Chapter 12

Metabolic Adaptation of Isocitrate Lyase in the Yeast Pathogen *Candida albicans*

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/62406

Abstract

The *ICL1* gene, which encodes the glyoxylate cycle enzyme isocitrate lyase (Icl1), is required for the growth of *Candida albicans* on non-fermentable carbon sources and for this yeast to be virulent. The aim of this study is to test the stability of the Icl1 enzyme in response to glucose. Glucose was found to trigger the degradation of the *ICL1* but the CaIcl1 was not destabilized by glucose. When CaIcl1 was expressed in *Saccharomycess cerevisiae*, it was not degraded in response to glucose, suggesting that CaIcl1 has lost the molecular signal that triggers destabilization in response to glucose. However, when ScIcl1 was expressed in *C. albicans* it was rapidly degraded in response to glucose indicating that *C. albicans* has retained the molecular apparatus for glucose-accelerated degradation of target proteins. ScIcl1 degradation was slowed in Ca*ubi4*/*ubi4* in which ubiquitin-mediated protein turnover is reduced. Furthermore, the addition of putative ubiquitination site to the carboxyl-terminus of CaIcl1 led to the glucose-accelerated degradation of this protein. *C. albicans* has retained the apparatus for ubiquitin-mediated degradation of target protein in response to glucose. However, CaIcl1 has lost the Ubi-site that mediate glucose accelerated protein degradation, thereby allowing *C. albicans* to simultaneously assimilate alternative carbon sources and glucose.

Keywords: Glyoxylate cycle, isocitrate lyase (Icl1), metabolic adaptation, virulence, pathogenicity

1. Introduction

Candida albicans is a major fungal pathogen of humans causes superficial and deep-seated candidiasis infections [1, 2]. *C. albicans* is an opportunistic pathogen residing as a commensal in the oral cavity and gastrointestinal and urogenital tracts of many individuals [3, 4]. The severity of candidiasis ranges from superficial mucosal infections to systemic or disseminated infections. In healthy individuals, *C. albicans* is relatively harmless. However, immunocom-

promised patients can get disseminated candidiasis in deep tissues that are difficult to diagnose and can result in death [5, 6].Treatment involves the use of antifungals such as fluconazole, amphotericin B and caspofungin [7, 8]. However, these treatments are not always successful [9].

This chapter addresses the metabolic adaptation of isocitrate lyase (ICL1) of this fungal pathogen in humans. This topic is important for studying the pathogenicity of *C. albicans* because this medically important fungus must grow to cause infections, and to grow it must assimilate carbon. *C. albicans* can occupy various diverse niches in humans, and many of these niches contain a range of different carbon sources. The question arises whether this pathogen is able to exploit this range of carbon sources if glucose happens to be present. This would not be the case in the model yeast *Saccharomycess cerevisiae* because various forms of glucose regulation inhibit the assimilation of alternative carbon sources in this model yeast [10 - 13]. Therefore, this first compares the impact of glucose on the assimilation of alternative carbon sources in these two yeasts and then examines whether molecular mechanisms exist in *C. albicans* to promote the rapid turnover of target proteins in response to glucose. Hence, this section focusses on commensalism, *C. albicans* infections, and antifungal therapies.

Candida infections have been reported for virtually every tissue of the human body, and they can be classified according to different criteria [14]. Superficial infections affect the skin and mucous membranes [15]. In contrast, invasive candidiases include candidemia, acute or chronic haematogenously disseminated candidiasis (infections of the bloodstream), and deep-seated infections of the internal organs [16].

C. albicans has evolved to become an effective commensal organism [17]. In the state of commensalism, *Candida* species live as relatively harmless members of the microflora of healthy individuals causing no discernible disease. *Candida* species are "carried" in the oral cavity, the GI tract, the anus and groin of healthy individuals, and also in the vaginal canal and vulva of healthy women [14, 18]. *Candida* is carried by the most of healthy individuals and can attain surprisingly high densities without symptoms of disease [19]. *C. albicans* is found in stools of about 50% healthy people in addition to the many bacteria that usually inhabit the GI tract [20]. The proliferation of pathogenic microorganisms such as *Candida* is inhibited partly by the growth of harmless bacteria in these niches [21]. The symbiosis of these microorganisms depends on the amount of mucus, the peristaltic behaviour of the bowel and the presence of specific host antibodies and on the capacity of these microbes to adhere to epithelial cells [20].

C. albicans is the most virulent *Candida* species; among the *Candida* species, *C. albicans* is the most commonly isolated yeasts from susceptible hosts [22 - 24]. Multiple risk factors play a role in increasing likelihood of *C. albicans* undergoing the transition from harmless commensal to a virulent pathogen. These risk factors include injuries or traumatic surgery; the presence of indwelling devices, such as catheters or prosthetic devices; antibiotic treatment, which reduces the competitiveness of bacterial species in host niches; and age, with new born babies or aged individuals displaying increased risk of infection [14, 24, 25].

Candidemia is defined as the isolation of *Candida* from at least one blood culture specimen. This type of infection is mainly acquired as the result of neutropenia, injuries caused by recent surgery or the presence of indwelling devices. Also the use of broad-spectrum antibiotics

represents another significant risk factor [16]. *Candida* not only often infects the livers of patients during systemic candidiasis but can also thrive in their spleen, brain or kidney [26].

C. albicans skin infections mostly occur in warm and moist niches such as the armpit, the perineum and skin folds. Similarly, *Candida* is a common cause of nappy (diaper) rash in infants, and it particularly affects obese and elderly adults, as well as the inframammary region of women. Itching and burning are the common symptoms of these types of infections [15]. Most women suffer from oral and vaginal infections (thrush) at least once in their life time [27]. Also, HIV and AIDS -patients suffered from oral thrush before the advent of the highly active anti-retroviral treatment (HAART), which includes a protease inhibitor that also inhibits an important virulence attribute of *C. albicans*- secreted aspartyl protease [28].

There are three main classes of clinically useful antifungal drugs: the polyenes, the azoles and the echinocandins. Amphotericin B is the main drug in the polyene family. It is thought to perturb the functionality of the fungal plasma membrane via interactions with ergosterol [29]. Unfortunately, the clinical utility of Amphotericin B is limited because it can cause nephrotoxicity in patients.

The azoles are an expanding family of compounds, as exemplified by the classic drug flucoazole. They target ergosterol synthesis, and hence the fungal plasma membrane [29]. Additional antifungal agents are being developed. These include triazoles such as posaconazole, ravuconazole and voriconazole. This strengthens the choice of azoles, which is the most successful antifungal class in the clinic since the late 1960s. Voriconazole is a broad spectrum drug that is fungicidal against some isolates of filamentous species [30]. Posaconazole also inhibits a broad spectrum of fungi, and has shown promising effects against Coccidioides in preclinical studies [31]. Meanwhile, ravuconazole has a long plasma half-life in humans that might improve its efficacy [32].

Echinocandins such as caspofungin, anidulafungin and micafungin target cell wall β-1 3-glucan synthesis [29]. The development of echinocandins represented a major advance in antifungal drug development because they targeted a new area of fungal cell biology -the cell wall [29]. *C. albicans* cells can become tolerant to echinocandin treatment via activation of the cell wall rescue pathway leading to elevated chitin synthesis [33].

2. Effect of glucose on alternative carbon sources in *Candida albicans*

The assimilation of carbon sources is fundamentally important for the growth of *C. albicans* and for the establishment of infections in the human host. As described earlier, to grow, a microbe must be able to assimilate carbon [34]. For most yeasts, glucose is generally a preferred carbon source and for *S. cerevisiae* the chosen mode of metabolism is often fermentative in the presence of excess glucose. This uses the Embden-Meyerhof or glycolytic pathway, resulting in the formation of ethanol. In the absence of glucose, *S. cerevisiae* adapts to utilise the alternative carbon sources that are available and switching to non-fermentable carbon metabolism.

Likewise, *C. albicans* alters the expression of its metabolic functions to facilitate cell survival [35]. *C. albicans* adjusts its metabolism to growth in biofilms by up-regulating amino acid biosynthesis genes [36]. When exposed to human neutrophils or cultured macrophages, *C. albicans* also up-regulates amino acid biosynthesis genes and displays a shift from fermentative to non-fermentative metabolism [35, 37, 38]. Importantly, the utilisation of non-fermentable carbon sources requires gluconeogenesis and the glyoxylate cycle [39]. Furthermore, the glyoxylate cycle is required for fungal virulence [40]. These examples illustrate the metabolic flexibility of this pathogen and the relevance of metabolic adaptation pathogenicity [41].

Our understanding of the physiology of *C. albicans* has been largely based on presumptions that the central carbon metabolism in *S. cerevisiae* and *C. albicans* is similar. In these fungi, the pathways of central carbon metabolism, including glycolysis, gluconeogenesis, the pentose phosphate pathway, the tricarboxylic acid (TCA) cycle and glyoxylate cycle, are highly conserved [14, 42]. However, metabolic differences do exist between *C. albicans* and *S. cerevisiae*, the most obvious of which relates to their patterns of sugar utilisation [41]. For example, *S. cerevisiae* belongs to the group that is called Crabtree-positive yeasts which have the ability to produce ethanol even in the presence of oxygen. In contrast, *C. albicans* is designated as a Crabtree-negative yeast [43] because it retains respiratory capacity in the presence of excess glucose [44].

As described earlier, pathways of alternative carbon assimilation in *S. cerevisiae* are subject of glucose repression study. The genetics of glucose repression have been studied, and the regulatory elements that drive this regulation have been described in *S. cerevisiae* [10, 12, 45, 46, 47]. These include co-regulatory mechanisms that act on common elements within the promoter sequences of gluconeogenic and glyoxylate cycle genes. The carbon source regulator elements (CSRE) in the promoters of the *S. cerevisiae PCK1, FBP1, MLS1* and *ACR1* are required for their transcriptional induction in the absence of glucose [48 - 52]. In addition the promoters of the *S. cerevisiae ICL1, MLS1* and *FBP1* genes contain binding sites for the transcription repressor Mig1 [53]. Mig1 represses the transcription of these genes in the presence of glucose, and the activity of Mig1 is being regulated by Snf1 (AMP kinase) signalling.

Transcript profiling of glucose responses in *C. albicans* and *S. cerevisiae* has shown that both yeasts are sensitive to very low levels of glucose [54, 55]. In *S. cerevisiae* and *C. albicans*, glycolytic genes were up-regulated and gluconeogenic and TCA cycle genes were down-regulated even when only 0.01% glucose was added to the growth medium. Yin *et al*. [54] also showed that *S. cerevisiae* ribosomal protein genes also respond to glucose but that they were less sensitive to glucose than the metabolic genes mentioned above. In *S. cerevisiae*, ribosomal protein gene expression was up-regulated following glucose addition at concentrations above 0.1% [54, 56].

