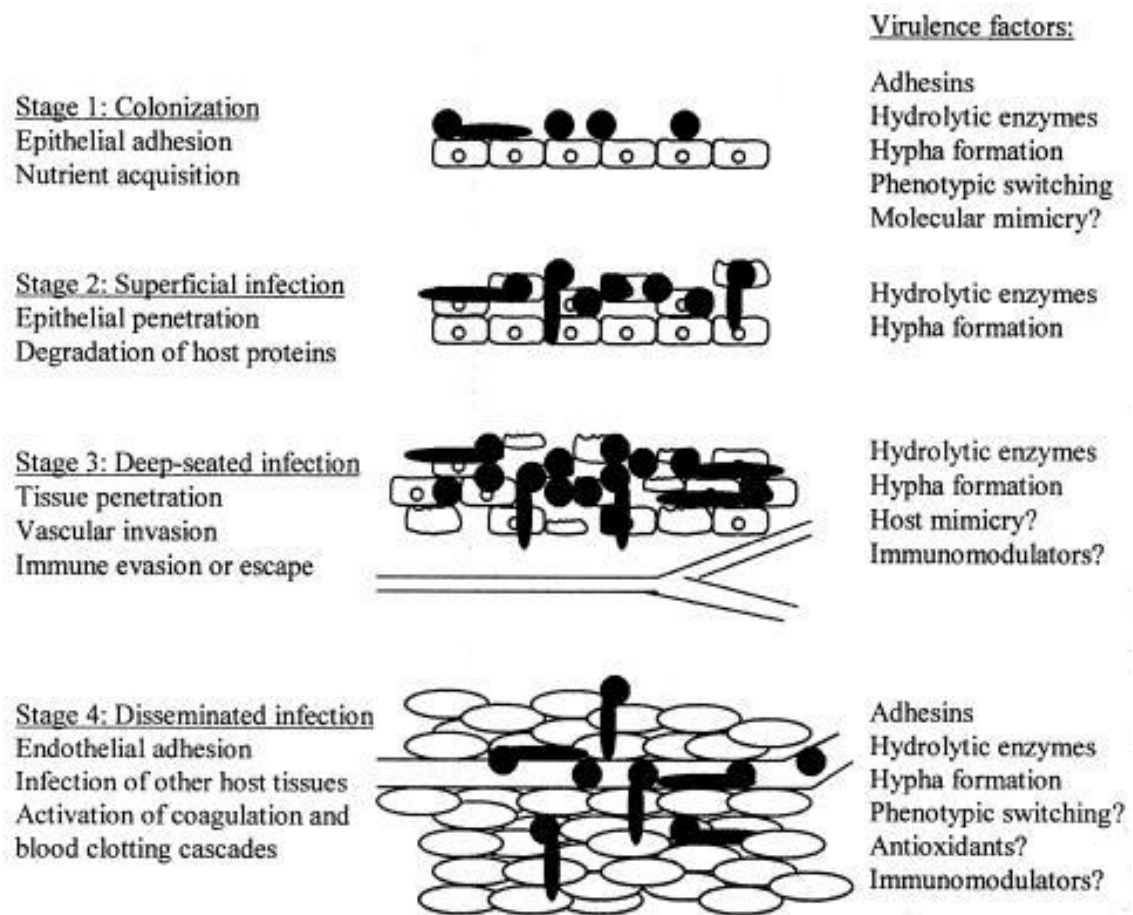
Figure 2. The four stages of *C. albicans* infections. Schematic diagram of the proposed stages of a *C. albicans* infection. The various virulence factors that have been implicated in each stage of infection are shown on the right side of the Figure. Figure taken from Naglik *et al*., 2003.

URA-blaster cassette has been used for constructing null mutants of various putative virulence factors. Each of these is discussed on a case by case basis in the subsequent sections.

1.3.1. Adhesins.

C. albicans expresses a range of adhesins encoded by both gene families and individual genes. Although complicated by functional redundancy a number of adhesins have been shown to be important in virulence, as strains lacking the ability to adhere to host cells have been shown to be attenuated in virulence (Calderone and Braun, 1991).

The Agglutinin-like sequence (ALS) family consists of nine genes which display homology to *S. cerevisiae* α-agglutinin that is required for mating (Hoyer, 2001). The Als proteins are secreted proteins and contain a hydrophobic carboxyl terminus end which suggests they are glycophosphatidylinositol (GPI) anchored to the plasma membrane or cell wall and they are heavily N- and O- glycosylated (Lu *et al.*, 1994). The ALS family is split into two subfamilies based on the sequence of their tandem repeat domain. These two subfamilies consist of *ALS1-4* and *ALS5-7* while *ALS8* and *9* are not part of either subfamily (Hoyer *et al.*, 1995; Gaur and Klotz, 1997; Hoyer *et al.*, 1998a; Hoyer *et al.*, 1998b; Hoyer and Hecht, 2000; Hoyer and Hecht, 2001; Kapteyn *et al.*, 2000). Regulation of the different *ALS* genes is complex and has been found to be dependent on a number of physiological conditions. These range from the growthstage (*ALS4*) to changes in growth medium (*ALS1*) and the morphological growth form (*ALS3/ALS8*) (Hoyer *et al.*, 1995; Hoyer *et al.*, 1998a; Hoyer *et al.*, 1998b; Hoyer and Hecht, 2000).

Experimental findings from oral and disseminated candidiasis models or from a buccal reconstituted human epithelium (RHE) tissue culture model have shown that the Als1-3 proteins are required for virulence. A mutant strain lacking the Als1 protein was observed to exhibit delayed pathogenicity in a immunocompetent mouse systemic candidiasis model while strains lacking the Als2 or Als3 proteins caused a decreased level of RHE destruction (Kamai *et al.*, 2002; Fu *et al.*, 2002; Alberti-Segui *et al.*, 2004; Zhao *et al.*, 2004; Zhao *et al.*, 2005). Disruption of the other six *ALS* genes resulted in no loss of adherence or destruction of the buccal reconstituted human epithelium

(RHE) or vaginal RHE tissues and are assumed to play a redundant role in the virulence of *C. albicans* (Zhao *et al*., 2005; Zhao *et al*., 2007a; Zhao *et al*., 2007b). All the *als* mutants discussed above contained a copy of the *URA3* gene. However, this *URA3* was inserted into the locus of the disrupted gene in all the mutants except the *als1* mutant where it was integrated into the RPS10 locus. As a result the defects observed for the *als2* and *als3* mutants could be attributed to variable OMPD levels and not the loss of the genes.

The hyphal wall protein 1 (Hwp1) is an outer surface mannoprotein that is only expressed in hyphal cells (Staab *et al*., 1996; Sundstrom, 1999; Nobile *et al*., 2006a). This strain also exhibits a five-fold reduction in stable adhesion to HBECs. Analysis of an *hwp1* null strain confirmed that Hwp1 is required for virulence in a murine systemic candidiasis model and it was also found that this strain germinated much more slowly in the kidneys of infected mice and caused less endothelial cell damage when compared to the wild type strain (Sundstrom, 1999; Staab *et al*., 1999; Tsuchimori *et al*., 2000). This defect cannot be attributed to variable OMPD levels as OMPD levels in the *hwp1* null strain were similar to those of wild type strains therefore Hwp1 is required for virulence. The Hwp1 protein has also been shown to be involved in biofilm formation and maturation (Nobile *et al*., 2006b; discussed in section 1.4.1.5).

The mannosyltransferase (Mnt) family is a family of type two membrane proteins that are required for N- and O-mannosylation of cell wall proteins. The family consists of five genes *MNT1-5* (Buurman *et al*., 1998). *In vitro* mannosyltransferase activity assays have implicated the Mnt1 and Mnt2 proteins in the O-mannosylation process while alcian blue binding assays have demonstrated that the Mnt3-5 proteins are involved in phosphomannam addition (Munro *et* al., 2005; Mora-Montes *et al*., 2010). Deletion of single genes as well as deletion of combinations of the *MNT* genes has resulted in strains with a range of defects. Deletion of the *MNT1* and *MNT2* genes results in a reduction in epithelial cell adherence by 75-90 % and 50-60 % respectively, when compared to the *URA3* positive CAI2-1 strain, as demonstrated by an *in vitro* buccal epithelial cell adherence assay (Munro *et al*., 2005). In a mouse systemic infection model neither the *mnt1/mnt1* or *mnt2/mnt2* mutants experienced a significant attenuation in their virulence while the virulence of a *mnt1 mnt2* double mutant was

significantly attenuated. The defects observed for the *mnt1 mnt2* double mutant could be attributed to the lack of *URA3* gene in this strain therefore these adherence and virulence assays require to be repeated using *ura* positive strains (Munro *et al.*, 2005). These results suggest that the O-mannosylation of cell wall proteins is required for adherence of the cells to the target surface and for virulence.

An additional family of mannosyltransferase proteins have been implicated in adherence of *C. albicans* cells to target cells using the RHE model and an engineered human oral mucosa adherence model (Rouabhia *et al.*, 2005). This family of enzymes is the protein-O-mannosyltransferase (Pmt) family. The Pmt family consists of five proteins, Pmt1-2 and Pmt 4-6. The *pmt1* and *pmt4-6* null mutants have all been shown to exhibit virulence defects in either a RHE oral candidiasis model or a disseminated infection mouse model. These defects range from slight attenuation (Pmt5) to being highly attenuated (Pmt1, Pmt4 and Pmt6) and are not caused by variable OMPD levels as the *URA3* gene was integrated into the highly expressed RPS10 locus in each strain (Timpel *et al.*, 1998; Timpel *et al.*, 2000; Prill *et al.*, 2005; Rouabhia *et al.*, 2005; Corbucci *et al.*, 2007). The *PMT*2 gene was found to be essential for vegetative growth, consequently no virulence assays were carried out (Prill *et al.*, 2005). It was also shown that *pmt1* and *pmt4* strains exhibited vastly reduced levels of mannoprotein, which has been shown to be important for *C. albicans* adherence to a range of different surfaces (Fukazawa and Kagaya, 1997).

Another adhesin important to *C.albicans* is the *INT*1 gene which encodes an integrin-like protein. Vertebrate leukocyte integrins are adhesin molecules that bind to extracellular matrix proteins, leading to the interaction of the integrin molecule with the epithelial cell, which results in morphological changes in some cell types such as endothelial cells undergoing angiogenesis (Ruoslahti and Pierschbacher, 1987; Clark and Brugge, 1995; Gumbiner, 1996). This was also seen when the *C. albicans INT1* was expressed in *S. cerevisiae* cells, resulting in the cells switching to a filamentous growth form that resembles *C. albicans* hyphae. Investigations also showed that these cells adhered to a monolayer of human cervical epithelial (HeLa) cells (Gale *et al.*, 1996). Disruption of both copies of the *INT*1 gene in *C. albicans* resulted in a strain that was extremely attenuated in virulence in a systemic candidiasis mouse infection model and

also showed a 40 % reduction in adherence to HeLa cells (Gale *et al.*, 1998). The *int1* null strain used in the virulence and adherence assays contained a *URA3* gene however the specific location of this gene was not described therefore it cannot be concluded that these defects are not the results of variable OMPD levels.

1.3.2. Biofilms.

Many microorganisms have the ability to form biofilm-like structures. Biofilms protect the organism from host defences, and in addition the cells within a biofilm often display different traits from those of normal planktonic cells, such as extreme resistance to antimicrobial drugs (Kumamoto, 2002; Douglas, 2003; Fux *et al.*, 2005; Ramage *et al.*, 2005). It is estimated that 50 % of all nosocomial *Candida* infections are caused by biofilm-based infections (Bertagnolio *et al.*, 2004; Kojic and Darouiche, 2004). *C. albicans* has been shown to form biofilms on tissues, catheters, prosthetic devices and other surfaces. Furthermore, these biofilms have been shown to be resistant to a range of antifungal therapies (Hawser and Douglas, 1994; Chandra *et al.*, 2001). The formation of a *C. albicans* biofilm consists of four main stages; adherence, filamentation and initiation, maturation of the biofilm and finally dispersal of yeast cells to form additional sites of infection (Figure 3, Finkel and Mitchell, 2011). As with the model for superficial and systemic infections, Figure 2, a number of different proteins have been implicated in biofilm formation (Figure 3). These proteins range from transcription factors to cell wall proteins and drug-efflux pumps (Figure 3).

The first stage of biofilm formation is the adhesion of the cells to the target surface. A number of different adhesin proteins have been studied in *C. albicans*. The Eap1 protein is one such adhesin. Eap1 is a GPI anchored cell wall protein that has been implicated in biofilm formation by a series of experimental findings (Li *et al.*, 2007; Richard and Plaine, 2007; Chaffin, 2008). Li and co-workers showed that a *C. albicans eap1* null mutant was found to be defective in biofilm formation in a catheter model both *in vitro* and *in vivo*. The authors also showed this strain to have reduced adherence to polystyrene and on introduction of the *EAP1* gene into non-adherent *S. cerevisiae* cells resulted in these cells becoming adherent to polystyrene (Li *et al.*, 2007). The initial colonisation of a target surface begins by the binding of additional cells to the target surface or to the already adhered cells which creates a layer of cells

called the basal layer.

The construction of the basal layer is followed by the initiation stage and during this stage expression of the biofilm regulator transcription factor *BCR1* is required. Bcr1 is required for expression of the *HWP1*, *ALS3* and other related cell surface proteins and deletion of *BCR1* results in a strain that is defective in biofilm formation. This defect can be recovered by increasing the expression of *ALS3* or *HWP1*, suggesting that Als3 and Hwp1 are required for biofilm formation (Nobile and Mitchell, 2005; Nobile *et al.*, 2006a; Nobile *et al.*, 2006b). Strains lacking either of these genes, as well as the *ALS1* gene, have been shown to form sparse and brittle biofilms (Garcia-Sanchez *et al.*, 2004; Nobile *et al.*, 2006b). In addition, during the initiation stage the cells start to form hyphae, which also contributed with the expression of hypha-specific genes such as *ALS3* and *HWP1*, and is required for biofilm integrity (Nobile *et al.*, 2006b).

The third stage of *Candida* biofilm formation is the maturation of the biofilm. During this stage the hyphae continue to grow out from the biofilm forming aerial hyphae and this allows the extracellular matrix layer (EML) to form (Figure 3). The EML is composed of a mixture of carbohydrates, proteins, and other compounds (Baillie and Douglas, 2000; Al-Fattani and Douglas, 2006). A number of proteins have been implicated in the production of EML, for example, in a study by Nobile and co-workers it was shown that the Zap1 transcription factor is a negative regulator of the EML formation as an *zap1/zap1* biofilm that formed on a rat catheter infection model contained vastly more EML when compared to a *ZAP1/ZAP1* strain. The authors calculated by analysing the soluble β-1,3 glucan in the serum removed from the model that the *zap1/zap1* biofilm produced 3 fold more soluble β-1,3 glucan than a *ZAP1/ZAP1* biofilm (Nobile *et al.*, 2009). Zap1 carries out this function by activating the expression of the *CSH1* and *IFD6* genes. Expression of these genes led to the decrease in soluble β-1,3 glucan levels, when compared to a *ZAP1/ZAP1* strain, in serum extracted from both an *in vivo* rat catheter model and an *in vitro* biofilm grown on spider media (Nobile *et al.*, 2009). This suggests that Csh1 and Ifd6 proteins play a role in negatively regulating matrix production. The defects observed for the *eap1*, *bcr1* and *zap1* null mutants are *bona fide* as these strains all had a copy of the *URA3* gene.
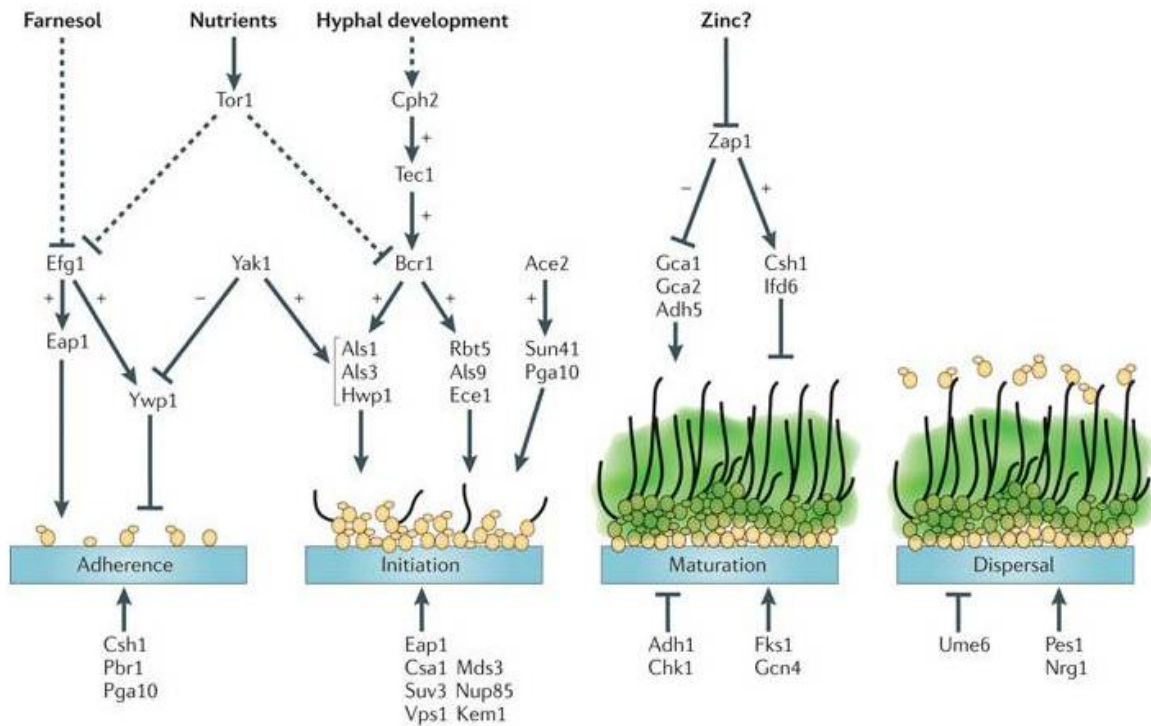
Figure 3. Biofilm formation by *C. albicans*. Schematic diagram of the four stages of biofilm formation. Dashed T-shaped bars indicate repression by an indirect mechanism. + indicates the protein stimulates the gene expression of the target while the – indicates a inhibition of gene inhibition. The extracellular matrix is represented in green. Figure taken from Finkel and Mitchell, 2011.

The final stage of a *Candida* biofilm is cell dispersal. The ability of cells to disperse from the biofilm allows the biofilm to act as a reservoir for seeding new infections. Two recent papers have studied this release of cells from *Candida* biofilms and have implicated three transcriptional regulators; Nrg1, Ume6 and Pes1 in the process (Uppuluri *et al*., 2010a; Uppuluri *et al*., 2010b). These studies found that overexpression of *PES1* or *NRG1* resulted in an increase in the release of cells from the biofilm while overexpression of the *UME6* gene had the opposite effect.

1.3.3. Degradative/Hydrolytic enzymes.

C. albicans possesses a range of extracellular hydrolytic enzymes. The three most commonly studied of these are lipases, phospholipases and aspartic proteinases (Hube and Naglik, Extracellular Hydrolases, *Candida* and Candidiasis. Ed. R.A. Calderone. 2002).

1.3.3.1. Phospholipases.

Phospholipases are a group of enzymes that hydrolyse one or more ester linkages in glycerophospholipids (Ghannoum, 2000). The phospholipase group consists of four classes of enzymes, A, B, C and D and they are all unique as they each cleave a specific ester bond. Phospholipase activity in *C. albicans* was first documented by Werner, in 1966, by analysing lipid breakdown in egg yolk or lecithin. The early research focussed on testing various clinical isolates to evaluate phospholipase levels and it was found, using *in vitro* egg yolk-based media assay, that the strains isolated from systemic infection had the higher phospholipase activity compared to those strains isolated from superficial infections. Egg yolk is used as a phospholipase activity indicator as it contains large amounts of phospholipids and a white zone forms around colonies of phospholipase positive *C. albicans* strains. To date two phospholipase B genes, *PLB1* and *PLB2* have been isolated in *C. albicans* and cloned (Hoover *et al*., 1998; Leidich *et al*., 1998; Sugiyama *et al*., 1999). It was previously thought that *C. albicans* was the only member of the pathogenic *Candida* genus to have phospholipases but recently it has been shown by studying lipid breakdown following growth on egg yolk-based media and also in acyl-CoA-oxidase based colometric assays that non-*C. albicans* species also secrete phospholipases but at much lower levels (Lane and Garcia, 1991; Ghannoum, 2000). Analysis of a *ura* positive *plb1* null strain has established that this

strain suffers from significantly attenuated virulence and a lower fungal burden in the kidneys of infected mice (Leidich *et al*., 1998; Ghannoum, 2000). The Leidich study also showed that the *plb1* null strain exhibited a defect in epithelial cell damage and hyphal penetration into target cells. These results suggest that the phospholipase B of *C.albicans* is required for epithelial penetration and breakdown of host proteins.

Costa and colleagues documented that they were also able to detected phospholipase A, C and D activity in some clinical isolates of *C. albicans* in an *in vitro* egg yolk-based plate assay (Costa *et al*., 1968). Further studies have shown that there are potentially three phospholipase C genes, *PLC1, PLC2* and *PLC*3 and one phospholipase D gene, *PLD1* in the *C. albicans* genome (Bennett *et al*., 1998; Hube *et al*., 2001; Andaluz *et al*., 2001). Unlike the Plb1 and Plb2 proteins, none of the other phospholipase proteins were detected within the media surrounding the cells which implies they are not secreted, suggesting a limited role in virulence (Schaller *et al*., 2005). Additionally the phospholipase C protein was detected in microsome fractions of the cytosol which points to the protein being intracellular (Mago and Khuller, 1990). However Hube *et al*., showed the Pld1 protein is required for virulence in two different models of systemic candidiasis, an immunosuppressed mouse model and a transgenic ε26 mouse model, but is not required in an oral model of candidiasis (Hube *et al*., 2001). The *pld1* null mutant contains a copy of the *URA3* gene integrated into one *PLD1* allele therefore OMPD levels might not be at the same level as in the wild type. This potential difference in OMPD levels could be the cause of the virulence defects of the *pld1* null strain. This evidence indicates that *PLD1* is required for disseminated infection and suggests a possible role in host defence evasion.

1.3.3.2. Extracellular lipases.

There are currently ten known lipase genes (*LIP1-10*) in the *C. albicans* genome (Fu *et al*., 1997; Hube *et al*., 2000). Northern blotting studies showed that the *LIP3-LIP6* genes are expressed in all the types of media tested while the *LIP2* and *LIP9* genes were only expressed in media without lipids (Hube *et al*., 2000). Analysis of gene expression, using rtPCR, of cells extracted from infected mucosal tissues (tongue, stomach and esophagus) from a mouse model of mucosal candidiasis found that the *LIP4-8* genes were expressed indicating that the Lip4-8 proteins either play a general

role in virulence or are required for general cell maintenance. Expression of the other five *LIP* genes except *LIP10* was detected by rtPCR in cells from the stomachs of infected mice from a systemic mouse virulence model but not in cells found in the infected tongues or esophagus of the mice, suggesting that expression of the various lipase genes is dependent on the environmental conditions in which the cells are grown (Stehr *et al.*, 2004; Schofield *et al.*, 2005). To date there are no reports of null mutants in any of the lipase genes.

1.3.3.3. Secreted aspartic proteinases.

The secreted aspartic proteinases (SAP) are the most comprehensively researched of the secreted enzymes of *C. albicans*. The SAP family consists of ten members, eight of which are secreted while two are membrane-anchored GPI proteins (Hube *et al.*, 1991; Wright *et al.*, 1992; White *et al.*, 1993; Miyasaki *et al.*, 1994; Monod *et al.*, 1994; Monod *et al.*, 1998; Schaller *et al.*, 2005). One member of the family, *SAP2*, has been extensively studied, and *in vitro* experiments have shown it is the most highly expressed gene of the family. The expression of the other SAPs has been found to be growth state dependant with *SAP1-3* predominantly expressed in yeast cells whereas *SAP4-6* have been associated with hyphal formation (Hube *et al.*, 1994; White and Agabian, 1995; Naglik *et al.*, 2008). The expression of the two GPI-anchored proteins Sap9 and Sap10 appears to be unaffected by the growth form of the cells (Hube and Naglik, Extracellular Hydrolases. *Candida* and Candidiasis. Ed. R.A. Calderone. 2002). Studies have found that treating *C. albicans* cells with the proteinase inhibitor pepstatin A resulted in cells that were unable to penetrate a range of different epithelial models (De Bernardis *et al.*, 1996; Fallon *et al.*, 1997; De Bernardis *et al.*, 1997; Schaller *et al*, 1999; Schaller *et al.*, 2000). Virulence models have shown that Sap1-3 are involved in the infections of mucosal surfaces while Sap4-6 are required for systemic infections. This suggests that Sap1-3 are involved in adhesion to mucosal surfaces or other cells while Sap4-6 play a role in tissue invasion (Hube *et al.*, 1997; Sanglard *et al.*, 1997; De Bernardis *et al.*, 1999; Schaller *et al.*, 1999). All the mutant strain used in these studies contain the *URA3* gene within the locus of the disrupted gene.

The evidence described above suggests that SAPs play a major role in the virulence of

C. albicans. However, two recent articles suggest that SAPs play minor roles in virulence (Lermann and Morschhaüser, 2008; Correia *et al.*, 2010). Both of these articles used SAP mutants generated using the SAT1-flipper cassette instead of the *URA*-blaster cassette that was used in previous studies. In the recent studies single null mutants were constructed for *SAP1-6* and two triple mutants, *SAP1-3* and *SAP4-6.* It was found that none of the eight mutants displayed any defect in RHE damage or invasion, suggesting that SAPs are not required for epithelial invasion (Lermann and Morschhaüser, 2008). The studies also assessed the mutants' virulence in a BALB/c mouse model and concluded that only the mutants lacking the *SAP5* gene (the *sap5* and *sap456* triple null mutants) had any virulence defect and this defect constituted only a slight extension of the survival time of the mice (Correia *et al.*, 2010). These two studies suggest that only *SAP5* plays a role in virulence and that the findings of previous groups were caused by variable OMPD levels as a result of the *URA3* gene being expressed in the locus of the disrupted gene and not in its native locus or a highly expressed locus.

1.3.4. Phenotypic switching.

C. albicans has the unique trait of being able to switch colony morphology from smooth colonies to rough colonies or even 'star, stippled, hat, irregular wrinkle and fuzzy' shaped colonies (Pomes *et al.*, 1985; Slutsky *et al.*, 1985). This switching is reversible and can be triggered by exposure to low doses of UV light. The most intensely studied version of phenotypic switching is white-opaque switching in the WO-1 strain (Slutsky *et al.*, 1987). White-opaque switching is referred to when white colonies switch to flatter grey colonies. The cells in these flatter grey colonies (opaque) are much more elongated than that of the traditional ovoid yeast cells of white colonies (Figure 4; Slutsky *et al.*, 1987). White-opaque switching can only occur in strains that are homozygous at the mating type locus (MTL). The MTL in *C. albicans* is located on chromosome 5 and growing *C. albicans* in sorbose media results in the loss of one copy of chromosome 5 (Janbon *et al.*, 1998; Magee and Magee, 2000). In non-switching strains, the MTL consist of two alleles, *MTL* **a** and *MTL* α, which produces a white-opaque switch repressor **a**1-α2 (Hull *et al.*, 2000; Miller and Johnson, 2002; Tsong *et al.*, 2003). This repressor acts by repressing the transcription factor Wor1 which regulates the white-opaque switch (Huang *et al.*, 2006; Srikantha *et al.*,

2006; Zordan *et al*., 2006). In strains that are homozygous at the MTL, Wor1 is not repressed therefore allowing white-opaque switching to occur. Additional regulatory mechanisms for this switching are present and include the positive regulators of the white to opaque switch, Czf1 and Wor2, and the positive regulator of the opaque to white switch, Efg1 (Sonneborn *et al*, 1999; Srikantha *et al*., 2000; Zordan *et al*., 2006; Vinces and Kumamoto, 2007; Ramirez-Zavala *et al*., 2008). In opaque cells Wor1 and its co-regulator Mcm1 represses *EFG1* expression by binding to its promoter region preventing expression, while in white cells Efg1 represses *WOR1* expression (Tuch *et al*., 2008).

The role of phenotypic switching in virulence has been studied for a number of years. Studies have shown that free oxidants and polymorphonuclear leukocytes (PMLs) stimulate white phase cells to switch to the opaque phase but not *vice versa* and that white phase cells are more resistant to killing by PMLs than opaque phase cells. (Kolotila and Diamond, 1990; Klar *et al*., 2001; Srikantha *et al*., 2001). Further research has shown, that both white and opaque phase cells are required for virulence, but in different types of infection. Kvaal *et al*., showed in their 1997 and 1999 papers that white phase cells are more virulent in systemic mouse models while opaque cells are more virulent in a cutaneous infection model. A number of clinical studies have shown that isolates from infections phenotypically switch at a higher rate that than of commensal isolates (Hellstein *et al*., 1993; Jones *et al*., 1994; Vargas *et al*., 2000).

1.4. Morphogenesis of *C. albicans*.

An important feature of *C. albicans* biology is the ability of the fungus to switch between its five growth morphologies, yeast, elongated pseudohyphae, parallel sided true hyphae, chlamydospores and the opaque form (Figure 4). The yeast, pseudohyphal and true hyphal forms are the most commonly studied forms, therefore the remainder of this section will concentrate on them. *C. albicans* can be induced to switch to any of these three growth forms by manipulation of the growth conditions. *C. albicans* grows preferentially in yeast form at 30˚C or in media with a pH around 4. Switching the cells to high phosphate or pH 6 media or increasing the temperature to 35˚C will result in pseudohyphae formation. Hyphal formation is triggered by growth at 37˚C, at neutral pH or in media containing serum or *N*-acetylglucosamine (Sudbery *et*
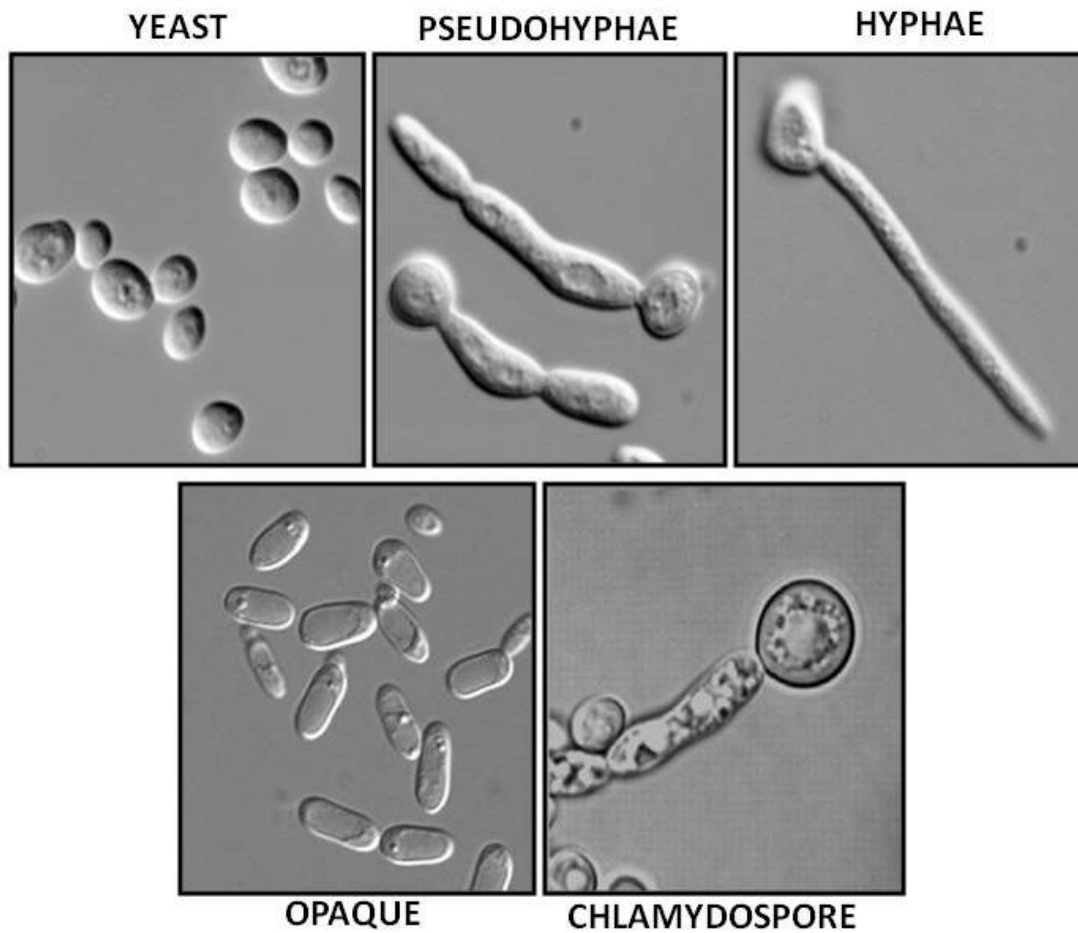
Figure 4. Five growth morphologies of *C. albicans*. The growth forms of *C. albicans*. The yeast form is the most common form along with the pseudohyphal and true hyphal forms. The opaque growth form only occurs in strains that are heterozygous for chromosome 5. The chlamydospore growth form is only seen when *C. albicans* cells are grown on media containing rice or cornmeal agar supplemented with Tween-80. Pictures provided by Dr Steve Bates.

Growth form	Site of nuclear division	Constrictions at site of septation	Cell separation	Typical filament width	Spitzenkörper present
Yeast	Mother/Daughter bud neck	Yes	Yes	NA	No
Pseudohyphae	Mother/Daughter bud neck	Yes	No	Minimum of 2.8 μm	No
True hyphae	About 10-15μm in hyphae	No	No	2.0 μm	Yes

Table 1. Features of the three main growth morphologies of *C. albicans*.

al., 2004).

1.4.1. The differences between the three major morphological growth forms.

The differing cell shapes are a very obvious feature of the different morphologies (Figure 4). Yeast cells are similar in size and appearance to those of the budding yeast *S. cerevisiae,* with obvious constrictions at the site of septation during budding and the separation of the mother and daughter buds after cytokinesis is completed (Table 1). Yeast cells are also easily distinguishable from the other two major growth morphologies by their ovoid shape and lack of obvious extended polarised growth. Early literature suggests that there was some confusion as to what defines a pseudohypha and a true hypha. Frank Odds, Pete Sudbery and colleagues have carried out detailed research to elucidate the physiological differences between the pseudohyphal and true hyphal growth states and have set out parameters for defining the two morphologies. From this research, it was concluded that true hyphae are narrow with a width of about 2.0 μm have parallel sides and no constrictions, while pseudohyphae have a minimum width of 2.8 μm and have obvious constrictions at the septal site as with budding yeast cells. However, unlike yeast cells, the mother and daughter cells do not separate after cytokinesis (Merson-Davies and Odds, 1989; Sudbery *et al*., 2004). In hyphal cells, it is known, that the Cdc28-Hgc1 complex phosphorylates Efg1 and this results in downregulation of the Ace2 controlled genes, *CHT3* and *SCW11*, and therefore no cell separation occurs following cytokinesis (Wang *et al*., 2009). The mechanism in pseudohyphal cells could be similar with the activation of Efg1 being controlled by another protein.

The location of nuclear division and the ultimate plane of septation are also different in the various morphologies. Division occurs about 10-15 μm into the true hyphae

while in yeast and pseudohyphae it occurs at the mother/daughter bud neck (Sudbery *et al.*, 2004). One final cellular landmark, which can be used to define a hypha from a pseudohypha, is the presence of a structure called a Spitzenkörper. A Spitzenkörper is an apical body that is found in hyphae of many filamentous fungi and its role is to direct secretary vesicles to the tip of growing hyphae and hence assist polarised growth (Crampin *et al.*, 2005; Virag and Harris, 2006). *C. albicans* hyphae possess a Spitzenkörper while pseudohyphae do not have this feature thus cells may be examined using the FM4-64 stain and fluorescence microscopy to establish the presence or absence of this structure (Sudbery *et al.*, 2004; Whiteway and Bachewich, 2007).

1.4.2. Morphogenesis signalling pathways.

Much research in *C. albicans* has focussed on the transition between the yeast and hyphal forms. So far four separate signalling pathways have been found which, when activated, initiate hyphal formation and one pathway that negatively regulates hyphal formation. At the end of each pathway there is a transcription factor; *EFG1*, *CPH1*, *CZF1*, *RIM101* and the negative regulator *TUP1* (Figure 5). The Efg1 and Cph1 pathways are very similar to the Ras-cAMP and MAP kinase morphology pathways in *S. cerevisiae*.

1.4.2.1. Cph1 MAP kinase pathway.

The Cph1 MAP kinase pathway was the first morphogenesis pathway deciphered in *C. albicans* and shows a large degree of similarity to the *S. cerevisiae* morphogenic pathway. Homologues of all members of the MAP kinase morphogenesis pathway in *S. cerevisiae* have been found in *C. albicans* except the Dig1/2 proteins and are shown in Figure 6. Epistasis experiments have been carried out that show the homologues of the four kinases Ste20 (Cst20), Ste11 (Ste11), Ste7 (Hst7), Kss1 (Cek1) function in the same order as in *S. cerevisiae* and that the Ste12 homologue Cph1 is located downstream of the final kinase Cek1 (Leberer *et al.*, 1996; Leberer *et al.*, 1997). Mutants in *CST20*, *HST7*, *CEK1* and *CPH1* have been constructed and shown to have no filamentation defect in the presence of serum, *N*-acetylglucosamine or proline, but morphogenesis is inhibited in media lacking nitrogen (Liu *et al.*, 1994; Köhler and Fink, 1996). The Cph1 pathway in *C. albicans* is negatively regulated by the Cpp1 protein.
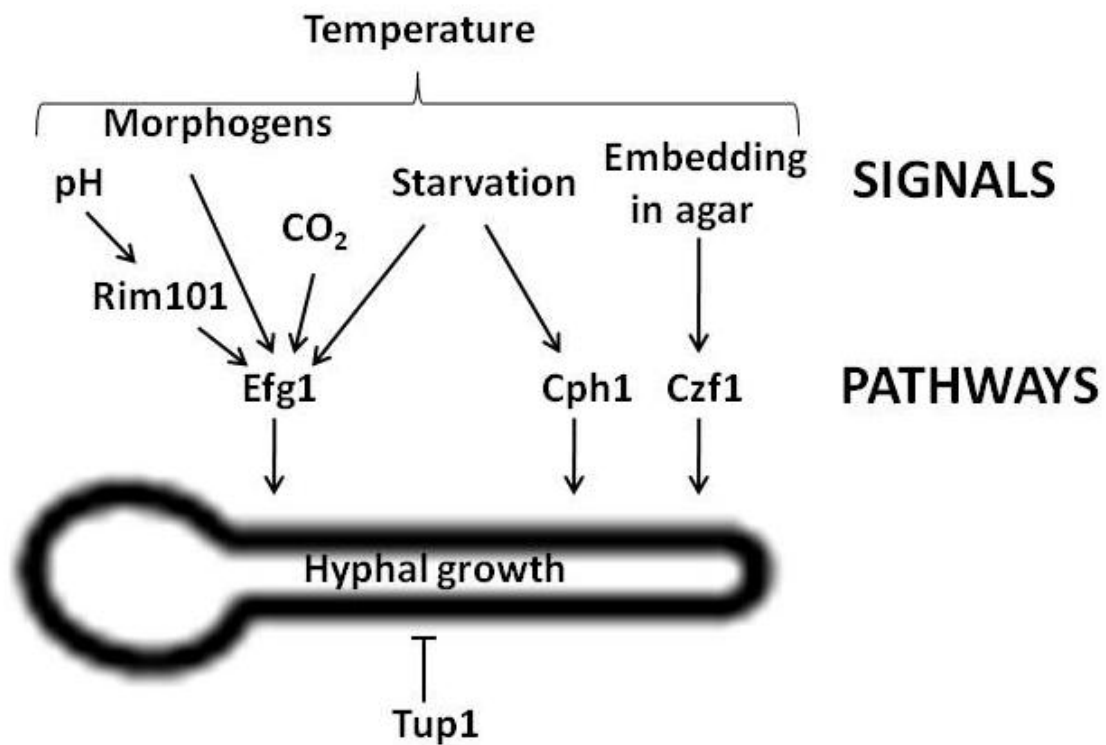
Figure 5. Yeast to hyphae signalling pathways. The signalling pathways controlling the yeast to hyphae transition in response to various different external conditions. The external signals include pH, 'morphogens' such as serum, CO_2, starvation and embedding in agar or another surface such as epithelial cells. Figure based on Figure 1 in Brown, Morphogenetic Signalling Pathways in *Candida albicans*. *Candida* and Candidiasis. Ed. R.A. Calderone. 2002 and updating results from Klengel *et al*., 2005.
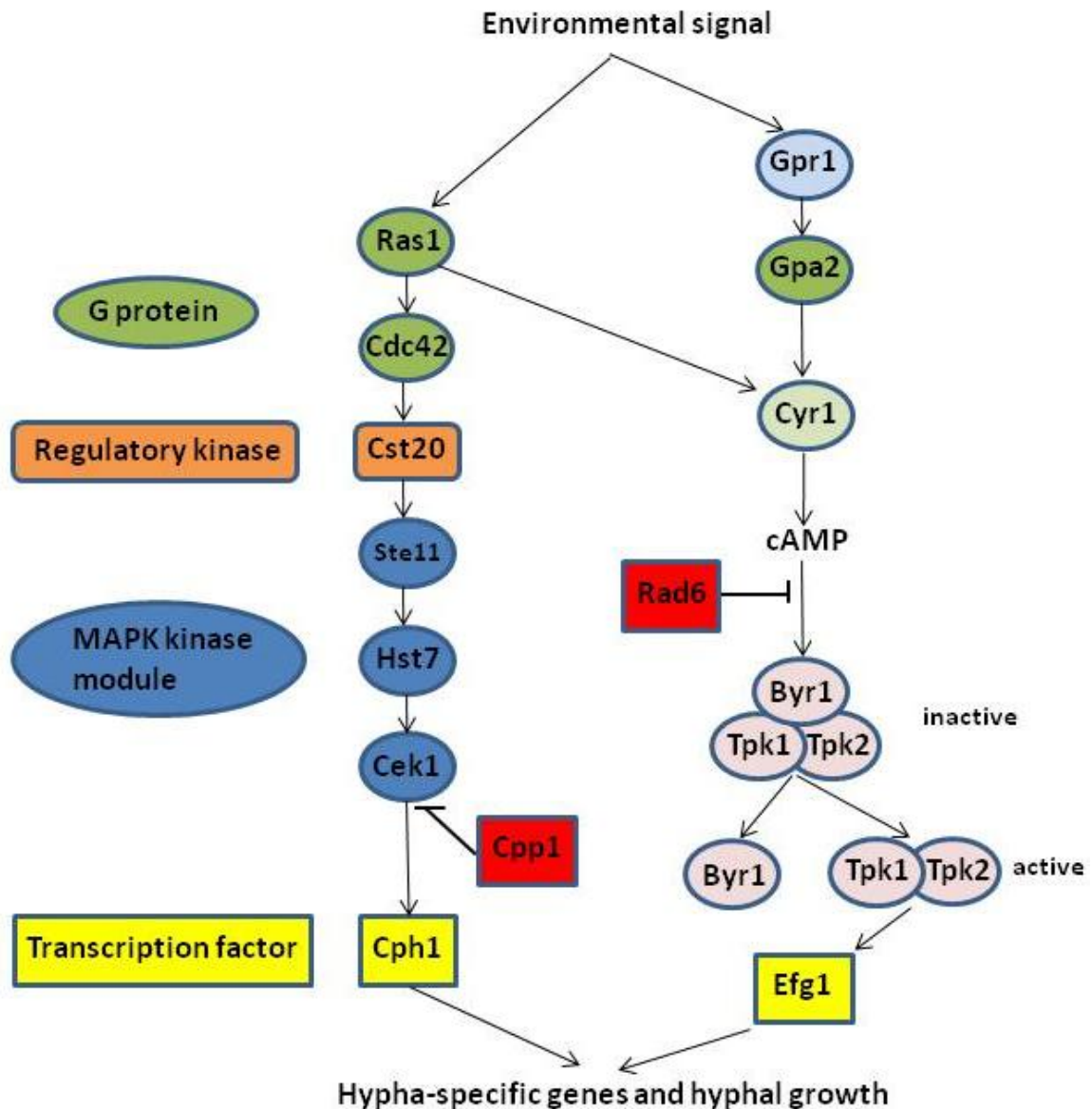
Figure 6. The cAMP and MAP kinase morphogenesis signalling pathways in *C. albicans*. The two major yeast to hyphae transition pathways, the cAMP and Efg1/cAMP kinase pathways. The activation of these pathways results in the activation of a transcription factor, Cph1 (MAP kinase pathway) and Efg1 (cAMP pathway) and expression of hypha-specific genes and hyphal formation. Adapted from Brown and Gow, 2001.

Csank *et al.,* (1997) showed that on solid yeast-inducing media a *cpp1* null mutant grew as hyphae. Yeast growth of the *cpp1* null mutant was restored by deleting both *CEK1* alleles suggesting that the Cpp1 protein represses the activity of Cek1 which in turn prevents the activation of Cph1 (Csank *et al.,* 1997). Csank *et al* showed that deletion of the *cpp1* gene results in hyphal formation even under repressing conditions and that this phenotype is dependent on the presence of an active Cek1 protein (Csank *et al.,* 1997; Csank *et al.,* 1998). This result suggests that Cpp1 negatively regulates the activity of Cek1 and hence the pathway and also provides further confirmation that the Cph1 MAP kinase pathway is involved in hyphal formation. In addition a recent study showed that constitutive expression of *CPH1* results in enhanced filamentous growth, further confirming the Cph1 pathway's role in hyphal formation (Huang *et al.,* 2008). All the mutants discussed in this section are *ura* minus however this had no effect on hyphae formation.

1.4.2.2. Efg1/cAMP pathway.

A second major pathway has been implicated in morphogenesis. This is the Efg1/cAMP pathway (Figure 6). The Efg1/cAMP pathway is very similar to the Ras-cAMP morphogenesis pathway in *S. cerevisiae* with homologues of all proteins present in *C. albicans* (Figure 6; Lo *et al.,* 1997; Stoldt *et al.,* 1997; Feng *et al.,* 1999; Mallet *et al.,* 2000; Sonneborn *et al.,* 2000; Sanchez-Martinez and Perez-Martin, 2002; Doedt *et al.,* 2004). The Efg1/cAMP pathway is split in to two sections. Section one is the two component section and is involved in sensing and responding to external environmental signals and results in the generation of cyclic adenosine monophosphate (cAMP). The second section of the pathway is cAMP activation of the hyphal formation process. The pathway has two components that are involved in its initiation, Ras1 and Gpr1-Gpa2, which both signal to adenyl cyclase. These two start point were deciphered using various epistasis experiments (Leberer *et al.,* 2001; Maidan *et al.,* 2005). Studies showed that deletion of any of these three proteins resulted in a hyphal defect on either serum (Ras1) or spider medium (Gpr1 and Gpa2) suggesting they all have a role in hyphal formation (Feng *et al.,* 1999; Miwa *et al.,* 2004). Adenyl cyclase is required to make cAMP from ATP and is encoded in *C. albicans* by the *CYR1/CDC35* gene. Deletion of both *CYR1/CDC35* alleles results in a strain that is defective in hyphal formation on serum, spider and Lee's medium (Rocha

et al., 2001). Furthermore this defect can be alleviated by the addition of exogenous cAMP. In addition the defects seen in the *ras1, gpr1, gpa2* and *cyr*1/*cdc35* null strains can also be overcome by the addition of exogenous cAMP confirming that the proteins function upstream of adenylate cyclase in the pathway (Whiteway, 2000; Rocha *et al*., 2001; Miwa *et al*., 2004). The *ras1*, *gpr1*, *gpa2* and *cyr1*/*cdc35* null mutants all contain an intact *URA3* gene integrated into the locus of the disrupted gene.

Following synthesis, cAMP activates the protein kinase A (PKA) catalytic subunits Tpk1 and Tpk2. The exact mechanism for this in *C. albicans* is yet to be deciphered however a study showed by using a phosphotransferase activity assay that the PKA activity of the Tpk1 subunit is dependent on the actions of cAMP and that this occurs in a Bcy1 independent manner (Cassola *et al*., 2004). Additional mechanisms of PKA activation by cAMP are possible as in *S. cerevisiae* cAMP binds to the PKA regulatory subunit Bcy1 causing it to dissociate from the Tpk1-Tpk2 complex (Toda *et al*., 1987). Activated Tpk1-Tpk2 complex then phosphorylates the transcription factor Efg1 leading to its activation (Bockmuhl and Ernst, 2001). A number of hypha-specific genes have been shown to be dependent on the activation of Efg1 such as *HWP1* and *HWP2* (Sharkey *et al*., 1999; Braun and Johnson, 2000; Leng *et al*., 2001; Sohn *et al*., 2003). Deletion of *tpk2* resulted in complete abolition of hyphal formation on solid spider or serum media but few defects were seen in liquid media (Sonneborn *et al*., 2000). This finding suggests that Tpk2 might also be part of the Czf1 cell embedded pathway as a double mutant lacking Efg1 as well as Cph1 is filamentous when grown embedded in a matrix (Brown *et al*., 1999). Deletion of the *EFG1* gene results in pseudohyphal formation when the cells are grown in the presence of serum and the same pseudohyphal growth is seen when *EFG1* is over-expressed (Lo *et al*., 1997; Stoldt *et al*., 1997). These findings led Stoldt and co-workers to suggest that Efg1 plays a double role as both a morphogenesis activator and repressor. Furthermore they suggested that the growth morphology is dependent on Efg1 levels. Indeed the action of Efg1 as a repressor is part of the Czf1 pathway and will be discussed further in the section below.

As with the Cph1 MAP kinase pathway the cAMP pathway contains a negative regulator. In the cAMP pathway this is Rad6 (Leng *et al*., 2000). Rad6 is a putative ubiquitin conjugating enzyme that was identified from a cDNA library using the *S.*

cerevisiae RAD6 as a probe. *RAD6* in *S. cerevisiae* was originally isolated by a screen of UV hypersensitive mutants (Cox and Parry, 1968). Deletion of *rad6* in a strain with a fully functional *EFG1* gene resulted in enhanced hyphal growth while over-expression was shown to inhibit hyphal formation. Additionally it was found that repressing expression of *RAD6*, using the regulatable *MET3* promoter, in a strain lacking *efg1* resulted in the cells growing as pseudohyphae while in a *cph1* null strain R*AD6* repression had no effect on growth type (Leng *et al.*, 2000). These results confirm that Rad6 is a negative regulator of the cAMP pathway and probably of Efg1 activity. The authors also suggested that when Rad6 is activated, it targets members of the cAMP pathway for ubiquitin-mediated degradation (Leng *et al.*, 2000).

All the various Efg1 cAMP pathway null mutants contain an intact *URA3* gene either integrated in the locus of the disrupted gene or as part of a regulatable promoter construct except the *tpk1* and *tpk2* mutants. Similarly to the Cph1 MAP kinase pathway mutants the *URA3* status does not affect hyphal morphogenesis as the mutants were able to form hyphae under most hyphae-inducing conditions but not in serum-containing media.

1.4.2.3. Rim101 pH sensing pathway.

The ability of *C. albicans* cells to sense environmental pH is vitally important. As discussed in section 1.5 different pHs trigger morphological change in *C. albicans* cells. In both *Aspergillus nidulans* and *S. cerevisiae* the pH sensing pathway revolves around the transcription factor Rim101 (PacC in *A. nidulans*) and is very well understood (Figure 7; Tilburn *et al.*, 1995; Bignell *et al.*, 2005). At acidic pH the PacC/Rim101 protein normally exists in its non-cleaved/closed (inactive) conformation while at alkaline pH the protein is found in a cleaved/open (active) form (Orejas *et al.*, 1995; Li and Mitchell, 1997). Studies have shown that processing of PacC/Rim101 requires the activities of a number of proteins: Rim13/PalB, Rim20/PalA, Rim8/PalF, Rim9/PalI, Rim21/PalH and, in *A. nidulans*, PalC (Su and Mitchell, 1993; Arst *et al.*, 1994; Denison *et al.*, 1995; Maccheroni *et al.*, 1997; Negrete-Urtasun *et al.*, 1997; Denison *et al.*, 1998; Negrete-Urtasun *et al.*, 1999; Xu and Mitchell, 2001). Overexpression of a C-terminal truncated PacC/Rim101 protein alleviates the consequences of the loss of any of the above genes (Xu and Mitchell, 2001). Homologs of all the members of the
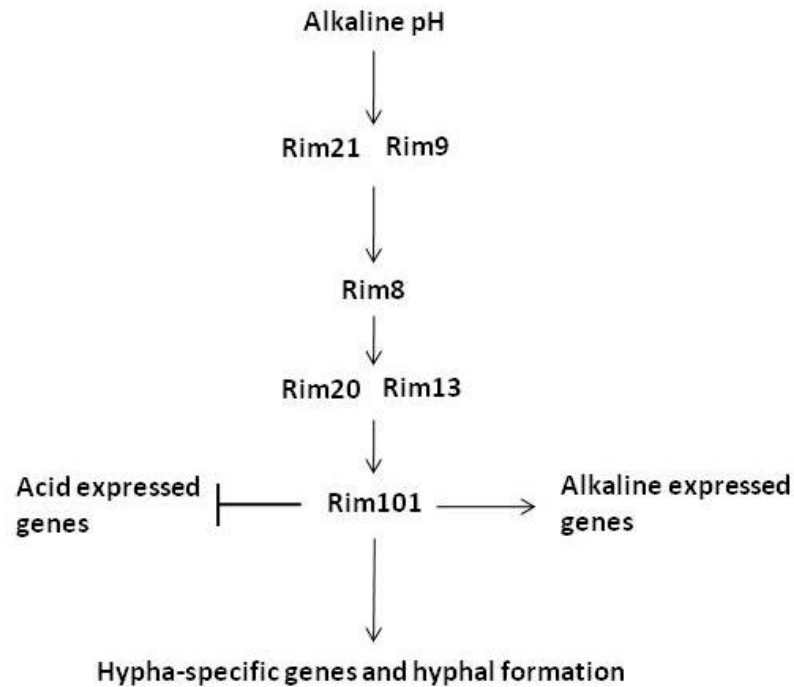
Figure 7. The Rim101 signalling pathway in *C. albicans*. The pH sensing pathway in *C. albicans* senses alkaline pHs by the membrane proteins Rim21 and Rim9. The activated pathway concludes with the truncation of the Rim101 transcription factor which activates alkaline expressed genes and represses expression of acid expressed genes. Figure adapted from Brown and Gow, 2001.

PacC/Rim101 pathway have been identified in *C. albicans* with the exception of PalC (Figure 7, Davis *et al.*, 2000). Mutant strains lacking any of these genes all exhibited hyphal formation defects under alkaline conditions but not under other hyphal inducting conditions such as serum (Davis *et al.*, 2000). All these mutants contained the *URA3* gene inserted into the disrupted gene. However, the potential variable OMPD levels in the Rim101 pathway mutants appears to have little or no affect on morphogenesis as the mutants can still form hyphae in serum. Ectopic expression of a C-terminally truncated Rim101 protein leads to the induction of hyphal formation at acidic pH (Porta *et al.*, 1999; Davis *et al.*, 2000; El Barkani *et al.*, 2000; Barwell *et al.*, 2005; Cornet *et al.*, 2009;). Interestingly a study showed that hyphal formation at acidic pH induced by truncated Rim101 is dependent on Efg1: possibly suggesting that Rim101 signals through Efg1 as well as activating alkaline expressed genes directly (El Barkani *et al.*, 2000). Additionally members of the endosomal sorting complex required for transport (ESCRT) have been implicated in PacC/Rim101 processing. Strains lacking the ESCRT I or II complex or the Vps20 and Snf7 proteins have been shown not to undergo PacC/Rim101 processing (Xu *et al.*, 2004). Snf7 is required to recruit Rim13 and Rim20 to Rim101 by acting as a scaffold protein. This all occurs at the endosomal membrane and is dependent on ESCRT I and II (Ito *et al.*, 2001; Bower *et al.*, 2004; Boysen and Mitchell, 2006; Blanchin-Roland *et al.*, 2008).

1.4.2.4. Czf1 pathway.

It was originally believed that the *efg1*/*efg1 cph1*/*cph1* double null mutant could not form hyphae, however, when this strain was grown on rough agar plates or on the tongues of germ free immunosuppressed piglets, hyphae formed (Riggle *et al.*, 1999). The pathway responsible for the hyphal formation is the Czf1 pathway, which was discovered through the screening of a DNA library to determine which gene was responsible for the *efg1*/*efg1 cph1*/*cph1* double null mutant becoming filamentous (Brown *et al.*, 1999). A *czf1* null mutant (*URA3* positive strain) displayed normal hyphal formation in response to *N*-acetylglucosamine, proline, serum and spider medium (Brown *et al.*, 1999). However, mutants lacking the *CZF1* gene or both the *CZF1* and *CPH1* genes were defective in filamentous growth when embedded in an agar matrix, but a *efg1*, *cph1* and *czf1* triple mutant (*URA3* positive) exhibited wild type filamentous growth under the same agar matrix conditions (Brown *et al.*, 1999; Giusani *et al.*,

2002). Giusani and colleagues also showed, by yeast 2 hybrid analysis, that Czf1 and Efg1 interact, and proposed that Efg1 represses hyphae specific gene expression in cells embedded in a matrix. Upon binding of Czf1 to Efg1, a conformational change in Efg1 occurs and this relieves the repression and allows hyphae to form. Later studies utilised northern blots to show that *CZF1* expression is dependent on the growth phase, temperature, and the carbon source, and identified the binding of both Efg1 and Czf1 to regulatory sequences in the *CZF1* promoter (Vinces *et al*., 2006). All the current evidence suggests that hyphal formation in embedded matrix is the result of Czf1 activity; however the pathway leading to its activation is yet to be fully elucidated.

1.4.2.5. Tup1 pathway.

In addition to positive regulatory pathways negative regulators including the transcriptional repressor Tup1 have been identified as playing a role in the yeast to hyphal transition. In *S. cerevisiae*, the Tup1 protein forms a complex with Ssn6 and this complex targets the promoters of hypoxic, glucose-repressed and cell type specific genes (Keleher *et al*., 1992; Treitel and Carlson, 1995; Gavin and Simpson, 1997; Redd *et al*., 1997; Deckert *et al*., 1998). This occurs with the complex binding to sequence-specific DNA binding proteins such as Nrg1 (Park *et al*., 1999; Smith and Johnson, 2000). *C. albicans* homologues of these three proteins have been identified and null mutants constructed (all *URA3* positive; Braun and Johnson, 1997; Murad *et al*., 2001; Hwang *et al*., 2003; Garcia-Sánchez *et al*., 2005). The *tup1* and *nrg1* null mutants constitutively grow as pseudohyphae with the phenotype being more pronounced in the *tup1* null strain. The latter strain is unable to form hyphae when grown in serum whilst the *nrg1* null strain is still able to do so (Braun and Johnson, 1997; Murad *et al*., 2001). Transcript profiling and northern blot analysis has shown that hyphal-specific genes are not repressed in these two mutants (Braun and Johnson, 2000; Braun *et al*., 2000; Murad *et al*., 2001; Sharkey *et al*., 1999). In addition to Tup1 another transcriptional repressor, Rfg1, has also been shown to contribute to repressing the yeast to hyphae switch. An *rfg1* null strain forms hyphae under normal yeast inducing conditions and hence may also be involved in the repression of hyphal formation (Khalaf and Zitomer, 2001).

It has been proposed that both Tup1 and Rfg1 are involved in recruiting the Tup1-Ssn6

complex to hyphal-specific genes, therefore repressing their expression and maintaining cells in the yeast growth form (Brown, Morphogenetic Signalling Pathways in *Candida albicans. Candida* and Candidiasis. Ed. R.A. Calderone. 2002). However, this repression is overcome under conditions promoting hyphal growth by the expression of *NRG1* being down-regulated (Murad *et al.*, 2001).

1.4.3. The role of morphogenesis in *C. albicans* virulence.

The general perception that morphogenesis is important for the pathogenicity of *C. albicans* is based on four main pieces of evidence. The first evidence came from histopathological studies, in which all three main growth forms of *C. albicans* have been observed in tissue samples taken from animal candidiasis models as well as from human Candidiasis patients (Calderone and Gow, Host recognition by *Candida* species, *Candida* and Candidiasis. Ed. R.A. Calderone. 2002). The second piece of evidence that points to morphogenesis as important in pathogenicity and virulence is the finding that an increasing number of strains that have morphogenesis defects are also attenuated virulence in murine infection models. Examples of this include the *tup1* null strain which is locked in the pseudohyphal growth form, and an *efg1/efg1 cph1/cph1* double mutant which is locked in the yeast form (Riggle *et al.*, 1999; Phan *et al.*, 2000). These findings suggest that it is the ability of *C. albicans* cells to switch morphology rather than any single growth form that determines its pathogenicity. Studies have also shown that several genes undergo different expression patterns that are dependant of the growth form of the cells. Many of these genes have been demonstrated to be virulence factors and include the hyphae-specific *SAP4-6* genes and a number of adhesion genes. The final piece of evidence is that morphogenesis plays a major role in biofilm formation. The basal layer of the biofilm consists of yeast cells together with a layer of aerial hyphae which produce various proteins and carbohydrates that together make up the EPS layer (Baillie and Douglas, 2000). Not only do biofilms act as large reservoirs of cells for potential infection but these cells often have a higher resistance to the commonly administered anti-fungal drugs (Baillie and Douglas, 2000).

Recently a study was published that suggests morphogenesis alone is not essential for virulence. In this study, 674 genes were deleted and 115 of these null mutants exhibited defects in infecting mice (Noble *et al.*, 2010). This study also found that 133

mutants were unable to form hyphae on spider medium but their ability to form hyphae *in vivo* was not tested. In the literature there is a strong relationship between virulence and morphogenesis, 106 mutants out of 130 published *C. albicans* mutant constructed using reverse genetics have both a virulence defect and an abnormal morphology. This would suggest that majority of the 133 mutants unable to form hyphae in the Noble study would have attenuated virulence however 80 of the strains were still pathogenic (Noble *et al.*, 2010). Of the 115 mutants which exhibited virulence defects only 15 of these also had a hyphal formation defect. Altogether these results suggest the pathogenicity of *C. albicans* is governed by a number of virulence factors and not by morphogenesis alone.

Many putative virulence factors have been proposed to be involved in the pathogenicity of *C. albicans* and have been discussed in the previous sections. The majority of the mutants used for studying these virulence factors were constructed utilising the *URA3* gene as the auxotrophic marker. In most cases the *URA3* gene was either recycled to leave the mutant strain without an intact *URA3* gene or left within the locus of the disrupted target gene. This makes interpreting the results of virulence assays difficult as strains with variable OMPD levels have virulence defects (Kirsch and Whitney, 1991; Laurenson and Rine, 1992; Cole *et al.*, 1995; Bain *et al.*, 2001; Cheng *et al.*, 2003; Staab and Sundstrom, 2003; Brand *et al.*, 2004). A recent study documented a methodology for studying gene essentiality during an infection without using the *URA3* gene as part of the strain construction (Becker *et al.*, 2010). In this study tetracycline-regulatable promoter-based conditional mutants underwent virulence testing using an immunocompetent murine systemic candidiasis model in which the mice had been pre-treated with doxycycline in the drinking water either 3 days prior to or 2 days post infection until the conclusion of the experiment (Becker *et al.*, 2010). These two different doxycycline treatment assays allow the role of the depleted protein in both early and late stages of infection to be studied. Within this study the authors found that of the 177 genes tested 102 were essential (101 of which were essential *in vitro*) and 29 not essential for virulence at either doxycycline treatment assay (Becker *et al.*, 2010). The remaining 46 mutants experienced attenuated virulence in which survival rates of the infected mice were equal or higher that 40 % but less than 80 %. During *in vitro* growth assays 19 of the 29 non essential genes

were found to be conditionally essential as they were not required for growth on YPD plus doxycycline media but required for growth on YNBD plus doxycycline media. These findings confirm that this method can be used for distinguishing genes which are essential for vegetative growth from those that are required virulence. Overall this study documents a method for re-evaluating all the proposed *C. albicans* virulence factors without the problems associated with the use of the *URA3* gene in gene disruption.

1.5. Overview of the cell cycle.

The ability of all cells to replicate in an error-free manner is extremely important. Much research has therefore focussed on this area of cell biology. Studies using autoradiographs have found that the cell cycle of eukaryotic organisms consist of four distinct phases; G_1, S, G_2 and M, each of which is important for a different aspect of cell growth and division (Figure 8A; Howard and Pelc, 1951). During the S phase DNA replication, chromosome duplication and tight linkage of sister-chromatids occurs and during M phase separation of the chromosomes into two nuclei is completed (mitosis). The G_1 and G_2 phases are both gap phases allowing cells extra time for growth. The G_1 phase is the point at which the cell controls progression into the next cell cycle by monitoring intra-cellular signals such as cell size and also extra-cellular signals including nutrient availability. While in G_1 phase, haploid yeast cells have three choices; enter the stationary G_0 phase in response to unfavourable growth conditions, enter the next cell cycle and divide, or to mate (Forsburg and Nurse, 1991). In *S. cerevisiae*, there is no clear G_2 phase and the cells have a longer G_1 phase, during which the primary regulation of the cell cycle occurs (Forsburg and Nurse, 1991).

The M phase of the cell cycle has been the most extensively studied. M phase consists of two major events; nuclear and cellular division. The nuclear division stage is called mitosis and consists of four stages; prophase, metaphase, anaphase and telophase (Figure 8B). In most fungi mitosis occurs within an intact cell nucleus and is therefore referred to as a closed mitosis whereas in animal cells the nuclear envelope breaks down prior to chromosome segregation (De Souza and Osmani, 2007). The overall role of mitosis, is to separate the duplicated chromosomes into a pair of daughter nuclei with one nucleus destined for the mother cell and the other for the daughter cell.
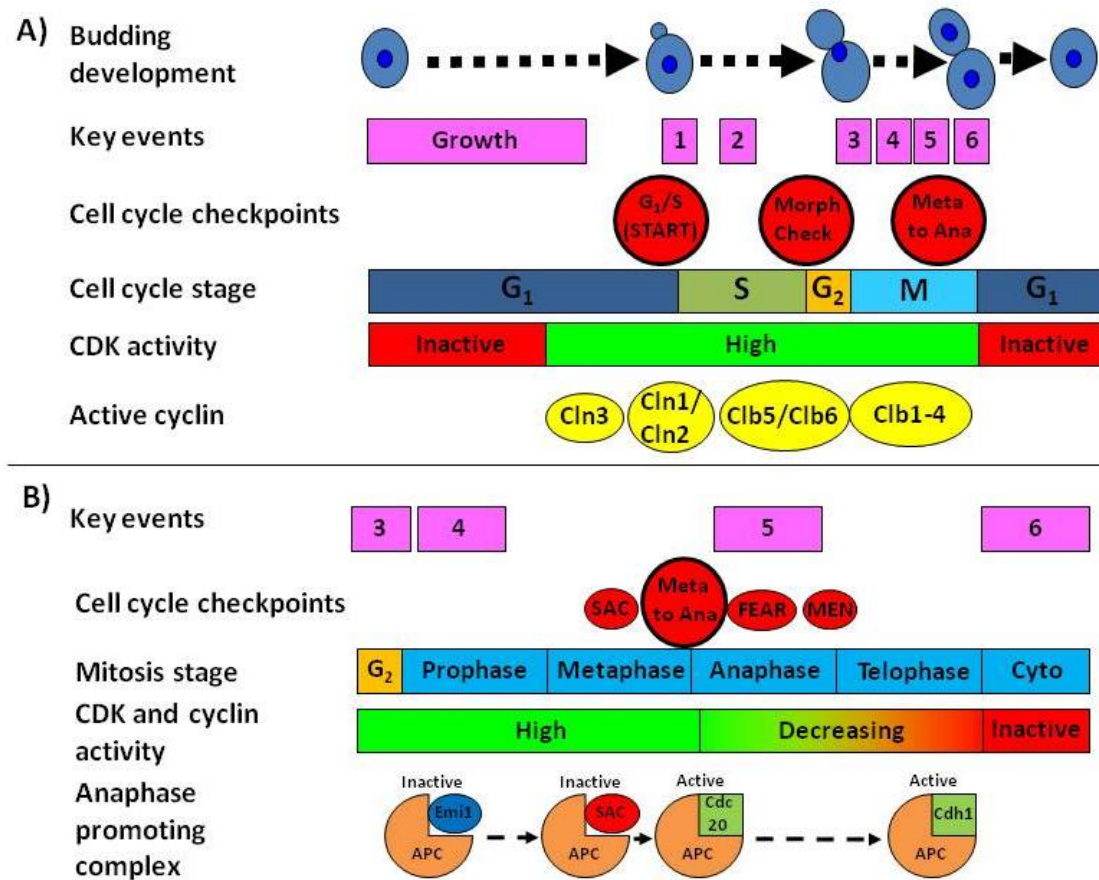
Figure 8. Key events and proteins involved in cell cycle progression in *S. cerevisiae*. (A) In all eukaryotic organisms progression through a cell cycle is driven by a set of kinases called cyclin dependant kinases (CDK) of which *S. cerevisiae* has one, Cdc28. Activity of the CDKs enzymes is controlled by the binding of various cyclin proteins. Each set of cyclin proteins is involved in initiating a series of different events and are each is active at a different stage of the cell cycle. The various CDK-cyclin complexes drive the cell through a number of events such as bud formation, SPB duplication and checkpoints in the G_1, S and G_2 cell cycle phases (A) as well as during mitosis (B). During the anaphase and telophase sections of mitosis the activity of CDK decrease as the last set of cyclins, Clb1-4 is targeted for degradation initially by the APC-Cdc20 complex following chromosome segregation. The final CDK-Clb1-4 complexes are destroyed through the actions of the mitotic exit network (MEN) activated APC-Cdh1 complexes and. CDK inhibitor protein Sic1. The complete destruction of the CDK-cyclin complexes at the end of telophase signals for cytokinesis and cell separation to occur. Key. 1 = spindle pole body duplication, 2 = DNA replication, 3 = nuclear migration, 4 = spindle formation, 5 = chromosome segregation, 6 and cyto = cytokinesis, APC = Anaphase promoting complex, SAC = spindle assembly checkpoint, FEAR = Cdc14 early anaphase release, MEN = mitotic exit network,

During mitosis, a number of processes occur which are required for mitotic progression including the attachment of the sister-chromatids to the spindle pole bodies, the destruction of the proteins that hold sister-chromatids together and the separation of these sister-chromatids into two daughter nuclei (Morgan, The Cell Cycle; Principles of Control, 2007). Cell division is the stage during which the cytoplasm of mother and daughter cells divide. This process is called cytokinesis and results in the deposition of new plasma membrane and cell wall material at the site of septation. The deposition of this new membrane is believed to be guided by the formation of a contractile actomyosin ring at the site of cytokinesis.

1.5.1. Cyclin-dependent kinase.

Progression through the various stages of the cell cycle is controlled by the cyclin-dependent kinase (CDK) enzyme. In *S. cerevisiae*, the CDK is encoded by the *CDC28* gene (also known as *CDK1*; Mendenhall and Hodge, 1998). CDK functions by phosphorylating substrates resulting in either changes in protein interactions or their enzyme activities. CDK substrates are known to include several key components of the cell cycle machinery, allowing CDK to control the initiation of various cell cycle events or to govern their progress towards conclusion.

The activity of CDK is controlled by two main factors. The first of these is the oscillation in cyclin expression, and the second is CDK inhibition by CDK inhibitor proteins e.g. Sic1 or ubiquitination by the anaphase promoting complex-Cdh1 protein complex (APC^{Cdh1}). Cyclins are a family of proteins which are involved in cell cycle progression by forming complexes with CDK and activating its kinase domain (Galderisi *et al.*, 2003). There are four distinct classes of cyclins and these are named after the phase of the cell cycle during which they are active, G_1, G_1/S, S and M. The G_1 cyclins, in both *S. cerevisiae* and *C. albicans* are encoded by the *CLN1-3* genes (Hadwiger *et al.*, 1989; Amon *et al.*, 1994; Dirick *et al.*, 1995; Futcher, 1996; Loeb *et al.*, 1999; Rupes, 2002; Wittenberg and La Velle, 2003; Chapa y Lazo *et al.*, 2005; Bachewich and Whiteway, 2005). The role of G_1 cyclins is to coordinate initiating cell division with environmental conditions by regulating entry into the cell cycle at the START checkpoint (Rupes, 2002; Wittenberg and La Velle, 2003). Cln3 is the first of these cyclins to play a part in cell cycle progression while the Cln1 and Cln2 proteins act at

the end of G_1 phase and fulfil the role of the G_1/S cyclins (Figure 8A). Deletion of the *cln3* gene in *S. cerevisiae* yields cells that exhibit a G_1 phase delay and are larger than wild type cells but nevertheless survive, while a triple mutant of all G_1 cyclins is non-viable; suggesting that there is functional redundancy with the Cln1 and Cln2 proteins. Late in G_1 phase, the activity of the Cln3-Cdc28 complex reaches a threshold activating the Swi4/6 cell cycle box binding factor (SBF) and *MluI* cell cycle box binding factor (MBF) transcription factors. The SBF and MBF transcription factors are then responsible for expression of the G_1/S and S cyclins (Futcher, 1996). Deletion of the *CLN1* and *CLN2* genes results in cells that also have a delay in the execution of START (Hadwiger *et al.*, 1989). Progress through the START checkpoint of cells lacking these two cyclins occurs at a greatly reduced rate when compared to wild type cells. In *S. cerevisiae* the Clb5 and Clb6 cyclins fulfil the role of the S cyclins (Nasmyth, 1993). The formation of the S cyclin-Cdc28 complex results in initiating a number of cell cycle events including DNA replication, spindle pole body (SPB) duplication, expression of the M cyclins (B type) and the inhibition of M cyclin proteolysis (Figure 8B; Dirick *et al.*, 1995).

The final class of cyclins are the M cyclins. There are two known B-type cyclins in *C. albicans*, Clb2 and Clb4, while *S. cerevisiae* possesses four, *CLB1-4* (Richardson *et al.*, 1992; Fitch *et al.*, 1992; Schwob and Nasmyth, 1993). The M cyclin-Cdc28 complexes are responsible for driving the cell cycle through mitosis. They carry out this function by initiating assembly of the mitotic spindle and alignment of sister-chromatid pairs on the mitotic spindle. These complexes also activate the Mcm1-Fkh transcription factor which stimulates the expression of the genes required for mitosis. During anaphase M cyclin-Cdc28 complexes are destroyed by the activated anaphase promoting complex coupled with Cdc20 (APC^{Cdc20}) and this destruction then signals for mitotic exit and the initiation of cytokinesis.

Functional complementation studies have shown *C. albicans* possesses homologs of *CLN1* (*CCN1*) and *CLN3* genes (Whiteway *et al.*, 1992; Sherlock *et al.*, 1994). Deleting these genes causes two different phenotypes. *C. albicans* cells lacking the *CCN1* gene progress through the cell cycle more slowly than wild type cells, with bud formation and nuclear division as the landmarks for cell cycle progression, while the cells lacking

Ccn1 were also defective in formation of hyphae on solid media (Loeb *et al.*, 1999). Cln3 was found to be essential and depletion of Cln3 caused the cells to arrest in G_1 phase whilst increasing in size before becoming filamentous; suggesting that Cln3 is also involved in the regulation of morphogenesis (Bachewich and Whiteway, 2005; Chapa y Lazo *et al.*, 2005). Furthermore, *C. albicans* possesses one more G_1 cyclin, Hgc1. Unlike Ccn1, Hgc1 expression is restricted to hyphal cells. *HGC1* expression is induced upon hypha formation early in G_1 phase before declining at the G_1/S transition. An additional spike in *HGC1* expression is seen during early M phase of subsequent cell cycles (Wang *et al.*, 2007). Deletion of *HGC1* results in cells that are defective in maintaining hyphal growth (Zheng *et al.*, 2004). These findings and subsequent characterisation of the Hgc1 protein in these studies indicate that Hgc1 is required for hyphal growth and that its expression is controlled by the cAMP morphogenesis pathway and is therefore not cell cycle regulated.

In addition to these G_1 cyclins *C. albicans* possesses two B-type cyclins, *CLB2* and *CLB4* (Bensen *et al.*, 2005). Depletion of the Clb2 cyclin results in the cells forming hyper-elongated pseudohyphae-like cells which have arrested in late mitosis with divided nuclei. It has been shown that Clb4 depleted cells also form hyper-elongated pseudohyphae-like cells. However the cells are able to remain viable and continue to grow and divide (Bensen *et al.*, 2005). No homologs of the *CLB5* and *CLB6* S cyclins have been isolated or characterised in *C .albicans*, though a recent study found that Clb4 has S phase activity and could complement *S. cerevisiae* cells lacking Clb5 cyclin (Ofir and Kornitzer, 2010). For *C. albicans* cell to exit from mitosis both Clb2 and Clb4 must be destroyed (Bensen *et al.*, 2005).

1.5.2. Cell cycle checkpoints.

The ability of cells to stop the cell cycle if they are not ready to proceed is very important. In *S. cerevisiae* this is controlled by three major checkpoints (Figure 8A).

The first of these is the G_1/S (START) checkpoint. The START checkpoint occurs during G_1 phase and is responsible for the commitment to undergo a new cell cycle. The checkpoint is activated in conditions where the cells are not the correct size for dividing or insufficient nutrients are available, resulting in decreased Cln3 levels

(Hartwell *et al*., 1974; Nurse and Bissett, 1981; Skotheim *et al*., 2008). For a cell to progress through this checkpoint the CDK, Cdc28, must be activated; first by the Cln3 cyclin and then by the Cln1 and Cln2 cyclins (Cross, 1995). The Cln3-Cdc28 complex activates transcription of over 200 genes and this is achieved by phosphorylation of the SBF and MBF transcription factors (G_1/S regulon; Figure 9; Spellman *et al*., 1998; Costanzo *et al*., 2004). In addition to phosphorylating the SBF and MBF transcription factors the Cln3-Cdc28 complexes also phosphorylates the SBF inhibitor protein Whi5, stimulating relocalisation of Whi5 from the nucleus to the cytoplasm (de Bruin *et al*., 2004; Costanzo *et al*., 2004). When Cln1 and Cln2 cyclins are expressed they activate Cdc28 and drive the cells through S phase and into M phase (Nasmyth, 1996). This is achieved by the phosphorylation and hence inactivation of Sic1. This in turn allows DNA replication, spindle maturation and chromosome segregation to occur (Figure 9; Schwob *et al*., 1994; Nasmyth, 1996).

The second cell cycle checkpoint is the morphogenesis checkpoint which occurs during the G_2 phase. The morphogenesis checkpoint monitors bud formation and septin and actin organisation. When triggered the checkpoint components stabilise the Swe1 kinase which in turn phosphorylates and inactivates Cdc28 (Booher *et al*., 1993; Lew, 2003). The Swe1 protein accumulates during late G_1 and S phases but by the start of mitosis all the Swe1 protein has been degraded (Sia *et al*., 1996; Sia *et al*., 1998). For Swe1 to be targeted for destruction the Hsl1 and Hsl7 proteins must bind to Swe1 (McMillan *et al*., 1999). The Hsl1 protein localises to septins prior to bud formation but is not in an active conformation. Following bud formation Hsl1 becomes activated and Hsl7 also localises to the bud neck (Theesfeld *et al*., 2003). This localisation of Hsl7 to the bud neck is closely followed by relocalisation of Swe1 to the bud neck from the nucleus, and is dependent on the presence of septin proteins at the bud neck (Shulewitz *et al*., 1999; Barral *et al*., 1999; Longtine *et al*., 2000). Disruption of bud neck localisation of the Hsl1 and Hsl7 protein or septin misorganisation results in a prolonged Swe1-dependent G_2 delay. This mechanism allows the cell cycle to become arrested in two circumstances; firstly if bud formation fails to occur or secondly if septin dynamics are impeded. Interestingly, following bud formation the morphogenesis checkpoint can be triggered by actin depolymerisation and osmotic stress. This is caused by Swe1 being displaced from the bud neck but the exact
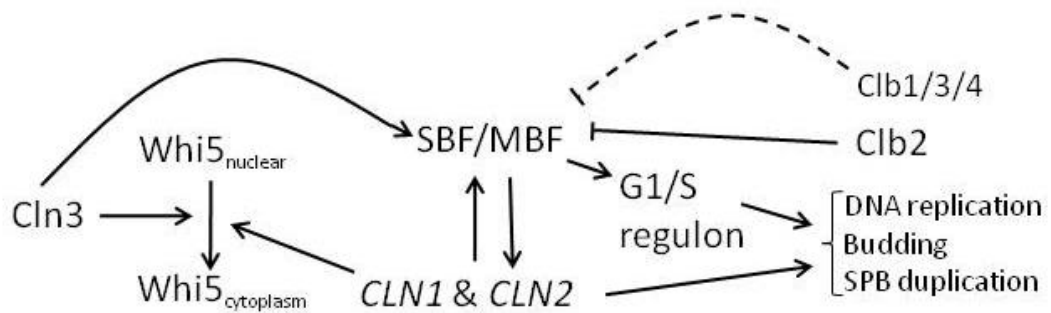
Figure 9. The START checkpoint in *S. cerevisiae*. The model for the START checkpoint. Activation of the Cdc28-Cln3 complexes leads to the activation of the SBF and MBF transcription factors and the relocation of the SBF inhibitor Whi5 from the nucleus to the cytoplasm. The SBF/MBF transcription factor activates the transcription of over 200 genes (G_1/S regulon) which results in the initiation of DNA replication, SPB duplication and budding. Dashed line shows suggested inhibition. Based on Figure 1 from Skotheim *et al*., 2008.

mechanism is currently unknown (Longtine *et al.*, 2000; Theesfeld *et al.*, 2003).

