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Transcriptional regulation of Iron and Copper

homeostasis in *Candida albicans*

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Abstract

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The ability of the human pathogen, *Candida albicans*, to acquire copper and iron from the uncongenial host environment is essential for virulence. Studies in our laboratory have led to identification of the copper-sensing transactivator CaMac1p that regulates genes involved in both copper and iron homeostasis. Described here are our findings of the molecular mechanism involved in the action of Mac1p. Experiments using yeast two-hybrid assays, suggests that the C-terminus domain is involved in protein - protein interactions and that CaMac1p can form homo-dimers in the presence of copper, however it does not undergo intramolecular interactions. The results also indicated that it is likely that the N-terminus domain increases the protein binding activity of the C-terminus domain. For the first time CaMac1 protein was successfully purified and using gel-shift assays CaMac1p was also demonstrated to bind to its own promoter which confirmed the hypothesis that *CaMAC1* is self-regulated. This copper-responsive regulation is a key difference when compared to the *ScMAC1* (*Saccharomyces cerevisiae MAC1*) gene, which is constitutively transcribed. The presence of one CuRE (copper response element) site allowed for optimal activation demonstrating the sites are not synergistically associated unlike in *S. cerevisiae*.

Although *CaMAC1* also plays a role in iron homeostasis, the exact mechanism of iron responsive gene regulation in C. albicans was as yet unknown; therefore we also studied the role of the putative transcription factor Sef1p as a potential regulator of iron acquisition genes. A novel role of *SEF1* in copper homeostasis was detected where Sef1p was found to be involved directly or indirectly in regulation of cupric (as well as ferric) reductase Ca*FRE7* and copper uptake gene Ca*CTR1*. Hence RT-PCR was also used to analyse the interactions of Sef1p with the other copper and iron responsive regulators - Mac1p and Sfu1p. The transcriptional activator Mac1p was observed to function in a manner different from its traditional role as a positive regulator. CaMac1p acted as a repressor and regulated *SFU1* in a copper responsive manner by directly binding to its promoter region. This is the first time that functional evidence (using EMSA) has been provided for this. Sef1p was also found to function together with Mac1p to co-regulate the expression of *CaMAC1* itself. This affirms that *C. albicans* portrays an interdependent regulatory circuit, referred to as the 'Feed forward loop'. This study elucidates the co-dependent role of these regulators linking iron and copper acquisition which play a vital role in the virulence of *C. albicans*.

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AD	Activation domain
ATP	Adenosine triphosphate
BCS	Bathocuproinedisulfonic acid
BPS	Bathophenanthrolinedisulfonic acid
bp	base pair
BSA	Bovine serum albumin
cDNA	copy DNA
CuRE	copper response element
CSPD	Disodium 3-(4-methoxyspiro (1,2-dioxetane-3,2'-(5'-chloro)tricycle[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate
DCytB	Cytochrome b reductase
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
DEPC	Di-ethyl-pyrocarbonate
DIG	Digoxigenin
d/W	Distilled water
EDTA	Ethylenediaminotetra acetic acid
EMSA	Electrophoretic mobility shift assay
FGSS	Fungal genetics stock centre
5-FOA	5-Fluoroorotic acid
FRE	Ferric reductase
LA	Luria Bertani agar

LB	Luria Bertani broth
LEU	Leucine
g	Grams
GlcNAc	N-acetylglucosamine
Hb	Haemoglobin
HCP	Haem carrier protein
HIS	Histidine
HO	Haem oxygenase
HEPES	N-(2-hydroxyethyl) piperazine N'-(2-ethanesulfonic acid)
IPTG	isopropyl-b-D-thiogalactopyranoside
kb	Kilobases
µM	Micromolar
mM	Millimolar
M	Molar
MD	Minimal defined
mets	methionine
MOPS	4-Morpholinepropanesulfonic acid
mRNA	Messenger ribonucleic Acid
nM	Nanomolar
NRC	National research council
ONPG	2-nitrophenyl β-D-galactopyranoside
ORF	Open reading frame

OD	Optical density
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
RIN	RNA integrity number
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT-PCR	Real-time PCR
RQ	Relative Quantitation
SDS	Sodium dodecyl sulphate
SEF	Suppressor of essential function
Srb	Suppressor of RNA
SSC	Standard saline citrate
SD	Synthetic defined
TAE	Tris acetate EDTA buffer
TBP	TATA-box binding protein
TE	Tris EDTA buffer
TEMED	N,N.N',N'-tetramethylethylenediamine
Tris-Cl	Tris (hydroxymethyl) aminomethane adjusted to pH with hydrochloric acid
TRKO	Transcriptional regulator knockout
TRP	Tryptophan

UV	Ultraviolet
Vac	Vacuole associated protein
v/v	Volume by volume
w/v	Weight by volume
YPD	Yeast Peptone Dextrose (media)
Y2H	Yeast two hybrid
ZF	Zinc finger

1. Introduction

This introduction, is a review of, iron and copper homeostasis in the pathogenic fungus *Candida albicans* and compares it with the information available on the much-more extensively studied, model yeast- *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* is a good model system since a lot is known about its genome. *Candida albicans'* genome shares a lot of similarities with it; however, the differences between the two yeasts are going to be highlighted in this Introduction. The introduction then covers the regulation of iron and copper acquisition systems and concludes with a background to the project, highlighting the project aims.

1.1. An overview of *Candida albicans*

Candida albicans is a eukaryotic micro-organism. It is a chemo-organotrophic, diploid fungus. *C. albicans* is an obligate commensal organism and is a part of the normal gut flora of most mammals. It is generally found in mucosal membranes of guts of humans where it shares a harmless relationship in most individuals. *C. albicans* mostly proliferates in the gastrointestinal tract, vagina and human mouth. However, it is also one of the most common opportunistic pathogen that causes systemic infections in immunocompromised hosts. Factors such as immunosuppressant drugs, surgery, radiation and intravascular cauterization, weaken the host's immune system and decrease the body's ability to fight against pathogens and infection (Tortorano AM *et al.*, 2004). In hosts such as cancer patients, AIDS patients or individuals having undergone organ transplantations, where the immune system is in a compromised state, *Candida albicans* can switch from a harmless commensal into a pathogenic organism.

All organisms require iron and copper for their survival and proliferation because they are essential cofactors of many important enzymes and metabolic pathways. In all organisms, strict control and regulation of iron and copper is essential because free cellular ions can be toxic for cells. For pathogens such as *C. albicans*, these homeostasis systems are even more important since iron is highly restricted *in vivo* and is often the growth limiting factor in the host environment. Copper is also restrictive in the host but can become elevated during inflammation. Also, iron restriction is used by many pathogens as a signal that they have entered a host. This results in them expressing their virulence factors. Therefore, the study of a pathogens iron and copper homeostasis system and their regulation will increase our understanding of the infection progress and their response to the *in vivo* environment. (Neppelenbroek *et al.*, 2013)

Unusually in *C. albicans*, the CUG codon which usually codes for Leucine, instead codes for Serine in this species. This shift from the universal genetic code may help the organism to induce a permanent stress response in certain host environments and additionally also adds another dimension to the molecular cloning techniques used in *C. albicans*, making them more complicated (Ohama *et al.*, 1993).

Previous work in our laboratory has identified the copper-responsive transcriptional activator CaMac1p (Metal-binding Activator), which increases transcription of the high affinity copper transporter gene *CaCTR1* in copper limited conditions (Marvin *et al.*, 2004). This project aims to further understand the molecular mechanism of action of this regulatory molecule in *C. albicans*, and describe how it differs from the mechanism of action of ScMac1p in the non-pathogenic yeast, *Saccharomyces cerevisiae*. The work described in this thesis also further characterises the functioning of putative transcriptional activator Sef1p (Suppressor of Essential Function) which may be involved in iron regulation. Through this work, we also propose a novel mechanism

involving multiple transcriptional regulators (Sfu1p, Sef1p, and Mac1p), linking the high affinity iron uptake system with the high affinity copper uptake system in *C. albicans*.

Mating

C. albicans was thought to be an obligate diploid but on analysis of the evolutionary history of *C. albicans*, a sexual cycle has been characterised (Bennett & Johnson, 2005). There are likenesses in mating in *C. albicans* and the model organism *S. cerevisiae* however the significant differences that are detected- may be adaption tools which give *C. albicans* an advantage and allow it to mate in human hosts. Similar to *S. cerevisiae*, *C. albicans* possesses two mating-type alleles at the MAT locus, a and α. In *S. cerevisiae* mating occurs in the haploid stage of its lifecycle however in the obligate diploid *C. albicans* only α homozygous and a homozygous strains can mate however a/α heterozygous strains are incapable of mating. In addition to being MAT homozygous, the strains must also switch from white to the opaque form. This switch cannot be made by the MAT heterozygous strains (Bennett & Johnson, 2005).

The white and opaque cell types are highly distinct in many aspects such as their cell and colony morphology, their mating pattern, the way in which they interact with the human host and their metabolic states. These two cell types are inherited for many generations and the switching between them is likely to be a random event, however, certain environmental signals can affect the rate at which switching occurs, significantly (Lohse & Johnson, 2009) Most of the clinical isolates that have been studied to date, have been found to be heterozygous (a/α) for the mating type locus. Strains that were heterozygous were found to be more virulent which appeared to

provide them with a competitive benefit (Lockhart *et al.*, 2005). Hence in nature, due to the prevalence of the a/α allele, the rate at which mating occurs is quite rare.

A close link between morphology and mating has been observed since the mating pheromones activate the same MAPK cascade which is also involved in regulation of morphogenesis (Bennett & Johnson, 2005). After mating, the organism can switch back to its diploid state via chromosomal reduction which can occur through a parasexual cycle (through chromosome loss) or through a sexual cycle (via meiosis). However, some vital elements of the genes that are mandatory for meiosis to occur, have been found to be missing from the genome in *C. albicans* and hence it has not been confirmed whether meiosis occurs, nevertheless, a para-sexual cycle has been characterised (Bennett & Johnson, 2005, Bennett & Johnson, 2003). Due to the apparent non-existence of meiosis in *C. albicans*, the typical yeast genetic techniques used in *S. cerevisiae* cannot be used in *C. albicans* which further complicates molecular investigation in *C. albicans*.

Morphology

There are various factors that play a role as virulence determinants in *Candida* and they vary among different strains. One of the most important virulence factors is the pleomorphic property of *C. albicans*. It can exist in three different morphological forms: unicellular budding yeast cells, multicellular hyphae which are a fungal form consisting of elongated cells or as a pseudohyphal form (Whiteway & Bachewich, 2007), which is an intermediate between the yeast and hyphal forms (Figure 1.1 and 1.2). However there is some debate as to whether pseudohyphae are a form of bud or whether they are a distinct form altogether (Woodacre A., Post-doc- personal communication). The pseudohyphal state is a type of incomplete budding where the

cells remain attached to each other after division but branch out in order to colonise (Berman J *et al.*, 2002, Sudbery P *et al.*, 2004). The organism has the ability to switch between the unicellular yeast form and invasive filamentous multicellular, pseudo-hyphae and hyphae forms via a complex pathway that involves many regulatory cascades that respond to various environmental signals, during infection (Dhillon NK *et al.*, 2003). To date, all the components of this complex cascade have not been fully characterised. A lot of the environmental signals and transcription factors that induce the morphological switch also regulate genes involved in virulence. It is not clear how the yeast-hyphal switch exactly contributes to survival but it may be an adaptation mechanism for persistence and propagation of this organism in the mammalian host and for its virulence causing ability (Lane *et al.*, 2001, Murad *et al.*, 2001). In a recent study in our lab, it was found that regulation of iron and copper homeostasis is different in the yeast form of *C. albicans* as compared to hyphae and hence morphology and the acquisition of these essential virulence determinants is linked (Jeeves RE *et al.*, 2011).

Factors such as changes in pH, methionine and glucose levels, nitrogen and amino acid starvation, changes in temperature, presence of *N*-acetylglucosamine (GlcNAc) and presence of serum can induce a morphological switch in *C. albicans.* Under laboratory conditions, it has been observed that at temperatures of 30°C or below pH 4. *C. albicans* grows in its unicellular yeast form, while at 35°C, a slightly less acidic pH 6 and in the presence of high phosphate conditions, *C. albicans* grows in its pseudohyphal form as elongated yeast cells. Interestingly when *C. albicans* is grown at 37°C, pH 7, in the presence of serum or *N*-acetylglucosamine, it switches to its long, continuous hyphae form (Sudbery P *et al.*, 2004).

Disease and Virulence

Although, *C. albicans* has great clinical relevance and hence its virulence is widely studied and it is of major interest to researchers, all the components involved in
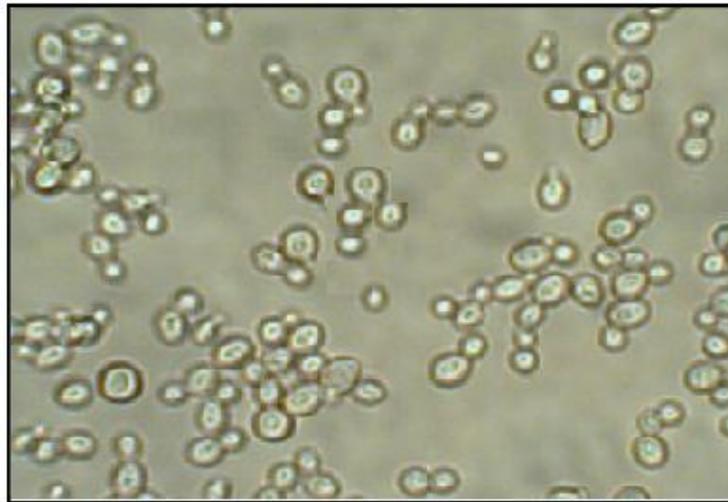
A. YPB without serum

Magnification: 1000x
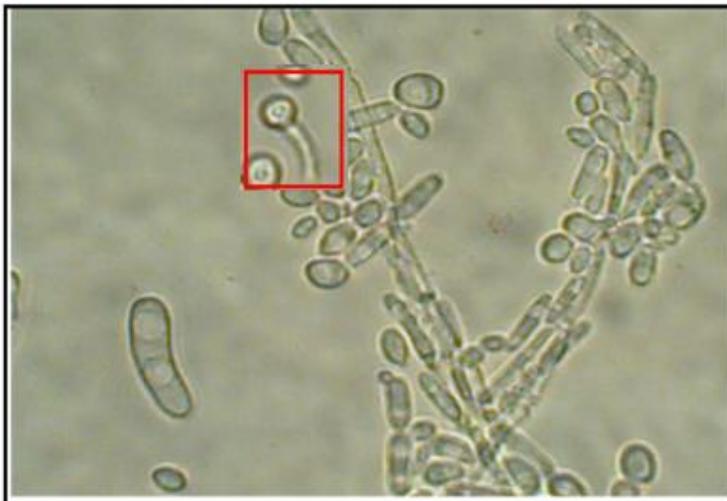
B. YPB with 20% serum

Figure 1. 1: Microscopic picture of C. albicans when grown in YPD

C. albicans cells were grown in YPD media for 6 hours at 30°C and were then inoculated into fresh YPB without serum (**A.**) and with 20% bovine serum (**B.**). The cells were then grown for a further 3 hours at 37°C before being suspended in YPB and observed under phase contrast microscope at 1000x magnification. In the absence of serum (**A.**) the cells grew in the unicellular budding yeast form. (**B.**) In the presence of serum the cells switched morphology and elongated fungal cells representing multicellular pseudohyphae and hyphae were observed. A true hyphae form is highlighted within the red box.
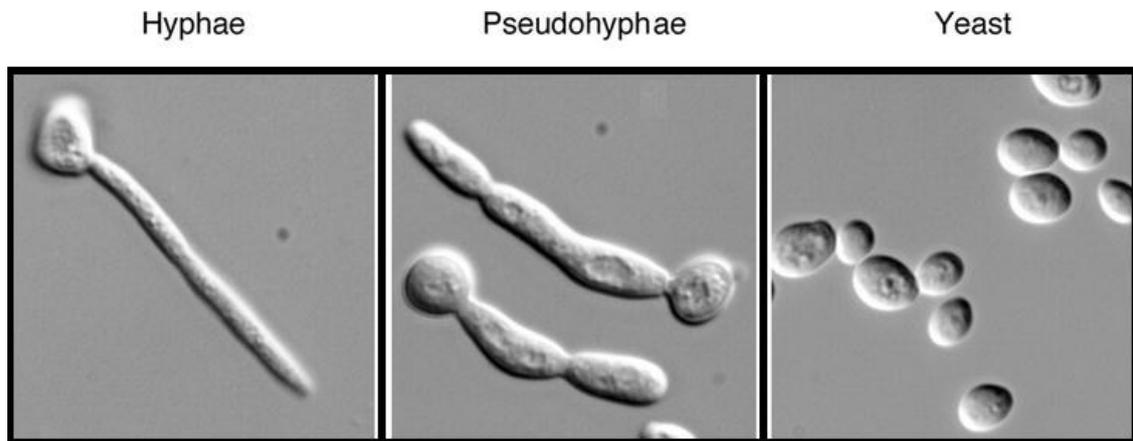
Figure 1. 2: Different morphological forms of *C. albicans* (taken from Wightman *et al.*, 2004))

Cells were grown in YPB at 30°C under constant shaking, until they reached stationary phase and then the cells were harvested and re-inoculated into fresh media and allowed to grow for a further 2 hours to form hyphae, pseudohyphae or yeast.

virulence have not yet been fully determined. *C. albicans* primarily causes superficial skin and mucosal membrane infections in immunocompetent persons known as Candidiasis. Infections are accompanied by redness, discomfort, itching and localized inflammation of the skin or mucosal membranes. Locations of infection include the oral cavity, the pharynx, the urinary bladder, the gastrointestinal tract, or the genitalia. Mycosis of the subcutaneous layer of the mouth and the vagina, caused by *C. albicans*, is commonly known as thrush or vaginitis respectively. Vaginal candidiasis has been found to occur quite frequently and around 75% of women would contract at least one such infection during their lifespan (Ferrer, 2000). In immunocompromised hosts (transplant, cancer and AIDS patients) *Candida* infections can affect the oesophagus resulting in a life-threatening systemic bloodstream infection termed candidaemia. In the USA, *C. albicans* is the fourth-most common pathogen responsible for causing bloodstream infections and the mortality rate for these was estimated to be 49% (Gudlaugsson O *et al.*, 2003, Wisplinghoff H *et al.*, 2004). Although oesophageal Candidiasis is highly common, Candidaemia can result due to invasion from any mucosal site (Woodacre A., personal communication). *C. albicans* is lightly intrinsically resistant to anti-fungal agents (Johnson *et al.*, 1995) and even after anti-fungal treatment it shows high survival rates of 55% (Weinberger M *et al.*, 2005). *C. albicans* has also been observed to acquire resistance to anti-mycotic agents, on being treated by the same drug, over a period of time. For example, over time, it can become resistant to the effect of anti-fungal treatments, such as the azole drug fluconazole which is commonly used in the treatment of candidiasis and thrush (Johnson *et al.*, 1995). It is this acquired resistance to antifungal drugs, combined with its intrinsic resistance that are the primary factors that gives *C. albicans* an advantage. It develops

the ability to survive treatment with antifungal agents and hence contributes to high mortality rates in infected immunocompromised patients.

C. albicans, unlike bacteria, does not develop resistance to anti-fungal agents by transferring mobile genetic elements, carrying resistant genes across populations but instead resistance develops within a lone population, over a period. (Cannon *et al.*, 2007b) The majority of *C. albicans* antifungals are constituted of polyenes (eg. Amphotericin B) - that function by binding to sterols and disrupting the lipid bilayers; echinocandins - that have been found to inhibit $(1,3)$-D-β-glucan biosynthesis and azoles- that affect the nucleic acid synthesis of *ERG11* gene involved in ergosterol biosynthetic pathway and production of 5-fluorocytosine thereby disrupting the fungal cell membrane (Sanglard & Odds, 2002, Cannon *et al.*, 2007a).

A number of factors contribute to the switch between a commensal organism and a pathogenic one; such as resistance to anti-fungal drugs, adherence to surfaces, morphology and iron and copper acquisition. The ability of *C. albicans* to be able to change its morphological form has been shown to be necessary for virulence (Braun BR *et al.*, 1997, Rocha CR *et al.*, 2001). The different morphological forms play a role during different stages of infection - the yeast is essential for dissemination of the organism in the host's blood stream while the hyphal form allows tissue and cell penetration utilising the pressure from the growing hyphal tip (Whiteway M *et al.*, 2004). Mating has also been observed to have an effect on virulence. In mouse models, homozygous strains; a/a or α/α were found to be less virulent when compared to a/α heterozygous strains (Lockhart *et al.*, 2005).

Relationship of Iron and Copper to Virulence

Iron and copper are metal ions that are indispensable to the survival of the organism extending from bacteria to mammals. They are required for respiratory reactions in the cell, to prevent oxidative stress, as pathogenesis factors and as co-factors of important enzymes such as cytochrome oxidase (Hwang CS *et al.*, 2002). Copper, for example, is vital in processes such as oxidative phosphorylation, signalling to the transcription and protein trafficking machinery, neuropeptide maturation, iron mobilization, and normal development of most bacterial organisms (Rees *et al.*, 2004). Iron is thought to be the fundamental agent that induces host-pathogen interactions. For example in a research carried out in Zimbabwe, on pregnant women who were receiving iron supplementation, the presence of ferritin was found to be an autonomous predictor of HIV viral load. Additionally, both iron-loading and deficiency of iron act as risk factors for human tuberculosis. The actions of IFN-γ and iron loading of macrophages has also been found to be directly repressed by iron; which eventually leads to inhibition of IFN-γ -mediated pathways and triggers intra-phagosomal mycobacterial growth (Doherty, 2007). Iron and copper are principally essential because they take part in critical electron transfer reactions within the organism. Proteins that are important for the virulence of the organism such as ferrous iron transport complex and superoxide dismutase also require copper (Hwang CS *et al.*, 2003). It has been previously observed, in our laboratory, that in *C. albicans* iron and copper homeostasis are interlinked and it has been shown that copper is essentially required for high affinity iron uptake (Marvin *et al.*, 2004, Knight SA *et al.*, 2002).

Previous experiments have demonstrated that the amount of free iron available in the host environment and uptake of this iron has been shown to be essential for virulence in bacterial and fungal organisms (Ratledge C *et al.*, 2000). It has been observed that CaFtr1p, which is involved in high affinity iron uptake in *C. albicans*, also plays an

important role in its virulence (Ramanan N *et al.*, 2000). Mammalian hosts restrict the amount of free iron and copper available and this has an impact on the ability of the pathogen to obtain the ions essential for its survival. In experiments with *Vibrio vulnificus*, when free iron was injected into infected mouse it significantly reduced their resistance to infection showing the importance of available iron in the spread of disease by the organism. It has also been observed that Leukaemia patients are likely to be more vulnerable to *C. albicans* infections, if they have an overload of free iron in their system (reviewed by (Bullen JJ, 1981). However contrarily restricted amounts of iron also acts as a signal for many pathogenic organisms to initiate transcription of their iron acquisition genes and virulent genes, since it's a mechanism of recognition of entry into host.

Furthermore it is known that in the presence of alkaline pH (Bensen ES *et al.*, 2004), macrophages and prostaglandins, in the environment, the expression of copper metabolism genes alters in *C. albicans*. In addition, defects in copper uptake system leads to impairment of high affinity iron uptake which was found to be essential for virulence in systemic mouse models of Candidiasis, carried out in our laboratory (Marvin *et al.*, 2004, Ramanan N *et al.*, 2000). All this evidence suggest that iron and copper may play an important role in the interactions between *C. albicans* and the human host and therefore help in establishment of the disease (Marvin *et al.*, 2003).

Mammalian hosts, like all other organisms, require iron for their survival but they have to limit the amount of free iron to prevent toxicity and proliferation of infectious pathogens. Hence iron is found tightly bound in the iron storage molecules ferritin or transferrin and lactoferrin which are single chain glycoproteins capable of binding two ferric iron ions. This appropriation of free iron also acts as a defence mechanism and prevents colonisation by pathogen. Additionally a hypoferraemic response is triggered

as soon as the host detects a pathogen, which further reduces the amount of free iron available in the host environment.

In mammals, iron homeostasis is mainly regulated by Hepacidin, secreted by the liver and also by inflammatory macrophages and monocytes. Iron is absorbed in duodenum and the upper jejunum where the haem carrier protein 1 (HCP1) takes up haem iron which is then oxidised in the cell by haem oxygenase to release ferrous iron. DcytB, a cytochrome b reductase, reduces non-haem iron which is then taken up by divalent metal transporter (DMT1), a ferrous iron transporter. Hephaestin, a copper-dependent ferroxidase, exports cellular ferrous iron utilising ferroporin. For transport in the bloodstream, iron is bound to transferrin, lactoferrin and lactotransferrin. Once iron enters cells via transferrin receptors, it is bound to haem and either incorporated into proteins or it is stored in ferritin (Figure 1.3), which is capable of binding more than 4000 ferric ions (Edison *et al.*, 2008).

Pathogenic organisms have to develop mechanisms by which they can obtain the essential metals from the iron and copper limited host environment. In the host, iron and copper are present as insoluble complexes linked with environmental ligands and hence the organism has well-developed iron and copper acquisition mechanisms that either produce and take up iron chelators called Siderophores or employ a high affinity reductive iron uptake system, which will be focussed on extensively in this study (Van Ho A *et al.*, 2002).   Iron and copper are highly reactive native elements. As a result, excessive iron and copper can lead to development of reactive oxygen species (ROS) within the cell, due to reactions with H_2O_2 in Fenton reactions (Figure 1. 4). These free radicals are toxic and can cause DNA damage. Hence the organism has to carefully monitor and regulate the concentration of these two metals *in vivo*. It is critical to maintain the concentration of these metals, within the cell, in a narrow range between

Figure 1. 3: Utilization of iron in Mammals.

In mammalian cells, haem iron is taken up into the cytosol by HAEM Carrier Protein (HCP1), and then oxidised by Haem Oxygenase (HO-1) to release iron in its ferrous form. Non-haem iron is reduced at the cell surface by a cytochrome b reductase (DCytB) and then taken up into the cytosol via a ferrous iron transporter DMT1. The ferrous iron taken up into the duodenal enterocyte is either stored in ferritin or transported out of cell, after being oxidised back to its ferric form, by a complex of Hephaesin with Ferroporin. The ferric iron is then integrated into transferrin for transport in the blood (adapted from Edison *et al.*, 2008).

Harber-Weiss-Fenton Reaction

A. $Fe^{2+}(Cu^{+}) + H_2O_2 \longrightarrow Fe^{3+}(Cu^{2+}) + OH^{-} + OH^{\bullet}$

B. $Fe^{3+}(Cu^{2+}) + H_2O_2 \longrightarrow Fe^{2+}(Cu^{+}) + {}^{\bullet}OOH + H^{+}$

C. $Fe^{2+}(Cu^{+}) + O_2 \longrightarrow Fe^{3+}(Cu^{2+}) + O_2^{\bullet}$
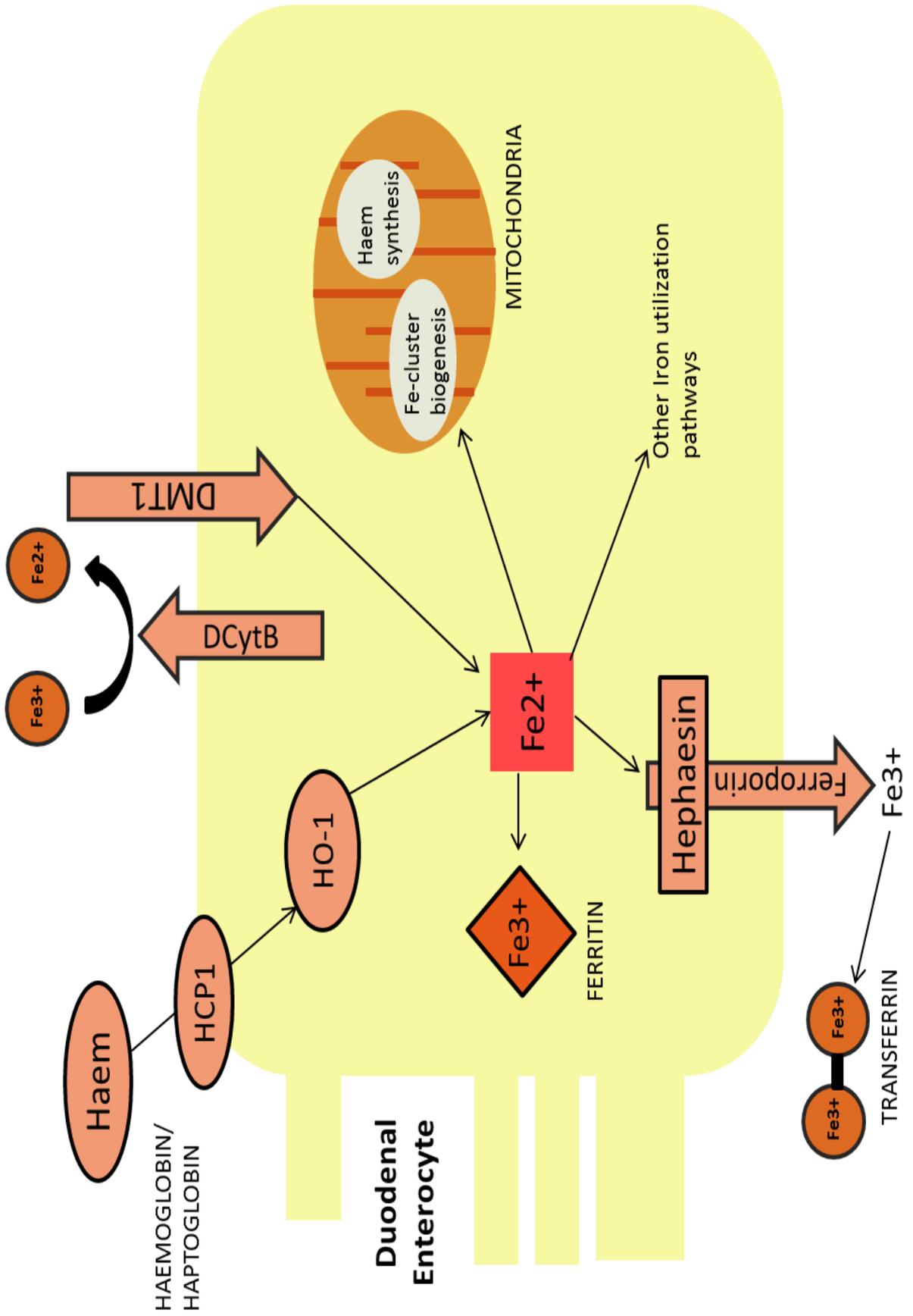
The oxidative reactions of both iron and copper produce toxic oxygen and hydroxyl free radicals. These radicals can damage most types of biological molecules within the cell including nucleic acids, lipids and proteins.

Figure 1. 4: Harber-Weiss-Fenton Reactions

utility and toxicity. A low iron environment is used as a trigger by many pathogens as an indicator of entry into a host and to express genes involved in virulence. For example, in bacteria Fur protein that regulates iron uptake systems and a number of virulence factors has been found to repress genes in high iron environments (Escolar *et al.*, 1999). The mechanism of iron and copper uptake has been well studied in *Saccharomyces cerevisiae* and since *C. albicans* shows a considerable amount of homology to *S. cerevisiae*, the iron and copper uptake mechanisms in *C. albicans* is also starting to be elucidated. However, their ion uptake regulation differs considerably; presumably due to their different environmental niches as *S. cerevisiae* is a non-pathogen whereas *C. albicans* is an opportunistic pathogen (Woodacre, 2007, Lan CY *et al.*, 2004).

This report gives a review of iron uptake, copper uptake and their regulation in *S. cerevisiae* as it is a model organism (used to study *C. albicans*) and mechanisms of iron and copper transport and toxicity are very well understood in it; and hence we draw comparisons with the information that is currently available on *C. albicans*. *C. albicans* has five main systems for acquisition of iron: a system for acquiring and utilising iron from haem, a system to acquire iron from Ferritin, a siderophore uptake system, a high affinity iron acquisition system, which is active in iron starvation conditions and a low affinity system, which is active in iron replete conditions (Van Ho A *et al.*, 2002, Almeida *et al.*, 2008). This study aims to gain a better understanding of the intricate principles of *C. albicans* pathogenesis because it is the most common fungal pathogen and causes significant morbidity and mortality worldwide. This will give us a greater understanding of the association between copper and iron acquisition and their regulation in *C. albicans* and we hope to elucidate the role these metals may play in the prevalence and spread of this pathogen in the human host.

1.2. Iron homeostasis in Fungi

Fungi obtain iron from various sources since iron is present in the environment in several forms such as ferrous, ferric and bound to low molecular weight iron-binding molecules known as siderophores (Van Ho A *et al.*, 2002). Iron is usually available in its insoluble ferric form in nature since Fe^{2+} binds much more strongly with environmental ligands and has to be converted into its more soluble ferrous form (which is quite unstable as it can get rapidly oxidised) (Dancis A *et al.*, 1990, De Freitas *et al.*, 2003). Ferric reductases have been identified in a range of fungi including *Candida albicans*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Aspergillus nidulans* (Hammacott *et al.*, 2000, Oberegger *et al.*, 2001, Crichton & Pierre, 2001, Jung & Kronstad, 2008).

Yeasts like *S. cerevisiae* and *C. albicans* have two well-characterised systems for acquiring iron, a low affinity system which becomes active in iron replete conditions and a high affinity system which is active in low iron conditions, such as in the host environment.

High affinity systems have been found to function in a variety of yeast and fungi where the homologues of the metal transporters have been identified, including *Candida albicans*, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Schizosaccharomyces pombe*, *Arxula adenivorans*, *Cryptococcus neoformans* and *Rhizopus oryzae* (Ramanan N *et al.*, 2000, Jung & Kronstad, 2008, Dancis A *et al.*, 1994, Eck *et al.*, 1999, Fu *et al.*, 1994, Paronetto *et al.*, 2001, Wartmann *et al.*, 2002).

High affinity iron uptake in *S. cerevisiae*

Iron is usually found in the environment in its stable ferric form however it needs to be converted into its highly reactive, unstable, but more soluble ferrous form before it can be transported across the cell membrane since the proteins involved in the high affinity uptake of iron have a specificity for 0.15 μM Fe^{2+} and they only become active in iron restricted conditions (Eide D *et al.*, 1992). In yeast, ferric reductases, that are cytochrome-like proteins, are responsible for bringing about this transition.