Therefore addition of glucose to *S. cerevisiae* cells growing on alternative carbon sources causes a rapid shift from non-fermentative to fermentative metabolism, in part through tight regulation of gene transcription. Glucose also regulates metabolic activity in *S. cerevisiae* at post-transcriptional levels. Glucose triggers the accelerated decay of gluconeogenic mRNA (*PCK1, FBP1*) [54]. Furthermore, glucose triggers the catabolite inactivation and degradation of gluconeogenic and glyoxylate cycle enzymes in *S. cerevisiae.* Fructose-1,6-bisphophatase (FBPase) is expressed when yeast cells are grown on non-fermentable carbon sources. When

the cells are then transferred to a glucose-containing medium, the cells rapidly degrade FBPase to inactivate gluconeogenic activity. This was shown by immunoprecipitation and Western blotting [57]. These authors found that the ubiquitin-conjugating enzyme Ubc8p contributes to glucose-induced ubiquitination of FBPase and that this ubiquitination proceeds the catabolite degradation of the enzyme via the proteasome [58], three other gluconeogenic and glyoxylate cycle enzymes were identified as additional targets of the catabolite inactivation machinery [59]. In addition, it was discovered that an amino-terminal proline residue is essential for the rapid degradation of FBPase in response to glucose. FBPase phosphorylation was not necessary for degradation to occur [59]. This amino-terminal ubiquitination target site on FBPase essentially functions as an autonomous, primary degradation signal.

We reasoned that glucose responses might have diverged significantly between *C. albicans* and *S. cerevisiae*. Our rationale was that the relaxation of glucose repression would confer an evolutionary advantage upon a yeast such as *C. albicans* by allowing this pathogen to continue to assimilate alternative carbon sources even when small amounts of glucose are present *in vivo.* This section describes the testing of this working hypothesis through comparison of the effects of glucose upon gluconeogenic and glyoxylate cycle gene expression in *C. albicans* and *S. cerevisiae.*

To understand carbon assimilation in *C. albicans*, growth on selected alternative carbon sources was first defined. Therefore analogous growth experiments were carried out for both *S. cerevisiae* and *C. albicans* in media containing glucose or alternative carbon sources. Lactic acid was chosen as one alternative carbon source because it is a three-carbon molecule of physiological relevance found in various host niches and in the bloodstream after exercise [60]. Also Aberdeen Fungal Group Laboratory has generated a considerable body of data on cells grown on lactate [54, 55].

Yeast cells were grown overnight in media containing 2% lactate or 2% (2% each or 1% + 1%) both glucose and lactate. These cells were then harvested and used to inoculate fresh media of the same composition and grown for 10 hours. Glucose and ethanol levels and growth absorbance (at OD_{340}) were measured.

As expected, both yeasts grew better on media containing glucose plus lactate, than on lactate alone. *C. albicans* grew faster than *S. cerevisiae* on both media under these conditions. However, both yeasts displayed similar rates of glucose consumption and different ethanol accumulation under these conditions. Glucose was utilised rapidly by both *S. cerevisiae* and *C. albicans*. Ethanol levels in glucose plus lactate cultures significantly increased in *S. cerevisiae*, but they remained similar during glucose assimilation by *C. albicans*. This indicated that most glucose was not fermented to ethanol under the experimental conditions examined in *C. albicans* (minimal medium; 30°C; 200 rpm).

The fatty acid i.e. oleic acid was chosen as the second alternative carbon source for analysis. A fatty acid was chosen because lipids represent a rich source of carbon in the host, *C. albicans* is known to secrete lipases [61], and *C. albicans* is known to induce fatty acid β-oxidation genes following phagocytosis by macrophages [62].

Once again, analogous growth experiments were carried out for both *S. cerevisiae* and *C. albicans* in media containing oleic acid or glucose plus oleic acid. Yeasts cells were grown overnight in media containing 0.2% oleic acid or 2% glucose plus oleic acid, and these cells were used to inoculate fresh media containing the same amount of carbon sources. Growth was monitored for 10 hours. Once again, glucose and ethanol levels and absorbance (OD_{340}) were measured.

Both yeasts grew on oleic acid or on oleic acid plus glucose. As expected more growth was observed for both yeasts on the glucose containing medium compared with the medium containing oleic acid alone. *C. albicans* grew more efficiently than *S. cerevisiae* on both media. Once again, the rates of glucose consumption and ethanol production were different for both yeasts. Both yeasts consumed glucose rapidly, but ethanol levels were accumulated significantly throughout the experiment in *S. cerevisiae*. This suggested that most glucose was not fermented to ethanol under these conditions in *C. albicans*. Less ethanol was generated during growth on oleic acid (about 2 mg/ml or 0.02%) compared to during growth on lactic acid (about 4 mg/ml or 0.04%).

Previous work by Yin *et al*. [54] using Northern blotting and transcriptomic analyses showed that transcripts encoding the gluconeogenic enzymes (*FBP1* and *PCK1*) are repressed by glucose in *S. cerevisiae*. To reconfirm this report and to compare it with the glucose responses of *C. albicans* more directly in this study, we first examined the responses of *S. cerevisiae* glyoxylate cycle (*ScICL1*) and gluconeogenic mRNAs (*ScPCK1*) using the following experimental approach.

S. cerevisiae cells were grown to mid-exponential phase in a minimal medium containing lactate or oleic acid as the sole carbon source and lactate + glucose and oleic acid + glucose. The levels of the *S. cerevisiae ICL1* and *PCK1* mRNAs were measured relative to the housekeeping β-actin gene (*ScACT1*), following the addition of glucose to a final concentration of 2%. Samples were collected and frozen immediately in liquid nitrogen for RNA extraction. *S. cerevisiae ICL1*, *PCK1* and *ACT1* primers [63] were then designed, and the expression of these genes was quantified using Syber green quantitative real-time polymerase chain reaction (qRT-PCR).

ScICL1 mRNA levels showed a dramatic decrease within 30 minutes of glucose addition to cells growing on lactate or oleic acid. Similarly, *ScPCK1* mRNA levels declined after glucose addition to cells growing on lactate or oleic acid media. This strong repression occurred 30 minutes after glucose addition. These results confirmed that in *S. cerevisiae*, the *ICL1* and *PCK1* transcripts are strongly repressed by glucose [54]. At least for the *PCK1* mRNA, this repression is mediated by transcriptional repression and accelerated mRNA degradation [54].

Aberdeen Fungal Group Laboratory has also described the global transcriptional responses of *C. albicans* to low (0.01%), medium (0.1%) and high (1%) glucose concentrations by microarray analysis [55]. The data indicated that a total of 347 *C. albicans* genes were up-regulated, and 344 genes were down-regulated in response to at least one of the glucose concentrations examined. There are170 of these genes that were up-regulated and 180 genes that were down-regulated by 0.01% glucose, indicating that about half of glucose-regulated genes are responsive to low glucose levels. Therefore, the authors concluded that like *S. cerevisiae*, *C. albicans* is

http://dx.doi.org/10.5772/62406

exquisitely sensitive to glucose, responding to concentrations as low as 0.01%. Hence, at the start of this study, an aim was to confirm the impact of glucose on specific mRNAs that encode enzymes required for the assimilation of alternative carbon sources. The transcripts encoding the glyoxylate cycle enzyme isocitrate lyase (*CaICL1*) and the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (*CaPCK1*) were the main focus here.

C. albicans cells were grown to mid-exponential phase in media containing lactate or oleic acid as the sole carbon source using the same procedures described for *S. cerevisiae* by Yin *et al*. [54]. Glucose was then added to a final concentration of 2%; samples were taken for RNA analysis at various times thereafter; the levels of the *CaICL1, CaPCK1* and *CaACT1* mRNAs were measured by qRT-PCR. The relative expression of *CaICL1* (compared with the internal *CaACT1* control) was high in lactate- and oleic acid-grown cells compared with the cells that were exposed to glucose. The *CaICL1* mRNA was strongly down-regulated within 60 minutes after glucose addition under both of these growth conditions. The *CaPCK1* mRNA was expressed at relatively high levels in lactate- and oleic acid-grown cells, and was also strongly down-regulated within 60 minutes of glucose addition. These results confirmed that in both *S. cerevisiae* and *C. albicans*, the *ICL1* and *PCK1* genes are strongly repressed by glucose [63].

The next step is to test the effects of glucose on the expression levels of the CaIcl1 and CaPck1 proteins in *C. albicans* and to compare this response with the corresponding situation *S. cerevisiae.* In *S. cerevisiae*, the effects of glucose on fructose-1,6-bisphosphatase (FBPase) have been intensively studied and it was reported that the FBPase protein (ScFbp1) is rapidly degraded upon the addition of glucose [58]. Also it has been reported that the levels of cytosolic malate dehydrogenase, fructose-1,6-bisphosphatase, isocitrate lyase and phosphoenolpyruvate carboxykinase are all low in *S. cerevisiae* after glucose addition [59]. Therefore, as a starting point, we tested for ourselves whether Icl1 and Pck1 decline in *S. cerevisiae* upon glucose addition.

To achieve this, the *S. cerevisiae ICL1* coding region was tagged at its 3′-end with Myc_9 and the *PCK1* coding region was tagged with HA6. Control Western blots with these tagged strains and their untagged parental strains demonstrated that ScIcl1-Myc_9 and ScPck1-HA6 were expressed during growth on non-fermentable carbon sources [63].

After confirming the validity of the ScIcl1-Myc_9 and ScPck1-HA6 tagging, the next step is to examine the effects of glucose upon the stability of these proteins following glucose addition to *S. cerevisiae* cells. Therefore, the epitope-tagged *S. cerevisiae* strains were grown on lactate or oleic acid, and then glucose was added to a final concentration of 2%. All experiments were performed on exponentially growing cells. Samples were prepared at various times thereafter, and the levels of the ScIcl1-Myc_9 and ScPck1-HA6 proteins were measured by Western blotting. Clearly, glucose addition led to the degradation of ScIcl1 and ScPck1. These results confirmed that in *S. cerevisiae* Icl1 and Pck1 are degraded in response to glucose. Interestingly, the degradation of ScPck1 appears to start about 2 hours after glucose addition, whereas ScIcl1 degradation starts earlier. This might reflect differences in the mechanisms of glucose-activated degradation of these proteins, via ubiquitin-mediated or vacuole-mediated pathways, as described by Regelmann *et al*. [64].