The final checkpoint is the metaphase to anaphase transition. This checkpoint occurs at the end of metaphase and if triggered leads to the inactivation of the APC-Cdc20 (APC^{Cdc20}) complex. This inactivation occurs through the spindle checkpoint. The APC and spindle checkpoint will be discussed in more depth in the next section.

1.5.3. Anaphase promoting complex.

The anaphase promoting complex (APC) is a large ubiquitin-protein ligase that consists of 13 subunits in *S. cerevisiae* and more in other eukaryotes (Lindon, 2008). The APC is an E3 ubiquitin ligase that targets substrates for degradation by the addition of an activated polyubiquitin chain. The APC acts as a platform allowing the E2 ubiquitin-conjugating enzyme to bind to the target substrate. This ubiquitination targets a protein for degradation by the 26S proteosome (Peters, 2006). The APC is active only when bound by one of its two co-factors, Cdc20 and Cdh1. Both *CDC20* and *CDH1* homologs are present in all eukaryotic genomes analysed to date (Peters, 2006). The APC catalyses ubiquitination of many different cell cycle proteins; the two major targets being the Pds1 protein and the S- and M-phase cyclins. Destruction of these cyclins results in the inactivation of the Cdk Cdc28 thereby allowing the cells to complete mitosis and undergo cytokinesis. The two co-factors Cdc20 and Cdh1, both contain a WD40 domain which is a 'propeller-shaped protein domain' that is made up of 40 amino acids sequence repeats (Peters, 2006). The WD40 domain of the co-factors is thought to target the activated APC complexes to their specific substrates by binding to one of two sequences motifs: the destruction box (RXXLXXXXN) or the KEN box (KENXXXN; Pfleger and Kirschner, 2000; Kraft *et al.*, 2005).

1.5.3.1. APC^{Cdc20}.

The APC^{Cdc20} complex becomes active during the metaphase to anaphase transition and is involved in the exit from mitosis. The Cdc20 protein accumulates during S phase and peaks at M phase before levels drop away in G$_1$ phase when it is degraded (Fang *et al.*, 1998; Prinz *et al.*, 1998; Pesin and Orr-Weaver, 2008). In addition, the APC^{Cdc20} is inhibited by the Emi1 protein of the END (Emi1/NuMA/dynein-dynactin) network binding to the APC complex or Cdc20 early in mitosis (Reimann *et al.*, 2001a; Reimann

et al., 2001b; Miller *et al.*, 2006; Ban *et al.*, 2007). Emi1 is expressed at the G_1/S transition and accumulates late in the S phase before being degraded in late prophase by the E3 ubiquitin ligase SCF$^{\beta\text{-}TrCP}$ complex following phosphorylation by the Polo-like kinase Cdc5 (Eldridge *et al.*, 2006). Following Emi1 degradation in late prophase the APC^{Cdc20} complex is maintained in its inactive state by the actions of the spindle assembly checkpoint (SAC) until the metaphase-anaphase transition. The SAC is a complex of proteins that can sense a single kinetochore that is not attached to the spindle microtubules. This results in blocking of mitotic progression until the kinetochore is reattached to the microtubules (Rieder *et al.*, 1994; Rieder *et al.*, 1995). During mitosis, the checkpoint proteins (Bub1, Bub1R, Bub3, Mad1-3 and Mps1) localise to any unattached kinetochores or a kinetochore that lacks tension (Jablonski *et al.*, 1998; Yu *et al.*, 1999; Chan *et al.*, 1999). In early anaphase, any unattached kinetochores trigger the SAC checkpoint by signalling for formation of the mitotic checkpoint complex (Lew and Burke, 2003). Two complexes have been identified in yeast; a Mad2-Mad3-Bub3-Cdc20 complex and a Mad1-Bub1-Bub3 complex (Brady and Hardwick, 2000; Fraschini *et al.*, 2001). These two complexes bind together and this results in the inhibition of the APC^{Cdc20} complex therefore inhibiting securin degradation and preventing cell cycle progression (Lew and Burke, 2003).

Following successful spindle assembly the APC^{Cdc20} signals for sister-chromatid separation by targeting the Pds1 (securin) protein for destruction. The Pds1 protein inhibits the protease, separase (Esp1) and in doing so maintains the proteins that are required to hold the sister-chromatids together. Upon destruction of Pds1, the activated Esp1 cleaves the Scc1 protein and this allows the sister-chromatids to separate. Activated Esp1 protein also initiates the release of a small quantity of the protein phosphatase Cdc14, by the FEAR pathway (Cdc14 early anaphase release) from the nucleolus into the nucleus (Harper *et al.*, 2002). The APC^{Cdc20} also destroys the bulk of the mitotic cyclins by targeting them for degradation (Shirayama *et al.*, 1999; Yeong *et al.*, 2000). Complete destruction of the M cyclins however requires activation of the APC^{Cdh1} complex. This activation only occurs when the Cdc14 protein is fully released into the cytoplasm and it activates the Cdk inhibitor Sic1 which results in a decrease in Cdh1 phosphorylation and formation of the APC^{Cdh1} complex (Visintin *et al.*, 1998; Jaspersen *et al.*, 1999).

1.5.3.2. APC^{Cdh1}.

The Cdh1 protein is the second of the APC co-factors. The Cdh1 protein is expressed throughout the cell cycle. However, its levels are highest during mitosis but subsequently fall during G$_1$ and S phases (Prinz *et al*., 1998; Kramer *et al*., 2000; Hsu *et al*., 2002). Throughout the majority of the cell cycle Cdh1, is maintained in a phosphorylated state by the cyclin-dependant kinases. Upon mitotic exit Cdh1 is dephosporylated by Cdc14 (Zachariae, 1998; Jaspersen *et al*., 1999; Lukas *et al*., 1999; Kramer *et al*., 2000; Huang *et al*., 2001; Sorensen *et al*., 2001; Keck *et al*., 2007). This dephosphorylation allows Cdh1 to bind to the APC and therefore activate the APC^{Cdh1} complex. The role of this complex is to destroy the remaining M cyclins (Lim *et al*., 1998; Baumer *et al*., 2000). As Cdh1 is expressed through the cell cycle its regulation is critical. This is carried out by maintaining Cdh1 in its phosphorylated state and by direct inhibition. For example, the Emi1 protein which is expressed in G$_1$ phase has been shown to inhibit Cdh1 as well as Cdc20 (Reimann *et al*., 2001b; Miller *et al*., 2006). The Acm1 protein also inhibits Cdh1 activity by acting as a pseudosubstrate and this inhibition is only relieved upon Acm1 ubiquitination by APC^{Cdc20} at the metaphase to anaphase transition (Martinez *et al*., 2006; Dial *et al*., 2007; Enquist-Newman *et al*., 2008). In mammalian cells, Cdh1 has been shown to bind to the Rae1-Nup98 complex and thus inhibit the ubiquitination of Pds1 (Jeganathan *et al*., 2005). This suggests that in mammalian cells APC^{Cdh1} and not APC^{Cdc20} is required for Pds1 destruction and sister-chromatid separation.

Overall both APC complexes are required for the completion of the cell cycle and the phosphatase Cdc14 plays an important role in regulating APC activity. In yeast Cdc14 is regulated by two pathways, the FEAR and MEN pathway which are discussed in more detail in the next two sections.

1.5.4. FEAR network.

Activation of the protein phosphatase Cdc14 is an integral part of anaphase progression and mitotic exit. Protein localisation studies, utilising GFP protein fusions, have shown that the Cdc14 protein in *S. cerevisiae* is released from the nucleolus in two distinct pulses during anaphase. These Cdc14 pulses are caused by the FEAR (Cdc **F**ourteen **E**arly **A**naphase **R**elease network) and the MEN (**M**itotic **E**xit **N**etwork). As a

result of the FEAR network the Cdc14 protein is released from the nucleolus into the nucleus while the MEN network releases Cdc14 from the nucleolus into the cell cytoplasm.

The FEAR network is responsible for the transient release of Cdc14 during the early part of anaphase. During the rest of the cell cycle, Cdc14 is sequestered in the nucleolus by the nucleolar located inhibitor Net1 (Shou *et al.*, 1999; Visintin *et al.*, 1999). The Net1 protein is maintained in its hypophosphorylated form by the protein phosphatase 2A bound to regulatory subunit Cdc55 (PP2A^{Cdc55}) and this prevents Cdc14 dissociation (Queralt *et al.*, 2006; Yellman and Burke, 2006). A number of FEAR network proteins have been identified in recent years (Figure 10, Cohen-Fix *et al.*, 1996; Ciosk *et* al., 1998; Alexandru *et al.*, 2001; Stegmeier *et al.*, 2002; Queralt and Uhlmann, 2008). Following the entry into anaphase the Pds1 protein is targeted for degradation by APC^{Cdc20} which activates the separase protein, Esp1. Following Esp1 activation, the separase binding protein Slk19 binds to Esp1 to form the Esp1-Slk19 complex which in turn activates the Zds1 and Zds2 proteins (Stegmeier *et al.*, 2002; Sullivan and Uhlmann, 2003; Queralt *et al.*, 2008). The activity of the Zds1 and Zds2 proteins inhibits PP2A^{Cdc55} by binding to Cdc55, resulting in Net1 becoming phosphorylated by Clb2-Cdc28, leading to Cdc14 release (Queralt *et al.*, 2006; Yasutis *et al.*, 2010). The second part of the FEAR network involves the Spo12 and Fob1 proteins. The exact mechanism of this phase is unknown but it is suspected that upon activation of the FEAR pathway, the Spo12 protein is dephosphorylated by the Cdc14 and sequestered in the nucleolus and then in turn it binds to the FEAR inhibitor protein Fob1 releasing Fob1 from binding to Net1; therefore acting as a positive feedback mechanism (Shah *et al*, 2001; Stegmeier *et al.*, 2002; Visintin *et al*, 2003; Stegmeier *et al.*, 2004; Tomson *et al.*, 2009). The Polo-like kinase Cdc5 has long been thought to be a member of the FEAR pathway but until recently its specific function in the pathway was not known. In 2009, Cdc5 was implicated in promoting the release of Cdc14 by stimulating the degradation of the Swe1 protein (Liang *et al.*, 2009). It was also shown that Swe1 is responsible for the decrease in phosphorylation of Net1 protein in a strain lacking *cdc5* (Liang *et al.*, 2009). These findings suggest that Swe1 as well as PP2A^{Cdc55} and Fob1 are all inhibitors of Cdc14 release during the cell cycle. The concentration of Cdc14 protein released by the FEAR pathway is not at a level high enough to initiate
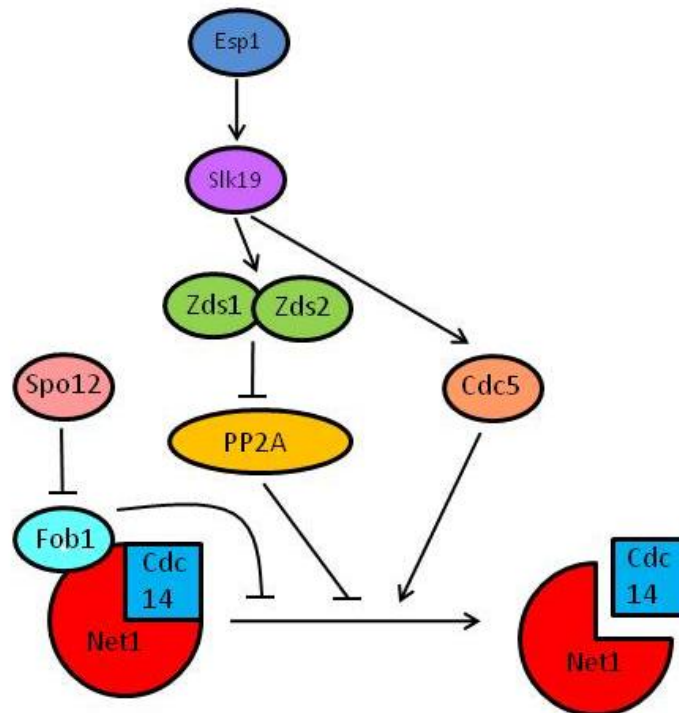
Figure 10. The FEAR network in *S. cerevisiae*. Upon Pds1 destruction at the metaphase-anaphase transition Esp1 initiates the activation of the FEAR network. Throughout the majority of the cell cycle Cdc14 is sequestered in the nucleolus by the Net1 protein (shown as the Net1-Cdc14 interlocked shape) and this is maintained by the Fob1 protein. Upon activation of the FEAR network Cdc14 is transiently released from Net1 and moves into the nucleus where it is involved in rRNA segregation and microtubule maintenance. The FEAR network consists of the Slk19, Spo12, Cdc5 proteins and the Zds1-Zds2 complex. The exact order of function is currently unclear. The role in the FEAR network played by the Cdc5 kinase is also currently unclear. Figure adapted from D'Amours and Amon, 2004.

the full mitotic exit machinery but it does play a role in parts of the chromosome segregation process. The first role Cdc14 plays is in the degradation of Pds1 (securin). At the metaphase to anaphase transition the Pds1 protein is phosphorylated by M cyclin-Cdc28 complex and this decreases the ability of APC^{Cdc20} to target Pds1 for ubiquitination. Some Pds1 is degraded and this allows the activation of the Esp1-Slk19 complex which leads to Cdc14 being released from the nucleolus. Cdc14 then phosphorylates Pds1 resulting in complete APC^{Cdc20}-dependant Pds1 degradation (Holt *et al*., 2008). This mechanism forms a feedback loop which hones the metaphase to anaphase transition. Studies have shown that some FEAR network proteins are involved in the generation and maintenance of the microtubules that pull one of the divided nuclei back into the mother cells by stabilising the microtubules which suggests the FEAR network is essential for nuclear segregation (Ross and Cohen-Fix, 2004).

1.5.5. Mitotic exit network.

The mitotic exit network (MEN) is a signal transduction cascade that signals for the cells to exit mitosis and initiates the activation of the cytokinesis machinery (McCollum and Gould, 2001). The MEN carries out this function by responding to the position of the anaphase spindle in relation to the bud neck, and when active it initiates the destruction of mitotic cyclins (Bosi and Li, 2005). The MEN consists of a GTPase protein Tem1 whose activity is regulated by the guanosine exchange factor (GEF) Lte1, and two GTPase activating proteins (GAPs) Bfa1 and Bub2. Tem1 then acts through two downstream kinases Cdc15 and Dbf2 (in association with Mob1) and the end point for the pathway is release of the protein phosphatase Cdc14 from the nucleolus into the cytoplasm (Figure 11; Hoyt *et al*., 1991; Schweitzer and Phillippsen, 1991; Toyn *et al*., 1991; Wan *et al*., 1992; Shirayama *et al*., 1994a; Shirayama *et al*., 1994b). Following its release into the cytoplasm, Cdc14 dephosphorylates Cdh1 and Sic1 which results in the degradation of the M cyclins as discussed previously (Moll *et al*., 1991; Knapp *et al*., 1996; Feldman *et al*., 1997; Skowyra *et al*., 1997; Toyn *et al*., 1997; Verma *et al*., 1997; Visintin *et al*., 1998; Zachariae *et al*., 1998; Jaspersen *et al*., 1999). The activation of the MEN pathway is thought to be controlled by spatial segregation of the component proteins. During the majority of the cell cycle the Bfa1-Bub2 complex and Tem1 are found on both spindle pole bodies (SPB) but upon entry of the daughter cell by the daughter bound SPB, the Lte1 protein is released from the daughter cell cortex
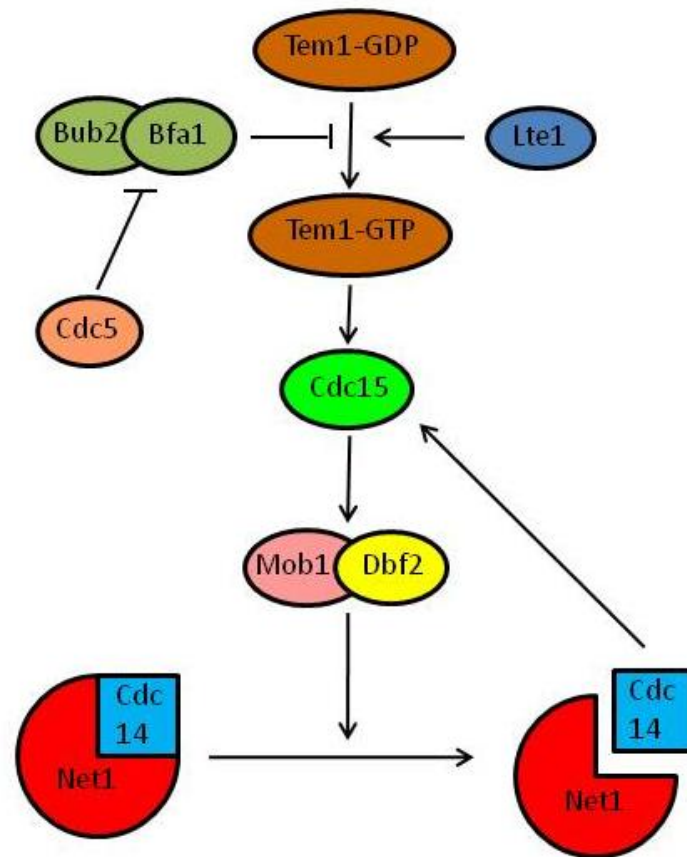
Figure 11. The Mitotic Exit Network in *S. cerevisiae*. In late anaphase the Mitotic Exit Network promotes the release of Cdc14 from the nucleolus (Net1 and Cdc14 interlocking shape) into the cytoplasm (Net1 and Cdc14 released shapes). This release of Cdc14 into the cytoplasm activate the Sic1 and Cdh1 proteins which in turn leads to the inactivation of the Cdc28-M cyclin complexes and signal the exit from mitosis. The MEN is a kinase cascade pathway which is initiated by the dissociation of the Bfa1-Bub2 GAP complex from the GTPase Tem1 which allows Tem1 to switch from it inactive GDP-bound form to the active GTP-bound form. The activated Tem1 in turn recruits the Cdc15 kinase, to the daughter-bound spindle pole body, and activate it which leads to the activation of the Dbf2-Mob1 complex. The final step in the MEN is the relocation of the Dbf2-Mob1 complex to the nucleolus where Dbf2 phosphorylates Cdc14 resulting in the release of Cdc14 into the cytoplasm. Based on Figure 1 from Piatti *et al*., 2006.

into the daughter cell cytoplasm. This release results in recruitment of the Bfa1-Bub2 complex and Tem1 to the daughter SPB (dSPB) which leads to activation of Tem1 and the other MEN components (Bardin *et al.*, 2000; Pereira *et al.*, 2000; Lee *et al.*, 2001; Jensen *et al.*, 2002; Molk *et al.*, 2004).

1.5.5.1. GTPase module.

Activation of the MEN pathway is controlled by the GTPase protein Tem1. Tem1 is a member of the Ras family of GTPases and was first isolated in *S. cerevisiae* as a suppressor of the cold-sensitivity phenotype of an *lte1* mutant (Shirayama *et al.*, 1994b). This study also found that the *TEM1* gene was essential and that cells that lack the Tem1 protein arrested as enlarged dumbbell-like cells with divided nuclei and high CDK activity which suggest that cell division is arrested during telophase. This study showed that the phenotype could be recovered by overexpression of the *CDC15* gene (Shirayama *et al.*, 1994b).

Regulation of Tem1 activity has been an area of extensive research. The majority of this research has focussed on determining the roles played by the putative GEF, Lte1, and GAPs Bfa1-Bub2. The Bfa1 and Bub2 proteins are both members of the spindle assembly checkpoint which has been shown not to inhibit the metaphase to anaphase transition but to inhibit mitotic exit (Alexandru *et al.*, 1999; Franchini *et al.*, 1999; Ro *et al.*, 2002; Kim *et al.*, 2008). During the cell cycle the Bfa1-Bub2 complex localises to both SPBs but during late anaphase they are recruited to the dSPB (Pereira *et al.*, 2000; Lee *et al.*, 2001; Fraschini *et al.*, 2006; Caydasi and Pereira, 2009; Geymonat *et al.*, 2009; Monje-Casas and Amon, 2009). This localisation is similar to that seen for Tem1 and is dependent on the actions of Kin4 and Lte1 in the mother and daughter cells respectively (Bardin *et al.*, 2000; Molk *et al.*, 2004, Caydasi and Pereira, 2009; Geymonat *et al.*, 2009). Bfa1 and Bub2 are homologs of Byr4 and Cdc16 GAP proteins in *S. pombe* and physically associate with each other during the cell cycle which suggests Bfa1 and Bub2 are components of a two protein GAP (Furge *et al.*, 1998; Lee *et al.*, 2001). This phenotype was later confirmed by Geymonat *et al.*, (2002) and it was also shown by co-immunoprecipitation that both proteins bind to Tem1p (Pereira *et al.*, 2000; Lee *et al.*, 2001). Additional studies have shown that of the two proteins Bfa1 is the stronger regulator of Tem1 and therefore of mitotic exit. Even in the

absence of Bub2 overexpression of Bfa1 results in cells arresting with a large budded morphology with elongated spindles (Ro *et al.*, 2002). It was also shown that only a partial inhibition of cell growth occurs when Bub2 is overexpressed, but only in the presence of Bfa1 (Ro *et al.*, 2002). Another study has found that a Bub2 protein containing the R85A mutation could not recruit Bfa1 to the SPB and had no GAP activity which confirms that Bub2 is required in the presence of Bfa1 for it to become a GAP for Tem1 (Fraschini *et al.*, 2006). These findings suggest that Bfa1 is responsible for the inhibition of Tem1 while Bub2 despite having GAP domains participates in this process by recruiting Bfa1 to the SPB. During the majority of mitosis the Bfa1/Bub2 complex is bound to Tem1 and this binding is thought to be assisted by the phosphorylation of Bfa1 by the spindle checkpoint protein Kin4 (D'Aquino *et al.*, 2005; Maekawa *et al.*, 2007). However, as Kin4 is maintained in the mother cell upon the relocalisation of the Bfa1-Bub2 complex and Tem1 to the dSPB this inhibition is lost. As a result, Bfa1 becomes dephosphorylated and the Polo-like kinase Cdc5 then phosphorylates Bfa1. This in turn impedes the interaction between Bfa1 and Tem1 and leads to the dissociation of the Bfa1-Bub2 complex from Tem1 (Hu *et al.*, 2001).

The ability of GTPase proteins to switch from being in the GDP-bound form to the GTP-bound form and *vice versa* is critical for the function of the GTPase protein. This switch-like on/off mechanism is considered the reason why GTPases are commonly located at the top of biochemical pathways. Interestingly, the Tem1 protein has been shown to exhibit a higher intrinsic GDP release rate than other GTPases, with the exception of the Sec4 protein (Kabcenell *et al.*, 1990; Geymonat *et al.*, 2002). Normally, locking a GTPase protein in its active GTP-bound state results in various growth defects but when Tem1 is locked in a GTP-bound state, by point mutations in the GTP binding domains, little effect on growth is seen (Shirayama *et al.*, 1994b). This finding suggests that the binding of Bfa1-Bub2 to Tem1 is the important step in controlling activity of Tem1.

The Lte1 protein has long been presumed to be the GEF for Tem1. Lte1 is a member of the Cdc25 family of guanosine nucleotide exchange factor proteins and has been shown to be required for growth at low temperatures (Keng *et al.*, 1994; Shirayama *et al.*, 1994b). During mitosis, Lte1 localises to the cortex of the daughter bud and this

localisation led researchers to predict that Lte1 is the GEF of Tem1, as Tem1 only becomes active when the daughter-bound SPB enters the daughter bud and hence comes into contact with Lte1. The Lte1 protein does possess a GEF domain but deletion of this domain has little or no effect on mitotic exit. Furthermore a recent *in vitro* study has shown Lte1 has no GEF activity (Bardin *et al.*, 2000; Pereira *et al.*, 2000; Jensen *et al.*, 2002; Yoshida *et al.*, 2003; Geymonat *et al.*, 2009). In addition to the Lte1 protein being essential at low temperatures, the lack of GEF activity of Lte1 also casts further doubt upon the hypothesis that GDP/GTP cycling controls Tem1 activity or that Lte1 is the GEF of Tem1.

A recently published study, suggests the relocalisation of the Bfa1/Bub2 complex from mSPB to dSPB is as a result of the actions of Lte1, and that this is Lte1's role in the MEN (Geymonat *et al.*, 2009). The authors found that mislocalisation of Lte1 in the mother cell caused Bfa1 to localise to the SPB closest to the daughter cell. This same mislocalisation of Lte1 has previously been shown to cause mitotic exit even if the nuclei are not segregated into the mother and daughter cells (Bardin *et al.*, 2000; Castillon *et al.*, 2003). These results lead to the following hypothesis; that upon entry of the dSPB into the daughter bud Lte1 recruits the Bfa1/Bub2 complex to the dSPB while Kin4 localises to the mSPB thereby preventing Kin4 from phosphorylating Bfa1. This results in dephosphorylation of Bfa1 which is then phosphorylated by Cdc5 resulting in the dissociation of the Bfa1/Bub2 complex from Tem1. The Tem1 then initiates mitotic exit by binding and activation of the kinase Cdc15. In addition, Lte1 is also known to interact with many proteins of the polarisome complex and the proteins kinase Cla4 which could link the MEN to cell polarity determinants (Höfken and Schiebel, 2002; Jensen *et al.*, 2002; Seshan *et al.*, 2002; Yoshida *et al.*, 2003; Seshan and Amon, 2005).

1.5.5.2. Kinase Cascade.

1.5.5.2.1. Cdc15.

The next protein in the MEN pathway is the kinase Cdc15 (Schweitzer and Philippsen, 1991; Shirayama *et al.*, 1994b; Shirayama *et al.*, 1996; Jaspersen *et al.*, 1998; Bardin *et al.*, 2000 Gruneberg *et al.*, 2000). Cdc15 has a high level of sequence similarity to the MAP kinase family of proteins (Hunter and Plowman, 1997). Throughout the cell cycle

the protein level and kinase activity of Cdc15 are consistent, but Cdc15 localisation to SPBs is regulated in a cell cycle dependent manner (Jaspersen *et al*, 1998; Cenamor *et al*., 1999). This localisation is controlled by active Tem1 recruiting the Cdc15 protein, and is impaired in strains lacking either Tem1 or the scaffold protein Nud1 or in which the outer plaque of the SPB is disrupted (Cenamor *et al*., 1999; Menssen *et al*., 2001; Visintin and Amon, 2001). Cells that lack the Cdc15 protein in *S. cerevisiae* arrest as dumbbell-like cells with divided nuclei similar to Tem1-depleted cells (Schweitzer and Philippsen, 1991). Later studies have shown that Tem1 and Cdc15 proteins physically interact, and that this is dependent on a specific 82 amino acid Tem1-binding domain of Cdc15 (Asakawa *et al*., 2001). It has been found that this domain is essential for the functionality of the Cdc15 protein (Bardin *et al*., 2000; Asakawa *et al*., 2001). The Cdc15 protein also contains a kinase domain and a kinase activity inhibitory domain (Bardin *et al*., 2003). The kinase domain is required for phosphorylating the next protein in the MEN pathway, Dbf2, and disruption of the kinase activity inhibitory domain results in the hyperactivation of the MEN pathway (Mah *et al*., 2001; Bardin *et al*., 2003).

1.5.5.2.2. Dbf2/Dbf20-Mob1.

As mentioned previously the next protein in the MEN pathway is the kinase Dbf2. The Dbf2 kinase was first identified by Johnston *et al*., (1990) and was found at constant level throughout the cell cycle even though its kinase activity fluctuates (Johnston *et al*., 1990; Toyn and Johnston, 1994; Luca *et al*., 2001). The Dbf20 kinase associates with Dbf2 throughout the cell cycle but has little kinase activity but deletion of this gene showed it had little effect on the activity of Dbf2 therefore either its function is as a binding protein or it has become functionally redundant (Toyn and Johnston, 1994). Inactivation of Dbf2 in a *dbf2* J114 temperature sensitive strain causes the cells to arrest in telophase suggesting that Dbf2 is involved in the events at the end of mitosis (Johnston *et al*., 1990; Toyn and Johnston, 1994). Like Cdc15, Dbf2 localises to SPBs during the late phases of mitosis and this action is dependent on the Nud1, Tem1 and Cdc15 proteins (Frenz *et al*., 2000; Visintin and Amon, 2001; Luca *et al*., 2001).

The kinase activity of Dbf2 is controlled by its binding protein Mob1, and activation of Dbf2 correlates with its localisation at the SPB. Mob1 and Dbf2 physically interact

together and phosphorylation of either protein by Cdc15 results in Dbf2 kinase activity being switched on (Luca and Winey, 1998; Komarnitsky *et al.*, 1998; Mah *et al.*, 2001). Research has determined that Mob1 is present throughout the cell cycle and preferentially localises to the SPB while a small quantity of protein is released from the SPB during anaphase (Luca *et al.*, 2001; Stoepel *et al.*, 2005). Studies have shown that truncating the N' terminal end of Mob1 causes the protein to relocate to the nucleus (Stoepel *et al.*, 2005). The truncated Mob1 protein, seen in the nucleolus was shown to co-localise with Cdc14 and this has led to the theory that the Mob1-Dbf2 complex relocates from the SPB to the nucleus upon Dbf2 activation and Mob1 phosphorylation by Cdc15 (Stoepel *et al.*, 2005). The Mob1 protein has also been shown to interact with the spindle assembly and spindle tension checkpoint protein Mps1 which suggests that Mob1 could also be the site of further regulation of the MEN pathway by these two checkpoints (Luca and Winey, 1998; Biggins and Murray, 2001; Yoshida and Toh-e, 2001).

1.5.5.3. Cdc14 protein phosphatase.

The final protein in the mitotic exit network is the protein phosphatase Cdc14. As mentioned in the previous sections on the Anaphase Promoting Complex and FEAR network, Cdc14 is required for cells to exit mitosis and for the initiation of cytokinesis. Overexpression of *CDC14* has been shown to suppress the cell cycle arrest phenotype of *dbf2-2* cells upon Dbf2 depletion, while cells lacking Cdc14 arrest in telophase with high mitotic CDK levels (Wan *et al.*, 1992; Visintin *et al.*, 1998; Grandin *et al.*, 1998).

During the majority of the cell cycle the Cdc14 protein is sequestered in the nucleolus by the Net1 protein but is released in two bursts during anaphase. The first burst is as a result of actions of the FEAR network and, as discussed previously, the released Cdc14 protein is involved in rDNA segregation and other anaphase specific processes. This Cdc14 is quickly re-sequestered as a result of the degradation of B cyclins, with the exception of Clb2, and it is thought that Cdc14 may play a role in its own return to the nucleolus (Manzoni *et al.*, 2010). During this release, Cdc14 has also been shown to dephosphorylate Cdc15 allowing it to become a better activator of mitotic exit through the MEN pathway (Jaspersen and Morgan; 2000). The second burst of Cdc14 release is much stronger and is caused by activation of the MEN pathway. This release

has been shown to be directly triggered by the actions of the Dbf2-Mob1 complex, which has been shown to co-localise with Cdc14 during telophase (Stoepel *et al.*, 2005; Mohl *et al.*, 2009). Analysis of the Cdc14 protein sequence has led to the discovery that it contains at least one nuclear localisation sequence (NLS) at its C' terminus and phosphorylation by Dbf2 in this region leads to Cdc14 release from the nucleus into the cytoplasm where it comes into contact with its target substrates which include CDK which it inactivates by dephosphorylation (Mohl *et al.*, 2009). Release of Cdc14 results in the accumulation of the Cdk inhibitor protein Sic1, through dephosphorylation of Sic1 and Swi5. This Sic1 accumulation also results in an increase in *SIC1* expression and a dephosphorylation of the Cdh1 protein which allows the APC^{Cdh1} complex to form (Moll *et al.*, 1991; Knapp *et al.*, 1996; Feldman *et al.*, 1997; Skowyra *et al.*, 1997; Toyn *et al.*, 1997; Verma *et al.*, 1997; Visintin *et al.*, 1998; Zachariae, 1998; Jaspersen *et al.*, 1999). Through the majority of the cell cycle Sic1 is undetectable as the Sic1 protein is destroyed following phosphorylation by the Cln1-Cdc28, Cln2-Cdc28 and Cln3-Cdc28 complexes. However, late in anaphase and in early G$_1$ phase *SIC1* is expressed and the Sic1 protein is active (Donovan *et al.*, 1994; Schwob *et al.*, 1994; Verma *et al*, 1997). The activation of Sic1 and the APC^{Cdh1} complex results in the destruction of the M cyclins, therefore inactivating the CDK-cyclin complexes (Amon *et al.*, 1994; Lim *et al.*, 1998; Baumer *et al.*, 2000; Yeong *et al.*, 2000). The APC^{Cdh1} complex has been shown to target the Spo12 and Cdc5 proteins for degradation but this is only one of a number of events that Cdc14 triggers to inhibit any further signalling by the MEN pathway (Charles *et al.*, 1998; Cheng *et al.*, 1998; Shirayama *et al.*, 1998; Shah *et al.*, 2001). Following Cdc14 release the Bfa1 and Lte1 proteins are dephosphorylated and this is thought to restore the GAP activity of the Bfa1/Bub2 complex towards Tem1 while Lte1 dephosphorylation causes it to disassociate from the bud cortex, thereby preventing it from activating Tem1 (Bardin *et al.*, 2000; Hu *et al.*, 2001; Jensen *et al.*, 2002; Pereira *et al.*, 2002; Seshan *et al.*, 2002; Geymonat *et al.*, 2003). The final control mechanism that Cdc14 is involved in is with signalling the initiation of expression of the Amn1 protein in the daughter cell. Amn1 binds to the Tem1 protein and this prevents Cdc15 binding Tem1 which in turn prevents addition Cdc14 being released from the nucleolus and allows the cells to exit from mitosis and reset ready for the next cell cycle (Wang *et al.*, 2003).

Following CDK inactivation and the completion of cytokinesis the APC^{Cdh1} complex, Amn1 and Bub2 proteins are involved in an unknown mechanism that results in Cdc14 return to the nucleolus (Visintin *et al*., 2008). This is probably caused by the activation of the NLS sequence of Cdc14 by dephosphorylation.

1.5.6. MEN proteins in cytokinesis.

In addition to their roles in signalling mitotic exit the Tem1, Cdc5, Cdc14, Cdc15, Dbf2 and Mob1 proteins have been implicated in the process of cytokinesis (Frenz *et al*., 2000; Song *et al*., 2000; Lippincott *et al*., 2001; Luca *et al*., 2001; Bembenek *et al*., 2005). Mutant strains of the various MEN components, that bypass the mitotic exit defect, have been shown to exhibit defects in actomyosin ring contraction, actin re-polarisation and cell separation (Lippincott and Li, 1998; Jimenez *et al*., 1998; Frenz *et al*., 2000; Lippincott *et al*., 2001; Yeong *et al*., 2002; Hwa Lim *et al*., 2003; Yeong 2005; Corbett *et al*., 2006). The *cdc15-2* mutant cells for example are unable to maintain a stable CAR and also exhibit abnormal deposits of chitin which suggests Cdc15 plays a role in regulating the activities of various cytokinesis proteins. The Tem1 protein has also been shown to regulate both CAR contraction and the splitting of the septin ring (Lippincott and Li, 1998; Lippincott *et al*., 2001; Yeong *et al*., 2002; Hwa Lim *et al*., 2003). As well as these defects the mentioned above, the MEN proteins have been shown to re-localise to the bud neck and occurs following the conclusion of anaphase in a MEN dependant fashion (Frenz *et al*., 2000; McCollum and Gould, 2001; Yeong *et al*., 2002; Balasubramanian *et al*., 2004).

The MEN components have also been implicated in regulating actomyosin ring contraction (Corbett *et al*., 2006). The Iqg1 protein in *S. cerevisiae* plays an important role in actomyosin ring contraction and cells that lack this protein have been found todie (Cheney and Mooseker, 1992). A study has shown that combining the *tem1-3*, *cdc15-1* or *dbf2-2 dbf20Δ* mutations with the *iqg1-1* mutation results in synthetic lethality of the strains at 32 ˚C. All the single mutation strains grow at 32 ˚C (Corbett *et al*., 2006). This finding suggests that the Tem1, Cdc15 and Dbf2 proteins are actively involved in actomyosin ring contraction.

In *S. cerevisiae,* the Hof1 protein negatively regulates cytokinesis and is involved in

septum formation (Vallen *et al.*, 2000; Blondel *et al.*, 2005; Ren *et al.*, 2005). Following mitotic exit the Hof1 protein becomes phosphorylated and this results in the protein becoming a target for degradation by the SCF^{Grr1} complex (Blondel *et al.*, 2005). The kinase that carries out this phosphorylation has yet to be identified. Studies have found that the MEN network is required for localising the Grr1 protein to the bud neck (Blondel *et al.*, 2005). Overall these studies have implicated the MEN network not only in signalling for Hof1 phosphorylation, but also for recruitment of the Grr1 protein which is partly involved in the degradation of Hof1 and thus the execution of cytokinesis.

The MEN pathway also plays a role in cell separation as Cdc14 signals for Ace2 to localise to the daughter nucleus and this leads to the expression of the cell separation genes *DSE1* and *SCW11* (Colman-Lerner *et al.*, 2001; Doolin *et al.*, 2001). The above evidence suggests that the MEN proteins play a number of roles in the process of cytokinesis but many of these are still to be elucidated.

1.6. Septation initiation network in *Schizosaccharomyces pombe.*

In the fission yeast *Schizosaccharomyces pombe* the septation initiation network (SIN) pathway operates at the end of mitosis (Figure 12). Orthologs of all the MEN proteins are involved in the SIN pathway with the exception of *LTE1* (Table 2). However the SIN pathway is involved in signalling septum formation and not mitotic exit. The SIN pathway also contains an additional kinase, Sid1, and its regulatory subunit Cdc14 (Fankhauser and Simanis, 1993; Guertin *et al.*, 2000). Mutant strains lacking the GAP proteins, Cdc16 and Byr4, undergo multiple rounds of septation without cell separation while the inhibition of the GTPase Spg1, or the downstream kinases or scaffold protein causes the cells to become multinucleated and elongated (Nurse *et al.*, 1976). All the proteins of the SIN network localise to the SPB at some point during mitosis (Sohrmann *et al.*, 1998; Cerutti and Simanis, 1999; Chang and Gould, 2000; Hou *et al.*, 2000; Li *et al.*, 2000; Salimova *et al.*, 2000; Krapp *et al.*, 2001). During interphase Spg1 is in the GDP-bound form and following entry into mitosis Cdc16 disassociates from the SPB and Spg1 converting it to its active GTP-bound form and interacts with Cdc7 (Sohrmann *et al.*, 1998; Cerutti and Simanis, 1999). During anaphase, the Cdc16-Byr4 complex reforms but only on the mother SPB while at the daughter SPB the Sid1-
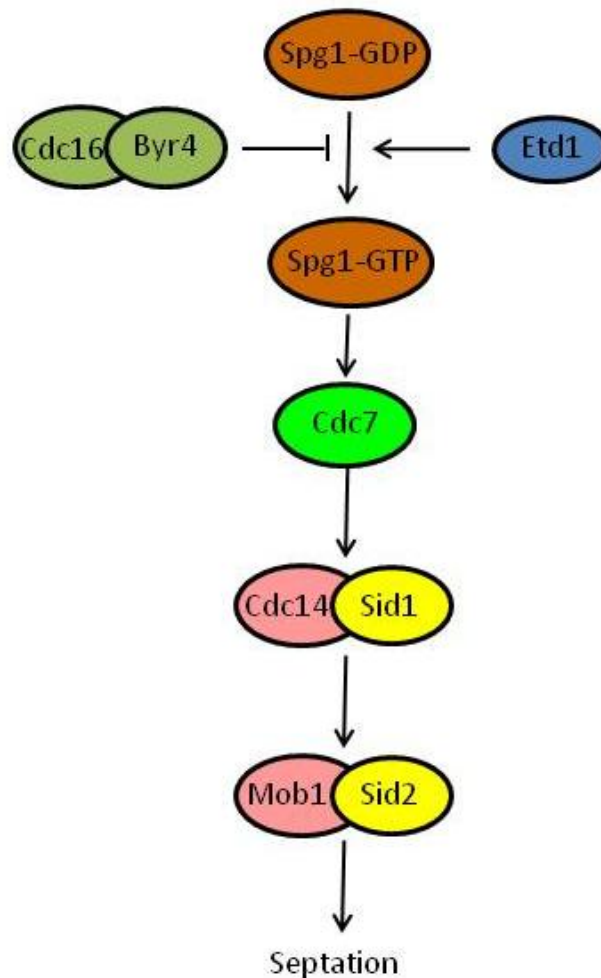
Figure 12. The Septation Initiation Network in *S. pombe.* The SIN is activated at the end of anaphase and is involved in signalling for septum formation and septation to occur. The SIN consists of the Spg1, Etd1, Cdc7 proteins along with the Cdc14-Byr4 GEF complex and the Sid1-Cdc14 and Sid2-Mob1 kinase complexes. All the proteins localise to the spindle pole body during mitosis. During early mitosis Spg1 is activated and interacts with the Cdc7 kinase however the remainder of the SIN becomes activated at the end of anaphase upon the inactivation of the CDK$^{M\ cyclin}$ complexes. At this time the Sid1-Cdc14 complex are recruited to the daughter-bound spindle pole body and become activated. This activation results in the relocalisation of the Sid2-Mob1 complex to the contractile actomyosin contractile ring and leads to septum formation and septation. Based on Figure 1 from Krapp *et al*., 2004.

S. cerevisiae	S. pombe	A. nidulans
LTE1	ETD1	AN3092
TEM1	SPG1	SPG A
BFA1	BYR4	BYR A
BUB2	CDC16	BUB 1
CDC15	CDC7	SEP H
DBF2	SID2	SID B
MOB1	MOB1	MOB A
CDC14	CLP1	AN5057
NUD1	CDC11	SEP K
NO HOMOLOG	SID1	SEP L
NO HOMOLOG	CDC14	SEP M
NO HOMOLOG	SID4	NO HOMOLOG

Table 2. MEN/SIN proteins in *S. cerevisiae*, *S. pombe* and *A. nidulans*. Based on Piatti *et al*,. 2006, and Kim *et al*., 2009.

Cdc14 complex is recruited but only after inactivation of the CDK^{Cdc2} (Guertin *et al*., 2000). Recruitment of the Sid1-Cdc14 complex results in relocalisation of the Sid2-Mob1 complex upon spindle breakdown to the CAR where it stays until cell separation is complete (Sparks *et al*., 1999; Hou *et al*., 2000; Salimova *et al*., 2000). This relocalisation to the CAR of Sid2-Mob1 complex also requires the Etd1 protein (Jimenez and Oballe, 1994; Daga *et al*., 2005). Further studies have suggested that Etd1 could be the GEF of Spg1. Evidence for this hypothesis includes homology between part of the *ETD1* gene sequence and the guanosine exchange factor domain (GEF) found in CDC25-related GEFs. Furthermore, localisation of Etd1 to the cell cortex during the cell cycle up to the point at which the SIN pathway is expected to be active reflects the localisation pattern of Lte1 in *S. cerevisiae* (Daga *et al*., 2005; Garcia-Cortes and McCollum, 2009). Unlike the MEN pathway which initiates the final destruction of the CDK-cyclin complexes the SIN pathway actually requires them to be degraded before they become activated. How the CDK-cyclin complexes accomplishes this function has yet to be elucidated (Krapp and Simanis, 2008).

1.7. MEN and SIN networks in other eukaryotes.

In a recent study Bedhomme and colleagues, investigated the degree of conservation

of the MEN and SIN pathways across the eukaryotic domain (Bedhomme *et al*., 2008). They concluded that the various components of the pathways are conserved at different levels. They found that homologs of the Dbf2/Sid2 and Mob1 proteins are conserved among the fungal, plant and animal kingdoms while homologs of the Tem1/Spg1 and Cdc15/Cdc7 proteins are found in the fungi, plants and also in the *Dictyostelium* genus but not in animals. Interestingly the Cdc14/Clp1 protein has homologs only in the fungal and animal kingdoms. These findings suggest that different groups of MEN/SIN components have evolved differently and therefore other proteins may fulfil the role of a protein that has been lost.

In the genome of *Arabidopsis thaliana* there are two paralogs of the *SPG1*, *CDC7*, *MOB1* genes and disruption of one of these paralogs does not cause any phenotypic effect (Bedhomme *et al*., 2008). Analysis of the *A. thaliana SGP1/2* (*SPG1*), *MAP3Kε1/2* (*CDC7*) gene sequences found that they all contain a large region of extension either within the gene or at amino- or carboxyl-terminal ends (Jouannic *et al*., 2001; Champion *et al*., 2004). These extensions are specific to plants and contain new phosphorylation sites. Complementation studies showed that *SGP1* and *SGP2* were able to rescue *spg1* mutants, while addition of the *MAP3Kε1* or *MAP3Kε2* to a *cdc7* null *S. pombe* strain resulted in increased production of septum material. However, in plants cytokinesis does not occur by actomyosin ring contraction but by targeted secretion at the plane of cell division (Waizenegger *et al*., 2000; Soyano *et al*., 2003; Bedhomme *et al*., 2008).. This suggests that the SIN homologs play a different role in plants cells to that in yeast.

In the filamentous ascomycete fungus *Aspergillus nidulans* homologs of the various MEN/SIN proteins have been identified (Table 2). This suggests a MEN/SIN like network exists within this species. However, in *Aspergillus nidulans* septation and mitosis are not coupled for the first two nuclear divisions, as upon conidium germination septation only occurs following the third round of nuclear division. Following this, septation is regulated by cell cycle progression. This suggests that the network is most likely involved in septation (Wolkow *et al*., 1996; Harris, 1997; Wolkow *et al*., 2000). To date only the SepH, SepK, SidB and MobA protein have been studied. These studies found that all four proteins are required for septation. However

they are all non-essential for growth (Bruno *et al.*, 2001; Kim *et al.*, 2006; Kim *et al.*, 2009). Like the MEN and SIN pathways the SidB (Dbf2/Sid2) and MobA (Mob1) proteins localise to the SPB early in mitosis then relocate to the site of septation (Kim *et al.*, 2006). The SidB/MobA ring also contracts with the actomyosin ring. In addition, the SepH protein has also been implicated in actomyosin ring formation as deletion of *SEPH* resulted in no actomyosin ring formation (Bruno *et al.*, 2001). This has not been seen in either *S. cerevisiae* or *S. pombe*. These findings suggest that SepH functions both upstream of the SIN network and also during cytokinesis. This data also suggests the differences that exist between the SIN network in *A. nidulans* and those found in *S. pombe.*

1.8. What is known in *Candida* about mitotic exit network components.

To date very little research has been carried to determine the means by which mitotic exit is regulated in *C. albicans*. Only the terminal phosphatase Cdc14 and more recently the kinase Dbf2 have been studied in detail (Clemente-Blanco *et al.*, 2006; González-Novo *et al.*, 2009).

1.8.1. *C. albicans* Cdc14.

To understand the role that *CDC14* plays in *C. albicans*, Clemente-Blanco *et* al. (2006) constructed a regulatable mutant using the *MET3* promoter. This strain grew under repressing conditions suggesting that *CaCDC14* is non-essential. To confirm non-essentiality two *cdc14*/*cdc14* null strains were constructed in different strain backgrounds and these were also shown to have no obvious growth defect. This finding is different from that in *S. cerevisiae* where *CDC14* is essential. This suggests that either Cdc14 is not required for mitotic exit in *C. albicans* or another protein complements the mitotic exit function of Cdc14.

Although not required for mitotic exit CaCdc14 expression is cell cycle regulated. The CaCdc14 protein was not detected in G1 cells but as the cells passed through the S and G2 phases and into mitosis the protein levels were seen to rise to a maximum level during mitosis. In terms of protein localisation, Cdc14 localised to the nucleus when cells began to bud rather than the nucleolus as seen in *S. cerevisiae* (Shou *et al.*, 1999; Visintin *et al.*, 1999; Traverso *et al.*, 2001; Clemente-Blanco *et al.*, 2006). Further

analysis of a Cdc14-YFP strain shows that during early mitosis the CaCdc14 localises to the SPB. A small proportion of the CaCdc14 then relocalises to the bud neck at the end of mitosis. This is similar to the localisation of the Sid2-Mob1 complex in *S. pombe*. In hyphal cells the CaCdc14 only localised to the nucleus of the apical compartment of the hypha and was not seen to localise at the site of septation. This localisation pattern in hyphae requires the Hgc1 protein (Clemente-Blanco *et al.*, 2006). The bud neck localisation of the CaCdc14 protein in yeast cells but not hyphal cells suggests that Cdc14 plays a role in yeast cell separation process that does not occur in hyphal cells. Microscope analysis of the *cdc14/cdc14* null strains showed that the cells clumped together and transmission electron microscopy (TEM) of the septal areas of these cells showed that the primary and secondary septa formed normally. Thus CaCdc14 is not required for cytokinesis but does input on cell separation. This cell separation defect has been shown to be due to a lack of expression of the Cht3 and Eng1 enzymes, which are required for this process (McCreath *et al.*,1995; Esteban *et al.*, 2005). This suggests a link between the MEN and RAM networks in *C. albicans*.

Non-essential, CaCdc14 was required for the normal timing of mitotic exit. This was demonstrated by studying the levels of the B cyclins, Clb2 and Clb4, in the *cdc14/cdc14* null cells. It was found that B cyclin levels were about 2-fold higher than wild type levels. The B cyclin levels normally increase during mitosis and decrease following mitosis but in the *cdc14/cdc14* null cells the decrease in B cyclin levels were not observed suggesting there is a delay in exit from mitosis.

The final finding was that the Cdc14 protein is required for hyphal growth and agar invasion. The *cdc14/cdc14* null strains were unable to form hyphae on Lee's, M199 or spider medium plates and exhibited delayed hyphal formation in serum media.

Overall the findings documented by Clemente-Blanco *et al.*, (2006), suggest that the primary role of Cdc14 in *C. albicans* is to co-ordinate cell separation following septation. This differs from the role of Cdc14 plays in mitotic exit in *S. cerevisiae* of and suggests that the role of the mitotic exit network in *C. albicans* may be different from that in *S. cerevisiae*.

1.9. Project objectives.

The overall aim of this project is to decipher how cell cycle progression and morphogenesis are linked. The particular focus of this study will be on the mitotic exit network in *C. albicans*. Previous work focussing on the terminal phosphatase Cdc14 found that Cdc14 was not essential for vegetative growth but is required for cell separation and hyphal growth. These findings suggest that the MEN in *C. albicans* may be required for morphogenesis and the output of the pathway would require differential regulation in the various morphological forms.

This project will focus on the GTPase protein Tem1 which is situated at the head of the MEN network. The project has three specific aims:

1) To construct *C. albicans tem1* mutant strains, and study the role of Tem1 in mitotic exit, cytokinesis and separation.

2) To study the regulation of Tem1 by identifying key protein interactions and following its spatial and temporal regulation in the different morphological forms.

3) To develop a set of new molecular tools that utilise the positive selection marker *NAT1* in order to achieve the above goals.

Chapter 2. Materials and Methods.

2.1. Yeast techniques.

2.1.1. Strains.

Strain Name	Genotype	Reference
C. albicans strain		
SC5314	Wild type clinical isolate	Gillum *et al*., 1984
CAI4	*ura3Δ::imm434/ura3Δ::imm434*	Fonzi & Irwin, 1993
CAI4 (CIp10)	*ura3Δ::imm434/ura3Δ::imm434, RP10/Δrp10::CIp10*	Murad *et al*., 2000
SN78	*leu2Δ/leu2Δ, his1Δ/his1Δ, ura3Δ::imm*[434]*/ura3Δ::imm*[434]*, iro1Δ::imm*[434]*/iro1Δ::imm*[434]	Noble & Johnson, 2005
THE1	As CAI4 but *ade2Δ::hisG/ade2Δ::hisG, ura3Δ::imm434/ ura3Δ::imm434, ENO1/eno1Δ::ENO1-tetR-ScHAP4AD-3HA-ADE2*	Nakayama *et al*., 2000
SWM4	As THE1 but *TEM1/tem1Δ::dpl200-URA3-dpl200*	This study
SWM5	As THE1 but *TEM1/tem1Δ::dpl200*	This study
SWM6	As THE1 but *URA3-TET1p-TEM1/tem1Δ::dpl200*	This study
SWM10	As SN78 but *LTE1/lte1Δ::LEU2*	This study
SWM17	As SN78 but *lte1Δ::HIS1/lte1Δ::LEU2*	This study
SWM52	As CAI4 but *TEM1-GFP-URA3/TEM1*	This study
SWM66	As THE1 but *URA3-TET1p-TEM1-V5-NAT1/tem1Δ::dpl200*	This study
SWM71	As SWM6 but *CDC3-GFP-NAT1/CDC3*	This study
SWM72	As SWM6 but *NAT1-ENO1p-CDC15/CDC15*	This study
SWM84	As SWM6 but *NAT1-ENO1p-DBF2/DBF2*	This study
SWM88	As SWM6 but *ABP1-GFP-NAT1/ABP1*	This study
SWM95	As SWM6 but *NAT1-ENO1p-CDC14/CDC14*	This study
SWM99	As THE1 but *LTE1/lte1Δ::dpl200-URA3-dpl200*	This study
SWM108	As CAI4 (CIp10) but *ACT1p-RFP-NAT1*	This study
SWM112	As THE1 but *LTE1/lte1Δ::dpl200*	This study
SWM113	As SWM6 but *TUB4-GFP-NAT1/TUB4*	This study
SWM114	As CAI4 (CIp10) but *TEM1-V5-NAT1/TEM1*	This study
SWM116	As CAI4 (CIp10) but *NAT1-ENO1p-CDC14/CDC14*	This study
SWM118	As CAI4 (CIp10) but *ACT1p-CFP-NAT1*	This study

Strain Name	Genotype	Reference
SWM121	As CAI4 but *LTE1-GFP-URA3/LTE1*	This study
SWM123	As CAI4 but *NAT1-ENO1p-TEM1-GFP/TEM1*	This study
SWM137	As CAI4 (CIp10) but *ACT1p-YFP-NAT1*	This study
SWM142	As SWM52 but *TUB4-RFP-NAT1/TUB4*	This study
SWM148	As SC5314 but *CDC3-GFP-NAT1/CDC3*	This study
SWM149	As CAI4 but *HEX1-V5-URA3/HEX1*	This study
SWM150	As CAI4 (CIp10) but *HEX1-V5-6xHIS-NAT1/HEX1*	This study
SWM151	As CAI4 (CIp10) but *HEX1-V5-6xHIS-NAT1/HEX1*	This study
SWM152	As CAI4 (CIp10) but *NAT1-ENO1p-HEX1/HEX1*	This study
SWM155	As CAI4 (CIp10) but *ACT1p-GFP-NAT1*	This study
S. cerevisiae strains		
tem1-3	*MATα, tem1-3, ade2-1, ura3-52, his3, leu2-3,112, trp1-1, can1-100*	Corbett *et al*., 2006
AH109	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ, MEL1*	James *et al*., 1996
SWM40	As *tem1-3* but pYES2.1-*ScTEM1*	This study
SWM81	As *tem1-3* but pYES2.1-*CaTEM1*	This study
SWM83	As *tem1-3* but pYES2.1-6x*HIS/lacZ*	This study
E. coli strains		
DH5α	F- φ80*lacZ*ΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(rk-, mk+), *pho*A, *sup*E44, *thi*-1, *gyr*A96, *rel*A1 λ-	Invitrogen
TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74, *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
Strataclone	Not known	

Table 3. The *C. albicans*, *S. cerevisiae* and *E. coli* strains used in this study.

2.1.2. Bacterial growth conditions and transformation.

Three different *Escherichia coli* strains were used during this project, DH5α, TOP10 (both Invitrogen, Paisley, UK) and StrataClone solo pack competent cells (Stratagene, Leicester, UK). All *E. coli* strains were grown in Luria Bertani broth at 37 ˚C (2 % (w/v) tryptone, 1 % (w/v) yeast extract and 2 % (w/v) NaCl pH 7.0) with either ampicillin (100

µg/ml) or kanamycin (50 µg/ml) added as the selection antibiotics. *E. coli* transformations were carried out as per the manufacturer's instructions.

2.1.3. Growth conditions for *C. albicans* and *S. cerevisiae.*

The *C. albicans* and *S. cerevisiae* strains used in this study are shown in Table 3. These strains were grown in YPD media (1 % (w/v) yeast extract, 2 % (w/v) mycological peptone and 2 % (w/v) glucose) at 30 ˚C unless otherwise stated. For all solid media 2 % (w/v) agar was added to media prior to sterilisation. For transformations utilising an auxotrophic marker SC media (0.67 % (w/v) yeast nitrogen base without amino acids and 2 % (w/v) glucose) with complete supplement mixture (Formedium, Hunstanton, UK) lacking uridine, histidine or leucine was used as required. For transformations conducted using the *NAT1* positive selection marker, cells were spread onto Sabouraud dextrose agar (Sigma-Aldrich, Gillingham, UK) containing 200 µg/ml Nourseothricin (Werner BioAgents, Jena, Germany).

2.1.4. Transformation of *C. albicans* and *S. cerevisiae.*

The transformation of *C. albicans* and *S. cerevisiae* cells with exogenous DNA was carried out using a modified version of the lithium acetate/single stranded DNA/polyethylene glycol (LiOAc/ssDNA/PEG) protocol (Gietz and Schiestl, 1991). Cells were inoculated into 50 ml of YPD to a density of 2.5×10^6 cells/ml from an overnight culture. Cells were then grown until the cell density reached 1×10^7 cells/ml, normally 4-5 hours, before harvesting by pelleting at 4000 rpm for 3 minutes and washing in 20 ml double distilled water (ddH$_2$O). The cells were re-pelleted, washed with 1 ml of 0.1M LiOAc, pelleted and resuspended in 1 ml 0.1M LiOAc. 100 µl of this cell suspension was centrifuged at 12000 rpm for 1 minute and the cell pellet resuspended in 240 µl of fresh 50 % PEG 3350 solution (Sigma-Aldrich, Gillingham, UK). In addition, 36 µl of 1M LiOAc, 10 µl of boiled single stranded salmon sperm DNA (10 mg/ml) and 75 µl of exogenous DNA were added to the resuspended cells. This suspension was then incubated at 30 ˚C for 40 minutes followed by heat shock at 42 ˚C for 20 minutes. For transformations utilising the *NAT1* marker, the cells were pelleted at 12000 rpm for 1 minute before being incubated in 1 ml of YPD for an additional 4 hours at 30 ˚C prior to plating. The cells were pelleted at 12000 rpm for 1 minute and resuspended in 400 µl of ddH$_2$O and spread on selective media. The plates were then incubated at

30 C for 2-4 days.

2.1.5. 5-FOA selection.

To recycle the *URA3* gene from chromosomally integrated mini-URA-blaster cassettes 5-FOA selection was used (Boeke *et al.*, 1984). 100 µl and 500 µl of an overnight YPD culture of a *URA3* positive transformant were pelleted at 12000 rpm for 1 minute and resuspended in 100 µl of ddH$_2$O. The cells were then spread on SD media supplemented with 2 mg/ml (w/v) 5-FOA and 50 µg/ml (w/v) uridine and grown at 30 ˚C for 3-5 days. 5-FOA resistant colonies were confirmed by PCR.

2.2. Molecular biology techniques.

2.2.1. Plasmid extractions.

The extraction of plasmids from 1.5 ml of *E. coli* overnight culture was routinely carried out during this project. This was carried out as per the manufacturer's instructions using either the QIAprep Spin Miniprep kit (QIAGEN, Crawley, UK) or the Spin column plasmid miniprep kit (NBS Biologicals, Huntingdon, UK). A list of plasmids used in this study is given in Table 4.

2.2.2. Oligonucleotide primer design.

The oligonucleotide primers used in this study are listed in Table 5 and were all manufactuered by Invitrogen (Paisley, UK). All the primers used were designed for this study unless otherwise stated in Table 5. The primers used for amplifying transformation cassettes contain 18-25 nucleotide of homology to the cassette and 74-81 nucleotides to target the amplified cassette to the specific target gene. Shorter primers for PCR screening and gene expression studies were designed to be between 18 and 25 nucleotides in length and have melting temperatures of between 58˚C and 62 ˚C.

2.2.3. Polymerase chain reaction.

The polymerase chain reaction (PCR) reactions were carried out using a Techne tc-412 thermocycler machine (Bibby Scientific Limited**,** Staffordshire, UK). Each reaction was set up to contain 25 µl of master mix (0.625 units Thermo Prime Taq DNA polymerase, 75 mM Tris-HCl, 20 mM (NH$_4$)$_2$SO$_4$, 1.5 mM MgCl$_2$, 0.01% Tween-20, 0.2 mM each

Plasmid Name	Details	Reference
pMG1506	*GFP-URA3*	Gerami-Nejad *et al.*, 2001
pMG1416	*CFP-URA3*	Gerami-Nejad *et al.*, 2001
pMG1683	*YFP-URA3*	Gerami-Nejad *et al.*, 2001
pMG2169	*RFP-URA3*	Gerami-Nejad *et al.*, 2009
pCIp10-V5-6x*HIS*	V5-6xHIS, *URA3*, *RPS10*	Bates, unpublished
pSWM84	pYES2.1-*ScTEM1*	This study
pSWM85	pYES2.1-*CaTEM1*	This study
pYES2.1/V5-6x*HIS*/*lacZ*	pYES2.1-V5-6x*HIS-lacZ*	Invitrogen
pJK795	*TEF1p-CaNAT1-TEF1*t	Shen *et al.*, 2005
pDDB57	*dpl200::URA3::dpl200*	Wilson *et al.*, 2000
pCAU99t	*URA3*-TETp	Nakayama *et al.*, 2000
pSWM90	*CFP-NAT1* in Strataclone vector	This study
pSWM93	*YFP-NAT1* in Strataclone vector	This study
pSWM157	*GFP-NAT1* in TOPO pCR2.1	This study
pSWM158	*NAT1-ENO1*p in TOPO pCR2.1	This study
pSWM159	V5-6x*HIS-NAT1* in TOPO pCR2.1	This study
pSWM160	GADT7-*BFA1*	This study
pSWM161	GADT7-*BUB2*	This study
pSWM162	GADT7-*TEM1*	This study
pSWM163	GADT7-*CDC15*	This study
pSWM164	GADT7-*DBF2*	This study
pSWM165	GADT7-*MOB1*	This study
pSWM166	GBKT7-*BFA1*	This study
pSWM167	GBKT7-*BUB2*	This study
pSWM168	GBKT7-*TEM1*	This study
pSWM169	GBKT7-*CDC15*	This study
pSWM170	GBKT7-*DBF2*	This study
pSWM171	GBKT7-*MOB1*	This study
pGADT7-t	GADT7-t	Clontech
pGBKT7-53	GBKT7-53	Clontech

Table 4. Plasmids used in this study.

dNTP, Abgene, Epson, UK), 100 pmol of each oligonucleotide primer and template DNA and made up to 50 µl with ddH$_2$O. The PCR protocol used consisted of the initial denaturation of the template DNA at 94 ˚C for 3 minutes, then 30 cycles of 1 minute at 94 ˚C (denaturation), 1 minute of 53 ˚C (primer annealing), and then 72 ˚C for 1 minute per 1 kb of the fragment (elongation). Following the 30 cycles a final elongation stage of 72 ˚C for 5 minutes was carried out. When extensor master mix (Abgene, Epson UK) was used the elongation temperatures were reduced to 68 ˚C. PCR products were confirmed by gel electrophoresis.

2.2.4. Fusion PCR.

Fusion PCR was used to construct molecular cassettes fused with the *NAT1* marker *e.g. GFP-NAT1*. This protocol consisted of two rounds of PCR. The first round of PCR involved the amplification of the *NAT1* marker and various epitope tags using primers with overlapping 5' regions (Table 5). The PCR products were then purified by gel extraction. The second round of PCR consisted of the fusion of two PCR products together using the flanking primers (Table 5).

2.2.5. Restriction endonuclease digestions, ligations and cloning.

Restriction endonuclease digestions, plasmid ligations and cloning were all carried out as per the manufacturer's instructions.

2.2.6. Electrophoresis.

During this study DNA electrophoresis was routinely carried out. For DNA electrophoresis, the DNA samples were analysed on 0.8 % or 2 % (w/v) agarose gels (depending on fragment size) in Tris-acetate-EDTA buffer (50x stock; 24.2 % (w/v) Tris base, 5.7 % (v/v) acetic acid, 10 % (v/v) 0.5M EDTA). The gels also contained ethidium bromide (0.5 µg/ml) to stain the DNA. For retrieval of DNA fragments the QIAquick gel extraction kit (QIAGEN, Crawley, UK) or the spin column gel extraction kit (NBS Biologicals, Huntingdon, UK) was used.

Name	Sequence	Reference
GENE DISRUPTIONS		
TEM1-KO-F	aagtctcaaaatgtaaacaaacttatttagtttcagtgaagtgtatgatgtg gaaacaaacaaaacgcactggttaTGTGAAATTGTGAGCGGATA	This study
TEM1-KO-R	ctacttatatatcaatatgggttcccccacgttaacaatttcatcaaggtttta actttaagtcaaacgctttagatGTTTTCCCAGTCACGACGTT	This study
TEM1-TET-F	aagtctcaaaatgtaaacaaacttatttagtttcagtgaagtgtatgatgtg gaaacaaacaaaacgcactggttaGTAATACGACTCACTATAGGG	This study
TEM1-TET-R	ctccagaatgaaggggtgataaattacatactggttattctgatcctgttga ttatcttcttccatattatgatctCTAGTTTTCTGAGATAAAGCTG	This Study
LTE1-KO-F	taatttgtttatatcttaatcttaatcatttaattcttatattcaatccaaaatt acattcccatccaattaatatGCTCGGATCCACTAGTAACG	This study
LTE1-KO-R	ctatttttgattatgttgaatacaatatttcatttcatcttcatccaaacttttg atataaagacatttcgataatCCAGTGTGATGGATATCTGC	This study
LTE1-URA-KO-F	taatttgtttatatcttaatcttaatcatttaattcttatattcaatccaaaatt acattcccatccaattaatatTGTGAAATTGTGAGCGGATA	This study
LTE1-URA-KO-R	ctatttttgattatgttgaatacaatatttcatttcatcttcatccaaacttt tgatataaagacatttcgataatGTTTTCCCAGTCACGACGTT	This study
LTE1-TET-F	taatttgtttatatcttaatcttaatcatttaattcttatattcaatccaaaa ttacattcccatccaattaatatGTAATACGACTCACTATAGGG	This study
LTE1-TET-R	tattattattattaccaatatcaccagtgagattggttgattctggattatc ttgtggcattgaactgttgCTAGTTTTCTGAGATAAAGCTG	This study
TEM1-SF	tagaaatgctggttggaatgc	This study
TEM1-SR	taagcgtattatgtctagttatg	This study
TEM1-TET-SR	acatacttccaccattagtgatg	This study
LTE1-SF	tcttcctatctttctttcatcg	This study
LTE1-SR	aagaatttcttgcagccaatgaccc	This study
LTE1-TET-SR	tatcattggtggtcggtaaac	This study
TET-S	ACATACTTCACCATTAGTGATG	This study
URA3-SF	TAGAAATGCTGGTTGGAATGC	This study
LEU2-SR	CCTGGGTATTGATATGTTGG	This study
HIS1-SR	GCAGACATTCACAGCACGC	This study

Name	Sequence	Reference
GFP and V5 tagging		
TEM1-GFP-F	taaagcgtttgacttaaagttaaaccttgatgaaattgttaacgtgggggaacccatattgatatataagGGTGGTGGTTGTAAAGGTGAAGAATTATT	This study
TEM1-V5-F	ttatattatctaaagcgtttgacttaaagttaaaccttgatgaaattgttaacgtgggggaacccatattgatatataagAAGGGCGAGCTTCGAGGTC	This study
TEM1-NAT1-R	ataataataaacaatgataagcgtattatgtctagttatgtaataaatagctattgaattgtttaaagaatggaaGGTACCGAATTCGAGCTCG	This study
TEM1-URA-R	aataataataaacaatgataagcgtattatgtctagttatgtaataaatagctattgaattgtttaaagaatggaaTCTAGAAGGACCACCTTTGATTG	This study
LTE1-GFP-F	atcgaaatgtctttatatcaaaagtttggatgaagatgaaatgaaatattgtattcaacataatcaaaaaGGTGGTGGTTCTAAAGGTGAAGAATTATT	This study
LTE1-URA-R	tttccaaatgtaaaatcctccacgattattattattagttattgttgttgttgttggtattaggtttgtattatTCTAGAAGGACCACCTTTGATTG	This study
CDC3-GFP-F	acaaaaattattaccacaagacccaccagcacaaccagctccacaaaagagtcgtaaaggattttttacgtGGTGGTGGTTGTAAAGGTGAAGAATTATT	Gerami-Nejad *et al.*, 2001
CDC3-NAT1-R	tactgacaattttttatacatcacaatatcaaattaaacaaacagattaacaaacaaataaactaaattaagttacataGGTACCGAATTCGAGCTCG	Gerami-Nejad *et al.*, 2001
ABP1-GFP-F	gtggcaaggaaaacattccaagacaggagaagtcggattgttccctgctaactatgttgtcttgaatgagGGTGGTGGTTCTAAAGGTGAAGAATTATT	This study
ABP1-NAT1-R	tggtgtagagatcgaataaaatgaaagctaatttaaaacagtaatccctgaaagctggctatagcaccaatttatcttCGTTAGTATCGAATCGACAGC	This study
TUB4-GFP-F	ggatgacctagaagatggtggtggtaatggtaatggttataacaatatagatgatgcagatatgggtataGGTGGTGGTTCTAAAGGTGAAGAATTATT	This study

81

Name	Sequence	Reference
TUB4-RFP-F	gatgacctagaagatggtggtggtaatggtaatggttataacaatatagat gatgcagatatgggtataGGTGGTGGTGATAACACTGAAGATG TTATT	This study
TUB4-NAT1-R	tgactccacaaccacaaaagttattcctcaactcggacattatcttttattc tatatacatttaaccttctcttacaCGTTAGTATCGAATCGACAGC	This study
TEM1-TAG-S	ttcggaagtgcgatgaaagc	This study
LTE1-TAG-S	gctaaatttagaacttcggttc	This study
CDC3-TAG-S	aagagaatgggtattgaacaag	This study
ABP1-TAG-S	ctacagctgaatacgattacg	This study
TUB4-TAG-S	gtttaatgaatctcgtgaagtg	This study
GFP-UP	CACCTTCACCGGAGACAG	This study
RFP-UP	ATAATCTGGAATATCAGCTGG	This study
V5-S	GAGGGCGTGAATGTAAGCG	This study

GENE OVEREXPRESSION

Name	Sequence	Reference
TEM1-ENO-F	aagtctcaaaatgtaaacaaacttatttagtttcagtgaagtgtatgatgtgg aaacaaacaaaacgcactggttaCGTTAGTATCGAATCGACAGC	This study
TEM1-ENO-R	aatggcattcctccagaatgaaggggtgataaattacatactggttattctg atcctgttgattatcttcttccatTGTTGTAATATTCCTGAATTATC	This study
CDC15-ENO-F	tggaacaaactttttgaagacaacaaaccaaatggaaagatgatcactaa agtagccttagatatatagtttttataaCGTTAGTATCGAATCGAC AGC	This study
CDC15-ENO-R	tggctcgtgtgtaaagttgtcctttcatttaaagcttctgtcttcaaatcaaa atcatttaaaaccatTGTTGTAATATTCCTGAATTATC	This study
DBF2-ENO-F	cagctcaactcaactcaactcaactcaactattacacaccatcttataagc ctaacaagaaaccacacccccacctccCGTTAGTATCGAATCGA CAGC	This study
DBF2-ENO-R	ttgttgttgttgttgttgaggattgttataagtaggcttagatgaacgataa ctagtatggatatccatcatTGTTGTAATATTCCTGAATTATC	This study
CDC14-ENO-F	aattttatccaattaactcaacgtctatcattcatttgtattttctacatata agtacattttgtctttcCGTTAGTATCGAATCGACAGC	This study

Name	Sequence	Reference
CDC14-ENO-R	ttgaagtagattttcccaacatacttttaagaaactctataagaggcaca tgaaccagtgaactatgcatTGTTGTAATATTCCTGAATTATC	This study
CDC15-ENO-S	ggtcactcccaactataatcgatgc	This study
DBF2-ENO-S	cccaggttgtggagaattacc	This study
CDC14-ENO-S	cattatatggtaatgcgtcttc	This study
ENO-SF	TTGATAATTCAGGAATATTACAAC	This study

HOMOLOG STUDY

Name	Sequence	Reference
CaTEM1-F	atcataatatggaagaagataatc	This study
CaTEM1-R	cttatatatcaatatgggttccc	This study
ScTEM1-F	ttaagctaagatggctacacc	This study
ScTEM1-R	tgtatattcgcccggcgatg	This study

RT-PCR

Name	Sequence	Reference
CCN1-F	tcaaatatggtcctccacatc	This study
CCN1-R	gacaatcttctaaagttggatg	This study
CLN3-F	atcaagttaggatgatgcaatc	This study
CLN3-R	cattgacgacccattctaatg	This study
CLB2-F	aatgagtttagacttactagatc	This study
CLB2-R	gatttcttatggtatgactgac	This study
CLB4-F	agtattgccaatttgagcagc	This study
CLB4-R	attggttgcatgggcacatg	This study
HGC1-F	caaccctcaagcttctggctccaaatcatttg	This study
HGC1-R	ggaccaagatatttcccaccacatccataac	This study

YEAST 2 HYBRID VECTOR CONSTRUCTION

Name	Sequence	Reference
BFA1-F*	gaaggatccatatggtatctgataaattaacgtta	This study
BFA1-R*	gaaggatccttactcttcaattatcattttacg	This study
BUB2-F*	gaaggatcctatggagcaagaattactcctc	This study
BUB2-R*	gaaggatccttattttttcttctaaaaatcttgcc	This study
TEM1-NDE1-F	ggaattccatatgatgatggaagaagataatcaacaggatcag	This study
TEM1-R*	gcgcggatccctacttatatatcaatatgggttcccccacgttaac	This study

Name	Sequence	Reference
CDC15-NDE1-F	ggaattc<u>catatg</u>atgatggttttaaatgatttt	This study
CDC15-NDE1-R	ggaattc<u>catatg</u>atgctactttagcaccagtaa	This study
DBF2-NDE1-F	ggaattc<u>catatg</u>atgatgatggatatcgatactagttatcgttcatctaagc ctacttataac	This study
DBf2-NDE1-R	ggaattc<u>catatg</u>atggctaatggatttaatctacttgatcgagaatttcc	This study
MOB1-NDE1-F	ggaattc<u>catatg</u>atgatggcacttttccagaatttcaatactcattcc	This study
MOB1-NDE1-R	ggaattc<u>catatg</u>atgttatctttgaagcatcgtgtctaccaa	This study

NAT1 CASSETTE CONSTRUCTION

Name	Sequence	Reference
GFP-F	GGTGGTGGTTCTAAAGGTGAAGAATTATT	Gerami-Nejad *et al.*, 2001
RFP-F	GGTGGTGGTGATAACACTGAAGATGTTATT	Gerami-Nejad *et al.*, 2001
V5-F	AACAGCTATGACCATGATTAC	This study
ENO1-F	**gcagggatgcggccgctga**CATTTGTATCTTTAGTAGACATG	This study
GFP-R	**gcagggatgcggccgctgac**ATATTTCAACGCCTTCCAGC	This study
V5-R	**gcagggatgcggccgctgac**TGCAGGGCCGCAGCTTGC	This study
ENO1-R	TGTTGTAATATTCCTGAATTATC	This study
NAT1-F	**gtcagcggccgcatccctgc**GATATCAAGCTTGCCTCGTC	This study
NAT1-R	CGTTAGTATCGAATCGACAGC	This study
ACT1-GFP-F	ctggttttctttctttcttagaaacattatctcgatattaatattaaaaaaatat aatcattcaaaatgGGTGGTGGTTCTAAAGGTGAAGAATTATT	This study
ACT-RFP-F	ctggttttctttctttcttagaaacattatctcgatattaatattaaaaaaatata atcattcaaaatgGGTGGTGGTGATAACACTGAAGATGTTATT	This study
ACT1-GFP-R	gtgtgtattattaatgtgacagtaacatcccaaacgagaaatattatgtcgac aacaaaaaagtttgatcCGTTAGTATCGAATCGACAGC	This study
HEX1-V5-F	acggggtttctcctttggtgccaaaatactgtttgctcaatccacacgcttgtg atttgtacaaaaatccaccagtttatAAGGGCGAGCTTCGAGGTC	This study
HEX1-NAT1-R	acttccgttccctttgagcacttagatagtgatatcgtatattttcttttccaaa ccatctattccagacacagatctCGTTAGTATCGAATCGACAGC	This study
HEX1-ENO-F	gcgttttatggtttaccccacaaaggtccgtgttttcaaaaatttctaaaagat agatctattaatgtggctagtCGTTAGTATCGAATCGACAGC	This study

Name	Sequence	Reference
HEX1-ENO-R	aaccttggcagcgtggacaaccacattgcacaaccaaagaagcaaatgaa	This study
	agataatcattttatctaacaccatTGTTGTAATATTCCTGAATTATC	
ACT1-SF	caccaagatttattgccaac	This study
ENO-SF	TTGATAATTCAGGAATATTACAAC	This study
HEX1-V5-SF	acaaggattccaacggacac	This study
HEX1-ENO-SR	accaagccatgtaatgctcc	This study
T7	gtaatacgactcactatagg	Wallace *et al.*, 1981

Table 5. The oligonucleotide primers used in this study. Primer sequences in lower case represent gene specific sequences while primer sequences in upper case correspond to cassette specific sequences. The nucleotides highlighted in bold correspond to the fusion section of the primer. The underlined nucleotide sequences of some of the above primer sequence correspond to the restriction enzyme site. * denotes primers that contain the *Bam*H1 restriction site.

2.2.7. *C. albicans* and *S. cerevisiae* genomic DNA extraction.

The extraction of genomic DNA from 1.5 ml of saturated *C. albicans* and *S. cerevisiae* culture (>1 x 10^8 cells/ml) were carried out using the Master Pure^{TM} DNA Purification kit (Epicentre Biotechnologies, Cambridge, UK) as per manufacturer's instructions.

2.2.8. Yeast colony PCR.

The yeast colony PCR protocol was used for confirming successful chromosome integration events (based on Linder *et al.*, 1996). Colonies from transformation plates were picked into 10 μl of dd H_2O. To maintain a stock of transformants 2 μl of this cell suspension was then spotted on to a selection media plate for maintenance of stock. The remaining 8 μl of the cell suspension was treated with 2 μl of the *Arthrobacter luteus* enzyme Lyticase (Sigma, Gillingham, UK, 25 units/μl in a solution of 100mM Sodium Phosphate buffer ph7.5, 10 % (w/v) Glycerol). This suspension was incubated at 37 ˚C for 10 minutes followed by 10 minutes at -80 ˚C. Following freezing the cell suspension was allowed to thaw at room temperature. Once thawed 2 μl of the solution was added to a 50 μl PCR reaction as the template DNA.

2.3. RNA analysis.

2.3.1. *C. albicans* RNA extraction.

The RNA extraction method used in this project is modified from Blackwell *et al.*, (2004). *C. albicans* cell pellets were collected from 50 ml cell cultures and frozen at

-80 ˚C. The thawed cell pellets were resuspended in 200 µl cold extraction buffer (100 mM EDTA pH 8.0, 100 mM NaCl, 50 mM Tris-HCl pH 8.0, 5 µl 10 % SDS). The cell suspension was then transferred to screw-capped Eppendorf tubes and 200 µl iso-amyl alcohol/ phenol/chloroform (ratio 1:24:25) was added as well as glass beads until a layer of dry beads was visible. The cells were then disrupted in a FastPrep machine (MP Biomedicals, Illkirch, France) 4 times at 6.5 speed for 20 seconds and incubated on ice between disruptions. Following cell disruption 800 µl of cold extraction buffer was added and the cell debris and beads were pelleted by centrifugation for 10 minutes at 6000 rpm. Iso-amyl alcohol phenol chloroform extraction of the aqueous layer of the supernatant was carried out four times. The RNA was then precipitated by incubation at -80 ˚C overnight following the addition of 0.6 volume of isopropanol and 0.1 volume of 3M sodium acetate to the RNA samples. The RNA was then pelleted by centrifugation at 13000 rpm for 15 minutes. The resultant pellets were then washed with 70 % ethanol, dried and resuspended in 50 µl dd H_2O. Potential DNA contamination was enzymatically removed using RQ1 DNase (Promega, Southampton, UK) following the manufacturer's instructions. The RNA concentration was then determined through measuring at absorbance 260 nm.

2.3.2. Reverse transcriptase PCR.

For reverse transcriptase PCR (RT-PCR) a two stage protocol was used. The first stage involved the synthesis of cDNA. This was carried out using the M-MuLV Reverse transcriptase enzyme (Fermentas, York, UK) as per the manufacturer's instructions. For the PCR reactions 2 µl of the synthesised cDNA was then used as the template DNA.