The *ScFET3* (Ferrous transport 3) gene is a part of the high affinity system and *fet3Δ* mutants have shown respiratory defects and are incapable of utilising non-fermentable carbon sources, while also showing reduced growth in iron and copper restricted conditions and possessing a defective high affinity iron uptake system (Askwith & Kaplan, 1998, Dancis A *et al.*, 1994). It has been found that Fet3p is not an iron transporter but is a multi-copper oxidase protein that has an affinity for ferrous iron and oxidises it to its ferric form while carrying it across the membrane. Fet3p requires four copper ions for its functioning which are loaded onto it in a post-Golgi compartment and this illustrates how copper and iron uptake is intrinsically linked in *S. cerevisiae* (Figure 1.7). As a result copper starvation conditions have been found to result in secondary iron starvation (Dancis A *et al.*, 1994, Yuan DS *et al.*, 1997). The multi-copper oxidase forms a complex with an iron permease protein which allows the transport of ferrous across the cell membrane and its simultaneous oxidation to ferric form (Stearman R *et al.*, 1996).

Two other multi-copper oxidase genes, *ScFET4* and *ScFET5*, have also been identified in *S. cerevisiae*. In a study, *ScFET5* (ferric reductase) on overexpression in a *Scfet3Δ* mutant, was found to re-establish wild-type growth in reasonable iron limited

conditions but it has been found to be less effective as compared to *ScFET3* and *ScFET4* (Spizzo *et al.*, 1997). Experiments have also shown that ScFet5p is capable of forming a complex with the vacuolar iron transporter ScFth1p, indicating it can be responsible for transporting iron in and out of the vacuolar membrane (Urbanowski & Piper, 1999).

Previous experiments have shown that in *Scfet3Δ*, permease protein ScFtr1p remains localised in the endoplasmic reticulum, indicating that its complex formation with the multicopper oxidase ScFet3p is essential for its localisation to the plasma membrane while Ftr1p is required for loading of copper on to ScFet3p. Additionally Ftr1p has been found to have conserved iron binding motifs and hence it can directly bind ferrous indicating that this multi-copper oxidase - iron permease complex is required for high affinity iron uptake to take place in *S. cerevisiae*. (Stearman R et al., 1996).

This oxidase/permease complex for transporting iron across the cell membrane has also been identified in other eukaryotes such as bacteria like the magnetotactic strain of marine *vibrio* (Dubbels *et al.*, 2004) and the algae *Chlamydomonas reinhardtiu* (La Fontaine *et al.*, 2002).

Fet3p has a specific reactivity towards ferrous iron (Fe^{+2}) as reducing substrate and it catalyzes the ferroxidase reaction.

$$4Fe^{2+} + 4H^+ + O_2 \rightarrow 4Fe^{3+} + 2H_2O$$
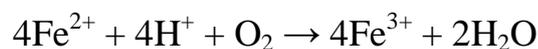
The ferric iron product of this Fet3p-catalyzed reaction is ligand for the iron permease Ftr1p, for iron uptake (Kwok *et al.*, 2006).

Low affinity iron uptake in *S. cerevisiae*

It has been observed that mutants that have a defective high iron uptake system are still able to grow normally on media supplemented with iron because of the existence of a low affinity iron uptake system which functions independently of the Fet3p-Ftr1p complex, required for high affinity iron transport (Dix D *et al,* 1997.).

The high affinity iron uptake system cannot function in anaerobic conditions because oxygen is required for the oxidase activity of Fet3p; hence the low affinity iron uptake system becomes active in the absence of oxygen. The low affinity plasma membrane iron transporter ScFet4p has been shown to have an affinity for iron at 30 μM as compared to an affinity of 0.15 μM (or lower) that activates the high affinity system. ScFet4p is able to transport iron in its ferrous form and hence requires reduction of the ferric iron by ferric reductases (Dix D *et al*, 2000.). ScFet4p can also transport nickel, cadmium, cobalt, zinc, manganese and copper (Hassett *et al.*, 1998).

Ferric reductases in *S. cerevisiae*

In *S. cerevisiae*, nine ferric reductase (*FRE*) genes have been identified and have been found to be involved in high and low affinity iron acquisition or in the reduction and acquisition of iron from siderophores produced by other microbes. They are capable of reducing ferric ion (Fe^{+3}) to ferrous ion (Fe^{+2}) as well as cupric ion (Cu^{+2}) to cuprous ion (Cu^{+1}). From previous experiments it is known that, in *S. cerevisiae*, Fre1p and Fre2p have been found to be the major cell surface ferric reductases (Figure 1.7). *fre1Δfre2Δ* mutants showed a deficit in high affinity uptake of iron (Dancis A *et al.*, 1990, Georgatsou E *et al.*, 1994) and it was observed that transcription of *FRE1* and *FRE2* increased under low iron conditions. Additionally, copper ions were also found

to play a role in transcription of *FRE1* and hence iron and copper homeostasis has been concluded to be intrinsically linked.

In addition to Fre1p and Fre2p, there are seven more reductases which are not as well studied but contain functional domains involved in reduction. It has been observed that transcription of *FRE3-6* is regulated by iron levels while transcription of *FRE7* is regulated by copper levels and transcription of *YGL160w* and *YLR047c* is not regulated by either iron or copper (Georgatsou E *et al.*, 1994, Martins LJ *et al.*, 1998). In contrast to Fre1p and Fre2p, the remaining reductases do not reduce ferric iron at the cell surface but appear to reduce alternative substrates or localise to different parts of the cell while Fre3p and Fre4p specifically reduce iron bound to siderophores (Yun CW *et al.*, 2001).

The plasma membrane protein, ScFre1p has been found to be responsible for 80% of the cell surface ferric reductase activity. Additionally when the *ScFRE1* gene was over expressed, it led to copper sensitivity in the strain (Dancis A *et al.*, 1990, Dancis A *et al.*, 1994). Another cell surface ferric reductase *ScFRE2* was found to be regulated only by iron in spite of being ferric as well as cupric reductase. Deletion of *ScFRE2* in a *Scfre2Δ* mutant leads to loss of the residual ferric reductase activity (Georgatsou E *et al.*, 1994). A discernible increase in expression of *ScFRE1-ScFRE6* was seen on addition of an iron chelator, bathophenanthroline sulfonate (BPS), to the growth medium while the expression of *ScFRE1* and *ScFRE7* was induced on addition of the copper chelator, bathocuproine disulphonate (BCS), to the media indicating that *ScFRE2-6* are regulated by iron while *ScFRE7* is regulated by copper and *ScFRE1* is induced in both iron and copper limited conditions (Georgatsou E *et al.*, 1994, Martins LJ *et al.*, 1998).

High Affinity Iron uptake in *C. albicans*

C. albicans shows presence of iron permease genes *CaFTR1* and *CaFTR2* which share a high level of homology with the *S. cerevisiae ScFTR1* and were found to be capable of rescuing *S. cerevisiae ftr1Δ* mutants indicating that the high affinity iron uptake system is similar in the two organisms. CaFtr1p contains five iron-binding domains (Glu-Xaa-Xaa-Glu) motifs and it was seen that on disruption of any one of these, the mutant could no longer grow on iron limited conditions (Fang & Wang, 2002). CaFtr1p has been observed to be the major iron permease in *C. albicans* and is also the functional homologue of ScFtr1p as *caftr1Δ/caftr1Δ* mutants show almost no iron uptake activity and low growth on iron deficient media in contrast to *caftr2Δ/caftr2Δ* mutants that showed wild-type levels of iron uptake. It was also found that transcription of *CaFTR1* was induced in low iron conditions and *caftr1Δ/caftr1Δ* mutants were also found to be incapable of establishing a systemic infection in a mouse model of candidiasis thereby demonstrating the importance of effective iron uptake in the growth and virulence of *C. albicans* (Ramanan N et al., 2000, Eck et al., 1999).

The copper transporter gene *CaCTR1* is required for effective iron uptake, confirming the need for copper which would be incorporated into the multi-copper oxidase for effective functioning of the iron transporter CaFtr1 (Marvin *et al.*, 2004). This suggests the existence of an oxidase-permease complex in *C. albicans* similar to the Fet3p-Ftr1p complex in *S. cerevisiae* (Braun BR *et al.*, 1991, Braun BR *et al.*, 2005). Five homologues of the *ScFET3* gene have been identified in the *C. albicans* genome; *CaFET31*, *CaFET33*, *CaFET34*, *CaFET35* and *CaFET99*. *CaFET34* has been recently demonstrated to be functional homologues to *ScFET3* and is the one most likely to be involved in formation of the oxidase-permease complex in *C. albicans*. The complex

brings about iron-trafficking by routing the CaFet34-generated-Fe^{+3} directly to CaFtr1p for transport into the cytosol (Ziegler *et al.*, 2011) (Figure 1.8).

In *C. albicans* in addition to the *ScFTR1* and *ScFTR2* homologues (*CaFTR1* and *CaFTR2* respectively), two *ScFTH1* (that localise to the vacuolar membrane) homologues (*CaFTH1* and *CaFTH2*) have also been identified (Van Ho A *et al.*, 2002).

C. albicans mutants that portrayed defects in uptake of iron via the high affinity system where found to be able to grow on media that contained large quantities of iron indicating that they possessed a low affinity iron transport system that was activated in iron replete conditions, however to date, no other experimental evidence has been found to support this (Ramanan N *et al.*, 2000).

Ferric reductases in *C. albicans*

Our laboratory was the first to provide experimental evidence for the presence of cell surface ferric and cupric reductase activity which was found to be activated in response to both copper and iron limitation (Morrissey *et al.*, 1996). In the following years, using complementation studies (Hammacott *et al.*, 2000) and on sequence analysis of *Candida* genome (Mason, 2006b, Jones *et al.*, 2004), 17 putative ferric reductase genes have been identified (Table 1.1). However, as yet the specific roles for their putative protein products have not yet been determined. The gene *CaFRE10* (orf19.1415, also called *CFL95*) has been demonstrated to be required for wild-type levels of high affinity iron uptake and work in our lab and others have determined it to be the major cell surface ferric and cupric reductase in *C. albicans*. The cell surface ferric reductase activity was observed to be decreased by 75% on deletion of the *CaFRE10* gene and the uptake of ferric iron was also found to be decreased by 77%. The uptake of iron from

transferrin was also seen to be reduced by 75% in a *Cafre10ΔΔ* double mutant indicating that *CaFRE10* is majorly (but not essentially) responsible for transferrin iron uptake (Knight *et al.*, 2005).

The *CaFRE1* (also called *CFL1*) and *CaFRE2* (also called *CFL2*) genes were found to be capable of complementing *S. cerevisiae fre1Δ/fre2Δ* mutants however the *C. albicans fre1ΔΔ* double mutant did not show any of the expected reductase-mutant phenotypes while in the *fre2ΔΔ* double mutant, the cell surface ferric reductase activity only decreased minimally (Hammacott *et al.*, 2000, Mason, 2006b). On bioinformatics analysis, the *CaFRE5* (also called *CFL4*) gene was found to contain a mitochondrial signalling sequence. Via microarray experiments, *CaFRE1*, *CaFRE2*, *CaFRE5* and *CaFRE31* (also called *CFL5*) showed increased expression in iron limited conditions (Lan CY *et al.*, 2004) while expression of *CaFRE2*, *CaFRE7*, *CaFRE9* and *CaFRP1* was found to be induced in alkaline environments (Bensen ES *et al.*, 2004). *CaFRE1* showed reduced expression in the presence of serum (Lesuisse E *et al.*, 1998) while copper was found to solely regulate the expression of *CaFRE7* (Jeeves RE et al., 2011). Hence it was realised that the transcription of ferric reductase genes is regulated not only by changes in iron and copper levels in the environment but pH changes and presence of serum also acts a vital trigger.

Work carried out in our laboratory demonstrated that *CaFRE10* acted as the major cell surface ferric as well as cupric reductase which majorly responded to changes in iron levels but also showed a slight increase in expression in response to changes in copper levels (Jeeves RE *et al.*, 2011, Mason, 2006b). Lately another reductase *CaFRE7* was found to contribute significantly to the cell's ferric and cupric reductase activity, however unlike *CaFRE10* which was regulated mainly in response to iron, *CaFRE7* was found to be regulated in response to changes in copper levels (Jeeves RE *et al.*,

2011, Jeeves, 2008, Woodacre *et al.*, 2008) (Figure 1.8). It was observed that *CaFRE10* and *CaFRE7* showed higher expression in yeast as compared to their expression in hyphae which led to a corresponding reduction in cell surface ferric (but not cupric) reductase activity. This confirmed the hypothesis that the two virulence determinants, the regulation of the reductive iron uptake system and the morphological form of *C. albicans*, were inter-linked since the hyphal or yeast-inducing conditions such as serum or alkaline pH affected regulation of metal homeostasis genes (Jeeves RE *et al.*, 2011).

These ferric reductases in *C. albicans* have been identified due to their homology with the major cell surface ferric reductase, *CaFRE10*, gene sequence (Knight *et al.*, 2005). There are many differences in nomenclature which leads to a problem in data comparison between different studies. The Table 1.1 highlights all the different names used for the same genes on various databases such as the National Research council (NRC), *Candida* database and the Stanford database.

C. albicans ORF 19 no.	Size (amino acids)	Chromosome	C. albicans ORF6 no.	NRC name	Candida database name	Standford sequence name
19.1415	706	4	6.6384	FRE10	RBT2	CFL95
19.8849	738	4	6.5731	FRE2	CFL2	CFL97
19.8848	760	4	6.5730	FRE1	CFL1	CFL1
19.2312	679	1	6.5538	FRE11	FRE42	FRE3
19.1930	710	5	6.1281	FRE31	CFL5	CFL90
19.1932	710	5	6.5606	FRE5	FRE32	CFL94
19.8856	727	4	6.2162	FRE3	CFL3	CFL99
19.3538	749	2	6.4284	FRE9	CFL12	CFL98
19.6138	730	R	6.5676	FRE6	FRE41	CFL93
19.701	739	Unmapped	6.3325	FRE8	CFL11	CFL96
19.9403/ 19.1844	751	R	6.5612	FRE4	FRE43	CFL91
19.6139	520	R	6.5675	FRE7	FRE30	CFL93
19.7077	586	7	6.8476	FRE12	FRE7	-
19.867	555	2	6.3547	FRE13	-	-
19.4843	593	1	6.7368	FRE14	-	-
19.5634	554	4	6.4503	FRP1	FRE5/FRP1	-
19.7112	592	7	6.8441	FRP2	FRP2	-

Table 1. 1: Open reading frames for putative ferric reductases in C. albicans and the different nomenclature.

These ferric reductases have been identified due to their homology with the major cell surface ferric reductase, *ScFRE1*, *CaFRE1* and *CaFRE10*, gene sequence using Blast searches (Knight *et al.*, 2005). There are many differences in nomenclature which leads to a problem in data comparison between different studies. This table highlights all the different names used for the same genes on various databases such as the National Research council (NRC), *Candida* database and the Stanford database. All of the proteins appear to localise to the plasma membrane which was determined by using WoLF PSORT (http://wolfpsort.seq.cbrc.jp/).

Acquisition of iron from Haem, Ferritin & Siderophores in *C. albicans*

C. albicans is capable of utilising transferrin, haem and haemoglobin as an exogenous source of iron, from the mammalian host's serum. It has been observed that growth deficiency experienced by *C. albicans* in iron deficient human serum, can be corrected by addition of haem, haemoglobin and/or iron obtained from red blood cells. It has also been observed that presence of haemoglobin in media causes the hyphal genes *HWP1* and *ECE1* to be up-regulated which induces hyphae formation and also increases expression of cell surface adhesins (such as fibronectin) which may help it to switch from commensal to a pathogen in the human host (Pendrak & Roberts, 2007). In *C. albicans* Rbt5p and Rbt51p have been implicated in haemoglobin utilisation and uptake. *RBT5* has been found to be negatively regulated by CaTup1p and is induced in iron starvation conditions (Braun BR *et al.*, 1997, Weissman Z *et al.*, 2004).

C. albicans, unlike *S. cerevisiae*, can also utilise ferritin, the mammalian iron storage molecule, as a sole source of iron. It was seen that only the hyphal form of *C. albicans* was capable of binding ferritin while the yeast form cannot and it is dependent on the Als3p adhesin which is specific to hyphae. *als3ΔΔ* double mutants were found be less damaging to epithelial cells. It has also been concluded that ferritin utilization depends on the high affinity iron reductive system because *Caaftr1ΔΔ* mutants are incapable of using ferritin as the sole iron source (Almeida *et al.*, 2008).

C. albicans, like *S. cerevisiae*, can also utilise siderophores produces by other organisms, as a source of iron. Many studies have shown that *C. albicans* can competently utilise ferrirubin, ferricrocin, hydroxamate siderophores ferrichrome, ferrichrysin and to some extent COP, ferrioxamine B, triacetylfusarinine C and

ferrioxamine E (Lesuisse E *et al.*, 1998, Ardon *et al.*, 2001). The siderophore iron transporter *CaARN1*, in *C. albicans*, shows 46% identity to *ScARN1* which is primarily responsible for the uptake of siderophores in *S. cerevisiae* and is specific for ferrichrome. The *CaARN1* gene has been found to be regulated by iron and CaArn1p is generally found in the plasma membrane but some protein is also found in the cytoplasm. This indicates that protein internalization could be the mechanism by which ferrichrome iron is transported across the cell membrane since higher amounts of CaArn1p is found within the cytoplasm corresponding to increasing concentrations of ferrichrome within the surrounding environment (Hu *et al.*, 2002). In reconstituted human epithelium cells, *Caarn1ΔΔ* mutants were incapable of causing infections hence indicating that iron transport via CaArn1p may play an important role in virulence of the organism and is important for epithelial invasion (Hu *et al.*, 2002, Heymann *et al.*, 2002).

In the cytoplasm, iron is also stored in a bound from attached to the siderophores. There is no concrete evidence demonstrating that *C. albicans* is capable of producing siderophores and this is still debatable but it can utilise siderophores from a variety of fungi as the sole iron source and can transport them across the cell membrane via reductive and non-reductive pathways (Lesuisse E *et al.*, 1998, Li L *et al.*, 2001). However, since *C. albicans* may not be able to produce iron-storage siderophores, it stores iron in vacuoles.

Storage of iron in vacuoles

In yeasts, iron storage within vacuoles helps to maintain iron and copper homeostasis and prevents toxic conditions from developing within the cell. During intracellular iron-replete conditions, the vacuoles start storing iron which is then released into the

cytoplasm when the cell faces iron starvation. ScCCC1p is responsible for transport of iron from the cell membrane to the vacuole. Over-expression of *ScCCC1* leads to depletion of iron in the cytosol (Li L *et al.*, 2001). The high affinity transport of iron across the vacuolar membrane, into the cytosol, requires reduction of ferric ion to ferrous ion by ScFRE6p (Figure 1.8). The reduced iron is then transported across the vacuolar membrane by ScFth1p-ScFet5p permease-multi-copper oxidase complex (Urbanowski & Piper, 1999).

In *C. albicans*, the cell surface iron permease-multi-copper oxidase complex has been identified as CaFtr1p-CaFet34p (Van Ho A *et al.*, 2002, Ziegler *et al.*, 2011). However the other homologues of iron transport proteins and multi-copper oxidases have not yet been characterised. A *ScCCC1* homologue has been found to be present in *C. albicans* genome (Braun BR *et al.*, 2005).

Regulation of iron uptake

Iron homeostasis has to be strictly regulated in *C. albicans* so that the organism has sufficient iron to carry out all cellular processes such as respiration while maintaining the levels of free iron below toxic levels. In *S. cerevisiae* Aft1p and Aft2p have been found to be the major iron-responsive regulatory proteins and are responsible for regulation of the majority of the genes involved in iron homeostasis (Rutherford JC *et al.*, 2003, Yamaguchi-Iwai Y *et al.*, 1995).

Transcriptional activators, such as Aft1p and Aft2p, lead to an increase in expression of target genes by recruiting an increased amount of RNA polymerase II and other general transcription factors which can further initiate more transcription. This process of transcription initiation is negatively suppressed by transcriptional repressors. TATA-

box binding protein (TBP) initially binds to TBP-associated proteins to form the general transcription factor TFIID. This is followed by employment of other general transcription factors such as IIB, IIE, IIF and IIH and RNA polymerase II which contribute to the formation of the transcriptional machinery. Specific transcription factors such as Aft1p are connected with the transcriptional machinery by and intermediate which is called as the Mediator complex that directly interacts with the C-terminal domain of RNA polymerase II and increases efficiency of transcription (Bjorklund & Gustafsson, 2005). Mediator without the Srb subunit 8-11 is employed specifically by transcriptional activators to increase expression of target genes since they lack the suppressor of RNA (Srb) subunit; while mediator with the 8-11 Srb subunits are employed by transcriptional repressors such as Tup1p to supress transcription of target genes.

Transcriptional activators and repressors usually regulate transcription of target genes by binding to specific DNA binding sequences present in the promoter regions via their highly precise DNA binding domains. The high degree of specificity is maintained due to the presence of an extensive range of DNA binding domains such as zinc finger, helix-turn-helix and leucine zipper structures which allow for targeted regulation of genes. External environmental conditions such as temperature, iron or copper concentrations lead to an alteration in the activity of the transcriptional protein, and as a consequence, they may undergo conformational changes from inactive to active form or post-translational modifications or may also be degraded (Woodacre, 2007).

In *S. cerevisiae* Aft1p acts as a positive regulator localised within the nucleus and activates transcription of target genes only under low iron conditions. Whereas, in high iron conditions, Aft1p interacts with the nuclear export receptor Msn5p and is rapidly

exported into the cytoplasm thereby preventing further gene activation by Aft1p (Ueta R *et al.*, 2007).

Aft1p and Aft2p have overlapping but distinctive roles in iron regulation and also regulate certain genes that are not involved in iron regulation. They both regulate genes such as *FIT1-3*, *FRE1*, *FET3* and *FTR1* which are required for high affinity iron uptake (Rutherford JC *et al.*, 2003). Deletion of the *AFT1* gene resulted in the expected defective-high affinity iron uptake- phenotypes such as slow growth on low iron, sensitivity to oxidative stress, respiratory deficiency, low levels of iron transport and low ferric reductase activity due to lower expression of genes like *FT1, FET3* and *FRE1*. An *aft2ΔΔ* mutant did not show the expected phenotypes associated with defective high affinity iron uptake but the *aft1Δ/aft2Δ* mutant was found to be more vulnerable to iron shortage and oxidative stress indicating that *AFT2* plays some role in iron homeostasis. When *AFT2* was overexpressed in the *aft1Δ/aft1Δ* mutants, they could be rescued due to increased expression of *FET3* and *FTR1* via Aft2p which suggested that the roles of the two transcription factors were partially redundant and *AFT2* could be an evolutionary product of the *Saccharomyces* branch whole genome duplication event (Rutherford JC *et al.*, 2001). The DNA binding core sequence in the promoter of target genes, is the same for the two proteins (GCACCC), however, Aft1p possesses a higher DNA binding affinity and binds to a slightly longer sequence (TGCACCCA) which makes it a stronger activator than Aft2p (Courel M *et al.*, 2005).

In our laboratory, two *C. albicans* homologues of the *S. cerevisiae AFT* have been identified however; the *Caaft1Δ/Caaft1Δ* mutant was found to grow competently in the presence of respiratory carbon sources and in iron restricted conditions, portraying no deficiency in its high affinity iron uptake system (Mason, 2006b). The *Caaft1Δ/Caaft1Δ* mutant also showed wild-type levels of iron uptake and ferric/cupric

reductase activity and the expression of ferric reductase genes was not significantly affected on deletion of *CaAFT1* although expression of *CaFRE5* and *CaFRE10* showed a slight increase (Mason, 2006b). This indicates that *CaAFT1* is not the functional homologue of *ScAFT1* gene but it may be playing an altered role in iron homeostasis in *C. albicans*.

In microarray study of *C. albicans*, iron responsive regulation has been observed where 526 ORFs showed increased expression in iron restriction and 626 ORFs showed increased expression in iron replete conditions. Iron restriction also triggered a stress response in *C. albicans* with increased expression of superoxide dismutase. Genes involved in iron utilization and production of iron-containing molecules, such as haem, were expressed at higher levels in high iron conditions The constituents of the high affinity iron uptake system, genes homologous to *S. cerevisiae* genes involved in transporting elements between mitochondria and cell membrane and the haem oxygenase gene were expressed at a higher level in low iron conditions. (Lan CY *et al.*, 2004).

Many putative virulence determinants and many genes involved in yeast to hyphal transition also showed increased expression in iron limitation. Low iron condition are a trigger to many pathogens and is indicative of host entry while the yeast to hyphae switch has also been previously associated with virulence (Lan CY *et al.*, 2004).

In many fungi, instead of activation, iron uptake genes are instead repressed in high iron conditions by a GATA-type repressor. Such a regulatory mechanism has previously been observed by Fep1p in *Schizosaccharomyces pombe*, Urbs1p *Ustilago maydis* and SreAp in *Aspergillus nidulans* (Oberegger *et. al.*, 2001; Pelletier *et al.*, 2002; Voisard *et al.*, 1993). A possible candidate that may regulate iron uptake genes

for high affinity iron acquisition in *C. albicans* is Sfu1p. *SFU1* encodes a GATA-type transcriptional repressor that was identified because it shares a large degree of homology with *URBS1* from *U. maydis* and the putative protein possesses two N-terminal zinc finger domains situated on either side of a cysteine-rich region containing four largely conserved cysteine residues which is characteristic of GATA-type transcriptional proteins (Lan CY *et al.*, 2004). In previous microarray studies, Sfu1p has shown to repress *CaFRE1, CaFRE2, CaFRE5, CaFRE31, CaFET34, CaFET35, CaFTR1, CaFTH1, CaCCC2* and *CaCTR1* in high iron concentrations (Lan CY *et al.*,). Furthermore, in the heterologous *S. pombe* system Sfu1p was shown to rescue a *fep1Δ* mutant and bind the consensus DNA sequence AGATAA (Pelletier *et. al.*, 2007). It was also shown to physically interact with the Tup11p and Tup12p global regulators from *S. pombe*, which are homologous to the *C. albicans* Tup1p, which has been previously shown to regulate *CaFRE10* expression (Knight SA *et al.*, 2005).

Work carried out in our laboratory demonstrated that in a *sfu1Δ/sfu1Δ* mutant, increased amounts of cell surface ferric and cupric reductase activity was observed in co-relation to the increased transcription of ferric reductase genes, *CaFRE5* and *CaFRE10*. We have also shown that in *sfu1Δ/sfu1Δ* mutant, the increased expression of the iron permease gene *CaFTR1* was also in correspondence to the increased amounts of ferric iron uptake (Jeeves, 2008, Mason, 2006a).

There have been previous reports implicating the Sfu1p-Tup1p transcription repressor system, however research is still under progress on this and Sfu1p has not been able to explain the iron uptake regulation in *C. albicans* adeptly; which indicates the presence of an additional regulator. It has been seen that Sfu1p is not the sole iron responsive regulator in *C. albicans* since a putative ferric reductase gene *FRP1* was shown to be regulated by iron independently of Sfu1p (Baek *et al.*, 2008). Also, to date, much

remains unknown about *C. albicans* iron regulation, particularly *in vivo* and for the low affinity systems. It is also unclear as to which regulatory protein is responsible and how exactly it regulates the genes involved in high affinity iron uptake in *C. albicans*.

Recent reports indicate that the putative transcription factor gene *SEF1* may represent a positive regulator of *C. albicans* iron acquisition (Homann OR *et al.*, 2009). Homologues of *SEF1* have also been identified in *Kluyveromyces lactis* and *Saccharomyces cerevisiae* where it was found to supress a mutation in an essential gene involved in synthesis of a yeast mitochondrial RNase P enzyme (Groom *et al.*, 1998). The putative regulator *SEF1* was identified in *C. albicans* in a screen for candidate virulence factors ((Noble *et al.*, 2010). The *SEF1* gene has shown expression changes in the presence of the iron/copper chelator BPS and a *sef1Δ/sef1Δ* displayed growth reduction on YEPD with either BPS or alkaline pH when compared to wild type. Additionally in microarray studies, up-regulation of Sef1p was observed in *suf1Δ/sfu1Δ* (Lan CY *et al.*, 2004). This evidence suggested that *SEF1* was required for growth in iron restricted media and Sef1p may be involved in iron regulation, to transcriptionally activate genes involved in iron acquisition. This hypothesis indicated a supplementary mechanism for regulation in addition to the prevailing model of iron regulation already described utilizing the transcriptional GATAA-type repressor Sfu1p. This data indicated that *C. albicans* had both an activator and a repressor acting to regulate expression of genes in varying iron concentrations and hence this was decided to be studied further, in this report.

1.3. Copper homeostasis in Fungi

In *C. albicans* copper uptake is intrinsically linked with iron uptake and hence the mechanism used for obtaining both the metals is similar. Similar to iron uptake, the organism has a high affinity (with affinity of less than 2 μM) and a low affinity (with affinity of 22 μM) copper uptake system for acquiring the essential metal (Hassett R *et al.*, 1995, Hassett R *et al.*, 2000). In this report the model organism *S. cerevisiae* is used to study and compare the highly conserved genes and proteins involved in the copper uptake system with the system in *C. albicans*.

Copper is generally found in the cupric (Cu^{2+}) state in the environment but it is taken up into the yeast cell in the form of Cu^+ and not Cu^{2+}; since high affinity copper uptake has been found to be linked with cell surface cupric reductase activity (Dancis A *et al.*, 1994, Hassett R *et al.*, 1995). 85-90% of the cell surface cupric reductase activity in *S. cerevisiae* has been found to be carried out by the ferric (as well as cupric) reductases ScFre1p and ScFre2p. However, ScFre1p is more active and is mainly responsible for the cell surface metal ion reductions (Georgatsou E *et al.*, 1994).

Copper transporter family and copper binding

S. cerevisiae shows the presence of a copper transporter gene *CTR1* which was initially identified while screening for mutants with defective high affinity iron uptake system since *ctr1* mutants showed slow growth in the presence of environmental copper and also had defective ferric iron uptake activity. This proved yet again that copper is essential for high affinity iron uptake (Dancis A *et al.*, 1990, Dancis A *et al.*, 1994) since the iron permease requires copper molecules for its activity. The *ScCTR1* gene contains 8 Met-X_2-Met repeated motifs in the N-terminal region. In many species of

bacteria, this met motif has been observed to be conserved in various copper transporter proteins. The gene functions as an oligomer and localises to the plasma membrane while maintaining its C-terminal domain, intracellular (Dancis A *et al.*, 1994).

Another copper transporter *ScCTR2* was identified and in *Scctr2Δ* homozygotes, loss of high affinity iron uptake was observed when *ScCTR2* was knocked-out (Portnoy *et al.*, 2001) and in spite of being regulated by iron limitation, the deletion also led to an accumulation of copper within the vacuole (Rees *et al.*, 2004). There is another high affinity copper transporter gene *ScCTR3* which is not generally expressed in most laboratory strains of *S. cerevisiae* due to transposon insertion in the promoter region of the gene. Double deletion mutants *ctr1Δ/ctr3Δ* were found to display the same phenotypes as the original *ctr1* mutant, such as slow growth in low copper or iron media, decreased or no uptake of radiolabelled copper and iron, sensitivity to oxidative stress and respiratory defects (Dancis A *et al.*, 1990, Dancis A *et al.*, 1994).

In *C. albicans*, homologues of *ScCTR1* and *ScCTR2*, *CaCTR1* and *CaCTR2* have been identified respectively. *CaCTR1* was able to rescue a *Scctr1ΔΔ* mutant and was also found to be regulated by copper restriction (Dancis A *et al.*, 1994, Marvin *et al.*, 2003, Marvin *et al.*, 2004). *CaCTR1* deficient mutants showed slow growth in copper restricted conditions, respiratory deficiency on non-fermentable carbon sources and increased vulnerability to oxidative stress indicating copper scarcity since copper is essentially required for these processes (Marvin *et al.*, 2003)

The Ctr family of copper transporters show presence of highly conserved regions from humans to yeast. Excluding Ctr3p of *S. cerevisiae*, N-terminal methionine motifs, C-terminal cysteine/histidine motifs and three transmembrane domains are maintained by all proteins (Marvin *et al.*, 2003, Puig S *et al.*, 2002).

Chemical and genetic analysis have determined that methionine motifs (mets) function as copper-binding sequences and each Ctr protein (except Ctr3) contains one to eight mets motifs in the N-terminal region (Marvin *et al.*, 2003, Puig S *et al.*, 2002, Jiang J *et al.*, 2005). Experiments using synthetic peptides containing 3 methionines showed that the motif could bind one Cu ion with the same dissociation constant (2.6 μM) as observed in yeast's high affinity copper uptake system (Jiang J *et al.*, 2005). It was also observed that on substitution of any two of the methionines in the peptide with nor-leucine, the motif completely lost its copper binding ability showing that in yeast the other copper ligands histidine and cysteine do not take part in copper binding.

Experiments performed on ScCtr1p via sequential removal of N-terminal mets motifs helped determine that only the last methionine of the mets motif was essential for copper uptake (Puig S *et al.*, 2002). Contrastingly work in our laboratory showed that mutation of the last methionine of the mets motif in CaCtr1p had no effect on copper uptake in *C. albicans* (Marvin et al., 2004).

Low affinity copper uptake

It has been observed that even in the presence of copper-replete conditions, *S. cerevisiae* and *C. albicans* strains that lack the high affinity copper transporter genes *CTR1, CTR3* and *CaCTR1* still show growth and hence this indicates that there exists an alternate low-affinity mechanism for transportation of copper on the cell surface in these yeasts (Knight SA *et al.*, 2002, Marvin *et al.*, 2003, Dancis A *et al.*, 1994). Studies on *S. cerevisiae* have shown that the low affinity ion transporter Fet4p which is capable of transporting Fe^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+} and Cu^{2+} plays a role in low affinity copper uptake. However it has been observed that the *ctr1Δctr3Δfet4Δ* mutants which did not show any observable radioactive Cu^{2+} uptake was still able to grow in copper

replete conditions indicating the existence of an additional copper uptake system or the presence of a mechanism by which the intracellular copper stores can be used (Hassett R *et al.*, 2000).

There have been theories about the low affinity metal transporter Smf1p which can transport divalent cations Fe^{2+} and Mn^{2+} and may also be able to transport copper in its divalent form. Overexpression of *SMF1* has been observed to cause copper toxicity which indicates that it may be responsible for copper uptake in oxidising conditions which would also explain why no radioactive Cu^+ uptake was detected in the triple mutant (Liu XD *et al.*,). Similar to iron, copper acquisition can also be brought about by a high affinity as well as a low affinity system in most organisms, including *S. cerevisiae*. ScFet4p has been found to be capable of transporting copper and has been demonstrated to be involved in low affinity copper transport (Hassett R *et al.*, 2000). However overexpression of another low affinity copper transporter, ScSmf1p, has found to lead to copper toxicity within the cell indicating that ScFet4p may not be the sole low affinity copper transporter in *S. cerevisiae* (Liu XD *et al.*, 1997)

Homologues of *ScFET4* have as yet not been identified in *C. albicans* however there is evidence to suggest the presence of a low affinity copper uptake mechanism because of survival and growth of strains on copper replete media in spite of lacking high affinity copper transporter proteins (Marvin *et al.*, 2004, Marvin *et al.*, 2003).

