

Functional analysis of the vacuole in *Candida albicans*

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by

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Marcus Marvin, Glen Palmer and Raymond Whyteman (Left to Right), Pike Market Place, Seattle 2000, during the Yeasty Boys Millennium US tour.

Functional analysis of the vacuole in *Candida albicans*

Glen Palmer

Abstract

The fungal vacuole is an acidic membrane bound compartment, containing a range of hydrolytic enzymes. Its functions include recycling of cellular proteins through degradation, storage of cellular metabolites, and homeostasis of the cytoplasmic environment. In the model eukaryote *Saccharomyces cerevisiae*, vacuolar function is non-essential for vegetative growth, since mutant strains deficient for vacuole function are viable. However, vacuolar function appears to be more important for survival under conditions of nutritional, osmotic, and temperature stress. Furthermore, mutants deficient in vacuolar hydrolase activity are unable to sporulate. This suggests that the vacuole plays an important role during processes of adaptation and differentiation. The vacuole has been well characterised in the model fungi *S. cerevisiae*, and *Aspergillus nidulans*, but to date little is known about the functions of the vacuole in the human fungal pathogen *Candida albicans*. It has been postulated that the vacuole is likely to play a central role in the adaptation of *C. albicans* to host environments during the process of infection. Moreover, the vacuole has previously been observed to undergo rapid expansion during the emergence of a germ-tube from a yeast cell, to occupy the majority of the parent yeast cell. This process of the yeast-hyphal switch has been implicated in virulence.

The class-C *vps* mutants of *S. cerevisiae* are defective in vacuole biogenesis and lack a vacuolar compartment. In this study *C. albicans* homologues of the *S. cerevisiae* class-C *VPS* genes, have been identified. Consistent with a role in vacuole enlargement during the yeast-hyphae switch, transcription of a *VPS18* like ORF (*CaVPS18*) was found to be increased during the process of germ-tube formation. Disruption of a *C. albicans VPS11* homologue (*CaVPS11*), resulted in a number of phenotypes similar to that of the class-C *vps* mutants of *S. cerevisiae*. Furthermore, a *cavps11* null strain was delayed in the emergence of germ-tubes upon serum induction of filamentous growth, and had a reduced apical extension rate compared to its parental strain. These results support a model whereby vacuole enlargement is necessary to support the rapid emergence and extension of the germ-tube from the parent yeast cell. Further analysis of vacuolar function in *C. albicans* should elucidate some of the processes underlying the yeast-hypha switch.

Abbreviations

AIDS	Acquired immuno deficiency syndrome
ARS	Autonomous replication sequence
bp	Base pairs
BPS	Bathophenanthroline disulphonic acid
BSA	Bovine serum albumin
CEN	Centromeric sequence
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide 5'-triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine 5'-triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
5-FOA	5-fluoroorotic acid
GADPDH	Glyceraldehyde-3-phosphate dehydrogenase
HGT	High gelling temperature
HIV	Human immunodeficiency virus
kb	Kilobase pairs
kDa	Kilodaltons
LA	Luria agar
LB	Luria broth
LGT	Low gelling temperature
M	Molar
MAP kinase	Mitogen-activated protein kinase
MOPS	3-(N Morpholino) propanesulphonic acid
mRNA	Messenger RNA
nt	Nucleotides
ORF	Open reading frame
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Synthetic defined
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
TE	Tris-EDTA buffer
TAE	Tris-acetate electrophoresis buffer
Tris	Tris(hydroxymethyl)aminomethane
v/v	Volume by volume
w/v	Weight by volume

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Chapter 1

Introduction

1.1 Overview

The number of infections caused by the dimorphic yeast *Candida albicans* have increased significantly over recent decades. This organism has now become the leading cause of fungal infection in humans. This has attracted an intense research effort to increase understanding of the epidemiology and pathogenesis of *C. albicans* infections. A number of 'virulence factors' that contribute to its disease causing capability have so far been identified, these include adherence to host tissues, the secretion of proteases, and the ability to produce different morphological forms. *C. albicans* cells readily switch between yeast, pseudohyphal and true hyphal forms, and this adaptive capacity plays a crucial role during infection and disease progression (Calderone and Fonzi, 2001).

The fungal vacuole is an acidic membrane bound compartment, with a wide range of functions including the degradation of cellular material, storage of metabolites, and homeostasis of the cytoplasmic environment. Whilst non-essential for vegetative growth in *Saccharomyces cerevisiae*, the functions of the vacuole are of increased importance for survival during periods of stress or adaptation (Klionsky *et al.*, 1990; Takeshige *et al.*, 1992). However, to date, little is known about the specific functions of the vacuole in the human pathogen *C. albicans*. Interestingly the membrane bound vacuole compartment has been observed to undergo rapid expansion during germ-tube emergence from *C. albicans* yeast cells (Gow and Gooday, 1982b). It was therefore postulated that a functional vacuole is likely to play a central role in the adaptation to, and colonisation of mammalian tissues by *C. albicans*. The purpose of this study was to assess the functional significance of the vacuolar compartment in the human pathogen *C. albicans*. In particular, the importance of the vacuole in supporting the transition from growth as ellipsoid yeast cells to filamentous hyphal forms.

The following sections of this chapter discuss current knowledge relevant to this research field and the approaches taken within this project. Firstly, the epidemiology of *C. albicans* infections, and some of its recognised virulence factors are reviewed. This is followed by a more detailed account of the underlying mechanisms and regulation of the yeast-hypha transition, with emphasis on the behaviour of the vacuole during this process. Subsequent

sections describe the general functions of the vacuole, delivery pathways to the vacuole, and some of the protein components required for vacuole delivery and biogenesis. Finally, previous research within our laboratory is described, and the specific aims and objectives of this project stated.

1.2 *Candida albicans*

1.2.1 Epidemiology of *Candida* infections

Candida albicans is a commensal organism which can be isolated from the mouth, vaginal tract, gastro-intestinal tract and skin of many healthy individuals (Odds, 1988). However, changes in the homeostasis at these sites can trigger *C. albicans* proliferation, resulting in an increase in *C. albicans* colonisation at mucosal membranes, leading to tissue invasion and damage. Such changes in homeostasis are often associated with underlying disease or treatment for an unrelated condition; as such *C. albicans* is often described as an opportunistic pathogen. The infections caused by *C. albicans* fall into two distinct categories, superficial muco-cutaneous infections such as oral and vaginal candidiasis, commonly known as thrush, and more serious systemic infections of internal organs and the blood.

Hospital acquired fungal infections have been steadily increasing over recent decades. Beck-Sague *et al.* (1993) reported that from 1980 to 1990 the rate of hospital acquired fungal infections throughout the U.S. rose from 2.0 to 3.8 infections per 1000 discharged patients. By far the most frequently isolated fungal pathogen was *C. albicans*, accounting for nearly 60% of fungal infections during this period. Moreover, the proportion of fungal infections caused by *C. albicans* increased from 52 to 63% from 1980 to 1990. Jarvis (1995) reported that from 1986 to 1990 *Candida* species collectively were the sixth most commonly isolated nosocomial pathogens in from United States hospitals, and the fourth from intensive care units (10.1%). In addition, *Candida* species were found to be the fourth most commonly isolated pathogen from nosocomial bloodstream infections (Jarvis, 1995). The increase of nosocomial *Candida* infections has coincided with improvements in the prognosis of seriously ill patients, increased use of broad-spectrum antibiotics, invasive procedures such as surgery, organ transplantation, aggressive anti-cancer therapies and an increase in the number of HIV infections.

There is a high mortality rate of 38-59% associated with candidemia (Komshian *et al.*, 1989; Wey *et al.*, 1988), and these infections correlate with a significant increase in the length of patient hospitalisation (Wey *et al.*, 1988). This is due in part to difficulties in diagnosing the systemic form. Blood cultures from patients with extensive invasive *Candida* infections are often negative (De Repentigny and Reiss, 1984). An alternative method for detection of systemic *Candida* based on the detection of the candida antigen enolase in patient's sera is also unreliable with just a 52% success rate (Walsh *et al.*, 1991). In the past tests based on the detection of antibodies against *Candida* species have proved equally inaccurate (Filice *et al.*, 1977). Coupled to the diagnostic difficulties are problems of effective treatment for candidiasis. Treatment of fungal infections is inherently difficult as both fungal and host cells are eukaryotic. It is therefore difficult to find compounds that act specifically as antifungals, and host toxicity is often a problem. The azole fungicides such as fluconazole provide effective treatment for mucocutaneous candidiasis, and have more recently been used to treat invasive infection without serious toxicity. These act by inhibiting the biosynthesis of ergosterol, leading to alterations in membrane structure and function (Georgopapadakou and Walsh, 1994). The current antifungal of choice for the treatment of systemic *Candida* infections is amphotericin B, which directly binds to membrane sterols leading to cytoplasmic leakage and cell death (Georgopapadakou and Walsh, 1994). Amphotericin B binds with a higher affinity to fungal ergosterol than mammalian cholesterol (Georgopapadakou and Walsh, 1994), but is toxic to the host (Edwards, 1991). Antifungal resistance has also been on the increase, particularly to the azole fungicides among isolates from AIDS patients (Matthews, 1994; White, 1997).

Current clinical opinion supports the hypothesis that the majority of hospital acquired *Candida* infections result from a strain endogenous to the patient (Wenzel, 1995; Powderley *et al.*, 1993; Voss *et al.*, 1994). Data supporting patient to patient transmission has also been reported (Vasquez *et al.*, 1993; Schmid *et al.*, 1992). The relative importance of colonisation versus cross-transmission is likely to differ according to environmental factors and the degree of patient contact.

1.2.2 Predisposing risks to candidiasis

A number of factors have been identified that predispose an individual to developing candidiasis. Many of these weaken the immunity of the host. A broad range of chemical,

physical and even emotional stress has also been noted to affect the susceptibility of a host to fungal infection (Mishra *et al.*, 1994). Mishra and colleagues (Reszel *et al.*, 1999) demonstrated that *C. albicans* was more likely to be isolated from vaginal swabs taken from women undergoing divorce and showing signs of depression than control patients. Precipitating antibodies were also significantly more common among the depressed patients.

Candida infections may often be indicative of an underlying (congenital or acquired) disease state. Mucocutaneous candidiasis is commonly associated with diabetes mellitus (Mishra *et al.*, 1994), and oral candidiasis can often be a sign that a HIV⁺ individual has developed full blown AIDS (Cannon *et al.*, 1995). Burns patients are also particularly susceptible to *Candida* infections (Jarvis, 1995). The administration of various medical procedures or therapeutic agents for an underlying condition has also been correlated with an increased risk of candidiasis. An increased risk of nosocomial *Candida* infections has frequently been associated with invasive techniques such as catheterisation (Wenzel, 1995), and surgery (Mishra *et al.*, 1994; Edwards, 1991). The use of multiple or broad-spectrum antibiotics presents a further risk (Wenzel, 1995) due to the depletion of other competing endogenous bacteria (Kennedy and Volz, 1985). Vancomycin has in particular been associated with an increase in the number of *Candida* organisms in stool samples (Jarvis, 1995), which in turn has been associated with an increased risk of blood borne *Candida* infections (candidemia). More severe therapies such chemo- and radiotherapy also result in the host's immune system being compromised, this explains the high incidence of *Candida* infections in patients with various cancers such as leukaemia and lymphoma (Meunier-Carpentier *et al.*, 1981). Mishra *et al.*, (1994) demonstrated that in an animal model irradiation or treatment with the chemo-therapeutic agent methotrexate, increased gastro-intestinal (G.I.) tract colonisation and systemic infections by *C. albicans*, resulting in a high mortality rate among these animals. Chemotherapeutic agents are also particularly active against the rapidly dividing cells of the gut mucosa, and the work carried out by Mishra *et al.*, supports idea that a damaged gut may act as a major portal of entry for *Candida* leading to its dissemination to other organs.

The relative importance of the various host defence mechanisms differ with different sites of *Candida* infection. Cellular immunity seems to be more important in combating superficial mucocutaneous infections (Matthews, 1994). For example, AIDS patients,

defective in cell-mediated immunity are prone to oral and oesophageal candidiasis, but rarely suffer systemic infections. Humoral, immunoglobulin mediated immunity appears to be more important in the prevention of systemic candidiasis (Matthews, 1994).

1.2.3 Putative *Candida albicans* virulence factors

Several features of *C. albicans* have been implicated in contributing to its disease causing ability (reviewed in Cutler 1991; Navarro-Garcia *et al.*, 2001). A virulence factor can be considered to be a gene or protein, the absence of which results in a reduction in the ability of a pathogen to cause disease. *C. albicans* appears to have a complex set of virulence factors that work collectively to allow this yeast to act as a pathogen. It also appears that the relative contribution of each virulence trait is highly strain dependent. The importance of a given virulence factor may also vary according to the site of infection, and with the course of disease. When present as a commensal organism, some of these virulence factors are likely to aid colonisation of healthy individuals. However, under various host compromising conditions, they act to facilitate the invasion of host tissues. Thus, it is the dynamic equilibrium between the host's immune defences and the pathogenic capability of a given *C. albicans* strain that determines the yeast's clearance, colonisation, or invasion at any given site.

Adherence

Adherence to host tissues is necessary for the initial colonisation of a host, to avoid the many 'flushing' mechanisms that operate in mammals, and to facilitate the invasion of host tissues. It is unlikely that *C. albicans* would be maintained as a commensal of the G.I. tract without a means of attachment. Kennedy and Volz (1985) demonstrated that the elimination of the indigenous microflora in the guts of Syrian hamsters resulted in increased colonisation and subsequent systemic infection by *C. albicans*. They suggested that this was due to an increase in the availability of adhesion sites usually occupied by the bacterial microflora. Mishra *et al.*, (1994) demonstrated that the administration of a chitin extract, known to inhibit adherence of *C. albicans*, reduced colonisation, systemic infection and mortality in a murine model of systemic infection originating from the GI tract. Segal *et al.*, (1988) also found that a chitin extract significantly reduced adherence and virulence in a mouse model of vaginitis. Thus it seems that adherence plays a key role in the establishment of candidiasis.

C. albicans has been shown to adhere to epithelial and endothelial cells which are likely to be important in mucosal and disseminated infections respectively (Cannon *et al.*, 1995; Klotz, 1992). *C. albicans* also specifically binds components of the host extra-cellular matrix (ECM) including fibronectin, laminin, and collagens I and IV (Klotz, 1992). Antibodies raised to *C. albicans* laminin and fibrinogen binding proteins have been used to demonstrate the presence of these receptors at both mucosal and systemic sites of infection (Lopez-Ribot *et al.*, 1996).

One class of adhesin found at the surface of *C. albicans* resembles mammalian integrins. Integrins are proteins which mediate the attachment of cells to the ECM, through the binding to specific target peptide sequences (Hynes, 1992). Monoclonal antibodies to human integrins were found to identify surface proteins on *C. albicans* (Gilmore *et al.*, 1988). In addition peptides containing the integrin recognition sequence (arginine-glycine-glutamine) inhibit adherence of *C. albicans* to the ECM components fibronectin, laminin, collagens I and IV, as well as to endothelial and epithelial cells (Klotz and Smith, 1990; Klotz and Smith, 1991; Frey *et al.*, 1990; Choi *et al.*, 1990). Furthermore the *C. albicans* integrin homologue *INT1* (Gale *et al.*, 1996), is sufficient to confer adherence to human epithelial cells on the normally non-adherent yeast *S. cerevisiae* (Gale *et al.*, 1998). Disruption of *INT1* in *C. albicans* reduced adhesion by nearly 40%, suppressed hyphal growth and reduced mortality in a mouse intra-venous model of infection.

The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been found at the cell surface of *C. albicans*, in addition to its usual cytoplasmic location, and may mediate fungal adhesion to the ECM. GAPDH binds to fibronectin and laminin, and anti-GAPDH antibodies inhibit *C. albicans* adhesion to these substrates *in vitro* (Gozalbo *et al.*, 1998). Another hyphal specific surface protein encoded by the *HWP1* gene appears to act as a substrate for mammalian transglutaminases. Hwp1p has been shown to mediate a stable covalent attachment to mammalian buccal epithelial cells, and *hwp1* homozygotes have attenuated virulence in a mouse model of systemic infection (Staab *et al.*, 1999). Finally two *C. albicans* genes *ALA1* and *ALS1*, have been isolated that confer adhesion on the non-adherent yeast *S. cerevisiae*, and share homology to the *S. cerevisiae* agglutinin AG α 1 (Lipke *et al.*, 1989). In *S. cerevisiae* agglutinins mediate cell-cell contact during

matting, whilst the *C. albicans* homologues *ALA1* (Gaur and Klotz, 1997), and *ALS1* (Fu *et al.*, 1998) mediate adhesion to mammalian ligands. *ALA1* confers adhesion to fibronectin, type IV collagen, laminin and buccal epithelial cells, while *ALS1* binds both epithelial and endothelial cells.

It is likely that each of the adhesins discussed here play an important role in the development of candidiasis at one or more sites of infection. Thus *C. albicans* possesses multiple adhesins that permit adherence to a range of mammalian hosts tissues.

Secreted aspartyl proteinases and phospholipases

C. albicans is known to secrete several enzymes including phospholipase, lipase, and aspartyl proteinases (Ruchel, 1981). Secretory Aspartyl Proteinase (SAP) antigen has been detected in patients infected with *C. albicans* (Ruchel *et al.*, 1988; De Bernardis *et al.*, 1990), and has been implicated in contributing to pathogenesis. SAP activity is induced when protein is the sole nitrogen source, but its broad substrate specificity is thought to be important in the dissolution of cell and basement membranes and facilitating hyphal penetration of host tissues (Ray and Payne, 1988). SAPs have been shown to degrade ECM proteins laminin and fibronectin (Morschhauser *et al.*, 1997), human secretory IgA (Ruchel, 1981), and possess a keratinolytic activity (Negi *et al.*, 1984) which may aid the initiation of cutaneous candidiasis. Most studies have concluded that these activities are likely to be particularly important at an early stage of infection (De Bernardis *et al.*, 1995; Ibrahim *et al.*, 1998). Proteinase expression has also been linked with phagocytosis of *C. albicans* blastoconidia, and it has been proposed that this may be important in the resistance to phagocytosis (Matthews, 1994). There is also some evidence for the involvement of SAP in adherence to particular host surfaces (Ray and Payne, 1988; De Bernardis *et al.*, 1995).

The importance of SAPs has been supported by the demonstration that virulence is reduced in various *in vitro* and *in vivo* models of oral, cutaneous, vaginal and systemic candidiasis by the proteinase inhibitor pepstatin A (Schaller *et al.*, 1999; Ray and Payne, 1988; De Bernardis *et al.*, 1999; Ruchel *et al.*, 1990). In addition to this, early studies correlated the virulence of various *C. albicans* isolates and derived mutant strains, with the level of SAP activity (Ross *et al.*, 1990). While it seems that all clinical isolates produce aspartyl proteinases, there seems to be considerable variation among strains with respect to the SAP

quantity and qualities (Ruchel *et al.*, 1982). *C. albicans* isolates from the vaginas of women suffering from candidal vaginitis have been found to produce more SAP than isolates from healthy carriers (Cassone *et al.*, 1987). Fungal isolates from HIV⁺ patients suffering from oral or vaginal candidiasis were also found to secrete up to eight times more proteinase and were significantly more pathogenic in animal models than isolates from carrier subjects (De Bernardis *et al.*, 1996; De Bernardis *et al.*, 1999). Thus high SAP production is associated with virulence in *C. albicans*.

Definitive experiments have only been possible with the development of targeted gene disruption techniques in this organism. In fact there appear to be at least nine SAP-like genes (*SAP1-9*) in *C. albicans* (Naglik *et al.*, 1999), and each appears to be differentially regulated in response to a number of *in vitro* conditions (Hube *et al.*, 1994). For example *SAP1* and *SAP3* are expressed in opaque but not white colony types of strain WO-1, while *SAP4-6* are induced during the yeast-hyphal switch (Hube *et al.*, 1994). In addition, Schaller *et al.* (1998) reported differential and temporal regulation of SAP gene expression in an *in vitro* model of oral candidiasis. There have also been reports of differential and temporal regulation *in vivo* (Schaller *et al.*, 1998; Naglik *et al.*, 1999; Staib *et al.*, 1999).

It seems likely that the relative importance of each SAP gene varies with the site of infection. Schaller *et al.* (1999), found $\Delta sap1$, $\Delta sap2$, and $\Delta sap3$ mutant strains caused less tissue damage than the parental strain, in an *in vitro* model of oral candidiasis. In contrast the virulence of a triple *SAP4-6* deletion strain appeared unimpaired, implying *SAP1-3* and not *SAP4-6* contributed to tissue damage in this model. However, Sanglard *et al.* (1997) reported that a *SAP4-6* triple mutant strain exhibited attenuated virulence in an animal model of systemic infection, implicating these isoenzymes in the normal progression of systemic candidiasis. Furthermore, *in vivo* expression of *SAP2* is dependent upon the site of infection, being induced during invasion of deep organs during disseminated candidiasis, but not in oesophageal infection (Staib *et al.*, 1999).

SAP seems to play a key role in the establishment of *Candida* infections. While there may be some degree of overlap, each of the SAP genes is subject to differential regulation and this is likely to reflect the specific role played by each isoenzyme.

A secreted phospholipase activity has also been linked to *C. albicans* adherence to epithelial cells and pathogenicity in a murine model of candidemia (Barrett-Bee *et al.*, 1985). Phospholipases are enzymes which degrade phospholipids, and it has been proposed that this activity may play a role in damaging host cell membranes. Furthermore, a *C. albicans* phospholipase B homologue (*caPLB1*), appears to aid the penetration of host cells, and is necessary for full virulence (Leidich *et al.*, 1998).

Phenotypic switching

Phenotypic switching can be observed in many strains of *C. albicans* as a reversible change in colony morphology, occurring at a relatively high frequency (10^{-2} / colony). The best characterised switching system is that of strain WO-1, in which cells switch from a white hemispherical (White phase) to a flat grey (Opaque phase) colony forming unit (Slutsky *et al.*, 1987). The variation in colony morphology appears to be the result of fundamental changes in cell structure. White phase cells are oval shaped with a budding pattern similar to the common yeast form, while opaque phase cells are much larger bean shaped cells with a pimpled surface and exhibit a polar budding pattern (Slutsky *et al.*, 1987; Anderson and Soll, 1987). In addition, each cell type exhibits a range of unique physiological characteristics and virulence determinants, suggesting that there is a global change in gene expression. It has been noted that white and opaque phase cells have a different propensity to form hyphal filaments (Slutsky *et al.*, 1987; Anderson *et al.*, 1989). In addition the switch type has been shown to effect adherence to host cells, surface antigens and susceptibility to antifungals (Anderson and Soll, 1987; Soll, 1992; White *et al.*, 1995). The fact that switching affects the expression of several putative virulence factors, indicates that it may be important in the pathogenesis of *C. albicans*, and this is supported by the finding that white phase cells are more virulent than opaques (Ray and Payne, 1991). However, opaque phase cells produce more proteinase and this appears to confer a higher degree of adherence in a murine model of cutaneous infection (Kvaal *et al.*, 1999). Importantly, phenotypic switching has been observed in both commensal and pathogenic strains at the site of infection (Soll *et al.*, 1989; Hellstein *et al.*, 1993).

In fact WO-1 is capable of forming at least three other colony morphologies (Slutsky *et al.*, 1987), indicating that a single strain of *C. albicans* may be capable of generating extensive variation. This may help *C. albicans* to evade the host's specific immune response, it may

also be important at different stages of infection and/ or for adaptation to different host sites.

Filamentation

Candida albicans is often described as dimorphic. In fact it is capable of forming at least three morphologically distinct forms, blastospores, pseudohyphae and true hyphae. Blastospores are the vegetative yeast form, which are unicellular and divide through budding. Blastospore can be induced to 'germinate', leading to the emergence of a filamentous or hyphal form. The primary hypha emerging from a parent yeast cell is often referred to as a 'germ-tube', and subsequent elongation and branching forms a fungal mycelium. Each filament is composed of uninucleated cells joined end to end which are defined by septal walls at regular intervals along the hypha. Pseudohyphae appear to arise when budding cells fail to separate following cytokinesis resulting in a chain of elongated cells. These can be distinguished from true hyphae as they have visible constrictions at each cell-cell junction, whereas true hyphae are unconstricted with parallel cell walls (Gow, 1997). Pseudohyphal cells are usually elongated to a variable extent, and it has been suggested that they represent a continuity of forms between blastophores and true hyphae (Cutler, 1991; Gow, 1997).

It seems likely that all three morphogenic forms contribute to the pathogenesis of *C. albicans* disease, as all three forms have been observed at sites of infection (Odds, 1988). However the importance of the yeast-hyphal transition in the ability of *C. albicans* to cause disease has been the subject of several investigations. Shepherd (1985) identified mutant strains which grew only as yeast, or only as hyphae, both of which caused mortality in a murine model of systemic infection, but to a lesser extent than the wild-type strain. Sobel *et al.*, (1984) reported that a *C. albicans* variant strain incapable of forming hyphae at 37°C was less likely to cause infection, and that when established, infections were less severe in a rat model of vaginal candidiasis, than a germ-tube producing strain. However, the variant and 'wild-type' strains used in these experiments were not isogenic, and may have resulted from multiple mutations. Thus the variant's reduced virulence could not be attributed with certainty to the morphogenesis defects. More recent studies have made use of targeted gene disruption strategies to create isogenic non-germinative mutant strains. Lo *et al.* (1997) constructed a *C. albicans* *cph1/cph1 efg1/efg1* double mutant strain, defective

in the two major hyphal signalling pathways (see section 1.3.1), and unable to form hyphae or pseudohyphae. This strain was found to be virtually avirulent in mice inoculated intravenously (Lo *et al.*, 1997). Moreover hyphae may contribute to virulence at specific tissue locations. For example while non-germinative mutants inoculated intravenously can initiate kidney disease in mice, colonisation is limited to the renal cortex, whereas germinative strains invade cortical, medullary, and pelvic regions of the kidney (Ryley and Ryley, 1990).

The growth of *C. albicans* hyphae can be influenced by a number of environmental factors, including surface contact (thigmotropism). Hyphae induced upon nucleopore membranes appear to 'invade' the pores and pass through to the other side of the membrane (Sherwood *et al.*, 1992). Hyphae also reorient to follow ridges or grooves (Gow *et al.*, 1994). Such behaviour may be advantageous for pathogenesis, using topographical cues to guide the hyphal tip through wounds or weakened points in epithelial, endothelial or skin surfaces, leading to the invasion of host surfaces (Gow *et al.*, 1997). Polarised growth and thigmotropism make hyphae ideally suited to mediate the penetration of host surfaces (Gow *et al.*, 1997), although this has not been proven unequivocally. The yeast-hyphal transition may also be important in evading host cell-mediated immunity. *C. albicans* blastospores have been observed to germinate upon phagocytosis, often leading to death of the phagocyte (Arai *et al.*, 1977). The signals leading to germination and the transition itself will be the subject of the next section.

1.3 The Yeast-Hypha switch

While the precise nature of the signal acting to induce hyphae *in vivo* remains obscure, *C. albicans* readily forms hyphae in response to a variety of *in vitro* conditions. These include physical conditions such as neutral pH and growth at 37°C, and chemical inducers such as N-acetylglucosamine and serum proteins. Many media that induce *C. albicans* filamentation are poor nitrogen sources. In this respect, this process is similar to the induction of pseudohyphae in *S. cerevisiae*, which also occurs in response to nitrogen starvation (Gimeno *et al.*, 1992).

In addition to the physical features, filamentation is accompanied by multiple changes in cellular physiology, affecting properties such as adherence, surface antigens, and cell wall

structure (Sobel *et al.*, 1984; Gilmore *et al.*, 1988). These changes are brought about by a complex alteration in the pattern of gene expression (Gow *et al.*, 1995; White *et al.*, 1995), and confer different properties upon hyphae including those relating to pathogenicity. For example, expression of *ALSI*, which encodes an agglutinin-like adhesin, is hyphal specific (Hoyer *et al.*, 1998), as is *HWP1*, also involved in *C. albicans* adherence (Staab *et al.*, 1999). The precise nature of the changes in gene expression may depend upon the signal used to induce the hyphae, and some are likely to be in response to a new nutritional environment rather than a direct result of the transition. Nevertheless there is a comprehensive change in gene regulation associated with hyphal formation.

1.3.1 Regulation of morphogenesis

Environmental signals that induce the formation of hyphae must effect the expression of a large number of target genes. These environmental signals seem to be conducted along signal transduction pathways, that are conserved throughout, and regulate differentiation in a broad range of fungal species. The conservation of signal transduction pathways between fungal species has facilitated rapid advances in the elucidation of these pathways in *C. albicans*, with the budding yeast *S. cerevisiae* proving a particularly fruitful model system. The diploid form of this yeast undergoes a yeast-pseudohyphal growth switch under conditions of nitrogen starvation, and it is thought that this aids the yeast to forage for nutrients (Kobayashi and Cutler, 1998). Heterologous gene expression in *S. cerevisiae* has led to the identification of several *C. albicans* genes that encode components of signal transduction pathways or affect morphogenesis.

While a number of genes have been identified that effect the regulation of the yeast-hyphal switch in *C. albicans*, an understanding of the way in which these interact to achieve this regulation is far from complete. In this section some of the major regulators of morphogenesis in *C. albicans* will be discussed, more comprehensive reviews have been provided by Brown and Gow, (1999); Kobayashi and Cutler, (1998).

In *S. cerevisiae* the pseudohyphal signalling pathway shares many components with the mating pheromone response pathway (Liu *et al.*, 1993). In haploids the peptide mating hormone signal is transmitted through a kinase cascade to the transcription factor, Ste12p which is required for mating-specific gene expression. In diploids the same kinase cascade

and Ste12p are required for filamentous growth (Liu *et al.*, 1993). A *C. albicans* *STE12* homologue, *CPH1*, was isolated as enhancing pseudohyphal formation when heterologously expressed in *S. cerevisiae*, it was also found to complement *S. cerevisiae* *ste12* mutants defective in mating (haploids) and filamentous growth (diploids) (Liu *et al.*, 1994). *C. albicans* *cph1/cph1* mutants were found to be suppressed in hyphal formation on solid spider medium, but formed hyphae in response to other stimuli such as serum (Liu *et al.*, 1994). Since then more components of the *C. albicans* mitogen activated protein kinase (MAPK) cascade have been identified, either through the complementation of *S. cerevisiae* mutants or through homology searches of the *C. albicans* genome sequence database. These include a MAPK homologue Cek1p (Csank *et al.*, 1998), a MAPK kinase homologue Hst7p (Leberer *et al.*, 1996), and a homologue of the *S. cerevisiae* Ste20p serine/threonine kinase, Cst20p (Leberer *et al.*, 1996). Mutation of each of these homologues gave a similar phenotype to that observed in *cph1/cph1* strains, suggesting that they operate in the same pathway. The current model of the *CPH1* dependent morphogenetic pathway (as described by Brown and Gow, 1999) has been devised through analogy to the *S. cerevisiae* MAP kinase cascade, and supported through epistasis studies. In this model Cst20p activates a MAPK module containing a putative Ste11p homologue (MAPK kinase kinase), Hst11p, Hst7p, and Cek1p (figure 1.1). Cek1p is thought to activate Cph1p, which then activates transcription of the target genes. A further level of control is provided by the phosphatase Cpp1p (Csank *et al.*, 1997), which acts on Cek1p, to repress hyphal growth (figure 1.1). *C. albicans* *cpp1/cpp1* mutant strains grow in the hyphal form under all conditions examined (Csank *et al.*, 1997). Therefore *C. albicans* possesses a MAPK based signal transduction pathway that activates morphogenesis through the putative transcription factor *CPH1*.

The events occurring upstream of Cst20p are unclear, however some insight can be gained from the model system in *S. cerevisiae*. Gimeno *et al.* (1992) found that constitutive activation of the *RAS2* gene, which encodes a surface bound GTPase, leads to enhanced filamentous growth in *S. cerevisiae*. In the current model of Ras2p mediated signal transduction, Ras2p interacts with the essential GTPase Cdc42p, which in turn activates either Ste20p or Cla4p (Holly and Blumer, 1999). Cla4p is a member of the Ste20p-like serine/ threonine kinases which are involved in triggering morphogenetic processes in a range of organisms; disruption of the *C. albicans* *CLA4* homologue leads to defects in hyphal formation (Leberer *et al.*, 1997). Interestingly this model fits in with the model of

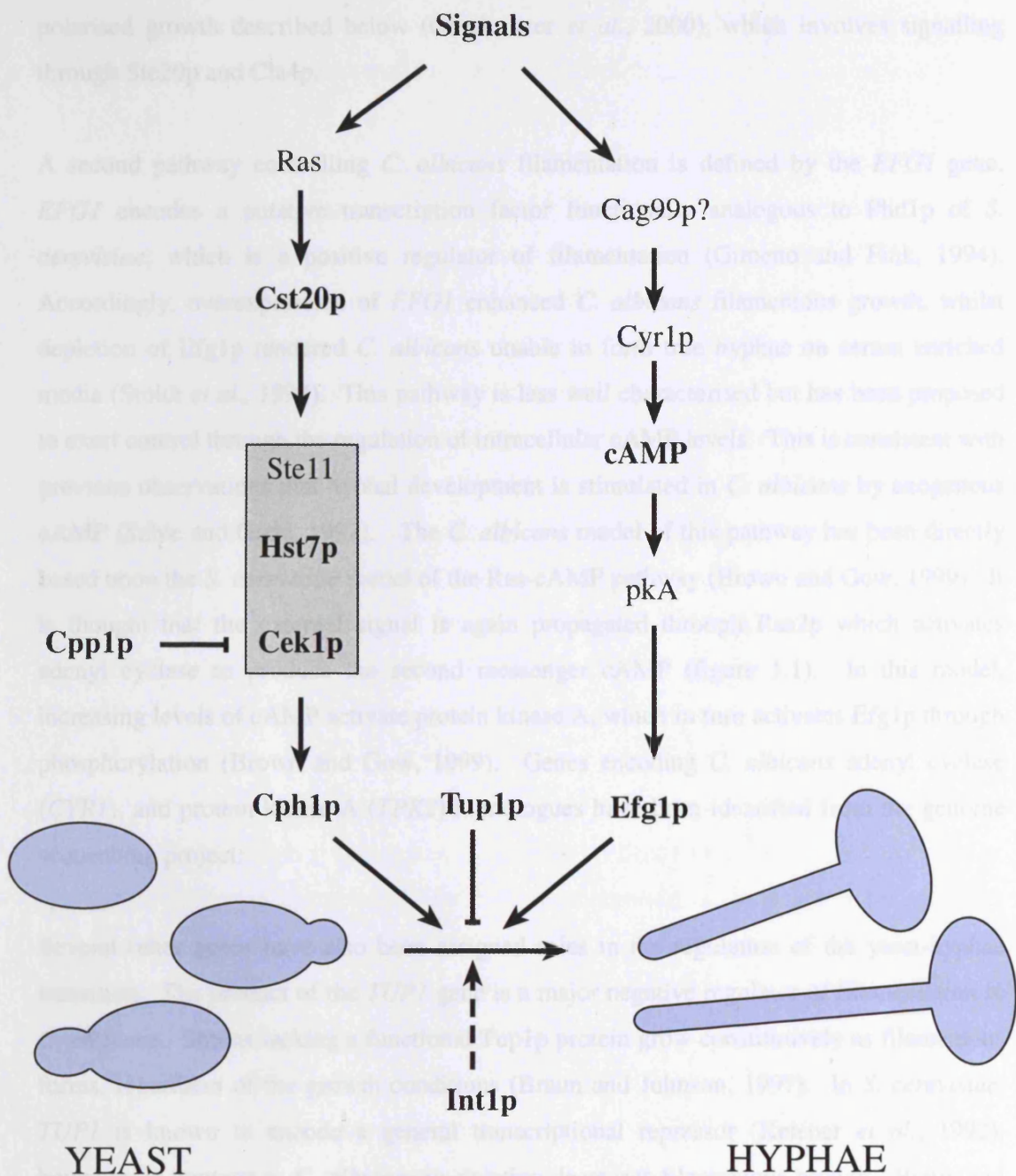


Figure 1.1. Regulatory networks controlling *Candida albicans* morphogenesis. At least two major signaling pathways defined by the transcription factors Cph1p and Efg1p, activate filamentous growth. A third pathway defined by Tup1p acts to repress filamentous growth. Components shown in bold type have been demonstrated to exert an effect upon filamentous growth in *C. albicans* and their positions in each pathway are shown based upon epistasis analysis. Those in plain type are proposed to have a role based upon similarity to related signal transduction pathways in *S. cerevisiae*. Arrows represent positive regulation, while blunt arrows represent negative regulation. The grey box encompasses a Mitogen Activated Protein Kinase (MAPK) module. Int1p exerts a positive effect upon morphogenesis but is presumed to act indirectly (dashed line). (Modified from Brown and Gow, 1999).

polarised growth described below (Oberholtzer *et al.*, 2000), which involves signalling through Ste20p and Cla4p.

A second pathway controlling *C. albicans* filamentation is defined by the *EFG1* gene. *EFG1* encodes a putative transcription factor functionally analogous to Phd1p of *S. cerevisiae*, which is a positive regulator of filamentation (Gimeno and Fink, 1994). Accordingly, overexpression of *EFG1* enhanced *C. albicans* filamentous growth, whilst depletion of Efg1p rendered *C. albicans* unable to form true hyphae on serum enriched media (Stoldt *et al.*, 1997). This pathway is less well characterised but has been proposed to exert control through the regulation of intracellular cAMP levels. This is consistent with previous observations that hyphal development is stimulated in *C. albicans* by exogenous cAMP (Sabie and Gadd, 1992). The *C. albicans* model of this pathway has been directly based upon the *S. cerevisiae* model of the Ras-cAMP pathway (Brown and Gow, 1999). It is thought that the external signal is again propagated through Ras2p which activates adenylyl cyclase to produce the second messenger cAMP (figure 1.1). In this model, increasing levels of cAMP activate protein kinase A, which in turn activates Efg1p through phosphorylation (Brown and Gow, 1999). Genes encoding *C. albicans* adenylyl cyclase (*CYR1*), and protein kinase A (*TPK2*) homologues have been identified from the genome sequencing project.

Several other genes have also been assigned roles in the regulation of the yeast-hyphae transition. The product of the *TUP1* gene is a major negative regulator of filamentation in *C. albicans*. Strains lacking a functional Tup1p protein grow constitutively as filamentous forms, regardless of the growth conditions (Braun and Johnson, 1997). In *S. cerevisiae*, *TUP1* is known to encode a general transcriptional repressor (Keleher *et al.*, 1992), however, in contrast to *C. albicans* its deletion decreases filamentous growth. Braun and Johnson (2000) found that *TUP1*, *EFG1*, and *CPH1*, all made additive contributions to the control of filamentation in *C. albicans*, suggesting that the genes function in three distinct pathways. The *C. albicans* Rad6p homologue, CaRad6p, has also been found to repress filamentation, and this repression is dependent upon Efg1p but not Cph1p (Leng *et al.*, 2000). *CaRAD6* expression is induced in response to UV, null strains being UV sensitive (Leng *et al.*, 2000). Therefore *CaRAD6* seems to play a role in protecting cells against UV damage and provides a checkpoint control for filamentation. A third repressor of hyphal

growth, *RBF1*, encodes a DNA binding protein (Ishii *et al.*, 1997). It is not yet known how this fits with the regulatory networks so far established.

Based on the observation that a triple *TUP1*, *EFG1*, *CPH1* mutant still retained a low residual level of filamentous growth, Braun and Johnson (2000) have suggested that there may be a fourth regulatory pathway that exerts control over filamentation in *C. albicans*. One possible explanation for there being multiple control pathways is that each may trigger hyphal growth in response to a different sub-set of environmental conditions (Mitchell, 1998). This is supported by the fact that *CPH1* null strains are defective in filamentation on solid spider media but remain competent to form hyphae in response to serum (Liu *et al.*, 1994). There is also evidence that each pathway may regulate a different set of target genes. For example, the expression of several structural genes associated with filamentation were found to be differentially affected in *tup1*, *efg1* and *cph1* mutant strains, and were differentially expressed in response to different inducing conditions (Sharkey *et al.*, 1999; Braun and Johnson, 2000). Thus there may be several distinct types of hyphae which are specifically adapted to the environmental conditions that trigger their formation.

Each of the pathways discussed above that lead to morphogenesis are also likely to regulate a number of other determinants including virulence traits. For example, while an *EFG1/efg1* heterozygote apparently forms hyphae normally *in vitro*, it is less virulent in animal models (Kobayashi and Cutler, 1998). This could suggest that Efg1p regulates genes other than those involved in morphogenesis, alternatively it may not form normal hyphae *in vivo*. Sharkey *et al.* (1999) demonstrated that the expression of *ECE1* is dependent upon Efg1p, although *ECE1* is not required for morphogenesis. Therefore, the regulatory role of Efg1p extends beyond the control of cell shape determinants. It is because the regulation of morphogenesis appears to be intimately linked to regulation of other virulence factors, that it has been difficult to establish the true contribution of morphogenesis alone in pathogenesis.

1.3.2 Apical growth in germ tubes

Once initiated germ tubes exhibit a highly polarised growth pattern, with expansion confined to the apical region of the hyphae (Staebell and Soll, 1985). In all filamentous fungi studied, cell wall biosynthesis and membrane expansion at the apex is supported by

the directed exocytosis of microvesicles, releasing cell wall biosynthetic enzymes to synthesise chitin, and providing additional cell membrane material (Gow, 1994). Polarised growth in *S. cerevisiae* is dependent upon the establishment of a polarised distribution of actin (Welsh *et al.*, 1994), which has been associated with sites of vesicle exocytosis (Mulholland *et al.*, 1994). During germination of *C. albicans*, actin is redistributed and becomes polarised at the apical region, forming spots and cables (Anderson and Soll, 1985). Furthermore, germ tube formation is dependent upon actin microfilaments, since agents which inhibit microfilament assembly such as Cytochalasin A, also inhibit germ tube formation (Akashi *et al.*, 1994). Type I myosins have also been implicated in vesicle endo- and exo-cytosis (Mermall *et al.*, 1998), and polarised growth in both *Aspergillus nidulans* and *S. cerevisiae* (Coluccio, 1997). Moreover, disruption of the *C. albicans* *MYO5* gene, encoding a type I myosin, results in a strain unable to form true hyphae (Oberholzer *et al.*, 2000). In the current model for apical extension, myosin I acts as a molecular motor, which is activated by the Cla4p/ Ste20p family of protein kinases (section 1.3.1). Once activated, myosin I moves along actin microfilaments with its attached 'cargo' to the site of active cell growth. In this way it has been suggested that type I myosin is responsible for translocating the Arp2p/3p complex (which nucleates actin assembly), and exocytotic vesicles, to sites of polarised growth (Oberholzer *et al.*, 2000) where vesicle fusion to the cellular membrane facilitates apical extension.

In summary, it appears that the regulation of morphogenesis in *C. albicans* is complex, integrating the regulation of multiple cellular processes such as polarised growth and the cell cycle. In addition to the process of morphogenesis itself it appears that the same regulators also control the expression of other virulence factors.

1.3.3 The role of the vacuole in the yeast-hyphal switch

Gow and Gooday (1982a) found that the growth kinetics of *C. albicans* germ tubes were unusual, in that the hyphal extension rate was constant, whereas in other species the hyphal extension rate was logarithmic. They also noted that there was a prolonged delay between septation and the formation of branches from a sub-apical compartment. Further microscopic observations of germinating *C. albicans* gave a surprising insight into cytological events during the yeast-hyphal switch. As the germ-tube emerged, a large refractile body identified as a vacuole, developed to fill almost the entire parent yeast cell

(Gow and Gooday, 1982b). The rapid expansion of the vacuole coincided with migration of parental cytoplasmic material into the hyphal tip (figure 1.2). As the germ tube extended, sub-apical compartments were also identified that were composed almost entirely of vacuole and containing little protoplasm, whereas the protoplasm seemed to migrate with the hyphal tip (figure 1.2). After a delay period of apparent inactivity, the highly vacuolated compartments became phase dark as the cytoplasm was regenerated. Formation of secondary germ tubes from mother cells or branches from sub-apical compartments only occurred after cytoplasmic regeneration (figure 1.2), when again the cytoplasm migrated into the newly synthesised hyphal tip, leaving behind a highly vacuolated parent cell. The delay between septation and branching most likely corresponds to the period during which the sub-apical compartment regenerates protoplasm and therefore its biosynthetic capacity (Gow and Gooday, 1984). In addition, the apparently constant volume of cytoplasm supporting the growth of the apex, accounts for the constant growth rate of *C. albicans* germ tubes (Gow and Gooday 1982b; Gow and Gooday 1984). This is in contrast with other filamentous fungi in which the extension rate is logarithmic owing to an increasing volume of biosynthetically active protoplasm able to support growth at the hyphal apex.

One major advantage of this mechanism maybe that it requires minimal amounts of *de novo* protein synthesis, but allows *C. albicans* to germinate rapidly and explore its environment or forage for nutrients under conditions limiting for nitrogen.

1.4 The fungal vacuole

1.4.1 Functions of the vacuole

Fungal vacuoles are often considered functionally analogous to the mammalian lysosome, as both are acidic compartments containing a variety of hydrolytic enzymes with broad substrate specificity. The vacuole also acts as a major storage site for a range of cellular metabolites, and is involved in homeostasis of the cytoplasmic environment. Despite the multiple functions of the fungal vacuole, it appears to be a non-essential organelle for vegetative growth, as a number of *S. cerevisiae* mutants deficient in vacuolar function are viable (Banta *et al.*, 1988). However, many such mutants are less viable under stressful conditions such as elevated temperature, and this probably reflects the importance of this organelle for adaptation to, or survival under such conditions. In this section some of the

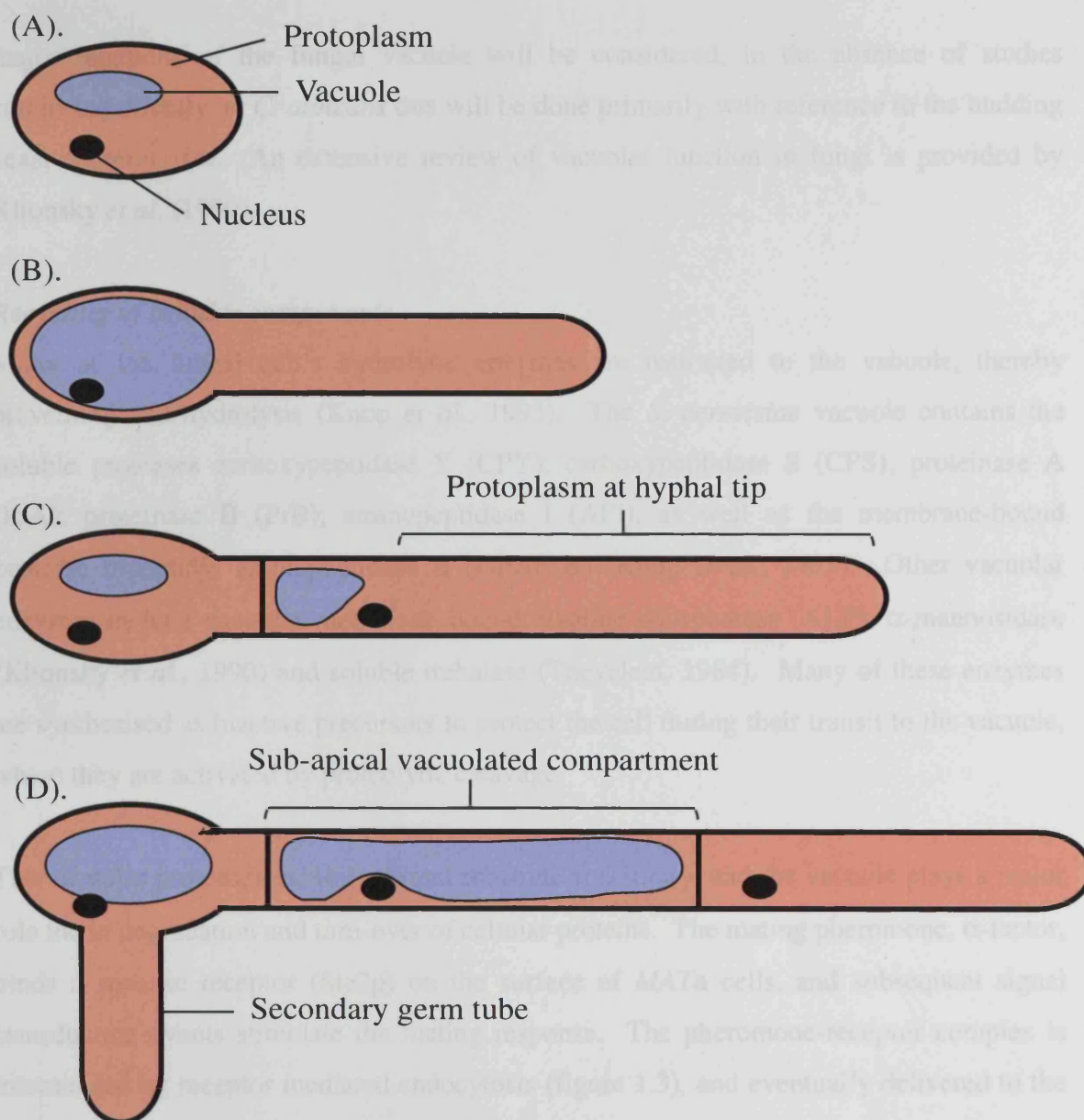


Figure 1.2. Model for germ-tube formation in *Candida albicans*. During outgrowth of the germ tube the protoplasm (pink) migrates into the hyphal tip, as the vacuole (blue) expands within the mother cell A and B. Following a period of apparent cell cycle arrest, the parent cell regenerates its cytoplasm, before the emergence of a secondary germ tube. Within the new hyphal filament the protoplasm continues to migrate forward with the hyphal tip, leaving highly vacuolated sub-apical compartment. Cytoplasmic regeneration is necessary in the sub-apical region before new branching can occur (Taken from Gow and Gooday, 1984).

major functions of the fungal vacuole will be considered, in the absence of studies conducted directly on *C. albicans* this will be done primarily with reference to the budding yeast *S. cerevisiae*. An extensive review of vacuolar function in fungi is provided by Klionsky *et al.* (1990).

Recycling of cellular components

Many of the fungal cell's hydrolytic enzymes are restricted to the vacuole, thereby preventing self-hydrolysis (Knop *et al.*, 1993). The *S. cerevisiae* vacuole contains the soluble proteases carboxypeptidase Y (CPY); carboxypeptidase S (CPS); proteinase A (PrA); proteinase B (PrB); aminopeptidase I (API), as well as the membrane-bound protease dipeptidyl aminopeptidase B (DPAP B) (Knop *et al.*, 1993). Other vacuolar enzymes include vacuolar membrane bound alkaline phosphatase (ALP), α -mannosidase (Klionsky *et al.*, 1990) and soluble trehalase (Thevelein, 1984). Many of these enzymes are synthesised as inactive precursors to protect the cell during their transit to the vacuole, where they are activated by proteolytic cleavage.

The vacuolar proteases exhibit a broad substrate specificity, and the vacuole plays a major role in the degradation and turn-over of cellular proteins. The mating pheromone, α -factor, binds a specific receptor (Ste2p) on the surface of *MATa* cells, and subsequent signal transduction events stimulate the mating response. The pheromone-receptor complex is internalised by receptor mediated endocytosis (figure 1.3), and eventually delivered to the vacuole via vesicle mediated transport, where its degradation is dependent upon the activity of PrA (Chvatchko *et al.*, 1986; Schandel and Jenness, 1994).

Autophagocytosis is dramatically stimulated under conditions of nitrogen starvation, and results in the delivery of a portion of the cytoplasm along with organelles including mitochondria and endoplasmic reticulum to the vacuole (Takeshige *et al.*, 1992). Degradation of this material provides the cell with amino acids for the synthesis of new cellular proteins (Teichert *et al.*, 1989), and in *S. cerevisiae* is dependent upon the activity of the vacuolar hydrolases PrA and PrB (Takeshige *et al.*, 1992; see section 1.5.3). This mechanism is non-selective and generally considered to account for the degradation of cellular proteins with a long half-life, while those with a shorter half-life are thought to be actively targeted for degradation by the cytoplasmic proteasome through poly-

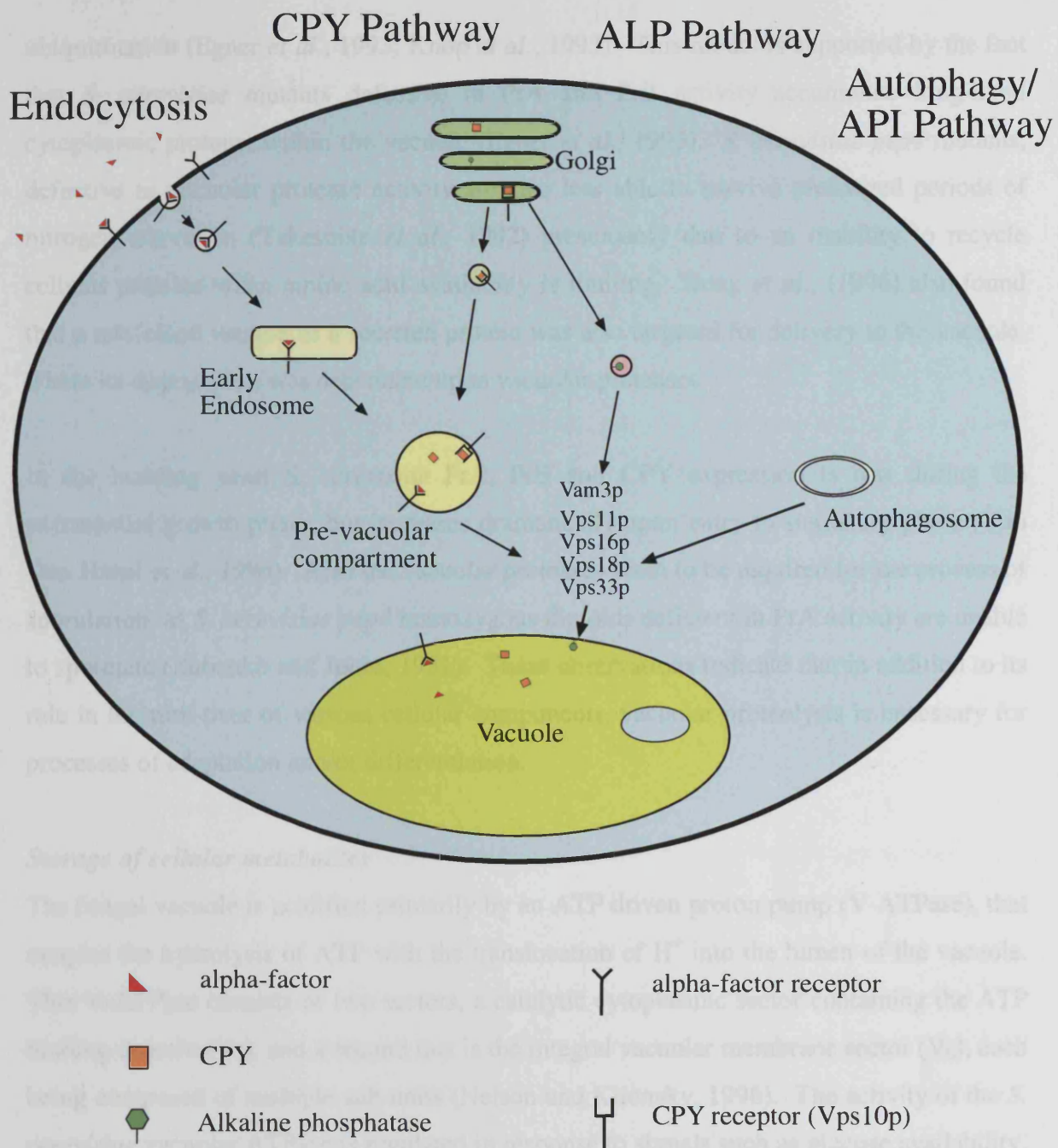


Figure 1.3. Delivery pathways to the vacuole in *Saccharomyces cerevisiae*. Multiple pathways act to deliver the vacuole's resident hydrolases, as well as material destined for degradation. Surface proteins are degraded in the vacuole following internalisation by endocytosis. Endocytosis can act on specific proteins such as alpha-factor during receptor mediated endocytosis, or internalise non-specific material in 'fluid phase' endocytosis. Endocytosed material transits through two intermediate compartments, the early endosome, and the pre-vacuolar compartment (PVC). Many of the resident vacuolar hydrolases travel through the early stages of the secretory pathway, and are sorted away from secretory proteins at a late compartment of the golgi apparatus. Two separate pathways from golgi to vacuole are defined by CPY, which reaches the vacuole via the PVC, and ALP which is independent of the PVC. During starvation bulk cytoplasmic material is delivered to the vacuole via a double membrane bound autophagosome for degradation during the process of autophagy. In a process related to autophagy, API is delivered to the vacuole from the cytoplasm.

ubiquitination (Egner *et al.*, 1993; Knop *et al.*, 1993). This model is supported by the fact that *S. cerevisiae* mutants defective in PrA and PrB activity accumulate long-lived cytoplasmic proteins within the vacuole (Egner *et al.*, 1993). *S. cerevisiae pep4* mutants, defective in vacuolar protease activity are also less able to survive prolonged periods of nitrogen starvation (Takeshige *et al.*, 1992) presumably due to an inability to recycle cellular proteins when amino acid availability is limiting. Hong *et al.*, (1996) also found that a misfolded version of a secreted protein was also targeted for delivery to the vacuole, where its degradation was dependent upon vacuolar proteases.