2.4. Strain construction.

2.4.1. Construction of knockout and conditional mutants in *C. albicans.*

During this study two different approaches were undertaken for the disruption of target genes in *C. albicans*. The first approach involved the disruption of one allele of the target gene by targeted insertion of the mini-URA-blaster (figure 15A) followed by replacing the promoter of the second allele with the regulatable TET promoter (figure 15B; Nakayama *et al*., 2000; Wilson *et al*., 2000). The mini-URA-blaster cassette is a PCR amplified gene disruption cassette which consists of the *C. albicans URA3* gene

flanked by two identical 200 bp segment of the *C. albicans URA3* (*dpl200-URA3-dpl200*; Wilson *et al*., 2000). The advantage of using this cassette is that the *URA3* marker can be recycled by growing the cells on media containing 5-fluoro-orotic acid (5-FOA). This results in OMPD converting 5-FOA into 5-fluoro-uricil, which is toxic to the cells, in the presence of an intact *URA3* gene and so induces homologous recombination between the two *dpl200* sequences and results in a *ura* minus strain which is able to grow on 5-FOA. This allows the marker to be reused to disrupt the other target allele. The second approach was by gene deletion through insertion of the *C. dubliniensis HIS1* and *C. maltosa LEU2* genes into the two alleles of the target gene in the SN78 strain (Noble and Johnson, 2005). In addition epitope tags were used extensively during this project. To generate disruption and tagging cassettes the vectors were amplified by PCR using primers containing 70-80 nt of homology to the target locus (Table 5).

Confirmation of the integration of a cassette into the correct locus was carried out using the colony PCR protocol. The primers were used included one that annealed to the transformed cassette and the other that annealed to target gene outside the integration region (Table 5).

2.4.2. Construction of the *S. cerevisiae* strain carrying the *C. albicans TEM1.*

The introduction of the *C. albicans* or *S. cerevisiae TEM1* gene into the *S. cerevisiae* temperature-sensitive *tem1-3* mutant was conducted using the yeast expression plasmid system (pYES2.1, Invitrogen, Paisley, UK, Shirayama *et al*., 1994a). The *C. albicans TEM1* was amplified from genomic DNA using the CaTEM1-F and CaTEM1-R primers, gel extracted and then cloned into the pYES2.1 vector as per the manufacturer's instructions. While the *S. cerevisiae* TEM1 was amplified from genomic DNA with the ScTEM-1 and ScTEM1-R primers. Insertion into the plasmid and correct orientation was confirmed by PCR using the CaTEM1-F and V5-S primers. The plasmids were then introduced into the *tem1-3* cells by transformation and the transformed cells were selected for on SC-U media. Successful transformation was confirmed by colony PCR using the CaTEM1-F or ScTEM1-F and V5-S screening primers. An additional *tem1-3* strain was constructed that carried the pYES2.1-*lacZ* control plasmid that is provided in the kit.

2.4.3. Construction of yeast 2-hybrid strains.

Yeast 2-hybrid (Y2H) screening is a technique developed in 1989 for identifying protein-protein interactions (Young, 1998). The system relies upon the fact that transcription factors contain a binding domain (BD) and an activating domain (AD). Both domains are essential for effective transcription of a set of genes, including reporter genes such as auxotrophic markers and the β-galactosidase gene *lacZ*. To assess if two proteins physically interact each protein is fused to one of the domains. If the proteins interact the binding and activating domains act in concert to effect expression of the reporter genes, allowing researchers to identify *bona fide* interactions. To conduct our yeast-2-hybrid analysis the Matchmaker *GAL4* two-hybrid system 3 was used (Clontech, Oxford, UK). To construct the various pGADT7 and pGBKT7 vectors used in this study the *BFA1*, *BUB2*, *TEM1*, *CDC15*, *DBF2* and *MOB1* genes were PCR amplified from *C. albicans* genomic DNA using the various primers listed in the yeast-2-hybrid section of Table 5. Either an *Nde*1 or *Bam*H1 restriction site was incorporated in to each primer to allow the genes to be cloned into the pGADT7 and pGBKT7 vectors. Following PCR amplification the genes were gel purified and then cloned into either the pGEM-T-Easy (Promega, Southampton, UK) or Strataclone (Stratagene, Leicester, UK) vectors. The ORFs were then sub-cloned in frame into the digested pGADT7 and pGBKT7 vectors. Conformation of the successful cloning of the target gene into the *E. coli* vectors and the yeast-2-hybrid vectors was confirmed by carrying out a restriction digest of the plasmid and also by PCR using the reverse primer for the gene and the T7 primer.

Each vector was transformed into the AH109 *S. cerevisiae* strain and transformant colonies were selected for on synthetic complete media lacking tryptophan (pGBKT7 plasmids) or leucine (pGADT7 plasmids). A second round of transformation was carried out on the strain containing one of the six pGBKT7 plasmids to transform the various pGADT7 plasmids. These transformations resulted in thirty six different strains containing the different plasmid combinations. A positive control strain of AH109 carrying the pGADT7-t and pGBKT7-53 plasmids was also constructed.

2.4.4. Yeast 2-hybrid growth assay.

A colony from the streak plate of each of the yeast-2-hybrid strains was selected and

used to inoculate 100 μl ddH$_2$O in a 96 well plate. Cells were spotted, using a 48 prong replicator, onto synthetic complete media lacking a combination of tryptophan, leucine, histidine or adenine (SC –trp/leu/his/ade, SC –trp/leu). Plates were incubated at 30˚C for 3-4 days.

2.4.5. Cell viability (Miles and Misra).

The effects of Tem1 depletion were studied using a series of time courses. Cells from overnight cultures, of the strains being assayed, were added to 50 ml of YPD (+/- 20 μg/ml doxycycline) to an OD$_{600}$ of 0.05 and incubated at 30 ˚C 200 rpm. To test the viability samples of the SWM6 and CAI4 (CIp10) cultures were taken at 24 hours post inoculation, harvested, washed with PBS, and the number of cells counted using a haemocytometer. 1 x 10^5 cells/ml were then added to 2 ml of fresh YPD. Four 10 times dilutions were then carried out and the entire 1 ml of each dilution were spread on to a YPD plate which was then incubated at 30 ˚C for 2 days. Each dilution was carried out in duplicate. Following the incubation the number of colonies was counted and the percentage viability of each strain in YPD and YPD plus doxycycline was calculated.

2.4.6. Virulence testing.

The CAI4 (CIp10), SWM6, SWM72, SWM84 and SWM95 strains were grown to mid-log phase in YPD. The cells were the harvested and washed three times with PBS before being normalised to a cell concentration of 1 x 10^7 cells/ml in PBS. 10 μl of this cell suspension was then injected into *Galleria mellonella* larvae (Livefoods UK, Sheffield, UK) through the last pro-leg (Morton *et al*., 1987; Cotter *et al*., 2000). 10 larvae were infected for each strain used. 10 larvae were also inoculated with 10 μl of sterile PBS as a control. The infected larvae were placed in petri dishes with filter paper inserts and incubated at 37 ˚C in the dark for up to 96 hours. Every 24 hours the numbers of live and dead larvae were counted.

2.5. Spot tests.

2.5.1. Gene essentiality study.

The CAI4 (CIp10) and SWM6 strains were grown in YPD media for 5 hours. Cell concentrations were normalised and spotted onto YPD, SC-U plates or YPD plates

containing either 5, 10, 15 or 20 % foetal calf serum (FCS), in the presence or absence of 20 µg/ml doxycycline using a 48 prong replicator. The YPD and SC-U plates were incubated at 30 ˚C for 2 days while for the YPD plus serum plates were incubated at 37 ˚C for 4 days.

2.5.2. *S.cerevisiae* homolog study.

The *tem1*-3, SWM40, SWM81 and SWM83 *S. cerevisiae* strains were grown in YPD media overnight. The cells numbers were normalised and spotted on to the required agar plates using a 48 prong replicator. The types of agar plates used for the *S. cerevisiae* ortholog study were YPD or YPGal (1 % (w/v) yeast extract, 2 % (w/v) mycological peptone, 2 % (w/v) agar number 2 and 2 % (w/v) galactose) plates. Plates were incubated at 30 ˚C for 2 days.

2.5.3. Cell wall sensitivity.

C. albicans strains were grown overnight at 30 ˚C. The cells were then diluted in ddH$_2$O to a cell density of 1×10^5 cells/ml. Further 10 fold dilutions were carried out in a 96 well plate. The cells were then spotted onto YPD plates containing specific compounds using a 48 prong replicator. The compounds used were calcofluor white, Congo red (at concentrations of 100 or 250 µg/ml), SDS (at concentrations of 0.005 or 0.01 %) and NaCl (at 1M or 2M concentrations) in the presence or absence of 0.625 µg/ml doxycycline. Plates were incubated at 30 ˚C for 2 days.

2.6. Protein techniques.

2.6.1. Soluble protein extraction.

For protein extractions cells, from 50 ml cultures were harvested by centrifugation at 4000 rpm for 5 minutes. For storage cell pellets were frozen at -80 ˚C. When required the pellets were thawed and the cells washed in 10 ml ice cold ddH$_2$O and re-pelleted at 4000 rpm for 5 minutes at 4 ˚C. Following pelleting the cells were washed with 1 ml of breaking buffer (100 mM Tris pH7.5, 0.01 % (w/v) SDS, 1 mM dithiothreitol (DTT), 10 % (v/v) Glycerol, 1 mM EDTA, plus Protease Inhibitors (Roche, Welwyn Garden City UK)) and transferred to a screwtop microcentrifuge tube where the cells were re-pelleted at 14000 rpm for 5 minutes at 4 ˚C. The cells were then resuspended in 250 µl breaking buffer and a pellet sized quantity of 0.45-0.52 µm glass beads was added.

The cells were then disrupted in a FastPrep machine (MP Biomedicals, Illkirch, France) four times at 6.5 speed for 20 seconds, with a 1 minute incubation on ice between disruptions. The cell debris and beads pelleted at 13000 rpm for 10 minutes at 4˚C. The protein extract was then clarified by transferring the supernatant to a new screwtop microcentrifuge tube and subjecting to centrifugation at 13000 rpm for a further 10 minutes at 4 ˚C. Protein concentrations were then determined by carrying out a Bradford assay (Sigma Aldrich, Gillingham, UK).

2.6.2. Western blotting.

Prior to analysis by western blotting 50 µg of Tem1-V5-6xHis protein was precipitated using nickel-nitrotriacetic acid agarose beads (50 µl, QIAGEN, Crawley, UK). The protein/beads mixture was incubated at 4 ˚C for 45 minutes on a rocker. The beads were then washed six times with breaking buffer, sample buffer added and the enriched proteins released from the beads by centrifuging the beads at 13000 rpm for 5 minutes. The enriched protein was then prepared for electrophoresis by adding 1 x sample buffer (Invitrogen, Paisley, UK) and 25 mM DTT and incubated at 70 ˚C for 10 minutes. Following the 70 ˚C incubation the proteins were separated on a NuPAGE 4-12 % gradient gel (Generon, Maidenhead, UK) in Tris-HEPES-SDS running buffer (100 mM Tris, 100 mM HEPES, 3 mM SDS). Following electrophoresis the proteins were transferred to a methanol activated PVDF membrane in transfer buffer (0.303 % (w/v) Tris base, 1.44 % (w/v) glycine, 20 % (v/v) methanol), using the X cell blot module (Invitrogen, Paisley, UK). The transfer of the proteins to the membrane was confirmed by staining the membrane with Ponceau S (Sigma-Aldrich, Gillingham, UK) as per the manufacturer's instructions. Subsequent to Ponceau staining the membrane was washed with tris buffered saline containing Tween-20 (TBS-T, 10 mM Tris-HCl pH 8, 137 mM NaCl, 0.1 % (v/v) Tween-20) and blocked in TBS-T + 5 % (w/v) BSA for 2 hours at room temperature. The blocked membrane was probed with an anti-V5 primary antibody (1/1000 dilution in TBS-T+BSA, Invitrogen, Paisley, UK) overnight at 4 ˚C and then washed with TBS-T four times prior to a one hour incubation at room temperature in TBS-T+BSA containing a 1/10000 dilution of horseradish peroxidase labelled anti-mouse secondary antibody (Cell Signalling Technologies, Hitchin, UK). Following this incubation the membrane was washed four times with TBS-T and the relevant protein bands visualised using the Lumiglo system (Cell Signalling

Technologies, Hitchin, UK) and a G box transilluminator (Syngene, Cambridge, UK).

2.6.3. *HEX1* assay.

The *HEX1* assay documented here is a modified version of the β-N-acetylglucosaminidase assay used by Cannon *et al*., (1994). In this assay the CAI4 (CIp10) and SWM152 strains were grown in both SC-GlcNAc (0.67 % (w/v) yeast nitrogen base, 0.077 % (w/v) complete supplement mixture, 25 mM N-Acetylglucosamine) and SC overnight at 30˚C and the proteins were extracted using the soluble protein extraction method. 10 µl of the protein sample was mixed with 390 µl of the substrate solution (1.25 mM pNitrophenol-GlcNAc in 0.1M citrate/KOH buffer, pH 4) and these reactions were incubated at 37˚C for 30 minutes. Following the incubation period the reactions were stopped by the addition of 1 ml of 1M Na$_2$CO$_3$. The absorbance of these reactions were measured at 420 nm and the concentration of nitrophenyl released was calculated using a nitrophenyl standard curve. The Hex1 activity was calculated as nmol pNP released/minute/mg protein.

2.7. Cell morphogenesis assay.

2.7.1. Liquid morphogenesis assays.

C. albicans cells were grown overnight in YPD media. To induce hyphal development 1 x 10^6 cells/ml were added to pre-warmed FCS media (5, 10, 15 and 20 % FCS in YPD) and the culture incubated at 37 ˚C in a static incubator. To induce pseudohyphal development 1 x 10^6 cell/ml were added to pre-warmed YPD media and the incubated at 37 ˚C in a static incubator. 1 ml samples were taken at set intervals and fixed using 100 % ethanol.

2.7.2. Spider media.

For growth on Spider media *C. albicans* cells were grown in YPD overnight and then normalised to a cell number of 1 x 10^9 cells/ml in ddH$_2$O. 5 µl sample of this cell suspension was then spotted by pippetting on to a solid Spider medium plate (1 % [w/v] nutrient broth, 1 % (w/v) mannitol, 0.2 % (w/v) di-phosphate hydrogen orthophosphate anhydrous, 2 % (w/v) purified agar, Liu *et al*, 1994). Plates were then incubated at 30 ˚C for between 4 and 9 days.

2.7.3. Lee's media.

C. albicans cells were grown overnight in YPD. The cells were normalised to a cell concentration of 1 x 10^5 cells/ml and 1/10 dilutions carried out in a 96 well plate. Cells were then spotted on to Lee's media plates (5 % (w/v) ammonium sulphate, 0.2 % (w/v) magnesium sulphate, 2.5 % (w/v) potassium hydrophosphate (anhydrous), 5 % (w/v) NaCl, 12.5 % (w/v) Glucose, 0.01 % (w/v) Biotin, 0.05 % (w/v) L-alanine, 0.05 % (w/v) L-leucine, 0.05 % (w/v) L-lysine, 0.01 % (w/v) L-methionine, 0.0071 % (w/v) L-ornithine, 0.05 % (w/v) L-proline and 0.05 % (w/v) L-threonine). Plates were incubated at 30˚C for 4 days.

2.8. Microscopy.

2.8.1. Fluorescence microscopy.

C. albicans cells were fixed with 70 % ethanol for 30 minutes and then washed three times with phosphate buffered saline (PBS). To visualise the nuclei and septa in these cells, 1 x 10^6 cells/ml were incubated in 1 μg/ml 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 40 min and 5 μg/ml calcofluor white (both Sigma-Aldrich, Gillingham, UK) for 30 min and then washed five times with PBS. Cells for differential interference contrast microscopy (DIC) were fixed not stained. Cells for protein localisation analysis were not fixed to preserve Gfp activity. 5 μl of cells were placed on a glass microscope slide and were visualised using an Olympus IX81 epifluorescence microscope utilising Olympus mirror units (Olympus, Southend-on-Sea, UK). The fluorescent mirrors used in this study were Gfp (Excitation (Ex) 460-480 nm, Emission (Em) 495-540 nm), Cfp (Ex 435-445 nm, Em 460-510 nm), Yfp (Ex 490-500 nm, Em 515-560 nm), Rfp (Ex 535-555 nm, Em 570-625 nm), DAPI (Ex 400-440, Em 475 nm). Images were acquired using a CoolSnap HQ^2 camera (Q imaging, Newcastle-under-Lyme, UK) and Metamorph 2 imaging software. Measurements were taken using the Image J software (http://rsbweb.nih.gov/ij/).

2.8.2. Scanning electron microscopy.

For SEM analysis the SWM6 strain was grown in 10 ml of YPD (+/- doxycycline) for 24 hours. The cells were then harvested by centrifugation at 6000 rpm for 3 minutes and fixed overnight at 4 ˚C in 3 % glutaraldehyde (Sigma-Aldrich, Gillingham, UK) in 0.1 M cacodylate buffer pH 7.2 (0.2 M (w/v) $(CH_3)_2AsO_2Na.3H_2O$, 0.2 % (v/v) HCl, 3 % (w/v)

sucrose, 0.1 % (w/v) CaCl). Following fixation the cells were washed three times with 0.1M cacodylate buffer for 10 minutes each time. After the last wash the cells were resuspended in 1 % osmium tetraoxide (Sigma-Aldrich, Gillingham, UK, in 0.1M cacodylate buffer) and incubated overnight at 4 ˚C. The samples were dehydrated by successive 10 minutes washes in 30 %, 50 %, 75 % and 90 % ethanol and then washed twice in 100 % ethanol solutions. The cells were adhered to 25 mm membrane filters (Osmonic Inc, Minnetonka, USA) and placed in baskets for the critical point dryer (E3000, Polaron Equipment Ltd, Watford, UK) and held in 100 % ethanol. The samples were then dried by substitution of the 100 % ethanol with liquid carbon dioxide over two hours while the chamber temperature and pressure was increased to 34-35 ˚C and 1200 psi respectively. Following drying the samples were transferred onto carbon tabs (Agar Scientific, Stansted, UK) which were placed on to Jeol SEM stubs and then coated with gold palladium using a VG microtech SC510 sputter coater (Fisons Instruments, Altrincham, UK). After coating the samples were analysed using a Jeol JSM 6390LV scanning electron microscope (Jeol UK, Welwyn Garden City, UK) operating at 10kV.

2.8.3. Transmission electron microscopy.

For TEM analysis, the SWM6 strain was grown in 10 ml of YPD (+/- doxycycline) for 24 hours. The cells were harvested by centrifugation at 6000 rpm for 3 minutes. The samples were then frozen at -182 ˚C in a RMC-JF-8000 propane jet freezer (Boeckeler, Tucson, USA). The samples were freeze-substituted using a RMC FS-7500 (Boeckeler, Tucson, USA) with 0.1 % uranyl acetate plus 0.1 % glutaraldehyde in 100 % dried acetone for 3 days at -90 ˚C and then 24 hours at -30 ˚C. Next the samples were washed with 100 % dried acetone four times and then infiltrated with increasing concentrations of Lowicryl HM20 resin (Polysciences, Northampton, UK, 2:1, 1:1, 1:2 and 100 % acetone to resin ratio) all carried out at -30 ˚C. Following an additional infiltration in 100 % resin overnight the samples were polymerized in BEEM capsules at -30 ˚C in a Cryocut E machine (Reichert-Jung, Seefeld, Germany). A final polymerisation at room temperature for two days was then carried out. Sections of the samples were then cut to a thickness of 60-80 nm with a diamond knife and put on a formvar coated copper grid and analysed using a Jeol JEM 1400 electron microscope (Jeol UK, Welwyn Garden City, UK) operating at 80 kV.

2.9. Bioinformatics.

2.9.1. Identification of putative *Candida albicans* homologs of *Saccharomyces cerevisiae* genes.

To identify putative *C. albicans* homologs of *S. cerevisiae* genes the sequences of target genes were retrieved from the *S. cerevisiae* genome database (www.yeastgenome.org) and entered into the BLAST tool on the *Candida* genome database (www.candidagenome.org; Arnaud *et al*., 2007). Where a *C. albicans* gene had already been annotated as the relevant homolog this was accepted. Where this was not the case the *C. albicans* gene, in the list of candidates returned by the BLAST tool, with the greatest similarity to the reference *S. cerevisiae* gene was selected.

2.9.2. Sequence alignment of *S. cerevisiae* and *C. albicans* proteins.

The *S. cerevisiae* and *C. albicans* protein sequences were retrieved from the genome database by selecting the ORF translation link on the 'retrieve sequence' dropdown menu located on the appropriate locus pages for each genome databases. The protein sequences were aligned using the Align program on the European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

2.9.3. Analysis of the Tem1 protein domains.

To identify protein domains and their location within the Tem1 protein the protein sequence of Tem1 was submitted to the conserved domain database program (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). To establish whether the Tem1 protein contains either a PEST or nuclear localisation sequence (NLS) the PESTfind (https://rostlab.org/owiki/index.php/PredictNLS)   and   NLS   predict (http://emboss.bioinformatics.nl/cgi-bin/emboss/pestfind) programs were used.

2.9.4. Tem1 phylogeny construction.

To construct the phylogenetic tree of the Tem1 protein across the fungal and plant kingdoms the homologs were identified using the gene by gene phylogeny pipeline program (Richards *et al*., 2009) using the *S. cerevisiae* Tem1p sequence as the query sequence (conducted by Dr. Darren Soanes). The pipeline facilitates BLAST searches of full genome sequences of six plants, five green algae and forty six fungal species. Additional yeast and plants sequences were retrieved using the protein BLAST tool on

the NCBI website with the *S. cerevisiae* Tem1 as the query sequence. All the protein sequences were aligned using the MUSCLE program on the EBI website (http://www.ebi.ac.uk/Tools/msa/muscle/). The MUSCLE program builds this multiple alignment in three stages. A tree is built, which is based on a pairwise distance matrix and then improved using the more accurate Kimura distance calculations. Finally the tree is split in two and the central and deleted sections realigned. This is repeated for each branch and sub-branch, starting at the edge and working in and re-alignments, which increase the score retained. A refined tree is produced which incorporates the improved alignments. To generate the phylogeny the aligned sequences were run through the PhyML program located on the phylogeny website (http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=phyml). The PhyML program generates phylogenies and branch lengths using maximum likelihood. The program works by constructing an initial tree from the aligned sequences and then running a series of refinement steps in which the tree likelihood increase until an optimum is reached. To visualise the phylogeny the newick file generated by PhyML was then used to construct a tree using the TreeDyn software also located on the phylogeny website (http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=treedyn).

Chapter 3-Characterisation of the *Candida albicans* GTPase *TEM1.*

3.1. Introduction.

The mechanisms that control the completion of mitosis in budding yeast are well understood. Integral to this is the role the Tem1 protein plays. The *S. cerevisiae TEM1* gene was first discovered during a *S. cerevisiae* genomic library screen to isolate genes that suppressed the cold sensitivity of an *lte1* mutant (Shirayama *et al.*, 1994b). Epistasis experiments have identified Tem1 as the GTPase protein which controls the initiation of the mitotic exit network. Tem1 carries out this role in signalling mitotic exit by recruiting the kinase Cdc15 to the spindle pole body and activating it (Cenamor *et al.*, 1999; Xu *et al.*, 2000; Menssen *et al.*, 2001; Visintin and Amon, 2001). Disruption of the Tem1 protein in *S. cerevisiae* by growing a temperature sensitive *tem1* mutant at restrictive temperatures results in arrest of the cells in telophase with divided nuclei (Shirayama *et al.*, 1994b).

A number of regulatable promoter systems are currently available for use in *C. albicans*, such as *GAL1*, *MAL1*, *PCK1* and *MET3* (Gorman *et al.*, 1991; Leuker *et al.*, 1997; Care *et al.*, 1999; Backen *et al.*, 2000). In this chapter we used the tetracycline regulatable promoter (TET-off promoter, Nakayama *et al.*, 2000) to construct a regulatable *TEM1* mutant. The TET promoter system consists of two main components; a transactivator domain and an operator domain. The transactivator domain is a fusion protein consisting of the *Escherichia coli* tetracycline repressor protein (TetR) and the activation domain of the *S. cerevisiae HAP4* transcriptional activator. The THE1 *C. albicans* strain was constructed to contain a copy of the transactivator domain integrated into the *ENO1* locus (Nakayama *et al.*, 2000). The operator domain consists of the *tetO* sequences and the *S. cerevisiae ADH1* terminator and is carried on the pCAU99t plasmid. The function of the *ADH1* terminator is to prevent any read-through from the native promoter sequences. The system functions by the binding of the TetR protein to *tetO* in the absence of tetracycline resulting in the expression of the target gene. When tetracycline binds to TetR it results in conformational changes in the TetR protein which prevents it from binding to *tetO*

therefore preventing expressing of the gene. This system allows for easy regulation of the expression of a gene of interest.

In this chapter we identified an ORF 46.5 % identical to the *S. cerevisiae TEM1* gene. We show through complementation analysis that this ORF is an ortholog of the *S. cerevisiae TEM1* gene. To assess the role *TEM1* plays in *C. albicans* we constructed a regulatable *TEM1* mutant strain and used this strain to study the effects of Tem1 depletion on growth, nuclear division and cytokinesis. Our results show that *TEM1* is essential in *C. albicans* and that Tem1 depletion results in the cells arresting in late anaphase with divided nuclei prior to the formation of highly polarised growth filaments which fail to undergo cytokinesis. Eventually Tem1 depleted cells were able to bypass the mitotic block, however they fail to undergo cytokinesis and lose viability.

3.2. Results.

3.2.1. Bioinformatics.

3.2.1.1. Identification of the *C. albicans TEM1* homolog.

To identify the homolog of the *S. cerevisiae TEM1* gene in the *Candida albicans* genome the *S. cerevisiae* Tem1 protein sequence, retrieved from the yeast genome database (www.yeastgenome.org), was entered into the BLAST tool on the *Candida* genome database (www.candidagenome.org; Arnaud *et al*., 2007). This search identified the best hit to be orf 19.3001 which had previously been annotated as *TEM1* (Bachewich *et al*., 2005). Alignment of the *S. cerevisiae* and *C. albicans* Tem1 protein sequences demonstrated that the CaTem1 protein is 49.1 % identical to the ScTem1 gene with a highly conserved central region (Figure 13A).

3.2.1.2 Analysis of the *CaTEM1* protein sequence.

Following the identification of the *TEM1* gene in the *Candida* genome, the DNA and protein sequences were analysed further. The *C. albicans TEM1* ORF, on chromosome 1, comprised of 681 nucleotides and contains an intron of 77 nucleotides. The gene encodes a putative protein that is 200 amino acids long (CaTem1) and when aligned with the *S. cerevisiae* Tem1 (ScTem1), using the align tool on the European Bioinformatic Institute website (www.ebi.ac.uk), is highly conserved through the majority of the protein except for the carboxyl terminal end where the *CaTEM1* has a

A)

```
                                               *******
S.cTEM1    1 MATPSTGANNSIPAVRNQVEVQVGLVGDAQVGKTSLMVKYVQNIYDKEYT
             |...:....|       |||.:::||:||:|:||||||||||:..:|::|.
C.aTEM1    1 MEEDNQQDQN------NQVALKIGLIGDSQIGKTSLMVKYVEGSFDEDYI

                                              ****
S.cTEM1   51 QTLGVNFLKRKVSIRSTDIIFSIMDLGGQREFINMLPIATVGSSVIIFLF
             |||||||:..:|:.||:|.|.|||.|||||:|||||||:..:..:..|:|:|
C.aTEM1   45 QTLGVNFMDKKIQIRNTTITFSIWDLGGQKEFINMLPLVSNDAVAILFMF

                                             ****
S.cTEM1  101 DLTRPETLSSIKEWYRQAYGLNDSAIPILVGTKYDLLIDLDPEYQEQISR
             ||||..||:|||||||||..|.|.:|||.||||||||..|||...:|.:|::
C.aTEM1   95 DLTRKSTLNSIKEWYRQVRGFNKTAIPFLVGTKYDQFIDLSFQDQIEITQ

S.cTEM1  151 TSMKYAQVMNAPLIFCSTAKSINIQKIFKIALAKIFNLTLTIPEINEIGD
             .:.|:...|.||:|||||:.|||:|||||.|:|.|:|.|.:.||..:|:
C.aTEM1  145 QAKKFGSAMKAPVIFCSTSHSINVQKIFKIILSKAFDLKLNLDEIVNVGE

S.cTEM1  201 PLLIYKHLGGQQHRHHNKSQDRKSHNIRKPSSSPSSKAPSPGVNT
             |:||||
C.aTEM1  195 PILIYK---------------------------------------
```

B)

Figure 13. Bioinformatics of the *C.albicans* Tem1 protein.

(A) An alignment of the *S. cerevisiae* Tem1 and *C. albicans* Tem1 proteins. * denotes the consensus GTP-binding domains. (B) A domain map of the *C. albicans* Tem1 proteins showing the presence of many domains, generated using the conserved domain database software (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The red pyramids indicate each amino acids within a domain motif.

39 amino acid truncation (Figure 13A). The *CaTEM1* protein also contains three GTP/Mg^{2+} binding domains; GDAQVGK (amino acids 21-27), DLGG (amino acids 69-72) and TKYD (amino acids 126-129, shown by the * on Figure 13A), which fit with the consensus GTP-binding sequences documented by Dever *et al.*, (1987).

To investigate other potential domains that the CaTem1 protein possesses the protein sequence was entered into the conserved domain database (CDD, www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, Figure 13B). The results show that the protein contains multiple domains which are suggested to be involved in protein interactions. The majority of these domains are located within the G domain which is highly conserved in all Ras superfamily GTPases. The G domain also contains all three consensus GTP-binding domains (Wennerberg *et al.*, 2005). These GTP-binding domains also overlap with the G1-G5 box domains which are important for GDP/GTP switching, GTP-induced conformational changes and GTP hydrolysis in other Ras family GTPases (Bourne *et al.*, 1991). The CaTem1 protein also contains multiple putative guanosine exchange factors (GEF) and guanosine nucleotide dissociation inhibitor (GDI) binding domains which are most likely involved in the activation and inactivation of the protein.

In all Ras super family GTPase proteins the binding of either GTP or GDP to the protein results in conformational changes in two domains, the switch I and switch II regions, and it is these changes that regulate the interactions between the GTPase protein and its effector proteins and hence these could be the Cdc15 interaction domains (Figure 13B; Bishop and Hall, 2000). Analysis of the CaTem1 protein sequence (NLS predict software https://rostlab.org/owiki/index.php/PredictNLS) found no nuclear localisation sequence but identified a region with weak homology to a PEST sequence (pestfind software, http://emboss.bioinformatics.nl/cgi-bin/emboss/pestfind).

3.2.1.3. The *Ca*Tem1 protein clusters with the other ascomycete yeast species.

To establish the relationships among various Tem1 protein homologs through the fungal and plant kingdoms we constructed a phylogenetic tree of these proteins (Figure 14). The phylogenetic tree shows the evolutionary relationships between a group of species or gene sequences. These relationships are derived from similarities
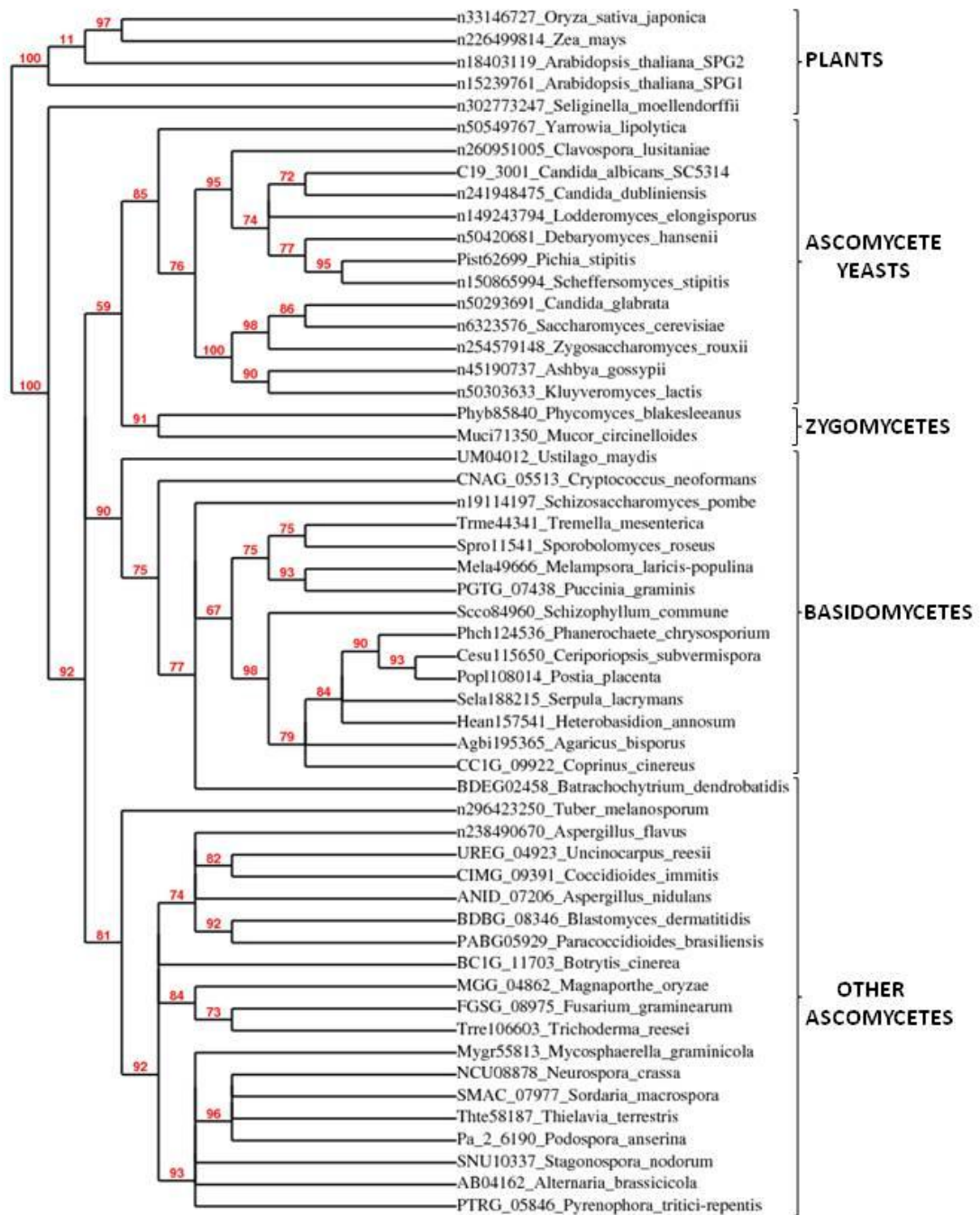
Figure 14. Phylogenetic tree of the Tem1 homologs within the plant and fungal kingdoms. Tem1 homologs were identified using the gene phylogeny pipeline (Richards *et al.*, 2009) and the protein BLAST tool on the NCBI website. The protein sequences were then aligned using the MUSCLE program and the phylogeny generated using the PhyML software which uses maximum likelihood instead of maximum parsimony. The tree was drawn using the TreeDyn program. The bootstrap values represent the maximum likelihood generated by the PhyML program.

and differences in the genetic and physical characteristics of these sequences. We identified putative Tem1 homologs within the genomes of 4 plant and 51 fungal species. From the phylogenetic tree (Figure 14) a number of observations can be drawn about these putative Tem1 homologs. Firstly, the tree of *TEM1* homologs largely conforms with studies of the evolutionary origins of the different species. Secondly, the plant homologs form the major out group of the tree with the exception of *Selaginella moellendorffii*, which diverges from the fungi later than the flowering plants. *Arabadopsis thaliana* possesses two homologs of the *TEM1*, *SGP1* and *SGP2*, which are thought to have arisen as a result of a genome duplication event (Bedhomme *et al.*, 2008).

It was also interesting that the ascomycete phylum was split into two groups, the yeast species and the other species. The yeast group itself split into two further groups, one containing species like *Saccharomyces cerevisiae* and *Ashbya gossypii* (*Saccharomyces* group) and the other containing species like *Candida albicans* and *Debaryomyces hansenii* (CTG group). The major difference between these two groups is that the Tem1 proteins of *Saccharomyces* group species have a C' terminal extensions of between six and thirty seven amino acids. The role this extension plays has yet to be investigated.

Surprisingly only two zygomycete species, *Phycomyces blakesleeanus* and *Mucor circinelloides*, possess homologs of the *S.cerevisiae TEM1* and these cluster closely with the various yeast species. This finding was not expected as zygomycete species normally branch off phylogenetic trees just prior to the divergence of the dikarya groups,the basidiomycetes and the ascomycetes (James *et al.*, 2006). Between the two groups of ascomycete fungi the basidiomycete fungi branched from the main tree. This finding might suggest differences in the role of the Tem1 proteins in the two groups.

3.2.1.4. Analysis of the anaphase signaling pathways

To evaluate the levels of conservation of the two anaphase signaling pathways described in budding yeast, the Cdc14 early anaphase release pathway (FEAR) and the mitotic exit network (MEN), a series of alignments of the proteins that are involved in

A)

Gene	Identify	Similarity	Gaps
ESP1	23.0 %	40.0 %	26.8 %
SLK19	15.8 %	28.2 %	45.6 %
ZDS1	17.4 %	26.6 %	51.9 %
ZDS2	-	-	-
CDC5	49.9 %	65.4 %	8.7 %
SPO12	15.5 %	25.7 %	49.7 %
FOB1	-	-	-

B)

Gene	Identity	Similarity	Gaps
LTE1	22.1 %	37.1 %	34.0 %
BFA1	21.1 %	34.0 %	34.7 %
BUB2	40.7 %	56.0 %	13.3 %
TEM1	46.5 %	62.0 %	18.4 %
CDC15	20.8 %	33.5 %	39.7 %
DBF2	45.1 %	59.2 %	20.4 %
MOB1	40.2 %	52.5 %	28.9 %
NET1	19.8 %	33.7 %	35.8 %
CDC14	49.5 %	64.3 %	11.6 %

Table 6. *C. albicans* does not possess a functional FEAR network. Results from the alignment comparison of the components of the FEAR (A) and MEN (B) pathways in *S. cerevisiae* and the *C. albicans* homologs carried out using the Align software on the European Bioinformatics Website (this software has now been replaced with the Pairwise Sequence Alignment software http://www.ebi.ac.uk/Tools/psa/). Dashes indicate that no alignment was carried out as no homolog was found within the *C. albicans* genome. Full protein alignments are shown in Appendix 1. The percentage identity and similarity shows the level of identical or similar amino acids at each position within the two aligned sequences. The percentage gaps refer to a single space or run of spaces in one sequence of the alignment. Gaps are caused by a number of events such as DNA slippage during replication, a single insertion or deletion mutation or the translocation of DNA between chromosomes.

each pathway in *S. cerevisiae* and the *C. albicans* homologs or best hits were conducted. Within the FEAR network proteins, Esp1 and Cdc5, are conserved between *S. cerevisiae* and *C.albicans* (Table 6A). However, both are known to have FEAR-independent roles in the cell cycle (Kitada, *et al*., 1993; Sullivan and Uhlmann, 2003).

The other proteins in the FEAR network have only distant homologs (*ZDS1* and *SPO12*), are not involved in mitosis (Slk19) or lack homologs altogether (*ZDS2* and *FOB1*; Galán *et al.*, 2004). This result suggests that *C. albicans* may not possess a FEAR network which fits with the differential localization of Cdc14 in *S. cerevisiae* and *C. albicans*.

The proteins within the MEN are relatively well conserved through-out the pathway with the exception of Net1. The role of Net1 in *C. albicans* is however questionable as *CDC14* is not essential for cell viability and the Cdc14 protein localizes to the nucleus throughout the majority of the cell cycle and not the nucleolus as in *S. cerevisiae* (Table 6B, Clemente-Blanco *et al.*, 2006). The presence of all important proteins involved in the MEN suggests that the pathway is intact in *C. albicans*. However these proteins are also homologs of the proteins involved in the septation initiation network in *S. pombe* so the *C. albicans* proteins could be involved in the signaling of septation and play no role in mitosis.

Gene disruption studies suggest the latter may be true as the Cdc14 protein is not essential in *C. albicans*. Therefore it appears that alternative signaling results in mitotic exit.

3.2.2. The *C. albicans TEM1* is a functional homolog of the *S.cerevisiae TEM1*.

To further investigate the relationship between the *ScTEM1* and *CaTEM1* genes the *CaTEM1* gene was expressed in a *S. cerevisiae* temperature sensitive *tem1-3* mutant (Corbett *et al.*, 2006) which had been constructed using oligonucleotide-directed *in vitro* mutagenesis. When grown at 37 ˚C the *tem1-3* cells arrest in anaphase as large dumbbells with divided nuclei (Shirayama *et al.*, 1994b). To express the *CaTEM1* gene in the *tem1-3* cells the yeast expression system plasmid (pYES2.1, Invitrogen, Paisley, UK) was chosen and the *CaTEM1* gene was PCR amplified from CAI4 (CIp10) genomic DNA, gel extracted and cloned into the pYES2.1 vector (Table 4 and 5). The cloning of the gene into this plasmid places gene expression under the control of the galacatose-inducible *GAL1* promoter. In addition a second plasmid was constructed containing the *ScTEM1* gene (Table 4 and 5). The successful insertion of the genes into the plasmid was confirmed by PCR using the V5-S primer and the forward primer used for amplifying the gene from the genomic DNA (Table 5). These two plasmids and the

manufacturer's expression control plasmid, pYES2.1-*LacZ,* were transformed into the *tem1-3* strain and the colonies were screened by colony PCR. Multiple versions of each strain were constructed and assayed but for presentation purposes we used the SWM81 (*tem1-3* pYES2.1-*CaTEM1*), SWM40 (*tem1-3* pYES2.1-*ScTEM1*) and SWM83 (*tem1-3* pYES2.1-*LacZ*) strains (Figure 15A and B). Following the construction of these strains they were spotted onto both YPD and YPgalactose agar plates and incubated at either 30 ˚C or 37 ˚C for two days. The BY4741 (wild type) and *tem1-3* strains were also spotted on to these plates as controls. At 37 ˚C neither the *tem1-3* or SWM83 strains grew, which confirms that the introduction of the pYES2.1 plasmid cannot complement the temperature sensitive defect of the *tem1-3* strain. At 30 ˚C all the strains grew, on both media, but when incubated at 37 ˚C the wild type strain BY4741, the SWM40 and SWM81 strains grew including on repressing media (Figure 15C). These results show that the introduction of either the *CaTEM1* or *ScTEM1* genes can recover the temperature sensitive defect of the *tem1-3* strain, therefore *CaTEM1* can be regarded as a true homolog of the *ScTEM1* gene. This growth of the SWM40 and SWM81 strains on YPD at 37 ˚C was surprising as expression of the genes should have been repressed by the glucose in the media (Figure 15C). However this expression system is known not to be under tight control. The growth on glucose also shows that at presumably low levels of *CaTEM1* expression growth is supported which further confirms that *CaTEM1* is a true functional *ScTEM1* homolog.

3.2.3. Construction of a regulatable *C. albicans TEM1* mutant.

The above results show that the *C. albicans* Tem1 protein is a functional homolog of the *ScTEM1*, therefore, as the *S. cerevisiae TEM1* gene is essential for cell viability we hypothesised that the *C. albicans TEM1* gene could also be essential. As a result we constructed a strain with *TEM1* under the control of a regulatable promoter. To carry this out the tetracycline-regulatable promoter was used (Nakayama *et al*., 2000). The THE1 strain was used as it expresses the transactivator protein, *Sc*Hap4. To construct the regulatable *TEM1* mutant strain two separate rounds of transformations were carried out. The first round of transformation involved transforming the *TEM1*-targeted mini URA blaster cassette, amplified from the pDDB57 plasmid using the TEM1-KO-F and TEM1-KO-R primers, into the strain, THE1. This transformation disrupts one of the two *TEM1* alleles (Table 5; Figure 16A). Four *URA3* positive
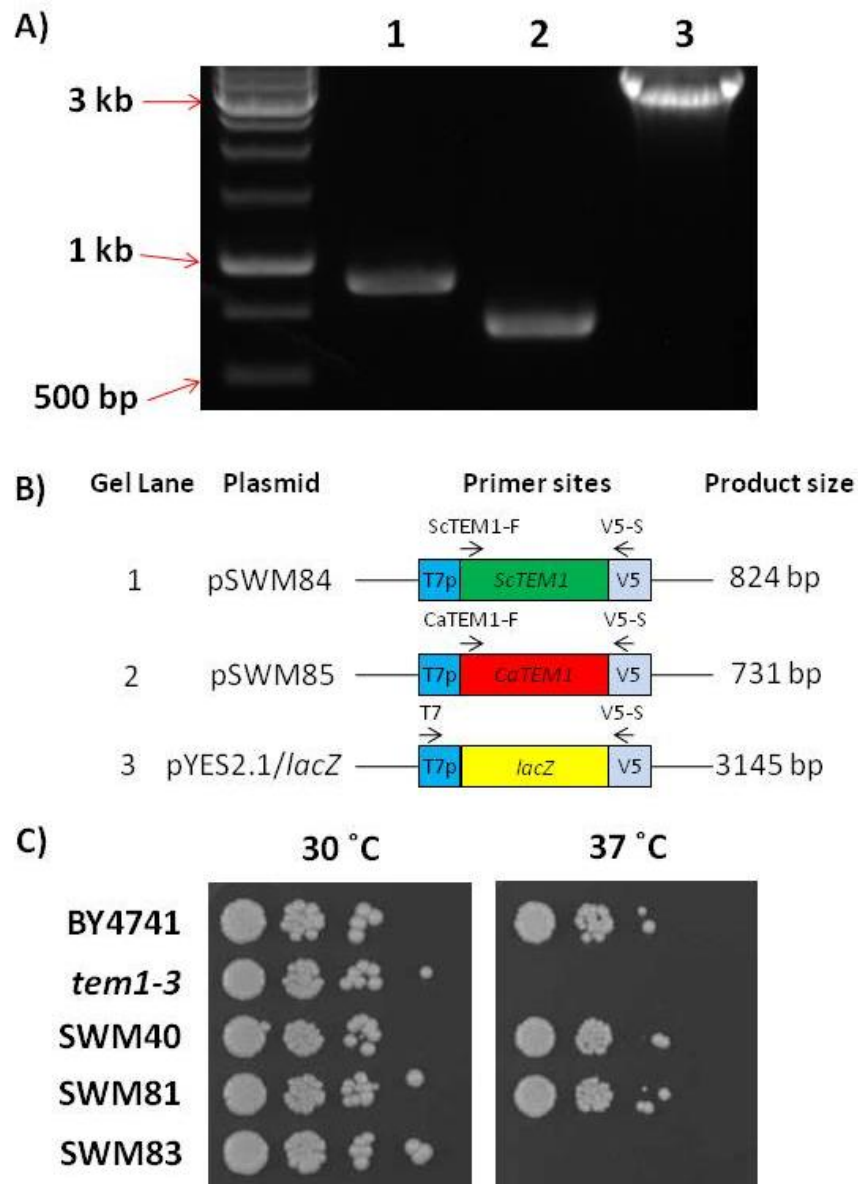
Figure 15. Construction and analysis of the SWM40, SWM81 and SWM83 strains. (A) Colony PCR conformation of the successful transformation of the *tem1-3* strain with either pYES2.1-*S.cTEM1* (lane1), pYES2.1-*C.aTEM1* (lane2) or pYES2.1-*lacZ* (lane 3) plasmids. The primers used in the PCR reactions were ScTEM1-F and V5-S primers (lane 1), CaTEM1-F and V5-S primers (lane 2), and the T7 and V5-S primers (lane 3). Primer annealing sites and predicted PCR product sizes (B). At 30 ˚C all the tested strains; BY4741, *tem1-3*, SWM40, SWM81 and SWM83, grew while at 37 ˚C only the strains containing a functional *ScTEM1* or *CaTEM1* gene; BY4741, SWM40 and SWM81, grew.
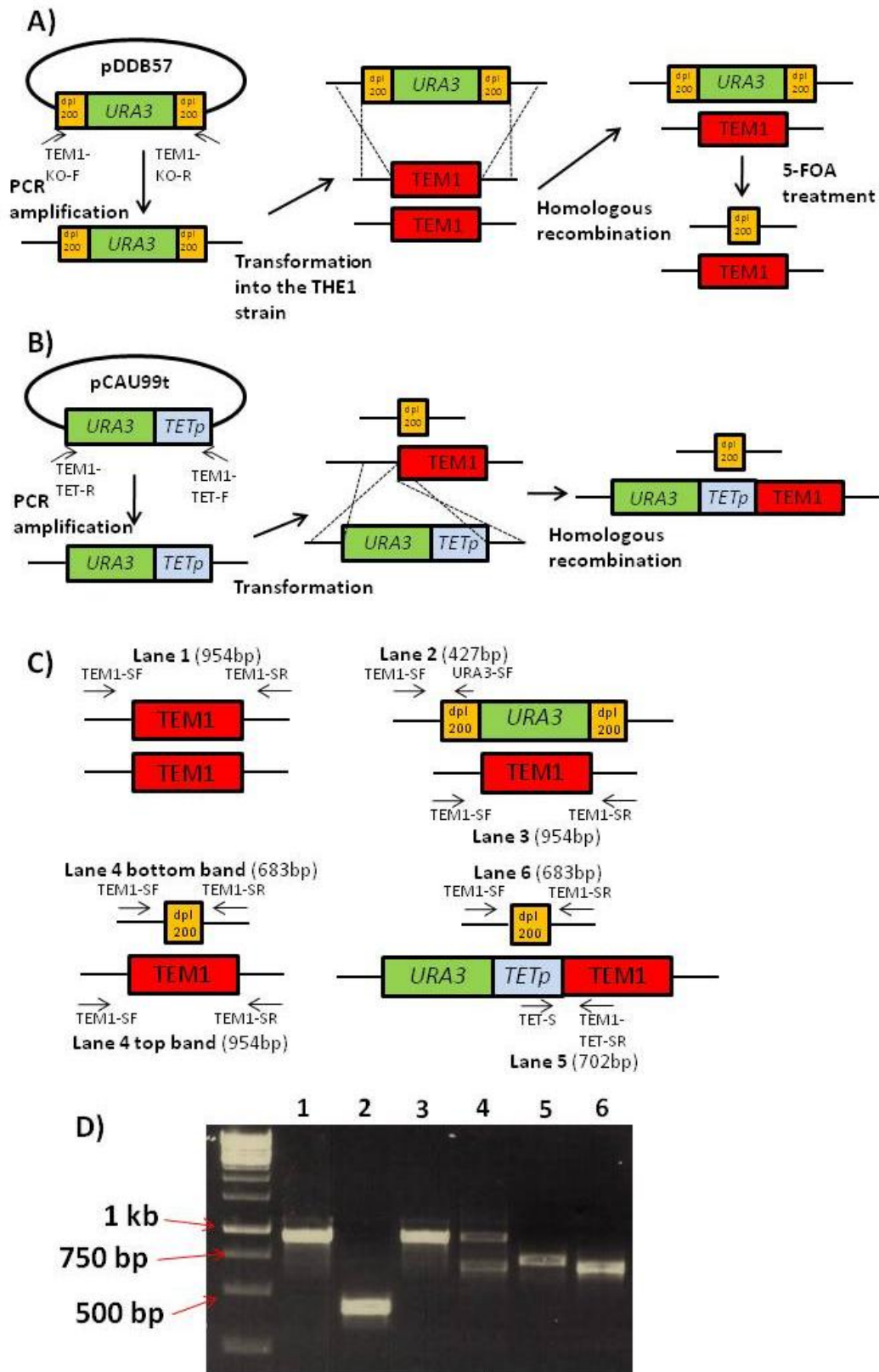
Figure 16. The construction of a tetracycline regulatable *TEM1* mutant. (A) To delete one of the *TEM1* alleles the mini-URA blaster cassette was amplified from the pDDB57 plasmids using the TEM1-KO-F and TEM1-KO-R primers to target the cassette to the *TEM1* locus. This cassette was then transformed into the THE1 *C. albicans* strain. The *URA3* gene was then recycled by selection on 5-FOA media. (B) The second *TEM1* allele was then placed under the control of the tetracycline regulatable promoter (TETp). The promoter cassette was amplified from the pCAU99t plasmid using the TEM1-TET-F and TEM1-TET-R

primers and transformed into the *ura* minus *TEM1* heterozygous strain (SWM5). (C) A schematic diagram of the construction of the SWM6 strain containing the primer annealing regions. The numbers in parentheses are the predicted PCR product size. (D) Colony PCR of the various stages of the construction of this strain. Lane 1 contains the PCR of the *TEM1* gene in the parent strain using the TEM1-SF and TEM1-SR. Lane 2 contains the PCR, using the TEM1-SF and URA3-SF primer, of the successful integration of the mini-*URA*-blaster cassette into one of the *TEM1* alleles (SWM4). Lane 3 contains the product of PCR, using the TEM1-SF and TEM1-SR, the presence of one wild type copy of the *TEM1* gene in the SWM4 strain. Lane 4 shows the PCR confirmation of the successful recycling of the *URA3* gene in the SWM5 strain using TEM1-SF and TEM1-SR primers. PCR confirmation (TEM1-SF and TEM1-SR primers) shows the integration of the tetracycline regulatable promoter cassette into the promoter region of the intact *TEM1* gene in the SWM5 strain to generate the SWM6 strain (Lane5). Lane 6 shows the PCR of the presence of the dpl200 sequence in the disrupted *TEM1* gene using the TET-S and TEM1-TET-SR primers.

TEM1/Δtem1 colonies formed and these transformants were confirmed by colony PCR using the TEM1-SF and URA-SF primers (Figure 16C; 16D lane 2 and 3). The positive PCR reactions generated a product of 427 nucleotides in length. A second PCR was carried out using the TEM1-SF and TEM1-SR primers to confirm the presence of a non-disrupted copy of the *TEM1* gene (Table 5; Figure 16C; 16D lane 1).

To allow the re-use of the *URA3* marker gene the *URA3* gene in the mini-URA blaster cassette was recycled. This was carried out by plating the *URA3* positive strain onto media containing 5-flouroorotic acid (5-FOA). This selects for strains where the *URA3* gene has been excised due to homologous recombination between the two *dpl200* sequences. Two of the four *URA3* positive *TEM1/Δtem1* strains (SWM4 and SWM7) underwent 5-FOA selection (section 2.1.5). Twenty nine colonies formed on the 5-FOA plates of the SWM4 strain while seven colonies formed on the SWM7 plates of which three were PCR positive for the recycling of the *URA3* gene (Figure 16A; 16C; 16D lane 4). This PCR was carried out using the TEM-SF and TEM-SR primers and produced two products: one of 954 nucleotides which correspond to the intact *TEM1* gene and a smaller band of 683 nucleotides which corresponds to *tem1::dpl200* allele (Table 5). Two of these *URA3* minus strains (SWM5 and SWM8) were selected and were transformed with the PCR amplified TET promoter cassette. The TEM1-TET-F and TEM1-TET-R primers were used to amplify the tetracycline-regulatable promoter cassettes from the pCAU99t plasmid (Table 5; Figure 16B). Two PCR positive colonies

(SWM6 and SWM9) formed as a result of this transformation. The TET-S and TEM1-TET-S primers were used to confirm the insertion of the TET promoter cassette into the target locus (Table 5; Figure 16C; 16D lane 5). An additional PCR was carried out using the TEM1-SF and TEM1-SR primers to confirm the cassette had inserted into the promoter region of the intact *TEM1* gene (Figure 16B; 16C; 16D lane 6). These two colonies provided two independent versions of the TET-TEM1 strain (SWM6 and SWM9) and were constructed from two independent *URA3* positive heterozygous *TEM1* mutants. The SWM6 strain was constructed from the SWM4 strain while the SWM9 strain was constructed from the SWM7 strain.

3.2.4. *TEM1* is essential for vegetative growth of *C. albicans*.

To test if *TEM1* an essential gene in *C. albicans* the THE1, SWM5 (*TEM1/tem1::dpl200*) and SWM6 (*URA3-TETp-TEM1/tem1::dpl200*) strains were grown on both YPD and synthetic complete (SC) medium in the presence or absence of 20 μg/ml doxycycline (Figure 17A and B). On the non-repressing medium (absence of doxycycline) all the strains grew. This was seen in all the assays in which these strains were used. However on the repressing medium the THE1 and SWM5 strains grew well but the SWM6 strain did not grow (Figure 17A and B). These results confirm that *TEM1* is essential for *C. albicans* growth in the yeast form.

Recently it has been observed that some genes are essential for growth in the yeast form but not essential in the hyphal form (Shen *et al*., 2008). As a result of this observation we set out to establish whether *TEM1* is essential in the hyphal growth form. This was carried out by growing the THE1, SWM5 and SWM6 strains on YPD agar containing 20 % serum plus or minus 20 μg/ml of doxycycline. All three strains grew on YPD + serum agar which lacked doxycycline, however the SWM6 colonies had the characteristic morphology associated with filamentous growth. The THE1 and SWM5 colonies had the appearance of colonies grown on normal YPD suggesting the strains were not as filamentous as the SWM6 strain (Figure 17C). This is likely to be caused by the lack of a *URA3* gene. In these strains this phenomenon has been witnessed before (Brand *et al*., 2004). However when 20 μg/ml of doxycycline was added to the YPD plus 20 % serum the SWM6 strain did not grow at all (Figure 17C). This finding confirms that *TEM1* is also essential in the hyphal growth form.
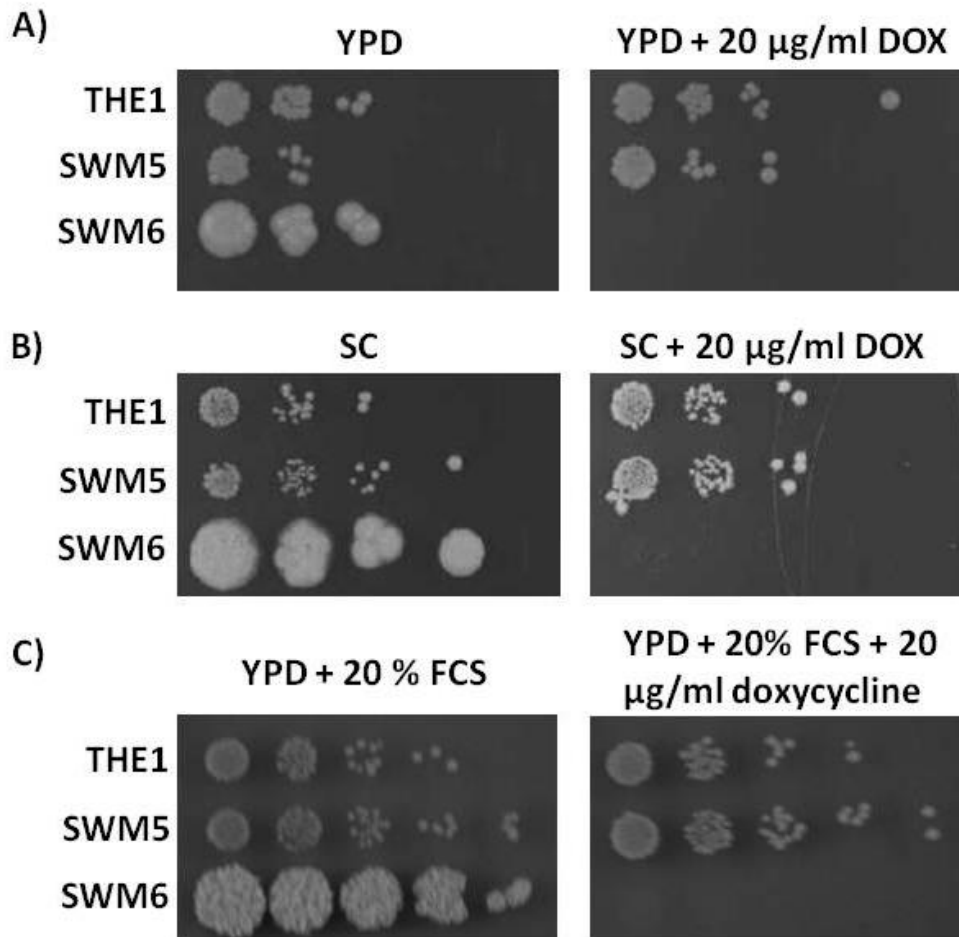
Figure 17. ***TEM1* is essential for vegetative growth in *C. albicans*.** The SWM5, SWM6 and THE1 strains were grown on YPD (A), synthetic complete media (B) and YPD containing 20 % serum all plus and minus the presence of 20 µg/ml of doxycycline (C). Serial dilutions (1/10) were carried out from 1 x 10^6 cells/ml down to 1 x 10^2 cells/ml. The SWM6 strain grew more quickly than the THE1 and SWM5 strains as the SWM6 strain possesses the *URA3* gene while the other two strains do not.

These results confirm that *TEM1* is essential for vegetative *C. albicans* growth on solid media. To asses if this is the same when the cells were grown in liquid media a growth curve assay was carried out. The growth curve assay consisted of growing the wild type CAI4 (CIp10) and SWM6 strains in 50 ml of YPD plus or minus 20 µg/ml doxycycline from an OD_{595} of 0.05 for 24 hours with a sample taken every hour and the OD_{595} reading taken. The CAI4 (CIp10) strain was chosen as the control for this experiment as this is a common control used by *Candida* researchers while the CAI4 (CIp10) and THE1 strains share a common parent strain. The CAI4 (CIp10) strain is also *ura* positive and therefore does not experience the growth defects of *ura* minus strains. Under non-repressing conditions (YPD) both CAI4 (CIp10) and SWM6 grew as yeast cells for the duration of the assay (Figure 18A). This was also seen for the CAI4 (CIp10) strain grown in the repressing media. This confirms that the presence of the doxycycline has no effect on the growth of the CAI4 (CIp10) strain.

Under repressing conditions the SWM6 strain (Tem1 depleted cells) grew at a normal rate for the first 5 hours of the assay but after 5 hours the growth rate of the Tem1 depleted cells slowed suggesting that the potential lack of Tem1 was beginning to affect the growth potential of the cells. The OD_{595} reading continued to increase for the SWM6 strain in the repressing media until the conclusion of the assay suggesting that growth of the cells was still ongoing but at a slower rate. However, the microscopic analysis of cell number in this culture levels out at approximately 5×10^6 cells/ml from 11 hours post inoculation to the end of the assay (24 hours post inoculation), and there was a distinct change in cellular morphology (discussed in 3.2.7.1.). The other three cultures continue to grow to a cell number of around 2×10^8 cells/ml at 15 hours post inoculation after which time the number of cells only very slowly increases further. Presumably the reason that the OD readings for the SWM6 culture continue to increase in the repressing media while the total number of cells did not increase was due to differential light scattering caused by the change in morphology.

3.2.5. Depletion of the Tem1 protein is cidal to *C. albicans* cells.

To investigate whether the cells in the Tem1 depleted culture were either dead (cidal) or in a state of no growth (static) a Miles and Misra type viability assay was carried out.
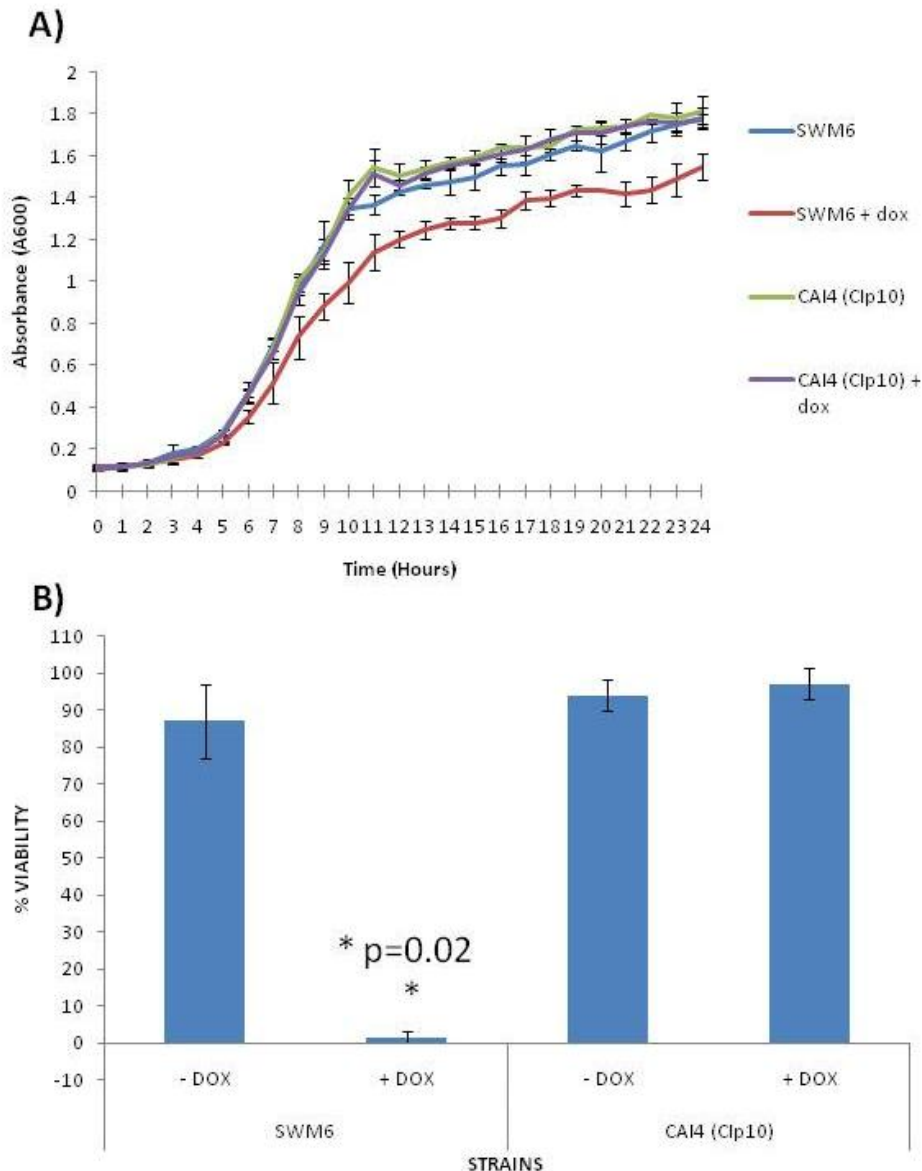
Figure 18. Tem1 depletion results in a growth defect and eventual cell death. SWM6 cells experience growth defects after 5 hours of growth in repressing media when compared to SWM6 cells grown in non-repressing media and CAI4 (CIp10) cells grown in both repressing and non-repressing media (A). No growth defects were observed throughout the 24 assay for the CAI4 (CIp10) cells grown in repressing media. All cultures were normalised to an optical density of 0.1 (A_{595}) and the average of 3 replicates taken and plotted. After 24 hours growth in the repressing media the % viability of the SWM6 cells was significantly lower than that of the CAI4 (CIp10) cells grown in the repressing media (B). 2 replicate samples were used to calculate the % viability of each strain grown in repressing and non-repressing media. P-value calculated using a student T-test. Error bars indicate the standard deviation of the replicates.

Both the strains were grown in the non-repressing and repressing media for 24 hours after which the cell numbers were normalised to 1×10^5 cells/ml and four 10 times serial dilutions carried out and the cells plated onto YPD plates. While the percentage viability of the SWM6 and CAI4 (CIp10) strains grown in the non-repressing media and also the CAI4 (CIp10) strain grown in the repressing media were all in excess of 85 %, the percentage viability of the Tem1 depleted cells was less than 2 % (Figure 18B). This is a significant difference (p-value=0.02) and confirms that the depletion of the Tem1 protein results in the cell death.

3.2.6. Tem1 depleted cells form long hyphal-like filaments.

In section 3.2.4 we documented that Tem1 depleted cells continue to grow throughout the 24 hour assay. However, cell numbers in the culture did not increase after 11 hours post inoculation. To investigate this difference between the findings we analysed the growth phenotype of the cells using both differential interference contrast (DIC) microscopy and scanning electron microscopy (SEM; Figure 19 and 20). When incubated in both repressing and non-repressing media the CAI4 (CIp10) cells grew as yeast cells (Figure 19A). The SWM6 cells also grew as yeast cells when incubated in non-repressing media (Figure 19B). However, the SWM6 cells become filamentous at around 8 hours post inoculation into the repressing media and these filaments continue to elongate until after the 24 hour time point (Figure 19C and 20). These filaments are wide during early development but do not have the constriction at the neck between the filament and the yeast cell which is typical of pseudohyphal cells. At the later time points, 16-24 hours, the filaments become thinner with parallel sided walls more typical of true hyphal cells (studied more in section 3.2.9; Figure 19 and 20). Prior to the emergence of the filaments the cells grew as yeast cells. Interestingly these filaments emerge from budding yeast cells with 33 % of the filament formed from the daughter yeast cell and 67 % emerged from the mother cell. Additionally these mother yeast cells were significantly enlarged from an average of 4.86 μm +/- 0.635 μm wide at 4 hours post inoculation to 5.96 μm +/- 1.04 μm wide at 8 hours post inoculation. At later time points filaments also formed from the mother cells. This suggests that the cells have encountered a mitotic blockage and the isotropic growth of the mother cell had continued (Figure 19; 20 and 21). By 13 hours post inoculation into repressing media all the SWM6 cells had become filamentous
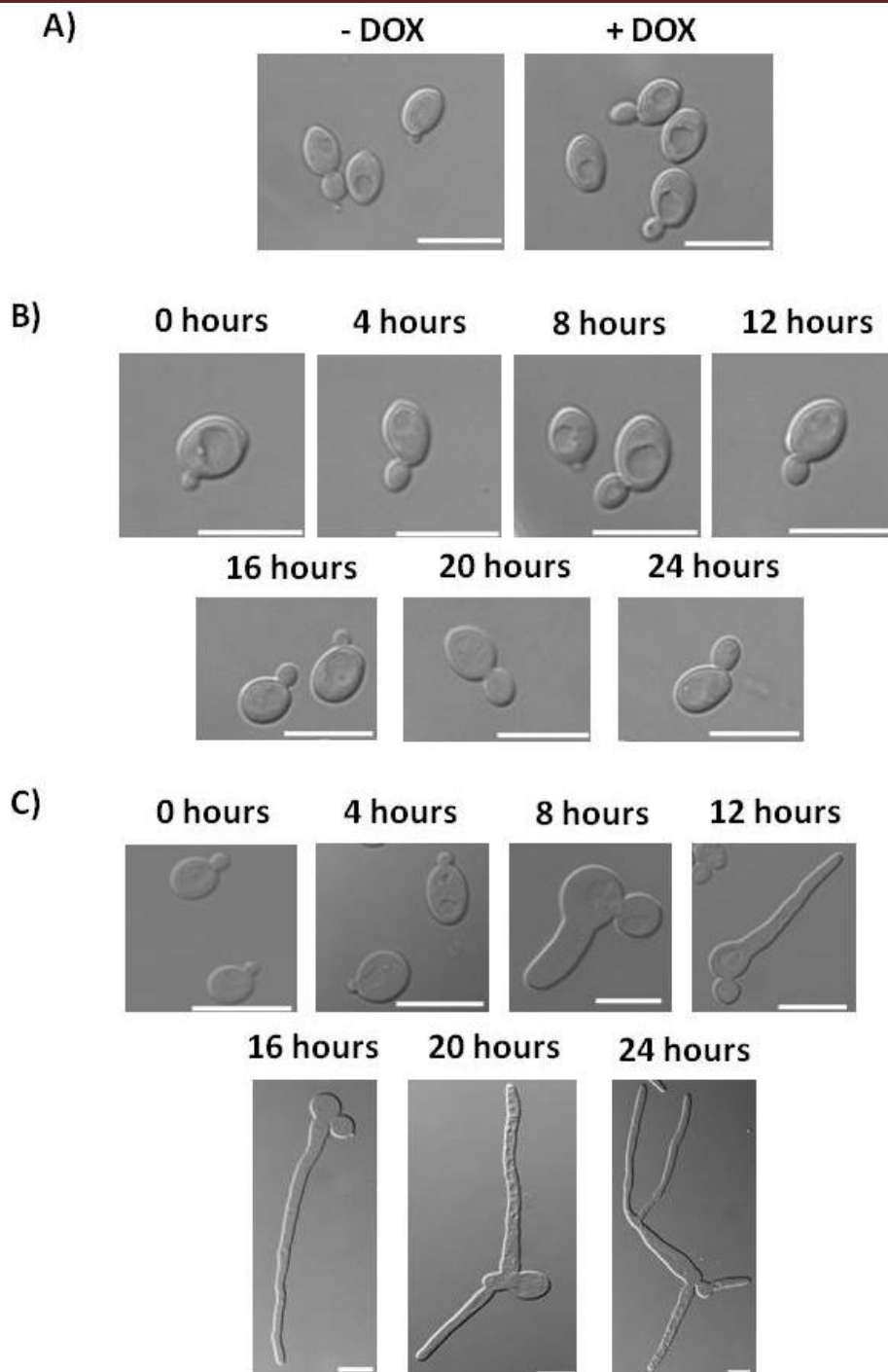
Figure 19. Differential interference contrast microscopy CAI4 (CIp10) and SWM6 cells grown in repressing and non-repressing media. CAI4 (CIp10) cells grew as yeast cells after 24 hours of incubation in both repressing (+DOX) and non-repressing media (-DOX, A). In non-repressing conditions the SWM6 cells grew as yeast cells throughout a 24 hours time course with sample taken and analysed at 4 hour intervals (B). In repressing media SWM6 cells initially grew as yeast cells (up to 4 hours) before the mother cells increased in size while still budding. The cells then spontaneously formed filaments which continued to elongate for the remainder of the 24 hours assay (C). At the later time points the Tem1-depleted cells became branched and formed additional filaments from the parent budding yeast cells. Scale bar on DIC images equals 10 µm.
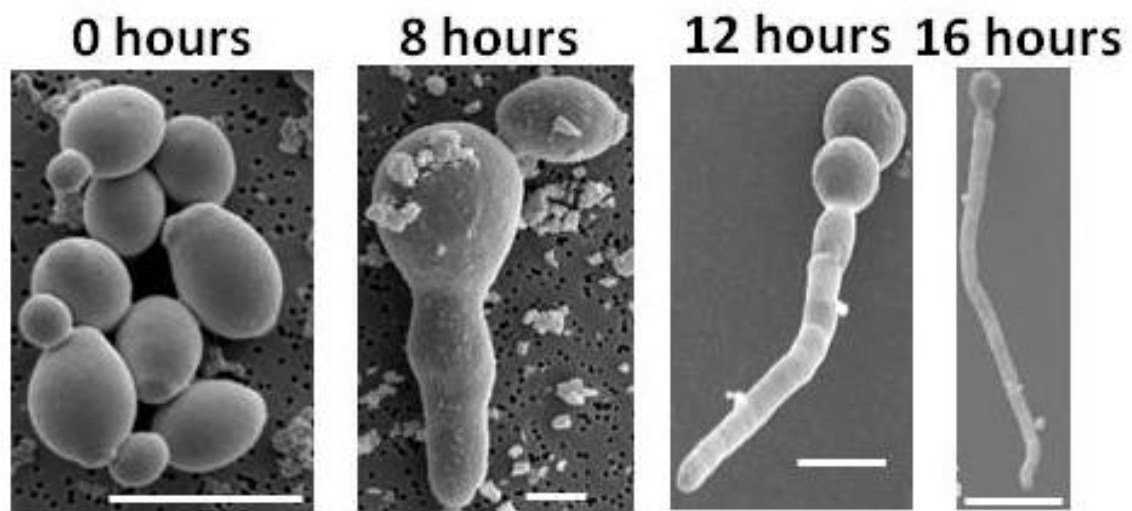
Figure 20. Scanning electron microscopy of SWM6 cells grown in repressing media. The SEM images show the filament formation observed in the DIC images when the SWM6 strain was grown in repressing media. Scale bar equals 10 µm.

(100 %) while the SWM6 cells grown in non-repressing media grew predominately (98 %) as yeast cells during the duration of the time course (Figure 18A and 21). Further experiments using the scanning electron microscope are required to draw firm conclusions. These include samples, taken at 0, 8, 12 and 16 hours post inoculation, of SWM6 cells grown in non-repressing media and also CAI4 (CIp10) cells grown in both repressing and non-repressing media.

To further confirm that the lack of viability of the Tem1 depleted cells and the timing of filamentous growth is a result of the depletion of the Tem1 protein *TEM1* was tagged with the V5-6x*HIS-NAT1* cassette. The *TEM1* targeted cassette was amplified from the pCR2.1 V5-6x*HISNAT1* plasmid using the TEM1-V5-F and TEM1-NAT1-R primers and transformed into the SWM6 strain (Table 5). Transformant colonies were selected for on SABDEX agar containing 200 µg/ml of nourseothricin. Five colonies formed of which one was PCR positive (SWM66; Figure 26A), using the TEM1-TAG-S and the V5-S primers. The TEM1-TAG-S primer was designed to confirm the cassette had integrated into the 3' end of the intact *TEM1* gene and not the deleted gene. To confirm that the TET promoter was switching off expression of *TEM1,* protein samples from cultures grown in repressing media for 0, 4, 8, 12 and 24 hours were analysed by western blotting. The western blot confirms that the Tem1-V5 protein was expressed and detected at time 0 but by the first sample point, post repression at 4 hours, Tem1-V5 protein levels had fallen below that detectable, suggesting that the TET promoter had completely shut off the expression the *TEM1* gene (Figure 22). This switch off of *TEM1* expression by 4 hours post inoculation corresponds to the slowing in the growth rate of the cells as they start and suggests there is a delay prior to the initiation of polarised growth. An additional western blot should be conducted which includes protein samples from SWM66 cells grown in non-repressing media for 0, 4, 8, 12 and 24 hours as a non-switch off control. The western blot finding also requires to be confirmed by RT-PCR to conclusively to rule out that a low level of expression, resulting in non-detectable level of protein expression is responsible for continued growth and the delay in the switch off and morphogenesis.
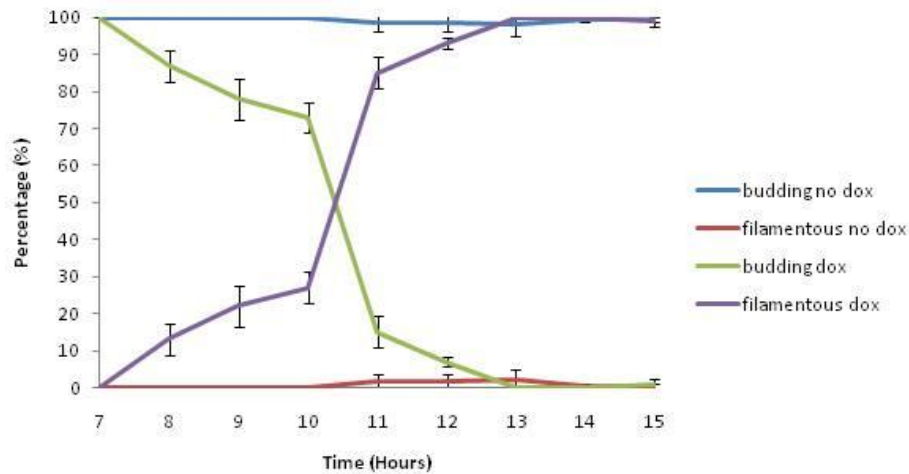
Figure 21. Tem1 depleted cells form filaments post Tem1 depletion. SWM6 cells all become filamentous by 13 hours of incubation in repressing growth media (filamentous dox). The CAI4 (CIp10) cells grown in both repressing (budding dox), non-repressing (budding no dox) as well as the SWM6 cells grown in the non-repressing (filamentous no dox) grow predominantly as yeast cells throughout the assay. Error bars represent the standard deviation between the 3 replicates for each time point.

Figure 22. Western analysis of Tem1 shut-off. The Tem1 protein levels in SWM66 cells grown in repressing media was detectable at 0 hours, however by 4 hours post inoculation no Tem1 protein was detected. No Tem1 protein was detected in the 8, 12 and 24 hour post inoculation samples. The Invitrogen anti-V5 antibody was used for detecting the Tem1-V5 protein fusion. The size of the band is about 25 kDa which is the similar size to the Tem1-V5-6xHIS fusion protein (24.6 kDa). Image was captured using a G Box transilluminator (Syngene, Cambridge, UK).

3.2.7. The filaments formed by Tem1 depleted cells develop into true hyphae with highly polarised actin.

In section 3.2.6, we commented on the observation that the growth filaments had the characteristics of pseudohyphal cells at 8 hours incubation in the repressing media while at the later time points the filaments had the appearance of true hyphae. There are a number of major differences that distinguish pseudohyphae from true hyphal cells. These include the presence of constrictions at the sites of septation in pseudohyphae which are lacking in true hyphae, and the average width of true hyphal cells is 2 μm while pseudohyphae have a minimum width of 2.8 μm. The width of the two filamentous forms of *C. albicans* cells is controlled by the type of growth the cells are undergoing. Yeast and pseudohyphal cells initially grow in a polarised fashion before switching to isotropic growth while true hyphae permanently grow in a polarised fashion and this polarised growth is controlled by polarisation of the actin cytoskeleton (Anderson and Soll, 1986; Hazan *et al*., 2002; Sudbery *et al*., 2004). This allows microscopic analysis of the growth mechanism of the cells to be carried out using the actin stain rhodamine phalloidin.