Intracellular copper transport (Copper chaperone proteins)

Intracellular copper levels are monitored strictly to avoid formation of toxic reactive free-oxygen radicals and the cytosol of *S. cerevisiae* has shown the presence of one free copper ion per cell (Rae TD *et al.*, 1999). Within the cytosol the major amount of

copper is found bound to copper chaperone proteins such as Atx1p (a copper ATPase), ScCcc2p and ScGef1p (a voltage regulated chlorine channel) for delivery to various cupro-proteins within the cell (Van Ho A *et al.*, 2002, Field LS *et al.*, 2002). All these copper binding proteins show the presence of one or more copper binding domains [M/L][T/S]CXXC. Ccc2p and Atx1p are both capable of binding one copper ion and it has been hypothesised that Atx1p transfers copper from plasma membrane copper transporter Ctr1p to the various cellular locations like the Golgi where it interacts with ATPase-Ccc2p which transports copper into the Golgi where it is utilised for the production of copper loaded-Fet3p (Yuan DS *et al.*, 1997).

Copper is transferred via a physical electrostatic interaction between the two proteins Atx1p and Ccc2p with the formation hydrogen bonds to stabilise the complex. Atx1p transfers the copper ion to the first amino terminal Atx1p-like domain in ScCcc2p. It has also been suggested that copper transport may occur even in the absence of Atx1p since Ctr1p is capable of exchanging copper ions with Ccc2p directly, and also deletion of *CCC2* leads to complete loss of high affinity iron uptake whereas deletion of *ATX1* only causes partial reduction of ferrous iron uptake (Lin SJ *et al.*, 1997, Xiao Z *et al.*, 2004). *C. albicans* also requires *CaCCC2* (*ScCCC2* homologue) for ferrous iron transport and hence the mechanism of copper-loading onto multicopper oxidase may be similar to that in *S. cerevisiae*. Deletion of *CaCCC2* led to loss of high affinity iron transport, however it did not affect virulence in a systemic mouse model of candidiasis *(Weissman Z et al.,)*. Virulence was possibly not affected on deletion of *CaCCC2* because iron uptake could have still occurred via an alternate pathway involving siderophore or haem utilisation.

Storage of copper in vacuoles

In yeasts, copper can be stored in vacuoles and transported across the vacuolar membrane during copper starvation conditions by high affinity copper transporter Ctr2p that requires reduction of Cu^{2+} in the vacuole by Fre6p before it can be transported into the cytoplasm. It has been suggested that the vacuole may play a likely role in copper homeostasis in *C. albicans* as increased sensitivity to copper toxicity was observed on disruption of the vacuole-associated protein (Vac1p) (Franke *et al.*, 2006). A homologue of the *ScCTR2* gene has been identified in the *C. albicans* genome which may have a copper transporter function similar to *ScCTR2*. However, further studies in *C. albicans* are required to experimentally characterise this gene.

Regulation of copper uptake

Copper homeostasis must be controlled and regulated strictly within the organism to ensure availability of optimal copper levels which allow the organism to carry out the essential cellular processes such as respiration but keep the copper levels below toxic concentrations. Regulation of copper metabolism occurs at number of points such as transcriptional, post-transcriptional, translational and post-translational regulation.

Transcriptional Regulation

Previous transcriptional profiling studies carried out in *S. cerevisiae* and *S. pombe* showed that not only the expression of the copper homeostasis genes was altered but also other cellular processes such as respiration, oxidative stress response, metabolism of other metal ions such as iron homeostasis, and transcription of a large number of genes with undetermined functions were affected due to fluctuations in extracellular copper levels (van Bakel H *et al.*, 2005, Gross C *et al.*, 2000). In *S. cerevisiae*

transcription of *AFT1* and the majority of Aft1p-regulated genes showed an increase in expression in copper restricted conditions which indicated an association between transcriptional response to copper and iron levels (van Bakel H *et al.*, 2005, Gross C *et al.*, 2000). Additionally the transcription of genes that encoded for copper and iron-containing proteins was also observed to be decreased in low copper conditions which suggested that copper starvation conditions within the cell lead to a secondary iron starvation which required an increase in iron uptake and regulated usage of the available iron within the cell (van Bakel H *et al.*, 2005, Gross C *et al.*, 2000).

ScMAC1 and ScMa1p regulon

The main copper-responsive transcription factor gene in *S. cerevisiae* is *ScMAC1* which was identified because it is highly similar to previously known copper-responsive transcription factors such as Ace1p and Amt1p in *S. cerevisiae* and *C. glabrata* respectively (Keller *et al.*, 2005, Graden *et al.*, 1996). Several functional domains such as the N-terminal DNA binding domain which is composed of approximately 40 amino acids, names as a copper-fist domain has been found to be conserved in transcription factors such as Ace1p, Amt1p and Mac1p and plays a vital role in the protein's DNA binding activity (Dameron CT *et al.*, 1991, Joshi A *et al.*, 1999). The copper-fist region contains extremely conserved histidine and cysteine residues and in Amt1p and Ace1p, these residues have been shown to be capable of directly binding single Zinc ions. The evolutionary conservation of this domain suggests that all such copper-responsive proteins are likely to be able to bind Zinc at this position (Farrell RA *et al.*, 1996). At the C-terminal end, another binding motif exists which binds to the minor groove of DNA of target genes (Koch KA *et al.*, 1996).

ScMac1p functions as a homo-dimer and binds to two CuRE (copper-responsive elements) sequences in target promoters of genes where the level of dimerisation is proportional to its activation activity (Joshi A *et al.*, 1999, Jensen LT *et al.*, 1998). Experiments were performed where the predicted D-helix (residues 388-406) which is required for the formation of homo-dimer and protein : protein interactions was deleted or mutated and it was observed that this mutated ScMac1p could not rescue a *mac1Δ* mutant indicating that dimerisation was essential for the functioning of ScMac1p *in vitro* in *S. cerevisiae* (Serpe M *et al.*, 1999).

ScMac1p has two cysteine-rich domains in the C-terminal portions referred as C1 and C2 respectively. These domains are also conserved in CaMac1p and SpCuf1p and possess the consensus sequence $CXCX_4CXCX_2CX_2H$ (Marvin *et al.*, 2004, Jensen LT *et al.*, 1998). The conserved cysteine and histidine residues in the C1 and C2 domain are involved in copper-responsive activation property of the protein. The C1 domain of ScMac1p was found to be able to directly bind four copper ions and when the last histidine residue was mutated to alanine, a loss of response to copper was detected for ScMac1p and resulted in a constitutively active mutant (Jungmann J *et al.*, 1993, Brown KR *et al.*, 2002). However when the C2 domain was mutated it led to a decline in activation property of the protein but it did not affect the regulation of the activity in response to extracellular copper indicating that the C2 domain is also able to bind four copper ions but it only functions to strengthen the activation activity of ScMac1p (Jensen LT *et al.*, 1998, Keller G *et al.*, 2000).

ScMac1p is a positive transcriptional regulator and has been observed to activate transcription of the ferric/cupric reductase gene *FRE1* in response to copper restrictive conditions (Jungmann J *et al.*, 1993). ScMac1p has also been shown to be indispensable for copper-responsive transcription of copper transporter genes *CTR1* and *CTR2* and the

ferric-reductase-like gene *FRE7* (Martins LJ *et al.*, 1998, Labbe S *et al.*, 1997). On further analysis it was seen that the promoters of all these genes contained a common promoter element possessing the sequence TTTGC(T/G)CA which was analogous to the sequence recognised by ScAce1p and CgAmt1p in *S. cerevisiae* and *C. glabrata* respectively (Martins LJ *et al.*, 1998, Labbe S *et al.*, 1997). ScMac1p bound to this sequence element during copper scarcity and two of these elements were necessary to be present in the promoter region of the target gene for nominal copper regulation of transcription and hence the element was called a copper response element or CuRE (Martins LJ *et al.*, 1998, Yamaguchi-Iwai Y *et al.*, 1995, Labbe S *et al.*, 1997). The strength with which ScMac1p binds to the CuRE is directly proportional to the level of its activation activity and increased binding and activation activity has been observed when the CuRE is preceded by TA instead of TT since ScMac1p shows greater binding to TA (Joshi A *et al.*, 1999). Using microarray technology, ChIP analysis and checking for the presence of CuREs in the promoter region helped identify additional genes in the ScMac1p regulon, however the genes *YFR055w* and *YJL217w* showed presence of only one CuRE in the promoter. As a result there is ambiguity as to whether these genes are truly transcriptionally activated by ScMac1p (Gross C *et al.*, 2000).

S. cerevisiae also shows the presence of a post-transcriptional regulation system that prevents the remaining Ctr1p from transporting possibly harmful amounts of copper within the cell. ScMac1p plays a role in the internalisation and degradation of Ctr1p, when copper concentrations within the cell reach 10 μM or more (Yonkovich J *et al.*, 2002, Ooi CE *et al.*, 1996). The mechanism by which ScMac1p helps in degradation is not known but possibly it activates certain proteins that maybe a part of the degradation pathway. However Ctr3p is not degraded even at high copper levels and maybe

maintained at the plasma membrane to prevent copper starvation when there is a sudden drop in extracellular copper levels (Pena MM *et al.*, 2000).

Electrophoretic mobility shift assays demonstrated that purified ScMac1p can bind to CuRE element having consensus sequence TTTGC(T/G)C(A/G). A point mutagenesis experiment, showed that deviation from the consensus CuRE sequence, led to target promoters showing reduced affinity for Mac1p and as a consequence, a decrease in reporter gene activity was observed (Labbe *et al.,* 1997). It was detected that at least two CuREs were required for wild-type expression levels of *CTR1, CTR3* and *FRE7* in *S. cerevisiae*.

In *S. cerevisiae* the *MAC1* gene is constitutively transcribed however when the intracellular copper levels are at 1 μM or higher, ScMac1p folds to allow an intramolecular interaction between its DNA binding and activation domains thereby effectively blocking the DNA binding and transactivation activity of the protein. As a result the target genes in the ScMac1p regulon are not transcribed at moderate copper levels of 1-10 μM and the protein is degraded when the copper levels exceed this range and becomes potentially toxic to the cell (Jensen LT *et al.*, 1998, Yonkovich J *et al.*, 2002). The organism shows a range of responses to nutritionally required and toxic copper levels and it was also found that native ScMac1p is phosphorylated and that phosphorylation of the protein was essential to induce DNA binding (Heredia J *et al.*,).

CaMAC1 **and CaMac1p regulon**

Previous work in our laboratory identified a sequence (orf 19.7068) on the *C. albicans* genome which showed significant identity with *ScMAC1* and the putative Mac1 protein sequence from *C. albicans* was found to share 35% similarity and 26.2% identity with ScMac1p. On further analysis it was observed that the CaMac1p sequence contained

specific conserved motifs that indicated that it was a functional homologue of ScMac1p. CaMac1p also shows the presence of a copper fist motif at the N-terminus. This amino motif had also been detected in several copper-responsive transcription factors, including Mac1p and Ace1p of *S. cerevisiae* (Jungmann J *et al.*, 1993) and Amt1p of *C. glabrata* (Zhou and Thiele, 1991) and Cuf1p of *Schizosaccharomyces pombe* (Labbe S *et al.*, 1997). In all these fungi, the motif was found to be responsible for protein - DNA binding in the presence of silver or copper (Jensen and Winge 1998). Further analysis showed the presence of two cysteine-rich segments having a consensus sequence of $CXCX_4CXCX_2CX_2H$ between residues 169 and 224 and residues 246 and 301 which shared significant identity with the C1 and C2 (or REP1 and REPII) motifs found in ScMac1p (Marvin *et. al.,* 2004). These motifs are involved in copper-responsive control of expression as they prevent binding of ScMac1p to CuREs in high-copper conditions thereby preventing activation of transcription of copper transporter genes (Labbe S *et al.*, 1997). The C1 and C2 motif is separated by 42 residues in ScMac1p whereas it is separated by 61 residues in CaMac1p. Analysis of the predicted CaMac1p amino acid sequence, carried out in our laboratory, indicated a 431-amino acid protein product that had 73.9% probability of being a nuclear protein (Marvin *et al.*, 2004).

Further work carried out in our laboratory, found that a *Camac1Δ/Camac1Δ* mutants showed phenotypes that were comparable to the *Scmac1Δ* mutant such as demonstrating respiratory defects, reduced growth in copper and iron deficient media and increased sensitivity to oxidative stress (Marvin *et al.*, 2004). It was found to regulate *CaCTR1* in response to low copper conditions and the regulation was shown to be dependent on three putative CuRE sequences that were identical to the ScMac1p CuRE sequence and were found upstream of the gene at -393, -275 and -273 of the
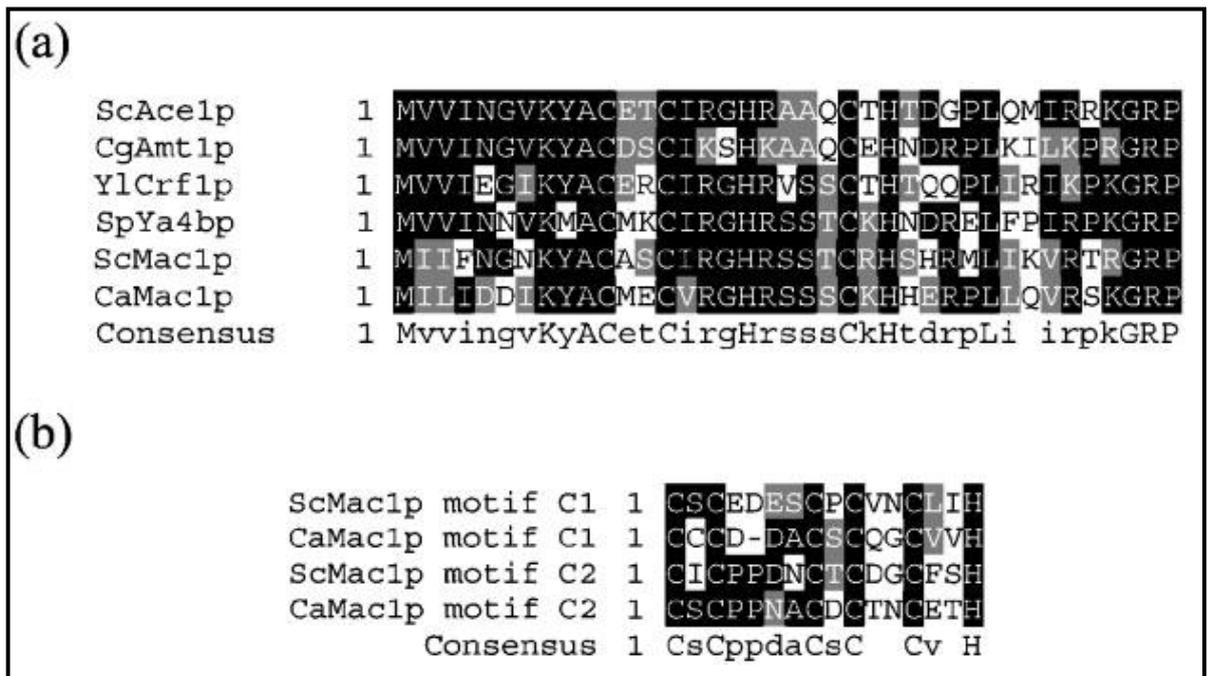
Figure 1. 5: Sequence comparisons

(**a.**) Comparison of the putative copper fist motif of CaMac1p with analogous motifs found in the copper-responsive transcription factors of *S. cerevisiae* - ScMac1p and ScAce1p, C. *glabrata* - CgAmt1p, *S. pombe* - SpCuf1p and *Y. lipolytica* - YlCrf1p. (**b.**) Comparing between the cysteine-rich copper-binding sites found in ScMac1p and putative copper binding sites of CaMac1p (Marvin *et al.*, 2004).
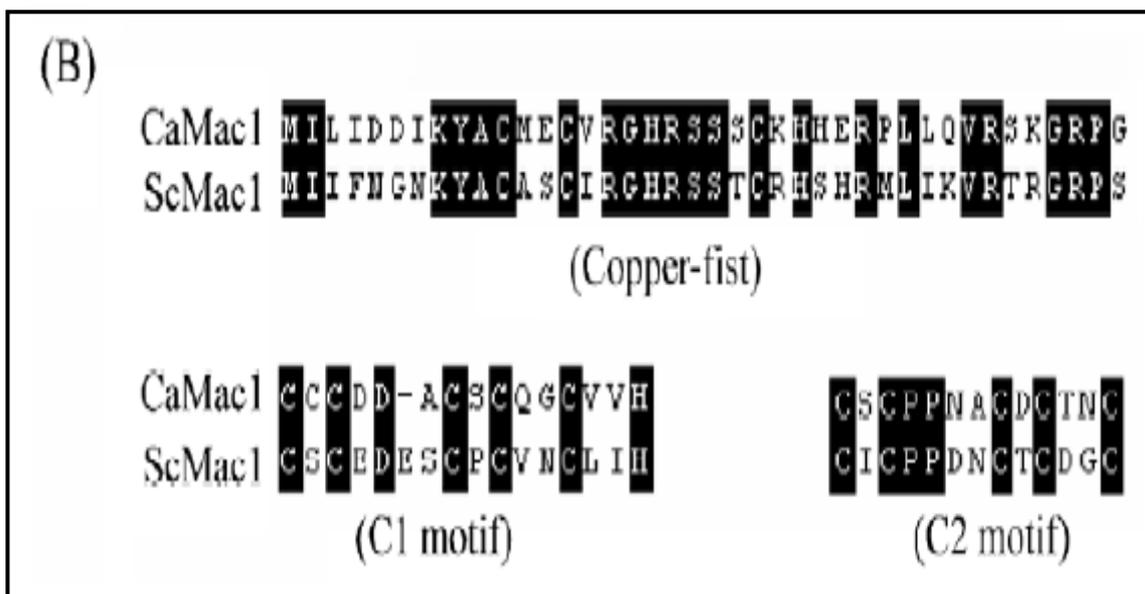
Figure 1. 6: Sequence alignment of C. albicans CaMac1 and S. cerevisiae ScMac1

Comparing the protein sequences between the three motifs of CaMac1p and ScMac1p (Hwang CS *et al.*, , Huang GH *et al.*,).

start codon. An additional sequence was also observed to be present in the *CaCTR1* promoter that contained the core sequence of GCTCA but the preceding TTT sequence was missing. When all three CuRE elements in the *CaCTR1* promoter were mutated, copper sensitive regulation of the *LacZ* reporter was lost. However mutation of only the -397 bp upstream CuRE led to a reduction in expression of the *LacZ* reporter, but up regulation in response to copper limitation was still present. When the -275 bp upstream CuRE was mutated it led to a 40% decline in the expression of the *LacZ* reporter in copper replete conditions but it showed activity comparable to the wild-type in copper restrictive conditions. When the -237 bp upstream CuRE was mutated, no significant changes in expression of the *LacZ* reporter were detected. Additionally in *Camac1Δ/Camac1Δ* mutant, the wildtype *CaCTR1* promoter showed absence of copper responsive regulation (Jeeves, 2008, Woodacre *et al.*, 2008).

CuREs were found to be present in the promoters of several genes including the putative ferric reductase *CaFRE7* (Woodacre *et al.*, 2008, Levitin A *et al.*, 2007). The promoter of *CaFRE7* contained two CuREs and the mutation of both of these resulted in a loss of copper sensitive regulation however when only -177 CuRE was mutated it resulted in an increase in *LacZ* reporter in low copper conditions, and when -132 CuRE was mutated it showed no significant difference from wild-type expression in low copper conditions. Also in *Camac1Δ/Camac1Δ* mutant copper responsive expression of the *LacZ* reporter gene under the native *CaFRE7* promoter was not observed indicating that CaMac1p is responsible for copper sensitive regulation of genes via the CuRE promoter sequence (Woodacre *et al.*, 2008, Levitin A *et al.*, 2007).

Figure 1. 7: A simplified diagrammatic representation of the iron and copper homeostasis in *Saccharomyces cerevisiae*

Not to scale. The nucleus is pale yellow and the vacuole is beige. Copper uptake is shown in blue and iron uptake is shown in red. Iron and copper are usually available in the host environment in their insoluble ferric and cupric forms and in limiting iron conditions they are converted to their more soluble ferrous and cuprous forms by the major cell surface ferric reductase proteins (**Fre1p, Fre2p** and **Fre7p**). Once reduced copper is transported within the cell by copper transporters (**Ctr1p** and **Ctr3p**) while iron is transported across the cell membrane via a multi-copper oxidase-permease complex (**Fet3p-Ftr1p**). Once inside the cell, copper is shuttled around by the copper chaperone protein (**Ccc2p**). The **Arn** proteins are involved in uptake and utilization of siderophores as an iron source while **Smf1p** and **Fet3p** are low affinity iron and copper transporters. The **Fit** proteins increase the association of iron with the cell membrane. The vacuole is used for storage of iron and ferrous iron is transported across the vacuolar membrane in iron replete conditions by an **Fth1p-Fet5p** complex or by **Ccc2p**. **Mac1p** regulates most copper-related and some iron-related genes in response to copper. **Aft1p** and **Aft2p** are the major iron-responsive gene regulators in *S. cerevisiae*.
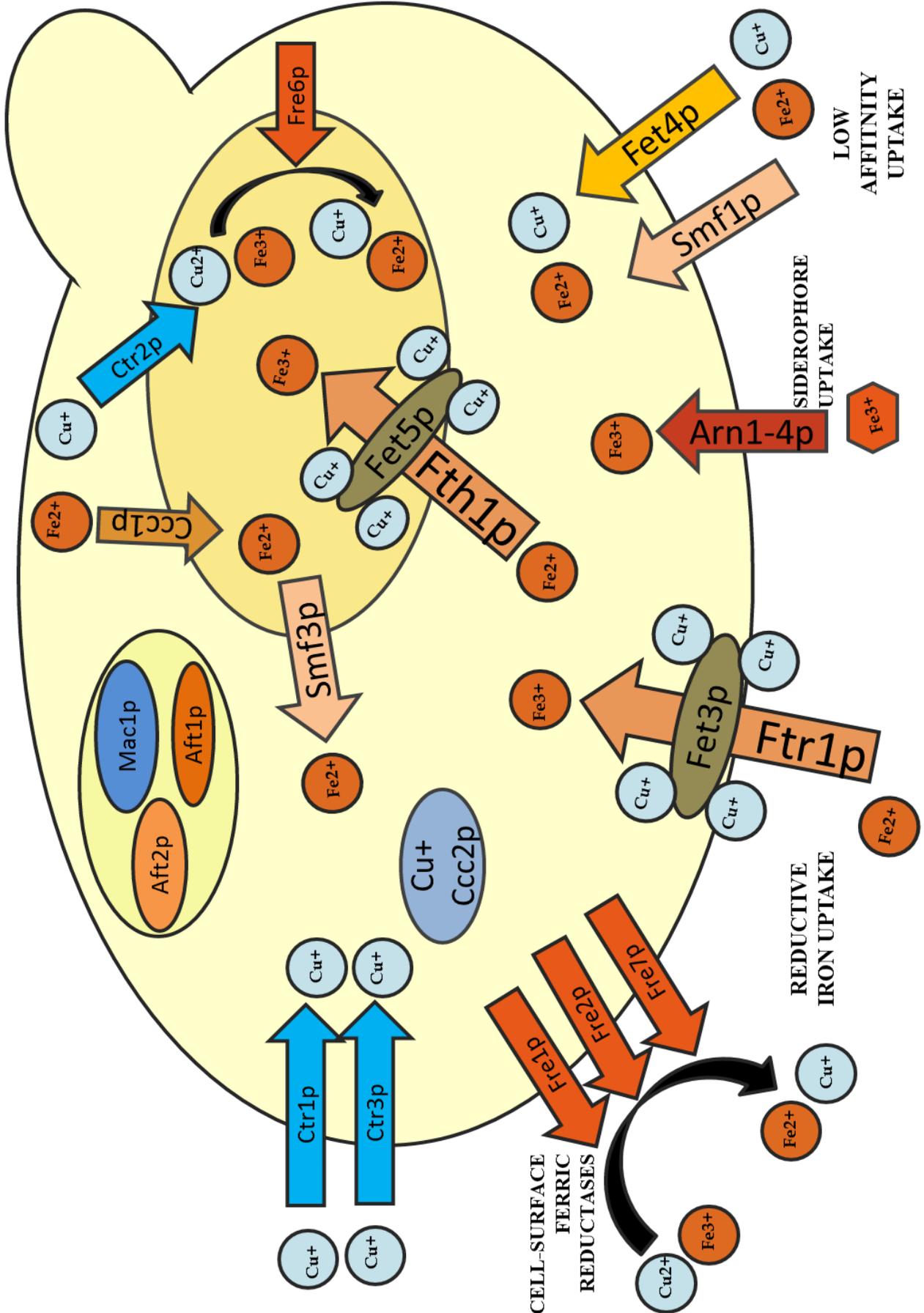
Figure 1. 8: A simplified diagrammatic representation of the iron and copper homeostasis in Candida albicans

Not to scale. The nucleus is cream and the vacuole is yellow. Copper uptake is shown in blue and iron uptake is shown in red. Iron and copper are usually available in the host environment in their insoluble ferric and cupric forms and in limiting iron conditions they are converted to their more soluble ferrous and cuprous forms by the major cell surface ferric reductase proteins (primarily by **CaFre10p** and also by **CaFre7p**). Once reduced, copper is transported within the cell by copper transporter (**CaCtr1p**) while iron is transported across the cell membrane via a multi-copper oxidase-permease complex (**CaFet34p-CaFtr1p**). Once inside the cell, copper is shuttled around by the copper chaperone protein (**CaCcc2p**). The **Arn** and **Sit1p** proteins are involved in uptake and utilization of siderophores as an iron source while **Rbt5p** is involved in utilization and uptake of haemoglobin from the host's serum, as a source of iron. **Als3p** is responsible for utilization and uptake of ferritin as a source of iron, after reduction at the cell membrane by the ferric reductases. The low affinity iron and copper transporters have not been identified, as yet. The proteins involved in employment of transferrin as an iron source, have not yet been characterised. The vacuole is used for storage of iron and ferrous iron is transported across the vacuolar membrane in iron replete conditions by a multicopper oxidase-iron permease complex which has not been identified or by **CaCcc2p**. **Mac1p** regulates most copper-related and some iron-related genes in response to copper. **Sfu1p** is a transcriptional repressor while **Sef1p** is a potential transcription activator. These are the two likely candidates that may play a role in regulation of major iron uptake and utilization genes in *C. albicans*.
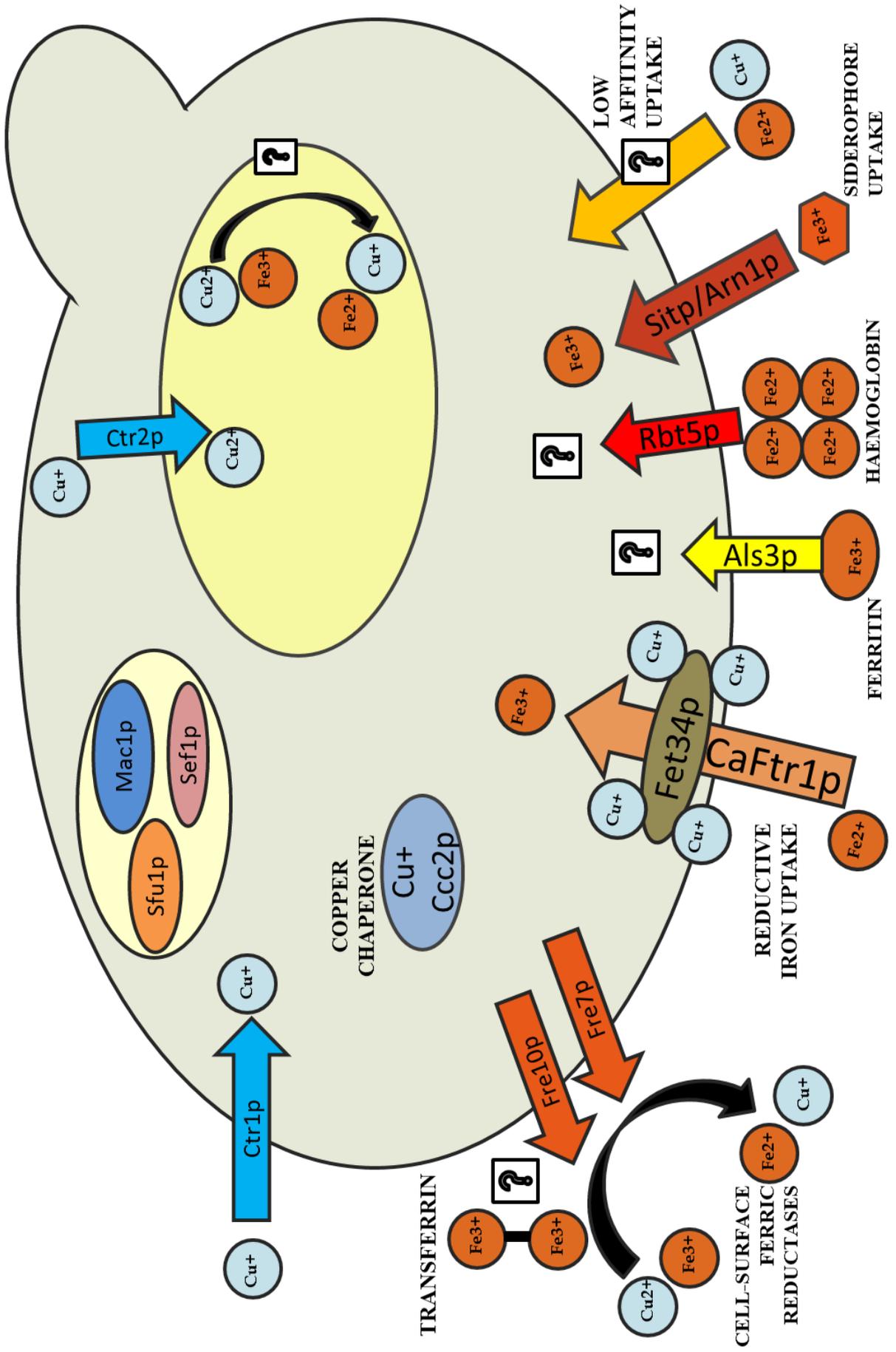
1.4. Background to project

The overall aim of this project was to investigate regulatory mechanisms involved in iron and copper acquisition in *C. albicans*. This section summarises the previous findings which proposed the questions that led to the individual aims of this study.

In *S. cerevisiae* the *ScMAC1* gene is constitutively transcribed but using a *LacZ* reporter it was shown that the *C. albicans CaMAC1* is activated in response to low copper conditions. The copper responsiveness is lost in a *Camac1Δ/Camac1Δ* mutant and when the single CuRE found in the *CaMAC1* promoter is mutated, copper responsive behaviour of the gene is abolished indicating that CaMac1p auto-regulates its own gene's (*CaMac1*) transcription in response to copper which has not been seen in *S. cerevisiae*. Work carried out in our laboratory led to the hypothesis that CaMac1p binds to the CuRE sequences in promoters of *CaCTR1*, *CaFRE7* and *CaMAC1*, activating their transcription in low copper conditions (Woodacre *et al.*, 2008). This led us to the question, whether the molecular mechanism of action of the CaMac1p was fundamentally different from the mechanism of action of the ScMac1p?

In *C. albicans* the positive regulator of the high affinity iron uptake system that may cause an increase of transcription initiation of the ferric and cupric reductase genes as well as the iron transporters in low iron conditions, has as yet not been identified. Further studies of the high affinity system are required to understand its regulation mechanism. However, in *sfu1Δ/sfu1Δ* mutant and in iron limiting conditions the expression of the putative iron responsive transcription factor *SEF1* was found to be up-regulated, indicating its expression is induced in the absence of Sfu1p (Lan CY *et al.*, 2004). Sef1p is a putative transcription activator with Zn-cluster DNA binding motif. Previous reports have also indicated that the transcription factor *SEF1* may represent a

positive regulator of *C. albicans* iron acquisition as it has shown expression changes in the presence of the iron/copper chelator BPS and additionally a *Casef1Δ/Casef1Δ* knockout mutant displayed growth reduction on YEPD with either BPS or alkaline pH when compared to wild type (Homann OR *et al.*, 2009) This led us to the question whether Sef1p is the most likely candidate that plays a potential role in activating the genes involved in high affinity iron uptake in low iron conditions in *C. albicans*?

SEF1 has been cited as the positive regulator of the iron acquisition genes in *C. albicans* and it may act as a regulator of the ferric reductases (Lan CY *et al.*, 2004, Homann OR *et al.*, 2009), however no *in vitro* functional analysis of this regulation had been carried out until the commencement of this study. Previously, work carried out in our laboratory has shown that Sfu1p is only involved in iron responsive regulation and may not be involved in copper sensitive regulation of the ferric reductases. It was also seen that Sfu1p repressed the cupric reductase activity but it was not responsible for the iron or copper responsive regulation of the cupric reductases in *C. albicans* (Jeeves R *et al.*, 2011). This led to the question, could the putative transcriptional regulator Sef1p be responsible for the copper responsive regulation of the ferric reductases and may also play a role in copper and iron responsive regulation of the cupric reductases?

Reports from work done in our laboratory also indicate that *SFU1* is also self-regulated and the regulation of the two transcription factors *SFU1* and *MAC1* may be intimately linked (Woodacre A *et al.*, 2008; Jeeves R *et al.*, 2011). As the iron uptake system is so closely linked with that of the copper uptake it would seem likely that copper sensitive regulation would correspondingly play a part in regulating high affinity iron uptake. It has been shown in our lab that *SFU1* itself is regulated by CaMac1p and is derepressed in a *mac1Δ/mac1Δ* mutant. In a *sfu1Δ/sfu1Δ* mutant an increase in *CaMAC1* expression was also observed (Jeeves, 2008, Woodacre *et al.*, 2008). This led us to question

whether the transcriptional regulators CaMac1p, Sfu1p and Sef1p are inter-related and work together to regulate the iron and copper homeostasis in *C. albicans*?

This work will also help us distinguish between the iron and copper homeostasis system in *C. albicans* from similar systems in the model yeast *S. cerevisiae*. These differences could enable *C. albicans* to respond more precisely to environmental changes, conferring an adaption to the human host that may be an advantage in the disease process. By gaining an understanding of the molecular mechanism of functioning of the regulatory proteins, we will increase our understanding of the specific role of iron and copper acquisition in the virulence of *C. albicans* and other such pathogenic fungi.