Having confirmed that glucose addition to cells growing on non-fermentable carbon sources leads to the degradation of Icl1 and Pck1 in *S. cerevisiae*, the next step is to test the effects of glucose upon the corresponding enzymes in *C. albicans.*

C. albicans strains expressing Myc_3-tagged Icl1 or Myc_3-tagged Pck1 were then used in an analogous experimental design to our previous *S. cerevisiae* protein analysis. *C. albicans* cells were grown in media containing non-fermentable carbon sources (lactate or oleic acid) as sole carbon sources, and then 2% glucose was added while cells were in the exponential growth phase. Cells were then harvested at various time periods, and their proteins were extracted for Western blotting. Proteins were loaded in equal amounts onto the SDS/PAGE gels, and expression of the Myc_3-tagged CaIcl1 and CaPck1 proteins was detected with anti-Myc antibodies and the images quantified using a phosphorimager.

The *C. albicans* Icl1 protein was expressed during growth on lactate or oleic acid. Interestingly, CaIcl1 was not destabilised by the addition of 2% glucose. Indeed, CaIcl1 protein levels were not significantly different from the control even after 4 hours. Likewise, CaPck1 expression levels were relatively high during growth on lactate or oleic acid, and CaPck1 was also not destabilised by glucose addition. These data suggested that glucose does not affect the stability of the CaIcl1 and CaPck1 proteins. This behaviour of *C. albicans* was in direct contrast to that observed in *S. cerevisiae*, in which the Icl1 and Pck1 proteins were destabilised by glucose [63].

Carbon assimilation is essential for the generation of new biomass (i.e. growth). Therefore, the growth of *C. albicans* in the immunocompromised host depends upon the assimilation of available carbon sources *in vivo*, and the fungus must adjust its metabolism to the microenvironments it occupies in the host [41].

Previous reports have suggested that *S. cerevisiae* might provide a reasonable metabolic paradigm for *C. albicans* as reviewed by Brown [41]. Certainly, many of the pathways of central metabolism are conserved in fungi, including the glycolytic, gluconeogenic and pentose phosphate pathways and the TCA and glyoxylate cycles [14, 42]. Pathways for the generation of storage and cell wall carbohydrates are also conserved. Furthermore, the pathways of amino acid, lipid and nucleotide catabolism and anabolism appear to be conserved. However, significant metabolic differences do exist between *C. albicans* and *S. cerevisiae*, as revealed by their different patterns of sugar utilisation. In fact, differences in the patterns of carbohydrate assimilation are used routinely to distinguish *C. albicans* from other microbes in the clinic [65, 66]. Also, significant differences in the regulation of carbon metabolism are emerging for *C. albicans* and *S. cerevisiae* [67, 68]. Based on these observations, we tested whether there are metabolic differences in carbon assimilation between these fungi. The approach here was to measure the effects of glucose upon the assimilation by *S. cerevisiae* and *C. albicans* of radiolabelled lactic or oleic acid into large molecular weight compounds.

To achieve this, *S. cerevisiae* cells were grown to exponential phase on media containing lactate or oleic acid as sole carbon source. These cells were then harvested and resuspended in equivalent media containing radiolabelled lactic or oleic acid. Glucose (2%) was added to test samples, and no glucose was added to control samples. The assimilation of ^{14}C-lactic acid or ^{3}H-oleic acid by *S. cerevisiae* cells into large molecular weight TCA-precipitable material was

then measured. In *S. cerevisiae*, both lactate and oleic acid assimilation were rapidly repressed by glucose. Therefore the *S. cerevisiae* cells stopped the assimilation of these secondary carbon sources and apparently switched quickly to glucose assimilation. This confirmed the generally held view that *S. cerevisiae* does not assimilate both glucose and secondary carbon sources at the same time.

The next step is to investigate the effects of glucose on carbon assimilation in *C. albicans*. Once again, the approach was to test the assimilation of radiolabelled carbon sources by the cell. The same procedures were followed as for *S. cerevisiae*, with *C. albicans* cells being grown on lactate or oleic acid and then the assimilation of ^{14}C-lactic acid or ^{3}H-oleic acid into TCA-precipitable material was measured after glucose addition [63].

Unlike *S. cerevisiae*, *C. albicans* was still able to assimilate both lactate and oleic acid for some hours after addition of glucose. Lactate metabolism appeared to continue relatively normal because only minor effects were observed in the lactate uptake following glucose addition. Tests with the other secondary carbon source (oleic acid) showed similar results. Therefore, *C. albicans* is able to assimilate lactate and glucose at the same time. Similarly, both oleic acid and glucose can be assimilated by *C. albicans* at the same time. This suggests that glucose has a minimal immediate impact on the ability of *C. albicans* to assimilate secondary carbon sources at least over the timescales examined [63].

Therefore, there are significant differences between *C. albicans* and *S. cerevisiae* with respect to the regulation of their assimilation of alternative carbon sources. Apparently in *S. cerevisiae*, glucose and secondary carbon sources are not assimilated at the same time. In contrast, in *C. albicans*, the continued stability of gluconeogenic and glyoxylate cycle enzymes after glucose exposure correlates with the ability of *C. albicans* cells to continue to assimilate alternative carbon sources, even after glucose addition. Hence, the hypothesis that significant metabolic differences exist between *C. albicans* and *S. cerevisiae* was confirmed [63].

3. Glucose-accelerated protein degradation in *Candida albicans*

The previous section examined the impact of glucose on Pck1 and Icl1 expression and the assimilation of alternative carbon sources such as lactic and oleic acid in *C. albicans*. So far, the author's data confirmed that transcript profiling results were described by Yin *et al*. [54] and Rodaki *et al*. [55]. Numerous *S. cerevisiae* transcripts, including those encoding gluconeogenic enzymes (*FBP1* and *PCK1*), are repressed by glucose [54]. Similarly, 180 genes in *C. albicans*, including gluconeogenic and glyoxylate cycle enzymes were, down-regulated, even in response to very low concentrations of glucose (0.01%) [55]. The results presented in the previous section confirmed that the *ScICL1* and *ScPCK1* genes in *S. cerevisiae* and the *CaICL1* and *CaPCK1* genes in *C. albicans* are exquisitely sensitive to glucose.

While *S. cerevisiae* and *C. albicans* displayed similar responses at the transcriptional level, they diverged significantly at the post-transcriptional and metabolic levels. The *C. albicans* Icl1 and Pck1 enzymes were expressed during growth on lactate and oleic acid and were not destabi-

lised by the addition of 2% glucose. In contrast, their orthologues in *S. cerevisiae* were rapidly destabilised by glucose. Consequently, this appeared to affect carbon assimilation, allowing *C. albicans* to continue to assimilate alternative carbon sources even after exposure to glucose. In contrast, following glucose addition to *S. cerevisiae* cells, Icl1 and Pck1 were degraded and the cells stopped assimilating lactic acid or oleic acid. These new findings showed that there are fundamental differences in the regulation of carbon assimilation in *C. albicans* compared to *S. cerevisiae* [63]. The next step is to examine the basis for these differences and in particular, possible differences in glucose-accelerated protein degradation between *C. albicans* and *S. cerevisiae.* The focus of these studies was on Icl1.

Entian and Schüller [46] reported the genetic characterisation of *C. albicans* gluconeogenic and glyoxylate cycle genes. The *C. albicans FBP1, PCK1, MLS1* and *ICL1* genes were all isolated by functional complementation of the corresponding *S. cerevisiae* deletion mutants. Remarkably, the regulation of the heterologously expressed *C. albicans* gluconeogenic and glyoxylate cycle genes in *S. cerevisiae* was similar to that of their *S. cerevisiae* orthologues. Therefore, in this project we expressed *C. albicans ICL1* in *S. cerevisiae* and tested whether CaIcl1 is destabilised by glucose in *S. cerevisiae.* The other aims of this section are to test whether *C. albicans* has retained the ability to destabilise target proteins in response to glucose and to examine the signals and mechanisms that trigger glucose-mediated destabilisation of target proteins in *C. albicans.*

To test whether *C. albicans* is able to degrade proteins in response to glucose, the *S. cerevisiae ICL1* gene was expressed in *C. albicans.* To achieve this, one *C. albicans ICL1* allele was replaced with a tagged *S. cerevisiae ICL1* ORF. The *ScICL1* locus in *S. cerevisiae* was first tagged using primers with Myc_3-URA3, and the genomic DNA from this tagged was PCR amplified using primers to create the *CaICL1p-ScICL1-MYC$_3$-URA3* cassette [63]. This cassette was transformed into the *CaICL1* locus in *C. albicans ICL1/ICL1*. Before going further, it was necessary to test whether the *ScICL1-MYC$_3$-URA3* sequence was integrated accurately into the *CaICL1* genomic locus. Three primer pairs were designed to amplify overlapping fragments of the *CaICL1p-ScICL1-MYC$_3$-URA3* locus based on the *in silico* sequence [63]. PCR amplification using these primers yielded the desired bands, establishing that the newly created strain *ScICL1-MYC$_3$-URA3* sequence had integrated correctly into the *CaICL1* locus in the *C. albicans* genome [63].

Then Western blots were performed to test whether the ScIcl1-Myc_3 protein was detectable in these *C. albicans* transformants. The two positive clones were grown to stationary phase overnight on an alternative carbon source in the absence of glucose. Controls were included to confirm expression of the tagged ScIcl1 in *C. albicans.* In both new strains, an Icl1 band of the predicted size (62 kDa) was observed, which was the right size compared to the controls.

To further ensure the correct replacement of the *C. albicans ICL1* ORF with the tagged *S. cerevisiae ICL1* ORF, the functionality of this *ScICL1* ORF was tested in *C. albicans.* The *ScICL1-MYC$_3$-URA3* cassette was amplified from genomic DNA and transformed into *C. albicans ICL1/icl1* cells selecting heterozygote for uridine prototrophs. Once again, correct insertion of the *ScICL1-MYC$_3$-URA3* sequence was confirmed by diagnostic PCR using the three primer pairs as before and also tested by another three primer pairs to confirm the construction of the *C. albicans ScICL1-MYC$_3$-URA3/icl1* strain [63].

http://dx.doi.org/10.5772/62406

Western blots were performed to confirm the expression of the Myc-tagged *ScICL1* ORF *S. cerevisiae* in *C. albicans ScICL1-MYC$_3$-URA3/icl1* background. Two positive clones were grown to stationary phase on lactate-containing medium and protein subjected to Western blotting. This showed the expression of the tagged ScIcl1 of about 62 kDa in *C. albicans.* As expected the ScIcl1 protein was not expressed during growth on glucose because it was expressed from the endogenous *CaICL1* promoter that is glucose repressed [63].