To study whether the localisation of the actin cytoskeleton in the Tem1 depleted cell changed between the 8 and 16 hours time points we collected cells at the 8, 12 and 16 hour post inoculation time points and fixed them before staining with rhodamine phalloidin. Repeatedly no fluorescence could be seen from any of these samples when they were examined microscopically, while wild type cells stained well. Therefore Tem1 depleted cells undergo changes resulting in lack of rhodamine staining (see section 3.2.11). As a result we decided to tag an actin associated protein with GFP. The protein that was chosen was the actin binding protein 1 (Abp1). To carry this out the *GFP-NAT1* cassette (constructed as part of chapter 5) was amplified to insert into the 3' end of the *ABP1* gene using the ABP1-GFP-F and ABP1-NAT1-R primers (Table 5). The amplified cassette was transformed into the SWM6 strain and one PCR positive colony formed (SWM88). The GFP-UP and ABP1-TAG-S primers were used to confirm the correct integration of the cassette (Table 5). The resulting SWM88 strain was then grown in repressing conditions for 8, 12 and 16 hours as well as in non-repressing, yeast and hyphae inducing conditions (YPD and YPD + 20 % serum respectively). Cells from the yeast sample were stained with rhodamine phalloidin and the localisation of

the Abp1-GFP was identical to the rhodamine phalloidin staining, therefore, confirming that Abp1-GFP is an acceptable substitute to staining the actin in cells (Figure 23A). In the yeast cells the Abp1-GFP localised as punctate spots to the edge of the daughter cells while in the hyphal cells the Abp1-GFP localised to an intense spot at the apical end of the hypha (Figure 23B).

After 8 hours of growth in repressing medium actin localised as patches in 97 % of the cells assayed (n=100) suggesting that the cells are undergoing isotropic growth (Figure 19B). In the 12 hour sample 50 % of cells displayed the actin localisation typically associated with polarised growth, an intense patch of Abp1-GFP at the tip of the growing cells, while the rest of the cells were undergoing isotropic growth. Thus, confirming that the cells are switching from isotropic to polarised growth (Figure 23B). Finally in the 16 hour sample 80 % of the cells were undergoing polarised growth (Figure 23B). These findings suggest that the filaments originally form as pseudohyphae but become more polarised as they grow longer and become true hyphae at the latter time points. To investigate this observation further we measured the average width of Tem1 depleted cells. For cells to be classed as pseudohyphae they should be at least 2.8 µm in width so using this as the cut off between what is a hypha or pseudohypha we quantified the different cell morphologies displayed by Tem1 depleted cells. At 8 hours the cells had an average cell width of 4.37 µm +/- 1.2 µm (n=40) which confirms that these cells can be classed as pseudohyphal and only 10 % of the cells measured could be classed as being true hyphae. After 12 and 16 hours of growth in repressing media the average cell widths were 2.29 µm +/- 0.53 µm (n=50) and 2.20 µ +/- 0.45 µm (n=50) respectively. From these samples 82 % of the 12 hours cells and 92 % of the 16 hours cells can be classed as true hyphae.

The final piece of evidence of this switch from isotropic to polarised growth came from studying *HGC1* expression. *HGC1* is the only identified hyphal specific cyclin gene in *C. albicans*, and as Hgc1 protein is required for hyphal development it is often used as an indicator for determining if a growth filament is a true hypha (Zheng *et al.*, 2004). In this study we used reverse transcriptase PCR (RT-PCR) to study the expression of *HGC1* using the HGC1-F and HGC1-R primers (Table 5). As a control the expression of the elongation factor-1 β (*EFB1*) experiment was also studied using the EFB1-F and EFB1-R
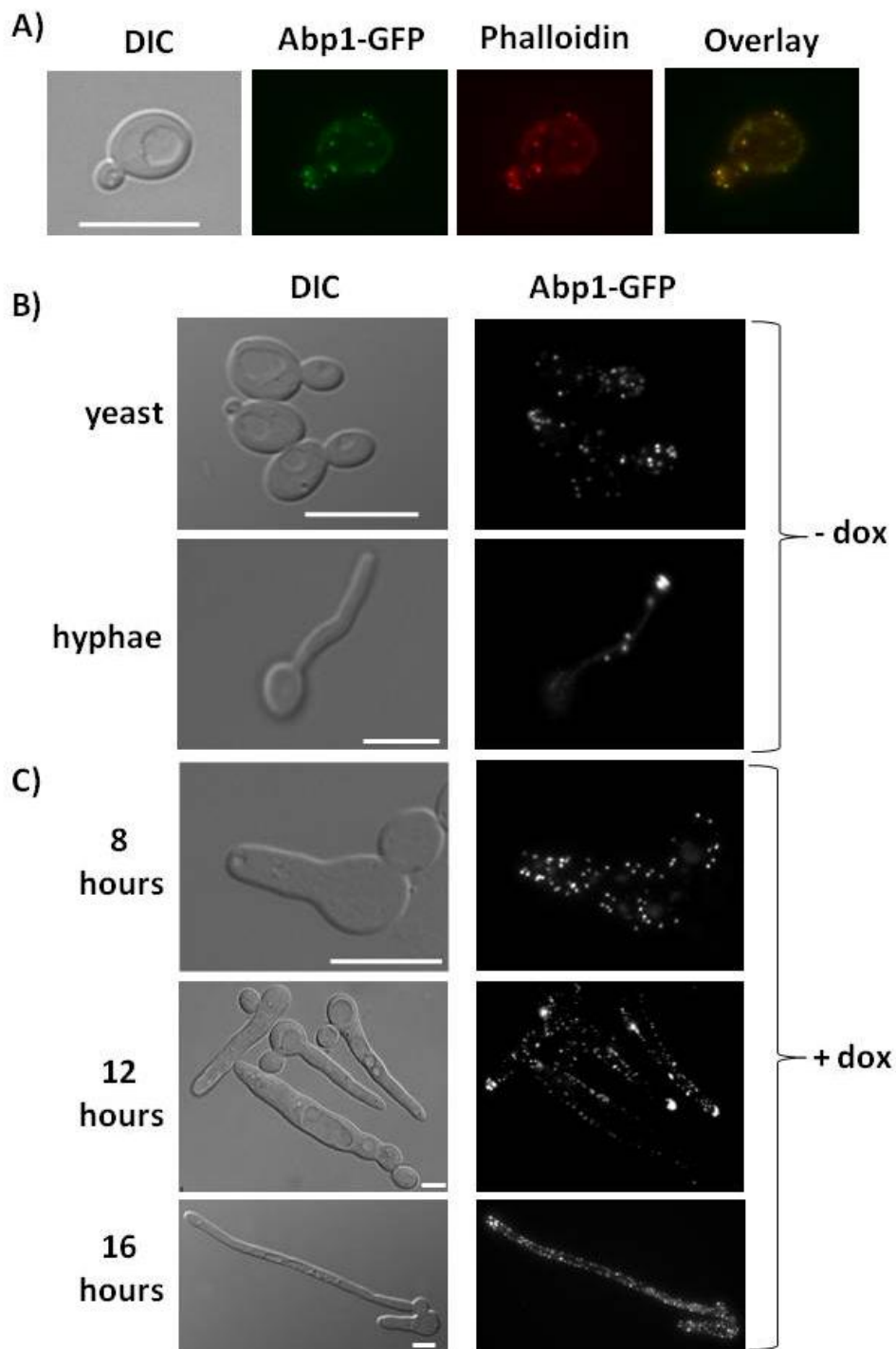
Figure 23. The actin cytoskeleton becomes highly polarised in Tem1 depleted cells. The actin binding protein-GFP fusion is an acceptable substitute for rhodamine phalloidin staining (A). Abp1-GFP localisation in the SWM88 grown in the yeast form (YPD - doxycycline for 24 hours) and hyphal form `(YPD+20 % serum – doxycyline for 75 minutes; B). In the hyphal cells Abp1-GFP localises in a polarised fashion at the tip of the hyphae while in the yeast cells the Abp1-GFP primarily localises in the growing bud. In SWM88 cell grown in repressing media the Abp-GFP localises in a highly polarised fashion at the tip of the filament (C). Scale bar equals 10 µm.

primers (Table 5). Expression of these two gene was investigated in SWM6 cells grown in repressing media for 24 hours with samples taken every 4 hours from time 0 (time of inoculation). *HGC1* and *EFB1* expression was also investigated in SWM6 cells grown in YPD media for 5 hours and YPD + 20 % serum media for 90 minutes to induce yeast and hyphal growth respectively. We observed that *EFB1* was expressed in all the samples while *HGC1* was only expressed in the 16 and 20 hours post inoculation into repressing media and in the hyphal sample (Figure 24). Interestingly the switch from pseudohyphal to true hyphal growth occurs 4 hours before *HGC1* expression was detected which fits with the literature which states that Hgc1 is required for maintaining but not initiating polarised growth (Zheng *et al.*, 2004). This result further confirms that Tem1 depleted cells originally form pseudohyphae which later become true hyphae.

3.2.8. Depletion of the Tem1 protein causes a delay in the exit from mitosis.

In *S. cerevisiae* the Tem1 protein is involved signalling mitotic exit and cytokinesis. To evaluate the role *CaTEM1* plays in mitotic exit we studied nuclear dynamics. Two approaches were undertaken to study the numbers and localisation of the nuclei in Tem1 depleted cells. The first approach focussed on the use of the molecular stain DAPI. DAPI binds to A-T rich regions of DNA and it is frequently used for visualising nuclei within both live and dead cells (Kapuscinski, 1995). Our initial attempt to stain the nuclei in Tem1 depleted cells was discontinued due to inconsistent staining patterns. As a result we decided to tag a nuclear landmark protein with GFP. Two proteins were selected, the H1 histone protein Hho1 and nuclear pore protein Nup49. Numerous attempts were made to tag the genes of these proteins with the *GFP-NAT1* cassette but they were all unsuccessful. A number of colonies screened as being positive by PCR but upon microscope analysis no fluorescence could be detected. Therefore we went back to the DAPI staining, which was ultimately successful.

To analyse the number and the localisation of the nuclei the SWM6 strain was grown in repressing media for 20 hours with samples taken at 8, 12, 16 and 20 hours. The SWM6 strain was also grown in non-repressing media for 5 hours to induce growth of the cells in the yeast form as an experimental control. Each sample was fixed, stained with DAPI (1 μg/ml) and the analysed under the microscope. For each time point the
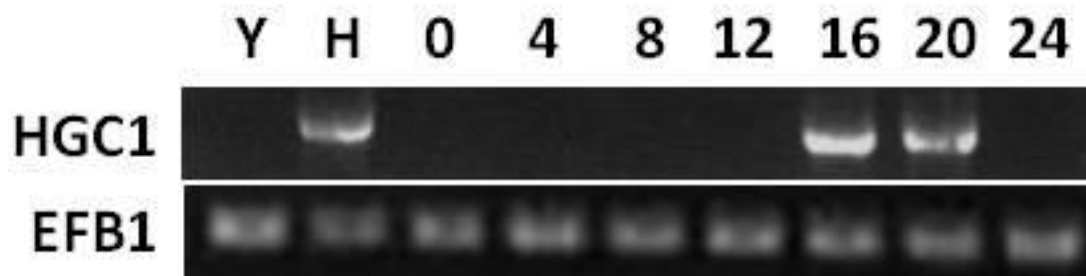
Figure 24. *HGC1* **expression in Tem1-depleted cells.** SWM6 cells were inoculated into repressing media for 24 hours. Samples were taken at the indicated times post inoculation and total cellular RNA extracted and the RT-PCR conducted to evaluate *HGC1* and *EFB1* expression. *HGC1* expression was only detected in the 16 and 20 hour post inoculation into repressing media sample plus the hyphal control sample while *EFB1* expression was detected in all the samples. Y denotes the SWM6 sample grown in the yeast form in non-repressing conditions (YPD without doxycycline) for 5 hours. H denotes the SWM6 sample grown in the hyphal form grown in YPD + 20 % serum without doxycycline. Three replicates were carried out.

number of nuclei within 75 cells were counted. The staining revealed that after 20 hours of incubation in repressing media 66 % of the filaments contained either one or two nuclei with few cells containing more than two nuclei (Figure 25A and B). At both 8 and 12 hours, post inoculation in to repression media, a single nucleus was observed to be located within each cell, mother and filament. At the later time points in 80 % of multi-nucleated cells the nuclei had migrated into the filaments. Ultimately 5 % of the cells were able to overcome this defect and re-enter the cell cycle. Within the sample grown in the non-repressing media almost 80 % of the cells contained a single nucleus while the remaining 20 % of cells were daughter buds which contained no nuclei as cells were undergoing nuclear division. Overall these findings further imply that the Tem1 depleted cells had undergone a single nuclear division but had arrested late in the cell cycle with divided nuclei. This is very similar to cells depleted of the M cyclin Clb2 which arrest with divided nuclei and are unable to re-enter the cell cycle therefore it can be proposed that Tem1 depleted cells fail to re-enter the cell cycle (Bensen *et al*., 2005). Additional experiments consisting of FACS analysis could be carried out to determine the nuclear content of these cells, however due to morphology this is not feasible but nuclear preparations could potentially be carried out instead.

To further investigate this mitotic arrest we tagged the γ-tubulin protein with GFP to label the SPBs. This was carried out by amplifying the *GFP-NAT1* cassette using the TUB4-GFP-F and TUB4-NAT1-R primers which targeted the cassette to integrate into the 3' end of the *TUB4* gene (Table 5). Colony PCR confirmed that one transformant colony (SWM113) contained the *TUB4* gene tagged with the *GFP-NAT1* cassettes. A Tem1 depletion time course was carried out using the SWM113 strain with samples taken at 8, 12, and 16 hours post inoculation into repressing media. Samples were not fixed but immediately analysed by microscopy, to prevent loss of the GFP signal, and the number of SPBs in each cell counted. As an experiment control the SWM113 cells were also grown in YPD media for 5 hours and in YPD + 20 % serum for 90 minutes to induce yeast and hyphal growth respectively (Figure 26A and B). Within the budding yeast culture about 70 % of the cells contained either one or two spindle poles while the hyphal cells contained two spindle poles which suggest the cells were actively dividing (Figure 26A and B). At both 8 and 12 hours post inoculation into repressing
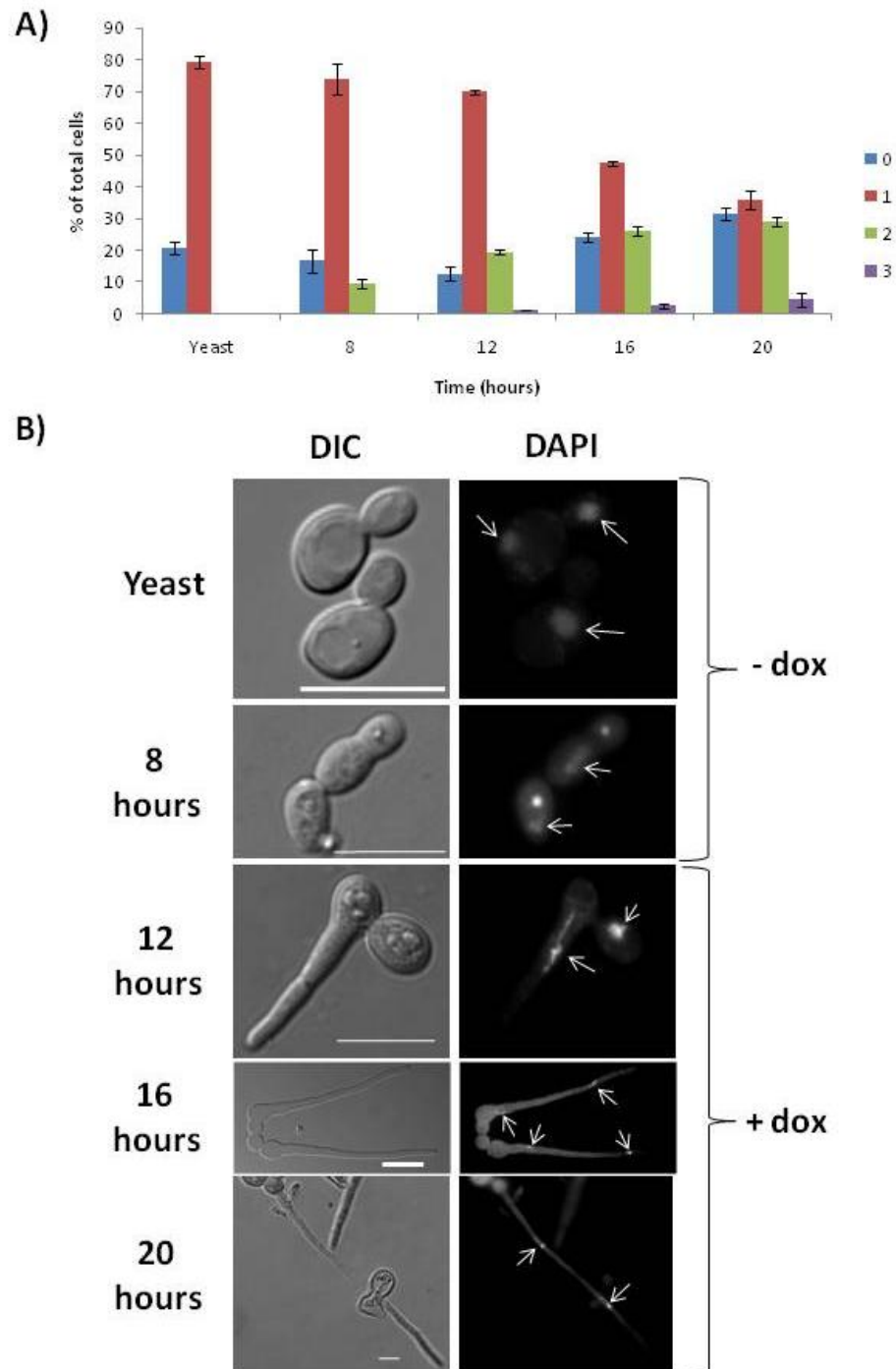
Figure 25. Nuclear division is impaired in Tem1-depleted cells. SWM6 cells were grown in non-repressing media for 5 hours and in repressing media for 20 hours. Samples were taken for the repressing media at 8, 12, 16 and 20 hours post inoculation. The cells were then fixed and stained with DAPI (1 µg/ml; B). The yeast sample was the SWM6 strain grown in YPD minus doxycycline for 5 hours. The numbers of nuclei within 75 cells were counted and converted into a percentage (A). Error bars indicate the standard deviation for the 2 replicates for each sample. Scale bars equal 10 µm. Arrow on the microscope images show the position of the nuclei within the cells.
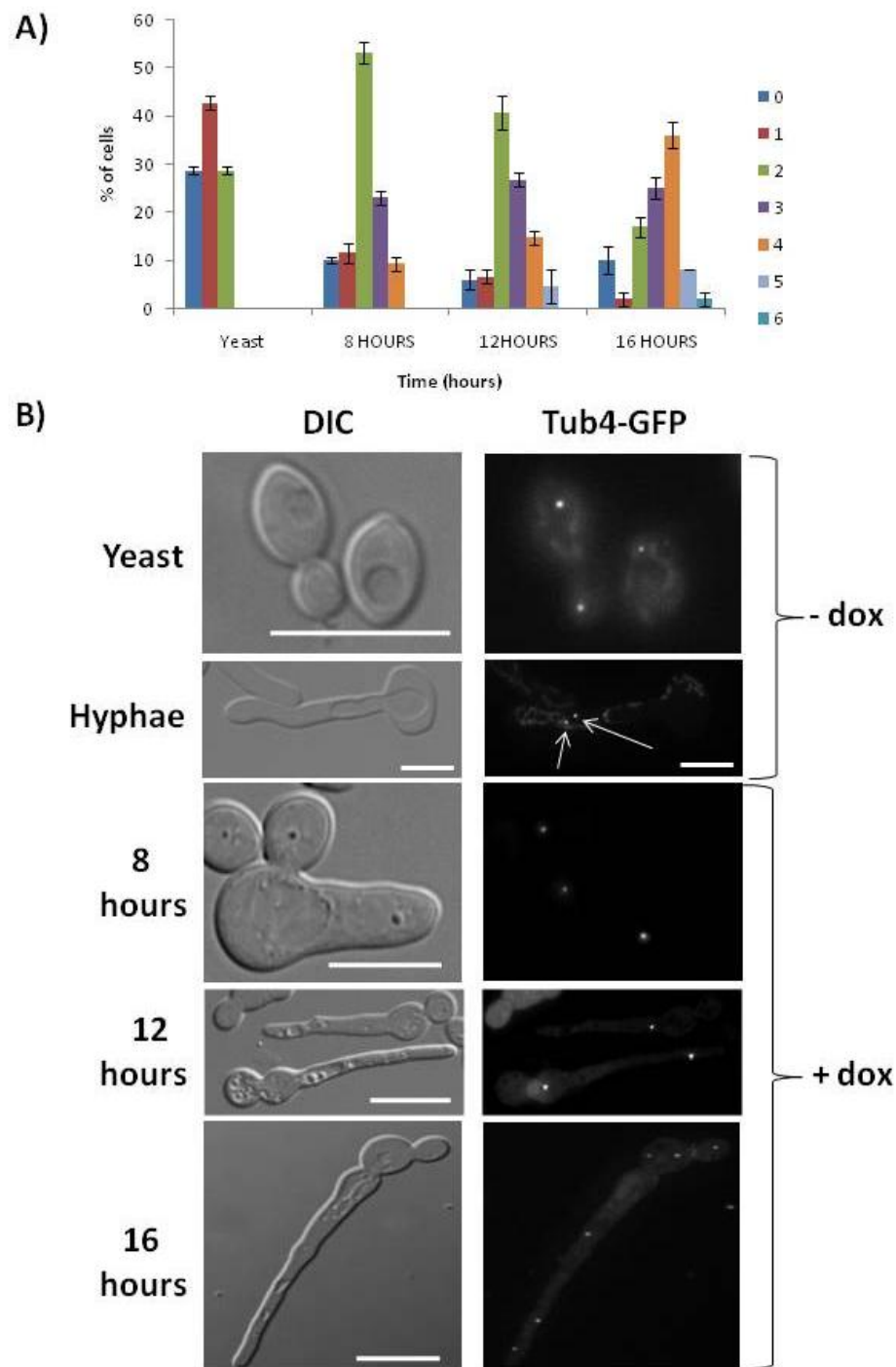
Figure 26. Tem1 depleted cells undergo a mitotic arrest. SWM113 cells were grown in repressing media for 16 hours. Samples were taken at 8, 12, and 16 hours post inoculation, washed with PBS and analysed by microscopy. The yeast sample was the SWM113 strain grown in YPD minus doxycycline for 5 hours, while the hyphal sample was the SWM113 strain grown in YPD + 20 % serum minus doxycycline for 75 minutes (B). The percentage of cells in the yeast, 8, 12 and 16 hour post depletion samples that contained 0, 1, 2, 3, 4, 5 or 6 Tub4-GFP spots (A). 75 cells were counted per sample and 2 replicates were carried out. Error bars indicate the standard deviation for the 2 replicates for each sample. Scale bars equal 10 μm. Arrows indicate the position of the Tub4-GFP fusion protein spots.

media the number of cells containing two spindle poles were between 40 and 50 % (Figure 26A and B). This suggests that the majority of the cells had stalled post nuclear segregation. Ideally, this would be confirmed by demonstrating the two SPBs could be shown to be still attached to each other by the elongated spindle. This could be done by carrying out the same experiment in a SWM6 strain carrying either α or β-tubulin (Tub1 or Tub2 proteins) tagged with GFP however this strain was not successfully constructed. At the 16 hour time point the majority of cells contain three or four SPBs which suggest that these cells have been able to overcome the mitotic arrest and have been re-entered the cell cycle. However, as ultimately *TEM1* is essential this bypass is not sufficient to restore viability.

Additionally we observed that the SPBs within the cells were normally separated from each other, 87 % of cells contain anaphase SPB localisation, and this confirms that the mitotic spindle can elongate in these cells therefore the Tem1 protein is not required for spindle elongation. This also suggests that SPB duplication was not occurred and provides further evidence of a cell cycle blockage (Figure 26B).

To observe gross changes in cyclin expression we carried out qualitative RT-PCR (Table 5). At 16 and 20 hour time points as expected we saw high levels of B cyclins and low levels of G_1 cyclins. This is indicative of a cell cycle arrest late in mitosis (Figure 27). However it is appreciated that this end point qualitative assay is not sufficiently sensitive therefore a more quantitative approach requires to be carried out.

Overall the findings documented in this section point to the conclusion that depletion of the Tem1 protein in *C. albicans* cells results in a blockage in mitosis which delays the cell's ability to exit from mitosis. Results from the nuclear localisation and SPB studies suggest that a small percentage of the cells (between 5 %) were able to overcome this blockage however this was not sufficient to restore viability of the cells. However further experiments such as FACS analysis and more time points in the Tem1 depletion time course are required to confirm this conclusion.

3.2.9. Tem1 depleted cells fail to septate.

In section 3.2.6 we showed that the depletion of the Tem1 protein resulted in the
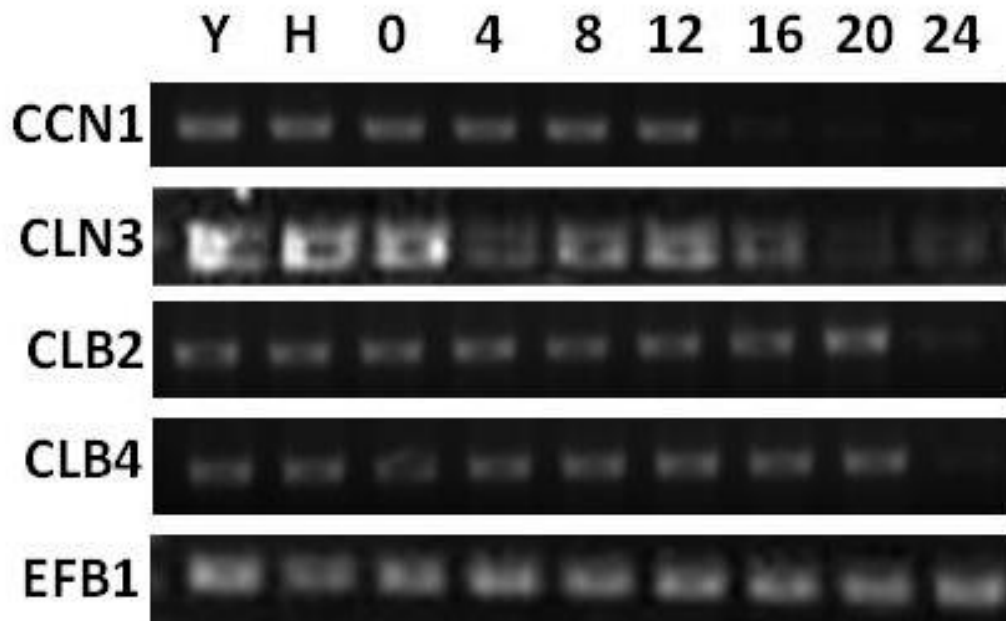
Figure 27. B cyclins are expressed at high levels in Tem1 depleted cells. SWM6 cells were inoculated into repressing media for 24 hours. Samples were taken at the indicated times post inoculation and total cellular RNA extracted and the RT-PCR conducted to evaluate expression of the G_1 cyclins (*CCN1* and *CLN3*), B cyclins (*CLB2* and *CLB4*) and the *EFB1* expression control. Cyclin expression was also assayed in SWM6 cells grown in non-repressing conditions to induce yeast (Y; YPD without doxycycline for 5 hours) and hyphal growth (H; YPD + 20 % serum without doxycycline for 2 hours). Expression of B cyclins was detected in Tem1-depleted cells throughout the assay apart from the 24 hour post inoculation sample. Expression of the G_1 cyclins was high in the 0, 4, 8, and 12 hour samples while in the last 3 samples (16, 20 and 24 hours) the expression was low. *EFB1* expression was detected in all the samples. Three replicates were carried out.

mother and daughter buds failing to separate which suggests a potential cytokinesis and septation defect. To further investigate this we stained Tem1 depleted cells with the chitin stain calcofluor white. During the cell cycle following assembly of the septin ring, the Bni4 protein recruits chitin synthase to the site of the septin ring and as a result an enriched chitin band is deposited on the cell surface (Rowbottom *et al.*, 2004). At 8, 12 and 16 hours post inoculation into repressing media, one chitin rich deposit was observed and this was located at the initial mother-daughter bud neck of each cell (Figure 28). No further chitin bands were visible within any of the filaments observed. As a control SWM6 cells were grown in the yeast form in YPD media and in these cells a chitin rich band was only present between the mother and daughter cells (Figure 28). In addition no actomyosin rings were observed at the mother-daughter bud neck of the Tem1 depleted cells suggesting the loss of the signal for actomyosin rings formation failed. To investigate this further we studied primary septum formation at the mother-daughter bud neck in Tem1 depleted cells. To carry this out we analysed these cells using a transmission electron microscope (TEM). As Figure 29 shows, the SWM6 cells grown in non-repressing media undergo primary septum formation at the mother/daughter bud neck while the SWM6 cells grown in the repressing media have no evidence of primary septum formation, therefore demonstrating a clear cytokinesis defect in Tem1 depleted cells. However this experiment should with further controls to strengthen the conclusions drawn. These controls would be the CAI4 (CIp10) cell grown in repressing and non-repressing media.

Septins are a highly conserved family of GTPase proteins that form hetero-oligomeric complexes such as hourglasses, rings and filaments. These complexes either associate with actin and tubulin or localise to part of the plasma membrane where they act as scaffold proteins for a number of cellular processes including cytokinesis (Oh and Bi, 2011). To further investigate the septin dynamics in Tem1 depleted cells we tagged the septin protein Cdc3 with GFP.

To generate a strain carrying the Cdc3 septin protein tagged with GFP the *GFP-NAT1* cassette was amplified with the CDC3-GFP-F and CDC3-NAT1-R primers and transformed into the SWM6 strain (Table 5). One PCR positive colony (SWM71) formed as a result of this transformation experiment. To assess the impact of Tem1
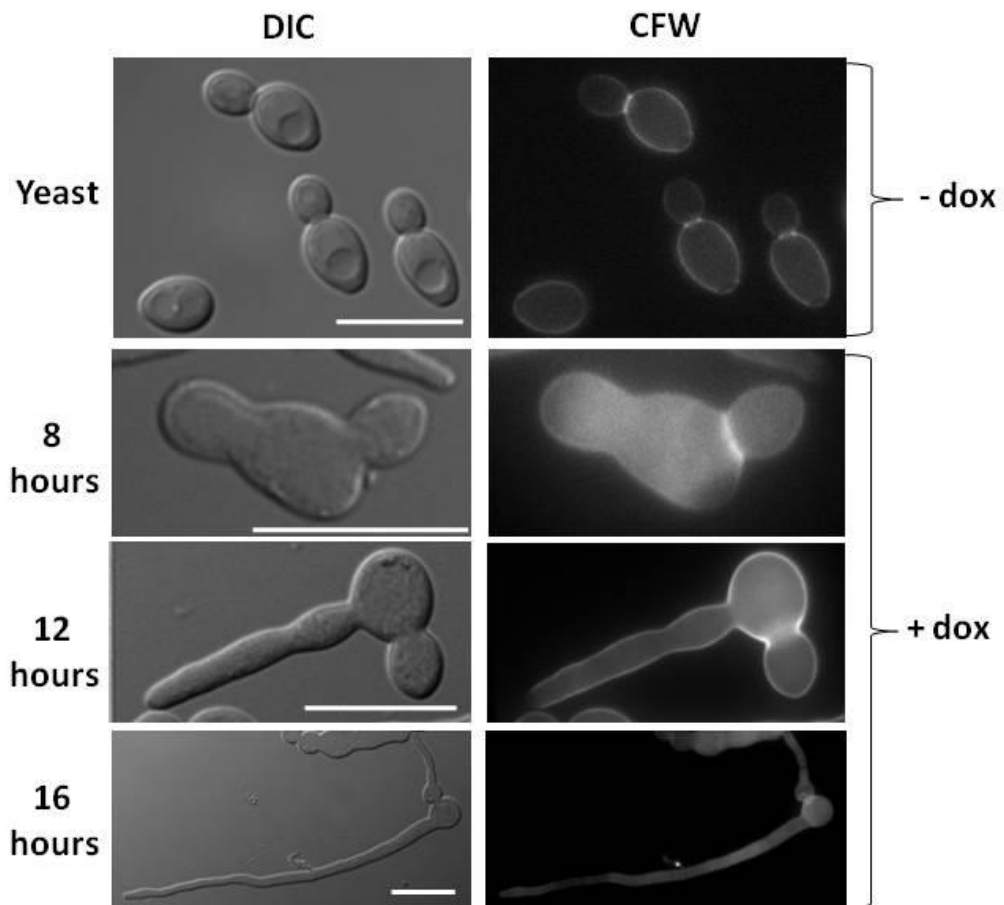
Figure 28. Tem1 depleted filaments do not contain a chitin band. SWM6 cells were grown in repressing media for 16 hours. Samples were taken at 8, 12, and 16 hours post inoculation, fixed, stained with calcofluor white (5 µg/ml) and analysed by microscopy. The yeast sample was the SWM6 strain grown in YPD minus doxycycline for 5 hours. In all the Tem1 depleted cells only 1 chitin band was visible and this was located between the mother and daughter buds just as in the cells in the yeast sample, with no chitin band being observed in any filaments. Scale bars equal 10 µm.
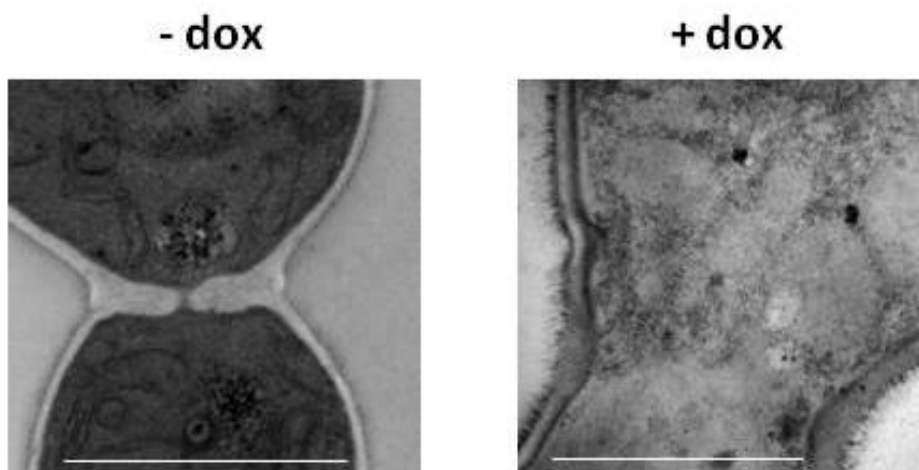
Figure 29. Tem1 depleted cells fail to form primary septa. SWM6 cells were grown in repressing and non-repressing media for 16 and 5 hours respectively. Samples were then fixed and prepared for visualisation using the transmission electron microscope. Analysis of the samples showed that the SWM6 cells grown in the repressing media failed to form a primary septa at the mother-daughter bud neck while the cells grown in the non-repressing media formed a normal primary septa. Scale bars equal 2 μm.

Strain	SWM71 (-dox)	SWM71 (+dox)
Average width of septin band	1.946 µm	3.257 µm
Standard deviation	0.330 µm	0.862 µm

p-value = 1.0608×10^{-14}

Table 7. Tem1 depleted cells have significantly larger septin rings. SWM71 cells were grown in repressing (+dox) and non-repressing (-dox) media for 12 and 5 hours respectively. The cells were fixed, washed and analysed by fluorescence microscopy. The width of the Cdc3 septin rings of 50 cells for each sample were measured using Image J software. The p-value was calculated by conducting a student t-test.

depletion on Cdc3p localisation the SWM71 strain was grown in repressing media for 20 hours with samples taken at 8, 12 and 16 hours post inoculation. As a control SWM71 cells were also grown under non-repressing conditions on; YPD or YPD + 20% serum to allow yeast and hyphal growth respectively. Interestingly the SWM71 cells depleted of Tem1 possessed only one Cdc3-GFP band and this was located between the initial mother and daughter yeast cells (Figure 30). No septin band was visible in any of the growth filaments at any of the time points observed even though some cells at late time points had bypassed the mitotic blockage. The septin band between the mother and daughter cells on the Tem1 depleted cells appeared to be wider than those found in the SWM71 cells grown in non-repressing conditions. To quantify this, the width of the Cdc3-GFP band in the SWM71 strain grown in both non-repressing media for 5 hours and in repressing media for 12 hours were measured. The 5 hour time point was chosen for the yeast samples to ensure the culture was undergoing exponential growth and had not entered stationary phase. These measurements confirmed that the average width of the septin band in the cells grown in repressing media was significantly wider at 3.257 µm +/- 0.862 µm (n=50) while the cells grown in the non-repressing condition had an average septin band width of 1.946 µm +/- 0.33 µm (n=50; p-value = 1.0608×10^{-14}; Table 7). Furthermore the septin band in the Tem1 depleted cells had the appearance of an hourglass structure and at no time point was the septin band observed to split into two bands. Overall these findings suggest that Tem1 is essential for cytokinesis and is required for septin ring reorganisation and actomyosin ring formation while also suggesting that Tem1 depleted cells arrest prior to cytokinesis.
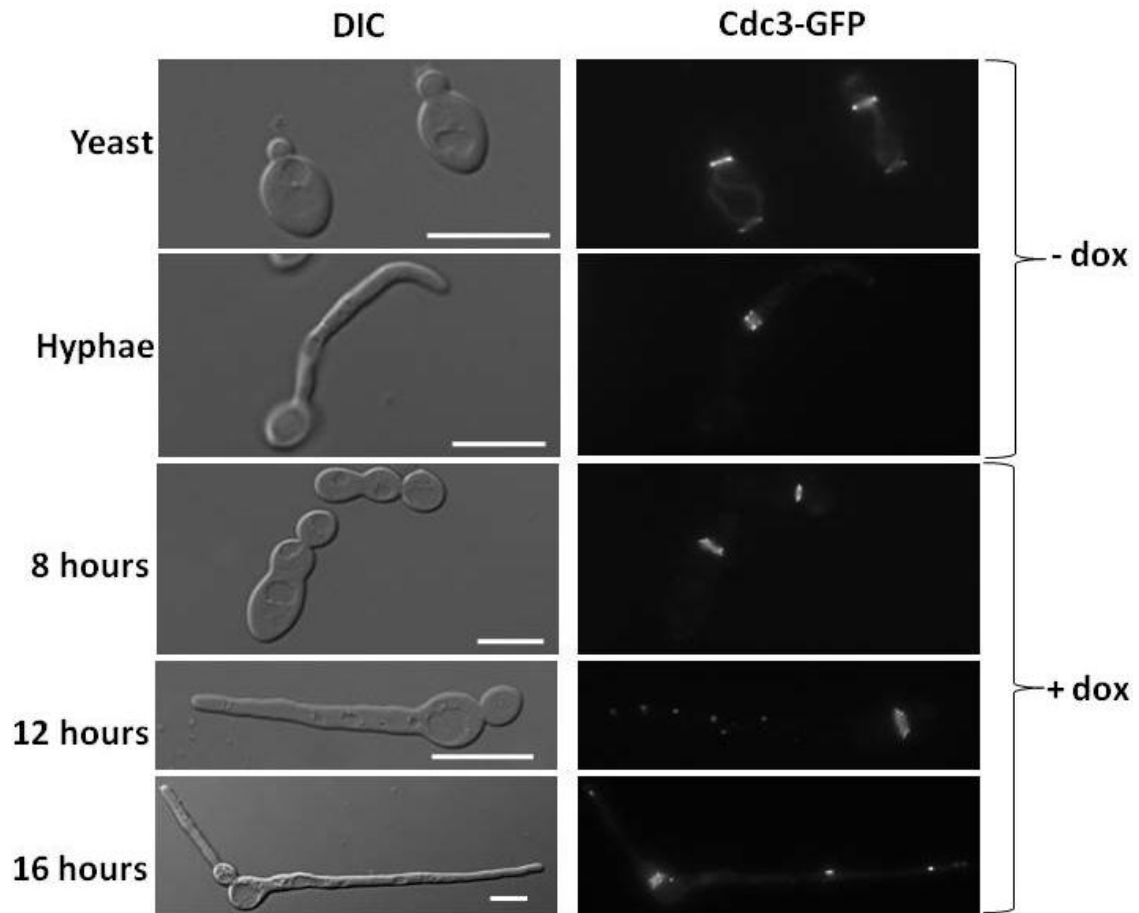
Figure 30. Tem1 depleted cells have impaired septin dynamics. SWM71 cells were grown in repressing media for 16 hours. Samples were taken at 8, 12, and 16 hours post inoculation, washed with PBS and analysed by fluorescence microscopy. As a control the SWM71 strain was grown in the yeast form in YPD minus doxycycline for 5 hours and in the hyphal form in YPD plus 20 % serum minus doxycycline. In both the control samples the dynamics of the Cdc3 rings occur as expected while in the Tem1 depleted cells a Cdc3 ring forms between the mother and daughter yeast cells however this ring is maintained in an hourglass-like structure and fails to separate into two rings. Scale bars equal 10 µm.

3.2.10. Tem1 depleted cells are hypersensitive to some cell wall perturbation agents.

During this project three main cellular stains were used; calcofluor white, DAPI and rhodamine phalloidin. The calcofluor white staining has worked well with each of the cell types on which it was used. However this has not been the case for the two other stains. There was no difficulty in staining yeast, hyphal or pseudohyphal cells with either DAPI or rhodamine phalloidin but unfortunately the staining of Tem1 depleted cells was problematic. These staining problems led us to hypothesise that the stains could not enter the cells because the depletion of the Tem1 protein resulted in changes to the cell wall structure and permeability. To investigate this we undertook two different approaches firstly a cell wall sensitivity assay and secondly TEM analysis of the outer layer of the cell wall.

To study the sensitivity of Tem1 depleted cells to cell wall perturbing agents we initially identified the concentration of doxycycline that would deplete *TEM1* expression levels to that which permitted a phenotype to be seen, but which did not inhibit cell growth and differentiation (suboptimal doxycycline concentration). To do this we carried out both liquid and plate growth assays and selected a doxycycline concentration of 0.626 μg/ml (Figure 31; YPD pictures). For perturbation agents we chose to use the cell wall intercalating agents Congo red and calcofluor white, the detergent SDS and sodium chloride (NaCl). When the suboptimal concentration of doxycycline was added to the media the wild type CAI4 (CIp10) strain could still grow under all conditions tested, however the SWM6 strain could only grow on YPD and YPD containing 1M NaCl (Figure 31). Hence, Tem1 depleted cells are hypersensitive to Congo red, SDS and calcofluor white, suggesting that overall cell wall architecture has been altered in this strain.

In order to visualise these changes in cell wall structure the SWM6 strain was grown in non-repressing media for 5 hours and the cells harvested and prepared for TEM analysis using the jet propane freeze substitution protocol (section 2.8.3) as were SWM6 cells grown in repressing media for 12 hours. TEM analysis of these cells showed that the cell wall of the Tem1 depleted cells was not as uniform as that of the cells grown in non repressing media (Figure 32). Further experimentation would confirm these findings with the additional samples, including the CAI4 (CIp10) strain
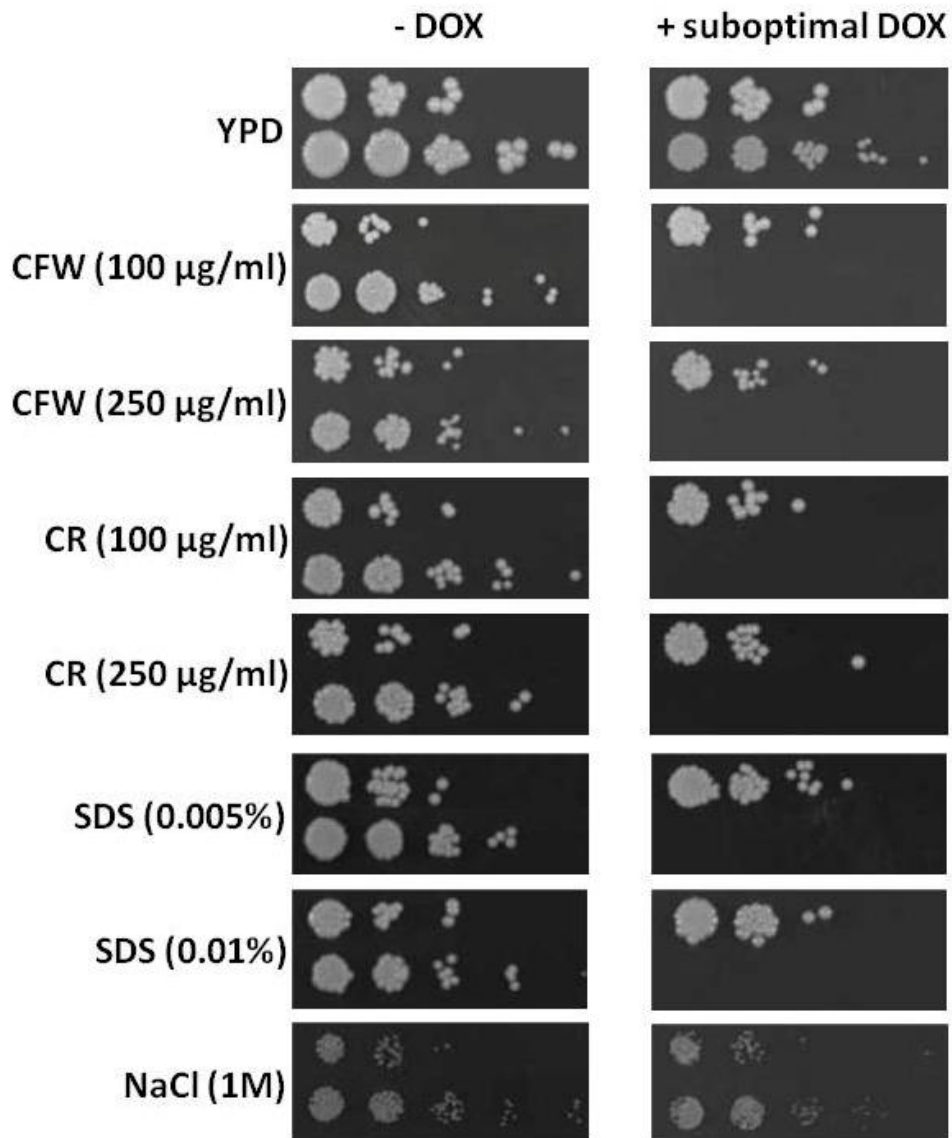
Figure 31. **Tem1 depleted cells are hyper sensitive to Sodium dodecyl sulphate, Calcofluor white and Congo red.** Serial dilutions (1/10), from 1×10^6 cells/ml down to 1×10^2 cells/ml, of the CAI4 (CIp10) (top row of colonies) and SWM6 (bottom row of colonies) strains were spotted on YPD agar plates (+/- dox) containing a cell wall perturbing agents calcofluor white (CFW), Congo Red (CR), sodium dodecyl sulphate (SDS) and salt (NaCl).
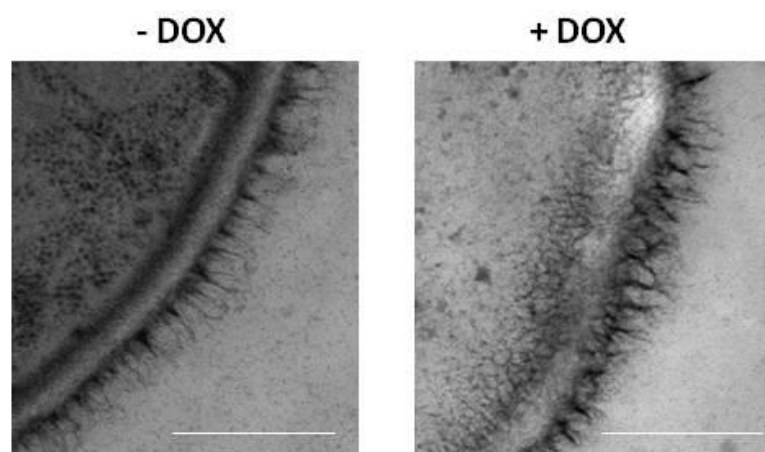
Figure 32. TEM analysis of the outer cell wall of the SWM6 cells grown in repressing and non-repressing conditions. SWM6 cells were grown in repressing and non-repressing media for 16 and 5 hours respectively. Samples were then fixed and prepared for visualisation using the transmission electron microscope. Analysis of the samples showed that the cell wall of the cells grown in the repressing media were not as uniform as those of the cells grown in the non-repressing media. Scale bars equal 1 µm.

grown in both repressing and non-repressing media and additional cuts of the previous SWM6 samples.

Overall these findings confirm that depletion of the Tem1 protein does result in changes to cell wall structure. These changes may be the cause of the problems encountered with cellular staining.

3.2.11. Overexpression study of Tem1.

To complement our work on the growth effects of Tem1 depletion we also investigated the effect of overexpressing *TEM1*. This work was carried out using the SWM6 strain as the tetracycline regulatable promoter system constitutively expresses the target gene at a high level (Nakayama *et al*., 2000). Protein analysis by western blotting showed that expression of Tem1 by its native promoter was undetectable (data not shown) while Tem1 expressed from the TET promoter was detected (Figure 22), however repetition is required to confirm this result. In addition quantitative rtPCR could be used to confirm the increase expression of *TEM1* from the TET promoter when compared to the native promoter. As Figure 18A shows the SWM6 stain maintains a similar growth rate with that of CAI4 (CIp10) throughout the 24 hour period of the growth assay. This confirms that overexpression of the *TEM1* gene in the SWM6 strain does not cause any major effect on cell growth.

3.2.11.1. SWM6 cells display slightly enhanced response to the growth environment.

To evaluate the effect of overexpression of *TEM1* on hyphal formation we spotted the CAI4 (CIp10) and SWM6 strains onto either YPD agar containing 5, 10, 15 or 20 % serum, or onto Lee's agar plates (Figure 33A and B). We observed that on all four serum concentrations the SWM6 colonies were wrinkled which suggests the cells were filamentous while the CAI4 (CIp10) colonies were only wrinkled on the 15 and 20 % serum medium (Figure 33A). Similarly on the Lee's media the SWM6 colonies produced a larger filament ring around the central colony than the CAI4 (CIp10) strain did (Figure 33B). On spider medium the single colony for each strain were of a similar size, however the central part of the CAI4 (CIp10) colony was easily distinguishable from the filamentous fringe while it is difficult to differentiate between the central part and the fringe in the SWM6 colony (Figure 33C). After 9 days of incubation the central
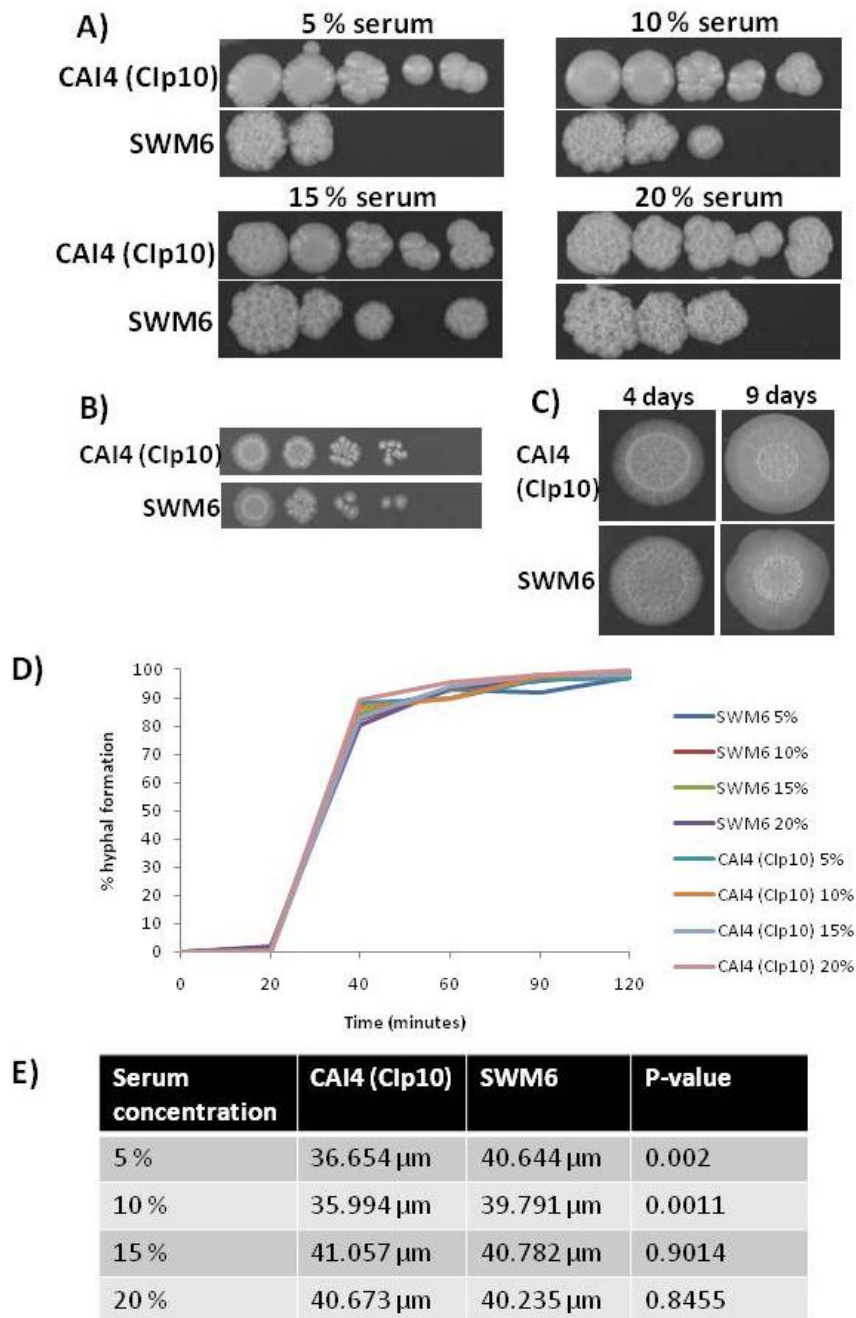
Figure 33. Cells overexpressing the *TEM1* gene have increase sensing of low serum concentrations. The growth of the CAI4 (CIp10) and SWM6 strains on hyphae-inducing serum after 3 days incubation at 37 ˚C (A), Lee's (B; 4 days at 30 ˚C) and spider (C; 9 days at 30 ˚C) media. Serial dilutions (1/10) were carried out from 1 x 10^6 cells/ml (YPD+serum) or 1 x 10^5 cells/ml (Lee's) and the cells were spotted onto the media using a 48 pronged replicator. For the spider media 5 x 10^6 cells/ml were pipette onto the media. CAI4 (CIp10) and SWM6 cells were grown in four different serum concentrations (5, 10, 15, 20 % serum) for 120 minutes and the percentage of cells which had formed hyphae were counted at 20 minute intervals. 100 cells were counted for each serum concentration at each time point. This was replicated three times and the average percentage was calculated (D). Cells from the 120 minute samples were fixed and the lengths of 50 hyphae were measured using the Image J software (E). P-values were generated using a student t-test.

part of the SWM6 colony was visible. However the filamentous fringes of the two strains had a different appearance. The fringe of CAI4 (CIp10) colony was circular while the SWM6 colony had a fringe that was undulate which suggest differential patterns of hyphal growth of the SWM6 strain. Overall these assays suggest that the SWM6 strain may have different sensitivity to various environmental hyphae inducing conditions than the CAI4 (CIp10) wild type strain.

In liquid medium assays we found that at 20 minutes post inoculation between 1 and 2 % of cells formed germ tubes for both strains but by 40 minutes this had risen to between 80 and 90 % and this was irrespective of serum concentration (Figure 33D). However we found that after 2 hours of growth in the 5 % serum medium the hyphae formed by the SWM6 cells (40.644 µm) were significantly longer than those formed by the CAI4 (CIp10) cells (36.654 µm; p-value 0.002; Figure 33E). This was also observed for the cells grown in 10 % serum (SWM6 39.791 µm; CAI4 (CIp10) 35.994 µm, p-value 0.0011), however in the 15 and 20 % serum media no significant difference was observed. These findings suggest that the SWM6 strain exhibits a slightly enhanced response to changes in environmental conditions.

3.2.11.2. The SWM6 strain is fully virulent in a *G. mellonella* virulence model.

To assess if overexpression of the *TEM1* gene had any effect on virulence we used an insect model: the larvae of the wax moth, *Galleria mellonella* (Cotter *et al.*, 2000). The *G. mellonella* virulence model was developed to provide an alternative to the murine virulence models. The *G. mellonella* model has advantages over the mouse models such as cost, length of assays and no home office licence is required to conduct the experiments. A virulence study carried out by the Kevin Kavanagh's group using a series of *C. albicans* mutants that influence the yeast to hyphae transition showed a good correlation between the virulence results seen in the *G. mellonella* assays to those seen in systemic mouse infections (Brennan *et al.*, 2002). However, the use of the *G. mellonella* model has been limited with the mouse models being preferentially used (Cotter *et al.*, 2000; Brennan *et al.*, 2002; Askew *et al.*, 2009; Fuchs *et al.*, 2010).

Each larva was injected with 1 x 10^5 cells of the CAI4 (CIp10) or SWM6 strains or PBS as a control and the number of dead larvae was counted every 24 hours for four days
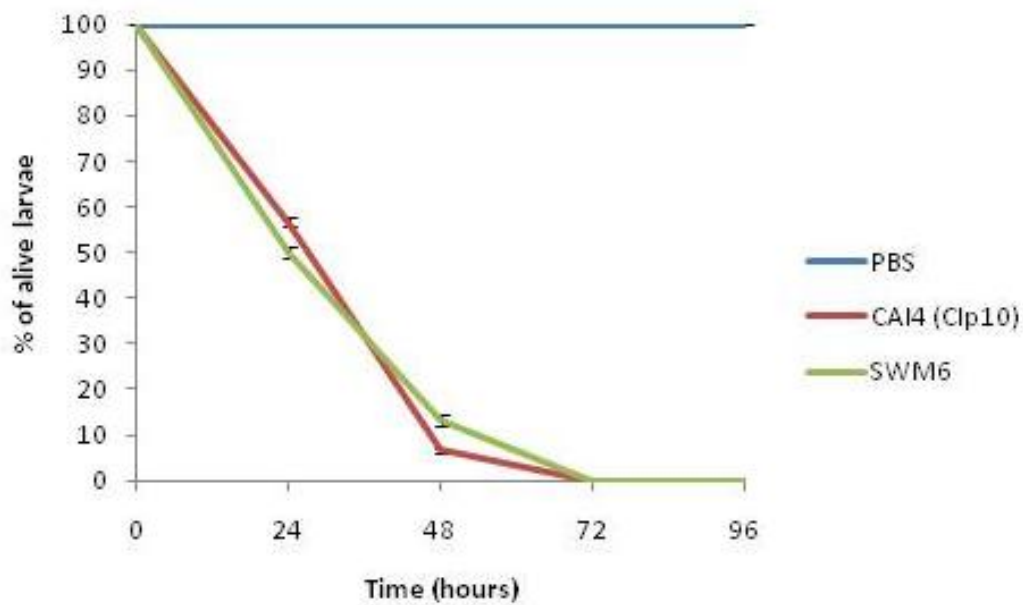
Figure 34. Overexpression of *TEM1* has no affect on virulence in a *Galleria mellonella* model. CAI4 (Clp10) and SWM6 cells were grown to mid-log phase in YPD prior to being washed with PBS and the cell number normalised. 1×10^5 cells were injected through a pro-leg of a *G. mellonella* larva. As a negative control 10 µl of PBS was injected into each of ten larvae were infected for each strain and incubated at 37 ˚C for 96 hours. The number of dead larvae were counted every 24 hours throughout the assay and converted into a percentage of total larvae. This was repeated in three replicates.

(Figure 34). None of the larvae injected with the PBS died by the end of the assay while all of larvae injected with the CAI4 (CIp10) cells died. These results confirm that the death of the larvae was caused by the *C. albicans* cells and not the process of injecting the larvae. We found that both the control strain CAI4 (CIp10) and the test strain SWM6 kill the larvae at a similar rate and by 72 hours all the infected larvae had died. This result confirms that overexpression of the *TEM1* gene has no effect on the ability of the cells to infect and kill the *G. mellonella* larvae. The effect of Tem1 depletion on virulence was not tested in this study due to two reason; the size and shape of Tem1 depleted cells makes it difficult to control dosage and also the kinetics of TET promoter repression within *G. mellonella* larvae were still being investigated at the conclusion of this study. However, a recent study found that Tem1 is required for virulence in a systemic mouse model (Becker *et al.*, 2010).

3.3. Discussion.

3.3.1. *C. albicans TEM1*.

The budding yeast *Saccharomyces cerevisiae* has two regulatory networks that control the progression of the cells through anaphase, the FEAR and MEN. The FEAR network is responsible for signalling the release of the Cdc14 protein from the nucleolus into the nucleus at the start of anaphase and results in the initiation of cyclin destruction and spindle midzone assembly (Rock and Amon, 2009). Homologs of the various FEAR components; the Polo-like kinase, the seperase protein and the protein phosphatase 2A complex, have been found in all eukaryotes (Rock and Amon, 2009). However no fully functioning FEAR network has been identified in any other eukaryote organism to date, and we found that many components of the network are absent in *C. albicans*. Homologs of all the MEN proteins can be found within the *C. albicans* genome, with varying degrees of conservation. This finding is not surprising as the MEN pathway is highly conserved through the fungal kingdom and even in some plants, however often under the name of the MENs sister pathway the SIN (Bedhomme *et al.*, 2008). Despite the MEN and SIN pathways signalling two different processes: mitotic exit and septation respectively, both pathways are composed of a majority of the same proteins and also the septin proteins are involved in both networks (Bardin and Amon; 2001; Simanis, 2003).

Here we document the identification of a *TEM1* homolog in *C. albicans*. The protein encoded by this gene is 200 amino acids in length and is 46.5 % identical to the *S. cerevisiae* Tem1 protein. Analysis of the *Ca*Tem1 protein sequence identifies it as a GTPase binding protein due to the presence of the three consensus GTP-binding domains. The *C.a*Tem1 protein sequence also contains six G box motifs which are conserved among all Ras family GTPase proteins (Bourne *et al.*, 1991).

Phylogenetic analysis of the *C.a*Tem1 protein and a number of homologs from across the fungal and plant kingdoms revealed that as expected the *C.a*Tem1 groups within the other ascomycete yeast species. Within the yeast grouping two specific groups emerged with *S. cerevisiae* in one group and *C. albicans* in the other. Despite this grouping the *C. albicans TEM1* is fully functional when expressed in *S. cerevisiae* cells and can complement the temperature sensitive *tem1-3* strains. This result was similar to that observed by Oh *et al.*, (2010) using a different overexpressing plasmid system (pAG416GPD-ccdB) introduced into a *tem1* null strain. The findings of Oh *et al.*, (2010) and this study confirm that the *C.aTEM1* gene is an ortholog of its *S. cerevisiae* counterpart. This suggests that Tem1 in *C. albicans* could be involved in both mitotic exit and cytokinesis.

Interestingly the phylogenetic analysis found that the ascomycete phylum is split into two groups, the yeast species and the other species. Aside from the obvious differences between the two groups, one grows predominantly as yeast cells and the other as filamentous cells. This split of the ascomycetes could reflect the divergence of the function of the *TEM1* homologs. Further phylogenetic and sequence analysis is required to be carried out to confirm this theory and further investigation may also identify protein differences that could explain the high intrinsic GTP hydrolysis of the *Sc*Tem1 protein and whether this is conserved in the *Ca*Tem1 protein.

3.3.2. *TEM1* is essential for *C. albicans* vegetative growth.

In *S. cerevisiae* the *TEM1* gene is essential for cell viability and the loss of Tem1 functionality results in the cells arresting as dumbbells with divided nuclei (Shirayama *et al.*, 1994b). This essentiality phenotype has also been seen in *Candida glabrata* where the cells lacking the *TEM1* gene arrest as dumbbells and also when the *SPG1*

gene is deleted in *S. pombe* (Schmidt *et al.*, 1997; Miyakawa *et al.*, 2009). As a result of *C. albicans TEM1* being able to complement the temperature sensitive *S. cerevisiae tem1-3* strain we hypothesised that *TEM1* in *C. albicans* is essential, we therefore constructed a regulatable *TEM1* mutant using the tetracycline regulatable promoter system. Analysis of this strain showed that *TEM1* is essential in both the yeast and hyphal growth forms of *C. albicans*. Thus suggesting the role of Tem1 is universal to all *C. albicans* growth forms. Depletion of Tem1 resulted in loss of viability by 24 hours in repressing media less than 2 % of the cells were viable, confirming that the loss of Tem1 has cidal effects which makes Tem1 an attractive potential antifungal target (Figure 18B).

3.3.3. Tem1 depletion results in Hgc1-independent filament formation.

One of the major effects of Tem1 depletion in *C. albicans* is that the cells form elongated filaments (Figure 19, 20 and 21). During the first 5 hours of incubation in repressing medium the cells grew as budding yeast cells, however after 5 hours this growth rate slowed and then the cells spontaneously formed filaments. These filaments emerge as wide non-parallel sided pseudohyphae before tapering down to resemble true hyphae at later time points. This was driven by a reorganisation of actin in the cells as a result of a switch from isotropic to polarised growth (Figures 23). The 8 hour delay between the start of the assay and the formation of the filaments is likely caused by the time required for the Tem1 protein to become depleted. The decreased growth rate coincides with the expression of the *TEM1* becoming undetectable by western blotting at 4 hours post inoculation into the repressing media.

The emergence of these filaments is independent of the actions of the hyphal-specific G_1 cyclin Hgc1, however *HGC1* was expressed at the 16 and 20 hour time points. This proves that the switch from pseudohyphal to true hyphal growth occurs prior to *HGC1* expression. Hgc1 has previously been shown not to be required for the initiation of hyphal growth, but it was shown to be essential for the maintenance of hyphal growth (Zheng *et al.*, 2004). Our findings suggest that Hgc1 may be involved in the maintenance of the polarised growth in the Tem1 depleted filaments.

The promotion of filamentous growth has previously been reported as a consequence

of cell cycle defects such as G_1 blockage caused by the repression of Cln3 expression (Chapa y Lazo *et al.*, 2005). This was also supported by the observation that when wild type cells were incubated in media containing the cell cycle inhibitors hydroxyurea or nocodazole they formed similar filaments (Bachewich *et al.*, 2003). We observed that the filaments formed by Tem1 depleted cells emerged from large budded cells (Figure 19). These filaments also resemble true hyphae that form during the latter stages of mitosis while the mother and daughter cells are still attached (Hazan *et al.*, 2002). Previous studies have shown; through FACS analysis, cyclin expression studies and microscope analysis utilising fluorescent dyes, that filaments forming as a result of an early cell cycle blockage emerge from unbudded yeast cells however if the cell cycle blockage occurs late in mitosis filaments emerge from large budding cells (Bachewich *et al.*, 2003; Wightman *et al.*, 2004; Bachewich and Whiteway, 2005; Chapa y Lazo *et al.*, 2005; Atir-Lande *et al.*, 2005; Bensen *et al.*, 2005; Anduluz *et al.*, 2006; Umeyama *et al.*, 2006). This leads us to conclude that Tem1 depleted cells undergo a blockage late in the cell cycle.

3.3.4. Tem1 is required for timely mitotic exit.

Exit from mitosis in *S. cerevisiae* requires the activation of *TEM1* which signals through the mitotic exit network for the phosphatase Cdc14 to be released from the nucleolus and this allows the execution of mitotic exit (Kitada *et al.*, 1993; Shirayama *et al.*, 1994a; Shirayama *et al.*, 1994b; Donovan *et al.*, 1994; Toyn and Johnston, 1994; Charles *et al.*, 1998; Komarnitsky *et al.*, 1998; Luca and Winey, 1998; Alexandru *et al.*, 1999; Fesquet *et al.*, 1999; Fraschini *et al.*, 1999; Li, 1999; Visintin and Amon, 2003; Bardin *et al.*, 2003; Mohl *et al.*, 2009). In this chapter we highlighted several observations that suggest the Tem1 protein in *C. albicans* is involved in mitosis. Firstly, as discussed above, cells depleted of the Tem1 protein form filaments which are typical of cells undergoing a cell cycle arrest. Secondly, Tem1 depleted filaments predominantly contain only one nucleus at 8 and 12 hours post depletion which suggests a blockage in the cell cycle (Figure 25B). This same phenotype has been seen in cells depleted of Cln3, Cdc5, Clb2 and Rad52 (Chapa y Lazo *et al.*, 2003; Bachewich *et al.*, 2003; Bensen *et al.*, 2005; Andaluz *et al.*, 2006). However, at 16 and 20 hours the vast majority of the cells contained either one or two nuclei which imply the cells have completed chromosome segregation but had not re-entered the cell cycle. This could

be further confirmed by carrying out FACS analysis to study the DNA content of the cells or alternatively be conducting quantitative analysis of the expression of cell cycle dependent genes. At 20 hours only a small number of cells had bypassed the cell cycle blockage. A theory has been proposed, in human cells, that once cells undergo a prolonged mitotic arrest the cells fate is determined by one of two pathways. These two pathways are programmed cell death and destruction of the B cyclins through an "adaptation" process consisting of the activation of CDK inhibitors and the inhibitory phosphorylation of CDK (Huang *et al*., 2010). The latter leads to a phenomenon called mitotic slippage. Mitotic slippage is a potential explanation for the few Tem1 depleted cells that were able to escape this mitotic blockage. This has been observed before in *C. albicans* cells. Bai *et al*., (2002) observed that a wild type *C. albicans* strain grown in presence of cell cycle inhibitor nocodazole was able to overcome this cell cycle arrest and enter into a new cell cycle. The trend of the cells undergoing a period of cell cycle arrest followed by some of the cells overcoming this was also observed when the number of spindle poles in the cells were counted (Figure 26). We also observed that these SPBs were separated from each other which confirmed that the Tem1 protein plays no role in spindle elongation and that the mitotic arrest caused by Tem1 depletion occurs after the spindle elongation in early anaphase.

The Tem1 protein is not the only MEN protein in *C. albicans* that has been shown to cause a delay in mitotic exit upon depletion. Deletion of the phosphatase protein Cdc14 has also been seen to cause a delay in mitotic exit. However unlike *TEM1 CDC14* is not an essential gene in *C. albicans* and this suggests that the essential function of *TEM1* is not completely dependent on Cdc14. This would suggest that the MEN is branched at some point in *C. albicans*, and that Tem1 signals for mitotic exit through other factors. Alternatively the main role of Tem1 in *C. albicans* may be cytokinesis and not mitotic exit. To identify if the main role of Tem1 is in signaling mitotic exit or cytokinesis a protein involved in mitotic exit downstream requires to be identified. Increasing the levels of this protein in cells would allow mitotic exit to occur in the absence of Tem1 and therefore allow the role of Tem1 in cytokinesis to be discovered.

3.3.5. Tem1 depleted cells are unable to form a primary septum.

The process of cytokinesis is the final part of mitosis and requires a number of different proteins in *S. cerevisiae*. One such protein is Tem1 which responsible for initiating actomyosin ring contraction and septin ring splitting (Lippincott *et al*., 2001). To assess the role Tem1 plays during cytokinesis in *C. albicans* we tagged the septin protein Cdc3 with GFP in the SWM6 strain (SMW71). In this chapter we showed that cells depleted of Tem1 fail to undergo cell separation and undergo a mitotic arrest (Figure 29). We found that Tem1 depleted cells contained one area of septin localisation. This was located at the initial mother bud junction. This finding was also seen when the cells were stained with calcofluor white (CFW) to stain the cellular chitin and is very similar to the observations documented by Hazan *et al*., (2002) for cells that formed hyphae while the cells were in the latter stages of mitosis.

The septin band present in Tem1 depleted cells was significantly wider than those found in the non-depleted cells. This is probably caused by the Tem1 depleted yeast cells undergoing an extended period of isotropic growth which results in these cells being significantly larger than normal yeast cells. In *S. cerevisiae* the septin proteins form an hourglass like structure during the S phase of the cells cycle through to the end of anaphase after which the septin band splits in two, actomyosin ring contraction occurs and the primary septum forms (Gladfelter *et al*., 2001; Oh and Bi, 2010). In all the SWM71 cells depleted of Tem1 the septin ring resembles an hourglass therefore suggesting Tem1 is required for septin ring splitting in *C. albicans*. This further supports the evidence gathered from SPB and nuclei localisation work that Tem1 depleted cells arrest late in anaphase.

In *S. cerevisiae* cells the septin rings persist at the site of budding/birth until the cell enters into the next cell cycle and a new ring forms (Gladfelter *et al*., 2001). In the Tem1 depleted cells only one site of septation was labelled with Cdc3-GFP or CFW, even when nuclear staining and SPB counts suggested that the cells have undergone two or more nuclear divisions (Figure 25, 26, 28, and 29). This is probably caused by the septin proteins failing to be recruited to a new division site presumably due to the lack of a signal for septin ring disassembly.

In conclusion, our research identified one homolog of *S. cerevisiae TEM1* in the *C. albicans* genome. We have shown that *TEM1* is essential in *C. albicans* and that the protein it encodes is required for both mitotic exit and cytokinesis.

Chapter 4- Protein interactions and regulation of Tem1 in

C. albicans.

4.1. Introduction.

The ability of GTPase proteins to switch from the inactive GDP-bound state to the active GTP-bound state and back again is critical to their roles in many cellular regulatory networks (Boguski and McCormick, 1993). In *S. cerevisiae* during the majority of the cell cycle the Tem1 protein is maintained in its inactive GDP-bound state by the two protein GAP complex Bfa1-Bub2 and the regulatory kinase Kin4 (Pereira *et al.*, 2000; Lee *et al.*, 2001; Geymonat *et al.*, 2002; Ro *et al.*, 2002; D'Aquino *et al.*, 2005). The Kin4 kinase is located in the mother cell cortex and prevents the Cdc5 kinase from phosphorylating Bfa1 while both SPB are within the mother cell (Geymonat *et al.*, 2003; D'Aquino *et al.*, 2005; Pereira and Schiebel, 2005). Upon entry of one SPB into the daughter cell the Cdc5 protein phosphorylates Bfa1 which results in the Bfa1-Bub2 complex dissociating from Tem1 and allowing Tem1 to become activated (Geymonat *et al.*, 2003). At the same time, the dSPB comes in contact with Lte1, which localise to the cortex of the daughter cell, and Lte1 recruits the Bfa1-Bub2 complex and Tem1 from the mother-bound SPB (mSPB) to the dSPB (Bardin *et al.*, 2000; Jensen *et al.*, 2002; Geymonat *et al.*, 2009). This recruitment of Tem1 from the mSPB results in an increase in activated Tem1 on the dSPB and this increases the MEN activation signal. Following activation Tem1 recruits Cdc15 to the dSPB and activates it (Cenamor *et al.*, 1999). The activated Cdc15 in turn recruits and activates Dbf2 which leads to release of Cdc14 from the nucleus and mitotic exit (Frenz *et al.*, 2000; Visintin and Amon, 2001; Luca *et al.*, 2001; Stoepel *et al.*, 2005; Mohl *et al.*, 2009). So overall, the MEN is regulated through the special regulation of the component proteins.

Yeast 2-hybrid (Y2H) systems are a frequently used tool for studying protein-protein interactions. The Y2H systems involves fusing a protein of interest to a DNA binding domain of a transcription factor such as the Gal4 transcription factor (bait), while fusing another protein of interest to the activator domain of a transcription factor (prey; Fields and Song, 1989). If the two proteins interact the DNA binding domain and the activator domain are brought into close proximity to each other and bind together

to form a functional transcription factor resulting in the expression of reporter genes (James *et al.*, 1996).

Here we show that the Tem1 protein localises to both spindle poles in a cell cycle dependent fashion. However, although essential for cytokinesis Tem1 does not relocate to the size of cytokinesis upon completion of mitosis. Additionally we show that the Bfa1-Bub2 complex binds to the Tem1 protein suggesting that the complex plays a role in Tem1 regulation, and that the essential function of Tem1 is signalled through the Cdc15 protein. We finally showed that Lte1 protein is likely to be essential for vegetative growth and that the protein localises to the bud cortex during mitosis.

4.2. Results.

4.2.1. Tem1 localisation.

4.2.1.1. Tem1 localises to the spindle pole bodies.

In *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* the Spg1/Tem1 proteins localise to spindle pole bodies (Sohrmann *et al.*, 1998; Bardin *et al.*, 2000; Simanis, 2003; Molk *et al.*, 2004). To study the localisation of the Tem1 protein in *C. albicans* we constructed a strain in which the *TEM1* gene was tagged with *GFP* under control of its endogenous promoter. This was achieved by amplifying the GFP-*URA3* cassettes from the pMG1509 plasmid using the TEM1-GFP-F and TEM1-URA3-R primers and transforming it into the CAI4 strain (Table 5). Successful integration of the cassette at the native *TEM1* locus was confirmed by colony PCR using the GFP-UP and TEM1-TAG-S primers (Table 5). One PCR positive transformant was produced (SWM52; Figure 35A and B).

The SWM52 strain was grown in YPD media for 5 hours and the cells washed three times with PBS. Following washing the cells were analysed using fluorescent microscopy. Tem1-GFP was seen to localise to one or two distinct spots reminiscent of SPB localisation in *S. cerevisiae* (Figure 35C; Kahana *et al.*, 1995; Yeh *et al.*, 1995; Bardin *et al.*, 2000; Molk *et al.*, 2004). In cells with small to medium sized buds these spots are found in the mother cells but in cells with a large bud one of the spots migrates to the daughter bud. To determine where Tem1 localises in pseudohyphal and true hyphal cells the SWM52 strain was grown at 37 ˚C in YPD or YPD + 20 %
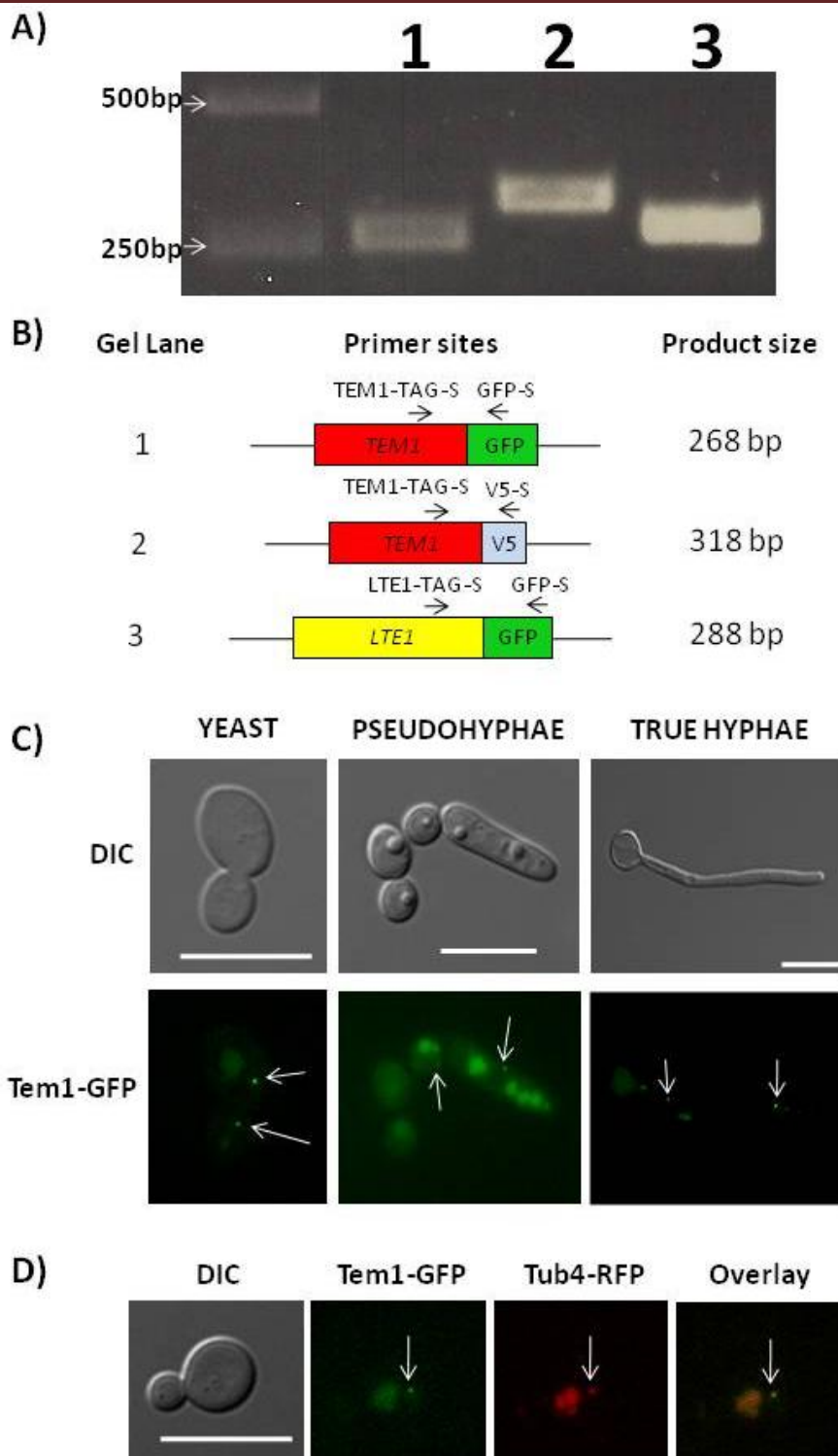
Figure 35. Tem1 localises to the spindle pole bodies. Colony PCR was carried out using a forward primer within the target gene ORF and a reverse primer within the tag codon DNA. Lane 1 confirms integration of *GFP-URA3* into the *TEM1* locus. Lane 2 confirms integration of V5-*NAT1* into the end of the *TEM1* locus. Lane 3 confirms integration of *GFP-URA3* into the end of the *LTE1* gene (A). PCR results are consistent with primer annealing sites and expected PCR product sizes (B). Tem1 localising to the SPB in yeast, pseudohyphal and true hyphal cells (C). Co-localisation of Tem1 with the SPB marker γ-tubulin Tub4 protein (D). The large fluorescent area in the cells is probably due to auto fluorescence from the vacuole. Scale bar equals 10 μm. Arrow indicate the localisation of protein-GFP fusion.

serum for 150 minutes or 90 minutes. As in yeast cells, Tem1-GFP localises to one or two distinct spots within the cells (Figure 35C).

