Translational and morphological effects of signalling alcohols on *C. albicans*

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences.

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<td>α</td>
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<tr>
<td>β</td>
<td>Beta</td>
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<td>∆</td>
<td>Delta</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>αa-tRNA</td>
<td>Amino acid-bound tRNA</td>
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<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<tr>
<td>ATF-4</td>
<td>Activating transcription factor 4</td>
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<td>4-[(2-hydroxyethyl)-1-piperazineethanesulfonic acid]</td>
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<td>M</td>
<td>Molar</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>mGTP</td>
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<td>Initiator methionyl-tRNA</td>
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<td>Multi factor complex</td>
</tr>
<tr>
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</table>
Mn  Manganese
Mnk  Mitogen activated protein kinase signal-integrating kinases
mRNA  Messenger RNA
mRNP  Messenger ribonucleoprotein
mTORC  Mammalian target of rapamycin complex 1
n  Nano (prefix)
N. crassa  Neurospora crassa
NIH  National institute of health
Nrf2  Nuclear factor (erythroid-derived 2)-like 2
OD  Optical density
ORF  Open reading frame
P  Phosphorylation
PABP  Poly(A)-binding protein
P-bodies  Processing bodies
PCR  Polymerase chain reaction
PEG  Polyethylene glycol
PERK/PEK  PKR endoplasmic reticulum eIF2α kinase
PKR  Double stranded RNA-activated protein kinase
PMSF  Phenylmethylsulfonylfluoride
Rev  Revolution
RLUC  Renilla luciferase
RNA  Ribonucleic acid
ROS  Reactive oxygen species
Rpm  Revolutions per minute
rRNA  Ribosomal RNA
RT-PCR  Real time-PCR
SC  Synthetic complete
S. cerevisiae  Saccharomyces cerevisiae
SD  Synthetic minimal
SG  Stress granule
siRNA  Small interfering RNA
Sod  Superoxide dismutase
Spp  Species
SUI  Supressor of initiation
TAE  Tris base, acetic acid, EDTA
TC  Ternary complex
TCA  Tricarboxylic acid
Tef1  Translation elongation factor 1
TEMED  Tetramethylethylenediamine
TOR  Target of rapamycin
tRNA  Transfer RNA
T_m  Melting temperature
uORF  Upstream open reading frame
UTR  Untranslated region
UV  Ultraviolet
v/v  Volume per volume
w/v  Weight per volume
WT  Wild-type
YEPD  Yeast extract peptone dextrose
2-DE  Two-dimensional gel electrophoresis
3AT  3-amino-1,2,4-triazole
4E-BP  4E-binding protein
5-FOA  5-fluoroorotic acid
Abstract

*Candida albicans* is a polymorphic yeast that can cause life threatening systemic infections in immunocompromised individuals. One key attribute of *C. albicans* that enhances its pathogenicity is the ability to switch morphologies between filamentous and vegetative modes in response to specific environmental conditions. Stressful changes in such cellular conditions commonly cause a rapid inhibition of global protein synthesis leading to altered programmes of gene expression. In *Saccharomyces cerevisiae*, fusel alcohols signal nitrogen scarcity and induce pseudohyphal growth enabling yeast colonies to spread towards nutrient replete areas. These alcohols also inhibit protein synthesis by targeting the translation initiation factor, eIF2B. eIF2B is the guanine nucleotide exchange factor for eIF2, which supports eIF2-GTP production and represents a key regulated step in translation initiation. eIF2-GTP interacts with Met-tRNA$_{Met}$ to form the ternary complex which is essential for translation initiation. Fusel alcohols target eIF2B leading to reduced levels of ternary complex and reduced protein synthesis.

In *Candida albicans*, a variety of cell biological and genetic assays suggest that fusel alcohols and ethanol inhibit protein synthesis by targeting the translation initiation factor, eIF2B, and they also induce hyphal/pseudohyphal growth, a process that is associated with pathogenesis in *C. albicans*. In contrast to fusel alcohols, farnesol, a quorum sensing alcohol, does not appear to impact upon eIF2B activity. Rather, biochemical and mass spectrometric analysis suggest farnesol affects the interaction of the mRNA with the small ribosomal subunit during translation initiation. Further elucidation of the effect of farnesol on *C. albicans* transcript levels and ribosome association by next generation sequencing gave insight into the genes that are differentially expressed following farnesol treatment. While genes involved in morphological differentiation were generally repressed, those involved in protein synthesis were upregulated, possibly as an adaptive response to inhibition of protein synthesis by farnesol. Intriguingly, the regulation of these functional categories of genes occurred in a co-ordinated manner at either the transcript level or at the level of ribosome association, but rarely was gene expression regulated at both transcriptional and post-transcriptional levels for the same gene.

Nkechi E. Egbe

The University of Manchester

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences. 2014

Translational and morphological effects of signalling alcohols on *C. albicans*
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Communications


Nkechi E. Egbe, Mark P. Ashe. Quorum sensing, protein synthesis and morphological transitions in Candida albicans. Poster presentation- SGM Autumn Conference UK 2013

Nkechi E. Egbe, Mark P. Ashe. Different alcohols inhibit translation initiation by distinct mechanisms in yeast correlating with effects on morphological transitions. Poster presentation-Translation UK 2013


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1. Introduction

1.1: The Problem of Fungal Pathogenicity

Yeast belong to the large and diverse fungal kingdom. These ubiquitous microorganisms appear in various forms and inhabit a whole range of different environments. Fungi are heterotrophic organisms, which possess a chitinous cell wall and sterol containing cell membranes. Several species are pathogens of human or other animals and plants. The most frequently diagnosed fungal infections of humans are caused by pathogens from the genera *Candida*, *Aspergillus* and *Cryptococcus* (Richardson 2005). Single-celled yeasts belong to the phylum Ascomycota and this phylum contains the order Saccharomycetales consisting of the most prominent genera *Saccharomyces* and *Candida*. The genus *Candida* was formerly of the order Cryptococcales. Traditionally, species within the genus have been classified and identified on the basis of a limited number of morphological features such as production of true hyphae, formation of chlamydospores and the assimilation of a range of nutrients as the sole source of carbon or nitrogen. The genus *Candida* is the form genus consisting of an array of yeast species in which no complete sexual cycle has been observed (Kwon-Chung and Bennett 1992; Forche et al. 2008). Although an elaborate mechanism for mating can still be operational (Kim and Sudbery 2011). In *C. albicans*, mating occurs between diploid mating type-like (MTL) a and α strains to generate an a/α tetraploid strain. The resulting tetraploids then undergo efficient, random chromosome loss resulting in diploid cells. (Forche et al. 2008). Mating occurs both under laboratory conditions and different invivo niches (Hull et al. 2000). Efficient mating requires that the diploid cells of opposite mating type first switch morphology from the more common ‘white’ phase to the ‘opaque’ phase and then undergo cell fusion (Miller and Johnson 2002).
Candida species is a normal part of the human microflora. They usually reside as commensal organisms on the skin, in the gastrointestinal tracts and/or in the genitourinary tracts of mammalian hosts. As such, they can be detected in approximately 50% of the human population in this form (Jarvis 1995).

However, Candida species especially C. albicans, is an opportunistic pathogen and if the balance of the normal microflora is disrupted or the immune system is compromised, as in cancer, transplant or AIDS patients, Candida infections pose a specific and major problem; as they can quickly become the cause of morbidity. Individuals with healthy immune systems limit Candida growth at mucosal sites. In contrast, a compromised immune system often leads to mucocandidiasis, oral thrush or systemic candidiasis in which the fungus can spread to all major organs of the body (Pfaller and Diekema 2007). Candidiasis (infection caused by Candida species) is increasing in an ever-expanding population of immunodeficient patients. As a result, Candida species now rank among the top four microbes isolated from clinical specimens (Pfaller and Diekema 2007) and mortality attributed to bloodstream infections caused by Candida species approaches 35% (Pittet and Wenzel 1995).

Common risk factors for bloodstream candidiasis include extremes of age (low-birth weight infants and the elderly), immunosuppression, malignancy with leukopenia, major abdominal surgery, trauma, burns, chemotherapy or radiotherapy as well as exposure to multiple antibacterial agents, central venous catheterization, prolonged length of stay in intensive care units and parenteral nutrition (Jarvis 1995).

The most common species that cause candidiasis is C. albicans, but others, such as C. glabrata, C. parapsilopsis and C. dublinensis, can be associated with disease; however, C. albicans is generally regarded as the most virulent of all Candida species. Pathogenesis of C. albicans requires differential expression of virulence factors at each new stage of the process. Some of these virulence attributes are the ability to exist in
various morphologies, expression of surface adhesion molecules to colonise epithelial surfaces, range of proteolytic enzymes such as secreted aspartyl proteinases (SAP) and lipases, that are required for nutrient acquisition and invasion of epithelial surfaces (Ross et al. 1990; Cutler 1991).

*Candida albicans* is a dimorphic pathogenic fungus alternating between the vegetative (yeast) form and filamentous (pseudohyphae or hyphae) form.

Fusel alcohols such as 1-butanol, isoamyl alcohol, active amyl alcohol are products of amino acid catabolism in yeast, and their production is associated with nitrogen starvation. Fusel alcohols have been shown to affect various cellular processes such as protein translation initiation in *S. cerevisiae* (Ashe et al. 2001) and morphogenesis (pseudohyphal formation) in *S. cerevisiae* and *C. albicans* (Dickinson 1996).

Farnesol, an acyclic sesquiterpene alcohol is produced and secreted predominantly by two species of *Candida*: *Candida albicans* and *Candida dubliniensis*. Apart from transcriptional activation, farnesol has been shown to prevent yeast-to-hyphae transition and most likely as a result inhibits the colonization of several matrices, such as polystyrene or tissues by *C. albicans* (Ramage et al. 2002; Kruppa 2009).

Ethanol is a byproduct of the metabolism of glucose in *S. cerevisiae*, *C. albicans* and other fungi. However *C. albicans* produce less ethanol than *S. cerevisiae*, as their metabolism is more geared towards respiration. Ethanol has been shown to inhibit protein translation in *S. cerevisiae* and induce germ tube formation in *C. albicans* (Pollack and Hashimoto 1985). Ethanol also has a wide use in medical practice due its antiseptic properties against a variety of micro-organisms including *Candida* species.
1.2: Cellular Morphogenesis

The ability to switch between unicellular yeast cells and filamentous forms, such as hyphae and pseudohyphae, in response to diverse stimuli is important for fungal pathogenicity (D'Souza and Heitman 2001). The hyphal form is particularly associated with virulence attributes, including passage through host tissues and defence against immune cells. (Fernandez-Arenas et al. 2007). However, there is no absolute relationship between hyphal growth and pathogenesis because many clinically relevant fungi including Histoplasma capsulatum and Coccidiodes imitis, are pathogenic in yeast form and saprophytic in hyphal form (Nemecek et al. 2006; Wang et al. 2012).

Candida albicans undergoes reversible morphological transitions between ovoid, unicellular budding cells (yeast cells) and chains of filamentous cells (pseudohyphae or hyphae). It is also capable of producing chlamydompires usually believed to be dormant growth forms (Odds 1988). Therefore, C. albicans has the ability to adopt a spectrum of morphologies and mechanisms responsible for morphological switching have received a great deal of attention (Reviewed in Sudbery et al. 2004).

During the switch from vegetative yeast-form growth to the hyphal form, the production of germ tubes appears to represent a key first stage (Biswas and Morschhauser 2005). The conversion of yeast form cells to pseudohyphae occurs as a result of polarized cell division when yeast cells growing by budding have elongated without detaching from adjacent cells. As a result, filaments composed of elongated cells with constrictions at the septa are formed. Sometimes the buds elongate so much that they resemble true hyphae. However as shown in Table 1.1, various subtle distinctions have been identified between hyphae and pseudohyphae (Sudbery et al. 2004).
Table 1.1 Distinguishing features of hyphae and pseudohyphae formed by *C. albicans* (Sudbery *et al.* 2004).

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Hyphae</th>
<th>Pseudohypha</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape</td>
<td>Sides parallel</td>
<td>Sides not parallel</td>
</tr>
<tr>
<td>Septa</td>
<td>No constrictions at septal junctions</td>
<td>Constrictions at mother-bud neck and subsequent septal junctions</td>
</tr>
<tr>
<td></td>
<td>First septum within the germ tube</td>
<td>First septum at the mother bud neck</td>
</tr>
<tr>
<td>Cell width</td>
<td>Width ~2μ</td>
<td>Minimum width ≥ 2.8μ</td>
</tr>
<tr>
<td>Septins</td>
<td>Septin ring within germ tube</td>
<td>Septin ring at mother-bud neck</td>
</tr>
<tr>
<td>Filaments</td>
<td>Less branches, cell cycle not synchronised</td>
<td>Highly branched with cell cycle synchrony</td>
</tr>
</tbody>
</table>

Despite the observed differences between hyphae and pseudohyphae, it is interesting that similar environmental conditions induce these morphologies, with the balance being tipped towards hyphae as the conditions become more extreme (Sudbery *et al.* 2004).

A second morphological switch involves the interconversion between white and opaque forms of *C. albicans*. This is an epigenetic switch that allows rapid and reversible switching between cells in white colonies that are round and cells in opaque colonies that are ellipsoidal (Slutsky *et al.* 1987). Cells from white colonies are similar in shape, size, and budding pattern to cells of common laboratory strains. In contrast, cells from opaque colonies are elongate, or bean shaped, and twice as large as the white cells. ‘Opaque’ cells also undergo filamentation, but they do so in response of different environmental cues than those of ‘white’ cells (Si *et al.* 2013).
Under extreme non-optimal growth conditions rather than forming hyphae or pseudophyphae, *C. albicans* can undergo the formation of chlamydospores, which are round, retractile spores with a thick wall. These are thought to function as dormant cellular forms (Raudonis and Smith 1982; Staib and Morschhäuser 2007).

The yeast to hyphae transition in *C. albicans* is induced in response to several environmental conditions. Parameters that promote hyphal development *in vitro* include temperatures above 37°C, a pH greater than 6.5, serum, proline or N-acetylglucosamine, low dissolved oxygen concentrations and elevated CO₂ concentrations, nitrogen or carbon starvation; while high cells densities inhibit hyphal development (Brown and Gow 1999; Klengel et al. 2005). It has become clear that an increase in temperature to about 37°C is particularly important, being required before most of the above conditions can impact on morphogenesis. An exception to this is embedded *C. albicans* cells which form hyphae even at 25°C (Brown and Gow 1999). The production of unicellular yeast forms is stimulated by lower temperatures and more acidic pH, absence of serum and high concentrations of glucose.

Recently, quorum sensing has been described as a phenomenon contributing to morphogenetic control in *C. albicans*. Dependence on cell density has been shown to be one of the effects that contribute to yeast to hyphae transition *in vitro*; and this has been termed the ‘inoculum effect’ (Kruppa 2009). The inoculum effect is observed when yeast cells are diluted to concentrations less than 10⁶ cells ml⁻¹ in culture medium; conditions which induce germ tube formation. However, when cells are inoculated at higher concentrations (>10⁶ cells ml⁻¹), they remain predominantly in a yeast state. The term inoculum effect has been associated with regulation by quorum sensing (Kruppa 2009) and one of the molecules that is considered important for this is farnesol, a quorum sensing molecule (QSM) of *C. albicans* (Hornby et al. 2001).
Thus the morphological transitions are often a response of the fungus to changing environmental conditions and it has been hypothesized that these form part of a mechanism permitting adaptation to a different biological niche. Evidence supporting this hypothesis includes the fact that strains of *C. albicans* defective in morphogenesis are attenuated in terms of virulence and hence their capacity to cause systemic candidiasis. These experiments correlated the avirulent phenotype of *C. albicans* mutants with that of a failure to form hyphae in a mouse model (Lo et al. 1997). However virulence is a complex trait with both the yeast and hyphal switching of *C. albicans* thought to play vital roles in pathogenesis, as mutants lacking genes responsible for the production of either trait are less virulent (Calderone and Fonzi 2001).

1.3: Morphogenetic signalling pathways in *Candida albicans*

Hyphal development in *C. albicans* is determined by a broad range of signals or culture conditions *in vivo*. According to Liu (2001) many signalling pathways or regulators have been found to influence filamentation in various *in vitro* hyphae-inducing conditions. These signalling pathways are complex and comprise several pathways encompassing crosstalk and feedback. Despite the diversity of external signals promoting dimorphic transition in several fungal species, pathways transducing these external signals to the cellular machinery are highly conserved (D'Souza and Heitman 2001). This has allowed the identification of components of these pathways based on the insight gained from studies of pseudohyphae formation in *S. cerevisiae* (Lengeler et al. 2000).

The major morphogenetic signalling pathways in *C. albicans* are: the Cek1p Mitogen-Activated Protein Kinase pathway, the cyclic Adenosine Monophosphate (cAMP)-dependent Protein Kinase A pathway, the Czf1p pathway (induced by embedding in a
solid matrix) (Brown and Gow 1999) and the Rim 101p pathway induced in response to pH alterations (Liu 2001). In addition, two negative regulators of filamentous growth act through the transcriptional regulator Tup1p (Braun and Johnson 1997) associated with Nrg1p (Braun et al. 2001) and Rfg1p (Kadosh and Johnson 2001) respectively.

1.3.1: The Mitogen-Activated protein kinase (MAP kinase) pathway in C. albicans.

The first morphogenetic signalling components to be identified in C. albicans were members of a mitogen activated protein (MAP) kinase pathway similar to the pheromone signalling and morphogenetic signalling pathway in S. cerevisiae (Monge et al. 2006). Mitogen-activated protein kinase signalling cascades are widely used mechanisms in eukaryotic cells coupling environmental cues to transcriptional regulation. Four MAPKs have been identified in C. albicans (Navarro-Garcia et al. 2005; Roman et al. 2009). They are the Cek1p-mediated pathway involved in morphogenesis and hyphal formation (Whiteway 2000), the Cek2p pathway with a role in mating (Chen et al. 2002). Others are the cell integrity pathway, culminating in the Mk1 MAPK, which plays a role in cell wall construction, the response to certain stress conditions (Navarro-Garcia et al. 2005) and biofilm formation (Kumamoto 2005) and the High Osmolarity Glycerol (HOG) pathway, which enables adaptation to both osmotic and oxidative stress (Alonso-Monge et al. 1999; Arana et al. 2005).

The morphogenetic or Cek1p MAPK cascade of C. albicans comprising the Cst20p (MAPK kinase kinase; PAK (P21-activated protein kinase) (Leberer et al. 1996); Hst7p (MAPK kinase; MEK (MAPK/ERK kinase), Cek1 (MAPK) (Liu et al. 1994; Leberer et al. 1996; Csank et al. 1997) are all required for hyphal morphogenesis, invasive hyphal growth and virulence.
1.3.1.1 The MAP kinase module

The core of the MAPK pathway is a Map kinase module (Cst20p, Hst7p, Cek1p) which is activated by Cst20p (Fig. 1.1). When upstream signals are fed into the MAPK kinase (Cst20p), it becomes phosphorylated and in turn phosphorylates the MAPK kinase, which in turn phosphorylates the MAPK. A MAPK cascade normally transmits and amplifies the signal to downstream transcription factors that generate a specific adaptive response. (Monge et al. 2006). The transcription factor, Cph1p, which is homologous to the Ste12p transcription factor in S. cerevisiae, lies downstream of the MAPK module. (Liu et al. 1994). Null mutants of the MAPK pathway (Cst20, Hst7 or Cek1) or the transcription factor Cph1 all display a defect in hyphal development on solid medium in response to many inducing conditions (Liu 2001).

In addition to these components, homologous MAP Kinase Phosphatases, Cpp1p and Cpp2p, have been identified which regulate filamentous growth in C. albicans (Csank et al. 1997). Disruption of both alleles of the CPP1 gene results in a hyperfilamentous phenotype. The cpp1/cpp2 mutant strains are also reduced for virulence in both systemic and localized models of candidiasis (Csank et al. 1997). Activation of MAPK pathway can occur through Cdc42p, which itself binds with high affinity to the Cst20 kinase (Leberer et al. 1997).

Cek1p MAPK activity and/or expression could also be regulated by quorum sensing (QS). Two QS molecules tyrosol and farnesol recently identified in C. albicans have been shown to be involved in morphogenesis (Hornby et al. 2001) and studies have shown that farnesol reduces the levels of both the HTS7 and CPH1 mRNAs (Sato et al. 2004).

However, a key consideration that was recognised in early studies of this pathway in C. albicans, is that in the presence of serum, all of the MAPK pathway mutants are able to undergo filamentous growth, thus indicating that this pathway is neither the only
mechanism leading to hyphal formation nor is it likely to be the main one in pathogenesis (Liu 2001).

1.3.2: The cAMP – dependent Protein kinase pathway in C. albicans.

The cAMP-PKA pathway also plays a crucial role in filamentation in S. cerevisiae, C. albicans and other filamentous fungi (Lengeler et al. 2000). In C. albicans, an increase in cAMP levels accompanies the yeast to hyphae transition, and inhibition of the cAMP phosphodiesterase induces this transition (Sbie and Gadd 1992).

A number of homologues of S. cerevisiae cAMP-dependent PKA pathway components have been identified in C. albicans including the transmembrane receptor Mep2p (Biswas and Morschhauser 2005), the G-protein coupled receptor Gpr1p, the Ga protein Gpa2p, the G-protein Ras1p, the adenylyl cyclase Cdc35p, the cAMP-dependent protein kinase A and the transcriptional factor Flo8p (Leberer et al. 2001); reviewed in Biswas et al. (2007) (Fig. 1.1).

1.3.2.1 Upstream signalling components

In S. cerevisiae, the cAMP-PKA pathway is activated by a G protein coupled receptor (GPRC) system Gpr1p and the Ga protein Gpa2p; and this system is required for the induction of pseudohyphal and invasive growth. More specifically, GPRI and GPA2 mutants are deficient in this growth switch and this deficiency can be suppressed by the addition of cAMP (Lorenz et al. 2000).

C. albicans GPA2 may function upstream of both the Cek1 MAPK pathway and the cAMP-PKA pathway (reviewed in Biswas et al. 2007). GPA2 (the Ga protein) or GPRI (the receptor) deletion strains of C. albicans show defects in morphogenesis which are reversed by overexpression of downstream components in the pathway or by addition of
dibutyryl-cAMP (dbcAMP). Epistasis analysis showed that GPA2 acts downstream of 
GPR1 in the cAMP-PKA pathway (Miwa et al. 2004).

Another potential upstream component that might signal through PKA is C. albicans 
Ras1p. By analogy with S. cerevisiae where Ras2p regulates morphogenesis pathways 
(Biswas et al. 2007), ras1 mutants of C. albicans are impaired in hyphal growth under 
a wide range of inducing conditions (Leberer et al. 2001) and this morphogenetic defect 
can be reversed by supplementing the growth medium with cAMP or by overexpressing 
components of the MAPK cascade (Leberer et al. 2001). This result highlights the 
crosstalk that occurs between these in morphogenetic differentiation in C. albicans.

1.3.2.2 Adenylase cyclase, Phosphodiesterases and cAMP levels

C. albicans CDC35 is a homologue of the S. cerevisiae adenylyl cyclase gene 
CYR1/CDC35 (Rocha et al. 2001). The presence of a Ras1p binding domain in C. 
albicans Cdc35p, suggests that, similar to other fungal isoforms, C. albicans Cdc35p 
could be regulated by Ras1p. C. albicans cells deleted for both alleles of CDC35 had no 
detectable cAMP levels, suggesting that this gene encodes the only adenylyl cyclase in 
this organism. C. albicans cdc35 mutants are avirulent in a mouse model of infection, 
are unable to form hyphae in most liquid and solid hyphae-inducing media; however, 
they are slow growing (Rocha et al. 2001). The morphogenetic defect can be rescued by 
exogenous cAMP, which points to a role for Cdc35p in the activation of the cAMP-
PKA pathway (Rocha et al. 2001).

Low and high-affinity phosphodiesterase genes (PDE1 and PDE2) have also been 
identified in C. albicans (Bahn et al. 2003; Jung et al. 2005). pde2 mutants have 
increased sensitivity to cell wall and membrane perturbing agents, fail to produce 
normal hyphae in liquid medium and form aberrant hyphae on solid medium (Jung et al.
2005). Under non-hyphae inducing conditions on agar media, the homozygous pde2 mutant forms wrinkled colonies consisting of mixtures of elongated yeast, pseudohyphae and true hyphae. Germ tube formation in liquid media is also accelerated in the pde2 mutant compared to wild-type strains. Therefore, Pde2p is viewed as a negative regulator of cAMP signalling.

1.3.2.3 Protein kinase A (PKA)

The cAMP-dependent protein kinase (PKA) is crucial for growth and cellular differentiation in eukaryotic cells. PKA consists of two catalytic subunits (Tpk1 and Tpk2) that are inactivated by the binding of a heterodimer of regulatory subunits (Bcy1). External signals increase intracellular levels of cAMP, which when bound to the regulatory subunits liberates and thereby activates the catalytic subunits. PKA is therefore thought to phosphorylate and activate the transcription factor Efg1 (Bockmühl and Ernst 2001). C. albicans has only two PKA catalytic subunits, Tpk1p and Tpk2p. Unlike S. cerevisiae in which only one of the three catalytic subunits is an activator and the other two are inhibitors of pseudohyphal growth, both PKA isoforms in Candida albicans are positive regulators of hyphal morphogenesis. However the phenotypic outputs of the two PKA mutants differ, while tpk1 mutants are defective in hyphal formation on solid media, tpk2 mutants are blocked for hyphal formation in liquid medium (Sonneborn et al. 2000). Epistasis analyses place both TPK1 and TPK2 downstream of RAS1 (Bockmühl et al. 2001). The regulatory subunits of PKA in S. cerevisiae and C. albicans are encoded by BCY1 genes. It has been shown that a Tpk1-GFP fusion is dispersed throughout the cell in the bcy1 tpk2 double mutant, while it is normally localised in the nucleus in the wild-type cells. This suggests that C. albicans Bcy1p may tether the PKA catalytic subunit to the
nucleus and thereby perform a vital role in regulating the enzymatic activity and availability of PKA in response to growth (Cassola et al. 2004).

**1.3.2.4 Downstream effectors**

Efg1p, a basic helix-loop-helix (bHLH) protein similar to Phd1p and Sok2p of *S. cerevisiae*, StuAP of *Aspergillus nidulans* and Asm1 of *Neurospora crassa* plays a major role in hyphal morphogenesis (Ernst 2000). Serum-induced morphogenesis, a process that is regulated by the PKA pathway, requires Efg1p. The *efg1* null mutant strain does not form hyphae under most inducing conditions and is defective in the induction of hyphae-specific genes (Liu 2001). Efg1p is therefore considered an activator of hyphal development as it is essential for the activation of hypha-specific genes in *C. albicans* (Sohn et al. 2003). An *efg1 cph1* double mutant strain has an extreme filamentous growth defect and is essentially avirulent in a mouse model of systemic infection (Lo et al. 1997). Another transcription factor, Flo8p, interacts with Efg1 in vivo and is required for both morphogenesis and for virulence (Cao et al. 2006).

**1.3.2.5 Transcriptional repressors (Tup1, Nrg1 and Rfg1) and hyphal/pseudohyphal growth**

As well as activators, transcriptional repressors are important downstream effectors of signalling pathways. Tup1p has been described by Braun and Johnson (1997) to negatively control filamentous growth in *C. albicans*. Tup1p in association with either Nrg1p or Rfg1p negatively regulates the expression of hypha-specific genes (Kadosh and Johnson 2001; Murad et al. 2001; Kadosh and Johnson 2005). Nrg1p mRNA is down regulated by serum and temperature (Braun et al. 2001); hence, the hypha-specific genes controlled by Nrg1p and Tup1p are likely induced as a result of this
down regulation. Thus a specific subset of \textit{C. albicans} genes involved in filamentation are repressed in a Tup1p dependent fashion, however; Nrg1p and another repressor Mig1p also repress other \textit{C. albicans} genes independently of Tup1p (Murad \textit{et al.} 2001). Cells lacking any of these repressors grow constitutively as pseudohyphae, as the expression of hypha-specific genes is constitutively derepressed (Sudbery 2011).

1.3.3 Tec1p and Cph2p: Convergent regulation of Cph1p and Efg1p

Tec1p, a member of the TEA/ATTS family of transcription factors, has been shown to regulate hyphal development in \textit{C. albicans} (Schweizer \textit{et al.} 2000). In \textit{S. cerevisiae}, Tec1p and Ste12p (a homologue of Cph1p in \textit{C. albicans}) form a transcription factor complex to specifically activate genes involved in pseudohyphal growth (Madhani and Fink 1997). In \textit{C. albicans} however; \textit{TEC1} transcription is regulated by Cph2p and Efg1p. Cph2p binds directly to two sterol regulatory element promoter elements upstream of the \textit{TEC1} gene. Furthermore, ectopic expression of \textit{TEC1} suppresses the defect of a \textit{CPH2} deletion in hyphal development.
Figure 1.1 Conserved MAP-kinase and cAMP-dependent PKA pathways regulate morphogenesis in *C. albicans*. Boxes represent transcriptional regulators, green colour represents positive regulators and red colour represents negative regulators. Arrows indicate positive regulation and bars negative regulation. Adapted from Sudbery (2011).
1.3.4: Hyphal Extention Pathway

The *C. albicans* epithelial escape and dissemination 1 (*EED1*) (Zakikhany *et al.* 2007) and *UME6* (Banerjee *et al.* 2008) play a crucial role in extension of germ tubes into elongated hyphae and also in the maintenance of filamentous growth (Martin *et al.* 2011). Expression of *UME6* depends on Eed1, which itself is a target of the transcription factor Efg1 (Doedt *et al.* 2004) (Figure 1.2). Mutants lacking eed1 failed to extend germ tubes into long filaments and switched back to yeast growth after 3 h of incubation; however overexpression of *UME6* restored the hyphal elongation. *UME6* was found to be *NRG1* repressed but under hyphae-inducing conditions may play a role in suppressing *NRG1* transcription (O'Connor *et al.* 2010; Martin *et al.* 2011). *EED1* expression is regulated by Nrg1 and Tup1 as the expression of *EED1* is upregulated in *nrg1Δ* and *tup1Δ* mutants (Doedt *et al.* 2004; Martin *et al.* 2011). *EED1* is a unique species-specific gene of *C. albicans* as no homologous gene was found in any genome sequence accessible via NCBI. Another important target of Ume6 is the kinase *HGC1* encoding a hypha-specific G1 cyclin (Zheng *et al.* 2004). *HGC1* was downregulated in *eed1Δ* and this downregulation was bypassed by overexpression of Ume6 in *eed1Δ*. Hgc1 is involved in the phosphorylation of Efg1.
1.3.4: Summary of Cellular Morphogenesis

A variety of pathways have been identified which impact on morphogenesis leading to alterations in the virulence of *Candida albicans*. Much of the work has relied upon direct comparisons with *S. cerevisiae* pseudohyphal growth. Therefore, signal transduction pathways that lead to changes in gene transcription during the switch to pseudohyphal growth are well studied. It yet remains to be revealed whether control of gene expression at the post-transcriptional level plays a role in this switch (Gancedo 2001), particularly in the human pathogen, *Candida albicans*. As will be covered in more detail in Section 1.10, a variety of alcohols which inhibit protein synthesis also cause changes to morphogenesis, although it is not entirely clear which pathway(s) are

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**Figure 1.2 Eed1 mediates the elongation of germ tubes to hyphae.** Environmental signals transduced via the signalling regulators like Ras1p, Cph1p and Efg1 convert yeast cells into germ tubes. Eed1 and Ume6 are required for the extension of the germ tube into long hyphae. Arrows indicate positive regulation and bars negative regulation Adapted from Martin *et al.* (2011).
involved and how the translational control connects with other aspects of the effects of alcohols on cells.

1.4: The Cell Wall Integrity Pathway in *C. albicans*

It is well established that alcohols have chaotropic effects on membrane structure (Ingram 1981; Ingram 1986). In *C. albicans*, several signaling pathways regulate cell wall and membrane integrity (Lengeler *et al.* 2000; Kruppa and Calderone 2006). Mkc1p (the homologue of *S. cerevisiae* Slt2p MAPK) is involved in regulating cell wall integrity and is required for growth at elevated temperatures in *C. albicans* (Navarro-Garcia *et al.* 1995). A *C. albicans* mkc1 null mutant exhibits increased susceptibility to cell wall perturbing agents and to some inhibitors of cell wall synthesis. The mutant also has defects in morphogenesis, has attenuated virulence and exhibits reduced cell viability at 42°C (Navarro-Garcia *et al.* 1995; Diez-Orejas *et al.* 1997; Zhao *et al.* 2007). In *S. cerevisiae*, the analogous slt2Δ mutant is unable to form pseudohyphae and to penetrate agar (Navarro-Garcia *et al.* 1998). The activation of Slt2p is triggered by various oxidants, certain osmotic stresses, antifungal drugs (targeted at cell wall and membrane synthesis), calcium ions, cold shock and alcohols (Martinez-Anaya *et al.* 2003; Navarro-Garcia *et al.* 2005). Mkc1p is phosphorylated in the presence of these stress conditions; mostly by Pkc1p, but under certain conditions Hog1p is required for Mkc1p phosphorylation, illustrating the interconnection between different MAPK pathways in *C. albicans* (Navarro-Garcia *et al.* 2005).

By analogy with *S. cerevisiae*, the Hog1p MAP kinase pathway is presumed to play a vital role in the biosynthesis of cell wall of *C. albicans* (Kruppa and Calderone 2006). The Hog1p protein is thought to be important in regulating the phosphorylation of the cell wall integrity MAP kinase Mkc1p (Arana *et al.* 2005). Finally, in reference to the work in this thesis, Slt2p (Mpk1p), the cell integrity MAP kinase has been implicated in
the response of *S. cerevisiae* to fusel alcohols and Slt2p is activated upon exposure to fusel alcohols (Martinez-Anaya *et al.* 2003).

### 1.4.1 Fusel Alcohols

Intriguingly, fusel alcohols impact on morphological transitions, the cell wall integrity pathway and protein synthesis simultaneously in *S. cerevisiae*. A number of studies have shown that the nutritional conditions that induce global regulation of protein synthesis are also cues for various forms of filamentous growth in yeasts. For instance, glucose depletion causes a rapid inhibition of translation initiation and also leads to haploid invasive growth (Ashe *et al.* 2000; Cullen and Sprague 2000). In addition, the effects of various alcohols and nitrogen limitation on translation initiation are accompanied by a switch to pseudohyphal growth in the yeast, *S. cerevisiae* (Lorenz *et al.* 2000; Ashe *et al.* 2001; Gancedo 2001).