In the budding yeast *S. cerevisiae* PrA, PrB and CPY expression is low during the exponential growth phase, but increases dramatically upon entry to stationary phase (Van Den Hazel *et al.*, 1996). Also the vacuolar proteases seem to be required for the process of sporulation, as *S. cerevisiae pep4* homozygous diploids deficient in PrA activity are unable to sporulate (Zubenko and Jones, 1981). These observations indicate that in addition to its role in the turn-over of various cellular components, vacuolar proteolysis is necessary for processes of adaptation and/or differentiation.

Storage of cellular metabolites

The fungal vacuole is acidified primarily by an ATP driven proton pump (V-ATPase), that couples the hydrolysis of ATP with the translocation of H^+ into the lumen of the vacuole. This V-ATPase consists of two sectors, a catalytic cytoplasmic sector containing the ATP binding domain (V_1), and a second that is the integral vacuolar membrane sector (V_0), each being composed of multiple sub-units (Nelson and Klionsky, 1996). The activity of the *S. cerevisiae* vacuolar ATPase is regulated in response to signals such as glucose availability, and this is achieved through the regulation of V_1 and V_0 interaction (Kane and Parra, 2000). A second enzyme bound at the vacuolar membrane has been identified in *S. carlbergensis* that couples a pyrophosphatase activity with the translocation of protons into the lumen of the vacuole (Lichko, 1995). Acidification of the vacuole is necessary for several vacuolar functions.

The vacuole is the main storage organelle for metabolically important compounds and ions, and contains a large pool of mainly basic amino acids. Proton translocation into the vacuole generates an electrochemical gradient across the vacuolar membrane, and the resulting proton motive force is utilised to transport cellular metabolites into the vacuole

against concentration gradients for storage. The transport of arginine, lysine, histidine, phenylalanine, tryptophan, tyrosine, glutamine, asparagine, isoleucine and leucine into the vacuole all appear to depend upon H^+ /amino acid antiport systems (Ohsumi and Anraku, 1981; Sato *et al.*, 1984), which mediate the exchange of vacuolar protons with cytoplasmic amino acids. The storage of basic amino acids in the vacuole, especially nitrogen rich arginine provides the cell with a nitrogen reserve. Similar H^+ antiport proteins are proposed to accumulate various metal ions including calcium, zinc, magnesium, strontium and manganese (Ohsumi and Anraku, 1983; Okorokov *et al.*, 1985; White and Gadd, 1987). The primary mechanism for transport of storage molecules into the vacuole appears to depend on the proton motive force, however one notable exception is iron. Iron is still able to accumulate in the vacuole of *S. cerevisiae* mutants defective in vacuole acidification (Bode *et al.*, 1995). In addition to amino acids and metal ions, the vacuole stores phosphate in the form of polyphosphate (Urech *et al.*, 1978). Negatively charged polyphosphate is proposed to act as a 'cation trap', and form complexes that are involved in metabolite retention (Durr *et al.*, 1979).

Cytoplasmic homeostasis

A functional vacuole is important for the homeostasis of the cytoplasmic environment. For example, mutants of *S. cerevisiae* that lack a normal vacuole are sensitive to external pH and osmotic pressure (Banta *et al.*, 1988). Nelson and Nelson (1990) also found that mutants defective in the V-ATPase only grew well in a narrow range of pH around pH 5.5. Furthermore, as a major storage organelle the vacuole is central to the regulation of the cytoplasmic concentration of nutrients including some amino acids, phosphate and physiologically important metal ions. It has been noted that while the size and composition of the cytoplasmic amino acid pool remains relatively constant, the vacuolar pool varies widely depending on nutrient availability (Kitamoto *et al.*, 1988), suggesting that the vacuolar store acts to 'buffer' amino acid concentrations in the cytoplasm. This necessitates mechanisms for release into the cytoplasm of compounds stored within the vacuole, under conditions where they become limiting. For example, during nitrogen starvation vacuolar stores of arginine are released, increasing the cytoplasmic concentration of arginine, a nitrogen rich amino acid (Kitamoto *et al.*, 1988). Iron can also be mobilised from the vacuole during the iron requiring process of mitochondriogenesis (Raguzzi *et al.*, 1988).

Thus a functional vacuole is central to the homeostasis of the cytoplasmic pH and osmolarity, as well as metal ion and amino acid content. In addition, the vacuole is a major site of general cytoplasmic protein (and organelle) turn-over, a process that seems to be particularly important under stressful conditions or during processes of adaptation or differentiation. Furthermore, as exemplified by α -factor, targeting to the vacuole can provide a specific mechanism for the elimination of exogenous or undesirable proteins (figure 1.3).

1.4.2 Inheritance of the fungal vacuole

During cell division, cytoplasmic organelles must be duplicated and partitioned between mother and daughter. In the yeast *S. cerevisiae*, a vacuole can be detected within the daughter at an early stage, when the bud is approximately 1% the size of the mother cell (Weisman *et al.*, 1987). In addition, experiments using stable fluorophores, have demonstrated the inheritance of at least some material from the mother vacuole to the bud vacuole (Weisman *et al.*, 1987; Weisman and Wickner, 1988). This occurs via a segregation structure, the nature of which is currently unclear, but transfer of material from parental to bud vacuole may occur as a result of vesicle mediated transport (Weisman and Wickner, 1988) or a tubular structure that directly links parent and bud vacuoles (Catlett and Weisman, 2000). A number of *vac* mutants have been identified as defective in vacuole partitioning (Weisman *et al.*, 1990; Wang *et al.*, 1998). Vacuole inheritance is also dependent upon actin-myosin mediated transport, since specific mutations in actin and *MYO2* result in a *vac* phenotype (Hill *et al.*, 1996; Schott *et al.*, 1999). Cells with defects in these components are Class I *vac* mutants, characterised by normal vacuoles in parents, but no vacuole in the buds (Wang *et al.*, 1998).

Class II *vac* mutants appear to arrest in segregation once a node forms on the parent vacuole near to the bud (Nicolson *et al.*, 1995). This node is likely to indicate a defect in the machinery responsible for the emergence of the vacuolar segregation structure. Class III *vac* mutants include *vac7*, *vac14*, and *fab1*, and lack segregation structures but have enlarged vacuoles which often extend from the parent cell into the bud (Bonangelino *et al.*, 1997). These mutants are proposed to be defective in vacuolar membrane fission (Catlett and Weisman, 2000).

1.5 Vacuole biogenesis

The vacuole is a highly dynamic organelle, and has several distinct pathways for the delivery of the resident hydrolases as well as cellular material destined for degradation in the vacuole (summarised in figure 1.3). Many of the vacuole's hydrolases are synthesised as inactive precursors, which are activated by proteolytic cleavage upon delivery to the vacuole, and this presumably represents a mechanism to protect the cell from self-hydrolysis during transit of these enzymes to the vacuole.

1.5.1 Golgi-to-vacuole transport

The CPY pathway

The majority of soluble vacuolar hydrolases including CPY, PrA and PrB as well as the vacuolar membrane proteins ALP and DPAP-B are delivered to the vacuole via the early stages of the secretory pathway (Klionsky *et al.*, 1990), where they transit with proteins destined for the cell surface. CPY delivery has been well characterised, and the model of delivery and processing of vacuolar hydrolases via the secretory pathway is largely based upon CPY transit. CPY is synthesised with an N-terminal signal peptide that targets the protein for translocation into the endoplasmic reticulum (ER) during or shortly after its translation (Blachly-Dyson and Stevens, 1987). The 20 amino acid signal peptide is cleaved upon entry to the ER, and the protein undergoes core N-linked glycosylation at four sites (Hasilik and Tanner, 1978). This ER form of CPY is known as p1CPY. The p1 form is then conveyed to the golgi apparatus by a vesicle mediated transport step, where further glycosylation with mannose residues results in a further gain in molecular mass, readily detected in western blots of protein extracts (Stevens *et al.*, 1982). The fully glycosylated golgi form of CPY is known as the p2 form. Upon delivery to the vacuole the N-terminal propeptide of the precursor CPY is proteolytically cleaved to yield the active mature form (Hasilik and Tanner, 1978). This cleavage occurs between amino-acid residues 111 and 112, and is dependent upon the activity of the *PEP4* gene, which encodes PrA (Hemmings *et al.*, 1981). Many other vacuolar hydrolases including PrB, ALP, API and PrA itself, are synthesised as inactive precursors, and are activated by the proteolytic removal of a propeptide sequence upon reaching the vacuole (Hemmings *et al.*, 1981). The nature of the propeptide seems to vary widely in terms of size and location within each hydrolase (Klionsky *et al.*, 1990). However, PrA and PrB seem to play a central role, being required for the maturation of several precursors (Van Den Hazel *et al.*, 1996). PrA

may undergo autocatalytic activation upon reaching the vacuole in response to the low pH and high salt concentration (Ammerer *et al.*, 1986; Van Den Hazel *et al.*, 1996). An 'activation cascade' has been proposed in which PrA is responsible either directly or indirectly, for the activation of vacuolar hydrolases including PrB, CPY, CPS, and API (Van Den Hazel *et al.*, 1996).

Sorting of vacuolar proteins from proteins destined for the cell surface occurs in a late compartment of the golgi apparatus (figure 1.3)(Graham and Emr, 1991). In addition to the signal peptide necessary for translocation into the ER, a soluble vacuolar hydrolase must contain additional information for sorting from secretory proteins and subsequent delivery to the vacuole. Vacuolar sorting signals have been defined for a number of hydrolases (Klionsky *et al.*, 1990). Gene fusion and mutational studies identified the N-terminal vacuolar targeting signal of CPY (Johnson *et al.*, 1987; Valls *et al.*, 1987), just C-terminal to the ER signal peptide and within the cleaved propeptide. Subsequent study has revealed that the CPY sequence QRPL is necessary and sufficient for vacuolar localisation (Chapman, 1994); the absence of this sorting information results in secretion of the golgi modified form from the cell, via the default pathway to the cell surface. Early studies indicated that CPY sorting in the *trans*-golgi was a receptor mediated event. For example the sorting mechanism was found to be saturable, in that overexpression of CPY caused mis-sorting of the p2 (golgi modified form of CPY), resulting in secretion from the cell (Stevens *et al.*, 1986). More recently the CPY sorting receptor has been identified, and is encoded by the *VPS10* gene (Marcusson *et al.*, 1994). Vps10p is a large integral membrane protein, localised to the *trans*-golgi, and has been shown to specifically interact with CPY at its QRPL sorting sequence (Marcusson *et al.*, 1994). Vacuolar sorting signals identified for other hydrolases bear little resemblance to that of CPY, and furthermore overexpression of CPY does not result in the mislocalisation of other hydrolases (Chapman, 1994). This has lead to the proposal that there are a family of vacuolar sorting receptors, each of which is responsible for the sorting of a specific hydrolase (Chapman, 1994). Vps10p is also required for targeting soluble misfolded proteins in the golgi for degradation in the vacuole, since $\Delta vps10$ strains of *S. cerevisiae* mis-sort a misfolded protein, targeting it to the cell surface (Hong *et al.*, 1996). In contrast to the soluble proteins, integral membrane proteins in the secretory pathway such as DPAP B, which lack

sorting information, are transported to the vacuole, indicating that for membrane proteins the default pathway leads to the vacuole (Roberts *et al.*, 1992).

In fractionation experiments Vida *et al.* (1993) detected the presence of p2CPY in a further membrane-bound compartment that was distinct from either the golgi apparatus or the vacuole. Pulse-chase experiments suggested that CPY delivery to this compartment occurred as an intermediate step between the golgi and the vacuole (figure 1.3), and lead to it being termed the Pre-Vacuolar Compartment (PVC) also known as the late endosome. Furthermore, a mutant CPY form lacking a functional vacuolar sorting signal was detected within the golgi but not the PVC (Vida *et al.*, 1993), supporting the idea that vacuolar protein sorting occurs before the PVC. The CPY sorting receptor Vps10p has been detected in the PVC (Cooper and Stevens, 1996) where it is proposed that CPY dissociates, and is transported on to the vacuole, while Vps10p is recycled to the *trans*-golgi through the activity of the golgi retention signal in its cytoplasmic domain (Chapman, 1994; Marcusson *et al.*, 1994). Several other vacuolar hydrolases also transit to the vacuole via the same route as CPY including PrA, PrB, and CPS (Bryant and Stevens, 1998). Furthermore, it was noted that internalised α -factor also co-fractionated with the same endosome-like compartment, indicating that endocytosed material also transits through the PVC before passing on to the vacuole (Vida *et al.*, 1993).

Transport from the PVC to the vacuole is also likely to be a vesicle mediated process, since the vacuolar t-SNARE Vam3p, involved in membrane fusion, is required for this final step (Darsow *et al.*, 1997). A number of other proteins required for this process have been identified and are discussed in section 1.6.

A large collection of *S. cerevisiae* mutants defective in the delivery of vacuolar hydrolases from the golgi have been isolated. These *vps* (Vacuolar Protein Sorting) mutants have been identified in genetic screens due to missorting of either CPY or a CPY-invertase fusion protein to the cell surface (Bankaitis *et al.*, 1986; Rothman *et al.*, 1989). A further set of *pep* mutants, previously identified as defective in CPY activity (Jones, 1977), were also found to mislocalise vacuolar proteases, and complementation analysis revealed there was a large degree of overlap between *pep* and *vps* mutants. In all, around 50 mutants have been identified that missort CPY, and most are also defective in the sorting and processing

of PrA and PrB. Microscopic analysis revealed that a number of *vps* mutants had an abnormal vacuolar morphology, and based upon this and other phenotypic characteristics these mutants have been classified into six groups A to F (Banta *et al.*, 1988; Raymond *et al.*, 1992). Class A *vps* mutants have a morphologically normal vacuole, with 1-3 large compartments per cell (Banta *et al.*, 1988). Class B *vps* mutants have a fragmented vacuole morphology with around 20 smaller compartments per cell, while class C mutants lack a vacuole compartment all together, accumulating a range of different sized vesicles (Banta *et al.*, 1988). Each class of *vps* mutant are likely to define a set of gene products that function at a common step of the VPS pathway.

The ALP pathway

The vacuolar membrane protein ALP is also sorted in the late golgi and transits on to the vacuole but does not pass through the PVC (figure 1.3)(Piper *et al.*, 1997). ALP is delivered and correctly processed in several *S. cerevisiae* mutants that are defective in the CPY pathway. For example, the class E *vps* mutants have an enlarged PVC that accumulates endocytosed material as well as CPY (Raymond *et al.*, 1992). These mutants are defective in the exit of proteins from the PVC, and are defective in CPY sorting but correctly deliver ALP to the vacuole (Piper *et al.*, 1997). Conversely *vps41* mutants accumulate ALP in a vesicle enriched cellular fraction, but are apparently unaffected in CPY delivery (Cowles *et al.*, 1997). Thus the transit of ALP from the *trans*-golgi to the vacuole occurs via a second pathway. While it appears that the set of cellular components required for each pathway (as defined by mutants defective in each pathway) is unique, there also appears to be some overlap. For example, Vps1p is involved in vesicle budding from the *trans*-golgi network, and is required for the vacuolar delivery of both CPY and ALP (Nothwehr *et al.*, 1995). Like CPY, ALP sorting is likely to be receptor mediated due to the saturable nature of the ALP pathway, excess ALP still reaches the vacuole but via the PVC (Cowles *et al.*, 1997). The 33 amino acid N-terminal cytosolic tail is necessary and sufficient to direct ALP away from the CPY pathway and into the alternative pathway from golgi to vacuole (Piper *et al.*, 1997), and is likely to contain a receptor binding site.

The CPY and ALP pathways apparently converge at the point of vesicle fusion to the vacuole, as inactivation of the vacuolar localised syntaxin homologue, Vam3p, results in the disruption of both pathways (Piper *et al.*, 1997). Similarly the small GTPase Ypt7p is

required for delivery of both ALP and PVC derived traffic to the vacuole (Schimmoller *et al.*, 1993). The function of both these proteins is discussed in section 1.6.

1.5.2 Plasma membrane to vacuole transport

Extracellular material at the cell surface and plasma membrane proteins are transported to the vacuole in membrane vesicles through the process of endocytosis (figure 1.3). Endocytosis can result in selective uptake in the process of receptor-mediated or ligand dependent endocytosis, or in the non-specific bulk flow of material through fluid-phase endocytosis. In *S. cerevisiae* fluid phase endocytosis can be observed using lipophilic dyes such as FM4-64 or lucifer yellow, which are taken up into membrane vesicles following membrane invagination and are later observed to accumulate within the vacuole (Vida and Emr, 1995). Receptor mediated endocytosis is readily studied through the internalisation of the mating pheromone receptors Ste2p and Ste3p (Chvatchko *et al.*, 1986).

Once the mating pheromone α -factor is bound to its receptor, Ste2p, the receptor undergoes hyperphosphorylation and ubiquitination within its cytoplasmic tail, a step necessary for its internalisation (Hicke and Reizman, 1996). Both yeast pheromone receptors (Ste2p and Ste3p), can also be constitutively endocytosed in a ligand independent pathway for degradation within the vacuole (Davis *et al.*, 1993). This mechanism is likely to be distinct from ligand dependent endocytosis since a different portion of the cytoplasmic tail is required for internalisation through this process (Davis *et al.*, 1993). The constitutive pathway is also dependent upon ubiquitination, and seems to take the same route to the vacuole (Roth and Davis, 1996). Indeed the vacuole seems to be involved more widely in the turn-over of proteins bound at the cell surface, and this has been demonstrated for the plasma membrane proteins uracil permease (Volland *et al.*, 1994), and the α -factor transporter Ste6p (Berkower *et al.*, 1994).

Endocytosed material proceeds to the vacuole via two intermediate compartments, the early and late endosomes (Singer and Riezman, 1990). The late endosome corresponds to the PVC compartment, and it is within this compartment that the CPY and endocytotic pathways converge (Vida *et al.*, 1993). The class-E VPS gene products, thought to mediate transport from PVC to vacuole are necessary for endocytosis and CPY delivery (Piper *et*

al., 1995). A group of *end* mutants defective in endocytosis have been isolated based on their inability to take-up the fluorescent dye lucifer yellow CH and accumulate it within the vacuole (Chvatchko *et al.*, 1986).

1.5.3 Cytoplasm-to-vacuole transport

The vacuole plays a central role in the recycling of proteins with a long half-life or under conditions of nutritional stress, providing amino acids for the synthesis of new polypeptides through the degradation of cytoplasmic proteins and organelles delivered via macroautophagy. This process is initiated when a portion of the cytosol is engulfed within a double membrane structure called an autophagosome (Takeshige *et al.*, 1992)(figure 1.3). The outer membrane of the autophagosome subsequently fuses with the vacuolar membrane to release a single membrane bound portion of cytoplasm, termed an autophagic body, into the vacuole for degradation. In *S. cerevisiae* this degradation is dependent upon the activity of PrA and PrB, and strains defective in these proteases accumulate autophagic bodies containing cytoplasmic proteins and organelles within the vacuole lumen (Takeshige *et al.*, 1992; Egner *et al.*, 1993). Two genetic screens have identified *S. cerevisiae* mutants that do not accumulate autophagic bodies within their vacuole upon nitrogen starvation. The *apg* mutants were identified as less able to survive during starvation (Tsukada and Ohsumi, 1993), whilst *aut* mutants are defective in the starvation induced degradation of fatty acid synthase (Thumm *et al.*, 1994).

Mechanisms also exist to specifically target cytoplasmic proteins for delivery to the vacuole. One well characterised example is that of the vacuolar resident hydrolase API. It was noted that pro-API lacks a signal peptide for ER translocation, and was found not to enter the early stages of the secretory pathway, but was found in the cytosol (Klionsky *et al.*, 1992). Like PrA and CPY, API has an N-terminal propeptide, that contains information required for vacuolar localisation and is cleaved upon reaching the vacuole (Oda *et al.*, 1996). However, the half-life for API maturation of 30-40 minutes, is significantly longer than proteins which travel via the secretory pathway (Klionsky *et al.*, 1992). Mutants defective in this sorting sequence accumulate pro-API within the cytoplasm (Oda *et al.*, 1996). Furthermore, whereas hydrolases which reach the vacuole via the ER and golgi are highly glycosylated, API is unglycosylated despite containing putative N-linked glycosylation sites (Klionsky *et al.*, 1992). Finally, many of the *vps*

mutants, isolated as missorting CPY are apparently unaffected in API processing (Klionsky *et al.*, 1992). This evidence strongly suggested that API does not transit to the vacuole via the secretory pathway but rather enters the vacuole from the cytoplasm. API has been observed to oligomerise into a dodecomeric form prior to vacuolar delivery (Kim *et al.*, 1997), and the large size of this oligomer supports a vesicle-mediated delivery model, rather than translocation via a proteinaceous pore (Klionsky, 1998). To investigate the nature of this import pathway, mutants were isolated that accumulate the precursor form of API (Harding *et al.*, 1995), termed *cvt* mutants (Cytoplasm to Vacuole Targeting). Investigations utilising some of these mutants revealed that API transits to the vacuole via a double membrane compartment in the cytoplasm, and is later found within a single membranous compartment within the vacuole (Scott *et al.*, 1997), a process paralleled by macroautophagy. It appears that the vacuolar membrane protein α -mannosidase I also takes a similar route from cytoplasm to vacuole (Yoshihisa and Anraku, 1990).

Both the API and macroautophagy pathways act to deliver cytoplasmic proteins into the vacuole lumen, however there are fundamental differences. The API pathway is essentially biosynthetic, acting to deliver a resident hydrolase, while autophagy is degradative. API is delivered specifically in a saturable manner indicating a possible API receptor, macroautophagy on the other hand is a non-selective, unsaturable mechanism for the bulk delivery of cytoplasmic proteins (Klionsky, 1998). API delivery occurs during vegetative growth and is much more rapid than that of autophagy which is mainly induced upon starvation (Scott *et al.*, 1996). Nevertheless, complementation analysis revealed that there is extensive overlap between the *cvt* and autophagy mutants (Scott *et al.*, 1996), indicating that elements of either pathway are shared. In fact under conditions of starvation API has been observed to be delivered within autophagosomes (Baba *et al.*, 1997).

1.5.4 Vacuole delivery pathways converge at the vacuolar membrane

Each of the delivery pathways discussed above are mediated by a unique set of gene functions. However, many of the mutants isolated for a given pathway were found to exhibit pleiotropic phenotypes, and be defective in multiple vacuolar delivery pathways. Complementation analysis also found varying degrees of genetic overlap between *vps*, *end*, and *cvt* mutants (Bryant and Stevens, 1998; Klionsky, 1998). For example, the class-E

VPS genes are required for vacuolar targeting of both endocytosed material, and proteins which transit through the CPY pathway (Piper *et al.*, 1995). The syntaxin homolog, Vam3p, is required for delivery via the CPY, ALP, endocytotic and CVT pathways (Darsow *et al.*, 1997; Wada *et al.*, 1997). Mutant strains lacking a functional Vam3p exhibit a range of phenotypes, are defective in the maturation of CPY, PrA, PrB, ALP, API, defective in autophagy, and exhibit a fragmented (class B) vacuole morphology. The primary defect is in the docking and/or fusion of transport intermediates to the vacuole, which results in the accumulation of a variety of membrane bound transport vesicles and autophagosomes within the cytoplasm (Darsow *et al.*, 1997). Vam3p is a low abundance protein and has been localised to the cytosolic face of the vacuolar membrane (Srivastava and Jones, 1998; Darsow *et al.*, 1997). The requirement of Vam3p for the docking and fusion of transport vesicles derived from several distinct trafficking routes indicates a convergence of the vacuolar delivery pathways at the vacuolar membrane.

1.6 Vesicle fusion and SNARE hypothesis

Much of the work on vesicle-target membrane fusion has been carried out through the reconstitution of transport in mammalian cell-free systems, and is supported by genetic evidence in yeast. This has uncovered a 'general fusion machinery' in eukaryotic cells, composed of the cytosolic fusion factor NSF (*N*-ethylmaleimide Sensitive Factor), and α -SNAP (Soluble NSF Attachment Protein), encoded by *SEC18* and *SEC17* respectively in yeast. These factors are required for each vesicle-target membrane fusion event within the cell (Graham and Emr, 1991). However, eukaryotic cells are characterised by several distinct membrane bound compartments, each of which carries out specialised functions, and contain a unique set of proteins. In order to maintain the functional and compositional integrity of each cellular compartment there must be some degree of specificity in the targeting of transport vesicles arising from each organelle. Studies of neurotransmitter release from mammalian neuron synapses have provided an insight as to the fundamental mechanism of eukaryotic vesicle targeting.

Neurotransmitters are stored in synaptic vesicles within the cytoplasm of neurons, and are released at the pre-synaptic membrane upon triggered exocytosis. SNARE (SNAP receptors) complexes from brain tissue were identified as complexing with NSF and α -SNAP (Sollner *et al.*, 1993). These complexes consist of two proteins located upon the

pre-synaptic membrane, syntaxin and SNAP-25 (Synaptosome Associated Protein, not to be confused with Soluble NSF Attachment Protein), and a Vesicle Associated Membrane Protein (VAMP), also known as synaptobrevin. Syntaxin and synaptobrevin are both integral membrane proteins, and the absence of other components suggest that these proteins are responsible for the docking of synaptic vesicles at the pre-synaptic membrane. A large number of syntaxin, SNAP-25, and synaptobrevin homologues required for particular vesicle mediated transport steps have since been identified in both mammalian and yeast cells, and localised to discrete cellular compartments (Ferro-Novick and Jahn, 1994). This led to the proposal of the 'SNARE hypothesis' (figure 1.4)(Sollner *et al.*, 1993b), in which each cellular compartment possesses on its cytoplasmic face one or more syntaxin and/ or SNAP-25 homologues, referred to as target-SNAREs (t-SNARE). In addition, the transport vesicles originating from each compartment present a membrane bound vesicle-SNARE (v-SNARE) on their cytosolic surface, similar to synaptobrevin on synaptic vesicles. Such v-SNAREs are derived from donor compartments where they are taken up into budding vesicles (for a review of vesicle budding see Rothman, 1994). The specificity of targeting is inherent in the specificity of v-SNARE/ t-SNARE pairing, and the programme of protein trafficking routes is established through the intracellular distribution of compatible v- and t-SNAREs. For example, v-SNAREs derived from ER transport vesicles will only interact efficiently with t-SNAREs present at the *cis*-golgi surface. More recent studies have shown the situation to be a little more complex than this early model. Cognate SNAREs can form complexes in *cis*-between members on the same membrane surface, or in *trans*-between one member on a target membrane and another on an incoming vesicle (figure 1.4)(Hayashi *et al.*, 1995). It is the *trans* complex which is proposed to be important in membrane fusion. Furthermore, a 'priming' reaction disassembles *cis*-SNARE complexes on both target and vesicle surfaces, and is necessary to make these SNAREs available to form *trans*-complexes. This disassembly is dependent upon ATP hydrolysis by the soluble protein NSF/ Sec18p, which is recruited to *cis*-SNARE complexes at some stage during docking by α -SNAP/Sec17p (Otto *et al.*, 1997). The ATPase activity of Sec18p is necessary for fusion and results in the release of the general fusion machinery (Sollner *et al.*, 1993).

Since the SNARE hypothesis was first put forward the precise role of SNAREs has been the subject of intense speculation. Reconstitution of v-SNAREs and t-SNAREs into

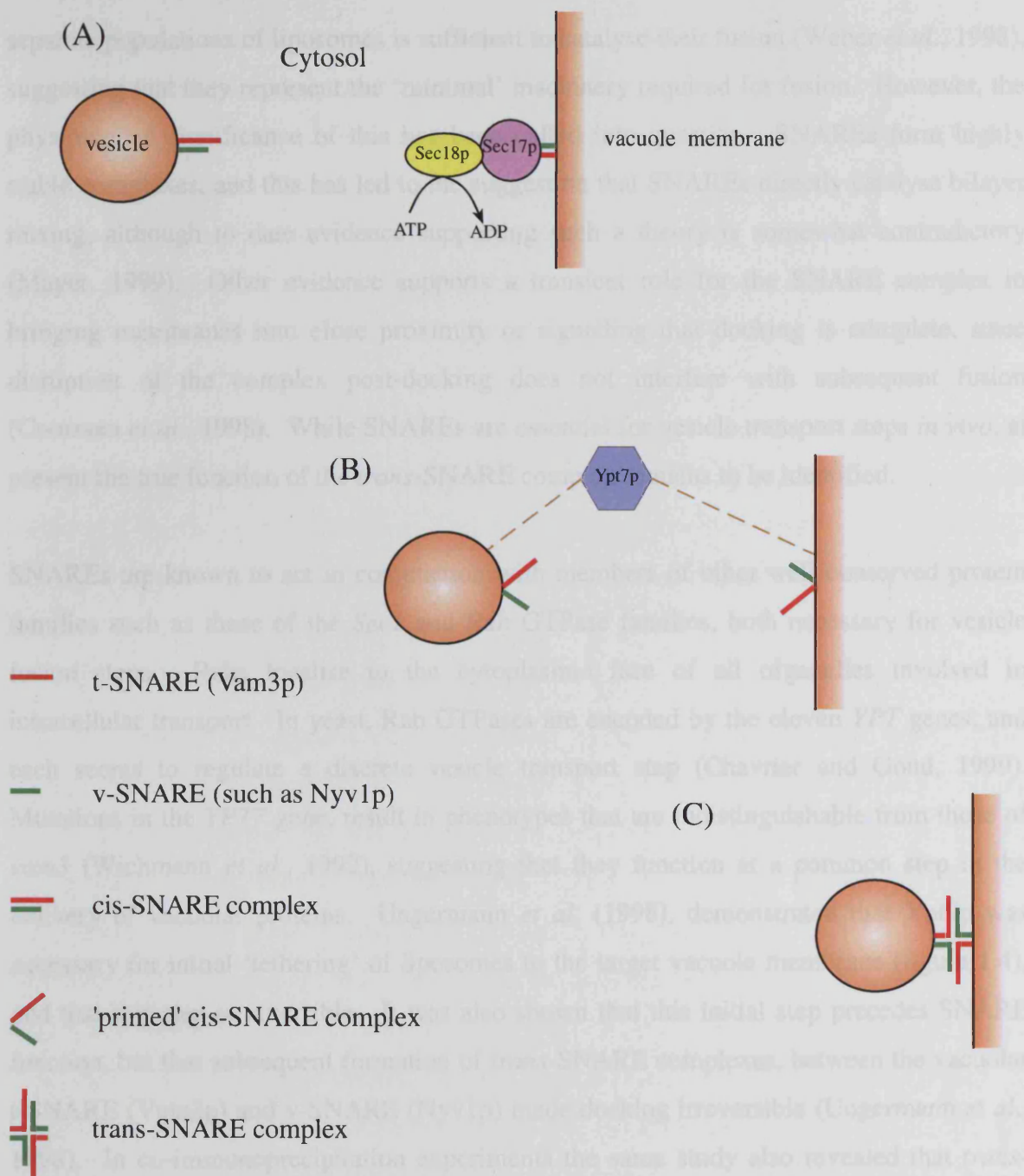


Figure 1.4. The function of the SNARE complex in vesicle docking with the vacuole. (A) Cognate v- and t- SNAREs are paired on both vesicle and target membranes (the cis-SNARE complex). The cis complex is disrupted in an ATP dependent 'priming' reaction catalysed by the NSF homologue, Sec18p, which binds the cis complex through its attachment factor Sec17p. This priming reaction is necessary for the initial and reversible tethering of the transport vesicle with the target vacuolar membrane. (B) Tethering is dependent upon Ypt7p, a member of the Rab GTPase family, and is required for subsequent pairing of v- and t- SNAREs in trans. (C) Formation of the trans-SNARE complex stabilises the docking complex. The precise role of the trans-SNARE complex in membrane fusion is still unclear, but it seems likely that it facilitates close membrane-membrane interaction prior to fusion.

separate populations of liposomes is sufficient to catalyse their fusion (Weber *et al.*, 1998), suggesting that they represent the 'minimal' machinery required for fusion. However, the physiological significance of this has been called into question. SNAREs form highly stable complexes, and this has led to the suggestion that SNAREs directly catalyse bilayer mixing, although to date evidence supporting such a theory is somewhat contradictory (Mayer, 1999). Other evidence supports a transient role for the SNARE complex in bringing membranes into close proximity or signalling that docking is complete, since disruption of the complex post-docking does not interfere with subsequent fusion (Coorssen *et al.*, 1998). While SNAREs are essential for vesicle transport steps *in vivo*, at present the true function of the *trans*-SNARE complex remains to be identified.

SNAREs are known to act in conjunction with members of other well conserved protein families such as those of the *Sec1* and *Rab* GTPase families, both necessary for vesicle fusion steps. Rabs localise to the cytoplasmic face of all organelles involved in intracellular transport. In yeast, Rab GTPases are encoded by the eleven *YPT* genes, and each seems to regulate a discrete vesicle transport step (Chavrier and Goud, 1999). Mutations in the *YPT7* gene, result in phenotypes that are indistinguishable from those of *vam3* (Wichmann *et al.*, 1992), suggesting that they function at a common step in the delivery of vacuolar proteins. Ungermann *et al.* (1998), demonstrated that Ypt7p was necessary for initial 'tethering' of liposomes to the target vacuole membrane (figure 1.4), and that this step is reversible. It was also shown that this initial step precedes SNARE function, but that subsequent formation of *trans*-SNARE complexes, between the vacuolar t-SNARE (Vam3p) and v-SNARE (Nyv1p) made docking irreversible (Ungermann *et al.*, 1998). In co-immunoprecipitation experiments the same study also revealed that *trans*-SNARE complexes formed before fusion occurs, and therefore constitute an intermediate reaction between tethering and fusion, possibly docking.

The precise molecular function of Sec1p like proteins remains unclear but they may regulate v-SNARE-t-SNARE interaction, or 'proof-read' SNARE complex formation (Pevsner *et al.*, 1994). Yeast cells possess four members of the *SEC1* family, each of which has been implicated in a different vesicle mediated transport step (Pfeffer, 1996). Two of these *SEC1* family members, *VPS45* and *VPS33*, are required for vacuolar protein delivery. Vps45p interacts in a complex with a PVC t-SNARE Pep12p and both are required for golgi to PVC trafficking (Burd *et al.*, 1997; Gerrard *et al.*, 2000). Vps33p is a

class-C *VPS* gene product, and has been implicated in PVC to vacuole transport (Wada *et al.*, 1997). *VPS33* mutants exhibit similar vacuolar protein sorting defects to those of *vam3* and *ypt7*, and *VPS33* has been demonstrated to genetically interact with *VAM3*, which encodes the vacuolar t-SNARE (Darsow *et al.*, 1997). This provides evidence that Vps33p interacts with Vam3p to mediate a common step of the docking/ fusion of vesicles at the vacuole membrane. Such an interaction has been supported by the demonstration that mammalian neuronal Sec1p binds directly to syntaxin *in vitro* (Pevsner *et al.*, 1994).

Rieder *et al.* (1997) also demonstrated that interaction between the vacuolar t-SNARE encoded by *VAM3*, and another class-C *VPS* gene. The CPY sorting defects of *vps18* are suppressed by the overexpression of Vam3p. This suggests that Vam3p and the class-C *VPS* gene products may function at a common step of vacuolar protein transport.

1.7 The class-C *VPS* genes

The four class-C *vps* mutants of *S. cerevisiae*, *vps11*, *vps16*, *vps18* and *vps33*, share common phenotypes; these are the most severe of all the *vps* mutants. These mutants exhibit a range of pleiotropic phenotypes, however the use of temperature sensitive alleles has demonstrated the primary defect to be in protein sorting to the vacuole (Rieder and Emr, 1997). Each mutant is completely blocked in the processing of CPY, PrA and PrB, and some missorting to the cell surface can be detected (Robinson *et al.*, 1988). This suggests a complete block in the CPY pathway. ALP is also rapidly missorted as an unprocessed form in class-C mutant strains (Preston *et al.*, 1991; Horazdovsky and Emr, 1993; Rieder and Emr 1997). While α -mannosidase (which utilises the Cvt pathway for its vacuolar localisation) is unaffected by the defects in most *vps* mutants, *vps11*; *vps16*, *vps18* and *vps33* mutants missort α -mannosidase, whose activity can be detected at the cell surface (Robinson *et al.*, 1988). These mutants are also defective in autophagy, and *vps18*^{ts} mutants accumulate double membrane autophagosomes within the cytoplasm at the non-permissive temperature (Rieder and Emr, 1997). Vacuolar accumulation of the fluorescent dye FM4-64 by endocytosis is blocked in *vps18* mutants, instead of a single fluorescent compartment of the parental strain, the mutant had a punctate staining pattern throughout the cytoplasm, possibly corresponding to endocytic intermediates (Rieder and Emr, 1997). Furthermore, mutations in the *VPS11* gene result in an inability to degrade exogenous α -factor via endocytosis (Dulic and Riezman, 1990). Therefore like Vam3p, the class-C *VPS*

gene products are required for delivery of proteins to the vacuole from the golgi apparatus, the cell surface and the cytoplasm.

These primary sorting defects result in a wide range of secondary phenotypes, most notably they are defective in vacuole assembly. Each mutant lacks a central vacuole and instead accumulates a range of smaller membranous compartments, presumed to be blocked transport intermediates destined for the vacuole (Banta *et al.*, 1988). Together with the known genetic interaction of *VPS18* and *VPS33* with *VAM3*, this evidence supports a role for the class-C gene products in the docking and fusion of multiple transport intermediates with the vacuolar membrane. Other phenotypes so far assigned to these mutants include sensitivity to osmotic stress (Banta *et al.*, 1988), deregulation of iron responsive gene regulation (Szczypka *et al.*, 1997), reduced intracellular pools of amino acids (Gent and Slaughter, 1998), inability to utilise non-fermentable carbon sources, and are unable to grow at higher temperatures (37°C) (Preston *et al.*, 1991; Banta *et al.*, 1990). Additionally homozygous diploids are also unable to sporulate (Preston *et al.*, 1991). Most of these phenotypes are unsurprising and simply reflect the loss of the vacuolar functions (reviewed in section 1.4.1). This supports the notion that the vacuole is non-essential for vegetative growth under laboratory conditions, but is essential for survival under more stressful conditions, such as starvation and growth at elevated temperatures or during processes of differentiation.

The *VPS11* ORF of *S. cerevisiae* is predicted to encode a very large, hydrophilic protein of 1030 amino acids (Dulic and Riezman, 1989). It shares a short region of homology with the ATP binding site of the α -subunit of *E. coli* H⁺-ATPase. This suggests it may itself be an ATP binding protein, however, this has yet to be confirmed. The putative protein encoded by *VPS18*, shares some similar features to that of *VPS11*. It is also a very large protein of 918 amino acids, essentially hydrophilic in nature. What is more, both proteins co-localise to the cytosolic face of the vacuolar membrane (Preston *et al.*, 1991), although neither possesses a putative transmembrane domain. Most notably both Vps11p and Vps18p are predicted to have cysteine rich zinc-binding domains close to their C-terminus. The proposed zinc-binding domain of *S. cerevisiae* Vps18p is CX₂CX₁₃CX₂C, (Preston *et al.*, 1991) and this sequence seems to be functionally important since a mutant in which the first cysteine of this motif was changed to a serine resulted in a temperature sensitive

mutant phenotype (Robinson *et al.*, 1991). The cysteines at the C-terminus of Vps11p are in a CX₂CX₁₂CX₇CX₁₆CX₂C arrangement (Dulic and Riezman, 1989; Robinson *et al.*, 1991), but it is unknown which of these, if any, are important for function. A further feature worthy of note is a putative cyclic-AMP binding motif near the middle of Vps18p. However, this sequence can be deleted by site directed mutagenesis without giving rise to a mutant phenotype, indicating that it is of no known functional significance (Preston *et al.*, 1991).

VPS16 encodes a putative protein of 797 amino acids (Horazdovsky and Emr, 1993). This product is also hydrophilic in nature, and contains no obvious membrane spanning domains. Cell fractionation experiments suggested that most of Vps16p is associated with a large sedimentable proteinaceous complex, and that this association was saturable (Horazdovsky and Emr, 1993). Protease treatment of gently lysed spheroplasts resulted in the degradation of Vps16p, conditions under which vacuole lumen proteins remained intact. This suggested that Vps16p is at least in contact with the cytoplasm.

Finally the *Sec1* homologue Vps33p is a hydrophilic protein of 691 amino acids (Banta *et al.*, 1990). Unlike other members of the Sec1p protein family, Vps33p has a functional ATP binding domain (Gerhardt *et al.*, 1998). The majority of this protein localises to a cytosolic cell fraction, with a smaller portion (20-40%) associated with a cellular fraction enriched for permeabilised membranes (Gerhardt *et al.*, 1998). Interestingly, depletion of cellular ATP increased the membrane association of this protein, but this shift was reversed upon regeneration of ATP. *In vitro* reconstitution of CPY transport revealed that while cytoplasm from a *vps33* strain can support transport between wild-type cellular membranes, wild-type cytosol cannot support transport between a membrane fraction of *vps33* mutant cells (Gerhardt *et al.*, 1998). This evidence suggests that it is the membrane associated fraction that functions in vacuole protein transport. Furthermore, to solubilise membrane bound Vps33p efficiently *in vitro*, requires ATP and cytosol. Thus Vps33p seems to cycle between the cytosol and a membrane fraction, and its release from the membrane fraction requires ATP. Interestingly, Vps45p, a Sec1p homologue required for transport to the PVC also has soluble and membrane bound fractions, but has no recognised ATP binding domain (Gerhardt *et al.*, 1998).

None of these class-C *VPS* proteins appear to be glycosylated and all four lack a putative signal peptide for translocation into the ER, suggesting that they do not enter the secretory pathway. Through Northern and immunoblot experiments all four class-C gene products were found to be expressed to a very low level (Dulic and Riezman, 1989; Preston *et al.*, 1991; Horazdovsky and Emr, 1993; Banta *et al.*, 1990).

Rieder and Emr (1997), first presented evidence of genetic interaction of *VPS16* with *VPS18* and *VPS33*. Then in chemical cross-linking experiments they demonstrated that Vps11p, Vps16p, Vps18p and Vps33p all co-immunoprecipitated with Vps11p specific antiserum (Rieder and Emr, 1997), indicating that each are components of a protein complex. Both Vps16p and Vps33p were less abundant in this complex than were Vps11p or Vps18p. This may represent the stoichiometry of each component within this complex, but in the case of Vps33p at least, this may reflect a transient association with the other members. Also such complexes isolated from cells deleted for *VPS16*, consisted of Vps11p and Vps18p only, which suggests that it is Vps16p that mediates the interaction of Vps33p with this complex (Rieder and Emr, 1997). In cell fractionation experiments Vps11p, Vps16p and Vps18p behaved as components of a sedimentable protein complex, a significant portion of which co-fractionated with vacuolar membranes, while the majority of Vps33p remained within the cytosol. These results suggest that the class-C Vps proteins form a very large complex on the vacuole membrane, but that Vps33p association is only transient. The same study demonstrated a genetic interaction between a *VPS18* and *VAM3*, hinting that this complex's function is related to the assembly of SNARE complexes, or regulation of their activity.

In summary, the four class-C *VPS* gene products form a complex at the vacuolar membrane, that interacts with the vacuolar t-SNARE to mediate the docking and fusion of multiple transport intermediates with the vacuolar membrane. This function is necessary for the maintenance of an intact vacuole compartment.

1.8 Background

A *S. cerevisiae vps18* mutant was isolated within our laboratory during a screen to detect mutants defective in iron responsive gene regulation. Given the vacuole biogenesis defect in *vps18* mutants (Banta *et al.*, 1988), this is consistent with the vacuole playing a central

role in cytoplasmic iron homeostasis. Whilst the functions of the vacuole have been well defined for the model fungi *S. cerevisiae* and *Aspergillus nidulans*, little is known about the role the vacuole plays in pathogenic species during the process of infection. This led us to initiate a study into aspects of vacuolar function in the human pathogen *C. albicans*.

The vacuolar hydrolases of *S. cerevisiae* are necessary for processes of cellular differentiation, including sporulation (Zubenko and Jones, 1981), and are of increased importance during adaptation to new or hostile conditions such as the onset of starvation. It was therefore postulated that a functional vacuole is likely to play a central role in the adaptation to, and colonisation of mammalian tissues by *C. albicans*. The vacuole of *C. albicans* has been observed cytologically to play a prominent role during the yeast to hypha transition (section 1.3.3; Gow and Gooday, 1984). Due to the extremely rapid emergence of germ-tubes upon hyphal induction in *C. albicans*, it is unlikely that this could be supported through the synthesis of new protoplasm. It is therefore possible that the vacuole acts as a 'space filling' compartment, to reduce or at least delay the requirement for protoplasm synthesis, while permitting rapid extension of the hyphal tip. It was therefore of particular interest to determine the effect of a defect in vacuolar biogenesis upon the yeast-hyphae switch.

1.9 Project aims

The aim of this project was to investigate the importance of a functional vacuole in *C. albicans*. Specific objectives were to:

- clone *C. albicans* genes involved in vacuolar biogenesis by the functional complementation of *S. cerevisiae* class-C *VPS* mutants, and/ or through sequence homology with these genes
- construct a *C. albicans* strain defective in vacuole biogenesis, by targeted gene disruption
- carry out phenotypic analysis of the *C. albicans* strain(s) defective in vacuolar biogenesis, in particular with respect to the yeast-hyphae transition

Chapter 2

Materials and Methods

2.1 Strains and plasmids

Table 2.1 *Saccharomyces cerevisiae* and *Candida albicans* strains used in this study

Organism	Strain	Genotype	Source/ Reference
<i>Saccharomyces cerevisiae</i>	S150-2B	<i>MATa, leu2-3, leu2-112; his3-Δ; trp1-289; ura3-52</i>	J. Hicks, Cold Spring Harbour Laboratory, New York
<i>Saccharomyces cerevisiae</i>	JM18	<i>MATa, leu2-3, leu2-112; his3-Δ; trp1-289; ura3-52; vps18</i>	Julie Morrissey, Department of Microbiology, University of Leicester
<i>Saccharomyces cerevisiae</i>	JMΔ18	<i>MATa, leu2-3, leu2-112; his3-Δ; trp1-289, ura3-52; vps18::Tn1000HIS3</i>	Julie Morrissey, Department of Microbiology, University of Leicester
<i>Saccharomyces cerevisiae</i>	SEY6211	<i>MATa, leu2-3,112, ura3-52, his3-Δ200, trp1-Δ901, ade2-101, suc2-Δ9, GAL</i>	Robinson <i>et al.</i> , 1988
<i>Saccharomyces cerevisiae</i>	<i>vps11</i>	<i>MATa, leu2-3,112, ura3-52, his3-Δ200, trp1-Δ901, ade2-101, suc2-Δ9, GAL, vps11</i>	Robinson <i>et al.</i> , 1988
<i>Saccharomyces cerevisiae</i>	<i>vps16</i>	<i>MATa, leu2-3,112, ura3-52, his3-Δ200, trp1-Δ901, ade2-101, suc2-Δ9, GAL, vps16</i>	Robinson <i>et al.</i> , 1988
<i>Saccharomyces cerevisiae</i>	<i>vps18</i>	<i>MATa, leu2-3,112, ura3-52, his3-Δ200, trp1-Δ901, ade2-101, suc2-Δ9, GAL, vps18</i>	Robinson <i>et al.</i> , 1988

<i>Saccharomyces cerevisiae</i>	<i>vps33</i>	<i>MATa</i> , <i>leu2-3,112</i> , <i>ura3-52</i> , <i>his3-Δ200</i> , <i>trp1-Δ901</i> , <i>ade2-101</i> , <i>suc2-Δ9</i> , <i>GAL</i> , <i>vps33</i>	Robinson <i>et al.</i> , 1988
<i>Candida albicans</i>	S01		R. Matthews, Department of Microbiology, University of Manchester, UK
<i>Candida albicans</i>	SC5314		Clinical isolate from patient with disseminated candidosis (Gillum <i>et al.</i> , 1984)
<i>Candida albicans</i>	CAF2	<i>Δura3::λimm434/URA3</i>	Fonzi and Irwin, 1993
<i>Candida albicans</i>	CAI4	<i>Δura3::λ:imm434/Δura3::λimm434</i>	Fonzi and Irwin, 1993
<i>Candida albicans</i>	BWP17	<i>ura3Δ::λimm434/ura3Δ::λimm434</i> <i>his1::hisG/his1::hisG</i> <i>arg4::hisG/arg4::hisG</i>	Aaron Mitchell, Columbia University (U.S.A.); (Wilson <i>et al.</i> , 1999).
<i>Candida albicans</i>	GPR1; GPR2; GPR3	Derived from BWP17; <i>CaVPS11/ cavps11::ARG4</i>	This study
<i>Candida albicans</i>	GPR1U1 GPR2U1 GPR2U2	Derived from BWP17; <i>cavps11::ARG4/</i> <i>cavps11::URA3</i>	This study

Table 2.2 *E. coli* strains used in this study

Organism	Strain	Genotype	Source or reference
<i>E. coli</i>	DH5 α	$\phi 80lacZ\Delta M15$, <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> (rK , m_K^+), <i>supE44</i> , <i>relA1</i> , <i>deo1</i> , $\Delta(lacZYA-argF)U169$	Hanahan, 1983.
<i>E. coli</i>	MH1599	<i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> (rK , m_K^+), <i>suoE44</i> , <i>relA1</i>	Sedgewick and Morgan, 1984.
<i>E. coli</i>	MH1578	<i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> (rK , m_K^+), <i>suoE44</i> , <i>relA1</i> , <i>Sm^r</i>	Sedgewick and Morgan, 1984.

Table 2.3 Plasmids and vectors used in this study

Plasmid	Genotype	Source or reference
YEp213	<i>amp^R</i> , <i>tet^R</i> , 2 μ M ORI, <i>LEU2</i>	Broach <i>et al.</i> , 1988
<i>C. albicans</i> genomic DNA Library (Strain S01)	<i>C. albicans</i> genomic DNA fragments cloned into the <i>Bam</i> HI site of YEp213	Peter Meacock, Department of Genetics, Leicester University
pRS415	<i>amp^R</i> , <i>ARSH4 CEN6</i> , <i>LEU2</i>	Stratagene
pUC19	<i>lacZ</i> , <i>amp^R</i>	New England Biolabs Inc.
pMB7	<i>hisGURA3hisG</i> cassette carried on pUC18	Fonzi and Irwin, 1993
pGEM-URA3	<i>amp^R</i> , <i>URA3</i>	Wilson <i>et al.</i> , 1999
pRS-ARG4 Δ SpeI	<i>amp^R</i> , <i>ARG4</i>	Wilson <i>et al.</i> , 1999
pGPT1	Isolated from <i>C. albicans</i> genomic library as partially rescuing <i>S. cerevisiae vps18</i> associated phenotypes	This study
pGPT2;	Isolated from <i>C. albicans</i> genomic	This study

pGPT3; pGPT4	library as rescuing <i>S. cerevisiae</i> <i>vps16</i> associated phenotypes	
pGPTA	pGPT3 minus 3.4kb <i>HindIII</i> fragment. This results in the loss of half the insert and 475nt of the YEp213 vector (from <i>HindIII</i> to <i>BamHI</i>).	This study
pGPTB	YEp213 carrying a 3.4kb <i>HindIII</i> band from pGPT3.	This study
pGPTAS	pRS415 carrying a 2.6kb <i>HindIII-SalI</i> fragment from pGPTA (entire pGPTA insert + 276nt of YEp213 vector from <i>BamHI</i> and <i>SalI</i> sites).	This study
pGPTAΔ1	pGPTA deleted for +189-+495 within <i>GPA1</i> ORF (= nt 562-868 of insert)	This study
pGPTAΔ2	pGPTA deleted for <i>GPA2</i> sequences, nt 1820-2328 of insert consensus	This study
pGPTAΔ3	pGPTA deleted for 2328nt (entire) insert sequence + 17nt of YEp213 vector sequence	This study
pGPA1A/B	YEp213 carrying <i>GPA1</i> ORF + 373nt upstream of ATG, and 83nt downstream of STOP; A and B have insert in either orientation	This study
pGP11	Isolated from <i>C. albicans</i> genomic library, through hybridisation to <i>CaVPS11</i> specific probe	This study
pGP182; pGP184; pGP186	Isolated from <i>C. albicans</i> genomic library, through hybridisation to <i>CaVPS18</i> specific probe	This study

pGPC185	pUC19 with <i>CaVPS18</i> 5' flanking sequence (-584 to -29) cloned between <i>Sph</i> I and <i>Sal</i> I sites.	This study
pGPC1853	pGPC185 with <i>CaVPS18</i> 3' flanking sequence (+2429-+3552) cloned between <i>Bam</i> HI and <i>Kpn</i> I sites.	This study
pGPCD18	pGPC1853 with pMB7 <i>hisGURA3hisG</i> cassette cloned between the <i>CaVPS18</i> 5' and 3' flanking sequences.	This study

2.2 Growth media and conditions

2.2.1 Bacterial media and growth conditions

Luria-Bertani medium (LB). 1% Bacto-tryptone (Oxoid); 0.5% Bacto-yeast extract (Oxoid); 0.5% sodium chloride; pH adjusted to 7.2 with sodium hydroxide. Antibiotics were added to the media as appropriate at the concentrations given in table 2.4.

Table 2.4 Antibiotic supplements

Antibiotic	Stock concentration	Media concentration
Ampicillin ammonium salt	25 mg/ml	25 µg/ml
Streptomycin	200 mg/ml	200 µg/ml

Solid media Bacto agar (Oxoid) was added at a concentration of 2% (w/v). All media was sterilised by autoclaving at 10 psi for 15 minutes.

Growth conditions All *E. coli* strains were grown at 37°. Liquid cultures were grown with continuous agitation (200 rpm).

Determination of cell titre The growth of a liquid culture was monitored using a spectrophotometer measuring optical density (OD) at a wavelength of 600 nm. One ml aliquots of growing cultures were taken for measurement. An OD₆₀₀ of 1.0 is approximately equal to a cell density of 8×10^8 cells per ml.

2.2.2 Yeast media and growth conditions

Yeast extract Peptone Glucose medium (YPD) 1% (w/v) yeast extract (Oxoid); 2% (w/v) Bactopeptone (Oxoid); 2% (v/v) glucose

YPG medium As YPD but with 3% glycerol instead of glucose

YPE medium As YPD but with 3% ethanol instead of glucose

Synthetic Glucose medium (SD) 0.67% (w/v) yeast nitrogen base with ammonium sulphate (Bio 101); 2% (v/v) glucose; appropriate amino acid and base supplements were added as indicated previously (Sherman *et al.*, 1986), see table 2.5.

SG medium As SD but with 3% glycerol instead of glucose

SE medium As SD but with 3% ethanol instead of glucose

Table 2.5 amino acid and base supplements

Supplement	Stock concentration	Media concentration
Adenine hemisulphate salt	2 mg/ml	20 µg/ml
Histidine	8 mg/ml	20 µg/ml
Leucine	12 mg/ml	30 µg/ml
Tryptophan	8 mg/ml	20 µg/ml
Uracil	2 mg/ml	20 µg/ml
Uridine	5 mg/ml	50 µg/ml

Solid media Bacto agar (Oxoid) was added at a concentration of 2% (w/v).

All media was sterilised by autoclaving at 10 psi for 10 minutes prior to use.

Copper enriched media BPS (Bathophenanthroline disulfonic acid; Sigma) was added to a concentration of 50 µM to molten SD agar supplemented with the appropriate amino acids. FeCl₃ was then added back to a concentration of 40 µM, and then CuSO₄ added back to the desired concentration, and the plates poured.

Hyphal induction in *Candida albicans* *C. albicans* cells grown overnight in YPD media at 30°C were sub-cultured to a cell density of approximately 2×10^7 cells/ml in fresh YPD medium supplemented with 10% (v/v) fetal calf serum. The cells were then grown at 37°C.

Growth conditions All *S. cerevisiae* and *C. albicans* strains were grown at 30°C (unless otherwise stated). Liquid cultures were grown with continuous agitation (200 rpm).

Determination of cell titre The growth of a liquid culture was monitored using a counting chamber with modified Thomas ruling. A 25 µl sample, diluted when necessary, was placed over the grid and covered with a counting chamber cover glass.

2.2.3 Phenotypic tests

The relative sensitivity of mutant and wild-type strains to a range of compounds was tested. Cells grown to mid log phase in SD minimal media supplemented with the appropriate amino acids (1×10^7 cells/ml) were harvested and resuspended in sterile distilled water (10^7 cells/ml). Then 1 ml of this suspension was mixed with 9 ml of molten low gelling temperature agarose (1.5% w/v in sterile distilled water) pre-cooled to 45°C, mixed by inversion and then poured over pre-poured solid media (either an SD or YPD plate). Once the agar suspension has set, a Whatman 3MM 30 mm paper disc was placed at the centre of the plate, and 5-20 µl of the test solution applied to the centre of the disc. Each plate was then incubated at 30°C for 2 days and photographed.

2.3 Transformation procedures

2.3.1 Transformation of *Escherichia coli* (Mandel and Higa, 1970)

Cultures grown to a titre of 0.45-0.55 (OD₆₀₀) were harvested by centrifugation at 4000 rpm for 5 minutes at 4°C, washed in 0.5 x culture volume of ice cold 1000 mM calcium chloride and again harvested by centrifugation. The cells were then resuspended in 0.5 x culture volume of ice cold 100 mM magnesium chloride and incubated on ice for 20 minutes. After harvesting again, the cells were resuspended in 0.05 x culture volume of ice cold 100 mM calcium chloride and incubated on ice or stored in 20% glycerol at -80°C until use.