In order to confirm Tem1-GFP is localised on the SPB we tagged a copy of the *TUB4* gene, encoding γ-tubulin, in the SWM52 strain with the *RFP-NAT1* cassette, yielding the SWM142 strain (Table 5). Microscopic analysis of the SWM142 strain shows that the Tem1-GFP fusion protein does localise to the SPBs as the Tem1-GFP co-localises with the Tub4-Rfp protein (Figure 35D). Analysis of the SWM52 strain using the same *RFP* filter and exposure time showed that the signal from the Tem1-GFP could not be detected using this filter. This confirms that the signal from the SWM142 cells when exposed to light from the *RFP* filter was the result of the Tub4-Rfp fusion protein and not bleed through from the Tem1-GFP proteins. Therefore, Tem1 is localised on the SPBs.

4.2.1.2. Tem1 localisation is cell cycle regulated.

To assess whether Tem1 localisation to the SPB is cell cycle regulated we grew the SWM52 strain in YPD for 4 hours. This resulted in an asynchronous culture containing cells at various different stages of the cell cycle. These cells were then microscopically analysed and scored for budding pattern and Tem1 localisation. In unbudded G_1 cells no Tem1-GFP signal was detected in the majority of the cells (60 %, n=80 cells) but as the cells entered into the G_1-S transition and buds appeared one distinct Tem1-GFP spot was detected in 65 % of the cells (Figure 36A and B; n=120 cells). This indicates that Tem1 is loaded onto the SPB early in the cell cycle, at around the time of S-phase initiation. As the cell cycle progressed and the daughter bud enlarged two Tem1-GFP spots were detected with one moving into the daughter bud at the later stages of the cell cycle (Figure 36A and B). Following the conclusion of cytokinesis and cell separation the Tem1-GFP signal disappeared from the cells and this suggests the protein is either destroyed or delocalised from the SPB in G_1-phase. Contrary to the findings in *S. cerevisiae* where Tem1 preferentially localises to the dSPB (Molk *et al.*, 2004) we observed that the Tem1-GFP signal was similar on both SPBs throughout the cell cycle. Quantification would need to be carried out but this suggests a potential regulation difference between the two species. This could be undertaken by carrying out GFP intensity studies at different stages of the cell cycle. Alternatively live cell
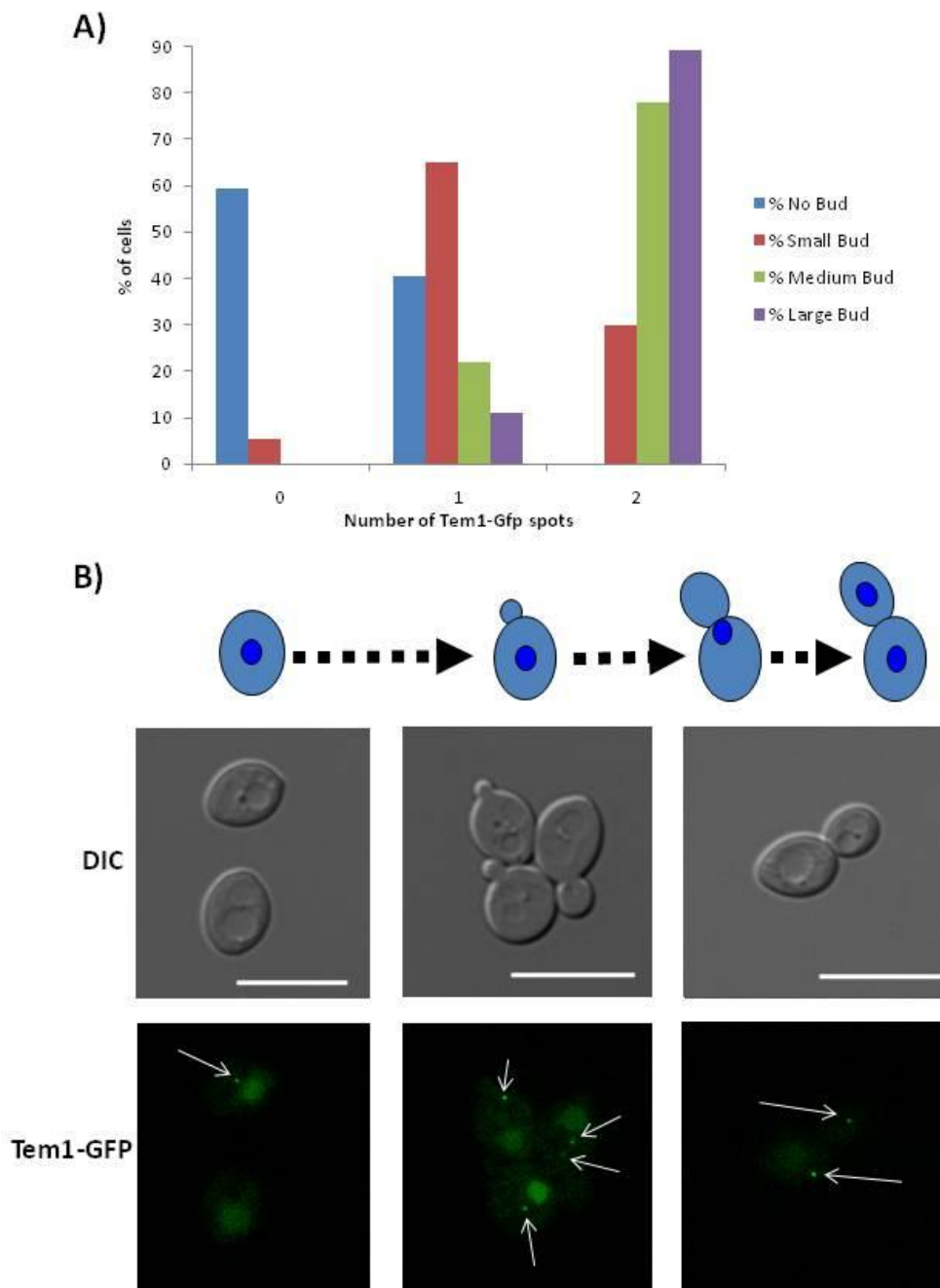
Figure 36. Tem1 localises to the spindle poles in a cell cycle dependent fashion. The number of Tem1 spots at different points of the cell cycle (n=203; A). Fluorescence microscope images showing the cell cycle nature of Tem1 spindle pole localisation (B). Scale bar equals 10 μm. Arrows indicate the localisation of the Tem1-GFP protein.

imaging could be carried out to detect any visual relocation of the Tem1-GFP from one SPB to the other. Within hyphal cells the duplicated spindle poles were observed to migrate into the hyphae prior to separation and this fits with the pattern of nuclear division seen in hyphal cells (Sudbery, 2001). The Tem1-GFP signal disappeared within hyphal cells post cytokinesis which matches what we observed in yeast cells. This finding confirms that Tem1 localisation is cell cycle regulated with Tem1 localising to the SPB at the onset of S-phase, and being lost from the SPB immediately following cytokinesis.

4.2.1.3. Tem1 does not localise to the bud neck.

The Tem1-GFP fusion protein was not detected at the mother daughter bud neck in any cells of the SWM52 strain. To investigate this further we constructed the SWM123 strain which would overexpress the *TEM1-GFP* fusion gene under the control of the constitutively active *ENO1* promoter (Staab *et al*., 2003). To construct this strain the *NAT1-ENO1*p cassette was amplified using the TEM1-ENO-F and TEM1-ENO-R primers and transformed into the SWM52 strain (Table 5). The successful integration of this cassette into the promoter region of the *TEM1-GFP* fusion gene was confirmed by PCR using the ENO-SF and GFP-UP primers (Table 5). Analysis of this strain showed that the Tem1p-GFP fusion protein did not localise to the mother-daughter bud neck. This confirms that as in *S. cerevisiae* the Tem1p protein in *C. albicans* only localises to SPBs (Bardin *et al*., 2000; Molk *et al*., 2004).

4.2.2. Lte1 protein.
4.2.2.1. Construction of an *lte1* null and conditional mutant.

In chapter 3 section 3.2.1.2 we showed that the *C. albicans* Tem1 protein possesses all the GTP/Mg^{2+} binding domains typically found in members of the Ras superfamily of GTPases (Figure 13A and B). We also showed that Tem1 contains four GEF domains. The GEF protein that is required for the GTPase activity of Tem1p in *S. cerevisiae* is Lte1. To evaluate the role the *LTE1* homolog (ORF 19.2238) plays in *C. albicans* we attempted to construct a null mutant of *lte1* in the SN78 parent strain. The first *LTE1* allele was disrupted using the *Candida maltosa LEU2* gene amplified from the pSN40 plasmid using the LTE1-KO-F and LTE1-KO-R primers (Table 5, Noble and Johnson, 2005). Eleven colonies formed one of which (SWM10) was confirmed by PCR as

containing the *CmLEU2* gene integrated into the *LTE1* gene. The second *LTE1* allele was disrupted with the *Candida dubliniensis HIS1* gene amplified using the LTE1-KO-F and LTE1-KO-R from the pSN52 plasmid (Table 5; Noble and Johnson, 2005). Nineteen colonies formed as a result of this transformation two of which were confirmed by PCR (SWM17 and SWM18). Additional PCR reactions were carried out on the SWM17 and SWM18 strains to confirm they both contained the *CmLEU2* and *CdHIS1* genes and had lost the *LTE1* gene using either the LEU2-SF, HIS-SF or LTE1-TAG-S primers in combination with the LTE1-SR primer (Table 5). However, the results of these PCR reactions demonstrate that a third copy of the *LTE1* gene was present in these strains (Figure 37A and B). This suggests that either there is a third chromosomal copy of the *LTE1* gene in *C. albicans* strain SN78, or that the *LTE1* gene is essential.

To confirm if the *LTE1* gene was essential for vegetative growth in *C. albicans* we attempted to construct a regulatable *LTE1* mutant. For this we decided to use two different promoter systems, the TET promoter and the *MET3* promoter (Care *et al*, 1999; Nakayama *et al*., 2000). To do this the first *LTE1* allele was deleted in the THE1 strain using the mini-URA-blaster cassette PCR amplified using the LTE1-URA-KO-F and LTE1-URA-KO-R primers (Table 5). Three PCR positive colonies formed (SWM98, 99, 100) of which the SWM98 and SWM 99 strains underwent 5-FOA selection to recycle the *URA3* gene from which one *URA3* minus colony formed (SWM112). The SWM112 strain was then transformed with either the amplified TET promoter or the *MET3* promoter cassettes. These two cassettes were amplified from pCAU99t plasmid (TET promoter) and the pFA-*URA3*-*MET3*p plasmid (*MET3*p) using the LTE1-TET-F and LTE1-TET-R primers or the LTE1-MET3-F and LTE1-MET3-R primers (Table 5). Despite numerous attempts no PCR positive transformants were identified for either cassette. This is may be because both of these promoter systems continually express the target gene, and our failure to construct the regulatable strain suggests that the expression of the *LTE1* gene is tightly regulated.

4.2.2.2. Lte1 localises to the bud cortex.

In both the mitotic exit and septation initiation networks the proposed GEF proteins (Lte1 and Etd1) localise to the cell cortex, the daughter cell cortex in *S. cerevisiae* and the cell cortex at both poles in *S. pombe* (Bardin *et al*., 2000; Pereira *et al*., 2000; Daga
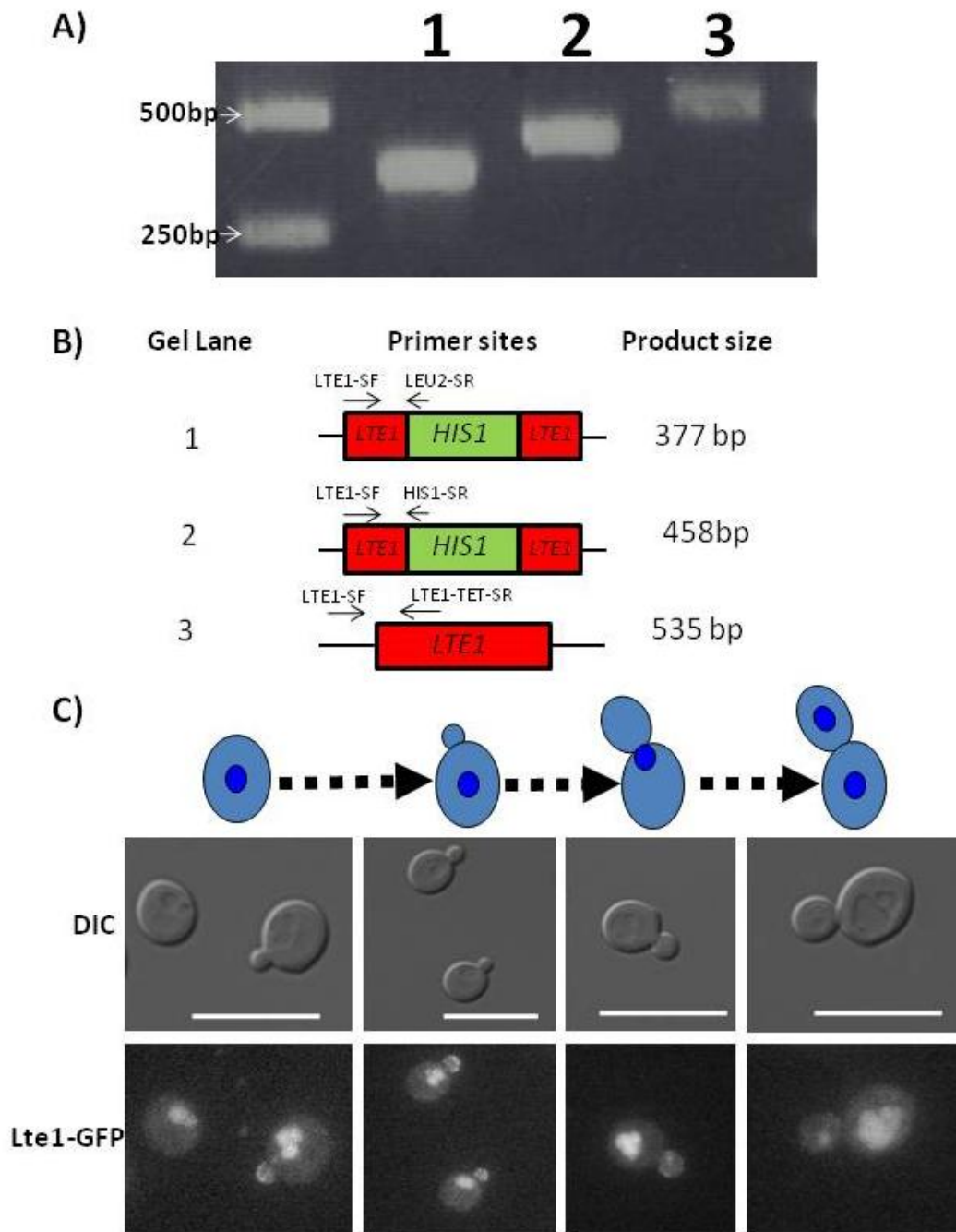
Figure 37. Lte1 localises to the bud cortex in a cell cycle dependant manner. PCR confirmation that the SWM18 strain contains the *LEU2* (lane 1) and the *HIS1* (lane 2) disruption cassettes integrated into the two *LTE1* genes. Lane 3 shows the presence of a third copy of the *LTE1* gene (A). PCR results are consistent with primer annealing sites and expected PCR product sizes (B). The cell cycle dependant localisation of Lte1-GFP to the bud cortex (C). Scale bar equals 10 µm.

et al., 2005; Geymonat *et al.*, 2009). We decided to investigate the localisation of the Lte1 protein in *C. albicans* cells so we constructed the SWM121 strain which has one of the *LTE1* alleles tagged with the *GFP-URA3* cassette. The cassette was amplified from the pMG1506 plasmid using the LTE1-GFP-F and LTE1-GFP-R primers and then transformed into the CAI4 strain (Gerami-Nejad *et al.*, 2001). Two PCR positive colonies formed (SWM120 and SWM121). The GFP signal was detected following microscopic analysis in the SWM121 strain (Figure 37C). In yeast cells of the SWM121 strain the Lte1-GFP fusion protein was not visible in unbudded cells but upon bud emergence the Lte1-GFP fusion protein localises to the cell cortex where it remains until prior to cytokinesis when the protein is released from the bud cortex and into the bud cytoplasm (Figure 27B). This suggests that the Lte1 protein localisation to the cell cortex is regulated in a cell cycle dependent fashion. The exact timing of the release of Lte1 from the cell cortex was not studied. The signal from the Lte1-GFP fusion protein was faint and was not detected in all budding cells. This localisation pattern is similar to that seen in *S. cerevisiae* (Bardin *et al.*, 2000; Pereira *et al.*, 2000; Geymonat *et al.*, 2009). Interestingly the Lte1-GFP fusion protein could not be localised within either pseudohyphal or true hyphal cells. This suggests that Lte1 is either not expressed in pseudohyphal and true hyphal cells or lacks a specific location. Western blotting analysis would confirm this finding.

4.2.3. The essential function of Tem1 is signalled through the Cdc15 protein.

Studies in *S. cerevisiae* showed that overexpressing *CDC15* was able to overcome the loss of either *lte1* or *tem1* (Shirayama *et al.*, 1994a; Shirayama *et al.*, 1994b). We decided to investigate this in *C. albicans* by overexpressing the *CDC15*, *DBF2* and *CDC14* genes in the SWM6 strain. To do this we integrated the *NAT1-ENO1*p cassette into the promoter region of these three genes within the SWM6 strain. The cassettes were targeted to the promoter region of each gene using the primers listed in the gene overexpression section of Table 5. Two PCR positive colonies formed for each target strain (Figure 38A and B). From these colonies the strains SWM72 (*ENO1*p-*CDC15*), SWM84 (*ENO1*p-*DBF2*) and SWM95 (*ENO1*p-*CDC14*) were selected and further analysed. To confirm that these strains overexpress *CDC15*, *DBF2* and *CDC14* we carried out RT-PCR. The results from the RT-PCR show that in the SWM6 strain the expression levels of these three genes was low while the mRNA levels in the SWM72,
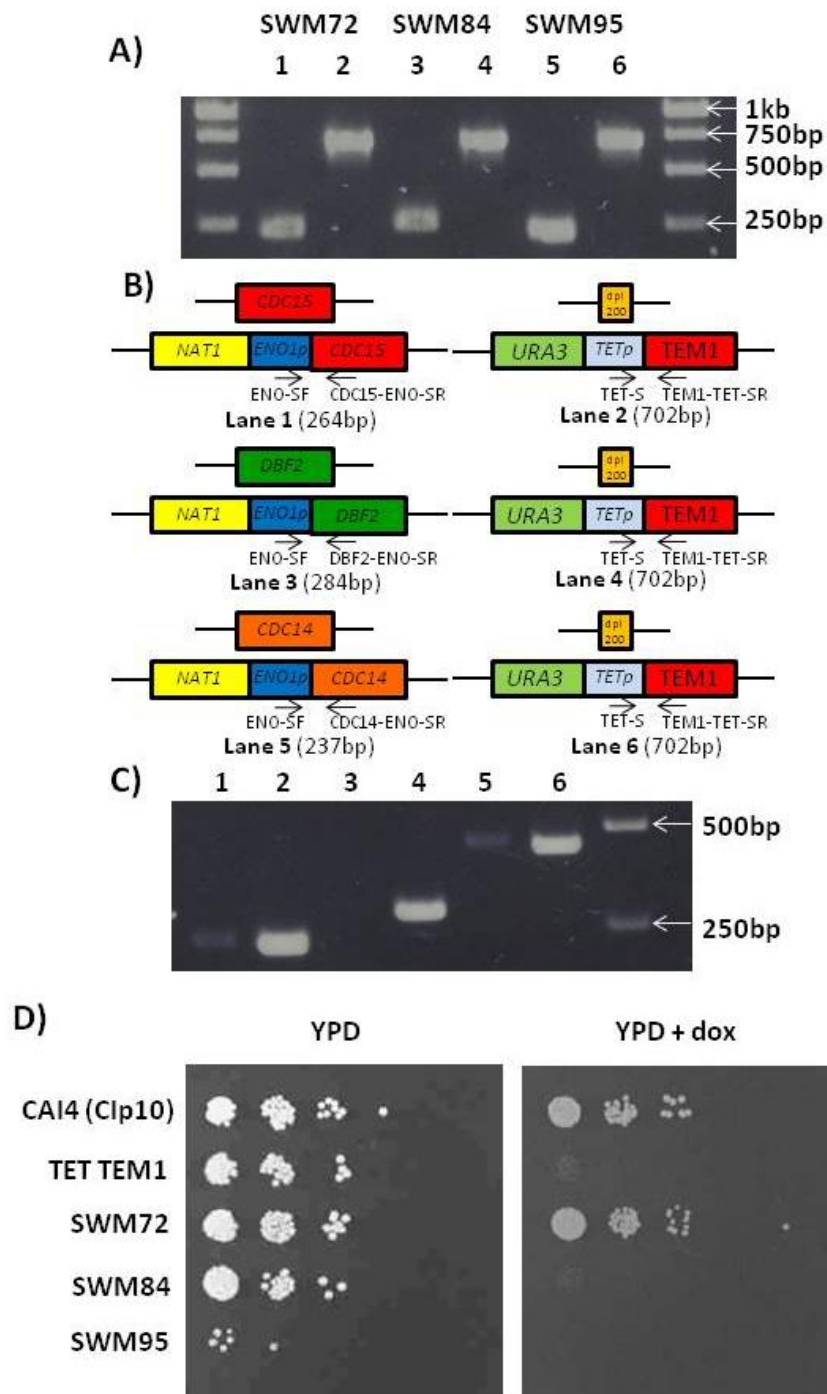
Figure 38. The essential function of Tem1 is signalled through the Cdc15 protein. PCR confirmation of the *ENO1*p cassette insertion into the promoter region of the *CDC15* (lane 1), *DBF2* (lane 3) and *CDC14* (lane 5) genes in the SWM6 strain. Lanes 2, 4 and 6 confirm that the cassettes were transformed into the SWM6 strain by PCR using the TET-S and TEM1-TET-SR primers (A). PCR results are consistent with primer annealing sites and expected PCR product sizes (B). RT-PCR analysis to confirms overexpression of the *CDC15* (lane2), *DBF2* (lane 4) and *CDC14* (lane 6) genes compared to the same genes in the CAI4 (CIp10) strain (lanes 1, 3 and 5 respectively; C). Overexpression of *CDC15* can restore growth in cells lacking the Tem1 protein (YPD + DOX plate; D).

SWM84 and SWM95 strains appeared to be much higher (Figure 38C). This result clearly confirms that these three genes were being overexpressed. To discover whether overexpressing any of these three genes can recover the essential requirement of the Tem1 protein in *C. albicans* the following strains were spotted onto repressing media: CAI4 (CIp10), SWM6, SWM72, SWM84 and SWM95 (Figure 38D). In addition to the wild type control the SWM72 strain also grew on repressing media, demonstrating that overexpression of *CDC15* can overcome the essential function of Tem1 (Figure 38D). Furthermore, analysis of cellular morphology clearly demonstrates that Cdc15 overexpression in Tem1 depleted cells restores wild type morphology. This genetic interaction suggests that the essential function of Tem1 is signalled through the Cdc15 protein. Overexpression of *DBF2* and *CDC14* is unable to rescue for the loss of the Tem1 protein.

Interestingly the SWM95 (TET-*TEM1*; *ENO1*p-*CDC14*) strain grew very poorly on the non-repressing media. To investigate this further we examined the growth phenotype of these cells. We found that around 60 % of the SWM95 cells grew as filaments in the non-repressing media (Figure 39). These cells resembled those formed by the Tem1p depleted cells. Both the SWM72 and SWM84 strains grew as budding yeast cells in non-repressing media which suggests that either the overexpression of *CDC14* by itself or in combination with the overexpression of *TEM1* resulted in this filament formation. To evaluate this further we constructed a strain in which *CDC14* was overexpressed using the same construct used to create the SWM95 strain but in the CAI4 (CIp10) background (SWM141 and SWM142). We found that these cells grew as yeast cells in the non-repressing media. This is consistent with the finding is also supported by the findings of Clemente-Blanco *et al.*, (2006) that overexpressing Cdc14 in *C. albicans*, using the *ACT1* promoter, had no apparent effect on cellular growth. Therefore we can conclude that the filamentous growth of the SWM95 strain in non-repressing media is caused by the simultaneous overexpression of both the *CDC14* and *TEM1* genes.

When the SWM72, SWM84 and SWM95 strains were grown in repressing media the SWM84 and SWM95 cells formed filaments. The SWM84 filaments resemble those formed by SWM6 cells in repressing media (Figure 19, 20 and 21). The SWM95
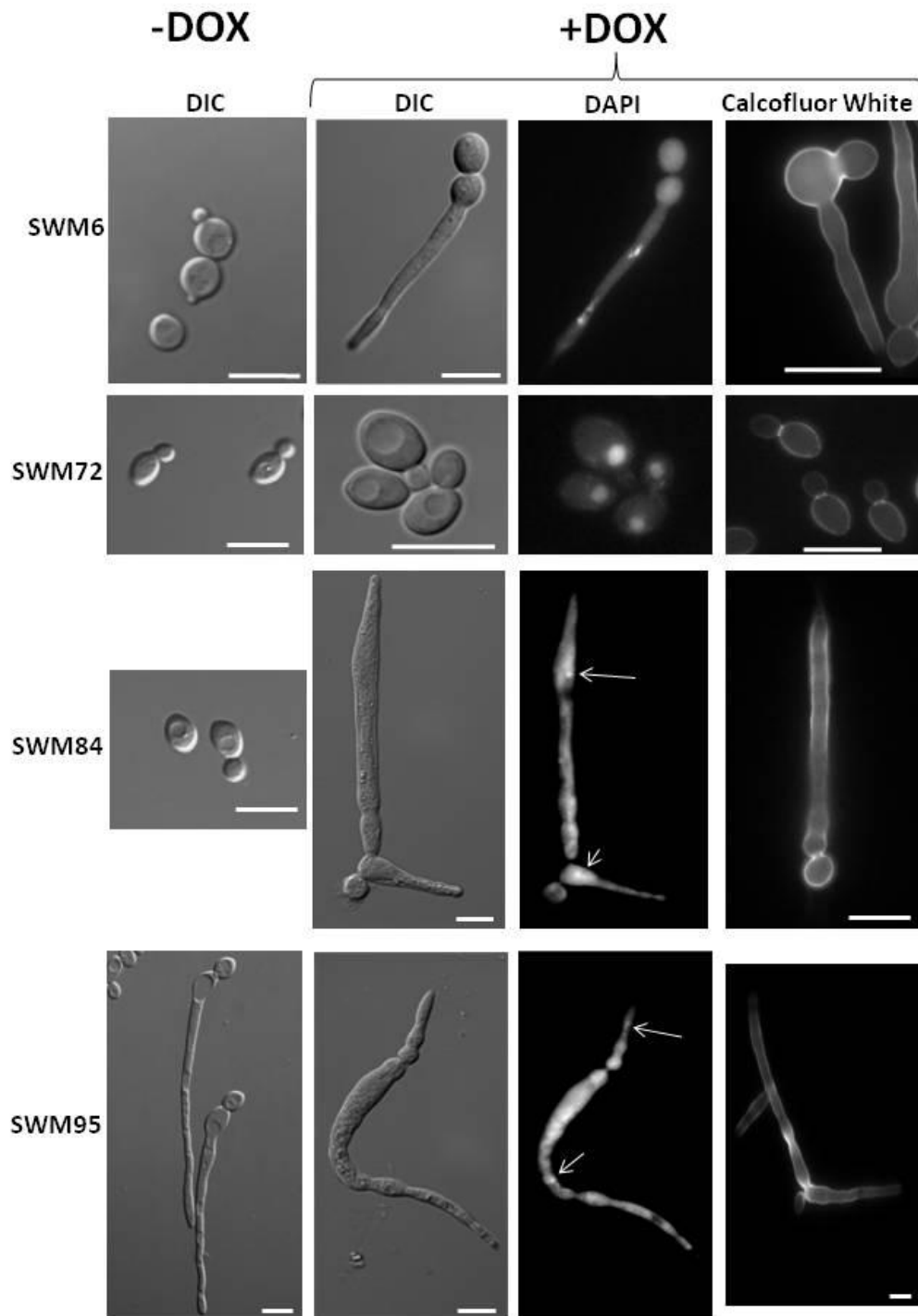
Figure 39. Overexpression of *DBF2* and *CDC14* in Tem1 depleted cells has no effect on filament formation. Microscopic analysis of SWM6, SWM72, SWM84 and SWM95 strains, grown in repressing and non-repressing conditions, using the calcofluor white and DAPI stains. SWM72 cells grown as budding yeast cells while the SWM6, SWM84 and SWM95 cells form filaments which contain a single chitin band and two nuclei. Scale bar equals 10 μm. Arrows indicate the position of the nuclei.

filaments were however branched and had undergone changes in the direction of growth. These changes in growth direction of the filament were not seen with either the SWM6 or SWM84 cells grown in repressing media as these filaments were typically very straight (Figure 19, 20 and 21). The SWM72 cells grew as yeast cells in the repressing media which supports the conclusion that the Tem1 carries out its cellular function through the Cdc15 protein. This is further confirmed by the observation that the SWM72 cells underwent successful nuclear division: no cell was observed that lacked a visible nucleus when stained with the DAPI stain. A strong chitin band was visible at the mother-daughter bud neck which is also seen in normally budding cells, indicating normal cell cycle progression. However in the SWM84 and 95 strains no more than two nuclei were observed with the filaments and a chitin band was only present at the mother–daughter bud neck of the parent yeast cells which suggests cells are undergoing a cell cycle arrest as a result of Tem1 depletion (Figure 39). These findings confirm that overexpressing either the *DBF2* or *CDC14* genes is not able to recover the defects caused by the loss of the Tem1 protein while overexpressing *CDC15* is able to fully recover the defects.

4.2.4. Yeast 2-hybrid analysis of the mitotic exit network components.

To study the interaction between the various proteins in the mitotic exit network we carried out a yeast two-hybrid (Y2H) analysis. This was carried out using the Matchmaker® *GAL4* Two-Hybrid system 3 (Clontech, Oxford UK).

4.2.4.1. Construction of the yeast 2-hybrid vectors and strains

To construct the various bait and prey vectors to be used in this Y2H study the following genes; *BFA1*, *BUB2*, *TEM1*, *CDC15*, *DBF2* and *MOB1*, were amplified from *C. albicans* genomic DNA (CAI4 (CIp10) strain) using the primers listed in the yeast 2-hybrid construction section of Table 5. Incorporated into these primers were restriction sites for either the *BamH1* or *Nde1* enzymes to allow the genes to be sub-cloned into the pGADT7 (Prey) and pGBKT7 (Bait) vectors. Following PCR amplification the genes were gel extracted and cloned into the Strataclone PCR cloning vector (pSC-A-amp/kan, Stratagene, Leicester UK). The successful cloning of the genes into the plasmid was confirmed by restriction digestion of the extracted plasmids. To construct the various pGBKT7-bait, and pGADT7-prey plasmids the genes were sub-cloned from

the various Strataclone plasmids into the pGBKT7 or pGADT7 plasmids. Successful sub-cloning was confirmed by carrying out restriction digests (Figure 40A). To confirm that the gene had inserted into the plasmid in the correct orientation, PCR reactions were carried out using the plasmids as template and utilising the forward primer used to amplify the gene from genomic DNA and the T7 primer. The full list of plasmids constructed is given in Table 4.

Following construction of the plasmids the pGBKT7-bait plasmids were transformed into the AH109 *S. cerevisiae* strain. Positive transformants were selected by growth on synthetic complete medium deficient in tryptophan (SC–trp). These transformations generated six strains each containing a different gene on the pGBKT7 plasmid. Each of these six strains was then transformed with the six pGADT7 plasmids and plated onto synthetic complete media deficient in tryptophan and leucine (SC–trp/leu). A positive control strain was also constructed which contained the pGBKT7-53 and pGADT7-T control plasmids transformed into the AH109 strain.

4.2.4.2. Conformation that the bait and prey constructs do not activate the reporter genes.

To confirm that the presence of either the binding domain—target protein fusion or the activator domain-target protein fusion proteins did not auto activate the expression of the reporter genes; *lacZ*, *HIS3*, *ADE2* or *MEL1*, the AH109 strain was transformed with each of the pGADT7-prey plasmids. The positive transformants were selected for on synthetic complete medium deficient in leucine (SC -leu). The resulting six strains were then spotted on to SC –leu/-his/-ade media while the six pGBKT7–bait strains were spotted onto SC-try/-his/-ade media. For each of these only the positive control strain grew confirming that there was no activation of the reporter genes by either the activation domain protein fusion or the binding domain protein fusions (Figure 40B). Additionally on media selecting for the presence of plasmids all strains grew well, confirming expression of bait/prey constructs was not toxic to the cells.

4.2.4.3. Bfa1 and Bub2 form a complex in *C. albicans*.

Following construction of the thirty six strains we investigated the interactions between components of the *C. albicans* MEN. To do this we spotted all the AH109
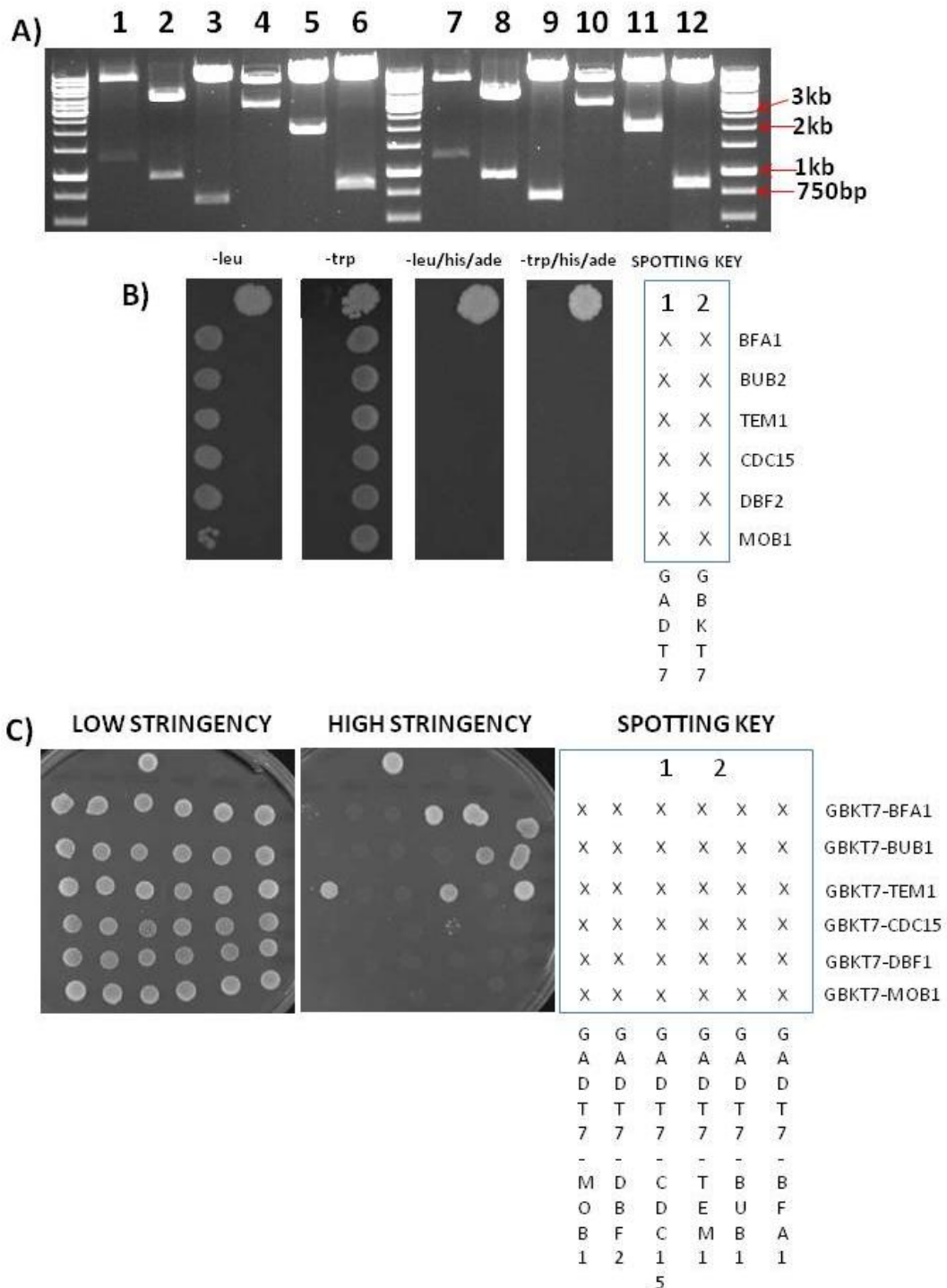
Figure 40. Yeast 2-Hybrid analysis of the Mitotic Exit Network components in *C. albicans*. Restriction digests of the successful sub-cloning of the *BFA1*, *BUB2*, *TEM1*, *CDC15*, *DBF2* and *MOB1* (in that order) into the GADT7 (lane1-6) and GBKT7 (lane 7-12) vectors (A). Plates showing that the various pGADT7-prey or PGBKT7-bait vectors do not cause auto-activation of the expression of the reporter genes *HIS3* and *ADE2* in the AH109 strain (B). Five interactions were detected on the high stringency medium. The low stringency plate shows each strain contains both plasmids. Spotting key shows which colony corresponds to which strain. 1= AH109 (negative control), 2= AH109 pGADT7-t pGBKT7-53 (positive control; C).

strains containing the various gene combinations onto two different types of media; low-stringency (SC-leu/trp) and high-stringency (SC deficient of adenine, histidine, leucine and tryptophan, SC-ade/his/leu/trp). A negative control strain, AH109 parent strain, and a positive control strain, AH109 (pGADT7-T pGBKT7-53), were also spotted on to each of these plates. We found that all the strains grew on the low-stringency media and this confirms that each strain contains a copy of both a pGADT7-prey and pGBKT7-bait plasmids (Figure 40C).

On the high stringency media we found that the Bfa1 and Bub2 proteins interact with each other (Figure 40C). We found this result in both combinations e.g. with Bfa1 as either the bait or prey protein. This confirms that the Bfa1 and Bub2 proteins form a complex in *C. albicans*. Interestingly we also found that both of these proteins bind to themselves. Therefore Bfa1 and Bub2 form a large complex which is likely required for the activation or function of either protein.

4.2.4.4. Bfa1 interacts with Tem1.

In *S. cerevisiae* the Bfa1 and Bub2 proteins both bind to Tem1 and this is important for the regulation of Tem1 (Pereira *et al*., 2000; Lee *et al*., 2001; Ro *et al*., 2002; Geymonat *et al*., 2002). We demonstrated that in *C. albicans* the Bfa1 protein does interact with Tem1, however no interaction could be seen between the Tem1 and Bub2 proteins (Figure 40C). This result suggests that Bfa1 is the protein in the complex that is required to recruit Tem1.

4.2.4.5. Tem1 interacts with itself.

In addition to binding to Bfa1 we also found that Tem1 binds to itself (Figure 40C). This finding leads us to suggest an exciting new theory about the regulation of Tem1: that the dissociation of Tem1 from the Bfa1-Bub2 complex through the actions of Lte1 and other proteins results in the Tem1 protein forming a self activating complex, causing Tem1 to undergo conformational changes and become GTP-bound and active.

In section 4.2.3, we showed that the overexpression of *CDC15* could recover the defect seen in Tem1 depleted cells, and hence *TEM1* and *CDC15* have a genetic interaction. However in the Y2H assay we saw no evidence of an interaction between the Tem1

and Cdc15 proteins. Since we already know there is a genetic interaction this perhaps suggests that the physical interactions is only transient and that Tem1 is only required to activate Cdc15 and that they do not form stable complex. Alternatively, Tem1 and Cdc15 could potentially interact through a partner on the SPB, as Cdc15 has been shown to localise to the SPB (Cheetham *et al.*, unpublished), or the interaction may require a conformational change in Tem1. However, it is also important to note that the Cdc15 sequence contains eight CTG codons, of which two are conserved, therefore *C. albicans* Cdc15 may not fold correctly in *S. cerevisiae*. Indeed it has also been shown that *CaCDC15* cannot rescue a *S. cerevisiae cdc15* temperature sensitive mutant. Alternatively Tem1 and Cdc15 do not interact with each other.

An interaction between Tem1 and Mob1 was detected when Tem1 was the prey protein but not when it was the bait protein, therefore we could not confirm a true interaction. Further investigation using protein pull downs needs to be carried out to confirm or rule out this interaction.

4.2.4.6. Dbf2 and Mob1 do not form a complex in *C. albicans*.

The binding of the Mob1 to the Dbf2 protein is important for the function of the Dbf2 protein in releasing Cdc14 from the nucleolus in *S. cerevisiae* (Komarnitsky *et al.*, 1998; Mah *et al.*, 2001). Interestingly in our Y2H assay we saw no interaction between the *C. albicans* Dbf2 and Mob1 proteins or between the Cdc15 and Dbf2 proteins (Figure 29C). This suggests that either: the Dbf2 and Mob1 proteins do not form a stable complex in *C. albicans* or that they must undergo activation and/or a conformational change to allow complex formation. This is also a possible reason why no Cdc15 and Dbf2 interaction was detected.

4.3. Discussion.

In *S. cerevisiae* regulation of the activity of the GTPase protein Tem1 is important as inactivation of the protein results in the cells arresting in late mitosis while early activation of the protein by mislocalisation of the Lte1 protein results in misaligned spindles and binucleated cells (Shirayama *et al.*, 1994a; Geymonat *et al.*, 2009). To study the regulation as well as the interactions of the Tem1 protein in *C. albicans* we carried out a series of experiments.

4.3.1. Tem1 localises to the spindle pole bodies in a cell cycle dependant fashion.

The first such experiment was to study the cellular localisation of Tem1 within yeast, pseudohyphal and true hyphal cells. We found that in all three growth forms the Tem1-GFP fusion protein localised to the spindle pole bodies in a cell cycle dependent fashion. This spindle pole localisation was confirmed by the co-localisation of the Tem1-GFP protein with the γ-tubulin protein Tub4. This lack of any obvious change in the localisation of the Tem1-GFP protein between the three growth forms suggests that the role of the Tem1 protein is the same or similar in each growth form. This was also seen in the finding that the *TEM1* gene is essential in both yeast and hyphal cells. This SPB localisation is similar to that seen for the Tem1 protein in *S. cerevisiae* and the Spg1 protein in *Schizosaccharomyces pombe* (Sohrmann *et al.*, 1998; Bardin *et al.*, 2000; Simanis 2003; Molk *et al.*, 2004). However, in *S. cerevisiae* the Tem1 protein is preferentially localised to the daughter SPB upon its passage through the mother-daughter bud neck and this is promoted by the Lte1 protein (Molk *et al.*, 2004). Recent studies have shown that in *S. cerevisiae* when the spindle has elongated correctly the Bfa1-Bub2 complex has a higher affinity for the dSPB and that Lte1 recruits the complex from the mSPB to the dSPB and this recruitment results in the activation of Tem1 (Geymonat *et al.*, 2009; Caydasi and Pereira, 2009; Monte-Casas and Amon, 2009). This theory is further supported by the observation that Tem1 localisation to the SPB is dependent on Bfa1 and Bub2 (Pereira *et al.*, 2000). However the recruitment of Spg1 from the mSPB to the dSPB has not been documented in *S. pombe* and we saw no evidence of it occurring in *C. albicans* as the intensity of the Tem1-GFP at both SPBs was similar. This difference between findings in *S. cerevisiae* and our findings in *C. albicans* suggests that the regulation of Tem1 could be different in the two species

4.3.2. Lte1 localisation and knockout construction.

To evaluate this potential regulation difference further we decided to study the proposed guanosine exchange factor protein for Tem1, Lte1. We attempted to construct two different *lte1* mutants, one a null mutant and the second a regulatable mutant. We were unable to generate a true null mutant as all the double mutants we constructed contained a third copy of the *LTE1* gene. This suggests that the *LTE1* gene is essential for vegetative growth in *C. albicans*. As a result attempts were made to

construct a heterozygous strain with the remaining copy of the *LTE1* gene under control of either the *MET3* or TET promoters. Unfortunately this strain was never successfully constructed. This is likely to be because both of these promoters result in overexpression. Overexpression of the Spg1 GEF *ETD1* has been shown to be lethal in *S. pombe* (Daga *et al.*, 2005; Garcia-Cortés and McCollum, 2009). Additionally in *S. cerevisiae* the mislocalisation of Lte1 to the mother cell results in premature mitotic exit (Geymonat *et al.*, 2009) so this could further explain our failure to construct a regulatable *LTE1* strain. To overcome this setback a non overexpressing promoter, such as the *MAL2* maltose inducible promoter (Backen *et al.*, 2000), will have to be used. Alternatively to assess if overexpression of *LTE1* is lethal, regulated overexpression could be carried out. This could be achieved by inserting the tetracycline-regulatable promoter into the promoter region of one of the *LTE1* alleles in the THE1 strain and selecting for positive transformants on repressing media. The resulting strain could then be grown in non-repressing media and the viability of the cells assayed.

The failure to construct any of the above strains has led us to believe that *LTE1* is essential in *C. albicans*. This however does not match what was seen in *S. cerevisiae* where *LTE1* is only essential at low temperatures. A study by Geymonat *et al.*, (2002) proposes that Lte1 is required for activating Tem1 at low temperatures, but at normal growth temperatures the high intrinsic GDP release of Tem1 is sufficient for self activation and Lte1 is obsolete. If our observation of *LTE1* being essential is validated this would suggest that Lte1 is required for Tem1 activation at all temperatures and in *C. albicans*, therefore that Tem1 has lower intrinsic GDP release than is required for self activation. Alternatively, Lte1 may play no role in Tem1 activation and its essential function is involved in other cellular processes.

As a result of this potential difference in essentiality of *LTE1* between *S. cerevisiae* and *C. albicans* we decided to study the localisation of the Lte1 protein to elucidate further the role Lte1 plays in *C. albicans*. We found that in yeast cells Lte1 localised to the cortex of the daughter bud in a cell cycle dependant fashion before being released into the bud cytoplasm late in the cell cycle. This localisation is similar to that seen in *S. cerevisiae* (Bardin *et al.*, 2000; Lee *et al.*, 2001; Jensen *et al.*, 2002). In *S. cerevisiae*

Cdc28 has been shown to phosphorylate Lte1 at the time of bud emergence resulting in the localisation to the bud cortex. The maintenance of Lte1 at the bud cortex thorough out the majority of the cell cycle has however been shown to involve a number of different proteins from Ras1 to the cell polarity proteins Cdc42 and Cla4, and the septin proteins Cdc10 and Sep7 (Höfken and Schiebel, 2002; Castillon *et al.*, 2003; Geymonat *et al.*, 2009). During anaphase, Lte1 is released from the bud cortex due to its dephosphorylation by Cdc14 (Jensen *et al.*, 2002). Interestingly we observed this same release of Lte1 from the bud cortex in *C. albicans*, which potentially forms part of a feedback loop. However, no localisation of Lte1 could be determined in pseudohyphae or true hyphae, which may suggest alternative factors are involved in activating Tem1 in these cell types.

4.3.3. *CDC15 and TEM1* have a genetic interaction.

In *S. cerevisiae* both the *LTE1* and *TEM1* genes were identified in screens to suppress defects caused by deletion or disruption of other genes (Shirayama *et al.*, 1994a; Shirayama *et al.*, 1994b). We undertook a similar approach to identify if the *C. albicans* homologs of the *CDC15*, *DBF2* or *CDC14* could suppress the defects of Tem1 depletion. We found that overexpressing the *CDC15* gene could fully recover the defects caused by the depletion of Tem1, and this confirms that the essential function of Tem1 is signalled through the Cdc15 protein and that Cdc15 acts downstream of Tem1 in signalling mitotic exit and cytokinesis. This corresponds to what was seen in *S. cerevisiae* when *CDC15* was overexpressed in the *tem1-3* mutant (Shirayama *et al.*, 1994b). When the Tem1 protein was depleted in the SWM84 (TET-*TEM1*, *ENO1*p-*DBF2*) and SWM95 (TET-*TEM1 ENO1*p-*CDC14*) strains the cells formed growth filaments. The filaments on average contained two nuclei and had no chitin deposits apart from the mother daughter bud neck and eventually died. Therefore we can conclude that overexpressing either the *DBF2* (SWM84) or *CDC14* (SWM95) genes had no effect on reversing the mitotic exit and cytokinesis defects of Tem1 depleted cells. The finding that overexpressing *CDC14* failed to recover the Tem1 depletion defect came as no surprise as *CDC14* has been shown to be non-essential in *C. albicans*, therefore the essential function of *TEM1* must be signalled through other factors.

Interestingly the combination of overexpressing *TEM1* and *CDC14* in the SWM95 strain

caused the cells to grow poorly and 60 % of these cells grew as growth filaments like the SWM6 strain in repressing media. We confirmed that this was not caused by overexpressing *CDC14* alone as a wild type strain overexpressing the *CDC14* gene grew as yeast cells, and it has previously been reported that *CDC14* overexpression has no affect on growth of the cells (Clemente-Blanco *et al*., 2006). When the SWM95 strain was grown in repressing media the cells all formed growth filaments as discussed above but these filaments were very irregular and often changed their growth direction and in general had the appearance of very sick dying cells unlike the Tem1p depleted cells that resembled normal true hyphae. The exact reason for the differences seen for the SWM95 strain in both repressing and non-repressing media are currently unknown therefore further research is required to uncover the cause of this phenomenon.

Recently a hyperactive *DBF2* gene has been constructed in *S. cerevisiae* that recovers the lethality defects of the *lte1Δ* and *tem1Δ* strains. This gene contains the substitution mutations: L12M, K85N, K335E, F349L, L384S, L434F and T528S (Geymonat *et al*., 2009). This suggests that the lack of recovery of the Tem1p depletion defects in the SWM84 strain when grown in repressing media could be caused by the lack of activation of the Dbf2 protein. To fully assess this, a hyper active Dbf2 protein will need to be constructed and tested in the SWM6 strain under repressing conditions.

4.3.4. Yeast 2-hybrid analysis of the mitotic exit network.

The final set of experiments we carried out was designed to study the protein interactions within the mitotic exit network. To do this we decided to utilise the *GAL4* Matchmaker yeast 2-hybrid system (Clontech, Oxford UK).

Our results suggest there are five Y2H detectable protein interactions within the mitotic exit network. Three of these interactions are where a protein interacted with itself. These were for the Bfa1, Bub2 and Tem1 proteins. The other two interactions were between Bfa1 and Bub2 and between Bfa1 and Tem1. These two interactions suggest that Bfa1 and Bub2 form a complex and as in *S. cerevisiae* this complex binds to and maintains Tem1 in its inactive GDP-bound state. The lack of an interaction

between Bub2 and Tem1 is surprising as Bub2 contains a TBC domain which is typically found in GAP proteins including the Bub2 protein in *S. cerevisiae* and the Cdc16 protein in *S. pombe* (Furge *et al.*, 1998; Geymonat *et al.*, 2002). This suggests that if Bub2 is a GAP protein for Tem1 it requires the Bfa1 protein to act as a scaffold/binding protein to allow Bub2 to inhibit Tem1 activity. This is also the case in *S. cerevisiae* where Bfa1 & Bub2 form a complex throughout the cell cycle and more Bfa1 binds to Tem1 than Bub2 does (Pereira *et al.*, 2000; Lee *et al.*, 2001; Geymonat *et al.*, 2002). The overexpression of *BUB2* in *S. cerevisiae* results in only a partial growth inhibition which is abolished in cells lacking Bfa1 which points to the conclusion that Bub2 requires Bfa1 to be present to be able to regulate Tem1. However it has also been shown that Bfa1 can also inhibit the function of Tem1 by preventing the interaction between Tem1 and Cdc15 and this occurs independent of the actions of Bub2 (Ro *et al.*, 2002). This suggests that both proteins can regulate the activity of Tem1 by alternative means. In *C. albicans* a mutant lacking the Bub2 protein arrests in anaphase as a result of the triggering of the spindle checkpoint (Finley *et al.*, 2008). All this suggests two possible theories for the maintenance of Tem1 in its inactive form in *C. albicans*, firstly that both Bfa1 and Bub2 inhibit Tem1 or that alternatively Bub2 inhibits Tem1 but requires Bfa1 to act as a binding protein between Tem1 and Bub2.

Interestingly we found that Tem1 can bind to itself. This allows us to suggest that the Tem1 protein in *C. albicans* will have a high intrinsic GDP release rate and therefore effectively self activate itself akin to the Tem1 protein in *S. cerevisiae*. This self-activation only occurs following the phosphorylation of Bfa1 by Cdc5 which leads to the dissociation of the Bfa1-Bub2 complex from Tem1 (Hu *et al.*, 2001; Geymonat *et al.*, 2002). Throughout the remainder of the cell cycle the Bfa1-Bub2 complex inhibits this self activating mechanism and this is maintained by the kinase Kin4 (D'Aquino *et al.*, 2005). A series of further experiments are required to confirm or discard this prediction; such as studying the GDP release rate of Tem1, discovering whether Bfa1 is phosphorylated by Cdc5 in *C. albicans* and to also study the role Kin4 plays.

A number of protein interactions that have been seen in *S. cerevisiae* and *S. pombe* were not seen in our yeast 2-hybrid analysis of the MEN components. The first such interaction is that between Tem1 and Cdc15. In both *S. cerevisiae* and *S. pombe* this

interaction has been observed by yeast 2-hybrid analysis and this is important for the initiation of the pathway (Schmidt *et al*., 1997; Asakawa *et al*., 2001). We showed in section 4.2.3, that a genetic interaction between *TEM1* and *CDC15* exists in *C. albicans*. Therefore an interaction between the two proteins was expected. The most likely explanation is drawn from the fact that *CDC15* contains eight CTG codons of which two are conserved. Therefore, Cdc15 protein might not be functional in *S. cerevisiae*, indeed it has been observed that *C. albicans CDC15* fails to rescue a temperature sensitive *cdc15-1* mutant (Cheetham *et al*., unpublished). Alternatively, the interaction between Tem1 and Cdc15 could be a transient interaction that was not detectable in our Y2H screen, but this is unlikely as the same interaction was detected by Asakawa *et al*., (2001) using the *S. cerevisiae* proteins in a Y2H screen. To overcome these issues this interaction needs to be investigated further by utilising co-immunoprecipitation and also the newly documented *Candida* 2-hybrid system (Stynen *et al*., 2010).

The interaction between Cdc15 and Dbf2 and also between Dbf2 and Mob1 were also not detected in our yeast 2-hybrid screen. So far we have no evidence that these interactions occur in *C. albicans*, however, these interactions occur in both *S. pombe* and *S. cerevisiae* (Luca and Winey, 1998; Komarnitsky *et al*., 1998; Hou *et al*., 2000;). This could be caused by the Cdc15 protein being non-functional in the Cdc15-Dbf2 interaction screen. In the case of the Dbf2-Mob1 interaction, this may require Dbf2 to become activated, thus could be assessed through the construction of a hyperactive Dbf2 protein as discussed previously.

In conclusion, we observed that Tem1 localises to both spindle pole bodies in a cell cycle dependant fashion while the GEF protein Lte1 localises to the daughter cell cortex, suggestive of spatial regulation of Tem1 in *C. albicans*. We also found that the essential function of Tem1 in *C. albicans* is signalled through Cdc15 however the final output has yet to be determined. Finally, we demonstrated that the Bfa1/Bub2 complex potentially plays a role in regulating Tem1 in *C. albicans*.

Chapter 5–The development of new molecular tools.

5.1. Introduction.

Candida albicans virulence mechanisms have been the focus of much scientific enquiry in recent years. Research into *C. albicans* has been restricted as *C. albicans* is a diploid organism, making classical genetic analysis extremely challenging. Also *C. albicans* and eight closely related species translate the CTG codon as serine and not leucine. This codon reassignment renders a great many previously developed disruption and tagging cassettes non-functional in these species (Scherer and Magee, 1990; Ohama *et al.*, 1993; Santos *et al.*, 1994; Santos and Tuite, 1995; White *et al.*, 1995). As a result of this alternative codon usage, research groups have had to adapt *S. cerevisiae* cassettes in order to use them in *C. albicans* and other CTG clade species. Adapted cassettes include the *URA*-blaster gene disruption cassette, which allows the selectable marker to be recycled (Alani *et al.*, 1987; Fonzi and Irwin, 1993).

The majority of the *C. albicans* cassettes utilise auxotrophic markers for selection and the *URA3* gene is by far the most commonly used. *URA3* encodes the enzyme orotidine-5′-phosphate decarboxylase (OMPD), which is involved in pyrimidine biosynthesis. Unfortunately there are some disadvantages to using the *URA3* gene as a selectable marker, for example *ura3* mutants are known to be highly attenuated in virulence. OMPD levels may be reduced, when compared to the SC5314 clinical isolate, in strains with the *URA3* gene restored but in a non-native locus (Lay *et al.*, 1998). Strains with variable OMPD levels have been shown to exhibit virulence defects in murine models therefore wild type expression levels must be restored (Kirsch and Whitney, 1991; Laurenson and Rine, 1992; Cole *et al.*, 1995; Bain *et al.*, 2001; Cheng *et al.*, 2003; Staab and Sundstrom, 2003; Brand *et al.*, 2004). This can be achieved by restoring the *URA3* at its native locus or inserting the gene at the *RPS10* locus (Brand *et al.*, 2004). The use of the *URA*-blaster cassette also requires recycling of the *URA3* gene, and this is carried out by growing the strain on 5-FOA-containing media. However exposing *C. albicans* cells to 5-FOA can result in unlinked chromosome rearrangements, non-lethal mutations and other types of genetic defects (Wellington and Rustchenko, 2005). Additional auxotrophic selection markers have more recently been developed including *ARG4*, *HIS1* and *LEU2,* and these have been used in

disruption and tagging cassettes (Gerami-Nejad *et al.*, 2001; Gola *et al.*, 2003; Noble and Johnson, 2005; Schaub *et al.,* 2006; Gerami-Nejad *et al.*, 2009). These markers utilise genes from other *Candida* species which have been shown to complement the *C. albicans* deficient strains. The benefits of using these markers are that, as the genes originate from other *Candida* species, they should not undergo homologous recombination with the chromosomal marker loci, and strains lacking any of these three markers exhibit no virulence defects when compared to wild type strains (Kirsch and Whitney, 1991; Alonso-Monge *et al.*, 1999; Noble and Johnson, 2005). However, the use of these three markers is limited to engineered laboratory strains and not clinical isolates, hence limiting their usefulness to researchers.

An increasing number of molecular tools are available to facilitate research into *C. albicans* virulence factors. These include reporter genes for studying gene expression and fluorescent protein genes for studying protein localisation. The *K. lactis* β-galactosidase *Lac4* gene was the first such reporter gene used in *C. albicans* and was utilised to study gene expression (Leuker *et al.*, 1992). This system had some drawbacks such as gene expression could not be detected if the gene was chromosomally integrated therefore limiting the studies to *Candida* plasmids and these plasmids were not stable. Other commonly used reporter genes are the *C. albicans URA3* and *XOG1* genes (but they can only be used in *ura3* or *xog1* delete strains), the luciferase gene from the sea pansy *Renilla reniformis* and *Streptococcus thermophilus LacZ* (Myers *et al.,* 1995; Gonzalez *et al.*, 1997; Srikantha *et al.*, 1996; Uhl and Johnson, 2001).

The reporter gene that is used most extensively is the *GFP* gene. The *GFP* gene was isolated from the jellyfish *Aequorea victoria* and it encodes the protein *p*-hydroxybenzylideneimadazolinone (Shimomura *et al.*, 1962; Shimomura 1979). Between the years of 1979 and 1991 little progress was made in developing *GFP* into a useful tool for scientists to use. 1992 was a breakthrough year for *GFP* researchers as the first report of the successful cloning of the gene was published and this was followed by expression of the gene in *Caenorhabditis elegans* under control of a tubulin promoter (Prasher *et al.*, 1992; Chalfie *et al.*, 1994). Many variants of *GFP* have subsequently been constructed such as these encoding blue, yellow and cyan

fluorescent proteins (BFP, YFP and CFP). These proteins each emit light of different wavelengths and therefore allow co-localisation studies to be carried out (Heim *et al.,* 1994; Heim *et al.*, 1995; Heim and Tsien, 1996; Ormö *et al.*, 1996).

The *GFP* gene has been adapted for use in *C. albicans* by creating two mutations, S65G and S72A, and replacing the one CTG codon with a TTG codon (Cormack *et al.*, 1997; Morschhaüser *et al.*, 1998). This adapted *GFP* gene is referred to as yeast enhanced *GFP* (yE*GFP*) and the protein excites at 488 nm light compared to wild type *GFP,* which excites at 390 nm, both proteins have an emission wavelength of 509 nm. Many new plasmids have been constructed which utilise yE*GFP*. These include plasmids in which *GFP* is expressed only under hypha-inducing conditions, under the control of one of a number of regulatable promoters, or as a reporter for gene expression (Staab *et al.*, 2003; Barelle *et al.*, 2004). Other types of plasmids act as template plasmids for PCR amplification of the *GFP* gene coupled with an auxotrophic selection marker. The *GFP*-marker cassettes can be targeted by homologous recombination to the end of the target gene to form a C-terminal fusion protein for localisation studies (Gerami-Nejad *et al.*, 2001; Gola *et al.*, 2003; Schaub *et al.,* 2006; Gerami-Nejad *et al.*, 2009).

In addition to *GFP* there are many other tools available for studying protein function such as chromatin immunoprecipitation coupled with microarray analysis (ChIP-chip) and tandem affinity purification (TAP), and also epitope tags. A number of epitope tags are also now available for use in *S. cerevisiae* and *C. albicans* including FLAG, haemagglutinin (HA), V5, and MYC (Schneider *et al.*, 1995; Lavoie *et al.*, 2008; Moqtaderi and Struhl, 2008). These epitope tags are most commonly used during western blotting and immunoprecipitation experiments, as antibodies to the epitope tags are all widely available and relatively inexpensive.

In many fungi positive selection markers are available and can be used during transformation experiments. Examples of the chemicals/drugs used are benomyl in *Neurospora crassa*, hygromycin B in *N. crassa* and *S. cerevisiae*, and cycloheximide and phleomycin in *Cryptococcus neoformans*, (Jimenez and Davies, 1980; Gritz and Davies, 1983; Orbach *et al.*, 1986; Varma and Kwon-Chung, 2000; Hua *et al.*, 2000). Unfortunately *C. albicans* is intrinsically resistant to all the above-named chemicals,

thus excluding their use for *Candida* experiments (Beckermann *et al.*, 2001). However, two positive selection markers have been developed in recent years for use in *C. albicans*: mycophenolic acid and nourseothricin (Köhler *et al.*, 1997; Beckermann *et al.*, 2001; Reuss *et al.*, 2004; Shen *et al.*, 2005). In addition a third marker has now been developed, hygromycin B (HyB), despite the earlier reports that *C. albicans* is resistant to hygromycin B (Beckermann *et al.*, 2001; Basso *et al.*, 2010). Basso and co-workers tested seven different *C. albicans* strains in three different media; YPD, YNB + glucose and buffered YNB + glucose (pH7.0, 0.15 M HEPES-NaOH) each containing HyB. They found that the strains all grew well in YNB-glucose with 1200 µg/ml HyB but that none grew in YPD with 600 µg/ml HyB or in buffered YNB-glucose with 1000 µg/ml HyB. The finding that sensitivity is media dependent may explain the initial reports of resistance. Alternatively another possible reason for differences in findings between the two groups could be the source of the HyB, as it has been noted by a group working on the plant fungal pathogen *Magnaporthe oryzae* that HyB sourced from different companies exhibit different growth effects on the fungus (N. Tongue, personal communication). Resistance to hygromycin B is conferred by the HyB gene which encodes the *Escherichia coli* hygromycin B phosphotransferase (Basso. *et al.*, 2010).

The first dominant marker documented for use in *C. albicans* was the mutated form of the *IMH3* gene from strain 1006 which conveys resistance to the growth inhibitor mycophenolic acid (MPA; Goshorn and Scherer, 1989; Wirsching *et al.*, 2000). The *IMH3* gene is found naturally in the genome of *C. albicans* and codes for inosine monophosphate dehydrogenase. The mutated *IMH3* gene possesses three mutations (I47V, S102A, and G482D) and one deletion, with two of the mutations being required for resistance (Beckerman *et al*, 2001). The mutated version of *IMH3* has been used as a selection marker for transformations in *C. albicans*, *C. tropicalis* and *C. parapsolosis* (Köhler *et al.*, 1997; Staib *et al.*, 2000; Beckerman *et al.*, 2001; Gacser *et al.*, 2007). However, there are some drawbacks to using this marker system, including the fact that the mutated *IMH3* gene preferentially integrates at the wild type *IMH3* locus at a very high frequency, effectively ruling out its use in PCR-mediated gene tagging or disruption strategies.

The second positive selection marker documented for *C. albicans* which confers

resistance to the streptothricin group antibiotic nourseothricin is the *NAT1*/*SAT1* genes (Krügel *et al*, 1988; Roemer *et al*, 2003; Reuss *et al*, 2004; Shen *et al.*, 2005). These genes encode the enzymes streptothricin acetyltransferase (*SAT1*) and nourseothricin acetyltransferase (*NAT1*) (Joshi *et al*, 1995; Shen *et al*, 2005). To date this resistance cassette has been used for two different purposes: as a disruption cassette and as a marker for regulatable promoter cassettes (Roemer *et al*, 2003; Reuss *et al*, 2004; Shen *et al.*, 2005; Schaub *et al*, 2006). The disruption cassette contains the *SAT1* gene flanked by two *FLP* site-specific recombinase genes, allowing efficient recycling of the *SAT1* gene following gene disruption. This *SAT1* recycling allows both copies of a gene to be disrupted with the same cassette, thus forgoing the need to use an auxotrophic marker (Reuss *et al*, 2004).

In this study we constructed a set of PCR-amplifiable cassettes for gene tagging and constitutive gene expression, utilising the nourseothricin acetyltransferase gene *NAT1* as the positive selection marker. We demonstrated that these cassettes are fully functional and can be utilised in clinical strains.

5.2. Results.

5.2.1. Cassette design.

At the beginning of this study we set out to construct a set of gene tagging and promoter cassettes for use in *C. albicans* strains. To achieve this we decided to use the *NAT1* positive selection marker instead of an auxotrophic marker to expand the range of *Candida* strains in which they can be utilised. The *NAT1* marker is a newly available marker system that has distinct advantages over the MPA marker system: using the *NAT1* marker avoids the issue of recombination into the native locus as the marker is not present in the *Candida* genome. The tags we chose to use in this study were carboxyl terminus tags; yEGFP*,* CFP*,* YFP*,* RFP for localisation studies, the V5-6xHIS epitope tag for protein expression, and the enolase promoter sequence for overexpression studies (Cormack *et al.*, 1997; Gerami-Nejad *et al.*, 2001; Staab *et* al., 2003; Gerami-Nejad *et al.*, 2009). Cassettes were designed to incorporate the *CaADH1* or the *ScCYC1* terminator sequence following the fluorescent protein genes or V5-6xHIS sequence respectively and the *NAT1* gene under the control of the *Ashbya gossypii TEF1* promoter and terminator. These control sequences were obtained from

previously reported cassettes (Gerami-Nejad *et al.*, 2001; Shen *et al*., 2005; Gerami-Nejad *et al.*, 2009). An important feature of the fluorescent protein cassettes is the presence of three glycine codons before the fluorescent protein genes as a spacer. These three codons were introduced by Gerami-Nejad and colleagues to assist folding of the fused protein. This feature was maintained in our cassettes because misfolding of a fused protein could result in mislocalisation of the protein or potential loss of protein function with a resulting risk of cell death.

5.2.2. New DNA fusion technology.

A number of different tagging cassettes have recently been documented in the literature; traditional sub-cloning methods were used to construct all of these cassettes. In this study we used a different method, fusion PCR. Fusion PCR is a two stage protocol that results in the fusion of two PCR products. The first stage of the *GFP-NAT1* cassette construction consisted of two separate PCR reactions: one to amplify the *GFP* gene using the GFP-F and GFP-R primers and the other to amplify the *NAT1* gene, using the NAT1-Fusion-F and NAT1-R primers (Figure 41A; Table 5). These PCR reactions produced the GFP and NAT1 fragments of length 1540 and 1224 nucleotides (nt) respectively. The GFP-R and NAT1-Fusion-F primers contain complimentary regions that are used in the second PCR for the fusion of the two genes. The second round of PCR utilises these regions to fuse the *GFP* and *NAT1* genes together to form the 2740 nt in length cassette (Figure 41B and C). The *CFP-NAT1* and *YFP-NAT1* cassettes were constructed using the same protocol and primers as the *GFP-NAT1* cassette and are the same length. The other three cassettes, *RFP-NAT1*, V5-6x*HIS-NAT1* and *NAT1-ENO1*p, were constructed by the same protocol. The first round of PCRs used the RFP-F and GFP-R primers to amplify the *RFP* gene, the V5-F and V5-R primers to amplify the V5-*6xHIS* sequence, the NAT1-Fusion-F and NAT1-R primers to amplify the *NAT1* gene, and the ENO1-F and ENO1-R primers to amplify the *ENO1* promoter sequence (Figure 41A; Table 5). These PCR reactions amplified fragments of 1216, 410, 1002 and 1224 nt for the *RFP*, V5-6x*HIS*, *ENO1*p and *NAT1* sequences, respectively. To construct the fusions the second round of PCR was carried out using the RFP-F and NAT1-R primers for the *RFP-NAT1* cassette, the V5-F and NAT1-R primers for the V5-6x*HIS-NAT1* cassette and the NAT1-R and ENO1-R for the *NAT1-ENO1*p cassette (Figure 41B). The sizes of the fused cassettes were 2416nt for the *RFP-NAT1*
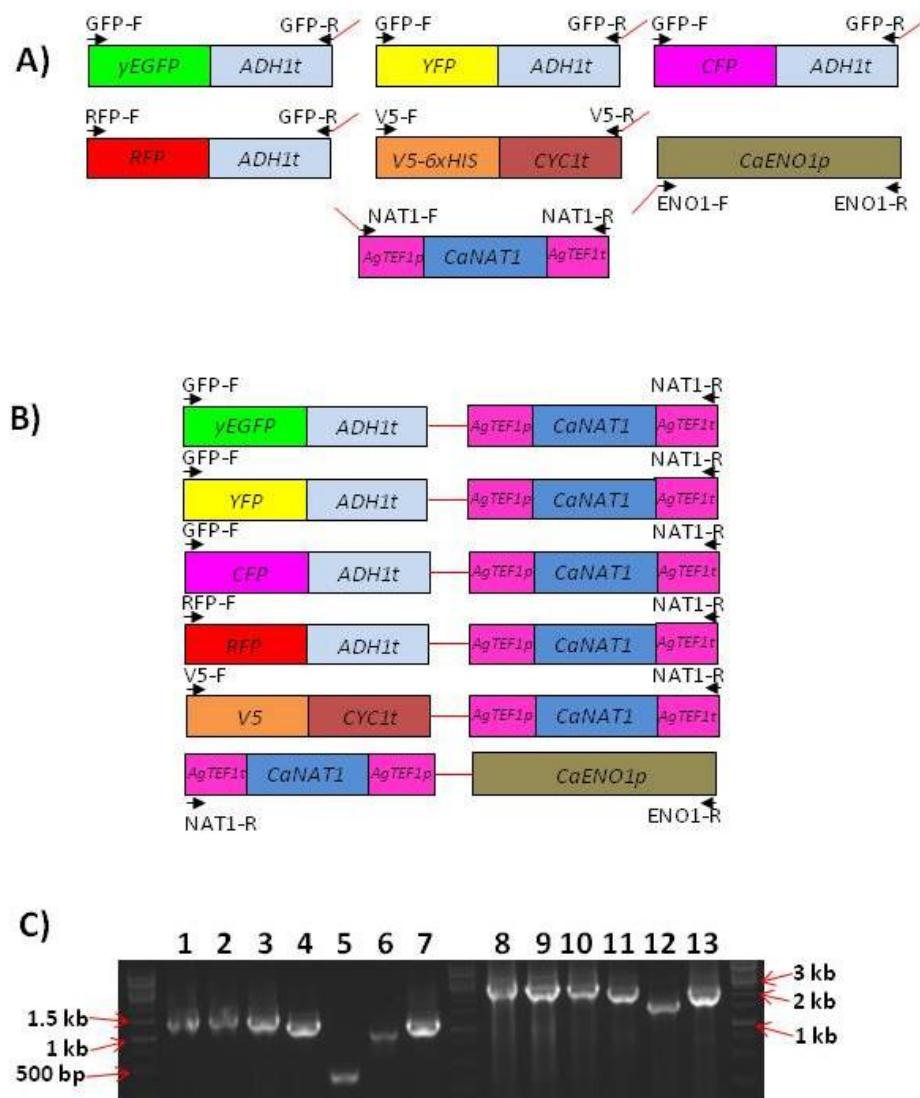
Figure 41 . **Construction of the *NAT1* fusion cassettes**. Schematic of the first round of the fusion PCR with primer names indicated. Previously reported plasmids were used as templates for PCR (Gerami-Nejad *et al*., 2001; Shen *et al*., 2005; Gerami-Nejad *et al*., 2009; A). Schematic of the second round of PCR with the various tagging genes/sequences and promoter sequences fused to the *NAT1* gene. Not drawn to scale. Primer sequences are given in Table 5 (B). DNA gel of the above PCR reactions. Lanes 1-7: round one of PCR (*GFP*, *YFP*, *CFP*, *RFP*, V5-6xHIS, *ENO1*p, *NAT1* for tagging cassettes and *NAT1* for the promoter cassettes in that order). In lanes 8-13 contain round two of the PCR, the fusion step in this order; *GFP-NAT1*, *YFP-NAT1*, *CFP-NAT1*, *RFP-NAT1*, V5-6xHIS-*NAT1* and *NAT1-ENO1*p (C).

cassette, 2206 nt for the *NAT1-ENO1*p cassette and 1607 nt for the V5-6x*HIS* cassette. Cassettes were then cloned into the TOPO2.1 vector (Invitrogen, Renfrew, UK) for the *GFP-NAT1* and V5-6xHIS-*NAT1* cassettes, and the Strataclone vector (Stratagene, UK) for the other four cassettes, to generate pSWM157, pSWM134, pSWM116, pSWM93, pSWM159 and pSWM158. Confirmation of successful cloning was carried out by PCR and restriction digests. These cassettes can be amplified by PCR for targeted integration in *C. albicans;* details of primer sequences are given in table 5.

5.2.3. Validation of the fluorescent protein-*NAT1* fusion cassettes.

To confirm that the four fluorescent protein-*NAT1* marker cassettes shown in Figure 30B are functional we integrated each cassette under the control of the constitutively active *ACT1* promoter (Figure 42A). A similar technique was used for testing *Ds*RFP and mCherryRFP fluorescence in *S. cerevisiae* and *C. albicans* (Keppler-Ross *et al*., 2008; Gerami-Nejad *et al*., 2009). Each cassette was PCR amplified to integrate by homologous recombination at the chromosomal locus of the *ACT1* gene immediately downstream of the *ACT1* start codon using the ACT1-GFP-F and ACT1-GFP-R primers for the *GFP-NAT1*, *YFP-NAT1* and *CFP-NAT1* cassettes and the ACT1-RFP-F and ACT1-GFP-R primers for the *RFP-NAT1* cassette, and transformed into the CAI4 (CIp10) strain (Table 5). Integration into the *ACT1* locus for *GFP*, *YFP* and *CFP* was confirmed by PCR using the GFP-UP and ACT1-S primers; integration of the *RFP-NAT1* cassette was confirmed using the RFP-UP primer instead of the GFP-UP primer (Figure 42A and B; Table 5). The positive transformants used in the microscope experiments were SWM155 (*ACT1-GFP-NAT1*), SWM137 (*ACT1-YFP-NAT1*), SWM118 (*ACT1-CFP-NAT1*) and SWM108 (*ACT1-RFP-NAT1*). All four fluorescent proteins were clearly visible, when expressed by the *ACT1* promoter, confirming that the proteins do fluoresce and are detectable using a fluorescence microscope system (Olympus IX81 microscope with a CoolSnap HQ^2 camera, Figure 42C). As a control we grew the parent strain, CAI4 (CIp10), for five hours in YPD and then viewed the cells microscopically whilst exposing them, in turn, to light which had passed through each of the four filters used in this study: green, yellow, red and cyan. No fluorescence was detected when the cells were exposed to the light from the green and yellow filter, while only low level background fluorescence from the vacuoles was detected with the red filter (Figure 42D). Filament-like structures were visible in these cells when light from the cyan filter was
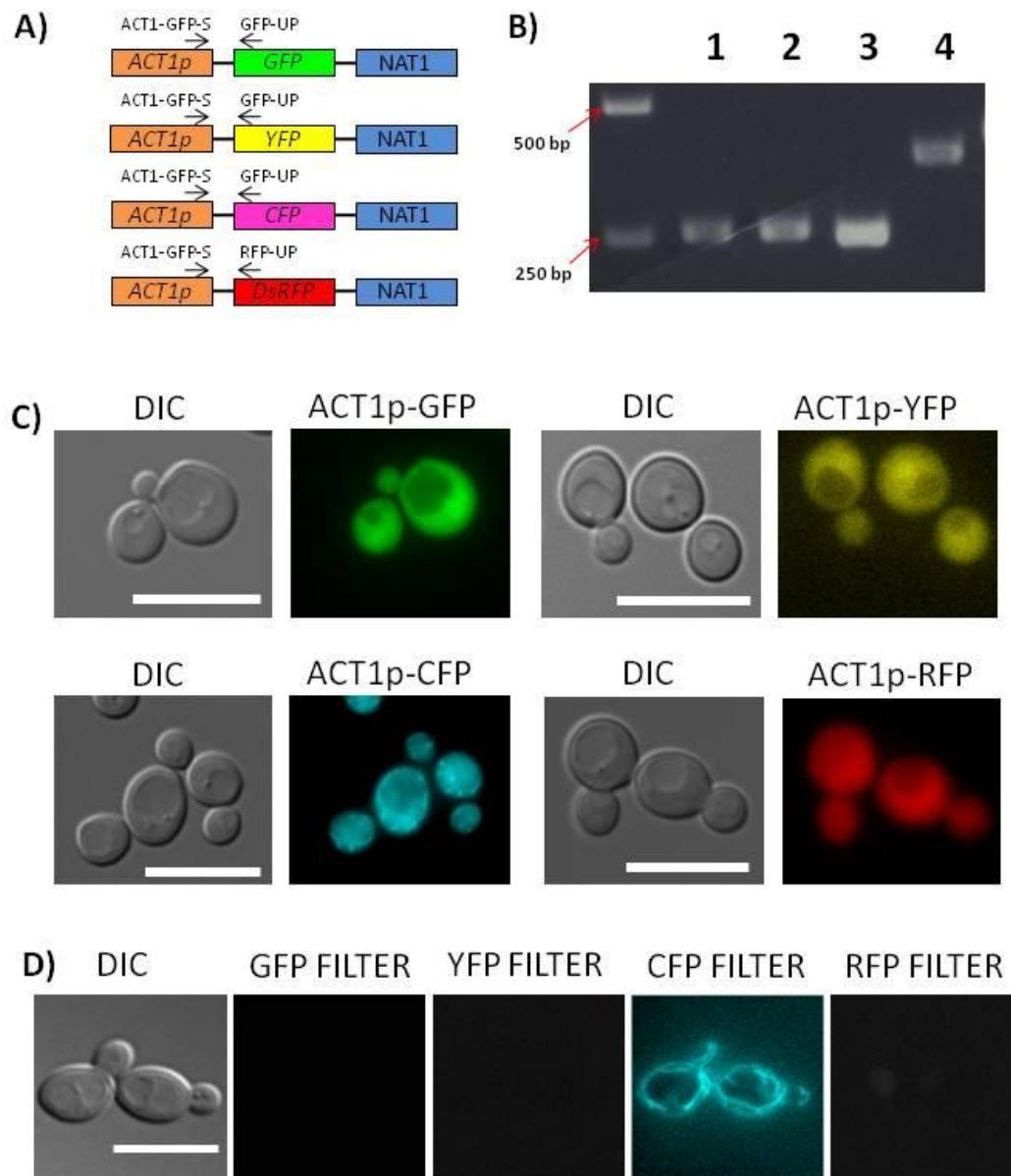
Figure 42. Validation of the four fluorescent protein *NAT1* fusion cassettes. (A) Schematic of the *ACT1* promoter fluorescent protein cassette constructs (not to scale). Positions of primers used for PCR confirmation are shown. (B) PCR confirmation of the successful integration of the cassette into the *ACT1* locus just downstream of the promoter region. Lane1 *GFP-NAT1*, Lane 2 *YFP-NAT1*, Lane3 *CFP-NAT1*, Lane 4 *RFP-NAT1*. Primers used in these PCR reactions were the ACT1-GFP-S and either the GFP-UP primer (*GFP*, *YFP* or *CFP* cassette) or RFP-UP primer (*RFP* cassette). (C) Microscopic analysis of the SWM155, SWM137, SWM118 and SWM108 strains (*ACT1-GFP, ACT1- YFP, ACT1-CFP*, and *ACT1-RFP* respectively). (D) Microscope analysis of the CAI4 (CIp10) strain using the four filters used in part B to confirm autofluorescence levels. Scale bar is 10 µm.

used, despite the absence of CFP protein in these cells, thus confirming that there was some auto-fluorescence under the cyan filter. This auto-fluorescence can also be seen in the *CFP* image in Figure 42B in the form of brighter dots. Notwithstanding the above, the overall conclusion was that *CFP-NAT1,* like the other three cassettes, fluoresces and is therefore functional. Attempts to construct and validate a mCherry-*NAT1* cassette were also made but no fluorescence was visible after a number of attempts to express this cassette under control of the *ACT1* promoter so this cassette was abandoned.