A number of signal transduction networks in *S. cerevisiae* have been elucidated which play a role in the switch from yeast form to pseudohyphal growth form (Gancedo 2001). As described above, similar signal transduction pathways have also been identified in *C. albicans* and other fungi. The question that remains to be addressed is whether control of gene expression at the level of protein synthesis plays a role in the morphological switch (Gancedo 2001).

Connections between protein synthesis and pseudohyphal growth have been suggested. It was observed in *S. cerevisiae* that a minimal cycloheximide treatment results in a lower proportion of pseudohyphal cells. The authors reasoned that the inhibition of protein synthesis might be contributing to the block in the conversion of vegetative cells to the pseudohyphal form (Kron *et al.* 1994). Ibrahimo *et al.* (2006), investigated whether the regulation of translation initiation could play a role in the *S. cerevisiae*
pseudohyphal response. They showed that the disruption of the genes for either of the yeast eIF4E binding proteins (Eap1p or Caf20p) or other key translational regulators (\textit{GCN2} or \textit{GCN4}) inhibits the capacity of yeast to undergo filamentous growth in response to nitrogen limitation. Mutations in the 4E binding domains in both genes prevent complementation of this phenotype, highlighting the importance of translational control (Ibrahimo \textit{et al.} 2006). To further highlight the connection between protein synthesis and morphological transition in fungi, (Tripathi \textit{et al.} 2002) reported that Gcn4p plays a central role in coordinating morphogenetic and metabolic responses to amino acid starvation in \textit{C. albicans}.

### 1.5: Protein Synthesis

Proteins comprise one of the essential molecules vital for life processes. They account for a large fraction of biological macromolecules, catalyse most of the reactions on which life depends and serve numerous structural, transportational, regulatory and other roles in all organisms. A high proportion of a cell’s energy budget and components are devoted to protein synthesis. For instance, in rapidly growing bacterium protein synthesis consumes 30-50\% of the energy generated (Mathews \textit{et al.} 2007). In addition, a rapidly growing yeast cell contains nearly 200,000 ribosomes occupying as much as 30-40\% of its cytoplasmic volume (Mathews \textit{et al.} 2007).

The transfer of genetic information from DNA to protein occurs via mRNA. In prokaryotes, co-transcriptional translation occurs, whereas in eukaryotes the two processes of transcription (production of mRNA) and translation (production of proteins) are separated as a consequence of eukaryotic cell having a defined nucleus. The process of protein synthesis in both prokaryotes and eukaryotes occurs in three steps: initiation, elongation and termination.
The requirements for high levels of energy, ribosomes, RNAs and other factors necessitates that a process such as protein synthesis be closely monitored and regulated by the cell in order to conserve energy and resources. Control occurs mostly at the initiation stage of protein synthesis. This allows rapid, reversible and spatial control of gene expression. In addition, response at this early stage means that cells respond rapidly to environmental changes in order to prevent further wastage of energy and the accumulation of intermediates as by-products.

1.6: Translation pathway overview
Translation is an extremely complex process by which the information content of mRNA is converted to proteins. The process starts with translation initiation, where the goal is to assemble a full ribosome on the mRNA with an initiator methionyl tRNA (Met-tRNA\textsubscript{i}) in the peptidyl transferase (P) site of the ribosome. Ribosomes are highly conserved between prokaryotes and eukaryotes and have three tRNA binding sites: the acceptor (A) site, the peptidyl transferase (P) site and the exit (E) site. The process by which ribosomes are recruited to mRNAs, however, varies between prokaryotes and eukaryotes. Polycistronic mRNAs are commonly found in prokaryotes. Prokaryotic mRNA contains a sequence called the Shine-Dalgarno sequence, which is complementary to the 5ʹ end of the 16S rRNA subunit and located upstream of the AUG start codon (Shine and Dalgarno 1975). In contrast, eukaryotic mRNA is modified by a 5ʹ cap and 3ʹ poly (A) tail. These modifications not only protect the mRNA from degradation but also facilitate its recognition by the translation initiation machinery.

In eukaryotes, at least 11 translation initiation factors are involved in assembling a translationally competent ribosome on the mRNA: while only three factors are involved in prokaryotic translation initiation. After initiation in both prokaryotes and eukaryotes, the appropriate aminoacylated tRNA enters the A site, a peptide bond is formed.
between the amino acids and the ribosome translocates one codon along the mRNA. In this manner, the mRNA is sequentially decoded into a chain of amino acids and ultimately a protein. This entire process termed translation elongation is GTP-dependent and, in eukaryotes, requires only three elongation factors (eEF1A, eEF1B, and eEF2) (Taylor et al. 2007). Translation is terminated when a stop codon is translocated into the ribosomal A site. The bond which links the last peptidyl-tRNA to the ribosomal P-site is hydrolysed to release the protein and this is facilitated by the release factor eRF1 (Kapp and Lorsch 2004). The process of eukaryotic translation is discussed in greater detail below, this discussion however, will focus mainly on the initiation stage of translation in eukaryotes as this is the stage where most regulation occurs and it is the stage targeted by alcohols in S. cerevisiae.

1.6.1: Translation Initiation - the mechanism of translation initiation in Eukaryotes

Translation initiation is the process that leads to the assembly of an elongation competent 80S ribosome on an mRNA in which the initiation codon is base paired with the anticodon loop of aminoacylated initiator methionyl-transfer RNA (Met-tRNA_{Met}) in the ribosomal P site (Pestova et al. 2007; Jackson et al. 2010). The process requires separated small (40S) and large (60S) ribosomal subunits, initiation factors and the nucleotide triphosphates (ATP and GTP) (Pestova et al. 2007).
Fig. 1.3. **Overview of Translation Initiation:** eIF2B catalyzes guanine nucleotide exchange to produce eIF2-GTP. eIF2-GTP binds to Met-tRNA$_{Met}$ to form a Ternary complex (TC). A multifactor complex made up of TC, eIFs 1, 1A, 3 and 5 binds to the 40S ribosomal subunit to form a 43S pre-initiation complex (PIC). The eIF4F (eIF4E, eIF4G and eIF4A) complex forms a closed loop complex with the mRNA via the interaction of 4G with 4E and Pab1p. PIC is recruited to mRNA by the eIF4F complex and possibly Pab1p to form 48S initiation complex. The 48S complex selects the appropriate AUG (initiation codon), followed by the joining of 60S ribosomal subunit to form 80S initiation complex.

The eukaryotic translation initiation mechanism most widely accepted is that based on the scanning model (Kozak 1991), however, in some eukaryotes and under some circumstances, another method of initiation has been proposed which is independent of...
the 5′ m^3G-cap structure. This method of translation initiation on a few mRNAs involves direct binding of the 40S ribosomal subunit to the mRNA at the internal ribosome entry site (IRESs) located at or near to an AUG codon (reviewed in Hellen and Sarnow (2001)).

Prior to translation, the mRNA transcript must be synthesized in the nucleus and processed by capping, splicing and polyadenylation, after which it is exported to the cytoplasm. In addition the post-termination ribosomal complexes, which comprise an 80S ribosome still bound to mRNA, deacetylated tRNA and eukaryotic release factor 1 (eRF1) (Jackson et al. 2010) must dissociate. Ribosomes are dissociated and are thought to be released from the mRNA as free 60S and factor-associated 40S subunits to provide a pool of separated ribosomal subunits needed for translation. This dissociation is aided by the eukaryotic factors eIF3, eIF1 and eIF1A (Kolupaeva et al. 2005).

1.6.1.1 Formation of the 43S pre-initiation complex

Initiation of protein synthesis begins with the assembly of eIF2, GTP, Met-tRNA$_{i}^{Met}$ into a ternary complex. eIF2 in the GTP bound form interacts specifically with the initiator methionyl tRNA. The resulting ternary complex is responsible for delivering the Met-tRNA$_{i}^{Met}$ to the 40S subunit to form the 43S pre-initiation complex. Formation of this complex is also promoted by eIF1, eIF1A and eIF3 (Hershey and Merrick 2000). Some of these factors bind the 40S subunit before the ternary complex, likely as a result of their prior role in subunit dissociation (Pestova et al. 2007). The initiation factors eIF1, eIF3, eIF5 and eIF2- Met-tRNA$_{i}^{Met}$ can also form a multifactor complex (MFC) which exists both free of and bound to the ribosome (Asano et al. 2000). Interactions between the MFC components co-operatively increase their affinity for the 40S subunit. The resulting 43S pre-initiation complex is competent for binding to the 5′ end of most mRNAs.
1.6.1.2 mRNA preparation and selection

Recruitment of the 43S preinitiation complex to mRNA is mediated by members of the eIF4F group of initiation factors, which is a heterotrimer containing eIF4A (an ATPase/RNA helicase of the DEAD box family), eIF4G (a large modular protein which functions as a scaffold that binds the other factors) and eIF4E (the cap binding protein) (Pestova et al. 2007). mRNA selection for translation is prompted by eIF4E and Poly (A) binding protein (PABP; Pab1p in yeast), which bind to the mRNA 5′ cap structure and 3′ poly(A) tail respectively. These proteins both interact with separate domains of eIF4G. eIF4G can therefore interact simultaneously with eIF4E and the Poly (A) binding protein bridging across the 5′ cap and 3′ poly (A) tail structures to form a closed-loop mRNP complex (Wells et al. 1998). This ‘circularisation’ of the mRNA likely explains the synergistic effect of the mRNA cap and poly (A) tail structures on translation initiation (Tarun and Sachs 1995; Wells et al. 1998). eIF4G also recruits the eIF4A RNA helicase which has been proposed to function in the removal of secondary structure from the 5′ end of the mRNA (Oberer et al. 2005).

1.6.1.3 43S preinitiation complex recruitment to the mRNA and mRNA scanning

One of the least well understood aspects of the translation initiation process is the mechanism by which the primed closed loop mRNA complex interacts with the 43S pre-initiation complex. In higher eukaryotes an interaction between eIF3 on the 43S complex and eIF4G on the mRNA is thought to be critical (Lamphear et al. 1995; Korneva et al. 2000). There is no report of similar interaction in S. cerevisiae. However, it has been reported that yeast eIF5 can interact with the carboxy-terminal half of eIF4G, and could act as a link between eIF3 and the mRNA (Asano and Hinnebusch 2001).
After the binding of the pre-initiation complex at the 5’ end of the mRNA, the complex scans the mRNA in a 5’ to 3’ direction until a ‘start’ codon is encountered (Kozak 2005). In eukaryotes, the start codon is usually the first AUG triplet that the ribosome encounters and is identified by virtue of complementarity to the Met-tRNAi anticodon (reviewed in Kapp and Lorsch 2004).

1.6.1.4 AUG recognition and 60S subunit joining

Binding of the 40S ribosomal subunit to the start codon results in a pause in scanning and triggers GTP hydrolysis on eIF2 by eIF5, a GTPase activating protein (GAP). This process is regulated by the initiation factors eIF1, eIF1A and eIF3 which prevent premature hydrolysis to limit initiation within poor sequence context or at non-AUG start codons (Majumdar and Maitra 2005). Specific mutants in eIF1 are deficient in discriminating correct start codons exhibiting increased initiation at UUG codons (Yoon and Donahue 1992). eIF1 dissociation from the pre-initiation complex mediates release of P_i following GTP hydrolysis on eIF2 upon AUG recognition, a process facilitated by eIF1A (Cheung et al. 2007). eIF2.GDP, eIF5, eIF1 and eIF3 are thought to be released from the complex following GTP hydrolysis, whereas eIF1A remains associated to recruit eIF5B.GTP. GTP hydrolysis on eIF5B promotes eIF1A dissociation from the ribosomal A site to drive 60S subunit joining leading to the formation of the 80S initiation complex. This process leaves the initiator methionyl tRNA in the ribosomal P site with its anticodon base-paired to the mRNA start codon (Lee et al. 2002). The ribosomal A site is now accessible to an incoming aminoacylated tRNA (aa-tRNA) during the elongation phase of translation (Hershey and Merrick 2000).
1.6.1.5 The eIF2 guanine nucleotide exchange cycle, eIF2B and the ternary complex

After hydrolysis of GTP to generate eIF2.GDP, the release of GDP from eIF2 is very slow, so to function in multiple rounds of initiation, eIF2 requires a guanine nucleotide exchange factor (GEF), eIF2B. The exchange factor increases the rate of exchange of GDP with GTP by at least ten-fold (Williams et al. 2001). Interestingly the guanine nucleotide exchange step can occur at a defined cytoplasmic focus within the cell, where eIF2B is a resident feature and eIF2 cycles rapidly through the focus (Campbell et al. 2005). One possibility is that the eIF2/eIF2B focus could serve to concentrate eIF2B, which is sub-stoichiometric to eIF2, to enable sufficient guanine exchange in rapidly growing yeast cells (von der Haar and McCarthy 2002; Campbell et al. 2005). After replacement of GDP with GTP on eIF2, the factor switches into a form with high affinity for Met-tRNA\textsubscript{Met} (Kapp and Lorsch 2004). The affinity of the initiator methionyl tRNA for eIF2.GTP is 15-fold tighter than for eIF2.GDP as measured using \textit{S. cerevisiae} components. This is important for release of the tRNA by eIF2.GDP during start codon recognition (Kapp and Lorsch 2004). However a new regulatory function of eIF5 in the recycling of eIF2 from its inactive eIF2.GDP form has recently been described (Jennings and Pavitt 2010). eIF5 acts as a GDP dissociation inhibitor (GDI) by stabilizing the binding of GDP to eIF2 and restricts the recycling of eIF2 to ternary complex.

1.6.2 Translation initiation via other mechanisms -cellular internal ribosome entry sites

During viral infection, apoptosis, heatshock or hypoxia, cap-dependent translation is often compromised (Johannes et al. 1999; Qin and Sarnow 2004; Bushell et al. 2006). Under such conditions, a subset of mRNAs are able to initiate translation in a 5' cap
independent manner, through secondary structure elements within their 5’ UTR called internal ribosomal entry sites (IRES). IRES elements are able to recruit the ribosome directly to, or in close proximity to the start codon and coordinate translation initiation with a limited number of translation initiation factors and IRES trans-acting factors (ITAFs). mRNAs where translation is instigated by an IRES are generally not translated efficiently under normal conditions, and may require a down regulation of cap-dependent translation for their expression (Merrick 2004; Qin and Sarnow 2004). In addition, many viral RNAs contain IRESs, which mediate their translation after viral infection. The host cell translation is shut-off as the viral RNA often encodes proteases that cleave initiation factors and the presence of the viral dsRNA triggers eIF2α phosphorylation by the eIF2α kinase, PKR (See Section 1.8.1.1). Different viral IRESs vary greatly in their translation initiation factor requirements. For instance, the EMCV (encephalomyocarditis virus) IRES minimally requires eIF4A and the central domain of eIF4GI, eIF2, eIF3 and eIF4B, whereas HCV (hepatitis C virus) IRES requires only eIF2 (Pestova et al. 1996; Pestova et al. 1998). In an extreme case the cricket paralysis virus IRES requires no canonical translation initiation actors, not even the initiator methionyl tRNA (Jan and Sarnow 2002). The differential factor requirements mean that IRES-containing mRNAs are often resistant to conditions that reduce global translation initiation. The fact that many of the proteins encoded by these mRNAs facilitate recovery from stress provides a rationale for this alternative mechanism for translation initiation.

1.6.3 Translation Elongation

Translation elongation is mediated by ribosomes and multiple soluble factors, most of which are conserved across bacteria and eukaryotes. During elongation, the ribosome catalyses the addition of new amino acids via the amino acylated-tRNA (aa-tRNAs) to a
growing polypeptide chain, according to the sequence of codons in the mRNA assisted by eukaryotic elongation factors (eEFs) (Taylor et al. 2007). At the onset of translation elongation, a Met-tRNA\textsubscript{i} is already present in the P site of the ribosome and the eukaryotic elongation factor 1A (eEF1A; EF-Tu in bacteria) binds and recruits aa-tRNAs to the A-site of the ribosome. The aa-tRNA, eEF1A and GTP exist as a ternary complex. As a result of stringent codon-anticodon base pairing, only the cognate aa-tRNA is accepted at the A site (Ogle et al. 2001). Codon-anticodon base pairing produces a conformational change in eEF1A that leads to GTP hydrolysis of the ternary complex. Upon GTP hydrolysis, the eEF1A.GDP complex is released from the ribosome, leaving the aa-tRNA in the A site. Spontaneous GDP dissociation from eEF1A is slow, so the eEF1Bαβγ complex, guanine nucleotide exchange factor, stimulates the exchange of GDP for GTP maintaining the level of active eEF1A.GTP (Slobin and Moller 1978). Peptide bond formation then occurs to leave the nascent peptide fleetingly associated with the A site tRNA (Rodnina and Wintermeyer 2001). Rapid translocation of the ribosome relative to the mRNAs and tRNAs leaves the A site free to accept the next aa-tRNA (Taylor et al. 2007). This translocation step is facilitated by eEF2 (EF-G in bacteria) which promotes ribosome movement along the mRNA (Taylor et al. 2007). Fungal translation elongation is striking in its absolute requirement for a third factor, the ATPase eEF3. eEF3 binds close to the E-site of the ribosome and has been proposed to facilitate the removal of deacylated tRNA from the E-site (Sasikumar and Kinzy 2014). The whole elongation cycle is repeated until a stop codon is reached.
Figure 1.4 Structure of the ribosome showing the large and the small subunits. The ribosomal tRNA binding sites are the aminoacyl (A), the peptidyl (P) and the exit (E). Each of the ribosomal subunits contributes to these EPA sites. Adapted from Russel P. J. (2010)

1.6.4 Translation Termination

Translation termination occurs when a stop codon is translocated into the ribosomal A site. There are three stop codons in eukaryotes, UAA, UAG, and UGA (Kapp and Lorsch 2004). The eukaryotic release factor 1 (eRF1) encoded by the SUP45 gene in S. cerevisiae recognises the stop codons. One model is that eRF1 is a functional mimic of the tRNAs and through its binding to the A site of the ribosome triggers the hydrolysis of the peptidyl-tRNA, resulting in the release of the peptide chain. A second eukaryotic release factor eRF3, encoded by the SUP35 gene in S. cerevisiae accelerates this process in a GTP-dependent manner (Baierlein and Krebber 2010; Khoshnevis et al. 2010). Additional factors have also been described to play roles in this process. For instance, the DEAD box RNA helicase Dbp5p has recently been shown to assist eRF1 in stop codon recognition and controls the subsequent eRF1-eRF3 interaction through its dissociation from eRF1 (Gross et al. 2007).
1.6.5 Ribosome recycling

At the end of the termination stage the ribosome exists as an 80S complex on the mRNA with a deacylated tRNA. For the ribosomal subunits to be used in another round of translation, they must be dissociated and be recycled. eIF3, one of the factors proposed to be involved in recycling, has anti-association activity in that it binds to the 40S subunit lowering the rate of association with 60S (Valasek et al. 2003). Under stress conditions where translation initiation is inhibited, the idle ribosomal subunits reassociate to form a large pool of non-translating 80S ribosomes. During recovery from these stress conditions, these inactive ribosomes need to be mobilised for translation restart. Dom34p-Hbs1p complex, together with the Rli1p NTPase have been implicated in the dissociation of such inactive ribosomes (van den Elzen et al. 2014).

1.7 Detailed steps in the Translation Initiation pathway

Two specific aspects of the translation initiation pathway are of particular importance in terms of both the work in this thesis and the regulation of translation initiation. These are the ternary complex cycle and the selection of mRNA for translation. Therefore these will be covered in greater detail in the following sections.

1.7.1 The ternary complex cycle

As described above, the ternary complex consists of eIF2.GTP bound to Met-tRNA\textsubscript{Met}. This complex delivers the initiator methionyl tRNA and provides chemical energy in the form of GTP for the structural rearrangements that occur following AUG recognition. eIF2.GDP is recycled back to eIF2.GTP via the guanine nucleotide exchange factor eIF2B.
1.7.1.1 Initiator tRNA

In all kingdoms of life, two different methionyl tRNAs are used in protein synthesis: elongator methionyl tRNA \(\text{Met-tRNA}_e\) and initiator methionyl tRNA \(\text{Met-tRNA}_i\). The Met-tRNA\(_i\) initiates protein synthesis, whereas Met-tRNA\(_e\) inserts methionine at internal sites in the growing polypeptide during elongation (Pestova et al. 2007). Initiator methionyl tRNA reads the start codon, allowing the initiating ribosome to begin translation in the correct location.

Initiator methionyl tRNA has a unique sequence and structural features that enable it to be specifically recognized by eIF2 and excluded from binding to elongation machinery (see 1.6.1.5). These features are important for productive binding in the P site (Pestova et al. 2007). The base pair at position 1:72 in the acceptor stem is an important contributor to initiator/elongator discrimination. In Met-tRNA\(_i\), the A1:U72 base pair at the top of the acceptor stem is crucial for binding to eIF2.GTP (Farruggio et al. 1996), while elongator tRNAs generally bear a G:C base pair in the 1:72 position. Two initiator-specific G:C base pairs in the anticodon stem are also important for the binding of the eIF2.GTP.MettRNA\(_i\) to the 40S subunit (Astrom et al. 1993).

1.7.1.2 eIF2

eIF2 is a highly conserved heterotrimeric complex consisting of \(\alpha\), \(\beta\), and \(\gamma\) subunits encoded by the yeast genes \(SUI2\), \(SUI3\) and \(GCD11\) respectively (Hershey and Merrick 2000). Table 1.2 shows the nomenclature used for eIF2, eIF2B subunits, protein kinase (Gcn2p) and transcription factor (Gcn4p) in both \(S.\ cerevisiae\) and \(C.\ albicans\). The \(\alpha\) subunit of eIF2 contains a highly conserved region in which Ser51 can be phosphorylated. A number of stress and metabolite-activated kinases phosphorylate eIF2\(\alpha\), resulting in a stable, unproductive interaction with eIF2B (see section 1.8.1.1). The \(\beta\) subunit of eIF2 is involved in binding to both eIF2B and eIF5. The catalytic
domain in the ε subunit of eIF2B and the C-terminus of eIF5 contain a conserved region which binds to the same sequence in their common substrate, eIF2β (Asano et al. 1999; Gomez and Pavitt 2000). The γ subunit has binding sites for GTP and Met-tRNA\textsubscript{i{\textsuperscript{Met}}} (Gaspar et al. 1994). Mutations of amino acids in eIF2γ proposed to be vital for tRNA binding result in reduced affinity for Met-tRNA\textsubscript{i{\textsuperscript{Met}}} (Kapp and Lorsch 2004).

1.7.2 eIF2B

eIF2B is a heteropentameric complex consisting of α, β, γ, δ, and ε, encoded by GCN3, GCD7, GCD1, GCD2 and GCD6 respectively. In S. cerevisiae all the eIF2B subunits, apart from the α subunit, are essential for growth and mutations in these subunits can lead to a temperature sensitive phenotype and derepression of GCN4 translation (Gcd-phenotype) indicative of reduced ternary complex levels (Krishnamoorthy et al. 2001). The eIF2B subunits are able to form two stable sub-complexes, a regulatory complex consisting of the α, β and δ subunits and a catalytic complex consisting of the ε and γ subunits (Pavitt et al. 1998). The regulatory subcomplex is able to bind eIF2, showing a higher affinity for the phosphorylated form of this protein; however it lacks guanine nucleotide exchange function. The catalytic subcomplex is sufficient for guanine nucleotide exchange only and in fact shows a faster rate of exchange than the wild type. This essential reaction is catalysed by the carboxy-terminal segment of eIF2Be, which interacts directly with the G domain of eIF2γ and with lysine-rich regions of eIF2β (Gomez and Pavitt 2000; Gomez et al. 2002). Therefore, it has been proposed that the regulatory subcomplex is required to mediate binding of eIF2 to eIF2B and to prevent exchange on phosphorylated eIF2 (Pavitt et al. 1998).

The activity of translation initiation factors in C. albicans is less well understood. However similar to S. cerevisiae only one C. albicans ORF (GCN2; orf19.6913) encodes a protein with significant sequence similarity to S. cerevisiae Gcn2p (37%
identity). The *C. albicans* Gcn2p protein sequence contains pseudo kinase, eIF2α kinase, and histidyl-tRNA synthetase-like domains. It also displays significant sequence similarity to *GCN2* eIF2α kinases from mammals, flies, and plants (Tournu et al. 2005).
Table 1.2 Protein subunits and gene names for eIF2, eIF2B, the protein kinase Gcn2p and the transcription factor Gcn4p

<table>
<thead>
<tr>
<th>Name of protein</th>
<th>Mass in kDa</th>
<th>Gene in S. cerevisiae</th>
<th>Gene in C. albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF2α/Sui2p</td>
<td>34.7</td>
<td>SUI2/YJR007W</td>
<td>SUI2/C1_06960W_A</td>
</tr>
<tr>
<td>eIF2β/Sui3p</td>
<td>31.6</td>
<td>SUI3/YPL237W</td>
<td>SUI3/C7_04130C_A</td>
</tr>
<tr>
<td>eIF2γ/Gcd11p</td>
<td>57.9</td>
<td>GCD11/YER025W</td>
<td>GCD11/C5_02170_A</td>
</tr>
<tr>
<td>eIF2βα/Gcn3p</td>
<td>34.0</td>
<td>GCN3/YKR026C</td>
<td>GCN3/C7_01220W_A</td>
</tr>
<tr>
<td>eIF2ββ/Gcd7p</td>
<td>42.6</td>
<td>GCD7/YLR291C</td>
<td>GCD7/C2_03990W_A</td>
</tr>
<tr>
<td>eIF2βγ/Gcd1p</td>
<td>65.7</td>
<td>GCD1/YOR260W</td>
<td>GCD1/CR_03990C_A</td>
</tr>
<tr>
<td>eIF2βδ/Gcd2p</td>
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<td>GCD2/YGR083C</td>
<td>GCD2/C3_07250W_A</td>
</tr>
<tr>
<td>eIF2βε/Gcd6p</td>
<td>81.1</td>
<td>GCD6/YDR211W</td>
<td>GCD6/C1_08600C_A</td>
</tr>
<tr>
<td>Gcn2p</td>
<td>190.1</td>
<td>GCN2/YDR283C</td>
<td>GCN2/C7_01330_A</td>
</tr>
<tr>
<td>Gcn4p</td>
<td>31.3</td>
<td>GCN4/YEL009C</td>
<td>GCN4/C2_09940W_A</td>
</tr>
</tbody>
</table>

**SUI** = Suppressor of initiation  
**GCN** = General control nonderepressible  
**GCD** = General control derepressed
1.7.3 mRNA selection pathway

As described above the selection of mRNA relies upon interactions of the mRNA cap and poly(A) tail with the translation machinery. eIF4E interacts with the cap and Pab1p with the poly(A) tail. eIF4G serves as a scaffold between the two and also interacts with the ATP-dependent RNA helicase eIF4A. In addition, eIF4E binding proteins (4E-BPs) can competitively inhibit the interaction between eIF4G and eIF4E to regulate translation initiation (see section 1.8.2). Table 1.3 shows the protein subunits and gene names of the eIF4F complex, as well as the interacting proteins Pab1 and the ribosomal protein Rps3 in *S. cerevisiae* and *C. albicans*.

1.7.3.1 eIF4E

The *S. cerevisiae* eIF4E is encoded by a single essential gene *CDC33* (Altmann *et al.* 1987). The structural features of eIF4E shed light on the biological properties of this factor. The amino terminal part of eIF4E is dispensable for cap recognition, for binding to eIF4G and 4E-BP, for stimulation of cap-dependent translation, and for in vivo functionality of the protein (Hernandez *et al.* 2005). On the other hand, the sequence and three dimensional structure of eIF4E carboxy-terminal part contains all the residues important for functionality and is highly conserved. Due to its central role in cap-dependent translation, regulation of eIF4E activity is critical to normal cell growth (Lachance *et al.* 2002; Richter and Sonenberg 2005). eIF4E binds directly to the cap structure at the 5′ end of the mRNA. The binding site contains two tryptophan residues which are stacked either side of the m7GTP nucleotide on the cap (Matsuo *et al.* 1997).
1.7.3.2 PABP

The single-copy essential gene \textit{PABI} encodes yeast PABP (Sachs 2000). In yeast cell-free extracts, a Pab1p-poly(A) complex interacts with eIF4G which in turn interacts with eIF4E bound to the mRNA cap (Tarun and Sachs 1996). Mutations within the yeast \textit{PABI} gene resulted in an inhibition of translation and cell growth (Sachs and Davis 1989). The poly(A) binding protein family carry four non-equivalent RNA-recognition motifs (RRMs) and a C-terminal domain. In yeast Pab1p, RRM3 and 4 and the C-terminus do not contribute to the translational activities of the protein (Kessler and Sachs 1998). Further dissection of eIF4G-Pab1p interaction revealed that the first and second RRM5 of Pab1p are needed for eIF4G binding and that substitution of as few as two amino acids within RRM2 of Pab1p can prevent this binding (Kessler and Sachs 1998; Otero et al. 1999). The consequence of the simultaneous association of eIF4E and Pab1p with eIF4G is that the mRNA could become circularised. As a result Pab1p would promote translation initiation by stabilisation of eIF4G on the mRNAs and hence increased recruitment of the 43S complex to the mRNA. The so-called ‘closed loop’ hypothesis is supported by the direct demonstration of circular polyribosomes in electron micrographs of serial sections through rough ER membranes (Tarun and Sachs 1996; Sachs 2000). It has also been suggested that Pab1p may mediate translational activation by inducing 60S subunit joining. The observation that 60S subunit protein mutants suppress \textit{pab1}Δ phenotypes provides some support for such a role (Sachs and Davis 1989; Searfoss et al. 2001).

1.7.3.3 eIF4A

eIF4A is the archetypal member of the ATP-dependent RNA helicase family (Schmid and Linder 1991; Schmid and Linder 1992). Conserved sequences within eIF4A couple ATP binding and hydrolysis to RNA helicase activity in order to disrupt RNA-RNA
interactions; thereby ‘melting’ secondary RNA structures within a transcript (Tanner et al. 2003; Marsden et al. 2006). The effect of mRNA secondary structure on translation initiation is varied but can have an inhibitory role by disrupting initiation factor function or ribosome transit on the RNA (Tanner et al. 2003; Marsden et al. 2006). An A66V substitution in yeast eIF4A, which prevents ATP-hydrolysis, abolishes its ability to rescue depleted translation extracts (Blum et al. 1992). The helicase activity of eIF4A is enhanced through its interactions with eIF4G. In S. cerevisiae however, eIF4A is not as stably associated with eIF4E and eIF4G. eIF4A interacts with the middle domain of eIF4G through interactions in both N-terminal and C-terminal domains. This interaction seems to stabilise the conformation of eIF4A and enhance the accessibility of its RNA and ATP-binding surfaces (Oberer et al. 2005). A number of conditions have been described that affect the association of eIF4A with the eIF4F complex or the helicase activity of eIF4A. For instance, glucose starvation in yeast has been shown to lead to the loss of eIF4A from the eIF4G-containing complex which is thought to inhibit global translation initiation (Castelli et al. 2011). (Cruz-Migoni et al. 2011) recently showed that the toxin produced by the bacterium, Burkholderia pseudomallei causes a translational block by inhibiting the helicase activity of eIF4A and this manifests in the human disease melioidosis. Furthermore during mitosis, intense phosphorylation of Ser1232 in eIF4G by the cyclin dependent kinase 1:cyclin B complex strongly enhances the interaction of eIF4A with the HEAT domain 2 of eIF4G and decreases the association of eIF4G/eIF4A with RNA. This results in the inhibition of eIF4A helicase activity leading to suppression of global translation (Dobrikov et al. 2013).

1.7.3.4 eIF4G

eIF4G is a large modular protein which in mammalian systems acts as scaffolding protein with binding sites for eIF4E, eIF4A, eIF3 and PABP. In S. cerevisiae, there are
two isoforms of eIF4G; eIF4G1 and eIF4G2, which are 50% identical and encoded by the genes TIF4631 and TIF4632. Deletion of both genes is lethal, but the presence of either gene alone is sufficient to support growth (Goyer et al. 1993). Yeast cells deleted for TIF4631, but not TIF4632 shows slow-growth (slg-) phenotype and translation initiation defect (Clarkson et al. 2010) consistent with the fact that eIF4G1 is expressed at higher levels than is eIF4G2 (Tarun and Sachs 1996; Clarkson et al. 2010). However at high cell density, eIF4G2 mRNA is expressed at higher levels compared to eIF4G1 which may imply a preferential role in nutrient limited cells (Gasch et al. 2000). Yeast eIF4G has binding sites for eIF4E, eIF4A and Pab1p, but not eIF3. In fact the eIF3ε subunit in mammalian cells that appears to be involved in binding to eIF4G is not conserved in yeast (LeFebvre et al. 2006). Therefore, direct interactions between eIF4G and eIF3 have not been shown, rather it has been suggested that eIF5 and eIF1 act as bridges between eIF4G and the 43S complex to promote 48S complex formation (He et al. 2003). The interaction between eIF4G and eIF4A is important in recruiting 43S complex to the cap. Mammalian eIF4G contains two eIF4A binding domains, one in the central domain and one in the C-terminal domain. The central domain binding site is conserved in yeast, but the C-terminal binding domain is absent (Imataka and Sonenberg 1997). The interaction between eIF4G and eIF4A is dependent on conserved residues within the yeast eIF4G binding site and the central domain binding site (Dominguez et al. 2001). It is becoming increasingly clear that eIF4A and possibly the interaction with eIF4G may be a site of regulation for protein synthesis (as described above).

C. albicans has only a single eIF4G gene encoded by TIF4631, and overexpression of this gene causes hyperfilamentation (Lee et al. 2005). Interestingly, Candida eIF4G is induced by conditions promoting hyphal growth and by macrophages (Lorenz et al. 2004).
The gene for *Candida* eIF4E has also been described (Lorenz *et al.* 2004; Lee *et al.* 2005; Feketova *et al.* 2010). This gene, *CaCDC33*, contains one CUG codon at position 116 of the amino acid residues (Feketova *et al.* 2010), but overall the sequence similarity between *CaCDC33* and *ScCDC33* is 54% identical at the amino acid level. Indeed a *S. cerevisiae cdc33* null mutant is complemented by *CaCDC33* in terms of lethality (Feketova *et al.* 2010). Interestingly, eIF4E can naturally exist as Ca4E116<sup>ser</sup> and Ca4E116<sup>Leu</sup> (CUG codon at position 116 is encoded as serine or leucine) in *C. albicans*. The mutation of the CUG codon into the universally serine-coding UCU triplet alleviates the temperature sensitivity of the Ca4E translation initiation factor in the *S. cerevisiae*. (Feketova *et al.* 2010).