For transformation, 200 µl of competent cells were mixed with 90 µl of 1 x TE (pH 8) and 1 µg of transforming DNA. A negative control was also set up using 1 x TE (pH 8) in place of the transforming DNA. The suspensions were mixed, incubated on ice for 30 minutes and then heat shocked for 2 minutes at 42°C, before incubating on ice for a further 20 minutes. Then 2 mls of LB was added to each cell suspension and then incubated at

37°C with agitation for 1 hour. The cells were then harvested by centrifugation (13000 rpm), resuspended in 100 µl LB and plated onto LA plates containing the appropriate antibiotic for selection of transformant colonies. Incubation was carried out at 37°C overnight until colonies appeared.

Transformation efficiencies of between 1×10^4 to 1×10^6 colony forming units per microgram of transforming DNA were obtained using this protocol.

2.3.2 Lithium acetate transformation of *Saccharomyces cerevisiae* (Gietz *et al.*,1992)

The cell titre of an overnight YPD culture was determined and diluted to a concentration of 2×10^6 cells/ml in 50 ml fresh pre-warmed YPD. Incubation was then continued until a cell titre of approximately $1-2 \times 10^7$ cells/ml was attained. The cells were harvested by centrifugation at 4000 rpm and the supernatant discarded. The cells were then washed by resuspension in first 1 ml of sterile distilled water and then 1 ml of TE-lithium acetate solution (1 x TE; 0.1 M lithium acetate, pH 7.5). The cells were then resuspended in 1 x TE-lithium acetate solution at a cell concentration of 2×10^9 cells/ml.

For transformation, a 50 µl aliquot of cell suspension was gently mixed with 1 µg of transforming DNA, 50 µg of denatured salmon sperm DNA and 300 µl of 40% PEG 3350 in 1 x TE-lithium acetate solution. A zero DNA control was also set up using sterile distilled water in place of DNA. The cells were incubated at 30°C for 30 minutes, and were then heat shocked at 42°C for 15 minutes and harvested at 13000 rpm. The supernatant was discarded, and the cells washed in 1 x TE. After harvesting the cells were resuspended in 500 µl 1 x TE, and 100 µl aliquots plated to selective SD plate. Transformation plates were then incubated at 30°C for 3-5 days until colonies were observed.

2.3.3 Lithium acetate transformation of *Candida albicans* (Gow *et al.*,1999)

Cultures growing exponentially in YPD media were harvested at 4000 rpm, washed in 0.5 volumes of 1 x TE-lithium acetate solution and then resuspended in 0.1 volumes of 1 x TE-

lithium acetate solution. The suspension was incubated at 30°C for 1 hour and 100 µl aliquots of the cells mixed with 100 µg of denatured salmon sperm DNA and 1-20 µg of transforming DNA. A zero-DNA control was also set up containing sterile distilled water in place of the transforming DNA. The cell suspension was then incubated at 30°C for 30 minutes and after this time 700 µl of 40% PEG 3350 in 1 x TE-lithium acetate solution was added and the suspension incubated for a further 1 hour at 30°C. The suspension was then heat-shocked at 42°C for 15 minutes and the cells harvested at 13000 rpm. The cells were washed in 500 µl of 1 x TE buffer, resuspended in 100 µl of 1 x TE buffer and dispensed onto selective SD plates. The plates were incubated at 30°C for 3-7 days until colonies appeared.

2.4 DNA and RNA preparations

2.4.1 Large scale DNA preparations from *Escherichia coli*

All large scale plasmid extractions were carried out using a Plasmid Midi (100) Kit from QIAGEN (available from QIAGEN Ltd.). The protocol provided by the manufacturers was followed. This method combines the alkaline lysis method (Birnboim and Doly, 1979) with ion exchange chromatography to isolate purified plasmid DNA from *E. coli*.

2.4.2 Small scale plasmid DNA preparations from *Escherichia coli* (Ish-Horowicz and Burke, 1981)

A 1.5ml overnight culture, grown with the appropriate selective antibiotic, was harvested, resuspended in 100 µl of solution I (50 mM glucose; 25 mM Tris-HCl, pH8; 10 mM EDTA, pH 8) and left at room temperature for 5 minutes. Then 200 µl of solution II (0.2 M sodium hydroxide; 0.1% SDS) was added with gentle mixing and the suspension placed on ice for 5 minutes. Next 150 µl of ice cold solution III (5 M potassium acetate) was added and the mixture placed on ice for 5 minutes, followed by centrifuging at 13000 rpm for 2 minutes. The supernatant was transferred to a fresh tube and the nucleic acids precipitated by the addition of 2 volumes of ethanol. The nucleic acids were pelleted by centrifugation at 13000 rpm and then washed in 70% ethanol before resuspending in 20 µl of sterile distilled water. This preparation results in the isolation of RNA as well as DNA, so RNase A was added to subsequent restriction enzyme digests.

2.4.3 Large scale preparation of total genomic DNA from *Saccharomyces cerevisiae* and *Candida albicans* (Cryer *et al.*, 1975)

A 50 ml overnight culture was harvested at 4000 rpm, resuspended in solution I (1.2 M sorbitol; 25 mM EDTA, pH8, 175 μ M DTT) and incubated at 30°C for 30 minutes. The cells were then harvested at 4000 rpm and resuspended in 5 ml solution II (1.2 M sorbitol; 0.1 M sodium citrate; 10mM EDTA, pH8) to which 1 mg of zymolyase was added. The cell suspension was incubated at 30°C for approximately 1 hour or until spheroplasts were observed and then harvested again, and washed gently in 1.2 M sorbitol to remove any traces of DTT. The cell pellet was resuspended in 2 ml of solution III (3% sarkosyl; 0.5 M Tris-HCl; 0.2 M EDTA, pH8) and 0.2 mg of proteinase K added. The suspension was incubated at 37°C for 45 minutes and then the volume made up to 5 ml with 1 x TE. Then 5 ml of phenol-chloroform was added and the mixture shaken and then centrifuged for 7 minutes at 13000 rpm. The aqueous phase was then removed and the extraction process repeated until white proteinaceous matter was no longer observed at the interface. A final extraction with chloroform was then carried out to remove any phenol contamination and the DNA was precipitated with 2 volumes of ethanol. The DNA was pelleted by centrifugation at 13000 rpm and washed in 70% ethanol before resuspending in 0.5 ml of sterile distilled water. In order to remove contaminating RNA, 200 μ g of RNase A was then added and the solution incubated for 1 hour at 37°C. The DNA was precipitated by the addition of 2 volumes of ethanol and pelleted and washed as before. Finally the DNA was resuspended in 100 μ l of sterile distilled water.

2.4.4 Preparation of genomic DNA from *Candida albicans* for colony PCR

This method was used for the rapid preparation of genomic DNA from *C. albicans* colonies for colony PCR screening. Cells were obtained from a colony by touching a yellow Gilson tip to the surface of the colony. The cells were resuspended in 10 μ l of incubation solution (1.2 M sorbitol; 10 mM Tris-HCl, pH 7.5) and 50 μ g of zymolyase added. The suspension was incubated at 37°C for 30 minutes, before 2 μ l of the suspension was used in a 50 μ l PCR reaction.

2.4.5 Small scale plasmid DNA preparations from *Saccharomyces cerevisiae* (Holm *et al.*, 1986)

The following method was used for the preparation of yeast genomic DNA enriched for plasmids for transformation into *E. coli*.

Overnight cultures of 5 ml were centrifuged at 4000 rpm and resuspended in 1ml sterile distilled water. The suspension was spun in a microfuge and the cells resuspended in β -ME buffer (50 mM sodium phosphate buffer, pH 7.5; 0.9 M sorbitol; 1 μ l β -mercaptoethanol per ml of buffer) to which 25 μ l of 10 mg/ml zymolyase was added. The suspension was incubated at 37°C for 30 minutes, checked for spheroplast formation, and then incubated at 70°C for 20 minutes. 200 μ l of potassium acetate was added and the suspension mixed by inversion. After incubation on ice for 45 minutes the suspension was centrifuged and the supernatant transferred to a fresh tube. 0.55 ml isopropanol was added and the mixture incubated at room temperature for 5 minutes. The DNA was then pelleted in a microcentrifuge at 13000 rpm for 15 minutes, and the supernatant removed. The pellet was washed in 70% ethanol and resuspended in 20 μ l sterile distilled water. Half of the suspension was used in subsequent transformations into *E. coli*.

2.4.6 RNA preparations from *Candida albicans* (Schmitt *et al.*, 1990)

A cell culture of 25-100ml was harvested by centrifugation, and washed with 25 ml DEPC (diethyl pyrocarbonate) treated water. The cell pellets were then resuspended in 400 μ l of AE buffer (50 mM sodium acetate, pH 5.3; 10 mM EDTA) and 80 μ l of 10% SDS added. The suspension was vortexed for 30 seconds prior to the addition of 480 μ l of phenol equilibrated with AE. The suspension was again vortexed for 30 seconds and then incubated at 65°C for 4 minutes followed by chilling in dry ice/ ethanol until phenol crystals appeared (about 3 minutes). This freeze-thaw process was repeated 3 times with a final four minutes incubation at 65°C. The aqueous layer was separated by centrifugation at 13000 rpm for 5 minutes and was removed into a separate tube. An equal volume of phenol/ chloroform was then added and the aqueous layer again separated by centrifugation. Phenol/ chloroform extractions were repeated until no proteinaceous matter was observed at the interface. The RNA was then precipitated by the addition of 2 volumes of absolute ethanol and was pelleted by centrifugation at 13000 rpm for 15

minutes. The RNA was resuspended in 50 µl of DEPC-treated water and the concentration determined by UV spectrophotometry at 260 nm (the extinction coefficient of RNA at this wavelength is 0.025).

DEPC-treated water DEPC (diethyl pyrocarbonate) was added to sterile distilled water at 0.1% v/v. The water was left overnight in a fume hood and then autoclaved the following morning. This water was used to prepare all solutions required for RNA work.

2.5 Bacterial transposon mutagenesis (Sedgewick and Morgan, 1994)

In order to pinpoint the location of a rescuing gene within the insert of a rescuing clone the bacterial mutagenesis method of Sedgewick and Morgan (1994) was employed. The plasmid of interest was transformed into *E. coli* strain MH1599 (*Sm^S*) harbouring the conjugative plasmid R388::*Tn1000(HIS3)*. Cointegrate formation between the two plasmids was then selected for by conjugal mating with a streptomycin resistant strain, MH1578 (*Amp^S, Sm^R*).

Donor and recipient cells were grown overnight in LB containing the appropriate antibiotics. Cells were then diluted to an OD₆₀₀ of 0.05 in 10 ml LB and allowed to grow with aeration until an OD₆₀₀ of 0.5 was reached. 0.5 ml of both donor and recipient cells were then mixed in a microcentrifuge tube and the cell suspension applied to a nitrocellulose filter (25 mm diameter, 0.45 µm pore) attached to a vacuum. The nitrocellulose filter was then placed on a pre-warmed LA plate and incubated for 1 hour at 37°C. The filter was then transferred to a microfuge tube, 0.5 ml of water added and the tube vortexed to detach the cells. The cells were then pelleted by centrifugation, resuspended in 100 µl of LB and plated onto LA plates containing ampicillin and streptomycin to select for recipient cells which had received the cointegrate from the donor cells.

2.6 DNA Manipulations

2.6.1 Restriction analysis

Restriction endonucleases were obtained from Gibco-BRL Ltd. Digestion of DNA was carried out using the React buffers supplied by the manufacturer at the recommended temperature following the manufacturers instructions.

Plasmid DNA (1-2 μg for analytical digests) was digested for 2-3 hours with 1 unit of enzyme per reaction. Yeast genomic DNA digestions were carried out in 20 μl volumes for 3 hours with 2 units of enzyme. This was followed by further digestion with an additional 1 unit for 2 hours. The reaction volume was increased to 30 μl using restriction buffer and distilled water.

2.6.2 DNA agarose gel electrophoresis

DNA was visualised and fragments were purified from gels made up and run in 1 x Tris acetate electrophoresis buffer (TAE) containing 0.5 $\mu\text{g/ml}$ ethidium bromide. Stocks of TAE were made up at 50 x concentrate (2 M Tris-base, 1 M Sodium acetate (trihydrate), 0.5 M EDTA, to pH8.2 using glacial acetic acid). Standard gels were made from 0.6-1.2% Seakem HGT agarose, depending on the fragments requiring separation.

10x loading buffer 0.4% Bromophenol blue
 50% glycerol

2.6.3 Recovery of DNA from agarose gels

Samples of restriction digested DNA were electrophoresed on a gel such that 5-10 μg of DNA was present in the band of interest (where possible). The band of interest was excised using a scalpel and placed in a microfuge tube. A QIAGEN gel extraction kit (available from QIAGEN Ltd.) was then used to recover the DNA from the gel according to the manufacturers instructions.

2.6.4 DNA ligation

Where it was necessary to phosphatase the vector prior to setting up the ligation reaction, shrimp alkaline phosphatase (USB) was used. Phosphatase reactions were carried out in volumes of 20 μl using 1 μg of vector DNA and 0.1 units of shrimp alkaline phosphatase.

The reaction buffer provided by the manufacturer was used. The reaction was incubated at 37°C for 30 minutes after which a further 0.1 units of enzyme were added, the reaction volume made up to 30 µl and incubated for a further 30 minutes at 37°C. The reaction was terminated by incubation at 65°C for 15 minutes.

T4 DNA ligase (Gibco-BRL, 1 unit/µl) was used. Reactions were carried out in a total volume of 20 µl, containing 1 µl T4 ligase, 4 µl 5x T4 ligase buffer (250 mM Tris-HCl, pH7.5; 50 mM MgCl₂; 50 mM DTT, 5 mM ATP, 125 µg/ml BSA) plus the DNA being ligated. Samples were incubated overnight at 4°C and then transformed into competent *E.coli* cells. Generally 3 reactions were set up in parallel, containing vector:insert in the molar ratio of 1:1, 1:3 and 1:10 (Dugaiczky *et al.*, 1975).

2.7 Southern, Northern and Colony blotting

2.7.1 Southern transfer (Southern, 1975; Wahl *et al.*, 1979)

The method used to transfer DNA to a nylon filter was essentially that of Southern (1975) with a depurination step before alkaline denaturation to facilitate the transfer of large DNA fragments (Wahl *et al.*, 1979).

After visualisation the gel was washed briefly in distilled water. It was then washed twice for 10 minutes in depurinating solution (0.25 M HCl) and twice for 10 minutes in denaturing solution (0.5 M NaOH, 1 M NaCl) with rinsing in distilled water between washes. Finally the gel was washed in neutralising solution (0.5 M Tris-HCl, pH7.4; 3 M NaCl) for 15 minutes and was then mounted on a glass sheet covered with Whatmann 3MM to act as a wick over a reservoir of 20X SSC. The edges of the gell were covered with plastic film in order to prevent 'short circuiting'. A Hybond-N+ nylon filter (Amersham Pharmacia Biotech) cut to the size of the gel was wetted in 6X SSC and then placed on the gel and covered with 5 sheets of quickdraw blotting paper (available from Sigma Chemicals Company Ltd.). A glass plate was placed on the top with a weight placed to distribute the weight evenly. This was left overnight to elute the DNA onto the filter.

After the transfer was complete the filter was removed and placed on a piece of Whatman 3MM paper and allowed to air dry. The DNA was then cross-linked to the membrane by

exposure to ultra violet light in a UV cross-linker (Amersham Life Science) at 254 nm and 70×10^3 microjoules/cm².

2.7.2 Denaturing gels for RNA separation

RNA for northern blotting was separated on Formaldehyde-denaturing gels. Gels were made containing 1.5% agarose in 1 x MOPS (3-[N-morpholino]propanesulphonic acid), 5% formaldehyde and were run in 1 x MOPS. RNA samples of 30 µg were prepared for loading by the addition of 2 µl of 5x MOPS, 10 µl of deionised formamide, 3.5 µl of 40% formaldehyde. The samples were then incubated at 65°C for 10 minutes in order to denature the RNA and then snap cooled on ice. Finally 2.5 µl of 10x loading buffer was added and the samples loaded onto the gel.

2.7.3 Northern transfer

The gel was mounted on a glass sheet covered with Whatman 3MM to act as a wick over a reservoir of 20x SSC. The edges of the gel were covered with plastic film in order to prevent 'short circuiting'. A nylon membrane (Hybond-N+, Amersham Pharmacia Biotech.) cut to the size of the gel was wetted with 6x SSC and placed on the gel and covered with 5 sheets of Quickdraw blotting paper (available from Sigma Chemicals Company Ltd.). A glass plate was placed on top with a weight placed on top to distribute the weight evenly. This was left overnight to elute the RNA onto the filter.

After transfer was complete the filter was removed and placed on a piece of Whatman 3MM paper and allowed to dry. The RNA was then cross-linked to the filter by exposure to ultra violet light in a UV cross linker (Amersham Life Sciences) at 254 nm and 70×10^3 microjoules/cm².

2.7.4 Colony lysis

The method used was based on that described by Sambrook *et al.* (1990). *E. coli* cells containing library clones were plated to fresh LA plates containing the appropriate antibiotic to give approximately 300 colonies per 90 mm (diameter) plate, or 1000 per 150 mm plate and grown overnight at 37°C. Colonies were then transferred to Hybond-N (Amersham Pharmacia Biotech) nylon membrane discs. A membrane disc was placed over the colonies on each plate, and left for one minute before being lifted with tweezers.

In order to locate putative positive colonies on the plate a number of reference points were marked on both the plate and filter. Each filter was then placed colony side up, on a sheet of 3MM Whatmann paper saturated with Denaturing solution (0.5 N NaOH; 1.5 M NaCl) for 7 minutes, before transfer to 3MM paper saturated with Neutralising solution (1.5 M NaCl; 0.5 M Tris-HCl, pH7.4) for 3 minutes. The filters were then transferred to 3MM saturated with Neutralising solution for a further 3 minutes, and then 2 x SSC for 5 minutes. Once the membranes had air dried the DNA was fixed to the filter by exposure to ultra violet light in a UV cross linker (Amersham Life Sciences) at 254 nm and 70×10^3 microjoules/cm².

2.8 Radioactive labelling and detection of probes

2.8.1 Preparation of labelled probe

Radiolabelled DNA probes were prepared for filter hybridisation using random hexamer priming (Feinberg and Vogelstein, 1983). α -³²PdCTP was incorporated into the DNA in the presence of the other unlabelled nucleotides.

The DNA to be labelled was denatured by boiling for 3 minutes and then snap cooled on ice. To 25 ng of the denatured DNA 5 μ l of oligo-labelling buffer (OLB; see below), 1 μ l of BSA (10 mg/ml), 1 μ l Klenow and 2.5 μ l α -³²PdCTP was added. The mixture was incubated at room temperature for 4 hours and then fractionated in order to separate the unincorporated nucleotides away from the labelled probe. The fractionation was carried out using a column made from a Pasteur pipette plugged with polymer wool containing Sephadex G-50 (medium) beads (Pharmacia) suspended in 1 x TE. The volume of the labelling reaction was made up to 100 μ l by the addition of 1 x TE and the solution added to the top of the column. Aliquots of 100 μ l of 1 x TE were then added to the top of the column and the individual 100 μ l fractions collected in microfuge tubes. Each aliquot was tested for radioactive emission using a Geiger counter and the first group of aliquots showing high emissions were pooled for use as the probe. Since Sephadex beads separate the oligonucleotides from un-incorporated nucleotides by the size exclusion principle the labelled probe passes through the column faster than the un-incorporated nucleotides and is found in the first peak of radioactive material collected from the bottom of the column. Finally the DNA was denatured by boiling for 3 minutes and was then added directly to the hybridisation chamber.

OLB was made up from solutions A, B and C in the ratio 2:5:3.

Solution A: 100 µl solution O (1.25 M Tris-HCl, pH8; 0.125 M MgCl₂), 18 µl β-mercapto-ethanol, 5 µl each of dATP, dTTP and dGTP.

Solution B: 2 M HEPES, titrated to pH 6.6 with 4 M sodium hydroxide.

Solution C: Hexadeoxynucleotides resuspended in 1x TE at 90 OD units/ml.

2.8.2 Filter hybridisation for Northern, Southern and Colony blots

Filters were incubated in 50 ml of prehybridisation solution (Southern blots) or 10 ml of Church-Gilbert buffer (Northern blots; Church and Gilbert, 1984) for 4 hours at 65°C in a hybridisation chamber in a rotating hybridisation oven. The prehybridisation was then replaced with 25 ml of the hybridisation solution (Southern blot only) and the denatured labelled probe added. Hybridisation was continued at 65°C overnight and stringency washes were carried out the following day. In general, three stringency washes (3 x SSC, 0.1% SDS; 1 x SSC, 0.1% SDS; and 0.5 x SSC, 0.1% SDS) were carried out at 65°C for 30 minutes each. The filters were then wrapped in Saran wrap, and placed in X-ray cassettes with X-ray film. The film was exposed at -80°C for 2-5 days prior to development.

Prehybridisation solution: 6 x SSC, 0.5% SDS, 5 x Denhardt's solution, 0.1 mg/ml denatured salmon sperm DNA

Hybridisation buffer: 6 x SSC, 0.5% SDS, 5 x Denhardt's solution, 10 mM EDTA, 0.2 mg/ml denatured salmon sperm DNA

50x Denhardt's solution: 1% (w/v) Ficoll (mw 40 000), 1% (w/v) bovine serum albumin, 1% (w/v) polyvinylpyrrolidone (mw 4000).

Church-Gilbert buffer: 0.5 M sodium phosphate, pH 7.4; 7% SDS; 1 mM EDTA.

2.9 DNA sequencing and polymerase chain reaction

2.9.1 DNA sequencing

Custom made primers were supplied by PNACL, Leicester University, as described in table 2.6. DNA was sequenced on an ABI model 373A sequencer by PNACL, Leicester University, following preparation using the PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit.

Sequencing reactions were carried out in a total volume of 20 μ l (overlaid with liquid paraffin) containing 8.0 μ l terminator premix, 200 ng template DNA and 30 pmol primer DNA. The cycling reaction was carried out in a Hybaid Omni-E cycler, and consisted of 45 cycles of a denaturation step (96°C, 30 seconds), an annealing step (54°C, 15 seconds), and an extension incubation (60°C, 4 minutes). After the PCR the reaction mix was isolated from under the paraffin layer by pipetting and the extension products precipitated by the addition of 50 μ l of absolute ethanol and 2 μ l 3 M sodium acetate (pH5.6). The precipitated DNA was washed twice with 70% ethanol and the dried pellet submitted to PNACL for analysis on the DNA sequencer.

Terminator premix: 1.58 μ M A-DyeDeoxy, 94.74 μ M T-DyeDeoxy, 0.42 μ M G-DyeDeoxy, 47.37 μ M C-DyeDeoxy, 78.95 μ M dITP, 15.79 μ M dATP, dCTP, dTTP, dGTP, 168.42 mM Tris-HCl (pH9), 4.21 mM NH_4SO_4 , 42.1 mM MgCl_2 , 0.42 units/ μ l AmpliTaq DNA polymerase.

2.9.2 Polymerase chain reaction

PCR was used for the amplification of DNA fragments and was performed using either a Hybaid Omni-E thermal cycler or a Sanyo MIR-D30 DNA amplifier. A typical reaction was carried out in a final volume of 50 μ l containing 200 ng of each oligo-nucleotide primer, 50 ng template DNA, 1 unit Taq polymerase and 5 μ l 10x reaction buffer. The reaction was overlaid with liquid paraffin to prevent evaporation during the course of the PCR temperature cycling. Custom made primers were made by PNACL, Leicester University and are described in table 2.6.

10x PCR buffer: 450 mM Tris-HCl pH8.8, 110 mM NH_4SO_4 , 45 mM MgCl_2 , 67 mM β -mercaptoethanol, 44 μ M EDTA pH8, 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP, 1.13 mg/ml BSA.

A typical reaction profile consisted of 35 cycles of a denaturation step (95°C, 1 minutes), an annealing step (50-60°C, 1 minutes) and an extension step (72°C). The length of the extension step was calculated using the rule of 1 minute per kb of DNA amplified. The products of PCR were visualised following agarose gel electrophoresis.

2.9.3 Reverse Transcriptase PCR

RNA templates were first DNase treated to remove contaminating DNA. 1 µg of RNA preparation was incubated at 37°C for 30 minutes with 1 unit RNase free DNase, 1 µl 10x reaction buffer and nuclease free water to 10 µl. The reaction was then terminated by the addition of 1 µl STOP solution, and incubation at 65°C for 10 minutes. RNase free DNase, reaction buffer and the STOP solution were provided by Promega Life Science.

A ProSTAR single step RT-PCR kit (Stratagene) was used for the detection of specific transcripts as per the manufacturers instructions. This system uses a single reaction tube for both Reverse Transcriptase (RT) cDNA synthesis, and PCR amplification. The reaction was carried out in a 50 µl volume with 200 ng of DNase treated RNA template. Each reaction was then overlaid with paraffin oil and subject to thermal cycling. Thermal cycling consisted of a single 37°C cycle for 15 minutes for RT dependent cDNA synthesis, followed by 40 cycles of a denaturing step (95°C, 30 seconds), an annealing step (60°C, 30 seconds) and an extension step (68°C, 2 minutes/kb of target sequence), this is the PCR amplification step. Finally a single cycle (68°C, 10 minutes) completed extension. Control reactions omitted RT, for the detection of any contaminating DNA. Samples (5 µl) from each reaction were then subject to analysis by agarose gel electrophoresis.

Table 2.6. Synthetic oligonucleotides used for sequencing and PCR in this study

Name	Sequence 5'→3'	Target
Sequencing of pGPTA Insert		
GP02	gca aag att gaa taa ggc gc	YEp213 nt10533-10552
GP03	cca aga ccc tac tat atg aaa cc	pGPTA insert 403-425
GP04	agt act tcc cac gtg agg	pGPTA insert 1966-1949
GP05	tta tac aaa ctt cgg agt gcc	pGPTA insert 1453-1433
GP06	aaa ggg gaa agg tta taa agc	pGPTA insert 882-902
GP07	gat cca cca cat taa tat ccc	pGPTA insert 552-532
GP08	gat gct tta taa cct ttc ccc	pGPTA insert 905-885
GP09	tag aat atg aca ggc act ccg	pGPTA insert 1421-1441
GP10	ccg taa aac tct cat aaa cac c	pGPTA insert 1787-1808
RT-PCR of pGPTA ORF		
TA-1	aaa aaa ctg tca ccg tat tcg	pGPTA insert 433-453
TA-2	gat gct tta taa cct ttc ccc	pGPTA insert 905-885

	Amplification of pGPTA ORFs	
TARF2-1	gcg cat ttt tct tca aag ctt tac g	YEp213 10549-10569 across <i>Hind</i> III site incorporated
TARF2-2	tat tta cat aag ctt gta atg aat agc tgg g	pGPTA insert 1649-1679 with <i>Hind</i> III site incorporated
GPTA01	ctg tgg cgc cgg gga tcc cgg cca cga tgc	425-395 YEp213 with <i>Bam</i> HI site incorporated
GPTA02	ctt caa ata aaa gga tcc atc act tgc ctt gc	pGPTA insert 1179-1148 with <i>Bam</i> HI site incorporated
	Disruption of pGPTA Insert	
TAD1	aaa aaa gga tcc gat cca cca cat taa tat ccc	pGPTA insert 552-532 with <i>Bam</i> HI site incorporated
TAD2	aaa aaa gga tcc gct gat ggg aac cca aag ggg	pGPTA insert 856-888 with <i>Bam</i> HI site incorporated
TAD3	aaa tct ttc gta aag ctt tga aga aaa atg c	YEp213 10551-10569 + 2329-2318 from pGPTA insert, with <i>Hind</i> III site incorporated
TAD4	ttg gtt taa taa aag ctt tgt ttt gag tgg	pGPTA insert 1836-1807 with <i>Hind</i> III site incorporated
TAD5	tac gcc gga agc ttc gtg gcc ggc atc acc	YEp213 386-415 with <i>Hind</i> III site incorporated
	Sequencing of pGPT1 Insert	
GPT1TN4501	cct tgt agg ggt ttg agg gcc	
GPT1TN4502	cca aat agg agt caa gat gag g	
	Sequencing Out of Tn1000	
TN1	tgc cag agg ata ttt taa aag ggg	Tn1000 5878-5901
TN2	tac aac ata cga aag ggt ccc	Tn1000 105-85
	Miscellaneous	
GPHISG	gga aga tga tct tcc ggg ggc	pMB7 4307-4328 (HISG region)
TETBGP	caa ccg cac ctg tgg cgc cgg	YEp213 434-414
18P5	ttt gaa gca cca aga gca agc	<i>CaVPS18</i> +1625-+1645
18P3	gaa cgc atg atg gca att tgg	<i>CaVPS18</i> +2372-+2352
	pGP184/ <i>CaVPS18</i> Sequencing	(from ATG Start)
CAPEP3-1	gat caa gcc tta gaa ctt gcc c	1872-1893
CAPEP3-2	cct tgt gga acg cat gat ggc	2379-2359
GP181	gaa aca ttg acg atg tga tgc	2083-2063
GP182	tga att gct tgc tct tgg tgc	1651-1631

GP183	ttt gaa gca cca aga gca agc	1625-1645
GP185	gct gac aac aca att act cgg	1285-1265
GP186	ctc att gaa tat ttg ttg gcc	1221-1241
GP187	att gca ttt cct cgt cgt tgg	732-712
GP188	acg acg agg aaa tgc aat tgg	715-732
GP189	cat tcg tta ctt tac cag gc	285-266
GP180	ggg gga gaa tta tgc gtt gcc	248-268
GP1811	cca atg ttg acc taa tac gca cg	-261- -283
GP1812	tat gcc acg ttt gtg tca ggg	-325- -305
GP1813	acc tat ttc aag cta aat ccc	-829- -809
GP1814	caa att gcc atc atg cgt tcc	2353-2373
GP1815	gcc atc atg cgt tcc ata agg	2359-2379
GP1816	gtc tgc caa ata ttt tcc ccc	2826-2806
GP1817	taa acc tta tgg agt taa gcc	2847-2867
GP1818	gaa tat ata acc att gtg cgg	3338-3358
GP1819	tat agt tgt att gtt ttc cgc	3375-3395
	Construction of <i>CaVPS18</i> Disruption Cassette	
CAP3-1	tat cag aaa acc gca tgc acg	Spans <i>SphI</i> site –584 in <i>CaVPS18</i> database sequence
CAP3-2	gtg gtt tag tcg aca tga atg gta agc	-21- -40 with <i>SalI</i> site incorporated
CAP3-3	taa tga ttt ttt gaa ttt ata gtc att cc	2414-2446 with <i>BglII</i> site incorporated
CAP3-4	ttc tgt tga aag cgg tac cgg	Spans <i>KpnI</i> site 3552 in <i>CaVPS18</i> database sequence
	pGPC10/<i>CaVPS11</i> Sequencing	(from ATG Start)
CAENDG11-1	cca tgc att gta ctc tga ccc	122-142
CAENDG11-2	gaa ttg tat tga atc att taa tcc	872-849
CAENDC10-1	cta acc att cct cct tgt cgc c	1221-1242
CAENDC10-2	ctt cca cca tga acc aac ccc	1981-1961
C103	ttt ggc att ggg ttc att agg	1949-1929
C104	tct tga ggc gag tct cga agc	2246-2266
C105	ctt tgt taa att aga tat att tgt agc	1514-1487
C106	tat aat tac taa att caa aga agc	1466-1488
C107	att att aac tcc cat tga att gg	2690-2712
C108	atg taa taa att cat tat cag gg	2754-2732
C109	cgc ttt att tct ttc ttc ggg	983-963
C1010	cga aga aag aaa taa agc ggg	965-985
C1011	caa tat aaa ctt gga gat gct gct ta	3168-3193
C1012	gat ctt cca tta cac ctt tac c	3297-3276

C1013	tga gaa cac caa gat ctg acg	3637-3617
C1014	tca tgc ccg ctt gaa cta ccg	1872-1892
C1015	act tat tgg att cat gcc cgc	1861-1881
A91	aat aac ttt acc att agt gta acc	593-570
A92	agt caa cga tca tag tag tgg	473-493
A93	aca ttg agt tgt att aca gcc	144-164
Disruption of <i>CaVPS11</i>		
11D5	ttc tcc ttc gag gac aat atc atc tcc tct att atc att atc atc atg gag aca gtt cca att gtt tga ttg tgg aat tgt gag cgg ata	17-87 <i>CaVPS11</i> (targeting sequence) + PCR annealing region (3979-3960 in pRSARG4-SpeI or 139-158 in pGEM-URA3)
11D3	cta ttg atc ttc cat tac acc ttt acc aat ata atc aga aac aaa ttt aaa ttt atc att act aga atg ctt tcc cag tca cga cgt t	3302-3233 <i>CaVPS11</i> (targeting region) + PCR annealing region (1821-1838 in pRSARG4-SpeI or 2896-2913 in pGEM-URA3)
11PB5	cct atc att ttc ggt gtt agg	-413- -393
11PB3	ggt gta atg aat gta att gcc	-10- -30
<i>CaVPS33</i> Probe Amplification and RT-PCR		(from ATG Start)
CAVPS33-1	gga ttg aga cta aac aaa ttg gc	1014-1036
CAVPS33-2	gtt ctt ctt tta tta cta ttc agt tcg	2115-2089
CAVPS33-3	atc tcc act ccc act gat ccc	1598-1578

2.10 Visualization of yeast vacuoles

2.10.3 Staining vacuoles of *Saccharomyces cerevisiae*

S. cerevisiae ade2⁻ strains are blocked in adenine production and when adenine is limiting accumulate the biosynthetic intermediate 4-aminoimidazole (Jones and Fink, 1982). A derivative of this compound accumulates in the vacuole, producing a stable red coloured compound, which is also visible by fluorescence microscopy (Weisman *et al.*, 1987) providing a convenient means of specifically staining vacuoles.

The method used was that of (Weisman *et al.*, 1987). Cells were grown in SD media with limiting amounts of adenine (12 µg/ml) overnight to induce the production of the *ade2⁻* endogenous fluorophore. Cells approaching stationary phase were mounted on a concanavalin-A coated slide (see below) and observed on a Zeiss confocal microscope, excitation 450-490 nm and viewing emitted light with a 520 nm cut-off filter.

Con-A coated microscope slide: Con-A coated slides were used to affix cells for analysis by fluorescence/ confocal microscopy. These were made by spreading 10 μ l of a 1 mg/ml solution of concanavalin-A onto a slide, which was allowed to air dry.

Chapter 3

Phenotypic Analysis of *Saccharomyces cerevisiae* Class-C *vps* Mutants

3.1 Introduction

Several *S. cerevisiae* mutants were identified within our laboratory as defective in the iron responsive regulation of a cell surface ferric reductase activity. The wild-type gene was cloned for one of these mutants by functional complementation, and identified as *VPS18*. This ORF was then disrupted through the insertion of a Tn1000:*HIS3* transposon and re-integrated at the *VPS18* loci to yield a *vps18*:Tn1000 (JMΔ18) mutant strain. In addition, all four class-C *vps* mutants originally isolated by Emr and colleagues (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988), and their parental strain (SEY6211) were obtained for analysis (from Martin Watson, University of Durham). Phenotypic analysis of these strains was performed, with a view to developing a screen for the isolation of *C. albicans* genomic DNA clones, through functional complementation of the *S. cerevisiae* class-C *vps* mutants.

3.2 JM18 and JMΔ18 exhibit similar phenotypes to previously studied class-C *vps* mutants

The *vps18* mutant strain identified during the study of iron responsive gene regulation (JM18), the transposon disrupted strain (JMΔ18), and the parental strain S150-2B, were first tested for phenotypes that had been previously associated with class-C *vps* mutants (Section 1.7). Both of the mutant strains exhibited the temperature sensitive growth phenotype reported for the previously characterised class-C *vps* mutants (Banta *et al.*, 1988)(figure 3.1). Both mutant and the parental strain grew at 28°C when streaked onto YPD agar. However, whilst the parental strain grew at 37°C, no growth was observed for either mutant. A second phenotype previously observed for each of the class-C *vps* mutants, was sensitivity to osmotic stress (Banta *et al.*, 1988). The mutant strains JM18 and JMΔ18, were compared to the parental strain, S150-2B, for their ability to grow under conditions of osmotic stress. Each strain was grown to log-phase ($1-2 \times 10^7$ cells/ml) in YPD media, and serial dilutions of each culture made with sterile water (between 1×10^7 and 3.2×10^3 cells/ml) in a microtitre dish. The diluted cells were then plated using a

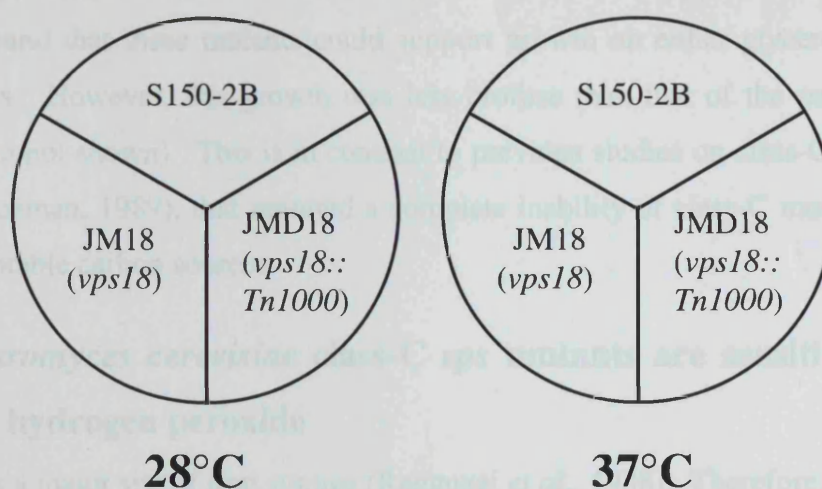
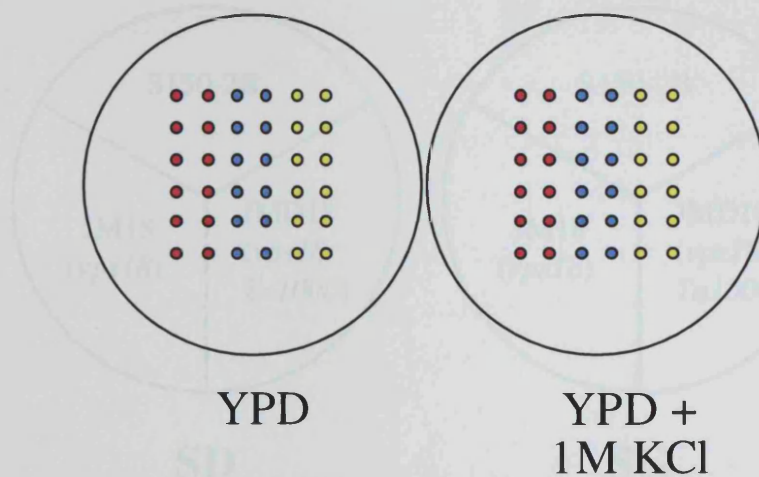
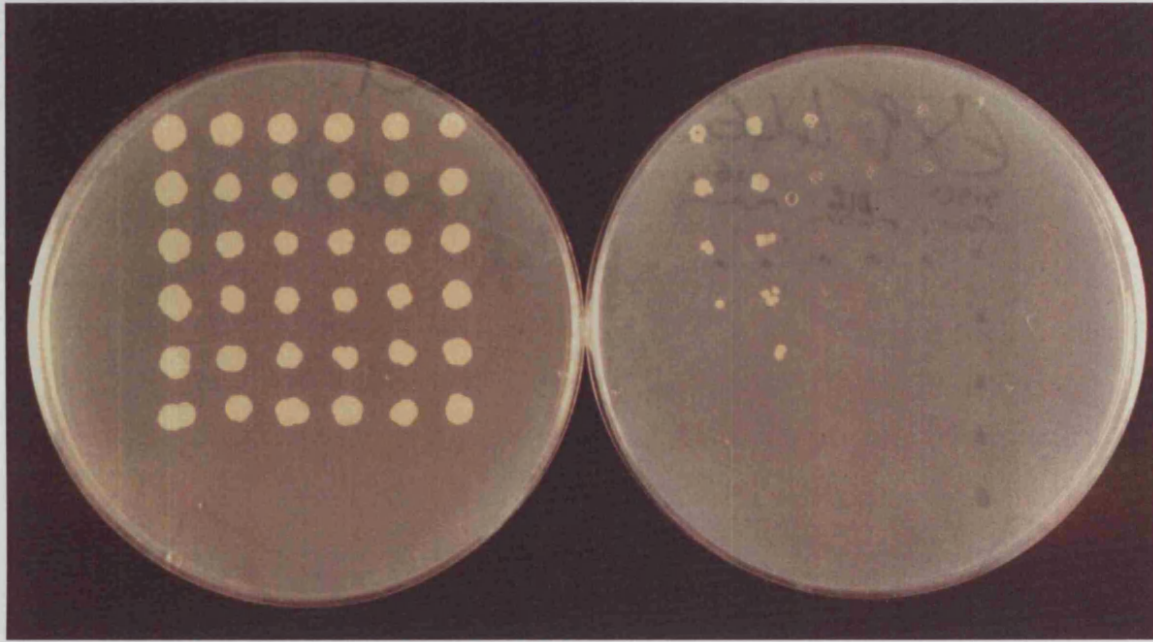


Figure 3.1. JM18 and JMD18 are temperature sensitive for growth. Single colonies of each strain were selected, re-streaked to fresh YPD medium, and incubated at either 28°C or 37°C for 3 days.

sterile ‘hedgehog’ to either YPD, or YPD supplemented with 1 M KCl. After 5 days incubation at 28°C, all strains had grown well on the YPD agar (figure 3.2). However, only the parental strain S150-2B had shown any detectable growth on the YPD media supplemented with 1 M KCl (figure 3.2). Finally, the ability of the mutant strains JM18 and JMΔ18, to utilise non-fermentable carbon sources was tested. Each strain was propagated on either YPD (glucose) or YPG (glycerol) or solid media, with incubation at 28°C. Then, single colonies of each strain from each media were re-streaked to fresh YP agar, supplemented with the same carbon sources. Growth was then assessed after 5 days incubation at 28°C. All strains grew well where glucose was the carbon source (figure 3.3). However, both mutant strains grew less well when glycerol was provided as the carbon source (figure 3.3). This suggests that JM18 and JMΔ18 can utilise glycerol as a carbon source, but less efficiently than the parental strain. Experiments with the class-C *vps* mutants *vps11*, *vps16*, *vps18* and *vps33* (parental strain SEY6211), (Robinson *et al.*, 1988), also found that these mutants could support growth on either glycerol or ethanol carbon sources. However, this growth was less profuse than that of the parental strain, SEY6211 (data not shown). This is in contrast to previous studies on class-C *vps* mutants (Dulic and Riezman, 1989), that reported a complete inability of class-C mutants to grow on non-fermentable carbon sources.

3.3 *Saccharomyces cerevisiae* class-C *vps* mutants are sensitive to iron, copper and hydrogen peroxide

The vacuole is a major site of iron storage (Ragguzzi *et al.*, 1988). Therefore, the defect in iron responsive gene regulation observed in JM18 (*vps18*) is most likely a consequence of the defect in vacuole biogenesis. Moreover, the observed deregulation of the surface ferric reductase, is likely to be indicative of an altered intracellular distribution of iron, and other transition metals such as copper. High concentrations of iron are cytotoxic, due to its ability to catalyse Haber-Weiss-Fenton chemistry (Halliwell and Gutteridge, 1999). This produces free radicals that damage cell membranes and can cause DNA strand breakage, leading to cell death (Weinberg, 1989). Therefore relative sensitivity of the class-C *vps* mutant and parental strains to extracellular iron chloride was examined. Each strain was grown to log phase ($1-2 \times 10^7$ cells/ml) in liquid YPD medium, and 1×10^7 cells mixed with 10 ml molten low-gelling-temperature agarose (preincubated at 37°C), and poured over a fresh YPD plate. Whatmann 3MM paper discs (12 mm diameter) were then placed



- S150-2B
- *vps18::Tn1000* (JMΔ18)
- *vps18* (JM18)

Figure 3.2. JM18 and JMΔ18 are sensitive to osmotic stress. Cells were grown to log phase in liquid YPD media at 28°C, diluted to known cell densities in a microtitre dish (between 1×10^7 and 3.2×10^3 cells/ml), and plated to either solid YPD or YPD supplemented with 1M KCl, using a 'hedgehog'. Plates were photographed after 5 days growth at 28°C.

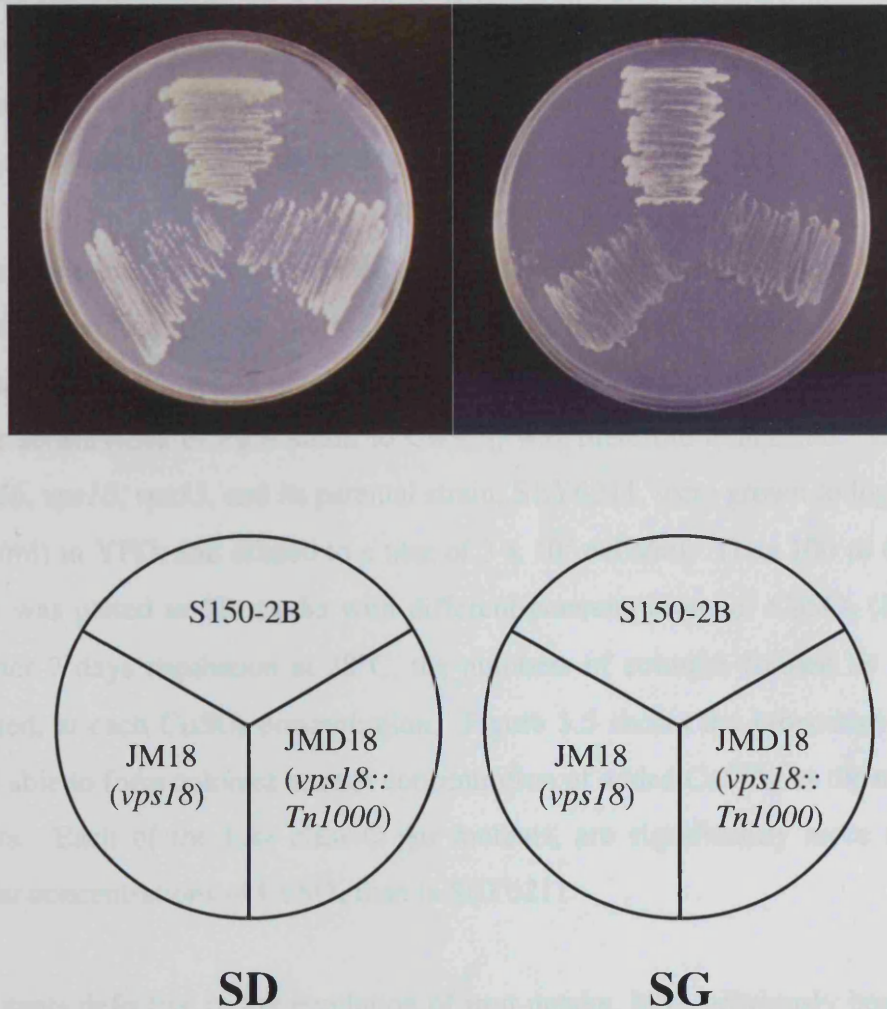


Figure 3.3. *Saccharomyces cerevisiae* class-C *vps* mutants grow less well than the parental strain on non-fermentable carbon sources. Cells were propagated on either SD (2% glucose), or SG (3% glycerol). Single colonies were then streaked to fresh SD or SG and incubated at 28°C for 5 days.

into the middle of each plate and 25 μ l of a saturated FeCl_3 solution (in 0.1 M HCl) deposited to the center of each disc. Growth was compared after two days incubation at 28°C (figure 3.4). The 'halo' of growth inhibition was much larger for both JM18 and JMA18, than the parental strain S150-2B (figure 3.4), indicating a greater sensitivity of these mutant strains to high concentrations of extracellular FeCl_3 . Similar results were found for each of *vps11*, *vps16*, *vps18* and *vps33* mutants (obtained from Martin Watson, University of Durham), when compared to their parental strain SEY6211 (data not shown).

In similar experiments to those described for FeCl_3 sensitivity (above), each of the class-C *vps* mutants were found to be more sensitive than the parental strain to CuSO_4 . The concentrations of CuSO_4 found to inhibit growth are significantly lower than for FeCl_3 , and the relative sensitivities of each strain to CuSO_4 , was therefore quantified. Each mutant *vps11*, *vps16*, *vps18*, *vps33*, and its parental strain, SEY6211, were grown to log phase ($1-2 \times 10^7$ cells/ml) in YPD, and diluted to a titre of 3×10^3 cells/ml. Then 100 μ l of each cell suspension was plated to SD media with different concentrations of CuSO_4 (See Section 2.2.2). After 7 days incubation at 28°C, the numbers of colonies formed by each strain were counted, at each CuSO_4 concentration. Figure 3.5 shows the proportion of cells of each strain able to form colonies at each concentration of added CuSO_4 , as the mean of two experiments. Each of the four class-C *vps* mutants, are significantly more sensitive to extracellular concentrations of CuSO_4 than is SEY6211.

Several mutants defective in the regulation of iron uptake, have previously been observed to be sensitive to the oxidative stress caused by hydrogen peroxide. This is most likely due to an increased cytoplasmic concentration of iron and other transition metals which catalyse the breakdown of H_2O_2 to the highly reactive $\cdot\text{OH}$ species. Each of the class-C *vps* mutants were therefore tested for sensitivity to H_2O_2 . Cells of each strain were prepared as for the CuSO_4 experiments (above), and plated on to minimal SD medium, supplemented with H_2O_2 (0-800 μM)(Sigma). After 4 days incubation at 28°C, the number of colonies formed by each strain, at each H_2O_2 concentration, were counted. The proportion of cells of each strain able to form colonies at each H_2O_2 concentration (for a single experiment) is shown in figure 3.6. Whilst the relative sensitivities of each strain remained similar, the actual level of H_2O_2 required for inhibition of growth, varied widely

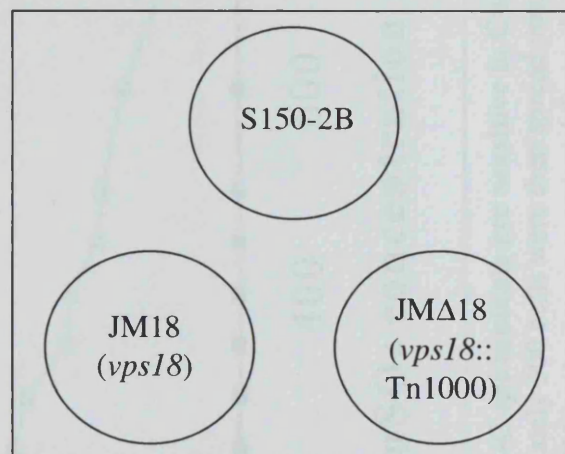
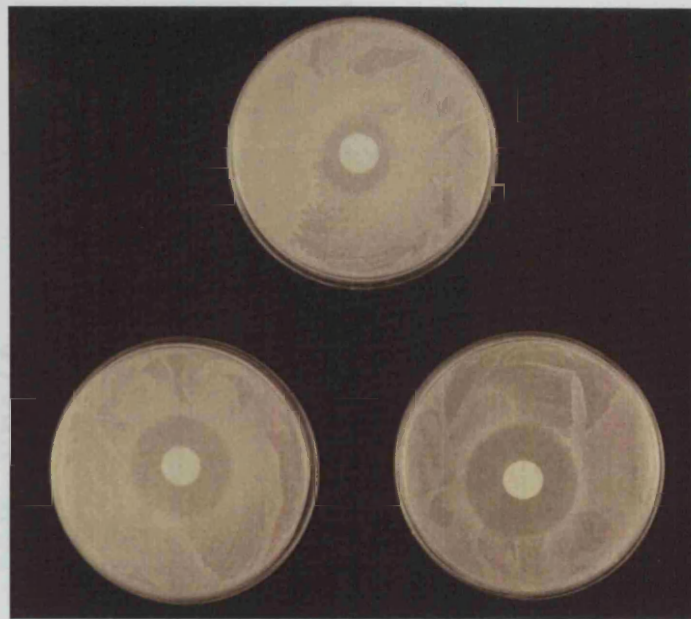


Figure 3.4. *Saccharomyces cerevisiae cerevisiae* class-C *vps* mutants are sensitive to FeCl_3 . Each strain was grown to log phase in liquid YPD ($1-2 \times 10^7/\text{ml}$), and 1×10^7 cells mixed in 10ml molten low gelling temperature agarose (37°C) before being poured over an SD plate supplemented with the appropriate nutrients. Once set, a whatman paper disc was applied to the centre of each plate and $25\mu\text{l}$ of a saturated FeCl_3 solution (in 0.1M HCl) added to the centre of each disc. Plates were incubated for 2 days at 28°C .

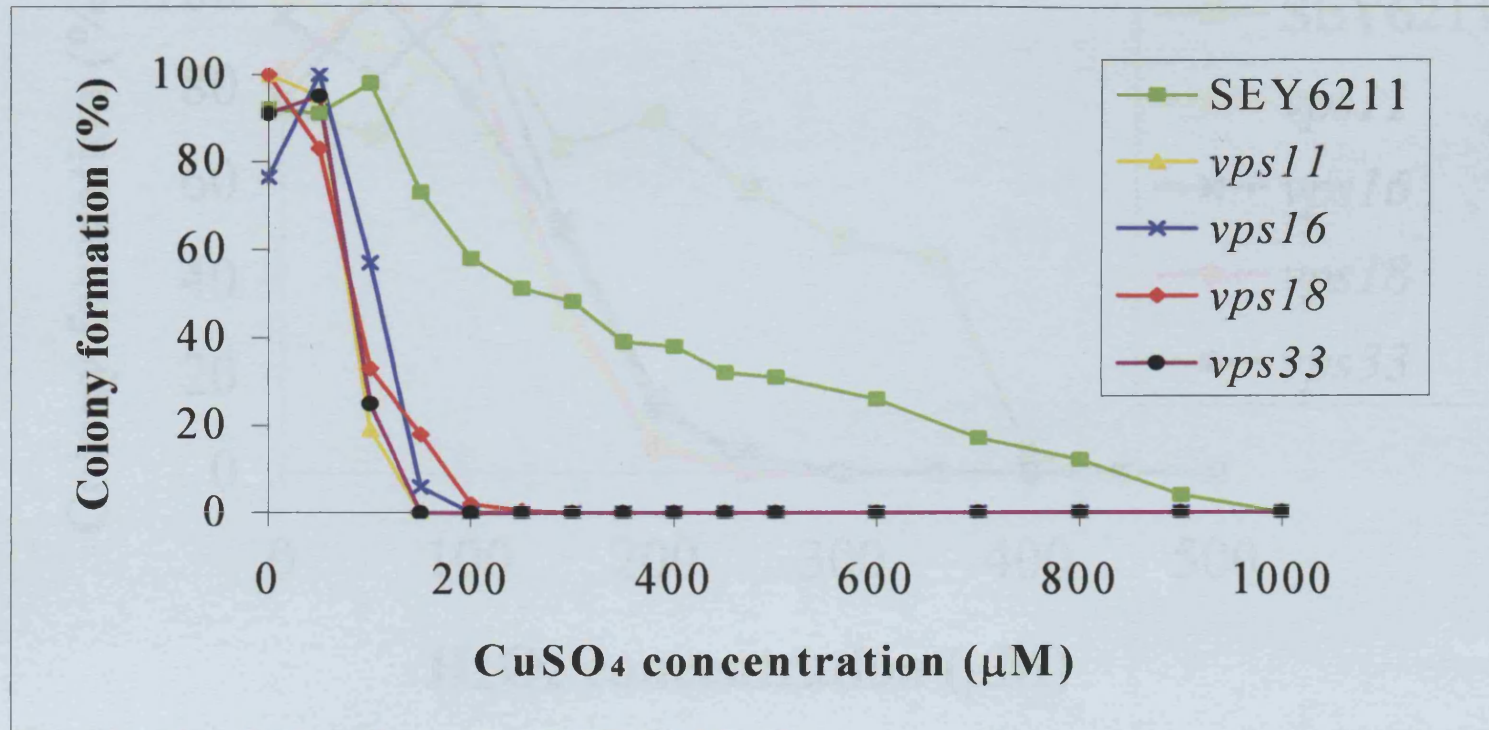


Figure 3.5. *Saccharomyces cerevisiae* class-C *vps* mutants are sensitive to CuSO₄. Each strain was grown to log phase ($1-2 \times 10^7$ cells/ml), in liquid YPD medium. Approximately 300 cells were then spread onto minimal SD agar plates supplemented with CuSO₄ (section 2.2.2). After 7 days incubation at 28°C, colonies were counted for each strain, at each CuSO₄ concentration. Colony formation was calculated as a percentage of the maximum number of colonies formed by any given strain. The data displayed is the mean of two independent experiments.