5.2.4. Validation of V5-6xHIS-*NAT1* and *NAT1-ENO1*p cassettes.

To investigate whether the V5-6xHIS-*NAT1* cassette was functional for use in protein studies we integrated the cassette at the C-terminus of one copy of the *HEX1* gene in the CAI4 (CIp10) strain (Figure 43A and B). A control strain was also constructed using the V5-6xHIS-*URA3* cassette in the CAI4 strain (S. Bates, personal communication). *HEX1* is the gene that encodes the enzyme *N*-acetylglucosaminidase. This enzyme is secreted by *C. albicans* cells and it hydrolyses *N-N'*-diacetylchitobiose, *N-N'-N''*-triacetylchitotriose and synthetic substrates such as *p*-nitrophenyl-β-*N*-acetylglucosamine (pNP-GlcNAc) or 4-methylumbelliferyl-*N*-acetyl-D-glucosamine (MUAG) to release *N*-acetylglucosamine (GlcNAc; Sullivan *et al*., 1984). *HEX1* expression is only induced in GlcNAc-containing media (Niimi *et al*., 1997). This regulatable expression along with the lack of a *HEX1* homologue in *S. cerevisiae* makes the *C. albicans HEX1* gene an effective reporter gene system for use in *S. cerevisiae* (Cannon *et al*., 1994; Niimi *et al*., 1998). The V5-*6*xHIS cassette was amplified by PCR to integrate by homologous recombination at the *HEX1* locus using the HEX1-V5-F and HEX1-NAT1-R primers. The *HEX1*-V5-6xHIS-*NAT1* transformants were screened by PCR using the V5-S primer within the V5-6xHIS sequences and the *HEX1*-specific *HEX1*-V5-SF primer (Figure 43B; Table 5). Two PCR positive *HEX1*-V5-6xHIS-*NAT1* colonies (SWM150 and SWM151) were chosen, along with one PCR positive *HEX1*-V5-6xHIS-*URA3* colony (SWM149), and grown in both synthetic complete medium (SC) and synthetic complete containing GlcNAc as the carbon source (SC-GlcNAc). The cells were harvested, the proteins extracted and analysed by western blot using a V5 antibody. The blot shows that none of the three strains expresses detectable levels of *N*-acetylglucosaminidase when grown in SC, but all expressed similar levels of both the
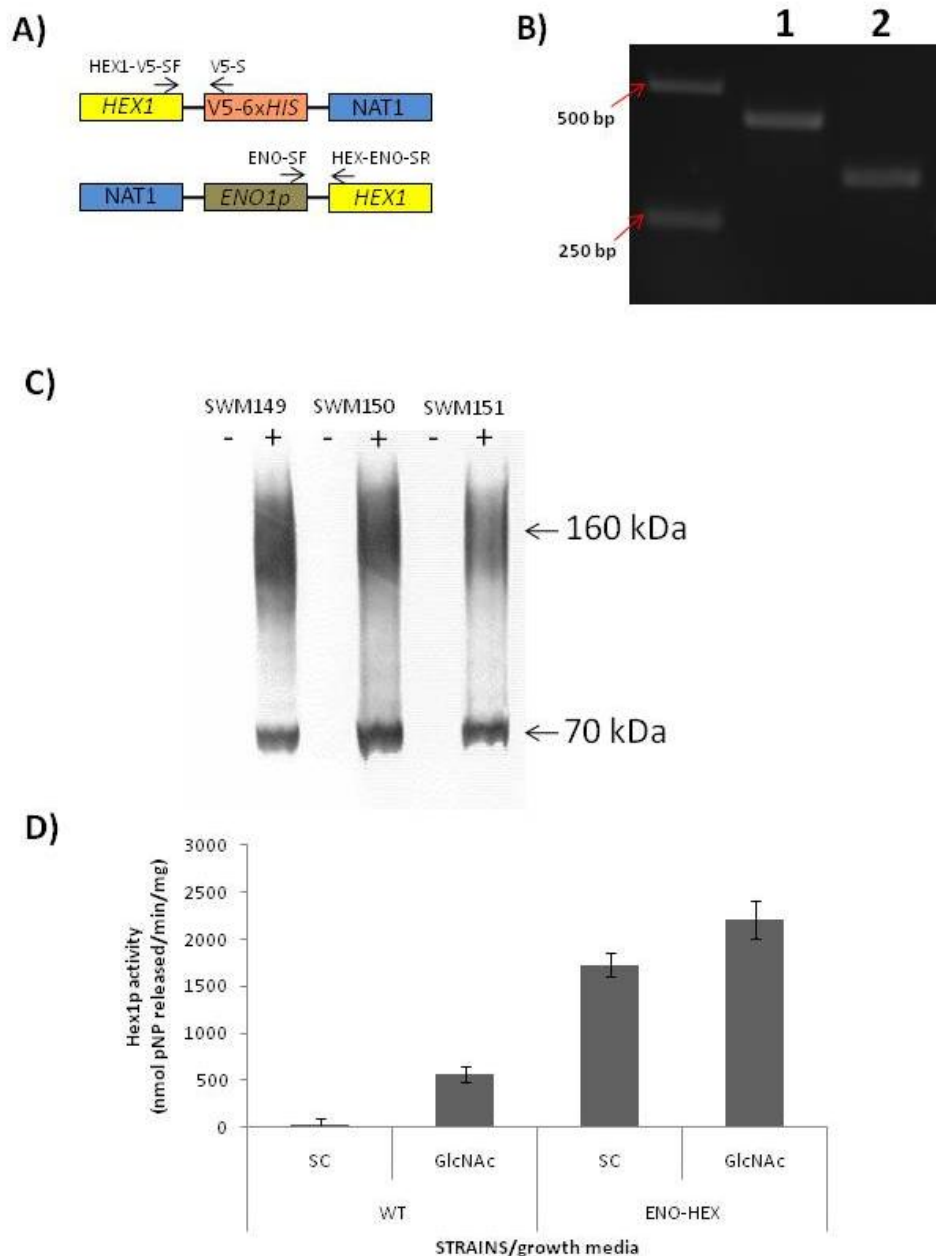
Figure 43. The validation of the V5-*NAT1* and *NAT1-ENO1p* cassettes. (A) Schematic of the V5-6x*HIS-NAT1* tagging and *NAT1-ENO1p* promoter replacement of the *HEX1* gene with screening primer sites shown. (B) PCR confirmation that each cassette has integrated at the *HEX1* locus. *NAT1-ENO1p* (lane 1) and V5-6xHIS-*NAT1* (lane 2). (C) Western blot analysis of Hex1-V5-6xHIS protein expression in SC medium (non-inducing) or SC-GlcNAc medium (Inducing) in the SWM149, SWM150 and SWM151 strains. (D) The Hex1p activity of two different strains in two different media. The nmol p-nitrophenol released per minute per mg of protein from a wild type CAI4 (CIp10) strain grown in SC +/- GlcNAc and the SWM152 strain containing the *NAT1-ENO1p* cassette expressing the *HEX1* gene. The construction of the V5-6x*HIS –NAT1* and *NAT1-ENO1*p cassettes and SWM149, SWM150, SWM151 and SWM152 *Candida* strains was carried out by Dr. Steven Bates. The validation of the V5-6x*HIS –NAT1* cassette was also carried out by Dr. Steven Bates.

unmodified form (70 kDa) and the glycosylated form (160 kDa) of *N*-acetylglucosaminidase in SC-GlcNAc (Figure 43C). This result confirms that the V5-6xHIS-*NAT1* cassette is fully functional and works well in *C. albicans*.

The *HEX1* gene was also used for validating the *NAT1-ENO1p* cassette. The *ENO1* promoter was chosen for this study as it has been shown to be constitutively active at high levels and therefore suitable for gene overexpression studies (Staab *et* al., 2003; Bates *et al.*, 2007). The validation of the *NAT1-ENO1p* cassette was carried out by replacing the native promoter of one of the *HEX1* alleles with this cassette in the CAI4 (CIp10) strain (Figure 43A and B). *NAT1-ENO1p-HEX1*-transformant colonies were screened by PCR using the ENO-SF primer which is located within the *ENO1* promoter sequence and the *HEX1* specific HEX-ENO-SR primer (Figure 32A; Table 5). Two independent PCR-confirmed *ENO1p-HEX1* colonies were identified following transformation of CAI4 (CIp10) with the *HEX1* specific *NAT1-ENO1p* cassette, SWM152 and SWM153 (Figure 43B). Only one of these colonies was assayed, SWM152. The SWM152 strain was grown in the same conditions and media as the SWM150 and SWM151 strains, cells were harvested and proteins extracted as documented in the methods section. The levels of *N*-acetylglucosaminidase were assayed using a modified version of the β-*N*- acetylglucosaminidase assay utilised by Cannon *et al.* (1994). The assay results confirmed that *N*-acetylglucosaminidase is only expressed in wild type cells in the presence of GlcNAc, which is consistent with the observations of Niimi and colleagues (Figure 43D; Niimi *et al.*, 1997). This finding also confirmed that replacing the endogenous *HEX1* promoter with the *NAT1-ENO1* promoter cassette resulted in over a thousand-fold increase in *N*-acetylglucosaminidase activity when grown in SC medium compared to the *N*-acetylglucosaminidase activity of the wild type strain grown in SC. The addition of GlcNAc to the medium instead of glucose resulted in *N*-acetylglucosaminidase expression at increased levels due to the presence of both the over-expressed and remaining wild type copy of the *HEX1* gene. These results confirm that the *NAT1-ENO1p* cassette is fully functional.

5.2.5. *NAT1* cassettes are fully functional in clinical strains.

The study of protein expression and localisation in clinical isolates of *C. albicans* is technically challenging due to a lack of available selectable markers. Successful

transformation of the *C. albicans* optimised *NAT1* gene into a selection of different *C. albicans* clinical isolates confirms the suitability of this gene as a marker for the cassettes we constructed in this study (Shen *et al.*, 2005). To test this hypothesis the *GFP-NAT1* cassette was PCR-amplified to tag the septin protein *CDC3* using the CDC3-GFP-F and CDC3-NAT1-R primers (Table 5). The PCR-amplified *GFP-NAT1* cassette was transformed into the SC5314 clinical isolate and nourseothricin resistant colonies began appearing after 24-48 hours. These colonies were screened by PCR using the *CDC3*-specific CDC3-TAG-S primer and the *GFP*-specific GFP-UP primer (Table 5). Three PCR-confirmed colonies were selected for microscopic analysis; SWM154, SWM155 and SWM156 (Figure 44). The Cdc3 protein is known to localise to the mother-bud neck in both *S. cerevisiae* and *C. albicans* (Longtine *et al.*, 1998; Field and Kellogg, 1999; Gerami-Nejad *et al.*, 2001). All three strains exhibited cell cycle-dependent Cdc3-GFP fluorescence at the mother-bud neck (Figure 44). This finding also confirms that these cassettes are viable for use in studies of clinical strains. The functionality of tagged proteins can be investigated further by disrupting the non-tagged allele and assessing cell viability. A low viability would suggest the tagged protein is not functional.

5.3. Discussion.

In this chapter we document the construction of a set of protein expression and localisation cassettes and an over-expression promoter cassette that utilise the *NAT1* gene as the selection marker. Both the *NAT1* and closely related *SAT1* genes have been adapted for use in *C. albicans.* These genes have been incorporated into a number of regulatable promoter cassettes and also a recyclable disruption cassette and this work expands the range of molecular tools available to *Candida* scientists (Roemer *et al.*, 2003; Reuss *et al.*, 2004; Shen *et al.*, 2005; Schaub *et al.*, 2006). The *NAT1/SAT1* markers have significant advantages over the other positive selection marker used in *C. albicans,* mycophenolic acid resistance, mutated *IMH3* gene, as the mutated *IMH3* gene preferentially integrate into the natural *C. albicans IMH3* locus therefore making it unsuitable for using in gene disruption or tagging experiments (Beckerman *et al.*, 2001). The hygromycin B resistance gene may also soon become another useful addition to the toolbox of *Candida* scientists, but this has only recently been adapted for use in *C. albicans* and is still being evaluated (Basso. *et al.*, 2010).
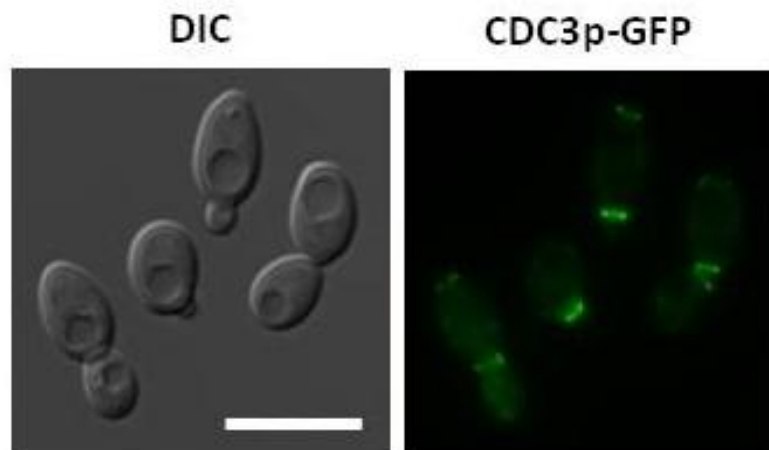
Figure 44. Evaluation of the *NAT1* tags in the clinical strain SC5314. Fluorescence microscopy of the mother-bud neck localisation of the Cdc3-GFP proteins in strain SWM154. Scale bar is 10 µm.

Interestingly, in relation to the use of the *NAT1* cassettes it was found that we needed to use 600 µg/ml nourseothricin in YPD plates to inhibit growth of a wild type *C. albicans* strain but that we were able to reduce this to 200 µg/ml by using Sabouraud dextrose (SABDEX) plates. This difference is possibly caused by reduced availability of nourseothricin in YPD compared to that in SABDEX. There is currently no consensus in the literature regarding the concentration of nourseothricin needed to inhibit cell growth on agar plates. Reported inhibitory concentrations vary from 200 µg/ml for the *SAT1* flipper cassettes to 400 µg/ml for the SAT-1-TET promoter. This difference cannot be explained by the use of nourseothricin from different sources as each group used nourseothricin purchased from Werner BioAgents (Jena, Germany). One possible explanation is that in the *SAT1* flipper experiments a lower cell density was plated when compared to our study.

The cassettes presented in this chapter were constructed utilising fusion PCR and not sub-cloning as has been used by many other groups. Fusion PCR has the advantage of being a relatively straightforward procedure consisting of just two PCR amplifications which may therefore be carried out more rapidly than the more time-consuming sub-cloning methods. The function of all the cassettes constructed was validated through control experiments. The fluorescent protein cassettes were expressed under the control of the *ACT1* promoter and the V5-6xHIS cassette by tagging the *HEX1* gene. The results of these experiments confirm that these cassettes all function properly and that the *GFP* cassette is the brightest of the fluorescent proteins tested. Western blot validation of the SWM150 and SWM151 strains showed two separate bands, a distinct lower band corresponding to the 70 kDa unmodified Hex1-V5-6xHIS protein and a upper smear corresponding to the glycosylated forms of the Hex1-V5-6xHIS protein which are around 160 kDa in size. These two bands were also present in the control therefore confirming that they are not artifactual and indicating that the cassette does not alter the protein and its function. The presence of both the modified and unmodified form of the Hex1 protein on the western blot shows that the V5-6xHIS tag is not cleaved off the target protein during post-translational modifications. The V5-6xHIS tagging cassette only adds 8.6 kDa to the size of the target protein and should not alter the activity of the protein and the 6xHis tags can be used for protein purification (validated by J. Cheetham, personal communication).

The above mentioned cassettes may be utilised in a range of other cell biology techniques beside those already discussed, including immunofluorescence. Protein co-localisation is another extremely useful technique in which the fluorescent protein cassettes could be utilised, as with previously published cassettes. In fact we were able to confirm the suitability of our cassettes for this purpose when we co-localised the Tem1 protein with the spindle pole body/γ-tubulin (section 4.2.1.1). The Tem1 protein was tagged using the *GFP-URA3* cassette while the γ-tubulin protein, Tub4, was tagged using the *RFP-NAT1* cassette. The results of that experiment were documented and discussed in the previous chapter.

The *HEX1* gene was also utilised for validating the *NAT1-ENO1p* cassette. This procedure inserted the cassette just upstream of the gene, so constructing a strain in which one copy of the *HEX1* gene was under the control of the *ENO1* promoter. Levels of the *HEX1* gene product *N*-acetylglucosaminidase were assayed using a modified version of the β-*N*-acetylglucosaminidase assay that Cannon *et al*. (1994) used to study *p*-nitrophenol release from *p*-nitrophenyl-β-*N*-acetylglucosamine molecules. The results show that in repressing conditions very little *N*-acetylglucosaminidase was expressed by a wild type strain while the SWM152 strain expresses large quantities of *N*-acetylglucosaminidase. This confirms that the *HEX1* gene is constitutively expressed in this strain and hence the *NAT1-ENO1p* cassette is fully functional.

The final experiment we carried out used the *GFP-NAT1* cassette to tag the septin protein Cdc3 in the SC5314 clinical strain, SWM158. Successful tagging of the Cdc3 protein confirms the finding of Shen *et al* (2005) that clinical strains of *C. albicans* are susceptible to nourseothricin and also that these molecular tools function well in a clinical strain. With the development of these cassettes there is now a wider range of cassettes available to *Candida* scientists to allow them to fully characterise a variety of genes in a broad range of strains without the need for an auxotrophic marker (Roemer *et al*., 2003; Reuss *et al*., 2004; Shen *et al*., 2005; Schaub *et al*., 2006). In addition, our cassettes, and others utilising the *NAT1/SAT1* marker system, are not restricted to use in *C. albicans*, but can be used in several other *Candida* species and potentially other fungi that are sensitive to nourseothricin (Shen *et al*., 2005).

Chapter 6–General discussion.

6.1. Overview.

During this project our objective was to understand the role that the GTPase Tem1 plays in signalling mitotic exit, cytokinesis and cell separation in *Candida albicans*. In order to achieve this goal we also had to develop a set of molecular tools. In this thesis we present data which indicates that the Tem1 protein is involved in signalling the conclusion of mitotis and is also involved in activating cytokinesis in *C. albicans*. Furthermore, we show that Tem1 localises to spindle pole bodies during the cell cycle but does not relocalise to the site of septation, suggesting that Tem1 carries out its mitotic exit and cytokinesis function from the spindle poles. Finally we showed that the essential function of *TEM1* is signalled through Cdc15 and that Tem1 also interacts with Bfa1.

6.2. *NAT1* cassettes.

Mounting evidence shows that the use of *URA3* as a marker results in various virulence defects (Kirsch and Whitney, 1991; Laurenson and Rine, 1992; Cole *et al.*, 1995; Bain *et al.*, 2001; Cheng *et al.*, 2003; Staab and Sundstrom, 2003; Brand *et al.*, 2004; Lermann and Morschhaüser, 2008; Correia *et al*., 2010; Noble *et al*., 2010). In addition the range of auxotrophic marker available for use in *C. albicans* is still limited. To combat this we constructed a set of cassettes for gene tagging and overexpression which do not utilise auxotrophic markers but instead use the *NAT1* positive selection marker (Shen *et al.*, 2005). These include the *GFP-NAT1*, *CFP-NAT1*, *YFP-NAT1* and *RFP-NAT1* cassettes for protein localisation and the V5-6xHIS cassette for protein expression studies. For gene overexpression studies we also constructed a cassette (*NAT1-ENO1p*) that utilises the constitutively active enolase promoter (*ENO1p*). All cassettes were validated for use in *C. albicans*, and may also function in other species displaying nourseothricin sensitivity. A number of other cassettes are currently available to researchers which combine either a regulatable promoter, a fluorescent protein gene or a epitope tag in combination with an auxotrophic marker (*URA3*, *HIS1* or *LEU2*, Gerami-Nejad *et al.*, 2001; Gola *et al.*, 2003; Schaub *et al.*, 2006; Gerami-Nejad *et al.*, 2009). However, to date only the *MAL2* and *MET3* regulatable promoters, the newly

manufactured venus yellow fluorescent protein gene and the *SAT1* flipper disruption cassette have been constructed to utilise nourseothricin resistance as the selectable marker (Reuss *et al.*, 2004; Schaub *et al.*, 2006; Reijnst *et al.*, 2011). Therefore, our cassettes complement those already available, and will allow researchers to utilise a wider range of strains for carrying out their studies. Additional cassettes could be constructed that contain either the mCHERRY fluorescent protein gene or the *HA*, *MYC* and *GST* epitope tags.

6.3. *TEM1* is essential in *Candida albicans*.

In this thesis we documented that *C. albicans* possesses one homolog of the *S. cerevisiae TEM1* gene and that this gene can complement the function of the *S. cerevisiae* gene. When expression of this gene was switched off we found that the cells lost viability, therefore marking *TEM1* as an essential gene in *C. albicans*. *TEM1/SPG1* genes are also essential in *S. cerevisiae, C. glabrata* and *S. pombe*, however, a recent study has found that in *Aspergillus nidulans* the *TEM1/SPG1* homolog *SPGA* is not essential for cell growth, and deletion of the *SPGA* gene causes no cytokinesis defects which suggest a potential difference in signalling mitotic exit and cytokinesis in yeast compared to filamentous fungi (Shirayama *et al.*, 1994b; Schmidt *et al.*, 1997; Miyakawa *et al.*, 2009; Kim *et al.*, 2011). Two constitutively active forms of the SpgA protein caused an increase in the frequency of cytokinesis therefore proving SpgA is involved in cytokinesis. These findings suggest that *A. nidulans* possesses an SpgA-independent cytokinesis pathway.

To investigate signalling downstream of Tem1 we overexpressed potential downstream effectors in cells depleted of the Tem1 protein and found that, as in *S. cerevisiae* and *S. pombe*, overexpression of the CDC15/*CDC7* gene bypasses the requirement for *TEM1/SPG1* (Shirayama *et al.*, 1994b; Schmidt *et al.*, 1997). This confirms that the Cdc15 protein is immediately downstream of Tem1 and that the essential function of Tem1 is signalled through Cdc15. Studies have found that in *C. albicans* the *CDC15* and *DBF2* genes are essential, while *CDC14*, *SOL1* (*SIC1* homolog) and CDH1, that are essential for Clb2 destruction and hence mitotic exit in *S. cerevisiae,* are not essential for growth in *C. albicans* (Cheetham *et al.*, unpublished; Clemente-Blanco *et al.*, 2006; Atir-Lande *et al.*, 2005; González-Novo *et al.*, 2009; Chou

et al., 2011). These results suggest that the MEN pathway is branched in *C. albicans* with two endpoints: Cdc14 and a yet to be identified protein. Overexpression of *DBF2* did not rescue Tem1 depleted cells therefore suggesting the branching of the MEN occurs at Cdc15. Future investigation in this area could focus upon identifying potential candidate proteins under the control of Tem1.

6.4. Depletion of the Tem1 protein results in cells arresting in late mitosis.

In *C. albicans*, the deletion or disruption of a number of genes resulting in cell cycle progression defects causes the cells to form filaments, which are often referred to in the literature as hyperpolarised buds. Based on the literature two different types of filaments were observed depending on the timing of cell cycle arrest. In cells that arrested early in the cell cycle, such as Cdc5 or Cln3 depleted cells, the filament formed from a single unbudded cell, while in those cells arresting late in mitosis, such as Clb2 or Sol1 depleted cells, the filaments form from budding cells (Bachewich *et al.*, 2003; Bachewich and Whiteway, 2005; Chapa y Lazo *et al.*, 2005; Bensen *et al.*, 2005; Atir-Lande *et al.*, 2005). We found that when Tem1 was depleted the cells formed filaments from budding yeast cells which suggests the cells arrested late in mitosis. This was confirmed by the fact that spindle poles in these Tem1-depleted cells had separated, indicating that spindle elongation had occurred and therefore that cells had progressed through the metaphase-anaphase transition prior to cell cycle arrest. Analysis of the number of nuclei in these cells further confirmed that the cells underwent a late anaphase arrest. Additional experiments, involving the use of fluorescence-activated cell sorting analysis would be ideal to examine the DNA content of these cells during the mitotic arrest, but these were not possible due to the cell morphology. The localisation of α-tubulin would also be desirable to confirm that nuclear separation is not complete. Interestingly, only some of the cells lacking the Sol1 and Cdh1 proteins form growth filaments, which implies these proteins are involved in mitotic exit in *C. albicans* in some capacity however this further indicates that the MEN is branched or a second pathway is involved in signalling mitotic exit (Atir-Lande *et al.*, 2005; Chou *et al.*, 2011). Overall our findings confirm that Tem1 is involved in signalling mitotic exit in *C. albicans*.

Of the cells that lack the Tem1, Cdc15, Dbf2 or Cdc14 proteins only the Tem1 depleted cells became filamentous. The cells lacking either Cdc15 or Dbf2 formed elongated chains of cells while Cdc14 depleted cells formed clumps (Cheetham *et al.*, unpublished; Clemente-Blanco *et al.*, 2006; González-Novo *et al.*, 2009). This difference is likely a result of the mitotic arrest caused by depletion of Tem1 as cells depleted of Cdc15, Dbf2 or Cdc14 complete nuclear division but display defects in either cytokinesis or cell separation. However, it is also possible is possible that Tem1 may play a role in pseudohyphae and true hyphae formation. This theory was supported by the observation that cells overexpressing the *TEM1* gene could form true hyphae at low serum concentrations and may therefore be more sensitive to external growth conditions.

6.5. Primary septum formation does not occur in Tem1 depleted cells.

In *S. cerevisiae*, the MEN proteins play various roles in cytokinesis (Jimenez *et al.*, 2005; Corbett *et al.*, 2006). In *C. albicans*, cells that were depleted of Cdc15, Dbf2 or Tem1 failed to form primary septa. In the Dbf2 depleted cells this was caused by the failure of the actomyosin ring to contract (Gonzalez-Novo *et al.*, 2009), while in cells depleted of Cdc15 (Cheetham *et al.*, unpublished) or Tem1 no actomyosin ring was seen at the mother-daughter bud neck. This lack of actomyosin ring function fits with the observation of septin rings failing to split in these cells. Further experiments are required to confirm if an actomyosin ring contraction defect is the cause of the lack of formation of primary septa in the Tem1p-depleted cells. These experiments would involve tagging either Hof1 or Mlc1 proteins with GFP in the SWM6 strain.

Aside from this likely actomyosin ring defect the Tem1p-depleted cells exhibit other cytokinesis defects. The first of these is that the septin ring that forms at the mother-daughter bud neck of the Tem1p-depleted yeast cells is maintained as an hourglass-like structure and failed to split into two rings. In *S. cerevisiae* the splitting of the hourglass septin structure into two septin rings occurs upon the completion of anaphase and prior to actin recruitment to the actomyosin ring. However these two events are uncoupled (Lippincott *et al.*, 2001; Gladfelter *et al.*, 2001; Oh and Bi, 2011). Significantly our observations of Tem1 playing a role in regulating septin dynamics was also seen in *S. cerevisiae* which proves that this function is conserved between the two

species (Lippincott *et al.*, 2001). Interestingly, in cells depleted of either, Tem1, Cdc15 or DBf2, the mitotic arrest is ultimately overcome but cytokinesis is not completed. This confirms that the MEN signals for both mitotic exit and cytokinesis, but these two functions can be separated.

6.6. Tem1 protein localisation and regulation.

A recent study questioned the mechanism of Tem1 activation by Lte1 in *S. cerevisiae* (Geymonat *et al.*, 2009). In this study they showed that Lte1 recruits the Bfa1-Bub2 complex bound from the mSPB to the dSPB and Lte1 had no GEF activity towards Tem1 (Geymonat *et al.*, 2009). We observed in *C. albicans* that Lte1 localised to the daughter cell cortex from bud formation through to the point, at the end of mitosis when Lte1 is released into the daughter cytoplasm. This is similar to the findings in *S. cerevisiae* and suggests Lte1 may play a similar role in *C. albicans* to that in *S. cerevisiae* (Bardin *et al.*, 2000; Jensen *et al.*, 2002; Geymonat *et al.*, 2009). However, we were unable to construct a null mutant in *LTE1*, suggesting it may play a more important role in the pathway in *C. albicans*. A yeast 2-hybrid analysis showed that the *C. albicans* Bfa1 and Bub2 proteins form a complex and that Bfa1 also binds to Tem1. This suggests that the Bfa1-Bub2 complex may negatively regulate Tem1 activity in *C. albicans* as it does in to *S. cerevisiae* (Ro *et al.*, 2002; Fraschini *et al.*, 2006). However, we observed Tem1 to localise to both SPB throughout the cell cycle with no detectable recruitment to the dSPB in anaphase, as was seen in *S. cerevisiae*, which calls into doubt the theory that Tem1 regulation in *C. albicans* is similar to that in *S. cerevisiae* (Molk *et al.*, 2004). Overall these findings suggest that the regulation of Tem1 is broadly similar to that seen in *S. cerevisiae*. Further research is required to confirm this conclusion. The research would include studying the localisation patterns of the Bfa1, Bub2 and also the regulatory kinase Kin4 to decipher if their localisation is similar to that in *S. cerevisiae*. In addition the successful construction and characterisation of a regulatable *LTE1* mutant should confirm the role of Lte1 in signalling mitotic exit in *C. albicans*.

The differential Tem1 localisation is not the only localisation difference of the potential MEN components between *C. albicans, S. cerevisiae* and *S. pombe*. It has been shown that the Cdc15, Dbf2 and Cdc14 proteins in *C. albicans* localise to both SPBs

throughout mitosis while in *S. cerevisiae* this localisation only occurs during late anaphase (Cheetham *et al.*, unpublished; Visintin and Amon, 2001; Yoshida *et al.*, 2002; Molk *et al.*, 2004; Clemente-Blanco *et al.*, 2006; González-Novo *et al.*, 2009). Interestingly in *S. pombe* the Cdc7 (Cdc15) protein localises to both SPBs during early mitosis but relocates to just one SPB in late mitosis while the Spg1 and Sid2 (Dbf2) proteins localise to both SPBs throughout mitosis (Sohrmann *et al.*, 1998, Schmidt *et al.*, 1997; Sparks *et al.*, 1999). The localisation pattern of the Clp1 (Cdc14) protein in *S. pombe* shares similarities with that of Cdc14 in *S. cerevisiae* which suggests that the Cdc14 and Clp1 proteins in *S. cerevisiae* and *S. pombe* have a different function than the Cdc14 protein in *C. albicans* (Cueille *et al.*, 2001; Trautmann *et al.*, 2001).

In *S. cerevisiae* Tem1 is known to display an unusually high intrinsic rate of GDP release, and it has been suggested that this is probably sufficient to activate itself and the MEN pathway (Geymonat *et al.*, 2002). The exact mechanism by which Tem1 releases the GDP is not known but we found that the *C. albicans* Tem1 protein is capable of binding to itself. This finding allows us to propose the exciting theory that upon dissociation of the Bfa1-Bub2 complex the Tem1 protein forms a homodimer, and this activates Tem1 hence allowing it to bind to Cdc15 and activate the MEN pathway. If this theory is correct it raises the question of what causes the Bfa1-Bub2 complex to disassociate.

6.7. Conclusion and future direction.

The *C. albicans* Tem1 GTPase plays an important role in regulating events at the conclusion of mitosis and the initiation of cytokinesis, and this is signalled through Cdc15. However our research and that carried out by other groups, suggests that the Cdc14 protein is only one of the primary end points for the MEN pathway. Ultimately, the pathway must be branched with an additional protein, yet to be identified, capable of instigating cyclin destruction in the absence of the Cdc14 protein. In addition our research showed that depletion of Tem1 resulted in cytokinesis and cell separation defects. Specifically we showed that Tem1 is required for regulating septin ring dynamics and therefore primary septum formation.

There are three main areas in which this project could be continued in the future;

(i) decipher the series of events leading up to mitotic exit, (ii) identify the branching point within the MEN pathway, (iii) understanding the signalling responsible for the filament formation of the Tem1 depleted cells.

The findings of this study, a partner study within our research group and published studies from the Correa-Bordes and Bachewich labs have led us to propose that mitotic exit in *C. albicans* is controlled in a different manner to that to in *S. cerevisiae*. In *S. cerevisiae* mitotic exit is controlled through the activation of Cdc14 and then subsequently Cdh1 and Sic1 (Amon *et al.*, 1994; Lim *et al.*, 1998; Baumer *et al.*, 2000; Yeong *et al.*, 2000). Homologs of these three proteins in *C. albicans* are not essential for mitotic exit but are, however are required for timely mitotic exit (Atir-Lande, *et al.*, 2005; Clemente-Blanco *et al.*, 2006; Chou *et al.*, 2011). We showed in this study that Tem1 is essential for both growth and mitotic exit therefore suggesting a branched mitotic exit network in *C. albicans*. Both mass spectrometry and protein pull-down experiments will be utilised to identify proteins which interact with Tem1, Cdc15 or Dbf2, and which could be part of the additional branch of the MEN. Identified proteins will then undergo overexpression studies in conditional *tem1*, *cdc15* and *dbf2* mutants in an attempt to identify the first protein involved in the pathway branch and also where in the network the branch occurs. To identify the other proteins involved in this new branch of the MEN the same protein interaction and overexpression experiments will be utilised.

Within this study (chapter 4) we began to investigate the regulation of the MEN. This work would be continued in three different areas. i) protein localisation, ii) null/conditional mutant construction and characterisation and iii) Tem1 GTP-GDP cycling. In *S. cerevisiae* the MEN is regulated through the cycling of Tem1 between its GTP (active) and GDP (inactive) bound forms. For the protein localisation studies the Kin4, Bfa1 and Bub2 proteins will be tagged with GFP. The localisation of each protein will be studied at various stages of the cell cycle progression. The second area of investigation will involve the characterisation of *kin4*, *bfa1*, *bub2* null/regulatable mutants to decipher the role the encoded proteins play in *C. albicans*. The localisation of the Tem1 protein will be studied within each mutant to identify whether Tem1 localisation is dependent on any of these three proteins. The ability of GTPase

proteins to switch from being in the GDP-bound form to the GTP-bound form and *vice versa* is critical for the function of the GTPase protein. However the *S. cerevisiae* Tem1 protein has an unusually high GDP dissociation rate. To determine if the *C. albicans* Tem1 possesses similar properties a nucleotide exchange assay will be run. To assess requirement for Tem1 to cycle between both inactive and active forms PCR mediated site directed mutagenesis will be conducted to construct two mutated forms of the *TEM1* gene which encode for a protein that is locked in either the GDP-bound or GTP-bound conformation. Each mutant gene will be introduced into the SWM6 strain on the CIp10-*NAT1* plasmid and the role the cycling of the activation state of Tem1 assessed upon repression of expression of the wild type *TEM1* gene.

The events of late metaphase and early anaphase are an additional area requiring extensive research to be conducted. Previous studies have illustrated that the APC subunit Cdc20 is not required for sister chromatid separation. This and the findings that *C. albicans* lacks the anaphase inhibitor protein securin (Pds1) and many components of the FEAR network, suggest that the metaphase to anaphase transition is different in *C. albicans* from that in *S. cerevisiae* (Chou *et al.*, 2011; this study). To investigate the events of early anaphase a seperase (Esp1) null mutant will be constructed and characterised. This characterisation will include; DAPI staining to study nuclear localisation, FACS analysis of the DNA content of the cells, rtPCR or northern blot analysis of cyclin expression, GFP or immunofluorescence analysis of spindle patterns.

In addition to the above discussed research studying the mechanisms of mitotic exit the role Tem1 plays in morphogenesis is to be investigated. This area is of particular interest as morphogenesis it thought to play a key role in the pathogenicity of *C. albicans*. As observed in this study cells that are depleted of Tem1 spontaneously form filaments that resemble true hyphae. Therefore Tem1 is involved in the negative regulation of hyphae formation. To investigate this further a series of experiments would be carried out to identify if Tem1 physically inhibits any member of the morphogenesis signalling pathways. These experiments include the identification of the protein interactions of Tem1 by protein pull-downs and mass spectrometry and yeast 2-hybrid analysis of any interactions highlighted by the previous two

experiments. The final set of experiments will involve the construction and characterisation a series of mutants depleted of the Tem1 protein and also lacking the gene for a key morphogenesis protein such as *EFG1* and *CPH1*.

Furthermore, some of experiments documented in this thesis may be repeated with additional controls. These experiments include the Tem1 expression shut off western blot (Figure 22), TEM analysis of the primary septa formation and cell wall architecture (Figure 29 and 32) and the SEM analysis of the growth phenotype of the Tem1 depleted cells (Figure 20). The experiment studying the shut off of expression of the *TEM1* gene in the SWM66 strain grown in doxycycline containing media may be repeated with a positive control, consisting of protein samples extracted from SWM66 cells grown in non-repressing media for 0, 4, 8, 12 and 24 hours. Also, coomassie staining of the SDS page gel would confirm equal quantities of proteins were loaded onto the gel. For the TEM experiments investigating primary septa formation and cell wall architecture within Tem1 depleted cells, samples of the SWM6 strain grown in repressing and non-repressing media were analysed (Figure 32). This was also the case for the SEM analysis of the growth phenotype of the Tem1 depleted cells experiments (Figure 20). Repeating these experiments an additional control of the CAI4 (CIp10) strain grown in repressing and non-repressing media would add weight to the conclusions drawn in this thesis. The CAI4 (CIp10) strain was used as the control strain for many of the experiments documented in chapter 3. This is strain is a common control utilised by *Candida* researchers as it has directed lineage to many of the strains used in the gene function studies and the strain is *ura* positive and therefore does not experience the growth defects of *ura* minus strains. An additional control strain could also be constructed for use in many of these experiments which would consist of the CIp10 plasmid chromosomally integrated into the RPS10 locus of the THE1 strain and therefore containing a highly expressed copy of the *URA3* gene.

References.

Alani, E., Cao, L. and Klechner, N (1987). A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. *Genetics*; **116(4)**: 541-545.

Alberti-Segui, C., Morales, AJ., Xing, H., Kessler, MM., Willins, DA., Weinstock, KG., Cottarel, G., Fechtel, K. and Rogers, B (2004). Identification of potential cell-surface protein in *Candida albicans* and investigation of the role of a putative cell-surface glycosidase in adhesion and virulence. *Yeast*: **21(4)**: 285-302.

Alexandru, G., Zachariae, W., Schleiffer, A. and Nasmyth, K (1999). Sister chromatid separation and chromosome re-duplication are regulated by different mechanism in response to spindle damage. *EMBO Journal*; **18(10)**: 2707-2721.

Alexandru, G., Uhlmann, F., Mechtler, K., Poupart, MA. and Nasmyth, K (2001). Phosphorylation of the cohsin subunit Scc by Polo/Cdc5 kinase regulates sister chromatid separation in yeast. *Cell*; **105(4)**: 459-472.

Al-Fattani, MA. and Douglas LJ (2006). Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *Journal of Medical Microbiology*; **55(8)**: 999-1008.

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

Amon, A., Irniger, S. and Nasmyth, K (1994). Closing the cell cycle circle in yeast: G_2 cyclin proteolysis initiated at mitosis persist until the activation of G_1 cyclins in the net cycle. *Cell*; **77(7)**: 1037-1050.

Andaluz, E., Coque, JJ., Cueva, R. and Larriba, G (2001). Sequencing of a 4.3 kbp region of chromosome 2 of *Candida albicans* reveals the presence of homologues of *SHE9* from *Saccharomyces cerevisiae* and of bacterial phosphatidyinositol-phospholipase C. *Yeast*; **18(8)**: 711-721.

Andaluz, E., Ciudad, T., Gomez-Raja, J., Calderone, R. and Larriba, G (2006). Rad52 depletion in *Candida albicans* triggers both the DNA-damage checkpoint and filamentation accompanied by but independent of expression of hypha-specific genes. *Molecular Microbiology;* **59(5)**: 1452-1472.

Anderson, JM. and Soll, DR (1986). Differences in actin localisation during bud and hypha formation in the yeast *Candida albicans. Journal of General Microbiology*; **132(7)**: 2035-2047.

Arnaud, MB., Costanzo, MC., Skrzypek, MS., Shah, P., Binkley, G., Lane, C., Miyasato, SR. and Sherlock, G (2007). Sequence resources at the *Candida* Genome Database. *Nucleic Acids*

Research; **35**: 452-456.

Arst, HN., Bignell, E. and Tilburn, J (1994). Two new genes involved in signalling ambient pH in *Aspergillus nidulans*. *Molecular and General Genetics*; **245(6)**: 787-790.

Asakawa, K., Yoshida, S., Otake, F. and Toh-e, A (2001). A novel functional domain of Cdc15 kinase is required for its interaction with Tem1 GTPase in *Saccharomyces cerevisiae. Genetics*; **157(4)**:1437-1450.

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

Atir-Lande, A., Gildor, T. and Kornitzer, D (2005). Role of the SCFCDC4 ubiquitin ligase in *Candida albicans* morphogenesis. *Molecular Biology of the Cell*; **16(6)**: 2772-2785.

Bachewich, C., Thomas, DY. and Whiteway, M (2003). Depletion of a polo-like kinase in *Candida albicans* activates cyclase-dependent hyphal-like growth. *Molecular Biology of the Cell*; **14(5)**: 2163-2180.

Bachewich, C. and Whiteway, M (2005). Cyclin Cln3 links G_1 progression to hyphal and pseudohyphal development in *Candida albicans*. *Eukaryotic Cell*; **4(1)**: 95-102.

Bachewich, C., Nantel, A. and Whiteway, M (2005). Cell cycle arrest during S or M phase generates polarised growth via distinct signals in *Candida albicans. Molecular Microbiology*; **57(4)**: 942-959.

Backen, AC., Broadbent, ID., Fetherston, RW., Rosamund, JD., Schnell, NF. and Stark, MJ (2000). Evaluation of the *CaMAL2* promoter for regulated expression of genes in *Candida albicans*. *Yeast*; **16(12)**: 1121-1129.

Bai, C., Ramanan, N., Wang, YM. and Wang, Y (2002). Spindle assembly checkpoint component CaMad2p is indispensable for *Candida albicans* survival and virulence in mice. *Molecular Microbiology*; **45(1)**: 31-44.

Baillie, GS and Douglas, LJ (2000). Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *Journal of Antimicrobial Chemotherapy*; **46(3)**: 397-403.

Bain, JM., Stubberfield, C. and Gow, NAR (2001). Ura-status-dependent adhesion of *Candida albicans* mutants. *FEMS Microbiology Letters*; **204(2)**: 323-328.

Balasubramanian, MK., Bi, E. and Glotzer, M (2004). Comparative analysis of cytokinesis in budding yeast, fission yeast and animal cells. *Current Biology*; **14(18)**: 806-818.

Ban, KH., Torres, JZ., Miller, JJ., Mikhailov, A., Nachury, MV., Tung, JJ., Rieder, CL. and Jackson, PK (2007). The END network couples spindle pole assembly to inhibition of the anaphase-promoting complex/cyclosome in early mitosis. *Developmental Cell*; **13(1)**: 29-42.

Bardin, AJ., Visintin, R. and Amon, A (2000). A mechanism for coupling exit from mitosis to partitioning of the nucleus. *Cell*; **102(1)**: 21-31.

Bardin, AJ. and Amon, A (2001). Men and sin: what's the difference? *Nature Reviews Molecular and Cell Biology*; **2(11)**: 815-826.

Bardin, AJ., Boselli, MG. and Amon, A (2003). Mitotic exit regulation through distinct domains within the protein kinase Cdc15. *Molecular Cell Biology*; **24(14)**: 5018-5030.

Barelle, CJ., Manson, CL., MacCallum, DM., Odds, FC., Gow, NAR. and Brown, AJ (2004). *GFP* as a quantitative reporter of gene regulation in *Candida albicans*. *Yeast*; **21(4)**: 333-340.

Barwell, KJ., Boysen, JH., Xu, W. and Mitchell, AP (2005). Relationship of *DFG16* to the Rim101 pH response pathway in *Saccharomyces cerevisiae* and *Candida albicans. Eukaryotic Cell*; **4(5)**: 890-899.

Barral, Y., Parra, M., Bidlingmaier, S. and Snyder, M (1999). Nim1-related kinase coordinate cell cycle progression with the organisation of the peripheral cytoskeleton in yeast. *Genes and Development*; **13(2)**: 176-187.

Basso, LR, Bartiss, A., Mao, Y., Gast, CE., Coelho, PS., Snyder, M. and Wong, B (2010). Transformation of *Candida albicans* with a synthetic hygromycin B resistance gene. *Yeast*; **27(12)**: 1039-1048.

Bates, S., de la Rosa, JM., MacCallum, DM., Brown, AJ, Gow, NAR and Odds, FC (2007). *Candida albicans* Iff11, a secreted protein required for cell wall structure and virulence. *Infection and Immunity*; **75(6)**: 2922-2928.

Baumer, M., Braus, GH. and Irniger, S (2000). Two different modes of cyclin clb2 proteolysis during mitosis in *Saccharomyces cerevisiae*; **468**: 142-148.

Beck-Sagué, C. and Jarvis, WR (1993). Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. National Nosocomial Infections Surveillance System. *Journal of Infectious Diseases*; **167(5)**: 1247-1251.

Beckerman, J., Chibana, H., Turner, J. and Magee, PT (2001). Single-copy *IMH3* allele is sufficient to confer resistance to mycophenolic acid in *Candida albicans* and to mediate transformation of clinical *Candida* species. *Infection and Immunity*; **69(1)**: 108-114.

Bedhomme, M., Jouannic, S., Champion, A., Simanis, V. and Henry, Y (2008). Plants, MEN and SIN. *Plants Physiology and Biochemistry*; **46(1)**: 1-10.

Bembenek, J., Kang, J., Kurischko, C., Li, B., Raab, JR., Belanger, KD., Luca, FC. and Yu, H (2005). Crm1-mediated nuclear export of Cdc14 is required for the completion of cytokinesis in budding yeast. *Cell Cycle*; **4(7)**: 961-971.

Bennett, DE., McCreary, CE. and Coleman, DC (1998). Genetic characterisation of a

phospholipase C gene from *Candida albicans*: presence of homologous sequences in *Candida* species other than *Candida albicans*. *Microbiology*; **144(1)**: 55-72.

Bensen, ES., Clemente-Blanco, A., Finley, KR., Correa-Bordes, J. and Berman, J (2005). The mitotic cyclins Clb2p and Clb4p affect morphogenesis in *Candida albicans*. *Molecular Biology of the Cell*; **16(7)**: 3387-3400.

Berg, FT. (1846). *Om Torsk Hos Barn*. L.J. Hjerta, Stockholm, Sweden.

Bertagnolio, S., de Gaetano Donati, K., Tacconelli, E., Scoppettuolo, G., Posteraro, B., Fadda, G., Cauda, R. and Tumbarello, M (2004). Hospital-acquired candidemia in HIV-infected patients. Incidence, risk factors and predictors of outcome. *Journal of Chemotherapy*; **16(2)**: 172-178.

Biggins, S. and Murray, AW (2001). The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint. *Genes and Development*; **15(23)**: 3118-3129.

Bignell, E., Negrete-Urtasun, S., Calcagno, AM., Arst, HN., Rogers, T. and Haynes, K (2005). Virulence comparisons of *Aspergillus nidulans* mutants are confounded by the inflammatory response of p47phox-/- mice. *Infection and Immunity*; **73(8)**: 5204-5207.

Bishop, AL. and Hall, A (2000). Rho GTpases and their effector proteins. *Biochemical Journal*; **348(2)**: 241-255.

Blackwell, C. Russel, C. L. Argimon, S. Brown, A. J. P. Brown, J.D. (2003). Protein A-tagging for purification of native macromolecular complexes from *Candida albicans*. *Yeast*. 20:1235-1241.

Blanchin-Roland, S., Da Costa, G. and Gaillardin, C (2008). Ambient pH signalling in the yeast *Yarrowia lipolytica* involves YlRim23p/PalC which interacts with Snf7p/Vps32p, but does not require the long C terminus of YlRim9p/PalI. *Microbiology*; **154**: 1668-1676.

Blondel, M., Bach, S., Bamps, S., Dobbelaere, J., Wiget, P., Longaretti, C., Barral, Y., Meijer, L. and Peter, M (2005). Degradation of Hof1 by SCF (Grr1) is important for actomyosin contraction during cytokinesis in yeast. *EMBO Journal*; **24(7)**: 1440-1452.

Bockmuhl, DP. and Ernst, JF (2001). A potential phosphorylation site for an A-type kinase in the Efg1 regulator protein contributes to hyphal morphogenesis of *Candida albicans. Genetics*; **157(4)**: 1523-1530.

Boeke, JD., LaCroute, F. and Fink, GR (1984). A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity activity in yeast: 5-fluoro-orotic acid resistance. *Molecular and General Genetics*; **197(2)**: 345-346.

Boguski, MS. and McCormick, F (1993). Proteins regulating Ras and its relatives. *Nature*; **366**: 643-54.

Booher, RN., Deshaies, RJ. and Kirschner, MW (1993). Properties of *Saccharomyces cerevisiae*

wee1 and its differential regulation of p34CDC28 in response to G_1 and G_2 cyclins. *EMBO Journal*; **12(9)**: 3417-3726.

Bosl, WJ. and Li, R (2005). Mitotic-exit control as an evolved complex system. *Cell*; **121(3)**: 325-333.

Bourne, HR., Sanders, DA. and McCormick, F (1991). The GTPase superfamily: conserved structure and molecular mechanism. *Nature*; **349**: 117-127.

Bowers, K., Lottridge, J., Helliwell, SB., Goldthwaite, LM., Luzio, JP. and Stevens, TH (2004). Protein-protein interactions of ESCRT complexes in the yeast *Saccharomyces cerevisiae*. *Traffic*; **5**: 194-210.

Boysen, JH and Mitchell, AP (2006). Control of Bro1-domain protein Rim20 localisation by external pH, ESCRT machinery, and the *Saccharomyces cerevisiae* Rim101 pathway. *Molecular Biology of the Cell*; **17**: 1344-1353.

Brady, DM. and Hardwick, KG (2000). Complex formation between Mad1p, Bub1p, and Bub3p is crucial for spindle checkpoint function. *Current Biology*; **10(11)**: 675-678.

Brand, A., MacCallum, DM., Brown, AJ, Gow, NAR. and Odds, FC (2004). Ectopic expression of *URA3* can influence the virulence phenotypes and proteome of *Candida albicans* but can be overcome by targeted reintegration of *URA3* at the RPS10 locus. *Eukaryotic Cell*; **3(4)**: 900-909.

Braun, BR. and Johnson, AD (1997). Control of filament formation in *Candida albicans* by the transcriptional repressor *TUP1*. *Science*; **277**: 105-109.

Braun, BR. and Johnson, AD (2000). *TUP1*, *CPH1* and *EFG1* make independent contributions to filamentation in *Candida albicans. Genetics*; **155(1)**: 57-67.

Braun, BR., Head, WS., Wang, MX. and Johnson, AD (2000). Identification and characterisation of *TUP1*- regulated genes in *Candida albicans*; **156(1)**: 31-44.

Brennan, M., Thomas, DY., Whiteway, M. and Kavanagh, K (2002). Correlation between virulence of *Candida albicans* mutants in mice and *Galleria mellonella* larvae. *FEMS Immunology and Medical Microbiology*; **34(2)**: 153-157.

Brown, DH., Giusani, AD., Chen, X. and Kumamoto, CA (1999). Filamentous growth of *Candida albicans* in response to physical environmental cues and its regulation by the unique *CZF1* gene. *Molecular Microbiology*; **34(4)**: 651-662.

Brown, AJ (2002). Morphogenetic Signaling Pathways in *Candida albicans*. *In* RA. Calderone (ed), *Candida and Candidiasis*, ASM Press. Washington, USA.

Brown, AJ. and Gow, NAR (2001). Signal transduction and morphogenesis in *Candida albicans*. *In* K. Esser and JW. Bennett (ed), *The Mycota*, Springer, Berlin, Germany

Bruno, KS., Morrell, JL., Hamer, JE. and Staiger, CJ (2001). SEPH, a Cdc7p orthologue from *Aspergillus nidulans*, functions upstream of actin ring formation during cytokinesis. *Molecular Microbiology*; **42(1)**: 3-12.

Buurman, ET., Westwater, C., Hube, B., Brown, AJ, Odds, FC. and Gow, NAR (1998). Molecular analysis of CaMnt1p, a mannosyl transferase important for adhesion and virulence of *Candida albicans*. *Proceedings of the National Academy of Science of United States of America*: **95**: 7670-7675.

Calderone, RA. and Braun, BC (1991). Adherence and receptor relationships of *Candida albicans*. *Microbiological Reviews*: **55(1)**: 1-20.

Calderone, RA. and Gow, NAR (2002). Host Recognition by *Candida* Species. *In* RA. Calderone (ed), *Candida and Candidiasis*, ASM Press. Washington, USA.

Cannon, RD., Niimi, K., Jenkinson, HF. and Shepherd, MG (1994). Molecular cloning and expression of the *Candida albicans* β-N-acetylglucosaminidase (*HEX1*) gene. *Journal of Bacteriology*; **176(9)**: 2640-2647.

Care, RS., Trevethick, J., Binley, KM. and Sudbery, PE (1999). The *MET3* promotes: a new tool for *Candida albicans* molecular genetics. *Molecular Microbiology*; **34(4)**: 792-798.

Cassola, A., Parrot, M., Silberstein, S., Magee, BB., Passeron, S., Giasson, L. and Cantore, ML. (2004). *Candida albicans* lacking the gene encoding the regulatory subunit of protein kinase A displays a defect in hyphal formation and an altered localisation of the catalytic subunit. *Eukaryotic Cell*; **3(1)**: 190-199.

Castillon, GA., Adames, NR., Rosello, CH., Seidel, HS., Longtine, MS., Cooper, JA. and Heil-Chapdelaine, RA (2003). Septins have a dual role in controlling mitotic exit in budding yeast. *Current Biology*; **13(8)**: 654-658.

Caydasi, AK. and Pereira, G (2009). Spindle alignment regulates the dynamic association of checkpoint proteins with yeast spindle pole bodies. *Developmental Cell*; **16(1)**: 146-156.

Cenamor, R., Jimenez, J., Cid, VJ., Nombela, C. and Sanchez, M (1999). The budding yeast Cdc15 localises to the spindle pole body in cell-cycle-dependent manner. *Molecular Cell Biology research Communications*; **2(3)**: 178-184.

Cerutti, L. and Simanis, V (1999). Asymmetry of the spindle pole bodies and spg1GAP segregation during mitosis in fission yeast. *Journal of Cell Science*; **112(14)**: 2313-2321.

Chaffin, WL (2008). *Candida albicans* cell wall proteins. *Microbiology and Molecular Biology Reviews*; **72(3)**: 495-544.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, WW. and Prasher, DC (1994). Green fluorescent protein as a marker for gene expression. *Science*; **263**: 802-805.

Champion, A., Jouannic, S., Guillon, S., Mockaitis, K., Krapp, A., Picaud, A., Simanis, A., Kreis, M. and Henry, Y (2004). AtSGP1, AtSGP2, and MAP4Kα are nucleolar plant proteins that can complement fission yeast mutants lacking a functional SIN pathway. *Journal of Cell Science*; **117**: 4265-4275.

Chan, GK., Jablonski, SA., Sudakin, V., Hittle, JC. and Yen, TJ (1999). Human BUBR1 is a mitotic checkpoint kinase that monitor CENP-E functions at kinetochores and binds the cyclosome/APC. *Journal of Cell Biology*; **146(5)**: 941-954.

Chandra, J., Kuhn, DM., Mukherjee, PK., Hoyer, LL., McCormick, T. and Ghannoum, MA (2001). Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture and drug resistance. *Journal of Bacteriology*; **183**: 5385-5394.

Chang, L. and Gould, KL (2000). Sid4p is required to localise components of the septation initiation pathway to the spindle pole body in fission yeast. *Proceedings of the National Academy of Science of the United States of America*; **97**: 5249-5254.

Chapa y Lazo, B., Bates, S. and Sudbery, P (2005). The G_1 cyclin regulates morphogenesis in *Candida albicans*. *Eukaryotic Cell*; **4(1)**: 90-94.

Charles, JF., Jaspersen, SL., Tinker-Kulberg, RL., Hwang, L., Szidon, A. and Morgan, DO (1998), The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in *S. cerevisiae*. *Current Biology*; **8(9)**: 497-507.

Chen, S., Slavin, M., Nguyen, Q., Marriott, D., Playford, EG., Ellis, D. and Sorrell, T (2006). Active surveillance for candidemia, Australia. *Emerging Infectious Diseases*; **12(10)**: 1508-1516.

Cheney, RE. and Mooseker, MS (1992). Unconventional myosins. *Current Opinions in Cell Biology*; **4(1)**: 27-35.

Cheng, L., Hunke, L. and Hardy, CF (1998). Cell cycle regulation of the *Saccharomyces cerevisiae* polo-like kinase cdc5p. *Molecular and Cell Biology*; **18(12)**: 7360-7370.

Cheng, S., Nguyen, MH., Zhang, Z., Jia, H., Handfield, M. and Clancy, CJ (2003). Evaluation of the roles of four *Candida albicans* genes in virulence by using gene disruption strains that express *URA3* from the native locus. *Infection and Immunity*; **71(10)**: 6101-6103.

Chou, H., Glory, A. and Bachewich, C (2011). Orthologues of the APC/C coactivators Cdc20p and Cdh1p are important for mitotic progression and morphogenesis in *Candida albicans*. *Eukaryotic Cell*; **10(5)**: 696-709.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M. and Nasmyth, K (1998). An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell*; **93(6)**: 1067-1076.

Clark, EA. and Brugge, JS (1995). Integrins and signal transduction pathways: the road taken. *Science*; **268**: 233-239.

Clemente-Blanco, A., Gonzalez-Novo, A., Machin, F., Caballero-Lima, D., Aragon, L., Sanchez, M., de Aldana, CR., Jimenez, J. and Correa-Bordes, J (2006). The Cdc14p phosphatase affects late cell-cycle events and morphogenesis in *Candida albicans*. *Journal of Cell Science*; **119(6)**: 1130-1143.

Cohen-Fix, O., Peters, JM., Kirschner, MW. and Koshland, D (1996). Anaphase initiation in *Saccharomyces cerevisiae* is controlled the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes and Development*; **10**: 3081-3093.

Cole, MF., Bowen, WH., Zhao, XJ. and Cihlar, RL (1995). Avirulence of *Candida albicans* auxotrophic mutants in a rat model of oropharyngeal candidiasis. *FEMS Microbiology Letter*; **126(2)**: 177-180.

Colman-Lerner, A., Chin, TE. and Brent, R (2001). Yeast Cbk1 and Mob2 activate daughter-specific genetic programs to induce asymmetric cell fates. *Cell*; **107(6)**: 739-750.

Colombo, AL., Nucci, M., Park, BJ., Nouér, SA., Arthington-Skaggs, B., de Matta, DA., Warnock, D. and Morgan, J (2006). Epidemiology of candidemia in Brazil: a national sentinel surveillance of candidemia in eleven medical centres. *Jornal of Clinical Microbiology*; **44(8)**: 2816-2823.

Corbett, M., Xiong, Y., Boyne, JR., Wright, DJ., Munro, E. and Price, C (2006). IQGAP and mitotic exit network (MEN) proteins are required for cytokinesis and re-polarisation of the actin cytoskeleton in the budding yeast, *Saccharomyces cerevisiae. European Journal of Cell Biology*; **85(11)**: 1201-1215.

Corbucci, C., Cenci, E., Skrzypek, F., Gabrielli, E., Mosci, P., Ernst, JF., Bistoni, F. and Vecchiarelli, A (2007). Immune response to *Candida albicans* is preserved despite defect in O-mannosylation of secretory proteins. *Medical Mycology*; **45(8)**: 709-719.

Cormack, BP., Bertram, G., Egerton, M., Gow, NA., Falkow, S. and Brown, AJ (1997). Yeast-enhanced green fluorescent protein (yE*GFP*) a reporter of gene expression in *Candida albicans*. *Microbiology*; **143(2)**: 303-311.

Cornet, M., Richard, ML. and Gaillardin, C (2009). The homologue of the *Saccharomyces cerevisiae RIM9* gene is required for ambient pH signalling in *Candida albicans*. *Research in Microbiology*; **160(3)**: 219-223.

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

Costa, AL., Amato, A. and Vermiglio, G (1968). Action *in vitro* of organic polycations on a pathogenic strain of *Candida albicans*. *Boll Soc Ital Biol Sper*; **44(8)**: 731-735.

Costanzo, M., Nishikawa, JL., Tang, X., Millman, JS., Schub, O., Breitkreuz, K., Dewar, D., Rupes, I., Andrews, B. and Tyers, M (2004). CDK activity antagonises Whi5, an inhibitor of G_1/S

transcription in yeast. *Cell*; **117(7)**: 899-913.

Cotter, G., Doyle, S. and Kavanagh, K (2000). Development of an insect model for the *in vivo* pathogenicity testing of yeasts. *FEMS Immunology and Medical Microbiology*; **27(2)**: 163-169.

Cox, BS. and Parry, JM (1968). The isolation, genetics and survival characteristics of ultraviolet light-sensitive mutants in yeast. *Mutation Research*; **6(1)**: 37-55.

Crampin, H., Finley, K., Gerami-Nejad, M., Court, H., Gale, C., Berman, J. and Sudbery, P (2005). *Candida albicans* hyphae have a Spitzenkorper that is distinct from the polarisome found in yeast and pseudohyphae. *Journal of Cell Science*; **118(13)**: 2935-2947.

Cross, FR (1995). Starting the cell cycle: what's the point? *Current Opinions in Cell Biology*; **7(6)**: 790-797.

Csank, C., Makris, C., Meloche, S., Schroppel, K., Rollinghoff, M., Dignard, D., Thomas, DY. and Whiteway, M (1997). Derepressed hyphal growth and reduced virulence in a VH1 family-related protein phosphatase mutant of the human pathogen *Candida albicans*. *Molecular Biology of the Cell*; **8(12)**: 2539-2551.

Csank, C., Schroppel, K., Leberer, E., Harcus, D., Mohamed, O., Meloche, S.,Thomas, DY. and Whiteway, M (1998). Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. *Infection and Immunity*; **66(6)**: 2713-2721.

Cueille, N., Salimova, E., Esteban, V., Blanco, M., Moreno, S., Bueno, A. and Simanis, V (2001). Flp1, a fission yeast orthologue of the *S. cerevisiae CDC14* gene, is not required for cyclin degradation or rum1p stabilisation at the end of mitosis. *Journal of Cell Science*; **114**: 2649-2664.

D'Amours, D. and Amon, A (2004). At the interface between signalling and executing anaphase-Cdc14 and the FEAR network. *Genes and Development*; **18(21)**: 2581-2595.

D'Aquino, KE., Monje-Casas, F., Paulson, J., Reiser, V., Charles, GM., Lai, L., Shokat, KM. and Amon, A (2005). The protein kinase Kin4 inhibits exit from mitosis in response to spindle position defects. *Molecular Cell*; **19(2)**: 223-234.

Daga, RR., lahoz, A., Munoz, MJ., Moreno, S. and Jimenez, J (2005). Etd1p is a novel protein that links the SIN cascade with cytokinesis. *EMBO Journal*; **24(13)**: 2436-2446.

Davis, D., Edwards, JE., Mitchell, AP. and Ibrahim, AS (2000). *Candida albicans RIM101* pH response pathway is required for host-pathogen interactions. *Infection and Immunity*; **68(10)**: 5953-5959.

De Bernardis, F., Chiani, P., Ciccozzi, M., Pellegrini, G., Ceddia, T., D'Offizzi, G., Quinti, I., Sullivan, PA. and Cassone, A (1996). Elevated aspartic proteinase secretion and experimental pathogenicity of *Candida albicans* isolates from oral cavities of subjects infected with human

immunodeficiency virus. *Infection and Immunity*; **64(2)**: 466-471.

De Bernardis, F., Boccanera, M., Adriani, D., Spreghini, E., Santoni, G. and Cassone, A (1997). Protective role of antimannam and anti-aspartyl proteinase antibodies in an experimental model of *Candida albicans* vaginitis in rats. *Infections and Immunity*; **65(8)**: 3399-3405.

De Bernardis, F., Mondello, F., Scaravelli, G., Pachi, A., Girolamo, A., Agatensi, L, L. and Cassone, A (1999). High aspartyl proteinase production and vaginitis in human immunodeficiency virus-infected women. *Journal of Clinical Microbiology*; **37(5)**: 1376-1380.

de Bruin, RA., McDonald, WH., Kalashnikova, TI., Yates, J. and Wittenberg, C (2004). Cln3 activates G_1-sepcific transcription via phosphorylation of the SBF bound repressor Whi5. *Cell*; **117(7)**: 887-898.

Deckert, J., Torres, AM., Hwang, SM., Kastaniotis, AJ. and Zitomer, RS (1998). The anatomy of a hypoxic operator in *Saccharomyces cerevisiae. Genetics*; **150(4)**: 1429-1441.

Denison, SH., Orejas, M. and Arst, HN (1995). Signalling of ambient pH in *Aspergillus* involves a cysteine protease. *Journal of Biological Chemistry*; **270**: 28519-28522.

Denison, SH., Negrete-Urtasun, S., Mingot, JM., Tilburn, J., Mayer, WA., Goel, A., Espeso, EA., Penalva, MA. and Arst, HN (1998). Putative membrane components of signal transduction pathways for ambient pH regulation in *Aspergillus* and meiosis in *Saccharomyces* are homologous. *Molecular Microbiology*; **30(2)**: 259-264.

De Souza, CP. and Osmani, SA (2009) Double duty for nuclear proteins-the price of more open forms of mitosis. *Trends in Genetics*; **25(12)**: 5545-552.

Dever, TE., Glynias, MJ. and Merrick, WC (1987). GTP-binding domain: three consensus sequence elements with distinct spacing. *Proceedings of the National Academy of Science of the United States of America*; **84(7)**: 1814-1818.

Dial, JM., Petrotchenko, EV. and Borchers, CH (2007). Inhibition of APCCdh1 activity by Cdh1/Acm1/Bmh1 ternary complex formation. *Journal of Biological Chemistry*; **282(8)**: 5237-5248.

Dirick, L., Bohm, T. and Nasmyth, K (1995). Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae. EMBO Journal*; **14(19)**: 4803-4813.

Doedt, T., Krishnamurthy, S., Bockmuhl, DP., Tebarth, B., Stempel, C., Russell, CL., Brown, AJ. and Ernst, JF (2004). APSES proteins regulate morphogenesis and metabolism in *Candida albicans. Molecular Biology of the Cell*; **15(7)**: 3167-3180.

Donovan, JD., Toyn, JH., Johnson, AL. and Johnston, LH (1994). P40SDB25, a putative CDK inhibitor, has a role in the m/G_1 transition in *Saccharomyces cerevisiae*. *Genes and Development*; **8**: 1640-1653.

Doolin, MT., Johnson, AL., Johnston, LH. and Butler, G (2001). Overlapping and distinct roles of the duplicated yeast transcription factors Ace2p and Swi5p. *Molecular Microbiology*; **40(2)**: 422-432.

Douglas, LJ(2003). *Candida* biofilms and their role in infection. *Trends in Microbiology*; **11(1)**: 30-36.

El Barkani, A., Kurzai, O., Fozi, WA., Ramon, A., Porta, A., Frosch, M. and Muhlschlegel, FA (2000). Dominant active alleles of *RIM101* (*PRR2*) bypass the pH restriction on filamentation of *Candida albicans. Molecular and Cellular Biology*; **20(13)**: 4635- 4647.

Eldridge, AG., Loktev, AV., Hansen, DV., Verschuren, EW., Reimann, JD. and Jackson, PK (2006). The evi5 oncogene regulates cyclin accumulation by stabilising the anaphase-promoting complex inhibitor emi1. *Cell*; **124(2)**: 367-380.

Enquist-Newman, M., Sullivan, M. and Morgan DO (2008). Modulation of the mitotic regulatory network by APC-dependent destruction of the Cdh1 inhibitor Acm1. *Molecular Cell*; **30(4)**: 437-446.

Estaban, PF., Rios, I., Garcia, R., Duenas, E., Pla, J., Sanchez, M., de Aldana, CR. and Del Rey, F (2005). Characterisation of the *CaENG1* gene encoding an endo-1,3-beta-glucanase involved in cell separation in *Candida albicans*. *Current Microbiology*; **51(6)**: 385-392.

Fallon, K., Bausch, K., Noonan, J., Huquenel, E. and Tamburini, P (1997). Role of aspartic proteases in disseminated *Candida albicans* infection in mice. *Infection and Immunity*; **65(2)**: 551-556.

Fang, G., Yu, H. and Kirschner, MW (1998). Direct binding of CDC20 protein family members activates the anaphase promoting complex in mitosis and G_1. *Molecular Cell*. **2(2)**: 163-171.

Fankhauser, C. and Simanis, V (1993). The *Schizosaccharomyces pombe cdc14* gene is required for septum formation and can also inhibit nuclear division. *Molecular Biology of the Cell*; **4(5)**: 531-539.

Feldman ,RM., Correll, CC., Kaplan, KB. and Deshaies, RJ (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyses ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell*; **91(2)**: 221-230.

Feng, Q., Summers, E., Guo, B. and Fink, G (1999) Ras signalling is required for serum-induced hyphal differentiation in *Candida albicans. Journal of Bacteriology*; **181**: 6339-6346.

Fesquet, D., Fitzpatrick, PJ., Johnson, AL., Kramer, KM., Toyn, JH. and Johnston, LH (1999). A Bub2p-dependent spindle checkpoint pathway regulates the Dbf2p kinase in budding yeast. *EMBO Journal*; **18(9)**: 2424-2434.

Field, CM. and Kellogg, D (1999). Septins: cytoskeletal polymers or signalling GTPases? *Trends in Cell Biology*; **9(10)**: 387-394.

Fields, S. and Song, O (1989). A novel genetic system to detect protein-protein interactions. *Nature*; **340**:245-246.

Finley, KR., Bouchonville, KJ., Quick, A. and Berman, J (2008). Dynein-dependent nuclear dynamics affect morphogenesis in *Candida albicans* by means of the Bub2p spindle checkpoint. *Journal of Cell Science*; **121(4)**: 466-476.

Finkel, JS. and Mitchell, AP (2011). Genetic control of *Candida albicans* biofilm development. *Nature Review Microbiology*; **9(2)**: 109-118.

Fitch, I., Dahmann, C., Surana, U., Amon, A., Nasmyth, K., Goetsch, L., Byers, B. and Futcher, B (1992). Characterisation of four B-type cyclin genes of the budding yeast *Saccharomyces cerevisiae*. *Molecular Biology of the Cell*; **3(7)**: 805-818.

Fonzi, WA. And Irwin, MY (1993). Isogenic strain construction and gene mapping in *Candida albicans. Genetics*; **134(3)**:717-728.

Forsburg, SL. and Nurse, P (1991). Cell cycle regulation in the yeasts *Saccharomyces cerevisiae and Schizosaccharomyces pombe*. *Annual Review of Cell Biology*; **7**: 227-256.

Fraschini, R., Formenti, E., Lucchini, G. and Piatti, S (1999). Budding yeast Bub2 is localised at spindle pole bodies and activates the mitotic checkpoint via a different pathway from Mad2. *Journal of Cell Biology*; **145(5)**: 979-991.

Fraschini, R., Beretta, A., Sironi, L., Musacchio, A., Lucchini, G. and Piatti, S (2001). Bub3 interaction with Mad2, Mad3 and Cdc20 is mediated by WD40 repeats and does not require intact kinetochores. *EMBO Journal*; **20**: 6648-6659.

Fraschini, R., D'Ambrosio, C., Venturetti, M., Lucchini, G. and Piatta, S (2006). Disappearance of the buddin yeast Bub2-Bfa1 complex from the mother-bound spindle pole contributes to mitotic exit. *Journal of Cell Biology*; **172(3)**: 335-346.

Frenz, LM., Lee, SE., Fesquet, D. and Johnston, LH (2000). The budding yeast Dbf2 protein kinase localises to the centrosome and moves to the bud neck in late mitosis. *Journal of Cell Science*; **113**: 3399-3408.

Fu, Y., Ibrahim, AS., Fonzi, WA., Zhou, X., Ramos, CF. and Ghannoum, MA (1997) Cloning and characterisation of a gene (*LIP1*) which encodes a lipase from the pathogenic yeast *Candida albicans*. *Microbiology*: **143(2)**: 331-340.

Fu, Y., Ibrahim, AS., Sheppard, DC., Chen, YC., French, SW., Cutler, JE., Filler, SG. and Edwards, JE (2002). *Candida albicans* Als1p: an adhesin that is a downstream effector of the *EFG1* filamentation pathway. *Molecular Microbiology*; **44(1)**: 61-72.

Fuchs, BB., Eby, J., Nobile, CJ., El Khoury, JB., Mitchell, AP. and Mylonakis, E (2010). Role of filamentation in *Galleria mellonella* killing by *Candida albicans*. *Microbes and Infection*; **12(6)**: 488-496.

Fukazawa, Y. and Kagaya, K (1997). Molecular bases of adhesion of *Candida albicans*. *Journal of Medical and Veterinary Mycology*; **35(2)**: 87-99.

Furge, KA., Wong, K., Armstrong, J., Balasubramanian, M. and Albright, CF (1998). Byr4 and Cdc16 form a two-component GTPase-activating protein for the Spg1 GFPase that controls septation in fission yeast. *Current Biology*; **8**: 947-954.

Futcher, B (1996). Cyclins and the wiring of the yeast cell cycle. *Yeast*; **12**: 1635-1646.

Fux, CA., Costerton, JW., Stewart, PS. and Stoodley, P (2005). Survival strategies of infectious biofilms. *Trends in Microbiology*; **13(1)**: 34-40.

Gacser, A., Trofa, D., Schafer, W. and Nosanchuk, JD (2007). Targeted gene deletion in *Candida parapsilosis* demonstrates the role of secreted lipase in virulence. *Journal of Clinical Investigation*; **117(10)**: 3049-3058.

Galderisi, U., Jori, FP. and Giordano, A (2003). Cell cycle regulation and neural differentiation. *Oncogene*; **22**: 5208-5219.

Galan, A., Casanova, M., Murgui, A., MacCallum, DM., Odds, FC., Gow, NAR. and Martinez, JP (2004). The *Candida albicans* pH-regulated *KER1* gene encodes a lysine/glutamic-acid-rich plasma-membrane protein that is involved in cell aggregation. *Microbiology*; **150(8)**: 2641-2651.

Gale, CA., Finkel, D., Tao, N., Meinke, M., McClellan, M., Olsen, J., Kendrick, K. and Hostetter, M (1996). Cloning and expression of a gene encoding an integrin-like protein in *Candida albicans*. *Proceeding of the National Academy of Science of United States of America*; **93(1)**: 357-361.

Gale, CA., Bendel, CM., McClellan, M., Hauser, M., Becker, JM., Berman, J. and Hostetter, MK (1998). Linkage of adhesion, filamentous growth, and virulence in *Candida albicans* to a single gene, *INT1*. *Science*; **279**: 1355-1358.

Garcia-Cortes, JC. and McCollum, D (2009). Proper timing of cytokinesis is regulated by *Schizosaccharomyces pombe* Etd1. *Journal of Cell Biology*; **186(5)**: 739-753.

Garcia-Sanchez, S., Mavor, AL., Russell, CL., Argimon, S, Dennison., P, Enjabert, B. and Brown, AJ (2005). Global roles of Ssn6 in Tup1- and Nrg1-dependent gene regulation in the fungal pathogen *Candida albicans. Molecular Biology of the Cell*; **16(6)**: 2913-2925.

Gaur, NK. and Klotz, SA (1997). Expression, cloning and characterisation of a *Candida albicans* gene, *ALA1*, that confers adherence properties upon *Sachharomyces cerevisiae* for extracellular matrix proteins. *Infection and Immunity*: **65**: 5289-5294.

Gavin, IM. and Simpson, RT (1997). Interplay of yeast global transcription regulators Ssn6p-Tup1p and Swi-Snf and their effect on chromatin structure. *EMBO Journal*; **16**: 6263-6271.

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

Gerami-Nejad, M., Dulmage, K. and Berman, J (2009). Additional cassettes for epitope and fluorescent fusion proteins in *Candida albicans. Yeast*; **26(7)**: 399-406.

Geymonat, M., Spanos, A., Smith, SJ., Wheatley, E., Rittinger, K., Johnston, LH. and Sedgwick, SG (2002). Control of mitotic exit in budding yeast. *In vitro* regulation of Tem1 GTPase by Bub2 and Bfa1. *Journal of Biological Chemistry*; **277**: 28439-28445.

Geymonat, M., Spanos, A., Walker, PA., Johnston, LH. and Sedgwick, SG (2003). *In vitro* regulation of budding yeast Bfa1/Bub2 GAP activity by Cdc5. *Journal of Biological Chemistry*; **278**: 14591-14594.

Geymonat, M., Spanos, A, de Bettignies, G. and Sedgwick, SG (2009). Lte1 contributes to Bfa1 localisation rather than stimulating nucleotide exchange by Tem1. *Journal of Cell Biology*; **187(4)**: 497-511.

Ghannoum, MA (2000). Potential role of phospholipases in virulence and fungal pathogenesis. *Clinical Microbiology Reviews*; **13(1)**: 122-143.

Gietz, RD. and Schiestl, RH (1991). Application of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. *Yeast*; **7(3)**: 253-263.

Gillum, AM., Tsay, EY. and Kirsch, DR (1984). Isolation of the *Candida albicans* gene for orotidine-5'phosphate decarboxylase by complementation of *S. cerevisiae ura3* and *E. coli pyrF* mutation. *Molecular and General Genetics*; **198(1)**:179-182.

Giusani, AD., Vinces, M. and Kumamoto, CA (2002). Invasive filamentous growth of *Candida albicans* is promoted by Czf1p-dependent relief of Efg1p-mediated repression. *Genetics*; **160(4)**: 1749-1753.

Gladfelter, AS., Pringle, JR. and Lew, DJ (2001). The septin cortex at the yeast mother-bud neck. *Current Opinions Microbiology*; **4(6)**: 681-689.

Gola, S., Martin, R., Walther, A., Dunkler, A. and Wendland, J (2003). New modules for PCR-based gene targeting in *Candida albicans*: rapid and efficient gene targeting using 100bp of flanking homology region. *Yeast*; **20**: 1339-1347.

Gonzalez, MM., Diez-Orejas, R., Molero, G., Alvarez, AM., Pla, J., Nombela, C. and Sanchez-Perez, M (1997). Phenotypic characterisation of a *Candida albicans* strain deficient in its major exoglucanase. *Microbiology*. **143(9)**: 3023-3032.

Gonzalez-Novo, A., Labrador, L., Pablo-Hernando, ME., Correa-Bordes, J., Sanchez, M., Jimenez J. and Vazquez de Aldana, CR (2009). Dbf2 is essential for cytokinesis and correct mitotic spindle formation in *Candida albicans. Molecular Microbiology*; **72(6)**: 1364-1378.

Gorman, JA., Chan, W. and Gorman, JW (1991). Repeated use of *GAL1* for gene disruption in *Candida albicans. Genetics*; **129(1)**: 19-24.

Goshorn, AK. and Scherer, S (1989). Genetic analysis of prototrophic natural variants of *Candida albicans*. *Genetics*; **123(4)**: 667-673.

Grandin, N., de Almeida, A. and Charbonneau, M (1998). The Cdc14 phosphatase isfunctionally associates with Dbf2 protein kinase in *Saccharomyces cerevisiae. Molecular and General Genetics*; **258**: 104-116.

Gritz, L. and Daies, J (1983). Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gen and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene*; **25**: 179-188.

Gruneberg, U., Campbell, K., Simpson, C., Grindlay, J. and Schiebel, E (2000). Nud1p links astral microtubule organisation and the control of exit from mitosis. *EMBO Journal*; **19**: 6475-6488.

Gumbiner, BM (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell*; **84(3)**: 345-357.

Guertin, DA., Chang, L., Irshad., Gould KL. and McCollum, D (2000). The role of the sid1 kinase and cdc14p in regulating the onset of cytokinesis in fission yeast. *EMBO Journal*; **19(8)**: 1803-1815.

Hadwiger, JA., Wittenberg, C., Richardson, HE., de Barros Lopes, M. and Reed, SI (1989). A family of cyclin homologs that control the G_1phase in yeast. *Proceedings of the National Academy of Science in the United States of America*; **86**: 6255-6259.

Harper, JW., Burton, JL. and Solomon, MJ (2002). The anaphase-promoting complex: it's not just for mitosis any more. *Genes and Development*; **16**: 2179-2206.

Harris, SD (1997). The duplication cycle in *Aspergillus nidulans*. *Fungal Genetics and Biology*; **22(1)**: 1-12.