The phenotype of this *C. albicans ScICL1-MYC$_3$-URA3/icl1* mutant was then tested by growing in different carbon sources: glucose, fructose, lactate, oleic acid, pyruvate and acetate. The mutants were compared with control *C. albicans ICL1/ICL1, ICl1/icl1* and *icl1/icl1* strains. The presence of the *ScICL1* gene in a *C. albicans icl1* background was sufficient to restore growth on lactate, oleic acid, pyruvate and acetate. This growth was comparable to the positive *ICL1/ICL1* control strain and contrasted with the negative *icl1/icl1* control, which was only able to grow on glucose and fructose. This indicated that the tagged *ScICL1* was functional in *C. albicans* [63].

Having confirmed the genotype, expression and functionality of the *ScICL1* ORF in *C. albicans*, the next step was to test the effects of glucose on the levels of the ScIcl1 protein when expressed in *C. albicans*. The *C. albicans ScICL1-MYC$_3$-URA3*//icl1 strain was first grown on alternative carbon sources (lactate or oleic acid), and then glucose was added to a final concentration of 2%. Samples were then taken at regular intervals, proteins extracted, and ScIcl1-Myc$_3$ levels examined by Western blotting. Control cultures to which no glucose was added, were also examined. This showed that ScIcl1 levels remained high in *C. albicans* cells grown on the alternative carbon sources. However, following the addition of 2% glucose to *C. albicans* cells, ScIcl1 was degraded. This indicates that *C. albicans* has retained the capacity to destabilise target proteins in response to glucose [63].

C. albicans is clearly capable of degrading target proteins following exposure to glucose. Therefore, why is CaIcl1 not degraded following glucose addition? Has CaIcl1 lost the specific signal that would target it for glucose-accelerated degradation? To test this, the CaIcl1 protein was expressed in *S. cerevisiae.*

To achieve this, the *S. cerevisiae ICL1* ORF was replaced with a Myc$_3$-tagged *C. albicans ICL1* ORF. The *C. albicans ICL1-MYC$_3$-URA3* was PCR amplified using the primers and transformed into *S. cerevisiae* strain (Ura$^-$, Leu$^-$) selecting for uracil prototrophs. Correct integration of the *CaICL1-MYC$_3$-URA3* cassette into the *ScICL1* locus was confirmed by diagnostic PCR using three primer pairs. In this way, a new *S. cerevisiae* strain was constructed containing a *ScICL1p-CaICL1-MYC$_3$-URA3* mutation [63].

Western blots were performed to test whether the CaIcl1-Myc$_3$ protein of the predicted 61 kDa size was expressed in *S. cerevisiae*. Cells were grown on alternative carbon sources in the absence of glucose and compared to positive and negative controls. These Western blots confirmed that CaIcl1 61 kDa was expressed in *S. cerevisiae* during growth on lactate or oleic acid and that CaIcl1-Myc$_3$ was in the correct size compared to the positive control in which CaIcl1-Myc$_3$ was expressed in *C. albicans.* Interestingly, CaIcl1-Myc$_3$ was expressed in *S.*

cerevisiae cells grown on lactate or oleic acid, but not in cells grown on glucose. This was to be expected when the *CaICL1-MYC*$_3$ ORF was expressed from the *ScICL1* promoter [63].

Having confirmed the genotype of the *S. cerevisiae* mutant expressing the *C. albicans ICL1* ORF, the next step was to test the effects of glucose on the CaIcl1-Myc$_3$ protein levels in *S. cerevisiae*. The *S. cerevisiae* strain was grown on lactate or oleic acid, and then glucose was added to a final concentration of 2%. Cells were harvested at various time periods thereafter, protein extracts prepared, and CaIcl1-Myc$_3$ protein levels measured by Western blotting. The results showed that CaIcl1-Myc$_3$ remained at high levels in *S. cerevisiae* during growth on the alternative carbon sources. Even upon the addition of 2% glucose, the levels of CaIcl1-Myc$_3$ remained high in *S. cerevisiae.* Minimal decay of the CaIcl1-Myc$_3$ protein was observed even 4 hours after glucose addition. These data suggest that the *C. albicans* Icl1 protein has lost the signals that trigger destabilisation in response to glucose [63].

The above work suggested that *C. albicans* has retained the ability to degrade target proteins in response to glucose, but that CaIcl1 has lost the specific signal(s) that trigger this glucose-accelerated protein degradation. What is the nature of this degradation signal that has been lost by CaIcl1?

Ubiquitination is known to play a role in the glucose-accelerated degradation of gluconeogenic enzymes in *S. cerevisiae* [57]. Previously, Entian and Barnett [45] demonstrated that Ubc8 functions in the catabolite degradation of fructose-1,6-bisphosphatase in *S. cerevisiae.* Earlier, Johnson *et al.* [69] showed that ubiquitin acts as a degradation signal in *S. cerevisiae.* Therefore, consensus ubiquitination target sites were examined in CaIcl1 and ScIcl1 using Ubpred (Predictor of protein ubiquitination site, from htt/www.ubpred.org/index.html) [70 - 73].

Based on this bioinformatic comparison, the ScIcl1 sequence contains strong consensus ubiquitination sites at amino acids 158 and 551, but there is a lack of high confidence ubiquitination targets in CaIcl1. This prediction was based on high level of confidence which is described in Ubpred system containing score range $0.84 \leq s \leq 1.00$, 0.197 for sensitivity and 0.989 for specificity. These included the hydrophobic nature of the ubiquitination target site for the high confidence prediction (TEDQFKENGVKK), which is contrast to the low- and medium- confidence sites that contain acidic and basic residues in the putative ubiquitination site (NGVKK; FNWPKAMSVD) [70 - 73]. Therefore, the presence of consensus ubiquitination sites in these proteins correlated with glucose-accelerated degradation.

Hence, the next step is to test whether ScIcl1 decay rates in *C. albicans* are affected by inactivation of polyubiquitin (*UBI4*).

To achieve this, the experimental goal was to introduce the Myc$_3$-tagged *S. cerevisiae ICL1* ORF into a *C. albicans ubi4*/*ubi4* mutant [74]. The *ScICL1-MYC*$_3$*-URA3* cassette was PCR amplified using primers and transformed into *C. albicans ubi4*/*ubi4* cells selecting for uridine prototrophs. The correct insertion of the *ScICL1-MYC*$_3$*-URA3* into the *CaICL1* locus was confirmed by diagnostic PCR with the same primer pairs as before. These amplified the conjoined sequence of the *CaICL1* promoter and the *ScICL1* ORF. The successful and accurate insertion of the *ScICL1-MYC*$_3$*-URA3* cassette into *C. albicans ubi4*/*ubi4* mutant was confirmed in this way.

Western blotting was then performed to test whether a ScIcl1-Myc_3 protein of the predicted size was expected in the *ubi4*/*ubi4* cells. Cells were grown overnight on the alternative carbon sources (lactate and oleic acid) in the absence of glucose. A new Myc_3-containing protein of 62 kD was observed indicating that the ScIcl1-Myc_3 protein was expressed on YPL and was the right size [63].

Having confirmed the genotype and the expression of the *ScICL1* ORF in *C. albicans ubi4*/*ubi4* cells, the next step was to test the effects of glucose on ScIcl1-Myc_3 protein levels. The *ubi4*/*ubi4* cells were grown on lactate or oleic acid and then glucose was added to a final concentration of 2%. Interestingly, ScIcl1-Myc_3 cells were more stable in *C. albicans ubi4*/*ubi4* cells than in wild type *C. albicans* cells after addition of 2% glucose. However, the inactivation of the *UBI4* (polyubiquitination) locus did not completely inhibit the degradation of the ScIcl1-Myc_3 protein such that it became as stable as ScIcl1-Myc_3 in wild type *C. albicans* cells in the absence of glucose. This might be because residual ubiquitination remains in *ubi4*/*ubi4* cells thanks to the presence of a second ubiquitin-encoding locus in *C. albicans* (*UBI3*) [74]. Nevertheless, it was concluded that the inactivation of *UBI4* (polyubiquitin) inhibits the glucose-accelerated degradation of ScIcl1 in *C. albicans.* Ubiquitination plays a role in glucose-accelerated protein decay in this fungus.

As stated above, ScIcl1 contains two high confidence putative ubiquitination sites located at residues 551 and 158, whereas CaIcl1 contains no such sites. Therefore, we reasoned that if ubiquitination plays a role in glucose-accelerated protein decay in *C. albicans*, then the addition of a ubiquitination site to CaIcl1 would confer glucose-accelerated degradation upon this protein. Therefore, the next experimental objective is to introduce the carboxyl-terminal ubiquitin site from ScIcl1 (TEDQFKENGVKK) into CaIcl1, together with the Myc_3 tag into wild type polyubiquitin containing *C. albicans* cells [63].

To achieve this, a *ScUBI*-site-*MYC_3-URA3* cassette was PCR amplified from *S. cerevisiae* genomic DNA and transformed into *C. albicans ICL1*/*ICL1* cells. To confirm the correct integration of this cassette at the 3′-end of the *CaICL1* ORF in these cells, uridine prototrophic transformants were subjected to diagnostic PCR using the same primer pairs as before and new primer pairs. This PCR amplification yielded the desired bands, establishing that the *ScUBI-MYC_3-URA3* was correctly integrated at the *C. albicans ICL1* locus [63].

Having established the genotype of the new strain (*C. albicans ICL1-ScUBI*-site-*MYC_3-URA3*), the next step is to confirm the expression of the CaIcl1 protein carrying the carboxyl-terminal ubiquitination site and the Myc_3 tag. Therefore, Western blots were performed to test the presence and size of the tagged protein. Five positive *C. albicans* clones were grown on an alternative carbon source (lactate) in the absence of glucose, protein extracts were made, and Western blots were performed, probing for the Myc epitope. A new Icl1-Myc_3 band of about 61 kD was observed, confirming that the Icl1 protein was expressed on YPL and that it had a similar size to the positive control ScIcl1-Myc_3 [63].

Having confirmed the genotype of the new strain and the expression of the CaIcl1 protein with the carboxyl-terminal ubiquitination site in *C. albicans* cells, the next step is to test the effects of glucose on the stability of this protein. The new strain was grown on lactic or oleic acid and glucose was added to a final concentration of 2%. Cells were harvested at various time periods thereafter; protein was extracted and these were subjected to Western blotting. Interestingly

the CaIcl1-Ubi-Myc_3 protein was rapidly degraded following glucose addition to cells grown on lactate or oleic acid. In conclusion, the addition of a ubiquitination site to CaIcl1 accelerates its degradation in response to glucose in *C. albicans* [63].