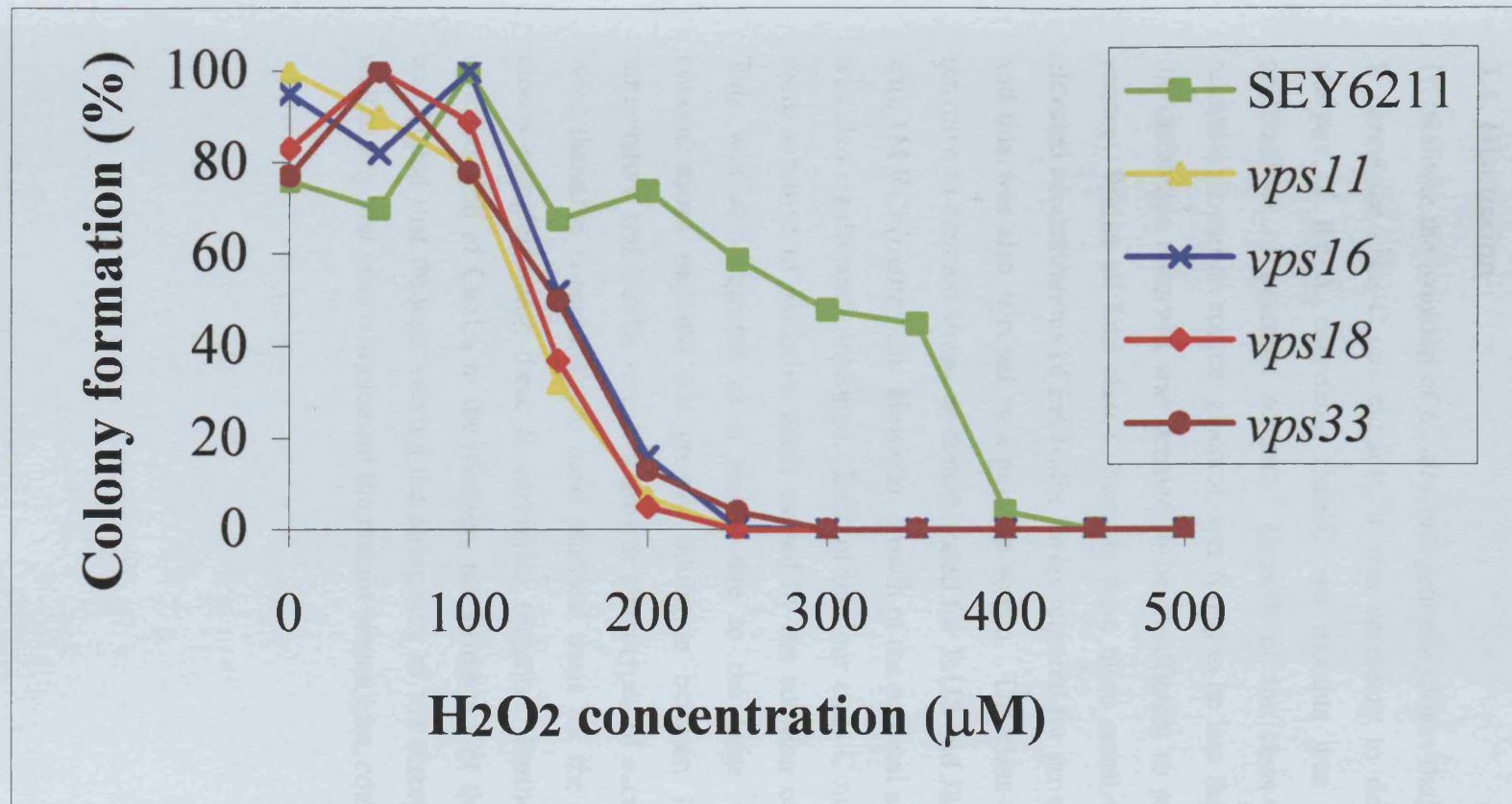


Figure 3.6. *Saccharomyces cerevisiae* class-C *vps* mutants are sensitive to hydrogen peroxide. Cells of each strain were grown to logarithmic phase ($1-2 \times 10^7$ cells/ml) in liquid YPD medium, and plated at approximately 300 cells per plate to SD media supplemented with H₂O₂. After 4 days incubation at 28°C, colonies were counted. 'Colony formation' at each H₂O₂ concentration was calculated as a percentage of the maximum colony count for each strain.

between three identical experiments, performed with separate batches of H_2O_2 . The unstable nature of H_2O_2 is likely to account for the observed variations in potency.

3.4. Discussion

To facilitate the isolation of *C. albicans* genomic clones that functionally complement the *S. cerevisiae* class-C *vps* mutants, it was necessary to develop a screen. Phenotypic analysis of the *S. cerevisiae* class-C *vps* mutants was carried out to evaluate the practicalities of potential screens. Growth of the class-C *vps* mutants on the non-fermentable carbon source, glycerol, was found to be less than parental strains. However, the difference observed was deemed to be insufficient to provide the basis of a reliable screen. Whilst all four class-C mutants were more sensitive than the parental strain to elevated concentrations of FeCl_3 , the levels required for growth inhibition were very high, and this was also rejected as a putative screen. The class-C *vps* mutants are also more sensitive to osmotic stress, as demonstrated for JM18 and JMA18 on media supplemented with 1M KCl (figure 3.2). However, growth of the parental strain (S150-2B) on this media was also significantly inhibited. Each of the four class-C mutant strains was found to be more sensitive to oxidative stress caused by the addition of H_2O_2 to the growth media. This was also rejected as a screen due to the large variation seen in the H_2O_2 concentrations required for growth inhibition between identical experiments. The temperature and CuSO_4 sensitive growth phenotypes of each of the class-C *vps* mutants, were therefore considered the most practical basis for the identification of *C. albicans* clones complementing these *S. cerevisiae* mutants. Furthermore, through altering the concentration of CuSO_4 in the medium, the stringency of the screen can be adjusted. It was hoped that through varying the stringency of the screen, clones that suppressed, as well as any that fully complement the mutant phenotypes, could be identified.

Chapter 4

Analysis of *Candida albicans* genomic DNA clones, which complement *Saccharomyces cerevisiae* class-C *vps* mutants

4.1 Introduction

One widely used strategy for the isolation of *C. albicans* genes is through their ability to complement mutant strains of the model eukaryote, *S. cerevisiae*. One advantage of isolating genes using this method is that it provides information about the function of the gene product. This chapter describes attempts to isolate class-C *VPS* functional homologues through the complementation of *S. cerevisiae* class-C mutants, with a multicopy *C. albicans* genomic DNA library.

4.2 Isolation of *Candida albicans* clones rescuing *Saccharomyces cerevisiae* class-C *VPS* mutants

The *C. albicans* genomic DNA library constructed in the multi-copy vector YEp213 (with an *S. cerevisiae* *LEU2* selectable marker), was used; it contains an average insert size of 7kb. The library DNA was transformed into each of the *S. cerevisiae* class-C *vps* mutant strains *vps11*, *vps16*, *vps18* and *vps33*, and the transformants screened for the rescue of mutant phenotypes. The following equation was used to calculate the number of colonies required to cover the whole genome:

$$N = \frac{\ln(1-p)}{\ln(1-a/b)}$$

where: N = the number of clones screened
 p = probability that any given gene is present in the library (0.95)
 a = average size of DNA inserts in library (7 kb)
 b = total size of genome (16×10^3 kb per haploid genome)

Therefore:

$$N = \frac{\ln(1-0.95)}{\ln(1-[7/16 \times 10^3])}$$

$$N = 6845 \text{ colonies}$$

Therefore approximately 7000 colonies need to be screened to give a probability of 95% of having screened the complete genome.

Initially the copper sensitive growth phenotype (Chapter 3) of the class-C mutants was utilised to identify rescuing clones. Growth on SD medium with additional copper was used as a screen, since increasing copper concentrations in successive screens, would allow control of the screen's stringency. This should permit the identification of clones that only partially rescued these mutants as well as any that fully rescued this phenotype. Library transformants of JMA18 (*vps18::Tn1000*) (parental strain S150-2B), were grown on selective media lacking leucine. In total 36000 transformants were subsequently screened through replica plating onto selective SD media enriched with either 400 or 500 μM CuSO_4 (see Materials and Methods), and incubated for 4 days at 28°C. Colonies which showed visible growth on this medium were picked and re-streaked on fresh SD media enriched with either 400 or 500 μM CuSO_4 . After 6 days incubation at 28°C, each transformant was assessed for growth. Seven *S. cerevisiae* transformants displaying significant growth on the copper enriched medium, were selected. The *C. albicans* genomic clones carried by these strains were isolated from the yeast and transformed into *E. coli* (DH5 α). Each plasmid was prepared and reintroduced into the original *vps18::Tn1000* mutant strain JMA18. The transformants were then again streaked on selective SD media enriched with either 400 or 500 μM CuSO_4 . Parental (S150-2B) and mutant (*vps18::Tn1000*) strains carrying vector (YEp213) alone, were also streaked on the same media, as positive and negative controls. However, none of these plasmids were found to reproducibly rescue the *vps18* mutant strain. Thus this screen resulted in the isolation of a high proportion of false positives. A second, and more stringent screen was then employed to screen library transformants.

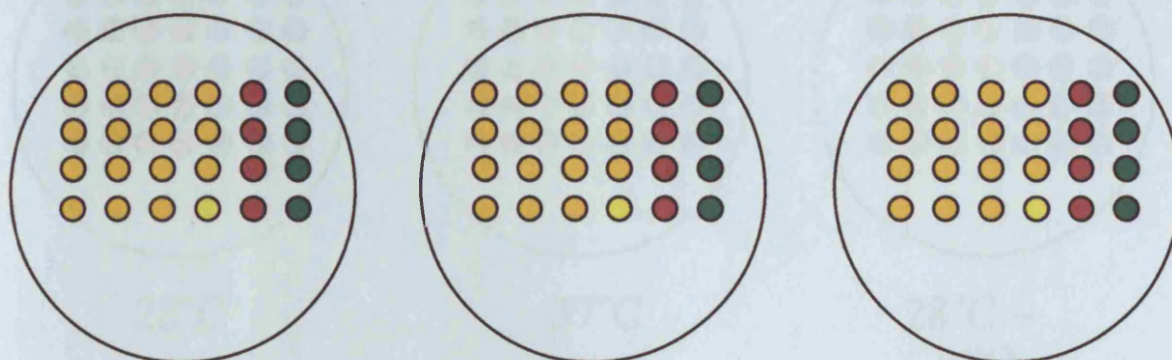
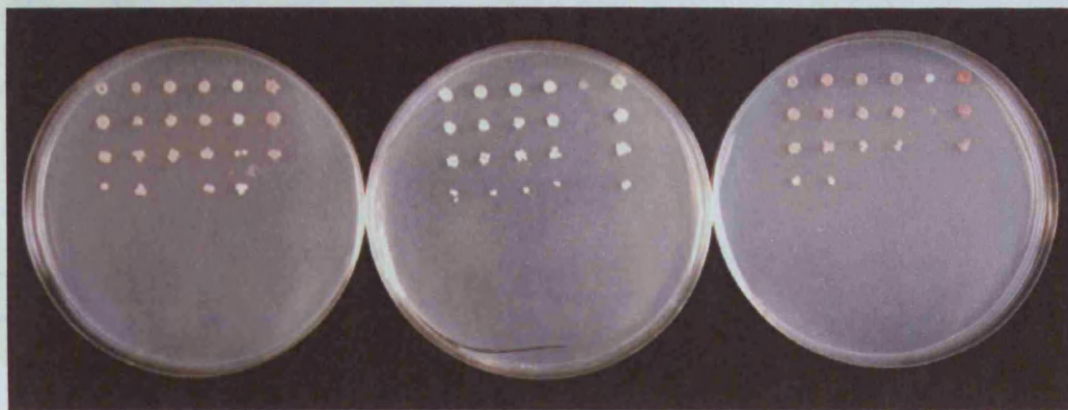
Library transformants of each of the four class-C mutants were replica plated this time onto fresh selective SD media lacking leucine, and incubated at 37°C. In total 11000 *vps11*, 26000 *vps16*, 55000 *vps18::Tn1000* and 16000 *vps33* transformants were screened ensuring a >95% confidence limit of genome coverage in each of these mutants. After 7-10 days colonies were selected which had grown significantly. Each was streaked on fresh selective SD media with parental strain + YEp213, and mutant strain + YEp213, as positive and negative controls. After 7 days incubation at 37°C, plasmids were prepared from those

strains which had formed colonies (see plasmid rescue protocol in Materials and Methods), and transformed into *E. coli* (DH5 α), to isolate the putative rescuing genomic clones. Each of these was purified, and re-transformed into the original *S. cerevisiae* mutant from which it was isolated. The transformants were then tested for ability to grow at 37°C, as above. This screen failed to identify any clones that restored the ability of the *vps11* or *vps33* mutants to form colonies at 37°C. Nine clones were identified that rescued the temperature sensitive growth phenotype of *vps16* (figure 4.1). In addition, one clone (pGPT1) was identified that partially restored the growth of *vps18::Tn1000* at 37°C (figure 4.2).

Mutant cells carrying the rescuing clones were grown to log phase (5×10^6 - 2×10^7 /ml), spotted in serial dilutions onto selective SD medium, and photographed after 4 days growth at either 28 or 37°C (figures 4.1 and 4.2). In the same experiments the cells were plated on SD medium enriched with 400 μ M CuSO $_4$ and grown at 28°C. Parental strains (SEY6211 or S150-2B) + YEp213 and mutant strain (*vps16* or *vps18*) + YEp213 were included as positive and negative controls. On SD media at the permissive temperature (28°C), all strains appeared to grow abundantly. At 37°C the parental strain grew abundantly, and the control *vps16* and *vps18* mutant cells failed to grow. The same pattern of growth was observed for parental and mutant strains grown on the copper enriched media. Each of the nine *vps16* isolated clones, appeared to restore growth of the mutant at both the non-permissive temperature and on copper enriched media to near parental levels (figure 4.1). The clone isolated as restoring the growth of *vps18::Tn1000* at 37°C (pGPT1), also improved *vps18::Tn1000* growth on copper enriched media. However, the level of growth observed for *vps18::Tn1000* + pGPT1, under both of these conditions was noticeably less than that of the parental strain (S150-2B).

4.3 *vps16* rescuing clones overlap a common genomic region

The nine *C. albicans* genomic clones identified as rescuing the *S. cerevisiae* *vps16* mutant were restriction mapped. This revealed that identical clones had been isolated more than once. The nine clones contained inserts of three types, designated pGPT2 (isolated five times), pGPT3 (isolated once) and pGPT4 (isolated three) (figure 4.3). Restriction analysis also revealed that these clones spanned a common region of the genome (figure 4.3). The insert of the smallest clone, pGPT3, was bisected at a *HindIII* site near its centre to make two further subclones in YEp213, pGPTA and pGPTB (figure 4.3). pGPTA contained a



28°C

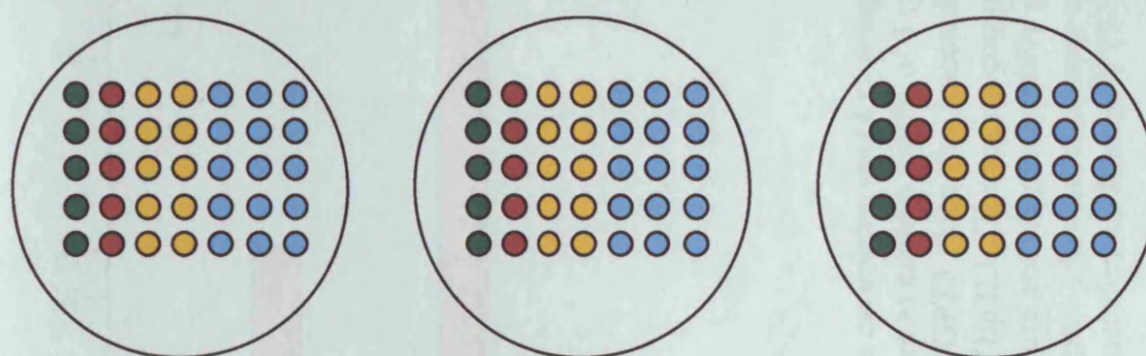
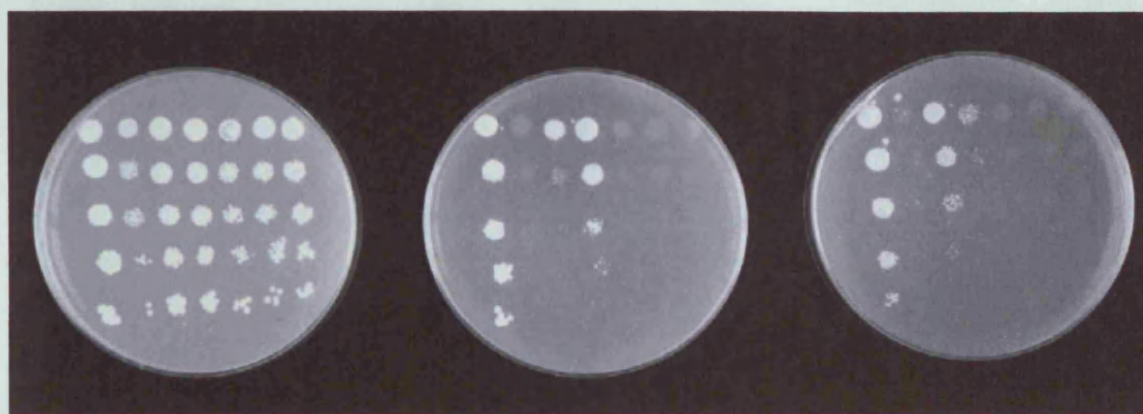
37°C

28°C +
CuSO₄

- SEY6211 + YEp213 (WT).
- *vps16* + YEp213.
- *vps16* + rescuing library clones.

Figure 4.1. *vps16* isolated clones rescue temperature and copper sensitive growth phenotypes.

Cells grown to log phase in selective minimal medium were plated as serial dilutions (1×10^7 , 2×10^6 , 4×10^5 , 8×10^4 cells/ml, as seen down the plate) and grown on SD plates at permissive (28°C), non-permissive (37°C) temperatures, and on SD media enriched with 400μM CuSO₄. Plates were photographed after 3 days. SEY6211 + YEp213 = positive control; *vps16* + YEp213 = negative control. The clones restore growth of the mutant strain at the non-permissive temperature, and on the copper enriched media. The plate grown at the permissive temperature revealed that while the *ade2* parental strain SEY6211 was red in colour, the mutant strain was white. However, the isolated clones also seem to restore some of the red pigmentation to the mutant (see section 4.4).



28°C

37°C

28°C +
CuSO₄

- S150-2B + YEp213 (WT).
- *vps18::Tn1000* + YEp213.
- *vps18::Tn1000* + pGPT1 (library clone).
- *vps18::Tn1000* + non-rescuing library clone.

Figure 4.2. *vps18* isolated clone, pGPT1, rescues temperature and copper sensitive growth phenotypes. Each strain was grown to log phase in selective minimal media and 5μl of cell dilutions plated (1×10^7 , 2×10^6 , 4×10^5 , 8×10^4 , 1.6×10^4 cells/ml, as seen down the plate) on SD media and grown at permissive (28°C), and non-permissive (37°C) temperatures, and on SD media enriched with 400μM CuSO₄. Plates were photographed after 3 days. S150-2B + YEp213 = positive control; *vps18::Tn1000* + YEp213 = negative control. *vps18::Tn1000* + pGPT1 strain is present twice on the above plates, and both show some degree of rescue of the copper and temperature sensitive growth phenotypes. However, when cells were grown and plated at mid-log phase (5×10^6 cells/ml), lane 4, the cells were better at growing at 37°C. When harvested at late log phase, lane 3, (2×10^7 cells/ml), while less able to grow at 37°, these cells grew better on the copper enriched media. pGPT1 does not appear to restore full growth to the *vps18* mutant under these conditions.

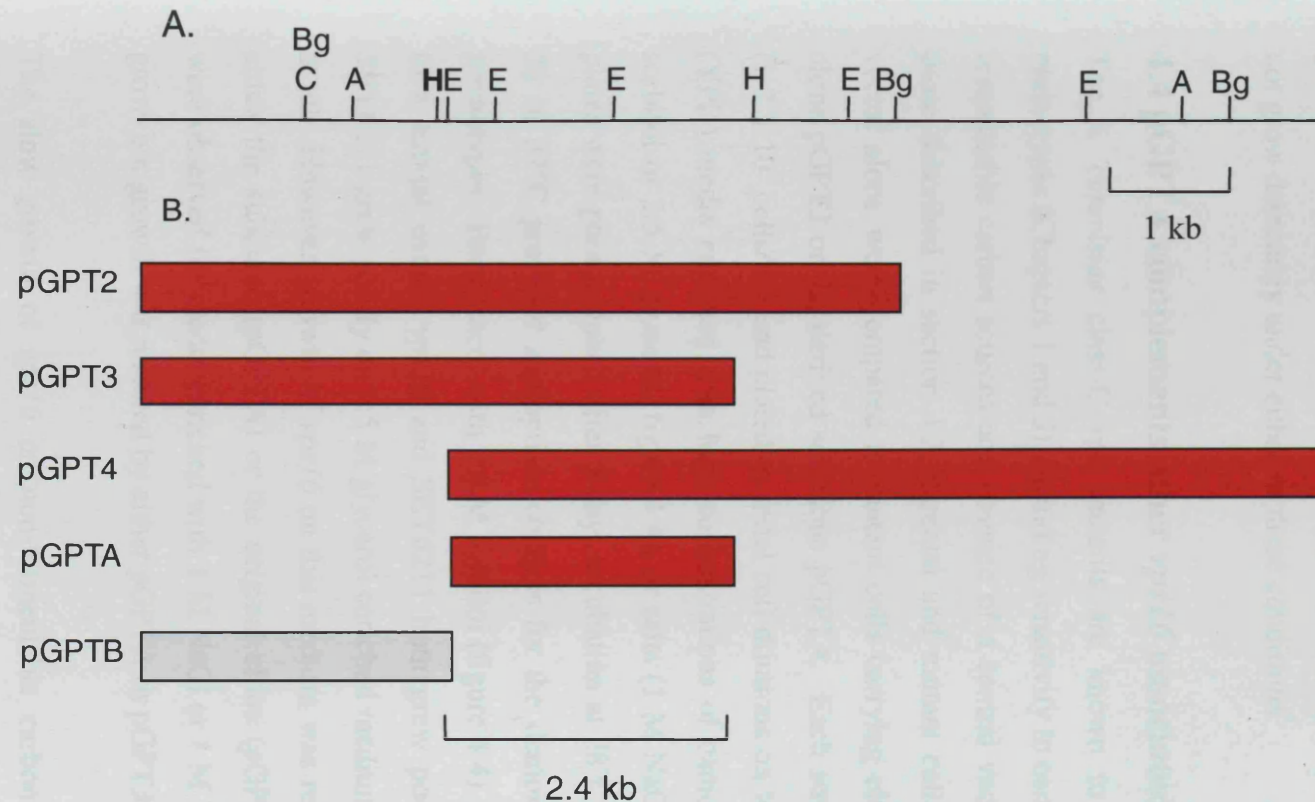


Figure 4.3. *Candida albicans* genomic clones rescuing *Saccharomyces cerevisiae vps16* overlap the same genomic region. Restriction mapping of the 9 *vps16* rescuing clones revealed them to contain inserts of 3 types (pGPT2, pGPT3 and pGPT4), which contained a common genomic region. The insert of the pGPT3 clone was bisected at the H (*HindIII*) site shown in bold type. Each of the resulting fragments was sub-cloned to YEp213. The region common to all three of the original clones (pGPTA) retained the rescuing activity of *vps16* temperature and copper sensitive growth. The other half of pGPT3 was cloned to pGPTB, which did not rescue these *vps16* phenotypes. (A) restriction map of the *vps16* rescuing region. (B) region spanned by each of the original clones and the subclones derived from pGPT3. *vps16* rescuing clones are shown in red, non-rescuing clones are shown in grey.

2.4 kb region common to all three of the original clones, while pGPTB contained the remainder of the pGPT3 insert. Both of the subclones were then introduced to the *S. cerevisiae vps16* mutant, and analysed for their ability to rescue of the temperature and copper sensitive growth phenotypes. The pGPTA subclone was sufficient to support the growth of the mutant to the same extent as the original clones, while *vps16* + pGPTB did not grow detectably under either of these conditions.

4.4 pGPTA complements other *vps16* associated defects

The *S. cerevisiae* class-C *vps* mutants are known to exhibit a range of secondary phenotypes (Chapters 1 and 3), including sensitivity to osmotic stress, slow growth on non-fermentable carbon sources and absence of a central vacuole. In similar experiments to those described in section 4.2, parental and mutant cells transformed with the YEp213 vector alone were compared to mutant cells carrying either the original *vps16* rescuing clone pGPT3 or the derived sub-clone pGPTA. Each strain was grown to mid-log phase ($1-2 \times 10^7$ cells/ml) and plated in serial cell dilutions on to selective (SD) or non-selective (YPD) media enriched with high concentrations of osmotic buffering compounds (1.4M sorbitol or 2.5 M glycerol, figure 4.4), or salts (1 M NaCl or 1 M KCl, figure 4.5). The plates were photographed after 3 days incubation at 28°C. Growth on SD media at either 28 or 37°C provided appropriate controls for the demonstration of parental and mutant phenotypes. Enrichment with 1.4 M sorbitol (figure 4.4), reduced the growth of all strains to a similar extent, *vps16* and SEY6211 both grew poorly. While the parental strain SEY6211 grew poorly on 2.5 M glycerol enriched medium, the *vps16* mutant did not grow at all. However, growth of *vps16* on this medium was restored to near parental levels by either the subclone (pGPTA) or the original clone (pGPT3)(figure 4.4). Similar results were observed for media enriched with 1 M NaCl or 1 M KCl. The *vps16* mutant failed to grow, but growth was restored by either pGPTA or pGPT3 (figure 4.5).

The slow growth of *vps16* on non-fermentable carbon sources, such as glycerol and ethanol, was also improved by pGPTA. In figure 4.6, each strain was propagated on either SD (glucose), SE (ethanol) or SG (glycerol), then streaked to fresh selective media with the same carbon source, and incubated at 28°C. On glucose containing media, all strains grew well; on both SE and SG, the parental strain and *vps16* carrying pGPTA grew to a similar extent, while the *vps16* mutant grew less well.

Figure 4.4. *vps16* rescuing region complements sensitivity to osmotic stress. Cells were grown to log phase in selective medium at 30°C, and plated to selective SD, or non-selective YPD media enriched with 2.5 M glycerol, 1.2/1.4 M sorbitol, or with no enrichment. Plates were photographed after 3 days growth at 28°C. Cells were grown on SD at 28°C as a control to check viability. Cells were grown on SD at 37°C, as a positive control for the rescuing activity of pGPT3/pGPTA. The SEY6211 (+ YEp213) parental strain grew under all conditions, however, the *vps16* (+ YEp213) mutant did not grow on media enriched with 2.5 M glycerol, or at the non-permissive temperature, 37°C. Growth of the mutant strain was restored to near 'wild-type' levels under both of these conditions by either pGPT3 or pGPTA. The media enriched with sorbitol did not seem to significantly reduce the growth of the *vps16* mutant.

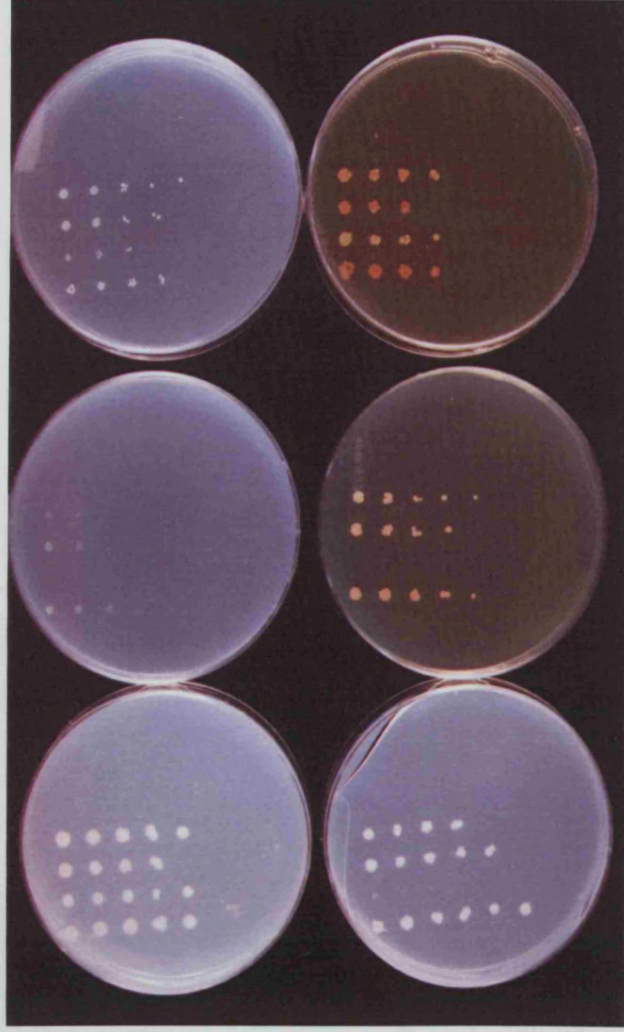
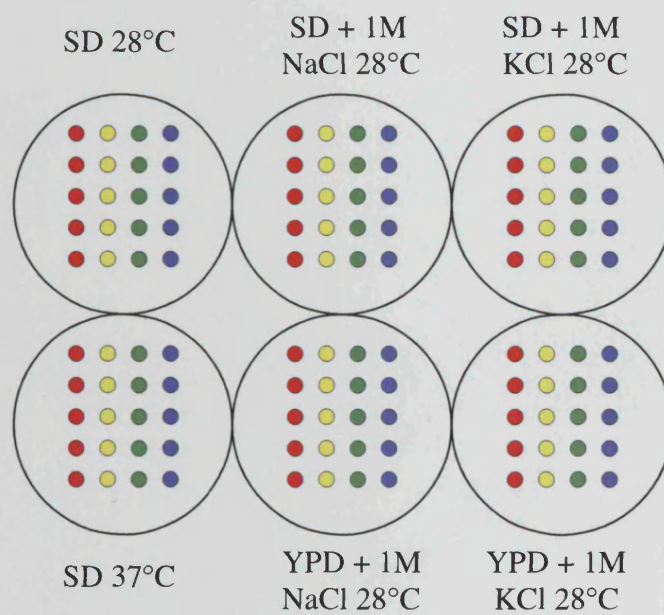
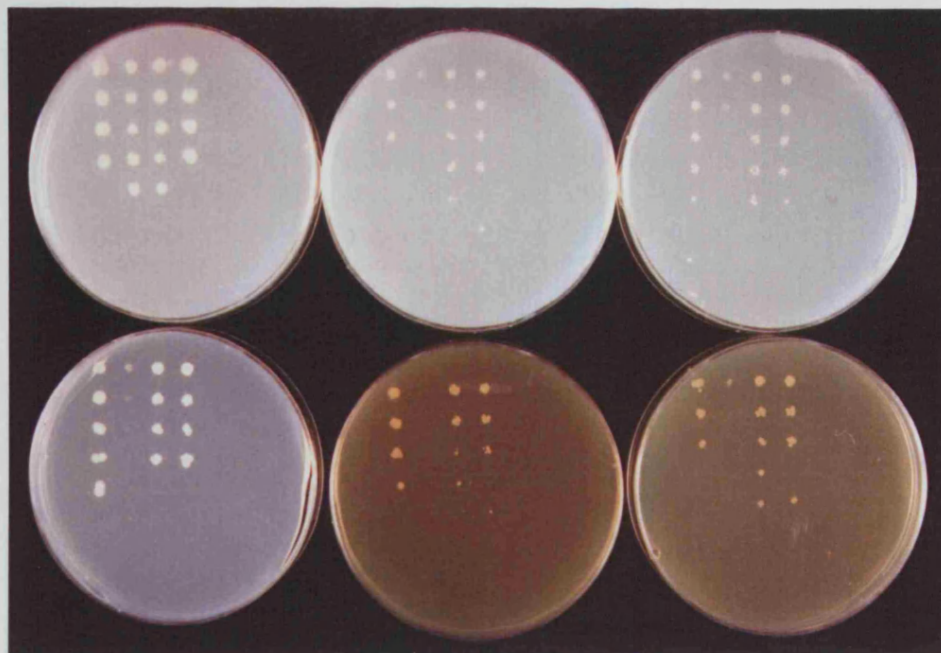
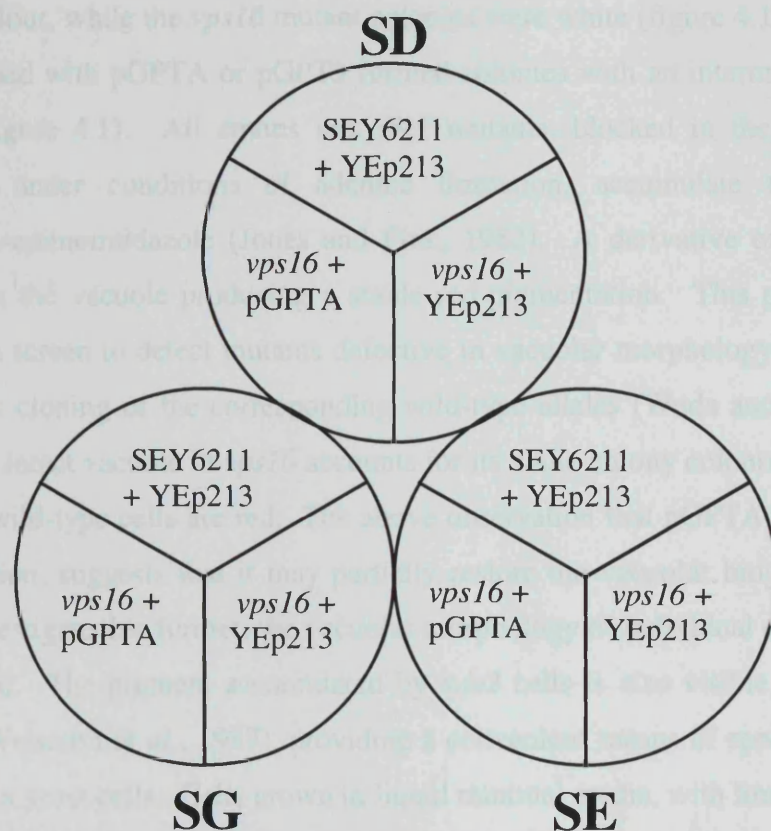


Figure 4.5. *vps16* rescuing region complements sensitivity to high concentrations of extracellular ions. Cells were grown to log phase in selective medium, and plated to selective SD, or non-selective YPD media enriched with 1 M NaCl, 1 M KCl, or with no enrichment. Plates were photographed after 3 days at 28°C. Cells were grown on SD at 28°C to check viability. Cells were grown on SD at the non-permissive temperature (37°C), as a positive control for the rescuing activity of pGPT3/pGPTA. While the SEY6211 (+ YEp213) parental strain grew under all conditions, the *vps16* (+ YEp213) mutant failed to grow on either the NaCl, or KCl enriched media, or at the non-permissive temperature 37°C. Growth of the mutant strain was restored to near 'wild-type' levels by both pGPT3 or pGPTA, under all of these conditions.



- SEY6211 + YEp213
- *vps16* + YEp213
- *vps16* + pGPT3
- *vps16* + pGPTA

Figure 4.6. pGPTA restores the ability of *vps16* to utilise non-fermentable carbon sources. Each strain was streaked to SD (2% glucose), SG (3% glycerol), or SE (3% ethanol) media, supplemented with the appropriate amino acids, and grown for 5 days at 28°C. Single colonies of each strain, on each media were then picked and re-streaked to fresh medium with the same carbon source. After 5 days at 28°C, the plates were photographed. SEY6211 + YEp213 = parental strain; *vps16* + YEp213 = mutant strain; *vps16* + pGPTA = test strain. On SD medium all strains grew comparably well. On media where glycerol or ethanol provided the carbon source, all strains grew less abundantly. However, the *vps16* mutant strain grew visibly less than the SEY6211 parental strain. The growth of *vps16* on these carbon sources was restored to near parental levels by pGPTA.



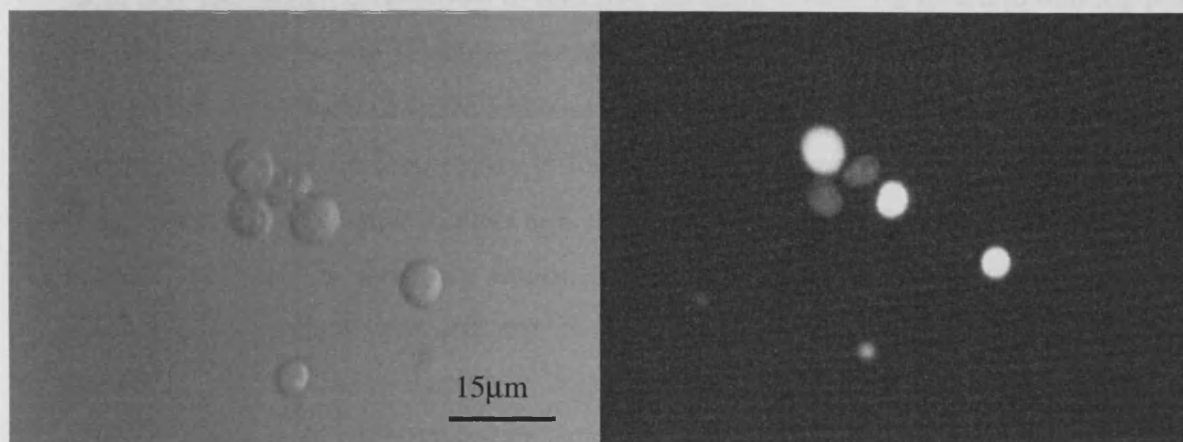
It was observed that wild-type cells grown on minimal media at 28°C formed colonies with a deep pink colour, while the *vps16* mutant colonies were white (figure 4.1). Mutant *vps16* cells transformed with pGPTA or pGPT3 formed colonies with an intermediate light pink colouration (figure 4.1). All strains are *ade2* mutants, blocked in the biosynthesis of adenine, and under conditions of adenine limitation, accumulate the biosynthetic intermediate 4-aminoimidazole (Jones and Fink, 1982). A derivative of this compound accumulates in the vacuole producing a stable red pigmentation. This pigmentation has been used as a screen to detect mutants defective in vacuolar morphology and to facilitate the subsequent cloning of the corresponding wild-type alleles (Wada and Anraku, 1992). The lack of an intact vacuole in *vps16* accounts for its white colony colouration on minimal media whilst wild-type cells are red. The above observation that pGPTA restores some of this pigmentation, suggests that it may partially restore the vacuolar biogenesis defect of *vps16*. To investigate this further, the vacuolar morphology of individual cells of each type were visualised. The pigment accumulated by *ade2* cells is also visible by fluorescence microscopy (Weisamn *et al.*, 1987), providing a convenient means of specifically staining vacuoles within yeast cells. Cells grown in liquid minimal media, with limiting amounts of adenine (see Materials and Methods, Chapter 2) were visualised using a confocal microscope (excitation 450-490 nm; 520 nm emission) (figure 4.7). Cells of the parental strain SEY6211 possessed intensely stained vacuoles, although there was some degree of variation within the population of cells (figure 4.7A). No fluorescence staining was detected for any of the *vps16* cells examined (figure 4.7B). However, the vacuolar morphology of the mutant was at least partially restored by pGPTA (figure 4.7C), although these cells stained less intensively than SEY6211. Fluorescence was only detected in approximately 60% of *vps16* + pGPTA cells. It is not known if this is due to variation in staining intensity as observed for the parental strain or if pGPTA is only able to restore vacuole biogenesis in a proportion of mutant cells. These results together with the intermediate colony pigmentation discussed above demonstrate that vacuole morphology of *vps16* + pGPTA is intermediate between that of *vps16* and SEY6211.

In addition to the temperature and CuSO₄ sensitive phenotypes, the *C. albicans* genomic insert within pGPTA also rescued the osmotic/ionic stress, carbon source utilisation, and vacuolar structure defects associated with the *vps16* mutant.

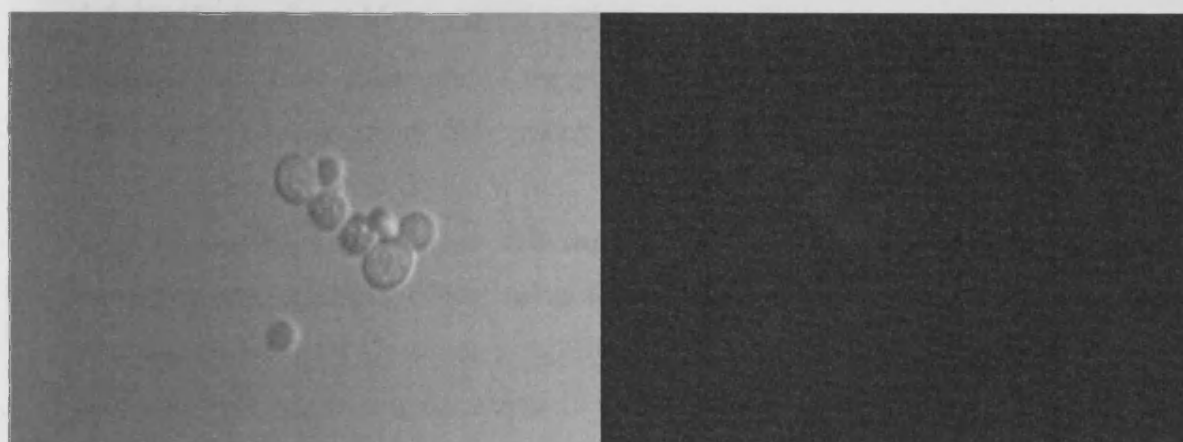
Figure 4.7. pGPTA restores the vacuole biogenesis defect in *vps16* mutants. Cells were grown in selective media at 30°C for 2 days, with limiting amounts of adenine (12µg/ml) to induce production of the *ade2* fluorophore, which specifically localises to the vacuole. Left panel: cells viewed with Normarski optics; Right panel: fluorescence image 450-490nm excitation and viewed with a 520nm cut-off filter. Magnification x1000.

The results in which the subcellular localization of the various proteins was determined are presented in Figure 4.10.

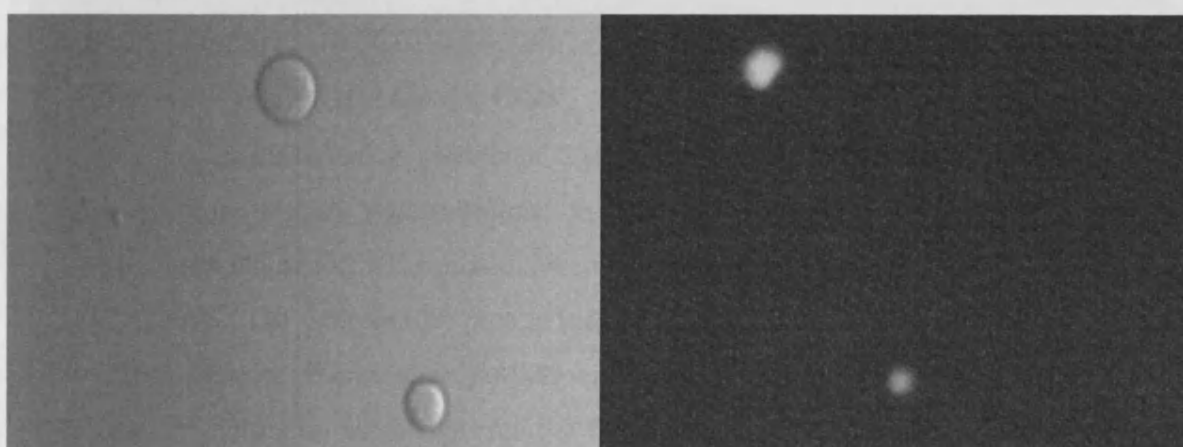
A. SEY6211 + YEp213



B. *vps16* + YEp213



C. *vps16* + pGPTA



was subjected to analysis by the method of percentage on the PSORT2 web site (http://psort.uconn.edu/psort). This analysis predicted a probability of 100% of known

The extent to which the sub-clone pGPTA rescued the *vps16* mutant phenotype was assessed by quantifying copper tolerance of parental, mutant, and mutant cells transformed with pGPTA. Again cells grown in selective SD culture were harvested in mid log phase, diluted, and plated to selective SD agar plates enriched with a range of CuSO₄ concentrations (0-1000 µM)(see Materials and Methods). After 4 days incubation (28°C), strains were scored for their ability to form colonies at each CuSO₄ concentration (figure 4.8). As found in chapter 3, there was a marked difference in copper tolerance between parental and *vps16* *S. cerevisiae* strains. The ability of *vps16* to form colonies at elevated CuSO₄ concentrations was restored by pGPTA (or pGPT3) to a similar level as the SEY6211 parent strain. Therefore based on CuSO₄ tolerance the pGPTA insert fully complements *vps16*, when present on a multi-copy vector.

4.5 Analysis of *vps16* rescuing region

Sequence data from either end of the pGPTA insert DNA were obtained using primers GP02 and TETBGP (table 2.6), which bind in the flanking vector sequences. New sequencing primers were designed from the acquired sequence to 'walk' across the insert on either strand. The sequence data derived from each of the individual reactions was assembled into a single contig using the GELASSEMBLE programme from the GCG computing package (Wisconsin suite)(figure 4.9). Where ambiguities in the sequence occurred, each of the original sequence traces were examined by eye, to determine the correct nucleotide at that position. The insert was 2328 nt and contained a single intact reading frame, designated *GPA1* (figure 4.9).

4.5.1 Analysis of the *GPA1* ORF

GPA1 encodes a putative protein of 234 amino acids. The predicted protein sequence derived from *GPA1*, was submitted in FASTA searches of the listed EBI databases (<http://www.ebi.ac.uk>). No proteins were identified as sharing significant similarity with Gpa1p. The most similar protein identified in these searches was the S-Antigen of the malarial parasite *Plasmodium falciparum*, which shares 39.2% identity, over 97 amino acids. To help gain an insight into the function of Gpa1p, the putative protein sequence was subjected to analysis by the package of programmes on the PSORT2 web site (<http://psort.nibb.ac.jp/psort>). This analyses protein sequences for the presence of known

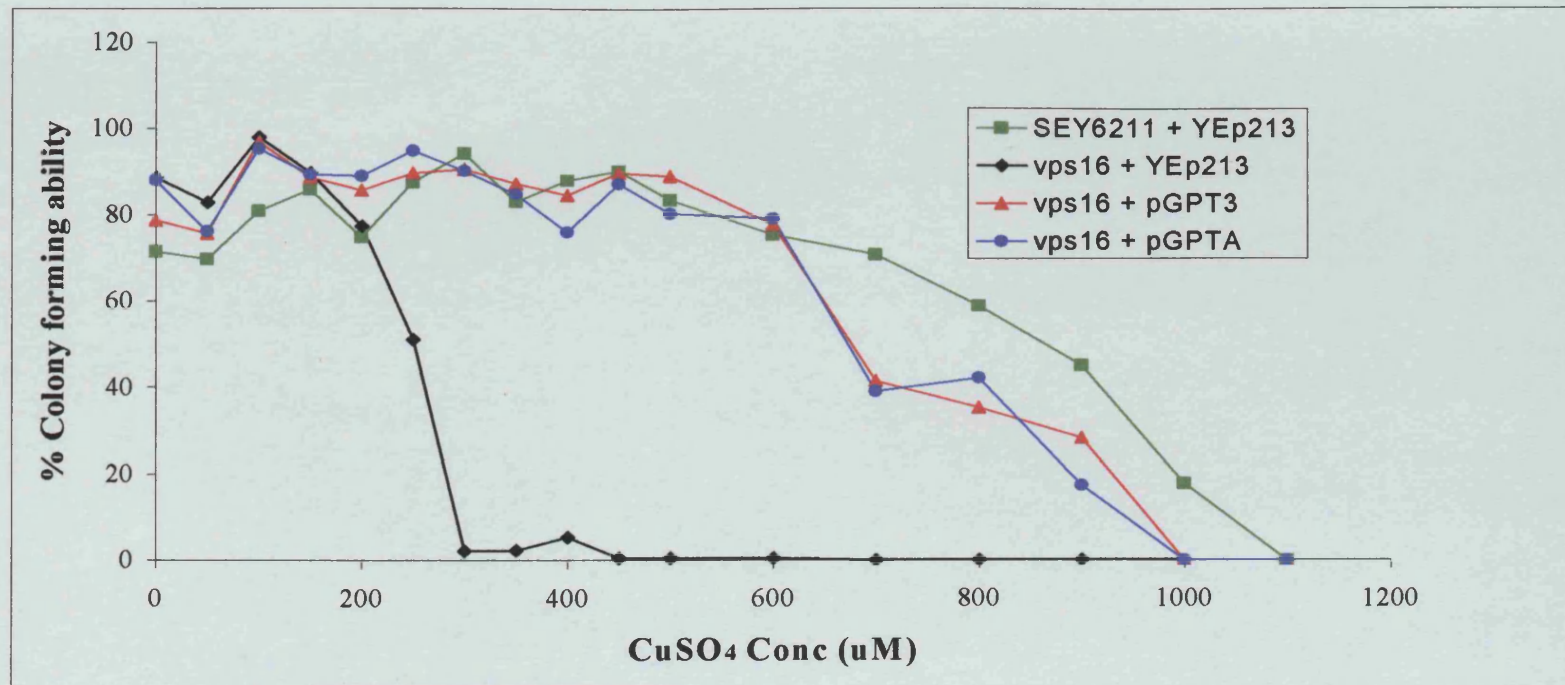


Figure 4.8. pGPTA restores copper tolerance of a *vps16* mutant. Cells were grown to log phase in selective media, plated to selective SD enriched with CuSO₄ and grown at 28°C. Strains were scored for their ability to form colonies at each CuSO₄ concentration after 4 days. Colony forming ability was calculated as the number of colonies at a particular CuSO₄ concentration divided by the maximum number of colonies observed for that strain (given as %). Values represented are the mean of 2 experiments. The original *vps16* rescuing clone pGPT3, and the derived subclone pGPTA, both complement the *vps16* mutants copper sensitivity to near parental levels (SEY6211).

Figure 4.9. Sequence analysis of the pGPTA *Candida albicans* genomic insert. (A) sequence of the pGPTA insert. The insert was 2328 nt in length. (B) represents the pGPTA insert (blue line), and the ORFs identified within its sequence. Only one complete ORF was identified within the insert (red arrow), encoding a 234 a.a. product. In addition 155 a.a. of a second ORF, truncated at the N-terminus by vector sequences was identified (green arrow). Predicted protein sequences for each of these ORFs is shown in (C).

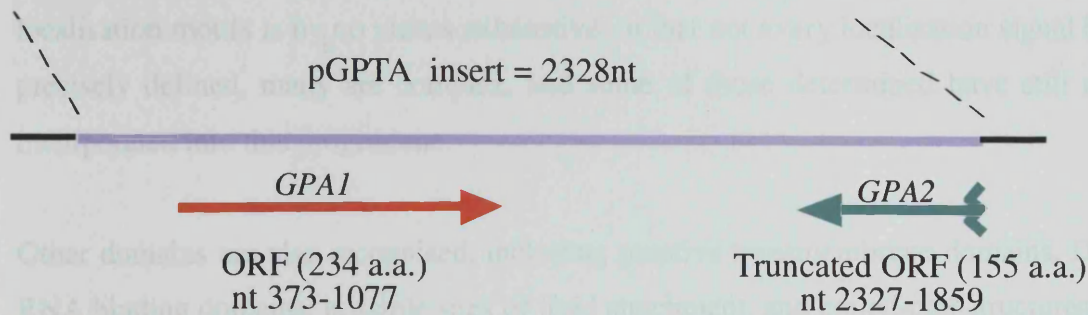
A.

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GGATCTTGGA AGGCTAGGTC CTACTAGATT TCATGAAATC AATCCCCTG
TTAGATGATA TAGGTATATG AATTTCTTCT ACTCGTTGAG TTAGTTCTAA
CATATGAATA CTAATTTTAA ATTCTAAAAA TAACTACACA TTAAAGACTA
GTCTAGATCA GCGCCTTTAT TTAAATGAGG ACGTGTATTT GTGTGCAAAA
AAAGAACAAT CTCTGATCAC AATTAGACAA AAAAAAATTG GATGAAAATC
AAACAACAAAT TGGAAGGAGG AAGGAAAAAA AAATCAATTT CAACAATATA
CAAGTTGAAG CTCACACAGTG ATCATTCCAA CTGTTATTAC TATTTCTTAT
TCATCTTAAT ATACTAGCAC ATATGCAAGG AAGTTACAAC AACAAATGGTC
TACCAAGACC CTACTATATG AAACCATCCA AAAAAAACT GTCACCGTAT
TCGAATTTGA AAGAACCAAT TGTATATTTC AATAACCCAA AAAGAAAATT
AGTGGGATAT ATAATAATGT TCAGTTTAAAT TGGGATATTA ATGTGGTGGA
TCTCTCAAGA TTTTAAAGGG AGACCAGAAG TCGAATATGA ATTAGTCAAG
AATGAAAAAT CAGGTGAAAA GAATACTCCA ATGGATCTTA AAAATAGTGC
CAATTTGGAC AAGATTGTGA ATGCAGTTGG TTCAAAAGCA GACAAGGAAA
GTGAAAATCT CGATTTAGCA GAAAATTTGT CAGAAGGTTT TAAAGGACAA
AAGGGAATTG GTGTAGCTGA AGCACCTAAA GGAGGAATAG CTAATGAGGC
ACCAAGTTGTT GGCAACGATG AAGAAGAATT GGTGGGTACT GGTAGGGTG
GACAAAAACA GCAAAAAGCT GATGGGAACC CAAAGGGGAA AGGTTATAAA
GCATCTATTG GAAAGACAGG TGATAAAGAT TCTGTTCTCG CTGGTGCTGG
CGCTCCTGCT GGTGTTGATA GCCCCAAGGG AAAAGCTGAA CCTGTACCTG
ATGTTGAAGA TGCTGCTGCT GCTGCAAGAC TATCTAATGA AGACACAGTT
CAACAGATTA TAGAAGATAC TGCATGATCT AATGAATTCG TTTTGTTTTT
TTCTTGTTCT TCATCATCAT TTATAGATCC ATTTTTCATA TATAACGACG
CTACTTTATT TACTGCATTT TTAATATATA GATATTAAAG TCAACACAGT
AAACATGTTT TGAATAAAAA AAACATAACA TTTGCAAGGC AAGTGATGGT
TTCTTTTATT TGAAGAGTAA AAAAACACTG TAGGTCCAAC TGGGAATCGA
ACCCAGGTTA CCGGAATCAG AAACCGATGT GATCGCCACT ACATATTGG
ACCACTAAAA CACCTTTGTT GGCCCTCAT AAAGTATAAT ACATGGCTAA
TAGCAATAAT TAAATGATGA TAGAATATGA CAGGCACTCC GAAGTTTGTA
TAATTATTAG TGTCAAATAGT TATTGATATG TATGAAAACA TTTTGAAACT
TAAAGAAGTG CGTTTAACAA TTCTAAATGG TGTGTTTGG TGGTTTATCA
GAGTCTACCA AAAGTCAGCG ATCTGTACGA TTTTATTCGT TCCAATGTGT
TCTACAAACC TAATATTCAA AATAAACTTG ACAAGTGAGT CTTCTATATA
TTTACATAAT AATGTAATGA ATAGCTGGGT AATAGGAATG ATTTTGTGG
TATTAGTAGC TACATAAAAC ATTTCAAGAT TTCAACAAAT GTTTTCTGA
AAAAATTTGC AATTTCTTGA ACATAAAAAA TTTACACCGT AAAACTCTCA
TAAACACCAC TCAAAACAAA CATTTTATTA AACCAATTAT AAACCTACTA
TTTATGTATC TACATGACAA TACATTTCTC ACCTGAATTA GCATTCTTTC
TTAATTCATT GTATCTTTCT TTGTCCCTCT CTCGTAATTT CTCTTTAACC
TCACGTGGGA AGTACTTCTT CATTAAATCC ATTGACTCCA AGTCCAAATT
AGAACTATCA GCATACAATA TAGCATTTC TTTTCTACAC AAGGGACAAC
TTGTCTTATC ATCTTGTTTC ATTTTGACTA AGCATCGAAC ACAAACAAA
TGTCACAAG ACAACGAAT TGGTTTATAC GCTATGTTCA TACAGATCGG
ACACGAATAA TCATCCAAC GTGGTATCAC TTTAATGATT GATTCTTGAA
TAATATAACA AATTGATTGA GCTAAAGAAG ACCCACTCAT GAAAAATGA
TCGTTGGAAA TCAATTTTGG GAAAGTTTTT TGAATTCCTA ATGAAGTCTG
CTTATCGAAT TTTTTCAAAA TCTTTCGT

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B.



C.

Gpa1p

MQGSYNNNGL PRPYMKPSK KKSSPYSNLK EPIVYFNNPK RKLVGYYIIMF
SLIGILMWWI SQDFKGRPEV EYELVKNEKS GEKNTPMDLK NSANLDKIVN
AVGSKADKES ENLDLAENLS EGSKGQKGIG VAEAPKGGIA NEAPVVGND
EELVGTGKGG QKQKADGNP KGKGYKASIG KTGDKDSVPA GAGAPAGVDS
PKGKAEPVPD VEDAAAAARL SNEDTVQQII EDTA•

Gpa2p (c-terminus)

RKILKKFDKQ TSLGIQKTFP KLISNDHIFM SGSSLAQSIC YIIQESIIVK
IPQLDDYSCP ICMNIAYKPI RLSCGHLFCV RCLVKMKQDD KTSCPLCRKE
NAILYADSSN LDLESMELMK KYFPREVKEK LRERDKERYN ELRKNANSGE
KCIVM•

An RT-PCR approach was used to detect the *GPA1* transcript in RNA prepared from *C. albicans* cells grown in mid-log phase (2×10^7 cells/ml) in YPD media (figure 4.10). These RNA samples were DNase treated and used as template to an RT-PCR reaction, with a primer set (TA-1 and TA-2) specifically designed to sequences within the *GPA1* ORF. A product of the expected size was amplified, confirming that *GPA1* is transcribed in *C. albicans* cells grown in log phase.

protein sorting signals, for entry into the secretory pathway, golgi retention, sorting to vacuoles, or targeting to the nucleus, peroxisomes or mitochondria. The search for localisation motifs is by no means exhaustive, in that not every localisation signal has been precisely defined, many are complex, and some of those determined have still not been incorporated into this programme.