Hartwell, LH (1974). *Saccharomyces cerevisiae* cell cycle. *Bacteriological Reviews*; **38(2)**: 164-198.

Hawser, SP. and Douglas, LJ (1994). Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. *Infection and Immunity*; **62(3)**: 915-921.

Hazan, I., Sepulveda-Becerra, M. and Liu, H (2002). Hyphal elongation is regulated independently of cell cycle in *Candida albicans*. *Molecular Biology of the Cell*; **13(1)**: 134-145.

Heim, R., Prasher, DC. and Tsien, RY (1994). Wavelength mutations and posttranslation autoxidation of green fluorescent protein. *Proceedings of the National Academy of Science of the United States of America*; **91**: 12501-12504.

Heim, R., Cubitt, AB. and Tsien, RY (1995). Improved green fluorescence. *Nature*; **373**: 663-664.

Heim, R. and Tsien, RY (1996). Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Current Biology*; **6(2)**: 178-182.

Hellstein, J., Vawter-Hufart, H., Fotos, P., Schmid, J. and Soll, DR (1993). Genetic similarity and phenotypic diversity of commensal and pathogenic strains of *Candida albicans* isolated from the oral cavity. *Journal of Clinical Microbiology*; **31**: 3190-3199.

Hofken, T. and Schiebel, E (2002). A role of cell polarity proteins in mitotic exit. *EMBO Journal*; **21**: 4851-4862.

Holt, LJ., Krutchinsky, AN, Morgan, DO (2008). Positive feedback sharpen the anaphase switch. *Nature*; **454**: 353-357.

Hoover, CI., Jantapour, MJ., Newport, G., Agabian, N. and Fisher, SJ (1998). Cloning and regulated expression of the *Candida albicans* phospholipase B (*PLB1*) gene. *FEMS Microbiology Letters*; **167(2)**: 163-169.

Hou, MC., Salek, J. and McCollum, D (2000). Mob1p interacts with the Sid2p kinase and is required for cytokinesis in fission yeast. *Current Biology*; **10(10)**: 619-622.

Howard, A. and Pelc, SR (1951). Synthesis of nucleoprotein in bean root cells. *Nature*; **167**: 599-600.

Hoyer, LL., Scherer, S., Shatzman, AR. and Livi, GP (1995). *Candida albicans ALS1*: domains related to a *Saccharomyces cerevisiae* sexual agglutinin separated by a repeating motif. *Molecular Microbiology*: **15(1)**: 39-54.

Hoyer, LL., Payne, TL., Bell, M., Myers, AM. and Scherer, S (1998a). *Candida albicans ALS3* and insights into the nature of the *ALS* gene family. *Current Genetics*: **33(6)**: 451-459.

Hoyer, LL., Payne, TL. and Hecht, JE (1998b). Identification of *Candida albicans ALS2* and *ALS4* and localisation of als proteins to the fungal cell surface. *Journal of Biotechnology*: **180**: 5334-5343.

Hoyer, LL. and Hecht, JE (2000). The *ALS6* and *ALS7* genes of *Candida albicans*. *Yeast*: **16(9)**: 847-855.

Hoyer, LL. and Hecht, JE (2001). The *ALS5* gene of *Candida albicans* and analysis of the Als5p N-terminal domain. *Yeast*: **18(1)**: 49-60.

Hoyer, LL (2001). The ALS gene family of *Candida albicans*. *Trends in Microbiology*: **9(4)**: 176-180.

Hoyt, MA., Totis, L. and Roberts, BT (1991). *S. cerevisiae* genes required for cell cycle arrest in

response to loss of microtubule function. *Cell*; **66(3)**: 507-517.

Hsu, JY., Reimann, JD., Sorensen, CS., Lukas, J. and Jackson, PK (2002). E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC(Cdh1). *Nature Cell Biology*; **4(5)**: 358-366.

Hu, F., Wang, Y., Liu, D., Qin, J. and Elledge, SJ (2001). Regulation of the Bub2/Bfa1 GAP complex by Cdc5 and cell cycle checkpoints. *Cell*; **107(5)**: 655-665.

Hua, J., Meyer, JD. and Lodge, JK (2000). Development of positive selectable markers for the fungal pathogen *Cryptococcus neoformans*. *Clinical and Diagnostic Laboratory Immunology*; **7(1)**: 125-128.

Huang, JN., Park, I., Ellingson, E., Littlepage, LE. and Pellman, D (2001). Activity of the APC(Cdh) form of the anaphase-promoting complex persists until S phase and prevents the premature expression of Cdc20. *Journal of Cell Biology*; **154(1)**: 85-94.

Huang, G., Wang, H., Chou, S., Nie, X., Chen, J. and Liu H (2006). Bistable expression of *WOR1*, a master regulator of white-opaque switching in *Candida albicans*. *Proceedings of the National Academy of Science of the United States of America*; **103**: 12813-12818.

Huang, H., Harcus, D. and Whiteway, M (2008). Transcript profiling of a MAP kinase pathway in *C. albicans. Microbiological Research*; **163(4)**: 380-393.

Huang, HC., Mitchison, TJ. and Shi, J (2010). Stochastic competition between mechanistically independent slippage and death pathway determines cell fate during mitotic arrest. *PLoS One*; **5**: e15724.

Hube, B., Turver, CJ., Odds, FC., Eiffert, H., Boulnois, GJ., Kochel, H. and Ruchel, R (1991). Sequence of the *Candida albicans* gene encoding the secretory aspartate proteinase. *Journal of Medical and Veterinary Mycology*; **29(2)**: 129-132.

Hube, B., Monod, M., Schofield, DA., Brown, AJ. and Gow, NAR. (1994). Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. *Molecular Microbiology*; **14(1)**: 87-99.

Hube, B., Sanglard, D., Odds, FC., Hess, D., Monod, M., Schafer, W., Brown, AJ. and Gow, NAR (1997). Disruption of each of the secreted aspartyl proteinase genes *SAP1*, *SAP2*, and *SAP3* of *Candida albicans* attenuates virulence. *Infection Immunity*; **65(9)**: 3529-3538.

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

Hube, B., Hess, D., Baker, CA., Schaller, M., Schafer, W. and Dolan, JW (2001). The role and relevance of phospholipase D1 during growth and dimorphism of *Candida albicans*. *Microbiology*; **147(4)**: 879-889.

Hube, B. and Naglik, JR (2002). Extracellular Hydrolases. *In* RA. Calderone (ed), *Candida and Candidiasis*, ASM Press. Washington, USA.

Hull, CM., Raisner, RM. and Johnson, AD (2000). Evidence for mating of the "asexual" yeast *Candida albicans* in a mammalian host. *Science*; **289**: 307-310.

Hunter, T. and Plowman, GD (1997). The protein kinases of budding yeast: six score and more. *Trends Biochemical Sciences*; **22(1)**: 18-22.

Hwa Lim, H., Yeong, FM. and Surana, U (2003). Inactivation of mitotic kinase triggers translocation of MEN components to mother-daughter neck in yeast. *Molecular and Cell Biology*; **14(11)**: 4734-4743.

Hwang, CS., Oh, J., Huh, WK., Yim, HS. and Kang, SO (2003). Ssn6, an important factor of morphological conversion and virulence in *Candida albicans*. *Molecular Microbiology*; **47(4)**: 1029-1043.

Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M. and Sakaki, Y (2001). A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proceedings of the National Academy of Science of the United States of America*; **98**: 4569-4574.

Jablonski, SA., Chan, GK., Cooke, CA., Earnshaw, WC. and Yen, TJ (1998). The hBUB1 and hBUBR1 kinases sequentially assemble onto kinetochores during prophase with hBUBR1 concentrating at the kinetochore plates in mitosis. *Chromosoma*; **107**: 386-396.

James, P., Halladay, J. and Craig, EA (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics*; **144(4)**: 1425-1436.

James, TY., Kauff, F., Schoch, CL., *et al* (2006). Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature*; **443**: 818-822.

Janbon, G., Sherman, F. Rustchenko, E (1998). Monosomy of a specific chromosome determines L-sorbose utilisation: a novel regulatory mechanism in *Candida albicans. Proceeding of the National Academy of Sciences of the United States of America*; **95(9)**: 5150-5155.

Jaspersen, SL., Charles, JF., Tinker-Kulberg, RL. and Morgan, DO (1998). A late mitotic regulatory network controlling cyclin destruction in *Saccharomyces cerevisiae. Molecular Biology of the Cell*; **9(10)**: 2803-2817.

Jaspersen, SL., Charles, JF. and Morgan, DO (1999). Inhibitory phosphorylation of the APC regulator Hct1 is controlled by the kinase Cdc28 and the phosphatase Cdc14. *Current Biology*; **9(5)**: 227-236.

Jaspersen, SL. and Morgan, DO (2000). Cdc14 activates Cdc15 to promote mitotic exit in budding yeast. *Current Biology*; **10(10)**: 615-618.

Jayatilake, JA. and Samaranayake, LP (2010). Experimental superficial candidiasis on tissue models. *Mycoses*; **53(4)**: 285-295.

Jeganathan, KB., Malureanu, L. and van Deursen, JM (2005). The Rae1-Nup98 complex prevents aneuploidy by inhibiting securing degradation. *Nature*; **438**: 1036-1039.

Jensen, S., Geymonat, M., Johnson, AL., Segal, M. and Johnston, LH (2002). Spatial regulation of the guanine nucleotide exchange factor Lte1 in *Saccharomyces cerevisiae*. *Journal of Cell Science*; **115**: 4977-4991.

Jimenez, A. and Davies, J (1980). Expression of a transposable antibiotic resistance element in *Saccharomyces*. *Nature*; **287**: 869-871.

Jimenez, J. and Oballe, J (1994). Ethanol-hypersensitive and ethanol-dependant cdc-mutants in *Schizosaccharomyces pombe. Molecular and General Genetics*; **245(1)**: 86-95.

Jimenez, J., Cid, VJ., Cenamor, R., Yuste, M., Molero, G., Nombela, C. and Sanchez, M (1998). Morphogenesis beyond cytokinetic arrest in *Saccharomyces cerevisiae. Journal of Cell Biology*; **143(6)**: 1617-1634.

Jimenez, J., Castelao, BA., Gonzalez-Novo, A. and Sanchez-Perez, M (2005). The role of MEN (mitosis exit network) proteins in the cytokinesis of *Saccharomyces cerevisiae. International Microbiology*; **8(1)**: 33-42.

Johnston, LH., Eberly, SL., Chapman, JW., Araki, H. and Sugino, A (1990). The product of the *Saccharomyces cerevisiae* cell cycle gene *DBF2* has homology with protein kinases and is periodically expressed in the cell cycle. *Molecular and Cell Biology*; **10(4)**: 1358-1366.

Jones, S., White, G. and Hunter, PR (1994). Increased phenotypic switching in strains of *Candida albicans* associated with invasive infections. *Journal of Clinical Microbiology*; **32(11)**: 2869-2870.

Joshi, PB., Webb, JR., Davies, JE. and McMaster, WR (1995). The gene encoding streptothricin acetyltransferase (sat) as a selectable marker for *Leishmania* expression vectors. *Gene*; **156(1)**: 145-149.

Jouannic, S., Champion, A., Segui-Simarro, JM., Salimova, E., Picaud, A., Tregear, J., Testillano, P., Risueno, MC., Simanis, V., Kreis, M. and Henry, Y (2001). The Protein kinases AtMAP3Kε1 and BnMAP3ε1 are functional homologues of *S. pombe* cdc7p and may be involved in cell division. *Plant Journal*; **26(6)**: 637-649.

Kabcenell, AK., Goud, B., Northup, JK. and Novick, PJ (1990). Binding and hydrolysis of guanine nucleotide by Sec4p, a yeast protein involved in the regulation of vesicular traffic. *Journal of Biological Chemistry*; **265**: 9366-9372.

Kahana, JA., Schnapp, BJ. and Silver, PA (1995). Kinetics of spindle pole body separation in budding yeast. *Proceedings of the National Academy of Science of the United States of*

America; **92**: 9707-9711.

Kamai, Y., Kubota, M., Kamai, Y., Hosokawa, T., Fukuoka, T. and Filler, SG (2002). Contribution of *Candida albicans ALS1* to the pathogensis of experimental oropharyngeal candidiasis. *Infection and Immunity*: **70(9)**: 5256-5258.

Kao, AS., Brandt, ME., Pruitt, WR., Conn, LA., Perkins, BA., Stephens, DS., Baughman, WS., Reingold, AL., Rothrock, GA., Pfaller, MA., Pinner, RW. and Hajjeh, RA (1999). The epidemiology of candidemia in two United States cities: results of a population-based active surveillance. *Clinical Infectious Disease*; **29(5)**: 1164-1170.

Kapteyn, JC., Hoyer, LL., Hecht, JE., Müller, WH., Andel, A., Verkleij, AJ., Makarow, M., Van Den Ende, H. and Klis, FM (2000). The cell wall architecture of *Candida albicans* wild-type cells and cell wall-defective mutants. *Molecular Microbiology*; **35(3)**: 601-611.

Kapuscinski, J (1995). DAPI: a DNA-specific fluorescent probe. *Biotechnic and Histochemistry*; **70(5)**: 220-233.

Keck, JM., Summers, MK., Tedesco, D., Ekholm-Reed, S., Chuang., LC., Jackson, PK. and Reed, SI (2007). Cyclin E overexpression impairs progression through mitosis by inhibiting APC(Cdh1). *Journal of Cell Biology*; **178(3)**: 371-385.

Keleher, CA., Redd, MJ., Schultz, J., Carlson, M. and Johnson, AD (1992). Ssn6-Tup1 is a general repressor of transcription in yeast. *Cell*; **68(4)**: 709-719.

Kelly, R., Card, D., Register, E., Mazur, P., Kelly, T., Tanaka, KI., Onishi, J., Williamson, JM., Fan, H., Satoh, T. and Kurtz, M (2000). Geranylgeranyltransferase I of *Candida albicans*: null mutants or enzyme inhibitors produce unexpected phenotype. *Journal of Bacteriology*; **182(3)**: 704-713.

Keng, T., Clark, MW., Storms, RK., Fortin, N., Zhong, W., Ouellette, BF., Barton, AB., Kaback, DB. and Bussey, H (1994). *LTE1* of *Saccharomyces cerevisiae* is a 1435 codon open reading frame that has sequence similarities to guanine nucleotide releasing factors. *Yeast*; **10(7)**: 953-958.

Keppler-Ross, S., Noffz, C. and Dena, N (2008). A new purple fluorescent color marker for genetic studies in *Saccharomyces cerevisiae* and *Candida albicans*. *Genetics*; **179(1)**: 705-710.

Khalaf, RA. and Zitomer, RS (2001). The DNA binding protein Rfg1 is a repressor of filamentation in *Candida albicans*. *Genetics*; **157(4)**: 1503-1512.

Kim, JM., Lu, L., Shao, R., Chin, J. and Liu, B (2006). Isolation of mutation that bypass the requirement of the septation initiation network for septum formation and conidiation in *Aspergillus nidulans. Genetics*; **173(2)**: 685-696.

Kim, J., Jang, SS. and Song, K (2008). Different levels of Bfa1/Bub2 GAP activity are required to prevent mitotic exit of budding yeast depending on the type of perturbation. *Molecular Biology of the Cell*; **19(10)**: 4328-4340.

Kim, JM., Zeng, CJ., Nayak, T., Shao, R., Huang, AC., Oakley, BR. and Liu B (2009). Timely septation requires SNAD-dependent spindle pole body localisation of the septation initiation network components in the filamentous fungus *Aspergillus nidulans. Molecular Biology of the Cell*; **20**: 2874-2884.

Kim, HR., Zeng, T. and Liu, B (2011). The small GTPase SPGA plays a critical role in septation in the filamentous fungus *Aspergillus nidulans*. 26th *Fungal Genetics Conference,* Asilomar, California, 15-20 March 2011: **Poster # 315**.

Kirsch, DR. and Whitney, PR (1991). Pathogenicity of *Candida albicans* auxotrophic mutants in experimental infections. *Infection and Immunity*; **59(9)**: 3297-3300.

Kitada, K., Johnson, AL., Johnston, LH. and Sugino, A (1993). A multicopy suppressor gene of the *Saccharomyces cerevisiae* G_1 cell cycle mutant gene *dbf4* encodes a protein kinase and is identified as *CDC5. Molecular Cell Biology*; **13(7)**: 4445-4457.

Klar, AJ., Srikantha, T. and Soll DR (2001). A histone deacetylation inhibitor and mutant promote colony-type switching of the human pathogen *Candida albicans. Genetics*; **158(2)**: 919-924.

Klengel, T., Liang, WJ., Chaloupka, J., Ruoff, C., Schroppel, K., Naglik, JR., Eckert, SE., Mogensen, EG., Haynes, K., Tuite, MF., Levin, LR., Buck, J. and Muhlschlegel, FA (2005). Fungal adenylyl cyclase integrates CO_2 sensing with cAMP signalling and virulence. *Current Biology*; **15**: 2021-2026.

Knapp, D., Bhoite, L., Stillman, DJ. and Nasmyth, K (1996). The transcription factor Swi5 regulates expression of the cyclin kinase inhibitor p40SIC1. *Molecular and Cellular Biology*; **16(10)**: 5701-5707.

Komarnitsky, SI., Chiang, YC., Luca, FC., Chen, J., Toyn, JH., Winey, M., Johnston, LH. and Denis, CL (1998). DBF2 protein kinase binds to and acts through the cell cycle-regulated MOB1 protein. *Molecular and Cell Biology*; **18(4)**: 2100-2107.

Kohler, JR. and Fink, GR (1996). *Candida albicans* strains heterozygous and homozygous for mutation in mitogen-activated protein kinase signalling components have defects in hyphal development. *Proceedings of the National Academy of Science in the United States of America*; **93**: 13223-13228.

Kohler, GA, White, TC. and Agabian, N (1997). Overexpression of a cloned IMP dehydrogenase gene of *Candida albicans* confers resistance to the specific inhibitor mycophenolic acid. *Journal of Bacteriology*; **179(7)**: 2331-2338.

Kojic, EM. and Darouiche, RO (2004). *Candida* infections of medical devices. *Clinical Microbiology Reviews*; **17(2)**: 255-267.

Kolotila, MP. and Diamond, RD (1990). Effects of neutrophils and *in vitro* oxidants on survival and phenotypic switching of *Candida albicans* WO-1. *Infection and Immunity*; **58(5)**: 1174-

1179.

Kraft, C., Vodermaier, HC., Maurer-Stroh, S., Eisenhaber, F. and Peters, Jm (2005). The WD40 propeller domain of Cdh1 functions as a destruction box receptor for APC/C substrates. *Molecular Cell*; **18(5)**: 543-553.

Kramer, ER., Scheuringer, N., Podtelejnikov, AV., Mann, M. and Peters, JM (2000). Mitotic regulation of the APC activator proteins CDC20 and CDH1. *Molecular Biology of the Cell*; **11(5)**: 1555-1569.

Krapp, A., Schmidt, S., Cano, E. and Simanis, V (2001). *S. pombe* cdc11p, together with sid4p, provides an anchor for septation initiation network proteins on the spindle pole bodies. *Current Biology*; **11**: 1559-1568.

Krapp, A., Gulli, MP. and Simanis V (2004). SIN and the art of splitting the fission yeast cell. *Current Biology*; **14(17)**: 722-730.

Krapp, A. and Simanis, V (2008). An overview of the fission yeast septation initiation network (SIN) . *Biochemical Society Transactions*; **36(3)**: 411-415.

Krugel, H., Fielder, G., Haupt, I., Sarfert, E. and Simon, H (1988). Analysis of the nourseothricin-resistance gene (nat) of *Streptomyces noursei*. *Gene*; **62(2)**: 209-217.

Kumamoto, CA (2002). *Candida* biofilms. *Current Opinion in Microbiology*; **5(6)**: 608-611.

Kvaal, CA., Srikantha, T. and Soll DR (1997). Misexpression of the white phase-specific gene *WH11* in the opaque phase of *Candida albicans* affects switching and virulence. *Infection and Immunity*; **65**: 4468-4475.

Kvaal, CA., Lachke, SA., Srikantha, T., Daniels, K., McCoy, J. and Soll, DR (1999). Misexpression of the opaque-phase-specific gene *PEP1* (*SAP1*) in the white phase of *Candida albicans* confers increase virulence in a mouse model of cutaneous infection. *Infection and Immunity*; **67**: 6652-6662.

Lane, T. and Garcia, JR (1991). Phospholipase production in morphological variants of *Candida albicans*. *Mycoses*; **34**: 217-220.

Laurenson, P. and Rine, J (1992). Silencers, silencing, and heritable transcriptional states. *Microbiological Reviews*; **56(4)**: 543-560.

Lavoie, H., Sellam, A., Askew, C., Nantel, A. and Whiteway, M (2008). A toolbox for epitope-tagging and genome-wide location analysis in *Candida albicans*. *BMC Genomics*; **9**: 578.

Lay, J., Henry, LK., Clifford, J., Koltin, Y., Bulawa, CE. and Becker, JM (1998). Altered expression of selectable marker *URA3* in gene-disrupted *Candida albicans* strain complicates interpretation of virulence studies. *Infection and Immunity*; **66(11)**: 5301-5306.

Leberer, E., Harcus, D., Broadbent, ID., Clark, KL., Dignard, D., Ziegelbauer, K., Schmidt, A., Gow, NAR., Brown, AJ. and Thomas, DY (1996). Signal transduction through homologs of the Ste20p and Ste7p protein kinases can trigger hyphal formation in the pathogenic fungus *Candida albicans*. *Proceeding of the National Academy of Science in the United States of America*; **93**: 13217-13222.

Leberer, E., Ziegelbauer, K., Schmidt, A., Harcus, D., Dignard, D., Ash, J., Johnson, L. and Thomas, DY (1997). Virulence and hyphal formation of *Candida albicans* require the Ste20p-like protein kinase CaCla4p. *Current Biology*; **7(8)**: 539-546.

Leberer, E., Harcus, D., Dignard, D., Johnson, L., Ushinsky, Thomas, DY. and Schroppel, K (2001). Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus *Candida albicans*. *Molecular Microbiology*; **42(3)**: 673-687.

Lee, SE., Frenz, LM., Wells, NJ., Johnson, AL. and Johnston, LH (2001). Order of function of the budding yeast mitotic exit-network proteins Tem1, Cdc15, Mob1, Dbf2, and Cdc5. *Current Biology*; **11(10)**:784-788.

Leidich, SD., Ibrahim, AS., Fu, Y., Koul, A., Jessup, C., Vitullo, J., Fonzi, WA., Mirbod, F., Nakashima, S., Nozawa, Y. and Ghannoum, MA (1998). Cloning and disruption of ca*PLB1*, a phospholipase B gene involved in the pathogenicity of *Candida albicans*. *Journal of Biological Chemistry*; **273**: 26078-26086.

Leng, P., Sudbery, PE. and Brown, AJ (2000) Rad6p represses yeast-hypha morphogenesis in the human fungal pathogen *Candida albicans. Molecular Microbiology*; **35(5)**: 1264-1275.

Lermann, U. and Morschhauser, J (2008). Secreted aspartic proteases are not required for invasion of reconstituted human epithelia by *Candida albicans*. *Microbiology*; **154(11)**: 3281-3285.

Leuker, CE., Sonneborn, A., Delbruck, S. and Ernst, JF (1997). Sequence and promoter regulation of the *PCK1* gene encoding phosphoenolpyruvate carboxykinase of the fungal pathogen *Candida albicans*. *Gene*; **192(2)**: 235-240.

Lew, DJ (2003). The morphogenesis checkpoint: how yeast cells watch their figures. *Current Opinions in Cell Biology*; **15(6)**: 648-653.

Lew, DJ. and Burke, DJ (2003). The spindle assembly and spindle position checkpoints. *Annual Reviews of Genetics*; **37**: 251-282.

Li, W. and Mitchell, AP. (1997). Proteolytic activation of Rim1p, a positive regulator of yeast sporulation and invasive growth. *Genetics*; **145(1)**: 63-73.

Li, C., Furge, KA., Cheng, QC. And Albright, CF (2000). Byr4 localises to spindle-pole bodies in a cell cycle-regulated manner to control Cdc7 localisation and septation in fission yeast. *Journal of Biological Chemistry*; **275**: 14381-14387.

Li, F., Svarovsky, MJ., Karlsson, AJ., Wagner, JP., Marchillo, K., Oshel, P., Andes, D. and Palacek, SP (2007). Eap1p, an adhesin that mediates *Candida albicans* biofilm formation *in vitro* and *in vivo*. *Eukaryotic Cell*; **6(6)**: 931-939.

Liang, F., Jin, F., Liu, H. and Wang, Y (2009). The molecular function of the yeast polo-like kinase Cdc5 in Cdc14 release during early anaphase. *Molecular Biology of the Cell*; **20**:3671-3679.

Lim, HH., Goh, PY. and Surana, U (1998). Cdc20 is essential for the cyclosome-mediated proteolysis of both Pds1 and Clb2 during M phase in budding yeast. *Current Biology*; **8(4)**: 231-234.

Linder,S., Schliwa, M. and Kube-Granderath, E (1996). Direct PCR screening of *Pichia pastoris* clones. *Biotechniques*; **20(6)**: 980-982

Lindon, C (2008). Control of mitotic exit and cytokinesis by the APC/C. *Biochemical Society Transactions*; **36(3)**: 405-410.

Lippincott, J. and LI, R (1998). Dual function of Cyk2, a cdc15/PSTPIP family protein, in regulating actomyosin ring dynamics and septin distribution. *Journal of Cell Biology*; **143(7)**: 1947-1960.

Lippincott, J., Shannon, KB., Shou, W., Deshaies, RJ. and Li, R (2001). The Tem1 small GTPase controls actomyosin and septin dynamics during cytokinesis. *Journal of Cell Science*; **114(7)**: 1379-1386.

Liu, H., Kohler, J. and Fink, GR (1994). Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. *Science*; **266**: 1723-1726.

Lo, HJ., Kohler, JR, DiDomenico, B., Loebenberg, D., Cacciapuoti, A. and Fink, GR (1997). Nonfilamentous *C. albicans* mutants are avirulent. *Cell*; **90(5)**: 939-949.

Loeb, JD., Sepulveda-Becerra, M., Hazan, I. and Liu, H (1999). A G_1 cyclin is necessary for maintance of filamentous growth in *Candida albicans*. *Molecular and Cellular Biology*; **19(6)**: 4019-4027.

Longtine, MS., Fares, H. and Pringle, JR (1998). Role of the yeast Gin4p protein kinase in septin assembly and the relationship between septin assembly and septin function. *Journal of Cell Biology*; **143(3)**: 719-736.

Longtine, MS., Theesfeld, CL., McMillan, JN., Weaver, E., Pringle, JR. and Lew, DJ (2000). Septin-dependent assembly of a cell cycle-regulatory module in *Saccharomyces cerevisiae*. *Molecular and Cell Biology*; **20(11)**: 4049-4061.

Lu, CF., Karjan, J. and Lipke, PN (1994). A pathway for cell wall anchorage of *Saccharomyces cerevisiae* α-agglutinin. *Molecular and Cellular Biology*: **14(7)**: 4825-4833.

Luca, FC. and Winey, M (1998). *MOB1*, an essential yeast gene is required for completion of

mitosis and maintance of ploidy. *Molecular Biology of the Cell*; **9(1)**: 29-46.

Luca, FC., Mody, M., Kurischko, C., Roof, DM., Giddings, TH. and Winey, M (2001). *Saccharomyces cerevisiae* Mob1p is required for cytokinesis and mitotic exit. *Molecular and Cell Biology*; **21(20)**: 6972-6983.

Lukas, C., Sorensen, CS., Kramer, E., Santoni-Rugiu, E., Lindeneg, C., Peters, JM., Bartek, J. and Lukas, J (1999). Accumulation of cyclin B1 requiers E2F and cyclin-A-dependent rearrangement of the anaphase-promoting complex. *Nature*; **401**: 815-818.

Lussier, M., Sdicu, AM., Shahinian, S. and Bussey, H (1998). The *Candida albicans KRE9* gene is required for cell wall β-1, 6-glucan synthesis and is essential for growth on glucose. *Proceedings of the National Academy of Science of United States of America*; **95**: 9825-9830.

Maccheroni, W., May, GS., Martinez-Rossi, NM. and Rossi, A (1997). The sequence of palF, an environmental pH response gene in *Aspergillus nidulans*. *Gene*; **194(2)**: 163-167.

Maekawa, H., Priest, C., Lechner, J., Pereira, G. and Schiebel, E (2007). The yeast centrosome translates the position information of the anaphase spindle into a cell cycle signal. *Journal of Cell Biology*; **179(3)**: 423-436.

Magee, BB. and Magee, PT (2000). Induction of mating in *Candida albicans* by construction of MTLa and MTLα strains. *Science*; **289**: 310-313.

Mago, N. and Khuller, GK (1990). Subcellular localisation of enzymes of phospholipid metabolism in *Candida albicans*. *Journal of Medical and Veterinary Mycology*; **28(5)**: 355-362.

Mah, AS., Jang, J. and Deshaies, RJ (2001). Protein kinase Cdc15 activates the Dbf2-Mob1 kinase complex. *Proceeding of the National Academy of Science of the United States of America*; **98**: 7325-7330.

Mallet, L., Renault, G. and Jacquet, M (2000). Functional cloning of the adenylate cyclase gene of *Candida albicans* in *Saccharomyces cerevisiae* within a genomic fragment containing five other genes, including homologues of *CHS6* and *SAP185. Yeast*; **16(10)**: 959-966.

Manzoni, R., Montani, F., Visintin, C., Caudron, F., Ciliberto, A. and Visintin, R (2010). Oscillation in Cdc14 release and sequestration reveal a circuit underlying mitotic exit. *Journal of Cell Biology*; **190(2)**: 209-222.

Martinez, JS., Jeong, DE., Choi, E., Billings, BM. and Hall, MC (2006). Acm1 is a negative regulator of the CDH1-dependant anaphase promoting complex/cyclosome in budding yeast. *Molecular and Cell Biology*; **26**: 9162-9176.

McCollum, D. and Gould, KL (2001). Timing is everything: regulation of mitotic exit and cytokinesis by the MEN and SIN. *Trends in Cell Biology*; **11(2)**: 89-95.

McCreath, KJ., Specht, CA. and Robbins, PW (1995). Molecular cloning and characterisation of

chitinase genes from *Candida albicans*. *Proceedings of the National Academy of Science in the United States of America*; **92(7)**: 2544-2548.

McMillan, JN., Longtine, MS., Sia, RA., Theesfeld, CL., Bardes, ES., Pringle, JR. and Lew, DJ (1999). The morphogenesis checkpoint in *Saccharomyces cerevisiae*: cell cycle control of Swe1p degradation by Hsl1p and Hsl7p. *Molecular and Cell Biology*; **19(10)**: 6929-6939.

Mendenhall, MD. and Hodge, AE (1998). Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews*; **62(4)**: 1191-1243.

Menssen, R., Neutzner, A. and Seufert, W (2001). Asymmetric spindle pole localisation of yeast Cdc15 kinase links mitotic exit and cytokinesis. *Current Biology*; **11(5)**: 345-350.

Merson-Davies, LA. and Odds, FC (1989). A morphology index for characterisation of cell shape in *Candida albicans*. *Journal of General Microbiology*; **135(11)**: 3143-3152.

Miller, DJ. Mejicano, GC (2001). Vertebral osteomyelitis due to *Candida* species: case report and literature review. *Clinical Infectious Disease*; **33(4)**: 523-530.

Miller, JJ., Summers, MK., Hansen, DV., Nachury, MV., Lehman, NL., Loktev, A. and Jackson, PK (2006). Emi1 stably binds and inhibits the anaphase-promoting complex/cyclosome as a pseudosubstrate inhibitor. *Genes and Development*; **20(17)**: 2410-2420.

Miller, MG. and Johnson, AD (2002). White-opaque switching in *Candida albicans* is controlled by mating-type locus homeodomain proteins and allows efficient mating. *Cell*; **110(3)**: 293-302.

Mio, T., Adachi-Shimizu, M., Tachibana, Y., Tabuchi, H., Inoue, SB., Yabe, T., Yamada-Okabe, T., Arisawa, M., Watanabe, T. and Yamada-Okabe, H (1997). Cloning of the *Candida albicans* homologs of *Saccharomyces cerevisiae GSC1/FKS1* and its involvement in β-1,3-glucan synthesis. *Journal of Bacteriology*; **179**: 4096-4105.

Miwa, T., Takagi, Y., Shinozaki, M., Yun, CW., Schell, WA., Perfect, JR., Kumagai, H. and Tamaki, H (2004). Gpr1, a putative G-protein coupled receptor, regulates morphogenesis and hypha formation in the pathogenic fungus *Candida albicans. Eukaryotic Cell*; **3(4)**: 919-931.

Miyakawa, Y., Hara, T. and Iimura, Y (2009). Establishment of a screening system for essential genes from the pathogenic yeast *Candida glabrata*: identification of a putative *TEM1* homologue. *Letters in Applied Microbiology*; **49(3)**: 317-323.

Miyasaki, SH., White, TC. and Agabian, N (1994). A fourth secreted aspartyl proteinase gene (*SAP4*) and a CARE2 repetitive element are located upstream of the *SAP1* gene in *Candida albicans*. *Journal of Bacteriology*; **176(6)**: 1702-1710.

Mohl, DA., Huddleston, MJ., Collingwood, TS., Annan, RS. and Deshaies, RJ (2009). Dbf2-Mob1 drives relocalisation of protein phosphatase Cdc14 to the cytoplasm during exit from mitosis. *Journal of Cell Biology*; **184(4)**: 527-539.

Molk, JN., Schuyler, SC., Liu, JY., Evans, JG., Salmon, ED, Pellman, D. and Bloom, K (2004). The differential roles of budding yeast Tem1p, Cdc15p and Bub2p protein dynamics in mitotic exit. *Molecular Biology of the Cell*; **15(4)**: 1519-1532.

Moll, T., Tebb, G., Surana, U., Robitsch, H. and Nasmyth, K (1991). The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the *S. cerevisiae* transcription factor SWI5. *Cell*; **66(4)**: 743-758.

Monje-Casas, F. and Amon A (2009). Cell polarity determinants establish asymmetry in MEN signalling. *Developmental Cell*; **16(1)**: 132-145.

Monod, M., Togni, G., Hube, B. and Sanglard, D (1994). Multiplicity of genes encoding secreted aspartic proteinases in *Candida* species. *Molecular Microbiology*; **13(2)**: 357-368.

Monod, M., Hube, B., Hess, D. and Sanglard, D. (1998). Differential regulation of *SAP8* and *SAP9*, which encode two new members of the secreted aspartic proteinase family in *Candida albicans*. *Microbiology*; **144(10)**: 2731-2737.

Moqtaderi, Z. and Struhl, K (2008). Expanding the repertoire of plasmids for PCR-mediated epitope tagging in yeast. *Yeast*; **25(4)**: 287-292.

Mora-Montes, HM., Bates, S., Netea, MG., Castillo, L., Brand, A., Buurman, ET., Diaz-Jimenez, DF., Kullberg, JB., Brown, AJ., Odds, FC. and Gow, NAR (2010). A multifunctional mannosyltransferase family in *Candida albicans* determines cell wall mannam structure and host-fungus interactions. *Journal of Biological Chemistry*; **285**: 12087-12095.

Moran, GP., Sullivan, DJ. and Coleman, DC (2002). Emergence of Non-*Candida albicans Candida* Species as Pathogens. *In* RA. Calderone (ed), *Candida and Candidiasis*, ASM Press. Washington, USA.

Morgan, DO. The Cell Cycle- Principles of Control. Oxford publishing. 2007.

Morschhauser, J., Michel, S. and Hacker, J (1998). Expression of a chromosomally integrated, single-copy *GFP* gene in *Candida albicans*, and its use as a reporter of gene regulation. *Molecular and General Genetics*; **257**: 412-420.

Morton, DB., Dunphy, GB. And Chadwick, JS (1987). Reactions of hemocytes of immune and non-immune *Galleria mellonella* larvae to *Proteus mirabilis*. *Developmental and Comparative Immunology*; **11(1)**: 47-55.

Munro, CA., Bates, S., Buurman, ET., Hughes, HB., Maccallum, DM., Bertram, G., Atrih, A., Ferguson, MA., Bain, JM., Brand, A., Hamilton, S., Westwater, C., Thomson, LM., Brown, AJ., Odds, FC. and Gow, NAR (2005). Mnt1p and Mnt2p of *Candida albicans* are partially redundant α-1,2-mannosyltransferases that participate in O-linked mannosylation and are required for adhesion and virulence. *Journal of Biological Chemistry*; **280(2)**: 1051-1060.

Murad, AM., Lee, PR., Broadbent, ID., Barelle, CJ. and Brown, AJ (2000). CIp10, an efficient and

convenient integrating vector for *Candida albicans. Yeast*; **16(4)**: 325-327.

Murad, AM., Leng, P., Straffon, M., Wishart, J., Macaskill, S., MacCallum, D., Schnell, N., Talibi, D., Marechal, D., Tekaia, F., d'Enfert, C., Gaillardin, C., Odds, FC. and Brown AJ (2001). *NRG1* represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans. EMBO journal*; **20**: 4742-4752.

Myers, KK., Sypherd, PS. and Fonzi, WA (1995). Use of *URA3* as a reporter of gene expression in *C. albicans*. *Current Genetics*; **27(3)**: 243-248.

Nagahashi, S., Mio, T., Ono, N., Yamada-Okabe, T., Arisawa, M., Bussey, H. and Yamade-Okabe, H (1998). Isolation of *Ca SLN1* and *CaNIK1*, the genes for osmosensing histidine kinase homologues from the pathogenic fungus *Candida albicans. Microbiology*; **144(2)**: 425-432.

Naglik, JR., Challacombe, SJ. and Hube, B (2003). *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiology and Molecular Biology Reviews*; **67(3)**: 400-438.

Naglik, JR., Moyes, D., Makwana, J., Kanzaria, P., Tsichlaki, E., Weindl, G., Tappuni, AR., Rodgers, CA., Woodman, AJ., Challacombe, SJ., Schaller, M. and Hube, B (2008). Quantitative expression of the *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. *Microbiology*; **154(11)**: 3266-3280.

Nakayama, H., Mio, T., Nagahashi, S., Kokado, M., Arisawa, M. and Aoki, Y (2000). Tetracycline-regulatable system to tightly control gene expression in the pathogenic fungus *Candida albicans. Infection and Immunity*; **68**: 6712-6719.

Nasmyth, K (1993). Control of the yeast cell cycle by the Cdc28 protein kinase. *Current Opinions in Cell Biology*; **5(2)**: 166-179.

Nasmyth, K (1996). At the heart of the budding yeast cell cycle. *Trends in Genetics*; **12(10)**: 405-412.

Negrete-Urtasun, S., Denison, SH. and Arst, HN (1997). Characterisation of the pH signal transduction pathway gene *palA* of *Aspergillus nidulans* and identification of possible homologs. *Journal of Bacteriology.* **179(5)**: 1832-1835.

Negrete-Urtasun, S., Reiter, W., Diez, E., Denison, SH., Tilburn, J., Espeso, EA., Penalva, MA. and Arst, HN (1999). Ambient pH signal transduction in *Aspergillus*: completion of genecharacterisation. *Molecular Microbiology*; **33(5)**: 994-1003.

Niimi, M., Niimi, K. and Cannon, RD (1997). Temperature-related expression of the vacuolar aspartic proteinase (*APR1*) gene and β-N-acetylglucosaminidase (*HEX1*) gene during *Candida albicans* morphogenesis. *FEMS Microbiology Letters*; **148(2)**: 247-254.

Niimi, K., Shepherd, MG., Cannon, RD (1998). *Candida albicans HEX1* gene, a reporter of gene expression in *Saccharomyces cerevisiae*. *Archives of Microbiology*; **170(2)**: 113-119.

Nobile, CJ. and Mitchell, AP (2005). Regulation of cell-surface genes and biofilm formation by the *C. albicans* transcription factor Bcr1p. *Current Biology*; **15**: 1150-1155.

Nobile, CJ., Andes, DR., Nett, JE., Smith, FJ., Yue, F., Phan, QT., Edwards, JE., Filler, SG and Mitchell, AP (2006a). Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation *in vitro* and *in vivo*. *PLoS Pathogens*; **2(7)**: e63.

Nobile, CJ., Nett, JE., Andes, DR. and Mitchell, AP (2006b). Function of *Candida albicans* adhesin Hwp1 in biofilm formation. *Eukaryotic Cell*; **5(10)**: 1604-1610.

Nobile, CJ., Nett, JE., Hernday, AD., Homann, OR., Deneault, JS., Nantel, A., Andes, DR, Johnson AD. and Mitchell, AP (2009). Biofilm matrix regulation by *Candida albicans* Zap1. *PLoS Biology*; **7(6)**: e1000133.

Noble, SM. and Johnson, AD (2005). Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans. Eukaryotic Cell*; **4(2)**: 298-309.

Noble, SM., French, S., Kohn, LA., Chen, V. and Johnson, AD (2010). Systemic screens of a *Candida albicans* homozygous deletion library decouple morphogenetic switching and pathogenicity. *Nature Genetics*; **42(7)**: 590-598.

Nurse, P., Thuriaux, P. and Nasmyth K (1976). Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe. Molecular and General Genetics*: **146(2)**: 167-178.

Nurse, P. and Bissett, Y (1981). Gene required in G_1 for commitment to cell cycle and in G_2 for control of mitosis in fission yeast. *Nature*; **292**: 558-560.

Odds, FC (1998). Should resistance to azole antifungals *in vitro* be interpreted as predicted clinical non-response? *Drug resistance Update*; **1(1)**: 11-15.

Ofir, A. and Kornitzer, D (2010). *Candida albicans* cyclin Clb4 carries S-phase cyclin activity. *Eukaryotic Cell*; **9(9)**: 1311-1319.

Oh, J., Fung, E., Schlecht, U., Davis, RW., Giaever, G., St Onge, RP, Deutschbauer, A. and Nislow, C (2010). Gene annotation and drug target discovery in *Candida albicans* with a tagged transposon mutant collection. *PLoS Pathogens*; **6(10)**: 1001140.

Oh, Y. and Bi, E (2011). Septin structure and function in yeast and beyond. *Trends in Cell Biology*; **21(3)**: 141-148.

Ohama, T., Suzuki, T., Mori, M., Osawa, S., Ueda, T., Watanabe, K. and Kakase, T (1993). Non-universal decoding of the leucine codon CUG in several *Candida* species. *Nucleic Acids Research*: **21**: 4039-4045.

Olaiya, AF. And Sogin, SJ (1979). Ploidy determination of *Candida albicans*. *Journal of Bacteriology*; **140(3)**: 1043-1049.

Orbach, MJ., Porro, EB. and Yanofsky, C (1986). Cloning and characterisation of the gene for β-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. *Molecular and Cell Biology*; **6(7)**: 2452-2461.

Orejas, M., Espeso, EA., Tilburn, J., Sarkar, S., Arst, HN. and Penalva, MA (1995). Activation of the *Aspergillus* PacC transcription factor in response to alkaline ambient pH requires proteolysis of the carboxy-terminal moiety. *Genes and Development*; **9**: 1622-1632.

Ormo, M., Cubitt, AB., Kallio, K., Gross, LA., Tsien, RY. and Remington, SJ (1996). Crystal structure of the *Aequorea Victoria* green fluorescent protein. *Science*; **273**: 1392-1395.

Pappas, PG., Rex, JH., Sobel, JD., Filler, SG., Dismukes, WE., Walsh, TJ. and Edwards, JE (2004). Guidelines for treatment of candidiasis. *Clinical Infectious Disease*; **38(2)**: 161-189.

Park, SH., Koh, SS., Chun, JH., Hwang, HJ. and Kang, HS (1999). Nrg1 is a transcriptional repressor for glucose repression of *STA1* gene expression in *Saccharomyces cerevisiae. Molecular and Cellular Biology*; **19(3)**: 2044-2050.

Pereira, G., Hofken, T., Grindlay, J., Manson, C. and Schiebel, E (2000). The Bub2p spindle checkpoint links nuclear migration with mitotic exit. *Molecular Cell*; **6(1)**: 1-10.

Pereira, G. and Schiebel, E (2005). Kin4 kinase delays mitotic exit in response to spindle alignment defects. *Molecular Cell*; **19(2)**: 209-221.

Pesin, JA. and Orr-Weaver, TL (2008). Regualtion of APC/C activators in mitosis and meiosis. *Annual Reviews Cell and Developmental Biology*; **24**: 475-499.

Peters, JM (2006). The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nature Reviews Molecular and Cell Biology*; **7(9)**: 644-656.

Pfaller, MA., Messer, SA., Hoolis, RJ., Jones, RN., Doern, GV., Brandt, ME. Hajjeh, RA (1999). Trends in species distribution and susceptibility to fluconazole among blood stream isolates of *Candida* species in the United States. *Diagnostic Microbiology and Infectious Disease*; **33(4)**; 217-222.

Pfleger, CM. and Kirschner, MW (2000). The KEN box: an APC recognition signal distict from the D box targeted by Cdh1. *Genes and Development*; **14(6)**: 655-665.

Phan ,QT., Belanger, PH. and Filler, SG (2000). Role of hyphal formation in interaction of *Candida albicans* with endothelial cells. *Infection and Immunity*; **68(6)**: 3485-3490.

Piatti, S., Venturetti, M., Chiroli, E. and Fraschini, R (2006). The spindle position checkpoint in budding yeast: the motherly care of MEN. *Cell Division*; **1(1)**: 2.

Pomes, R., Gil, C. and Nombela, C (1985). Genetic analysis of *Candida albicans* morphological mutants. *Journal of General Microbiology*; **131(8)**: 2107-2113.

Porta, A., Ramon, AM, Fonzi, WA (1999). *PRR1*, a homolog of *Aspergillus nidulans* palF, controls pH-dependent gene expression and filamentation in *Candida albicans*. *Journal of Bacteriology*; **181**: 7516-7523.

Prasher, DC., Eckenrode, VK., Ward, WW., Prendergast, FG. and Cormier, MJ (1992). Primary structure of the *Aequorea Victoria* green fluorescent protein. *Gene*; **111**: 229-233.

Prill, SK., Klinkert, B., Timpel, C., Gale, CA., Schroppel, K. and Ernst, JF (2005). PMT family of *Candida albicans*: five protein mannosyltransferase isoforms affect growth, morphogenesis and antifungal resistance. *Molecular Microbiology*; **55(2)**: 546-560.

Prinz, S., Hwang, ES., Visintin, R. and Amon, A (1998). The regulation of Cdc20 proteolysis reveals a role for APC components Cdc23 and Cdc27 during S phase and early mitosis. *Current Biology*; **8**: 750-760.

Queralt, E., Lehane, C., Novak, B. and Uhlmann, F (2006). Downregulation of PP2A (Cdc55) phosphatase by seperase initiates mitotic exit in budding yeast. *Cell*; **125(4)**: 719-732.

Queralt, E. and Uhlmann, F (2008). Seperase cooperates with Zds1 and Zds2 to activate Cdc14 phosphatase in early anaphase. *Journal of Cell Biology*; **182(5)**: 873-883.

Ramage, G., Saville, JP., Thomas, DP. and Lopez-Ribot, JL (2005). *Candida* biofilms: an update. *Eukaryotic Cell*; **4(4)**: 633-638.

Ramirez-Zavala, B., Reuss, O., Park, YN., Ohlsen, K. and Morschhauser J (2008) Environmental induction of white-opaque switching in *Candida albicans*. *PLoS Pathogens*; **4(6)**: e1000089.

Redd, MJ., Arnaud, MB. and Johnson, AD (1997). A complex composed of tup1 and ssn6 represses transcription *in vitro. Journal of Biological Chemistry*; **272**: 11193-11197.

Reijnst, P., Walther, A. and Wendland, J (2011). Dual-colour fluorescence microscopy using yEmCherry-/*GFP*-tagging of eisosome components Pil1 and Lsp1 in *Candida albicans*. *Yeast*; **28(4)**: 331-338.

Reimann, JD., Freed, E., Hsu, JY, Kramer, ER., Peters, JM. and Jackson, PK (2001a). Emi1 is a mitotic regulator that interacts with Cdc20 and inhibits the anaphase promoting complex. *Cell*; **105(5)**: 645-655.

Reimann, JD., Gardner, BE., Margottin-Goquet, F. and Jackson, PK (2001b). Emi1 regualtes the anaphase promoting complex by a different mechanism than Mad2 proteins. *Genes and Development*; **15**: 3278-3285.

Ren, G., Wang, J., Brinkworth, R., Winsor, B., Kobe, B. and Munn, AL (2005). Verprolin cytokinesis function mediated by Hof one trap domain. *Traffic*; **6(7)**: 575-593.

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

Richard, ML. and Plaine, A (2007). Comprehensive analysis of gylcosylphosphatidylinositol-anchored proteins in *Candida albicans*. *Eukaryotic Cell*; **6(2)**: 119-133.

Richards, TA., Soanes, DM., Foster, PG., Leonard, G., Thornton, CR. and Talbot, NJ (2009). Phylogenomic analysis demonstrate a pattern of rare and ancient horizontal gene transfers between plants and fungi. *Plant Cell*; **21(7)**: 1897-1911.

Richardson, H., Lew, DJ., Henze, M., Sugimoto, K. and Reed, SI (1992). Cyclin-B homologs in *Saccharomyces cerevisiae* function in S phase and in G_2. *Genes and Development*; **6(11)**: 2021-2034.

Rieder, CL., Schultz, A, Cole, R. and Sluder, G (1994). Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors kinetochore attachment to the spindle. *Journal of Cell Biology*. **127(5)**: 1301-1310.

Rieder, CL., Cole, RW., Khodjakov, A. and Sluder, G (1995). The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *Journal of Cell Biology*; **130(4)**: 941-948.

Riggle, RJ., Andrutis, KA., Chen, X., Tzipori, SR. and Kumamoto, CA (1999). Invasive lesions containing filamentous forms produced by a *Candida albicans* mutant that is defective in filamentous growth in culture*. Infection and Immunity*; **67(7)**: 3649-3652.

Ro, HS., Song, S. and Lee, KS (2002). Bfa1 can regulate Tem1 function independently of Bub2 in the mitotic exit network of *Saccharomyces cerevisiae*. *Proceeding of the National Academy of Science of the United States of America*; **99(8)**: 5436-5441.

Rocha, CR., Schroppel, K., Harcus, D., Marcil, A., Dignard, D., Taylor, BN., Thomas, DY., Whiteway, M. and Leberer, E (2001). Signalling through adenylyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus *Candida albicans. Molecular Biology of the Cell*; **12(11)**: 3631-3643.

Rock, JM. and Amon, A (2009). The FEAR network. *Current Biology*; **19**:1063-1068.

Roemer, T., Jiang, B., Davison, J., *et al*., (2003). Large-scale essential gene identification in *Candida albicans* and applications to antifungal drug discovery. *Molecular Microbiology*; **50(1)**: 167-181.

Ross, KE. and Cohen-Fix, O (2004). A role for the PEAR pathway in nuclear positioning during anaphase. *Developmental Cell*; **6(5)**: 729-735.

Rouabhia, M., Schaller, M., Corbucci, C., Vecchiarelli, A., Prill, SK., Giasson, L. and Ernst, JF (2005). Virulence of the fungal pathogen *Candida albicans*requires the five isoforms of protein mannosyltransferases. *Infection and Immunity*; **73(8)**: 4571-4580.

Rowbottom, L., Munro, CA. and Gow, NAR (2004). *Candida albicans* mutants in the *BNI4* gene have reduced cell-wall chitin and alterations in morphogenesis. *Microbiology*; **150(10)**: 3243-

3252.

Ruoslahti, E. and Pierschbacher, MD (1987). New perspectives in cell adhesion: RGD and integrins. *Science*; **238**: 491-497.

Rupes, I (2002). Checking cell size in yeast. *Trends in Genetics*; **18(9)**: 479-485.

Salimova, E., Sohrmann, M., Fournier, N. and Simanis, V (2000). The *S. pombe* orthologue of the *S. cerevisiae mob1* gene is essential and functions in signalling the onset of septum formation. *Journal of Cell Science*; **113(10)**: 1695-1704.

Sanchez-Martinez, C. and Perez-Martin, J (2002) Gpa2, a G-protein α-subunit required for hyphal development in *Candida albicans. Eukaryotic Cell*; **1(6)**: 865-874.

Sanglard, D., Hube, B., Monod, M., Odds, FC. and Gow, NAR (1997). A triple deletion of the secreted aspartyl proteinase genes *SAP4*, *SAP5* and *SAP6* of *Candida albicans* causes attenuated virulence. *Infection Immunity*; **65(9)**: 3539-3546.

Santos, MA., el-Adlouni, C., Cox, AD., Luz, JM., Keith, G. and Tuite, MF (1994). Transfer RNA profiling: a new method for the identification of pathogenic *Candida* species. *Yeast*; **10(5)**: 625-636.

Santos, MA. and Tuite. MF (1995). The CUG codon is decoded *in vivo* as serine and not leucine in *Candida albicans*. *Nucleic acid research*; **23(9)**: 1481-1486.

Schaller, M., Korting, HC., Schafer, W., Bastert, J., Chen, W. and Hube, B (1999). Secreted aspartic proteinase (Sap) activity contribute to tissue damage in a model of human oral candidosis. *Molecualr Microbiology*; **34(1)**: 169-180.

Schaller, M., Schackert, C., Korting, HC., Januschke, E. and Hube, B. (2000) Invasion of *Candida albicans* correlates with expression of secreted aspartic proteinases during experimental infection of human epidermis. *Journal of Investigative Dermatology*; **114(4)**: 712-717.

Schaller, M., Korting, HC., Borelli, C., Hamm, G. and Hube, B (2005). *Candida albicans*-secreted aspartic proteinases modify the epithelial cytokine response in an *in vitro* model of vaginal candidiasis. *Infection Immunity*; **73(5)**: 2758-2765.

Schaub, Y., Dunkler, A., Walther, A. and Wendland, J (2006). New pFA-cassettes for PCR-based gene manipulation in *Candida albicans*. *Journal of Basic Microbiology*; **46(5)**: 416-429.

Scherer, S. and Magee, PT (1990). Genetics of *Candida albicans*. *Mocrobiology Reviews*; **54(3)**: 226-241.

Schmidt, S., Sohrmann, M., Hofmann, K., Woollard, A. and Simanis, V (1997). The Spg1 GTPase is an essential, dosage-dependent inducer of septum formation in *Schizosaccharomyces pombe. Genes and Development*; **11**: 1519-1534.

Schneider, BL., Seufert, W., Steiner, B., Yang, QH. and Futcher, AB (1995). Use of polymerase chain reaction epitope tagging for protein tagging in *Saccharomyces cerevisiae*. *Yeast*; **11**: 1265-1274.

Schofield, DA., Westwater, C., Warner, T. and Balish, E. (2005). Differential *Candida albicans* lipase gene expression during alimentary tract colonisation and infection. *FEMS Microbiology Letters*; **244(2)**: 359-365.

Schweitzer, B. and Philippsen, P (1991). *CDC15*, an essential cell cycle gene in *Saccharomyces cerevisiae*, encodes a protein kinase domain. *Yeast*; **7(3)**: 265-273.

Schwob, E. and Nasmyth, K (1993). *CLB5* and *CLB6*, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae*. *Genes and Development*; **7(7)**: 1160-1175.

Schwob, E., Bohm, T., Mendenhall, M. and Nasmyth, K (1994). The B-type cyclin kinase inhibitorp40SIC1 controls the G_1 to S transition in *S. cerevisiae*. *Cell*; **79(2)**: 233-244.

Seshan, A., Bardin, AJ. and Amon, A (2002). Control of Lte1 localisation by cell polarity determinants and Cdc14. *Current Biology*; **12**: 2098-2110.

Seshan, A. and Amon, A (2005). Ras and the Pho effector Cla4 collaborate to target and anchor Lte1 at the bud cortex. *Cell Cycle*; **4(7)**: 940-946.

Shah, R., Jensen, S., Frenz, LM., Johnson, AL. and Johnston LH (2001). The Spo12 protein of *Saccharomyces cerevisiae*: a regulator of mitotic exit whose cell cycle-dependent degradation is mediated by the anaphase-promoting complex. *Genetics*; **159(3)**: 965-980.

Sharkey, LL., McNemar, MD., Saporito-Irwin, SM., Sypherd, PS. and Fonzi, WA (1999). *HWP1* functions in the morphological development of *Candida albicans* downstream of *EFG1*, *TUP1* and *RBF1. Journal of Bacteriology*; **181**: 5273-5279.

Shen, J., Guo, W. and Kohler, JR (2005). Ca*NAT1*, a heterologous dominant selectable marker for transformation of *Candida albicans* and other pathogenic *Candida* species. *Infection and Immunity*; **73(2)**: 1239-1242.

Shen, J., Cowen, LE., Griffin, AM., Chan, L. and Kohler, JR (2008). The *Candida albicans* pescadillo homolog is required for normal hypha-to-yeast morphogenesis and yeast proliferation. *Proceeding of the National Academy of Science of the United States of America*; **105**: 20918-20923.

Sherlock, G., Bahman, AM., Mahal, A., Shieh, JC., Ferreira, M. and Rosamond, J (1994). Molecular cloning and analysis of *CDC28* and cyclin homologues from the human fungal pathogen *Candida albicans*. *Molecular and General Genetics*; **245(6)**: 716-723.

Shimomura, O., Johnson, FH. and Saiga, Y (1962). Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *Journal of Cellular and Comparative Physiology*; **59**: 223-239.

Shimomura, O (1979). Structure of the chromophore of *Aequorea* green fluorescent protein. *FEBS Letters*; **104**: 220-222.

Shirayama, M., Matsui, Y., Tanaka, K. and Toh-e, A (1994a). Isolation of a CDC25 family gene, *MSI2/LTE1*, as a multicopy suppressor of *ira1*. *Yeast*; **10(4)**: 451-461.

Shirayama, M., Matsui, Y. and Toh-e, A (1994b). The yeast *TEM1* gene, which encodes a GTP-binding protein, is involved in termination of M phase. *Molecular Cell Biology*; **14(11)**: 7476-7482.

Shirayama, M., Matsui, Y. and Toh-e, A. (1996). Dominant mutant alleles of yeast protein kinase gene *CDC15* suppress the *lte1* defect in termination of M phase and genetically interact with *CDC14*. *Molecular and General Genetics*; **251(2)**: 176-185.

Shirayama, M., Toth, A., Galova, M. and Nasmyth, K (1999). APC(Cdc20) promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. *Nature*; **402**: 203-207.

Shou, W., Seol, JH., Shevchenko, A., Baskerville, C., Moazed, D., Chen, ZW., Jang, J., Shevchenko, A., Charbonneau, H. and Ceshaies, RJ (1999). Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell*; **97(2)**: 233-244.

Shulewitz, MJ., Inouye, CJ. and Thorner, J (1999). Hsl7 localises to a septin ring and serves as an adapter in the regulatory pathway that relieves tyrosine phosphorylation of Cdc28 protein kinase in *Saccharomyces cerevisiae. Molecular and Cell Biology*; **19(10)**: 7123-7137.

Skotheim, JM., Di Talia, S., Siggia, ED. and Cross, FR (2008). Positive feedback of G_1 cyclins ensures coherent cell cycle entry. *Nature*; **454**: 291-296.

Sia, RA., Herald, HA. and Lew, DJ (1996). Cdc28 tyrosine phosphorylation and the morphogenesis checkpoint in budding yeast. *Molecular Biology of the Cell*; **7(11)**: 1657-1666.

Sia, RA., Bardes, ES. and Lew, DJ (1998). Control of Swe1p degradation by the morphogenesis checkpoint. *EMBO Journal*; **17**: 6678-6688.

Simanis, V (2003). Events at the end of mitosis in the budding and fission yeasts. *Journal of Cell Science*; **116**: 4263-4275.

Skowyra, D., Craig, KL., Tyers, M., Elledge, SJ. and Harper, JW (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell*; **91(2)**: 209-219.

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

Slutsky, B., Staebell, M., Anderson, J., Risen, L., Pfaller, M. and Soll, DR (1987). "White-opaque transition": a second high-frequency switching system in *Candida albicans*. *Journal of*

Bacteriology: **169(1)**: 189-197.

Smith, RL. and Johnson, AD (2000). Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. *Trends in Biochemical Science*; **25(7)**: 325-330.

Sohn, K., Urban, C., Brunner, H. and Rupp, S (2003). *EFG1* is a major regulator of cell wall dynamics in *Candida albicans* as revealed by DNA microarrays. *Molecualr Microbiology*; **47(1)**: 89-102.

Sohrmann, M., Schmidt, S., Hagan, I. and Simanis, V (1998). Asymmetric segregation on spindle poles of the *Schizosaccharomyces pombe* septum-inducing protein kinase Cdc7p. *Genes and Development*; **12(1)**: 84-94.

Song, S., Grenfell, TZ., Garfield, S., Erikson, RL. and Lee, KS (2000). Essential function of the polo box of Cdc5 in subcellular localisation and induction of cytokineitc structures. *Molecular and Cell Biology*; **20(1)**: 286-298.

Sonneborn, A., Tebarth, B. and Ernst, JF (1999). Control of white-opaque phenotypic switching in *Candida albicans* by the Efg1p morphogenetic regulator. *Infection and Immunity*; **67(9)**: 4655-4660.

Sonneborn, A., Bockmuhl, DP., Gerads, M., Kurpanek, K., Sanglard, D. and Ernst, JF (2000). Protein kinase A encoded by *TPK2* regulates dimorphism of *Candida albicans. Molecular Microbiology*; **35(2)**: 386-396.

Sorensen, CS., Lukas, C., Kramer, ER., Peters, JM., Bartek, J. and Lukas, J (2001). A conserved cyclin-binding domain determines functional interplay between anaphase-promoting complex-Cdh1 and cyclin A-Cdk2 during cell cycle progression. *Molecular and Cell Biology*; **21(11)**: 3692-3703.

Soyano, T., Nishihama, R., Morikiyo, K., Ishikawa, M. and Machida, Y (2003). NQK1/NtMEK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated MAPK cascade and is required for plant cytokinesis. *Genes and Development*; **17(8)**: 1055-1067.

Sparks, CA., Morphew, M. and McCollum, D (1999). Sid2p, a spindle pole body kinase that regulates the onset of cytokinesis. *Journal of Cell Biology*; **146(4)**: 777-790.

Spellman, PT., Sherlock, G., Zhang, MQ., Iyer, VR., Anders, K., Eisen, MB., Brown, PO., Botstein, D. and Futcher, B (1998). Comprehensive identification of cell cycle-regulated genes of the yest *Saccharomyces cerevisiae* by microarray hybridisation. *Molecular Biology of the Cell*; **9(12)**: 3273-3297.

Srikantha, T., Klapach, A., Lorenz, WW., Tsai, LK., Laughlin, LA., Gorman, JA. and Soll, DR (1996). The sea pansy *Renilla reniformis* luciferase serves as a sensitive bioluminescent reporter for differential gene expression in *Candida albicans*. *Journal of Bacteriology*; **178(1)**: 121-129.

Srikantha, T., Tsai, LK., Daniels, K. and Soll DR (2000). *EFG1* null mutants of *Candida albicans*

switch but cannot express the complete phenotype of white-phase budding cells. *Journal of Bacteriology*; **182(6)**: 1580-1591.

Srikantha, T., Tsai, LK., Daniels, K., Klar, AJ. and Soll, DR (2001). The histone deacetylase genes *HDA1* and *RPD3* play distinct roles in regulation of high-frequency phenotypic switching in *Candida albicans*. *Journal of Bacteriology*; **183**: 4614-4625.

Srikantha, T., Borneman, AR., Daniels, KJ., Pujol, C., Wu, W., Seringhaus, MR., Gerstein, M., Yi, S., Snyder, M. and Soll DR (2006). *TOS9* regulates white-opaque switching in *Candida albicans*. *Eukaryotic Cell*; **5(10)**: 1674-1687.

Staab, JF., Ferrer, CA. and Sundstrom, P (1996). Developmental expression of a tandemly repeated , proline- and glutamine-rich amino acid motif on hyphal surfaces on *Candida albicans*. *Journal of Biological Chemistry*; **271(11)**: 6298-6305.

Staab, JF., Bradway, SD., Fidel, PL. and Sundstrom, P (1999). Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science*; **283**: 1535-1538.

Staab, JF. and Sundstrom, P (2003). *URA3* as a selectable marker for disruption and virulence assessment of *Candida albicans* genes. *Trends in Microbiology*; **11(2)**: 69-73.

Staab, JF., Bahn, YS. And Sundstrom, P (2003). Integrative, multifunctional plasmids for hypha-specific or constitutive expression of green fluorescent protein in *Candida albicans*. *Microbiology*; **149(10)**: 2977-2986.

Staib, P., Michel, S., Kohler, G. and Morschhauser, J (2000). A molecular genetic system for the pathogenic yeast *Candida dubliniensis*. *Gene*; **242**: 393-398.

Stegmeier, F., Visintin, F. and Amon, A (2002). Seperase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localisation during early anaphase. *Cell*; **108(2)**: 207-220.

Stegmeier, F., Huang, J., Rahal, R., Zmolik, J., Moazed, D. and Amon A (2004). The replication fork block Fob1 functions as a negative regulator of the FEAR network. *Current Biology*; **14(6)**: 467-480.

Stehr, F., Felk, A., Gacser, A., Kretschmar, M., Mahnss, B., Neuber, K., Hube, B. and Schafer, W. (2004). Expression analysis of the *Candida albicans* lipase gene family during experimental infections and in patient samples. *FEMS Yeast Research*; **4**: 401-408.

Stoldt, VR., Sonneborn, A., Leuker, CE. and Ernst, JF (1997). Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. *EMBO Journal*; **16(8)**: 1982-1991.

Stoepel, J., Ottey, MA., Kurischko, C., Hieter, P. and Luca, FC (2005). The mitotic exit network Mob1-Dbf2p kinase complex localises to the nucleus and regulates passenger protein localisation. *Molecular Biology of the Cell*; **16**: 5465-5479.

Stynen, B., Van Dijck, P. and Tournu, H (2010). A CUG codon adapted two-hybrid system for the pathogenic fungus *Candida albicans*. *Nucleic Acids Research*; **38**: e184.

Su, SS. and Mitchell, AP (1993). Molecular characterisation of the yeast meiotic regulatory gene *RIM1*. *Nucleic Acids Research*; **21**: 3789-3797.

Sudbery, PE (2001). The germ tubes of *Candida albicans* hyphae and pseudohyphae show different patterns of septin ring localisation. *Molecular Microbiology*; **41(1)**: 19-31.

Sudbery, PE., Gow, NAR. and Berman, J (2004). The distinct morphological states of *Candida albicans*. *Trends in Microbiology*; **12(7)**: 317-324.

Sugiyama, Y., Nakashima, S., Mirbod, F., Kanoh , H., Kitajima, Y., Ghannoum, MA. and Nozawa, Y (1999). Molecular cloning of a second phospholipase B gene, ca*PLB2* from *Candida albicans*. *Medical Mycology*; **37(1)**: 61-67.

Sullivan, PA., McHugh, NJ, Romana, LK. and Shepherd, MG (1984). The secretion of N-acetylglucosaminidase. *Journal of General Microbiology*; **130(9)**: 2213-2218.

Sullivan, M. and Uhlmann, F (2003). A non-proteolytic function of seperase links the onset of anaphase to mitotic exit. *Nature Cell Biology*; **5(3)**: 249-254.

Sundstrom, P (1999). Adhesins in *Candida albicans*. *Current Opinion in Microbiology*; **2(4)**:353-357.

Theesfeld, CL., Zyla, TR., Bardes, EG. and Lew, DJ (2003). A monitor for bud emergence in the yeast morphogenesis checkpoint. *Molecular and Cell Biology*; **14(8)**: 3280-3291.

Tilburn, J., Sarkar, S., Widdick, DA., Espeso, EA., Orejas, M., Mungroo, J., Penalva, MA. and Arst, HN (1995). The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. *EMBO Journal*; **14(4)**: 779-790.

Timpel, C., Strahl-Bolsinger, S., Ziegelbauer, K. and Ernst, JF (1998). Mutliple functions of Pmt1p-mediated protein O-mannosylation in the fungal pathogen *Candida albicans*. *Journal of Biological Chemistry*; **273**: 20837-20846.

Timpel, C., Zink, S., Strahl-Bolsinger, S., Schroppel, K. and Ernst, JF (2000). Morphogenesis, adhesive properties, and antifungal resistance depend on the Pmt6 protein mannsyltransferase in the fungal pathogen *Candida albicans*. *Journal of Bacteriology*: **182(11)**: 3063-3071.

Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, JD., McMullen, B., Hurwitz, M., Krebs, EG. and Wigler, M (1987). Cloning and characterisation of *BCY1*, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in *Saccharomyces cerevisiae*. *Molecular and Cell Biology*; **7(4)**: 1371-1377.

Tomson, BN., Rahal, R., Reiser, V., Monje-Casas, F., Mekhail, K., Moazed, D. and Amon A

(2009). Regulation of Spo12 phosphorylation and its essential role in the FEAR network. *Current Biology*; **19(6)**: 449-460.

Toyn, JH., Araki, H., Sugino, A. and Johnston, LH (1991). The cell-cycle-regulated budding yeast gene *DBF2*, encoding a putative protein kinase, has a homologue that is not under cell-cycle control. *Gene*; **104(1)**: 63-70.

Toyn, JH. and Johnston, LH (1994). The Dbf2 and Dbf20 protein kinases of budding yeast are activated after the metaphase to anaphase cell cycle transition. *EMBO Journal*; **13(5)**: 1103-1113.

Toyn, JH., Johnson, AL., Donovan, JD., Toone, WH. and Johnston, LH (1997). The Swi5 transcription factor of *Saccharomyces cerevisiae* has a role in exit from mitosis through induction of the cdk-inhibitor Sic1 in telophase. *Genetics*; **145(1)**: 85-96.

Trautmann, S., Wolfe, BA., Jorgensen, P., Tyers, M., Gould, KL. and McCollum, D (2001). Fission yeast Cip1p phosphatase regulates G_2/M transition and coordination of cytokinesis with cell cycle progression. *Current Biology*; **11**: 931-940.

Traverso, EE., Baskerville, C., Liu, Y., Shou, W., James, P., Deshaies, RJ. and Charbonneau, H (2001). Characterisation of the Net1 cell cycle-dependent regulator of the Cdc14 phosphatase from budding yeast. *Journal of Biological Chemistry*; **276**: 21924-21931.

Treitel, MA. and Carlson, M (1995). Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. *Proceeding of the National Academy of Science of the United States of America*; **92(8)**: 3132-3136.

Tsong, AE., Miller, MG., Raisner, RM. and Johnson, AD (2003). Evolution of a combinatorial transcriptional circuit: a case study in yeasts. *Cell*; **115(4)**: 389-399.

Tsuchimori, N., Sharkey, LL., Fonzi, WA., French, SW., Edwards, JE. and Filler, SG (2000). Reduced virulence of *HWP1*-deficient mutants of *Candida albicans* and their interactions with host cells. *Infection and Immunity*; **68(4)**: 1997-2002.

Tuch, BB., Galgoczy, DJ., Hernday, AD., Li, H. and Johnson, AD (2008). The evolution of combinatorial gene regulation in fungi. *PLoS Biology*; **6(2)**: e38.

Uhl, MA. and Johnson, AD (2001). Development of *Streptococcus thermophilus lacZ* as a reporter gene for *Candida albicans*. *Microbiology*; **147(5)**: 1189-1195.

Umeyama, T., Kaneko, A., Niimi, M. and Uehara, Y (2006). Repression of *CDC28* reduces the expression of the morphology-related transcription factors, Efg1p, Nrg1p, Rbf1p, Rim101p, Fkh2p and Tec1p and induces cell elongation in *Candida albicans*. *Yeast*; **23(7)**: 537-552.

Uppuluri, P., Chaturvedi, AK., Srinivasan, A., Banerjee, M., Ramasubramaniam, AK, Kohler, JR., Kadosh, D. and Lopez-Ribot, JL (2010a). Dispersion as an important step in the *Candida albicans* biofilm development cycle. *PLoS Pathogens*; **6(3)**: e1000828.

Uppuluri, P., Pierce, CG., Thomas, DP., Bubeck, SS., Saville, SP. and Lopez-Ribot, JL (2010b). The transcription regulator Nrg1p controls *Candida albicans* biofilm formation and dispersion. *Eukaryotic Cell*; **9(10)**: 1531-1537.

Vallen, EA., Caviston, J. and Bi, E (2000). Roles of Hof1p, Bni1p and myo1p in cytokinesis in *Saccharomyces cerevisiae. Molecular Biology of the Cell*; **11(2)**: 593-611.

Varma, A. and Kwon-Chung, KJ (2000). Characterisation of the *L41* gene in *Cryptococcus neoformans*: its application as a selectable transformation marker for cycloheximide resistance. *Yeast*; **16**: 1397-1403.

Vargas, K., Messer, SA., Pfaller, M., Lockhart, SR., Stapleton, JT., Hellstein, J. and Soll, DR (2000). Elevated phenotypic switching and drug resistance of *Candida albicans* from human immunodeficiency virus-positive individuals prior to first thrush episode. *Journal of Clinical Microbiology*; **38(10)**: 3595-3607.

Verma, R., Feldman, RM. and Deshaies, RJ (1997). SIC1 is ubiquitinated *in vitro* by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. *Molecular Biology of the Cell*; **8(8)**: 1427-1437.

Vinces, MD., Haas, C. and Kumamoto, CA (2006). Expression of the *Candida albicans* morphogenesis regulator gene *CZF1* and its regulation by Efg1p and Czf1p. *Eukaryotic Cell*; **5(5)**: 825-835.

Vinces, MD. and Kumamoto, CA (2007). The morphogenetic regulator Czf1p is a DNA-binding protein that regulates white opaque switching in *Candida albicans*. *Microbiology*; **153(9)**: 2877-2884.

Virag, A. and Harris, SD (2006). The Spitzenkorper: a molecular perspective. *Mycological Research*; **110(1)**: 4-13.

Visintin, R., Craig, K., Hwang, ES., Prinz, S., Tyers, M. and Amon, A (1998). The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. *Molecular Cell*; **2(6)**: 709-718.

Visintin, R., Hwang, ES. and Amon, A (1999). Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. *Nature*; **398(6730)**: 818-823.

Visintin, R. and Amon, A (2001). Regulation of the mitotic exit protein kinases Cdc15 and Dbf2. *Molecular Biology of the Cell*; **12(10)**:2961-2974.

Visintin, R., Stegmeier, F. and Amon, A (2003). The role of the polo kinase Cdc5 in controlling Cdc14 localisation. *Molecular Biology of the Cell*; **14(11)**: 4486-4498.

Visintin, C., Tomson, BN., Rahal, R., Paulson, J., Cohen, M., Taunton, J., Amon, A. and Visintin, R (2008). APC/C-Cdh1-mediated degradation of the Polo kinase Cdc5 promotes the return of Cdc14 into the nucleolus. *Genes and Development*; **22(1)**: 79-90.

Waizernegger, I., Lukowitz, W., Assaad, F., Schwarz, H., Jurgens, G. and Mayer, U (2000). The *Arabidopsis* KNOLLE and KEULE genes interact to promote vesicle fusion during cytokinesis. *Current Biology*; **10**: 1371-1374.

Wallace, RB., Johnson, MJ., Suggs, SV., Miyoshi, K., Bhatt, R. and Itakura, K (1981). A set of synthetic oligodeoxyribonucleotide primers for DNA sequencing in the plasmid vector pBR322. *Gene*; **16**: 21-26.

Wan, J., Xu, H. and Grunstein, M (1992). *CDC14* of *Saccharomyces cerevisiae*. Cloning, sequence analysis, and transcription during the cell cycle. *Journal Biological Chemistry*; **267**: 11274-11280.

Wang, Y., Shirogane, T., Liu, D., Harperm JW. and Elledge, SJ (2003). Eixt from exit: resetting the cell cycle through Amn1 inhibition of G protein signalling. *Cell*; **112(5)**: 697-709.

Wang, A., Lane, S., Tian, Z., Sharon, A., Hazan, I. and Liu, H (2007). Temporal and spatial control of *HGC1* expression results in Hgc1 localisation to the apical cells of hyphae in *Candida albicans*. *Eukaryotic Cell*; **6(2)**: 253-261.

Wang, A., Raniga, PP., Lane, S., Lu, Y. and Liu, H (2009). Hyphal chain formation in *Candida albicans*: Cdc28-Hgc1 phosphorylation of Efg1 represses cell separation genes. *Molecular and Cellular Biology*; **29**: 4406-4416.

Wellington, M. and Rustchenko, E (2005). 5-Fluoro-orotic acid induces chromosome alterations in *Candida albicans*. *Yeast*; **22(1)**: 57-70.

Wennerberg, K., Rossman, KL. and Der, CJ (2005). The Ras superfamily at a glance. *Journal of Cell Science*; **118(5)**: 843-846.

White, TC., Miyasaki, SH. and Agabian, N. (1993). Three distinct secreted aspartyl proteinases in *Candida albicans*. *Journal of Bacteriology*; **175**: 6126-6133.

White, TC., Andrews, LE, Maltby, D. and Agabian, N (1995). The "universal" leucine codon CTG in the secreted aspartyl proteinase 1 (*SAP1*) gene of *Candida albicans* encodes a serine *in vivo*. *Journal of Bacteriology*; **177(10)**: 2953-2955.

White, TC. and Agabian, N. (1995). *Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. *Journal of Bacteriology*; **177**: 5215-5221.

Whiteway, M., Dignard, D. and Thomas, DY (1992). Dominant negative selection of heterologous genes: isolation of *Candida albicans* genes that interfere with *Saccharomyces cerevisiae* mating factor-induced cell cycle arrest. *Proceedings of the National Academy of Science in the United States of America*; **89**: 9410-9414.

Whiteway, M (2000). Transcriptional control of cell type and morphogenesis in *Candida albicans. Current Opinions in Microbiology*; **3(6)**: 582-588.

Whiteway, M. and Bachewich, C (2007). Morphogensis in *Candida albicans*. *Annual Reviews of Microbiology*; **61**: 529-553.

Wightman, R., Bates, S., Amornrrattanapan, P. and Sudbery, PE (2004). In *Candida albicans*, the Nim1 kinases Gin4 and Hsl1 negatively regulate pseudohypha formation and Gin4 als control septin organisation. *Journal of Cell Biology*; **164(4)**: 581-591.

Wilson, RB., Davis, D., Enloe, BM. and Mitchell, AP (2000). A recyclable *Candida albicans URA3* cassette for PCR product-directed gene disruptions. *Yeast*; **16(1)**: 65-70.

Wirsching, S., Michel, S., Kohler, G. and Morshhauser, J (2000). Activation of the multiple drug resistance gene *MDR1* in fluconazole- resistance, clinical *Candida albicans* strains is caused by mutations in a trans-regulatory factor. *Journal of Bacteriology*; **182(2)**: 400-404.

Wittenberg, C. and La Valle, R (2003). Cell-cycle-regulatory elements and the control of cell differentiation in the budding yeast. *Bioessays*; **25(9)**: 856-867.

Wolkow, TD., Harris, SD. and Hamer, JE (1996). Cytokinesis in *Aspergillus nidulans* is controlled by cell size, nuclear positioning and mitosis. *Journal of Cell Science*; **109(8)**: 2179-2188.

Wolkow, TD, Mirabito, PM., Venkatram, S. and Hamer JE (2000). Hypomorphic bimA (APC3) alleles cause errors in chromosome metabolism that activate the DNA damage checkpoint blocking cytokinesis in *Aspergillus nidulans. Genetics*; **154(1)**: 167-179.

Wright, RJ., Carne, A., Hieber, AD., Lamont, IL., Emerson, GW. and Sullivan, PA. (1992) A second gene for a secreted aspartate proteinase in *Candida albicans*. *Journal of Bacteriology*; **174**: 7848-7853.

Xu, S., Huang, HK., Kaiser, P., Latterich, M. and Hunter, T (2000). Phosphorylation and spindle pole body localisation of the Cdc15p mitotic regulatory protein kinase in budding yeast. *Current Biology*; **10(6)**: 329-332.

Xu, W. and Mitchell, AP (2001). Yeast PalA/AIP1/Alix homolog Rim20p associates with a PEST-like region and is required for its proteolytic cleavage. *Journal of Bacteriology*; **183**: 6917-6923.

Xu, W., Smith, FJ., Subaran, R. and Mitchell, AP (2004). Multivesicular body-ESCRT components function in pH response regulation in *Saccharomyces cerevisiae* and *Candida albicans. Molecular Biology of the Cell*; **15(12)**: 5528-5537.

Yellman, CM. and Burke, DJ (2006). The role of Cdc55 in the spindle checkpoint is through regulation of mitotic exit in *Saccharomyces cerevisiae. Molecular Biology of the Cell*; **17(2)**: 658-666.

Yasutis, K., Vignali, M., Ryder, M., Tameire, F., Dighe, SA., Fields, S. and Kozminiski, KG (2010). Zds2p regulates Swi1-dependent polarised cell growth in *Saccharomyces cerevisiae* via a novel Cdc55p interaction domain. *Molecular Biology of the Cell*; **21**: 4373-4386.

Yeh, E., Skibbens, RV., Cheng, JW., Salmon, ED. and Bloom, K (1995). Spindle dynamics and cell cycle regulation of dynein in the budding yeast, *Saccharomyces cerevisiae*. *Journal of Cell Biology*; **130(3)**: 687-700.

Yeong, FM., Lim, HH., Padmashree, CG. and Surana, U (2000). Exit from mitosis in budding yeast: biphasic inactivation of the Cdc28-Clb2 mitotic kinase and the role of Cdc20. *Molecular Cell*; **5(3)**: 501-511.