Table 1.3 The protein subunits and gene names of the eIF4F complex, as well as the interacting proteins Pab1 and the ribosomal protein Rps3

<table>
<thead>
<tr>
<th>Name of protein</th>
<th>Mass in kDa</th>
<th>Gene in <em>S. cerevisiae</em></th>
<th>Gene in <em>C. albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4G/Tif4631p</td>
<td>107</td>
<td><em>TIF4631/YGR162W</em></td>
<td><em>TIF4631/C2_08760C_A</em></td>
</tr>
<tr>
<td>eIF4E/Cdc33p</td>
<td>24.2</td>
<td><em>CDC33/YOL139C</em></td>
<td><em>EIF4E/CR_10490W_A</em></td>
</tr>
<tr>
<td>eIF4A/Tif1p</td>
<td>44.6</td>
<td><em>TIF1/YKR059W</em></td>
<td><em>TIF1/C1_01350C_A</em></td>
</tr>
<tr>
<td>Pab1p</td>
<td>64.3</td>
<td><em>PAB1/YER165W</em></td>
<td><em>PAB1/C1_03370W_A</em></td>
</tr>
<tr>
<td>Rsp3p</td>
<td>26.5</td>
<td><em>RPS3/YNL178W</em></td>
<td><em>RPS3/CR_04810W_A</em></td>
</tr>
</tbody>
</table>
1.8: Regulation of protein translation initiation

The regulation of protein synthesis allows a rapid and dynamic cellular response to stresses such as amino acid starvation, glucose starvation, viral infection, hypoxia, heat shock and exposure to heavy metals. The vast majority of such stress conditions regulate protein synthesis by targeting the translation initiation step (Simpson and Ashe 2012). This global inhibition has an immediate effect on protein levels and allows the cell to re-channel resources toward stress survival (Yamasaki and Anderson 2008; Simpson and Ashe 2012). Mechanisms by which translation initiation can be regulated fall into two main categories: the control of eIF2 or of eIF4F, often by reversible protein phosphorylation (Jackson et al. 2010) (Fig. 1.5).
Figure 1.5. Translational initiation control via: a, eIF2α phosphorylation and b, binding of the hypophosphorylated 4E-BP (the cap-binding protein). Phosphorylation of 4E-BPs and their regulation by mTOR has been identified in mammalian systems, whereas in *S. cerevisiae* TOR targets the eIF2α kinase, Gcn2p. Figure adapted from Gebauer and Hentze (2004).

1.8.1: Regulation by activation of the stress responsive eIF2α kinases

As described above in detail, eIF2 forms a complex with Met-tRNA$_{\text{Met}}$ and GTP and in conjunction with the 40S ribosomal subunit, participates in the selection of the start codon. In *S. cerevisiae* there is just a single eIF2α kinase, Gcn2p, which is activated in response to a number of stress conditions including amino acid starvation (Dever *et al.* 1992), purine starvation (Rolfes and Hinnebusch 1993), rapamycin (Kubota *et al.* 2003) and peroxide (Shenton *et al.* 2006). Following amino acid starvation, there is an accumulation of uncharged tRNA, which binds to the regulatory histidyl-tRNA...
synthetase-like (HisRS) domain of Gcn2p: located C-terminal to the protein kinase domain (Hinnebusch 1994; Wek et al. 1995). It has been proposed that this binding induces a conformational change in Gcn2p thereby activating the adjacent protein kinase domain to induce phosphorylation of eIF2α (Wek et al. 1995). The phosphorylated eIF2α binds tightly to and sequesters the guanine nucleotide exchange factor, eIF2B, thus prevents the recycling of eIF2-GDP to the active eIF2-GTP that is required for subsequent rounds of translation (Dever et al. 1995; Hinnebusch 2000).

Similar to *S. cerevisiae*, *C. albicans* only expresses the Gcn2 eIF2 kinase which can phosphorylate eIF2α in response to amino acid starvation conditions (Tournu et al. 2005). Furthermore, *C. albicans* can mount a GCN (general control nonrepressible) response to amino acid starvation; where it induces the expression of most amino acid biosynthetic pathways, via Gcn4 transcription factor (Tripathi et al. 2002).

### 1.8.1.1 Mammalian eIF2α kinases

In mammalian cells, four different Serine Threonine protein kinases induce eIF2α phosphorylation. They include; haem-regulated inhibitor of translation (EIF2AK1 or HRI), double-stranded RNA activated kinase (EIF2AK2 or PKR) important in the antiviral response; the PKR-like endoplasmic reticulum kinase (PERK or EIF2AK3), a transmembrane ER enzyme that is activated by ER stress due to misfolded proteins in the ER lumen; and a homologue of the eIF2α kinase in yeast, Gcn2 (EIF2AK4 or GCN2) which is activated in response to amino acid starvation and ultraviolet (UV) light (Dever 2002; Hinnebusch 2005; Jiang and Wek 2005).

HRI is the principal kinase in erythroid cells where it is activated under conditions of haem deprivation. The primary function of HRI in erythroid cells is to coordinate globin synthesis with available iron by blocking protein synthesis when haem is scarce (Han et al. 2001; Lu et al. 2001). Haem binds to and inactivates HRI, but when haem
availability is limited, it is released, activating HRI. HRI has also been shown to respond to other stress conditions when present in other cell types (Lu et al. 2001).

Expression of PKR is induced by interferon, and the kinase is activated by binding double stranded RNAs, as part of the cellular antiviral response. Two dsRNA-binding motifs (dsRBMs) precede the protein kinase domain and it has been suggested that these promote dimerization and kinase activation in the presence of dsRNA (Lee et al. 1993; Garcia et al. 2007). Increased activation of HRI and PKR in cells can dramatically inhibit almost all protein synthesis in the affected cells (Pavitt 2005).

PERK, a transmembrane ER (endoplasmic reticulum) spanning enzyme is activated by ER stress due to misfolded proteins in the ER lumen. In response to the accumulation of unfolded proteins in the ER, PERK activation should limit translation of proteins destined for the ER, and other signals generated as part of the unfolded protein response lead to activation of ER chaperone genes.

1.8.1.2 eIF2α dephosphorylation

Phosphorylated eIF2α is also targeted by protein phosphatases and it is possible that regulation exists at this level too. In mammalian cells, the phosphatase PP1c (Latreille and Larose 2006) and GADD34, dephosphorylate eIF2α in a negative feedback loop (Novoa et al. 2003) as part of the integrated stress response. Indeed GADD34 is a transcriptional target of ATF4 (see section 1.8.3). In an analogous manner, in yeast the essential phosphatase Glc7p has been shown to antagonise Gcn2p activity and repress GCN4 when over-expressed (Wek et al. 1992). It has also been suggested that type II phosphatases may also be capable of dephosphorylating eIF2α (Taylor et al. 2010).
1.8.1.3 Other mechanisms for eIF2B regulation

In mammalian cells, direct phosphorylation of eIF2B has also been identified as an important regulatory mechanism. For example in the absence of insulin, the kinase GSK3 phosphorylates eIF2B, inhibiting its activity. Insulin stimulates eIF2B activity by relieving this inhibition through inactivation of GSK3. GSK3 phosphorylation sites have been identified within the catalytic domain of eIF2Bε at Ser535 and Ser539 (Welsh et al. 1998; Wang et al. 2001). In addition, in both yeast and higher cells, volatile anaesthetics appear to inhibit protein synthesis via eIF2B (Palmer et al. 2006). Furthermore, in yeast, fusel alcohols have been shown to inhibit translation initiation via a mechanism involving eIF2B (Ashe et al. 2001).

In humans specific mutations in any of the genes encoding the five subunits of eIF2B lead to the onset of a fatal autosomal recessive brain disease known as CACH (childhood ataxia with central nervous system hypomyelination) also called vanishing white matter leukodystrophy (Fogli et al. 2002; van der Knaap et al. 2002). The disease is chronic, progressive and fatal but has a wide clinical spectrum. Often symptoms become apparent after a head trauma or following stress conditions that may down-regulate eIF2B function (Fogli et al. 2004). All the model systems developed to study the disease, suggest that eIF2B activity is reduced in affected patients, with the degree of reduction showing some correlation with the severity of the disease (reviewed in Pavitt (2005).

1.8.2 Regulation by eIF4E-binding proteins

Cap-dependent translation can be regulated by the association of eIF4E with the eIF4E-binding proteins (4E-BPs). There are three functionally equivalent 4E-BPs in mammals; 4E-BP1, 4E-BP2, and 4E-BP3 (Jackson et al. 2010), S. cerevisiae has two 4E-BPs and C. albicans has a single 4E-BP. In mammalian cells, when hypo-phosphorylated, 4E-
BP1 binds to eIF4E (in a binary complex), which prevents eIF4E’s association with eIF4G and therefore prevents translation initiation (Jackson et al. 2010). Phosphorylation of 4E-BP1 on multiple sites, mainly by mammalian target of rapamycin (mTOR), releases eIF4E for assimilation into eIF4F. Rapamycin addition and nutrient stress inhibit the mTOR pathway leading to dephosphorylation of 4E-BP, sequestration of eIF4E and a general down-regulation of 5’ cap-dependent protein synthesis (Richter and Sonenberg 2005). These translational regulators are relevant to human disease. For example, LRRK2, which is mutated in Parkinson’s disease, has been shown to be a 4E-BP1 kinase. The altered LRRK2 activity may affect synapse structure and function in Parkinson’s disease. Mutated forms of this protein show increased kinase activity resulting in hyper-phosphorylation of 4E-BP1.

Other 4E-BP binding proteins that are mRNA-specific have been found to play a role in transcript-specific translational regulation. For instance, Maskin in *Xenopus laevis* and Cup in *Drosophila melanogaster* are two such 4E-BPs that are targeted to specific mRNAs by interaction with mRNA-binding proteins that are targeted to specific 3’UTR sequences (Richter and Sonenberg 2005).

*S. cerevisiae* has two eIF4E binding proteins, Caf20p and Eap1p whose binding sites are on the dorsal surface of eIF4E (Cosentino et al. 2000). Caf20p is a phosphoprotein of about 20kDa, which shares a conserved region of eIF4G required for binding eIF4E. Studies show that it competes with eIF4G for the same eIF4E binding site and inhibits cap-dependent but not cap-independent translation (Altmann et al. 1997). Caf20p is phosphorylated to various degrees under different conditions, suggesting a role in translation control analogous to the mammalian 4E-BPs. Disruption of *CAF20* has been shown to stimulate a slight increase in the growth of yeast cells, but overexpression causes a mild slow growth phenotype in yeast, consistent with a negative role in translation (Altmann et al. 1997).
Eap1p also blocks Cap-dependent translation in yeast via competition with eIF4G. Though Caf20p and Eap1p translationally regulate some mRNAs they are unlikely to act as global translational regulators (Ibrahimo et al. 2006). Both of the yeast eIF4E-binding proteins, are required for pseudohyphal growth as the mutants were shown to be deficient in pseudohyphal growth in *S. cerevisiae* (Ibrahimo et al. 2006).

While cap-independent translation does occur, cap-dependent translation is believed to be the major pathway for translation initiation in eukaryotes (McCarthy 1998). Intriguingly in *C. albicans*, deletion of genes involved in catalyzing the formation of mRNA cap structure including triphosphatase-encoding gene (*CET1*) and the mRNA methyltransferase encoding gene (*CCM1*) does not cause lethality (Dunyak et al. 2002), unlike the parallel mutations in *S. cerevisiae* (Shibagaki et al. 1992; Tsukamoto et al. 1997). Therefore the m7GpppN mRNA cap structure is not absolutely required in *C. albicans* and the mRNA cap probably contributes to efficiency but is not essential (Dunyak et al. 2002).

*C. albicans* harbours just a single eIF4E binding protein, Caf20p, which represses CAP-dependent translation and the protein is enriched during stationary phase (Kusch et al. 2008).

1.8.3 Amino acid starvation and *GCN4* regulation

Probably the best characterised translational regulatory circuit in *S. cerevisiae* is the general amino acid control pathway that is activated in response to amino acid starvation, although other cellular stresses can also have an impact. In stressed yeast cells Gcn2p phosphorylates eIF2α to reduce eIF2B activity and thereby lower the abundance of Ternary Complex (TC) in the cell. The consequent reduction in TC formation by eIF2α phosphorylation paradoxically stimulates translation of *GCN4*
mRNA while decreasing the rate of general translation. Gcn4p is a transcription factor which up-regulates transcription of over 500 genes including many that encode amino acid biosynthetic enzymes (Natarajan et al. 2001). This pathway is referred to as the general amino acid control (GAAC) pathway (Hinnebusch 2005). The induction of \textit{GCN4} translation is determined by four short open-reading frames (ORF) in the \textit{GCN4} mRNA leader region (Fig. 1.6 a,b) (Hinnebusch et al. 2004). According to the scanning mechanism of translation initiation, each of the four upstream AUGs in the \textit{GCN4} mRNA leader should be selected as initiation sites prior to the start codon of the \textit{GCN4} ORF. Control of \textit{GCN4} mRNA translation is thought to occur via a reinitiation model. The AUG start codon in uORF1 is the first to be recognised and translated. The termination context of uORF1 is unusual and allows the ribosome to remain bound to the mRNA after termination and to continue scanning (Grant and Hinnebusch 1994). In non-starved cells, scanning ribosomes select the first AUG, translate the uORF1 and resume scanning downstream.
Figure 1.6 a. A model for translational control of yeast GCN4 under non starvation condition. GCN4 mRNA is shown with uORFs 1 and 4 and the GCN4 coding sequence indicated as a box. Shaded 40 S ribosomal subunits are competent to initiate translation as they are associated with the ternary complex. Unshaded 40S ribosomal subunits lack TC and thus cannot reinitiate. Following the translation of uORF1, 50% of the 40S ribosomes remain attached to the mRNA and resume scanning. Under non-starvation conditions, the 40S subunit quickly rebinds the TC before reaching uORF4, because the TC concentration is high, translate uORF4, and dissociate.
Figure 1.6 b. A model for translational control of yeast GCN4 under starvation condition. GCN4 mRNA is shown with uORFs 1 and 4 and the GCN4 coding sequence indicated as a box. During amino acid starvation, eIF2α is phosphorylated by the protein kinase Gcn2p, thus converting eIF2 from substrate to inhibitor of the GEF eIF2B and reducing the eIF2-GTP level in the cell. As a result, TC levels are reduced. Following the translation of uORF1, 50% of the 40S ribosomes remain attached to the mRNA and resume scanning. Under starvation conditions, ~ 50% of the rescanning 40S ribosomes fail to rebind the TC until scanning past uORF4, because the TC concentration is low and reinitiate at GCN4. (Adapted from Hinnebusch 2005).

Since eIF2B activity is high and TC is abundant, all of the scanning 40S ribosomes rebind TC before reaching uORF4, which permits its recognition and translation (Fig. 1.6 a). Unlike the uORF1, the termination context of the uORF4 termination codon promotes dissociation of the ribosome, thereby preventing the ribosome from scanning to the downstream GCN4 ORF (Grant and Hinnebusch 1994). However under
starvation conditions, there is a lower abundance of TC as eIF2B is sequestered by phosphorylated eIF2 (Dever et al. 1995). While there remains a sufficient amount of TC to permit translation of uORF1, it takes much longer to recruit TC onto the 40S ribosomes scanning downstream from uORF1. This delay in the TC recruitment enables about 50% of the 40S subunits to bypass uORF4, rebind TC in the interval between uORF4 and GCN4. This permits recognition and reinitiation of translation at GCN4 (fig. 1.6b). Genetic evidence indicates that reinitiating ribosomes bypass uORFs 2 to 4 under derepressing conditions because the distance between uORF1 and uORF4 is not large enough to ensure that they rebind the factors required for reinitiation before reaching uORF4 (Hinnebusch 2005).

It is important to note that conditions which induce eIF2α phosphorylation are not the only means by which GCN4 can be translationally induced. Indeed any alteration in the rate of ternary complex formation will cause such induction. Hence, a number of Gcn2p-independent mechanisms for the regulation of GCN4 translation have been discovered (Ashe et al. 2001). For example inhibition of eIF2B activity by fusel alcohols in S. cerevisiae occurs independently of eIF2α phosphorylation, this also leads to reduced TC levels and derepression of GCN4 translation (Ashe et al. 2001).

In the human pathogenic yeast C. albicans, the GCN4 mRNA leader contain three uORFs. Intriguingly, recent findings show that a single inhibitory uORF (uORF3) is sufficient for translational regulation of GCN4 in C. albicans (Sundaram and Grant 2014).

In mammals, similar mechanisms to GCN4 regulation exist; such that as well as inhibiting general translation, the phosphorylation of eIF2α in response to a range of stress conditions induces the translation of specific mRNAs. For example, the ATF4, ATF5, CHOP and IBTKα mRNAs are induced by virtue of uORFs in a manner analogous to GCN4 (Harding et al. 2000; Vattem and Wek 2004). The protein ATF4
forms part of an integrated stress response where it is involved in the feedback regulation of protein synthesis via activation of the phosphatase GADD34 (see 1.8.1.2) and it activates a cascade of transcriptional effects, for instance the transcription factor CHOP is activated. In addition, ATF4 alters the transcription of many other genes involved in amino acid metabolism, the response to oxidative stress and regulation of apoptosis (Harding et al. 2000; Harding et al. 2003).

1.8.4: Translational response to membrane stress

Another pathway of translational regulation that could prove relevant to the cellular response to alcohols is the response to membrane stress. The transport of lipids and proteins between intracellular organelles is a highly regulated process. Perturbations to this transport pathway result in changes to the lipid composition and mis-localisation of membrane proteins. Exposure of S. cerevisiae to membrane stress causes the cells to repress the transcription of genes encoding rRNA, tRNA and ribosomal proteins (Li et al. 1999). In addition, perturbation of this pathway using the drug Chlorpromazine causes a rapid and specific inhibition of translation. This regulation of translation involves both eIF2α phosphorylation and the eIF4E binding protein Eap1p. GCN4 translation is upregulated in response to blocks in the membrane transport pathway; however the mechanism by which eIF2α phosphorylation is induced is not clear. It has been shown that eIF2α phosphorylation under membrane stress is not dependent on Gcn2p because there is no accumulation of uncharged tRNAs in the cell. Therefore it has been suggested that a protein phosphatase could be inhibited, giving rise to increased levels of phosphorylated eIF2α (De Filippi et al. 2007). However, the capacity of alcohols to trigger these effects has not been studied and it seems highly unlikely that fusel alcohols are acting in a similar manner to Chlorpromazine as they lead to a dephosphorylation of eIF2α (Taylor et al. 2010).
1.9 Localisation of translation initiation factors

1.9.1 Stress granules and processing bodies (P-bodies)

A variety of translation initiation factors and factors involved in mRNA maintenance have been found to localise to a number of discrete cellular foci under different conditions. Stress granules and processing bodies (P bodies) are two well described examples of such bodies. Cellular stress induces the formation of stable mRNA and mRNP aggregates, termed stress granules (SGs), that are induced in an eIF2α phosphorylation dependent or independent manner (Anderson and Kedersha 2006). In mammalian cells, eIF2α phosphorylation independent formation of SGs can be brought about by drugs that affect either eIF4A’s or eIF4G’s activity (Low et al. 2005; Kim et al. 2007). Stress granules harbour ribosomal proteins and early translation initiation factors such as eIF2, eIF3, eIF4E and eIF4G (Kedersha et al. 1999). Stress granules are dynamic and interact with other cytoplasmic proteins and also with processing bodies (Kedersha et al. 1999). Distinct granules known as P bodies are found in both yeast and mammalian systems. P-bodies have been defined as cytoplasmic bodies that harbour many of the enzymes involved in the 5’ to 3’ pathway of mRNA decay and are presumed to sort mRNA content according to cellular requirement (Sheth and Parker 2003; Kedersha et al. 2005). Stress granules are distinct from P-bodies in their composition and function, however, several factors populate both structures in vivo (Cougot et al. 2004).

1.9.2 eIF2B Bodies

Recent work has shown that eIF2B and eIF2 reside in a large cytoplasmic body termed the eIF2B body (2B body) and this body represents a site where guanine nucleotide exchange of eIF2-GDP to eIF2-GTP can occur (Campbell et al. 2005; Taylor et al. 2010). This localisation is specific for these factors and requires active translation.
Other initiation factors studied were found to be dispersed throughout the cytoplasm during log-phase of growth in complete media (Taylor et al. 2010). Addition of cycloheximide, which acts to inhibit translation initiation and elongation leads to the eradication and dispersal of the eIF2/eIF2B foci, therefore indicating the requirement for active translation for their formation.

Quantitative analysis of the proportion of cellular eIF2B and eIF2 found in these foci showed that under unstressed conditions around 20% of eIF2 and 40% of eIF2B is found within the eIF2B body. The dynamic cycling of eIF2 through the 2B body can be assessed using FRAP (fluorescence recovery after photobleaching). FRAP analysis showed that eIF2B remains a largely resident feature, whilst eIF2 shuttled through the focus. This could indicate that these foci are sites of guanine nucleotide exchange. Using FRAP, it was shown that amino acid starvation leads to a significant increase in the half-time of recovery after photobleaching for eIF2 (Campbell et al. 2005). It can be deduced from this that a stress known to reduce GEF activity of eIF2B, reduces the rate of eIF2 shuttling onto foci (Campbell et al. 2005). In addition to this, the recovery of gcn2Δ strain was unaffected when a similar experiment was carried out with the strain. Butanol treatment also causes an increase in the half-time of recovery after photobleaching, but the magnitude of this change is very much smaller than that observed after amino acid starvation. Other conditions that inhibit guanine nucleotide exchange, for example a GCN2-constitutive mutant, also reduce the rate of eIF2 shuttling (Campbell et al. 2005).

The 2B body was also shown to traverse almost all regions of the cytoplasm. Time lapse microscopy was used to investigate the dynamics of the 2B body. It appears that the body can cycle between two phases: a tethered non-mobile phase and a mobile diffusing phase (Taylor et al. 2010). Treatment with fusel alcohols increases the length of time
that the body remains in a tethered state, thus reducing the movement of the body around the cell (Taylor et al. 2010).

1.10 Fusel alcohols

It is well-established that *Saccharomyces cerevisiae* produces fusel alcohols. 1-butanol, isoamyl alcohol and isobutanol are end products of catabolism of threonine, leucine and valine respectively (Webb and Ingraham 1963; Dickinson 2000). Fusel alcohol production from amino acids proceeds via the Ehrlich pathway. This pathway consists of three enzymatic steps:

1. transamination to form an α-keto acid

2. decarboxylation to yield an aldehyde

3. reduction to a fusel alcohol

The decarboxylation step for the different fusel alcohols is carried out by different pyruvate decarboxylases (Dickinson 2000) and a range of alcohol dehydrogenases (*ADHI, ADHII and ADHVI*) with reductase activity have been implicated in the final reduction of aldehydes to fusel alcohols (Dickinson 2000; Larroy et al. 2003).
**Figure 1.7 Ehrlich pathway for fusel alcohol production from branched chain amino acids.** Catabolism of branched-chain amino acids leads to the formation of fusel alcohols.

1.10.1 The impact of fusel alcohols on protein translation

A range of fusel alcohols including n-butanol and isoamyl alcohol have been shown to rapidly inhibit translation initiation in yeast through an eIF2B-dependent yet eIF2α
phosphorylation independent mechanism (Ashe et al. 2001). The studies defined a role for eIF2B in regulating translation in response to butanol. Two variants of the S. cerevisiae W303-1A strains were identified which respond differently to the addition of butanol and were termed But² (butanol sensitive) and But¹ (butanol resistance) strains. The difference between the strains was traced to a mutation in the GCD1 gene, which results in a P180S variation in the Gcd1p protein (the γ subunit of eIF2B) (Ashe et al. 2001). The inhibition of translation initiation exerted by the addition of butanol is extremely rapid occurring within one minute as determined by polysome analysis and a 35S-methionine incorporation assay (Ashe et al. 2001). Intriguingly, this regulation of translation inhibition does not occur via previously described mechanisms. This was demonstrated by the use of mutants: a strain bearing an non-phosphorylatable form of eIF2α (SUI2-S51A) and a strain without the eIF2α kinase (gcn2Δ), which are translationally resistant to amino acid starvation; and TOR1-S1971I, which is resistant to rapamycin treatment. These mutant strains were both translationally sensitive to butanol in the But² strain, thus indicating that the amino acid starvation and exposure to rapamycin pathways of translational control are not the same as the butanol-dependent pathway. Furthermore, it has been shown that fusel alcohols bring about a decrease in the phosphorylation of eIF2α, in contrast to the effects of amino acid starvation (Taylor et al. 2010), but similar to the effect of volatile anaesthetics (Palmer et al. 2005; Palmer et al. 2006); however, this dephosphorylation is not essential for the inhibition of translation by fusel alcohols (Ashe et al. 2001). Gene expression profiling has revealed that as well as translation of GCN4 mRNA, other sets of genes are also upregulated in response to butanol. These include genes that are required for the cell cycle, protein synthesis, cellular transport and nitrogen metabolism. Therefore there is a correlation between the physiological impact of butanol and the genes that are upregulated in response to it (Smirnova et al. 2005). Furthermore, fusel alcohols were shown to
prevent the movement of this eIF2B body through the yeast cell cytoplasm, perhaps because the alcohols stabilize a tethered form (Taylor et al. 2010).

All of the work performed on the impact of alcohols, particularly fusel alcohols, on protein synthesis has centred on the budding yeast, *S. cerevisiae*. Therefore, the question as to how these alcohols affect a Crabtree negative yeast like the human pathogen, *Candida albicans* is unresolved.

### 1.10.2 Effect of alcohols on *Candida albicans*

There is increasing evidence that alcohols have profound effects on some clinically relevant properties of *Candida* species and other pathogenic microorganisms. Fusel alcohols are by products of amino acid catabolism in yeast and their production is associated with nitrogen scarcity. In both *S. cerevisiae* and *C. albicans*, as well as ethanol, fusel alcohols including isoamyl alcohol, isobutanol, active amyl alcohol and 1-butanol are also formed (Webb and Ingraham 1963). *C. albicans* when grown in poor nitrogen conditions can also utilize aromatic amino acids by the Erlich pathway and form aromatic alcohols as a byproduct (Ghosh et al. 2008). Ethanol is one of the products of the metabolism of glucose in *C. albicans*, but as a Crabtree negative yeast species the production of biomass via respiration is the favoured metabolic route rather than ethanol production from fermentation (Pronk et al. 1996; Piskur et al. 2006). Hence, while *C. albicans* can utilize ethanol as a sole source of carbon, it is effectively inhibited by quite low concentrations of ethanol compared to *S. cerevisiae* (Zeuthen et al. 1988).
1.10.3 Fusel alcohols induce morphological changes in yeast

As already mentioned in section 1.4.1, a number of connections have been described between the regulation of protein synthesis and filamentous growth in fungi. It was found that minimal cycloheximide treatment which will normally cause a cessation of protein synthesis also results in a lower proportion of pseudohyphal cells (Kron et al. 1994). In addition, studies have shown that nutritional conditions that elicit global effects on protein synthesis also impact upon morphological transitions in yeast (Ashe et al. 2000; Ashe et al. 2001; Gancedo 2001).

The formation of pseudohyphae can be induced in S. cerevisiae exposed to fusel alcohols. Isoamyl alcohol and 1-butanol have been shown to induce pseudohyphal formation in yeast and this may function as a foraging mechanism (Dickinson 1996). At a concentration of 0.5% (v/v) in complex media, isoamyl alcohol was reported to induce the formation of hypha-like extensions in haploid and diploid strains of S. cerevisiae, however at a lower concentration of isoamyl alcohol (0.25%)(v/v) the cells formed pseudohyphae (Dickinson 1996). The mechanism of fusel alcohol dependent filamentous growth is quite different from that occurring during nitrogen-limited growth because a distinct set of genes are involved. For example, butanol-induced pseudohyphal growth is induced by the Swe1p-dependent morphogenesis checkpoint, (Martinez-Anaya et al. 2003), and is independent of genes such as FLO8 and FLO11 that are required for pseudohyphal induction during nitrogen limitation (Lorenz et al. 2000). The morphogenesis checkpoint is a unique cell cycle checkpoint in budding yeast that coordinates bud formation with mitosis and ensures that mitosis only occurs after bud formation (Martinez-Anaya et al. 2003). It was also demonstrated that fusel alcohol-induced filamentous growth is dependent on Pkc/Slt2p (Mpk1p), the cell integrity MAP kinase and Slt2p is activated upon exposure to isoamyl alcohol (Martinez-Anaya et al. 2003).
1.10.4 Alcohols induce morphological changes in *Candida albicans*

One of the major predictors of *C. albicans* virulence is the ability to switch from yeast to pseudohyphal or hyphal morphology. The yeast to hyphal morphogenesis is induced in response to various environmental cues (Lengeler et al. 2000). Alcohols have been implicated in the induction of pseudohyphal morphogenesis in *Candida albicans* and other pathogenic *Candida* species. Isoamyl alcohol was reported to induce the formation of pseudohyphae in *C. albicans* when the cells are grown in YEPD containing 0.6% (v/v) isoamyl alcohol, about 75% of the *C. albicans* cells formed pseudohyphae within 24 h (Dickinson 1996). Another study suggests that tyrosol, an aromatic alcohol secreted by *Candida albicans* during growth can reduce the lag phase and accelerate germ tube formation in dilute cultures of *C. albicans* (Chen and Fink 2006), while other aromatic alcohols tested (tryptophol and phenylethanol) failed to stimulate filamentation in *C. albicans*. However these aromatic alcohols stimulated morphogenesis in *S. cerevisiae* cells by inducing the expression of FLO11 through a Tpk2p-dependent mechanism (Chen and Fink 2006). Studies carried out using *C. albicans* mutants defective in cAMP-dependent PKA pathway (efg1/efg1), MAP kinase pathway (cph1/cph1), or both (cph1/cph1 efg1/efg1), showed that while their parent strain (CAI-4) formed pseudohyphae in the presence of 100µM tyrosol, the mutant strains failed to form pseudohyphae under the same conditions (Ghosh et al. 2008). This observation suggests that the cAMP-PKA kinase and MAP kinase pathways are involved in the induction of pseudohyphae by aromatic alcohols in *C. albicans*. Furthermore, the observation that aromatic alcohols activate Gcn4p through an eIF2α independent manner (Ghosh et al. 2008) correlates with the findings of Ashe et al. (2001) using n-butanol in *S. cerevisiae*.

Ethanol has been reported to induce a number of morphological changes in fungi including germ tube formation in *C. albicans* and this occurs without the addition of
nitrogen-containing nutrients (Pollack and Hashimoto 1985). In another study, four strains of C. albicans were used to determine the concentration of ethanol that will induce germ tube formation, the results show that germ tubes are formed by all four strains at concentrations of ethanol of 0.25-1% (Zeuthen et al. 1988). At a concentration of 4.0% ethanol or below 0.015% no germ tubes were formed. The effect of ethanol on germ tube formation is connected to protein synthesis as it was observed that exposure of cells to ethanol depressed normal protein synthesis, but increased the synthesis of stress proteins (Zeuthen et al. 1988).

1.11 Farnesol a seisquiterpene alcohol

Fungi produce a whole range of volatile organic compounds comprising monoterpenes, sesquiterpenes and diterpenes. Volatile sesquiterpenes have almost exclusively been reported from Ascomyta and Basidiomycota. (Hibbett et al. 2007). Sesquiterpenes are usually formed by fungi in the late growth phase. The formation of volatile sesquiterpenes is often based on enzymes expressed in differentiated cells. Only a few sesquiterpene alcohols have been reported from fungi, however, for several of them some specific ecological functions are known.

Farnesol is a natural 15-carbon acyclic sesquiterpene alcohol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) produced from 5-carbon isoprene compounds in both plants and animals. It has three carbon-carbon double bonds and exists in four isomers but only the E,E isomer has QS molecule activity (Shchepin et al. 2005). Farnesol is produced by a few fungal species: Candida albicans, (Hornby et al. 2001; Langford et al. 2009), Candida dubliniensis, (Hornby et al., 2001), Aspergillus fumigatus (Dichtl et al. 2010) and Ceratocystis coerulescens (Sprecher and Hanssen 1983).
1.11.1 Farnesol and quorum sensing in *C. albicans*

*Candida albicans* has the capacity to switch from a yeast form to a hyphal form. One of the parameters that impacts upon the yeast-to-hyphal switch *in vitro* is cell density. This phenomenon has been associated with regulation by Quorum sensing (QS) (Hornby *et al.* 2001).

Quorum sensing is the regulation of gene expression and group behaviour in response to changes in cell population density. QS molecules were first discovered in bacteria. They can mediate the sensing of cell density and control group behaviour like virulence, antibiotic production, conjugation and biofilm formation (Miller and Bassler 2001).

In *C. albicans*, two studies identified two related compounds, farnesoic acid [(2E,6E)-3,7,11-trimethylundeca-2,6,10-trienoic acid] and farnesol [(2E,6E)-3,7,11-trimethylundeca-2,6,10-trien-1-ol], as QS regulators of the yeast-to-hyphal switch (Hornby *et al.* 2001; Oh *et al.* 2001). Farnesoic acid was only produced in *C. albicans* 10231, the strain background used in the study by Oh *et al.* (2001). Farnesoic acid was shown to have only 3.3% of the activity (compared with farnesol) on farnesol-producing strains of *C. albicans* (Shchepin *et al.* 2003). *C. albicans* produces farnesol from an intermediate of the sterol biosynthetic pathway, farnesyl pyrophosphate (FPP). Compounds that block sterol pathway beyond FPP, such as zaragozic acids, were shown to cause an eightfold increase in intracellular and extracellular farnesol levels. (Hornby...
et al. 2003) showed that FPP was converted to farnesol by incubating [1-\textsuperscript{3}H] (E,E)-FPP with a \textit{C. albicans} cell homogenate in a modification of allylpyrophosphatase assay. No farnesol was made when the cell extract was heated to 95°C for 10 min. This shows that \textit{C. albicans} possesses the enzymatic machinery to convert FPP to farnesol. (Hornby \textit{et al.} 2003). Also, treatment with the antifungal, fluconazole (another sterol inhibitor), resulted in a 13-fold increase in the amount of farnesol produced by \textit{C. albicans} (Hornby \textit{et al.} 2003).