The aims of this project are:-

- Analyse the molecular mechanism of action of CaMac1p in *C. albicans*.

- Identify and characterise the role of Sef1p in high affinity iron uptake in *C. albicans*.

- Characterise the regulatory link between iron and copper homeostasis systems in *C. albicans*.

2. Materials and Methods

2.1. Strains used during this study

Bacterial strains

All bacterial transformations were carried out using chemically competent TOP10 *E. coli* purchased from Invitrogen.

Genotype:

(F- mcrA D(mrr-hsdRMS-mcrBC) F80lacZDM15 DlacX74 recA1 araD139 D(ara-leu)7697 galU galK rpsL (Str[R]) endA1 nupG)

Yeast strains

Strain	Genotype	Reference
SC5314	Clinical isolate	Gillium *et al.*, 1984
CAF-2	As SC5314 but *ura3Δ::λimm434/URA3*	Fonzi & Irwin, 1993
CAI-4	As CAF-2 but *ura3Δ::λimm434/ura3Δ::λimm434*	Fonzi & Irwin, 1993
BWP17	As CAI-4 but *ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	Wilson *et. al.*, 1999
SN152	As SC5314 but *URA3/ura3Δ, argΔ/argΔ, leuΔ/leuΔ, his1Δ/his1Δ, IRO1/iro1Δ*	Noble & Johnson, 2005
Mem-m2	As BWP17 but *mac1Δ::URA3/mac1Δ::ARG4; his1::hisG/his1::hisG::HIS1*	Marvin *et al.*, 2004
CNA6	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG sfu1Δ::URA3/ sfu1Δ::HIS1*	Lan CY *et al.*, 2004
sef1Δ/sef1Δ	As SN152 but *sef1Δ::HIS1/sef11Δ::LEU2*	Homann OR *et al.*, 2009
AWP1	BWP17 with *RPS10*::plac-poly/*RPS10*	Woodacre *et al.*, 2008
AWC1	BWP17 with *RPS10*::pAWC1/*RPS10*	Woodacre *et al.*, 2008

AWC1.1	Mem-m2 with *RPS10*::pAWC1/*RPS10*	Woodacre *et al.*, 2008
A2	BWP17 with *RPS10*::pA2/*RPS10*	This study
A3	BWP17 with *RPS10*::pA3/*RPS10*	This study
A4	BWP17 with *RPS10*::pA4/*RPS10*	This study
A5	BWP17 with *RPS10*::pA5/*RPS10*	This study
S. cerevisiae Y187	*gal4-542, gal80-538, LYS2:GAL1$_{UAS}$-GAL1-lacZ, mat α, his3-200, leu2-3, 112, trp1-901, met-, ade2-101, ura3-52*	Gietz *et. al.*, 1997 (Y2H host strain)
S. cerevisiae CG1945	*gal4-542, gal80-538, LYS2:GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, URA3::GAL4 17 MERS (x3) –CYC1$_{TATA}$-lacZ, mat α, his3-200, leu2-3, 112, trp-901, cyh2-R, lys2-801, ade2-101, ura3-52*	Gietz *et. al.*, 1997 (Y2H host strain)

Table 2. 1: Yeast strains used during this study

Candida albicans laboratory strains lineage

SC5314 → CAF2-1 → CAI4

CAI4 → CAI8

CAI4 → RM1000/RM1000#2

RM1000/RM1000#2 → BWP17

RM1000/RM1000#2 → SN78 → SN87

RM1000/RM1000#2 → SN76 → SN95

SN76 → SN148 → SN152
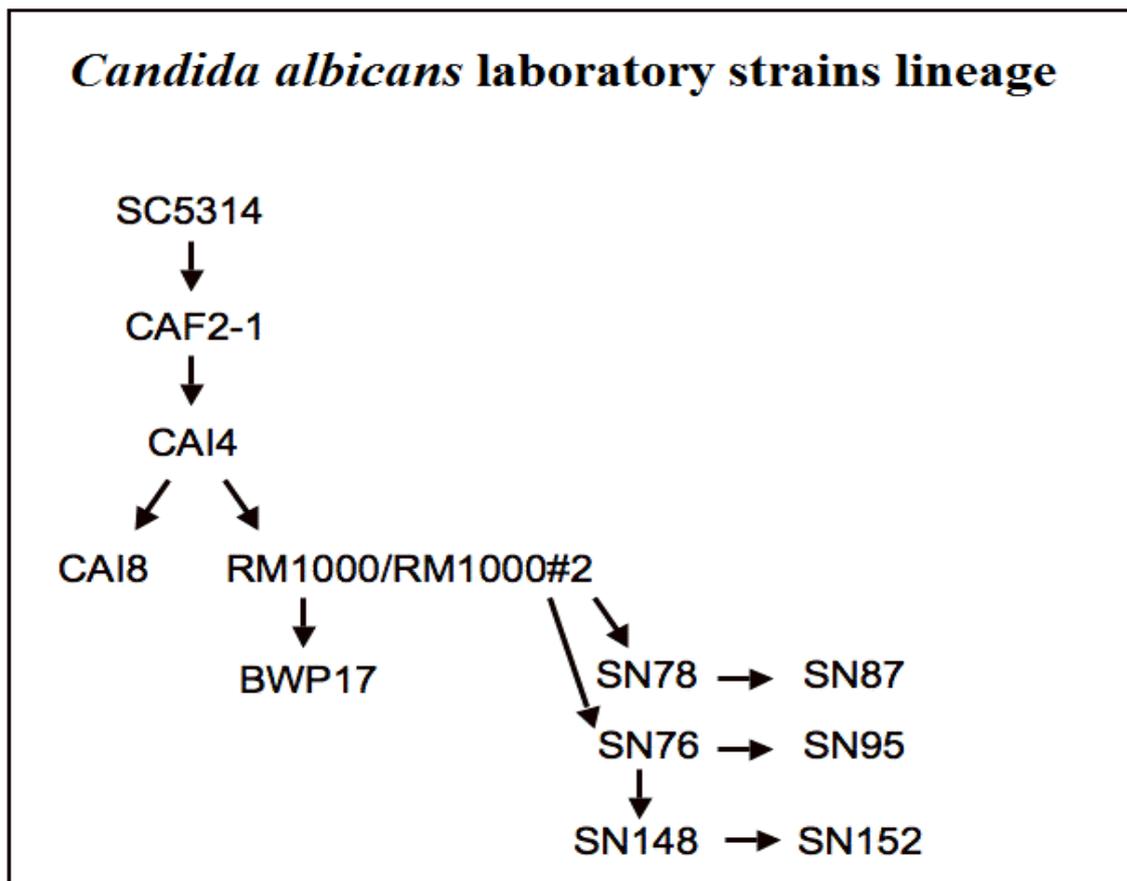
2.2. Media and growth conditions

Growth of *E. coli* strains

E. coli were grown in Luria Bertani (LB) medium at 37˚C overnight with liquid cultures undergoing shaking at 200 rpm. The components of LB were 1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride, adjusted to a pH 7.2 with sodium hydroxide. Solid media contained 2 % (w/v) Bactoagar and all media components were purchased from Oxoid. Ampicillin was used as selective agent at a final concentration of 100 μg/ml.

Growth of *C. albicans* strains

All strains were grown at 30˚C, with liquid cultures undergoing continuous agitation at 200 rpm.

Yeast Peptone Glucose Media (YPD)

C. albicans strains were routinely grown in Yeast Peptone Glucose Media made with 1% (w/v) yeast extract, 2% (w/v) Bactopeptone, 2% (w/v) glucose and 50 μg/ml uridine. Solid media also contained 2% (w/v) Bactoagar and all media components were purchased from Oxoid.

Synthetic Defined Media (SD)

Synthetic Defined Media contained 2% (w/v) Bactoagar, 0.67% (w/v) yeast nitrogen base without amino acids (Bio 101), 2% (w/v) glucose and was supplemented with amino acids at the concentrations shown in Table 2.2.

Minimal Defined Media (MD)

Wickerham's nitrogen base recipe was modified to develop the Minimal defined media with modifications made by Eide and co-workers. (Wickerham *et*. al., 1951; Eide *et*.

al., 1992) The media contains 10% (v/v) salt and trace solution, 0.1% (v/v) glucose and 1.25% (w/v) Bacto-agar (Oxoid) in solid media. The specific compositions of the salt and trace solution and vitamin solution have been defined below.

Table 2. 2: Amino acid supplements

Amino acid	Stock concentration	Final concentration
Arginine	4 mg/ml	20 μg/ml
Histidine	8 mg/ml	20 μg/ml
Uridine	50 mg/ml	50 μg/ml

Table 2. 3: Salt and Trace solution

Component	Stock concentration	Final concentration
Ammonium sulphate	75.7 mM	7.57 mM
Potassium di hydrogen orthophosphate	50.2 mM	5.02 mM
Di-potassium hydrogen orthophosphate	9.2 mM	0.92 mM
Magnesium sulphate	20.3 mM	2.03 mM
Sodium chloride	17.1 mM	1.71 mM
Boric acid	1.62 μM	162 nM
Potassium iodide	0.6 μM	60 nM
Zinc sulphate	2.44 μM	244 nM

Table 2. 4: Vitamin solution

Component	Stock concentration	Final concentration
d-biotin	8.19 μM	8.19 nM
Thiamine hydrochloride	1.19 mM	1.19 μM
Pyridoxine hydrochloride	1.95 mM	1.95 μM
Myo-inositol	11 mM	11 μM
d-pantothenic acid calcium salt	0.84 mM	0.84 μM

2.3. Plasmids used during this study

Plasmid	Genotype	Reference
pGAD424	*2μ ori*; *ori; amp^R; LEU2; GAL4$_{AD;}$* (Y2H prey vector)	GenBank
pGBT9	*2μ ori*; *ori; amp^R; TRP3; GAL4$_{DBD;}$* (Y2H bait vector)	GenBank
pET28α	F1 *ori*, *ori¸kan^R; LacL*	GenBank
plac-poly	*ori*; *amp^R; CaRPS10; CaURA3; LacZ*	Brown et al., 2007
pAWC1	plac-poly with 776 bp *CaCTR1* promoter	Woodacre *et al.*, 2008
pA2	plac-poly with 501 bp *CaCTR1* promoter containing CuRE1	This study
pA3	plac-poly with 623 bp *CaCTR1* promoter containing two copies of CuRE1	This study
pA4	plac-poly with 745 bp *CaCTR1* promoter containing three copies of CuRE1	This study
pA5	plac-poly with 378 bp *CaCTR1* promoter containing no copies of CuREs	This study
pET28α-CaMAC1	pET28α but with *CaMAC1* gene inserted within *EcoRI* and *XhoI* sites.	(Woodacre A.) for this study

Table 2. 5: Plasmids used in this study
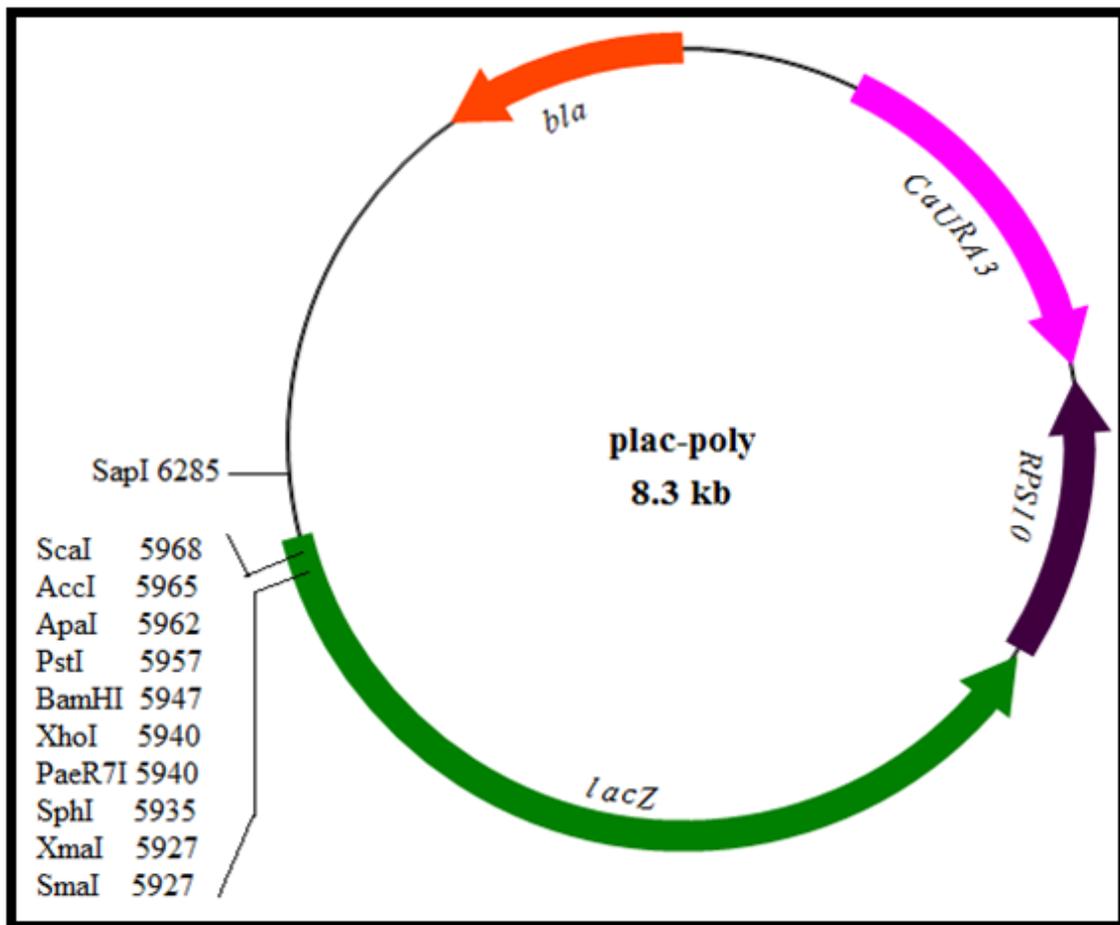
Figure 2.1: Plasmid map of plac-poly

The plasmid plac-poly has a *LacZ* reporter gene that has been derived from *Streptococcus thermophilus*. It carries the *bla* gene encoding for ampicillin resistance in *E. coli* and the *CaURA3* nutritional marker that allows yeast auxotrophs carrying plac-poly to grow on limiting synthetic medium lacking uridine and helps in selection of transformants in *C. albicans*. After restriction digestion with *StuI*, the *RPS10* gene is used for targeted integration within the *C. albicans* genome.
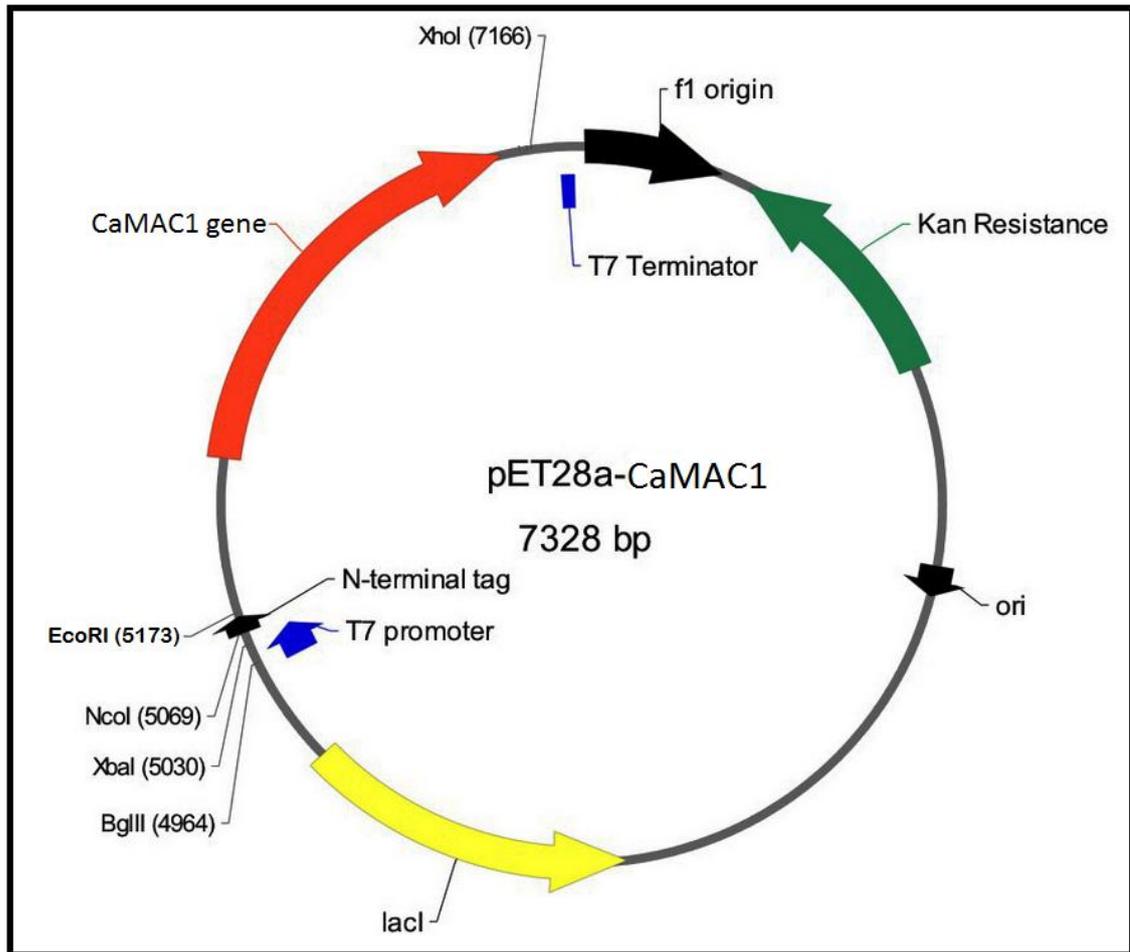
Figure 2. 2: Plasmid map of pET28α-CaMAC1

The Pet28α vector carries an N-terminal His●Tag® configuration plus an optional C-terminal His●Tag® sequence. The cloning/ expression region of the coding strand transcribed by T7 RNA polymerase is shown. The f1 origin is oriented as shown above (GenBank). *CaMAC1* gene has been cloned within the *EcoRI* and *XhoI* site within the expression vector. The vector has a kanamycin resistance gene and an *ori* for replication in *E. coli*.
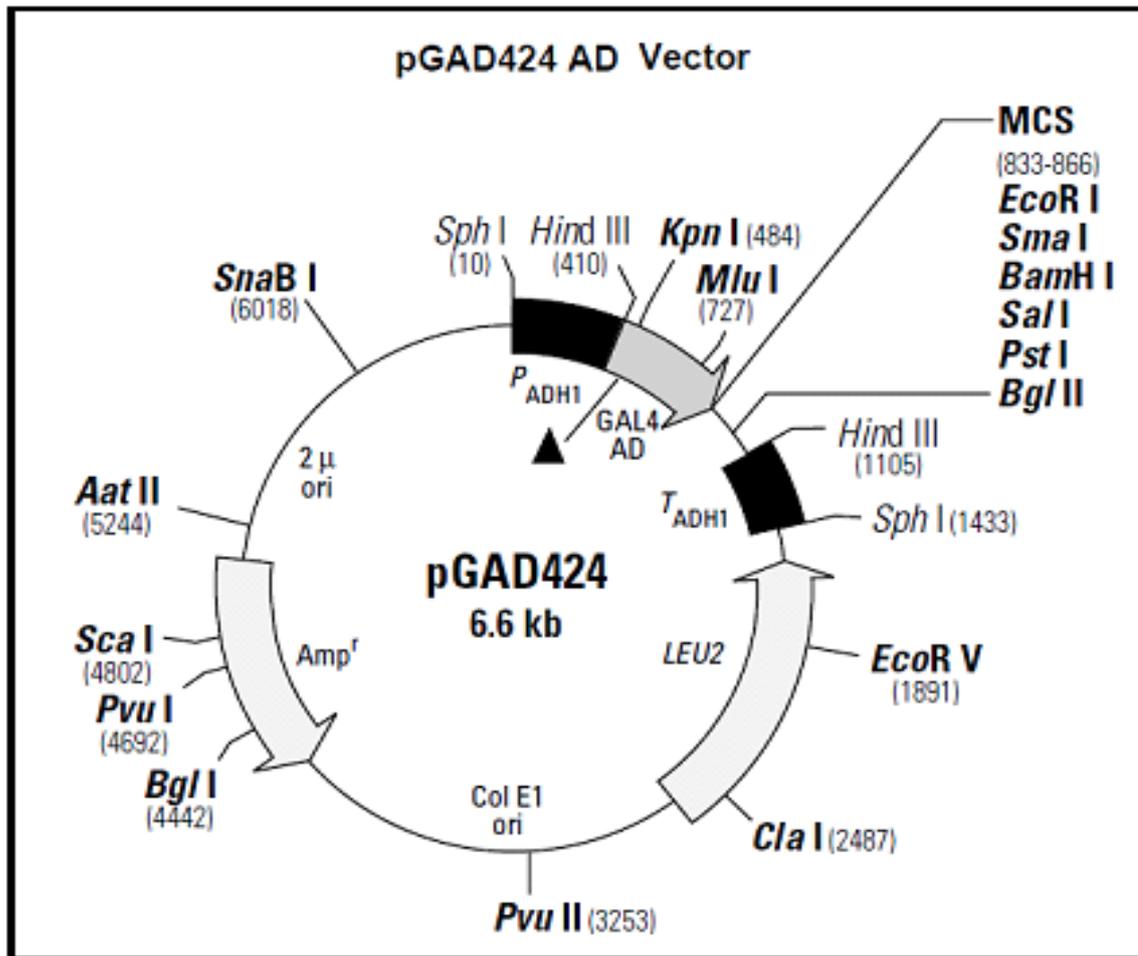
Figure 2. 3: Plasmid map of pGAD424 (GenBank) – Yeast two Hybrid Prey vector

pGAD424 generates a hybrid protein that contains the sequences for the *GAL4* activation domain (AD; 768–881 aa). pGAD424 has unique restriction sites located in the MCS region at the 3'-end of the open reading frame for the *GAL4*-AD. For the construction of a hybrid protein, the gene encoding the protein of interest is ligated into the MCS in the correct orientation and in the correct reading frame such that a fusion protein is generated. The fusion protein is expressed at high levels in yeast host cells from the constitutive ADH1 promoter; transcription is terminated at the ADH1 transcription termination signal. The hybrid protein is targeted to the yeast nucleus by nuclear localization sequences that have been added to the AD sequence from a heterologous source. pGAD424 is a shuttle vector that replicates autonomously in both *E. coli* (*ori*) and *S. cerevisiae* (2μ *ori*). It carries the *bla* gene (for ampicillin resistance in *E. coli*) and the *LEU2* nutritional marker that allow yeast auxotrophs carrying pGAD424 to grow on limiting synthetic medium lacking leucine. The direction of transcription is specified by an arrow within each gene. DNA fragments can be inserted into the multicloning site shown below each plasmid. The restriction sites and the translation frames are also shown. Other restriction sites for each plasmid are also illustrated.
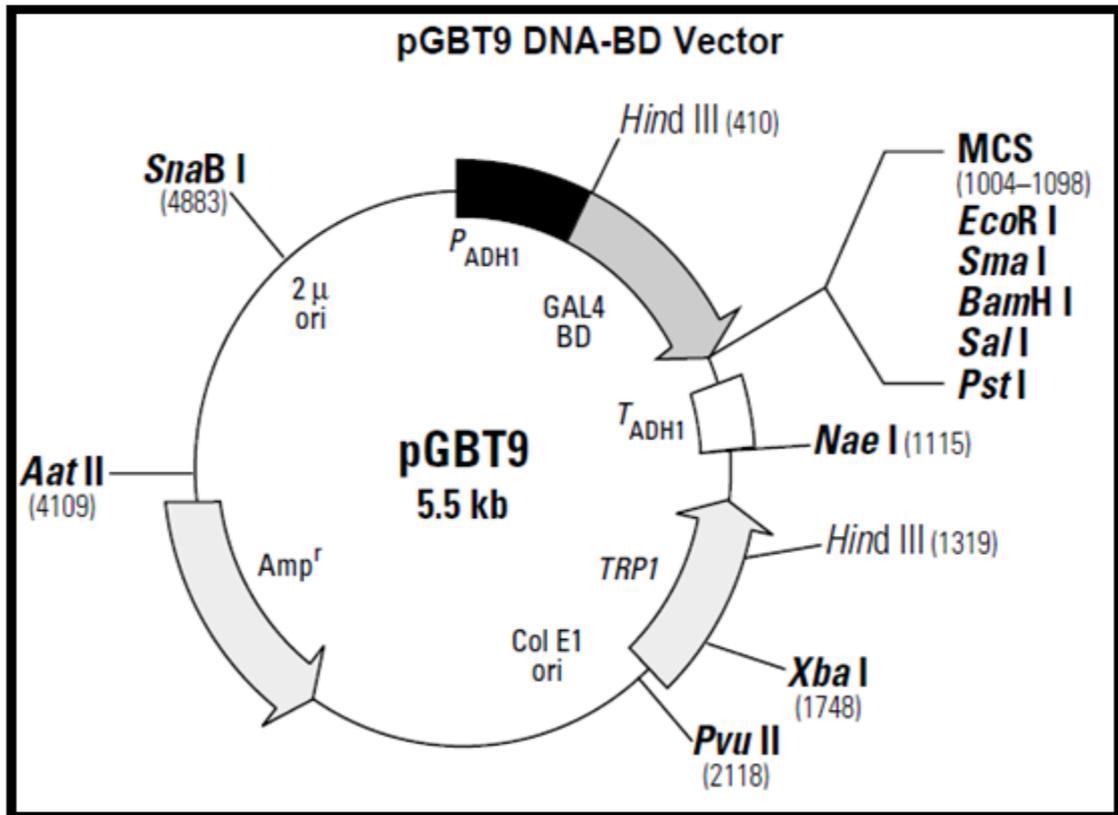
Figure 2. 4: Plasmid map of pGBT9 (GenBank) – Yeast two hybrid bait vector

pGBT9 generates a hybrid protein that contains the sequence for the *GAL4*-DNA binding domain (DNA-BD; 1-147 aa). For the construction of a hybrid protein, the gene encoding the protein of interest is ligated into the MCS in the correct orientation and in the correct reading frame such that a fusion protein is generated. The fusion protein is expressed at high levels in yeast host cells from the constitutive ADH1 promoter; transcription is terminated at the ADH1 transcription termination signal. The hybrid protein is targeted to the yeast nucleus by nuclear localization sequences that are intrinsic part of the GAL4 DNA-BD. pGBT9 is a shuttle vector that replicates autonomously in both *E. coli* (*ori*) and *S. cerevisiae* (2μ *ori*). It carries the *bla* gene (for ampicillin resistance in *E. coli*) and the *TRP1* nutritional marker that allow yeast auxotrophs carrying pGBT9 to grow on limiting synthetic medium lacking tryptophan.

2.4. Preparation and manipulation of nucleic acids

Preparation of plasmid DNA

Plasmids used in this study are listed in were pGAD424 and pGBT9 which were obtained from CLONTECH Laboratories (Refer fig. 2.1 and 2.2). Plasmid DNA was prepared using the Ezna Plasmid Mini Prep kit I (Omega), following manufacturer's instructions.

Preparation of *C. albicans* genomic DNA

An overnight culture in 10 ml of YPD broth was harvested by centrifugation at 3000 rpm for 5 minutes. The supernatant was discarded and cells were resuspended in 0.5 ml distilled water and transferred to a screw cap microcentrifuge tube. The resuspended cells were centrifuged at 13000 rpm for 1 minute and the supernatant discarded. The pellet was disrupted by vortexing for 10 seconds and was resuspended in 200 µl of breaking buffer (2% (v/v) Triton X, 1% (v/v) SDS, 100 Mm NaCl, 10mM TrisCl pH 8.0, and 1 mM EDTA, pH 8.0). An approximately equal volume (200 µl) of acid washed glass beads (Sigma) were added to the tube, along with 200 µl phenol:chloroform:isoamylalcohol (25:24:1 ratio). The tubes were vortexed for 7 minutes using multi-tube vortexer and 200 µl of TE was added (10 mM TrisCl pH 8.0, 1 mM EDTA pH 8.0). After centrifugation at 13000 rpm for 5 minutes, the aqueous layer was transferred to a fresh tube containing 500 µl chloroform:isoamylalcohol (24:1 ratio). This tube was vortexed briefly before centrifugation at 13000 rpm for 5 minutes and transfer of the aqueous layer to a clean microcentrifuge tube. The DNA was

precipitated by the addition of 1 ml 100% ethanol and incubation at -20°C for at least 2 hours. The DNA was pelleted by centrifugation at 13000 rpm for 25 minutes and the pellet was resuspended in 0.4 ml TE (pH 8.0). RNase A was added to a final concentration of 2.5 mg/ml and the contaminating RNA was digested for at least 1 hour at 37°C. The DNA was again precipitated by the addition of 1 ml of 100% ethanol and 40 µl of 3M sodium acetate (pH 5.2) and incubation at -20°c for at least 2 hours. The DNA was pelleted by centrifugation at 13000 rpm for 25 minutes and the pellet was air-dried before resuspension in 100 µl deionised distilled water.

RNA extraction (Schmitt *et al.*, 1990)

10 ml of YPD was inoculated with the culture using a single colony from a YPD plate and incubated for 6 hours at 30°C with shaking. Then the culture was harvested at 4000 rpm for 5 minutes and the culture was washed twice with sterile distilled water before being resuspended in a final volume of 1 ml sterile distilled water. The O.D.$_{600}$ was determined and cell density was estimated on the basis of previous growth curve experimental data. Approximately 3×10^4 cells were inoculated in 50 ml of MD containing high iron and high copper and were allowed to grow at 30°C overnight on the shaker. The cultures were then harvested the next day and resuspended in a final volume of 1 ml distilled water just like before. This overnight culture was used to scale-up and inoculate 100 ml of MD in flasks containing different supplements to a cell density of 2×10^6 cells/ml. The cultures were allowed to grow at 30°C for 5 hours on shaker until they reached log growth phase with a cell density of around 1×10^7 cells/ml. The culture was then harvested for RNA extraction.

Exponentially growing cultures were harvested by centrifugation at 4000 rpm for 5 minutes, washed in 1 ml distilled water and resuspended in 400 µl RNase free AE buffer [50 mM sodium acetate (pH 5.3), 10 mM EDTA]. Then 80 µl 10% (w/v) SDS was added and the mixture was vortexed for 30 seconds to allow cell disruption and an equal amount (480 µl) of phenol (pre-equilibrated with AE buffer) was added and vortexed again for 30 seconds. Samples were then incubated at 65°C for 4 minutes they were snap-frozen in dry ice for 3 minutes. This process was repeated 3 more times with a final incubation at 65°C for 4 minutes. The samples were next centrifuged at 13000 rpm for 5 minutes and the aqueous layer was carefully transferred to a fresh RNase-free tube containing 500 µl of pre-prepared phenol:chrlorofom:isoamylalcohol (25:24:1). The tubes were then vortexed for 5-10 seconds and centrifuged at 13000 rpm for 10 minutes at 4°C. The step was repeated a number of times (3-4) till the contamination was miniscule and the aqueous layer was transferred to a fresh RNase-free tube. 40 µl 3 M sodium acetate and 2 volumes of 100% ethanol were added to the solution and this reaction was incubated at -80°C overnight, to allow RNA precipitation. The next day the tubes were centrifuged at 13000 rpm at 4°C for 25 minutes and the pellet was then washed with 500 µl 80% ethanol. The pellets were then air-dried and resuspended in 50 µl DEPC-treated water. RNA was then stored at -80°C.

Agarose gel electrophoresis of DNA

DNA was separated and visualised using gels made from agarose (Seakem LE agarose) dissolved in 1 x TAE (Tris acetate EDTA) buffer with 25 µg/ml of ethidium bromide. Loading buffer (15% (w/v) Ficoll 400, 0.06% (w/v) bromophenol blue, 0.06% (w/v) xylene cyanol FF, 30 Mm EDTA) was added to DNA samples at a 1/6 dilution.

Samples were loaded onto the gel before electrophoresis in 1 x TAE buffer. Electrophoresis was typically performed at 10 volts per centimetre of gel and gels were visualised using a UV transilluminator.

Recovery of DNA from Agarose gels

After gel electrophoresis the gel was then visualised under UV light and the band containing the DNA of interest was excised using a sterile scalpel. The DNA was then extracted and purified using QIAquick gel extraction kit (Qiagen), following the manufacturer's instructions.

Formaldehyde denaturing gel electrophoresis for RNA

Denaturing gels for RNA were made up by dissolving 1.8 g agarose in 130.5 ml DEPC-treated d/W and mixed with 3 µl 10 mg/ml ethidium bromide, 15 ml 10 x MOPS solution and 4.5 ml 37% (v/v) formaldehyde solution (Sigma). The gels was then poured into RNase-free gel cassettes. RNA preparations (usually 30 µg RNA in 5 µl volume) were treated before electrophoresis, with 10 µl deionised formamide, 2 µl 10 x MOPS and 3.5 µl 40% formaldehyde. The samples were then incubated at 65°C and chilled on ice for 5 minutes. The RNA samples were mixed with ethidium bromide at final concentration of 4 µg/ml and 2.5 µl of 10 x loading buffer before being loaded on the formaldehyde denaturing gel and being run in 1 x MOPS buffer for approximately 3 hours at 110 volts. The gel was finally visualised and photographed using UV light and UV transilluminator system (Syngene). Size estimations were made by using 4 µl RNA ladder (Lonza)

Restriction enzyme digestion of DNA

Restriction enzymes were purchased from New England Biolabs Ltd. and all digestions were carried out using the buffers supplied and following the manufacturer's instructions. Plasmid DNA and PCR products were typically digested for 3 hours using 10 units of restriction enzyme for every 5 μg of DNA.

Ligation of DNA

Phosphate groups were removed from vectors prior to ligation using Antarctic Phosphate (New England Biolabs Ltd.) using the buffer supplied and following the manufacturer's instructions. Vector and insert DNA were quantified and a molar ratio of insert:vector of 3:1 was used in the ligation, typically using 50 ng of vector. Ligations were performed in a total volume of either 10 μl or 30 μl. The vector, insert and sterile distilled water were incubated at 65˚C for 5 minutes and then chilled on ice for 5 minutes. 400 units of T_4 DNA ligase (New England Biolabs Ltd.) and 1 x T_4 DNA ligase reaction buffer were then added before incubation overnight at 16˚C.