The above observations strongly suggest that specific proteins can be targeted for degradation in *C. albicans* following exposure to glucose, and these proteins are degraded via ubiquitination. If this is the case, it should be theoretically possible to detect ubiquitinated forms of these proteins. Hence immuno-precipitation experiments were then performed in an attempt to demonstrate ubiquitinated forms of the CaIcl1-Myc_3 protein in *C. albicans.* Proteins were extracted from *C. albicans ICL1-UBI*-site-*MYC_3-URA3* cells 20, 40 and 120 minutes after glucose addition. Analogous control extracts were also prepared from *S. cerevisiae* and *C. albicans* cells expressing ScIcl1+Myc_3, CaIcl1+Myc_3 and untagged parental strains grown on lactate plus glucose and lactate alone. These extracts were immunoprecipitated with an anti-Myc antibody that was predicted to precipitate Icl1 proteins having carboxyl-terminal Myc_3 tags. These immunoprecipitates were then subjected to Western blotting with an anti-ubiquitin antibody to test whether any of these Myc_3-tagged Icl1 proteins carry ubiquitin sequences. The Western blots were also probed with the anti-Myc antibody to confirm that Myc-tagged Icl1 proteins had been immunoprecipitated. This was the case. Interestingly, a weak ubiquitin-containing band of a length consistent with Icl1-Myc_3 proteins was detected. Such bands were observed in three replicate experiments. These weak bands were observed for the ScIcl1-Myc_3 CaIcl1-Ubi-Myc_3 proteins following glucose addition (both proteins carry ubiquitination sites). However, no ubiquitination of these proteins was observed in the absence of glucose, or for the CaIcl1-Myc_3 protein (which lacks a strong ubiquitination site) or for the untagged control cells [63].

These data suggest that when *S.cerevisiae* Icl1 or an artificial *C. albicans* Icl1 carrying a ubiquitination signal is expressed in *C. albicans*, it becomes ubiquitinated and destabilised in response to glucose. However, the native *C. albicans* Icl1 protein is not destabilised by glucose. Therefore, in response to glucose, target proteins became ubiquitinated and then degraded in *C. albicans* [63].

What natural *C. albicans* proteins might be subjected to ubiquitin-mediated, glucose-accelerated protein degradation? To address this, bioinformatic tools were used to predict possible ubiquitination sites in glycolytic, gluconeogenic and glyoxylate cycle enzymes in *S. cerevisiae* and *C. albicans.* Five enzymes were selected for analysis: Fbp1, Pck1, Mdh1, Eno1 and Mls1. Based on this analysis, *S. cerevisiae* Fbp1, Pck1 and Eno1 appear to carry strong ubiquitination sites, while only Eno1 *C. albicans* appears to have a high confidence ubiquitination site. In conclusion, while these central metabolic pathways are highly conserved between *S. cerevisiae* and *C. albicans,* these organisms appear to display significant differences in the presence of ubiquitination sites in the orthologous enzymes [63].

4. Overview of Metabolic Adaptation in *Candida albicans*

A previous study suggested that *C. albicans* might be capable of using more than one carbon source at the same time during growth in specific niches in the host [34]. This study was based

on the analysis of specific promoter-green fluorescent protein (GFP) fusions during systemic candidiasis. Almost all *C. albicans* cells infecting the kidney expressed GFP fusions with glycolytic promoters (*PYK1*, *PFK2*). Meanwhile one third to one-half of cells infecting the kidney also expressed *ICL1*- and *PCK1*-GFP fusions, suggesting that anabolic and catabolic pathways might be expressed at the same time. If this is the case, in principle, this would allow this pathogenic yeast to better utilise the complex mixture of available carbon sources in host niches. This working hypothesis would be consistent with earlier studies, suggesting that *C. albicans* is a glucose Crabtree-negative yeast. In other words, this pathogen retains respiratory activity even following exposure to glucose [44]. During growth on glucose, *ADH1* mRNA levels rise to maximum levels during late exponential growth phase and then decline to low levels in stationary phase [75]. The *ADH1* mRNA is relatively abundant during growth on galactose, glycerol, pyruvate, lactate or succinate, and less abundant during growth on glucose or ethanol. However, alcohol dehydrogenase levels do not correlate closely with *ADH1* mRNA levels. This locus may be controlled at both transcriptional and post-transcriptional levels, or other differentially regulated *ADH* loci may exist in *C. albicans* [75].

Interestingly, a significantly smaller proportion of glucose is fermented to ethanol by *C. albicans* than by *S. cerevisiae* [75]. This is consistent with the low amounts of ethanol produced by *C. albicans* observed in this study.

S. cerevisiae is not able to assimilate both non-fermentable carbon sources and glucose at the same time because of glucose repression. Hence, we predicted that these yeasts have evolved different responses to glucose. Therefore, in this study, I analysed the regulation of carbon assimilation in *C. albicans* focussing on genes/enzymes involved in gluconeogenesis and the glyoxylate cycle. I tested *ICL1* and *PCK1* gene expression, Icl1 and Pck1 protein stability and the impact of glucose on the assimilation of non-fermentable carbon sources. The author compared their *C. albicans* responses to those of *S. cerevisiae* under equivalent conditions. The following conclusions can be drawn from these findings.

First, gluconeogenic and glyoxylate cycle mRNAs are sensitive to glucose in both *C. albicans* and *S. cerevisiae*. This reconfirmed previous findings from Aberdeen Fungal Group Laboratory [54, 55] and other laboratories [76]. Dramatic decreases in *ICL1* and *PCK1* mRNA levels were observed in *C. albicans* cells after exposure to 2% glucose. This glucose concentration is higher than the levels of glucose homeostatically maintained in human blood (about 0.1%). However, it is already known that *C. albicans* responds to lower glucose concentrations within the physiological range of blood glucose [54, 55]. Therefore, *C. albicans* is able to respond to blood glucose levels during disseminated haematological infections. Interestingly, patients with diabetes who often have elevated blood glucose levels, have a higher risk of systemic *Candida* infections [14], and dietary glucose enhances *C. albicans* colonisation and invasion [77].

The second main observation was that the Icl1 and Pck1 proteins are stable in *C. albicans* following glucose exposure. The addition of 2% glucose to *C. albicans* cells growing on lactate or oleic acid did not trigger the degradation of the Icl1 and Pck1 proteins, at least within the 4 hours examined. This is in contrast to the situation in *S. cerevisiae*, where the addition of 2% glucose triggered the rapid degradation of the Icl1 and Pck1 proteins. The estimated half-lives for these proteins in *S. cerevisiae* are more than 20 hours [78] indicating that these proteins are

very stable. This probably represents a significant difference in the physiological responses of these pathogenic and benign yeasts to glucose. *C. albicans* is able to establish infections in complex niches, many of which contain a rich mixture of alternative carbon sources [34]. The stability of the Icl1 and Pck1 proteins in *C. albicans*, even in the presence of glucose, provided the first clue that this pathogen might be able to assimilate alternative carbon sources at the same time as glucose in these carbon-rich niches.

The third conclusion was that *C. albicans* is able to assimilate both glucose and alternative carbon sources at the same time. It was shown that glucose addition has no major impact on the assimilation of the alternative carbon sources (lactate and oleic acid). The maintenance of gluconeogenic and glyoxylate cycle enzymes, therefore, appears to allow *C. albicans* to continue to assimilate alternative carbon sources, even following glucose addition. Therefore, during a transient exposure to glucose in the bloodstream, for example, *C. albicans* would be able to maintain anabolic metabolism. Also, after phagocytosis, when the genes of glyoxylate cycle and gluconeogenesis have been induced [34], these pathways probably remain active some time afterwards because of the stability of their enzymes. It was reported that the glyoxylate cycle helps to protect *C. albicans* against host anti-microbial defences by facilitating anabolic metabolism in the absence of fermentable carbon sources [79]. Barelle *et al*. [34] indicated that the pathogen *C. albicans* regulates central carbon metabolism in a niche-specific manner during disease establishment and progression. These authors reported two stages *C. albicans* activate the glyoxylate cycle and gluconeogenesis in response to phagocytosis during the early stage of infection and this is followed by glycolytic metabolism when the fungus colonises tissue. This metabolic flexibility is thought to increase the biological fitness of this pathogen within its host. It is conceivable that the prolonged activity of the anabolic pathways might further increase the fitness of this pathogen.

In conclusion, the regulation of central carbon metabolism in *S. cerevisiae* and *C. albicans* seems to have evolved in ways that reflect their different biological niches. *S. cerevisiae* has adapted to grow rapidly when high concentrations of sugars become available from fruit (*from feasts to famine*) [10], whereas *C. albicans* appears to have adapted to utilise the complex mixtures of carbon sources that are available in the GI tract or the bloodstream for example. The next step in this study is to test whether *C. albicans* has retained the ability to target accelerated protein degradation in response to glucose.

Next, the reverse cloning was done by replacing the *S. cerevisiae ICL1* ORF in *S. cerevisiae* with a Myc_3-tagged *C. albicans ICL1* ORF. The aim was to test whether the CaIcl1 protein was destabilised by glucose when expressed in *S. cerevisiae.* This revealed that the CaIcl1 protein was not destabilised in response to glucose when expressed in *S. cerevisiae*. This indicated that the *C. albicans* Icl1 protein has lost the signal that triggers destabilisation in response to glucose.

The *S. cerevisiae* Fbp1 protein is destabilised by glucose via ubiquitination [64]. Therefore, we reasoned that the CaIcl1 protein might have lost ubiquitination signals, which could account for the stability of the CaIcl1 protein in *S. cerevisiae* following glucose addition. Indeed, a bioinformatic analysis revealed that while ScIcl1 carries ubiquitination sites, CaIcl1 does not.

To test whether ubiquitination might play a role in glucose-accelerated protein degradation in *C. albicans*, the impact of *UBI4* inactivation upon ScIcl1 degradation was tested. Interestingly,

inactivation of the polyubiquitin gene slowed ScIcl1 degradation in *C. albicans* in response to glucose. This was consistent with the idea that ubiquitination contributes to glucose-accelerated protein degradation in *C. albicans.*

If this was the case, the addition of a ubiquitination signal should confer glucose-accelerated degradation upon the stable CaIcl1 protein. This was tested, showing that a carboxyl-terminal ScIcl1 ubiquitination signal was sufficient to trigger the rapid degradation of CaIcl1 following glucose addition to *C. albicans* cells [63].