According to Hornby \textit{et al.} (2001), the properties of farnesol as a QS molecule are consistent with those expected of a fungal QS molecule. (a) It is extracellular, diffusible and can be removed from cells by washing. (b) It is produced continually during growth in amounts roughly proportional to cell mass. (c) It inhibits the formation of germ tubes under germ tube inducing conditions. (d) It alters cell morphology but does not alter cell growth at the physiological concentration (below 40µM). Thus its mode of action is likely more specific than just general inhibition. (e) It is produced at all growth temperatures and its production is dependent on cell growth and not on a particular carbon or nitrogen source.

\textit{C. albicans} produces farnesol as an extracellular autoregulatory compound and when farnesol accumulates above a threshold level, it inhibits yeast-to-hyphal switch as well as biofilm formation (Hornby \textit{et al.} 2001; Ramage \textit{et al.} 2002; Martins \textit{et al.} 2007). In their study, (Ramage \textit{et al.} 2002) observed that budded yeast cells in a mature biofilm could not germinate in culture media containing high concentrations of farnesol. This observation suggested that at certain farnesol concentration, mycelia development is inhibited and the resulting yeast cells are prone to detach from the biofilm. However farnesol had minimal effect on mycelia formation or biofilm growth if introduced after any of these processes has already begun. Thus farnesol inhibits hyphal initiation but
does not suppress hyphal elongation (Mosel et al. 2005). Farnesol can also affect the expression of virulence genes (Cao et al. 2005).

Low concentrations of farnesol (twenty to fifty micromolar) inhibits or kills a range of fungi, opaque C. albicans cells, several mammalian cell lines and some bacteria (reviewed in Langford et al. 2009). The report that C. albicans can exhibit exceptional tolerance to farnesol (Hornby et al. 2001) was challenged by Shirtliff et al. (2009) who showed that C. albicans were killed at concentrations of farnesol as low as 40µM. However the differences in growth conditions used in these studies were shown to be responsible for the discrepancies observed with growth sensitivities of C. albicans to farnesol (Langford et al. 2009). Log phase cells or cells grown in minimal media were shown to be more sensitive to farnesol than stationary phase cells or cells grown in rich media (Langford et al. 2009). To gain insight into the response of C. albicans to farnesol, global gene expression analysis were performed (Cao et al. 2005; Cho et al. 2007). Although various experimental approaches were used in these studies, farnesol commonly affected the genes that belong to functional categories such as stress response, heat shock, drug resistance, amino acid and carbon metabolism, iron transport, cell wall and cell cycle.

1.11.2 Mechanism of inhibition of hyphal growth by farnesol.

Sato et al. (2004) suggested that the farnesol-dependent inhibition of hyphal formation involves the mitogen-activated protein kinase pathway, which regulates the expression of transcription factor Cph1p and the MAPKK Hst7p. This was determined by reduction in the transcript levels of the genes for the proteins involved in this pathway in the presence of farnesol. However farnesol inhibited the yeast-to-hypha transition in a cph1/cph1 mutant, suggesting that CPH1 is not a primary but rather a secondary target of farnesol (Davis-Hanna et al. 2008). Other morphogenetic regulators may play a role
in the *C. albicans* response to farnesol. For instance Chk1p, a two-component signal transduction histidine kinase has been implicated in the farnesol mediated inhibition of hyphal growth (Kruppa *et al.* 2004). Chk1p could function via the Hog1p MAP kinase, which was shown to be phosphorylated in the presence of farnesol (Kruppa *et al.* 2004). Tup1p has also been implicated in the response to farnesol. It was shown that expression of *TUP1* was slightly increased in response to farnesol and strains lacking *TUP1* or *NRG1* did not respond to farnesol but generated higher levels of farnesol than the wild type cells (Kebaara *et al.* 2008). In addition, the Ras1p-cyclic AMP-protein kinase signalling pathway has been identified in the farnesol response. Farnesol treatment increased the mRNA levels of cAMP-repressed genes, suggesting that cAMP levels were reduced in the presence of farnesol (Davis-Hanna *et al.* 2008). Furthermore, Hall *et al.* (2011), showed that farnesol can directly affect the activity of the *C. albicans* adenylyl cyclase, thereby reducing the cAMP output to inhibit morphogeneis. With regard to the yeast-to-hyphae transition, it appears that the response to farnesol is multifactorial as there is no specific signalling pathway controlled in this response and a single receptor for farnesol has yet to be identified.

### 1.12: Outline of Research

A starting point for the work in this thesis was provided by the observations that fusel alcohols induce morphological differentiation in *Candida albicans*, whereas farnesol blocks this process. Morphological transition is one of the key virulence factors in the *Candida albicans*, a human pathogen and a relative of non-pathogenic yeast, *S cerevisiae*. The mechanism through which fusel alcohol and farnesol induce these changes have not been characterised in *C. albicans*. During the course of the studies in this thesis, both fusel alcohols and farnesol were found to inhibit global translation initiation in *Candida albicans*. The research presented takes both biochemical and cell
biological approaches to follow the mechanism by which alcohols exert translational control in *C. albicans*, by mainly focusing on the effects on translation initiation factors. Furthermore alterations in transcription and translation of *C. albicans* exposed to farnesol are studied using RNA sequencing of Total and polysome associated RNA, respectively. In addition, the mechanisms by which alcohols mediate morphological transition in *C. albicans* will be investigated. It is hoped that this will lead to a better understanding of the link between alcohol induced translational control and morphological transition in *C. albicans*. Given the connection between virulence and morphogenesis it is possible that this work may ultimately inform potential treatment strategies.
2. Materials and Methods

2.1 Culture conditions

2.1.1 Strains and growth conditions

The *Candida albicans* CAI4 strain (Fonzi and Irwin 1993), an isogenic derivative of the parental wild-type clinical isolate (SC5314) (Gillum et al. 1984), was used throughout this study unless otherwise indicated (see Table 2.2). The CAI4 strain is a *URA3* auxotroph, but has been used extensively in studies of stress responses in *C. albicans* without significant difference when compared to the parent strain (SC5314) except in animal model of systemic candidiasis assays where absence of *URA3* affected virulence of the CAI4 strain (Staab and Sundstrom 2003). *Candida* strains were routinely cultured at 30°C in YPD (1% (w/v) Yeast Extract, 2% (w/v) Bacto peptone and 2% (w/v) glucose) to mid-logarithmic phase (~1.0x10^7 cells/ml or optical density at 600 nm of 0.7). The alcohols (butanol, ethanol and farnesol) were added for 10 or 15 min to particular concentrations in liquid or solid culture as stated in the results.

For radiolabelling experiments, *C. albicans* strains were grown in Synthetic Complete Media (SCD) (Guthrie and Fink 1991) composed of 0.67% (w/v) Yeast Nitrogen Base without amino acids (Formedium), 2% (w/v) glucose. The medium was supplemented with synthetic complete amino acid drop out mix lacking methionine (Formedium). Solid SCD and YPD agar plates were prepared as for liquid media, except for the addition of 2% (w/v) Bacto Agar.

2.1.2 Monitoring the growth of yeast cultures

The growth of *Candida albicans* and *Saccharomyces cerevisiae* strains was determined by measuring the optical density of the culture at a wavelength of 600 nm. For routine
OD measurements, 1ml of the culture was aliquoted into a disposable plastic cuvette and the OD$_{600}$ was measured using an Eppendorf Biophotometer plus

2.1.3 Bacterial strain and growth conditions

The DH5α *Escherichia coli* strain was used to amplify plasmid DNA for PCR and yeast transformation. Cells were grown in Luria-Bertani (LB) media (10g/l Bacto-trypone and sodium chloride, 0.5g/l Bacto yeast extract) at 37°C. Plasmid DNA was isolated using the Qiagen Spin® Miniprep alkaline lysis kit according to manufacturers’ instructions. Selection of plasmids was achieved by growth on LB+Carbenicillin plates. Carbenicillin (Sigma) was added to LB at a concentration of 50µg/ml and 2% (w/v) Bacto Agar (Melford) was included.

2.1.4 Alcohol tolerance

Strains were grown overnight to OD$_{600}$ of 0.1 in YPD. 50ml cultures were set up in 250ml Erlenmeyer flasks containing butanol (0.5%, 1% and 2%); ethanol (2%, 4%, 6% and 8%) and farnesol (40µM, 100µM, 200µM and 300µM). Control flasks had no alcohol. The cultures were incubated on an orbital shaker (New Brunswick) for 6 h at 30°C. Growth was assessed at hourly intervals by measuring the OD$_{600}$.

2.2 Morphogenesis assays

2.2.1 Colony morphology in liquid media

Overnight exponential *C. albicans* cultures were harvested, washed once in water and resuspended in water to give an OD$_{600}$ of 1.0. 50µl of the cell suspension was diluted into 5ml YPD and the required concentrations of the appropriate alcohol (butanol,
ethanol and farnesol) were added. As controls, cells prepared in the same manner were inoculated into 10% serum medium and into YPD without any of the alcohols. All of the tubes were incubated at 37°C and 200 rpm on an orbital shaker (New Brunswick Scientific). At various times during incubation (1 h, 2 h 4 h, 8 h and 24 h), cells were mounted onto a microscopy slide and the morphology of the cells was monitored using a Nikon Eclipse E600 a Nikon x10 and x40 objective and Axiocam MRm camera. Images were acquired using Axiovision 4.5 software. The percentage of the cells forming germ tubes or pseudohyphae were counted using a haemacytometer. Each count was repeated for three different experiments and the mean of the three counts recorded.

2.2.2. Colony morphology on solid media

To determine colony morphology on solid media, YPD agar supplemented with or without the appropriate alcohol was used. Alcohols were added just before the agar solidified to reduce their volatility. YPD agar with 10% serum was also prepared to serve as a positive control. Log phase cells washed in water were diluted to give 10^4/ml-10^4/ml of cells. 100μl of the lowest two dilutions were spread on the plates using sterile glass beads. The plates were incubated at 37°C until colonies appeared. Colony morphology was monitored using a Nikon Eclipse E600 with a Nikon x10 and x40 objective and Axiocam MRm camera. Images were acquired using Axiovision 4.5 software.
2.3 Manipulation of protein in vitro

2.3.1 Preparation of C. albicans cell extracts for Western analysis

50ml of an OD$_{600}$ 0.7 YPD-grown yeast culture was harvested in a Sigma 4K15 centrifuge at 5000xg for 5 min. The cell pellet was resuspended in 1ml of chilled 1 x Buffer A (30mM Heps.KOH pH 7.5, 100mM Potassium Acetate, 2mM Magnesium Acetate, 1mM phenylmethylsulfonyl fluoride) and the cells harvested at 4°C in an Eppendorf 5415D microfuge at 10,000xg for 2 min. The cell pellet was resuspended in 100μl of chilled 1 x Buffer A prior to the addition of 200μl of glass beads (425-600μm, Sigma-Aldrich). The samples were mixed vigorously on a vortex mixer for 15 sec then incubated in iced water for at least 40 sec. This step was repeated five times except the final mix was extended to 25 sec. The samples were centrifuged at 10,000xg for 15 min at 4°C. The final supernatant was collected and the protein concentration was measured (see section 2.3.2). Protein extracts were routinely stored at -20°C in 2 x SDS loading buffer (10% (v/v) Glycerol, 5% (v/v) mercaptoethanol, 3% (w/v) SDS, 62.5mM Tris-Hcl pH 6.8, Bromophenol blue) and boiled at 95°C for 5 min prior to loading.

2.3.2: Determination of protein concentration

The total protein concentration of whole cell extracts was determined using the Bradford method (Bradford 1976). This method is based on the fact that protein-dye complexes cause a shift in the dye (Coomassie) absorption maximum from 465 to 595 nm. Absorption at 595 nm is proportional to protein concentration. A standard curve was prepared using dilutions of bovine serum albumin (Sigma). 200μl of BioRad Bradford reagent was added to 800μl of each sample and mixed thoroughly. Absorbance readings were measured at OD$_{595}$ and the values obtained were plotted against the protein concentration to obtain a standard curve. To measure the protein concentration of yeast samples, 10μl of a 1:10 dilution of yeast protein extract was
added to 790µl water and 200µl Bradford reagent. The OD$_{595}$ of samples was measured and protein concentration determined based on the standard curve.

2.3.3: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)
Protein electrophoresis was performed as described by Laemmlli (1970), Sambrook and Russel, (2001) using the BioRad Protean III mini gel apparatus. 10% polyacrylamide resolving gels (364mM Tris-HCL pH 8.8, 0.1% (w/v) SDS), were routinely used. The stacking gel composition was 125mM Tris-HCL pH6.8, 0.1% (w/v) SDS, 4% (v/v) polyacrylamide (Sigma-Aldrich). Polymerisation was promoted by adding 11µl TEMED (Sigma) and 30µl (10mg/ml) ammonium persulphate to a total gel volume of 5.5ml immediately prior to pouring. Gels were run at 130 V for 1 h in a reservoir of 1 x protein running buffer (0.19M glycine, 25mM Tris base, 1% (w/v) SDS). Precision plus protein All Blue standards (BioRad) were routinely used as size standards throughout this study.

2.3.4 Coomassie stain
SDS PAGE resolving gels were removed from the gel running apparatus and soaked in Simply Blue SafeStain (Invitrogen) for 1 h, then washed in water.

2.3.5: Western blotting
SDS PAGE resolving gels were removed from the gel running apparatus and immersed in 1 x transfer buffer (20% (v/v) methanol, 25mM Tris base, 192mM glycine, 0.1% (w/v) SDS). The blotting apparatus, consisting of four sponges, blotting paper and Hybond-ECL nitrocellulose membrane (Pharmacia), was also soaked in 1 x transfer buffer. These were assembled in the BioRad electroblotting apparatus such that proteins
would be transferred from the gel to the membrane. Electroblotting was performed at 25 V in 1 x transfer buffer for 2 h, cooled with an ice chamber. The membrane was removed and immersed in blocking solution (1 x PBS (137mM sodium chloride, 10mM sodium phosphate buffer pH 7.4, 2.7mM potassium chloride), 0.1% (v/v) Tween 20 and 5% (w/v) skimmed milk) for 1 h at room temperature. The membrane was subsequently immersed in the same solution containing an antibody to the protein of interest (see table 2.5) for at least 1 h. The membrane was then washed three times for 15 min each in 1 x PBS Tween solution (137mM sodium chloride, 10mM sodium phosphate buffer pH 7.4, 2.7mM potassium chloride and 0.1% (v/v) Tween 20). A source specific horseradish per oxidise (HRP) conjugated secondary antibody, typically a 1:10,000 dilution in 1 x PBS Tween + 5% (w/v) skimmed milk was added for 1 h with rocking. The membrane was then washed for 3 x 15 min in 1 x PBS Tween before addition of equal volumes of ECL detection reagent (Thermo Scientific). The membrane was overlaid with a strip of blotting paper then wrapped with the Saran wrap before exposure to autoradiography film (Fuji, Japan).

2.3.6 $^{35}$S methionine incorporation assay

*C. albicans* strains were grown to OD$_{600}$ of 0.7 in SCD medium lacking methionine (0.67% (w/v) Yeast nitrogen base without amino acid (Formedium), 2% (w/v) glucose and 0.2% synthetic Complete Amino acid Drop out-Met (Formedium). The culture was split into two flasks and an appropriate quantity of a particular alcohol was added to one of these. The two flasks were incubated for 10 min at 30°C, then methionine was added to a final concentration of 60ng/ml, of which 0.5ng/ml was $^{35}$S methionine (cell-labelling grade 1175 Ci/mmol; New England Nuclear, Boston, MA). Samples (1ml) were taken and processed as described previously (Ashe et al. 2000). Briefly, 1ml samples were added to 1ml of 20% trichloroacetic acid (TCA) at the various time points.
and heated at 95°C for 20 min in a water bath. The protein precipitate was collected on GFC filters (Whatman, GE Healthcare Buckinghamshire, UK), washed with 2ml of 10% TCA and then with 2ml of 95% ethanol, and counted in Scintisafe (Fisher, Leicestershire, UK) scintillation fluid using the Liquid Scintillation Analyzer (Packard).

2.3.7 GCN4 Reporter assays

Luciferase assays to evaluate expression from the GCN4 and the GCRE reporters were performed essentially as described by Srikantha et al. (1996). Briefly, cells were harvested and extracted using RLUC buffer (0.5M NaCl, 0.1M K$_2$HPO$_4$ (pH 7.6), 1mM Na$_2$ EDTA, 0.6mM sodium azide, 1mM phenylmethysulfonyl fluoride, 0.02% bovine serum albumin). Luciferase assays were started by adding 1.25µM coelentrazine h (Promega USA) to cell extracts, and activity was measured using GloMax 20/20 luminometer (Promega). Luciferase activity (RLU) is expressed as relative luminescence per 10 sec/mg protein.

2.3.8 Preparation of yeast cell extracts for Tandem Affinity Purification

1 litre of S. cerevisiae TAP-tagged strains at mid-log phase was harvested at 8,000xg for 4 min at 30°C in an Avanti J-20 centrifuge (Beckman Coulter). The supernatant was removed and the yeast cell pellet was resuspended in 50ml of SC-His containing 3% glucose. The cells were then transferred to a 50ml tube and pelleted at 5,000xg for 5 min at 4°C in a clinical centrifuge. All media was removed and yeast cells were flash frozen in liquid nitrogen. 5ml of LOLA140 complete lysis buffer (20mM Tris-HCl (pH 8.0), 140mM NaCl, 1mM MgCl$_2$, 0.5% NP40, 0.5mM DTT, 1mM PMSF, phospho inhibitors (5mM NaF, 100µM Na$_3$VO$_4$) and protease inhibitor cocktail) was added to
the frozen cells, re-frozen again and stored at -80°C prior to processing. Cells were ground to a fine powder in liquid nitrogen in a freezer mill (Spec 6870, SPEX, UK). The powder was allowed to thaw on ice and clarified by centrifugation at 15000xg for 10 min at 4°C in a microcentrifuge (Eppendorf, Model 5414D) to give the whole cell extract.

### 2.3.9 Tandem Affinity Purification of proteins

200µl of an IgG bead slurry (Dynabeads M-280 Tosylated - Invitrogen) was washed twice in LOLA140 complete buffer. Then 10mg of whole cell extract was incubated with the beads on a rotating wheel in the cold room for 20 min. Following this, 50µl of supernatant (flowthrough) was removed for SDS-PAGE analysis. The conjugated beads were washed 3 times for 15 min using 1ml of LOLA140 complete. NP40 was not included in the buffer for the final wash step as the detergent interferes with mass spectrometry. Bound proteins were eluted using the elution peptide (O(PEG)4-DCAWHLGELVWCT) (Peptide Protein Research Limited). 200µl of elution buffer (40mM Tris-HCl (pH8, 100mM NaCl, 1mM EDTA, 0.01% (v/v) Tween 20, 5% ethanol) containing 2.1mM final concentration of the peptide was mixed with the beads after the final wash and incubated with rotation at room temperature for 15 min. The eluate was collected and concentrated using a YM13 ultracentrifugal filter (Millipore). Samples were mixed with 2x SDS loading buffer and run on SDS-PAGE gel for either Western analysis or stained with Coomassie for mass spectrometry.

### 2.3.10 Preparation of yeast cell extracts for FLAG-Affinity purification

4litre of mid-log phase *C. albicans* FLAG-Gcd1p strain was harvested at 8,000xg for 4 min at 30°C in an Avanti J-20 centrifuge (Beckman Coulter). All supernatant was
removed and the yeast were resuspended in 50ml of SC containing 3% glucose. The suspension was transferred to 50ml tube and yeast were pelleted at 5,000xg for 5 min at 4°C in a clinical centrifuge. The spent media was removed and the yeast cells were frozen in liquid nitrogen. 5ml of lysis buffer (30mM HEPES, 800mM KCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5mM NaF, 1mM PMSF, 5mM β-mercaptoethanol, 1% (v/v) Calbiochem Set IV Protease Inhibitor Cocktail, 1µg/ml pepstatin, 1µg/ml leupeptin, 1µg/ml aprotinin in dH2O pH 7.5) was added to the frozen cells, re-frozen and stored at -80°C prior to processing. Cells were ground to a fine powder in liquid nitrogen in a freezer mill (Spec 6870, SPEX, UK). The powder was allowed to thaw on ice and 2ml of lysis buffer per gram of cell powder was added. The resulting mixture was clarified by centrifugation at 5,000xg for 20 min at 4°C and the supernatant collected into a new tube. The supernatant was further clarified by centrifugation at 20,000xg for 30 min at 4°C in a Biofuge stratos (Heraeus Instruments) to give the whole cell extract.

2.3.11 FLAG Affinity purification of proteins

400µl of ANTI-FLAG M2 Magnetic beads (Sigma) was pre-washed twice with 1ml lysis buffer. Whole cell extract (10g of total protein) was added to the beads and incubated with rotation for 2 h at 4°C. The supernatant (flowthrough) was removed from the beads, which were further washed twice in 25ml lysis buffer and twice in 25ml low salt buffer (30mM HEPES, 100mM KCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5mM NaF, 1mM PMSF, 5mM β-mercaptoethanol, 1% (v/v) Calbiochem Set IV Protease Inhibitor Cocktail, 1µg/ml pepstatin, 1µg/ml leupeptin, 1µg/ml aprotinin in dH2O pH 7.5). Bound proteins were eluted by incubation with 500µl elution buffer (30mM HEPES, 100mM KCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5mM NaF, 1mM PMSF, 5mM β-mercaptoethanol, 1% (v/v) Calbiochem Set IV Protease Inhibitor
Cocktail, 1µg/ml pepstatin, 1µg/ml leupeptin, 1µg/ml aprotinin in dH2O pH 7.5, 0.1mg/ml 3X FLAG peptide (Sigma) for 30 min at 4°C with constant rotation and the resulting eluate was collected.

2.4 Polysome analysis

2.4.1: Preparation of *C. albicans* cell extracts for polysome analysis

*C. albicans* cultures were grown to an OD_{600} of 0.7 and 50ml aliquots were either maintained as untreated or a particular alcohol was added for 10 min. Cultures were transferred to a pre-chilled tube containing 1ml of 50mg/ml cycloheximide (Calbiochem). The cells were harvested by centrifugation at 4,000xg for 5 min at 4°C in a clinical centrifuge (Sigma). Cells were washed in 25ml pre-chilled lysis buffer (20mM HEPES pH7.4, 2mM Magnesium Acetate, 100mM Potassium Acetate, 1mg/ml cycloheximide, 0.5mM dithiothreitol), pelleted by centrifugation at 4,000xg for 5 min and resuspended in 800µl lysis buffer. Cells were pelleted at 10,000xg for 30 sec at 4°C and resuspended in 200µl lysis buffer. 200µl of 425-600µm acid-washed glass beads (Sigma-Aldrich) were added and the tubes were mixed vigorously for 6 x 20 sec allowing cooling for at least 40 sec in iced water between each mixing step. The resulting cell extract was centrifuged twice at 10,000xg for 5 min and 15 min at 4°C in a microfuge and the OD_{260} was measured using NANODROP-8000 Spectrophotometer (Thermo Scientific). The extract was snap frozen in liquid Nitrogen and stored at -80°C.

The same protocol was used for the preparation of *S. cerevisiae* polysomes extracts, except that the concentration of cycloheximide required to prevent ribosomal run-off was significantly lower. Hence, 500µl of 10mg/ml cycloheximide was added to the chilled falcon tubes and the samples were left to chill on iced-water for 1 h before the cells were harvested.
2.4.2: Preparation of sucrose gradients

15-50% (w/v) sucrose gradients were prepared using filter-sterilized diethyl pyrocarbonate (DEPC)-treated sucrose solution (60% stock, made with water containing 200μl/L DEPC), and 10 x polysome buffer (100mM Tris Acetate, pH 7.4; 700mM ammonium acetate, 40mM magnesium acetate). Individual solutions were prepared as follows:

Table 2.1 Sucrose solutions for gradient preparation

<table>
<thead>
<tr>
<th></th>
<th>50%</th>
<th>42%</th>
<th>33%</th>
<th>24%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>60% sucrose solution (ml)</td>
<td>41.6</td>
<td>35.0</td>
<td>27.3</td>
<td>20.0</td>
<td>12.5</td>
</tr>
<tr>
<td>10% Polysome buffer (ml)</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
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<td>DEPC (ml)</td>
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<td>10.0</td>
<td>17.3</td>
<td>25.0</td>
<td>32.0</td>
</tr>
</tbody>
</table>

2.25 ml amounts of the different sucrose solutions were dispensed, starting with 50% solution, then 42%, 33%, 24% and finally 15% into SW41 polyallomer centrifuge tubes (Beckman coulter) and flash frozen in liquid nitrogen between each layer. Gradients were stored at -80°C and defrosted for 12 h at 4°C prior to loading of the samples (Luthe 1983).

2.4.3: Sedimentation of polyribosomes

A volume equivalent to 2.5 OD$_{260}$ units of the *C. albicans* or *S. cerevisiae* polysome extract was layered onto the top of a 15-50% sucrose gradient. The gradients were centrifuged in a SW41 rotor (Beckman Instruments) in an L-90K ultracentrifuge (Beckman Instruments) for 2.5 h at 40,000xg. Gradients were collected and the absorbance was continuously measured at 254 nm to generate polysome profiles using a UV/Vis detector (ISCO UA-6) attached to a pump (Tris TM) and an Optical unit type II
(Teledyne ISCO). An example polysome trace is shown in the figure below with the various ribosomal peaks labelled.

**Figure 2.1** An example of a polysome trace from actively translating cells and following inhibition of translation. A. The 40S small ribosomal subunit, 60S large ribosomal subunit, 80S monosome and polysomes are labelled. A trace from yeast inhibited at the translation initiation step would show a redistribution from the polysome peaks to the 80S/monosome peak. B. Possible changes in profile following blocks to either initiation or elongation/termination.
2.5 Transformations

2.5.1 Transformation of bacteria with plasmid DNA

0.5-1µl of purified plasmid was added to 70µl of thawed competent DH5α E. coli cells and incubated on ice for 3 min. The bacterial cells were incubated at 42°C for 1.5-2 min, then 600µl of LB media was added and the mix was incubated at 37°C for 30 min. The cells were pelleted at 5000xg in a microfuge for 15 sec. Excess LB was removed to leave 100-200µl, which was spread on LB carbenicillin agar plate.

2.5.2 Transformations into C. albicans

C. albicans cells were transformed using the lithium acetate procedure (Wilson et al. 1999). 50ml cultures were grown at 30°C in YPD to the equivalent of 5x10^6 cells/ml. Cells were pelleted at 5000xg for 5 min in a clinical centrifuge and washed in 5ml Lithium acetate (LATE) buffer (0.1M lithium acetate, 10mM Tris HCl (pH 7.5), 1mM EDTA) before being resuspended in 500µl of LATE buffer. A transformation mix consisting of 100µl cell suspension, 5µl salmon sperm DNA (10mg/ml) and 80µl PCR product was prepared and incubated in a shaker incubator at 30°C for 30 min. Before salmon sperm DNA was added to the transformation mix, the DNA was denatured by boiling for 5 min then chilling on ice. Then 700µl of PLATE buffer (40% PEG4000 in LATE buffer) was added to the culture, mixed briefly and incubated at 30°C overnight. The samples were incubated at 42°C for 1 h before 3ml YPD media was added and the cell suspension was incubated overnight at room temperature. The cells were pelleted and most of the supernatant was tipped off, leaving 200µl, which was spread onto selective solid media. Plates were incubated at 30°C for 3-5 days and transformants were selected from these plates.
2.6 Manipulation of DNA in vitro

2.6.1 Extraction of genomic DNA from yeast

*C. albicans* was grown in appropriate media at 30°C to stationary phase. 5ml was harvested by centrifugation at 16,000xg for 2 min in a microfuge. Pellets were resuspended in 1ml of a pre-spheroplasting mix (1M sorbitol, 1mM EDTA and 30mM dithiothreitol), then pelleted by centrifugation at 16,000xg for 5 min. The cells were resuspended in 500µl of spheroplasting mix (1M sorbitol, 1mM EDTA, 15mM β-mercaptoethanol and 0.235mg/ml lyticase), then incubated at 37°C for 20 min before 55µl of stop solution (3M NaCl, 100mM Tris pH7.5, 20mM EDTA) with 30µl of 20% (w/v) SDS was added and mixed. An equal volume of phenol chloroform (1:1 (v/v) Tris HCl, pH8.0 buffered) was added to the extract, mixed and the aqueous phase was separated from the organic phase by centrifugation at 13,000xg for 2 min in a microfuge. The aqueous layer was collected and the phenol-chloroform step was repeated. An equal volume of chloroform was added, samples were mixed and centrifuged at 16,000xg for 2 min in a microfuge. The aqueous layer was collected and nucleic acids were precipitated by the addition of 2 volumes of ethanol. The samples were centrifuged at 16,000xg for 15 min and the pellet was resuspended in 50µl of TE buffer (10mM Tris pH7.5, 1mM EDTA pH8.0).

2.6.2 Amplification of DNA by polymerase chain reaction

Polymerase chain reaction (PCR) using an Expand High Fidelity kit (Roche) was carried out to amplify cassettes used for genomic epitope tagging. Verification of genomic integration was carried out using the same kit. Oligonucleotides used in this study are listed in table 2.6.
In vitro amplification of DNA using the Expand High Fidelity kit were performed in 0.5ml thin walled microfuge tubes by adding 1µl of the desired DNA template (5-50ng) with 1 x PCR buffer 2 (containing MgCl₂), 2.5mM dNTPs, 0.2µM of each oligonucleotide primer, 3.4 U of DNA Expand polymerase and sterile distilled water to a final volume of 50µl. Samples were then placed in a Biometra® T3 thermocycler and subjected to the following reaction conditions:

1. Initial denaturation at 95°C for 3 min

2. Denaturation at 95°C for 15 sec

3. Annealing at the appropriate temperature for the oligonucleotide primers for 30 sec

4. Extension at 72°C for 1 min

5. Final extension at 72°C for 7 min

Stages 2-4 were repeated 30 times. Melting temperature ™ of the oligonucleotides was estimated using the following formula; Tm=2[(A+T) +2(G+C)] where A, T, G and C refer to the base composition of the oligonucleotide. If the Tm for two oligonucleotides used in one reaction varied the lower value was used.

2.6.3 Agarose gel electrophoresis

DNA was separated according to its size via electrophoresis on agarose gels (1% (w/v) agarose in 1 x TAE buffer (40mM Tris base, 20mM acetic acid, 1mM EDTA pH8.0) containing 0.5µg/ml of Safe view nucleic acid stain (NBS Biologicals, UK). DNA samples were mixed with 5µl of orange loading dye (25% Ficoll, 10mM EDTA and 25µg/ml orange G) and loaded onto the gel. Electrophoresis was carried out in a BioRad electrophoresis chamber at 100V for 60 min DNA was visualized with a UV transilluminator.
2.7 Microscopic analysis

2.7.1 Slide preparation

Cells were grown to an OD$_{600}$ of 0.6 in YPD and harvested by centrifugation for 1 min at 5000xg. Cell pellets were resuspended in 1x PBS containing particular concentrations of alcohol or untreated and incubated for 15 min at 30°C. 1ml of cells was pelleted, the supernatant was removed leaving approx. 100µl of culture. 5µl of cell suspension was applied to a 0.01% (w/v) poly-lysine (Sigma) coated glass slides. A cover slip was carefully lowered onto the slide and the edges sealed with a solvent based nail polish.

2.7.2 Epifluorescent microscopy

Real-time 2D deconvolved projections generated from continuous z-sweep acquisition were generated using a Delta Vision RT microscope with an Olympus 100X/1.4 NA differential interference contrast (DIC) oil Plan Apo objective and Roper Coolsnap HQ (Photometrics) camera using Applied Precision Softworx 1.1 software and 2X2 binning at room temperature. Z-sweep acquisition allowed fast visualisation of all planes whilst minimizing fluorescent bleaching. Time course experiments were performed by acquiring images every 5 sec over a two min time period. ImageJ was used to track and quantitate the movement of the eIF2B body in the images acquired during the time course. The values calculated for the mean total distance moved by the eIF2B body in Chapter 4 were subjected to a two-sample t test to determine if the mean total distance moved by the eIF2B body in cell treated with either 1% (v/v) butanol or 100µM farnesol was significantly different from the movement of eIF2B body in untreated cells. Where p<0.05 the null hypothesis that there was no significant difference between the means of the samples was rejected. Where p>0.05 there was no evidence to reject the null hypothesis.
2.8 Formaldehyde cross-linked polysome analysis

2.8.1 Extract preparation

Extracts for cross-linked sucrose density gradient analysis were made using the method described by (Nielsen et al. 2004). 80ml of yeast culture grown to OD$_{600}$ 0.6 in YPD was added to 15ml of crushed, frozen YPD media and 2.16ml of 40% formaldehyde. After 1 h on ice, glycine was added to 0.1M. Cross-linked cells were harvested by centrifugation and polysome extracts made as described in section 2.4.1, except that cycloheximide was omitted from the buffers.

2.8.2 Gradient fractionation and protein purification

7.5 Abs$_{260}$ units of cross-linked cell extract were separated on 15-50% (w/v) sucrose gradients using the method described in section 2.4.1. 650µl gradient fractions were collected using a Foxy Jr fraction collector (Isco) while the Abs$_{254}$ was continuously measured. Individual fractions were precipitated with 20% (w/v) tricarboxylic acid, washed with 500µl acetone and resuspended in 50µl Laemmli buffer. Proteins were analysed by SDS-PAGE and western blotting.

2.9 RNA sequencing of polysome fractions

2.9.1 Preparation of C. albicans extracts

C. albicans extracts for polysome analysis for RNA sequencing cultures were prepared as was described in section 2.4.1 except that 100ml of cell culture was used. 1/4 of the polysome extracts (approx. 100µl) was made up to 300µl with the polysome lysis buffer and stored in the -80°C freezer to serve as the input fraction.

10 A$_{260}$ units of the remaining extract (approx. 300µl) were separated on 15-50% (w/v) sucrose gradients as described above except that the sensitivity was on the ISCO UA-6 detector was set at 1. 600µl gradient fractions were collected into tubes.
15 harboured the polysomal material and were used to prepare RNA. The polysomal samples were pooled after precipitating with isopropanol.