2.5. Transformation procedures

Electroporation of electrocompetent TOPO10 *E. coli*

An overnight culture of TOPO10 *E. coli* in LB was diluted one hundred fold in fresh LB and grown for 2-3 hours until exponential phase was reached (OD_{600} of approximately 0.5). The cells were chilled on ice for 10 minutes before harvesting by centrifugation at 4000 rpm for 10 minutes at 4˚C. The pellets were washed two times in 1 volume of ice-cold distilled water. Cells were harvested by centrifugation at 4000

rpm for 8 minutes at 4°C and the pellets were washed twice in 1/2 volume and 1/20 volume of ice-cold 10% glycerol with a final suspension in 1/200 volume of ice-cold 10% glycerol and stored at -80°C.

Transforming DNA was dialysed and then incubated with 50 µl of competent cells on ice for 30 minutes. The transformation samples were electroporated at 25 µF - 1000 ohms - 1.5 volts before adding 1000 µl LB and incubation at 37°C for 60 minutes. The cells were harvested by centrifugation at 13000 rpm for 1 minute at room temperature and the pellets resuspended in 1000 µl of fresh LB. 200 µl of each transformation was spread onto LA plates containing 100 µg/ml ampicillin and incubated at 37°C.

Lithium acetate transformation of *C. albicans* (Short transformation protocol)

A 1-2 day old culture of *C. albicans* strain (to be transformed) in 5 ml of YPD was used and the cells were harvested by centrifugation at 4000 rpm for 5 minutes at room temperature. The pellet was resuspended in 100 µl of One Step buffer (50% PEG, 1M lithium acetate, 1M DTT). 3 µl (50 ng- 1 µg) of plasmid DNA was added to the transforming DNA and 50 µg of single stranded salmon sperm DNA. The transformation DNA was incubated at 42°C for 30 minutes. The cells were then harvested by centrifugation at 4000 rpm for 30 minutes at room temperature. The pellet was washed with 1 ml of sterile distilled water and finally resuspended in 200 µl of fresh sterile distilled water. This was spread onto selective SD plates and incubated at 30°C for 1-3 days.

2.6. DNA sequencing and polymerase chain reaction

DNA sequencing

The BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used to sequence plasmid DNA. The terminator pre-mix was diluted 1/8 with 5 x Sequencing buffer (Applied Biosystems) before use. Sequencing reactions comprised of 4 μl diluted terminator pre-mix, 100 ng plasmid template DNA and 2 pmoles of primer in a total volume of 10 μl distilled deionised water. Primers used for sequencing are shown in Table 2.3. The reaction comprised of one cycle at 94°C for 0.1 minutes, 50°C for 0.05 minutes and 60°C for 4 minutes. Completed reactions were purified using Performa® Gel Filtration Catridges (Edge BioSystems). The purified reactions were analysed by the University of Leicester's Protein and Nucleic Acid Chemistry Laboratory using an Applied Biosystems 3730 sequencer.

Polymerase chain reactions

PCR was carried out using Bio-X-Act Long DNA polymerase (Bioline) with 2 x Premix D buffer. Typical reactions contained 0.5 units/μl of polymerase, 1 x buffer and 1 pmol/μl of forward and reverse primers. Reactions contained 50-100 ng of template DNA in a total volume of 20-50 μl, made up with distilled deionised water. A typical reaction involved 30 cycles of a denaturation step at 92°C for 1 minute, an annealing step at 50-70°C for 30 seconds and an extension step at 68°C for 1 minute; per 1 kb of PCR product.

Oligonucleotide primers were purchased from Invitrogen or Sigma and are shown in Table 2.6.

Primer name	Sequence
NDEL F	GATCGGATCCATCCAGGTGTTTATGCCAATGGG
NDEL R3	GATCGTCGACGTTATTTGGTCTTTTTTGAGCAAC
CDEL F2	GATCGGATCCGCATGATACTAATAGATGATATC
CDEL R2	GATCGTCGACCCGTATTTTCCGATGGCGGTTGC
pGAD424 sequencing F	TACCACTACAATGGATG
pGAD424 sequencing R	ACGATGCACAGTTGAAG
CaCTR1 probe F	GGTAACGTCGGCCACTT
CaCTR1 probe R	CTCTAGAAATAGTTGATGCC
MAC1-F567	GGAGGACCATCAACTATACG
MAC1-R846	AGACGAATCTGCTGGAGGAA
SEF1-F998	CACCAAGATCAACTCATGTT
SEF1-R1446	TGAACCAGCACGATTCAGAG
CaMAC1-899	GATCGTCGACCATGTCATTCTGGTAGCTAG
CaMAC1+1443	GATCGTCGACTAATTGGTGCTGTATGCAGG
SEF1-F1261	GGGTTGCCACCTTCAATAAA
SEF1-R1340	CGGAAATTTTTAGGCAACGA
SEF1-F1200	GTGGAGGACTCGTTCATGGT
ACT1-F116	TTGGTGATGAAGCCCAATCC
ACT1-R180	CATATCGTCCCAGTTGGAAACA
PMA1-F1382	TTGAAGATGACCACCCAATCC
PMA1-R1440	GAAACCTCTGGAAGCAAATTCG
Mac1 Seq 7	GAAATGATAAATTTGCGCAT
Mac1 Cdel R comp	GATCAAGCTTTTACGTATTTTCCGATGGCGGTTGC
Mac1 Cdel term F	GATCAAGCTTTGAACTGTATATGTAAATATTTT
Mac1 Ndel F comp	GATCAAGCTTCCAGGTGTTTATGCCAATGGG
Mac1 Ndel promo R	GATCAAGCTTCATTCCTTATTCAGTCTTG
Sef1 F	TACGGGTACCGTATATATCTAGCTAACAAAAG
Sef1 R	TACGCTCGAGGTACCTACCCATCTGAGCT
Sef1 SEQ1	GAATTGGCGATGCATTGCT
Sef1 SEQ2	GACTCGTTCATGGTTAGCA
Sef1 SEQ3	GTCTAGTAACGCTAATACCC
Sef1 SEQ4	GAATGAGGTCACATTTGACA
Sef1 SEQ5	GGTGATAATTTTATATTATTCC
Sef1 SEQ6	GATCAACAAACAAGTGGATG
FRE5 probe 1 F	GGGATGCATGGACGAGTT
FRE5 probe 1 R	GCAAACTGTCTGATAAACTT
FRE7 probe F	GATTATAAATTAATTTGTAATGAG
FRE7 probe R	GCTTACAATTACTTAAATACAT
FRE10 probe 1 F	CAATTCATTATTATTTGCTGC
FRE10 probe 1 R	GTATTTTCTGAACGCATTAGA
SEF1 probe F	GCTGAAAGCAAAGGAACAAA

SEF1 probe R	GAATTTGAAAACTACTTTCTG
CTR probe F	CTCACCTCATCTTTACTTTTC
CTR probe R	GAATGTTAAATGAATGAATATGG
MAC1 probe F	GGATCTCTTGGATTTCAT
MAC1 probe R	GTGACATGTAAACATAGACTT
SFU1 probe F	GTCCTGTAAATCTTAAACGG
SFU1 probe R	GATGATTGCATTGGTGAATG
CaCTR1 +60 probe	GGCAATGTCGGCGAATTCAGC
CaCTR1 +568 probe	CTCTAGAAATAGTTGATGCC
Cdel Rstop	GATCGTCGACTTACGTATTTTCCGATGGCGGTTGC
T7 pGEM F-2983	GTAATACGACTCACTATAGGG
SP6 pGEM R-145	CTATTTAGGTGACACTATAG
pGEM+HISI seq F 5444	ACAGTCAATGAAGCCCCATC
M13 205-221	CAGGAAACAGCTATGAC
FRE10 forward1056	GCTTTGGTCGCCATGTCAAT
FRE10 forward1156	GCCATTGCTGGTACTTGGAT
FRE10 reverse1242	ACGATCGAAAACCCAAACAG
SEF1 ORF F564	TCCACAACGTGCATTGTCATATACG
SEF1 ORF R783	GTTTAATGGCAAAGTAACGTCACCC
FRE43.5f-F388	GGGATGGCCATGTCTTCTTA
FRE43.5f-R543	GGTGCCAAATGTAGCAGGTAA
FRE7-F224	CCTCCAACTTTGGAACCTCA
FRE7-R399	GAAATGCGGTACAAGGCAAT
FRE4-F1833	GTAGTGATGATGCTGGTTCC
FRE4-R2024	CAGGAGACAAATGCGACAGA
ACT1 ORF F	GTGGTACTACCATGTTCCCAGG
ACT1 ORF R	GATAGAACCACCAATCCAGACAGAG
FRE1 ORF forward981	CGACAGGACTGGTATTTTAGGAAC
FRE1 ORF reverse1113	ACGGGAAATCCATCTGTGATAC
FRE2 ORFforward642	GTTCCCTCGCTTAACCAACTACT
FRE2 ORFreverse791	AATCTAGTAGGGACAAGACCATCG
FRE2 ORFforward642	GTTCCCTCGCTTAACCAACTACT
FRE2 ORFreverse791	AATCTAGTAGGGACAAGACCATCG
CFL2 ORF forward	GTTCCCTCGCTTAACCAACTACT
CFL2 ORF reverse	AATCTAGTAGGGACAAGACCATCG
CaCTR1 776 F	GATCGTCGACGTCCCTACAGTAAACAAGGC
CaCTR1 387 F	GATCGTCGACCTCATCAATCTCTTACCAATCC
CaMAC1 683 F	GATCCTCGAGCGGCATTGTTTAAGACTGAAATTGGG
CaMAC1 683 R	GATCCCCGGGTCCTTATTCAGTCTTGCTTTTTGGAGG
RP10 F	CCAGCTCTCACAGATACTC
URA3 R	GGTGATGGATTAGGACAAC
LacZ seq F	GATCGCTGGTACTGAGCTCACGC
LacZ seq R	TGAGCGTCAACAGTATTAAC

Table 2. 6: Primers used in this study

2.7. ONPG assay of *β*-Galactosidase activity

A single colony was used to inoculate 10 ml of SD Leu Trp drop-out media and the culture was incubated overnight at 30˚C with shaking until it reached exponential growth and a concentration of approximately 1×10^7 cells/ml. The next morning the culture was harvested by centrifugation at 6000 rpm for 10 minutes and the pellet was washed twice in sterile distilled water and resuspended in a final volume of 1 ml of chilled Z buffer. The absorbance at 600 nm was determined for the cell suspension. The diluted cells were then permeabilized by adding 100 μl chloroform and 50 μl 0.1% SDS. The tubes were vortex for 15 seconds and then equilibrated for 5 minutes in 28˚C water bath. The reaction was started by addition of 200 μl freshly prepared *o*-nitrophenyl-β-D-galactoside (ONPG; 4mg/ml). The tubes were vortexed for 15 seconds and incubated at 28˚C. The reaction was stopped after sufficient yellow colour was developed by adding 500 μl 1M Na_2CO_3. The tubes were vortexed again and then allowed to centrifuge at 13000 rpm for 5 minutes to get rid of debris and chloroform. The supernatant was transferred to a fresh eppendorf tube and the optical density at 420 nm was recorded for each reaction tube. The negative control sample containing only Z buffer, ONPG and Na_2CO_3 was used as blank. Units of *β*-Galactosidase activity was calculated using the formulae given below.

$$\beta\text{-galactosidase activity} = \frac{1000 \times OD_{420}}{V \times t \times OD_{600}}$$

where V = the volume of cells (ml) and t = the incubation time in min

2.8. Northern blot analysis

DEPC treated water (RNase free)

0.1% (v/v) DEPC (di-ethyl-pyrocarbinate) (Sigma) was added to Duran bottles containing d/W in a fume hood and the bottles were shaken thoroughly until DEPC was completely suspended. The bottles were allowed to remain in the fume hood overnight with a loose lid to allow evaporation of DEPC and next day this DEPC-treated water was autoclaved at 120°C for 15 minutes at 1 atm pressure; to allow destruction of any left over DEPC. All solutions that needed to be RNase free were made using this water.

AE buffer (RNase free)

50 mM sodium acetate (pH 5.3) was added to 10 mM EDTA in 500 ml DEPC-treated water.

10 x MOPS [3-(N-Morpholino)propanesulfonic acid] (RNase free)

The required reagents were added to DEPC-treated water to achieve final concentrations of 0.2 M MOPS (pH 7.0) (Sambrook), 50 mM sodium acetate and 1mM EDTA.

Sephadex column elution buffer (50 ml)

The required reagents were added to highly pure d/W (pH 7.0) to achieve final concentrations of 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.1% (w/v) SDS.

3 x SSC (Sodium chloride sodium citrate), 0.1% SDS

The required reagents were added to 420 ml of pure d/W to achieve final concentrations of 3 x SSC (using pre-available 20 x SSC) and 0.1% SDS (using pre-prepared 10% SDS).

Church & Gilbert's buffer

The reagents 51.11 g Na_2HPO_4, 21.84 g NaH_2PO_4, 70 g SDS and 2 ml 0.5 M EDTA were added to 998 ml d/W and the buffer was heated to 65°C every time, before use.

5 x RNA loading buffer (RNase free)

RNA loading buffer was prepared in 10 ml of RNase-free water, using 200 µl saturated bromophenol blue, 720 µl 37% formaldehyde, 3.084 ml formamide, 80 µl 500 mM EDTA (pH 8.0), 2 ml glycerol and 10 ml 10 x MOPS.

Northern blotting

20 x SSC was used as the blotting solution and was filled in a plastic rectangular container up to a depth of 2 cm. A plastic gel tray was placed upside down in this solution, acting as a base onto which a wick made from 3 mm chromatography Whatman paper was placed in such a way that the base was covered and the edges of the paper dipped into the 20 x SSC solution. Air bubbles were smoothed out using a glass pipette and the formaldehyde gel was placed onto this paper-covered base. A nitrocellulose membrane was cut-out in the same size as the gel and pre-soaked in 2 x

SSC before being placed on top of the gel. Another piece of 3 mm paper (pre-soaked in 2 x SSC) was then placed on top of the membrane and air bubbles were removed once again. Clingfilm was used to separate the wick and lower tray from coming in contact with the membrane directly. 30-50 blotting paper towels were placed on top of the exposed membrane to initiate the blotting process via capillary action. A glass plate carrying weight was placed on top of these papers to assist capillarity. The transfer was carried out overnight and then the set-up was dismantled. The gel along-with the membrane was transferred to a dry piece of 3 mm paper where the well positions were marked to aid orientation. The gel was then removed and discarded. An Ultraviolet Cross Linker (Amersham Life Science) at 70,000 μJ/cm^2, was used to cross-link the membrane and fix the RNA.

Pre-hybridisation of membrane

The cross-linked membrane was pre-hybridised by placing it in glass tubes containing 1/3rd Church Gilbert's buffer at 65°C, with constant turning in a dual hybridisation chamber (Hybaid), for 2-12 hours. The side containing the RNA was kept facing the buffer.

Radioactive labelling of probes and probing technique

Probes were generated using standard PCR with specific probe primers and the PCR products were purified using a Cycle Pure PCR clean up kit (E.Z.N.A). The probes were diluted to give a concentration of 30 ng in 16 μl. The samples were then denatured by boiling for 5 minutes and chilling on ice for 5 minutes. The probes were then

labelled by adding 1 μl (10 ng) BSA (New England Biolabs), 1 μl Klenow fragment, 5 μl OLB (Oligolabelling buffer) and 2.5 μl of α-^{32}P dCTP (specific activity of 0.925 Mbq) and incubated at 37 °C for 1-2 hours. Three solutions (A, B and C) were used to make the Oligolabelling buffer in the ratio 2:5:3. Solution A contained 1 ml Solution 0 (1.25 M Tris-Cl, pH 8.0 and 0.125 M Magnesium chloride), 5 μl of each dATP, dTTP and dGTP (100 μM each) and 18 μl β-mercaptoethanol. Solution B was composed of 2 M HERPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 6.6) and Solution C contained random hexamers (GE healthcare) re-suspended in TE (ph8.0) at a concentration of 90 OD$_{595}$ units/ml.

Probes were labelled for an hour and the unincorporated nucleotides were removed using Illustra NICK ^{TM} columns (GE healthcare). Columns were equilibrated, using the manufacturer's directions, using elution buffer and the probe was recovered. The eluted solution that gave the highest radioactivity count, was used as the probe. The probe was boiled for 10 minutes at 95°C in a heating block and then chilled on ice for 10 minutes before being added to the hybridisation tubes containing the membranes in Church Gilberts buffer. The hybridisation reaction was allowed to proceed at 65°C in the hybridisation chamber with gentle rotation, overnight.

Washing blots and Autoradiography

The blots were washed at 65°C 3 x SSC/0.1% (w/v) SDS with constant rotation and 3-5 changes of wash buffer until no further radioactivity was detected in the washed-off solution. The blots were then dried using 3 mm paper and wrapped in Clingfilm. The membrane was then placed into a cassette containing intensifier screens and a X-ray

film (Super RX Fuji medical X-ray film) and exposed at -80°C for 5-15 days and the blots were then developed.

Stripping the blots

The membranes were washed with 2-3 changes of boiling 0.1% (w/v) SDS for 15 minutes followed by a final rinse with 65°C 2 x SSC to strip the probes off them. They were then pre-hybridised and probed once again (with different probes) as described above.

2.9. Protein expression and purification

Equilibration buffer

Equilibration buffer contained a final concentration of 50 mM sodium phosphate and 0.3 M sodium chloride at pH 8.

Wash Buffer

Wash buffer contained a final concentration of 50 mM sodium phosphate, 0.3 M sodium chloride and 5 mM imidazole at pH 8.

Elution buffer

Elution buffer was composed of a final concentration of 50 mM sodium phosphate, 0.3 M sodium chloride and 250 mM imidazole at pH 8.

10 X SDS PAGE running buffer

144 g glycine, 30.2 g Tris and 10 g SDS were suspended in 1000 ml distilled water and stored at room temperature. The pH was adjusted to 8.5 and the buffer was diluted to 1x using distilled water, just before being used in electrophoresis.

SDS buffer B

15.1 g Tris and 1 g SDS was dissolved in 500 ml water. The pH was adjusted to 6.8 and the buffer was stored at room temperature.

Coomassie brilliant blue stain (100 ml)

0.25 g Coomassie R250 (Sigma) was suspended in 90 ml 50% (v/v) methanol and 10 ml glacial acetic acid and it was then stored at room temperature.

SDS de-stain

SDS de-stain was composed of 7.5% (v/v) glacial acetic acid and 20% (v/v) methanol in distilled water.

CaMac1-His-tagged fusion protein expression and purification

CaMAC1 sequence has previously been subcloned (by Woodacre A.) into pET28α expression vector in between sites *EcoRI* and *XhoI* (Figure 2.2) and was then transformed into *E. coli* RIL [BL21(DE3) CodonPLus-RIL] cells for recombinant

protein expression. Due to the presence of a non-universal coding system in *C. albicans* where CTG codes for serine instead of leucine, all CTG codon sequences were initially mutated to universal serine codon sequences (TCG) before being cloned into *E. coli* pET28α expression vector, to ensure expression and correct folding of CaMac1p in *E. coli*. pET28α carries an N-terminal His-Tag coding sequence generating a N-terminal fusion of the His-tag with CaMac1 on expression. *E. coli* strain RIL cells were grown in 250 ml Luria broth at 37 °C for 2 hours and 70 µl of 1M isopropyl-b-D-thiogalactopyranoside (IPTG) was added to induce production of the protein. 1 hour after induction, the cells were collected by centrifugation and His-select binding buffer, protease inhibitors and 100 mg/ml lysozyme was added to the cells and they were left on ice for 1 hour. Finally cells were lysed via repeated sonication. The lysate was centrifuged at 4 °C for 30 min at 4000 rpm, and the supernatant was collected and filtered through a 0.45-mm filter. The CaMac1-His tag protein was purified using His-select spin columns (Sigma). The extract was loaded onto the 5ml His crude column and washed with 25 ml of water and was equilibrated with wash buffer containing 50 mM Imidazole and the sample was reapplied to the column once. The CaMac1-His tag protein was eluted with a 100–500 mM imidazole linear gradient in elution buffer. The eluted proteins were monitored by SDS-polyacrylamide gel electrophoresis. The CaMac1-His tag protein was ultimately concentrated to a final volume of 15 µl using concentration columns that were centrifuged at 4000 rpm for 30 minutes.

SDS PAGE mini-gels

The expressed proteins were separated on a SDS mini-gel by electrophoresis (SDS-PAGE). 2.7 ml buffer A, 1.83 ml of acrylamide mix (37.5:1 acrylamide:bisacrylamide),

765 µl of distilled water and 190 µl 1% (w/v) APS (ammoniumpersulphate) were mixed to prepare 5.5 ml of 10% (w/v) separating gel. 15 µl TEMED (N,N.N',N'-tetramethylethylenediamine) was added specifically right before the gel was poured. The surface of the gel was then covered with distilled water to allow polymerisation to occur after which the water was decanted and the excess was dried out using blotting paper. A 5% stacking gel was then layered on top of the separating gel and a comb was placed to allow well formation. 2 ml of 5% stacking gel was composed of 330 µl acrylamide mix (as described above), 616 µl distilled water, 50 µl 1% (w/v) APS and 1 ml Buffer B. 10 ml TEMED was once again added right before the gel was poured. Once the stacking gel had polymerised, the wells were rinsed with distilled water and the protein samples were loaded. Protein ladders (FAVORGEN) were employed to estimate band sizes.

2.10.   Real time PCR (RT-PCR)

RiboPure^{TM} –Yeast RNA extraction protocol

Cells were inoculated and grown similar to the first section of the RNA extraction protocol mentioned in Section 2.4. Then RNA for RT-PCR analysis was extracted from 1×10^7 yeast cells, using the RiboPure protocol which came with the RiboPure-Yeast kit. Zirconia beads, lysis buffer, 10% SDS and Phenol:Chloroform:IAA was used for the initial cell disruption and purification step. Binding buffer and 100% ethanol was added to initially draw the sample through the filter cartridge. The sample retained within the filter was then sequentially washed with Wash solution 1 and Wash solution 2/3. RNA was eluted using preheated Elution solution (heated to ≈95°C). RNA was

then subjected to DNase I treatment adding 10 x DNase I buffer and DNase I enzyme and incubating reaction at 37°C for 30 minutes. The reaction was terminated by addition of DNase inactivation reagent and the supernatant was transferred to a fresh RNase-free tube. RNA samples were stored at -20°C for up to 1-2 months and transferred to -80°C for longer storage.

Assessing RNA yield and quality

RNA concentration and purity was determined by running it on a Nanodrop analyser. $A_{260/280}$ ratio in the range of 1.8-2.1 represented a highly pure sample that could be used for Northern blotting, RT-PCR and RNase protection assay. Overall quality of RNA was assessed by electrophoresing the samples in a denaturing agarose gel as mentioned above.

RNA samples were also tested for purity and integrity on the Agilent 2100 Bioanalyser using the Agilent RNA 6000 Nano kit protocol. Electropherograms were generated at the end of the run and an RNA integrity number (RIN) was obtained for each sample, where an RIN of 7-10 represented a good quality RNA sample that could be used for microarray of RT-PCR analysis.

cDNA synthesis

Applied Biosytems High Capacity RNA-to-cDNA kit was used for cDNA synthesis and reactions were set up, according to the manufacturer's protocol. 2 µg total RNA was used to set up each 20 µl reaction and the reverse transcription reactions were set up in

a thermal cycler. The cDNA was then analysed via agarose gel electrophoresis and stored in freezer at -20°C.

RT-PCR reaction using Fast SYBR® Master mix

2 x Fast SYBR® Green master mix, forward and reverse primers and the cDNA template were added to each 20 µl reaction well in a 96-well plate, following the manufacturer's instructions (Applied Biosystems). The plate was vortexed briefly on a plate vortex and the contents were spun down to eliminate air bubbles before sealing the plate with optical adhesive RT-PCR suitable seal. Four replicates of each reaction were set up. Figure 4.10 illustrates an example of a reaction plate set-up. The reaction plates were then run within 20 minutes on Applied Biosystems 7500 Fast Real-Time PCR system.

After the reaction run, the data was analysed. The Sequence Detection system (SDS) software was used to automatically detect the baseline and threshold values and C_T was calculated. The relative quantification ($\Delta\Delta C_T$) comparative method was used to analyse the results (Refer section 4.6).

2.11.   Radioactive iron uptake assay

Dancis *et. al.* (1990) have previously reported a technique which allows us to measure the amount of $^{55}FeCl_3$ accumulation within a cell. This technique was adapted and used to measure the rate of iron uptake by cells by measuring the amount of radioactive Fe present intracellularly. Cells were allowed to grow overnight in YPD media at 30°C on a shaker and were then harvested, washed and used to inoculate 10 ml of fresh YPD

media up to a cell density of 1×10^6 cells/ml. Cultures were allowed to grow until they reached a final cell density of 1×10^7 cells/ml and were harvested, washed 2 times with 20 ml ice-cold water and re-suspended in 1 ml of ice-cold assay buffer (10 mM sodium citrate pH 6.5 and 5% (v/v) glucose). Cells were diluted using 1 ml of chilled assay buffer to re-obtain a final cell density of 1×10^7 cells/ml and then incubated at 30°C for 15 minutes. After incubation, 1µM of $^{55}FeCl_3$ was added to the cells and they were incubated at 30°C for further 30 minutes in a shaking water bath. A vacuum manifold was used for the final harvest of the cells onto glass filters and then the cells were washed 2 times with 10 ml chilled 0.25 M EDTA (pH 6.5) followed by 2 times wash with 10 ml ice-cold distilled water. The filters were then allowed to air-dry and were placed in scintillation vials containing 5 ml scintillation fluid. Re-hydration of the filters was allowed to proceed for 30 minutes and then the β-emissions were counted in the scintillation counter which allowed us to determine the amount of $^{55}FeCl_3$ incorporated within the cells.

2.12.   Ferric and cupric reductase assay

Cells were grown in 10 ml YPD at 30°C for approximately 6-8 hours and were then harvested and used to inoculate 10 ml of MD media to obtain a cell density of 3.6×10^4 cells/ml. The cultures were allowed to grow overnight and were then harvested, washed thrice with distilled water and used to inoculate 10 ml of the fresh (required) media up to a cell density of 2×10^6 cells/ml. Cultures were then allowed to grow for 5-6 hours until they reached exponential growth phase and they were then harvested, washed with 5 ml of chilled distilled water and resuspended in 1 ml of ice-cold assay buffer (50 mM sodium citrate, pH 6.5 and 5% (v/v) glucose). 50 µl cells were then transferred to a fresh tube and incubated at 30°C for 10 minutes. A control reaction was also included

which contained no cells. The assay was carried out by addition of 800 µl reductase buffer (50 mM sodium citrate pH 6.5, 1 mM BPS, 5% (v/v) glucose and 1mM ferric chloride) to the sample and control tubes; followed by incubation at 30 °C for 5 minutes. The cells were then harvested by centrifugation at 13000 rpm for 3 minutes. The supernatant was transferred to a cuvette and absorbance for the solutions was measured at 520 nm.

A curve was generated, using the O.D. at 520 nm and the concentration of Fe^{2+} ions. For the standard curve, several reactions were set up which contained reductase buffer and a range of known concentration of ferrous chloride. The reactions were processed as described above and the observations were used to plot the standard curve. The standard curve was then used to extrapolate and determine the amount of iron reduced by the (test) sample cells.

3. Analysis of the molecular mechanism of action of CaMac1p in *C. albicans*

3.1. Introduction

Work carried out in our laboratory led to the hypothesis that CaMac1p binds to the CuRE (copper response element) sequences in promoters of the copper transporter gene- *CaCTR1*, reductase gene- *CaFRE7* and its own gene- *CaMAC1* (self-regulation), activating their transcription in low copper conditions (Woodacre, 2008). It is possible that either CaMac1p could be directly binding to the CuRE sequences or an intermediate transcription factor may also be involved. In a *Camac1Δ/Camac1Δ* mutant the basal levels of *CaMAC1-lacZ* transcription has been observed to be higher than basal levels of *CaFRE7* or *CaCTR1* transcription indicating that an additional transcription factor may be involved in regulation of *CaMAC1* (Woodacre, 2007).

Previous experiments on *MAC1* in *Saccharomyces cerevisiae* have shown that the two cysteine-rich motifs present on the C-terminal of the protein are capable of binding eight cuprous ions and this binding induces a molecular switch which results in an intramolecular interaction between the N-terminal and C-terminal domains of Mac1p. It was also observed that this copper-induced intramolecular interaction, repressed the DNA binding as well as transactivation properties of the protein. It was then theorised that during this intramolecular interaction, the N-terminal domain binds to and masks the C-terminal domain and thereby prevents the protein from undergoing DNA binding (via the N-terminal) and protein interactions (via the C-terminal) (Jensen LT *et al.*, 1998). It is also known that optimal transcription activity by the *S. cerevisiae* Mac1p

requires the presence of two CuRE elements in the promoters of target genes (Labbe S *et. al.*, 1997), which could be because it forms homo-dimers and every one monomer of ScMac1p fixes to one CuRE sequence in the DNA (Joshi A *et al.*, 1999). Further evidence for the formation of ScMac1p homo-dimers was provided by the discovery of a Mac1p-Mac1p intermolecular interaction using yeast two-hybrid based approach (Joshi A *et al.*, 1999). The protein interacting domain was mapped to a minimal fragment containing residues 388-406, which are predicted to form a helix. This intermolecular interaction is copper independent but is negatively regulated by the N-terminal DNA-binding domain (Serpe M *et. al.,* 1999).

The ability to form Mac1p-Mac1p interaction is essential as mutations within the dimerisation domain abolish the ability of Mac1p to activate transcription of genes *in vitro* in *S. cerevisiae* (Joshi A *et al.*, 1999). This supports the theory that Mac1p homo-dimers are the active complexes that induce transcription of genes. However it was found that the promoters of *CaFRE7* and *CaMAC1* containing only one CuRE were able to mediate copper-responsive transcription at the same level as full-length wild-type promoter signifying that only one CuRE is required for wild-type activation by CaMac1p. This suggests that the formation of CaMac1p homo-dimers in *C. albicans* may not be required for activation activity of CaMac1p (Woodacre, 2008) or dimer binding may require the presence of only one CuRE.

Since the *CaMAC1* in *Candida albicans* shares 35% sequence homology with the *MAC1* in *Saccharomyces cerevisiae* we decided to study the protein interactions and compare it with the information available about ScMac1p to gain a better understanding of the molecular mechanism of action of CaMac1p. Through this study we aimed to show how CaMac1p is similar to and how exactly it is diverse from ScMac1p. It was possible that CaMac1p may not be capable of forming homo-dimers and hence

dimerisation may not be essential for gene activation *in vivo* in *C. albicans* by CaMac1p; or that dimers may not be the active functional units of CaMac1p. This theory was tested in our laboratory using yeast-two hybrid studies.

In *Saccharomyces cerevisiae*, experiments were also performed using *CTR1* promoter/*lacZ* hybrid genes containing 0-4 CuRE elements and tested for activity by measuring *lacZ* expression, in cells containing intact *MAC1*. Compared, to the control vector containing no upstream activation sequences, low level activation was seen with a single CuRE element while a promoter *lacZ* fusion containing two CuRE elements showed approximately 8-fold greater *lacZ* expression than that for a single element. The presence of three CuRE elements increased expression nearly 13-fold more than the level of promoter/*lacZ* fusion with one element and the promoter fusion containing four CuRE elements was about 15-fold higher than the expression in promoter/fusion containing only one CuRE element. The increase in expression in each case was more than additive and suggested that the requirement for two CuRE elements in the promoter of Mac1-regulated genes was due to synergism between the sites (Jensen *et al.*, 1998).

However by experiments performed in our lab (Woodacre, 2007), it was seen that the presence of a single copy of CuRE1in the *CaCTR1* promoter, was adequate for copper-dependent regulation by CaMac1p but CuRE 1 and 2 were essential for wild-type intensities of promoter activity. However, CuRE1 sequence seemed vital for CaMac1p binding. CuRE 3 did not appear to be essential for copper-dependent regulation but was able to alter overall levels of *β*-galactosidase activity (Woodacre, 2007). Hence it was thought that it would be interesting to see whether the presence of more than one copy of CuRE1 in the promoter of Mac1-regulated genes would show a synergistic increase in *lacZ* expression, similar to what was observed in *Saccharomyces cerevisiae*.

Reporter system for *C. albicans*

In *C. albicans* there is non-conventional codon usage, where CTG codon (CUG on mRNA) codes for serine instead of leucine. This means that genes from other organisms, containing CTG codons are transcribed but the proteins cannot be synthesised correctly since leucine would be replaced with serine in its primary structure leading to mis-folding of proteins. The *E. coli β*-galactosidase reporter gene *lazZ*, which is commonly used, cannot be expressed in *C. albicans* as it contains a very high number of CTG codons (51) in the gene sequence (Leuker *et al.*, 1992). The *lacZ* gene from *Streptococcus thermophiles* was identified as a potential reporter gene which could be modified to be used in *Candida albicans*. Uhl and Johnson used *S. thermophiles lacZ* to construct a number of reporter plasmids with *lacZ* fused to a number of promoters that were integrated into the *C. albicans* genome at a single locus. The *S. thermophiles lacZ* gene showed the presence of only one CTG codon which was found in a region of the gene that has not been conserved between *β*-galactosidases in other organisms and hence it was assumed not to be indispensable for protein function (Uhl & Johnson, 2001). It was then observed that mutation of the CTG codon did not increase *lacZ* activity and hence the wild-type *S. thermophilus lacZ* could be used as a reporter system in *C. albicans* (Woodacre, 2007, Uhl & Johnson, 2001).