Other domains are also recognised, including putative transmembrane domains, DNA and RNA binding domains, possible sites of lipid attachment, and coiled-coil structures. No N-terminal signal peptide was recognised in Gpa1p, suggesting that it is unlikely that this putative protein enters the secretory pathway. Two potential nuclear targeting sequences were identified, PKRK (a.a. 39-42), and PSKKKSS (a.a. 18-24) in the Gpa1p amino acid sequence (for review of nuclear localisation signals see Boulikas, 1993). PSORT2 also utilises Reinhardt's method (Reinhardt and Hubbard 1998), which predicts the sub-cellular location of a protein based on its amino acid composition. Proteins within four cellular compartments, nucleus, mitochondria, cytoplasm and extracellular space, have characteristic amino acid compositions. This information is used to predict the most likely location of a query protein. Using this method, Gpa1p was predicted to be a nuclear protein with 89% reliability. On the basis of this analysis and target sequence data, it seems likely that Gpa1p is localised to the nucleus. One further feature of Gpa1p was identified by PSORT2. A single putative transmembrane domain spanning amino acids 44-60, suggests that Gpa1p could be an integral membrane protein. On the basis of net charge difference of the 15 residues flanking the putative TM domain on either side, it was predicted, using PSORT, that the N-terminal end of Gpa1p (amino acids 1-44) would reside in the cytoplasm.

An RT-PCR approach was used to detect the *GPA1* transcript in RNA prepared from *C. albicans* cells, grown to mid-log phase (2×10^7 cells/ml), in YPD media (figure 4.10). These RNA samples were DNase treated and used as template in an RT-PCR reaction, with a primer set (TA-1 and TA-2) specifically designed to sequences within the *GPA1* ORF. A product of the expected size was amplified, confirming that *GPA1* is transcribed in *C. albicans* cells grown to log phase.

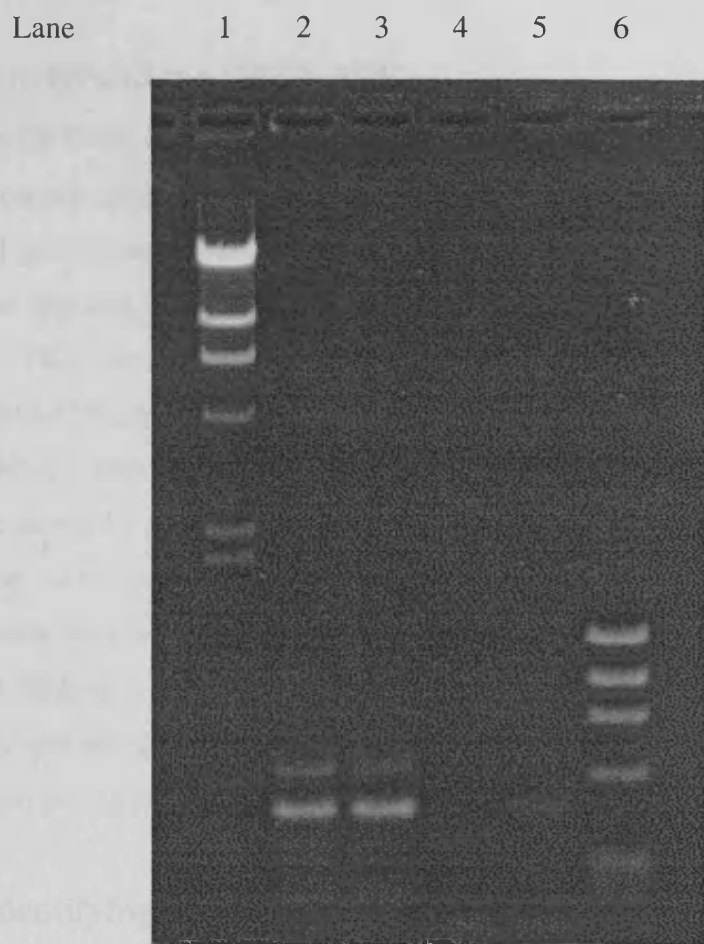


Figure 4.10. *GPA1* is expressed in log phase *Candida albicans* cells. Primers designed to the *GPA1* ORF (TA-1 and TA-2) were used for RT-PCR, using a DNase treated RNA template from log phase *C. albicans* cells grown in YPD. The predicted extension product of 452nt, was present when RNA from either S01 or SC5314 was used as a template, confirming that this ORF is transcribed to an RNA product in both strains. The control reaction for each was the omission of the reverse transcriptase (RT). In control reactions RT was omitted and no extension product was observed, therefore the product must be derived from an RNA template via a cDNA intermediate.

Lanes (left to right): 1= λ _{HindIII}; 2=S01 RNA template; 3=SC5314 RNA template; 4=S01 RNA minus RT control; 5=SC5314 RNA minus RT control; 6= ϕ X174_{HaeIII}.

4.5.2 Analysis of the *GPA2* ORF

A second ORF was identified within the pGPTA insert sequence, and called *GPA2*. *GPA2* was 155 codons long and truncated at the N-terminus by vector sequences (figure 4.9). The predicted protein sequence was compared to putative protein sequences contained on the *S. cerevisiae* genome sequence database. No highly significant matches were identified, however, *YER116c* an ORF of unknown function was the most similar, sharing 32% identity and 45% similarity over 71 amino acids. The same sequence was analysed using the PSORT2 programme (<http://psort.nib.ac.jp/psort>). One feature of the available sequence is worthy of note, the predicted Gpa2p product contains a putative C₃HC₄ type zinc-finger. This could indicate that *GPA2* encodes a DNA binding protein, alternatively this domain may be important in protein-protein interactions. The 155 amino acids available contains a bipartite nuclear localisation motif (KKYFPREVKEKLRERDK, a.a. 120-136), and using Reinhardt's method, the amino acid composition supports a nuclear localisation of this protein.

4.5.3 Identifying the *vps16* rescuing ORF

Three approaches were taken in an attempt to identify the ORF responsible for *vps16* rescuing activity. Firstly, a DNA fragment comprising *GPA1*, the upstream 373 nucleotides of insert sequence, upto the insert/vector boundary of pGPTA, plus 29nt of the YEp213 vector, and 83nt downstream of the STOP codon, was amplified by PCR, using specific primers (GPTA01 and GPTA02) with *Bam*HI sites engineered into them. The product was then cut with *Bam*HI and introduced back into the *Bam*HI site of YEp213, in both orientations. The resulting constructs (pGPA1A and pGPA1B) were then transformed into the *vps16* mutant strain. Surprisingly, both pGPA1A and pGPA1B, failed to complement the temperature sensitive growth defect of *vps16*. This may suggest that *GPA1* is not responsible for *vps16* rescue. Alternatively while a polymerase with proof-reading activity was used, it is possible that PCR generated 'mutations' could have been introduced into a functionally important part of *GPA1*, thereby inactivating its product. Alternatively, it is possible that while containing all insert sequences upstream of *GPA1*, the region selected for amplification may have lacked a functionally important sequence downstream of the ORF. Otherwise, *GPA1* may have been expressed fortuitously from the vector sequences of pGPTA, perhaps by read through from the *LEU2* promoter. This may have been disrupted in the pGPA1 constructs if there were changes in the spacing/

orientation of the vector promoter and the insert ORF. For example, pGPA1 contained *GPA1* and all insert sequences upstream of *GPA1* in the same orientation to the vector as pGPTA, however the cloned PCR fragment also contained a duplication of 29nt of the flanking vector DNA.

A second approach to identify the *vps16* rescuing ORF was undertaken, namely transposon mutagenesis (Sedgewick and Morgan, 1994). If transposon insertion into the rescuing ORF occurs, it will functionally inactivate the ORF and its rescuing activity. Once such a clone has been identified, it can be restriction mapped to determine the position of the transposon within the clone. Sequence data of DNA flanking the transposon may also be obtained using primers that bind to the end of the transposon. The system used consists of an *E. coli* donor strain (MH1578), which is streptomycin sensitive, and carries the conjugative plasmid R388 which contains the *Tn1000* sequences. The target plasmid is transformed into this strain. Duplicative transposition transiently links R388 and the target plasmid, to form a cointegrate. This is then mated with a recipient strain of *E. coli* (MH1599) which is streptomycin resistant, and the cointegrate transfers by conjugation. Recipient cells carrying the cointegrate are selected as resistant to streptomycin, and the antibiotic resistance marker present on the target plasmid. A site specific recombination event between the two copies of the transposon in the cointegrate, releases R388 and the target plasmid which now carries a copy of *Tn1000*. As the site of insertion is largely random a population of target plasmids with *Tn1000* insertions at different sites can be attained. In this way 120 pGPTA transposon disrupted plasmids were prepared from *E. coli* and transformed back into the *S. cerevisiae vps16* mutant. Surprisingly all plasmids seemed to rescue the temperature sensitive growth of *vps16*, indicating that the sequences responsible had not been disrupted by *Tn1000HIS3* insertion in any of these plasmids. Thirty-six of these plasmids were then restriction mapped to determine the position of the transposons. All 36 had transposon insertions within the YEp213 vector sequences, indicating a strong bias for the insertion of *Tn1000HIS3* into the vector.

Finally, a third approach was taken to identify the ORF responsible for pGPTA rescuing activity of *vps16*. Two divergent primers (TAD-1 and TAD-2) were designed within the *GPA1* ORF, each with an engineered *Bam*H1 site. These were used in an inverse PCR reaction, with a proof-reading polymerase (BIO-EXACT, BIOLINE Ltd), using pGPTA as a template. The resulting product was cut at its *Bam*H1 ends, and the linear fragment

recircularised by ligation of its sticky ends. The result was a plasmid containing all the YEp213 vector sequences, and the pGPTA insert minus nts 189-495 of the *GPA1* ORF (868 nts in length). This plasmid (pGPTAΔ1), was transformed to *S. cerevisiae vps16*, and the temperature sensitivity of the strain examined (figure 4.11). It seems that pGPTAΔ1 fully retained the ability to rescue the temperature-sensitive growth defect of *vps16*. This suggests that *GPA1* is not necessary for the rescuing activity of pGPTA. It was therefore suggested that the truncated ORF, *GPA2*, may be fortuitously expressed as a fusion protein, driven from a promoter in the YEp213 vector sequences. A second set of primers for the deletion of *GPA2* (TAD-3 and TAD-4), were designed to incorporate *HindIII* sites, and used in an inverse PCR approach, with proof reading. The PCR product was cut with *HindIII* and ligated to create pGPTAΔ2. This again possessed all the YEp213 vector, and most of the pGPTA insert sequences, but lacked 507 bp within the *GPA2* ORF (upto the *HindIII* vector/insert boundary). Again this construct was transformed into the *vps16* mutant and the temperature sensitivity of the resulting strain determined (figure 4.11). pGPTAΔ2 also restored growth of *vps16* at 37°C, indicating that the *GPA2* sequences were also not required for pGPTA rescuing activity. A third pGPTA deletion plasmid was amplified by inverse PCR using primers TAD-3 and TAD-5. The PCR product was cut at the *HindIII* primer incorporated ends, and then recircularised to form pGPTAΔ3. This contained all the YEp213 sequences of pGPTA (minus nucleotides 378-395 of YEp213), but the entire insert sequence was deleted. When introduced into *vps16*, pGPTAΔ3 did not restore the mutant's ability to grow at 37°C (figure 4.11). This confirms that pGPTA rescuing activity is dependent upon insert sequences, rather than pGPTA vector sequences.

In order to investigate if 'gene dosage' was important for the rescuing activity of pGPTA (multi-copy), the insert sequences were cloned into the CEN-ARS based single copy vector pRS415. pRS415 possesses the same selectable marker (*LEU2*) as the 2 μM plasmid based multi-copy vector YEp213. A 2.6 kb *HindIII*-*SalI* fragment from pGPTA, containing the whole insert and 276 nt of YEp213 sequence (from *Bam*H1-*SalI* sites of YEp213) was cloned into pRS415. The resulting plasmid, pGPTAS, was then examined for its ability to complement the temperature sensitive growth phenotype of *vps16* (figure 4.12). Growth at 37°C of the mutant carrying either the multicopy pGPTA or single copy pGPTAS was indistinguishable, with both strains growing similarly to the parental strain (SEY6211). It seems that gene dosage of this sequence does not affect rescue of *vps16*.

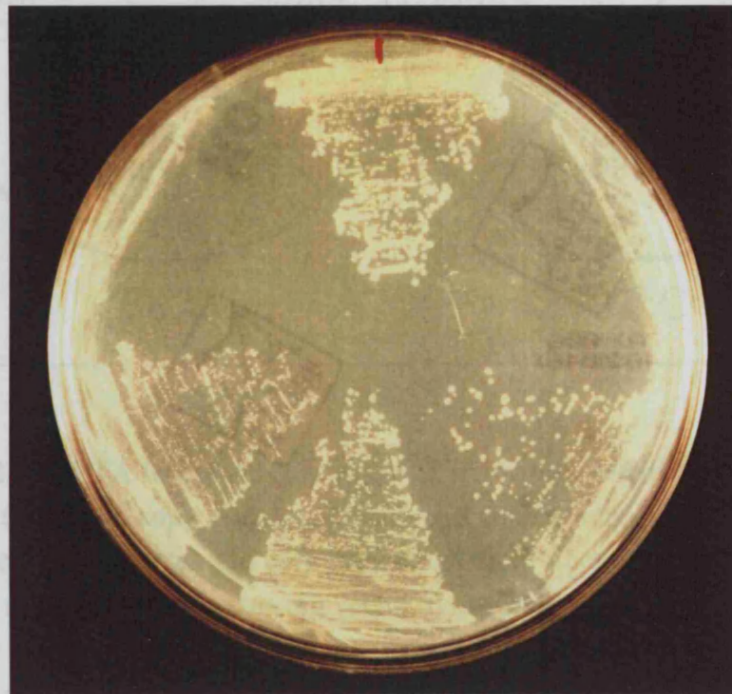
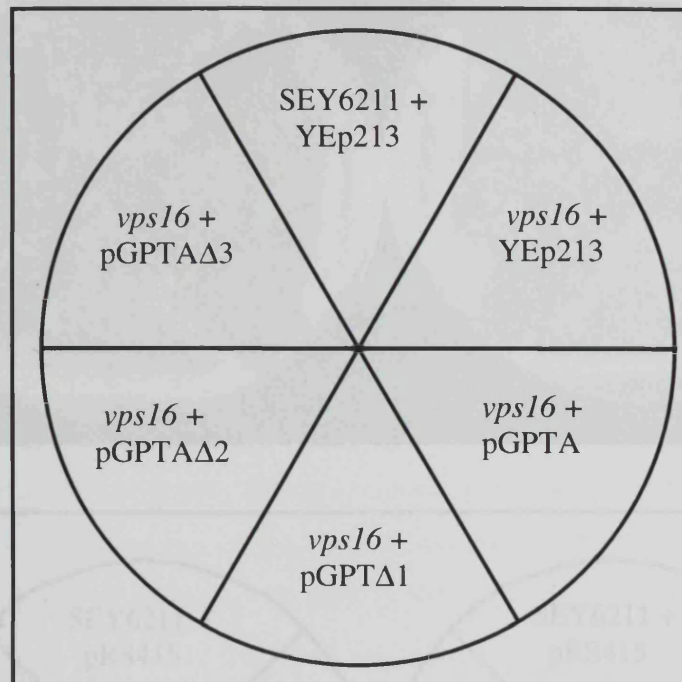


Figure 4.11. pGPTA rescue of *vps16* is not dependent upon *GPA1* or *GPA2* ORFs. Each strain was streaked to fresh selective SD media, and incubated for 3 days at 37°C. pGPTA Δ 1 is deleted for sequences within the *GPA1* ORF. pGPTA Δ 2 is deleted for sequences within the truncated ORF *GPA2*. pGPTA Δ 3 is deleted for the entire pGPTA insert, this no longer supported growth at 37°C.

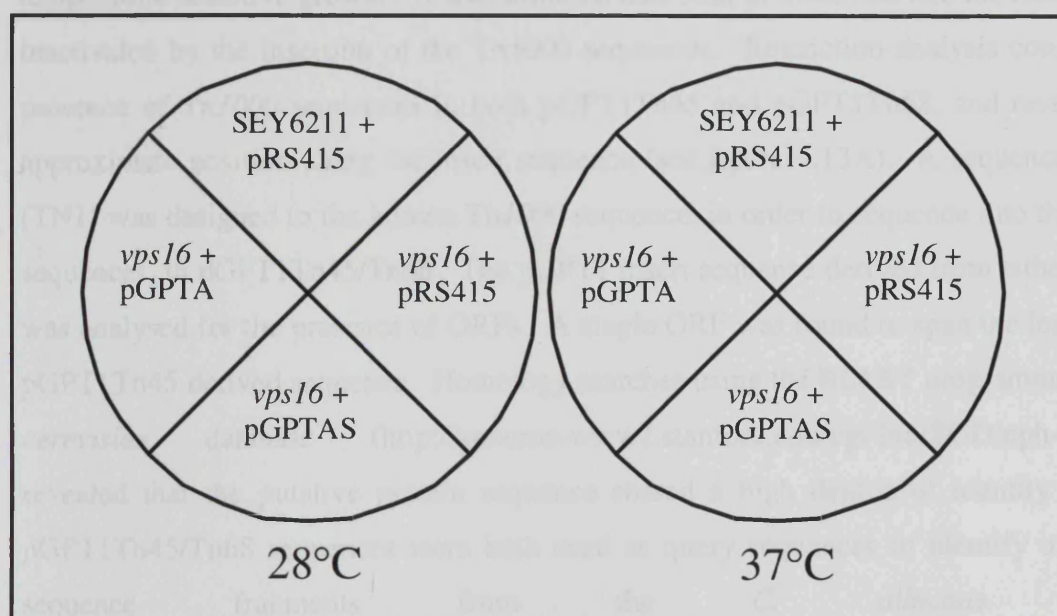
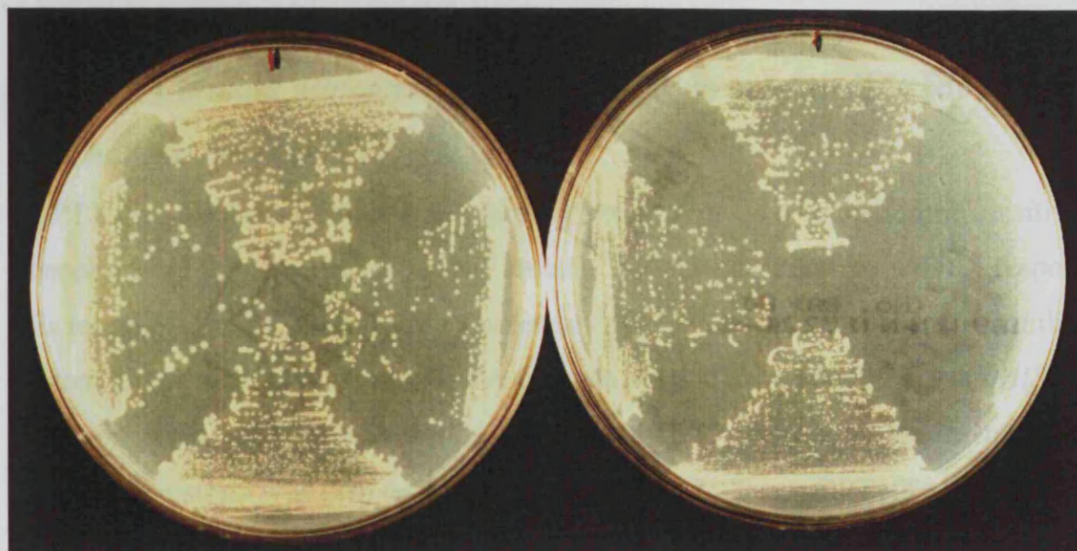


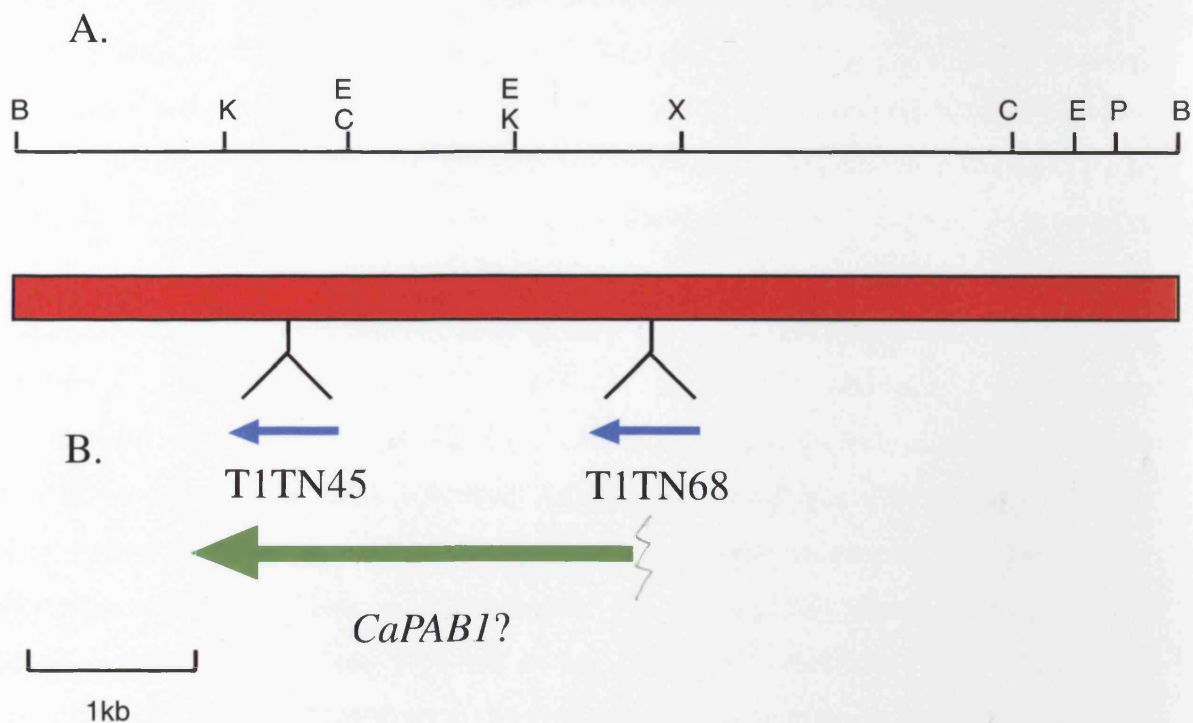
Figure 4.12. pGPTA insert rescues *vps16* temperature sensitive growth when present on the single copy vector pRS415. All strains were streaked to fresh (minus leucine) selective SD plates and incubated at either 28°C or 37°C for 3 days. Parental (SEY6211) and mutant cells carrying vector alone provided positive and negative controls for growth at 37°C. pGPTAS was constructed by inserting a 2.6kb *HindIII*-*SalI* fragment from pGPTA, which contained the entire insert sequence, into the single copy vector pRS415, which has a *LEU2* marker gene.

4.6 A *Candida albicans* PAB1-like ORF is responsible for the rescue of *Saccharomyces cerevisiae* *vps18*

pGPT1 was isolated as 'partially' rescuing the copper, and temperature sensitive growth phenotypes of the *S. cerevisiae* *vps18* mutant. In order to identify the ORF responsible for this rescuing activity, transposon mutagenesis of pGPT1 was undertaken, using the Tn1000 system as described above for pGPTA (section 4.5). Thirty pGPT1:Tn1000HIS3 plasmids were prepared, and transformed back into the *S. cerevisiae* *vps18* mutant. Each transformant strain was then tested for growth at 37°C. Two pGPT1:Tn1000HIS3 plasmids (pGPT1Tn45, and pGPT1Tn68) were identified as having lost the ability to rescue *vps18*, temperature sensitive growth. It was assumed that both of these had had the rescuing ORF inactivated by the insertion of the Tn1000 sequences. Restriction analysis confirmed the presence of Tn1000 sequences in both pGPT1Tn45 and pGPT1Tn68, and revealed their approximate position along the insert sequence (see figure 4.13A). A sequencing primer (TN1) was designed to the known Tn1000 sequence, in order to sequence into the flanking sequences, in pGPT1Tn45/Tn68. The pGPT1 insert sequence derived from either plasmid, was analysed for the presence of ORFs. A single ORF was found to span the length of the pGPT1Tn45 derived sequence. Homology searches using the BLAST programme, to the *S. cerevisiae* database (<http://genome-www2.stanford.edu/cgi-bin/SGD/nph-blast2sgd>) revealed that the putative protein sequence shared a high degree of identity to Pab1p. pGPT1Tn45/Tn68 sequences were both used as query sequences to identify overlapping sequence fragments from the *C. albicans* database (<http://alces.med.umn.edu/gbsearch/ybc.html>). Each additional sequence in turn was used as a query, to identify any further overlapping sequences, until no more sequences were recognised. All sequences were then assembled into one contiguous sequence using GELASSEMBLE (contig 18R appendix A). This contig was found to code for a single ORF truncated at the N-terminus. However, due to the poor quality of sequence from the database, a number of ambiguous nucleotides were present in the contig 18R, these also resulted in some ambiguous amino acids in the translated protein (these residues are represented as X in figure 4.13C).

The predicted protein sequence (figure 4.13C) was compared to sequences in the *S. cerevisiae* database, using the FASTA programme and was found to share 63.5% identity

Figure 4.13. pGPT1 encodes a Pab1p homologue. (A) Restriction map of pGPT1 insert. B = *Bam*H1; C = *Cla*I; E = *Eco* RI; K = *Kpn*I; P = *Pst*I; X = *Xba*I. (B) Transposon mutagenesis of pGPT1 was undertaken to identify the ORF responsible for rescuing the *S. cerevisiae vps18*. Two pGPT1:Tn1000 plasmids were identified as lacking the ability to rescue *vps18* (T1TN45 and T1TN68). Primers to the known transposon sequences, were used to sequence into the DNA flanking each transposon. Both T1TN45 and T1TN68 were found to reside within the coding sequence, of an ORF similar to *S. cerevisiae PAB1*. Searches of the *C. albicans* database, identified overlapping sequences which were assembled into a sequence contig (18R) of this *PAB1* like ORF, (appendix A). The approximate position of the *CaPAB1* ORF on the pGPT1 insert is displayed. (C) Protein sequence predicted from the *CaPAB1* ORF constructed from the database aligned to the *S. cerevisiae* Pab1p using PILEUP. *CaVPS18* was truncated in the 5' region. X marks the position of ambiguous amino acids, due to the poor quality of sequence on the database. *CaPab1p* shares 63.5% ID and 71.5% similarity over the whole length of the ScPab1p (619 a.a.). Identity is highlighted in black, similarity is highlighted in grey. The four RNA binding domains appear to be conserved. The RNP1 motif of each RNA binding domain is highlighted in red, RNP2 motifs highlighted in blue.



C.

scPab1p	1	~ ~ ~ M A D I T D K T A E Q L E N L N I Q D D Q K Q A A T G S E S Q S V E N S S A S L Y V G D L E P S V S E A H L Y D I
caPab1p	1	W C S K V D I G S T T E E Q S A A A A T T T A D Q S A E E Q G E S S G V A E N S A S L Y V G E L N P S V N E A T L F D I
scPab1p	58	F S P I G S V S S I R V C R D A I T K T S L G Y A Y V N F N D H E A G R K A I E Q L N Y T P I K G R L C R I M W S Q R D
caPab1p	61	F S P I G Q V S S I R V C R D A V S K K S L G Y A Y V N Y H K Y E D G E K A I E E L N Y N L I E G R P C R I M W S Q R D
scPab1p	118	P S L R K K G S G N I F I K N L H P D I D N K A L Y D T F S V F G D I L S S K I A T D E N G K S K G F G F V H F E E E G
caPab1p	121	P S A R R S G D G N I F I K N L H P A I D N K A L H D T F S A F G K I L S C K V A T D E F G Q S K C F G F V H Y E T A E
scPab1p	178	A A K E A I D A L N G M L L N G Q E I Y V A P H L S R K E R D S Q L E E T K A H Y T N L Y V K N I N S E T I D E Q F Q E
caPab1p	181	A A E A A I E N V N G M L L N D R E V F V G K H I S K K D R E S K F E E M K A N F T N I Y V K N I D L N Y S E S F E K
scPab1p	238	L F A K F G P I V S A S L E K D A D G K L K G F G F V N Y E K H E D A V K A V E A L N D S E L N G E K I Y V G R A Q K K
caPab1p	241	L F S P F G K I T S I Y L E K D Q D G K S K G F G F V N E E D H E S A V K A V E X L N D K E I N G Q K I Y V G R A Q K K
scPab1p	298	N E R M H V L K K Q Y E A Y R L E K M A K Y Q G V N L F V K N L D D S V D D E K L E E E F A P Y G T I T S A K V M R T E
caPab1p	301	R E R L E E L K K Q Y E A V R L E K L A K Y Q G V N L F V K N L D D T I D S E K L E E X F K P F G T I T S A K V M V D E
scPab1p	358	N G K S K G F G F V C F S T P E E A T K A I T E K N Q Q I V A G K P L Y V A I A Q R K D V R R S Q L A Q Q I Q A R N Q M
caPab1p	361	A G K S K G F G F V C F T T P E X X T K A I T E M N T R M I N G K P L Y V A L A Q R K D V R R S Q L X Q Q I Q A R N Q M
scPab1p	418	R Y Q Q A T A A A A A A A G M P G Q F M P P M F Y . . . G V M P P R G . . . V P F N G P N P Q Q M N P M G G M P . K N
caPab1p	421	R M Q N A A A G G L P G Q F I P P M F Y G Q Q G F F P N G K G N A P Y P X P N P Q M M R G R C Q P F X E
scPab1p	471	G M P P Q F R N G . . . P V Y G V P P Q G G S P R N A N D N N Q
caPab1p	475	Q W P R P G P N G Q P V F V Y G I P P Q F Q Q D F N G Q N M R P Q Q Q Q Q Q P R G G Y P N R N Q T S K R D L A A I I
scPab1p	500	. . F Y Q Q K Q R Q A L G E Q L Y K K V S A . . K T S N E E A A G K I T G M I L D L P P Q E V F P L L E S D E L F E Q H
caPab1p	535	S S V P O D Q Q R I L G E E L Y P K I V A T G K A Q E P E A A G K I T G M M L G L E N Q E I L D L L E D D E L F N N H
scPab1p	556	Y K E A S A A Y E S F K K E Q E . . Q Q T E Q A
caPab1p	595	F E D A L T A F E E Y K K S E A A G N A E E Q A

and 71.5% similarity (using BESTFIT programme in the GCG analysis package) to *S. cerevisiae* Pab1p (619 a.a.) over its entire length. Pab1p is the poly-A binding protein, which attaches to the poly-A tails of mRNAs. It mediates the physical interaction between the poly-A tail and the 5' mRNA cap (Tarun and Sachs, 1996), which is thought to stimulate translation initiation, and it may aid the export of mRNAs from nucleus to cytoplasm. Pab1p also affects the stability of mRNA, as it has been shown to inhibit decapping at the 5' end of mRNAs (Caponigro and Parker, 1995), a step which leads to the *XRN1* 5'-3' nuclease degradation of transcripts (Hsu and Stevens, 1993). In this way a transcript cannot be decapped until its poly-A tail has been removed to an extent which no longer permits Pab1p to bind. The rate of poly-A removal is specific to each mRNA, although Pab1p has been shown to be necessary for deadenylation mediated by the *PAN1* and *PAN2* encoded poly-A nuclease (Boeck *et al.*, 1996). *S. cerevisiae* *pab1* mutants have been shown to have decreased poly-A tail degradation, and mRNAs are decapped with long poly-A tails (Caponigro and Parker, 1995). Bound to poly-A tails, Pab1p plays an important role in determining the stability of mRNA.

Figure 4.13B illustrates the position of Tn45 and Tn68 on the pGPT1 insert. It also shows how the contig derived from the database sequence aligns to pGPT1, and the approximate position of the truncated *CaPAB1* ORF identified. Assuming ScPab1p and CaPab1p to be of similar size, it is likely that the *CaPAB1* ORF identified from the database sequence (on 18R) is truncated by just the first few codons. Due to the position of *CaPAB1* on the pGPT1 insert, pGPT1 is likely to contain the whole *CaPAB1* coding sequence and any associated promotor sequences. Complete sequencing of the *CaPAB1* ORF on the pGPT1 insert was not undertaken. However the incomplete protein sequence derived from the 18R contig was aligned to ScPab1p using the PILEUP programme on GCG (figure 4.13C), and annotated using the boxshade server (<http://www.ch.embnet.org/software/BOX-form.html>). ScPab1p has been shown to possess four repeated RNA recognition motifs (RRM) of around 92 amino acids at its N-terminus (Sachs *et al.*, 1986; Adam *et al.*, 1986). Each consists of two short highly conserved core sequences (RNP1 and RNP2; RNP = ribonucleoprotein), embedded in a more weakly conserved sequence. RNP2 consists of the consensus sequence L/I-F/Y-V/I-G/K-N/G-L/I (Burd and Dreyfuss, 1994; Birney *et al.*, 1993), while downstream RNP1 has a consensus R/K-G-F/Y-G/A-F/Y-V-X-F/Y (Burd and Dreyfuss, 1994; Birney *et al.*, 1993). Each RNP1/2 group (representative of each RRM) is highlighted in figure 4.13. In fact all four of the proposed ScPab1p RNA binding domains

(Adam *et al.*, 1986), are largely conserved between the ScPab1p and the *C. albicans* homologue identified here (figure 4.13C). In particular the RNP1 and RNP2 portions of these domains are almost entirely conserved.

4.7 Discussion

Functional complementation of *S. cerevisiae* mutants with a *C. albicans* genomic DNA library has been successfully used in the isolation of many *C. albicans* genes (De Backer *et al.*, 2000). This approach is not always successful, as the promoter of the heterologous *C. albicans* gene may not be recognised by the hosts transcriptional machinery. Alternatively the protein product may be expressed but fail to fold correctly, interact with the appropriate proteins, or just lack functional activity in *S. cerevisiae*. In particular this may be a problem due to the non-universal genetic code of *C. albicans* (CTG codes for serine instead of leucine), which can result in mis-translation of *C. albicans* protein products expressed in *S. cerevisiae*.

The sequences within pGPTA were isolated (on more than one clone) and fully rescued *S. cerevisiae vps16* associated defects, even when carried on the single copy vector pRS415. A single complete ORF of 234 amino acids was identified on pGPTA and designated *GPA1*, however no *VPS16* homologue was identified within the insert sequence. A second ORF of 155 amino acids, truncated at its N-terminus was present on the insert and designated as *GPA2*. Attempts to precisely define the sequences responsible for the *vps16* rescuing activity failed, but it seems that *GPA1* and its upstream sequences aren't sufficient for rescue of *vps16*. Surprisingly sequences within either *GPA1* or *GPA2* aren't necessary for the rescuing activity, as determined by deletion analysis. Deletion of the whole pGPTA insert, and transfer of the insert to pRS415, confirmed that the rescuing activity was associated with the *C. albicans* genomic DNA insert rather than any vector sequences of pGPTA. Further analysis is required to precisely define the minimum sequence necessary and sufficient for the rescue of *vps16*. This could be investigated by using inverse PCR in a sequential deletion strategy, the resulting clones being tested for their ability to complement the *vps16* temperature sensitive growth defect.

The *C. albicans* genomic sequences within pGPTA were apparently sufficient for the complementation of *vps16* mutant phenotypes. Despite this, no *VPS16* homologue was

identified within the *C. albicans* genomic insert. It therefore seems unlikely that pGPTA directly rescues the primary *vps16* defect i.e. through interaction with, and restoring the function of the class-C docking complex. It is more likely that it functions via an indirect mechanism to help ameliorate these phenotypes. The inability of *vps16* to grow at 37°C, or under conditions of osmotic or ionic stress could suggest an inability to induce stress responses, or perhaps the associated signalling pathways. However, the overexpression of a particular cellular component may result, either directly or indirectly, in the over stimulation of an alternative stress signalling pathway. The resulting induction of a stress response could permit the growth of *vps16* under conditions usually too hostile. If pGPTA was acting through such an indirect mechanism to suppress the *vps16* phenotypes, we would expect it to rescue the other class-C *vps* mutants of *S. cerevisiae*. However, pGPTA failed to complement any of the other 3 class-C *vps* mutants strains.

There is no obvious explanation as to why *CaPAB1* suppresses the temperature and copper sensitive growth phenotypes of *S. cerevisiae vps18*. It seems likely that CaPab1p acts indirectly to repress these phenotypes. Perhaps the overexpression of CaPab1p from the multi-copy vector YEp213 affects the stability of mRNAs. It may cause an increase in deadenylation of mRNA transcripts, and this could decrease their stability. Alternatively, CaPab1p may bind the poly-A tails of *S. cerevisiae* transcripts but not be compatible with the *S. cerevisiae* PAN2/3 poly-A nuclease. Failure to recruit PAN2/3 would decrease poly-A degradation, possibly increasing mRNA stability, and in turn affect a global change in gene expression. Altering the expression level of key genes, could affect a range of cellular functions, and perhaps a stress response is induced by the rescuing clone, this could permit some degree of tolerance of increased stress. If this were the case then as above, one might expect pGPT1 to rescue all four class-C *vps* mutants, but *vps11*, *vps16* and *vps33* transformed with pGPT1 had no detectable growth at 37°C. Further analysis is required to determine the mechanism by which *CaPAB1* is able to suppress these *S. cerevisiae vps18* phenotypes. It would be interesting to see if the overexpression of the *S. cerevisiae* Pab1p ameliorates the *vps18* phenotypes. *ScPAB1* could be cloned in to a multi-copy vector, and *vps18* cells carrying this construct screened for growth at 37°C and on high copper medium.

Chapter 5

Isolation and analysis of *Candida albicans* *VPS11* and *VPS18* like ORFs

5.1 Introduction

Attempts to clone *C. albicans* class-C *VPS* like genes by a functional complementation of *S. cerevisiae* class-C mutants were unsuccessful (Chapter 4). However, the *C. albicans* genome sequencing project (<http://alces.med.umn.edu/candida.html>) has identified fragments of *C. albicans* sequence which share homology to the coding regions of some of the class-C genes of *S. cerevisiae*. This chapter describes how this sequence information was used to isolate *C. albicans VPS18* (*CaVPS18*), and *VPS11* (*CaVPS11*) homologues. The putative protein products are compared to their *S. cerevisiae* homologues, and transcription of *CaVPS11* and *CaVPS18* analysed.

5.2 Analysis of class-C *VPS*-like gene sequences in the *Candida albicans* genome database

The *C. albicans* genome sequencing project (Stanford Sequencing and Technology Center) has used a 'shotgun' approach for the sequencing of strain SC5314. Random fragments of genomic sequence approx 250-600 base pairs long are available on the database (<http://alces.med.umn.edu/candida.html>). As progressively more sequences are made available, sequences overlapping a common genomic region can be identified and assembled into contiguous fragments of the genome (contigs).

One sequence identified as showing similarity to *S. cerevisiae VPS18/PEP3*, was designated as *PEP3* by the Stanford sequencing project. Further sequences which shared homology with *S. cerevisiae VPS11* and *VPS33* ORFs, were identified through BLAST searches using the *S. cerevisiae* DNA sequence to the database. No *VPS16* like fragments were found. Each of these class-C like sequences were then used as the query sequence in BLAST searches using the on-line facilities (<http://alces.med.umn.edu/gbsearch/searches.html>), to find additional overlapping sequences. Each new sequence was in turn used in additional BLAST searches until no additional overlapping sequences were found. The sequences associated with each class-C

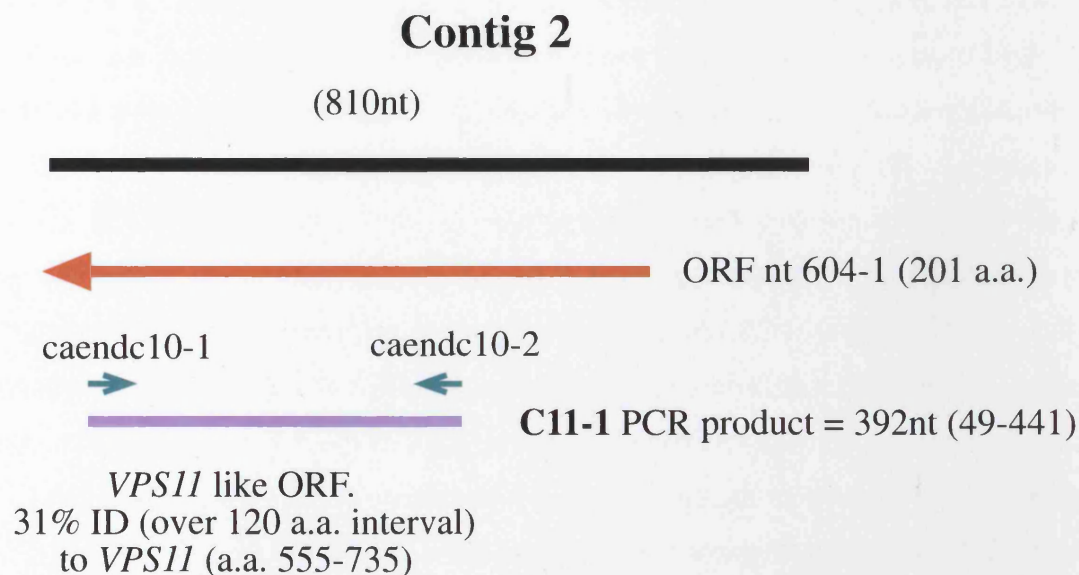
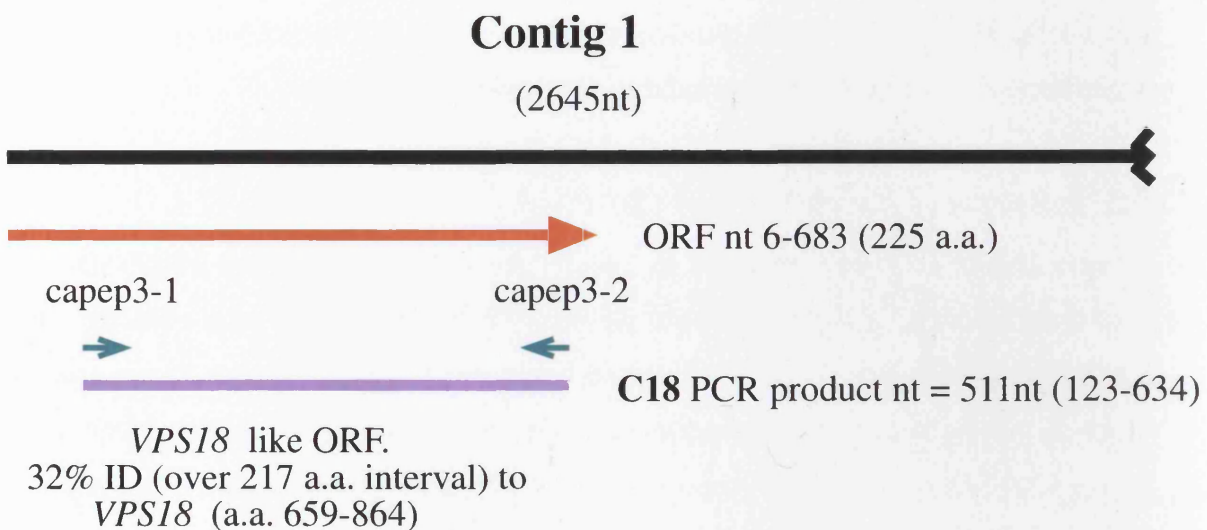
like gene were then aligned into contigs using the GELASSEMBLE program in the GCG package. Two contigs, of 810 and 1174 nucleotides, were constructed containing the *VPS11* like ORF. Also one contig of 2645 nucleotides containing a *VPS18* like ORF, and one contig of 2393 nucleotides containing a *VPS33* like ORF were also assembled (figure 5.1). However, none of these four contigs contained the whole of these ORFs, being truncated at either the 5' or 3' ends. The putative gene products encoded by these ORF 'fragments' shared homology to the *S. cerevisiae* gene products, with identity ranging from 26 to 38% (see figure 5.1). Contigs 3 and 4 (figure 5.1) each contain 2 *VPS11* and *VPS33* like ORFs, respectively, but in different reading frames. One possible explanation is the occurrence of sequencing errors, resulting in apparent 'frameshifts'.

5.3 Isolation of *CaVPS11* and *CaVPS18*

Primers (figure 5.1) were designed to amplify the *VPS11*, *VPS18* and *VPS33* like ORF fragments by PCR, using *C. albicans* genomic DNA (strain S01) as a template. Each fragment was radioactively labelled (see Materials and Methods Chapter 2) and used as a probe in colony hybridisation experiments, against a *C. albicans* genomic DNA library (strain S01) in *E.coli* (DH5 α). Approximately 8000 colonies were screened for each of the four probes, to give a 95% confidence level of genome coverage. Potential positives were picked and used in a second hybridisation screen, using the same probe. Plasmids from the positive clones identified in this secondary screen were then isolated, and used in PCR reactions with the above primer sets (figure 5.1) to confirm the presence of the desired sequences. No positive clones were identified using the *CaVPS11-2* (C11-2) or the *CaVPS33* (C33) probes. Three positives were isolated using the *CaVPS18* (C18) probe, and two positives with the *CaVPS11-1* (C11-1) probe (table 5.1). Using the genomic library as a template, PCR reactions were run with the primer sets used to amplify each probe. There was an abundance of product for *CaVPS18* and *CaVPS11-1* primer sets, less abundant product for *CaVPS33*, and no product for *CaVPS11-2*. These results suggest that the *CaVPS11-2* genomic region may not be covered by our library, and that the *CaVPS33* region is present but perhaps only in low abundance.

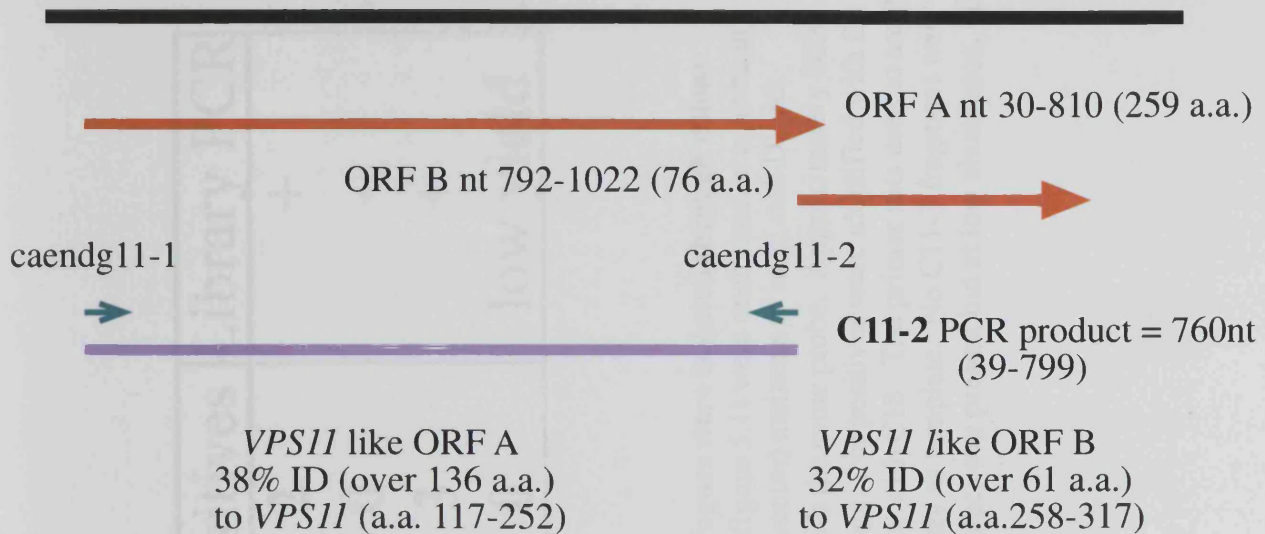
Subsequent restriction mapping analysis revealed that the two *CaVPS11* clones contained identical inserts (figure 5.2). However, the three *CaVPS18* clones, all had different

Figure 5.1. Class-C VPS like ORF fragments identified from the *Candida albicans* sequence database. Fragments of overlapping sequence were constructed into contigs (black line). For each of the homologous ORFs identified (red arrows) the % ID shared to the *S. cerevisiae* class-C VPS gene product is shown below. The position of primers (green arrows) designed for the amplification of DNA probes are given below, with the corresponding PCR product (blue line). No *VPS16* like ORFs were identified. Contig 1 was identified as containing a *VPS18* like ORF. Contigs 2 and 3 were identified as homologous to two separate regions of *VPS11*. Contig 4 contains a *VPS33* like ORF.



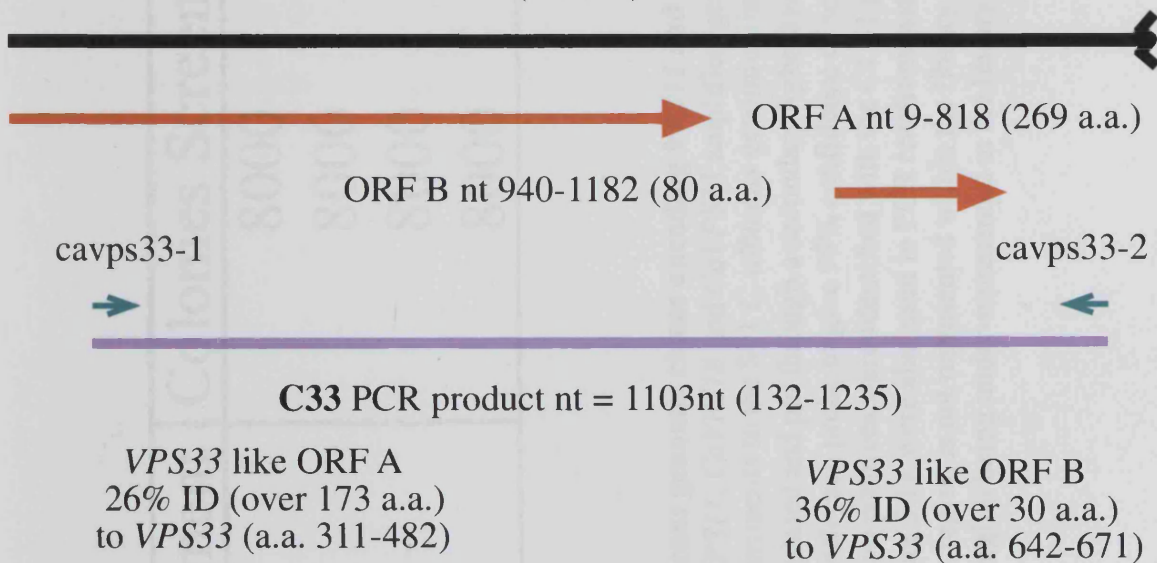
Contig 3

(1174nt)



Contig 4

(2393nt)



Probe Fragment	Colonies Screened	Positives	Library PCR
C11-1	8000	2	+
C11-2	8000	0	-
C18	8000	3	+
C33	8000	0	low yield

Table 5.1. *Candida albicans* genomic clones encoding *VPS11* and *VPS18* homologues were isolated through colony hybridisation. The *CaVPS11*, *CaVPS18* and *CaVPS33* amplified gene fragments (figure 5.1) were each used as a probe in colony hybridisation experiments to the S01 *C. albicans* genomic library (YEp213 vector) maintained in *E. coli* DH5 α . Positives were picked, replated and put through a secondary hybridisation screen, with the same probe. Approximately 8000 colonies were screened with each probe to give 95% confidence of sequence coverage. No positives were identified with the C11-2 or C33 probes. Two positives were identified with the C11-1 probe, and three with C18. The primer sets used to amplify the probe fragments (figure 5.1), were also used in PCR reactions with the S01 library as template. No C11-2 fragment was amplified, perhaps as this region is not represented within the library. The C33 product was present but at low abundance, this may be as a result of the region being under-represented in the library.

Lane 1 2 3 4 5 6 7 8 9 10 11

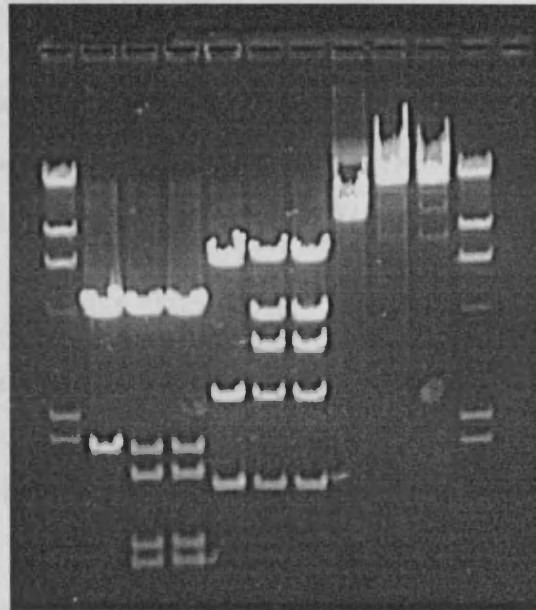


Figure 5.2. Restriction analysis of the two *CaVPS11* positive plasmids revealed them to contain identical inserts. Plasmids prepared from either positive clone were digested and run on a 1% agarose gel. The identical restriction fragment pattern indicates that the same plasmid was isolated twice in the primary hybridisation screen.

Lanes (left to right): 1= $\lambda_{HindIII}$; 2=YEp213/*EcoRI*; 3=pGP11-1/*EcoRI*; 4=pGP11-2/*EcoRI*; 5=YEp213/*Clal*; 6=pGP11-1/*Clal*; 7=pGP11-2/*Clal*; 8=YEp213/*HindIII*; 9=pGP11-1/*HindIII*; 10=pGP11-2/*HindIII*; 11= $\lambda_{HindIII}$.

restriction patterns (data not shown), indicating that each contained a different fragment of *C. albicans* genomic sequence.

5.4 Sequence analysis of *CaVPS11* and *CaVPS18*

The *CaVPS11* clone (pGP11), and one of the *CaVPS18* clones (pGP184) were sequenced. The PCR primers used to generate the *CaVPS11* and *CaVPS18* probes were used to initiate sequence analysis; sequence data derived from these reactions were used to design new forward and reverse primers. In this way, new sequence was generated by 'walking' along the inserts, and the sequence data was checked by designing reverse primers to carry out sequence analysis of the complementary strand. All sequence data accumulated for each clone was assembled into contigs using the GELASSEMBLE programme in the GCG analysis package. Where ambiguities in sequence data were observed, the original electrophoretograms were viewed and the data corrected accordingly.

A total of 3170 bp of sequence was determined for the pGP11 insert (figure 5.3A). This encoded a large ORF of 892 codons truncated in the 5' region (figure 5.3B). The predicted protein product for the available sequence was aligned to the *S. cerevisiae* Vps11p sequence using PILEUP in the GCG package (see figure 5.3C). The two products shared 35% identity and 48% similarity over their length. It has been postulated that a cysteine rich region near the C-terminus, may form a functionally important domain of ScVps11p (Robinson *et al.*, 1991). This region is very similar to the ScVps18p putative C-terminal zinc binding domain. Some of these cysteines, plus two additional histidines are conserved between the ScVps11p and CaVps11p (figure 5.3D), in a shared CX₂CX₁₂CXHX₂HX₂C sequence. A very similar cysteine-histidine rich region appears to be conserved near the C-terminal of Vps18p (discussed later in section). This supports the suggestion that this may form a functionally important domain, perhaps facilitating protein-protein interactions.

For the *CaVPS18* clone selected for sequencing (pGP184), 4962 nt of sequence data was assembled (figure 5.4A). This was found to encode a single large ORF of 810 codons (figure 5.4B). An alignment of the predicted ScVps18p and CaVps18p sequences using PILEUP is shown in figure 5.4C. The proteins share approximately 30.7% identity and 45% similarity. Again at least some of the cysteines and histidines, proposed to form a functionally important domain in ScVps18p, are conserved between the two homologues

Figure 5.3. Sequence analysis of *CaVPS11*. Sequencing of the pGP11 clone (A) revealed the *VPS11* like ORF (red nts) was truncated at the N terminus. (B) shows the position of the *CaVPS11* ORF (red arrow) within this sequence. Vector sequence for pGP11, is shown as a black line. (C) alignment of the complete *S. cerevisiae* and available *C. albicans VPS11* products using the PILEUP programme from the GCG package, which share 35% identity and 48% similarity. Identity is shown by black box-shading, and similarity by gray shading. (D) an alignment of the C-terminal cysteine/ histidine rich domains of these two proteins. Identical residues are shown in red, similar residues in blue, and conserved cysteine/ histidine residues are highlighted in gray.

A.