### 2.9.2 Extraction of RNA from the input and polysome fractions.

900µl of Trizol (Invitrogen) was added to the input fraction, whereas for the polysome fractions, samples were split into two 300µl amounts and 900µl of Trizol was added to each. The samples were stored in the -20°C freezer overnight, then thawed on ice, mixed vigorously for 1 min and left at room temperature for 5 min. 0.2ml of chloroform per 1ml of Trizol was added to the samples and mixed vigorously for 5 min then left at room temperature for 10 min. Samples were then centrifuged at 15,000xg for 15 min at 4°C in a microfuge to separate the phases. The aqueous layer was transferred to a fresh tube and 1µl of glycoblue was added (glycogen is co-precipitated with the RNA, but does not inhibit first strand synthesis at concentrations ≤4mg/ml and does not inhibit PCR). 500µl of 100% isopropanol for 1ml of Trizol reagent used was also added. Samples were incubated at room temperature for 10 min and then centrifuged at 15,000 xg for 10 min at 4°C. The resulting pellet was washed in 1ml of 70% (w/v) ethanol in DEPC treated water. The wash process was repeated, the samples were dried and then resuspended in 10µl of nuclease free water. The RNA suspension was heated to 55°C for 10 min and the RNA concentration was determined using a NanoDrop 8000 Spectrophotometer (Thermo Scientific).

### 2.9.3 Ribodepletion treatment to remove ribosomal RNA from the sample

#### 2.9.3.1 Magnetic Beads preparation

Ribo-zero™ magnetic Gold kit for yeast was used for the ribodepletion. The magnetic core kit was left at room temperature to equilibrate. For each Ribo-Zero reaction, 225µl
of the magnetic beads suspension was aliquoted into an RNase-free tube. The tube was placed on magnet stand and the supernatant was removed. The bead was then washed twice with 225µl of RNase-free water and resuspended in 65µl of bead resuspension solution.

2.9.3.2 Treatment of the Total RNA sample with Ribo-Zero rRNA removal solution

To each reaction set 1-5µg sample RNA, 8-10µl rRNA Removal solution and 4µl Reaction buffer were added. The reaction was then made up to 40µl with RNase-free water, mixed by pipetting and incubated first at 65°C for 10 min then at room temperature for 5 min.

The volume of Ribo-Zero rRNA Removal solution used was calculated based on:

<table>
<thead>
<tr>
<th>Amount of input total RNA</th>
<th>Max volume of total RNA</th>
<th>Volume of removal solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2.5µg</td>
<td>28µl</td>
<td>8µl</td>
</tr>
<tr>
<td>2.5-5µg</td>
<td>26µl</td>
<td>10µl</td>
</tr>
</tbody>
</table>

2.9.3.3 Magnetic Bead Reaction and rRNA Removal

The entire reaction was added to a tube containing the washed magnetic beads and immediately mixed by pipetting. Following incubation at room temperature for 5 min, the reaction was mixed vigorously for 10 sec and then incubated at 50°C for 5 min. Samples were placed in the magnetic stand and the supernatant (rRNA-depleted sample) was removed to a fresh RNase-free tube.
2.9.3.4 Purification of the rRNA-Depleted Sample

The volume of each sample was adjusted to 180µl using RNase-free water and the RNA precipitated with 18µl of 3 M Sodium acetate, 2µl of 10mg/ml glycobule and 600µl of ice-cold 100% ethanol at -20°C for 1 h followed by centrifugation at 15,000xg in microfuge for 30 min. The supernatant was carefully removed and the RNA pellet washed with ice-cold 70% ethanol then centrifuged at 15,000xg for 5 min. This wash step was repeated and any residual supernatant was removed. The RNA pellet was dried at room temperature for 5 min then dissolved in RNase-free water and stored at -80°C.

2.9.4 Assay for ribodepletion from RNA samples using Agilent RNA 6000 Nano Kit

2.9.4.1 Preparing the gel-dye mix

The RNA 6000 Nano dye was allowed to equilibrate at room temperature for 30 min, and mixed well. For each assay, 1µl of dye was added to 65µl of filtered RNA 6000 Nano gel matrix in a 0.5ml RNase-free tube. The solution was mixed well and centrifuged at 15,000xg for 10 min.

2.9.4.2 Agilent RNA 6000 Nano Gel Electrophoresis

Electrophoresis was performed as described in the manufacturers (Agilent) manual using the RNA 6000 Nano-chip. Briefly, 9µl of the gel-dye mix was loaded into the appropriate wells, 5µl of RNA 6000 Nano marker was loaded in the sample wells and the ladder well. 1µl of the prepared ladder was also loaded in the ladder well and 1µl of the sample was loaded in each of the sample well on the chip. The chip was mixed horizontally on the IKA vortexer (Agilent) for 1 min at 2400xg and run in the Agilent 2100 bioanalyzer for 5 min.
The ribodepleted RNA samples were then sent to the Genomic facilities of the University of Manchester for RNA sequencing using the Illumina Hiseq RNA sequencing technique. Briefly to generate the RNA-seq libraries, the RNA samples were fragmented, then double-stranded complementary DNA (cDNA) was generated from the fragmented RNA. Adapter sequences were added at either end of each cDNA molecule using the TruSeq stranded mRNA LT sample Prep kits and RNA Adapter indices (AR001-AR016, AR018-AR023, AR025, AR027 (Illumina, USA)

The cDNA that have adapter molecules on both ends are then selectively enriched and amplified by PCR using PCR Primer cocktails that anneal to the ends of the adapters. Next, the cDNA library is validated to check the purity of the sample by running 1µg of sample on an Agilent Technologies 2100 Bioanalyzer using the Agilent DNA 1000 chip and then by quantified by qPCR. The cDNA library is sequenced using illumina HiSeq sequencing platform.

2.9.4.3 Data analysis

Illumina HiSeq data was initially trimmed by removing low quality bases (quality scores < 20) from the 3’ end of all reads. Trimming was conducted using TRIMMOMATIC (Bolger et al. 2014). Trimmed reads were mapped (aligned) to Candida albicans SC5314 genome assembly and annotations (www.candidagenome.org.Assembly version 21) using STAR mapper (Dobin et al. 2013). The mapped reads were counted for all annotated genes using bedtools (Quinlan and Hall 2010). More specifically, two equations were obtained and these were applied to arrive at a set of differentially expressed genes. For the change in transcript, \( \log_2([TF/TU]) \) and for the change in translation \( \log_2([PF/TF]/[PU/TU]) \), with a cut off value of ±1.0 for alteration in both the transcript and translation.
2.9.4.4 Quantitative real-time PCR

RNA samples from input and polysome fractions were prepared as in section 2.9.2. except that the samples were spiked with Luciferase RNA to serve as a reference. RNA (1-6µl of each sample) was reverse transcribed into cDNA using a Protoscript Moloney murine leukemia virus (M-MuLV) RT-PCR kit and random hexamer primer (New England Biolabs). Oligonucleotide primers (Table 2.6) were designed to specific transcripts that are either differentially regulated transcriptionally or translationally in the presence of farnesol. Oligonucleotide primers were designed using the Primer3 software. Quantification was performed using the CFx Connect Real-Time system with iTaq Universal SYBR Green Supermix (BioRad Laboratories). 10ng of cDNA was added to 1x SYBR green RT-PCR buffer, 30nM of forward and reverse primer, and nuclease-free water up to a volume of 10µl. Reactions were run with initial denaturation of 95°C for 3 min, 5 sec at 95°C, 30 sec at 60°C and 40 cycles of 95-60°C and finally a melting curve. LUC was used as reference for this study. Samples were run in triplicate and in each case signals were normalized to the untreated sample for each primer pair. Data was analysed manually using the 2-\Delta\DeltaCT method (Livak and Schmittgen 2001).

2.10 Mass spectrometric analysis

SDS PAGE resolving gels were removed from the gel running apparatus and soaked in Simply Blue SafeStain (Invitrogen) for 1 h, then washed in water. Bands stained with Coomassie were excised from SDS PAGE gels and dehydrated using acetonitrile followed by vacuum centrifugation. Dried gel pieces were reduced with 10mM dithiothreitol and alkylated with 55mM iodoacetamide. Gel pieces were then washed alternately with 25mM ammonium bicarbonate followed by acetonitrile. This was repeated, and the gel pieces dried by vacuum centrifugation. 100ng sequencing grade
trypsin (Sigma), diluted in 50mM ammonium bicarbonate, was added and the gel samples were incubated overnight at 37 °C. Following digestion, the supernatants were removed to a fresh tube.

Digested samples were analysed by LC-MS/MS using an UltiMate® 3000 Rapid Separation liquid chromatography (RSLC, Dionex Corporation, Sunnyvale, CA) coupled to a LTQ Velos Pro (Thermo Fisher Scientific, Waltham, MA) mass spectrometer.

Peptide mixtures were separated using a gradient from 92% solution A (0.1% formic acid (FA) in water) and 8% solution B (0.1% FA in acetonitrile) to 33% solution B, in 44 min at 300nl min$^{-1}$, using a 250mm x 75μm i.d 1.7μM BEH (Ethylene Bridged Hybrid) C18, analytical column (Waters). Peptides were selected for fragmentation automatically by data dependent analysis.

2.10.3 Data Analysis

Data produced were searched using Mascot (Matrix Science UK), against the Uniprot database with taxonomy of [S. cerevisie and C. albicans] selected. Data were validated using Scaffold (Proteome Software, Portland, OR).
### Table 2.2 Candida albicans strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAI4/YMK 1766</td>
<td>ura3/ura3::λimm434/ura3::λimm434</td>
<td>(Fonzi and Irwin 1993)</td>
</tr>
<tr>
<td>HTC52/YMK1767</td>
<td>ura3/ura3::λimm434/ura3::λimm434 gen2::hisG/gen2::hisG</td>
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<td>YMK 1863</td>
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### Table 2.3 S. cerevisiae strains

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Table 2.4 Plasmids used in this study

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<td>pV5-NAT1/BMK668</td>
<td>V5</td>
<td>(Milne et al. 2011)</td>
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<td>This study</td>
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Table 2.5 Primary antibodies used in this study

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Table 2.6 Oligonucleotides used in this study

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3 Alcohols inhibit translation initiation in *Candida albicans* yet have opposing effects on morphological transition

3.1 Introduction

In budding yeast, amino acid starvation elicits a well-characterised mechanism of translational control involving phosphorylation of eukaryotic initiation factor 2\(\alpha\) (eIF2\(\alpha\)) via activation of eIF2\(\alpha\) (Gcn2p) kinases (Dever *et al.* 1992). Phosphorylated eIF2\(\alpha\) interacts tightly with eIF2B and inhibits its guanine nucleotide exchange function. This ultimately leads to the translation of *GCN4* mRNA while decreasing the rate of general translation. GCN signalling (or general amino acid control) has been best characterized in *Saccharomyces cerevisiae* (Hinnebusch 1988), although the GCN response has been described for *Candida albicans* where it is involved in both morphogenesis and biofilm formation (Tripathi *et al.* 2002).

*C. albicans* gcn2/gcn2 null mutants exhibit no detectable eIF2\(\alpha\) kinase activity indicating that *GCN2* encodes the major eIF2\(\alpha\) kinase activity in this fungus (Tournu *et al.* 2005). Moreover the eIF2\(\alpha\) kinase activity increased in wild-type cells on exposure to 3AT (3-Amino triazole) suggesting that this Gcn2p kinase is activated in response to amino acid starvation (Tripathi *et al.* 2002).

In *S. cerevisiae*, fusel alcohols also target eIF2B to bring about an inhibition in translation initiation. However this regulation of translation initiation by fusel alcohols is independent of the Gcn2p kinase (Ashe *et al.* 2001). In fact fusel alcohols lead to a Sit4p-dependent dephosphorylation of eIF2\(\alpha\) in *S. cerevisiae* rather than an increase in phosphorylation (Griffiths and Ashe 2006; Taylor *et al.* 2010).

Fusel alcohols as well as ethanol have also been shown to induce morphological changes in specific strains of *S. cerevisiae* (Dickinson 1996; Lorenz *et al.* 2000) and *C. albicans* (Pollack and Hashimoto 1985).
In contrast, farnesol, is a long chain quorum sensing or signalling alcohol that has been shown to block the switch from yeast to pseudohyphae or hyphae in *Candida albicans*, (Hornby *et al.* 2001). Therefore, farnesol elicits an opposing effect to butanol in terms of morphological switching in this important human pathogen. An important question is whether butanol and farnesol affect translation initiation in *Candida albicans* and if so, is this inhibition is independent of Gcn2 kinase, as was observed in *S. cerevisiae*.

In this chapter, the translational response of *C. albicans* to fusel alcohols, ethanol and farnesol is investigated, and the inhibitory effects of farnesol on filament formation in *C. albicans* in the presence of serum and butanol are analysed.

### 3.2 Results

#### 3.2.1 Different alcohols inhibit growth of both CAI4 and gcn2Δ strains of *Candida albicans*

Fusel alcohols and ethanol have been shown to inhibit growth of *Saccharomyces cerevisiae* in a dose dependent manner (Griffiths and Ashe 2006). This growth inhibition was shown both in the CAI4 and gcn2 null mutant. Alcohols are the product of fermentative growth and one possibility is that crabtree positive yeast species (yeast that preferentially ferment glucose) might present different alcohol sensitivities to crabtree negative yeast (species that preferentially respire carbon sources). On this account we set out to investigate the impact of alcohols on the growth of the crabtree negative yeast species *Candida albicans*, both the CAI4 strain and the gcn2Δ mutant. Three different alcohols were selected. Firstly ethanol as the major product of fermentative growth. Secondly, 1-butanol (n-butanol) as a fusel alcohol that induces pseudohyphal and hyphal-like growth both in *S. cerevisiae* and *C. albicans*. Finally, farnesol was selected as a quorum sensing long chain alcohol that inhibits filamentous
growth in both yeasts. Cultures of the CAI4 and gcn2Δ C. albicans strains were grown to mid-log phase (OD$_{600}$ of 0.7) in rich media. The cells were diluted into pre-warmed media and varying concentrations of each of the alcohols were then added to the cultures. As shown in figures 3.1, 3.2 and 3.3 each of the alcohols gave a concentration dependent inhibition of growth. More specifically, in the CAI-4 strain of Candida albicans, growth was mildly inhibited by butanol concentrations of 0.5% butanol (v/v), while 2% (v/v) butanol completely prevented the growth of the strain (Figure 3.1). Higher concentrations of ethanol (6% and 8% (v/v)) were required to bring the same level of growth inhibition (Figure 3.2). Much lower farnesol concentrations, e.g. 100µM, were required to cause growth inhibition. Interestingly, it was observed that the same degree of growth inhibition observed in the wild type was also evident in the gcn2Δ mutant. Finally, at very high concentrations of alcohol, the OD$_{600}$ even decreased slightly. One possible explanation for this, is that the higher alcohol concentrations could be chaotropic to cell membranes and hence cause cell lysis (Salgueiro et al. 1988; Bhaganna et al. 2010).
Figure 3.1 Butanol inhibits growth of *Candida albicans* CAI4 and mutant (gcn2Δ) strains in a dose dependent manner. The CAI4 strain and the mutant gcn2Δ strain were grown to OD$_{600}$ of 0.1 and were treated with various concentrations of butanol as indicated, OD$_{600}$ was taken hourly for 6 hr. (A) Line graphs for CAI4 and gcn2Δ strains, (B) Plot of growth rate versus alcohol concentration.
Figure 3.2 Ethanol inhibits growth of *Candida albicans* CAI4 and mutant (gcn2Δ) strains in a dose dependent manner. The CAI4 strain and the mutant gcn2Δ strain were grown to OD$_{600}$ of 0.1 and were treated with various concentrations of ethanol as indicated, OD$_{600}$ was taken hourly for 6 hr. (A) Line graphs for CAI4 and gcn2Δ strains, (B) Plot of growth rate versus alcohol concentration.
Figure 3.3  The quorum sensing molecule, farnesol inhibits growth of *Candida albicans* CAI4 and mutant (gcn2Δ) strains in a dose dependent manner. The CAI4 strain and the mutant gcn2Δ strain were grown to OD$_{600}$ of 0.1 and were treated with various concentrations of farnesol as indicated, OD$_{600}$ was taken hourly for 6 hr. (A) Line graphs for CAI4 and gcn2Δ strains, (B) Plot of growth rate versus alcohol concentration.
3.2.2 Fusel alcohol and ethanol induce pseudohyphae formation in *C. albicans*, while farnesol blocks it.

In addition to causing inhibition of growth, alcohols have also been shown to induce morphological changes (pseudohyphae and germtube formation) in various fungal species including various strains of *Saccharomyces cerevisiae* and *Candida albicans*. For instance, *S. cerevisiae* in the Δ1278B background exhibits filamentous growth in the presence of fusel alcohols (Dickinson 1996; Lorenz et al. 2000). Ethanol has also been shown to induce pseudohyphal formation in *Candida albicans* (Zeuthen et al. 1988). It has been suggested that the fusel alcohols might signal nitrogen scarcity to the cell (Dickinson 1996). Hence, pseudohyphae and germtube formation may allow these non-motile organisms to forage for limiting nutrients (Gimeno et al. 1992). In addition, for *Candida* species, it has been suggested that such growth patterns may provide a means of evading the immune responses of their host (Lorenz et al. 2000).

We studied the morphology of *C. albicans* in the presence of the selected exogenous alcohols in both liquid and solid media. Resting phase cells were inoculated into rich media (YPD) at 37°C, with butanol, ethanol or farnesol added to concentrations of 0.5% (v/v), 2% (v/v) and 40µM respectively. As a positive control, 10% serum was used to induce hyphae, whereas cells growing in YPD alone remained in the yeast form at 37°C (Figures 3.4 and 3.5).

Interestingly, addition of butanol, and ethanol to the growth media of both the CAI4 and *gcn2Δ* mutant *C. albicans* strains induced morphological differentiation both in liquid and solid growth media as presented in figures 3.4 and 3.5. Furthermore, about 50% of the cells exposed to ethanol or butanol formed pseudohyphae in less than 4 h of incubation in the liquid media as presented in figure 3.5B. However, for the farnesol treated cells no germ tubes or pseudohyphae were formed.
Figure 3.4 The effect of farnesol on filament formation in *C. albicans* predominates that of serum, butanol or ethanol. Serial dilutions of overnight cultures of the CAI4 strain was spread onto YPDA medium containing 0.5% (v/v) butanol, 2% ethanol or 10% serum agar plates for filamentation assay (A) and 10% serum agar supplemented with 150µM farnesol or YPDA containing 0.5% (v/v) butanol or 2% (v/v) ethanol supplemented with 70µM farnesol for competition assay (B). Day 1 and 2 colonies were examined by microscopy and representative images are shown as in Panel A and B.
Figure 3.5 The effect of farnesol on filament formation in *C. albicans* predominates that of serum, butanol or ethanol. Overnight cultures of the CAI4 strain was inoculated into prewarmed YP medium containing 0.5% (v/v) butanol, 2% ethanol or 10% serum for filamentation assay (A) and 0.5% (v/v) butanol or 2% (v/v) ethanol supplemented with 70µM farnesol or 10% serum supplemented with 100µM or 150µM farnesol for competition assay (B, C and D). Cultures were grown in shaker incubator at 37°C for 6 hr. Samples were taken every 2 hr and 4 hr to count the cells using a cell counting chamber. Percentages given are an average (±SE) calculated from 3 different counts. F means farnesol.
3.2.3 Farnesol’s mode of action is predominant over butanol or serum

A number of previous studies have shown that farnesol blocks germ tube formation in C. albicans (Hornby et al. 2001; Nickerson et al. 2006). We set out to test if the morphogenetic signals generated by serum or butanol can override farnesol’s action and vice versa. Cells were grown in 10% serum at 37°C for 4 h and the percentage of cells exhibiting yeast, pseudohyphal and hyphal forms was quantified. 10% serum alone gave over 90% hyphal growth, while the addition of farnesol blocked the switch from yeast to hyphae (Figures 3.4A and 3.5A). However, the amount of farnesol required to inhibit hyphal growth was dramatically increased in the presence of serum (from 40µM to 150µM) (Figure 3.5C). In contrast, for the butanol- farnesol competition assay 70µM farnesol was sufficient to block pseudohyphal formation in the butanol supplemented media. This shows that farnesol’s effect was dominant to that of butanol as the cells remained in yeast morphology when farnesol is introduced. (Figure 3.4 and 3.5). However, the serum farnesol signalling pathways appear to be competing more intensely. Throughout these assays the gc2Δ strain responded similar to the wild type in terms of the effects of serum, farnesol and the alcohols (data not shown).

3.2.4 Inhibition of protein synthesis is a common response to alcohol

Cells respond to changes in the extracellular environment by adjusting the levels of proteins in the cell. A variety of alcohols have been shown to impact on synthesis of proteins. For instance, following the addition of 0.5% isoamyl alcohol or 1% butanol to yeast cells, there was 2-to-3-fold decrease in the rate of incorporation of [35S]methionine (Ashe et al. 2001). The inhibition of growth of the CAI4 and gc2Δ mutant strains of C. albicans by the alcohols in this study (Figures 3.1, 3.2 and 3.3) prompted us to examine the rate of protein synthesis following alcohol treatment. To assess whether protein synthesis is inhibited upon treatment with butanol, farnesol or ethanol,
the incorporation of exogenously added radiolabelled methionine ([\(^{35}\)S]-methionine) was evaluated in the CAI4 and mutant strains of \(\textit{C. albicans}\). The data obtained shows that both butanol (2% v/v) and ethanol (8% v/v) caused a 10-fold inhibition in methionine incorporation to protein in both the CAI4 and \(\textit{gcn2}\Delta\) strains (Figures 3.6A, B and C). Intriguingly, farnesol (300\(\mu\)M) also caused a 10-fold inhibition of protein synthesis in the wild type, but had a more drastic effect on the \(\textit{gcn2}\Delta\) strain; inhibiting methionine incorporation up to 30-fold (Figure 3.6B and C). Overall, these results suggest that all of the alcohols used in this study dramatically inhibit protein synthesis at very early stages after addition.
Figure 3.6 Inhibition of protein synthesis is a common response to alcohol

CAI4 and gcn2Δ cells were grown to exponential phase in SC-Met media and treated with various alcohols as above for 15 min. Protein synthesis was measured by pulse labelling with $^{35}$S methionine for 10 min and sample was taken every 2 min and processed. Each treatment was analysed in triplicate. A and B are representative graphs for butanol treatment. The CPM has been normalised to 1 by dividing by the value of the untreated sample at 10 min. C is a summary plot that shows the ratio of the treated to the untreated. Error bars indicate ± SEM.
Figure 3.7 Translation initiation is inhibited by alcohols in the CAI4 strain. Figure shows polysome analyses assessing the effect of alcohols on translation initiation in the CAI4 strain of *C. albicans*. Yeast were grown in YPD and various concentrations of alcohols were added as indicated for 15 min prior to extract preparation. Extracts were sedimented on 15-50% sucrose gradients and the absorbance at 254nm was continuously measured. The position of 40S, 60S and 80S peaks are labelled and the direction of sedimentation is indicated.
3.2.5 Fusel alcohols and farnesol inhibit translation initiation in a process that is independent of the eIF2α kinase.

To further investigate the stage of protein synthesis that is targeted by the alcohols used above and the mechanism through which these alcohols inhibit protein synthesis in *C. albicans*, polysome profiling was used (See section 2.4. Materials and Methods). Polysome profiling not only allows the level of protein synthesis to be investigated, but can also pinpoint the step where translation is regulated (Ashe *et al.* 2001; Taylor *et al.* 2010).

Analysis of polysome distribution across a sucrose gradient for extracts from the CAI4 strain revealed that with increasing concentrations of either butanol, farnesol or ethanol, a change in the polysome profile was observed (Figure 3.7). The 80S peak increased dramatically and the polysome peaks were reduced. This change in profile is characteristic of an inhibition of the translation initiation step (Hartwell and McLaughlin 1969; Ashe *et al.* 2001). As indicated by the redistribution of area under the polysome peaks to the 80S peak, the addition of butanol and farnesol effectively inhibit translation at 2% (v/v) and 300µM respectively, whereas concentrations of ethanol up to 8% (v/v) are required to inhibit translation initiation in this strain. The correlation between the concentrations of alcohol required to inhibit growth and translation (Figures 3.1, 3.2 and 3.3) is suggestive that the inhibition of protein translation at the initiation stage could be the cause of growth inhibition of *C. albicans* cells exposed to alcohols. It is also possible based on disparity in the concentration of the different alcohols required to cause an inhibition of translation initiation that these agents act by different mechanisms.

Given that all of the alcohols target translation at the initiation step and a predominant mechanism of translation regulation that is conserved across all eukaryotes is the
activation of eIF2α kinases, the role of the only eIF2α kinase in *C. albicans*, Gcn2p was investigated.

The *S. cerevisiae* Gcn2p homologue has been shown to mediate a number of responses to cellular stress (Palam et al. 2011). A number of cellular stress conditions including amino acid starvation (Dever et al. 1992), purine starvation (Rolfes and Hinnebusch 1993) and rapamycin (Kubota et al. 2003) activate the Gcn2p kinase to phosphorylate the α subunit of the eIF2. The phosphorylated eIF2 binds tightly to and sequesters eIF2B (the guanine nucleotide exchange factor) thereby preventing guanine nucleotide exchange on eIF2 and ultimately inhibiting translation initiation (Dever et al. 1995; Hinnebusch 2000). It has been shown previously that the regulation of translation by butanol in *S. cerevisiae* targets eIF2B but does so in a Gcn2p-independent manner (Ashe et al. 2001; Taylor et al. 2010). By using the gcn2Δ mutant of *C. albicans*, the requirement for the Gcn2p kinase for alcohol dependent inhibition of translation can be assessed.

A comparison of the polysome profiles from the gcn2Δ mutant relative to the CAI4 strain shows that there is no obvious difference in terms of inhibition of translation by butanol, farnesol and ethanol (Figures 3.7 versus 3.8). Quantitation of the polysome/monosome ratios confirms that there is little difference in the polysome profiles of the CAI4 and gcn2Δ strains of *C. albicans* (Figure 3.9). This shows that the Gcn2 kinase plays little or no role in the inhibition of translation initiation by alcohols in *C. albicans*. In fact for farnesol, the gcn2Δ mutant was even more inhibited than the wild type (cf Figure 3.8C with Figure 3.7C, Figure 3.9). This difference also highlights the possibility that these alcohols act on cells in very different fashions.
Figure 3.8 Translation initiation is inhibited by alcohols in a \textit{gcn2A} strain. Figure shows polysome analyses assessing the effect of alcohols on translation initiation in a \textit{gcn2A} strain of \textit{C. albicans}. Yeast were grown in YPD and various concentrations of alcohols were added as indicated for 15 min prior to extract preparation. Extracts were sedimented and gradients collected as in Figure 3.7.
Figure 3.9 Polysome/monosome ratio of CAI4 and gcn2Δ (mutant) strains shows similar pattern of inhibition of translation for the two strains. The areas within the monosomal and the polysomal regions were measured using ImageJ software (NIH). The values calculated were plotted as shown in the figures.
Figure 3.10 Effect of farnesol and butanol on different transcriptional regulator mutants. Polysome analysis was used to assess the effect of alcohols on translation initiation in a *efg1Δ* and *cph1Δ* strains of *C. albicans*. Yeast were grown in YPD and various concentrations of the various alcohols were added as indicated for 15 min. Extracts were sedimented on 15-50% sucrose gradients and the absorbance at 254nm was continuously measured.
3.2.5 Does the signalling pathway that regulate morphogenesis also play a role in translation initiation in *C. albicans*?

One of the key virulence factors of *C. albicans* is its ability to undergo reversible morphological transitions from the vegetative form to the filamentous form (Odds 1994; Lo *et al.* 1997). Several signalling pathways control this morphogenesis in *C. albicans* (See Section 1.3 in the introduction). These include the MAPK pathway, which is dependent upon Cph1p; a homologue of *S. cerevisiae* Ste12p (Liu *et al.* 1994); and the Ras-cAMP signalling pathway, which requires functional Efg1p (Bockmühl and Ernst 2001). Both pathways are thought to activate filamentous growth in response to starvation or serum addition. Under most experimental conditions, the transition from yeast to hyphae is blocked in the *cph1 efg1* double mutant (Lo *et al.* 1997), indicating that the transduction of most environmental signals that control morphogenesis is dependent upon Ras-cAMP and/or MAPK signalling.

In order to probe whether there is a connection between these signal transduction pathways and the translational response of *C. albicans* to alcohols, we exposed the *efg1Δ* and the *cph1Δ* strains of *C. albicans* to varying concentrations of butanol or farnesol and assessed the translational response using polysome profiling. In terms of the response to butanol, neither mutant showed any difference to the CAI4 strain in terms of the redistribution of polysomes (cf Figure 3.7 with Figure 3.10). Therefore it appears that neither Efg1p nor Cph1p is involved in the translational response of *Candida albicans* to fusel alcohols.

Interestingly for farnesol, the *efg1Δ* strain showed some translational resistance to the effects (Figure 3.10), whereas the *cph1Δ* strain was inhibited in the same manner as the wild type (See Figure 3.7 for comparison). This resistance highlights a role for the Ras-cAMP pathway in the inhibition of translation initiation following farnesol treatment. Indeed, farnesol has been shown to target the Ras1-Cdc35-PKA-Efg1 dependent
pathway to inhibit transition from yeast to hypha in *C. albicans* (Davis-Hanna *et al.* 2008). It has also been shown that farnesol directly targets the adenyl cyclase, Cyr1p, to repress hypha formation and that exogenous cAMP restored filamentation in response to host environmental cues (Hall *et al.* 2011). It yet remains to be assessed the effect of exogenous cAMP on the translational resistance exhibited by the *efg1Δ* strain to farnesol. In summary though, the differential sensitivity of the mutants highlights a distinction between the responses of *C. albicans* to the different alcohols used in this study.

### 3.2.6 Dephosphorylation of eIF2α by fusel alcohols and farnesol in *C. albicans*

Fusel alcohols have been shown to bring about an inhibition of translation initiation in *S. cerevisiae* and *C. albicans* in a Gcn2p-independent manner (Ashe *et al.* 2001), (Figure 3.8). Paradoxically, it was reported by Taylor *et al.* (2010), that fusel alcohols lead to a dephosphorylation of eIF2α in *S. cerevisiae* rather than an increase in phosphorylation. Initially, an attempt was made to detect phosphorylated eIF2α in *C. albicans* by probing the whole cell extracts with phosphospecific antibodies to phosphoserine 51 on eIF2α, but no signal was detected on western blots (e.g. Figure 3.11A, upper panel). Upon longer exposure, however, a basal signal that could be phosphorylated eIF2α is just detectable (Figure 3.11A, middle panel). In response to butanol treatment the level of this signal did not increase, in fact it was reduced. This is reminiscent of the situation in *S. cerevisiae* where fusel alcohol treatment leads to eIF2α dephosphorylation.

A variety of conditions are known to induce phosphorylation of eIF2α at serine 51 via the kinase Gcn2p; such as the addition of rapamycin, amino acid starvation and treatment with various antioxidants (Dever *et al.* 1992; Kubota *et al.* 2003; Mascarenhas *et al.* 2008). On account of the low basal level, and to further explore the possibility
that eIF2α is dephosphorylated in response to butanol, the cells were exposed to 1mM cadmium sulphate to induce phosphorylation of eIF2α (Mascarenhas et al. 2008). Under these conditions, a robust signal was observed on the western blot that was not observed for gcn2Δ strains. This band was the same size as the weak band observed in untreated cells, suggesting that the weak band is indeed phosphorylated eIF2α. To explore whether butanol induces dephosphorylation of eIF2α, cultures of a CAI4 strain and gcn2Δ strain were first treated with 1mM Cadmium sulphate for 60 min to induce detectable eIF2α phosphorylation, then treated with butanol for 15 min. As shown in figure 3.11, high concentrations of butanol cause dephosphorylation of eIF2α. These same high concentrations of butanol are required to give the maximal inhibition of protein synthesis as judged by the polysome analysis (Figure 3.7). Therefore, the inhibition of translation initiation by fusel alcohols is accompanied by and correlates with a dephosphorylation of eIF2α.

In stark contrast to the effects of butanol on eIF2α dephosphorylation, concentrations of ethanol that inhibit protein synthesis do not cause eIF2α dephosphorylation either with or without the pretreatment with Cadmium (Figure 3.12). Once again this highlights a difference between the response to these alcohols and suggests that they probably act by different mechanisms.