In this study we used the *Streptococcus thermophilus* β-galactosidase reporter system since it has been previously used and optimised in our laboratory (Woodacre, 2007). Episomal plasmids have been found to be unstable in *C. albicans* and they also show a variance in copy number which affects host phenotypes. Instead, integrating plasmids are much more stable and function optimally in *C. albicans*. (De Backer *et al.*, 2000).
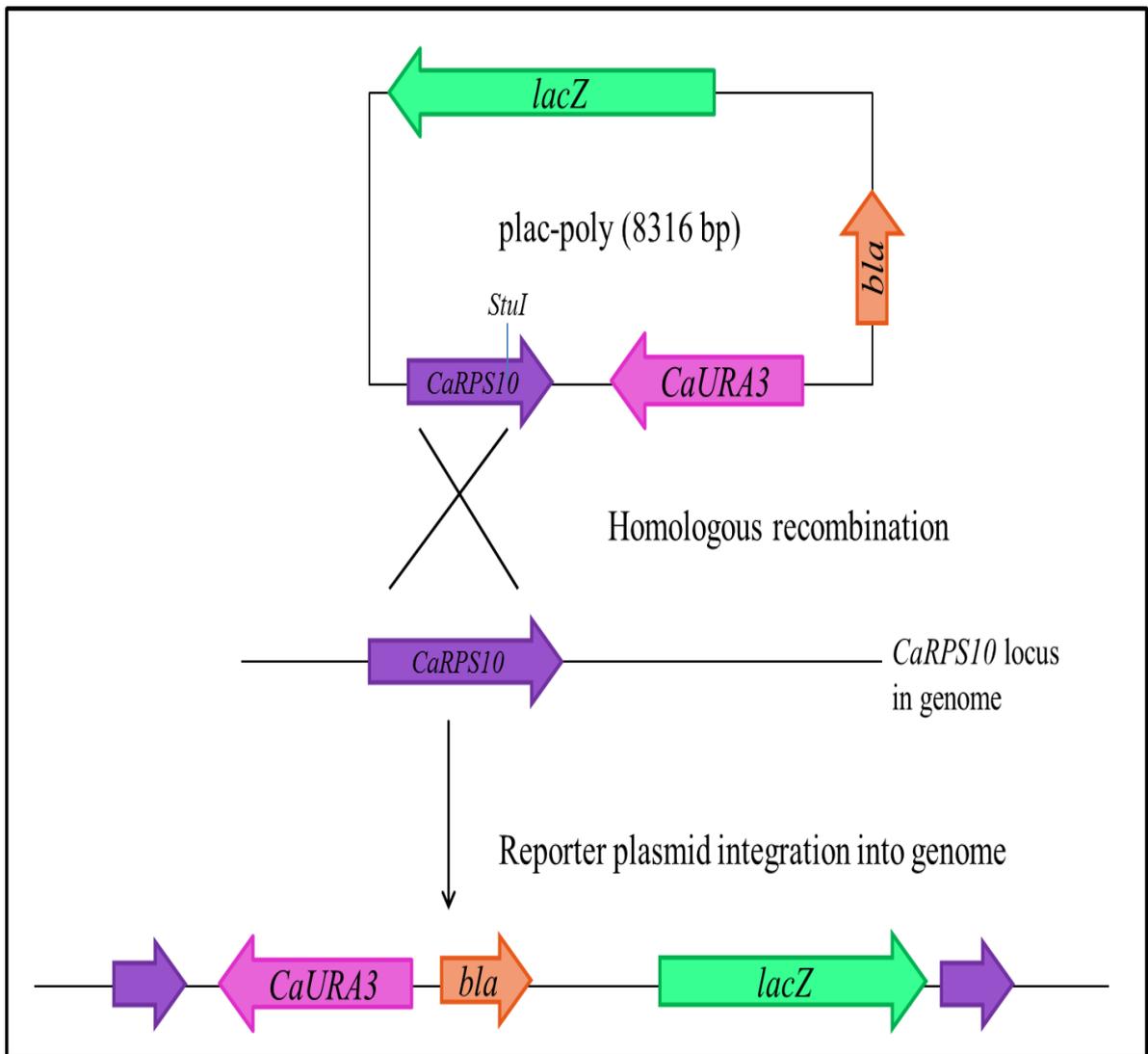
Figure 3.1: Targeted integration of plac-poly into C. albicans genome (adapted from Woodacre, 2007)

The copy of *CaRPS10* in the vector plac-poly contains an artificially inserted *StuI* restriction site. On digestion with the *StuI* restriction enzyme, the vector linearises and the two halves of *CaRPS10* undergo homologous recombination with the genomic copy of *CaRPS10* which leads to the plasmid containing the reporter gene of interest to become integrated into the genome at the highly expressed *CaRPS10* locus.

We decided to use the reporter plasmid plac-poly in this study which has been previously used in our laboratory (Woodacre A *et al.*, 2008, Jeeves R *et al.*, 2011). As mentioned in (Woodacre A, 2007), plac-poly was originally adapted from the CIp10 integrating plasmid, at the University of Aberdeen (Murad *et al.*, 2001, Murad *et al.*, 2000). CIp10 contains an ampicillin resistance *bla* gene which allows selection and maintenance in *E.coli* and *CaURA3* gene which is used as an auxotrophic marker and allows selection in *C. albicans*. Via Southern blot analysis, it was found that *C. albicans* shows the presence of two copies of a highly expressed, ribosomal protein gene *CaRPS10*, present at two separate loci on the genome. Hence it was understood that this locus could be disrupted without effecting growth while genes that would integrate at this locus, would be highly expressed and hence *CaRPS10* was used for targeted integration into *C. albicans* genome. CIp10 also shows a higher transformation rate as compared to other plasmids (Murad *et al.*, 2000). Therefore the plac-poly plasmid contains a copy of the *CaRPS10* gene with an added unique *StuI* restriction site. On digestion with *StuI*, the plasmid would linearize and integrate into the *C. albicans* at the *CaRPS10* locus via homologous recombination. In the plac-poly reporter plasmid, *Streptococcus thermophiles lacZ* gene has been inserted in CIp10 in between *bla* and *CaRPS10* genes in the same orientation while the multiple cloning site is located immediately upstream of the translation start of *lacZ* as shown in Figure 3.1 (Woodacre, 2007).

Using yeast-two hybrid assay to analyse protein interactions

A molecular genetic screen was developed by Fields and Song (1989) to analyse protein interactions. *Saccharomyces cerevisiae* is used as the test organism, where the

analysis is carried out. The yeast gene *GAL4* is used, that produces a protein with two functional domains that trigger transcription of genes involved in galactose metabolism. The promoter region of *GAL1* interacts with the DNA binding domain (DBD) of the Gal4 protein while the transcriptional activating domain (AD) of the Gal4p kindles transcription by employing the transcription machinery and interacting with activation domain of the reporter gene fused to *GAL1*-promoter. Separate plasmids carrying the sequences coding for (A) the DNA binding domain of *GAL4* (GAL4-DBD) and (B) the transcriptional activating domain of *GAL4* (GAL4-AD) are fused in frame to DNA sequences coding for the protein products of interest (Gietz RD *et al.*, 1997) *i.e.* *CaMAC1* with N-terminal DNA binding domain or C-terminal region with transactivation domain. These two plasmids are then co-transformed into a *S. cerevisiae* strain containing a 'reporter gene'. The reporter gene is transcribed only when the two test proteins present on different fusion plasmids, undergo interaction and complete the transcription circuit. Several versions of the yeast two-hybrid system are used. In this study, we used two different *S. cerevisiae* strains; Y187 which contained the *lacZ* as reporter gene and CG1945 which contained the *HIS3* reporter gene.

The *lacZ* reporter gene from *E. coli* is fused to the *GAL1* promoter in *S. cerevisiae* Y187. Interaction between the two proteins of interest, allows the *GAL4* activating domain to activate the transcription of the *lacZ* gene. The resulting β-galactosidase activity (indicating protein - protein interaction) can be detected by the appearance of blue colouration in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal), and also by assay of cell free extracts.

The selectable reporter gene *HIS3* is also fused to the *GAL1* promoter in *S. cerevisiae* CG1945. The DNA binding domain plasmid carries the yeast *TRP1* gene and the sequence coding for the protein of interest (truncated CaMac1-DBD) fused to GAL4-

DBD. The activating domain plasmid carries the yeast *LEU2* gene and the sequence coding for the other protein of interest (truncated CaMac1-ADp) fused to GAL4-AD. These plasmids are co-transformed into yeast strains which require leucine, tryptophan and histidine (*trp1*, *leu2*, *his3*) for growth and contain *HIS3* fused to *GAL1* promoter (*GAL1-HIS3*). If the binding domain and activating domain fusion proteins interact, the *GAL1-HIS3* reporter is transcribed and the yeast cell will grow on Synthetic Complete (SC) medium lacking all three amino acids- Tryptophan, Leucine, and Histidine (Gietz RD *et al.*, 1997). Thus the presence of co-transformants is selected for as a result of activating-domain fused test protein that positively interacts with the binding-domain fused protein (Figure 3.2).
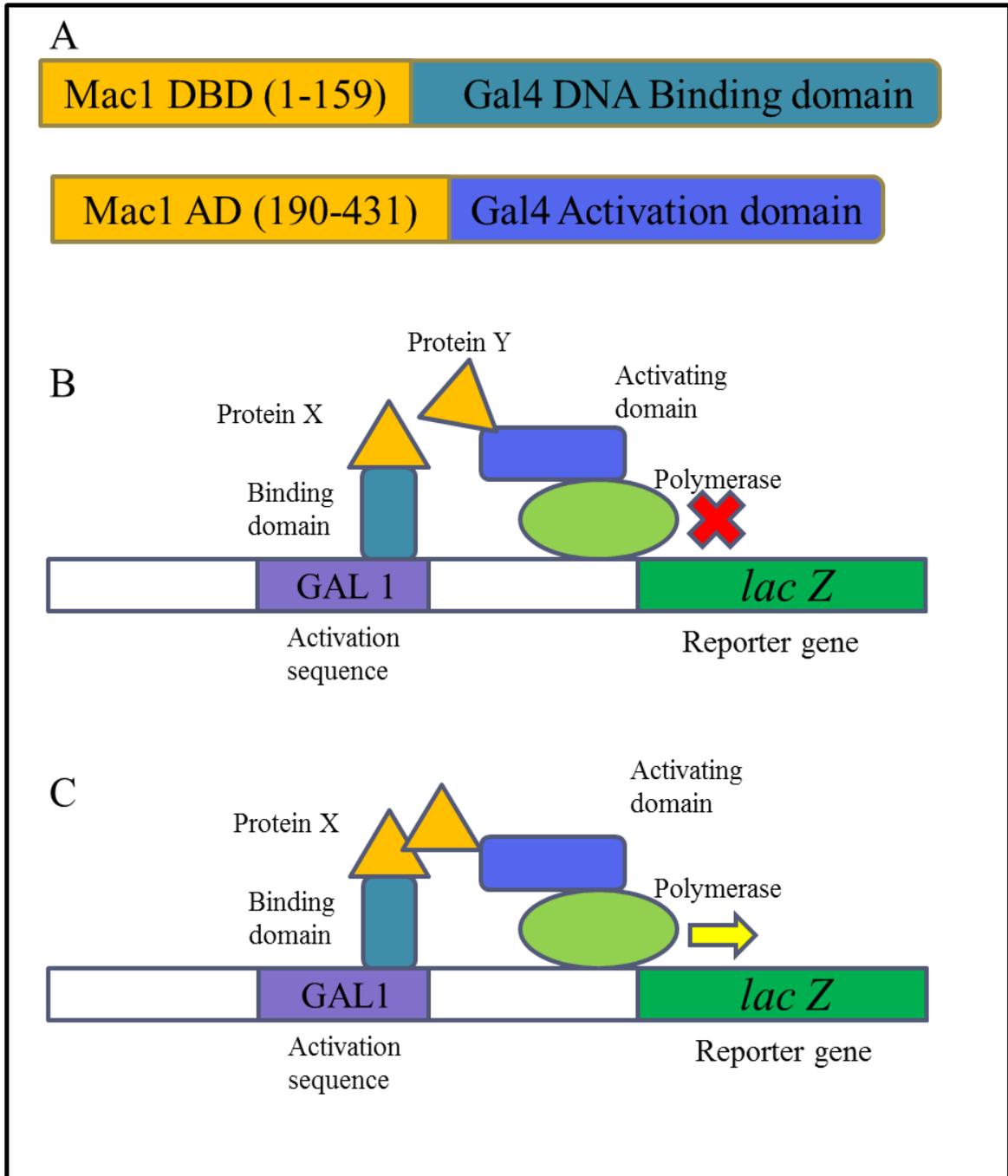
Figure 3.2: A diagrammatic representation of the yeast two-hybrid assay performed in S. cerevisiae.

(A) The two different vectors on expression give two different fusion proteins; one containing the Gal4 DNA binding domain fused to the N-terminus domain of CaMac1p and other containing the Gal4 activation domain fused to the truncated C-terminus domain of CaMac1p in the correct frame. (B) If the two fusion proteins do not interact then the reporter gene *lac Z* is not transcribed. (C) If the two fusion proteins undergo interaction then the reporter gene *lac Z* is transcribed and can be measured as β-galactosidase activity.

3.2. Analysis of intramolecular interactions of CaMac1p

Work carried out in our laboratory, has shown that the Mac1p sequence from *C. albicans* shared 35.0% similarity and 26.2% identity with ScMac1p (using the GAP Needleman & Wunsch algorithm alignment). The CaMac1 protein sequence was found to show the presence of a 'copper fist' motif at the amino terminus (N-terminal). This N-terminal 40-residue segment of CaMac1p is also homologous to the DNA binding zinc module found in many copper-activated transcription factors such as Mac1p and Ace1 (*Saccharomyces cerevisiae*), Amt1 (*Candida glabrata*), Cuf1p (*Schizosaccharomyces pombe*) and Crf1p (*Yarrowia lipolytica*). In each organism, the motif assists the binding of protein to target DNA in the presence of copper or silver.

On further analysis of the C. albicans Mac1p sequence two highly conserved cysteine-rich segments terminating with a histidine, between residues 169 - 224 and residues 246 – 301 were also identified. These segments showed significant identity to the C1 (203 – 286) and C2 motifs (289 - 417) found in ScMac1p with a consensus sequence of CXCXXXXCXCXXCXXH (where X = any amino acid). These motifs enable the copper-responsive control of expression by preventing the binding of ScMac1p, in high-copper conditions, to copper-responsive elements (CuREs) found in the target gene promoters. The spacing between the Rep1 and RepII motifs differs in both ScMac1p and CaMac1p. The RepI in ScMac1p is located at 264 - 279; while in CaMac1p it is located at 209 - 223. The RepII in ScMac1p is located at 322 - 337; while in CaMac1p it is located at 285 - 300 being separated by 42 residues in ScMac1p

Figure 3. 3: Diagrammatic representation of CaMac1 protein.

(**A**) *C. albicans* Mac1p is a 431 amino acid protein and contains a copper fist DNA binding domain in the N terminus region. It also shows presence of two conserved cysteine rich domains (**C1/RepI** and **C2/RepII**). (**B**) CaMac1p shares 35% sequence homology with ScMac1p and based and sequence analysis the N-terminal DNA binding domain (1-168 residues) and the C-terminal activation domain (169-431 residues) truncations that were created for our experiment have been highlighted. These truncated CaMac1p fragments were cloned into the yeast two hybrid prey and bait vectors and were co-transformed in *S. cerevisiae*, where the yeast two hybrid assays was carried out to detect intramolecular interaction within CaMac1p (Section 3.2).

and 61 residues in CaMac1p (Figure 3.3). Analysis of the CaMac1p predicted amino acid sequence using PSORT II revealed a 431-residue protein product that had a 73.9% probability of being retained in the nucleus (Marvin *et al.*, 2004).

To test for an intramolecular interaction between the N-terminal DNA Binding Domain and the potential C-terminal activation domain, a yeast two-hybrid-based assay was used. Similar to experiments carried out in *S. cerevisiae*, the Mac1 DBD (residues 1–159) was fused to the minimal Gal4 DBD (lacking any transactivation activity) in the first expression vector [Mac1–Gal4DBD]. The second vector consisted of the Mac1 AD (residues 190-431) fused to the Gal4 activation domain [Mac1–Gal4AD]. The DNA binding domains residues and the activation domain residues were decided by sequence analysis and comparing the *CaMAC1* sequence with the *ScMAC1* sequence. The 40 amino acid zinc finger motif which was essential for DNA binding was included in the Mac1 DBD while the cysteine-rich repeats C1 (209-223) and C2 (285-300) which are potentially essential for transactivation activity were included in the Mac1 AD.

The *CaMAC1* gene which was previously cloned in vector pGAD424 (Marvin *et al.*, 2004) was amplified to obtain the respective DNA binding domain and activation domain truncations of *CaMAC1* using primers MAC1 F1 (with artificial *BamH1* site) and MAC1 168R1 (with artificial *Sal1* site) for the Mac1(1-168) fragment; and primers MAC1 169F1 (with artificial *BamH1* site) and MAC1 R1 (with artificial *Sal1* site) for generating the Mac1(169-431) fragment. Additional nucleotides were added to the primers (Refer table 2.6) so that on expression, the truncated protein would be in the correct reading frame with the fusion protein.

The PCR products were electrophoresed on 1% agarose gel and single discrete bands corresponding to the expected size of *CaMAC1* truncated DNA fragments with deletions in C-terminal (Mac1 1-168) or N-terminal region (Mac1 169-431) were

observed, indicating that the PCR was successful and *CaMAC1* with the desired truncations was obtained (Figure 3.3).

Cloning the Y2H co-transformants in *S. cerevisiae* Y187

To perform yeast-two hybrid assay, the coding sequence for DNA binding domain of *MAC1* has to be cloned into bait vector (pGBT9) containing the Gal4-DNA binding domain (1-147 residues) and the yeast *TRP1* gene for selection. The coding sequence for activation domain of *MAC1* had to be cloned into prey vector (pGAD424) containing the Gal4-AD binding domain (768-881 residues) and the yeast *LEU2* gene for selection, maintaining the correct reading frame and orientation (Figure 2.3).

The vectors pGBT9 and pGAD424 containing the Gal4 DNA binding domain and activating domain respectively were extracted from *Escherichia coli* via plasmid mini prep and electrophoresed to confirm presence of plasmid.

The *CaMAC1* gene fragments containing truncations (Mac1 1-168 or Mac1 169-431) and the extracted pGBT9 or pGAD424 plasmid respectively were then double digested using *Bam*HI and *Sal*I and ligated overnight in a ratio of 1:3 (vector : insert). The ligation mixture was then dialysed to remove salts and impurities and transformed into *E. coli* via the electroporation method (Section 2.5). The transformants were plated on Luria agar plates containing ampicillin which is a selective marker for the plasmid as it contains a gene for ampicillin resistance. The colonies observed on the plates after incubation at 37˚C overnight, were screened for transformants. Plasmids were extracted from the potential transformant colonies and (Mac1 1-168)–Gal4DBD and (Mac1 169-431)-Gal4AD transformant constructs were identified via double digestion using *Bam*HI and *Sal*I. Two distinct bands of expected size, corresponding to the plasmid and

the truncated gene were observed, thereby leading to identification of the transformant. The truncations of the *CaMAC1* gene and the fusions with the vectors were also confirmed via sequencing. The inserts were sequenced using sequencing primers (Table 2.6). It was determined that the truncated gene sequences had been cloned within the pGAD424 plasmid vector or the pGBT9 vector containing the sequence for the *Gal4* activation domain or DNA binding domain accordingly, in the correct orientation and the correct reading frame. The modified gene constructs were then transformed in *S. cerevisiae* (using the Lithium Acetate method) where the yeast two-hybrid assay was performed. The Mac1(1–168)–Gal4 DBD vector was co-transformed into yeast strain Y187 with AD vectors encoding Mac1(169–431)–Ga14AD or the empty plasmid. Expression of the *GAL1–lacZ* reporter gene was assessed.

The co-transformants were grown either in the presence of BCS, which is a copper chelator, to generate copper deficient conditions or they were cultured in presence of $CuCl_2$ to generate copper replete conditions. β-galactosidase assay was carried out (Section 2.7).

β-galactosidase activity was calculated using the following formula:

$$\beta\text{-galactosidase activity} = \frac{1000 \times OD_{420}}{V \times t \times OD_{600}}$$

Where V = the volume of cells (ml) and t = the incubation time in min

This experiment was repeated independently as three biological replicates each having three technical replicates. The average *β*-galactosidase readings corresponding to protein : protein interaction were calculated for cells containing different vector fusions and have been summarised in (Figure 3.4).

A very low level (< 0.7) of β-galactosidase activity was detected in cells cultured under copper-deficient conditions, giving a value only slightly above blank. The vectors containing only the Gal4 DNA binding domain and activation domain were also co-transformed and the β-galactosidase activity was measured to ensure there were no false positives and the *lazZ* was transcribed only in the presence of an interaction between the proteins of interest. As expected, the β-galactosidase activity for the vector controls was no greater than the activity observed for the copper-deficient cells. However with the addition of $CuCl_2$, slight increase in β-galactosidase activity was observed in cells that had been co-transformed with the (Mac1 1-168)–Gal4DBD and (Mac1 169-431)-Gal4AD, but this increase was not statistically significant. This indicated that the protein fusions containing the N-terminal DNA binding domain and the C-terminal activation domain, did not undergo a protein interaction. It was observed that copper was incapable of inducing an intramolecular interaction within the CaMac1p in *C. albicans*.

The figure 3.4 shows the mean results of three separate experiments of the β-galactosidase assays, with error bars of standard deviation. The experiment was repeated by swapping the CaMac1p DNA binding domain and the activation domain with the bait and prey vectors and similar results were observed indicating that these results weren't false positives or dependent on the plasmid used for their fusions.
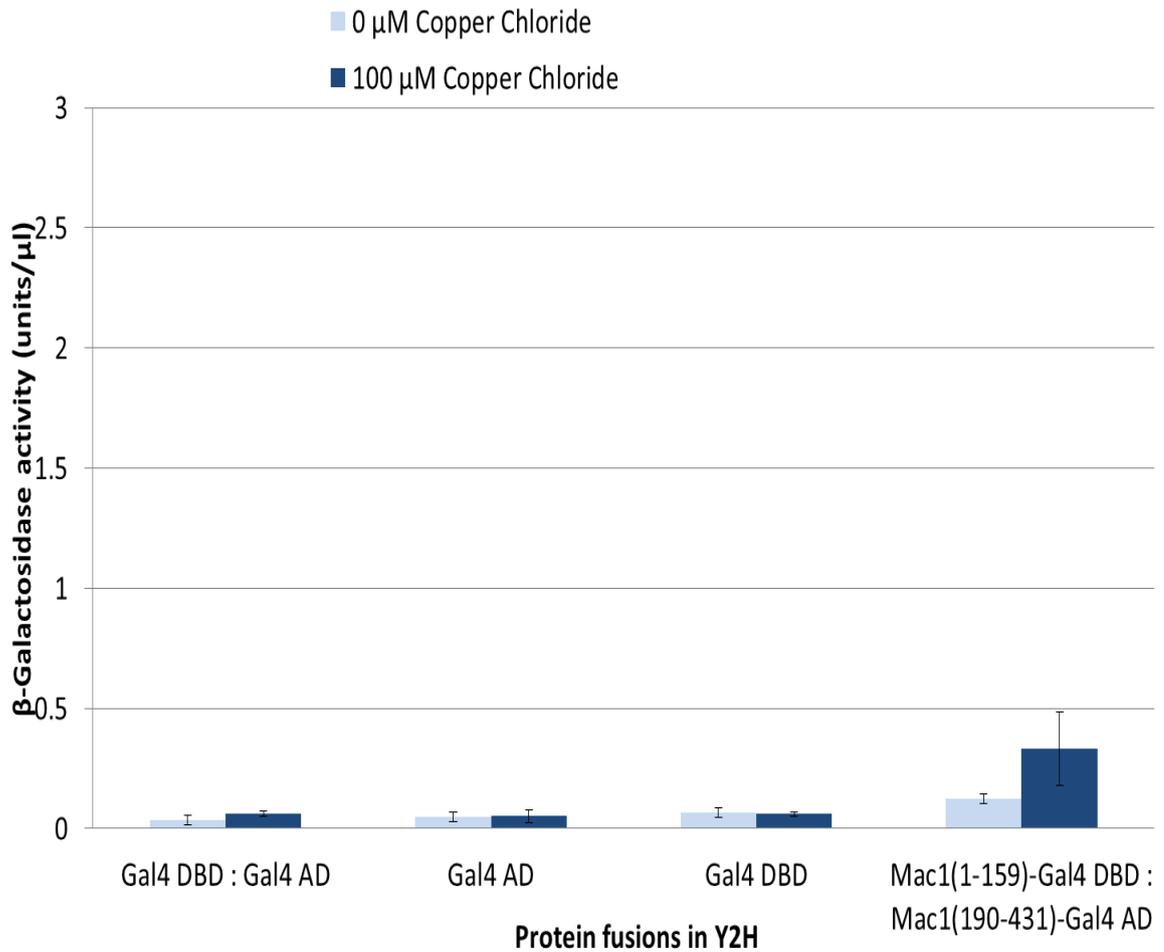
Figure 3. 4: Yeast-two hybrid assay to detect intramolecular interaction in CaMac1p

S. cerevisiae Y187 containing vector fusions with either the Gal4 DNA binding domain or the Gal4 activation domain fused to *C. albicans* Mac1p containing either the N-terminal DNA binding domain or the C-terminal activation domain were grown in excess copper and copper deficient conditions at 30°C on the shaker overnight. β-galactosidase activity was then measured as described in Section 2. . This figure shows the mean results of three separate experiments with error bars of standard deviation. Copper replete conditions were generated by supplementing media with 100 μM $CuCl_2$ +100 μM $FeCl_3$, whereas Copper deficient conditions were generated by supplementing media with 100 μM $FeCl_3$ + 100 μM BCS.

3.3. Analysis of intermolecular interactions of CaMac1p

To determine the domain involved in protein : protein interactions in CaMac1p, constructs containing the *CaMAC1* gene with deletions in the N-terminus or the C-terminus domain were created. These deletions were determined by analysing the sequence and comparing it to the *S. cerevisiae MAC1* sequence.

The ORF for the Mac1p was derived from the *CaMAC1* gene which was previously cloned in vector pGAD424. The *CaMAC1* gene was amplified using primers MAC1 F1 (with artificial *Bam*HI site) and MAC1 R1 (with artificial *SalI* site) to obtain the ORF for the wild-type Mac1p, which gave the Mac1(1-431) fragment. The *CaMAC11* gene was amplified using primers NDEL F1 (with artificial *Bam*HI site) and MAC1 R1 (with artificial *SalI* site) to obtain a 40 residue deletion in the N-terminal region of *CaMAC1* which gave the ΔZF(41-431) fragment. The *CaMAC11* gene was also amplified using primers NDEL F2 (with artificial *Bam*HI site) and NDEL R2 (with artificial *SalI* site) to obtain a 168 residue deletion in the N-terminal region of *CaMAC1* which gave the C1C2(169-431) fragment.

Similarly the gene was also amplified using primers CDEL F1 (with artificial *BamHI* site) and CDEL R1 (with artificial *SalI* site) to obtain a 186 residue deletion of the C2 motif in the C-terminal region of *CaMAC1* which gave the C1(169-245) fragment. The ORF for C2(246-431)p was constructed using primers CDEL F2 (with artificial *BamHI* site) and CDEL R2 (with artificial *SalI* site) to obtain a 77 residue deletion of the C1 motif in the C-terminal region of *CaMAC1*. Additionally a ΔC1C2 or NT(1-168) was generated using primers MAC1 F1 (with artificial *BamHI* site) and CDEL R3 (with artificial *SalI* site) to obtain a 263 residue deletion of the C1 and C2 motif in the C-
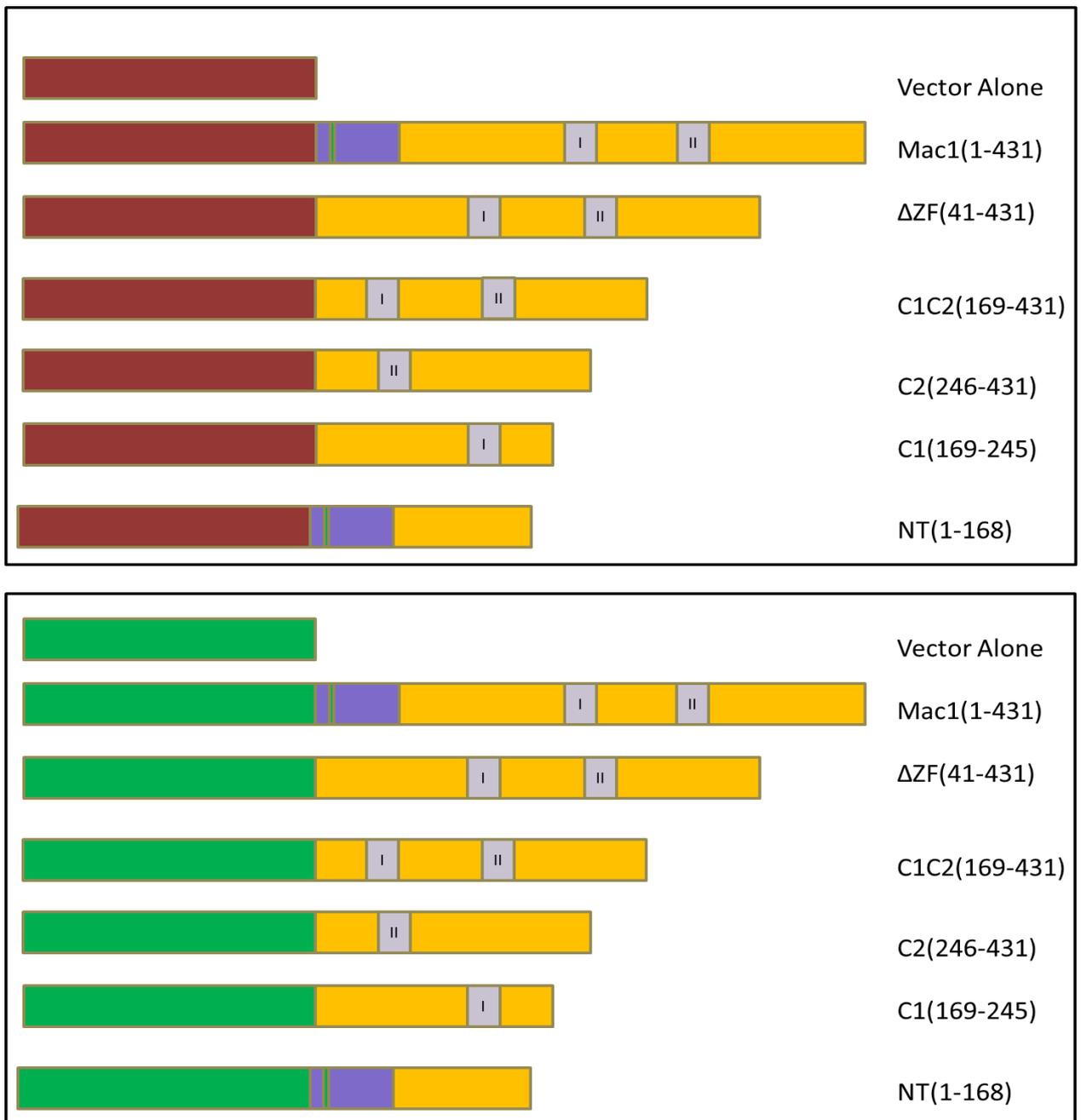
Figure 3. 5: Diagrammatic representation of the different deletions, truncations and CaMac1 protein fusions with the prey (pGAD424) or bait (pGBT9) vectors.

This pictorial diagram represents the different deletions of CaMac1p that were cloned into the prey and bait vectors and co-transformed into *S. cerevisiae* Y187. The co-transformants were allowed to grow at 30°C overnight and the β-galactosidase assays were carried out as described in Methods and materials section. The maroon boxes represent the prey vector, while the green boxes represent the bait vector. The dark purple box represents the Cu-fist motif in the N-terminal, while the light purple rectangles represent the RepI and RepII motifs respectively. These different deletions were used in combination, to analyse the intermolecular interactions of CaMac1p.

-terminal region of *CaMAC1*. Additional nucleotides and START or STOP codons were added to the primers wherever necessary to ensure that on expression, the deleted ORF would be in the correct reading frame to generate the respective fusion proteins. See Table 2.6 for primer sequences.

All PCR-generated fragments and reading frames in the plasmid constructs were confirmed by electrophoresis and sequencing to ensure that PCR was successful and *CaMAC1* ORFs with the desired deletions were obtained. The PCR products and the prey pGAD424, bait pGBT9 vectors were double digested with *BamH1* and *Sal1* and the fusion plasmids were constructed by ligating the deleted fragments into the prey and bait vectors respectively, for two-hybrid analysis. The ligation mixture was then dialysed to remove salts and impurities and transformed into *E. coli* via the electroporation method (Section 2.5). The transformants were plated on Luria agar plates containing ampicillin which is a selective marker for the plasmid as it contains a gene for ampicillin resistance. The colonies observed on the plates after incubation at 37°C overnight, were screened for transformants. Plasmids were extracted from the potential transformant colonies and deletions of the *CaMAC1* gene and the fusions with the vectors were confirmed via sequencing. It was determined that the deleted gene sequences had been cloned within the pGAD424 plasmid vector or the pGBT9 vector (Figure 3.5) containing the sequence for the *Gal4* activation domain or DNA binding domain accordingly, in the correct orientation and the correct reading frame.

Qualitative assay to analyse protein interaction

Saccharomyces cerevisiae was transformed with different combinations of ORF constructs coding for fusion proteins. The bait pGBT9 construct containing the *GAL4*

DNA binding domain fused to the wild-type *CaMAC1* sequence *i.e.* Gal4DBD-Mac1p was initially transformed (via Short transformation protocol- refer 2.4) into *S. cerevisiae* CG1945 and plated onto tryptophan drop-out plates (minimal media lacking tryp). These positive transformants were then passed through a second round of transformation with pGAD424 containing the *GAL4* activation domain fused to the wild-type *CaMAC1* sequence *i.e.* Gal4AD-Mac1p and plated onto leucine and tryptophan drop-out plates. These yeast-two hybrid co-transformants containing both the *GAL4* DNA binding and activation domains; were then streaked onto histidine, leucine and tryptophan triple drop-out plates and the wild-type CaMac1p interactions were studied. 3-AT is added to the yeast culture. This allows increased amounts of *HIS3* expression which allows the positive yeast-two hybrid transformant to survive in selective media containing no histidine. Growth on these triple drop-out minimal media plates is indicative of successful transcription of the reporter *HIS* gene which allows synthesis of histidine and hence is also indicative of protein interaction.

After 48 hours incubation of the histidine-leucine-tryptophan drop-out plate at 30°C, no growth was observed for the vector only control (as expected), visible growth was observed for (2) and (3) and very little growth was observed for 4 (Figure 3.6). No growth for (**1**) the Gal4AD-Mac1 and the Gal4DBD without any fused ORF, indicated that the reporter gene was not being auto-activated. Growth for (**2**) the Gal4AD-Mac1 and the Gal4DBD-Mac1 *i.e.* Mac1p fused to both the plasmids, indicated that CaMac1p could interact with itself while growth for (**3**) containing the Gal4AD-Mac1 and the Gal4DBD-C1C2(169-431), indicated that the deleted protein with 168 amino acid region deletion in the N-terminus could still interact with the wild-type CaMac1p. Very little growth for (**4**) the Gal4AD-Mac1 and the Gal4DBD-ΔC1C2(169-431) indicated that the protein : protein interaction between the wild-type CaMac1p and the deleted

CaMac1p (with 262 amino acid region deletion in the C-terminus) was substantially affected.

These transformants were re-inoculated into broth and allowed to grow at 30˚C overnight. The cultures were allowed to grow until a cell density of 1 x 10^7 cells/ ml was achieved and then 2-folds serial dilution was carried out six times and they were transferred to a fresh histidine-leucine-tryptophan drop-out plate using a 48-pin replicator.