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CGAGGGGCTA AACAAAGATT AATTTATAAT AGTGGTAACA ATGATCCAAT
AACCGGAGTA CAATTTAATG AAACCTGAACA AGTATTATAT GTAACCTACCA
CTGAAAAAATT GTTAACGGTA GCGACAACGG GGAGAAATCA TGAAAAACCA
TTGAAAATAT TATCCAATAA ATATGGAGCC GATTTGAATT GTACTGATAT
TGACGAATCA AATCAAAATT TAATTGTTGG ATTAAATGAT TCAATACAAT
TCTATGATTG TTTCACATAA ATACTGACAA TTAATTTCAA ACTCGCTAAA
TCAAGAATTA TCAAAATGCAA ACAATATTTA TTGATTGTGA GTCCCGAAGA
AAGAAATAAA GCGGGAGTAT CTGAAACCGA TCATGGCATT GATAAATCAT
TAATGTCGAG AATAGTTATT TTGGATTAG TCAACAACCA TATTTCTGTTT
AATCTACTTA TTTCTGACAG TTCAATTTCT CATGCATTTA GTTTATCAAA
TGGAACATTC TTGCTATTGA CTACCGATGG AGTATTGTAT AAAATCAATG
AAAAATCAAT CAACCAACAA ATTGAAATTG TTCTACAAAG AGAATTGTTT
TCCGTTGTCAT TCAATTTGGG CCAACAATAT AAATTACCTA ATGAAACATT
ATTAAGAATT CAAATATTAC ATGGTGATTA TTTATATGAT CAGAATAAAT
TTGACGAGGC AATCGATGTG TATATAAAAT GCCTTGAATT GTTTAAAAAG
AGTGGCAAGA TAGTAGAACA GAAACAGAAG AATAATCGAC TGGACAATCA
AACTGAAGTT GAAGAAGAAG AAGATATTGA TGAATTCATT ATCAATATAA
TTACTAAATT CAAAGAAGCT ACAAATATAT CTAATTTAAC AAAGTTTTTG
ATTCGATTAT ATGAAAAATC ACTTGCTAAT ATTGATCATA TTACTTTATT
ATTATGTTGC TATTGTAAGT TAAAGAAGAT TGACAATCTA AACGAATTTA
TCGATGAATT GGATTTATCA ATTGAAAATT TGCAAGAATT GAATTATGAA
TTAATTATTA ATTTATTTAA AGAATGTGGA TTTTATGATC AAGTGTTAAA
ATTACTTTAT AAATTGAACC AACCCAATTT AATTGTCGAT ATTCAATTGA
ATGATCTTCA TAAACCGAAA TTGGCATTGA ACTATATGAA GACATTAACC
ATTGATGATT TGTTATTAAT TTTAATCGAT CATTCAACAA ACTTATTGGA
TTCATGCCCC CTTGAAACTA CCGAATTATT GATTAATGTG TTTACTGGTA
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TAGATAAAGC CGAAACATTA ACACGAGATT ATAACCAATT ATTAGATAAT
CAAGCATTGT TATTGTTGAG TCATATTTAT AATTTCAAAC CTGGTGAAGT
GATTGCTCAA GAGAAAAGTG GGAATGAAAT AGATTTGTTT ATGAGTTGTC
AATTGAACCA AGACGTGGAA GGATGTTTGA AAATTTTAGA AAAATATGGT
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GAAAACCATA TTTGATCAGA TTAAATATGA AGATATCCAA GTAATACTTG
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TTAACAGAAA ACCCTGATAA TGAATTTATT ACATTAGGAT TAGTTAAAGA
TTATTTTATT GATTATTTCC AACACAAAC TAAAGAAATT TCCAATAATA
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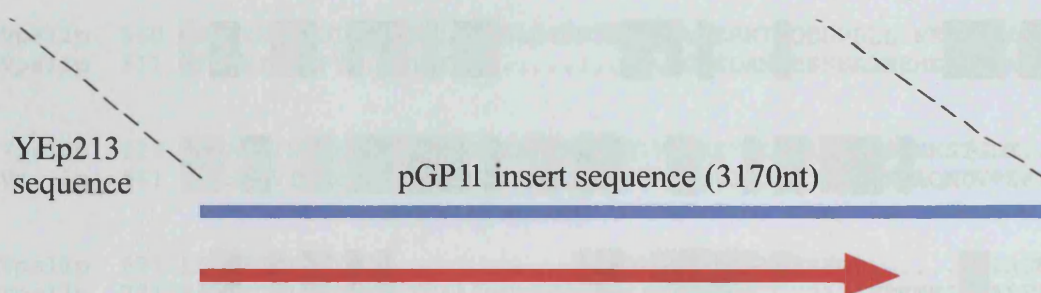


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CATTAATTCA TTGCATTCTA GTAATGATAA ATTTAAATTT GTTTCTGATT
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AGAATAAAAT AAAATTTAGT

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B.



C.

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CaVps11p 1 ~~~~~
ScVps11p 1 MSLSSWRQFQLFENIPIRDPNFGGDSLLYSPTLCAATIVDPQTLIIAVNSNIIKVVKLN

CaVps11p 1 ~~~~~MPSITKLDISKLNANNENND.SKNSKIQSEYFEFQTQVL
ScVps11p 61 QSQVIHEFQSFPDFQITFLAVINGEFVALAESIGKPSLIRVYKLEKLPNEQLY....

CaVps11p 45 VNDHSSGTTGIGDNSYPISCFKFNYDLTCLATGYTNGKVLVRGDLDRSAKQRIIYNS
ScVps11p 117 ...HSQVELKNENNTYPISVVSIISNDLSCTVVGTEINGKIIIRGDISRDRGSOQRIIYED

CaVps11p 105 GNNDPITGVQFNETEQLVYVTTEKLTVAITGRNHEKLPKILSNKYCADINCTDIDESN
ScVps11p 174 PSKEITATFLNDATAACAATTSRIILFNITGRNRGRPSLVLSKNGLDLNGGSFNPAT

CaVps11p 165 ONLIVGNDSCQYDCFTKISTINFKAKSRIIKL..KQYLIIVSPERNKAGVETDHDG
ScVps11p 234 NEFICCSNFIEETESSGKKHQFADSLRKRIEVDNDHILIVL.EETGVPTTETISVNE

CaVps11p 223 LDKSLMSRIVTILVNNHISFLLISDESISHAESLS....NGTFLLITTDGVIYKINEE
ScVps11p 293 LSPTILNRIIFILAKNKIISLNFVVS.SALIDISTQSCKNITY.LLTSEGVHATPN

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continued...

CaVps11p 279 S I N Q I E F I Q R E I S V A F N I G Q Y K P N E T L L R I Q I L H D Y L Y D Q N K F D E A I V Y I K K L
ScVps11p 351 S I E N Q I N I I I K E I Y P F L D L K K H S S P L D V Q E I H K K Y G D Y L K K G L R K E A T Q Y I Q E I

CaVps11p 339 E I F K K S G K I V E Q K Q K N N R S D N Q T E F E E E D I D E F I I N I I T K F K E A T N I S N I T K F I R I Y E
ScVps11p 411 D V V E T S E I I S K G V K ... E V P P E S Y R N I A D Y W S I K

CaVps11p 399 K S E A N I D H I T L L L C C Y C K L K E N T N E F I D E L L S I E N I Q E L N Y
ScVps11p 446 N S I S R D H V T L L L I V L I K L D V E G I D T E I Q H F R K G I W N E G V V M D M D D V T E F Y S D N D F F

CaVps11p 443 .. S I L I N I F K E C G E Y D Q V L K L Y L N Q P N I I V D I Q L N D L K K L E I N I M K T L T I D L L
ScVps11p 506 D I D L L E M K E S D K R L S Y R A K Y S K D S I I V D I L L N L L H N E V K A I K Y I K S L P I D E T L R

CaVps11p 500 I I I D H S T N L L D S C F L E T T E L L I N V F T G K Y Q F N E P N A K Y V E D I S N N G N T S I M N Q P Q N K P E K
ScVps11p 566 C V T Y S K K L L E E S N E I N A L L I E F T G K E K S T E V D L D R D T T G

CaVps11p 560 E S E R E K S I D I S N Y R A L N Y I S L Q S E E T D N S D N Y E S K T Q E N P S L L S N K P T Y L P P K N
ScVps11p 611 P F S N I R T V F Y S Y K T E F N Y M N S N G I S D A M S E S S E A S H E H E P T Y H P P K S

CaVps11p 620 L I Y A S E T N H P K E F V I F L E S E A F N K D G N I V K R E T L L L E I Y I S N K S T G D K ... E V
ScVps11p 661 I V E S E V T K P F E F V F L E A C A C Y Q Q Y E G F D E R Q V I T P Y D I Y I N A Q N D V P E R I D E N

CaVps11p 677 L D K E T I T D Y N Q L U N Q A L L L S H Y F K P G E V I A Q E K S G
ScVps11p 721 R S R A T G V L E S N K V Y S A A S N N T S K R E D M S I N L L H M D S S A S A K D K T K I I A S F A N D N

CaVps11p 718 N E I D L ... L M S C Q L N Q V E G F E I E K Y D T K E Q L Y E M K E I I S S T I E D Q I K Y E D I..
ScVps11p 781 P E M D L S T E R A M T L N E P S T L K F E K Y G T E E K L Q V A S Y F V N S L I P K E M G G N E V L K

CaVps11p 773 Q V I L E Q I K I Y K L T P I E L Y D I I T E N P D N E I T L G I K Y F I Y F Q Q Q T K E I S N N T K I T E K
ScVps11p 841 E K Y R P I I E G E R M P L L D I I K A S R T N V A H ... G L I Q I I I I H V K T E D T I K R M E K L I E S

CaVps11p 833 Y S Q S T K N S F K I S E L ... S K I F . V I Q N K S G N K L D E P N I H F K C K H S F H Q K C I S T N L
ScVps11p 898 E K E L K E K N K I K N T I N S D Q L H V P K K T C F M C R I T L D I P V V E F K C G H I Y H Q H C L N ...

CaVps11p 888 I A T S T E S T S S T P I S S T S M N P N N N A V I N H N D D D K T C I I T H K F N I K N A K L N Q Y K G
ScVps11p 955 E E E D I L E S E R K E K E K N I ... V L E T S N ... K I F

CaVps11p 948 D I A N Q N K N E V F ... E N S L H S S N K F E V S D Y I G K E V E D Q Q ~ ~ ~
ScVps11p 984 E I Q H E V V K N D L N F A I N S E E G R R E F K V I T I G R A I S Y S D I T I

D.

ScVps11p	764	C F M C R L T L D I P V V F F K C G H I Y H Q H C	788
CaVps11p	929	C S G C N L K L D F P V I H F K C K H S F H Q K C	953

Figure 5.4. Sequence analysis of pGP184. (A) Sequence derived from pGP184, *CaVPS18* ORF highlighted as red nucleotides. (B) Diagrammatic representation of the pGP184 insert (blue line), and the *CaVPS18* ORF (red arrow). (C) Alignment of ScVps18p (918 a.a.) and CaVps18p (810 a.a., as predicted from pGP184 sequence, using the *C. albicans* genetic code), using the PILEUP programme. The predicted products share 30.7% ID and 45.2% similarity. Identity is shown by black box-shading, similarity by gray shading. (D) Alignment over the C-terminal cysteine/ histidine rich region, of ScVps18p, CaVps18p and the *D. melanogaster* protein Dor. Identical residues are shown in red, similar residues in blue, and conserved cysteine/ histidine residues are highlighted in gray. (E) a 399 nt region (nt 3090-3489 from consensus in (A)) sequenced from pGP182, pGP184, and pGP186. While the sequence for pGP182 and pGP186 were identical, there were some differences to pGP184. These differences are at the positions marked by N. The predicted protein product is shown beneath the nucleotide sequence. Only one of the 8 observed nucleotide changes over this region results in an amino acid substitution, isoleucine in pGP184 to valine in pGP182/ pGP186 (PURPLE amino acid) at amino acid 710 of CaVps18p. Below is a list of the 8 differences in order with the pGP184 nucleotide, followed by the pGP182/ pGP186 nucleotide at the corresponding position.

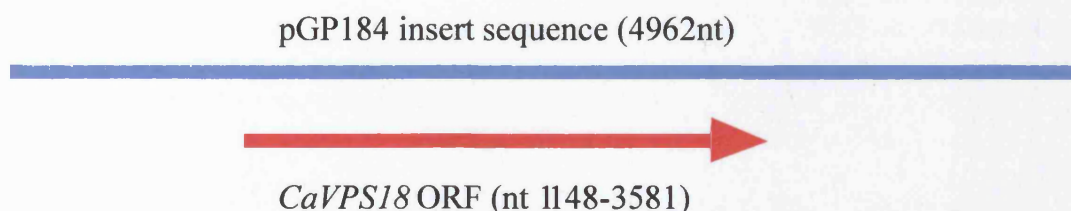
A.

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GAGGGTATTT	ATACCCTAGT	ATCAGTGGCG	TGGCGATAGT	CCGATTACTG
AGGAATACGT	GGACACTAGA	TAGGATTGAT	AAAGTACCTA	ACACCTCATA
TGTATAGTTT	TATATATGTT	TTCTTGAATC	ACTCTATCGA	TCAACGAACG
TTTTATAATT	CAAAATATATA	ACTTTATATT	TAGTTAACTT	GACTACTTAG
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CTCGTCTTTG	CAATGATATT	TACGTAATAT	CAAATGAGGG	ATATTCAATT
AAGATGACCA	ATTAATAGGG	GAAAATCATC	AATTGTTTAT	TTGAAGTGAA
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TAACCTTAGT	ATCTCTAGTA	AGACATCGAT	AATTCAATTA	TGGCAAGTTG
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CCAATGTTTG	CATTGATATC	CTATTTTCTG	GCAAGAAAAA	ATAATTACAA
CCAGACAAC	CACCTCTGTT	GTTTATGCCA	CGTTTGTGTC	AGGGAAATAT
CACCTTGATA	TAAGTCGTGC	GTATTAGGTC	AACATTGGTG	AAAAATTTGC
ACTCATCGAG	AGCCAGGAAT	TAGTATAAAA	AGAAGAGAAA	AGAAAGATAT
TTAGGATATT	TATTATATAG	AGACCGAGTT	TCAATAGACA	CTTTTAGTGG
GCGTAAACTT	CATTTACTCT	GTGTTTTGCT	TATTACAAAT	TATCACCTAT
CGTGTACTAG	GACTAATTCT	CACGAATATT	CCGTGTATAC	AAACACACTC
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GTCATCCAAC	GGGCATAAAA	CCAAATCTAT	AAAGTCGGAA	ACCAATTCGT
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GATGCCTGCA	TTAATTTGTC	ATTGAAACAT	TATCCAAACG	CATGAATGCT
CATAAATTCC	GTTTGATGTG	TCACTATATA	TATGGTTAGG	AGCAACACCG
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ATTGCTGCT TATAATTGA TGCTGCAATT CAATCTGATT AACGTTTTCA
CGCCTTTCTT TT

B.



C.

CaVps18p 1 MSSNGHKTKSIKSETNSSYTVHSLDQQQASICDNLQQAKFETEPVHLOFSLSN.KNKLL
ScVps18p 1 ~~~~~MIKTRIFEVQLQFLTGNTEITHK

CaVps18p 60 YSSNIMYILLANVYKIDLGNFSSQVENYALF.....GKVTNAWLNFNGQTIIVQIEKQAY
ScVps18p 25 YSNDQIIVTTQRTIYRINIQDPAIVNHFDCELSKELETIMNVHVSIMGSVLLIRTNFGRY

CaVps18p115 YTIKKVYKLLKFKNIEVTSIAFSNH.NMVVG.TKDGITIIYKSLKQV.....Y....K
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CaVps18p164 VDSPVQGVMFSSNDYSQINALANSLYTWCFDTSYV.....ELQKVFKQTAPVIASINP.P
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CaVps18p218 GIE...TSN.....PQNYVYISSNNEIITNDE...EMILRVVEEFSQI
ScVps18p205 GLFAWVTSNGIVFGDLKEKQMEKDPASNNFGKFLSSSKVLLNFELPDYNDK..HLIKDI

CaVps18p256 ALTSHHLIGIEGNSIKTIYNKLNK.....DIQELSLSN.KIRGIAVNLFNITYW
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ScVps18p383 EHSAAQTLGSMKDISHFGEIALNFTQI...KDYNDLNVILIKQLDNVPWKSTQVSSWI

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ScVps18p556 ILLIINSIEATVSTVWKKIDDPNKLIETILKFFFTNWQNSKLITNISEYPENYSITYIKW

CaVps18p556 VIMDKHYKKKQNNAYICILITKGTI....KQIIEKFINFTSN.FDTNFILRLCTSHEN
ScVps18p616 CVREVPKMCPIVYSIYYMITDARNMILENDIIEKMKSNENKILNFQLRISKFKK

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ScVps18p726 LKIAKHLLSMKDIDIKQLIRTIINDSNEIITIKDILLPFENEYTTIANLKEELIKFLENH

CaVps18p727 NKRIVHISLDMNNSSEHLRMKKKIYNKKTNVAIIEPGEPCRKUGKLLVQENFVYFPN
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CaVps18p787 CHHAFHKEEMKNQCCLCNDELNI~~~~~
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CaVps18p811 ~~~~~
ScVps18p901 KIDQPISIDETELAKWNE*

D.

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(continued over page)

E.

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| 1 | | | | | | |
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K Y L I D K L D D L N E T L H H I V N V S M L D L K
160 170 180 190 200 210 220 230
| 2 3 | 4 | 5 | | | |
AAGACCTTTTNCNNTTATTTCGGAACGNTACTGATTAATAATTTNAAAGACGAGATAGTTGAATCACTAAATGAGT
D L L P L F P E T V/I L I N N F K D E I V E S L N E Y
240 250 260 270 280 290 300 310
6 | | 7 | | | | |
ANAATAAACGAATTGTTTCATTTGTCGTTNGATATGAATAACTCGTCTGAGCATTGCGGGAAATGAAGAAAAAAGTGA
N K R I V H L S L D M N N S S E H L R E M K K K V I
320 330 340 350 360 370 380 390
| | 8 | | | | | |
TTTATAACAAAAAGAAAACAAATGTGGCNATAATAGAACCAGGTGAACCGTGTGCGCAAATGTGGCAAATGTTAGTAC
Y N K K K T N V A I I E P G E P C R K C G K L L V Q

AAGAGAACT

E N

- N1 = C or T

N2 = A or G

N3 = G or A

N4 = A or G
- N5 = T or C

N6 = C or T

N7 = A or G

N8 = T or C

(figure 5.4.D). Previously the suggested zinc-binding domain structure was $CX_2CX_{13}CX_2C$ (Preston *et al*, 1991). The structure was subsequently extended to include additional cysteines in a $CX_2CX_{13}CX_2CX_{38}CX_2C$ arrangement (Robinson *et al*, 1991). However, only the first cysteine (C₈₂₆) has been shown to be functionally important, through site-directed mutagenesis (Robinson *et al*, 1991). A different cysteine-rich structure was identified at the C-terminal end of the *Drosophila melanogaster* Dor protein, which shares homology to ScVps18p (Shestopal *et al*, 1997). In addition to developmental defects the *dor* mutation results in a deep orange eye colour, and is thought to play a role in intracellular vesicular transport (Shestopal *et al*, 1997). The proposed zinc finger domain of DOR incorporates 3 histidines in a $CX_2CX_{13}CXHX_2HX_2CX_2H$ motif, which is entirely conserved in ScVps18p excepting the last histidine residue. Analysis of conserved residues between ScVps18p, CaVps18p, and the *D. melanogaster* DOR protein suggests that a different pattern of cysteines and histidines may be important. The *CaVPS18* product has a series of cysteines and histidines near its C terminus ($CX_2CX_{14}CXHX_2HX_2C$), which aligns to similar sequences in ScVps18p ($CX_2CX_{13}CXHX_2HX_2C$), and DOR ($CX_2CX_{13}CXHX_2HX_2C$) (figure 5.4D). Thus, on the basis of conserved residues between these homologues, it would appear that such a C-terminal zinc-binding domain may have a different structure to that previously proposed. A similar cysteine/ histidine rich motif has been identified at the C-terminal of the Vps11p homologues discussed above ($CX_2CX_{12}CXHX_2HX_2C$). This could suggest that such a motif forms an important module within a general membrane fusion machinery.

It was considered that within the three *CaVPS18* clones isolated, different alleles may be present, and there may be differences between the chromosomal copies of *CaVPS18*. In order to investigate if there were any significant differences between the three clones, a region within the ORF of 399bp was sequenced for all three *CaVPS18* clones. The two primers designed for the amplification of the original *CaVPS18* probe (CAPEP3-1 and CAPEP3-2) were used to sequence complementary strands in this region. Two of the clones, pGP182 and pGP186, had identical sequences. The third, pGP184, contained 8 mismatches to the other two over the 399 nt interval (figure 5.4E). This could be because one chromosomal copy is present on pGP182 and pGP186, and the other chromosomal copy on pGP184, in which case there would appear to be a relatively high degree of heterogeneity between the two chromosomes at this locus. The amino acid sequence encoded by each of these two *CaVPS18* copies differ at just one residue of the 132 that

were available for comparison (amino acid 710 is a isoleucine in pGP184 and a valine in pGP182/186). The one observed amino acid difference involves amino acids which are both hydrophobic and share a similar hydrocarbon side chain. It is therefore not clear if both isoforms are functional. However, there is at least one amino acid difference in the encoded proteins, and there may be further differences between the two *CaVps18p* isoforms. It is possible that only a single functional copy of *CaVPS18* is necessary for viability in *C. albicans*.

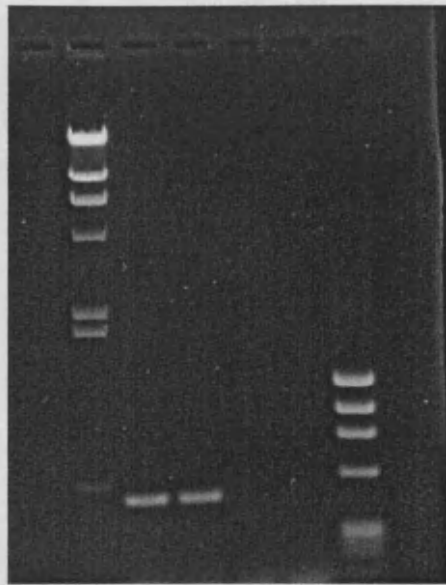
5.5 *CaVPS18* does not rescue the *Saccharomyces cerevisiae vps18* mutant

All three *CaVPS18* clones isolated in the multicopy vector YEp213, were transformed into the *S. cerevisiae vps18::Tn1000* mutant strain (JMΔ18). Transformants were then tested for growth on selective SD medium at 37°C and growth on SD media enriched with 400 μM copper sulphate (28°C), with positive (S150-2B + YEp213) and negative (*vps18::Tn1000* + YEp213) controls. No rescuing activity was observed for either *vps16* phenotype, with any of the clones. This explains why the original complementation approach (Chapter 4) was unsuccessful, i.e. the *C. albicans* class-C homologues do not function in *S. cerevisiae*. In *C. albicans* CTG is a rare codon which codes for serine instead of leucine, defined by the 'universal' genetic code. *CaVPS11* codes for seven CTG codons and therefore would be expected to have seven amino acid changes (serine to leucine) when heterologously expressed in *S. cerevisiae*. *CaVPS18* has eight such codons, which would lead to mistranslation. This is a significant number of substitutions when one considers that any one of them alone could result in a total loss of function. Mis-translation may account for the inability of *CaVPS18* to complement *S. cerevisiae vps18* mutants.

5.6 *CaVPS11* and *CaVPS18* are expressed in log phase *Candida albicans*

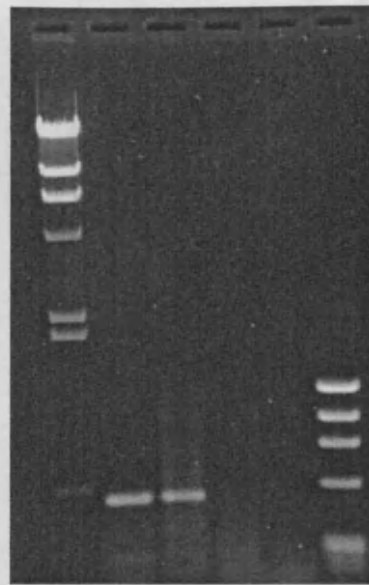
RNA samples prepared from *C. albicans* cells grown to log phase (2×10^7 cells/ ml) in YPD, were analysed for the presence of *CaVPS11* and *CaVPS18* transcripts using an RT-PCR approach. Pairs of primers (figure 5.5) internal to each ORF were used to prime the synthesis of a complementary cDNA strand, and subsequent amplification of the defined cDNA fragments. Negative controls were set-up with the omission of the reverse transcriptase (RT). This was a control for the detection of any of contaminating gDNA, which could serve as a template for synthesis of the extension product. Samples of each reaction were subject to agarose gel electrophoresis. Extension products of the expected

Lane 1 2 3 4 5 6



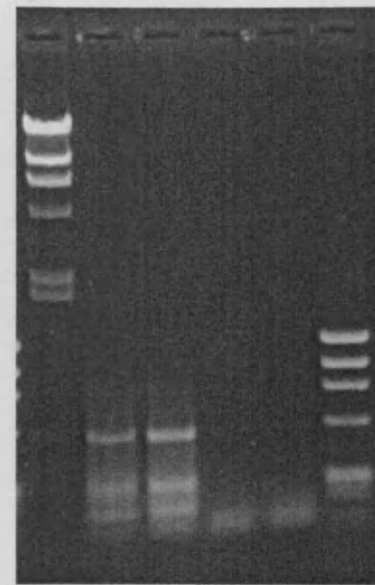
A.

1 2 3 4 5 6



B.

1 2 3 4 5 6



C.

Figure 5.5. *CaVPS11*, *CaVPS18* and *CaVPS33* are expressed in log phase *Candida albicans* cells. Primers designed to *CaVPS11*, *CaVPS18* and *CaVPS33* ORF's were used for RT-PCR, using a DNase treated RNA template from log phase *C. albicans* cells, grown in YPD. The predicted extension products (*CaVPS11*=508, *CaVPS18*=509 and *CaVPS33*=584nt), were present, confirming transcription of each of these genes. The control reaction for each was the omission of the reverse transcriptase (RT). Thus if contaminating DNA is acting as a template for amplification of these products then it should occur in the absence of RT. (A) RT-PCR using *CaVPS11*, (B) *CaVPS18*, (C) *CaVPS33* specific primer sets: *CaVPS11*= CAENDC10-1, CAENDC10-2; *CaVPS18*=CAPEP3-1, CAPEP3-2; *CaVPS33*= CAVPS33-1, CAVPS33-3. Lanes for A,B and C (left to right): 1= λ_{HindIII} ; 2=S01 RNA template; 3=SC5314 RNA template; 4=S01 RNA -RT control; 5=SC5314 RNA -RT; 6= $\phi\text{X174}_{\text{HaeIII}}$. Each experiment was repeated at least twice to confirm reproducibility.

sizes (*CaVPS11*= 509 nt; *CaVPS18*= 508 nt) were observed with primer sets to either gene, when RNA prepared from either S01 or SC5314 was used as a template (figure 5.5A and B). The extension product was dependent on the presence of RT, as these fragments were not amplified in its absence, and must therefore be derived from RNA via a cDNA intermediate. These experiments were repeated with identical results using RNA samples prepared on separate occasions, and in the case of *CaVPS18* using an alternative primer set, to confirm reproducibility. These results demonstrate that both *CaVPS11* and *CaVPS18* are expressed in *C. albicans* cells grown to log phase.

Similar experiments were performed on the same RNA samples using a primer set (CAVPS33-1 and CAVPS33-3) specific to the *CaVPS33* sequences identified from the database (section 5.2). The expected 584 nt fragment was observed with both S01 and SC5314 RNA templates, but only in the presence of the RT (figure 5.5C). This confirmed that the *C. albicans VPS33* like ORF is also expressed.

The pattern of codon usage for *CaVPS11* and *CaVPS18* was compiled using the CODON FREQUENCY programme in the GCG computing package. These patterns were compared to that of three genes known to be highly expressed in *C. albicans*, *ENO1* (Franklyn and Warmington, 1993), *HSP70* (La Valle *et al.*, 1995) and *TEF3* (Di Domenico *et al.*, 1992) using CORRESPOND (GCG). This revealed that there was a greater difference between the codon usage pattern of these *VPS* genes and the group of highly expressed genes, than there was within the group of highly expressed genes (data not shown). This could indicate that *CaVPS11* and *CaVPS18* have a higher proportion of codons with less abundant tRNA species, and may indicate a lower level of expression. This is consistent with the low level of expression observed for the *S. cerevisiae* class-C gene products (Chapter 1).

5.7 *CaVPS18* transcription increases during hyphal formation

The vacuole of *C. albicans* has been observed to expand dramatically within the parent cell during germ-tube outgrowth (Gow and Gooday, 1984). Each sub-apical compartment is also extensively vacuolated for some time before cytoplasm is regenerated. This expansion in vacuolar volume is likely to be fuelled by a net gain in membrane material from the fusion of transport vesicles at the vacuolar membrane. The class-C *VPS* gene products of *S. cerevisiae* play a critical role in the fusion of transport vesicles to the vacuolar

membrane and moreover vacuole biogenesis. If the *C. albicans VPS18* homologue described here is involved in vacuolar membrane fusion or expansion during germ-tube outgrowth, it might be expected to be up-regulated during this process. Expression of *CaVPS18* was therefore analysed through the yeast-hyphae transition.

SC5314 was grown to saturation in YPD media overnight at 30°C. Aliquots (2.5 ml) of this culture were then sub-cultured into non-inducing medium (YPD) and grown at 30°C, or inducing medium (YPD + 10% fetal calf serum) and grown at 37°C. RNA was prepared from the original overnight culture (0 minutes), and from both the inducing and non-inducing cultures at 30, 60 and 180 minutes after sub-culturing. Cells taken at each of these time points were also scored by eye to determine the proportion exhibiting a germ-tube, or hyphal growth. Samples of 30 µg of RNA were size fractionated by agarose gel electrophoresis, and transferred to a Hybond N+ nylon membrane by Northern blotting. The RNA was UV cross-linked to the membrane, and hybridised to a ³²PαdCTP labelled *CaVPS18* specific probe. The probe was a 747 nt fragment internal to the *CaVPS18* ORF, and was amplified from a SC5314 genomic DNA template using 18P5 and 18P3 primers. As a loading control a second probe specific to the housekeeping gene *CaURA3* was ³²PαdCTP labelled and hybridised to the membrane. Stringency washes were performed before the membrane was subject to autoradiography (figure 5.7) and phosphoimager analysis (table 5.2).

A 2.6 kb transcript was detected, consistent with the size of the *CaVPS18* ORF (2430 nt). A second transcript of approximately 1.2 kb was also detected corresponding to the *CaURA3* mRNA. The *CaURA3* loading control used here was not expressed at 0 minutes (figure 5.7), this is most likely because these cells are at or approaching stationary phase and therefore not actively growing. However, the *CaVPS18* transcript was detected even at 0 minutes, indicating that this mRNA is either very stable or actively transcribed in stationary phase cells. The quantity of *CaVPS18* RNA (as detected by phosphoimager analysis) was divided by the quantity of *CaURA3* RNA, in order to standardise data for comparison (table 5.2). This revealed that there was a 3.3 fold increase in the amount of *CaVPS18* mRNA, after 30 minutes under inducing compared to non-inducing conditions. At 60 minutes there was a 2.7 fold difference, and by 180 minutes the *CaVPS18* transcript was 5.8 times more abundant under inducing than non-inducing conditions. Cells

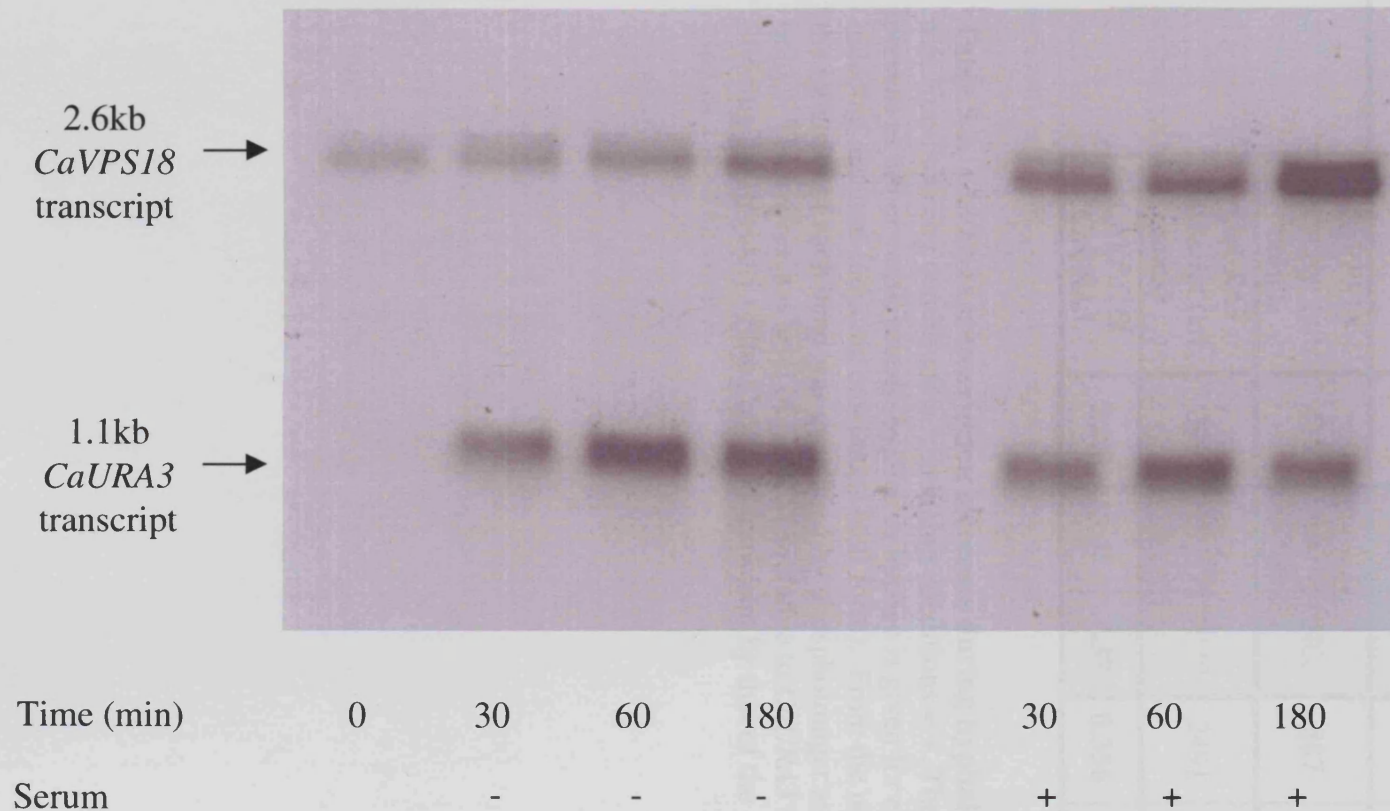


Figure 5.6. *CaVPS18* expression is up-regulated through the yeast to hyphae transition in *Candida albicans*. SC5314 cells were grown in YPD at 30°C to saturation overnight. Cells were then subbed back in 50 ml of either hyphae inducing (YPD + 10% FCS) media and grown at 37°C, or non-inducing (YPD) media and grown at 30°C. Cells were taken at 0, 30, 60 and 180 minutes from either culture, and RNA prepared. RNA samples were size fractionated by gel electrophoresis, transferred to a nylon membrane by northern blotting, and then probed with a ^{32}P -dCTP labelled *CaVPS18* specific probe. Each transcript was then detected by autoradiography. A *CaURA3* specific probe was also hybridised to act as a loading control, however it seems that this gene was expressed to a much lesser extent in stationary phase cells (t = 0 min).

Time (min)		0	30		60		180	
Inducing conditions		-	-	+	-	+	-	+
Cell Form (%)	Yeast	100	94	2.5	86.5	1.5	56	0
	Pseudo-hyphae	0	4	0	11.5	0	44	0
	Hyphae	0	2	97.5	2	98.5	0	100
<i>CaVPS18</i> transcript (arb units)		415	626	1406	887	1456	1174	3146
<i>CaURA3</i> transcript (arb units)		204	1501	1029	2491	1539	2324	1082
<i>CaVPS18</i> / <i>CaURA3</i>		2.03	0.41	1.37	0.356	0.946	0.505	2.91

Table 5.2. *CaVPS18* transcription increases during hyphal formation. From figure 5.6. Non-inducing conditions = -; inducing conditions = +. The percentage of cells present as either yeast, pseudo-hyphae or hyphae is given for each time point in both inducing and non-inducing conditions (cell form). From the northern blot (figure 5.6) the intensity of each band was quantified by phosphoimager analysis (given as arbitrary units). The expression level of *CaVPS18* relative to *CaURA3* was then calculated by dividing the intensity of the *CaVPS18* transcript by that of the *CaURA3*, which was used as a loading control.

Figure 5.7. Induction of *Candida albicans* germ tubes in serum containing media. From figure 5.6, and table 5.2. *C. albicans* strain SC5314 was grown overnight in YPD at 30°C and then 2.5ml sub-cultured into 50ml either fresh YPD medium and grown at 30°C (non-inducing conditions), or YPD + 10% FCS and grown at 37°C (inducing conditions). (a) Cells photographed before sub-culturing (0 min). (b), (d), and (f) are cells from the non-inducing culture 30, 60 and 180 minutes respectively after sub-culturing. (c), (e) and (g) are cells from the inducing culture taken at same time points.



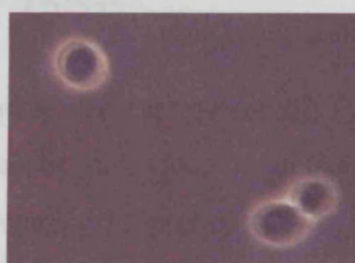
(f).



(b)



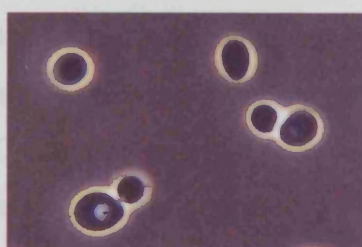
(d).

^e

(b).



©



(a.)

representative of each time point are shown in figure 5.8, and the proportion of cells exhibiting hyphal growth in table 5.2. The serum conditions used induced hyphae with nearly 100% efficiency, however some elongated forms appeared at a lower frequency under the non-inducing conditions. These forms were distinct from those found under inducing conditions as they were much shorter, and were thicker than the hyphal forms observed under inducing conditions. They also possessed constrictions at regular intervals along their length, appearing as a chain of elongated ellipsoid cells. These features are characteristic of pseudo-hyphae, and made up a significant portion of the cells observed under non-inducing conditions; 2, 13.5 and 44% at 30, 60 and 180 minutes, respectively.

5.8 Discussion

Sequence data encoding for putative class-C *VPS* homologues was identified on the *C. albicans* database. Although the complete sequence of the ORFs were not yet available, primers were designed for the amplification of these DNA fragments, and the products used to probe a *C. albicans* genomic DNA library. The probes allowed identification of one positive clone for *CaVPS11*, and three positives for *CaVPS18*. No positives were identified using the *VPS33*-like probe (C33), or the *CaVPS11-2* (C11-2) probe. This could be due to the C11-2 and C33 regions being under-represented in the library used. Sequence data from the *CaVPS11* positive clone, pGP11, revealed that the ORF was truncated in the 5' sequences.

Sequence comparisons of CaVps11p with ScVps11p and CaVps11p with ScVps11p revealed that there is conservation of at least some of the cysteines and histidines, previously proposed to form zinc-finger like domains at the C-terminus (Preston *et al.*, 1991). This motif has been suggested to have a role in protein-protein interactions. However, to date, only one of these cysteines (C₈₂₆) has been shown to be required for the function of ScVps18p, through site directed mutagenesis (Preston *et al.*, 1991). Here, alternative cysteine-rich structures have been proposed, on the basis of their conservation between the *S. cerevisiae* and *C. albicans* putative proteins, and in the case of Vps18p, the identification of a similar cysteine rich motif in the *D. melanogaster* DOR protein. The suggested Vps11p (CX₂CX₁₂CXHX₂HX₂C) and Vps18p (CX₂CX₁₃₋₁₄CXHX₂HX₂C) conserved motifs share a similar structure. In *S. cerevisiae* the two proteins are known to physically interact to mediate the docking and/or fusion of transport vesicles to the

vacuolar membrane (Rieder and Emr, 1997). The conservation of this cysteine/ histidine rich region in these proteins, may point to it forming an important 'module' in a general fusion machinery.

A large degree of heterogeneity between the two chromosomal copies of *CaVPS18* was observed over a small region. Heterogeneity has also been observed at the *C. albicans* *ERG16* (also known as *ERG11*) loci. Two nucleotide changes within the 1587 nucleotide ORF of *ERG16*, occurred between alleles in a single clinical isolate of *C. albicans* (White 1997). Neither resulted in a change in amino acid sequence. This is compared to 8 changes within 399 bp of the *CaVPS18* ORF observed between the alleles in strain S01. From this data it would appear that the *CaVPS18* loci described here have much greater allelic differences than the previously described *ERG16* gene. In addition one of the nucleotide changes results in an amino acid substitution - residue 710 was either an isoleucine or valine, both hydrophobic amino acids with hydrocarbon side chains. This conserved substitution may not significantly affect the function of this protein, however, only a short stretch of sequence data was analysed for each chromosomal copy of *CaVPS18*; thus there is likely to be many more nucleotide, and possibly additional amino acid differences, between the two chromosomal copies of *CaVPS18*. As such it is not clear if both alleles will be functional.

The inability of the *CaVPS18* positive clones to rescue the *S. cerevisiae* *vps18* mutant, still leaves the question of whether these two genes are true functional homologues. It is perhaps not surprising that *CaVPS18* does not rescue the *vps18* mutant. Even if the gene is heterologously expressed in *S. cerevisiae*, the 'non-universal' genetic code of *C. albicans* would result in 8 non-conserved amino acid substitutions in the heterologous protein. Any one of these changes could result in inactivation of this protein's function. Thus in order to assess whether *CaVPS11* or *CaVPS18* are true functional homologues of *S. cerevisiae* *VPS11* and *VPS18* requires the phenotypic analysis of *C. albicans* strains disrupted in these genes.

That the *CaVPS11* and *CaVPS18* genes are actively transcribed in logarithmically growing *C. albicans*, was confirmed through RT-PCR experiments. The level of expression is predicted to be low due to significant differences in the pattern of codon usage between

these genes and highly expressed *C. albicans* genes. This is similar to the situation in *S. cerevisiae*, where all four class-C gene products are expressed at a low level.

It is not known if the *S. cerevisiae* class-C *VPS* genes are regulated in response to developmental processes. However the *CaVPS18* transcript was more abundant during the process of hyphal formation, and this is consistent with a role for CaVps18p in the rapid vacuolar expansion observed during germ-tube outgrowth.

Chapter 6

Targeting *CaVPS18* for disruption

6.1 Introduction

The *CaVPS18* gene was isolated on the basis of sequence similarity to the *S. cerevisiae* *VPS18* (Chapter 5). It was found not to complement the defects of the *S. cerevisiae* *vps18* mutant strain. Therefore, except from being transcribed (Chapter 5), no information was available about *CaVPS18* function. One method of gaining an insight into the function of *CaVPS18*, is through the construction of a *C. albicans* strain deleted for *CaVPS18*. Since *C. albicans* is an obligate diploid, lacks a recognised sexual cycle, and there are a limited number of auxotrophic marker genes available, genetic manipulation of this organism is problematic. However, a range of techniques have emerged to overcome these difficulties. One approach to gene disruption in *C. albicans* utilises the URA-blasting system, in which the *URA3* marker gene can be recycled multiple times.

6.2 The URA blasting method

The 'URA-blasting' technique developed by Fonzi and Irwin (1993), utilises an auxotrophic *C. albicans* strain, CAI4, which is deleted for its *URA3* alleles. The 'URA blasting' cassette consists of the wild-type *C. albicans* *URA3* gene, which is the selectable marker, flanked on either side by two direct repeats of the *S. typhimurium* *hisG* gene sequence. This cassette is inserted between two sequences flanking the gene of interest, which target the construct to the desired chromosomal locus. The *hisG* sequences provide homologous regions, to allow subsequent excision of the *URA3* marker gene, through homologous recombination between these sequences. Such *ura⁻* recombinants are selected by growing the *URA⁺* transformants in the presence of the 5-FOA (5'-fluororotic acid) (Boeke *et al.*, 1984). 5-FOA is an analog of orotic acid, an intermediate in the pyrimidine biosynthetic pathway; its metabolism and entry into the pyrimidine biosynthetic pathway is dependent on the *URA3* gene product (orotidine-5' phosphate decarboxylase), which converts it to 5' fluorodeoxyuridylate. This compound permanently inhibits thymidylate synthase, resulting in pyrimidine biosynthesis defects, and halting cell growth. This only occurs in *URA3⁺* cells and thus provides a selection for *ura3⁻* cells, which have lost the *URA3* gene. *ura3⁻* cells generated through homologous recombination at the *hisG* sequences, result in one copy of *hisG* remaining in the target chromosome sequence (figure

6.1). The region of the ORF of interest that is deleted through this process depends upon the homologous sequences selected for targeting of the cassette, but in addition to the deletion of some/ all of the target ORF, any remaining sequences are disrupted by the integrated *hisG* sequence (figure 6.1). Thus after this ‘first round’ of transformation, *URA*⁺ selection, and 5’FOA counter selection, a strain deleted for one of its chromosomal copies of the target gene is created, and this strain is once more *ura3*⁻. Recycling of the *URA3* marker allows it to then be used for the ‘second round’ transformation, to disrupt the second chromosomal copy of the target gene. In this way the *URA3* gene can be recycled for a number of ‘rounds’ of gene disruption. For the disruption of *CaVPS18* it was proposed to leave the *URA3* gene in after the second round of disruption. The resulting *cavps18:hisG/cavps18:hisG-URA3-hisG; ura3⁻/ura3⁻* strain could then be compared to CAF2, a SC5314 derived strain which only has one functional copy of *URA3*, in phenotypic analysis.

6.3 Targeting *CaVPS18* for disruption

The *CaVPS18* sequence described in Chapter 5 was from the strain S01. Comparison of this sequence to that available for strain SC5314 (<http://alces.med.umn.edu/candida.html>), revealed that there were a significant number of base substitutions between the sequences of these strains, some of which resulted in changes in restriction sites available for the required cloning steps. This is consistent with previous findings of significant differences in the sequence of a particular gene between strains of *C. albicans* (White, 1997; De Backer *et al.*, 2000). Furthermore, Saporito-Irwin *et al.* (1995) found sufficient variation between the sequence of *PHR1* in different strains to significantly reduce homologous integration of a disruption cassette (De Backer *et al.*, 2000). The CAI4 *ura*⁻ strain used for the *URA* blasting technique, is derived from SC5314. It was thus decided that a disruption cassette would be constructed based on *CaVPS18* sequence of SC5314, rather than our lab strain S01. Fortunately the sequence data of the full *CaVPS18* ORF, and some flanking regions, was now available on the database for SC5314. Figure 6.2 is a schematic diagram of how the *CaVPS18* disruption cassette was constructed. An *SphI* site was identified 584 nts upstream of the *CaVPS18* ATG start and a primer (CAP3-1, figure 6.2A) spanning this site was designed. A second primer (CAP3-2, figure 6.2A) was designed in the opposite orientation, 29 nt upstream of the ATG start codon, and with a *SalI* site engineered into its sequence. This primer pair was used to amplify a 555 bp region upstream of the *CaVPS18*

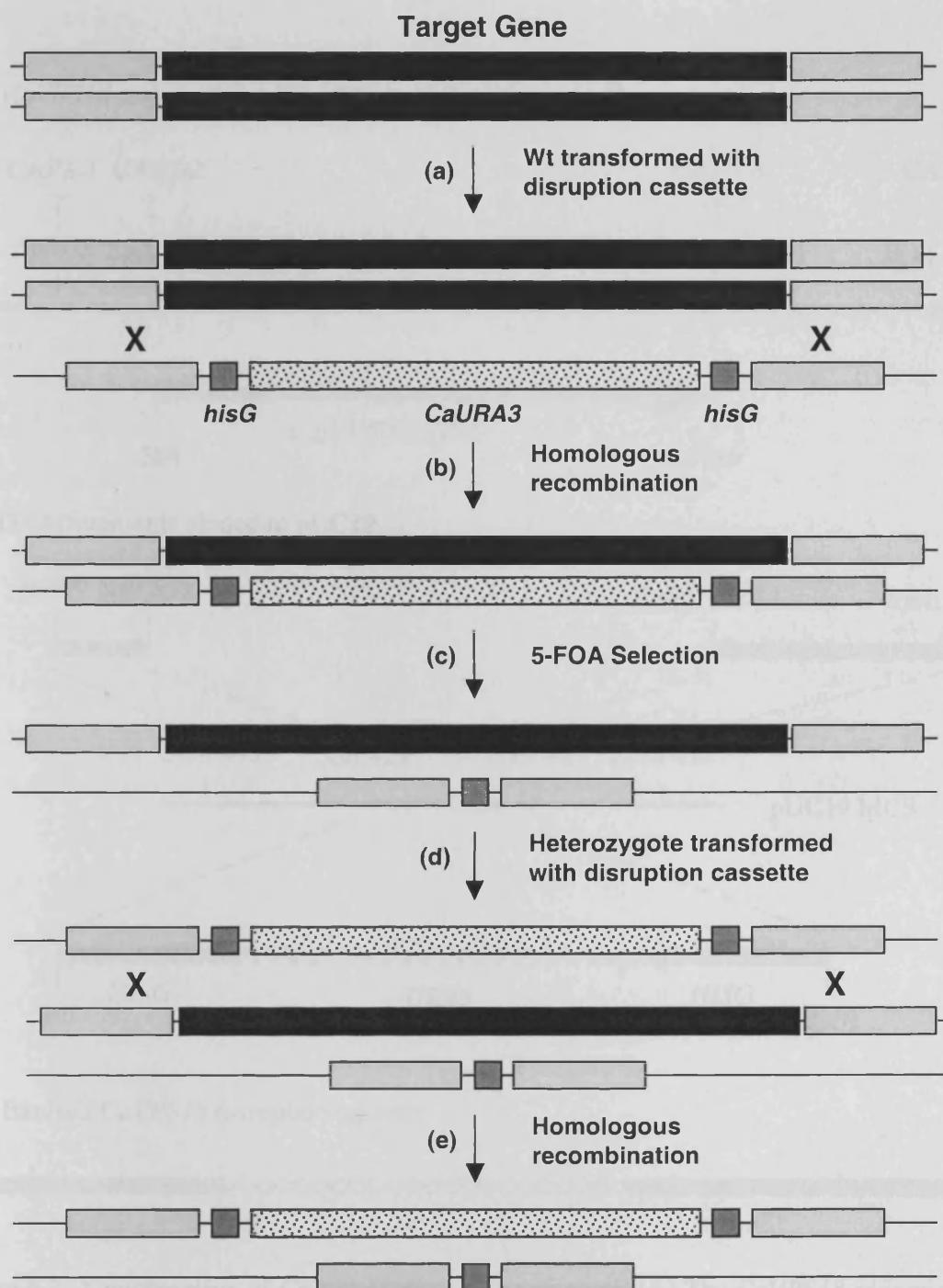
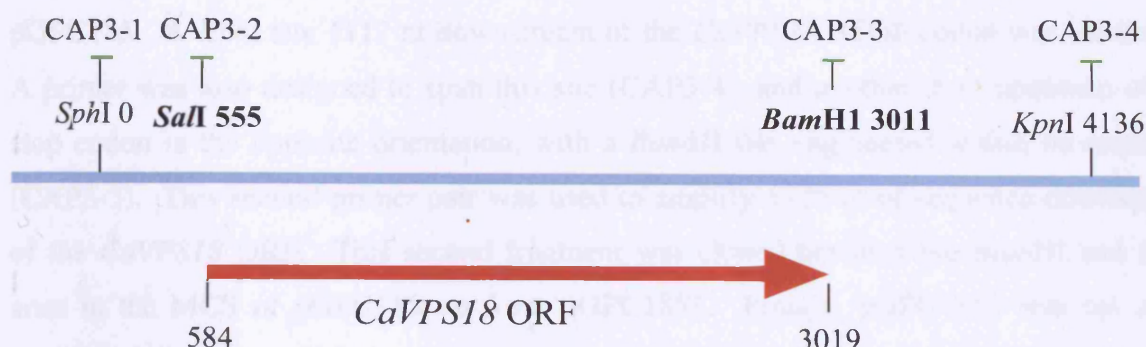
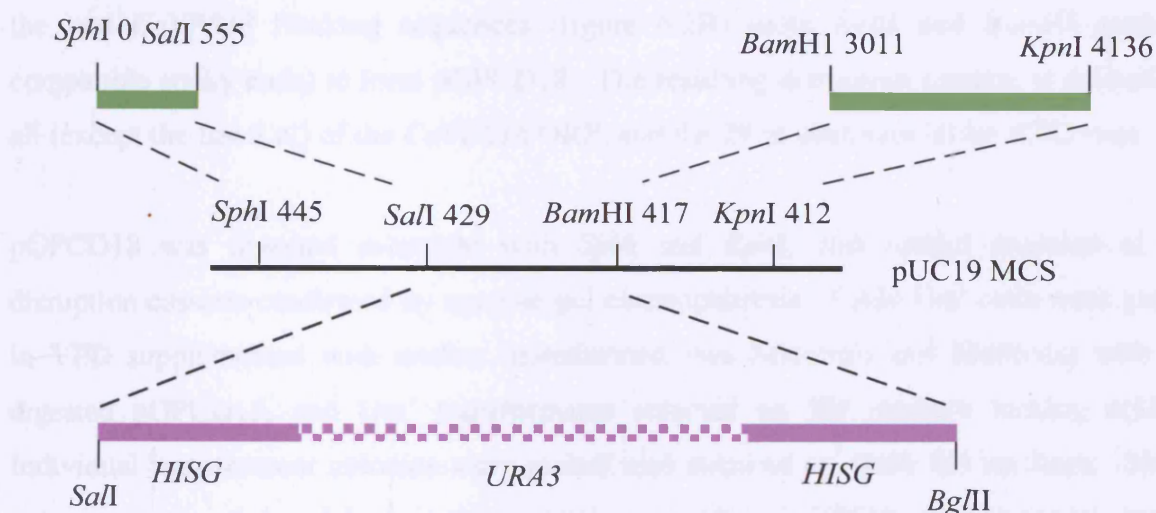


Figure 6.1. Targeted gene disruption using the 'Ura-blasting' method. (a) *C. albicans* is transformed with with a gene disruption cassette consisting of a target gene disrupted by a *hisG*-*CaURA3*-*hisG* cassette. (b) The integration of the cassette by homologous recombination results in the deletion of one chromosomal copy of the target gene. (c) Selection of the heterozygote on 5-FOA results in the loss of *CaURA3* by recombination between the *hisG* sequences. (d) The heterozygote can then be transformed with the same disruption cassette. (e) Homologous recombination may then result in the replacement of the second wild type allele.

A. *CaVPS18* region of *C. albicans* (strain S01).



B. DNA fragments cloned to pUC19



C. Excised *CaVPS18* disruption cassette



Figure 6.2. Construction of *CaVPS18* disruption cassette. (A) The *CaVPS18* genomic region, as determined from the genome sequencing project (strain SC5314) (contig 2, appendix C). The red arrow represents the *CaVPS18* ORF. Primer pairs were designed to amplify sequences flanking the ORF on either side (green lines). The outer primers, CAP3-1 and CAP3-4 spanned *Sph*I and *Kpn*I sites within the 5' and 3' flanking regions respectively. The inner primers, CAP3-2 and CAP3-3 had *Sal*I and *Bam*HI sites engineered (shown in bold type) into their sequences. (B) Each primer pair was used to amplify a *CaVPS18* flanking region from SC5314 genomic DNA (green blocks). The PCR products were then digested with appropriate restriction enzymes and each fragment cloned in turn to the multiple cloning site (MCS) of pUC19. The resulting plasmid was then cut between the two *CaVPS18* flanking sequences (at *Sal*I and *Bam*HI sites), and the 'URA blasting' cassette (purple) isolated from p5921 with *Sal*I and *Bgl*III (which gives *Bam*HI compatible ends), cloned in to give pGPCD18. (C) The *CaVPS18* disruption cassette was then excised from pUC19 using *Sph*I and *Kpn*I, and transformed into the CAI4 Ura⁻ recipient strain.

ORF, using SC5314 genomic DNA as a template, with a proof reading polymerase (figure 6.2B). The PCR product was then cut at the *Sph*I and *Sal*I sites at either end, and cloned between the *Sph*I and *Sal*I sites in the multiple cloning site (MCS) of pUC19 to form pGPC185. A *Kpn*I site 1117 nt downstream of the *CaVPS18* STOP codon was identified. A primer was also designed to span this site (CAP3-4), and another, 8 nt upstream of the stop codon in the opposite orientation, with a *Bam*HI site engineered within its sequence (CAP3-3). This second primer pair was used to amplify 1125 nt of sequence downstream of the *CaVPS18* ORF. This second fragment was cloned between the *Bam*HI and *Kpn*I sites in the MCS of pGPC185, to form pGPC1853. Finally, pGPC1853 was cut at its *Bam*HI and *Sal*I sites (between the two cloned *CaVPS18* flanking sequences) and the 3938 bp *Bgl*III-*Sal*I p5921 fragment, containing the URA blasting cassette was ligated between the two *CaVPS18* flanking sequences (figure 6.2B) (note *Bgl*III and *Bam*HI generate compatible sticky ends) to form pGPCD18. The resulting disruption cassette is deleted for all (except the last 8 nt) of the *CaVPS18* ORF, and the 29 nt upstream of the ATG start.

pGPCD18 was digested overnight with *Sph*I and *Kpn*I, and correct excision of the disruption cassette confirmed by agarose gel electrophoresis. CAI4 Ura⁻ cells were grown in YPD supplemented with uridine, transformed (see Materials and Methods) with the digested pGPCD18, and Ura⁺ transformants selected on SD medium lacking uridine. Individual transformant colonies were picked and streaked on fresh SD medium. Single colonies were then picked and integration at the *CaVPS18* chromosomal region investigated by either colony PCR or southern blotting.