Finally, the impact of farnesol on eIF2α dephosphorylation was assessed (Figure 3.13). Similar to butanol, in the experiment where cells are pretreated with Cadmium to induce high levels of phospho-eIF2α, high concentrations of farnesol possibly cause some dephosphorylation of eIF2α. It should be noted however, that only 300µM farnesol gave any observable effect. In contrast, from the polysome profiling maximal inhibition of protein synthesis was already evident at 200µM farnesol. This suggests that even though farnesol has a potential weak effect on eIF2α dephosphorylation, it is not connected to the inhibition of protein synthesis.
Overall, these results confirm the *gen2Δ* mutant data, in that none of the alcohols tested causes an inhibition of translation initiation as a result of inducing eIF2α phosphorylation. In fact, butanol and possibly farnesol act to induce dephosphorylation of eIF2α. Therefore, the possibility exists that these alcohols may be activating some phosphatase to dephosphorylate eIF2. Indeed, in *S. cerevisiae* a mutant in *SIT4*, that encodes a type 2A-related phosphatase exhibited virtually no dephosphorylation after exposure to fusel alcohols (Taylor *et al.* 2010). It is possible that a similar mechanism exists in *C. albicans*. While this may be interesting in terms of signal transduction, it is unlikely to have any bearing on translational regulation as dephosphorylation of eIF2 is activatory in terms of translation (Ashe *et al.* 2001; Taylor *et al.* 2010). Therefore, it appears butanol and farnesol induce an inhibitory mechanism that dominates the effects of eIF2α dephosphorylation.
A. **CAI4**

<table>
<thead>
<tr>
<th>Cd(^{2+}) pretreatment</th>
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<th>-</th>
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<tbody>
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<td>1%</td>
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<tr>
<td><strong>10 min exposure</strong></td>
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<td>+</td>
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<tr>
<td><strong>30 min exposure</strong></td>
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**Phospho-eIF2\(\alpha\)**

**Tef1p**

---

B. **\(gcn2\Delta\)**

<table>
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<tr>
<th>Cd(^{2+}) pretreatment</th>
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<td>+</td>
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<tr>
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**Figure 3.11 Butanol causes a dose dependent decrease in eIF2\(\alpha\) serine 51 phosphorylation (eIF2\(\alpha\)-P) in the CAI4 strain.** Protein extracts from the CAI4 and the \(gcn2\Delta\) strains were blotted and probed with antibodies to Tef1 and a phosphospecific antibody to phosphoserine 51 on eIF2\(\alpha\). Strains were grown on YPD to OD\(_{600}\) of 0.7, pretreated with 1mM Cadmium for 1 hr. Various concentrations of butanol (v/v) were then added for 15 min after which protein extracts were made.
A. CAI4

<table>
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<th>Tef1p</th>
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B. gcn2Δ

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<td>6%</td>
<td>8%</td>
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<td>8%</td>
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</table>

Figure 3.12 Ethanol seems to have no effect on the level of eIF2α serine 51 phosphorylation (eIF2α-P) in the CAI4 strain. Protein extracts from the CAI4 and the gcn2Δ strains were blotted and probed with antibodies to Tef1 and a phosphospecific antibody to phosphoserine 51 on eIF2α. Strains were grown on YPD to OD₆₀₀ of 0.7, pretreated with 1mM Cadmium for 1 hr. Various concentrations of ethanol (v/v) were then added for 15 min after which protein extracts were made.
Figure 3.13 Farnesol causes a dose dependent decrease in eIF2α serine 51 phosphorylation (eIF2α-P) in the CAI4 strain. Protein extracts from the CAI4 and the gcn2Δ were blotted and probed with antibodies to Tef1 and a phosphospecific antibody to phosphoserine 51 on eIF2α. Strains were grown on YPD to OD₆₀₀ of 0.7, pretreated with 1mM Cadmium for 1 hr. Various concentrations of farnesol (v/v) were then added for 15 min after which protein extracts were made.
3.3 Discussion

The results described in this chapter show that a range of alcohols inhibit growth and protein synthesis in strains of *C. albicans* in a dose-dependent manner. The inhibition of protein synthesis occurred at the level of translation initiation as shown by the polyribosomal profiles. There is a correlation in the concentrations required to inhibit growth and translation initiation in the two strains of *Candida albicans* studied, as shown in the growth curves and the polyribosomal profiles. Gcn2p is not involved in this regulation of translation both in *C. albicans* and *S. cerevisiae*; thus there is an inhibitory pathway that is independent of the eIF2α kinases. Previous work in *S. cerevisiae* has identified a rapid inhibition of translation initiation caused by fusel alcohols, which is accompanied by a characteristic change in polysome profile (Ashe *et al.* 2001). As initiation is inhibited, ribosomes can no longer load onto transcripts and ribosomes completing a translational cycle dissociate. This leads to a decrease in heavy, polysome peaks with a concomitant rise in free ribosomes (Hartwell and McLaughlin 1969). Fusel alcohols target eIF2B to cause inhibition of translation initiation in *S. cerevisiae* and this occur in a Gcn2p-independent manner (Ashe *et al.* 2001).

Butanol and possibly farnesol cause a dose dependent dephosphorylation of eIF2α in *C. albicans*. This observation mirrors what was previously described for *S. cerevisiae* following butanol treatment (Griffiths and Ashe 2006). However, ethanol seems not to cause dephosphorylation, this finding serves to separate distinct modes of action for the alcohols on protein translation.

Finally, alcohols also cause a range of effects on the morphology of *C. albicans*. Butanol and ethanol induce a switch from vegetative growth to filamentous (germ-tube and pseudohyphal) growth. Farnesol blocks filament development initiated by any of three chemical triggers used in this study (serum, butanol and ethanol). Pseudohyphal
formation in *S. cerevisiae* in response to fusel alcohols have been described (Dickinson 1996; Lorenz *et al.* 2000).

An assessment of the translational response of key transcriptional mutants in the morphological differentiation pathway to butanol and farnesol, revealed that cAMP-PKA pathway could be playing a major role in response of *C. albicans* to farnesol. Previously it has been shown that farnesol acts via RAS- cAMP-PKA pathway to inhibit morphogenesis in *C. albicans* (Davis-Hanna *et al.* 2008; Hall *et al.* 2011).
Table 3.1 Summary of effects of various alcohols on the physiology of *C. albicans* strains

<table>
<thead>
<tr>
<th>Cellular effects</th>
<th>Butanol</th>
<th>Ethanol</th>
<th>Farnesol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of translation initiation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GCN2-dependent inhibition</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>eIF2αP dephosphorylation</td>
<td>+</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Induction of morphological differentiation</td>
<td>+</td>
<td>+</td>
<td>—</td>
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</table>
4 Effects of various alcohols on the eukaryotic initiation factor 2B (eIF2B)

4.1 Introduction

Eukaryotic initiation factor 2B is the guanine nucleotide exchange factor for eIF2. It promotes the exchange of GDP for GTP on eIF2. The resulting eIF2.GTP has the capacity to interact with the initiator methionyl tRNA to form the ternary complex that is a critical part of the translation initiation process (Proud 2005). Recognition of the AUG start codon results in eIF5-dependent GTP hydrolysis on eIF2. eIF2B is required to recycle the resulting eIF2.GDP back to its translationally competent GTP bound form. eIF2B is therefore a key target of translational control mechanisms, as a decrease in its activity leads to a depleted pool of GTP bound active eIF2 in the cell and therefore a decrease in the rate of translation initiation (Dever et al. 1995).

As a result, several cellular stress conditions that inhibit the activity of eIF2B lead to reduced ternary complex and the inhibition of protein synthesis. Paradoxically, such stress conditions lead to the activation of the translation of specific mRNAs via short upstream open reading frames (uORFs) (see Introduction 1.8.3). For instance, in S. cerevisiae amino acid starvation leads to the phosphorylation of eIF2α by Gcn2p, which converts eIF2.GDP from a substrate to an inhibitor of eIF2B to impede the formation of ternary complex. As a result global protein synthesis is inhibited, while GCN4 mRNA translation is activated via a complex mechanism involving four uORFs (See Section 1.8.3) (Hinnebusch 1993).

Studies from the Ashe laboratory in S. cerevisiae have shown that fusel alcohols, such as 1-butanol, inhibit protein translation and activate GCN4 mRNA translation by a different mechanism (Ashe et al. 2001). Instead of activating the Gcn2p kinase to indirectly regulate eIF2B, these alcohols appear to impact on eIF2B more directly in a Gcn2p independent manner. This proposed mechanism of action for the alcohols has
stemmed from a number of key observations. Firstly, mutant strains such as *SUI2*-S51A and *gcn2Δ*, that are translationally resistant to amino acid starvation, are still sensitive to fusel alcohols. In addition, a mutation in the γ subunit of eIF2B (*GCD1*-P180 to *GCD1*-S180) was identified that confers greater sensitivity to fusel alcohols (Ashe *et al.* 2001). Subsequently, a number of other mutations in the different eIF2B sub-unit genes have been shown to impact on the resistance and sensitivity of yeast to fusel alcohols; (Richardson *et al.* 2004; Taylor *et al.* 2010). Finally, a direct evaluation of the precise levels of ternary complex after treatment with butanol shows that ternary complex levels are reduced in butanol sensitive strains but unaffected in butanol resistant strains (Taylor *et al.* 2010).

Other studies from the Ashe lab have focussed on the cellular localisation of eIF2B and its G-protein eIF2 (Campbell *et al.* 2005; Campbell and Ashe 2006; Taylor *et al.* 2010). It appears that both eIF2B and eIF2 co-localise to a discrete cytoplasmic focus within the cell that has been termed the eIF2B body (Campbell *et al.* 2005). This localisation is specific to these two initiation factors, as other initiation factors are found dispersed throughout the cytoplasm (Campbell *et al.* 2005). The eIF2B body is thought to provide a locally increased concentration of eIF2B and thus serves as a site for focussed guanine nucleotide exchange on eIF2 (Campbell *et al.* 2005). Intriguingly, butanol addition to *S. cerevisiae* cells at concentrations sufficient to inhibit protein synthesis has a negative effect on the movement of the eIF2B body. This effect was more severe in butanol sensitive relative to resistant strains, suggesting a role for the movement of the eIF2B body in the response to butanol (Taylor *et al.* 2010).

Attempts have also been made in the Ashe lab to identify fusel alcohol dependent covalent modifications in yeast eIF2B via mass spectrometric analysis. Such analyses have identified a number of potential phosphorylation sites in eIF2Bɛ, eIF2Bα and
eIF2Bγ and mutations in one of the eIF2Bδ phosphorylation sites altered the sensitivity of yeast to butanol (Taylor et al. 2010).

Given the results described above for S. cerevisiae, this chapter describes the study of the effects of butanol, ethanol and farnesol on the activation of GCN4 mRNA, the integrity and modification status of the eIF2B complex, and the presence/ movement of the eIF2B body in the related pathogenic yeast, C. albicans.

4.2 Results

4.2.1 Butanol and ethanol induce the translation of GCN4 mRNA in a Gcn2p independent manner in C. albicans

In S. cerevisiae, cellular stresses that inhibit translation initiation by reducing ternary complex also lead to the translational induction of the GCN4 mRNA. A similar system has been identified in C. albicans (Tripathi et al. 2002; Sundaram and Grant 2014). Therefore, as alcohols caused an inhibition of translation initiation in C. albicans (Section 3.2.5), the possibility exists that these alcohols could also act via a reduction of the ternary complex and hence induce translation of the GCN4 mRNA. In S. cerevisiae a GCN4-lacZ reporter construct containing the GCN4 promoter and the 5′-untranslated region driving expression of the lacZ gene was previously used to test whether Gcn4p is induced upon butanol treatment (Ashe et al. 2001). The expression of the GCN4-lacZ reporter was observed to increase ~3 fold following butanol treatment for 2 h. This experiment formed part of the evidence that fusel alcohols target eIF2B to reduce ternary complex levels.

Reporter genes that encode bioluminescence proteins, like luciferases, have provided a very rapid method for analysing the regulation of gene expression (Bronstein et al. 1994). Srikantha et al. (1996) reported the development of a reporter system for C.
*Candida albicans* using the luciferase gene *RLUC* of the sea pansy *Renilla reniformis*. The reporter contains no CUG codons in its open reading frame, as *Candida albicans* and other members of the CTG clade exhibit non-traditional codon use (Rocha *et al.* 2011); they decode CUG as serine rather than leucine. Furthermore, the choice of Renilla Luciferase as the reporter for this assay was made based on the fact that the reporter is ATP-independent (Lorenz *et al.* 1991; Wurdinger *et al.* 2008; Huang *et al.* 2011). Therefore RLUC reporter is well suited for use in *C. albicans* considering that the filamentation system is regulated by cAMP. As a result, this cassette has been adapted in two reporter constructs for use in the study of *GCN4* regulation in *C. albicans*. Firstly, a reporter has been constructed with five copies of the *GCRE* (General Control Response Element) upstream of a basal promoter and the *RLUC* reporter gene. This reporter allows the induction of Gcn4p responsive genes to be followed (Sundaram and Grant 2014). Secondly, a *GCN4-RLUC* reporter has been developed in which the 5′-leader sequence of *GCN4* was cloned upstream of a luciferase reporter gene. This reporter more directly assesses the translational regulation of the *GCN4* mRNA (Sundaram and Grant 2014).

Previously both the *GCRE* and *GCN4* reporters have been integrated specifically into the *Candida albicans* genome of CAI4 and gcn2Δ strains (Sundaram and Grant 2014). These strains have been used here to assess how alcohols (1% butanol, 6% ethanol and 100µM farnesol) impact on this pathway. The rationale behind the concentrations of alcohols used was that at these concentrations the alcohols do not completely inhibit translation initiation so any induction of *GCN4* should be observable based on similar observations in *S. cerevisiae*. Indeed for the ethanol and butanol treatments there was ~3.6 and ~2.6-fold increase in *GCRE*-Luc expression, respectively after two hours. However, for farnesol, no significant alteration relative to the basal level of *GCRE*-Luc expression was observed (Figure 4.1A). For the *GCN4*-Luc reporter strain, ethanol and
butanol treatment once again gave an induction of ~1.65 and ~2.4-fold respectively, whereas farnesol treatment gave no induction (Figure 4.2A). All of the increases observed following butanol and ethanol treatment were also observed in the gcna mutant strain (Figure 4.1B and 4.2B).

These results are suggestive that the inhibition of translation initiation by ethanol and butanol results from a decrease in the level of ternary complex. One possible explanation for this is that, in an analogous manner to the action of fusel alcohols on *S. cerevisiae*, ethanol and butanol act to inhibit eIF2B directly in a mechanism that does not involve eIF2α phosphorylation. In contrast, for farnesol, the mechanism of translational regulation appears to not involve changes in the activity of eIF2B and the levels of the ternary complex. As the results from the ethanol and butanol experiments thus far are very similar, in subsequent experiments a decision was made to focus on butanol and compare the results directly to the impact of farnesol.
Figure 4.1 Alcohols impact on the general control pathway in *C. albicans* in a Gcn2p independent manner. The CAI4 (A) and the *gcn2Δ* mutant (B) strains were treated with the indicated concentrations of alcohol for 2 h, and luciferase activity was measured from the *GCRE*-Luc reporter gene. Error bars indicate ± SE.
Figure 4.2 Alcohols induce *GCN4* expression in *C. albicans* in a Gcn2p independent manner. The CAI4 (A) and the *gcn2*Δ mutant (B) strains were treated with the indicated concentrations of alcohol for 2 h, and luciferase activity was measured from the *GCN4*-Luc reporter gene. Error bars indicate ± SE.
4.2.2 Effect of alcohols on the cellular localisation of eIF2B

4.2.2.1 Generating strains

Recent findings from the Ashe laboratory have shown that eIF2B exists in a large cytoplasmic body in *S. cerevisiae*, which has been termed the eIF2B body (Campbell *et al.* 2005). This body is mobile within the cell traversing almost all regions of the cytoplasm. Intriguingly, the addition of butanol to cells, at concentrations sufficient to inhibit protein synthesis, prevents the movement of the eIF2B body (Taylor *et al.* 2010). Furthermore, in a specific butanol resistant strain treated with the same concentration of butanol, the eIF2B body moves freely. Moreover, in other butanol resistant eIF2B mutant strains, the eIF2B body is absent (Taylor *et al.* 2010). These results suggest a connection between the eIf2B body and the regulation of translation initiation by fusel alcohols in *S. cerevisiae*.

Therefore, to investigate the localisation of the eIF2B, in the diploid *C. albicans* CAI4, one copy of the gene for the γ subunit of eIF2B (*GCD1*) was C-terminally tagged with GFP using a standard PCR-mediated epitope tagging strategy (Knop *et al.* 1999). In this method a cassette is generated with long primers that direct GFP (or other epitope tags) such that they are directly integrated by homologous recombination into the genome in frame with appropriate open reading frames. Positive transformants are selected by virtue of the marker gene also present on the cassette. In this case the nourseothricin acetyltransferase (*NAT1*) gene from *Streptomyces noursei* was used which gives resistance to the antibiotic nourseothricin. Integration of the GFP-tag into the genome at the correct site was verified using a PCR strategy on genomic DNA prepared from potential transformants. The outline of this diagnostic PCR scheme is shown in figure 4.3, along with a table of expected product sizes. PCR products of the expected size were obtained, indicating that the integration of the GFP-tag had occurred at the correct site (Figure 4.3).
Western blotting was also used to confirm the GFP-tagging of the *GCD1* gene across a number of different potential transformants. Protein extracts were made from the transformants and compared to those from an untagged control strain, and the blot was probed using an antibody against the GFP protein. The GFP signal was detected at the correct size in the GFP-tagged strain but no signal was detected in the untagged CAI4 strain (Figure 4.4 A).

Epiflourescence microscopy was used to visualize the tagged strain and the image shown in Figure 4.4 B clearly shows that the majority of the cells harboured an eIF2B body. It was also observed that the body was motile under untreated conditions. These results almost precisely parallel what has been found in *S. cerevisiae* (Campbell *et al.* 2005; Taylor *et al.* 2010).
Figure 4.3 Verification of GFP tagged CAI4 strain. Strains were generated harbouring a genomically GFP-tagged copy of \textit{GCD1} using the standard PCR mediated epitope tagging method. A. Schematic of PCR amplifications to verify the correct integration of the GFP tagging cassette into the \textit{GCD1} locus. B. Expected PCR sizes and C. an agarose gel electrophoresis of PCR products generated by the amplification are shown.
Figure 4.4 GFP tagging of \textit{GCD1} shows that eIF2B\(\gamma\) is present in an eIF2B body. A) Western analysis was probed with antibodies raised against GFP to confirm the tagging and Tef1p was used as a loading control. B) Strain bearing the GFP-tagged \textit{GCD1} was visualised with an epifluorescent microscope showing a single large cytoplasmic body.
4.2.3 Alcohols do not affect the localisation of eIF2B subunits to the eIF2B body

Previous studies of translational stresses, such as amino acid starvation, in *S. cerevisiae* have shown that despite an effect on shuttling of eIF2 through eIF2B bodies, they do not impact on the ability of eIF2 or eIF2B to localise to the foci (Campbell *et al.* 2005). In contrast, mutants in the α subunit of eIF2B have been described that lead to a complete loss of the eIF2B body in *S. cerevisiae*. These mutants are also resistant to both fusel alcohols and amino acid starvation. In this section, the effect of alcohols on the localisation of eIF2B to eIF2B bodies was investigated using the *GCD1*-GFP tagged strain.

Live cell epifluorescent microscopy was performed on mid-log phase *C. albicans* strain CAI4 bearing a GFP tag on one copy of *GCD1*, to count and score cells for the absence or presence of an eIF2B body. Figure 4.5A shows representative images of the localisation pattern of the eIF2B subunit. Quantitation of the average number of cells which contained an eIF2B body in either untreated cells or cells treated with 1% (v/v) butanol or 100µM farnesol for 15 min shows that the treatments have no significant effects on the capacity of the eIF2Bγ subunit to localise to eIF2B bodies (Figure 4.5B). These results suggest that the integrity of the eIF2B body is unaffected by concentrations of either butanol or farnesol that inhibit translation initiation.

4.2.4 Effect of alcohols on the dynamics of the eIF2B body

To investigate the impact of alcohols on the movement of eIF2B bodies, cells were grown to logarithmic phase and briefly treated with a concentration of the various alcohols that has been previously shown to inhibit translation initiation (see Figure 3.7). Epiflourescence microscopy time-lapse experiments were performed by acquiring images every 5 sec over a 2 min period. The movement of the eIF2B body across the images was tracked and the total distance (µ) moved was calculated. Figure 4.6 shows...
individual stills from a single time lapse microscopy experiment and an overlay of the images from all 25 time points; this gives a representation of the extent of movement over the time course. Quantitation of the average displacement shows that 1% butanol causes total eIF2B body movement to drop by approximately 50%, while farnesol does not appear to impact on this movement (Figure 4.7). As the eIF2B body moves less after exposure to concentrations of fusel alcohols that also inhibit translation initiation, it seems possible that as has been suggested in *S. cerevisiae* (Taylor et al. 2010), at least part of the translational inhibition relates to increased tethering of the eIF2B body. It is possible that the capacity of the eIF2B body to move rapidly around the cell may allow a sustained level of translation initiation by maximising levels of ternary complex throughout the cell.

4.2.5 Purification of eIF2B and eIF2 subunits from *C. albicans* for Mass spectrometry

The cell biological approach described above further implicates eIF2B and/ or eIF2 in the regulation of translation initiation by butanol but not by farnesol. In order to further explore the impact of butanol on eIF2B, a biochemical approach was attempted. In the first instance, the goal was to purify eIF2B and/or eIF2 from *Candida albicans* cells, and a variety of different strategies were attempted.
Figure 4.5 Alcohols do not affect the localisation of eIF2Bγ subunits to eIF2B bodies. Cells bearing GFP-tagged GCD1 were visualised with an epifluorescent microscope. Cells were either untreated or treated with 1% (v/v) butanol or 100µM farnesol for 15 min and the number of cells with eIF2B body were counted against a total of 100 cells. Data represents quantification from 3 biological repeats. Error bars indicate ±SE.
Figure 4.6 Butanol impedes the movement of eIF2B bodies. Cells were grown to mid-log phase in YPD and either maintained as untreated, or treated with 1% (v/v) butanol or 100µM farnesol for 15 min. Epifluorescent real-time 2D deconvolved projections were generated from continuous z-sweep acquisition. Images of cells were taken every 5 s over a 2 min time period. Representative images are shown on the left and an overlay showing the position of the body over the 2 min time course is shown on the right.
Figure 4.7  Butanol impedes movement of eIF2B bodies. Images of cells were taken as described in 4.6 and used to calculate the total distance moved by the eIF2B body over a 2 minute period using ImageJ software package (NIH). A bar chart shows the mean total distance moved by the eIF2B bodies in µ. Values shown are calculated from ≥ 20 cells ± SE. (* denotes a significant difference from untreated sample p<0.003)
4.2.5.1 Strategy for V5S-His6x tagging of GCD1 in C. albicans

The first approach taken was the use of a combined V5-6xHis epitope tag (Milne et al. 2011) to tag the eIF2Bγ and eIF2β proteins encoded by the GCD1 and SUI3 genes, respectively. The double epitope tag combines the benefits of efficient detection through the V5 epitope and protein purification using the 6xHis epitope. The V5-6xHis-NAT1 cassette was targeted to the relevant position on the GCD1 and SUI3 genes via homologous recombination in the CAI4 strain of C. albicans (Figure 4.8A). Correct integration was confirmed by PCR screening (Figure 4.8A, B and C). Western blotting was also used to confirm tagging of the correct genes in the strain. The protein blots were probed using antibodies raised against the V5 epitope (Figure 4.7 D), and bands of the expected size (56.8kDa and 33.6kDa for GCD1 and SUI3 respectively) were detected while no signal was detected in extracts from the untagged parent strain.

4.2.5.2 Purification of proteins from V5-6xHis tagged strains.

Repeated attempts were made to purify the tagged proteins using the V5 purification method. V5 resin was used to bind the tagged protein and elution was carried out by competition with the V5 peptide (Figure 4.9A). As this did not yield the expected set of bands, protein purification was also carried out using the 6xHis tag. Ni-NTA agarose beads were utilised and bound proteins were eluted with high concentrations of imidazole. Repeated purifications using this strategy also did not yield the expected sized bands (Figure 4.9B). Furthermore none of the subunits of eIF2B or eIF2 was detected by mass spectrometry from purified samples.

There are a number of possible explanations that could explain the difficulty in purifying the eIF2B and eIF2 proteins including insufficient protein levels or inaccessibility of the epitope tag. In S. cerevisiae, an eIF2B purification system has been developed, where all five eIF2B subunits are overexpressed from two plasmids,
and FLAG epitopes on eIF2Bγ allow purification (Mohammad-Qureshi et al. 2007). *C. albicans* is an obligate diploid and autonomous plasmid systems developed for routine use are unstable in this organism (Goshorn et al. 1992). Therefore, tagging just a single copy of a gene, as for the V5-6xHis system above, will not permit maximal protein expression, as the organism will also be expressing the untagged allele of the gene of interest.

In an attempt to circumvent some of these potential bottlenecks, a different tagging system was designed, similar to that which has been previously described in *S. cerevisiae*. Firstly, to maximise tagged protein expression levels, it was decided that both copies of the eIF2Bγ gene should be tagged. Secondly, the purifying epitope was switched to FLAG and four copies were used in order to increase the binding of the Flag antibody to the gene of interest. In order to genomically tag both alleles, a strategy was adopted making use of a mini *URA3* blaster (*URA3-dpl200*) cassette. This mini *URA3* blaster has short flanking repeats that permit PCR amplification of the cassette for transformation, as well as homologous excision of the *URA3* marker gene, such that reuse is possible. Although the mini *URA3* cassette was initially designed for gene deletions in *C. albicans* (Wilson et al. 2000), here it was adapted for the sequential FLAG-epitope tagging of two alleles of the *GCD1* gene in *C. albicans*. 

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Figure 4.8 Verification of *GCD1*-V5-6xHis-tagged and *SUI3* V5-6xHis-tagged CAI4 strains. Strains were generated harbouring genomically V5-6xHis tagged copy of *GCD1* and *SUI3* using the standard PCR mediated epitope tagging method. A. Schematic of PCR amplifications to verify the correct integration of the GFP tagging cassette into the *GCD1* locus. B. Expected PCR sizes for *GCD1* and *SUI3* tagged genes respectively and C. Agarose gel electrophoresis of PCR products generated by amplification are shown. D. Western analysis of the extracts from the tagged strain. The immunoblots were probed with antibodies against V5.
Affinity purification of V5-6xHis tagged eIF2B. Affinity purified eIF2B was separated by Polyacrylamide gel electrophoresis and stained with Coomassie. Affinity purification was done with (A) V5 resin and (B) Ni-NTA agarose beads. Molecular weight of V5-6xHis tag is 2.4kDa.
4.3.5.3 Construction of the 4X-FLAG-URA3dpl tagging cassette

A cassette was therefore designed with four FLAG epitopes and URA3 as the selectable marker. The URA3 marker was flanked by a 200bp segment of C. albicans URA3 3\’ sequences to allow marker removal via homologous integration between the repeats. The cassette, 4X-FLAG-URA3dpl200, was synthesized commercially (by Biomatik, USA) and cloned into a standard vector (pBMH). A standard PCR based epitope tagging strategy was taken to tag the GCD1 gene with the 4X-FLAG-URA3dpl cassette. In short, primers were designed with regions complementary to an appropriate region of the GCD1 gene, as well as a region that allows amplification of the 4X-FLAG-URA3dpl cassette. An overview of the strategy for tagging both alleles of the GCD1 gene is shown in Figure 4.10, while the precise site of integration for the PCR products is shown in Figure 4.11A.

For the first GCD1 allele, correct integration of the Flag-tag into the genome was verified using PCR reactions on genomic DNA purified from potential transformants (Figure 4.11B). In addition, Western blotting was used to verify that a tagged protein of the correct size (60 kDa) is present in protein extracts from the transformed strain but not in extracts from the parental strain (Figure 4.12B).

In order to facilitate tagging of the second allele of the GCD1 gene, the URA3 marker gene was first excised through homologous recombination between the 200bp flanking. URA3 is a counter-selectable marker in that its loss can be selected for using 5-fluoroorotic acid (5FOA). Therefore, single colonies where the URA3 gene has potentially been excised were selected on 5FOA plates to yield Urα strains and URA3 loss was verified using PCR on genomic DNA (Figure 4.11B).

The resulting strain was used in a second transformation with the 4X-FLAG-URA3dpl200 cassette. Selection of transformants and verification of the correct integration of the Flag-tag in the second allele of the gene was again performed using
PCR on genomic DNA preparations from potential transformants. The expected PCR products generated from strains having two Flag-tagged alleles of GCD1 were obtained—the lane labelled ‘1st and 2nd’ contains a DNA band for the first tagged allele, a DNA band for the second tagged allele yet no DNA band where the untagged allele migrates (Figure 4.12A). Western analysis using an antibody raised against Flag protein also showed approximately 2-fold higher levels of protein from the strain in which the two GCD1 alleles were tagged relative to the strain where only one allele of the gene is tagged (Figure 4.12B).
Figure 4.10 Strategy for tagging the two alleles of *C. albicans* genes using the mini Ura-blaster technique. (A) 4XFLAG-*URA3*-dpl200 cassette, the locations of the epitope tag (stripped box), ScCYC1 terminator (grey box), URA ORF (white box) and duplicated 3' sequences (hatched boxes) are shown. (B) The schematic of the sequential tagging of the two alleles of a gene.
Figure 4.11 Verification of genomic C-terminal FLAG tagging of the first allele of *GCD1* in CAI4 strain. A. Schematic of PCR strategy used to verify genomic integration of the FLAG-tag and the expected PCR product sizes. B. Agarose DNA electrophoresis of PCR products from 3 transformants. (a) refers to the transformants bearing the whole tagging cassette, while (b) refers to transformants that have lost the *URA3* gene.
Figure 4.12 Verification of genomic C-terminal 4X FLAG tagging of the second allele of GCD1 in CAI4 strain. A. Agarose DNA electrophoresis of PCR products generated by amplification of genome extracts. Product sizes are as presented in Figure 4.9. B. Immunoblots on extracts generated from the indicated strains. Immunoblots were probed with antibodies generated against Flag protein. (1/2 and 2/2 refer to one and two alleles of genes tagged with 4xFLAG epitope).
4.2.5.4 FLAG affinity purification of eIF2B for analysis by mass spectrometry

Protein extracts were made from cultures of the Flag-tagged strain and an untagged strain and affinity purified using a modification of the FLAG affinity purification method (Mohammad-Qureshi et al. 2007). In brief, extracts were incubated with a Flag-agarose resin, washed and bound proteins were eluted using a Flag peptide (for full purification methodology see Materials and Methods, section 2.3.10). The purified proteins were separated by SDS PAGE alongside whole cell extract samples from the tagged and untagged strains and visualized by staining with coomassie blue (Figure 4.13). From the gel, some of the protein bands are of the correct size for subunits of eIF2B. In particular, it seems possible that the bands at ~80kDa and ~55kDa could be eIF2Bε and eIF2Bγ, especially given that eIF2Bγ is the tagged subunit and work across eukaryotes has shown that this subunit forms a catalytic sub-complex with eIF2Bε (Koonin 1995).

In order to clarify which eIF2B subunits were present in the samples, immunoprecipitated samples were briefly run into an SDS PAGE gel, stained and protein bands were excised from the gel and analysed by mass spectrometry. Table 4.1 shows that eIF2B subunits were enriched in the sample, as shown by the number of peptides present in samples generated from the tagged strains relative to untagged. In particular, the eIF2Bγ and ε subunits, which are known to form the catalytic sub-complex, are heavily enriched in the tagged samples. Therefore, these data shows that the double tagging of the two GCD1 alleles with 4xFLAG epitope has successfully allowed at least a sub-complex of eIF2B to be purified.

The initial goal of the eIF2B purification was to ascertain whether treatment with 1% (v/v) butanol affected either the level of the eIF2B subunits or the interaction of the eIF2B subunits with other factors. To this end, Flag affinity chromatography was carried out on protein extracts from the Flag tagged strain either untreated or from a
culture to which 1% (v/v) butanol had been added for 15 min. The resulting purified proteins were run a little into SDS PAGE gel and protein bands were excised then analysed by mass spectrometry. Table 4.2 shows that butanol did not have any observable effect on the level of the eIF2B subunits as the level of the subunits remained the same in the untreated and the 1% (v/v) butanol treated samples.

Table 4.1 Identification of the eIF2B subunits by mass spectrometry (MS) analysis

<table>
<thead>
<tr>
<th>Identified proteins (45/56)</th>
<th>Mol. weight</th>
<th>NE1</th>
<th>NE2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>untagged</td>
<td>tagged</td>
<td></td>
</tr>
<tr>
<td>Orf19.407 GCD6</td>
<td>82kDa</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>Orf19.481 GCD1</td>
<td>54kDa</td>
<td>2</td>
<td>31</td>
</tr>
<tr>
<td>Orf19.6776 GCD2</td>
<td>56kDa</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Orf19.6904 GCN3</td>
<td>35kDa</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Orf19.825 GCD7</td>
<td>41kDa</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

The values shown are the exclusive peptide count indicating the confidence of the identification. It can be seen that there are significantly more peptides identified in sample NE2 for GCD6 and GCD1 indicating these proteins are enriched in sample NE2 relative to sample NE1.
Figure 4.13 FLAG affinity purification of eIF2B. Coomassie stained gels of FLAG affinity purified eIF2B from strains bearing one copy of the 4xFLAG tag and strains bearing two copies of the 4xFLAG tag.
Table 4.2 Quantitative measure of the total spectrum counts shows that the levels of the various eIF2B subunits were not altered by butanol treatment

<table>
<thead>
<tr>
<th>Identified proteins</th>
<th>Mol weight</th>
<th>untreated</th>
<th>treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orf19.407 <em>GCD6</em></td>
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<td>269</td>
<td>298</td>
</tr>
<tr>
<td>Orf 19.481 <em>GCD1</em></td>
<td>54kDa</td>
<td>202</td>
<td>254</td>
</tr>
<tr>
<td>Orf 19.825 <em>GCD7</em></td>
<td>41kDa</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Orf 19.6776 <em>GCD2</em></td>
<td>56kDa</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

The values shown are the total spectrum count of each protein, which includes data from peptides that have been analysed multiple times and is the most relevant value for relative quantification. To be considered significant, there needs to be a difference of at least 2-fold change and an absolute difference of 10 peptides between conditions.
4.3 Discussion

The data presented in this chapter show that fusel alcohol and ethanol most likely inhibit protein synthesis in a mechanism that involves the regulation of eIF2B, whereas farnesol appears to act via a different pathway. Firstly, ethanol and fusel alcohols both induce in a Gcn2p independent manner the translation of \textit{GCN4} mRNA. This assay is often used as an \textit{in vivo} indicator of ternary complex alterations and the guanine nucleotide exchange reaction facilitated by eIF2B is critical for ternary complex formation. On the other hand, farnesol has no effect on the translational activity of \textit{GCN4} mRNA. Additionally, fusel alcohols impede the movement of the eIF2B body by about 50% compared to the untreated sample, while farnesol had no apparent effect on these dynamics.

In an effort to characterise the impact of fusel alcohols on eIF2B a biochemical strategy was used to purify the eIF2B subunits from \textit{C. albicans} by tagging the two alleles of \textit{GCD1} with a 4xFLAG epitope tag. While subunits of eIF2B were identified in the purifications, little evidence for changes in subunit abundance or covalent modifications of eIF2B as a response to fusel alcohols was obtained. However, the peptide coverage of the eIF2B complex was sparse, especially for the eIF2Bα, β and δ subunits, which form the regulatory sub-complex; so any alteration to a specific covalent modification on eIF2B might easily have remained undetected.