Yeong, FM (2005). Severing all ties between mother and daughter: cell separation in budding yeast. *Molecualr Microbiology*; **55(5)**: 1325-1331.

Yoshida, S. and Toh-e, A (2001). Regulation of the localisation of Dbf2 and mob1 during cell division of *Saccharomyces cerevisiae*. *Genes and Genetic Systems*; **76(2)**: 141-147.

Yoshida, S., Ichihashi, R. and Toh-e, A (2003). Ras recruits mitotic exit regulator Lte1 to the bud cortex in budding yeast. *Journal of Cell Biology*; **161(5)**: 889-897.

Young, KH (1998). Yeast two-hybrid: so many interactions, (in) so little time... *Biology of Reproduction*; **58**: 302-311.

Yu, HG., Muszynski, MG. and Kelly Dawe, R (1999). The Maize homologue of the cell cycle checkpoint protein MAD2 reveals kinetochore substructure and contrasting mitotic and meiotic localisation patterns. *Journal of Cell Biology*: **145(3)**: 425-435.

Zachariae, W., Schwab, M., Nasmyth, K. and Seufert, W (1998). Control of cyclin ubiquitination by CDK-regualted binding of Hct1 to the anaphase promoting complex. *Science*; **282**: 1721-1724.

Zhao, X., Oh, SH., Cheng, G., Green, CB., Nuessen, JA., Yeater, K., Leng, RP., Brown, JA. and Hoyer, LL (2004). *ALS3* and *ALS8* represent a single locus that encodes a *Candida albicans* adhesin; functional comparisons between Als3p and Als1p. *Microbiology*: **150(7)**: 2415-2428.

Zhao, X., Oh, SH., Yeater, KM. and Hoyer, LL (2005). Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. *Microbiology*: **151(5)**: 1619-1630.

Zhao, X., Oh, SH. and Hoyer, LL (2007a). Deletion of *ALS5*, *ALS6* or *ALS7* increases adhesion of *Candida albicans* to human vascular endothelial and buccal epithelial cells. *Medical Mycology*: **45(5)**: 429-434.

Zhao, X., Oh, SH., Jajko, R., Diekema, DJ., Pfaller, MA., Pujol, C., Soll, DR. and Hoyer, LL (2007b). Analysis of *ALS5* and *ALS6* allelic variability in a geographically diverse collection of *Candida albicans* isolates. *Fungal Genetics and Biology*. **44(12)**: 1298-1309.

Zheng, X., Wang, Y. and Wang, Y (2004). Hgc1, a novel hypha-specific G_1 cyclin-related protein regulates *Candida albicans* hyphal morphogenesis. *EMBO Journal*; **23(8)**: 1845-1856.

Zordan, RE., Galgoczy, DJ. and Johnson, AD (2006). Epigenetic properties of white-opaque switching in *Candida albicans*are based on a self-sustaining transcriptional feedback loop. *Proceeding of the National Academy of Science of the United States of America*; **103**: 12807-12812.

Appendix 1

1.1. Alignments of the various FEAR proteins in *S. cerevisiae* and their *C. albicans* homologs.

1.1.1. Alignment of the *S. cerevisiae* and *C. albicans* seperase proteins.

```
S.cESP1     1 MMVKQEEPLNEISPNTPMTSKSYLLNDTLSK-----VHHSGQTRPLTSVL    45
                              ::::|.:        :......::||:...
C.aESP1     1 ----------------------MDNSLDQKLLAFISSQSSSKPLSPSK     26

S.cESP1    46 SGD------ASSNSIGILAMHNNIIRDFTKIASNNIDLAIEDITTVDHSL    89
                |.:      .|..|:.:.::  :.:.:.....:||..|.::.:..:.:.|
C.aESP1    27 SNNLPKADIKSLESLCVTSL-DTLLNNIGGSTTNNSILPLDSLDAIEVRL    75

S.cESP1    90 NSIYSLL-----KSHHMWGHINSTVKQHLMIIVKLINNNALGLASSEIIF   134
                ..:|.:.     ||..:      :::|.:.|||||.|      ..:|.:
C.aESP1    76 RGLYLIYADDPRKSFDI-------LRKHQLYIVKLFN-------IKQIDY   111

S.cESP1   135 LFNETNLFQAHSLK--------------NILLADFSTWND---YYLSNLK   167
                ::..|::.|...|              .|...||:.|||    ::..|.|
C.aESP1   112 VYQNLNVLLAQINKVFKIEFTSKSTIFTGIEFTDFNNWNDDTSFFNVNRK   161

S.cESP1   168 ILALQIILKRKLVDEYLPHILELFSHDKRYLLKDPNLKAHALTKIVLSFF   217
                .:...||....||.:::       .:|:.|  |||:..:.|   ..|.
C.aESP1   162 DITQLIIAFHFLVLQWI----------AQYVSK--NLKSILMQK--NQFI   197

S.cESP1   218 SVTTSCKV--LFGLKFLQYIKQFKLPFKKFISNITVECFSKNLLHKNYLE   265
                |:...|.:  :|  |....:.|...||       ||..:..::.|.:.   |.
C.aESP1   198 SLDEFCNIPQMF-LNSSNFRKWQLLP-----SNENIGKYNTNNVK---LI   238

S.cESP1   266 MGPNKIYLNSFYLSYSM-LYDGLDKIMLLDILSYEETTE-----------   303
                .|..|::  .|..:|.|: .|.||...:.|.:   ||.|.:
C.aESP1   239 SGFIKVF-QSIKVSSSLSKYVGLTNCLKLKL--YELTKDESLLESIGFSS   285

S.cESP1   304 -----VQRAIKSKKEF--------NEYCNMSENRLLWSCISVDDLNVILE   340
                :..|..:..||         :|..|..:.|||.|....|.|
C.aESP1   286 NLIPFINDANININEFIRKHVPIESEGSNELQTRLLKSPQLVTD------   329

S.cESP1   341 NATNFLQNKGKHISATL-KCLVCLWSTIRLEGLPKNKDILRQFDCTVI-Y   388
                ::||:|........:: ||..:.:|    ..|...|....:::  .
C.aESP1   330 --SDFLRNLQNDSQLSVDKCQIIISS---FNGFKDNLKTLSNQQLSILDL   374

S.cESP1   389 INSNIKSINDESAAALLSELLGVLSEICIDYKEPKRLSNIISVLFNASVL   438
                |..::|...|||..:||:...:|:....:.|.   :|..||:.||.::|||..
C.aESP1   375 ITIHLKDSLDESVVPILSQSFQIYSH----FKLMKRMRNISNLLFNLG--   418

S.cESP1   439 FKSHSFLLKTANLE----ISNVLISNDS-KTSHRTILK------------   471
                .|.|:.:.:...::     |..:..:||: |..:..:.:
C.aESP1   419 NKVHNEIYRNLAVDYECAIFGISPNNDNFKQLYNKLTRTQVTGSVLGNVL   468

S.cESP1   472 --FEKFISSAQSAQKKIEIFSCLFNVYCMLRNDTLSFV-----------F   508
                ||.:||...:  |...|...:.....|...:.|.:|.                :
C.aESP1   469 QCFETYISLTDN--KDDHINGTIIQFICKTLSQTSNFASLNNIKNENLKY   516

S.cESP1   509 DFCQNAFIHCFTRLKITKFIEFSNSSEIMLSVLYGNSSIENIPSENWSQL   558
                |...:.|         :|:|.||:::....:. ||.||   |.:|   .
C.aESP1   517 DIVNDIF----------QFMEKSNNTAEKTKIC--NSIIE---STSW--C   549

S.cESP1   559 SRMIFC----------SLRGIFDLDPLELNN-------TFDKLHLLNKYE   591
                :::|.|          .|....||:....:|         .|.:|......|
C.aESP1   550 NQLIECKIYYQYYNVNGLEKYLDLEEKSCDNQLVTSGIQFQRLVTFGWNE   599

S.cESP1   592 LLI-----RIVYLLNLDMSKHLTTNLSKITKLYINKWLQKSDEKAERISS   636
                .|:      .:.|.||.:.|:                          ...
C.aESP1   600 TLLVECLNNLKYGLNYNSSE-------------------------FDL    622

S.cESP1   637 FEMDFVKMLLCYLNFNN-FDKL--SIELSLCIKSKEKYYSSIVPYA----   679
                ||::.:|.:|.|:..:. .|:|  .||:||....|:.:.:..:.
C.aESP1   623 FELNIIKQVLLYIKYNGMIDELVDIIEISLAKSIKDEQFEKFLKFEYCRA   672

S.cESP1   680 ------DNYLLEAYLSLYMIDDALMMKNQLQKT----MNLSTAKIEQALL   719
                :|...:..:|  |..:|.:  :.||......|:    :.||.....|
C.aESP1   673 LLSQTNNNPTNDVKFSQIFLD--LKKRSQNWKSFDDIIQLSLLQLQYCIY   720

S.cESP1   720 HASSLINVHLWDSDLTAFQIYFGKTLPAMKPELFDINNDHNLPMSLYI--   767
                ..:|           :.|....:.|.....:|...|:|:...||||:...:
C.aESP1   721 AKNS----------IKAKDRFNGILQTLLKKPQFNISKSKNLPIVSKLQN   760
```

```
S.cESP1   768 -----KVILLNIKIFNESAKLNIKAGNVISAVIDCRKAQNLALSLLKKK-  811
              |..||...:.|......|:|.|.|...|        .|..|::||.
C.aESP1   761 FLILGKFQLLACHVNNHIIHNKIEAFNNIKTGI------QLLYSIVKKLP  804

S.cESP1   812 ---NK-LSQGSRLALLKSLSFSFFQLIKIHIRIGSARDCEFYSKELSRII  857
              ||  |.|....:.:..|:...|.:.|.||.:||..:..|...:.
C.aESP1   805 TNINKTLWQELNWEITRLLFDSYKLAIDLSIDIGISRDIPLFLNEWVKLN  854

S.cESP1   858 SDLEE--PIIVYRCLHFLHRYYMITEQTCLQNITLGKANKAFDYLDAEAD  905
              :.::.  ||:              .|:....:|:..
C.aESP1   855 NSIDNDVPIV----------------NCINEFEIGRYG-----------  876

S.cESP1   906 ITSLTMFLYDNKEFVKLEQSLVLYFGDQLEKTFLPNLWKLHLGKDIDDSI  955
              |..|.||.|..:......|..|.|. .:..:.::.:|.||.|
C.aESP1   877 -------LLSNNEFQKYIRIAQGRLGYSLVKN--NSAVQQYINRDRDDEI  917

S.cESP1   956 C---LSEYMPKNVINRVHNMWQKVMSQLEEDPFFKGMFESTLGIPSSLPV 1002
              |    .|....|::::.:.|.  ...:|:|.:            |:.:
C.aESP1   918 CGHASSSRRLKSLVRTIFNS-DNSLSELSK---------------SVQL  950

S.cESP1  1003 IPSTMPNNILKTPSKHSTGLKLCDSPRSSSMTPRGKNIRQKFDRIAAISK 1052
              :|                   |....||:|..:|           .:.|
C.aESP1   951 LP-------------------CIIGDSSTMCSKG-----------LLDK  969

S.cESP1  1053 LKQMKELLESLKLDTLDNHELSKISSLS-----------SLTLTILSNIT 1091
              |.|:|..:       |...:|:|  |.||||          :...:||::|
C.aESP1   970 LVQLKNEI----LTEVTNYE--KSSSLSLNQQQQLINNLNQVVCLLSSLT 1013

S.cESP1  1092 SIHNAESSLITNFSLTDLPRHMPLLFDKVLNNIDNKNYREFRVSSLIAPN 1141
              :....:..|...:.|.|...|::|...::.|  :|:....|   |:.:.|:
C.aESP1  1014 -LFKGDGLLSEIYYLQDYVRNLPFANERKL--MDSSKQDE---SNNLLPH 1057

S.cESP1  1142 --NISTITESIRVSAAQKDLMESNLNIN--------VITIDFCPITGNLL 1181
              :.:.:.|....:.....::.|:::.        ::|:|.|..||:||
C.aESP1  1058 ALDFNQVVEDPSNNTINNSMIDFNVDLQLYLPHNWILVTLDICQNTGDLL 1107

S.cESP1  1182 LSKLEPRRKRRTHLRLPLIRSNSRDLDEVHLSFPEATKKLLSIINESNQT 1231
              :|||........:||||.|..|        .|.|.|..::..||::||.:
C.aESP1  1108 ISKLTKGSPNPIFMRLPLSRFPS------SLGFQQLMQNFEKIIDDSNLS 1151

S.cESP1  1232 TSVEVTNKIKTREERKSWWTTRYDLDKRMQQLLNNIENSWFNG-VQGFFS 1280
              |..:|:||.|.:.||.||.::|.||.:.:||.:.||.|.:|  :.|.|:
C.aESP1  1152 TKRKTTSKILTVEDRKQWWRSRFTLDFQLQDILHHVESKWFGGFISGIFT 1201

S.cESP1  1281 PE-VVDN---SLFEKFKDKFYEILHQNLPSRKLYGNPAMFIKVEDWVIEL 1326
              .: .|:|    ::|.|||....:||...|.......|...|:..:::...
C.aESP1  1202 NDNDVENESKNVFHKFKQDLMKILKDCLTVSDDKSNIERFLQFNEFIYYC 1251

S.cESP1  1327 FLKLNPQEIDFLSKMEDLIYFVLDILLFHGE-ENAYDEIDFSMLHVQLEE 1375
              |..:.....:.    ::||||.|:...:..||. .|....:..:||     |
C.aESP1  1252 FYSMEEYNYEL---VDDLIKFITINMNSHGRIVNFGTNVKINKLH----E 1294

S.cESP1  1376 QIKKY---------------------------RATMTTNSIFHTFLVV 1396
              .|||:                          .....:|:..|..|:.
C.aESP1  1295 LIKKFDDKVIKTNQNVTSNNNNNNNNNNTNNNNNNNNSNSNNSQHIVLIP 1344

S.cESP1  1397 SSSCHLFPWECLSFLKDLSITRVPS-YVCLNKLLSRFHYQLPLQVTIEDN 1445
              :::|..||||.:.|:.:.||:|:|| ::.|.:.:.:....|::..|.
C.aESP1  1345 NANCSNFPWESMEFLRSKSISRMPSIHMLLDLVKSNTNNKNKLMFVDKSN 1394

S.cESP1  1446 ISMILNPNGDLSRTESKFKGMFQ-------KIIDAKPSSQLVMNEKPE-E 1487
              :..::||:||||.|:||||.|:|:::||.::|:        :|         .:|..||..: :
C.aESP1  1395 LYYLINPSGDLIRSENRFKKLFESNHLWRGEI------GKLSSNEHEDYQ 1438

S.cESP1  1488 ETLLKMLQNSNLFVYIGHGGGEQYVR-SKEIKKC-------TKIAPSFLL 1529
              :::|..:.:|:||||||||||.:||:: ||..||       .|:.||.||
C.aESP1  1439 DSILCEILKSHLFVYIGHGGCDQYIKVSKLFKKCGNNQDLSNKLPPSLLL 1488

S.cESP1  1530 GCSSAAM-----KYYGK-LEPTGTIYTYLLGGCPMVLGNLWDVTDKDIDK 1573
              ||||..:      .|... |:|:.|||.:|....|:|||||||||||||.
C.aESP1  1489 GCSSVKLDNCNYNYNSSMLQPSGNIYNWLNCKSSMILGNLWDVTDKDIDI 1538

S.cESP1  1574 FSEELFEKMGFRCNTDDLNGN-------SLSVSYAVSKSRGVCHLRYLNG 1616
              |:..|.:|.|.  .||.||:       .|.::...|:||.|.|.:||||
C.aESP1  1539 FTLSLLQKWGL---IDDYNGSGHDYGMKKLDLTNCVVQSRSKCTLKYLNG 1585

S.cESP1  1617 AAPVIYGLPIKFVS 1630
              :||||:|||||:...
C.aESP1  1586 SAPVVYGLPMYLK- 1598
```

1.1.2. Alignment of the *S. cerevisiae* and *C. albicans* Slk19 proteins.

```
S.cSlk19    1 MNEVPTTPVRLILGQAQQREQNSENCSQERN-PRTFNSEPDSSFNSPGSS    49
                         .|:.:. |:....|.........|.
C.aSlk19    1 -----------------------MSETKEAPKPTKQESQGILKKLTSG    25

S.cSlk19   50 QFVIHPHEPLEKEKDEKQDLDRSIDYGRSSALNN-----KNNANP-LE--    91
                ...:.|......|:|.|.:::..::.|.:.:    |.:.:| ||
C.aSlk19   26 DTWVSPFRSQASEEDPKKKINLYKQFKESNKIEHIKVPVKFSYHPTLEAE    75

S.cSlk19   92 -------NIDINKMFDDKKSDSGT--NDDKGGASTSDKHVLALNYSPIRV   132
                     ..:..|..:.|.:.:.|  .|.|....|..:.|....:.:.
C.aSlk19   76 AKKKKAAEAEAKKAKETKATVTSTEAKDAKEETETKAEKVEEKLETDAKE   125

S.cSlk19  133 EMNSSEKRSDKNVDV--------DENDKEGSHINKKLKLQLES------V   168
                .:....|.:.:..||.         ||::.|.|:::..::.:.:||.     |
C.aSlk19  126 PLIEDETKEEAKVDTEAKEPLIEDEDKQEPANEAEEKEAELEDKAEEKLV   175

S.cSlk19  169 PDLKQSSTKDIINDKEEIMSSPMAIDMIETNISPNKFIINDGVERNDSFN   218
                .|..:...:..||...:|.:....:|.:.:.:  |.||
C.aSlk19  176 TDQPKDEEIEVAEPKEVSAASEVEEQKLEKDAAEPE--IQDG--------   215

S.cSlk19  219 INTDTLKLENDINEKQQEEDFIKSNSNNVVNIDNAYKEKEDEENDITNSH   268
                :.||.:::| |||   ||.|...:|...:.:.    |...:.|..
C.aSlk19  216 ---EALKGDDNI-EKQ-----IKENPIELVDQPSQFKY---EPVELPNKE   253

S.cSlk19  269 -INRL--TPLYETSARESNSNEEG--RNDYDDDNQL-----DIRHDNFQ-   307
                :::|  .|:.....:|.|:...|  .|..:|.|:: .:|....|
C.aSlk19  254 TLDKLQDKPVLLKHYQELNAIGVGSIANRIEDPNKVIELGSGLRMTQQQL   303

S.cSlk19  308 --IVAKR--------NEELT-----DQIYHLNQMLNSLISKNESLSFQYE   342
                  |.|||         |:|::   |:|.. .|.|::.:.|:||
C.aSlk19  304 LDIAAKRVAPVITNINDEVSKTRQEDEIKR-QQGLDNKVKKHES------   346

S.cSlk19  343 KLNKNHQLLIDLTNEKLDKLNTERESDIAKVEKFKKRIK------ELNTE   386
                ||..:.:.:...:|.||.|.|.|..:|..|...|:..       |..|:
C.aSlk19  347 KLQSDFEKYLGKIGKKKDKFNKEIEEKLANFETMIKKSNNTAIEFEKTTK   396

S.cSlk19  387 IKVLNSNQKILQEKFDASITEVNHIKGEHENTVNTLQQN-EKILNDKNVE   435
                .:::.:|::  .|.:.|...|        :|.....|:::|  |::|..|.:|
C.aSlk19  397 GEIETANKE--YEEREAKAVE------QHATDKETIEKNHEELLATKKQE   438

S.cSlk19  436 LENMKAELKGNN------DKLSEYETTLNDLNSRIVQLNDKIESTDIVLK   479
                ||:.|.    |..      ::|.:.:|.|:|.||    :|.||||.....|.
C.aSlk19  439 LEDAKT---GQETATQEIEELKDKKTELDDKNS---ELADKIEQLTAQLN   482

S.cSlk19  480 SKENELDNLKLSLKETLSISKDFNDSDLIGQINELISTKNNLQQKMDDLN   529
                :::..:||:||.:.||     .:|..:|::|        :.|..|
C.aSlk19  483 TELAKLDDLKANHKE----HQDSIDANL--------TKKKEL--------   512

S.cSlk19  530 NLNDDNLKVVQDKLIKNEETLKLKEAEI--------------DSLNSEMD   565
                ||||.::::..|...:|..|...||:             .||:|:.:
C.aSlk19  513 ---DDNLTIIRKDLSDKKEKHKGLSAEVGVLAGSAAAYAARLTSLHSDKE   559

S.cSlk19  566 ELKKQITSKDDEFKMW-QSKYETVEDEAKIRNAEVTELNGDIEDLKESKL   614
                :...|::....|:...| :.|.|..|..|.:.....:...:.|.|
C.aSlk19  560 DRAKRLAEAKDKHASWLKEKRELAEQTARDHERKRLEATHEYETQK----   605

S.cSlk19  615 HLEETITELENKVHKLENECELEKQKFE----KTSLELESLQLKNSNIQA   660
                |||   |||.:...|.|.| ..||::.|       :...:|...::... :|
C.aSlk19  606 HLE----ELERQKQKEEELE-RKEKEEAEERERQAKIEAEERERKEKE-EA   649

S.cSlk19  661 EHIKELENLHENLISLQNELKISSDRITTLTKENEV-------LMEQNNN   703
                |....||.......:.:.|||.:.:....|:|:|      ::.:.:
C.aSlk19  650 EEKARLEKEQAEKEAKEKELKEQQEKEEAQKKKNDVEYQVSQAILSRETD   699

S.cSlk19  704 NN-------NSVTLSNDQKDRDDEKIKSLGKQVQDWK-EKYEAKEKDTNK   745
                ..          .|...:|::|.::.|....|.:::|.| ||.:|.|.:..:
C.aSlk19  700 QKKFEEERAESERIYNERKAKEKEETDRLLKEIEDLKAEKEKAAELEREE   749

S.cSlk19  746 RLKLLAEDL--YIQYSSKHEQKVKLLKKGYENKYQNKFDQLNLENKTLSE   793
                ..|:....| ..:.....:||.|.|| ::|.:...||:.:.|.|
C.aSlk19  750 AEKVAQAKLAQIEKLKQEHEAKTKL--------FNDKLEFENLQKQRLEE   791

S.cSlk19  794 EIEQLNKQLSSEREEKQELLK------LLENEKK---------------   821
                |:...| |:::...||||..|..     .||.||
C.aSlk19  792 EVANL-KKIRELREEKARLASEQYKDPELERVKKLIQERELEVDKLTKVI   840

S.cSlk19  822 --------------------------------------------------   821


C.aSlk19  841 EFEKPEQTSSEGGHFLGISNDSKSGAIPVIKSKDVVVGNNENSHSKSVNT   890
```

```
S.cSlk19   822 -------------------------------------------------   821

C.aSlk19   891 GNEAKTENESDDKKKDHHYGGAALGIAGGILAAAGAGALAAGSAGKNAVS   940

S.cSlk19   822 -------------------------------------------------   821

C.aSlk19   941 GIASGVSNAVPSGNKDVQLPSNGSGTQGNTTTSAPGQSQSSSPLATTTEP   990

S.cSlk19   822 -------------------------------------------------   821

C.aSlk19   991 SGSAASIPTSSNTSTISSKKNAASVTPTPVEAQRTNSLSDKFKGWGRKLS  1040

S.cSlk19   822 -------------------------------------------------   821

C.aSlk19  1041 KKDKSKDVNSAAGETTAETGVIAKDDDKPVGGITALDAEKNTVVARDAKP  1090

S.cSlk19   822 -------------------------------------------------   821

C.aSlk19  1091 IGGVTALDAEKNTVVARDTKPIGGINANDIEEKPSVGIFVSKNVSSNKTS  1140

S.cSlk19   822 -------------------------------------------------   821

C.aSlk19  1141 TDADDESFEIQSVYEVVSDSEYEANKHNPNYFEVSPEEFEKHKNLENKVV  1190

S.cSlk19   822 -------   821

C.aSlk19  1191 LIEKISE  1197
```

1.1.3. Alignment of the *S. cerevisiae* and *C. albicans* Zds1 proteins.

```
S.cZds1    1 MSNRDNESMLRTTSSDKAIASQRDKRKSEVLIAAQSLDNEIRSVKNLKRL    50
               .:|.:.:..|.      |....|.|.|:.|.:|..||||
C.aZds1    1 -----------MSSPNTSFHSD-----SNFESAVQDLEQEKKMVAALKRL    34

S.cZds1   51 SIGSMDLLIDPEL------DI-KFGGESSGRRSWSGTTSSSASMPSDTTT    93
               |||.| :..||:|      || .|...::...:.....:......|:.
C.aZds1   35 SIGHM-MQYDPDLPPGSMDDIDPFANNNNNSNTASNNNHYNGHTRDHTSN    83

S.cZds1   94 VNNTRYSDPTPLENLHGRGNS---------GIESSNKTKQGNYLGIKKGV   134
               .|||....|....| |.||.|      .|...|:.|:..:........
C.aZds1   84 NNNTHNHSPNSKLN-HHRGQSPYDEDLIPQNIHRSHSTRSRSKSHSTSPS   132

S.cZds1  135 HSPSRKL-------------------------------NANVLKKN---   149
               .||..|.                              |::||...
C.aZds1  133 TSPQHKQQQQQQPQPFPHEPQTPPYNKSPSPVKRRSFYDNSSVLTSESHD   182

S.cZds1  150 ---------------LLWVPANQHPNVKPDNFLELVQDTLQNIQLSDNGE   184
                              |||||||.||.|.|::|..|::
C.aZds1  183 IFFDAEDEVYDSSSPLLWVPANSHPQVNPESFKSLIK-------------   219

S.cZds1  185 DNDGNSNENNDIEDNGEDKESQSYENKENNTINLNRGLSRHGNASL----   230
                     ..:|...|.|.|:      .:||:....|||..:.|.
C.aZds1  220 ---------TQVEEILERKLSR------KSTISRKSTLSRSSSTSTKETL   254

S.cZds1  231 -------------IRRPSTLRR----SYTEFDDNEDDDNKGDSASETVNK   263
                           :..||.:|:      |.:::.|||...|.|.|:..:.:.
C.aZds1  255 APEPEISPESECDVSPPSPVRKSSLSSSSQQNQNEDVSRKSSSSVSSTSP   304

S.cZds1  264 VEE---------RISKIKERPVSLRDITEELTKISNSAGLTDNDAITLAR   304
               .::         ..||....| |||::|.||..::|..||:..|||:||||
C.aZds1  305 QKDPAKRESWYFNNSKRYSNP-SLRELTSELEQLSKMAGMDKNDAVTLAR   353

S.cZds1  305 TLSMAG-SYSDKKDQPQPEGHYDEGDIGFSTSQANTLDD----GEF-ASN   348
               |||...  .|:|.:...       :||.|....:|:.|.|.:   |.: ::|
C.aZds1  354 TLSAQSLGYTDVEKLA-----FDELDSSQTTATATTPNSSGSPGSYDSAN   398

S.cZds1  349 MPINNTMTWPERSSLRRSRFNTYRIRSQEQEKEVEQSVDE--------MK   390
               .|...:...:|     .:.:|...:|::    |:|.|:|...        :.
C.aZds1  399 PPRTTTLHLQQR---LQHQFQQAQIKA---EREAERSTRHQQSEQQWPVS   442

S.cZds1  391 NDDEER-------------------------LKLTKNTIKVEIDPHKS   413
               |||..:                         ||.::.|
C.aZds1  443 NDDSHKSSSQLTASEGGSTANAFTSAGSGADFALKRSRRT----------   482

S.cZds1  414 PFRQQDEDSENMSSPGS-------IGDFQDIYNHYR---------QSSG   446
               .:|:::.||:..:|..|      :..:.|.::|:.:         .|..
C.aZds1  483 DYRKKETDSKQKTSNNSPPTRKYNVRNSQLLFNYKKPVDSPSSSPSPSPS   532

S.cZds1  447 EWEQEMG--IEKEAEEVPVK--VRNDTVE-QDLE-------------LRE   478
               .::..||  ::.:.:.:.|::  :.|..:: .|:.            .|.
C.aZds1  533 TSQSMMGHRVKHKKSQKPLEAALANPMMDGSDMSHNPYPTASTTIDFSRM   582
```

```
S.cZds1   479 GTTDMVKPSATDDN---------KETKRHRRRNGWT--------------    505
              |......:.|.:.:|          .|.|.||    |::
C.aZds1   583 GAKKSARQSLSPENAMDGRSRTKPENKTHR---GYSHQERSHPYHQQPQP    629

S.cZds1   506 --------------WLNNKMSREDDNEENQG------------------D    523
                            ..:.:.:|..|..:.|                  .
C.aZds1   630 QVQPQTRQQLPPAQQAHRQSTRQTHNHPSTGVEKHHRQDNKRVMSSASNT    679

S.cZds1   524 DENEENVDSQRMELDNSKKHYISLFNGGEKTEVSNKEEMNNSSTSTATSQ    573
              |.|:....|.:.:.:.:.|....:.:.:||.....|:.....|.||..|.
C.aZds1   680 DINDFMAQSNQFQTNGTRNHRYDNLHKKDKTAFLPNEDHQRKSHSTRNSN    729

S.cZds1   574 TRQKIEKTFANLFRRKPHHKHDASSSPSSSPSSSPSIPNNDAVHVRVRKS    623
              .|        ||......|.|...|:.|.:|.|.....|.|.:....:.
C.aZds1   730 VR--------NLSSSSQQHLHQPYSTTSVAPKSRQLHQNLDKLRSEINEF    771

S.cZds1   624 KKLGNKS---GREPV--------EPIVLRNRPRPHRH--------HHSRH    654
              |:..|||    |.|..        :.....|.||.|.:|         |:.||
C.aZds1   772 KESLNKSELPGEESKREHRSRHDQHHQQRQRPAPSQHQLEPRNYNHNDRH    821

S.cZds1   655 GSQKISVKTLKDSQPQQQIPLQP-----------QLEGAIEIEK------    687
              ..|:       .:..||||..|||.            .:|..:.||:
C.aZds1   822 QRQQ-----HEHVQPQQVQPLQSDTSFDISYQDLSVEDQLGIEQEALREL    866

S.cZds1   688 -KEESDSESL---------------------------------------    696
               ||:..|..:
C.aZds1   867 GKEKGHSHEIDIDDAFDENLKXSPINERHGSQFTLDHDILDSFNLVDNQL    916

S.cZds1   697 -----------------------PQLQ----------------------    700
                                     ||.|
C.aZds1   917 VGSADEGIDNLKGKNEIPVGRQQPQQQRQQPRAASPPSSQQYLGHDELHL    966

S.cZds1   701 -----------PAVSV---------------------------------S    706
                         |.:|:                                  :
C.aZds1   967 QQGKDTNKKVGPRLSIDTLQNKPIHPEETATGFGMNALPSPTLHLDESQN   1016

S.cZds1   707 STKSNSR-----------------------------------------    713
              ||..:||
C.aZds1  1017 STPGHSRKASNSASYDDYYNIADKSSTAGTPKTKKETKVKTKLFNKDPNL   1066

S.cZds1   714 ---------------------------------------DREEEEAKKK    723
                                                     |..|.|..||
C.aZds1  1067 EIIDSDNYKEKMGIETSNNKKLKKKKSFGLLSTTSSVGANDTSENEGPKK   1116

S.cZds1   724 NKKRS--------------------------------NTTEISNQQHSK    740
              .||:.                                :...|.|..||.|
C.aZds1  1117 LKKKKSWGWLRERSASASSADINNLPPLPLDKLPTRSFSNPETSTDQHQK   1166

S.cZds1   741 H------------------------------------------------    741
              |
C.aZds1  1167 HDLENGSDLERELEHEPELELELESDLEFDYEQQRKHQDASMVNDSSFAV   1216

S.cZds1   742 -------VQKEN-------------------------------------    746
                     ..|||
C.aZds1  1217 DSISMKSTDKENVLSKFFKKKAKVPGSSSQSVFSFESKGSGASVDYESDN   1266

S.cZds1   747 ---------------------------------------------TDE    749
                                                           |.|
C.aZds1  1267 DAKSIKKKGNNSSRLFKKKSRAKLSEQENSVNKEKLRPLNLVSNESQTIE   1316

S.cZds1   750 QKAQL----------QAPAQEQVQTSVPVQAS-----------------    771
              :|..|          :..:|||.:...||.:|
C.aZds1  1317 EKENLRQSNGTRKAERVESQEQQEEQFPVTSSPIHQFNIEHLKDDFVTLG   1366

S.cZds1   772 -----------------------------APVQNSAP---------    779
                                          .|:||:..
C.aZds1  1367 EKDDVLDSGTDDLVEDVRSRNIQSTIVIVDEDETPIQNNNDNKDLGMLKV   1416

S.cZds1   780 ------------------------VQTSAPVEASAQTQAPAAPPLKHT    803
                                      ..|:..|...........:..|.:..
C.aZds1  1417 DELSKKKSISRKKRNNMQKKNLSTELTDTNKEVVEEVLATEQSVKPSQGE   1466

S.cZds1   804 SIL----PPRKLTFAD-----VKKPDKPNSPVQFTDSAFGFPLPLLTVST    844
              .:|    ...||..:     :|:..:.|.|.::||||||.|.::.||
C.aZds1  1467 DLLSKNEDKEKLDIQEKLKKSIKRTSRANQPIEFTDSAFGFPLPPPSQST   1516

S.cZds1   845 VIMFDHRLPINVERAIYRLSHLKLSNSKRGLREQVLLSNFMYAYLNLVNH    894
              ::|.|:|.|::|||||||||||||||:|.||.|||||||||||||||||||:|
C.aZds1  1517 LVMLDYRFPVHVERAIYRLSHLKLANPKRSLREQVLLSNFMYAYLNLVDH   1566
```

```
S.cZds1   895 TLYMEQVAHDKEQQQQQQQQP--------------------------- 915
              ||::||.....|...|.::..
C.aZds1  1567 TLHLEQQNMSSEDGDQMERDDDEEEEMTDTDEKDMIFGESNIADDDDLIP 1616

S.cZds1   916 --------------------------- 915

C.aZds1  1617 EEANGDSIGINLDMDGLHRKQHHQSGIEV 1645
```

1.1.4. Alignments of the *S. cerevisiae* and *C. albicans* Cdc5 proteins.

```
S.cCdc5     1 ---MSLGPLKAINDKQLNTRSKLVHTPIKGNTADLVGKENHFKQTKRLDP  47
              :...||::|..||.|:..: ||:|.....  |||      :.|
C.aCdc5     1 MSALRSQPLQPLNSGQLNARANNI-TPVKSIRRP---KEN-------VQP  39

S.cCdc5    48 NNDHHHQPAQKKKREKLSALCKTPPSLIKTR-GKDYHRGHFLGEGGFARC  96
              .:.      .||||:|||||||||||::|| |:||.||:|||||||||
C.aCdc5    40 TDQ-----KQKKKKEKLSALCKTPPSIVRTRNGRDYRRGNFLGEGGFARC  84

S.cCdc5    97 FQIKDDSGEIFAAKTVAKASIKSEKTRKKLLSEIQIHKSMSHPNIVQFID 146
              ||:||.||:|:||||||||||||:|||:.||||||:|||:.|||||.|:|
C.aCdc5    85 FQMKDASGQIYAAKTVAKASIKNEKTKTKLLSEIKIHKSLRHPNIVNFVD 134

S.cCdc5   147 CFEDDSNVYILLEICPNGSLMELLKRRKVLTEPEVRFFTTQICGAIKYMH 196
              |||||.|||||||||||||.|||||.||.::||||||..||.||||||:|
C.aCdc5   135 CFEDDVNVYILLEICPNQSLMELLKTRKRVSEPEVRFFMVQIVGAIKYLH 184

S.cCdc5   197 SRRVIHRDLKLGNIFFDSNYNLKIGDFGLAAVLANESERKYTICGTPNYI 246
              ||||||||||||||||||...||||||||||:||.:.:...|||||||||
C.aCdc5   185 SRRVIHRDLKLGNIFFDPEMNLKIGDFGLASVLPSTDSRKYTICGTPNYI 234

S.cCdc5   247 APEVLMGKHSGHSFEVDIWSLGVMLYALLIGKPPFQARDVNTIYERIKCR 296
              ||||||.||::||||||||||:::|:|:||||:|||||||||:|||.|||||..
C.aCdc5   235 APEVLGGKNTGHSFEVDIWAIGIMMYALLVGKPPFQAKDVNVIYERIKKT 284

S.cCdc5   297 DFSFPRDKPISDEGKILIRDILSLDPIERPSLTEIMDYVWFRGTFP-PSI 345
              ::.||.|||||:...|.||.|:|||||:.||::.||:.|.||:|.|| .:.
C.aCdc5   285 EYYFPEDKPISESAKQLIKDLLSLNPLHRPTIDEILSYDWFKGPFPDKTY 334

S.cCdc5   346 PSTVMSEAPNFEDIPEEQSLVNFKDCMEKSLLLESMSSDKIQRQKRDYIS 395
              ..:.:..|...:.|...||..|||...:.:.:...:.:...::.|..|
C.aCdc5   335 EVSLQTLPPGLDQITRAQSSANFKYAKDSAGIYTPKNKNPVEILRADLHS 384

S.cCdc5   396 SIKSSIDKLEEYHQNRPFLPHSLSPGGTKQKYKEVVDIEAQRRLNDLARE 445
                          .|.|..||.|||||..|:.||:|||          |..:.
C.aCdc5   385 ------------EQPRTLLPSSLSPNDTRNKYQEV----------DPGQL 412

S.cCdc5   446 ARIRRAQQAVLRKELIATSTNVIKSEISLRILASECHLTLNGIVEAEAQY 495
              .|.||          :..|:|........|..:..|..:..:......:
C.aCdc5   413 GRPRR----------VNFSSNFSTAIKRLNQVCFETYQTMRRLEHS---- 448

S.cCdc5   496 KMGGLPKSRLPKIKHPMIVTKWVDYSNKHGFSYQLSTEDIGVLFNNGTTV 545
              :...:..|..|..::|.:::||||||||||:||||||:..:|||||||:...|:
C.aCdc5   449 RHDEVDASEFPNCENPTLISKWVDYSNKYGFSYQLNNDDIGVLFNDENTL 498

S.cCdc5   546 LRLADAEEFWYISYDDREGWVASHYLLSEKPRELSRHLEVVDFFAKYMKA 595
              |:...::.|..:.|.:.|||......|:..|.::..|.||:|||||||||.:
C.aCdc5   499 LKRHNSDRFLELIYHENEGWTCIENSLANPPAQAKRQLEIVDFFAKYMNS 548

S.cCdc5   596 NLSRVSTFGREEYHKDDVFLRRYTRYKPFVMFELSDGTFQFNFKDHHKMA 645
              |||:||..    |..|:.|||||||.....:|||:::|||:::.||||||||:.
C.aCdc5   549 NLSKVSEI---EERKETVFLRRYTRTPDYIMFEMTNGNFQFNFKDHHKIC 595

S.cCdc5   646 ISDGGKLVTYISPSHESTTYPLVEVLKYGEIPGYPESNFREKLTLIKEGL 695
              ||..|..:|:|||....:.||..|.|:.||||..........||.:||:.
C.aCdc5   596 ISKTGLAITHISPGRLTQTQPLISVLKQGNFQHENVPDCMEKIMVIKEAI 645

S.cCdc5   696 KQKSTIVTVD    705
              |:|:....
C.aCdc5   646 KKKAFKEA--    653
```

1.1.5. Alignment of the *S. cerevisiae* and *C. albicans* Spo12 proteins.

```
S.cSpo12    1 MSNKASDQSARTASILKTDITRENTITRSSSSNNDNYHHHNNINNYNESA  50

C.aSpo12    1 -------------------------------------------------   0

S.cSpo12   51 KTGEDANKENIPNLEEEIAAFRIFRKKSTSNLKSSHTTSNLVKKTMFKRD 100
              ..||...|.|.....:        :|:...|.....|.||.: :.:|.|.
C.aSpo12    1 MIGEIVVKLNCKKRKPK-------KKEKNMNTNFLQTLSNPI-RGIFNRK  42
```

```
S.cSpo12  101 LLKQDPKRKLQLQ-QRF---ASPTDRLVSPCSLKLNEHKVKMFGKKKKVN  146
              ..||:.::|:.|: ||:  .||:|:...|.|.::|.:.....||.|.|
C.aSpo12   43 KNKQNQEKKILLKDQRWLFTQSPSDKTTKPKSKRINNYNTFRSRKKSKKN   92

S.cSpo12  147 ----------PMKLNFKGNLAADSEDVEIDEDEEYFY  173
                        |:...|
C.aSpo12   93 KRAWYLCIIMPLGSLF--------------------  108
```

1.1.6. No Fob1 homolog was found in the *C. albicans* genome.

1.1.7. No Zds2 homolog was found in the *C. albicans* genome.

1.2. Alignments of the various MEN proteins in *S. cerevisiae* and their *C. albicans* homologs.

1.2.1 Alignments of the *S. cerevisiae* and *C. albicans* Lte1 proteins.

```
S.cLte1    1 --------------------------------------------------    0

C.aLte1    1 MPQDNPESTNLTGDIGNNNNNNNKGEQQSPLSGQDIAKNNQDHDNIQEIL   50

S.cLte1    1 --------------------------------------------------    0

C.aLte1   51 QSDSIFKTPEQVETHDILSTLTSNCYNEQGQELNLQGHQQINIHPDFYYN  100

S.cLte1    1 ---MEIFSQKDYYP-TPSSN----VISYESDCVSKPVNSADLPALIVHLS   42
               ..||..:..:| .|::|    ::.|: :.....:.|.|.:.|||:.::
C.aLte1  101 KHNESIFDDERIFPCLPTTNDNEDLVKYD-EFNPELINHATIKALIIQMT  149

S.cLte1   43 SPLEGVDYNASADFFLIYRNFITPQDLHDLLIYRFRWCIREITTNAAKAK   92
             || |.:||:...||||.||.|....:::||:.|..|.::.:  |:....
C.aLte1  150 SP-EIIDYSLICDFFLTYRMFSNSNEIMNLLLTRLIWALQYL--NSVSED  196

S.cLte1   93 RRRIGEVALVRTFVLLRHSILNYFVQDFLPN-------------ITLRLR  129
             ..:||::.|||||||:|||.|:||||.||..|          |||...
C.aLte1  197 NIKIGKLVLVRTFVVLRHWIINYFVDDFNNNNELCDYFTNTINKITLESN  246

S.cLte1  130 LI-------EFLNDKHIEQYPKIISSCIINLKKNWVHCSKLVWENIELNE  172
             ||       ..::..|.....||:.  :||.:|:  |::     ||
C.aLte1  247 LIPQQQEPTNKVDSNHSLFTRKILG----DLKIHWL---KII------NE  283

S.cLte1  173 PDKLDFDAWLHYSLKD-------------FTQLESLHKRGSRLSIYARQS  209
             ...||.|  :|..|.:              ...::.|.|..:..||.
C.aLte1  284 FWLLDLD--IHSMLTNNSAYDYILPLTSTLNNIKKLSKSNTEASIR----  327

S.cLte1  210 FASPDFRNQSVLSLYKTSDVFRLPEKL------QSSNSSKN-QRSPSMLL  252
             ::|.:|..::||||..    |:..|:      .|:|..:| |.|.:.||
C.aLte1  328 -SNPSYRRSAMLSLYDQ----RIQHKMLIFDDTSSNNQQENPQFSINNLL  372

S.cLte1  253 FPDNTSNV--------------------YSKHRIAKEPSVDNES-----  276
             ...::|.|                    .|...|.|.|.:.|.:
C.aLte1  373 LHHHSSRVSINNKIKTIQQQKKLLKSPTFSSSFNIPKSPLISNSNLANIP  422

S.cLte1  277 ----------------EDMSDSKQ-------------KISHLSKVTLVST  297
                             ...|::|:          |.|.|......:|
C.aLte1  423 SPTPAPTHTLTTPTRLHSRSNNKRALMQSNRHNHMNIKDSSLDLKKTTTT  472

S.cLte1  298 LMKG-----------------------VDYPSSYAVDK---------  312
             :.:.                       ||:...:.|.
C.aLte1  473 ISRNFDRTDIVEQEGDTDFDEEKENEETEEVDFQVHDIDNDKSPESQFS  522

S.cLte1  313 -------------------------IMPPTPAKKVEFILNSLYIPEDL  335
                                      |:||||.||:|    ....|.:|:
C.aLte1  523 ESESKRYSQVGFSTNGNIKLPTSKVTVIVPPTPVKKME---KPPLIEDDI  569

S.cLte1  336 NEQSGTLQGTSTTSSLDNNSNSNSRSNTSSMSVLHRSAI-GLLAKWMKNH  384
             ...|.|.............:|||  ..|..:.:..|.:| .|:..|.|:.
C.aLte1  570 QPSSMTPPKLYHPQFQPPPSSSNS-IRTENEDLNRRGSIRKLMDNWKKSL  618

S.cLte1  385 NRHDSSNDKKFMSAIKPANQKP---EMDAFVKYVVSISSLNRKSSKEEEE  431
             ..|..|:....|....:.:||     ::|..:...:|: :|.|:
C.aLte1  619 IVHKVSSSATDMPINRTMSTKPSTEDLDRLINNAMSV--MNEKT------  660

S.cLte1  432 EFLNSDSSKFDILSARTIDEVESLLHL-----QNQLIEKVQTHS------  470
             ....:.|:|||||.|||:|.|:..      .::|:|::..|.
C.aLte1  661 ----VIGKRVDVLSARIIDELEYLIRYYISSESSSVIHEMNYHDDTGDDM  706
```

```
S.cLte1   471  -----NNNRGPTVNVDCERREH-----------IHDIKILQQNSFKPSND  504
               |:|.......|.:.|.|              :|.|::      .|:..
C.aLte1   707  EMEVINDNDNDLEEEDDDDRPHRRTRQDKGDVDLHPIEV------SPTRA  750

S.cLte1   505  NFSAMDNLDLYQTVSSIAQSVISLTNTLNKQLQNNESNMQPSPSYDALQR  554
               :...||..||...        :::.:.|.:|....:|..::
C.aLte1   751  DEIEMDINDLSDL------NIVKIDNLINDTTADNSHSV-----------  783

S.cLte1   555  RKV-KSLTTAYY-NKMHGSYSAESMRLFDKDN----SSSRTDENGPQRLL  598
               || ::::|:|: :::  ....:.|..|.||    .||.|....|..:.
C.aLte1   784  -KVPRNISTSYFLDEI--DIGNDNDRDNDNDNENVKESSSTSFQSPISIN  830

S.cLte1   599  FHETDKTNSEAITNMTPRRKNH------------SQSQKSMTSSPLKNVL  636
               :::.|..|.:......||..:.            :.:.:.:||:..|...
C.aLte1   831  WNDQDNINLDGSIEKTPLEEEFEGFEFSQEITTTTNNNNNSTSNDKKQTQ  880

S.cLte1   637  PDLKE-------SSPLNDSREDTESITYSYDSELSSSSPPRDTVTKKSRK  679
               ||..|       |...:.|:..:.|.::|...|.||.|.| ..:|:...:
C.aLte1   881  PDYSEPNFSFQHSQSQSYSQFQSHSHSHSKSHETSSISTP-SNITQYDAE  929

S.cLte1   680  VRNIVNNTDSPTLKTK-TGF---LNLREFTFEDTKSLDEKKSTIDGLEKN  725
               :..:........::|.|  ..|   .|:....|....|::.|..:.:..:
C.aLte1   930  IEELGIAMSPQSIKPKRISFCENTNINSAGFNKRLSVWSKNSITNSISNS  979

S.cLte1   726  YDN------KENQES--EYESTKKLDNSLDASSEANNYDI---TTR----  760
               ..|       :::.:|   .|:|...:.|..:.:|..|::.|    ||.
C.aLte1   980  NSNGSIAFKRDSMKSYVSYDSAFSVFNESNGNSNGNSFKIENTTTEYNGL 1029

S.cLte1   761  KKHSSCNHKIKQAVVRPASGRISISRVQSIAITPTKELSIVDPEQNKSNS  810
               ||.::.|:  .:::|..:.:...|::..        ..:|...||.
C.aLte1  1030  KKKTAFNN--LRSIVNNSGDGINLENA---------IRVVSSNQNH---  1064

S.cLte1   811  VIEEISEIEPLNLEYNKKSALYSDTSSTVISISTSKLFESAQNSPLKQTQ  860
                                          :|:|.|
C.aLte1  1065  ---------------------------LSLSLS---------------  1070

S.cLte1   861  NPQREFPNGTSVSETNRIRLSIAPTIESVVSDLNSITTGSTVETFETSRD  910
                 |....|:........|.       |.|.::|             :
C.aLte1  1071  ---RHSSKGSGSGSRKSVRF---------STLCALT-----------E  1095

S.cLte1   911  LPVPHQRIINLREEYQRGNQDIISNTSSLHELKTIDLSDSNNDLESPSTH  960
               ||. |:.|....|.....|.::.:|.::.:.|    |.|:.|
C.aLte1  1096  LPF-HEHIAIHNETIISTNANVATNNNNCNHSK----SSSSGD------- 1133

S.cLte1   961  AKNNKYFFSPDDGSIDVASPMKNVEELKSKFLKNESETNSNISGSVLTMD 1010
               |.|:..|                 .|..|..:...|
C.aLte1  1134  AANSSIF------------------SLAMKSRKSSIRT----------- 1153

S.cLte1  1011  DIDINDTSSARNTRRANSES---AFTGSLNKKNLNEIANMLDDSI--NDD 1055
                :|..||.::|...||.:   |..| ::.....|.|:|.:.|:::   .:|
C.aLte1  1154  ---VNKESSTQSTAFTNSSNNSVAIPG-ISSYALKELAAIPDETMLSTED 1199

S.cLte1  1056  PITVALMKLEG-----------------------TYEKIPEKPENTK-- 1079
               ||..||.||||              .|.|.|...::|:
C.aLte1  1200  PIQYALHKLEGKSSSKSTLKRKDDDDDDNDDDDDDEYLKQPHIDDDTQDI 1249

S.cLte1  1080  --------SSDAIGIKTSKLADEVEMLNLNNLP---SFQNSPAEKRKSLL 1118
               :.||||:..:.: |:..:.....|   |.:||.::..|
C.aLte1  1250  LNEINNANTEDAIGLSNTSI--EISQVDPPLTPIRMSCRNSNSDMTES-- 1295

S.cLte1  1119  IERRRQTIMNIPFTPDQSEK------------EGFTSSSPEKIDVSAN-V 1155
               :.....|..|..:..:|.::            :.|....|.|.:.|.. .
C.aLte1  1296  -DPITSTPRNSQYHENQQQQNHNHNGGDNNVIDTFDFQQPSKPNESTTPP 1344

S.cLte1  1156  DVAVQAAQIQELIGQYRIHDSRLMISN---NESHVPFILMYDSLSVAQQM 1202
               .:..|:.:|  ::..|......|.:||    |::|:.|:|.||||.|:|...
C.aLte1  1345  SLEYQSPKI--ILDNYSPSSDLLSVSNVLFNDAHISFVLSYDSRSLADHF 1392

S.cLte1  1203  TLIEKEILGEIDWKDLLDLKMKHEGPQVISWLQLLVRNETL---SGIDLA 1249
               |:||||::|.|||||||||::||...|....|.|||::::.:.   .|::|.
C.aLte1  1393  TMIEKDMLQEIDWKDLIELKWNKELTPVNSWLEIIVNDDYYIENKGVNLV 1442

S.cLte1  1250  ISRFNLTVDWIISEILLTKSSKMKRNVIQRFIHVADHCRTFQNFNTLMEI 1299
               |:||||.|:|||||||||||:|...:.:|.||||:|.||.||..|||.|||:
C.aLte1  1443  IARFNLMVNWIISEILLTQSPNERIHLISRFIHIAQHCLELQNFATLMQI 1492

S.cLte1  1300  ILALSSSVVQKFTDAWRLIEPGDLLTWEELKKIPSLDRNYSTIRNLLNSV 1349
               ||||:|..:||....|:.:.|||:|.::.|||:|.:.:|::.|..:|..:.:
C.aLte1  1493  ILALTSQRIQKLKQTWKDLIPGDILLLKNLEELASPLKNFLNIRLTINQI 1542
```

```
S.cLte1 1350 NPLVGCVPFIVVYLSDLSANAEKKDWILE-------------------DK 1380
             .|..||:||:.:|||||:.|||:...|.|                    |.
C.aLte1 1543 KPSKGCIPFVGLYLSDLTFNAERASIIKEHNHNLSGDGDGDGEGEGEGDT 1592

S.cLte1 1381 VVNYNKFDTNVQIVKNFIQRVQWSKFYTFKVNHELLSKCVYISTLTQEEI 1430
             ::|:.||.|:|.|||:..|..|.::||.|.|.:.:||||:||.:|.:::|
C.aLte1 1593 LINFAKFRTSVHIVKSLSQCIEWSSNYKFDIQPDLLSKCLYIKSLDEDEM 1642
S.cLte1 1431 NELST---- 1435
             .....
C.aLte1 1643 KYCIQHNQK 1651
```

1.2.2. Alignments of the *S. cerevisiae* and *C. albicans* Bfa1 proteins.

```
S.cBfa1    1 -MSIRPLTLNGLDEPETSFEELNTTLPRFQSHETLTLEENVPPLSTSTYI   49
             :|.:..:|....|.::.|.:::..  .:...|||.:.:...|.|:
C.aBfa1    1 MVSDKLTLLRQFSEEDELFGDIEGI--DYHDGETLKINKFSFPSSASS--   46

S.cBfa1   50 PPPSSVGTSDTGTVFSNSTSAFWSNKQADDDQDMEVDQDDEFLNDFQEFQ   99
             ||...|..:.:.|.:...........::..::.|.|...||.:  |||:
C.aBfa1   47 --PSFAITGQSPNMRSINGKRITRETLSEYSEENETDLTSEFSD--QEFE   92

S.cBfa1  100 NKKDDFDDAIKTNFHLRNGCRTGPFKNDIFAEEFDRKL----------SL  139
             .|.|:                   ||....::.:::|          :.
C.aBfa1   93 --WDGFN-----------------KNQSIYQQMNQRLIATKVAKQREAE  122

S.cBfa1  140 EDKPRLKQ-------PRSMMELKPKRKLSNSVTSRNLRSGNSVRFKKSMP  182
             .:::..|.|        |...:.||...||:|.
C.aBfa1  123 REQRELMQKRHKDYDPNQTLRLKDFNKLTNE------------------  153

S.cBfa1  183 NLALVNPAIREEEEDEEREREDQREFNYKIDNDTQDTILAKFSSDDEGDF  232
             ||.|::             :.:|::..||            :::..||..||
C.aBfa1  154 NLTLLD------------QLDDEKTVNY-----------EYVRDDVEDF  179

S.cBfa1  233 LTGFE-----ELEGEAIDETISSNDKESADHPRFLKKSSSSLPLKISPAQ  277
             ..||:     :|..:...|:.||...        |||..|
C.aBfa1  180 AQGFDKDFETKLRIQPSMPTLRSNAPT-------LKKYKS----------  212

S.cBfa1  278 YDIVKHDELLTPGLHRRQRDWNTQQELDSFKEKRSVR---------HCSN  318
             |...|.|..:...|.|....:|..|.|.|.||||.:|..     .|.|
C.aBfa1  213 YGEFKCDNRVKQKLDRIPSFYNKNQLLSKFKETKSYHPHHKKMGTVRCLN  262

S.cBfa1  319 QNVQLNGPAKIKTIKQQIDHNTPMKKGSMIYNPKTMKWEGNENVLSKFSD  368
             .|.:: |....:|        .:|..|.:..:|||||:..|.:|
C.aBfa1  263 NNSEV--PVTYPSI------------SNMKLNKEKNRWEGNDIDLIRF--  296

S.cBfa1  369 VDTANRKALLIKNKLQRDADSKKQKYSDLQHARATSRNQKVIGNMILDEQ  418
             .|..||.:   :..:||::            |||:.|||
C.aBfa1  297 -----EKPSLITHK---ENKTKKRQ-----------------GNMVYDEQ  321

S.cBfa1  419 NLRWVSVSEEEADPFAGIPEINLPPVGKSMKKRSSSPFLRSKSQVNTPFV  468
             ||||::: |.|.|.|..||::.:        |:..|| :|..|| |.
C.aBfa1  322 NLRWINI-ESEHDVFDDIPDLAV--------KQLQSP-VRGLSQ----FT  357

S.cBfa1  469 SNDNDGVYQSTAAQARLRKYHSMRTLNGTTETPEISSTFHLSSRALEKFY  518
             .........:||........|              |.|.:|.:.::||.
C.aBfa1  358 QRTTSTTATATAPSKNNETQH---------------SDFEISRKLVDKFQ  392

S.cBfa1  519 HEENRWCKKLASWFIPRDETIISVDEETIMDESTVNSKRKSYMYEIRNMV  568
             .|:.:..||:..|||                .|.:......::|||.|:
C.aBfa1  393 KEQAKIEKKINHWFI-----------------DTTSEFNTDHYWEIRKMI  425

S.cBfa1  569 INSTKD    574
             |..
C.aBfa1  426 IEE---    428
```

1.2.3. Alignment of the *S. cerevisiae* and *C. albicans* Bub2 proteins.

```
S.cBub2    1 -----------MTSIEDLISNPPLLLHSSLSQLRYLILSEGLPISEDKQQ   39
                         ..|||..||...|.|.:.|||||||::|.|||...|...|
C.aBub2    1 MEQELLLKGRKKDSIEQFISGSQLFLENGLSQLRYMVLVEGLSTPEGYDQ   50

S.cBub2   40 QRTRCYVWTVLSQTSMEASTQRYLALLKLG----PPSTTIYQKIKNDTSR   85
             ...|.|||::|.:.. .:.|::||.||:..     |....| |||||||.|
C.aBub2   51 CPYRSYVWSILCRVP-PSPTEQYLQLLETSSQTLPDELLI--KIKNDTFR   97

S.cBub2   86 TFQTDPNFRNRVSEDALIRCLSCFAWQTQQRRQKTRFGRIPVSTYVQGMN  135
             |...|..|.::|:|::|||.|||...|:          ..||||:|
C.aBub2   98 TLMNDKKFHSKVNEESLIRILSCIGITTE------------VGYVQGLN  134
```

```
S.cBub2   136 VLLAPLLYSC-PSEPMAYQLFTKLCYEMIPTYLTKNLNGAQNGAKLLDIS    184
              ||||||:.|.| .|||.|:.:...|...:||.|:|.|:.|...|.:|:|..
C.aBub2   135 VLLAPIAYVCHKSEPQAFAILHNLITKQIPLYITPNMEGVHTGLQLVDAV    184

S.cBub2   185 LRIIDPKLSKFLSDNLLTAEIYGMPSILTLSSCNKPLDQVIKLWDFMFAY    234
              |:|||||.||.:|....|.|:|||..||||||.:|..||..|:|||||:|||
C.aBub2   185 LKIIDPVLSDYLDSKFLKAKIYAFPSILTLCACTPPLKSVLKLWDFLFAY    234

S.cBub2   235 GFHMNILFVVAFLVKMRSKVFKSDSPVNLLRQFPDFDADEIIRLGVGFIA    284
              |.|:|:||||||.|:..||.:.:|:.|:.:||.:|:.:.:|||:|.:.||.
C.aBub2   235 GTHINVLFVVAQLIICRSSILESEQPMKILRTWPNLEENEIIKLSLSFIP    284

S.cBub2   285 KIPAQIYDLLVDHLTDPDIYI---------    305
              ::|.:.||||:...|..|..:..
C.aBub2   285 ELPEKLYDLIARHGYDRKVPKELARFLEEK    314
```

1.2.4. Alignment of the *S. cerevisiae* and *C. albicans* Tem1 proteins.

```
S.cTem1     1 MATPSTGANNSIPAVRNQVEVQVGLVGDAQVGKTSLMVKYVQNIYDKEYT    50
              |...:....|        |||.|.:::||:||:|:|||||||||:..:|::|.
C.aTem1     1 MEEDNQQDQN------NQVALKIGLIGDSQIGKTSLMVKYVEGSFDEDYI    44

S.cTem1    51 QTLGVNFLKRKVSIRSTDIIFSIMDLGGQREFINMLPIATVGSSVIIFLF   100
              |||||||:..:|:.||:|.|.|||.||||||:||||||||||..:..:.:|:|:|
C.aTem1    45 QTLGVNFMDKKIQIRNTTITFSIWDLGGQKEFINMLPLVSNDAVAILFMF    94

S.cTem1   101 DLTRPETLSSIKEWYRQAYGLNDSAIPILVGTKYDLLIDLDPEYQEQISR   150
              ||||..||:|||||||||..|.|.:|||.||||||||||||..|||..:.|.:|::
C.aTem1    95 DLTRKSTLNSIKEWYRQVRGFNKTAIPFLVGTKYDQFIDLSFQDQIEITQ   144

S.cTem1   151 TSMKYAQVMNAPLIFCSTAKSINIQKIFKIALAKIFNLTLTIPEINEIGD   200
              .:.|:...|.||:||||||:.|||:||||||||.|:|.|:|.|.:.||..:|:
C.aTem1   145 QAKKFGSAMKAPVIFCSTSHSINVQKIFKIILSKAFDLKLNLDEIVNVGE   194

S.cTem1   201 PLLIYKHLGGQQHRHHNKSQDRKSHNIRKPSSSPSSKAPSPGVNT       245
              |:||||
C.aTem1   195 PILIYK---------------------------------------       200
```

1.2.5. Alignment of the *S. cerevisiae* and *C. albicans* Cdc15 proteins.

```
S.cCdc15    1 --------------------------------------------------    0

C.aCdc15    1 MVLNDFDLKTEALNERTTLHTSQNASSKNGSNKESVLAKYEETNQNDEIE    50

S.cCdc15    1 --------------------------------------------------    0

C.aCdc15   51 GFENLNDLSFSKYSKFSKASIIVGSDPKSRTTTLDSPFAKKLKEDLNVEF   100

S.cCdc15    1 --------------------------------------------------    0

C.aCdc15  101 TSDNTKPYYPTVYPIYEFDNNDENSDSPFLDYQNEKGNIPQKLSGQKLRK   150

S.cCdc15    1 --------------------------MNSMADTDRVNLTPIQRASEKS     22
                                        .:.....:.:|:||||.|:..::
C.aCdc15  151 QQQQQQQQSEAEENFKQSNQYARLQSTPKHQRTSSVKVSLTPAQKFEKRT   200

S.cCdc15   23 V--------QYHLKQVIGRGSYGVVYKAINKHTDQVVAIKEVVYENDEEL    64
              .          .:.|..::|.|.|::..||:|:|..|:|||:|||::..|.|.:::
C.aCdc15  201 STRSHNALDNFELTTLVGNGAFASVYRAVNLKTNQVIAIKQIRIEKDQDV   250

S.cCdc15   65 NDIMAEISLLKNLNHNNIVKYHGFIRKSYELYILLEYCANGSLRRLISRS   114
              ..:|.||.||||.|.|.||||||||:::.:.|.:|||||..||||||:|..:.
C.aCdc15  251 GVLMGEIDLLKILKHRNIVKYHGFVKTATSLNVLLEYCEGGSLRQLYKKL   300

S.cCdc15  115 STGLSENESKTYVTQTLLGLKYLHGEGVIHRDIKAANILLSADNTVKLAD   164
              ..||.|.:....||.|.|.||.||||:||:|||:|||||:||||:...|||||
C.aCdc15  301 KKGLPEYQIINYVRQILEGLNYLHEQGVVHRDVKAANVLLTDKGDVKLAD   350

S.cCdc15  165 FGVSTIVNSSALTLAGTLNWMAPE-ILGNRGASTLSDIWSLGATVVEMLT   213
              |||:|.|.|.|..||:.||.||||||| :||..|..|||||||:||::|:.|
C.aCdc15  351 FGVATKVTSQHLTVVGTPNWMAPETVLGGEGICTASDIWSLGATIIELFT   400

S.cCdc15  214 KNPPYHNLTDANIYYAVENDTYYP-PSSFSEPLKDFLSKCFVKNMYKRPT   262
              .||||||:|......:|:..|.:.| |.:.|...||||.:||.|.....|.:
C.aCdc15  401 TNPPYHDLNAMATLHAIGTDEHPPLPKNMSTLAKDFLLECFQKQANLRIS   450

S.cCdc15  263 ADQLLKHVWINSTENVKVD----------KLNKFKEDFTDADYHWDADFQ   302
              |..||||.|:|.|....|.      :|...|......|.:||.||:
C.aCdc15  451 AKLLLKHKWLNQTATAKTSMATLLRQPSRELRSIKIYSESNDENWDEDFE   500
```

```
S.cCdc15   303 EEKLNISPSKFSLAAAPAAWAENNQELDLMPP-TESQLLSQLKSSSKPLT   351
               |.|:    |||..:.:..        ::|.|. ||.|.  :|...|| .
C.aCdc15   501 EIKV----SKFQNSTSSI--------IELKPDNTEKF--KLTEYSK--K   534

S.cCdc15   352 DLHVLFS---------------VCSLENIADTIIECLSRTTVDKRLITA   385
               ||..||:              ...|..:|:::.|......:|
C.aCdc15   535 DLLTLFADEKEDTSGNFEFETIGPNKLAVVAESMDESDPFLNID------   578

S.cCdc15   386 FGSIFVYDTQHNSRLRLKFIAM--------------GGIPLIIKFE---   418
                |..:||.....:.:::::.:         ..:|.::|..
C.aCdc15   579 ---IENFDTNELEVQSKMEYLVVRLSRKLEQVHLGYEDAVPALVKVTGRM   625

S.cCdc15   419 -HLAKEFVIDYPQTLIECGIMYPPNFASLKTPKYILELVYRFYDLTSTAF   467
                ||.|::.:.:...:.:.|::           .:|||:..|.|:.|...
C.aCdc15   626 LHLVKKYPVSHDTLIRDHGVL------------SLLELLESFQDIPSQQQ   663

S.cCdc15   468 WCRWCFKHLDISLLLNNIHERRAQSILLKLSSYAPWSFEKILPSLIDSKL   517
               ....|..      :||::.|.         .|.:.....:.|...:|::..
C.aCdc15   664 LWYHCLS------ILNHVFES-------DLGTLENFCFLGGIPTVAHF--   698

S.cCdc15   518 KKKILISPQITYVVFKSINYMITTNDDKIHKSAIPSSSSLPLSS------   561
               :......|:...|.|.|..:   |..:...|...|...|.|.|
C.aCdc15   699 -RSATYEVQVRLQVAKFIGIL---NLSEKALSMFVSCGGLRLVSKFVEED   744

S.cCdc15   562 -SPTRNSPVNSVQSPSRSPVHSLMATRPSSPMRHKSISNFPHLTISSKSR   610
               ..|...|:.:::|     :|:::|        |.:|.      |||.
C.aCdc15   745 FDTTPTFPLIAIES-----IHNILA---------KDLSR-------SKSD   773

S.cCdc15   611 LL-IELPEGFFTWLTSF------FVDMAQIKDLS-------------VLK   640
               |. |....|...||...       :.....||::|            ::|
C.aCdc15   774 LCRILSKHGVIFWLVVLLNRLVKYDQKPSIKNVSKEQVEITIERIIDIIK   823

S.cCdc15   641 YF----TKLCYLTVHINSTFLNDLLDNDAFFAFIRNID-----TIIPFID   681
               ||    ||   :.:.|.|..|..|:      |....|:.      |:::.|:.
C.aCdc15   824 YFSQSETK---VRISIGSIDLFKLI-----FKLFDNLKLSHQLTLLKFVK   865

S.cCdc15   682 DAKTAAFIWK-----QITAICVEMSLD-----------MDQMSASLF-ST   714
               .....:.:.|       :|....|::...            ::.::..|: |.
C.aCdc15   866 SMSCISEVLKNLYHAEILEFLVKLLKSYIPSKGNYKEIINVLAPILYNSL   915

S.cCdc15   715 AMNFIRKKNNTSISGLEII--LNCLHFTLRNVNDDVAPTVGSSESHSVFL   762
               |:|..|:....::.||..:  |.:.:...|..   :.|.:...
C.aCdc15   916 ALNHRRESEFVNLGGLPYLKTLSIINLPFRQF---ILPIICEF-------   955

S.cCdc15   763 IKVNNDAAI--ELPIDQLVDLFYALNDD---------------------   788
                |:.||::   ||..:.:|.::|.|..|
C.aCdc15   956 --VHCDASVVNELKKNDIVKIYYNLLLDPYWQSNALDSLHCWYKLEPSYI  1003

S.cCdc15   789 DVNLSKLISIFTKICSLPGFENL--TINIIF-------------HPNFY   822
               |:|...:........||...|| |:.|.|                :.|..
C.aCdc15  1004 DLNSPMAVDCLVGGFLLPKVSNLESTLEIYFKLLTNNLPLTRDMSNMNVI  1053

S.cCdc15   823 EKI---VSFFDTYFNSLLIQIDLLKFIK-----LIFSKSLLKLYDYTGQP   864
               ..|   :|..|.  .:.:||:..||.:|      |:.|||.|..      .
C.aCdc15  1054 NSILIKLSLHDK--KNPVIQLSYLKVLKCLINYLVDSKSSLPF------A  1095

S.cCdc15   865 DPIKQTEPNRRNKATVFKLRAILVQITEFLNNNWNKDVPKRNSNQVGGDS   914
               .|:..|..:.:...:...:.|...:.|.
C.aCdc15  1096 KPVVNTLQSLKSRQSSLLIEEVTTELLSVLK-------------------  1126

S.cCdc15   915 VLICQLCEDIRSLSKKGSLQKVSSVTAAIGSSPTKDERSNLRSSKDKSDG   964

C.aCdc15  1127 -------------------------------------------------  1126

S.cCdc15   965 FSVPITTFQT   974

C.aCdc15  1127 ----------  1126
```

1.2.6. Alignment of the *S. cerevisiae* and *C. albicans* Dbf2 proteins.

```
S.cDbf2      1 -----------------------MLSKSEKNVDLLAGNMSNL------S    20
                                      .:|.|.:||.:.:..|.::     |
C.aDbf2      1 MTNFFNRSPKHQSHHYQPHQQDVTDISYSMENVSISSNAMMDIDTSYRSS   50

S.cDbf2     21 FDGHGTP-----------GGTGLFPNQNITKRRTRPAGI-NDSPSPVKPS   58
               ...:.:.|           ....||..:|||...:....| ::||...|.|
C.aDbf2     51 KPTYNNPQQQQQQQQQQQQAQNNLFNKENITPLNSPTKSILHNSPQQAKSS  100

S.cDbf2     59 FFP-----------YEDTSNMDIDEVSQPDMDVSNSPKKLPP--------   89
               ..|           |...|......:.|.:.:.:|:..:|.|
C.aDbf2    101 TSPQHLYNKLVNANYNGNSPQPGIQQQQNNRALQNNINQLQPPLNKRYKL  150
```

```
S.cDbf2    90  ---KFYERATSNKTQRVVSVCKMYFLEHYCDMFDYVISRRQRTKQVLEYL  136
               :||.:|..:.|.::|:|:.|:.::|||::||||||||||:||:||..|.:.|
C.aDbf2   151  TEAEFYAKANSARTKRLTSIAQLYFLDYYCDMFDYVINRRERTAIVEKNL  200

S.cDbf2   137  QQQSQLPNSDQIKLNEEWSSYLQREHQVLRKRRLKPKNRDFEMITQVGQG  186
               .......|  |..|...||.:|:.||..:|||||||||::|||||||:|||
C.aDbf2   201  LTDPMYKN-DITKQQFEWKNYIGRERALLRKRRLKPKHKDFEMITQIGQG  249

S.cDbf2   187  GYGQVYLARKKDTKEVCALKILNKKLLFKLNETKHVLTERDILTTTRSEW  236
               ||||||:|:||:||:|:|||||||||||||.||:||:||||||||||.|||:|
C.aDbf2   250  GYGQVFLSRKRDTREICALKILNKKLLIKLDETRHVLTERDILTNTRSDW  299

S.cDbf2   237  LVKLLYAFQDLQSLYLAMEFVPGGDFRTLLINTRCLKSGHARFYISEMFC  286
               |||||||||||.:.::||||||||||||||.||..|...||||||||||.
C.aDbf2   300  LVKLLYAFQDQEKVFLAMEFVPGGDFRTLLNNTGYLIPPHARFYISEMFA  349

S.cDbf2   287  AVNALHDLGYTHRDLKPENFLIDAKGHIKLTDFGLAAGTISNERIESMKI  336
               |||:||:||:|||||||||||:||||||||||||:.|:||||||||
C.aDbf2   350  AVNSLHELGFTHRDLKPENFLIDSKGHIKLTDFGLAAGTVCNDRIESMKI  399

S.cDbf2   337  RLEKIKDL--------------EFPAFTEKSIEDRRKMYNQLREKEINY-  371
               :|:..|:|               :.|:   ..|.:|:|::.|.:::::..
C.aDbf2   400  KLQNFKNLNILNDDSNNDNRHYQVPS---SLIYERQKIFKQSQQQQQQQN  446

S.cDbf2   372  -----ANSMVGSPDYMALEVLEGKKYDFTVDYWSLGCMLFESLVGYTPFS  416
                    |||:||||||||||||||||.|:::|:|||||||||||:|.||.|||
C.aDbf2   447  SNNTTANSIVGSPDYMALEVLEGKNYNYTIDYWSLGCMLFEALCGYPPFS  496

S.cDbf2   417  GSSTNETYDNLRRWKQTLRRPRQSDGRAAFSDRTWDLITRLIADPINRLR  466
               ||..:|||.||:.||..|||:..|||..|||||.|||||||::||.|.|||:
C.aDbf2   497  GSKQDETYYNLKHWKTALRRPQTKDGRYVFSDRTWNLIIKLIASPNNRLQ  546

S.cDbf2   467  SFEHVKRMSYFADI-NFSTLRSMIPPFTPQLDSETDAGYFDDFTSEADMA  515
               :|:.|::.|||:||  ::..||...|||||||:|.|||||||..::.|.
C.aDbf2   547  NFKQVQQQSYFSDIKDWGNLRQKTPPFTPQLDNEEDAGYFDDFEDDEMMM  596

S.cDbf2   516  KYADVFKRQDKLTAMVDDSAVSS---------------------KLVGF  543
               ||.|||.||::....::.|..::                    ..:||
C.aDbf2   597  KYKDVFARQEQNEQLLEKSNTTTTTTTTTTTKNGKRFSPGSKFNDNFIGF  646

S.cDbf2   544  TFRHR-------------------NGKQGSSG--ILFNGLEHS------  565
               ||:|:                   ||...::|  .|.|.:|:.
C.aDbf2   647  TFKHKSNPNNKFTNGSGNTGRYGNGNGNNNNNGEINLLNMVENGNGIGNG  696

S.cDbf2   566  -------DPFSTFY  572
                      :|.:|.|
C.aDbf2   697  NSRSSRLNPLATLY  710
```

1.2.7. Alignment of the *S. cerevisiae* and *C. albicans* Mob1 proteins.

```
S.cMob1     1  MSFLQNFHISPGQTIRSTRGFKWNTANAANN----AGSVSPTKATPHNN-   45
               |:..|||:.  .::||||||...::..::   .||.|  .||.:|
C.aMob1     1  MALFQNFNT---HSLRSTRGFKLKQSSPISSPQPIGNSVLP--QTPFDNS   45

S.cMob1    46  --------------TINGNNNNANTINNRADFTNNPVNGYNESDHGRMSP   81
                             |||.||.|
C.aMob1    46  QRPHLDQTEQNPYQTINFNNGN--------------------------   67

S.cMob1    82  VLTTPKRHAPPPEQLQNVTDFNYTPSHQKPFLQPQAGTTVTTHQDIKQIV  131
                                              |..|::|:||:...
C.aMob1    68  ----------------------------------GQPVSSHKDIRNYA   81

S.cMob1   132  EMTLGSEGVLNQAVKLPRGEDENEWLAVHCVDFYNQINMLYGSITEFCSP  181
               |.|||||:..|.||||||||.||.||||||:|.||||||||||||||:|||||||
C.aMob1    82  EQTLGSDNALIQAVKLPRDEDVNEWLAIHVVDFYNQINMLYGAITEFCSP  131

S.cMob1   182  QTCPRMIATNEYEYLW----------AFQKGQPPVSVSAPKYVECLMRWC  221
               .|||||||||.||||||           ..|..:.|||:.|.:|:|.||.|.
C.aMob1   132  ATCPRMIATEEYEYLWQESAPTNQDGTVQSPKRPVSLPACEYIENLMNWV  181

S.cMob1   222  QDQFDDESLFPSKVTGTFPEGFIQRVIQPILRRLFRVYAHIYCHHFNEIL  271
               |:.||:::||:|:...||..|  .:::.|.:||||:|||||||||||||:|.
C.aMob1   182  QNFFDNDNIFPTKIGAPFPHQF-PTLVKTIFKRLFRIYAHIYCHHFHEVS  230

S.cMob1   272  ELNLQTVLNTSFRHFCLFAQEFELLRPADFGPLLELVMELRDR   314
               ||.||:.|||||.:|:.|||.||:|:.....:|||.:||..:.|
C.aMob1   231  ELGLQSHLNTSLKHYVLFANEFQLISRKDYGPLEDLVDTMLQR   273
```

1.2.8. Alignment of the *S. cerevisiae* and *C. albicans* Cdc14 proteins.

```
S.cCdc14    1 MRRSVYLDNTIEFLRGRVYLGAYDYTPEDTDELVFFTVEDAIFYNSFHLD    50
              |..|......||||:.|:||||||:...||::|.:||||||:.||:|::|
C.aCdc14    1 MHSSSVHVPLIEFLKNRIYLGAYDHHKRDTEDLAYFTVEDALPYNAFYMD    50

S.cCdc14   51 FGPMNIGHLYRFAVIFHEILNDPENANKAVVFYSSASTRQRANAACMLCC   100
              |||:||||||||||:.|:.||:.....|.:|.|||.|.::|||.||||||
C.aCdc14   51 FGPLNIGHLYRFAVLLHKKLNEDSTQGKGLVIYSSTSPKERANLACLLCC   100

S.cCdc14  101 YMILVQAWTPHQVLQPLAQVDPPFMPFRDAGYSNADFEITIQDVVYGVWR   150
              ||||:|.|.||||||||:||:.||...||||||||:||:||||||||.:||
C.aCdc14  101 YMILLQNWAPHQVLQPIAQITPPLQAFRDAGYSSADYEITIQDVVYAMWR   150
S.cCdc14  151 AKEKGLIDLHSFNLESYEKYEHVEFGDFNVLTPDFIAFASPQEDHPKGYL   200
              |||:|:|||..|:|:.||:||.|:.||||||::.|||||||||:
C.aCdc14  151 AKERGMIDLAKFDLDEYEQYERVDQGDFNVISKDFIAFASPQQ-------  193

S.cCdc14  201 ATKSSHLNQPFKSVLNFFANNNVQLVVRLNSHLYNKKHFEDIGIQHLDLI   250
              :|...||:||:.||.|:|..||||||||||||||||:.|.|....|:|:|:|
C.aCdc14  194 -SKRGGLNEPFQKVLEYFVENNVQLVVRLNSHLYDAKEFTKRNIKHIDMI   242

S.cCdc14  251 FEDGTCPDLSIVKNFVGAAETIIKRGGKIAVHCKAGLGRTGCLIGAHLIY   300
              |:|||||.|...|:.:.|:||||.||.:||||||||||||||||||||||||
C.aCdc14  243 FDDGTCPTLEYVQKFIGAAECIINKGGKIAVHCKAGLGRTGCLIGAHLIY   292

S.cCdc14  301 TYGFTANECIGFLRFIRPGMVVGPQQHWLYLHQNDFREWKYTTRISLKPS   350
              |:|||||||||.::|.||||||||||||||||.:||.|::|..:.::|.
C.aCdc14  293 THGFTANECIAYMRMIRPGMVVGPQQHWLYLHHDDFRSWRHTMIVDNRPD   342

S.cCdc14  351 EAIGGLYPLISLEEYRLQKKKLKDDKRVAQNNIEGELRDLTMTPPSNGHG   400
              ..||.|:||.|.|:|    |::||:.||..:.::.:|    |.|.....
C.aCdc14  343 PLIGNLFPLCSYEDY---KQRLKEAKRKERLQLQQQL-----TSPLADSS   384

S.cCdc14  401 ALSARNSSQPSTANNGSNSFKS---SAVPQTSPGQPRKGQNGSNTIEDIN   447
              .::.      |......|.:..|    :.||..||||||||....|..|::
C.aCdc14  385 VINT-----PIRRRKVSGALASKIQTVVPIESPGQPRKYFEDSEDIDEV-   428

S.cCdc14  448 NNRNPTSHANRKVVIESNNSDDE-SMQD-TNGTSNHYPKVSRKKND----   491
                         ::|   ||||||  :|||   ...:...|..|:.|..|
C.aCdc14  429 -----------EMV---NNSDDENTMQDIIQSSPARYDSVTPKTKDNSDW   464

S.cCdc14  492 -----ISSASSSRMEDNEPSATNINNAADDT-----------ILRQLLPK   525
                   ||:.:.|..:.......|......::|            :||....:
C.aCdc14  465 RVLRSISTNNVSSQQSIHIIKTTTTKTVNETLSSPPGTSPTNVLRVSKAR   514

S.cCdc14  526 NR-RVTSGRRTTSAA--GGIRKISGSIKK-    551
              ::  |:.||...||.|   ||:||:||   ||
C.aCdc14  515 SKNRIASGNSQTSRAHSGGVRKLSG--KKH  542
```