6.4 URA-blasting cassette is not targeted to *CaVPS18*

A colony PCR approach was used to confirm the presence of the disruption cassette in the Ura⁺ transformants. The correct localisation of the cassette to the *CaVPS18* loci was then examined by Southern blot hybridisation. A crude cell lysate was prepared as a template for colony PCR, and each reaction contained three primers: CAD1 which binds within the *CaVPS18* ORF 332 nt upstream of the stop codon (not present in the disruption cassette), and primes DNA synthesis into the 3' region of *CaVPS18*; CAD2 which binds to the complementary strand in the region 3' 471 nt downstream of the stop codon. The expected PCR product for the parental *CaVPS18* locus, primed by CAD1 and CAD2, is 803 nt (figure 6.3), and provides a positive control for the PCR reaction; the third primer, GHISG,

binds specifically to the *hisG* sequences in the URA blasting cassette. The expected PCR product derived from the GHISG, CAD2 primer pair, is 527 bp (figure 6.3), and should be synthesised in the presence of the disruption cassette. The parental CAI4 strain, with two parental *CaVPS18* loci would thus be expected to produce a single PCR product of 803 bp. However, if the disruption cassette has integrated within the genome, the resulting strain should produce two products of 803 and 527 bp. A total of 105 CAI4 Ura⁺ transformants were analysed by colony PCR, all were found to contain a single PCR product of approximately 800 bp, identical to the parental CAI4 strain (figure 6.4).

The *CaVPS18* loci of 24 of the Ura⁺ transformants was also examined by Southern blot hybridisation. Genomic DNA was prepared from each transformant, digested with *EcoRI*, the products separated on a 1% agarose gel, and transferred to Hybond-N⁺ (Amersham Pharmacia Biotech) nylon membrane by Southern blot transfer. A 528 bp probe fragment was amplified from DNA 3' to *CaVPS18*, using CAI4 genomic DNA template and the primer pair GP1817 and GP1819 (figure 6.3). The probe fragment was labelled with ³²PαdCTP and hybridised to the nylon membrane. Figure 6.3 shows the position of *EcoRI* sites at the parental *CaVPS18* locus (for strain SC5314) and on the disruption cassette. From this information we would expect the probe to detect a 2537 bp restriction fragment for the parental locus, and a 3486 bp fragment for a disrupted *CaVPS18* allele. Of the 24 transformants analysed, a single fragment of approximately 2.5 kb was detected for each, the same as for the CAI4 parental control (figure 6.5). This indicates that the disruption cassette has integrated at some site other than *CaVPS18* within the genome. Thus the *CaVPS18* disruption cassette constructed here appears not to target the *CaVPS18* locus in CAI4 by homologous recombination.

6.5 *Candida albicans* may possess multiple copies of *CaVPS18*

Since the work to disrupt the *CaVPS18* locus began, there have been significant advances in the amount of *C. albicans* sequence data available in the genome database. The disruption cassette was designed based on the sequence of a single *CaVPS18* spanning contig (referred to here as Contig 2) which was available on the database at the time. However, since then another three *CaVPS18* spanning contigs have become available on the database. All four of these contigs are distinct in sequence, both within the *CaVPS18* ORF, and flanking sequences. These four copies could represent two alleles present at two

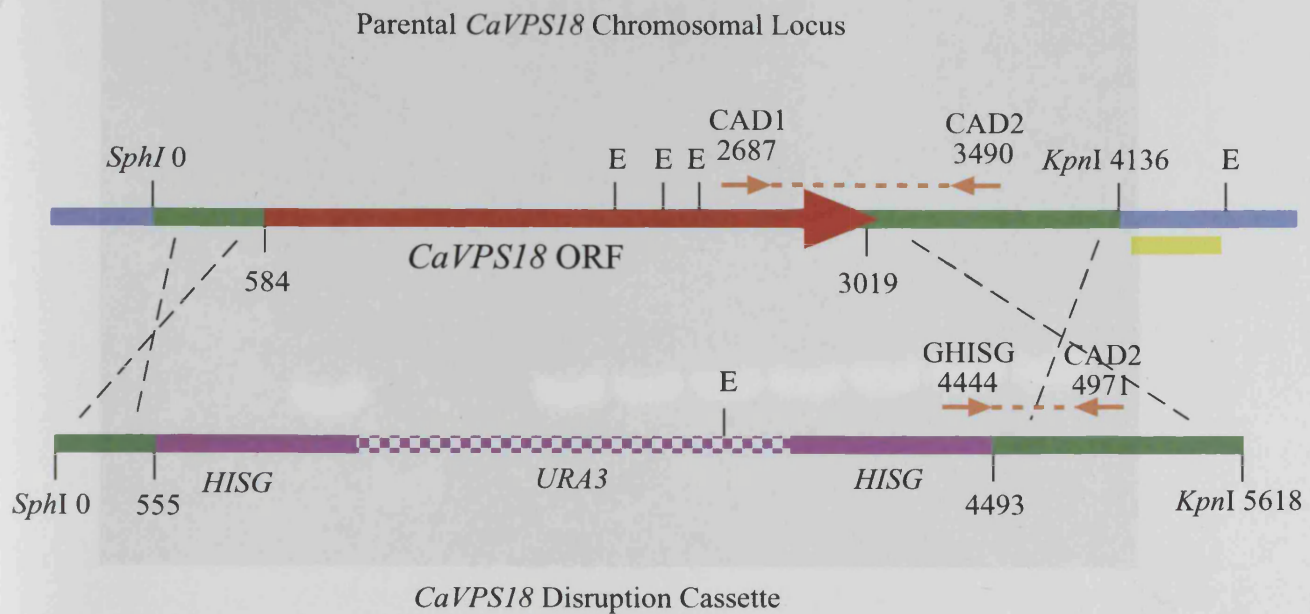


Figure 6.3. Strategies for detecting integration of the 'URA blasting' cassette. Both colony PCR and southern blot hybridisation approaches were used in order to detect disruption of *CaVPS18* with the 'URA blasting' cassette. The parental *CaVPS18* locus is shown (top), with the disruption cassette below. The ORF is shown as the red arrow, homologous flanking sequences in green, non-homologous flanking sequences in blue, and the 'URA blasting' cassette in purple. The three primers CAD1, CAD2 and GHISG, used in colony PCR are shown as orange arrows, and the expected PCR products as orange dotted lines. The expected parental product was 803nt long, the cassette primed product is expected to be 527nt. Southern blots were performed on CAI4 Ura⁺ transformants genomic DNA, digested with *Eco*RI. The DNA fragment used as a probe is shown as the yellow line. The parental or band size expected is 2537nt, the disruptant band is expected to be 3486nt. E= *Eco*RI sites.

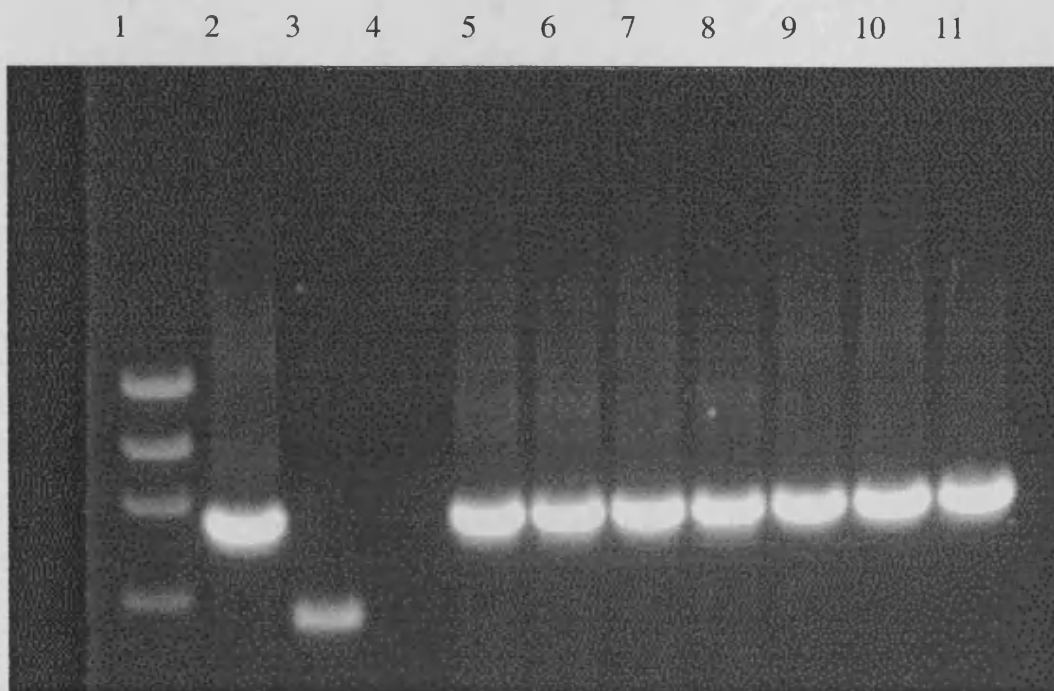


Figure 6.4. Colony PCR at the *CaVPS18* locus of CAI4 Ura⁺ transformants.

Single Ura⁺ transformant colonies were picked and treated with zymolyase. The crude lysate was then used as template in PCR reactions with a diagnostic primer set spanning the *CaVPS18* locus (CAD1, CAD2 and GHISG; figure 6.3). CAI4 lysate was used as a control template to represent the parental bands, and pGPCD18 plasmid DNA to represent the expected cassette band. However, out of 105 transformants screened by this method, all lacked the 527nt band that should have been primed by the intact disruption cassette. Lanes: 1= ϕ X174/*Hae*III marker DNA; 2=CAI4 lysate; 3=10ng pGPCD18 template DNA; minus DNA control; 4-11=transformant colonies 1-7.

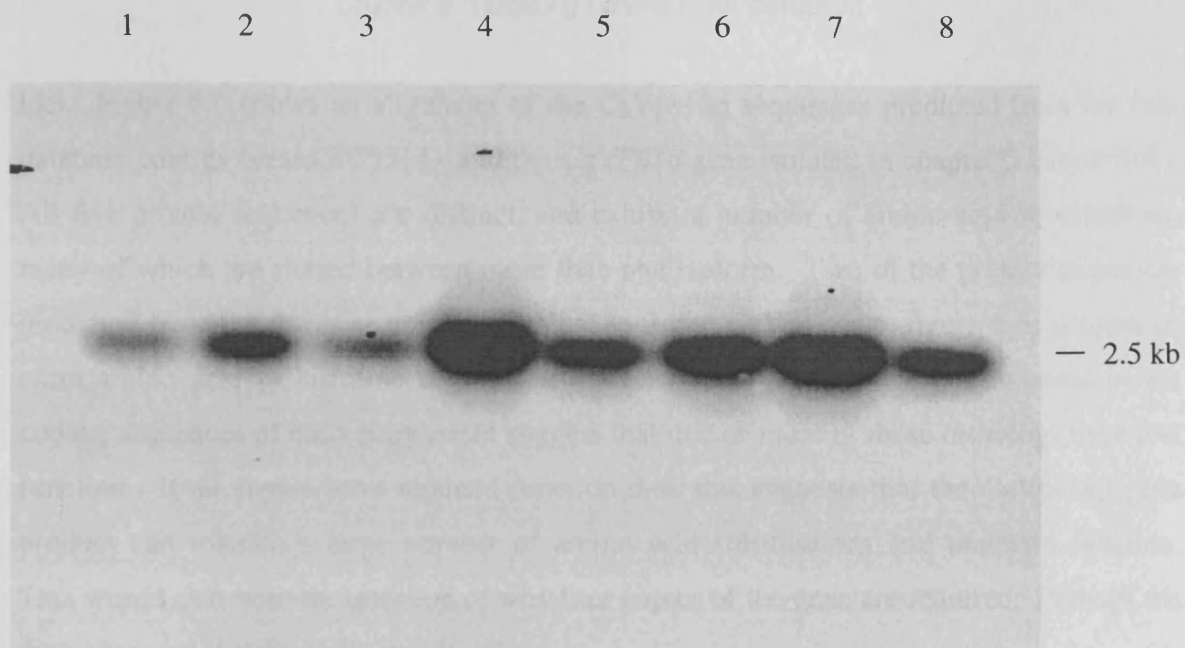


Figure 6.5. Analysis of the *CaVPS18* locus of CAI4 Ura⁺ transformants. Genomic DNA was isolated from CAI4 Ura⁺ transformants selected after transformation with the *CaVPS18* disruption cassette. This genomic DNA was digested with *Eco*RI, separated by agarose gel electrophoresis, and the resulting DNA fragments transferred to a nylon membrane by southern blot transfer. The membrane was then probed using a ³²PdCTP labelled *CaVPS18* specific probe (figure 6.3). The parental fragment predicted from the database sequence (section 6.4) is 2761bp; the fragment resulting from the correct integration of the disruption cassette at one of the chromosomal *CaVPS18* loci would be approximately 3.4-3.5kb (see figure 6.3). No difference was noted between the fragments identified for the parental strain (lanes 1 and 8), and any of 24 Ura⁺ transformants, all strains having a band of around 2.7kb. This supported the findings of previous colony PCR experiments, and suggested the disruption cassette was not integrating at the *CaVPS18* loci. Lanes: 1=CAI4 gDNA; 2-7 Ura⁺ transformants 1-6; 8=CAI4.

loci. Figure 6.6 shows an alignment of the *CaVps18p* sequences predicted from the four database contigs (strain SC5314), and the *CaVPS18* gene isolated in chapter 5 (strain S01). All five protein sequences are distinct, and exhibit a number of amino acid substitutions, many of which are shared between more than one isoform. Two of the protein sequences predicted from the database contigs (*CaVps18p*-3 and *CaVps18p*-4, figure 6.6) possess an extra amino acid (a histidine at position 690). The large number of differences in the coding sequences of each copy could suggest that one or more of these orthologs have lost function. If all copies have retained function then this suggests that the *CaVps18p* gene product can tolerate a large number of amino acid substitutions and maintain function. This would also raise the question of why four copies of the gene are required. Perhaps the four genes are differentially regulated.

The presence of multiple copies of *CaVPS18*, each of which has diverged significantly may have interfered with the construction and/ or proper integration of the disruption cassette described in this work. The primers used to amplify the *CaVPS18* flanking sequences incorporated into the cassette were designed based on just one of the database contigs. However, there is a large degree of divergence between the *CaVPS18* flanking regions in each of the four database contigs. Each flanking sequence was examined for the presence of the primer binding sites in order to determine which *CaVPS18* flanking sequences are likely to have been amplified and so incorporated into the disruption cassette. This analysis indicated that while the *CaVPS18* 5' region from contig 2 would be specifically amplified and incorporated into the disruption cassette, it is possible that the 3' region from any one of the four contigs may have been cloned into the cassette. If the 3' region from contig 1, 3 or 4 was cloned then this would result in the construction of a 'hybrid' cassette. The sequence upstream of the *CaVPS18* ORF on contig 2, is unlike that from that in the other three contigs, and therefore the 5' targeting sequence of the cassette is specific for this copy. The *CaVPS18* 3' regions (corresponding to the region incorporated into the disruption cassette) in contigs 2, 3 and 4 share between 89 and 96% identity over 1125 nucleotides (using BESTFIT analysis). Thus the 'chimeric' disruption cassette may have targeting sequences with insufficient homology to promote homologous integration at any one of the *CaVPS18* loci.

It has been predicted that the probe used in the Southern hybridisation experiments to detect the *CaVPS18* fragments would detect the sequences present on at least three of the

Figure 6.6. *Candida albicans* may possess multiple and distinct copies of *CaVPS18*.

Alignment of predicted *CaVps18p* sequences, from the cloned gene from S01 (*CaVps18p*; chapter 5), and the four contigs identified on the *C. albicans* sequencing database as containing a *VPS18* like ORFs (Jan 2001) (*CaVps18p*-1-4, strain SC5314). Identity is shown by **black** boxing. Similar residues are shown by **grey** boxing. Alignments were compiled using PILEUP.

CaVps18p	1	MSSNGHKTKSIKSETNSSYTVHSLDQQQASICDNLQQAQFELEPVHLQFSLSNKLNKLLV
CaVps18p-2	1	MSSNGHKTKSIKSETNSSYTVHSLDQQQASICDNLQQAQFELEPVHLQFSLSNKLNKLLV
CaVps18p-1	1	MSSNGHKTKSIKSETNSSYTVHSLDQQQASICDNLQQAQFELEPVHLQFSLSNKLNKLLV
CaVps18p-3	1	MSSNGHKTKSIKSETNSSYTVHSLDQQQASICDNLQQAQFELEPVHLQFSLSNKLNKLLV
CaVps18p-4	1	MSSNGHKTKSIKSETNSSYTVHSLDQQQASICDNLQQAQFELEPVHLQFSLSNKLNKLLV
CaVps18p	61	SSNIMYILLANVVYKIDLGNSQVENYALPGKVTNAWLNPNQGTLIVQIEKQAYYTLEKK
CaVps18p-2	61	SSNIMYILLANVVYKIDLGNSQVENYALPGKVTNAWLNPNQGTLIVQIEKQAYYTLEKK
CaVps18p-1	61	SSNIMYILLANVVYKIDLGNSQVENYALPGKVTNAWLNPNQGTLIVQIEKQAYYTLEKK
CaVps18p-3	61	SSNIMYILLANVVYKIDLGNSQVENYALPGKVTNAWLNPNQGTLIVQIEKQAYYTLEKK
CaVps18p-4	61	SSNIMYILLANVVYKIDLGNSQVENYALPGKVTNAWLNPNQGTLIVQIEKQAYYTLEKK
CaVps18p	121	VFKLLKFKNIEVTSIAFSNHNMVVGTKDGIIVYEKSLKQVYKVDSPVQGVMFSNDYSQI
CaVps18p-2	121	VFKLLKFKNIEVTSIAFSNHNMVVGTKDGIIVYEKSLKQVYKVDSPVQGVMFSNDYSQI
CaVps18p-1	121	VFKLLKFKNIEVTSIAFSNHNMVVGTKDGIIVYEKSLKQVYKVDSPVQGVMFSNDYSQI
CaVps18p-3	121	VFKLLKFKNIEVTSIAFSNHNMVVGTKDGIIVYEKSLKQVYKVDSPVQGVMFSNDYSQI
CaVps18p-4	121	VFKLLKFKNIEVTSIAFSNHNMVVGTKDGIIVYEKSLKQVYKVDSPVQGVMFSNDYSQI
CaVps18p	181	NALANSLYTWDCFDTSYVELQKVFKQTAPVIKSINPPGIFTSPNQNVYISSNEIITND
CaVps18p-2	181	NVLANSLYTWDCFDTSYVELQKVFKQTAPVIKSINPPGIFTSPNQNVYISSNEIITND
CaVps18p-1	181	NVLANSLYTWDCFDTSYVELQKVFKQTAPVIKSINPPGIFTSPNQNVYISSNEIITND
CaVps18p-3	181	NALANSLYTWDCFDTSYVELQKVFKQTAPVIKSINPPGIFTSPNQNVYISSNEIITND
CaVps18p-4	181	NVLANSLYTWDCFDTSYVELQKVFKQTAPVIKSINPPGIFTSPNQNVYISSNEIITND
CaVps18p	241	EEMQLDRVDEEFSQIALTSHHHLIGIEGNSLKIYNKLNKQLQELSLSENKIRGIAVDNLFN
CaVps18p-2	241	EEMQLDRVDEEFSQIAETPHHLIGIEGNSLKIYNKLNKQLQELSLSENKIRGIAVDNLFN
CaVps18p-1	241	EEMQLDRVDEEFSQIAHTPHHLIGIEGNSLKIYNKLNKQLQELSLSENKIRGIAVDNLFN
CaVps18p-3	241	EEMQLDRVDEEFSQIALTSHHHLIGIEGNSLKIYNKLNKQLQELSLSENKIRGIAVDNLFN
CaVps18p-4	241	EEMQLDRVDEEFSQIALAPHHLIGIEGNSLKIYNKLNKQLQELSLSENKIRGIAVDNLFN
CaVps18p	301	TYWVYTKNSIYEFVIENESISVWYDYYKMGKYSEALKYLEDDEANFSKRDLVLIKQGYD
CaVps18p-2	301	TYWVYTKNSIYEFVIENESISVWYDYYKMGKYSEALKYLEDDEANFSKRDLVLIKQGYD
CaVps18p-1	301	TYWVYTKNSIYEFVIENESISVWYDYYKMGKYSEALKYLEDDEANFSKRDLVLIKQGYD
CaVps18p-3	301	TYWVYTKNSIYEFVIENESISVWYDYYKMGKYSEALKYLEDDEANFSKRDLVLIKQGYD
CaVps18p-4	301	TYWVYTKNSIYEFVIENESISVWYDYYKMGKYSEALKYLEDDEANFSKRDLVLIKQGYD
CaVps18p	361	YLQGGFGISSDDLSQLIQGIQILAKSTEPFEKVCLMLLNHKQSDALLIEYLLAKLNKKN
CaVps18p-2	361	YLQGGFGISSDDLSQLIQGIQILAKSTEPFEKVCLMLLNHKQSDALLIEYLLAKLNKKN
CaVps18p-1	361	YLQGGFGISSDDLSQLIQGIQILAKSTEPFEKVCLMLLNHKQSDALLIEYLLAKLNKKN
CaVps18p-3	361	YLQGGFGISSDDLSQLIQGIQILAKSTEPFEKVCLMLLNHKQSDALLIEYLLAKLNKKN
CaVps18p-4	361	YLQGGFGISSDDLSQLIQGIQILAKSTEPFEKVCLMLLNHKQSDALLIEYLLAKLNKKN

CaVps18p	421	KVRIVLSAWIIELMVRNDDSRVYEFIKTNYKLLDRPTMYQILNSEKLIFYAELIEDYNF
CaVps18p-2	421	KVRMIVLSAWIIELMVRNDDSRVYEFIKTNYKLLDRPTMYQILNSEKLIFYAELIEDYNF
CaVps18p-1	421	KVRMIVLSAWIIELMVRNDDSRVYEFIKTNYKLLDRPTMYQILNSEKLIFYAELIEDYNF
CaVps18p-3	421	KVRIVLSAWIIELMVRNDDSRVYEFIKTNYKLLDRPTMYQILNSEKLIFYAELIEDYNF
CaVps18p-4	421	KVRMIVLPAWIIELMVRNDDSRVYEFIKTNYKLLDRPTMYQILNSEKLIFYAELIEDYNF

CaVps18p	481	ILKYYIDKKNWTLAVKTLIKLYTKGDIETVYENATILLMNYPKVTETWLKLDLEYEKLLP
CaVps18p-2	481	ILKYYIDKKNWTLAVKTLIKLYTKGDIETVYENATILLMNYPKVTETWLKLDLEYEKLLP
CaVps18p-1	481	ILKYYIDKKNWTLAVKTLIKLYTKGDIETVYENATILLMNYPKVTETWLKLDLEYEKLLP
CaVps18p-3	481	ILKYYIDKKNWTLAVKTLIKLYTKGDIETVYENATILLMNYPKVTETWLKLDLEYEKLLP
CaVps18p-4	481	ILKYYIDKKNWTLAVKTLIKLYTKGDIETVYENATILLMNYPKVTETWLKLDLEYEKLLP

CaVps18p	541	ALLKHQEQAIHFLQQVIMDKHYKKNQQLNNAYLCLLITKPGTDKQIIKFINFSTSNFDTNF
CaVps18p-2	541	ALLKHQEQAIHFLQQVIMDKHYKKNQQLNNAYLCLLITKPGTDKQIIKFINFSTSNFDTNF
CaVps18p-1	541	ALLKHQEQAIHFLQQVIMDKHYKKNQQLNNAYLCLLITKPGTDKQIIKFINFSTSNFDTNF
CaVps18p-3	541	ALLKHQEQAIHFLQQVIMDKHYKKNQQLNNAYLCLLITKPGTDKQIIKFINFSTSNFDTNF
CaVps18p-4	541	ALLKHQEQAIHFLQQVIMDKHYKKNQQLNNAYLCLLITKPGTDKQIIKFINFSTSNFDTNF

CaVps18p	601	ILRLCISHEKFHFAVLIYIEIGLFDQALELALKHDLTSLAEFILNKYDEDKQVEGIKLED
CaVps18p-2	601	ILRLCISHEKFHFAVLIYIEIGLFDQALELALKHDLTSLAEFILNKYDEDKQVEGIKLED
CaVps18p-1	601	ILRLCISHEKFHFAVLIYIEIGLFDQALELALKHDLTSLAEFILNKYDEDKQVEGIKLED
CaVps18p-3	601	ILRLCISHEKFHFAVLIYIEIGLFDQALELALKHDLTSLAEFILNKYDEDKQVEGIKLED
CaVps18p-4	601	ILRLCISHEKFHFAVLIYIEIGLFDQALELALKHDLTSLAEFILNKYDEDKQVEGIKLED

CaVps18p	661	ANYNVKRKLWLKFAKYLIKSDDDLNETLHHIVNVSMGLDKDLLPLFPETISINNFKDEI
CaVps18p-2	661	ANYNVKRKLWLKFAKYLIKSDDDLNETLHHIVNVSMGLDKDLLPLFPETISINNFKDEI
CaVps18p-1	661	ANYNVKRKLWLKFAKYLIKSDDDLNETLHHIVNVSMGLDKDLLPLFPETISINNFKDEI
CaVps18p-3	661	ANYNVKRKLWLKFAKYLIKSDDDLNETLHHIVNVSMGLDKDLLPLFPETISINNFKDEI
CaVps18p-4	661	VNYNVKRKLWLKFAKYLIKSDDDLNETLHHIVNVSMGLDKDLLPLFPETISINNFKDEI

CaVps18p	720	VESLNEYNKRIVHLSLDMNNSSEHLREMKKKVLYNKKKTNVAIEPGEPCRKCCKGLLVQE
CaVps18p-2	720	VESLNEYNKRIVHLSLDMNNSSEHLREMKKKVLYNKKKTNVAIEPGEPCRKCCKGLLVQE
CaVps18p-1	720	VESLNEYNKRIVHLSLDMNNSSEHLREMKKKVLYNKKKTNVAIEPGEPCRKCCKGLLVQE
CaVps18p-3	721	VESLNEYNKRIVHLSLDMNNSSEHLREMKKKVLYNKKKTNVAIEPGEPCRKCCKGLLVQE
CaVps18p-4	721	VESLNEYNKRIVHLSLDMNNSSEHLREMKKKVLYNKKKTNVAIEPGEPCRKCCKGLLVQE

CaVps18p	780	NFVYFPNCHHAFHKECMKKNQCLLCNDFLNL
CaVps18p-2	780	NFVYFPNCHHAFHKECMKKNQCLLCNDFLNL
CaVps18p-1	780	NFVYFPNCHHAFHKECMKKNQCLLCNDFLNL
CaVps18p-3	781	NFVYFPNCHHAFHKECMKKNQCLLCNDFLNL
CaVps18p-4	781	NFVYFPNCHHAFHKECMKKNQCLLCNDFLNL

above contigs, as there was significant homology between the chosen probe sequence and each contig sequence. Despite this, only parental fragment sizes (2.5-2.8 kb) were detected in Southern blot experiments with genomic DNA of *Ura*⁺ transformants, suggesting that the cassette was not integrating at any of the *CaVPS18* loci. The *Ura*⁺ transformants obtained may have resulted from integration of the *URA3* selectable marker by non-homologous recombination at a genomic loci other than *CaVPS18*.

6.6 Identification of further *Candida albicans* class-C VPS homologues

The *C. albicans* genome sequencing project has revealed genes sharing homology to the other three class-C VPS genes of *S. cerevisiae*, *VPS11*, *VPS16* and *VPS33*. If these genes represent true class-C functional homologues, then disruption of any of these ORFs would be expected to give rise to phenotypes associated with defects in vacuole biogenesis, as is the case in *S. cerevisiae*. *CaVPS11*, *CaVPS16* and *CaVPS33*, may be present as only two alleles at a single locus, and therefore provide targets easier to disrupt than *CaVPS18*.

The complete *CaVPS11* gene is present on one database contig, and encodes for a 1100 amino acid product, compared to a 1029 residue product from the *S. cerevisiae* *VPS11* gene. This revealed that the ORF sequenced in section 5.4 was truncated in its 5' region by 114 codons. The predicted protein sequences of the *VPS11*-like ORFs identified from strains S01 (Chapter 5) and SC5314 (database), differed at 10 amino acids (over the 985 available for comparison). The complete *CaVps11p* sequence predicted from SC5314, shares 36.8% identity and 49.9% similarity to the *S. cerevisiae* *VPS11* gene product (BESTFIT analysis).

The *C. albicans* *VPS16*-like gene encodes a 946 amino acid product which shares 30.8% identity and 43.7% similarity (BESTFIT) to the *S. cerevisiae* gene product (797 amino acids) (figure 6.7). The *VPS33*-like ORF, *CaVPS33*, encodes a 698 amino acid product that shares 27.6% identity and 40.3% similarity to *S. cerevisiae* *Vps33p* (see figure 6.8). An ATP-binding consensus has been identified in *S. cerevisiae* *Vps33p* (Banta *et al.*, 1990). This is proposed to consist of two motifs, amino acids 480-498, and 661-679. Despite the apparent importance of this ATP-binding domain in *S. cerevisiae* (Gerhardt *et al.*, 1998), it seems not to be highly conserved in the *CaVPS33* gene product. Now that these whole ORF sequences are available, genetic manipulation of these ORF's is possible.

Figure 6.7. Alignment of *Saccharomyces cerevisiae* VPS16 and *Candida albicans* VPS16 like gene products. The two proteins share 30.8% identity (highlighted with black shading), and 43.7% similarity (grey highlighting). Alignment was created using the PILEUP programme in the GCG package.

```

ScVps16p 1 ~MKNPSFDWERLKDVFYRSR.AIGEKKMPTQ..YEEKCALSL..LTWIAVEIQ.....DF
CaVps16p 1 MPSPNSFNWIKIQNVYINIRTCYDEMDSIDNLYSNLVSLSPNTTIIAASKSAPHPNL

ScVps16p 50 IQVYNYFGQLLGKINLQRIHEDIHK.FEFDKDEKLIIVTKSS.....IKIVKGLSP....
CaVps16p 61 IEIYSNSGHLWSVYVNSTHNDYIQSFHFTSDEDLITVLNNGRYRHYDILGSENEYNFV

ScVps16p 100 ...LTIESMPLQDPTIDTIWDYHNGIMHA.....KSRDIYKL.....NGNEW.....E
CaVps16p 21 EDLVTMTNHSQRHNTGDKIDANPDNTRITNLENNQIEIHKIEVKTWNQILVVRLESK

ScVps16p 141 LLYENKDKKYN.LLAKNHKSCND.....DSITLLDGHVYQV
CaVps16p 181 LIVSDLDSTNYAISLASYSASDFECFSIQAKTGTKKAVTIQLGYRKILSDVD..FDL

ScVps16p 177 STSNGALLKLITDSSVHKVTISSRG.FICLNNMKDNKIQFRDP.ARIILMEH...NLDST
CaVps16p 239 A.SYEILDQELTDGPSSSAISPSQQLISLENQQLKKIFVISNKFQDQILLYDTSNESSD

ScVps16p 232 PDDLCWCGNDTVACSFDEIKLGGPDGLYVTFYYP.....
CaVps16p 298 PYQVEWCGNDALVLSFKDEIKLIGPGQQSISFFYDIEDDDEFDLNLMVRKDNKNTDYKK

ScVps16p 267 .....ETVTNLRAEVDGLKVITTEKIYFLSRVQPTS.NIBRGSTEEGAIL...VDSFS
CaVps16p 358 FDDLLEFTIPILQTSIDGLKIVTINKIQFLSRV.PETSVEMIQVGSTAESSILADCDKFS

ScVps16p 318 LLEDHAPKAIEILK.NFVLEKGVLDCTAAAIDEFEPKLOMLINAAISGKASLQYKSFDA
CaVps16p 417 ANASKADSNISLLRADNTLLKAMEDCQVALDEFSPDWQRRALKAVSESGKVYYD.DYFDS

ScVps16p 377 SIFVNACNTIKLLNCFRS..FGIFLTVEEYRCI.SLKGVIDRLLYHYEYECQICKLAN
CaVps16p 476 DKFVSVVDIILKVLNLIIRSPELGFLTYQQIEKIGGWEEVIKMLLRNHDLSLDIDKLN

ScVps16p 434 ERFLLGYVFTETAKDKIKGSPDMEDDILLDKIKSL..SVIDMTDTQMVAIAKVAYLEG
CaVps16p 536 LGNAKPLVIHWCCYKIRKELDMSDILFKLSENLISSSTKTRVNYSTEQISETAHEEG

ScVps16p 492 RFQISRNIALLEKNEEARTEQLYNLDSDIALKECIKVQNYSLISLIALSKKLINSQL
CaVps16p 596 REEICKFILDLEPSVIKKIGKILDFDETEALIKSFQNGNYDLSTLALLYLQDNLTTSQF

ScVps16p 552 TKILL.....IIDMFNNP..YLYYMR.....MDKAYLYDF
CaVps16p 656 FKILSQTESVLKKNSTKQTIQKLDIDYKSEYMQVTGEVVGHAWEEIAKNVSPRLIETV

ScVps16p 580 YRQTDRFIDLAHVLLQOGKEQQSLH..SLPQIKDIYQVQNSEVVTNIEQLORQEKIW
CaVps16p 716 LKHEDRVNELNIFKIOOFKRDPELHKDNVYELYKSLAKAINRSTNRISKALQRETQVL

ScVps16p 638 IYQESLGKRFAISF.TNMTLDQTLISKIETGQDKQVKETVKKFKISEKKLYHLKCKTLVE
CaVps16p 776 ELQKKLSETYLTDFYQEKTLMAILKRMIAMNQIKPAKKIVANFQISVEKFWYVLNITMD

```

ScVps16p 750 LKWECRGRDAIQLAGKEKDIPGLKETYNIIP.PNEPETKAMANETVSR~~
CaVps16p 896 MF1KNDDYESALEAFRNKDIDILKSLLOKIPNSNQADIQ.VVNSYQQGY