After 48 hours incubation of the histidine-leucine-tryptophan drop-out plate at 30˚C, maximum growth indicating protein : protein interaction was observed for the wild-type CaMac1p : wild-type CaMac1p (Row 1) however the wild-type CaMac1p : CaMac1p containing 162 residue deletion in N-terminus, showed lesser growth (Row 2) and the wild-type CaMac1p : CaMac1p containing 262 residue deletion in C-terminus, showed barely any growth or protein interaction (Row 3 in Figure 3.6 B). To quantify these results more accurately, we used the other *S. cerevisiae* Y187 strain which has the *lacZ* reporter gene from *E. coli* fused to the *GAL1* promoter.

Figure 3.6: Yeast two-hybrid assay carried out in *Saccharomyces cerevisiae*

CG1945, plated on minimal media lacking leucine, histidine and tryptophan for 3-5 days at 30°C.

A. Y2H co-transformants containing (**1**) the Gal4 activating domain and DNA binding domain without any fused Mac1 protein sequence (control) (**2**) entire CaMac1p fused to both the prey and bait vector (**3**) entire CaMac1p on one vector with CaMac1p containing 168 residue deleted N-terminus region on other vector (**4**) entire CaMac1p on one vector with CaMac1p containing 262 residue deleted C-terminus region on other vector.

B. contains the serial dilutions for Y2H transformant containing (**Row 1)**) entire CaMac1p fused to both the prey and bait vector (**Row 2**) entire CaMac1p on one vector with CaMac1p containing 168 residue deleted N-terminus region on other vector (**Row 3**) entire CaMac1p on one vector with CaMac1p containing 262 residue deleted C-terminus region on other vector.

Growth is indicative of positive protein - protein interactions.

A. Y2H co-transformants streaked out on minimal media plate lacking leucine, histidine and tryptophan.
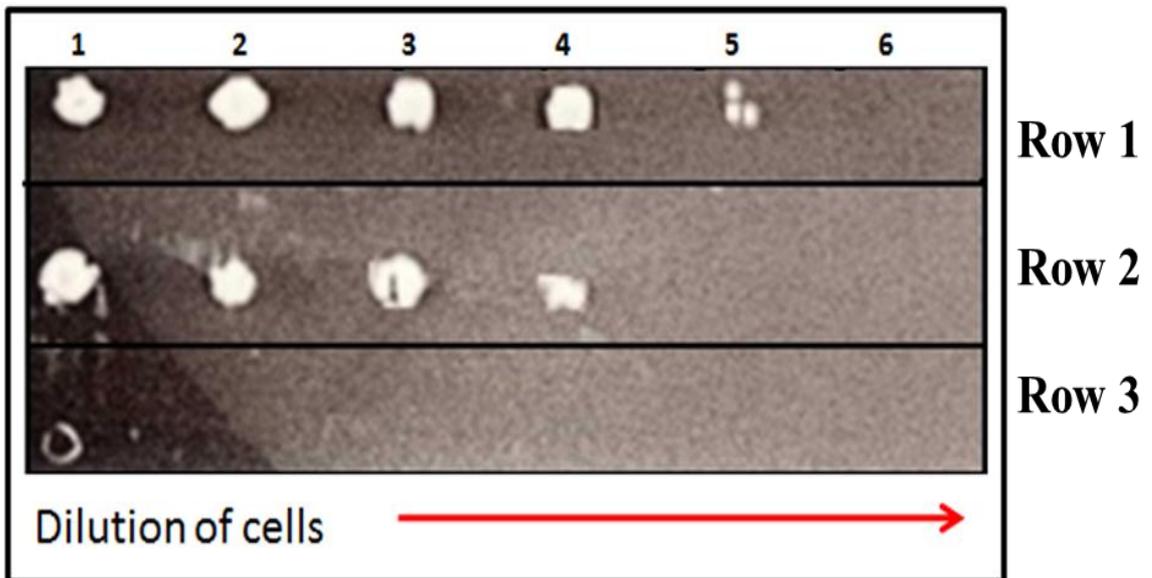
B. Serial dilutions of Y2H co-transformants, spot-platted on minimal media lacking leucine, histidine and tryptophan.

Qualitative β-galactosidase filter paper assay to analyse protein interaction

The gene constructs were then transformed in *S. cerevisiae* strain Y187 (using the Lithium Acetate method) where the yeast two-hybrid assay was performed. Expression of the *GAL1–lacZ* reporter gene was assessed.

The co-transformants were grown either in the presence of BCS, which is a copper chelator, to generate copper deficient conditions or they were cultured in presence of $CuCl_2$ to generate copper replete conditions. β-galactosidase assays carried out using prey and bait vectors transformed without containing the protein coding ORFs were included as negative control to determine the background amounts of β-galactosidase activity.

S. cerevisiae Y187 was transformed with different combinations of construct coding for fusion proteins as described earlier in the Drop-out plate assay section. The yeast-two hybrid positive co-transformants from the leucine, tryptophan drop-out plates, containing both the *GAL4* DNA binding and activation domains; were replicated onto a Whatman #1 filter paper and fixed using liquid nitrogen. Filter assay for β-galactosidase activity (Section 2.7) was carried out and the CaMac1p interactions were studied by appearance of blue colour in the presence of X-Gal after 48 hours incubation at 37˚C.

After 48 hours incubation of the filter paper at 37˚C, no blue colouration was observed for (**1**) the Gal4AD-Mac1 and the Gal4DBD without any fused ORF, indicating no protein - protein interaction which confirmed that the reporter gene was not being auto--activated in the absence of protein interaction. Blue colouration was observed on the filter paper, for (**2**) the Gal4AD-Mac1 and the Gal4DBD-Mac1 *i.e.* Mac1p fused to both the plasmids and (**3**) containing the Gal4AD-Mac1 and the Gal4DBD-C1C2(169-
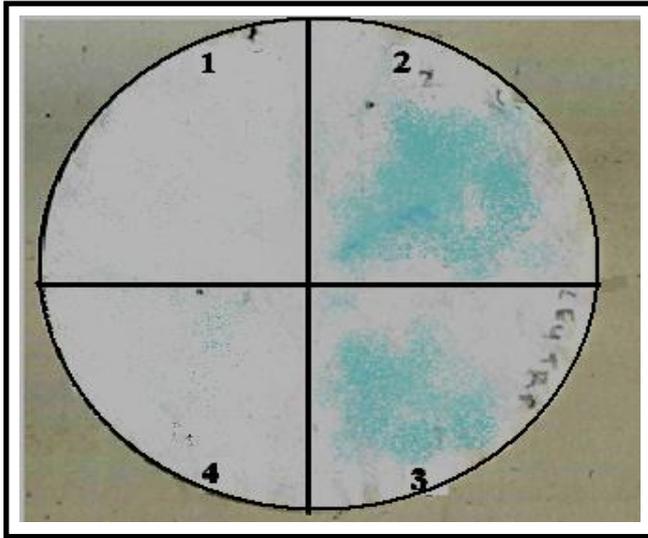
Figure 3.7: Yeast two-hybrid assay carried out in *Saccharomyces cerevisiae* Y187

Y2H co-transformants containing (**1**) the Gal4 activating domain and DNA binding domain without any fused Mac1 protein sequence (control) (**2**) entire CaMac1p fused to both the prey and bait vector (**3**) entire CaMac1p on one vector with CaMac1p containing 168 residue deleted N-terminus region on other vector (**4**) entire CaMac1p on one vector with CaMac1p containing 262 residue deleted C-terminus region on other vector. The co-transformants were grown in the presence of either BPS or $CuCl_2$ and plated onto leucine as well as tryptophan drop-out plates and allowed to grow at 30°C for 2-5 days. The growth was then replica plated onto filter paper and exposed to X-Gal, overnight at 37°C. The blue colouration is indicative of protein : protein interactions.

431) [*i.e.* Mac1p with 168 residue deleted N-terminus region] and comparatively slight blue colouration was observed for (**4**) the Gal4AD-Mac1 and the Gal4DBD-ΔC1C2(169-431) [*i.e.* Mac1p with 262 residue deleted C-terminus region] (Figure 3.7). These results were comparable to the ones obtained in the drop-out plate assay (Figure 3.6) however the levels of β-galactosidase activity could not be measured accurately. A more quantifiable measurement of β-Galactosidase activity was required and was obtained by assaying the hydrolysis of ONPG using the ONPG assay of β-galactosidase activity (next section).

Quantitative assay using ONPG to analyse protein interaction

As mentioned earlier (Drop-out plate assay), *S. cerevisiae* Y187 was transformed with different combinations of construct coding for fusion proteins. The yeast-two hybrid co-transformants (from leucine, tryptophan drop-out plates) containing both the *GAL4* DNA binding and activation domains; were then inoculated in liquid media and allowed to grow at 30˚C on a shaker, overnight. The overnight cultures were used to assay the *β*-Galactosidase activity using the ONPG quantitative assay as described earlier (Section 2.7 and 3.2).

This experiment was repeated independently as three biological replicates each having three technical replicates *i.e.* the experiment was repeated on three separate days utilising new starting cultures each having three replicates. The average *β*-galactosidase readings corresponding to protein : protein interaction were calculated for different combinations of deleted CaMac1p on both bait or prey vectors and have been summarised in (Figure 3.8 and 3.9). The most significant readings showing the interaction of deleted CaMac1p (on bait vector) with wild-type CaMac1p (on prey

vector), have been highlighted in Table 3.1. In copper deficient conditions ($0\mu M$ $CuCl_2$), negligible β-galactosidase activity was measured however in excess copper conditions ($100\mu M$ $CuCl_2$), a significant level of β-galactosidase activity was measured suggesting that wild-type CaMac1p interacted with another whole CaMac1p leading to a copper induced homo-dimerisation. The fold decrease in interaction between the different versions of deleted CaMac1p : wild-type CaMac1p as opposed to wild-type : wild-type interaction have also been calculated. When the zinc-finger DNA binding motif in the N-terminus was removed, there was a 1.2-fold decrease in the level of β-galactosidase activity as compared to the wild-type protein however when the C2 domain in the C-terminus was removed, a massive 28-fold decrease in the levels of β-galactosidase activity was observed suggesting that the C2 domain in the C-terminus, was mainly responsible for protein : protein interactions.

The readings obtained were plotted on graph (Figure 3.9) and the error bars as well as standard deviation was calculated. The readings were then analysed using a one-tailed Student's T test and the C2 domain deletions readings, were found to be statistically significant ($P < 0.05$).
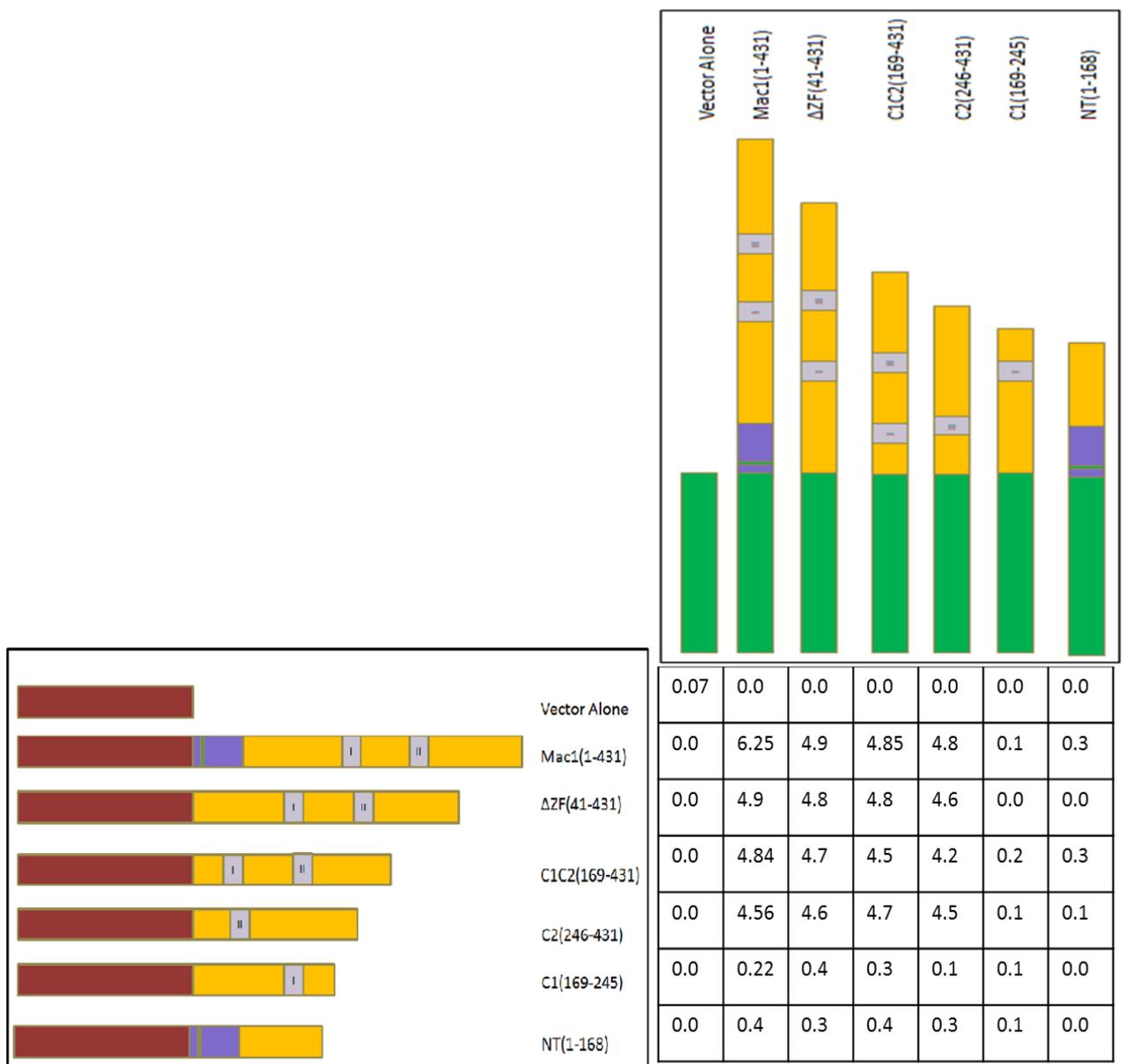
Figure 3. 8: Yeast two-hybrid assay to determine protein - protein intermolecular interaction domain in Mac1p.

This pictorial diagram illustrates the different deletions of CaMac1p that were cloned into the prey and bait vectors and co-transformed into *S. cerevisiae* Y187 (Refer figure 3.5). The co-transformants were allowed to grow at 30°C overnight and β-galactosidase assays were carried out as described in Methods and materials section. These deletions were used to analyse the intermolecular interactions of CaMac1p. Average *β*-galactosidase readings corresponding to each combination of vector transformants, depicting protein - protein interactions in the presence of copper, have been shown in the table.
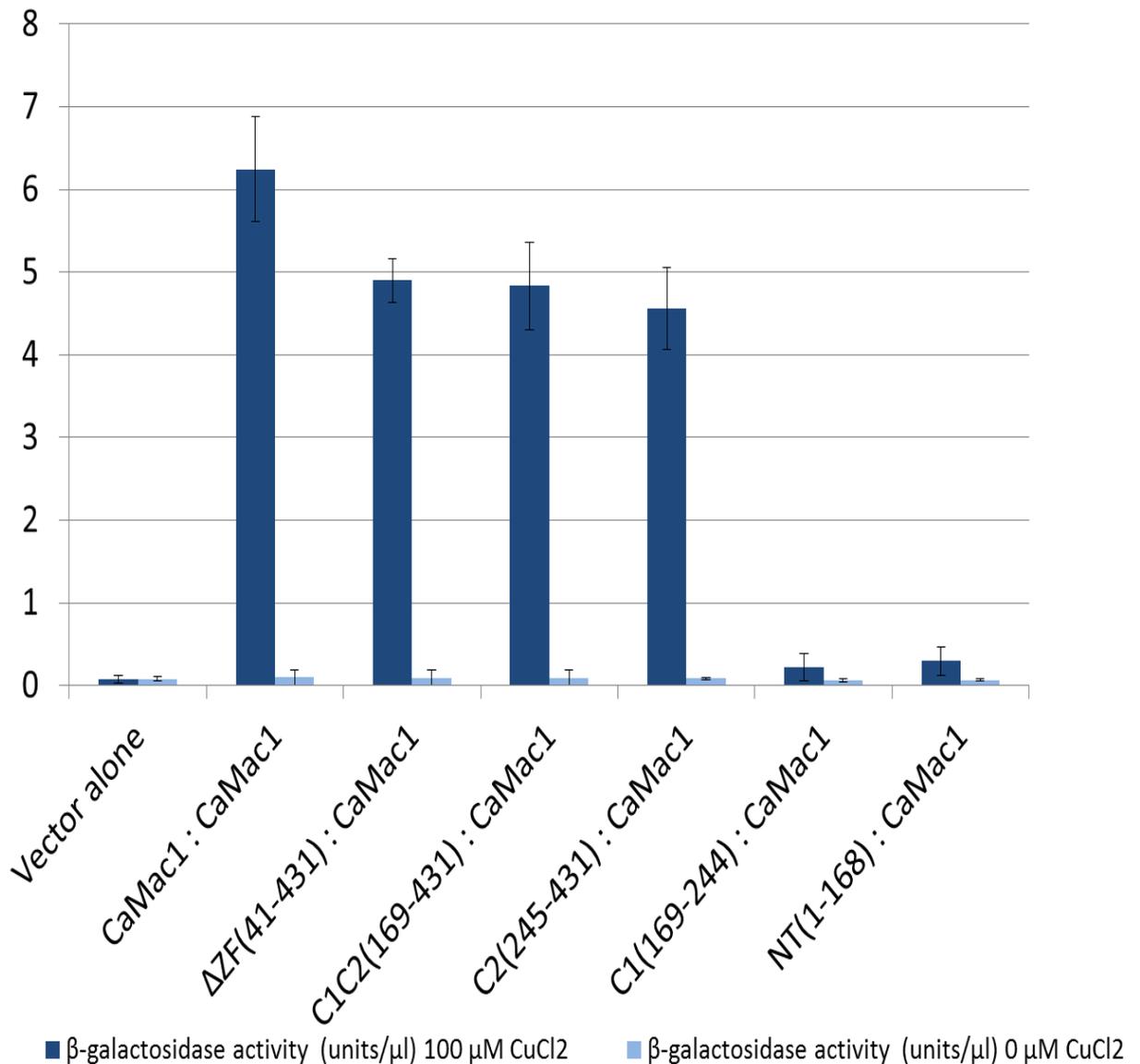
Figure 3. 9: Average β-galactosidase readings corresponding to protein : protein interaction obtained using yeast-two hybrid assay

S. cerevisiae Y187 containing wild-type CaMac1 protein-*lacZ* fusion in combination with either wild-type CaMac1 protein-*lacZ* fusion, or deleted CaMac1p-*lacZ* fusion with deletions in Zinc-finger motif, C1 domain, C2 domain, N-terminus or C-terminus region. Co-transformants were grown in high copper media containing 100 μM CuCl₂ or low copper media containing 100 μM BCS, overnight at 30°C and β-galactosidase assays were carried out as described in Methods and Materials 2.7. This figure shows the mean results of three separate experiments with error bars of standard deviation.

Vector/protein fusions & interactions	β-galactosidase activity (units/μl)	Difference from wild-type interaction (p value)	Fold decrease from Wild-type interaction
Vector alone	0.073	0.001	85.181
CaMac1 : CaMac1	6.246	n/a	n/a
ΔZF(41-431) : CaMac1	4.901	0.058	1.274
C1C2(169-431) : CaMac1	4.836	0.075	1.291
C2(245-431) : CaMac1	4.556	0.059	1.370
C1(169-244) : CaMac1	0.221	0.001	28.393
NT(1-168) : CaMac1	0.199	0.002	31.285

Table 3. 1: Summary of β-galactosidase assays

The results of all β-galactosidase assays carried out in figure 3.9 is summarised in this table, showing the statistical significance of each construct relative to the wild-type CaMac1p : CaMac1p interaction. The results of a one-tailed student's t-test are shown as P values ($\alpha = 0.05$). Results are deemed to be statistically significant if the P value is less than 0.05.

3.4. Analysis of synergism between Copper response sites of CaMac1p

CaCTR1 promoter/*lacZ* hybrid genes containing one, two, three or no CuRE elements were constructed. The *CaCTR1* promoter from -776bp to -3bp (relative to ATG translation site) was originally amplified from *C. albicans* genomic DNA using primers CaCTR1 776 F and CaCTR1 776 R (Woodacre, 2007), which incorporated restriction sites for *SalI* and *XmaI*. The PCR product and plac-poly were digested with *SalI* and *XmaI* and the fusion plasmid was generated by ligating the *CaCTR1* promoter to the MCS of plac-poly. The fusion plasmid was then linearized by digesting it with *StuI* and it was transformed into *C. albicans* strain BWP17 (wild type). *CaURA3* was used as the selectable marker to identify transformants in the parental strain BWP17 (*ura3/ura3ΔΔ*). Plac-poly without a promoter inserted in it, was used as a negative control to determine the background level of *β*-galactosidase activity.

We used the reporter plasmid pA1 (776 bp *CaCTR1* promoter) which contains -776 to -3 of *CaCTR1* promoter which was originally generated in the (Woodacre, 2007) study (referred to as pAWC1). Reporter plasmid pA2 (501bp promoter) containing sequences -776 to -275 was generated. A promoter containing two CuRE1 elements (pA3; 623bp promoter) was generated by PCR mutagenesis using a mutagenic oligonucleotide, which inserted a copy of sequences -397 to -275 (which includes a copy of CuRE1) to the CuRE1 element present in pA2. The spacing between the first and second copy of the CuRE1 element was 111bp which is similar to that seen between CuRE1 and CuRE2 in the wild-type *CaCTR1* promoter. pA4 (745bp promoter) containing three copies of CuRE1 was constructed in a similar manner. The artificial promoters were designed in a way so as to ensure they weren't significantly different

from the wild-type promoter. A reporter plasmid pA5 (378bp promoter) containing no CuRE elements was also generated containing sequences -776 to -398. BWP17 transformants containing single copies of the reporter plasmids were grown in high and low copper media and β-galactosidase activity was measured as described in section 2.7.

The 776bp wild-type promoter containing CuRE1, CuRE2 and CuRE3 showed wild-type levels of β-galactosidase activity while the deleted 501bp promoter containing only one copy of CuRE1 showed a slight decrease in β-galactosidase activity (Figure 3.10). The β-galactosidase activity of the 623bp promoter containing two copies of CuRE1 was found to increase and was almost equal to the wild-type levels shown by intact promoter containing CuRE1, CuRE2 and CuRE3. The promoter containing three copies of CuRE1 showed an increase in β-galactosidase expression as compared to the wild-type promoter. The increase in expression is each case was additive but was not exponential like in *Saccharomyces cerevisiae*, indicating that the activation by CaMac1p via the CuRE sites was not synergistic and binding of CaMac1p to CuRE was not dependent on the presence of more than one CuRE sequence.
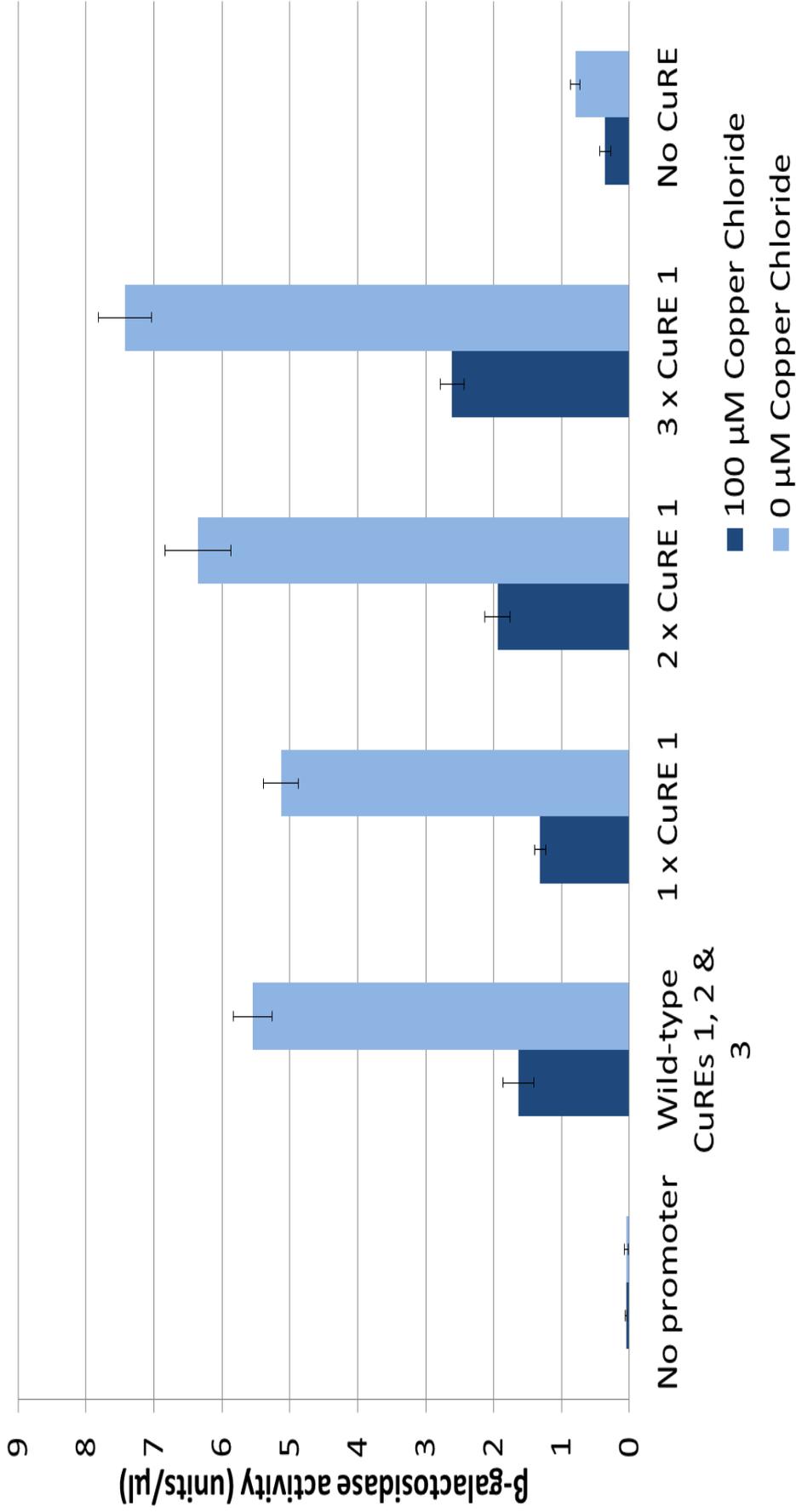
Figure 3. 10: Average β-galactosidase activity of CaCTR1-lacZ fusions with one or more copy of copper response element CuRE1

C. albicans BWP17 containing either wild-type *CaCTR1* promoter-*lacZ* fusion, or mutant *CaCTR1-lacZ* fusion with one, two or three copies of CuRE1 indicated, were grown in high and low copper media and β-galactosidase assays were carried out as described in Methods and Materials **2.**. This figure shows the mean results of three separate experiments with error bars of standard deviation.

Strain	CuRE mutations	High copper		Low copper		Fold increase in low copper	Difference between copper levels (p value)	Fold increase in low copper as compared to 1 x CuRE
		β-galactosidase activity (units/μl)	Difference from wild-type (p value)	β-galactosidase activity (units/μl)	Difference from wild-type (p value)			
pA0	No promoter	0.05	0.003	0.048	0.0005	0.951	0.462	n/a
pA1	Wild-type CuREs 1, 2 & 3	1.634	n/a	5.548	n/a	3.395	0.002	n/a
pA2	1 x CuRE 1	1.316	0.034	5.129	0.122	3.898	0.0004	n/a
pA3	2 x CuRE 1	1.942	0.040	6.349	0.011	3.270	0.002	1.238
pA4	3 x CuRE 1	2.611	0.007	7.427	0.009	2.845	0.001	1.448
pA5	No CuRE	0.357	0.002	0.797	0.0004	2.232	0.017	0.155

Table 3. 2: Summary of β-galactosidase assays

The results of all β-galactosidase assays carried out in figure 3.10 is summarised in this table, showing the statistical significance of each construct relative to the wild-type *CaCTR1-lacZ* promoter fusion, containing CuREs 1 2 & 3. The results of a one-tailed student's t-test are shown as P values ($\alpha = 0.05$). The relative increase in activity in low copper is also shown with the statistical significance of this increase. Results are deemed to be statistically significant if the P value is less than 0.05.

3.5. CaMac1 protein purification, expression and analysis

It had been previously hypothesised that CaMac1p binds to CuRE sequences in promoters of target genes such as *CaCTR1*, *CaFRE7* and *CaMAC1* itself to regulate their transcription in response to copper levels (Woodacre, 2007, Woodacre *et al.*, 2008). However there was no functional evidence available for this before. Hence we decided to purify CaMac1p and use the EMSA (electrophoretic mobility shift assay) technique to demonstrate CaMac1p:DNA binding. It is based on the principle that when subjected to non-denaturing polyacrylamide, protein:DNA complexes migrate more slowly than free linear DNA fragments. The rate of migration of DNA through a gel decelerates, when bound to protein. This technique is also called gel-retardation or a gel-shift assay.

Gel-shift assay

A His-tagged CaMac1 protein was expressed using the pET28α vector as described in Methods and materials Section 2.9 and despite low concentration; functional protein was purified and used in a gel-shift assay (Figure 3.12 B). The probes for gel-shift assays for *CaCTR1* (295 bp), *CaFRE7* (197 bp) and *CaMAC1* (221 bp) promoters were amplified using primers CTR1 probe F and CTR1 probe R; MAC1 probe F and MAC1 probe R respectively (Table 2.6) The probe oligonucleotides were electrophoresced (Figure 3.12 A), gel-extracted, purified and labelled with DIG. A DIG-labelled PCR probe of the *CaCTR1*, *CaFRE7* or *CaMAC1* promoter was then incubated with purified CaMac1-His1 protein in the presence of either 30 µM BCS (a copper chelator) or 100 µM CuCl$_2$. Samples were run on a native gel, blotted to a membrane and detected with

anti-DIG Fab fragments and CSPD. Protein-probe binding was seen as an additional distinctive band on the blot indicative of the retarded mobility on the gel of a protein : DNA complex. *CaCTR1* transcription was found to be induced in copper restricted conditions (Figure 3.12 B) and initial gel-shift data suggested that this was due to CaMac1 directly binding the *CaCTR1* promoter to initiate transcription (Figure 3.12 B). In addition, the previously described *CaMAC1* auto-regulation is likely accomplished through a similar mechanism*,* as the recombinant CaMac1-His1 protein bound the *CaMAC1* promoter in copper restrictive conditions. Interestingly a second fragment indicative of CaMac1p binding to *CaFRE7* promoter, was not observed in the gel-shift indicating the CaMac1p does not directly bind *CaFRE7*.
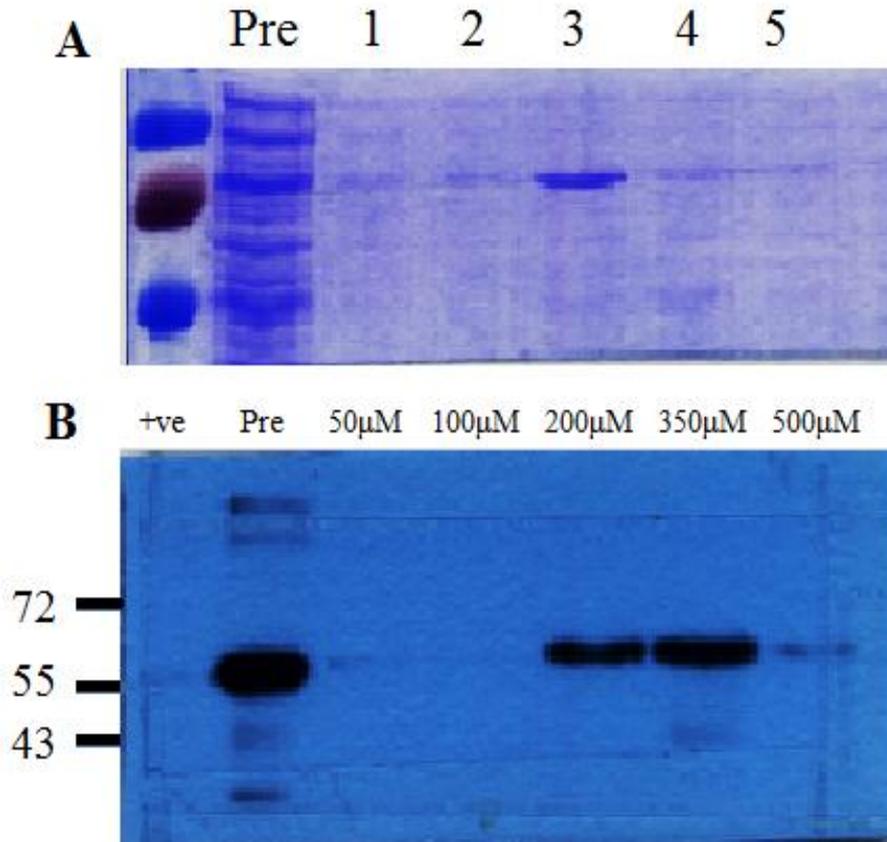
Figure 3. 11: SDS-polyacrylamide gel electrophoresis and Western of CaMac1-His tagged fusion protein

CaMAC1 sequence was transformed into *E. coli* RIL cells for recombinant protein expression. Cells were grown in 250 ml Luria broth at 30 °C for 2 hours and 70 µl of 1M IPTG to induce production of protein. The CaMac1-His tag protein was subsequently purified using His-select columns. The extract was loaded onto 5ml His crude column and washed with 25 ml of water and Imidazole. The sample of eluted protein from each wash was monitored by SDS-polyacrylamide gel electrophoresis (**A.**). The CaMac1-His tag protein was eluted with a 100–500 mM imidazole linear gradient in elution buffer. The eluted proteins were monitored by Western blotting (**B.**) as described in Methods and materials section, and a single band at the correct molecular weight for the recombinant protein fusion was observed.
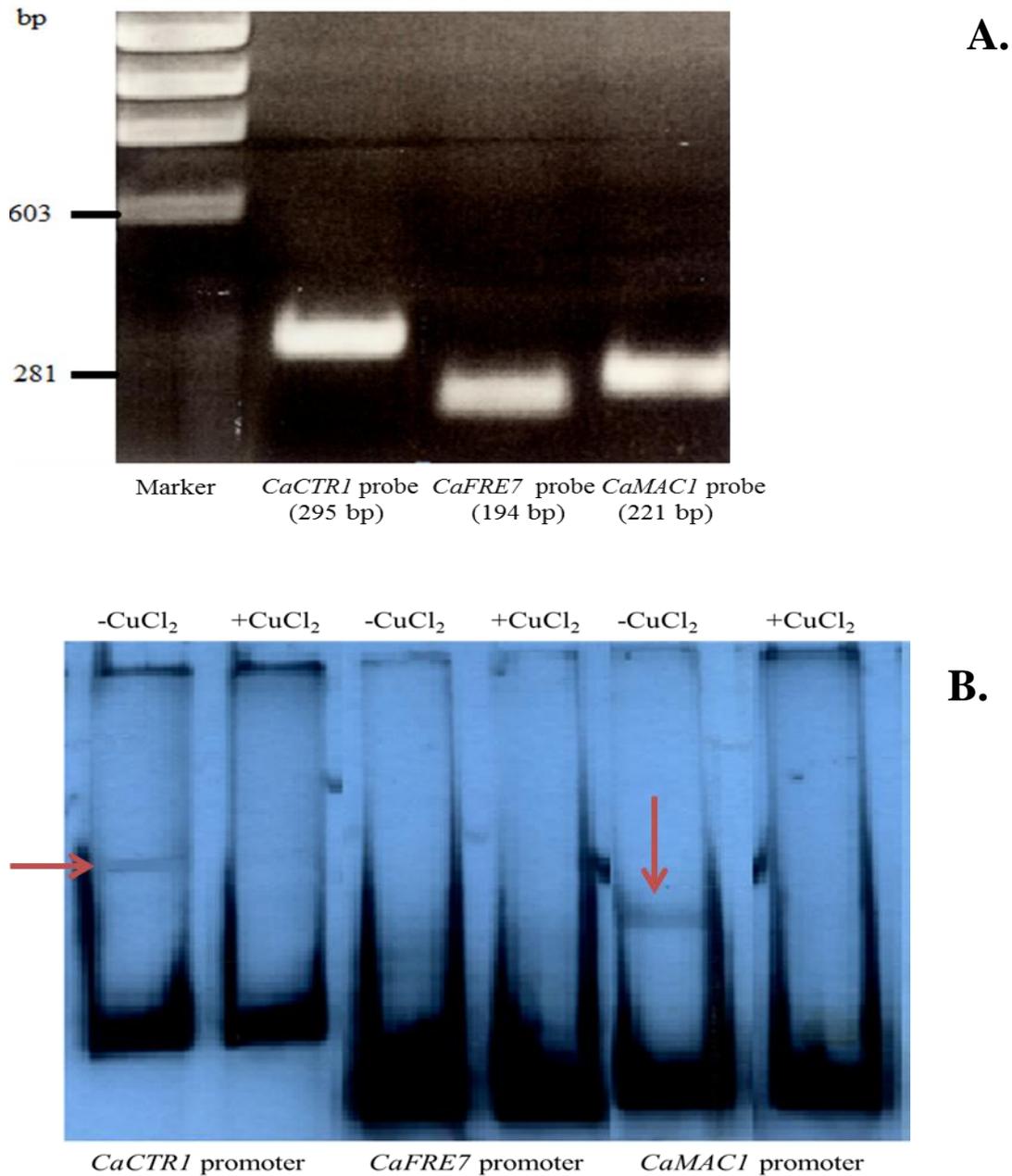
Figure 3. 12: Gel Shifts of CaCTR1 and CaMAC1 Promoters with the CaMac1 Protein.