Finally, direct biochemical evidence for the involvement of ubiquitination in glucose-accelerated protein degradation in *C. albicans* was obtained by showing that ubiquitin co-immunoprecipitated with the ScIcl1 and CaIcl1-Ubi proteins in *C. albicans*, but only when cells were exposed to glucose [63].

In conclusion, *C. albicans* is capable of destabilising target proteins in response to glucose, and this destabilisation is mediated by ubiquitination. The lack of ubiquitination sites on the *C. albicans* Icl1 and Pck1 proteins probably accounts for the observation that these glyoxylate cycle and gluconeogenic enzymes are not destabilised by glucose in *C. albicans.*

5. Conclusion and Future Perspectives

Overall, the topic was to explore the impact of glucose on the assimilation of alternative carbon sources and catabolite inactivation in *C. albicans.* To achieve this, the effects of glucose on the transcriptional and post-transcriptional regulation of key genes and enzymes were studied, and the molecular mechanisms that trigger protein destabilisation in response to glucose were examined. These effects were then compared with glucose responses in *S. cerevisiae.*

Gene expression in both *C. albicans* and *S. cerevisiae* is sensitive to glucose. In both yeasts, *ICL1* and *PCK1* mRNA expression levels were down-regulated in response to glucose. This confirmed the previous microarray studies in *S. cerevisiae* [54] and in *C. albicans* [55].

ICL1 and *PCK1* are involved in the utilisation of alternative carbon sources such as organic and fatty acids, and these genes are repressed by glucose in *S. cerevisiae*. Indeed gluconeogenic and glyoxylate cycle genes have been shown to be repressed by glucose concentrations as low as 0.01% in *S. cerevisiae* [54]. Similarly, *C. albicans* genes involved in central carbon metabolism respond rapidly to the addition of glucose. Indeed the *PCK1* and *ICL1* genes have been shown to be repressed by glucose at levels as low as 0.1% [55]. These observations imply that the utilisation of alternative carbon sources could be repressed by glucose in *C. albicans*. Given that if *C. albicans* genes involved in the utilisation of alternative carbon sources are glucose- regulated in a similar fashion to those in *S. cerevisiae*, then it might be expected that these genes would be repressed during systemic infections. However, during phagocytosis by macrophages, *C. albicans* cells appear to up-regulate the glyoxylate cycle, as the expression of the isocitrate lyase (*ICL1*) and malate synthase (*MLS1*) genes is induced [80]. The same applies to the gluconeogenic gene, *PCK1* [34]. Furthermore both *ICL1* and *PCK1* appear to be expressed in some *C. albicans*

cells during systemic kidney infections [34]. Various studies have shown that genes involved in gluconeogenic and glyoxylate cycle contribute to fungal virulence [34, 62, 79].

C. albicans often occupies niches that contain complex mixtures of carbon sources. Our initial working hypothesis, therefore, was that *C. albicans* might exploit many of these diverse carbon sources rather than focussing on glucose alone. Therefore, the tight glucose repression of the *PCK1* and *ICL1* genes seemed somewhat surprising [54, 55, 76] because this was inconsistent with our working hypothesis. Our subsequent discovery that the Pck1 and Icl1 enzymes were not destabilised by glucose was more consistent with this working hypothesis. Catabolite inactivation – the rapid degradation of specific enzymes following glucose exposure – is a well-defined phenomenon in *S. cerevisiae.* In the yeast, the gluconeogenic enzyme Fbp1 is rapidly degraded when cells are shifted from media with poor carbon sources to rich media containing glucose [59]. Consistent with this, in this project it was found that both the Icl1 and Pck1 proteins were rapidly degraded in *S. cerevisiae* in response to glucose. This was the case when cells were pre-grown on lactic acid or oleic acid. In contrast, the *C. albicans* Icl1 and Pck1 proteins were not destabilised in response to glucose when cells were exposed to glucose. The stability of the CaIcl1 and CaPck1 enzymes in the presence of glucose suggested that *C. albicans* might be capable of continuing to utilise alternative carbon sources even when glucose became available. This suggestion was more consistent with our working hypothesis that *C. albicans* has evolved to utilise carbon sources simultaneously in complex niches.

To examine this, the impact of glucose on the ability of *C. albicans* to assimilate radiolabelled lactic acid or oleic acid was tested and compared with that of *S. cerevisiae.* These incorporation studies confirmed the divergent behaviour of these yeasts with respect to their patterns of carbon assimilation. In *S. cerevisiae*, the assimilation of lactic and oleic acid was repressed by glucose and subsequent growth was reliant on glucose. In contrast, in *C. albicans*, the utilisation of lactic and oleic acid was not repressed by glucose. Hence, the utilisation of alternative carbon sources by *C. albicans* was not repressed by glucose, and thus the subsequent growth of this pathogen was supported by assimilating both glucose and alternative carbon sources [63].

In summary, both *C. albicans* and *S. cerevisiae* displayed similar responses to glucose at the transcriptional level, but their responses at post-transcriptional and metabolic levels differed significantly. Therefore, *C. albicans* can assimilate both glucose and alternative carbon sources at the same time, whereas *S. cerevisiae* is not able to do so (Fig. 1). This is predicted to play a significant role in the growth of this fungus during infection in the human host [41]. This could be tested by reprogramming the Icl1 and Pck1 enzymes to be glucose- sensitive in *C. albicans.* The prediction is that this engineered *C. albicans* strain would grow less well on mixed carbon sources containing glucose, and hence would display attenuated virulence. Additional experiments that could be carried should test the impact of carbon sources other than glucose, (such as lactose, galactose or fructose) [Ting and Sandai, unpublished] on the stability of gluconeogenic and glyoxylate cycle enzymes in *C. albicans.* Fructose, lactose and galactose are commonly found in the diet, and significant differences are thought to exist between *C. albicans* and *S. cerevisiae* with regard to the regulation of galactose utilisation [81, 82].

Another additional experiment could involve the broad screening of central metabolic enzymes in *C. albicans* for consensus ubiquitination target sites, beyond the preliminary screen

http://dx.doi.org/10.5772/62406

performed here. This would help to extend the predicted impact of glucose on central metabolic pathways. This preliminary bioinformatic screen of gluconeogenic and glyoxylate cycle enzymes has indicated that the *S. cerevisiae* Icl1, Fbp1, Eno1 and Pck1 proteins carry high confidence ubiquitination sites (Fig. 2). Previous studies have shown that ScFbp1 is catabolite inactivated in a glucose-dependent manner via ubiqutination and proteasomal degradation [59]. In contrast, in *C. albicans*, only Eno1 carries a high confidence ubiquitination site. This bioinformatic analysis is consistent with the suggestion that gluconeogenesis and the glyoxylate cycle are insensitive to glucose or at least glucose stimulated, ubiquitin-mediated protein degradation in *C. albicans* [63].

The next main step in this project involved testing whether *C. albicans* has retained the molecular capability of destabilising target proteins in response to glucose. Examinations of Icl1 protein levels in both *S. cerevisiae* and *C. albicans* revealed that while Icl1 is rapidly degraded in response to glucose in *S. cerevisiae*, Icl1 remains stable in *C. albicans*. To test whether the *C. albicans* Icl1 protein has lost the signal that triggers destabilisation in response to glucose, the *C. albicans ICL1* ORF was expressed in *S. cerevisiae.* Interestingly, the *C. albicans* Icl1 protein was not destabilised in response to glucose when it was expressed in *S. cerevisiae*. This was consistent with the idea that the *C. albicans* Icl1 protein has lost the signal required to trigger glucose-accelerated degradation [66].

The next step is to test whether *C. albicans* has retained the ability to degrade target proteins in response to glucose. To investigate this, the tagged *S. cerevisiae* Icl1 protein was expressed in *C. albicans.* This tagged *S. cerevisiae* Icl1 protein was rapidly degraded when the *C. albicans* cells were exposed to glucose. This indicated that *C. albicans* has retained the molecular apparatus that mediates glucose-accelerated protein decay (or "catabolite inactivation").

What apparatus mediates this glucose-accelerated degradation? In *S. cerevisiae,* ubiquitination plays an important role in the rapid proteasome-mediated degradation of Fbp1 in response to glucose [57]. Therefore, the amino acid sequences of ScIcl1 and CaIcl1 were screened for consensus ubiquitination sites. This revealed that while the ScIcl1 sequence has strong ubiquitination sites, the CaIcl1 sequence does not (Fig. 3). Therefore, to examine whether ubiquitination plays a role in glucose-accelerated protein degradation in *C. albicans*, the impact of a polyubiquitin (*ubi4*/*ubi4*) null mutation [74] on the degradation of ScIcl1 in *C. albicans* was tested. The tagged *ScICL1* ORF was expressed in this *C. albicans ubi4*/*ubi4* mutant and its decay rate was measured in the presence and absence of glucose. Compared to the controls, the degradation of ScIcl1 was relatively slow in the *C. albicans ubi4*/*ubi4* mutant. This suggested that ubiquitination plays a role in glucose-accelerated protein degradation in *C. albicans.*

To test this further, we investigated the effects of introducing a *S. cerevisiae* ubiquitination site on to the carboxyl-terminus of the stable CaIcl1 protein. This CaIcl1-Ubi protein was destabilised in response to glucose in *C. albicans* (Fig. 3). This confirmed that ubiquitination plays a key role in glucose-accelerated protein degradation in *C. albicans.* It is also confirmed that *C. albicans* has retained the molecular apparatus that destabilises target proteins in response to glucose. In summary, *C. albicans* cells have retained the molecular apparatus that degrades target proteins in response to glucose, but CaIcl1 has lost the signal that triggers this destabilisation. As a result, the stability of CaIcl1 enzyme in the presence of glucose allows *C.*

albicans cells to continue to use alternative carbon sources such as lactic and oleic acid rather than switching to glycolysis.