The response of \textit{C. albicans} to butanol treatment is therefore similar to what has been observed in \textit{S. cerevisiae}. For instance, using a \textit{GCN4}-LacZ reporter construct in \textit{S. cerevisiae}, (Ashe et al. 2001), showed that butanol induces the expression of \textit{GCN4} mRNA independently of the Gcn2p kinase. Interestingly, in \textit{C. albicans}, treatment with 4-6% ethanol also elicits a similar response to butanol, whereas farnesol does not seem to induce the translation of \textit{GCN4} mRNA, highlighting the difference between the action of farnesol and the other alcohols.
In *S. cerevisiae*, eIF2B is present in a large cytoplasmic body termed the ‘eIF2B body’ (Campbell *et al.* 2005). While the integrity of this body is not affected by butanol, the dynamics of its movement around the cytoplasm are significantly reduced. In mutants that are resistant at the level of growth and translation to butanol, either the eIF2B body is absent or its movement is unaffected by butanol (Taylor *et al.* 2010). In the data presented here, *C. albicans* was also shown to harbour an eIF2B body and its movement was significantly reduced by butanol. Once again this suggests that the regulation of eIF2B to inhibit translation as a response to fusel alcohols is conserved between *S. cerevisiae* and *C. albicans*. In contrast, to the results with fusel alcohols, neither the ability of eIF2B to localise to the eIF2B body nor the dynamics of the body are affected by farnesol. Similarly, in *S. cerevisiae* other stresses that are known to inhibit translation initiation, such as amino acid or glucose starvation, do not affect either the integrity or movement of the eIF2B body (Taylor *et al.* 2010).

Previously, it has been suggested that the function of the eIF2B body could be to provide a concentrated site of guanine nucleotide exchange. This hypothesis was supported by FRAP data showing that eIF2 shuttles through the body but that the rate of shuttling in response to stresses targeting eIF2B, such as amino acid starvation, decrease this shuttling (Campbell *et al.* 2005). The mechanism by which the eIF2B body moves through the cell under normal conditions is not well understood, however one possibility is that a concentrated core of eIF2B moves through the cell by simple diffusion from areas of low to high eIF2.GDP. The decrease in eIF2B activity seen in response to butanol could be due to the inhibition of movement of the eIF2B body or alternatively the decrease in the movement of the eIF2B body may be the consequences of a decrease in eIF2B activity. For example if movement of the eIF2B body was dependent in some way on eIF2B activity, a reduction in eIF2B activity in response to butanol would lead to a decrease in eIF2B body movement.
Overall, it appears in *C. albicans* that butanol targets eIF2B to cause an inhibition in translation initiation. However, the biochemical purifications do not reveal how this regulation is brought about. For farnesol, it seems that the regulatory mechanism is distinct from that of the other alcohols and does not appear to act via eIF2B or the regulation of ternary complex.
5 Farnesol inhibits translation initiation in *Candida albicans* in a pathway that targets some aspect of 48S preinitiation complex formation

5.1 Introduction

The isoprenoid alcohol, farnesol, a *C. albicans* cell-signalling molecule that participates in the control of morphology also affects protein synthesis at the level of translation initiation in a Gcn2p-independent manner (as shown in chapters 3 and 4). So a *gcn2Δ* mutant was equally responsive to the effects of farnesol both in *S. cerevisiae* and in *C. albicans*. However while other alcohols such as butanol cause an up-regulation of *GCN4* translation by inhibiting the activity of the eukaryotic initiation factor 2B (Ashe *et al.* 2001), farnesol does not lead to the translational up-regulation of *GCN4*. Seemingly then, farnesol does not appear to target eIF2B to cause an inhibition in translation (see chapter 4).

Highly conserved mechanisms allow eukaryotic cells to globally reduce the level of protein synthesis (Jackson *et al.* 2010). Besides the key eIF2B control, another step where translation initiation is regulated across eukaryotes is at the mRNA selection stage (Pavitt 2005; Richter and Sonenberg 2005; Wek *et al.* 2006).

The selection of mRNA relies on the interaction of a closed loop complex on the mRNA with the 43S complex to form the 48S preinitiation complex and this requires protein-protein interactions between components of the closed loop and 43S complexes (Jackson *et al.* 2010). A number of stress conditions have been shown to affect the formation of the 48S preinitiation complex. For instance in *S. cerevisiae*, glucose starvation leads to a loss of eIF4A from the translation initiation machinery and translational inhibition at a step after 48S complex formation (Castelli *et al.* 2011). Furthermore, one of the yeast eIF4E binding proteins, Eap1p was also shown to be involved in global mechanism targeting translation initiation as a result of oxidative stress (Mascarenhas *et al.* 2008).
The mechanism by which farnesol acts on the translational machinery is currently unknown. Though Uppuluri et al. (2007), reported a down regulation of genes regulated by Gcn4p following treatment with 40µM of farnesol, and the level of the transcription factor ATF4 is increased in human lung carcinoma H460 cells treated with farnesol (Joo et al. 2007). Both of these effects could relate to transcriptional regulation, but might also be explained by effects of farnesol on translational regulation. As farnesol does not appear to regulate translation via eIF2B, the most logical next step in the translation initiation pathway to investigate is the formation of 48S preinitiation complex.

5.2 Results

5.2.1 MLY61 strain of S. cerevisiae exhibited similar translational responses to fusel alcohol and farnesol as C. albicans

One of the limitations of molecular studies in C. albicans is that many of the reagents for protein studies are not readily available. For instance, while many of the antibodies for studies on protein synthesis are available for the model yeast, S. cerevisiae, most of these antibodies do not cross react with C. albicans translation factors and hence are not suitable for the study of protein synthesis in C. albicans. Therefore, in order to further investigate the step in the translation pathway that is targeted by farnesol, a decision was made to work initially in S. cerevisiae. The MLY61 strain of S. cerevisiae is a wild type a/α diploid of Σ1278b genetic background (Lorenz et al. 2000). The MLY61 strain was selected for this investigation, as like C. albicans it is a diploid yeast that forms pseudohyphae in the presence of butanol. However, in order to validate the use of this strain, we first needed to compare the effects of farnesol on translation initiation in MLY61 and C. albicans using a range of translation assays.
The sensitivity of the MLY61 *S. cerevisiae* strain to both butanol and farnesol was investigated using polysome analysis. Similar to the *C. albicans* CAI4 strain, the MLY61 *S. cerevisiae* strain was sensitive to butanol at 2% (v/v) and to farnesol at a concentration of 100μM (Figure 5.1). Furthermore, an investigation of the impact of these alcohols on the levels of phosphorylated eIF2α revealed a similar pattern of dephosphorylation in the *S. cerevisiae* MLY61 strain to that observed in *C. albicans* following treatment with 2% butanol or 100μM farnesol after pretreatment with CdSO₄ to induce initial phosphorylation (Compare Figure 5.2 with Figures 3.11 and 3.13). As both the inhibition of translation initiation and the dephosphorylation of eIF2α in *S. cerevisiae* and *C. albicans* is similarly dependent on the dose of butanol or farnesol, it seems reasonable that the translational responses to butanol and farnesol are mechanistically conserved across these species.
Figure 5.1 Translational initiation is inhibited by farnesol similarly in CAI4 strain of C. albicans and MLY61 strain of S. cerevisiae. Polysome analysis was used to assess the effect of farnesol on translation initiation a CAI4 and MLY61 strain. Yeast were grown in YPD and various concentrations of farnesol were added as indicated for 15 min prior to extract preparation. Extracts were sedimented on 15-50% sucrose gradients and the absorbance at 254nm was continuously measured.
**Figure 5.2 Butanol and farnesol cause a dose dependent decrease in eIF2α serine 51 phosphatase (eIF2α-P) in the wild type *S. cerevisiae* MLY 61 strain.** Protein extracts from the wild type were blotted and probed with antibodies to Tef1p and a phosphospecific antibody to phosphoserine 51 on eIF2α. Strains were grown in YPD to OD$_{600}$ of 0.7, treated with 1mM Cadmium for 1 h. Various concentrations of butanol (v/v) or farnesol (µM) were then added for 15 min after which protein extracts were made.

<table>
<thead>
<tr>
<th>Cd$^{2+}$ pretreatment</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
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</tr>
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<tbody>
<tr>
<td>Butanol</td>
<td>UT 2% 0% 0.5% 1% 2%</td>
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<td></td>
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</tr>
<tr>
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<td></td>
<td>Tef1p</td>
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<td>Cd$^{2+}$ pretreatment</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Farnesol (µm)</td>
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<tr>
<td></td>
<td>Tef1p</td>
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</table>
5.2.2 Farnesol causes resedimentation of specific translation factors in MLY61 strain of *S. cerevisiae*

The ‘formaldehyde-polysome’ method has been used previously for analysing translation complexes involved in the initiation stage of protein synthesis within the cell (Nielsen *et al.* 2004; Hoyle *et al.* 2007; Castelli *et al.* 2011). Here cross-linking with formaldehyde prior to cell lysis ensures that protein factors are maintained in complexes during the subsequent polysome analysis (Figure 5.3). This stabilisation of weakly interacting proteins allows the resolution of complexes that might otherwise be disrupted by cellular lysis and sedimentation.

Mid log phase MLY61 *S. cerevisiae* cells grown in YPD were analysed using this formaldehyde-polysome analysis. Continuous $A_{254}$ measurements reveal polysome profiles similar to those obtained using the more classical cycloheximide-dependent polysome profiling technique (compare Figure 5.1 and Figure 5.4). Similarly, the treatment of the cells with 100µM of farnesol for 15 min caused the same degree of polysome run-off that was observed previously. The gradients were separated into fractions, which were used for the production of protein samples for Western blotting (Figure 5.3). These blots were probed using specific antibodies for *S. cerevisiae* translation initiation factors.

It was noted that across the gradients the degree to which the eIF4G associates with the small ribosomal subunit (40S) decreases early after farnesol treatment (Figure 5.4 fractions 1-3 untreated and 100 µM farnesol). In previous studies from our laboratory, glucose starvation caused an accumulation of eIF4G in this region of the gradient at early time points, whereas longer stress periods caused eIF4G and other components of the closed loop complex to fall away from the ribosomal subunits to the top of the gradient (Hoyle *et al.* 2007; Castelli *et al.* 2011).
During translation initiation the eIF4F factors (eIF4E, eIF4G and eIF4A), the mRNA and Pab1p are thought to form a closed loop complex (Wells et al. 1998) and promote formation of the 48S preinitiation complex. The data presented here for farnesol suggest that this alcohol causes an alteration in protein-protein interactions that lie upstream of 48S complex formation. It is entirely possible that the observed depletion of eIF4G results in reduced 48S formation resulting and an inhibition of translation initiation.
Figure 5.3 Protocol overview for formaldehyde crosslinking

Grow to mid-log phase.
Exposed to environmental stress or untreated and crosslinked with formaldehyde
Lysed by vortexing/ RNA extraction

Separation of fractions along 15-50% sucrose gradients by centrifugation at 40,000rpm for 2.5 hours

Run on polysome machine and collect fractions

TCA precipitation

Western analysis
Figure 5.4 Farnesol affects the association of the specific translation factors with the ribosomes.
Sucrose density gradient analysis on extracts from strain MLY61 treated with 100µM farnesol for 15 min. Immunoblots on gradient fractions probed using antibodies against the indicated proteins are shown below the traces.
5.2.3 Tandem affinity purification (TAP) of eIF4G1

In order to further investigate how farnesol affects the formation of the 48S complex and investigate how eIF4G becomes depleted from this region of a polysome gradient, we undertook a modified tandem affinity purification strategy using TIF4631-TAP (eIF4G1-TAP) tagged strain of *S. cerevisiae*.

The TAP method was developed by the Seraphin lab to allow the affinity purification of specific proteins from whole cell extracts (Puig *et al.* 2001). In this method extracts are sequentially passed over an IgG column, then eluted with Tev protease and further purified on a Calmodulin column. In our lab a minimised form of this purification, where just the first column is used and elution relies upon a peptide (Castelli *et al.* 2011). This minimises the time of incubation preserving weaker interactions. This method was used to purify eIF4G1-TAP and eIF4E-TAP from *S. cerevisiae* strains where the endogenous genes are TAP-tagged. Affinity purified and input samples were analysed by Western blotting using antibodies against Rps3p. The rabbit HRP-conjugated secondary antibody also detects the TAP-tagged protein by virtue of the protein A moiety. The levels of Rps3p over background observed co-purifying with either eIF4G or eIF4E remained similar in both the untreated and 100µM treated samples (Figure 5.5A and B). Therefore, there was little evidence from this immunopurification to support the reduced association of eIF4G with the 48S complex, as observed on the formaldehyde sucrose density gradients. However, a substantial contamination of the eluted samples with heavy and possibly light chain antibody proteins was also observed (Figure 5.5A and B), which makes interpretation of these data very difficult.

Therefore, in order to resolve whether farnesol leads to reduced 48S complex formation, the experimental strategy was modified. Immunopurified samples from the eIF4G1-TAP tagged strain of *S. cerevisiae* were analysed by mass spectrometry. Figure 5.6
shows that the relative number of peptides observed in these samples for eIF4G, eIF4E, eIF4A and Pab1p; factors involved in the formation of the closed loop complex, were largely unaffected by farnesol treatment. Interestingly, however, the number of peptides recovered for the ribosomal proteins as well as eIF3, which are only associated with the closed loop complex as part of the 48S complex or 80S complex is reduced dramatically after farnesol treatment. These data support a model where farnesol targets the formation of the 48S preinitiation complex to inhibit protein synthesis.
Figure 5.5 Effect of farnesol on translation factors eIF4G and eIF4E was not observed in immunoprecipitations.

Protein extracts were made from 1 litre cultures of strains harbouring the (A) eIF4G-TAP or (B) eIF4E-TAP gene grown to mid-log phase. Purified samples were resolved by SDS-PAGE and electroblotted onto nitrocellulose membrane. Blots were probed with antibodies raised against (A and B) Rps3.
Figure 5.6 Farnesol causes the depletion of ribosome associated translation factors in immunoprecipitation of eIF4G1. Protein extracts from eIF4G1-TAP tagged strain treated with 100 µM farnesol or untreated were resolved on SDS-PAGE gels, stained with coomassie and sent for mass spectrometry. The figure shows no of unique peptides that have been matched to the identified protein in the sample. Untreated is normalised to 1, treated is fold change.
5.3 Discussion

The studies presented in this chapter have shown that farnesol produced by only a few species of *Candida* (Hornby *et al.* 2001), also has a repressive effect on protein synthesis in yeast. Various studies into the effects of farnesol in other organisms have revealed that farnesol impacts upon various developmental processes across *Candida* species and other pathogenic fungi including *Aspergillus* spp. (Langford *et al.* 2009). In addition, farnesol has also been shown to lead to cell-cycle arrest and growth inhibition (Machida *et al.* 1999), increased ROS production and effects on mitochondria (Machida *et al.* 1999), and cell death (Fairn *et al.* 2007). These effects have also been demonstrated in *C. albicans* (Shirtliff *et al.* 2009). Dichtl *et al.* (2010) further suggest that farnesol interferes with the cell wall integrity pathway and many of the components of this pathway are conserved across the fungal kingdom. Therefore, it is perhaps not surprising to discover that farnesol similarly inhibited translation initiation in the MLY61 strain of the budding yeast, *S. cerevisiae* (Figure 5.1). Equally this strain showed a similar response to the effect of farnesol on eIF2α dephosphorylation as was previously described for *C. albicans* (Figure 3.13 relative to Figure 5.2). In addition to exhibiting similar translational responses to farnesol, some key physiological attributes of MLY61 strain are similar to that of *C. albicans* (see section 5.2.1). These properties of the MLY61 provide justification for its use to further elucidate the target of farnesol in terms of the translational machinery.

Using the MLY61 strain, the mechanism by which farnesol elicits a global down-regulation of translation initiation appears to involve alterations in the formation of the 48S preinitiation complex. There are precedents for regulatory mechanisms targeting this step. For instance glucose starvation in yeast led to a decrease in eIF4E, eIF4G and Pab1p cosedimentation with ribosomal components, which could be interpreted to mean a decrease in the association between closed-loop complex and ribosomal species.
(Hoyle et al. 2007). More recently Castelli et al. (2011) also showed that glucose starvation in yeast leads to the loss of eIF4A from the eIF4G-containing preinitiation complex explaining the resulting translational inhibition. Therefore, the effects of farnesol that are described above, suggest that any alteration in the formation of the 48S complex or its interaction with the mRNA ultimately leads to rapid inhibition of translation at the initiation stage.

Data from mass spectrometry analysis of the eIF4G affinity purified samples also provided significant insight into the physiological consequences of farnesol treatment. A significant number of proteins were differentially present in the affinity purified eIF4G as demonstrated by the peptide count (Figure 5.6). While the factors of the closed-loop complex (eIF4A, eIF4G, eIF4A and Pab1p) remain similar in level, anything associated with the ribosome goes down. Given that the closed loop complex is perceived to be associated with high levels of translation initiation and, therefore, is a target for translational regulation (Richter and Sonenberg 2005), these observations suggest that farnesol targets the association of the ribosome with the mRNA, most likely at the 48S preinitiation complex formation step of translation initiation.
6 RNA sequencing reveals a global change in gene expression following treatment with farnesol in *C. albicans*

6.1 Introduction

The previous results chapters have revealed that farnesol inhibits morphological growth as well as protein synthesis at the level of translation initiation in *C. albicans*. The global level of protein synthesis occurring in a cell can be investigated by carrying out polyribosomal analysis. This technique can highlight the stage of translation that is regulated (Ashe *et al.* 2001), but reveals nothing about the identity of the mRNAs that are being translated under different conditions. Furthermore, it is well-established that farnesol impacts upon the transcriptome of both *C. albicans* and *S. cerevisiae*, so an in-depth understanding of gene regulation requires both the transcriptional and translational impact of farnesol to be monitored at the level of individual genes and mRNAs.

The recently developed RNA sequencing (RNA-seq) technology is revolutionizing our ability to analyse transcriptomes (Wang *et al.* 2009). It is based on next-generation sequencing platforms that were initially developed for high-throughput sequencing of genomic DNA (Westermann *et al.* 2012). Previously, both microarray analysis and RNA sequencing have been applied in the analysis of differential gene expression in hyphae and biofilm formation by *C. albicans* cells exposed to farnesol (Cao *et al.* 2005; Uppuluri *et al.* 2007; Nobile *et al.* 2012). RNA-seq technology generates data that is more reproducible and sensitive than the various microarray platforms, therefore resulting in more accurate measurements in gene expression changes (Xiang *et al.* 2010). Moreover the gene expression studies previously undertaken in *C. albicans* following farnesol treatment only considered the transcriptional landscape. In this chapter, RNA-seq has been used to analyse the impact of farnesol treatment at both the transcript and translation level. The data revealed that mRNAs involved in oxidative stress and translation were upregulated, while mRNAs involved in positive regulation of
filamentation were downregulated. Furthermore, farnesol appears to co-ordinately regulate the genes involved in these processes, either affecting their transcription or translation; or sometimes leading to reciprocal regulation of these processes.

6.2 Results

6.2.1 RNA isolation from input and polysome samples
To study the transcriptional and translational impact of farnesol on C. albicans, cell extracts were made from exponential C. albicans cultures either untreated or treated with 100µM farnesol for 15 min. From these, total and ribosome associated RNA samples were generated. RNA purified from polysome-associated fractions from across a polysome sucrose gradient was used as the ribosome associated RNA. This procedure is outlined in figure 6.1 and was performed in triplicate.

6.2.2 Enrichment of mRNA and processing of enriched sample for RNA sequencing
It is often necessary to enrich specific classes of RNA in the samples to be sequenced. Total RNA recovered by cell lysis and extraction with organic solvents (trizol) consists of at least 80% ribosomal RNA (Raz et al. 2011), so if rRNA were not removed, the majority of the final sequence reads would be from rRNA. Therefore, in order to deplete rRNA from the RNA samples, a method was adopted where non-target RNAs were removed by hybridization. Although selection of target RNA via hybridization to oligo-dT was the preferred method for mammalian systems, yeasts, including C. albicans mRNA have shorter poly(A) tails than mammalian mRNAs (Keller and Minvielle-Sebastia 1997), therefore hybridization to oligo-dT is not suitable for enriching yeast mRNA for sequencing. Furthermore while the use of oligo-dT to recover mature
mRNAs reduces the proportion of RNA classes that do not have long poly-A stretches, removal of ribosomal sequences via hybridization preserves non-polyadenylated RNAs allowing one to investigate broader classes of RNAs.

The mRNA enrichment protocol used in this study is ribodepletion using the Ribo zero rRNA removal kit (Epicentre, Illumina). This technique uses oligos that are complementary to highly conserved ribosomal RNA sequences to bind and remove the rRNA. As shown in Figure 6.2, the ribodepleted RNA samples were then fragmented and converted to double stranded cDNA. Sequencing adapters were then ligated to both the 3’ and 5’ ends of the double stranded cDNA and subsequently the cDNA library was amplified by PCR using a primer cocktail that anneals to the ends of the adapters (Illumina). The cDNA library was validated to check the purity of the sample by running 1µg of sample on a DNA chip and then sequenced using high-throughput illumina Hiseq sequencing.
Figure 6.1 **Strategy of experiment for RNA sequencing of polyribosomal fractions.** Yeast were grown in YPD and treated with 100µM of farnesol or untreated for 15 min prior to cycloheximide addition and extract preparation. Extracts were split into input and polyribosomal fractions. Following polyribosome separation and RNA extraction, the samples were ribodepleted and sent for illumina Hiseq sequencing.
Figure 6.2 Flow chart for RNA sequencing

Cells were grown to OD$_{600}$ of 0.7 and treated with 100µM farnesol or untreated. Input and polysome fractions from treated and untreated samples were processed for RNA. The RNA samples were ribodepleted using Ribo zero kit and the purity checked with Agilent Bioanalyzer. Ribodepleted samples were sent to the Genomic facility of the University of Manchester for DNA library generation, sequencing and initial analysis of sequencing data.
6.3 Analysis of gene expression from the sequencing data

The sequencing data was analysed as described in materials and methods, section 2.9.4.3. RPKM (reads per kilobase per million mapped reads) values were determined for all genes in each of the conditions tested. In order to assess the sequencing reads mapping to different RNA types in the samples. Mapped reads per million per base pair of RNA types in the sequenced data was calculated. The data as shown in table 6.1 suggests that the fractionation has been successful and gives evidence that the different runs are reproducible. The input samples are highly enriched for the various RNA species (snoRNA, ncRNA, tRNA and snRNA) compared to the polysome fractions where these RNAs are more depleted.

Table 6.1 The depth of coverage per million sequenced reads for the RNA type.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>rRNA</th>
<th>snoRNA</th>
<th>ncRNA</th>
<th>tRNA</th>
<th>snRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>InputUT1</td>
<td>20.6266</td>
<td>4.186745</td>
<td>11.85231</td>
<td>0.339016</td>
<td>15.87573</td>
</tr>
<tr>
<td>InputUT2</td>
<td>38.14159</td>
<td>3.136478</td>
<td>10.74961</td>
<td>0.290844</td>
<td>11.35054</td>
</tr>
<tr>
<td>InputUT3</td>
<td>21.94216</td>
<td>2.194619</td>
<td>10.24618</td>
<td>0.454221</td>
<td>7.34394</td>
</tr>
<tr>
<td>Input100uM-Farnesol1</td>
<td>39.14896</td>
<td>2.137437</td>
<td>12.54479</td>
<td>0.087663</td>
<td>9.903528</td>
</tr>
<tr>
<td>Input100uM-Farnesol2</td>
<td>42.3765</td>
<td>2.31221</td>
<td>14.19559</td>
<td>0.110736</td>
<td>10.62073</td>
</tr>
<tr>
<td>Polysome UT1</td>
<td>5.638171</td>
<td>0.705433</td>
<td>1.388306</td>
<td>0.010395</td>
<td>0.670995</td>
</tr>
<tr>
<td>Polysome UT2</td>
<td>2.697695</td>
<td>0.592301</td>
<td>1.062401</td>
<td>0.008674</td>
<td>0.592433</td>
</tr>
<tr>
<td>Polysome UT3</td>
<td>6.398139</td>
<td>0.880681</td>
<td>1.731622</td>
<td>0.013273</td>
<td>0.921996</td>
</tr>
<tr>
<td>Polysome 100uM-Farnesol2</td>
<td>8.23574</td>
<td>1.806252</td>
<td>2.371609</td>
<td>0.007332</td>
<td>2.010198</td>
</tr>
<tr>
<td>Polysome 100uM-Farnesol3</td>
<td>8.756714</td>
<td>2.041127</td>
<td>3.42422</td>
<td>0.009148</td>
<td>3.149637</td>
</tr>
</tbody>
</table>

This is the depth of coverage per million sequenced reads for the RNA type. The mapped reads per million per bp of xRNA was calculated thus:

Reads mapping to xRNAs X 1,000,000/total reads in experiment.
The value was then divided by the total length of xRNAs in the genome.
The normalisation to per million reads was done because the number of sequenced reads varies from experiment to experiment.

In order to assess the impact of farnesol on gene expression, the sequencing data were processed as described in materials and methods to generate two parameters: the change in transcript level \( \log_2[\text{Total F}/\text{Total U}] \) and the change in translation state \( \log_2[\text{polysome F}/\text{Total F}] / \log_2[\text{Polysome U}/\text{Total U}] \) following farnesol treatment. A 2-fold cut-off was applied to these ratios to define mRNAs that are significantly up regulated or down regulated at the transcript level and/or at the translation level. At the transcript level a total of 796 mRNAs were significantly altered, \( \geq 2 \)-fold with 330 up regulated and 466 down regulated. In addition, at the translation level, a similar number of mRNAs were significantly altered in their polysome association following treatment with 100\( \mu \)M farnesol, \( \geq 2 \)-fold (477 up and 476 down) giving a total of 953 mRNAs that were altered translationally. Scatter plots were used to visualise these data (Figure 6.3A and B).

To further investigate the physiological consequence of farnesol treatment on \( C. \) albicans, the Gene Ontology (GO) slim mapper (Candida Genome Database) tool was used to provide functional classifications of the transcriptionally and translationally altered mRNAs. This analysis revealed gene categories that were differentially altered. Some of these gene categories exhibit responses at both transcriptional and translational level, especially ‘the response to stress’, ‘translation’, ‘filamentous growth’, ‘cellular respiration’, ‘cell cycle’, ‘signal transduction’ and ‘cytokinesis’. (Figure 6.3A,B and 6.4A and 6.4B).

Interestingly, as a functional class mRNAs involved in translation were upregulated either transcriptionally or translationally. Given that in \( C. \) albicans farnesol inhibits protein synthesis (see section 3.2.4) such that global protein production is repressed by about 10-fold (see figure 3.6), the upregulation of mRNAs involved in translation may
serve as a feedback control mechanism. Such an idea has precedence in the literature: for instance, under various stress conditions that inhibit global translation, cells up-regulate the translation of specific mRNAs in order to overcome the inhibitory effect (Natarajan et al. 2001).

Three other classes of mRNA were down-regulated at either the transcriptional or translational level: those involved in filamentation, biofilm formation and cytokinesis (Figure 6.4 A abd B). This observation perfectly correlates with the established effects of farnesol on morphological transition and biofilm formation in *C. albicans* (Hornby et al. 2001; Ramage et al. 2002; Uppuluri et al. 2007). Of particular interest is the effect of farnesol on the *TUP1* mRNA. Farnesol caused up-regulation of *TUP1* mRNA at the transcriptional level: an observation that has been made in previous studies using microarrays (Cao et al. 2005; Kebaara et al. 2008). Tup1p is a transcriptional repressor of filamentation genes, so the fact that the signalling molecule farnesol up-regulates this gene is entirely consistent with the negative impact of farnesol on the transition to filamentous growth in *C. albicans*. Other mRNAs that are required for morphological differentiation were down-regulated transcriptionally by farnesol. For instance, mRNAs encoding the secreted aspartyl proteinases (*SAP8, SAP98*), (also reported by Décanis et al. (2011) and Cao et al. (2005)), the nucleotide phosphodiesterase (*PDE1*), the G-protein receptor (*GPR1*), the transcription factor (*TEC1*), and the adenylate cyclase (*CYR1*). A complementary set of mRNAs that encode factors involved in filamentation were down-regulated at the level of translation. For instance, mRNAs encoding the cAMP dependent protein kinase catalytic subunit (*TPK2*), G-protein α subunit (*GPA2*), agglutinins (*ALS2* and *ALS4*) the transcriptional regulators (*EFG1, FLO8* and *CFZ1*) Also the newly characterised gene, *DEF1* or *EED1*, crucial for extension and maintenance of filamentous growth and plays a role in epithelial cell escape, dissemination in a tissue culture model and is regulated by Efg1p (Martin et al. 2011).
Interestingly, the *EFG1* and *EED1* mRNAs are translationally down-regulated along with mRNAs involved in biofilm formation, cytokinesis and cell cycle. Farnesol induced growth inhibition in *S. cerevisiae* cells has been characterised to consist of cell cycle arrest concomitant with the downregulation of cell cycle gene expression (Machida et al. 1999). So the observation of the general downregulation of the mRNAs involved in cytokinesis and cell cycle at the translation level could indicate that farnesol affects cell cycle in *C. albicans*, as was previously observed in *S. cerevisiae* (Machida et al. 1999). Also downregulation of mRNAs involved in signal transduction was observed both at the transcriptional and translation level, this finding could be connected to the effect of farnesol on filamentation. A puzzling aspect of the dataset is the fact that the *TUP1* mRNA appears translationally down-regulated even though transcript levels increase. A further analysis of this mRNA is presented in section 6.5.

Among the mRNAs involved in stress response that were up-regulated transcriptionally are those involved in oxidative stress response (*SOD3, SOD4, SOD5, PRX1*) and heat shock proteins (*HSP104, HSP12, HSP21* and *HSP78*) while the translation of the *SOD4, TRX1*, and *ATX1* mRNAs appears up-regulated. Farnesol has been shown to cause the upregulation of proteins involved in protein folding and protection against environmental and oxidative stress (Shirtliff et al. 2009). Exposure of cells to farnesol causes the accumulation of reactive oxygen species (ROS) within 3 h (Shirtliff et al. 2009). Westwater et al. (2005) demonstrated that pre-treatment of *C. albicans* yeast cell with farnesol led to increased survival to oxidative stress generated by H₂O₂ due to increased expression of genes involved in oxidative stress resistance. *C. albicans SODs* are the primary enzymatic antioxidants involved in protection against ROS (Zhu et al. 2011). Therefore, these data correlate well with the observation that farnesol induces the up-regulation of mRNA involved in the oxidative stress response both at levels of transcription and translation.
In addition, the upregulation of mRNAs involved in cellular respiration both at the level of transcript and translation level could represent a response to the high levels of ROS that have been described to result from farnesol treatment. For instance, the level of ROS in farnesol-treated *S. cerevisiae* cells increases five to eight fold and this increase was not observed in the respiration-deficient petite mutant (*rho*<sup>0</sup>). These results illustrate the role of the mitochondrial electron transport chain in facilitating growth in the presence of farnesol (Machida *et al.* 1998).

Previous studies have shown that in response to stresses such as glucose starvation, amino acid starvation and rapamycin treatment in *S. cerevisiae* (Castelli *et al.* 2011) a specific subset of genes is co-regulated at both the transcriptional and the translational level. This phenomenon has been termed ‘potentiation’ (Preiss *et al.* 2003). For the farnesol experiments described here, however, only 6 such mRNAs were identified as coregulated at both stages in the gene expression pathway, and many mRNAs were increased transcriptionally while decreasing translationally (Figure 6.3A and B). One possibility is that these transcriptionally induced mRNAs could serve as a store of genetic material that could be rapidly mobilised should the cellular conditions improve. Further analysis of these set of mRNAs using the CGD GO Slim mapper (Figure 6.4C) shows that some of these mRNAs are those related to protein catabolic processes, drug response, filamentation, stress response and biofilm formation: functions that are consistent with the mRNA storage hypothesis.
Figure 6.3A Illumina Hiseq sequencing of input (total) RNA shows global changes at the transcriptional level following farnesol treatment of *C. albicans* culture. Figures show graphical plots depicting the transcript level \( \log_2 [\text{TF/ TU}] \). A. Transcriptionally upregulated mRNAs in red and transcriptionally downregulated mRNAs in green. A cutoff value of \( \pm 1.0 \) for the change in transcript level was used. B. As A, except the mRNAs defined as translationally regulated are highlighted on the plot: translationally up-regulated in red and down-regulated in green.
Figure 6.3B Illumina Hiseq sequencing of the input and polyribosomal fractions shows global changes at the translation level following farnesol treatment of *C. albicans* culture. Figure shows a graphical plots depicting the change in translation state (\(\log_2[PF/TF]/\log_2[PU/TU]\)) following farnesol treatment. A. Translationally upregulated mRNAs in red and downregulated mRNAs in green. Cutoff value of 1.0 for the change in translation level was used. B. As A, except the mRNAs defined as transcriptionally regulated are highlighted on the plot: transcriptionally up-regulated in red and down-regulated in green.
Figure 6.4A Functional annotation of transcripts reveals differential regulation of certain categories of genes. Data from RNA seq were categorised based on differential regulation of transcription. The different categories were annotated using the CGD Gene ontology Slim Mapper according to functional categories for transcriptionally up and down regulated.
Figure 6.4B Functional annotation of genes at translational level reveals differential regulation of certain categories of genes. Data from RNA seq were categorised based on differential regulation of translation. The different categories were annotated using the CGD Gene ontology Slim Mapper according to functional categories for translationally up and down regulated.
Figure 6.4C Functional annotation of mRNAs that are not coregulated at the transcription and translation level. mRNAs that were transcriptionally up regulated as well as translationally downregulated were annotated according to functional categories using the CGD Gene ontology Slim Mapper.
6.4 Clarifying the translational regulation of TUP1 mRNA by q RT-PCR.

Of particular interest with regard to the impact of farnesol on filamentation is the observation that TUP1 is up-regulated at the transcript level, yet appears down-regulated at the level of translation. A quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis was used to further investigate the effect of farnesol on TUP1 mRNA across the polysome. The qRT-PCR data showed redistribution of the mRNA from the polysomal region to the sub-polysomal region following treatment with 100µM farnesol (Figure 6.5). This agrees with the translational down-regulation of TUP1 mRNA observed from the RNA-seq datasets. However, transcription of the TUP1 mRNA is increased 2.7-fold. It is possible this excess of mRNA in farnesol treated cells is translated less well than the lower levels of mRNA in untreated cells simply as a consequence of the overall level of mRNA. In this case, the transcriptional response would play a more dominant role in determining the level of Tup1p protein in cells.