Figure 6.8. Alignment of the *Saccaromyces cerevisiae* Vps33p sequence to CaVps33p. Identity is highlighted with **black** shading. Similar amino acids are shown by **grey** shading. The alignment was performed using PILEUP. The proteins share 27.6% identity, and 40.3% similarity.

```

ScVps33p      1 ~~~~~~MNRFWNTKKFSLTNAAGLCATNEISQNDEVLVWQPSVLPVLNSLLTFQDLT
CaVps33p      1 MKDINATENNLLASKFQRFNDKTLNENLNQTSKIYSSNNLLVLDVLSPLINSLTNETKLEK

ScVps33p     53 QSTPVRKITLLDDQLSDDLPSALGSVPQMDLTFLLIDVRTSLREPPQLLDAAQKHNLSLH
CaVps33p     61 ELGKFQNIIVWLDNELVSIGSSVLAKYASLIIVVPTSSLENINLNKLTHSQPDLHSLKLN

ScVps33p    113 ILYCRWKPSFQNTLEDTEQWQKDGFDINSKKTHFPNVIESQLKELSNEYTYLPWDLLEFP
CaVps33p    121 IIVKDLKMSFMYQNKV...FNGI.VNFESILSPEKYQKPI.SVTSRIKIYNKTSPI.

ScVps33p    173 QIDENMLITHSYNMENVNMYYPN.LRSLOSATESILVDDMVNSLQSLIFETNSIHTNV
CaVps33p    174 YTDE.IILMT.EINKFNGIDDFSKPLKQVNLSEA.LMQLLFMGICNGKHDHLFKIRNTY

ScVps33p    232 SIGNLSK.RCSHLKKRIDELHQTENDLFTIKGTLYGERTNCGLEMDLITLERNTPITPL
CaVps33p    231 GKGNHSLKLLIDQLQNSKIPEFMNENMTTLEIDFYSDVLHS..NTDLVVLERNLDFTSCIF

ScVps33p    291 TQLTYAGIIDDLY..EFNSGIKIKEKDMNFNYKEDKIWN.DLKFLNFGSIGPQNLKLAK
CaVps33p    289 NQLNYHGIIDDMP.SIKFENIEQLSNBESKSLANDKLYSQDLKHLNFSISIGLRNLKLAKY

ScVps33p    348 TQTQIDT....RHKAES...VHETKEFVDSLGLQQRQAFLEKNHTTSSDVEKVDET..E
CaVps33p    349 TQQQKTSQAQNSSSSEPNTS.IKQLVSNLGLLSIQQLVKKHTITGEEVVEKKNKYE

ScVps33p    399 EGGF.NKILELELEILCNTLNNDIEDITILELQYQYEVQKKIIRLICLLSLCKNSIRE
CaVps33p    409 QLLEFQNDIFEMTYKSQYAK.....GRFV.NCMYPIEF....VLSAILLIGYLNDGISN

ScVps33p    458 KEYEYLRTPMIDSGKEKCFQLES.....AELGFFTSKTKGT.....
CaVps33p    459 KEIIVLSLEMQDNVGLAEVFTLEKLIKFKMIRVIHGSSVDFSSLGFTSQKKKPTTHSQS

ScVps33p    496 ....DLHTTSKSTRG.QKEYRYISQWNTVPIED.....EHAADKITNENDDSSE
CaVps33p    519 EFEDLKNIGISGSGD.FRNNTLINKVNLHPLDDESLSQNLTESGSELTNLLDLASN

ScVps33p    542 AFAYSG.VVPLTMREVMQMLYDRSIFHNYSSQPFIISREPRVSQTEDLIEQLYGDSHA
CaVps33p    579 PSFTLPGNTVPLIYRFVEALYFRDPLKHKPIN.....LKRRPN.....WDNLG

ScVps33p    601 DEESTVPGTITKKINASIKNRRSIDGSNGTFHAAEDIALVVFLGGVTMGEAIMKHL
CaVps33p    623 V.NTMEAGKTIDLND...NVNDDRS.....KYLVIIVVIGGVTRSEITCFKYL

ScVps33p    661 QKILGKKGINKRFIIADGLINGTRIMNSIS~
CaVps33p    667 QERFRKQGKTKEIIVSNGIVNSSKIMCFMSN

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6.7 Discussion

Attempts to disrupt *CaVPS18* using the URA blasting method were unsuccessful. It is possible that there is only one functional copy of *CaVPS18*, and if *CaVPS18* is an essential gene then deletion of this copy would be lethal. This would particularly be a problem if the cassette constructed preferentially integrated at this locus. In this case it would not be possible to obtain a disruption strain and an alternative approach would be needed to demonstrate the essential nature of the gene. However, *S. cerevisiae* haploid, or homozygous diploid *vps18* mutants, are viable despite a range of cellular defects. Further problems may have prevented the correct integration of the *CaVPS18* disruption cassette described above. For example, the homologous targeting regions of the disruption cassette may have been of insufficient length to direct homologous recombination into any of the *CaVPS18* chromosomal regions. This is unlikely as similar sized targeting sequences have been routinely used for disruption of *C. albicans* genes (Fonzi and Irwin, 1993). The colony PCR analysis of Ura⁺ transformants suggested the structural integrity of the cassette was not maintained upon integration. It is possible although unlikely, that sequences within the cassette make it prone to degradation upon transformation.

At the time of design, the strategy was based on a single *CaVPS18* encoding contig available on the database. It was assumed that there would be two alleles of the gene and the flanking sequences would be similar. The resulting disruption cassette was predicted to target to either chromosomal copy, with similar efficiency. However, sequence data currently available on the database (January 2001) suggests there maybe up to four distinct *CaVPS18*-like genes in *C. albicans*, each displaying a significant degree of sequence divergence. As discussed in section 6.4 this may have lead to the construction of a 'chimeric' disruption cassette, containing the 5' flanking region of one allele, and the 3' flanking region of another. Given the high divergence observed in the *CaVPS18* flanking regions this may yield a cassette with insufficient homology to target efficiently to any one of the four genes. This perhaps provides the most likely explanation for failure of the *CaVPS18* disruption cassette to correctly integrate.

It still remains to be established whether the four *CaVPS18* contigs on the database represent four genuine copies of *CaVPS18* in *C. albicans* SC5314. This could be tested

using a PCR approach. A specific primer could be designed to each contig sequence within the highly divergent upstream region, and a second to a sequence within the ORF. The primer sets, could then be used in amplifications using SC5314 genomic DNA as a template. The presence of extension product would confirm whether or not the database contig sequence is contiguous within the genome, and represents a genuine *CaVPS18* loci. In the event that the database sequence does represent four copies of *CaVPS18*, each with significant divergence within its flanking regions, then an alternative approach to the disruption of *CaVPS18* function may be necessary.

The four genes are predicted to encode distinct gene products, with multiple changes in amino acid sequence (figure 6.6); if these contigs represent four true *CaVPS18* sequences within the genome. One or more copies may be non-functional since there is significant divergence between the coding sequence of each *CaVPS18* gene. An RT-PCR approach could be used to examine the expression of each of these genes individually. There is sufficient divergence between each ORF's nucleotide sequence to allow the design of primers for the specific amplification of each gene's transcript. This could be used in order to determine which of these genes are transcribed under a range of conditions.

The *CaVPS18* gene(s) may prove to be difficult to target irrespective of the disruption method used. In this case, other *C. albicans* class-C *VPS* homologues, may provide a more convenient target for disruption. In *S. cerevisiae* each class-C gene deletion strain is phenotypically indistinguishable, due to their gene products functioning at a common point in the vacuolar transport vesicle docking/ fusion pathway (Rieder and Emr, 1997). This may also be the case in *C. albicans*, but some genes maybe targeted for inactivation with greater efficiency than others. Since the initial attempts to disrupt *CaVPS18* outlined above were made, the genome sequencing project has advanced significantly and is nearing completion (January, 2001). This has revealed further *C. albicans* ORFs which share homology to the *S. cerevisiae* class-C genes. Each of these would provide a potential target of interest for disruption, and it has been shown that at least *CaVPS33* is transcribed in *C. albicans* (figure 5.5).

It is still unclear as to whether the *C. albicans* class-C *VPS* gene homologues identified on the genome database and through the hybridisation experiments (Chapter 5) represent true

functional homologues of the *S. cerevisiae* genes. This may be investigated by disruption of these *C. albicans* genes.

Chapter 7

Disruption of *CaVPS11*

7.1 Introduction

Attempts to disrupt a *C. albicans* *VPS18* homologue using the 'URA-blasting' method, (described in the previous chapter) were unsuccessful. This may have been due to the presence of multiple heterogeneous *CaVPS18* genes, interfering with the construction or integration of the disruption cassette. Due to the difficulties encountered, it was decided that an alternative approach was needed in order to construct a *C. albicans* strain defective in vacuolar biogenesis. A different target gene was selected, and an alternative method for targeted gene inactivation utilised.

As part of this study a truncated *C. albicans* ORF had been isolated, which shares homology to the *S. cerevisiae* *VPS11* gene (Chapter 5). Since then, the sequence of a contig from strain SC5314 has been made available which contains the whole of this *CaVPS11* ORF (<http://www.sequence.stanford.edu/group/candida/search.html>). In *S. cerevisiae* Vps11p and Vps18p physically interact to mediate a common step in vacuole biogenesis, and *vps11* and *vps18* are phenotypically indistinguishable (Rieder and Emr, 1997). It was therefore decided that the *CaVPS11* gene was an appropriate target for inactivation.

7.2 PCR mediated gene disruption

Mitchell and colleagues have described a new method for targeted gene disruption in *C. albicans* (Wilson *et al.*, 1999). This method utilises up to three selectable markers, therefore abrogating the need for marker recycling. BWP17 is a strain derived from SC5314, which is deleted for all *URA3* and *ARG4* sequences, and carries a partial deletion in both *HIS1* alleles. This auxotrophic strain therefore requires supplements of uridine, arginine, and histidine for growth. Each selectable marker gene, *URA3*, *ARG4* and *HIS1*, has been cloned into either pGEM3 or pRS314 plasmid vectors, and these plasmids are used as templates for the amplification of the disruption cassettes. A synthetic oligonucleotide primer pair are designed with 50-70 bases of homology to the target sequence (at 5' end of each primer), followed by a shorter stretch (3' region of primer)

with homology to sequences within the marker plasmids. The primer pair can then be used to amplify the above *URA3*, *ARG4* or *HIS1* marker genes. The resulting products consist of each marker gene, flanked on either side by a short stretch of homologous targeting sequence (50-70 bp). One of the PCR products is then transformed into BWP17, and the appropriate prototrophs selected. Transformants are then screened for correct integration at the target loci by either colony PCR, and/ or Southern blot hybridisation. The 'single disruptant', is then subject to a second round of disruption with a different marker gene. The *URA3* and *ARG4* genes are usually used for the two rounds of disruption. This is because some *HIS1* sequences are present in BWP17, and therefore a large proportion of *HIS1* disruption cassettes can mis-target to this locus. For this reason, the *HIS1* marker is usually used for re-introduction of the 'wild-type' target gene back into the *his1* locus of the double disruption strain. This is carried out in order to confirm that the observed 'mutant' phenotypes are attributable to the target gene.

7.3 Construction of a *CaVPS11* null strain

In the previous attempt to disrupt *CaVPS18* (Chapter 6), sequences flanking the ORF were used as homologous targeting sequences, in order to completely delete the coding sequences. However, it was later observed that the four putative copies of *CaVPS18* each had highly divergent flanking sequences, which may have complicated the construction of the disruption cassette, and its integration. It was therefore decided that sequences internal to the *CaVPS11* ORF would be used to target integration, as these are likely to be more highly conserved between alleles than the flanking sequences.

Primer 11D5 (table 2.6) provides the homologous targeting region to the 5' region of *CaVPS11* (figure 7.1). In its 5' sequence, 11D5 is identical to nucleotides +17 to +87 (from the ATG start codon) of *CaVPS11*, followed by the plasmid amplification sequence (5'-TGTGGAATTGTGAGCGGATA-3'). The reverse primer 11D3, provides the homologous targeting sequence to the 3' region of *CaVPS11* (figure 7.1). In its 5' sequence, 11D3 is identical to that of nucleotides +3302-+3233 on the reverse (non-coding) strand of *CaVPS11*. Its 3' region comprise 18 nts for marker amplification (5'-TTTCCCAGTCACGACGTT-3'). This primer pair was used in PCR amplifications, with either pRSARG4ΔSpeI or pGEM-URA3 as template. The resulting products were observed by agarose gel electrophoresis, before the *ARG4* cassette was transformed into

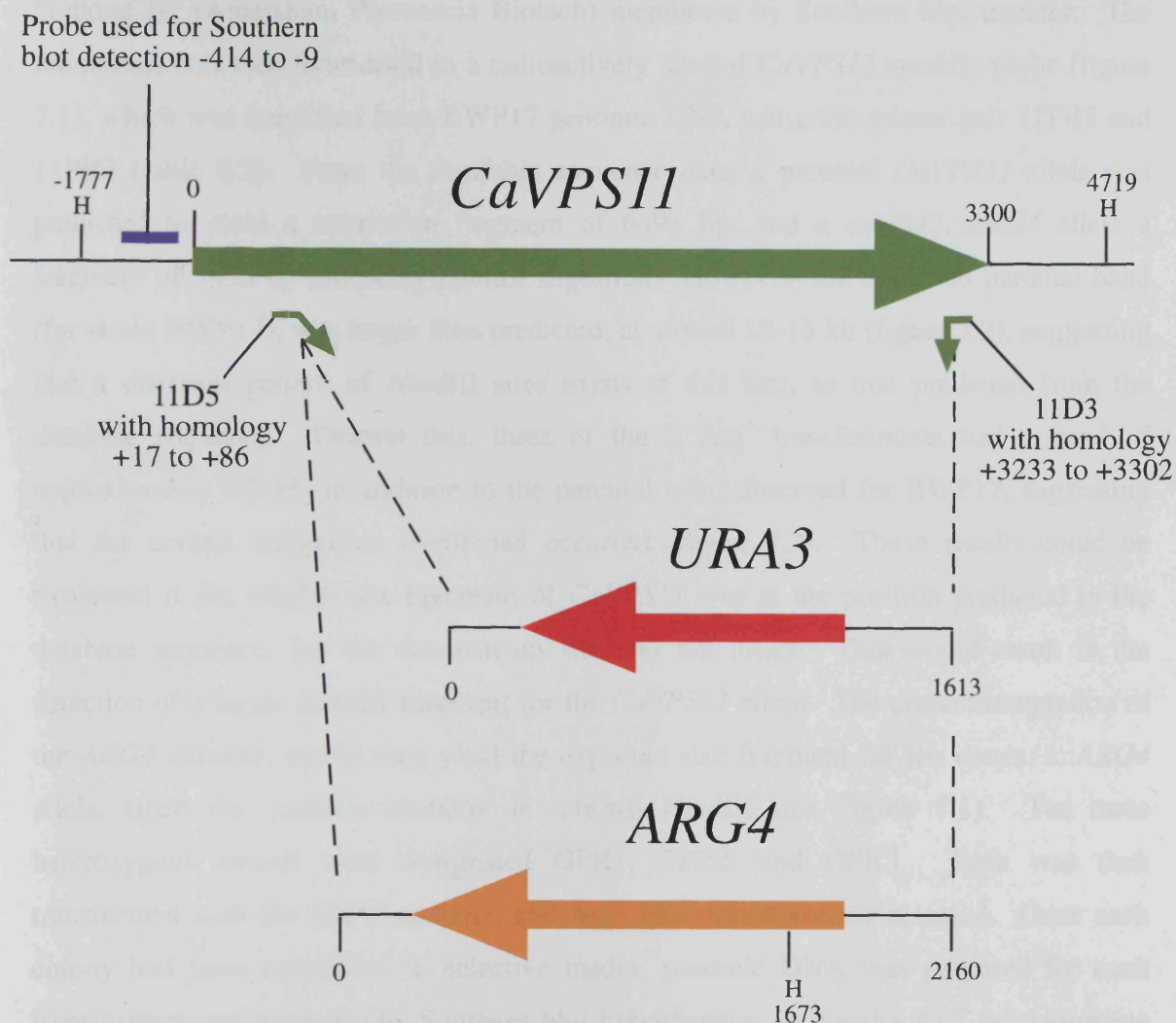


Figure 7.1. PCR mediated *CaVPS11* disruption strategy. The primer pair 11D5 and 11D3 are homologous to sequences within the *CaVPS11* ORF as shown. The 3' ends of these primers contain sequences for the amplification of either the *URA3* or *ARG4* disruption cassettes from the plasmid templates of pGEM-*URA3* or pRS-*Arg4*Δ*SpeI*. The resulting products consist of these selectable marker sequences flanked on either side by 70 base pairs of *CaVPS11* sequence. Blue line represents the *CaVPS11* specific probe used in Southern blot analysis. The predicted fragment sizes (from the database sequence) detected in *HindIII* cut genomic DNA are: *CaVPS11* = 6498bp; *cavps11::ARG4* = 3536bp; *cavps11::URA3* = 4914bp. H = *HindIII* site.

the recipient strain BWP17. Transformants were selected on arginine deficient media, and single colonies picked and re-streaked to fresh selective media. Genomic DNA was prepared from 5 Arg⁺ prototrophs and 1 µg of each was digested with *Hind*III. The resulting fragments were separated by agarose gel electrophoresis, and transferred to Hybond N⁺ (Amersham Pharmacia Biotech) membrane by Southern blot transfer. The membrane was then hybridised to a radioactively labeled *CaVPS11* specific probe (figure 7.1), which was amplified from BWP17 genomic DNA using the primer pair 11PB5 and 11PB3 (table 6.2). From the available sequence data, a parental *CaVPS11* allele was predicted to yield a restriction fragment of 6498 bp, and a *cavps11::ARG4* allele a fragment of 3536 bp following *Hind*III digestion. However, the observed parental band (for strain BWP17), was larger than predicted, at around 12-15 kb (figure 7.2), suggesting that a different pattern of *Hind*III sites exists at this loci, to that predicted from the database sequence. Despite this, three of the 5 Arg⁺ transformants had a band of approximately 3.5 kb, in addition to the parental band observed for BWP17, suggesting that the correct integration event had occurred (figure 7.2). These results could be explained if the *Hind*III site upstream of *CaVPS11* was at the position predicted in the database sequence, but the downstream site was not intact. This would result in the detection of a larger *Hind*III fragment for the *CaVPS11* allele. The correct integration of the *ARG4* cassette, would then yield the expected size fragment for the *cavps11::ARG4* allele, since this cassette contains an internal *Hind*III site (figure 7.1). The three heterozygous strains were designated GPR1, GPR2, and GPR3. Each was then transformed with the *URA3* cassette, and Arg⁺ Ura⁺ transformants selected. Once each colony had been restreaked to selective media, genomic DNA was prepared for each transformant and screened by Southern blot hybridisation, as for the Arg⁺ transformants above. Three colonies were identified in which the remaining parental band had shifted to a lower molecular weight (>10 kb, figure 7.2). While the *cavps11::ARG4* allele produced the expected size fragment, the observed *cavps11::URA3* band was larger than predicted from the database sequence. This was expected, as unlike the *ARG4* cassette, the *URA3* cassette does not contain an internal *Hind*III site. Thus the increase in the parental band size will directly affect the size of the *cavps11::URA3* band. These three 'double' mutant strains were called GPR1U1 (derived from the GPR1 single mutant), and GPR2U1 and GPR2U2 (both derived from the GPR2 single mutant). All three double mutants were observed to grow significantly slower than either the single mutant or parental strains.

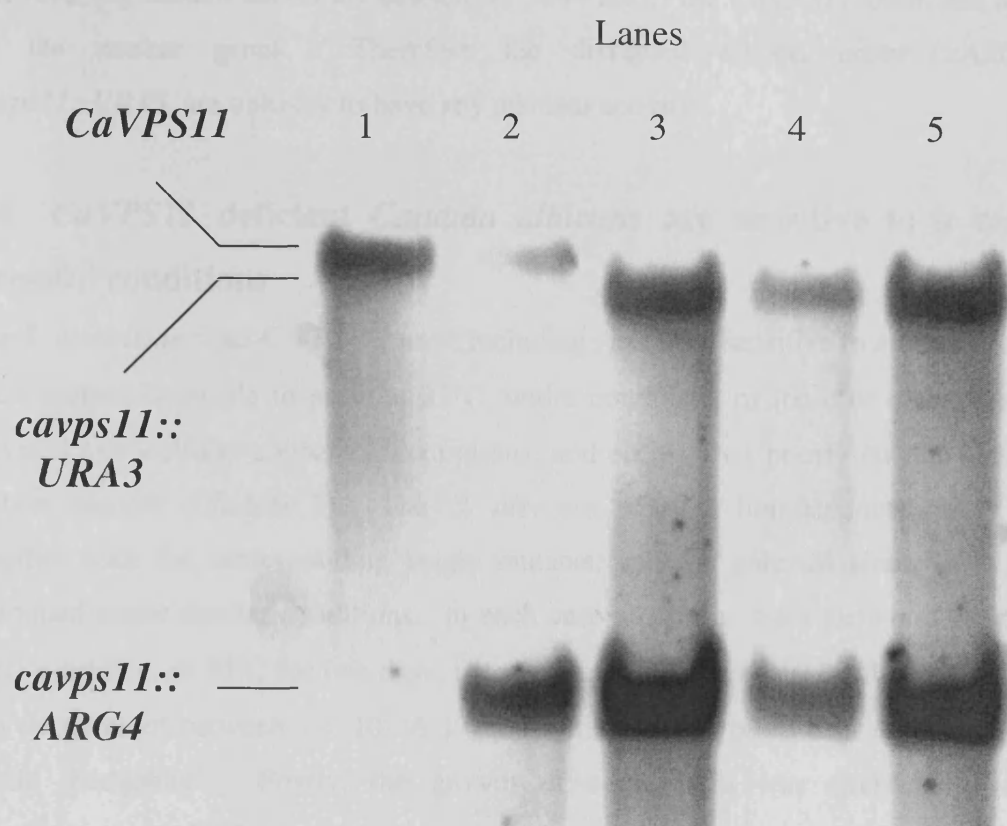


Figure 7.2. Integration of disruption cassettes at *CaVPS11*. Lanes: 1 = BWP17 (*CaVPS18/CaVPS18*); 2 = GPR1 (*CaVPS18/cavps18:ARG4*); 3 = GPR1U1 (*cavps11:ARG4/cavps11:URA3*); 4 = GPR2U1 (*cavps11:ARG4/cavps11:URA3*); 5 = GPR2U2 (*cavps11:ARG4/cavps11:URA3*).

Genomic DNA from the parental (*arg⁻ ura⁻*) strain (lane 1), *Arg⁺* (first round-lane 2), and *Arg⁺ Ura⁺* (second round-lanes 3-5) transformants was digested with *Hind*III, and separated on a 0.65% agarose gel. The DNA fragments were then transferred to Hybond N⁺ membrane, UV crosslinked, and hybridised to a *CaVPS11* specific probe at 65°C. The membrane was washed with 0.1% SDS, 3 x SSC, and bound probe detected by autoradiography. From sequence data the expected band sizes for *CaVPS11*, *cavps11::ARG4* and *cavps11::URA3* are 6498, 3536 and 4914 nucleotides respectively. A 3.5 kb fragment was detected, corresponding to that of *cavps11::ARG4*. However, the fragment detected in the parental strain (*CaVPS11*) was considerably larger than expected (~12-15kb), possibly due to alternative restriction site positions to that predicted from the available sequence. This would be expected to increase the size of the *cavps11::URA3* band due to the absence of *Hind*III sites within the *URA3* disruption cassette, and this was observed (>10kb).

The resulting mutant alleles are deleted for 3144 nts of the *CaVPS11* ORF, and disrupted by the marker genes. Therefore the disrupted alleles, *cavps11::ARG4* and *cavps11::URA3*, are unlikely to have any residual activity.

7.4 *CaVPS11* deficient *Candida albicans* are sensitive to a range of stressful conditions

The *S. cerevisiae* class-C *VPS* mutants, including *vps11* are sensitive to a range of stresses. Each mutant is unable to grow at 37°C, under conditions of ionic or osmotic stress, at elevated extracellular copper concentrations, and each grows poorly on non-fermentable carbon sources (Chapter 3). The *C. albicans* *cavps11* homozygous mutant strains, together with the corresponding single mutants, and the parental strain BWP17 were examined under similar conditions. In each case all strains were grown to saturation in YPD + uridine, at 30°C for two days, before serial dilutions of each culture were made to cell densities of between 1×10^7 to 1.6×10^4 /ml in a microtitre dish and plated using a sterile 'hedgehog'. Firstly, the growth of each strain was analysed at elevated temperatures, on YPD (+ uridine) media (figure 7.3). At 30 and 37°C all strains grew well. At 42°C, BWP17 and the single mutant strains grew less well, whilst the double mutant strains GPR1U1, GPR2U1 and GPR2U2, failed to grow at all (figure 7.3). That the non-permissive temperature for *C. albicans* *cavps11* null mutants (42°C) is higher than that of *S. cerevisiae* *vps11* mutants (37°C), is likely to reflect the fact that *C. albicans* is better adapted for growth at higher temperatures than *S. cerevisiae*. Even wild-type *S. cerevisiae* cannot grow at 42°C.

Under conditions of osmotic stress, on YPD supplemented with 2.5 M glycerol media or SD supplemented with 1 M NaCl or KCl (figures 7.4 and 7.5) the BWP17 and the single mutant strains grew less well than on the control plates. The double mutant failed to grow at all, indicating that it is more sensitive to osmotic stress than is the parental strain. In addition, like the *S. cerevisiae* *vps11* mutant, the *cavps11/cavps11* strains were more sensitive to elevated levels of extracellular copper, than either single mutant or the parental strain (figure 7.6). Importantly, the significantly lower concentrations of copper required to inhibit growth than either NaCl or KCl suggests that its toxicity is not simply due to an osmotic effect.

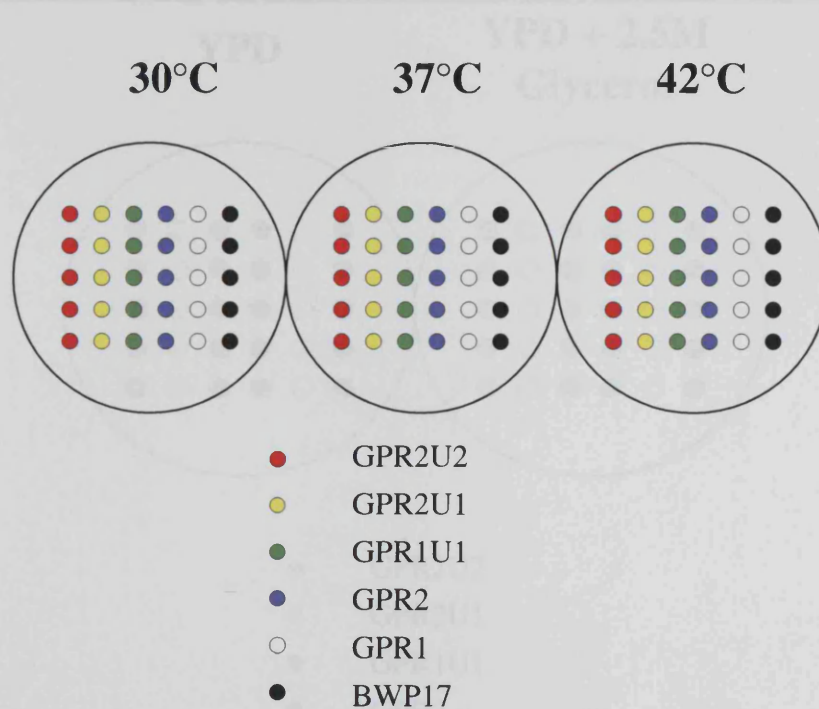
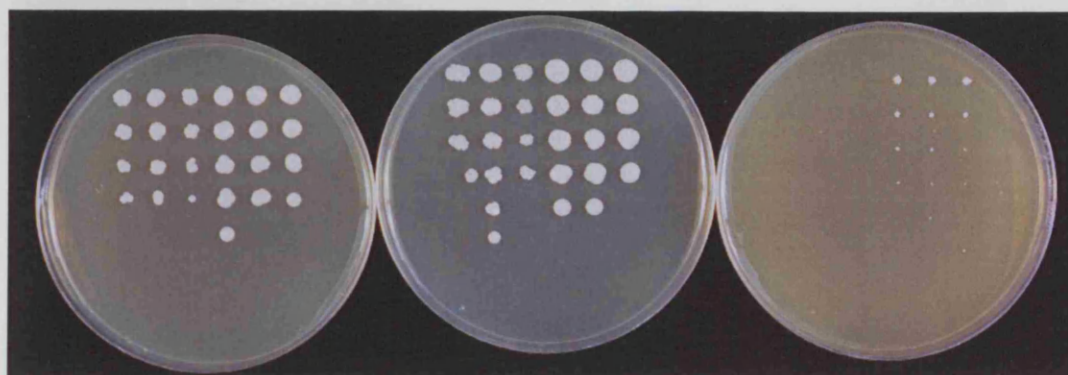
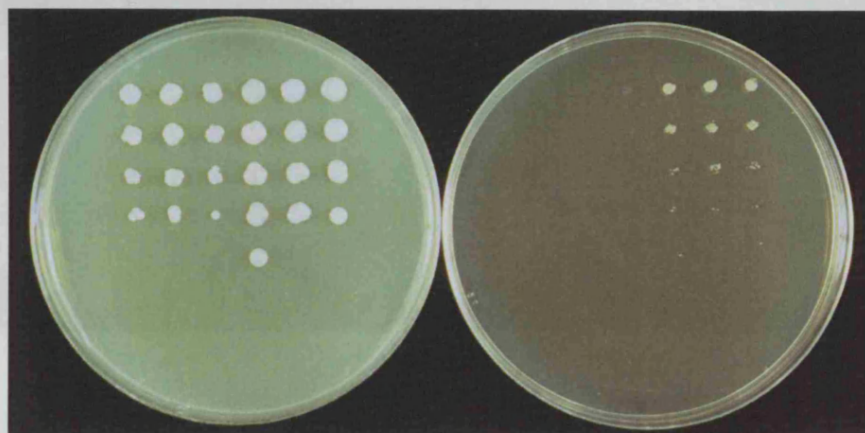
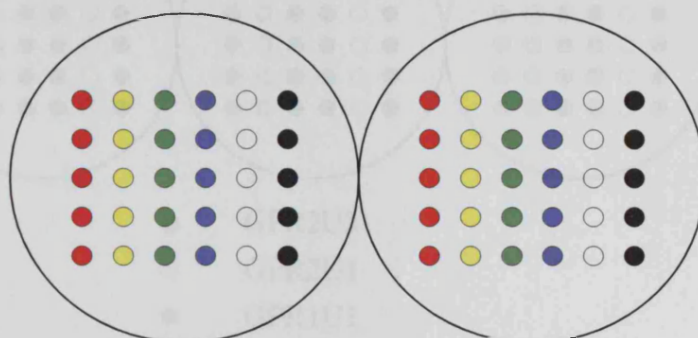


Figure 7.3. *Candida albicans* *CaVPS11* deletion strain is sensitive to growth at 42°C. Each strain was grown to stationary phase in YPD + uridine at 30°C. Serial dilutions of each culture were made to known cell densities, and plated onto YPD agar. The plates were then incubated at either 30, 37, or 42°C for 3 days. BWP17 = *CaVPS11*/*CaVPS11*; GPR1 and GPR2 are *CaVPS11*/*cavps11::ARG4* derivatives of BWP17; GPR1U1 is a *cavps11::ARG4/cavps11::URA3* derivative of GPR1; GPR2U1 and GPR2U2 are *cavps11::ARG4/cavps11::URA3* derivatives of GPR2



YPD

YPD + 2.5M
Glycerol



- GPR2U2
- GPR2U1
- GPR1U1
- GPR2
- GPR1
- BWP17

Figure 7.4. *Candida albicans* *cavp11* double mutant is sensitive to osmotic stress. Stationary phase cells were diluted to known cell densities and plated to YPD or YPD + 2.5M glycerol (both supplemented with uridine). The plates were incubated at 30°C for 3 days, and photographed.

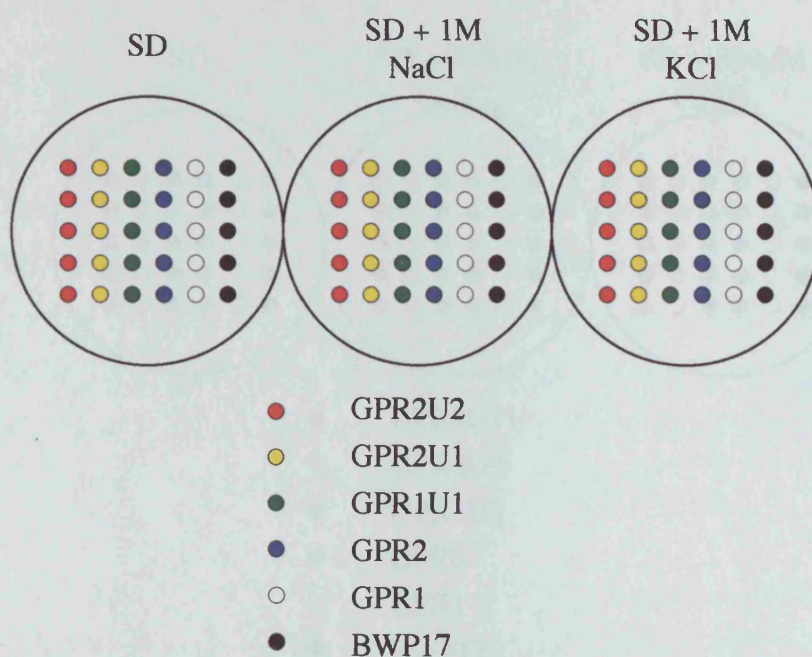
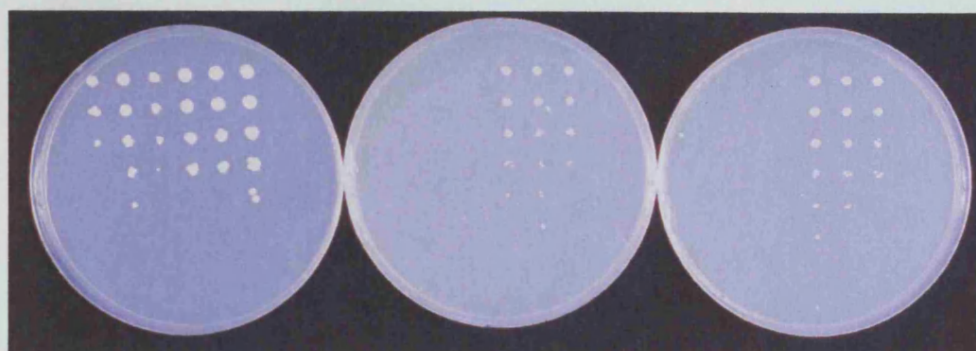


Figure 7.5. *Candida albicans* *CaVPS11* disruption strains are sensitive to ionic stress. Each strain was grown to stationary phase in YPD + uridine medium at 30°C. Serial dilutions of each culture were made to known cell densities, and plated on to SD; SD + 1M NaCl; or SD + 1M KCl, all supplemented with uridine, arginine and histidine. The plates were incubated at 30°C for 3 days.

A further observation was made regarding the colony morphology of parental and mutant strains. Delayed growth on YPD (+ uridine) media was seen in BWP17 and the single mutant strains GPR1, GPR2 and GPR3 all formed smooth, hemispherical colonies typical of *C. albicans*.

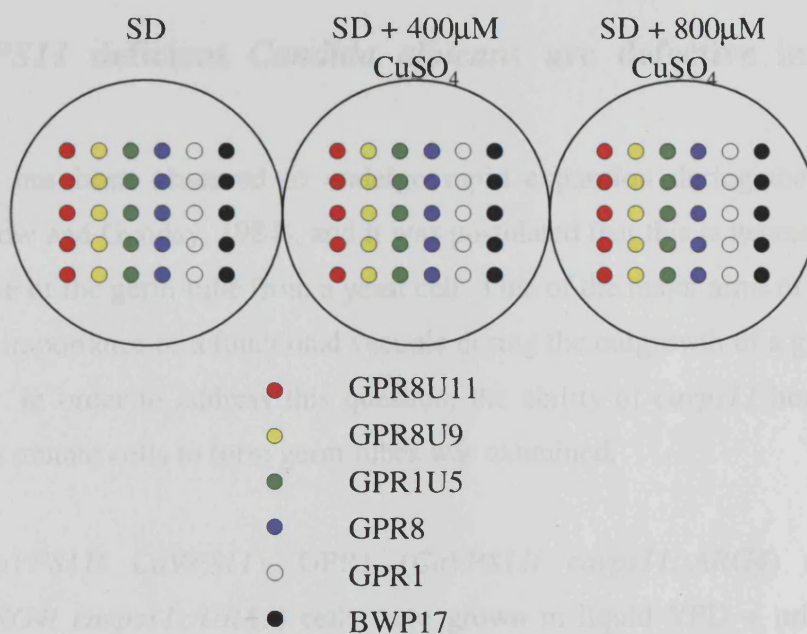
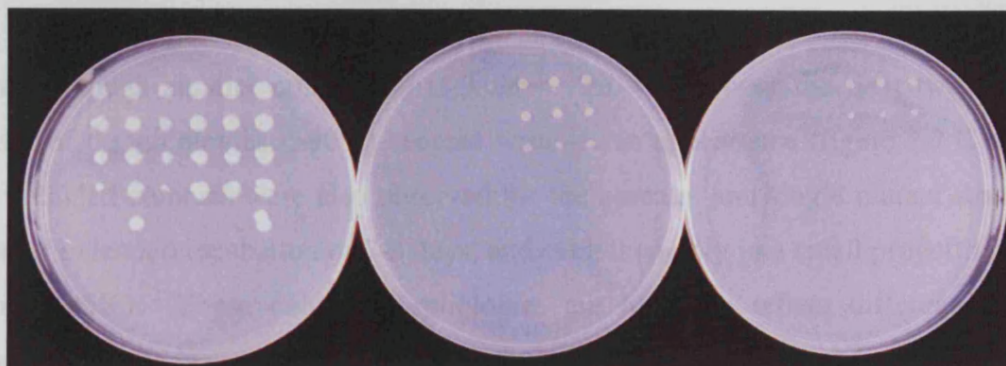


Figure 7.6. *Candida albicans* *CaVPS11* deletion strains are sensitive to elevated CuSO₄ concentrations. Each strain was grown to saturation in YPD + uridine media for 2 days. Cells were then plated to SD, SD + 400μM CuSO₄ or SD + 800μM CuSO₄ and grown at 28°C for 5 days..

A further observation was made regarding the colony morphology of parental and mutant strains. During growth on YPD (+ uridine) solid media, both BWP17 and the single mutant strains GPR1, GPR2 and GPR3 all formed smooth hemispherical colonies typical of *C. albicans* (figure 7.7 A and B). The double mutant strain also initially formed similar, although smaller colonies after 3 days. However, over the next two days the majority of these colonies (>90%) became wrinkled in appearance (figure 7.7 C and D). Such wrinkled colonies were also observed for the parental and single mutant strains but only after extended incubation of 7-8 days, and even then only in a small proportion of the colonies (<5%). These colony morphologies are likely to reflect differences in the proportion of cells in each of the cellular forms, yeast, pseudohyphae and true hyphae.

7.5 *CaVPS11* deficient *Candida albicans* are defective in germ-tube formation

The vacuole has been observed to undergo rapid expansion during the yeast-hyphae transition (Gow and Gooday, 1984), and it was postulated that this is necessary to support the emergence of the germ-tube from a yeast cell. One of the major aims of this study was to assess the importance of a functional vacuole during the outgrowth of a germ-tube from a yeast cell. In order to address this question, the ability of *cavps11* homozygous and heterozygous mutant cells to form germ-tubes was examined.

BWP17 (*CaVPS11/ CaVPS11*), GPR1 (*CaVPS11/ cavps11::ARG4*) and GPR1U1 (*cavps11::ARG4/ cavps11::URA3*) cells were grown in liquid YPD + uridine media at 30°C, to stationary phase. A 2.5 ml sample of each culture was then sub-cultured to 50 ml YPD + uridine + 10% Fetal Calf Serum (FCS) and cultured at 37°C to induce the formation of germ-tubes. Samples of each culture were removed and viewed by phase contrast microscopy before sub-culturing (t = 0), and 30, 60, 120, and 180 minutes after sub-culturing (figure 7.8). The behaviour of both BWP17 and GPR1 was essentially identical, and similar to that expected from a wild-type strain. The vast majority of cells were in the yeast cell form prior to sub-culturing (figure 7.8A), but germ-tubes rapidly emerged upon serum induction and were readily visible in nearly every cell by 30 minutes. These germ-tubes quickly extended into long parallel sided hyphae by 60 minutes, and at 120 minutes some cells were beginning to produce secondary germ tubes (figure 7.8A). The *cavps11* double mutant, GPR1U1 behaved differently. Notably cells grown in YPD +

(A).



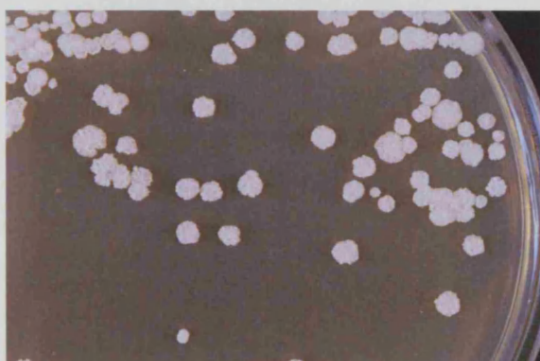
BWP17

(B).



GPR1

(C).



GPR1U1

(D).



Figure 7.7. *Candida albicans* *CaVPS11* deletion strains exhibit a wrinkled colony morphology. Each strain was streaked to fresh YPD agar, and incubated at 30°C for 5 days. (A). BWP17 = *CaVPS11*/*CaVPS11*; (B). GPR1 = *CaVPS11*/*cavps11::ARG4*; (C). GPR1U1 = *cavps11::ARG4*/*cavps11::URA3*. GPR1U1 initially appeared as normal hemispherical colonies after 3 days, which altered to the wrinkled form by 5 days. (D). Enlargement of GPR1U1 colonies.

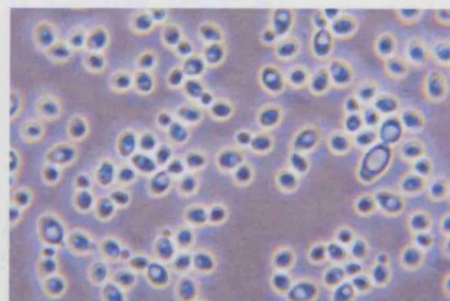
(A).

0 min

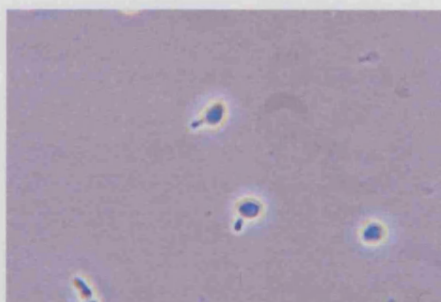
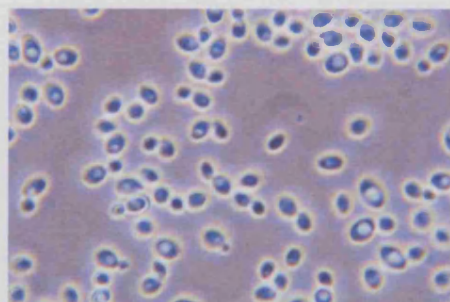
30 min

60 min

BWP17



GPR1



GPR1U1



**Figure 7.8. *cavps11*
homozygous mutants are
defective in filamentation.**

Strains BWP17, GPR1 and
GPR1U1, were grown to
stationary phase in YPD +
uridine media, and 2.5ml of each
culture sub-cultured into 50ml
YPD + uridine + 10% FCS. (A)
cells from each culture were
viewed before sub-culture (0
min), and 30, 60, 120 and 180
minutes after sub-culture.
GPR1U1 cells were also
examined 240 minutes after
induction (B), and 20hrs after
induction (C). 1000X
magnification

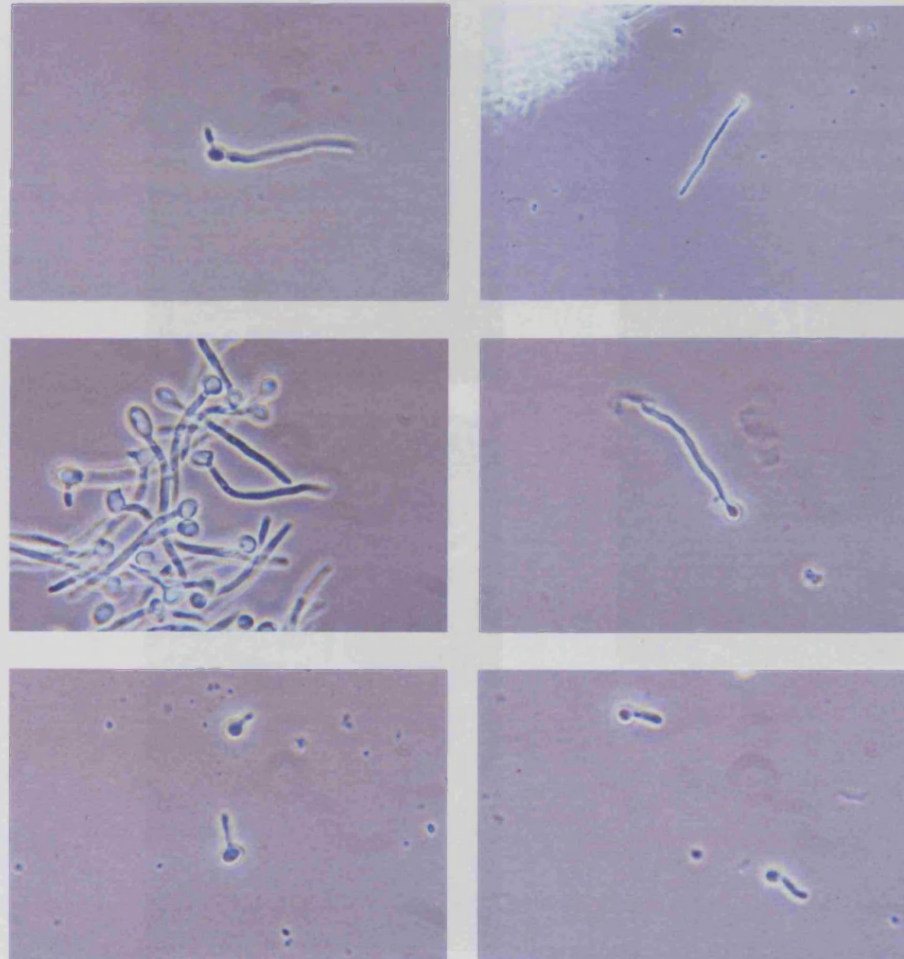
BWP17

GPR1

GPR1U1

120 min

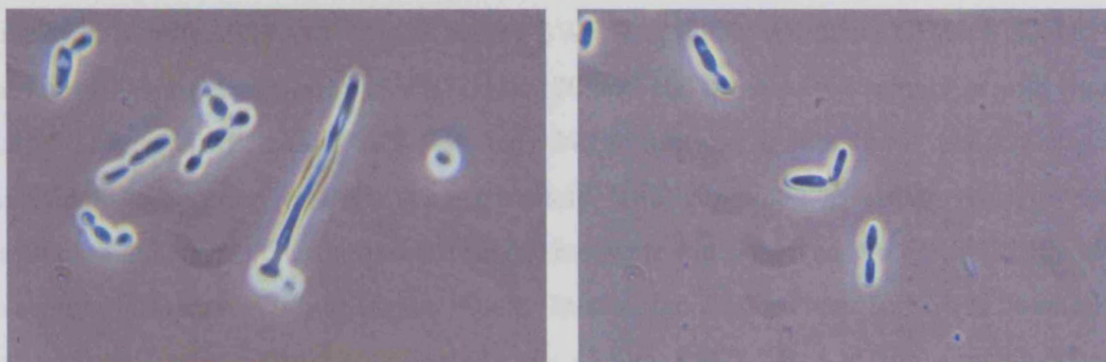
180min



(B).



(C). increased in 2004 as compared with 2003. Despite this, in the latter year of these years,



uridine ($t = 0$) were in morphological forms that ranged from yeast, to elongated unicellular forms, and extensively branched pseudohyphae (figure 7.8A). In addition this strain only reached a cell density of approximately 25% that of the parental or single mutant strains. Upon serum induction there was no noticeable change in the range of forms at 30 and 60 minutes than at 0 minutes. At 60 minutes 99% of BWP17, and 96% of GPR1 cells had visible parallel sided germ tubes. In contrast just 5.5% of GPR1U1 cells had structures resembling germ-tubes, the majority of which lacked the characteristic parallel cell walls of true germ-tubes and may have been mis-classified aberrant elongated forms, similar to those observed at $t = 0$. However, after 120 minutes a proportion of the cells were observed to have true germ-tubes (figure 7.8A), and by 180 minutes germ-tubes had emerged in 50% of GPR1U1 cells. Despite this, the elongation rate of these germ-tubes was significantly slower than that of BWP17 or GPR1 (compare GPR1 at 30 and 60 minutes [30 minute period] vs GPR1U1 at 120 and 180 minutes [60 minute period] figure 7.8A). Figure 7.8B shows GPR1U1 cells 240 minutes after induction. At this stage a significant proportion of cells (approximately 50%) remained as either yeast or short pseudohyphal forms. Furthermore, true hyphae were not observed in GPR1U1 cells after overnight induction (20 hrs) (figure 7.8C). Instead the predominant form was bi-cellular, consisting of two conjoined elongated cells (figure 7.8C). In addition, unicellular and budding yeast, and pseudohyphal forms were present. Occasional cells were observed to possess germ-tubes, but these were no more elongated than those observed at $t = 240$ minutes. However, this was not due to a general arrest in cell growth, as an increase in cell density was observed in the overnight induction culture, suggesting that the mutant cells continued to divide. These observations suggest that *CaVps11p* deficient cells exhibit a delay in germ-tube formation following serum induction. Furthermore, the extension rate of the germ-tube is significantly impaired in *cavps11* mutants, which also appear to be unable to sustain true hyphal growth beyond the initial emergence of the germ-tube. Due to strict time constraints, a detailed analysis of the filamentation defects associated with *CaVps11p* depletion was not possible.

7.6 Discussion

The strategy taken to inactivate *CaVPS11* was successful. Both heterozygous, and homozygous mutant strains were constructed using the PCR mediated gene disruption method (Wilson *et al.*, 1999). The *cavps11* double mutant was viable, but grew

significantly slower than either the single mutant, or parental strains. In addition, a number of phenotypes are manifest in this mutant that closely resemble those associated with *S. cerevisiae vps11* mutants. These include sensitivity to osmotic stress, elevated levels of extracellular CuSO₄, and growth at high temperatures. Furthermore, mutations in both *CaVPS11* and *S. cerevisiae VPS11* are recessive to the WT alleles. This supports the idea that a *C. albicans cavps11* mutant strain has similar defects to those of *S. cerevisiae vps11* mutants, and are thus likely to be defective in vacuolar biogenesis, although this needs to be confirmed through morphological analysis of the vacuole in the homozygous mutant. Nonetheless, the CaVps11p deficient strain GPR1U1, was found to be defective in the yeast-hyphae transition. Upon serum induction of hyphal growth both the parental (BWP17) and heterozygous strain (GPR1), rapidly produced germ-tubes, in contrast there was a significant delay of 60-120 minutes before the appearance of germ-tubes in the double mutant GPR1U1. Moreover, germ-tube formation occurred in >95% of BWP17 and GPR1 cells (after 60 minutes of induction), but in only 50% of the double mutant GPR1U1 (after 180 minutes of induction). The observed germ-tube extension rate was much reduced in GPR1U1, and no mature hyphae were observed, even after overnight serum incubation. These results are consistent with the hypothesis that the vacuole plays an important role in the yeast-hyphal transition. One possible explanation for the observed defects in germination is that the rapid expansion of the vacuole is necessary in order to support the rapid emergence of the germ-tube in inducing conditions. The rapid nature of germ-tube emergence may be advantageous during infection for adaptation to new host environments. *C. albicans* yeast cells have also been observed to undergo this transition in response to attempted ingestion by host phagocytes, which often coincides with the death of the phagocyte (Arai *et al.*, 1977). Thus the rapid emergence of a germ-tube may provide an escape mechanism from phagocytosis for *C. albicans* cells. However, it is unlikely that the extension rate of the germ-tube could be supported through the synthesis of new protoplasm alone. The vacuole may therefore act as a space-filling compartment to allow the rapid emergence and extension of the germ-tube, ahead of protoplasm biosynthesis. Assuming that the *CaVPS11* null strain described here has similar vacuole biogenesis defects to those of the *S. cerevisiae vps11* mutants, then the observed defects in the yeast-hyphae transition are consistent with this model. An alternative explanation is that the defects in filamentation are due to the absence of other vacuolar functions. For example, in *S. cerevisiae* the process of sporulation is dependent upon the function of the vacuolar hydrolases, which are important in the recycling of

cellular protein (section 1.4.1). It is possible that such hydrolases are important in a wide range of differentiation and adaptation processes, and this may extend to germ-tube formation in *C. albicans*. Indeed, a further study of interest would be to investigate if the vacuolar hydrolases of *C. albicans* are essential for the yeast-hyphae transition. This may be achieved through the cloning and disruption of a *C. albicans* *PEP4* (encoding PrA) or *PRB1* (encoding PrB) homologue. In *S. cerevisiae* such mutants are defective in the maturation of multiple vacuolar hydrolases and in sporulation (sections 1.4.1 and 1.5.1), but have morphologically normal vacuoles. Such an investigation would resolve whether it is the defect in vacuole expansion, or vacuolar hydrolase activity that is responsible for the defects in germ-tube formation observed in the *cavps11* mutant.

Due to time constraints, a comprehensive analysis of the phenotypes associated with *CaVPS11* disruption was not completed. The phenotypes observed for the *cavps11* null strains, are consistent with a defect in vacuolar biogenesis. An immediate priority for future work would be to confirm that the *cavps11* homozygous mutant strain is defective in vacuolar biogenesis. This could be examined through staining with the vacuole specific fluorescent dye, carboxy-fluorescein diacetate. Vacuolar morphology could then be determined by fluorescence microscopy. In addition, a more detailed analysis of the defects in germ-tube formation is required in order to quantify the delay in germ-tube emergence and reduced extension rate. The observation of single cells following induction by time-lapse photography could provide such detailed information, and may also indicate at which point germ-tube growth can no longer be supported in *cavps11* mutants. Finally reintroducing a 'wild-type' copy of *CaVPS11* into the homozygous mutant using the *HIS1* marker gene, is necessary in order to show that all the observed phenotypes can be unequivocally assigned to mutation of *CaVPS11*.

Chapter 8

General Discussion

The aim of this study was to investigate the functions of the vacuole in the pathogenic yeast *Candida albicans*. It was of particular interest to assess the importance of vacuole biogenesis in the formation of germ-tubes. The vacuole had previously been observed to undergo a rapid expansion during the emergence of germ-tubes from yeast cells (Gow and Gooday, 1984).

To address these questions a genetic approach was taken to identify *C. albicans* genes involved in vacuole biogenesis. This made use of the model eukaryote *Saccharomyces cerevisiae*, for which a large number of genes involved in vacuolar biogenesis have been isolated. The well characterised class-C *vps* mutants, *vps11*, *vps16*, *vps18* and *vps33* which lack a vacuole compartment, were selected for functional complementation with a *C. albicans* genomic DNA library. One genomic region was identified that complemented the *vps16* phenotypes including temperature sensitive growth and defective vacuole biogenesis. Sub-cloning reduced the complementing region to 2.4 kb, but attempts to precisely define the sequences responsible for this activity were unsuccessful. The rescuing activity associated with these clones is specific for *vps16*, and does not complement any of the other class-C *vps* mutants. Furthermore, the rescuing sequence is not a multi-copy suppresser of *vps16*, as it rescues when present on a single copy vector. These two facts suggest that the rescuing factor acts via a direct mechanism, substituting for Vps16p function. However, no *VPS16* homologue was present in the *C. albicans* sequence.

Using the functional complementation approach, a clone was also identified that partially rescued the temperature and copper sensitive growth phenotypes of *vps18*. Transposon mutagenesis identified a *PAB1* homologue as the ORF responsible for this activity. *PAB1* encodes a poly-A tail binding protein, important in the regulation of mRNA stability. It is not clear how such a protein might act to specifically ameliorate the phenotypes associated with *vps18*.

These screens did not yield any class-C *VPS* homologues. A *C. albicans* cDNA library expressed from an endogenous *S. cerevisiae* promoter may be a better approach, since this would guarantee expression of all coding sequences, whereas some of the *C. albicans* promoters would not be able to support expression in *S. cerevisiae*.

A second approach utilised data from the *C. albicans* genome sequencing project (<http://alces.med.umn.edu/candida.html>). Fragments of ORFs sequence were constructed from the database (March, 1999) that putatively encoded proteins homologous to the *S. cerevisiae* class-C proteins, Vps11p, Vps18p and Vps33p. These DNA fragments were amplified and used as probes for the isolation of *C. albicans* genomic clones containing these sequences. Three clones containing a *VPS18* homologue, and one containing a *VPS11* homologue were identified. More recently, (January, 2001), sequence encoding homologues of all four class-C genes have become available on the *C. albicans* sequence database. The proteins encoded by these ORFs all share a similar degree of identity to their *S. cerevisiae* relatives, between 28 and 37%. The previously proposed zinc finger like domains near to the C-terminus of Vps11p and Vps18p (Robinson *et al.*, 1991) were largely conserved in the *C. albicans* homologues, although protein sequence alignments between these homologues suggest that this motif consists of an alternative arrangement of cysteine and histidine residues. Despite the conservation of this functionally important domain, the *CaVPS18* genomic clones isolated by hybridisation failed to functionally rescue the *S. cerevisiae* *vps18* mutant. This finding was consistent with the failure in this study to identify class-C homologues through the complementation studies (Chapter 4). This may have been due to the *C. albicans* promoter being incompatible with the *S. cerevisiae* transcription machinery. Alternatively, the eight CUG codons present in *CaVPS18* would result in eight Serine→Leucine substitutions in the heterologously expressed protein, and this may have resulted in its inactivation. It was thus unclear if these genes represent true functional homologues of the class-C *VPS* genes.

Sequence analysis of the three *CaVPS18* positive clones also revealed a high degree of heterogeneity between alleles. The two alleles isolated contained 8 nucleotide differences over 399nt, one of which resulted in an amino acid substitution. Subsequent advances in the genome sequencing project also suggest that there is a high degree of divergence between *CaVPS18* sequences. There are now four contigs on the database (March, 2001)

that encode *CaVPS18*-like ORFs, and the proteins encoded differ in multiple residues (figure 6.6); two copies code for an extra amino acid. It has not yet been shown whether these contigs represent four genuine copies of *CaVPS18*, and it is possible that some of the observed differences are due to the incorporation of poor quality sequence. However, the sequencing performed in this study also found a large degree of heterogeneity at this loci. Such a high degree of divergence is surprising within a single strain, and it is not clear if these differences would result in the functional inactivation of any of these ORF i.e. they are pseudogenes.

Disruption of *CaVPS18* using the URA-blasting method was unsuccessful. The disruption cassette was designed based upon the single *CaVPS18* encoding contig available on the database at the time. However, three more *CaVPS18* containing contigs have since been made available on the database. Each of these contigs has significant divergence within the *CaVPS18* flanking sequences, affecting the regions that were used for the targeting of the cassette. The cassette may have failed to target correctly if a 'chimeric' cassette was constructed with the 5' flanking sequences of one allele, and the 3' sequences of another. While flanking sequences have been used successfully to target integration of the URA blasting cassette at some loci, it is perhaps more reliable to use sequences within the ORF for cassette targeting, since these sequences are likely to be more highly conserved between alleles.

The PCR mediated method of Mitchell and colleagues (Wilson *et al.*, 1999), was used to target *CaVPS11* for inactivation, using sequences within the ORF. Inactivation of both *CaVPS11* alleles resulted in a number of phenotypes that closely resembled those of *S. cerevisiae vps11* mutants. *CaVPS11* null strains were sensitive to osmotic stress, could not grow at 42°C, or on media with increased concentrations of CuSO₄. These phenotypes are consistent with a reduced homeostatic capacity in these cells caused by disruption of vacuolar function.

The *cavps11* double mutant strain GPR1U1 was also found to be defective in its ability to form hyphae in response to serum induction. The emergence of germ-tubes from the mutant yeast cell was significantly delayed, and only occurred in approximately 50% of cells. In addition, the tip extension rate was much slower than the parental, or single

mutant strains, and hyphal growth was not sustained since no mature hyphae were observed. Assuming that the *cavps11* homozygous strain lacks a vacuole compartment, as does the *S. cerevisiae vps11* mutant, then these defects suggest that the substantial vacuole expansion observed is necessary to support the rapid emergence of the germ-tube, and possibly its rapid extension rate. This supports the previously suggested model (Section 7.6; Gow and Gooday, 1984), whereby the vacuole provides 'bulk' during the initial burst of hyphal extension, permitting an extension rate above that which could be supported through cytoplasmic biosynthesis alone. Such a developmental model is unusual in filamentous fungi and may be a mechanism that is unique to *C. albicans*. However, the observed defects could be due to the absence of other vacuolar functions within the *cavps11* mutant. For example, it is likely that these mutants are deficient in vacuolar hydrolase activity, as is the case in *S. cerevisiae* class-C *vps* mutants. In *S. cerevisiae* vacuolar hydrolases are required for the process of sporulation (Zubenko and Jones, 1981). It is possible that the vacuolar hydrolases are also required in other processes of differentiation, to degrade and recycle cellular components, and this could be important during the yeast-hyphae development. This is perhaps a less likely explanation for the observed filamentation defects of the *cavps11* double mutant, since the rapid nature of germ-tube emergence seems unlikely to depend upon the relatively slow process of vacuole mediated recycling of cellular components (Egner *et al.*, 1993). Nonetheless, the importance of the vacuolar hydrolases in the yeast-hyphae transition could be evaluated through the construction of a *C. albicans* strain defective in their activity. *S. cerevisiae* mutants deficient in PrA activity fail to process, and are deficient in the activity of multiple vacuolar hydrolases, which also results in an inability to sporulate (Ammerer *et al.*, 1986). The sequence of a *C. albicans* ORF which encodes a product that shares 69% identity to *S. cerevisiae* PrA, has recently become available on the *C. albicans* database. Disruption of this ORF may yield a strain with a morphologically normal vacuole but lacking multiple vacuolar hydrolase activities. An assessment of the ability of this mutant strain to form germ-tubes would then allow us to distinguish whether it is the physical expansion of the vacuole or its hydrolase activities that are required for the normal germ-tube formation.

S. cerevisiae class-C *VPS* gene products form a complex required for the docking and/ or fusion of multiple transport intermediates with the vacuolar membrane (Rieder and Emr, 1997). During filamentation in *C. albicans* the rapid increase in vacuolar volume must

result from a net gain of membraneous material at the vacuole membrane. The incorporation of new membrane material at the vacuole membrane during expansion may well depend on the fusion machinery encoded by the class-C *VPS* homologues identified in this study. Therefore in addition to maintaining the structural integrity of the vacuole in the yeast form, the *C. albicans* class-C homologues may be required to facilitate vacuolar expansion upon hyphal induction. Consistent with this hypothesis, *CaVPS18* expression was found to increase several fold following induction of hyphal growth in serum culture. One approach to investigate the role of the class-C products in vacuole expansion would be to produce a temperature sensitive *CaVPS11* allele, which could be re-introduced in to the *cavps11/cavps11* strain described in this study. This may be attempted through the site directed mutation of the first cysteine residue of the proposed zinc-binding domain at the C-terminal of CaVps11p. Substitution of this residue with Serine in the closely related *S. cerevisiae* Vps18p C-terminal motif, yielded a temperature sensitive allele (Rieder and Emr, 1997). Cells of the *cavps11^{ts}* strain could be grown at the permissive temperature (e.g. 30°C) and shifted to the non-permissive temperature (e.g. 37°C) upon hyphal induction (in a similar way to the protocol used in Chapter 7). This strain would be expected to maintain a morphologically normal vacuole with wild-type hydrolase activities at the permissive temperature, and for some time after the temperature shift as is observed in *S. cerevisiae* *vps18^{ts}* mutants (Rieder and Emr, 1997). However, if CaVps11p plays a role in vacuole expansion, then the shift to the non-permissive temperature should result in an inability to expand the vacuole, thus true hyphae may not be formed.

The importance of vacuolar inheritance during hyphal development has been the subject of a separate investigation (Barelle *et al.*, 2000). In this study a *C. albicans* *VAC8* homologue was cloned and a *cavac8/cavac8* mutant strain constructed. In *S. cerevisiae*, Vac8p is localized to the surface of the vacuole, where it mediates interaction between the vacuole and the actin cytoskeleton (Wang *et al.*, 1998). Myo2p acts as a molecular motor, and provides the motive force for vacuole movement along the actin filaments and segregation structure emergence (as reviewed in Catlett and Weisman, 2000). *S. cerevisiae* *vac8* mutants are unable to form a vacuolar segregation structure, and are defective in vacuolar inheritance. The *C. albicans* *cavac8/cavac8* mutant was also defective in vacuole inheritance when grown in the budding yeast form, approximately 40% of buds lacked a vacuole compartment. Surprisingly, this seemed to have little effect

on germ-tube formation or extension rate, but resulted in an increase in branching frequency (Barelle *et al.*, 2000). These results suggest that vacuole biogenesis, rather than inheritance, is important during the yeast-hyphae switch. Indeed, vacuole inheritance seems to be more important once hyphal growth is established, in determining at which point a cell may form branches (Barelle *et al.*, 2000). Vacuole inheritance at the hyphal apex is non-symmetrical, with the apical compartment receiving the majority of cytoplasm, while the sub-apical compartment is extensively vacuolated (section 1.3.3). An extended G₁ phase is required in sub-apical compartments, to resynthesise cytoplasm before branching can occur (Gow and Gooday, 1984). The observed non-symmetrical vacuole segregation may be important for the regulation of G₁-to-branch formation switch in sub-apical compartments (Barelle *et al.*, 2000).

The observations made by Gow and Gooday (1984), raise several important questions. Firstly, from where is the extra membrane derived which is incorporated into the vacuole during germination? Is there a 'reservoir' of membrane vesicles that fuse to the vacuole upon hyphal induction, or is membrane material derived from other membrane bound organelles such as the PVC, golgi apparatus, or ER? This latter possibility could be investigated using a biochemical approach. Vacuoles could be isolated by density gradient centrifugation following serum induction, and examined for the presence of marker proteins that characterise these compartments. Secondly, how is this process of vacuolar expansion regulated? This may prove more difficult to investigate, and may only be resolved with further elucidation of the signal transduction pathways that regulate morphogenesis (see Section 1.3.1). While this study was in progress, it was reported in the literature that a *C. albicans* *VPS34* homologue (*CaVPS34*) had been disrupted (Bruckmann *et al.*, 2000). The *cavps34* null mutant was phenotypically very similar to the *cavps11* null strain described here. The *cavps34* null strain is hypersensitive to temperature and osmotic stress, is delayed in the emergence of germ-tubes, and only a small proportion of cells form true germ-tubes in response to serum induction (Bruckmann *et al.*, 2000). However, there are several differences between this *cavps34* mutant and the *cavps11* mutant analysed in this study. Firstly the pseudohyphal forms observed for the *cavps34* mutant are considerably more elongated than those observed for *cavps11* (Bruckmann *et al.*, 2000; fig 7.8). Also it was not reported that the *cavps34* homozygous strain has a reduced germ-tube extension rate. Finally, while the *CaVPS11* deficient strain is expected to lack a central vacuole, the *cavps34* null strain had a grossly enlarged

vacuole, occupying approximately 80% of cell volume (Bruckmann *et al.*, 2000). In *S. cerevisiae* Vps34p forms a membrane bound complex with Vps15p, essential for golgi to vacuole transport via the CPY pathway (Stack and Emr, 1994). Vps15p is proposed to activate Vps34p by phosphorylation, which in turn possesses a Phosphatidyl-Inositol (PI) kinase activity (Stack *et al.*, 1995). The production of PI(3)P by Vps34p, is thought to regulate golgi-PVC traffic by stimulating the production of transport vesicles in the *trans*-golgi, possibly by directly initiating localised membrane curvature (Marcusson *et al.*, 1994). A second *S. cerevisiae* gene *FAB1* has been implicated in the regulation of vacuolar membrane turnover. Mutations in this gene cause defects in vacuole segregation, and result in a greatly enlarged vacuole (Benangelino *et al.*, 1997). *FAB1* encodes a kinase located on the vacuole membrane, that phosphorylates PI(3)P to PI(3,5)P₂ (Cooke *et al.*, 1998). While the production of PI(3)P by Vps34p is required for the delivery of vacuolar hydrolases, this process is unaffected by the depletion of PI(3,5)P₂ in *fab1* mutants (Gary *et al.*, 1998). It therefore seems likely that Vps34p is essential for trafficking of membrane and protein cargo to the vacuole, while Fab1p may play a compensatory role in regulating the exit of membrane from this compartment (Gary *et al.*, 1998). The relative activities of these two proteins may therefore determine whether there is a net gain or loss of vacuolar membrane, and thereby the size of this compartment. It is not yet clear whether such a regulatory mechanism is responsible for controlling the rapid vacuolar expansion seen during germ-tube outgrowth, however the phenotypes observed in the *cavps34* null strain are consistent with this idea. The *cavps34* mutant strain would be deficient in PI(3)P synthesis, and therefore also PI(3,5)P₂. This is predicted to cause defects in anterograde and retrograde traffic to the vacuole. Thus upon the induction of hyphal growth, it is predicted that the vacuole would neither expand, nor contract. This may account for the observed defects in filamentous growth.

At present the mechanism by which vacuole expansion occurs during germ-tube formation, and its regulation, remain obscure. However, this study has indicated that this process is likely to play an essential role in the yeast-hyphae switch of *C. albicans*, a property that is central to the pathogenicity of this organism.

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Appendices

Appendix A

Contig 18R. *CaPAB1* ORF is underlined.

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TTTGGTGCTC AAAAGTTGAC ATTGGTTCAA CTAAGTGAAGA ACAATCAGCT
GCTGCTGCTA CTACCACTGC TGATCAATCA GCTGAAGAAC AAGGAGAATC
ATCTGGTGTT GCCGAGAATT CTGCCTCCTT GTACGTTGGT GAATTGAACC
CATCTGTTAA TGAAGCTACC TTGTTTCGACA TCTTTTCTCC AATCGGTCAA
GTTTCCTCTA TCAGAGTTTG TCGTGATGCT GTCTCTAAAA AATCTTTAGG
TTATGCTTAC GTCAACTACC ACAAGTACGA AGATGGTGAA AAGGCTATTG
AAGAATTGAA CTACAACCTG ATCGAAGGTC GTCCATGTCG TATCATGTGG
TCTCAAAGAG ACCCATCTGC TAGAAGATCT GGTGATGGTA ATATTTTCAT
CAAGAATTTG CATCCAGCCA TCGATAACAA AGCTTTGCAT GACACCTTTT
CTGCTTTTGG TAAAATTTTG TCTTGTAAGG TTGCCACCGA TGAATTTGGT
CAATCAAAGT GTTTTGGTTT TGTCCACTAT GAAACTGCTG AAGCTGCTGA
AGCTGCCATT GAAAATGTCA ATGGTATGTT ATTGAATGAT CGTGAAGTTT
TCGTTGGTAA GCACATTTCT AAAAAAGACC GTGAATCTAA GTTTGAAGAA
ATGAAAGCCA ACTTCACTAA CATTTATGTT AAAACATTG ACTTGAACATA
TTCAGAAGAA AGCTTTGAAA AATTGTTTTT TCCATTCCGGT AAGATTACTT
CCATTTACTT GGAAAAAGAC CAAGATGGGA AATCTAAAGG TTTTGGTTTT
GTTAATTTTG AAGATCATGA ATCTGCTGTT AAGGCTGTTG AARAATTGAA
CGATAAGAA ATCAACGGTC AAAAGATCTA CGTTGGTAGA GCACAAAAGA
AAAGAGAAAG ATTGGAAGAA TTGAAGAAAC AATACGAAGC TGTTAGATTA
GAAAAATTGG CCAAATACCA AGGTGTCAAC TTGTTTGTTA AGAATTTGGA
TGACACTATT GATTCTGAAA AATTAGAAGA AGARTTCAAA CCATTTGGTA
CCATTACATC TGCCAAGGTT ATGGTTGATG AAGCTGGTAA ATCAAAAGGT
TTTGGTTTCG TTTGCTTCAC AACCCAGAA RAARCCACCA AGGCTATCAC
TGAAATGAAC ACCAGAATGA TTAACGGCAA GCCATTGTAT GTTGCTTTGG
CTCAACGTAA GGATGTTAGA CGCTCTCAAT TARAACAACA AATTCAAGCC
AGAAACCAAA TGAGAATGCA AAATGCTGCT GCTGGTGGTT TACCTGGTCA
ATTCATTCCA CCAATGTTCT ACGGTCAACA AGGCTTTTTC CCACCAAATG
GCAAAGGTAA CGCTCCATAC CCARGTCCTA ATCCACAAAT GATGATGAGA
GGTAGAGGTC AACCATTTCNC AGAACAATGG CCAAGACCAG GTCCAAATGG
CCAACCAGTT CCTGTCTACG GTATTCCACC TCAATTTCAA CAAGACTTTA
ACGGTCAAAA CATGAGACCT CAGCAACAAC AACAACAACA ACCAAGAGGT
GGATACTATC CAAACCGTAA CCAAACCAGC AAGAGAGACT TGGCTGCTAT
CATTTCTAGT GTTCCACAAG ATCAACAAAA GAGAATTTTG GGTGAAGAAT
TGTATCCAAA GATTGTTGCT ACCGGTAAGG CTCAAGAACC AGAAGCTGCT
GGTAAATCA CTGGTATGAT GTTAGGTTTA GAAACCAAG AAATTTTGGA
TTTGTTAGAT GATGATGAAT TGTTCAATAA CCATTTTCGAA GATGCTTTGA
CTGCTTTTGA AGAGTACAAG AAGTCTGAAG CTGCCGGTAA TGCTGAAGAG
CAAGCTTAAA AGACTTGATT GTTATTGCAG AGAGGAGATG TTGTAACGGA
ATTTATGAAA GTGGGAGATG TCCAAGAATT AATCTAGATT ATAAAGAGAT
TGGGTTTCATA TTTTTTATTN TACTGATATT TCTNTATTAT CGTTGCTTAT
TGTATTCTAT TTGCTACACT TCTTG

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Appendix B

Codon usage of *CaVPS11* and *CaVPS18*

CaVPS11

AmAcid	Codon	Number	/1000	Fraction
Gly	GGG	3.00	3.36	0.11
Gly	GGA	12.00	13.42	0.44
Gly	GGT	8.00	8.95	0.30
Gly	GGC	4.00	4.47	0.15
Glu	GAG	8.00	8.95	0.12
Glu	GAA	60.00	67.11	0.88
Asp	GAT	48.00	53.69	0.83
Asp	GAC	10.00	11.19	0.17
Val	GTG	8.00	8.95	0.24
Val	GTA	9.00	10.07	0.26
Val	GTT	14.00	15.66	0.41
Val	GTC	3.00	3.36	0.09
Ala	GCG	3.00	3.36	0.12
Ala	GCA	7.00	7.83	0.29
Ala	GCT	9.00	10.07	0.38
Ala	GCC	5.00	5.59	0.21
Arg	AGG	0.00	0.00	0.00
Arg	AGA	9.00	10.07	0.64
Ser	AGT	13.00	14.54	0.21
Ser	AGC	1.00	1.12	0.02
Lys	AAG	13.00	14.54	0.16
Lys	AAA	66.00	73.83	0.84
Asn	AAT	63.00	70.47	0.74
Asn	AAC	22.00	24.61	0.26
Met	ATG	7.00	7.83	1.00
Ile	ATA	18.00	20.13	0.23
Ile	ATT	50.00	55.93	0.63
Ile	ATC	11.00	12.30	0.14
Thr	ACG	5.00	5.59	0.09
Thr	ACA	16.00	17.90	0.30
Thr	ACT	23.00	25.73	0.43
Thr	ACC	10.00	11.19	0.19
Trp	TGG	1.00	1.12	1.00
End	TGA	0.00	0.00	0.00
Cys	TGT	13.00	14.54	0.76
Cys	TGC	4.00	4.47	0.24

End	TAG	1.00	1.12	1.00
End	TAA	0.00	0.00	0.00
Tyr	TAT	34.00	38.03	0.94
Tyr	TAC	2.00	2.24	0.06
Leu	TTG	39.00	43.62	0.33
Leu	TTA	56.00	62.64	0.47
Phe	TTT	27.00	30.20	0.63
Phe	TTC	16.00	17.90	0.37
Ser	TCG	6.00	6.71	0.10
Ser	TCA	26.00	29.08	0.41
Ser	TCT	14.00	15.66	0.22
Ser	TCC	3.00	3.36	0.05
Arg	CGG	1.00	1.12	0.07
Arg	CGA	4.00	4.47	0.29
Arg	CGT	0.00	0.00	0.00
Arg	CGC	0.00	0.00	0.00
Gln	CAG	7.00	7.83	0.16
Gln	CAA	36.00	40.27	0.84
His	CAT	14.00	15.66	0.88
His	CAC	2.00	2.24	0.12
Leu	CTG	6.00	6.71	0.05
Leu	CTA	6.00	6.71	0.05
Leu	CTT	9.00	10.07	0.08
Leu	CTC	3.00	3.36	0.03
Pro	CCG	4.00	4.47	0.15
Pro	CCA	8.00	8.95	0.31
Pro	CCT	7.00	7.83	0.27
Pro	CCC	7.00	7.83	0.27

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AmAcid	Codon	Number	/1000	Fraction
Gly	GGG	8.00	9.86	0.35
Gly	GGA	7.00	8.63	0.30
Gly	GGT	7.00	8.63	0.30
Gly	GGC	1.00	1.23	0.04
Glu	GAG	22.00	27.13	0.40
Glu	GAA	33.00	40.69	0.60
Asp	GAT	36.00	44.39	0.82
Asp	GAC	8.00	9.86	0.18
Val	GTG	12.00	14.80	0.26
Val	GTA	12.00	14.80	0.26
Val	GTT	16.00	19.73	0.34

Val	GTC	7.00	8.63	0.15
Ala	GCG	6.00	7.40	0.19
Ala	GCA	10.00	12.33	0.31
Ala	GCT	8.00	9.86	0.25
Ala	GCC	8.00	9.86	0.25
Arg	AGG	0.00	0.00	0.00
Arg	AGA	3.00	3.70	0.23
Ser	AGT	4.00	4.93	0.09
Ser	AGC	2.00	2.47	0.04
Lys	AAG	19.00	23.43	0.23
Lys	AAA	63.00	77.68	0.77
Asn	AAT	44.00	54.25	0.66
Asn	AAC	23.00	28.36	0.34
Met	ATG	15.00	18.50	1.00
Ile	ATA	25.00	30.83	0.36
Ile	ATT	35.00	43.16	0.51
Ile	ATC	9.00	11.10	0.13
Thr	ACG	4.00	4.93	0.12
Thr	ACA	13.00	16.03	0.39
Thr	ACT	8.00	9.86	0.24
Thr	ACC	8.00	9.86	0.24
Trp	TGG	8.00	9.86	1.00
End	TGA	0.00	0.00	0.00
Cys	TGT	9.00	11.10	0.82
Cys	TGC	2.00	2.47	0.18
End	TAG	1.00	1.23	1.00
End	TAA	0.00	0.00	0.00
Tyr	TAT	33.00	40.69	0.72
Tyr	TAC	13.00	16.03	0.28
Leu	TTG	41.00	50.55	0.37
Leu	TTA	33.00	40.69	0.30
Phe	TTT	22.00	27.13	0.67
Phe	TTC	11.00	13.56	0.33
Ser	TCG	7.00	8.63	0.16
Ser	TCA	17.00	20.96	0.38
Ser	TCT	11.00	13.56	0.24
Ser	TCC	4.00	4.93	0.09
Arg	CGG	2.00	2.47	0.15
Arg	CGA	4.00	4.93	0.31
Arg	CGT	3.00	3.70	0.23
Arg	CGC	1.00	1.23	0.08

Gln	CAG	10.00	12.33	0.27
Gln	CAA	27.00	33.29	0.73
His	CAT	13.00	16.03	0.65
His	CAC	7.00	8.63	0.35
Leu	CTG	8.00	9.86	0.07
Leu	CTA	7.00	8.63	0.06
Leu	CTT	18.00	22.19	0.16
Leu	CTC	3.00	3.70	0.03
Pro	CCG	5.00	6.17	0.25
Pro	CCA	9.00	11.10	0.45
Pro	CCT	6.00	7.40	0.30
Pro	CCC	0.00	0.00	0.00