What is the apparatus required to trigger glucose accelerated decay? Ubiquitin- mediated protein degradation occurs via the proteasome [83]. This is a conserved molecular machine comprised of numerous protein subunits. Proteins are targeted to the proteasome via ubiquitination, which involves several steps (Fig. 4). Ubiquitin is activated in a two-step process involving the E1 and E2 enzymes and the hydrolysis of ATP. The primed ubiquitin molecule, once attached to an E2 enzyme via a thioester linkage, is then ligated to the target protein via an E3 ligase which provides the substrate specificity. A previous PhD student in the Aberdeen Fungal Group Laboratory [84] has already shown that proteasomal subunits are highly conserved across the fungal kingdom and are conserved in *C. albicans* (Fig. 4). Furthermore, E1, E2 and E3 enzymes are conserved in *C. albicans* (Fig 4) [85], which are consistent with the ubiquitination apparatus being retained in this pathogen. Presumably, the inactivation of components of this system could block glucose-accelerated protein degradation in *C. albicans.*

In *S. cerevisiae*, Ubc8 appears to be the ubiquitin-conjugating enzyme involved in glucose-accelerated protein degradation. Ubc8 has been described as a ubiquitin-conjugating enzyme that negatively regulates gluconeogenesis by mediating the glucose-induced ubiquitination of Fbp1 [57, 64, 85, 86]. Interestingly the Ubc8 protein appears to be conserved in *C. albicans* [87]. Additional ubiquitin-conjugating enzymes exist in *C. albicans,* such as Ubc4 and Ubc6, and these might also be involved. However, all these *UBC* genes remain uncharacterised in *C. albicans*, and therefore it is not yet known whether these genes play a role in glucose-accelerated protein degradation. Interestingly however, *UBC8* is the only ubiquitin-related gene that is up-regulated at the transcriptional level following glucose exposure in *C. albicans* (Table 1). Therefore, Ubc8 is an excellent candidate for the ubiquitin-conjugating enzyme that mediates glucose-accelerated protein degradation in *C. albicans*. Clearly, a future experiment that could be carried out would be to create a *C. albicans ubc8*/*ubc8* mutant and to test whether this mutation blocks glucose-accelerated protein degradation in *C. albicans*. Other *UBC* genes might also be inactivated to test the possibility that they are not involved in glucose-accelerated protein degradation [63].

These findings are relevant to our understanding of *C. albicans* growth and survival in the host, and hence to *C. albicans* pathogenicity. *C. albicans* persists as commensal during colonisation in the healthy human GI tract where monosaccharides (such as glucose, fructose and galactose) and disaccharides (such as sucrose and lactose) can be abundant [88]. These sugars probably help to sustain the fungus, for example, during the first 6 months of a host's life when new born infants consume milk and hence lactose [89]. Both disaccharides and monosaccharides may supply a carbon-rich environment for microbes such as *C. albicans*, and they are also absorbed into the bloodstream [82]. However, during systemic infections *C. albicans* cells invade the bloodstream and often internal organs such as kidney. Glucose is present in the bloodstream but appears to become limited in systemic microenvironments that are colonised during infection of organs. At this point, the fungal cells probably switch to gluconeogenesis and glyoxylate cycle metabolism to utilise the available alternative carbon sources [34]. Also, during phagocytosis, by macrophages and neutrophils, *C. albicans* cells switch to the assimilation of alternative carbon sources, activating genes such as *ICL1* [34, 62, 79, 89] that are required for full pathogenicity [34, 79]. It would appear from the findings in this chapter, that

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C. albicans has evolved in such a manner that this fungus can continue to assimilate these alternative carbon sources, even after exposure to glucose. The presumption is that this ability to use several of carbon sources in these complex and carbon-rich microenvironments contributes to the growth and pathogenicity of *C. albicans* in these microenvironments. Possibly the greatest challenge for the future is to elucidate exactly what carbon sources individual *C. albicans* cells assimilate *in vivo* during commensalism, mucosal infection and systemic candidiasis and to elucidate the contribution of transcriptional and post-transcriptional control mechanisms to this regulation.

This carbon metabolism of *C. albicans* might be investigated further by examining the ability of mutant *C. albicans* cells that carry the *ICL1-ubi* allele to grow and assimilate carbon *in vivo*. These *C. albicans* cells express Icl1 that is destabilised by glucose because of the addition of the carboxyl-terminal ubiquitination site. In principle, Icl1 would be degraded and cells no longer able to metabolise via the glyoxylate cycle following glucose addition. As a result, those cells would presumably be less able to course infections and would be less able to compete for available nutrients against other microorganisms such as endogenous bacteria in the GI tract compared to their wild type. It is likely to show less successful colonisation, virulence and fitness due to this defect in its ability to assimilate both glucose and alternative carbon sources at the same time [63].

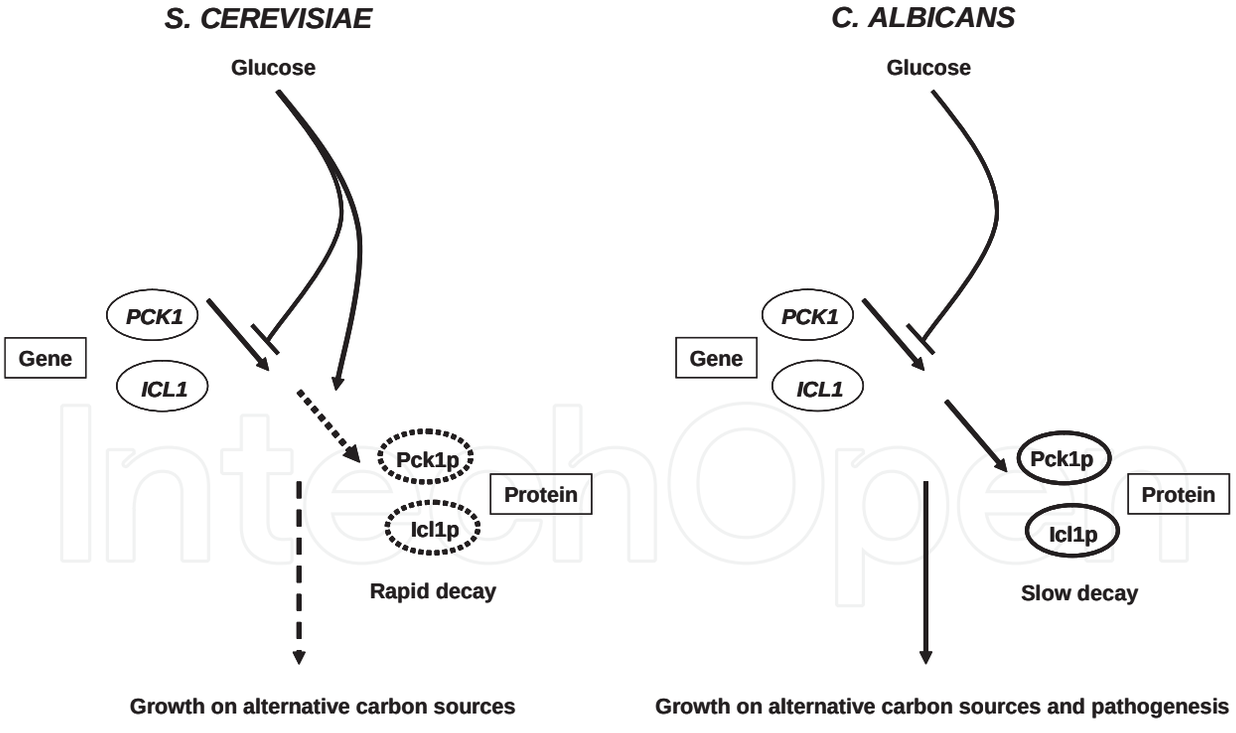

Figure 1. Proposed model of the impact of glucose on the assimilation of alternative carbon sources by *S. cerevisiae* and *C. albicans.* In *C. albicans* and *S. cerevisiae* transcription of both *ICL1* and *PCK1* are repressed by glucose. However, their regulation at post-transcriptional levels and metabolism diverge significantly. The *S. cerevisiae* Icl1 and Pck1 proteins are rapidly destabilised in response to glucose and the assimilation of alternative carbon sources is repressed by glucose. In contrast, in *C. albicans* the Icl1 and Pck1 proteins decay slowly in response to glucose and continue to assimilate both glucose and alternative carbon sources at the same time.

In addition, it would be interesting to conduct further investigations of the impact of other carbon sources such as galactose, fructose, sucrose, fatty acid and amino acid on carbon assimilation in *C. albicans*. For example galactose might also trigger glucose-like repression in *C. albicans* [82]. The behaviour of *C. albicans* towards other carbon sources differs from *S. cerevisiae*. It would be interesting to define the effects of these carbon sources on the levels and activities of glycolytic, gluconeogenic, glyoxylate cycle and fatty acid β-oxidation enzymes. Which other carbon sources trigger the ubiquitination-mediated degradation of these proteins in *C. albicans*? How do these mechanisms affect the ability of *C. albicans* to assimilate lipid, carbohydrate, protein and other organic acids in their human host during commensalism and infection? Such studies will provide a more complete physiological understanding of *C. albicans* for the future research.