6.5 Validation of some of the transcriptional changes that are relevant to filamentation and stress response using qRT-PCR

A qRT-PCR analysis was also used to further investigate the mRNAs that exhibited altered profiles in the RNA-seq data at the transcript level. The analysis was performed on a total of 6 genes and LUC mRNA was used as ‘spike-in’ control to allow normalisation. TUP1 and EFG1 exhibited increased transcript levels following treatment with 100µM farnesol, (Figure 6.6). PDE1 exhibited decreased transcript level, whereas TRX1 and TIF4631 remained the same transcriptionally (Figure 6.6).

These data show that the trends evident in the RNA-seq datasets can be reproduced using a qRT-PCR approach.
Figure 6.5 Farnesol causes redistribution of TUP1 mRNA from the polysome to the submonosomal fraction. RNA was extracted from polyribosomal fractions from A untreated sample and B sample treated with 100µM farnesol. qRT-PCR was carried out on the cDNA samples using CFx Connect Real-Time system with iTaq Universal SYBR Green Supermix (BioRad Laboratories). C Percentage of mRNA in the monosome versus polysome fractions was quantified. Error bars indicate ± SE.
Figure 6.6 Verification of gene expression analysis by quantitative real-time PCR (qRT-PCR). Data generated by RNA-seq was plotted with calculations done for the same gene using qRT-PCR. Values shown for the qRT-PCR are the means of three determinations and error bars indicate ±SE.
6.5 Discussion

*C. albicans* responds to farnesol, in part, by changing gene expression (Cao et al. 2005). Extensive studies on the effects of farnesol on hyphal growth and biofilm formation have generally focussed on the levels of mRNA transcripts (Cao et al. 2005; Cho et al. 2007; Uppuluri et al. 2007). However, as shown in Chapter 3, farnesol also impacts on protein synthesis. Therefore in order to elucidate the likely impact of farnesol on cells and provide insight into gene expression changes at both the transcript and translation level following farnesol treatment, a global high throughput RNA sequencing analysis of total and polysome associated RNA was undertaken.

RNA-seq analysis of *C. albicans* cells exposed to 100µM farnesol revealed a significant number of mRNAs to be differentially expressed at the transcript and differentially polysome associated. Most notable among the down-regulated mRNAs at both the transcript (*SAP8, SAP98, PDE1 GPR1, TEC1, CYR1*) and polysome association (*TPK2, EFG1, FLO8, GPA2 CZF1, NDT80, DEF1, ALS2,ALS4*) are those that play positive roles in filamentation, and also biofilm formation reflecting the effect of farnesol as an inhibitor of filament formation in *C. albicans*. This is consistent with previous findings that farnesol prevents hyphal formation in a dose dependent manner (Hornby et al. 2001; Ramage et al. 2002). The results provide evidence that several hyphal associated genes are differentially expressed both at the transcriptional and translational level. The concept of potentiation was first described by Preiss et al. (2003) for *S. cerevisiae*. Potentiation was not observed from the RNA seq data of the transcript and translation state following farnesol treatment in *C. albicans*. Rather genes appear to be either transcriptionally or translationally controlled. This perhaps provides a flexible and co-ordinated way to regulate the response to farnesol. Interestingly, *TUP1* was observed to be up-regulated at the level of transcription and it seems this may dominate over a down-regualtion in polysome association. *TUP1* encodes a global transcriptional co-
repressor. Deletion of the *TUP1* gene causes hyper-filamentation under conditions that favour yeast growth (Braun *et al.* 2000), therefore up-regulation of *TUP1* may contribute to the inhibition of filament formation in *C. albicans*.

mRNAs involved in protein synthesis were observed to be up-regulated both at the transcriptional and at the level of polysome association. One possibility is that up-regulation of the protein synthesis gene expression would serve an adaptive role allowing cells to overcome the impact of farnesol as a translational inhibitor.

Overall, mRNAs involved in the response to stress were down-regulated, however, some key mRNAs involved in the response to oxidative stress were up-regulated. This is consistent with the observation that farnesol induces mild levels of oxidative stress in *C. albicans* (Shirtliff *et al.* 2009; Zhu *et al.* 2011). Therefore, up-regulation of these mRNAs could suggest that the farnesol treated cells are experiencing a specific oxidative stress response and this could also account for the upregulation of mRNAs involved in cellular respiration both at the level of transcript and translation level.

Overall, the results presented in this chapter suggest that as for many other stresses or stimuli, the response to farnesol involves co-ordinate regulation of gene expression at both the transcript level and at the level of translational control. Such a co-ordinated response provides cells with greater flexibility in terms of the targeted regulation of specific pathways or processes.
7 General discussion

A range of alcohols have been described as signalling molecules or metabolic end-products across a variety of yeast species (Hornby *et al.* 2001; Chen *et al.* 2004; Chen and Fink 2006). The aim of this research at the outset was to characterise the impact of these alcohols on protein synthesis and filamentation in the pathogenic yeast species *Candida albicans*. Once it became apparent that these alcohols all inhibit protein synthesis yet have grossly different effects on filamentation, the precise mechanisms by which these alcohols act became a priority study area.

The regulation of protein synthesis at the translation initiation stage, allows cells to promptly modify protein levels and adapt to cellular stress. Previously it has been shown that the target of fusel alcohols in *S. cerevisiae* is the eukaryotic initiation factor 2B, which is the guanine nucleotide exchange factor for eIF2 (Ashe *et al.* 2001; Taylor *et al.* 2010). eIF2B has also been implicated in the translational inhibition caused by a variety of cellular stress conditions such as amino acid starvation (Dever *et al.* 1992); purine starvation (Rolfes and Hinnebusch 1993) and rapamycin (Kubota *et al.* 2003). However, while these cellular stresses target eIF2B in a Gcn2p-dependent manner, the mechanism by which fusel alcohols target eIF2B to inhibit translation initiation in *S. cerevisiae* is Gcn2p-independent. The effect of fusel alcohols on translation initiation in the human fungal pathogen, *C. albicans* therefore forms a key part of this thesis.

Another key regulated step in translation initiation is the formation of the 48S preinitiation complex. eIF4E and Pab1p select mRNA via interaction with the 5ʹ cap and 3ʹ poly(A) tail, respectively. eIF4G interacts with both factors, promoting a closed loop mRNP (Sachs 2000), which recruits the small ribosomal subunit via interactions with eIF3, eIF5 and eIF1 to form the 48S preinitiation complex. A variety of stress conditions have been shown to target these steps in the initiation pathway leading to
translational shut-off: for example glucose starvation (Hoyle et al. 2007) and inactivation of mammalian target of rapamycin (Richter and Sonenberg 2005).

Investigations in this thesis into the impact of farnesol, a quorum sensing alcohol, showed that farnesol caused an inhibition of translation initiation by a mechanism that did not target eIF2B. Furthermore, mass spectrometry studies suggest that farnesol targets formation of the 48S preinitiation complex. Finally, the consequences of farnesol regulation in terms of both transcription and translation were investigated using next generation sequencing technologies. These data suggest a co-ordinate regulation of individual genes involved in processes such as filamentation and stress responses, where genes are regulated either at the level of transcription or translation but not both, or individual genes are reciprocally regulated such that when transcription is high translation is low and vice versa.

7.1 Alcohols inhibit translation initiation in \textit{C. albicans} in addition to exerting opposing effects on growth morphology

After the initial observation that a variety of alcohols lead to the inhibition of translation initiation (albeit at different concentrations) in \textit{C. albicans}, a key question related to how the alcohols were exerting these effects. A variety of stresses across eukaryotic organisms lead to the inhibition of translation initiation via the activation of an eIF2\(\alpha\) kinase. Like \textit{S. cerevisiae}, \textit{C. albicans} harbors a single eIF2\(\alpha\) kinase, Gcn2p. This kinase phosphorylates the \(\alpha\) subunit of eIF2 in response to a range of stress conditions including amino acid starvation in \textit{S. cerevisiae} and the oxidative stress caused by the addition of Cadmium in \textit{C. albicans} (Sundaram and Grant 2014). Phosphorylated eIF2\(\alpha\) interacts tightly with and sequesters eIF2B causing it to become limiting and this is ultimately inhibitory to protein synthesis. Even though fusel alcohol-dependent
translational inhibition in *S. cerevisiae* appears to target eIF2B, the process does not rely upon Gcn2p or eIF2α phosphorylation (Ashe *et al.* 2001). So, both the *SUI2* *SSIA* strain, which is translationally resistant to amino acid starvation, and a *gcn2Δ* mutant were still inhibited at the translational level by the addition of butanol (Ashe *et al.* 2001). Similarly in the present study, the CAI4 and *gcn2Δ* mutant strains of *C. albicans* are both completely sensitive to butanol, farnesol and ethanol (Figures 3.6, 3.7, 3.8), showing that the inhibition of translation initiation by alcohols in *C. albicans* does not require the eIF2α kinase pathway. However, eIF2B has been demonstrated to be a key player in the fusel alcohol response in *S. cerevisiae*. Firstly, various eIF2B mutants have been shown to exhibit butanol-dependent phenotypes (Ashe *et al.* 2001; Richardson *et al.* 2004; Taylor *et al.* 2010). Secondly, the level of a ternary complex that lies downstream of eIF2B was reduced significantly by butanol in manner that correlated with the relative sensitivity of eIF2B mutants (Taylor *et al.* 2010). Finally, butanol caused increases in an eIF2B dependent *GCN4-lacZ* reporter system. A similar analysis using *GCRE*-luciferase and *GCN4*-luciferase reporters shows that all of the alcohols barring farnesol lead to increases in this system (Figures 4.1 and 4.2). Taken together, these results are suggestive that short chain alcohols, particularly, butanol could be targeting eIF2B to exert an effect on translation initiation in both *S. cerevisiae* and *C. albicans*.

One possibility is that butanol and other alcohols could be exerting these effects via a signalling pathway that involves eIF2B. Alternatively, it is possible that the alcohols are acting in a more allosteric fashion by interacting directly with eIF2B to impact upon activity.
7.1.1 Role of Sit4p in eIF2αP dephosphorylation

Studies from the Ashe laboratory have revealed some puzzling findings regarding the dephosphorylation of eIF2α in the presence of fusel alcohols in *S. cerevisiae* (Taylor *et al.* 2010). The effects observed run counter to the known mechanism by which eIF2α phosphorylation causes the inhibition of protein synthesis. The observed dephosphorylation of eIF2α in response to alcohols occurs in a manner that correlates with the inhibition of translation (Griffiths and Ashe 2006), so initially a link between the two effects of the alcohols was considered. A deletion mutant in *SIT4* (encoding a type 2A-related phosphatase) was identified that exhibit virtually no eIF2α dephosphorylation after cells have been exposed to fusel alcohols in *S. cerevisiae* (Taylor *et al.* 2010). However, in this mutant the alcohols still cause the full inhibition of protein synthesis. These data provided a direct demonstration that there is not a link between the eIF2α dephosphorylation and the inhibition of protein synthesis (Taylor *et al.* 2010).

In order to assess the level of similarity between the previous studies in *S. cerevisiae* and the impact of alcohols in *C. albicans*, studies on the dephosphorylation of eIF2α were undertaken. Butanol and farnesol were found to cause a dose-dependent dephosphorylation of eIF2α in *C. albicans* (Figures 3.11 and 3.13), it is likely that this effect could be either due to the inhibition of a kinase activity or the induction of a phosphatase. Indeed given the homology of the *C. albicans* and *S. cerevisiae SIT4* (89%), (Arndt *et al.* 1989; Lee *et al.* 2004) and the observation that *C. albicans SIT4* complements a *SIT4* deletion in *S. cerevisiae* (Lee *et al.* 2004), it is most likely that Sit4p is involved in alcohol-induced dephosphorylation as was observed in *C. albicans*.

An intriguing correlation with the published literature lies in studies on the effects of volatile anaesthetics on translation initiation in both yeast and mammalian cells (Palmer *et al.* 2005), where striking similarities can be found to the observed effects of fusel
alcohols on *C. albicans* and *S. cerevisiae*. The volatile anaesthetic, isoflurane, was observed to cause a 15-fold reduction in the level of phosphorylated eIF2α in the yeast within 15 min of incubation. Also a decreased phosphorylation of the ribosomal protein S6 (rpS6) and the kinase that phosphorylates rpS6 (p70\(^{60k1}\)) was observed in mammalian liver cells after exposure to the anesthetic, halothane (Palmer *et al.* 2006).

Intriguingly however, addition of ethanol concentrations that inhibit protein synthesis, showed no dephosphorylation of eIF2α in *C. albicans* (Figure 3.12). This observation provides further support for the interpretation that the dephosphorylation of eIF2α is unrelated to the impact of the alcohols on translation initiation.

### 7.1.2 Alcohols affect morphogenesis

Another observation with regard to the alcohols relates to their ability to impact upon morphological transitions in *C. albicans*. A number of suggestions have been previously made, regarding the physiological rationale underlying pseudohyphal growth and germ tube formation. The altered growth pattern may allow yeast to forage for nutrients under nutrient limiting conditions (Gimeno *et al.* 1992), it could facilitate escape from accumulating toxic end-point metabolites (Gancedo 2001), 2001) and for *Candida species*, it may provide a means to evade the host immune response. Here 0.25- 0.5\%(v/v) butanol was found to induce 50% pseudohyphae formation in *C. albicans* within 4 hours, while 1- 3\%(v/v) of ethanol was required to elicit a similar effect (Figure 3.5B and C). Studies presented here and elsewhere have shown that farnesol blocks germ tube formation in *C. albicans* (Hornby *et al.* 2001; Ramage *et al.* 2002). One intriguing question was whether the morphogenesis induced by serum, butanol or ethanol can override farnesol’s inhibitory activity or vice versa. 150µM farnesol effectively blocked the filamentation induced by serum, whereas lower concentrations were sufficient to block ethanol- or butanol-induced filamentation (Figure 3.5B and C). These data are
consistent with previous work in *C. albicans* where morphogenesis induced by the aromatic alcohol, tyrosol was blocked by farnesol in a dose-dependent manner (Ghosh *et al.* 2008).

Intriguingly, deletion of the *EFG1* gene, that encodes a transcriptional regulator involved in the cAMP-PKA morphological differentiation pathway, exhibited some resistance to the translational effects of farnesol in *C. albicans*. Previous studies have found that the cAMP-PKA kinase and MAP kinase pathways are important for pseudohyphal growth in response to aromatic alcohols in *C. albicans* (Ghosh *et al.* 2008). In addition, Sit4p was shown to play important roles during hyphal growth in *C. albicans* by regulating protein translation (Lee *et al.* 2004). Therefore, it is at least plausible that the translational and morphological impact of alcohols in *C. albicans* are connected. However, it is important to note that for all of the alcohols used in this study, the translational shut down occurs at higher concentrations than are required to cause effects on the growth morphology. Therefore, key questions for the future will be how alcohols induce morphological changes during growth and how this relates to their effects on protein synthesis.

### 7.2 Movement of the eIF2B body is impeded by fusel alcohol, but not farnesol

In order to further investigate how the various alcohols bring about inhibition of translation initiation, cell biological studies were initiated by comparison to what is already known in *S. cerevisiae*. eIF2B and its G protein eIF2 have been shown to colocalise to a discrete cytoplasmic foci in *S. cerevisiae*, termed the eIF2B body (Campbell *et al.* 2005). In response to fusel alcohols the dynamic movement of this body throughout the cell was inhibited in a manner that correlated both with the concentration of the alcohol and the alcohol sensitivity of the strains at the level of translation (Taylor *et al.* 2010). In this thesis, the data show that eIF2B also localises to...
eIF2B bodies in *C. albicans*. Furthermore, the addition of butanol, ethanol or farnesol at concentrations that inhibit translation initiation, did not impact on the ability of eIF2B subunits to localise to these foci (Figure 4.5). However, butanol and ethanol both affected the movement of the eIF2B body in a manner which correlated with their effects on translation initiation (Figures 4.6 and 4.7). The mechanistic relationship between alcohol dependent translational inhibition and the decreased movement of eIF2B body remains unclear. One possibility is that the eIF2B serves as a centre for guanine nucleotide exchange and inhibiting the movement of the body prevents the factor being exposed to high concentrations of its substrate eIF2GDP. Alternatively, as the movement in *S. cerevisiae* has been found to occur as a result of diffusion and where the 2B body remains static for periods it is likely due to tethering, it is possible that the alcohols favour the tethered form of the 2B body, which for some reason precludes guanine nucleotide exchange (Taylor *et al.* 2010). A similar pattern of movement has been described for insulin granules (Ivarsson *et al.* 2004), movement of these granules is sensitive to cooling, and a reduction in movement leads to a reduction in insulin granule secretion, providing an example where the rate of movement of the granules within the cell is necessary for cellular function (Ivarsson *et al.* 2004).

The inhibition of eIF2B body movement is not a general consequence of the translational shut-down, as an inhibition was not observed following farnesol (Figures 4.6 and 4.7). The fact that farnesol treatment does not induce the expression of *GCN4*, combined with its inability to regulate the movement of eIF2B bodies suggests that farnesol does not target eIF2B to regulate translation initiation.

### 7.2.1 Biochemical analysis of eIF2B

To further investigate the possibility that butanol could be affecting the interaction of eIF2B with other factors or that butanol treatment affected the level of eIF2B subunits,
strains of *C. albicans* wild type bearing a combined V5-6XHis epitope tag on *GCD1* and *SUI3* genes were constructed. The aim of the epitope tagging was to purify eIF2B and eIF2, but this strategy could not be pursued further as repeated attempts to purify the tagged proteins failed. A tagging strategy to maximise the purification of the tagged proteins by tagging the two copies of the gene (*GCD1*) with 4x FLAG epitope was therefore designed largely based on purification schemes from *S. cerevisiae* where Flag-tagged eIF2B subunits are overexpressed from plasmids (Mohammad-Qureshi *et al.* 2007). Mass spectrometry on the immunopurified eIF2B samples identified the eIF2B subunits, particularly the eIF2Bγ and ε subunits, which were over 30-fold enriched in the tagged samples relative to samples from the untagged strain (Table 4.1). Therefore the 4xFlag epitope tagging reagents for double tagging the two allelles of a gene developed in this study could be adapted for epitope tagging of other genes in *C. albicans*. However, because the other three subunits of eIF2B (α, β and δ) were not consistently enriched, a biochemical analysis of the guanine nucleotide exchange activity of the eIF2B complex was not possible. However, it may be possible in the future to measure this activity for the γ/ε sub-complex and assess the impact of butanol treatment on this activity. The finding that this catalytic sub-complex does not appear to co-purify stiochiometrically with the regulatory sub-complex may also have biological significance which could be explored in future experiments. For instance in *S. cerevisiae* two of the three regulatory eIF2B genes are essential and the third is required for translational control: Is this also true in *Candida*?

### 7.3 The formation of the 48S preinitiation complex is affected by farnesol treatment.

The data in this thesis also provide insights into the mechanism by which farnesol elicits an inhibition of translation initiation. As described above, the *GCN4*-luciferase reporter
experiments and 2B body studies are suggestive that eIF2B is not the regulatory target for farnesol. Therefore, in an attempt to further understand the stage of translation initiation regulated by farnesol, ribosomal complexes formed during translation initiation were investigated using the formaldehyde-polysome analysis. For this analysis a decision was made to switch to the MLY61 strain of *S. cerevisiae* due to the absence in *Candida* of antibodies with which to detect the components of the individual translational complexes. Therefore, to validate this approach, experiments were conducted showing that farnesol has a very similar impact on protein synthesis in the MLY61 strain of *S. cerevisiae* (Figures 5.1 and 5.2). The subsequent formaldehyde-polysome analysis revealed that the degree to which the eIF4G is associated with the small ribosomal subunit (40S) decreases substantially following treatment with 100µM farnesol (Figure 5.4). These data were verified and extended using an immunoprecipitation strategy combined with a mass spectrometric analysis. More specifically, following farnesol treatment immunopurified TAP-tagged eIF4G1 protein co-purifies with many components of the closed loop complex, that interacts with mRNA e.g. eIF4E and Pab1p, but fails to co-purify with the ribosomal proteins and eIF3 (Figure 5.6), that are only associated with the closed loop complex as part of the 48S complex or 80S complex.

Many studies have reported translational control targeting steps upstream of mRNA recruitment to the 43S ribosome complex to ultimately reduce 48S preinitiation complex formation. For instance, glucose starvation causes a reorganisation of the closed loop mRNP translation complex, whereby the cosedimentation of eIF4E, eIF4G and Pab1p with ribosomal complexes is compromised (Hoyle *et al.* 2007). In addition, phosphorylation of ribosomal protein L13a has been shown to inhibit 43S subunit recruitment to mRNAs bearing the γ interferon inhibitor of translation element in rabbit reticulocyte lysates (Kapasi *et al.* 2007). Patemine A (PatA) is a natural marine product...
and has been shown to target eIF4A to disrupt the eIF4F complex by decreasing the interaction between eIF4A and eIF4G while promoting the formation of a stable complex between eIF4A and eIF4B. As a consequence, PatA blocks translation initiation primarily by stalling the initiation complexes on mRNA (Low et al. 2005). Furthermore another study showed that hippuristanol, another natural marine product, inhibits translation initiation as a result of impaired eIF4A helicase activity and its ability to interact with eIF4G (Lindqvist et al. 2008). Cruz-Migoni et al. (2011) recently showed that the *Burkholderia* lethal factor 1, a toxin produced by the bacterium, *Burkholderia pseudomallei* causes a translational block by inhibiting the helicase activity of eIF4A and this manifests in the human disease melioidosis. Therefore, the observed depletion of eIF4G from the 40S region of the gradient combined with the mass spectrometric analysis of eIF4G containing complexes lend support to a model where farnesol targets the formation of the 48S preinitiation complex to inhibit protein synthesis.

7.4 Transcriptomic analysis reveals a global change in gene expression following farnesol treatment of *C. albicans*

Advances in transcriptomic technology offer great promise in understanding the molecular basis of stress responses in living cells. Previous global studies on the effect of farnesol exposure at the level of gene expression have been based on alterations in the level of transcripts alone (Cao et al. 2005; Uppuluri et al. 2007). As part of this thesis, farnesol has been shown to cause a rapid inhibition of protein synthesis. This means that in order to understand the changes in gene expression caused by farnesol a decision was made to assess the level of polysome association as well as the level of each transcript.
The data obtained from the transcriptomic and polysomal sequencing revealed that genes associated with the functions in hyphal growth, biofilm formation and cell cycle were down-regulated in response to farnesol (Figure 6.4A and B). One intriguing aspect of these data is that many genes implicated in these functions appear to be either regulated at the translational level or at the level of the transcript but not both. For some genes there is even a reciprocal regulation of these processes. This contrasts with data in *S. cerevisiae* for heat shock, rapamycin treatment, amino acid starvation and glucose starvation where individual genes have been viewed as co-ordinately regulated in a process that has been termed ‘potentiation’ (Preiss *et al.* 2003; Smirnova *et al.* 2005; Castelli *et al.* 2011). The reason for this distinction is unclear, but may relate to the fact that farnesol is more of a signalling molecule where a programmed response might be required, whereas nutritional stresses require a ‘knee-jerk’ instantaneous and robust response.

The *TUP1* gene proved particularly interesting as it appeared up-regulated at the level of transcription but down-regulated in terms of polysome association. These rather puzzling data were confirmed using qRT-PCR. It seems possible that the increases at the level of transcription overwhelm the translational machinery such that not all of the new mRNA can be simultaneously assimilated for the production of protein. Hence, it may appear that polysome association has been down-regulated. The anticipated outcome under such circumstances would still be higher levels of the global transcriptional co-repressor Tup1p protein. Previous studies have found that *TUP1* deletion causes hyper-filamentation under conditions that favour yeast growth (Braun *et al.* 2000), therefore an up-regulation of *TUP1* in response to farnesol may contribute significantly to the inhibition of filament formation in *C. albicans* by repressing transcription of a range of filamentation genes. However, it remains unclear how genes are regulated at the translational level in response to farnesol.
Three other functional classes of mRNA; those whose gene products are involved in translation, cellular respiration and the response to oxidative stress were also up-regulated in response to farnesol (Figure 6.4A and B). Once again, the mRNAs are regulated either at the transcript level or at the level of polysome association, but rarely both. As farnesol causes a 10-fold decrease in protein synthesis (see section 3.2.4), the up-regulation of genes involved in protein synthesis could represent part of an adaptive response by the cells to overcome the effect of farnesol on translation. In terms of oxidative stress, Shirliff et al. (2009) and Westwater et al. (2005), have previously shown that exposure of C. albicans cells to farnesol caused the accumulation of reactive oxygen species (ROS). Therefore, once again the up-regulation of genes involved in the response to oxidative stress could represent an adaptive response to farnesol treatment.

Upregulation of mRNAs, whose gene products are involved in cellular respiration, both at the transcript and translation level shows that farnesol could be impacting on the respiratory machinery of C. albicans as was previously observed in S. cerevisiae (Machida et al. 1998).

7.5 Concluding remarks and future perspectives

The data presented in this study show that alcohols inhibit translation initiation in C. albicans via different mechanisms. Fusel alcohols and ethanol appear to target eIF2B, whereas 48S preinitiation complex formation is the likely target of farnesol. These range of alcohols also differentially impact on one of the key virulence factors of this important human fungal pathogen: morphological transition from yeast to hyphae or pseudohyphae. Farnesol blocks the transition from yeast to hyphae even in the presence of serum, whereas fusel alcohols and ethanol promote pseudohyphae and germ tube formation. Future studies to investigate the connection between alcohol-induced inhibition of protein synthesis and morphological differentiation will involve screening
deletion mutants in the morphological differential pathway or in translation initiation factors for their translational or growth responses respectively in the presence of the alcohols.

Farnesol has been shown to target some virulence features of *C. albicans* and as such has been studied as a possible agent for antifungal therapy. Data obtained here from RNA sequencing has revealed that genes involved in filamentation were down-regulated at either the transcriptional or translational level. Part of this regulation could be due to the up-regulation of the *TUP1* gene, which explains the transcriptional response, however, an explanation for the translational regulation of these genes is still outstanding. Mapping strategies will be required to precisely define the RNA sequences that dictate the regulation of translation. These sequences can then be used to identify either based on prior knowledge of RNA binding specificity, or experimentally via affinity chromatography the proteins that interact with the sequence. Deletion mutation would then allow the regulatory impact of these factors to be assessed following farnesol treatment.

Interestingly the range of alcohols studied in this work are produced by *C. albicans* and could be playing various *in vivo* roles in the adaptation, survival and perhaps virulence of this opportunistic pathogen. Therefore elucidating mechanisms by which this range of alcohols affect protein synthesis and morphological transition may help to advance research on the development of novel antifungal agents that switch on endogenous cell inhibitory mechanisms in this human pathogen.
Figure 7.1 Model for the impact of various alcohols on protein synthesis in *C. albicans* and *S. cerevisiae*. The various alcohols had similar impact on protein synthesis in *C. albicans* and *S. cerevisiae*. However, *S. cerevisiae* being a Crabtree positive yeast exhibited more tolerance to the translational effects of ethanol. In addition, while mild inhibition of translation initiation seems to induce pseudohyphal formation, farnesol inhibited the process.
8. Bibliography


Johannes, G., Carter, M. S., Eisen, M. B., Brown, P. O. and Sarnow, P. (1999). "Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF-4F"


Sundaram, A. and Grant, C. M. (2014). "A single inhibitory upstream open reading frame (uORF) is sufficient to regulate Candida albicans GCN4 translation in response to amino acid starvation conditions." RNA.


# 9. Appendix

Transcriptionally upregulated genes

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<th>GO-Slim term</th>
<th>Cluster frequency</th>
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<td>response to stress</td>
<td>34 out of 312 genes, 10.9%</td>
<td>APR1, C1_02390W_A, C1_06600W_A, C1_07980C_A, C2_04700C_A, C3_02290W_A, C3_04810C_A, C4_00390W_A, C4_01720C_A, C4_07010C_A, C5_02110W_A, C5_05060C_A, CH12, EAF7, EFG1, HML1, HSP104, HSP12, HSP21, HSP78, MAL2, MDJ1, MEP2, PGAL14, PGA34, PKX1, RPB4, SOD4, SOD5, SPT6, TIF34, TUP1, VPD27, VRP1</td>
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<td>generation of precursor metabolites and energy RNA metabolic process</td>
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Transcriptionally down regulated genes

**Response to stress**

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</tr>
<tr>
<td><strong>translation</strong></td>
<td>45 out of 476 genes, 9.5%</td>
<td></td>
</tr>
<tr>
<td><strong>response to stress</strong></td>
<td>42 out of 476 genes, 8.8%</td>
<td></td>
</tr>
<tr>
<td><strong>RNA metabolic process</strong></td>
<td>40 out of 476 genes, 8.4%</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GO Tree View</th>
<th>1.2%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>respiration</strong></td>
<td>1.2%</td>
</tr>
<tr>
<td><strong>hyphal growth</strong></td>
<td>4 out of 338 genes, 1.2%</td>
</tr>
<tr>
<td><strong>CLN3, ENA2, TOM22, WSC1</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Translationally up regulated genes**

- **C1_00980W_A, C1_01580W_A, C1_02330C_A, C1_03620C_A, C1_04820C_A, C1_05230C_A, C1_06470W_A, C2_05410W_A, C3_06240C_A, C4_00660W_A, C4_04390W_A, C5_00320W_A, C5_05250C_A, C5_06470W_A, C6_00850W_A, C6_03210C_A, C7_01020C_A, CR_02950C_A, CR_04580W_A, CR_08480C_A, MRPL6, MTG1, RPL10A, RPL11, RPL12, RPL14, RPL17B, RPL20B, RPL23A, RPL29, RPL32, RPL37B, RPL38, RPL39, RPL42, RPL9B, RPL9A, RPS12, RPS13, RPS21B, RPS22A, RPS28B, RPS3, RPS30, RPS6A, SUI1, UBI3**

**Response to stress**

- **ADE1, AHP1, AQY1, ARCA4, ATX1, BIF1, C1_01300W_A, C1_02700C_A, C1_07280C_A, C1_11160C_A, C1_128405W_A, C2_05060C_A, C2_07200W_A, C3_06590W_A, C3_06860C_A, C3_07670W_A, C6_00850W_A, C8P1, ECC1, ESS1, FFT33, FGR24, FGR39, FGR46, GCN4, GLN1, GLX3, GPA1, HSP21, HTA1, NCE4, PHB1, PHO112, RAE1, RDI1, RHB1, SKP1, SMX3, SOD1, TRX1, WH1, WSC1**

**RNA metabolic process**

- **C1_05230W_A, C1_05420W_A, C1_07280C_A, C1_08660C_A, C1_11160C_A, C1_14410W_A, C2_03880C_A, C2_06300W_A, C2_07200W_A, C3_04380C_A, C4_05040W_A, C5_05920W_A, C6_03210C_A, C6_03910C_A, CR_01780W_A, CR_06690C_A, CR_07080W_A, ESS1, HHF1, HHF22, HTA3, KTI11, MSS116, NHP6A, PZ1, REX2, RFA12, RPB5, RPC10, RPF2, RPS13, RPS21B, RPS28B, RPS6A, RRP42, SMD2, SMX4, SUI3, UBI3**
### Translationally down regulated genes

<table>
<thead>
<tr>
<th>GO-Slim term</th>
<th>Cluster frequency</th>
<th>Genes annotated to the term</th>
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</thead>
<tbody>
<tr>
<td><strong>Response to stress</strong></td>
<td>83 out of 476 genes, 17.4%</td>
<td>ARP4, ATG2, BNA4, BNI4, C1_02390W, A C1_0798 0C, A C2_06530W, A C2_08630C, A2_10850C, A C3_08570C, A C3_05970C, A, C3_06630W, A, C4_00 030C, A, C4_01720C, A, C4_03810W, A, C4_03850W, A, C4_05350W, A, C4_07220C, A, C7_08810W, A 7 01130C, A, CAS1, CAS5, CCT8, CDC27, CKA2, C NHI1, CPP1, CRZ2, CR_00600C, A, CR_06780W, A, CZF1, EAF3, EAF7, ECM25, EFG1, EPL1, ESC4, EFG4, R47, FGR50, GPA2, GSG1, GUP1, GZF3, HAP4, HAP5, HIMS1, HR2, LTV1, MEP3, NFB2, NTD80, O P11, PHO23, PIN4, PSY2, RAD3, RN1, RFG1, RGD1, RIM8, RPB4, Rpg1, RVS161, SET1, SET3, SUF1, SGT1, SKO1, SNT4, SPF20, SPF5, SPF6, SN5, TC_0889, TFG1, TPK2, TUP1, UCL1, VID21, VID27, VR1, WAL1, YAF9</td>
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<tr>
<td><strong>Filamentous growth</strong></td>
<td>76 out of 476 genes, 16%</td>
<td>AAF1, AIS2, ALS4, ARG81, ARG83, BAS1, BEM1, BNA4, BNI4, C1_06090C, A, C1_14190C, A, C3_0657 0C, A, C3_06790W, A, CAS5, CCA1, CCT8, CPP1, C ZF1, DBF2, DEFI1, ECM25, EFG1, ESG4, FGR4, F GR47, FGR50, FLO8, GPA2, GUP1, GZF3, HAP5, H MS1, HXK1, KEL1, KIN2, KIS1, MEF2, MFG1, MS S1, MS5, NBP2, NTD80, PCL5, PEA2, PGS9, PIN 4, RFG1, RGD1, RGT1, RIM8, ROP1, RPB4, RVS161, SET1, SET3, SFL1, SGT1, SKO1, SL1, SNT1, SPF 5, SPF6, SN5, TCC1, TEA1, TFG1, TPK2, TUP1, U EC1, VID27, VRP1, WAL1, YAH1, YAK1, ZCF7, ZC F8</td>
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<tr>
<td>Process</td>
<td>GO Terms</td>
<td>Total Genes</td>
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<td>Response to chemical growth</td>
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<tr>
<td>Response to drug</td>
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<tr>
<td>Cytoskeleton organization</td>
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<tr>
<td>Signal transduction</td>
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<tr>
<td>Biofilm formation</td>
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<tr>
<td>Cytokinesis</td>
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<tr>
<td>Hyphal growth</td>
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<tr>
<td>Carbohydrate metabolic process</td>
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<td>12</td>
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<tr>
<td>Growth of unicellular organism as a thread of attached cells</td>
<td></td>
<td>11</td>
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<tr>
<td>Gene(s)</td>
<td>Count</td>
<td></td>
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<td>------------------</td>
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<tr>
<td>C3_00790W_A, EFG1, GPA2, MEP2, PEA2, WHI3</td>
<td>6 out of 476 genes, 1.3%</td>
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</tbody>
</table>

**Pseudohyphal growth**