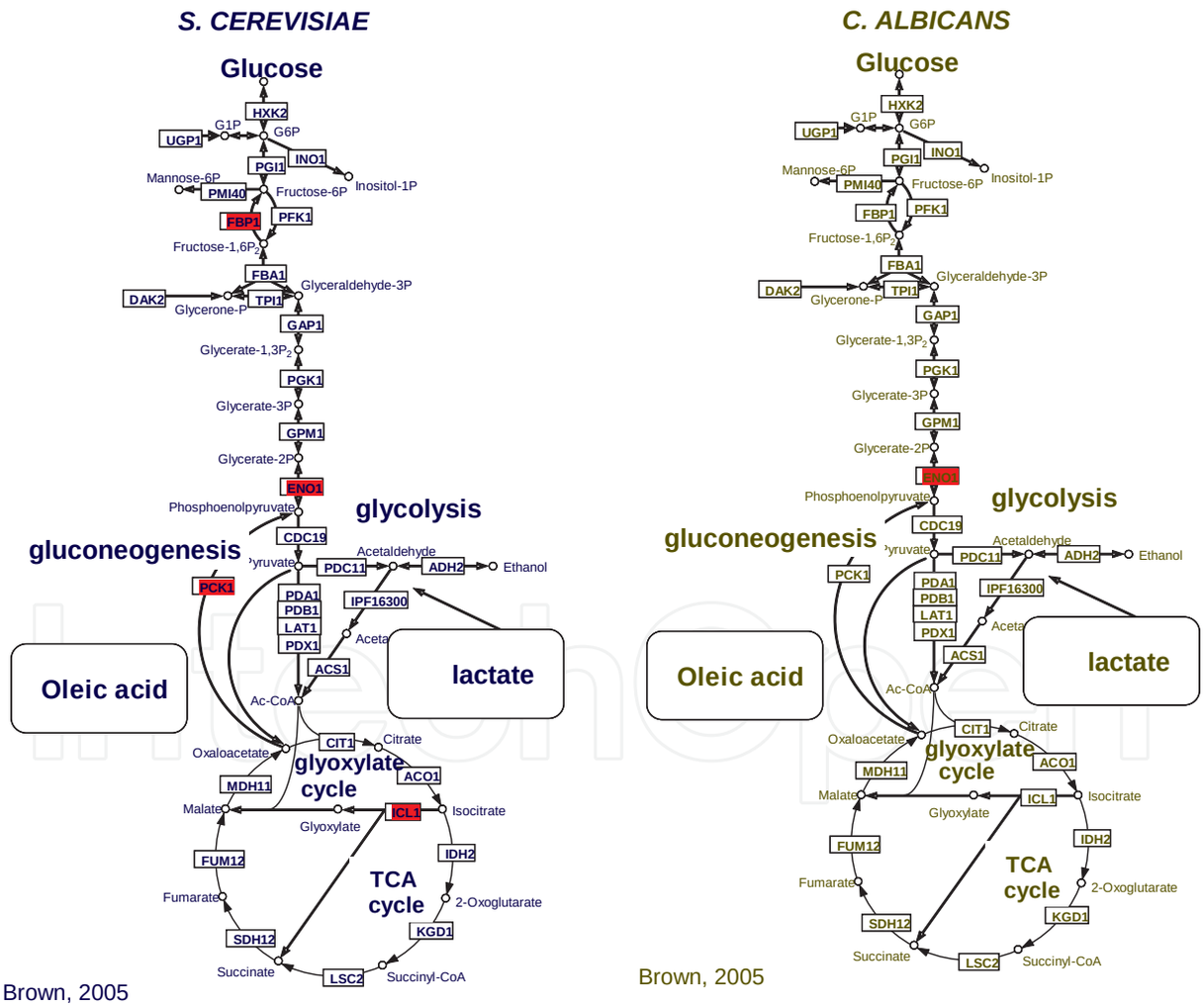

Figure 2. Bioinformatic prediction of ubiquitination sites in glycolytic, gluconeogenic and glyoxylate cycle enzymes from *S. cerevisiae* and *C. albicans*. Icl1, Pck1, Eno1 and Fbp1 were examined. Those carrying high confidence ubiquitination sites are highlighted in red.

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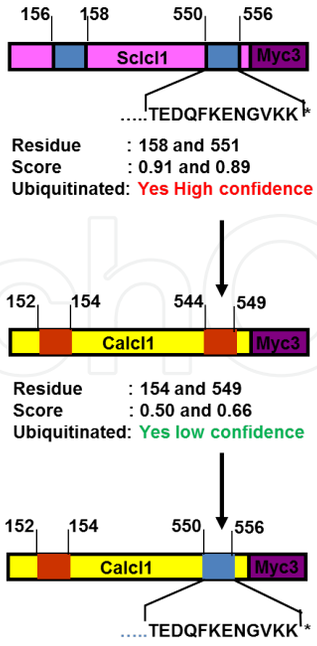

Figure 3. Role of ubiquitin-mediated protein degradation in enzyme destabilisation in C. albicans in response to glucose. Bioinformatics comparison of CaIcl1 and ScIcl1 reveals a lack of putative ubiquitination target in CaIcl1. Introduction of carboxyl-terminal ScUbi-site in CaIcl1 plus Myc-tagging protein and testing the impact of glucose on the turnover of CaIcl1 with C-terminal ScUbi-site. Adding back a Ubi-site accelerates degradation in response to glucose.

Candida dataset			Expression	*Saccharomyces dataset*		Expression	Description
Candida gene			Ratio glucose	S. cerevisiae		Ratio glucose	
Systematic	orf19Number	common	Concentration (%) 0.0 0.01 0.1 1.0	gene homology Systematic	common	Concentration (%) 0.0 0.01 0.1 1.0	
CA5729	orf19.7438	*UBA1*	-1.2 1.0 -1.2 -1.1	YKL210W	*UBA1*	1.0 -1.4 -1.1 -1.2	Ubiquitin-activating enzyme (by homology)
CA3898	orf19.5074	*UBA2*	-1.3 -1.0 -1.2 -1.2	YDR390C	*UBA2*	1.0 -1.2 1.1 1.0	Ubiquitin-activating -like enzyme (by homology)
CA2926	orf19.4209	*UBA3*	1.1 -1.0 1.0 -1.1	YPR066W	*UBA3*	1.0 -1.1 1.3 1.4	Ubiquitin-like protein activating enzyme
CA0531	orf19.8686	*UBC1*	1.2 1.0 1.4 1.2	YDR177W	*UBC1*	1.0 -1.2 1.1 1.1	Ubiquitin-conjugating enzyme (by homology)
CA5977	orf19.7571	*UBC4.3*	1.3 1.1 1.1 1.1	YBR082C	*UBC4*	1.0 1.1 1.7 1.7	E2 ubiquitin-conjugating

Candida dataset *Candida* gene Systematic orf19Number		common	Expression Ratio glucose Concentration (%) 0.0 0.01 0.1 1.0	*Saccharomyces dataset* S. cerevisiae gene homology Systematic	common	Expression Ratio glucose Concentration (%) 0.0 0.01 0.1 1.0	Description
							enzyme, 3-prime end
CA5648	orf19.7347	*UBC6*	-1.0 1.0 1.1 -1.0	YER100W	*UBC6*	1.0 1.0 1.2 1.6	E2 ubiquitin-conjugating enzyme (by homology)
CA4199	orf19.4540	***UBC8***	**1.3 2.1 3.6 2.5**	YEL012W	*UBC8*	1.0 1.1 -1.0 -1.1	Ubiquitin-conjugating enzyme (by homology)
CA5109	orf19.6424	*UBC9*	1.0 1.2 1.1 1.0	YDL064W	*UBC9*	1.1 1.3 1.2 -1.1	E2 ubiquitin-conjugating enzyme (by homology)
CA5769	orf19.5411	*UBC12*	-1.1 1.0 -1.1 1.1	YLR306W	*UBC12*	1.0 -1.6 -1.3 -1.5	E2 ubiquitin-conjugating enzyme (by homology)
CA0417	orf19.2225	*UBC13*	1.2 1.1 1.1 1.1	YDR092W	*UBC13*	1.1 -1.4 -1.3 -1.2	E2 ubiquitin-conjugating enzyme (by homology)
CA3263	orf19.2697	*UBR12*	-1.2 1.0 -1.1 -1.1	YLR024C	*UBR2*	1.0 -1.6 -1.7 -1.6	Ubiquitin-protein ligase (by homology)
CA3262	orf19.2695	*UBR11.3*	-1.1 1.0 -1.1 -1.1	YGR184C	*UBR1*	1.0 -1.4 -1.4 -1.3	Ubiquitin-protein ligase, 3′ end (by homology)
CA1279	orf19.3628	*RSP5*	-1.0 1.1 1.2 1.2	YER125W	*RSP5*	1.0 1.1 1.0 -1.1	Ubiquitin-protein ligase (by homology)
CA5435	orf19.3237	*UFD4*	-1.1 1.3 1.3 1.4	YKL010C	*UFD4*	1.0 -1.4 -1.4 -1.3	Ubiquitin fusion degradation protein (by homology)

Candida dataset			Expression	*Saccharomyces dataset*		Expression	Description
Candida gene			Ratio glucose	S. cerevisiae		Ratio glucose	
Systematic orf19Number			Concentration (%)	gene homology		Concentration (%)	
common			0.0 0.01 0.1 1.0	Systematic common		0.0 0.01 0.1 1.0	
CA2803	orf19.5776	*IPF11711*	-1.1 1.3 1.3 1.3	YDR457W	*TOM1*	1.0 1.2 1.1 1.1	Ubiquitin-protein ligase (by homology)
CA6150	orf19.5892	*IPF1857*	-1.3 1.4 -1.1 1.1	YJR036C	*HUL4*	1.0 -1.8 -1.9 -2.9	similar to Saccharomyces cerevisiae Hul4p hect domain E3 ubiquitin-protein ligase (by homology)

Table 1. Expression of *C. albicans* and *S. cerevisiae* homologues involved in protein ubiquitination [55].

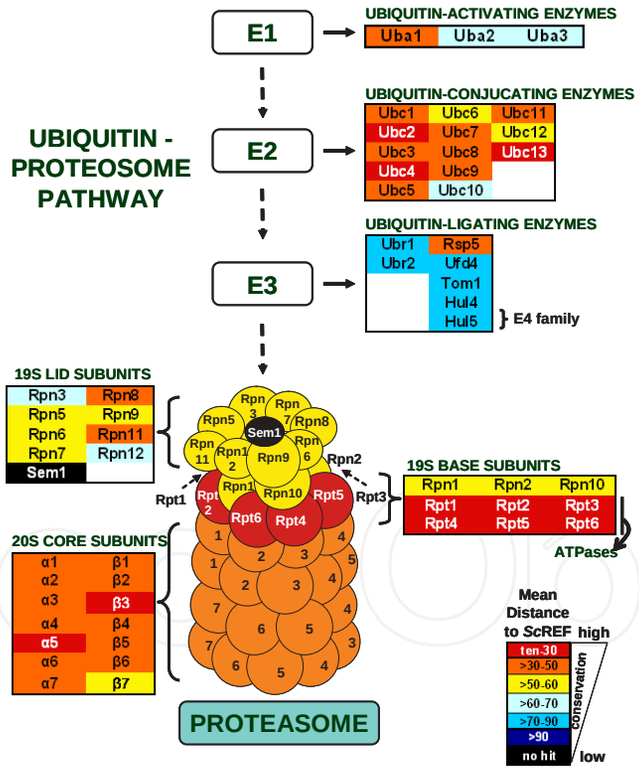

Figure 4. Protein conservation in the proteasome. Each protein is colour-coded according to its mean distance to *Sc*REF scores (scale bottom right: high *Sc*REF scores represent low sequence conservation). Each subunit of the actual machine (20S core particle and 19S cap) has been colour -coded based on the mean of the mean distance to *Sc*REF values for the proteins that form it in all species. Rpn13 (*Sc*REF distance = 82) is not included in the picture. Ubiquitin-related enzymes have been reviewed by Hochstrasser (1996), and protein component of the machine was identified based on the review by Sharon and co-workers (2006). In the ubiquitin-ligating enzymes, except for the Ubr1 and Ubr2 enzymes, all the others are hect-domain proteins [84].

Acknowledgements

I would like to thank Professor Alistair JP Brown for his advice and support throughout this project. I would also like to thank the Malaysian Ministry of Higher Education and Universiti Sains Malaysia for providing financial support. I am grateful to the members of Aberdeen Fungal Group, Institute of Medical Sciences, University of Aberdeen, UK and the staff of Advanced Medical and Dental Institute, Universiti Sains Malaysia for all their support and friendship. Thanks to my family and friends for their love and support.

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