 (A.) The PCR probe amplicons were electrophoresced on 1.5% agarose gel and the expected size probe fragments were observed. These fragments were gel extracted and purified. (**B.**) DIG-labelled PCR probes of the *CaCTR1*, *CaFRE7* or *CaMAC1* promoter was incubated with purified CaMac1 protein in the presence of either 30 μM BCS (a copper chelator) or 100 μM CuCl$_2$. Samples were run on a native gel, blotted to a membrane and detected with anti-DIG Fab fragments and CSPD. Protein-probe binding is seen as an additional distinctive band on the blot which is characteristic of the retarded mobility in the gel of protein - DNA complexes.

3.6. Discussion

The objective of the work described in this chapter was to gain a better grasp of the molecular mechanism of action of CaMac1p and to identify, as well as highlight the dissimilarities between the Mac1 proteins in *C. albicans* and *S. cerevisiae*. Using yeast-two hybrid assays, it was demonstrated that Mac1 DBD (residues 1–168) fused to the Gal4 DBD and the C-terminus activation domain of Mac1 (residues 169–417) fused to the Gal4 AD did not result in an *in vivo* protein–protein interaction as determined by lack of expression of the *GAL1–lacZ* reporter. CaMac1p does not undergo a copper induced intramolecular interaction, and the N-terminal DNA binding domain of the protein does not associate with the C-terminal transactivation domain leading to the absence of a heterodimer between the two separate fusion proteins, in copper replete conditions.

However, using yeast-two hybrid assays, it was seen that CaMac1p can bind with itself and is capable of an intermolecular self-interaction and homo-dimerisation. This finding was able to alleviate the previously considered theories of, Mac1p in *C. albicans* being incapable of self-association. It was also seen that when the zinc-finger DNA binding motif in the N-terminus was truncated, there was a 1.2-fold decrease in the level of β-galactosidase activity as compared to the wild-type protein however when the C2 domain in the C-terminus was truncated, a massive 28-fold decrease in the levels of β-galactosidase activity was observed. This data suggest that C2 domain in the C-terminus of CaMac1p was responsible for protein : protein interactions while the N-terminus domain is likely helping to increase binding activity of C-terminus domain.

However when a similar experiment was performed in *S. cerevisiae* (Serpe M *et al.*, 1999), they observed slightly different results. It was observed that on deletion of N-

terminus domain in ScMac1p, the truncated protein showed increased interaction via C-terminus. They predicted that could be happening as a consequence of the way in which the protein was folding. They proposed that the N-terminal domain of the protein was probably undergoing an intramolecular interaction with the C-terminal domain and therefore masking its protein binding ability. As a result, on deletion of N-terminus domain, the interaction via the C-terminus increased. They also noticed an copper dependent intramolecular interaction between the N-terminus DNA binding domain and C-terminus activation domain in ScMac1p which was absent in CaMac1p.

Our contradictory results in *C. albicans* indicate that it is likely that CaMac1p folds in a different manner than ScMac1p. The lack of intramolecular interaction in *C. albicans* CaMac1p appears to be of a beneficial nature and not of an inhibitory nature as is the case with the presence of repressive intramolecular interaction in *S. cerevisiae*. In *C. albicans*, the lack of intramolecular interaction within CaMac1p allows the C2 domain in C-terminal to be better exposed and hence allows for increased protein interactions, in the presence of copper.

Using *CaCTR1*-promoter/*lacZ* fusions, it was found that the wild-type promoter containing CuRE1, CuRE2 and CuRE3 showed high levels of β-galactosidase activity while the truncated promoter containing only one copy of CuRE1 showed a slight decrease in β-galactosidase activity. This was consistent with previous results indicating that CuRE1 has the most effect on activation by CaMac1p and it is sufficient for copper dependent regulation of the *CaCTR1* promoter (Woodacre *et al.*, 2008). The β-galactosidase activity of the promoter containing two copies of CuRE1 was found to increase and was almost equal to the wild-type levels shown by intact promoter containing CuRE1, CuRE2 and CuRE3. The promoter containing three copies of CuRE1 showed a greater increase in β-galactosidase expression as compared to the

wild-type promoter. Promoter fusions containing two CuRE1 elements, showed a 1.2-fold increase while promoter fusions containing three CuRE1 elements, showed a 1.4 fold increase as compared to promoter fusions containing a single CuRE1 element. Inserting multiple copies of CuRE1 sequence also tested the theory that it is the number of CuREs present that affects expression of lacZ reporter and not the specific sequences of the CuREs (which are not all exactly the same) or the promoter DNA surrounding the CuREs (personal communication, Woodacre A.).

Similar experiments in *S. cerevisiae* gave dissimilar results as there was a massive 8-fold and 13-fold increase in expression when the number of CuREs were increased to two and three, respectively, which indicated that the CuRE sites showed synergism. The increase in expression is each case in *C. albicans* was additive but was not exponential like in *S. cerevisiae*, indicating that the activation of target genes in *C. albicans* by CaMac1p via the CuRE sites was not synergistic and hence presence of more than one CuRE was not crucial. This is consistent with our previous results and hypothesis that indeed CaMac1p does not need to function as a dimer *in vivo* and hence one CuRE is sufficient for wild-type levels of transcription of target genes.

Repeated CuRE elements are not necessary for high affinity DNA binding based on *CaCTR1*-promoter/*lacZ* fusion data. This data is yet again contrasting to the observations made in *S. cerevisiae* where minimum of two CuRE elements was found to be necessary for high level *in vivo* transcriptional activation and increasing the number of CuRE elements in promoter/*lacZ* fusions from one to four showed "a more than additive effect on *lacZ* expression levels consistent with synergism" (Jensen *et al.*, 1998). The data in this chapter suggests that in *C. albicans*, there exists no synergism between the CaMac1 molecules bound to multiple sites and hence more than one CuRE is not essential for high level expression of CaMac1p-regulated genes.

EMSA data showed protein-probe binding as an additional distinctive fragment on the blot indicative of the retarded mobility on the gel of a protein:DNA complex. *CaCTR1* was found to be induced in copper restricted conditions (Figure. 3.12) and gel-shift data gave us functional evidence for CaMac1p directly binding the *CaCTR1* promoter to initiate transcription. In addition, the previously described *CaMAC1* auto-regulation is likely accomplished through a similar mechanism, as the recombinant CaMac1 protein also bound to the *CaMAC1* promoter in copper restrictive conditions. Interestingly a second retarded fragment indicative of CaMac1p binding to *CaFRE7* promoter, was not observed on the EMSA suggesting that CaMac1p does not directly bind *CaFRE7* promoter. This result is conflicting to what was supposed due to work carried out in our laboratory (Woodacre, 2007) where it was hypothesised that CaMac1p binding to the CuREs in *CaFRE7* promoter, led to activation of its transcription.

The gel-shift data from this study indicates that it is plausible that an intermediate transcription factor may be involved in the regulation of *CaFRE7* via *CaMAC1*. Hence CaMac1p does not directly bind to *CaFRE7* promoter in order to regulate its transcription but employs a co-transcription factor. An alternate reasoning for these contrary results could be that the purified CaMac1 protein was of very low concentration and hence the gel-shift was unable to detect the very low level of CaMac1p binding to *CaFRE7* promoter-probe. A number of different techniques and columns were used but CaMac1p continued to show extremely low solubility and the protein yield could not be increased. Therefore the small concentration of functional protein that could be obtained- was used for the gel-shift study in this chapter.

To summarise the data obtained in this chapter, it was found that in *C. albicans* the intermolecular self-association between the entire Mac1p-Mac1p is quite strong and not weak as was the case in *S. cerevisiae*. The interaction of complete Mac1 proteins in *S.*

cerevisiae showed a negligible amount of β-galactosidase activity (0.4 units) (Serpe M *et al.*, 1999) while the interaction of entire CaMac1 proteins in *C. albicans* showed 15-times more significant amount of β-galactosidase activity (6.246 units- refer Table 3.1) which yet again indicated a difference in folding of the quaternary structure of the proteins in the two organisms. This is also supported by the yeast-two hybrid assay which was used to detect intramolecular interactions and showed an absence of interaction between two halves of CaMac1p. It was also seen that on deletion of N-terminal domain the protein interaction reduced significantly. This indicates that the presence of the N-terminal domain is beneficial to protein-protein interactions. The lack of intramolecular interaction between the N-terminal and C-terminal domains allows the protein interacting C2 domain in C-terminus to be uncovered which allows maximum protein interaction to occur, via it.

In *C. albicans* the intermolecular self-interaction is stronger in the presence of copper, as observed in this study. This indicates that in the presence of copper the CaMac1p does not undergo an intramolecular interaction whereby the lack of binding of N-terminal to C-terminal causes the protein to be able to associate with other Mac1p and form dimers and hence the dimers cannot bind to target genes. This is consistent with the significant copper-dependent, CaMac1p self-interactions observed via the C-terminus.

An explanation for the observed results in this chapter is that in *C. albicans*, in copper replete conditions, the CaMac1 protein molecules that were already present do not undergo a copper-induced intramolecular interaction. The lack of N-terminal binding to the C-terminal promotes protein interactions which lead to CaMac1p forming dimers in its unused state. Since N-terminal also seems to play a role in dimerisation, it is likely that the CaMac1p dimers interact with each other in a way different from the ScMac1p
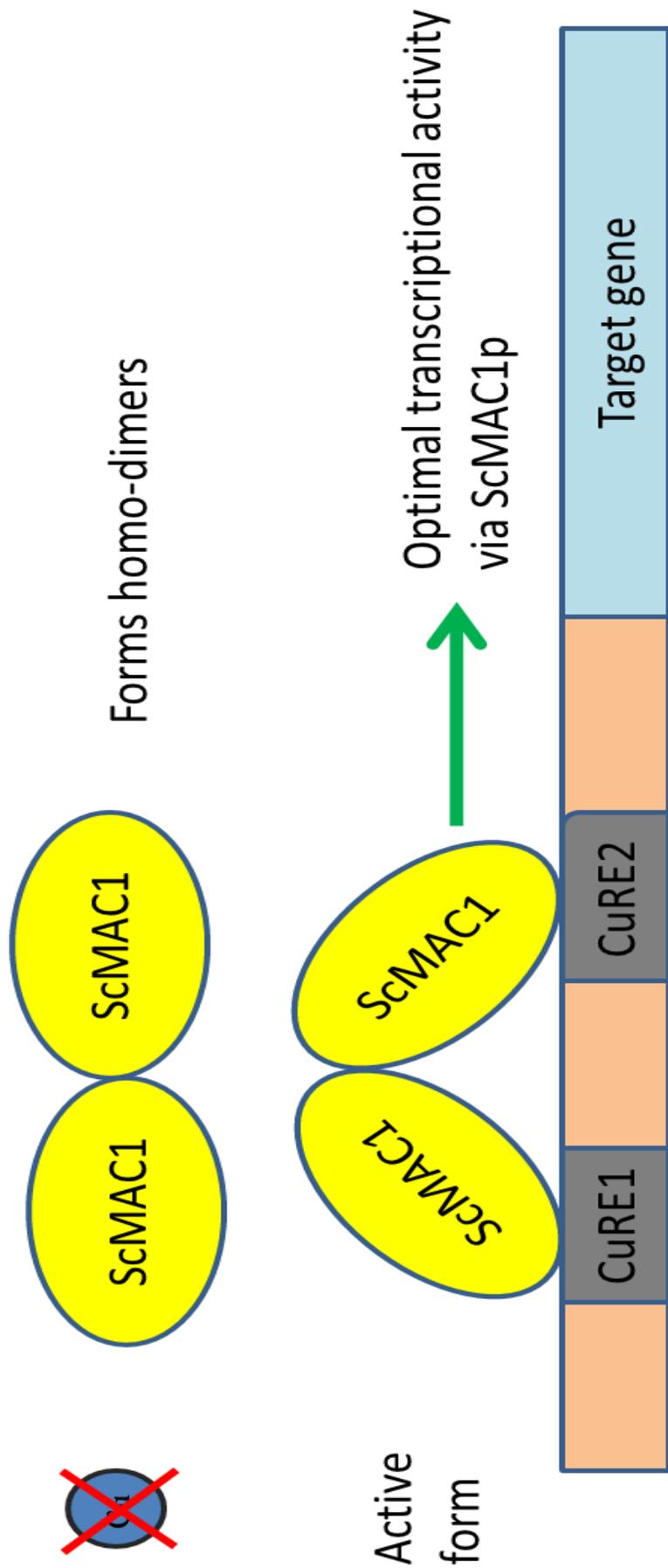
Figure 3. 13: Diagrammatic representation of molecular mechanism of action of ScMac1p.

ScMac1p forms homodimers in the absence of copper. This homodimer is the active transcriptional unit in *S. cerevisiae*, which then binds to two CuRE sequences in promoters of target genes to bring about optimal transcription.
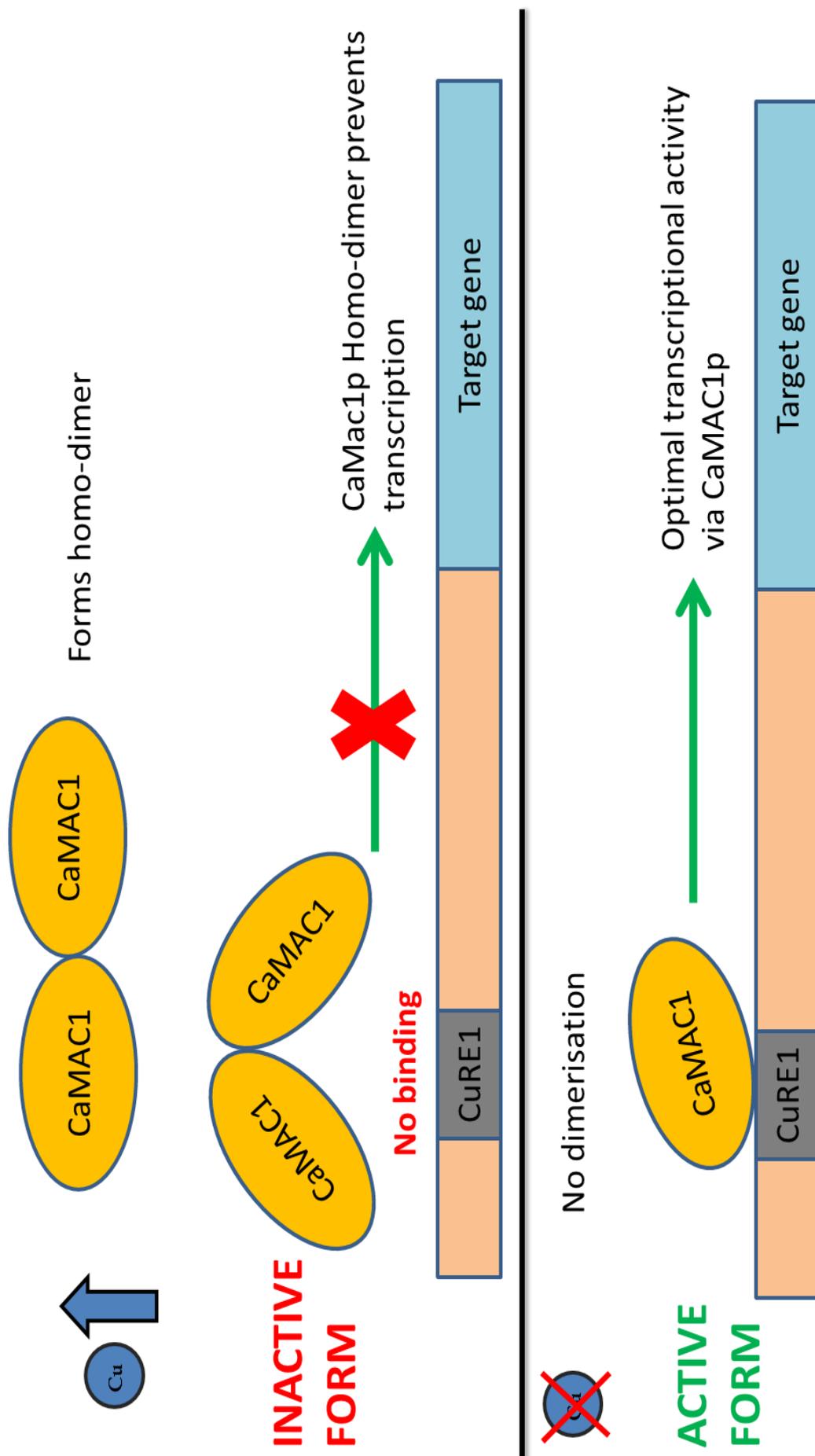
Figure 3. 14: Diagrammatic representation of molecular mechanism of action of CaMac1p.

CaMac1p forms homodimers in the presence of copper. This homodimer is the inactive transcriptional unit in *C. albicans* and does not bind to CuRE sequences in promoters of target genes, including its own gene (*CaMAC1*), thereby constitutively transcribing its gene only in the absence of copper. In absence of copper, single CaMac1p (active transcriptional unit), binds to a single CuRE sequence in promoters of target genes to bring about optimal transcription.

self-interactions. Dimerisation in *C. albicans*, increases in copper awash conditions, and possibly the folding of the protein to incorporate dimerisation, leads to the DNA binding domain of the protein as well as the activation domain being concelaed, thereby preventing the dimer from forming Mac1-DNA complex with the promoters of target genes. This prevents their transcription and also preventing its own transcription. As a result *CaMAC1* is not constitutively transcribed, and in copper replete conditions, no new protein is produced. In contrast, under copper scarce conditions, the lack of a repressive intermolecular interaction between two CaMac1 proteins, allows the activation domain to be exposed and it can then initiate transcription of target genes.

We can hypothesise that the lack of intramolecular interaction in presence of copper leads to dimerisation of the protein due to improved exposure of the C2 domain in C-terminus which allows greater protein-protein interactions, which in turn conceals the DNA binding and activation domains, leading to lack of transcription of *CaMAC1* via CaMac1p, in copper replete conditions thereby making the transcription of its own gene, constitutive.

The work demonstrated in this chapter suggests that in spite of there being sequence similarities between the Mac1 proteins in *S. cerevisiae* and *C. albicans*, its mechanism of action and functioning differs consistently *in vivo*, between the two organisms. The data presented, helps us distinguish between the copper homeostasis systems in *C. albicans* from similar system in the model yeast *S. cerevisiae*. These differences may enable *C. albicans* to respond more precisely to environmental changes (such as changes in copper levels), conferring a quicker adaption to the human host that leads to an advantage in the disease-causing process.

4. Role of Sef1p in copper and iron uptake systems in *C. albicans*

4.1. Introduction

SEF1 was first recognised in *Kluyveromyces lactis* and its *Saccharomyces cerevisiae* homologue was also identified (Groom *et al.*, 1998). In *K. lactis* and *S. cerevisiae*, *SEF1* was found to be capable of supressing a mutation in an essential gene *RPM2* which codes for the protein subunit of a yeast mitochondrial RNase P enzyme; responsible for 5' maturation of mitochondrial tRNAs. Hence it was given the name *SEF1* (Suppressor of Essential Function). When the DNA sequence was analysed, it was also observed that it showed presence of the Zn(2)-Cys(6) binuclear motif which is associated with a lot of yeast transcription factors (Groom *et al.*, 1998). A homologue of *SEF1* also acts in a regulatory manner in riboflavin synthesis in *Candida famata*, however its exact mechanism of action, has not yet been determined.(Dmytruk *et al.*, 2006)

Before the beginning of this study, there were recent reports that identified a novel putative transcription factor Sef1p in *Candida albicans*. The putative regulator gene,

SEF1 was originally identified in a screen to determine the target genes of Sfu1p (Lan CY *et al.*, 2004) and later appeared once again in a screen for candidate virulence factors ((Noble *et al.*, 2010). The *SEF1* gene has shown expression changes in the presence of the iron/copper chelator BPS and a *sef1Δ/sef1Δ* displayed growth reduction on YEPD with either BPS or alkaline pH when compared to wild type (Homann OR *et al.*, 2009). Additionally in microarray studies, up-regulation of Sef1p was observed in

suf1Δ/sfu1Δ (Lan CY *et al.*, 2004). *sef1ΔΔ* mutants showed low growth in low iron media (Lan CY *et al.*, 2004, Homann OR *et al.*, 2009). It was indicated that *SEF1* may be playing a role in regulation of iron homeostasis in *C. albicans* (Homann OR et al., 2009) and hence *SFU1* may not be the only iron responsive gene regulator. This data indicated that *C. albicans* had both an activator and a repressor acting to regulate expression of genes in varying iron concentrations and hence this was decided to be studied further, in this study.

On DNA sequence analysis of *SEF1*, the predicted Zn(2)-Cys(6) DNA binding fungal cluster was identified (Chen C *et al.*, 2011) and it was found to encode a 917 amino acid residue. pSORT analysis carried out during this study revealed three nuclear localisation signals (KRKSAASTPGNESKKSR at site 38, RKSAASTPGNESKKSRK at site 39 and KKMGLKCEIDPEFRPRK at 114 site) in the N-terminus domain of the protein and showed that there is a 73.9% chance of it being a nuclear protein. The fungal Zn(2-Cy(6) binuclear cluster domain (SCTFCRQHKIKCNASDNYPNPCERCK -KMGLKC) was found at site 89 and a possible vacuolar targeting motif (ILPK at site 10) was also found. The aim of this part of the study was to analyse a *sef1Δ/sef1Δ* homozygous mutant and to provide functional evidence for the role of Sef1p as a regulator of high affinity iron uptake in *C. albicans*.

Creation of *sef1Δ/sef1Δ* double knock-out strain

A sef1-double knockout strain was obtained from the Fungal Genetics Stock Centre (FGSC http://www.fgsc.net/). The strain was originally created by the Homann group who constructed it for use in a study to characterise a phenotypic profile of the *Candida albicans* regulatory network (Homann OR *et al.*, 2009). The technique used for creation of the transcriptional regulator knockout (TRKO) strain was adopted from Noble &

Johnson (2005) who described a new procedure using fusion PCR that allows a competent and swift generation of homozygous knockout mutant strains. (Homann OR *et al.*, 2009, Noble *et al.*, 2010, Noble & Johnson, 2005)

The deletion mutant was constructed in the background strain SN152 which has originally been derived from the clinical isolate SC5314 which is our "wild-type" strain. The background strain was *URA3/ura3Δ, argΔ/argΔ, leuΔ/leuΔ, his1Δ/his1Δ, IRO1/iro1Δ*, where on targeted site gene disruption with the auxotrophic marker cassettes, the *HIS1* and *LEU2* functions were restored in the TRKO strain. In SN152 strains *URA3* has been previously restored to its original gene locus (Noble & Johnson, 2005).

A two-step fusion PCR method was used for the generation of a disruption cassette where the selectable marker (*HIS1* or *LEU2*) was surrounded by lengthy sequences sharing homology to upstream and downstream sequences of the target ORF. This aids in homologous recombination and disruption of the target gene. The first step consisted of three PCRs which amplified the upstream and downstream sequences of the target gene and the selectable marker gene. The internal primers used in the first step had complementary tails which allowed the three fragments to be fused together in the second step, resulting in a disruption cassette with a long region, homologous to the contiguous region of the target gene. In the parental strain SN152, the two alleles of the target gene were then sequentially disrupted using disruption fragments (mentioned above) with *HIS1* and *LEU2* (Figure 4.1). The transformants were then screened on plates lacking histidine and leucine and the His+ Leu+ transformants were determined by colony PCR to check for the absence of the target ORF and the four anticipated gene disruption regions (Homann OR *et al.*, 2009). The resultant *sef1Δ/sef1Δ* strain is a *arg4Δ/arg4Δ, sef1Δ::HIS1/ sef1Δ::LEU2, URA3/ura3Δ, IRO1/iro1Δ*. It was previously

also confirmed that the reference strain that was used (*leu2Δ/leu2Δ*, *his1Δ/his1Δ*, *arg4Δ/arg4Δ*, *his1Δ/his1*), showed wild-type virulence in mouse models ensuring that the targeted site disruption using these cassettes was not influencing virulence or complicating the analysis of our deletion mutant (*sef1Δ/sef1Δ*) (Noble & Johnson, 2005).
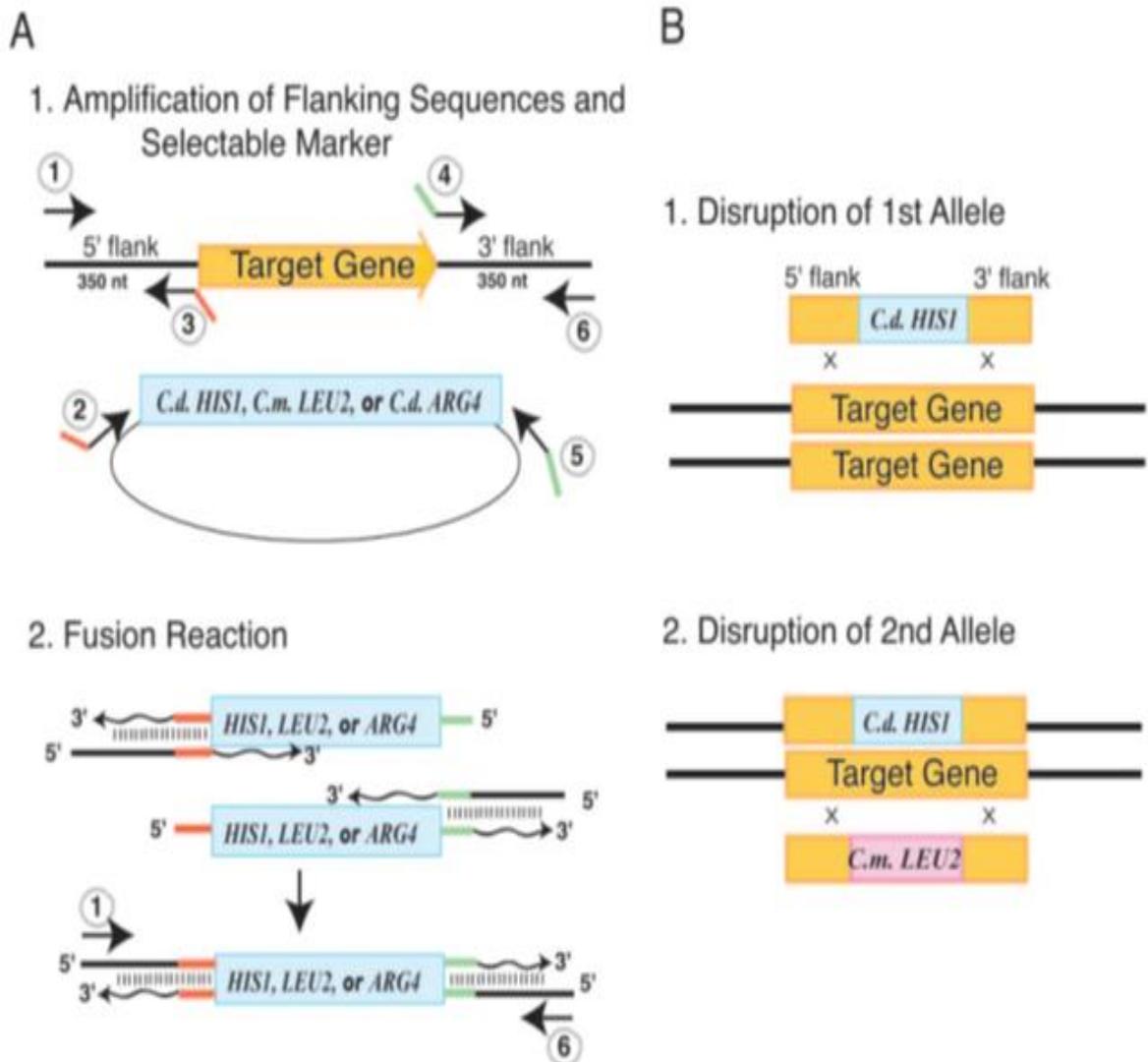
Figure 4. 1: A diagrammatic representation of the gene disruption protocol used for generation of the *sef1Δ/sef1Δ* mutant strain (Noble & Johnson, 2005)**.**

(**A**) Fusion PCR consists of two PCR steps. In the first step, primers 1 and 3 are used to amplify genomic DNA on the 5' side of the target gene while primers 4 and 6 are used to amplify the 3' side of the target gene and primers 2 and 5 are used to amplify the selectable marker. The complementary sequences that are used for fusing the three fragments in the second round of PCR are denoted by the red and green primer tails. (Noble & Johnson, 2005). (**B**) This disruption cassette is then used for sequential disruption of both alleles of *SEF1*.

4.2. Phenotypic analysis of *sef1Δ/sef1Δ* strain

Verification of *SEF1*-knockout by PCR

The *sef1ΔΔ* mutant containing a deletion in the *SEF1* gene was confirmed via PCR analysis and as well as sequence analysis. This was carried out by our undergraduate project student William Clare, as part of this study (Figure 4.2). Wild-type and *sef1ΔΔ* were grown in liquid media in the presence of iron and copper for 6 hours on shaker at 30°C and genomic DNA was extracted from both strains. Internal primers were designed for *MAC1* gene which was to be used as a control and *SEF1* gene. Refer Table 2.6 for primer sequence. As expected the right size DNA fragment was observed for the *MAC1* gene in both strains indicating that the extracted genomic DNA was undamaged. No amplicon was observed for the *SEF1* gene in the *sef1ΔΔ* strain; while the expected fragment was observed for the gene in wild-type strain indicating that the primers and PCR was functioning optimally and absence of fragment was indeed indicative of absence of gene. This in addition with the sequence analysis data confirmed that this strain was a *sef1Δ/sef1Δ* strain.

Testing the *sef1Δ/sef1Δ* mutant for response to serum

In a recent study in our lab, it was found that regulation of iron and copper homeostasis is different in the yeast form of *C. albicans* as compared to hyphae and hence morphology and the acquisition of these essential virulence determinants is linked (Jeeves RE *et al.*, 2011). This means that defects in potential iron regulator *SEF1* may have an effect on the morphogenesis capability of *C. albicans*. The mutant was therefore tested
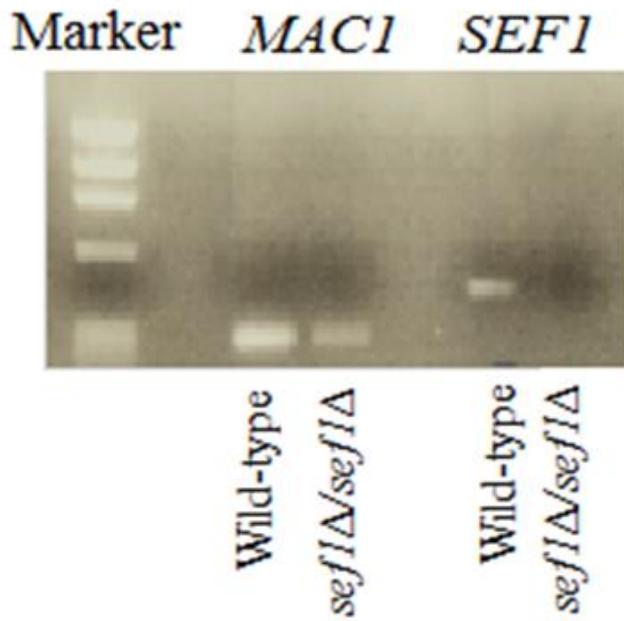
Figure 4. 2: Verification PCR to confirm the absence of SEF1 in *sef1ΔΔ*

Genomic DNA was extracted from the wild-type strain as well as *sef1ΔΔ* mutant and PCR was performed on both DNA samples, for internal amplicons within the *CaMAC1* gene (control) and the *CaSEF1* gene. The PCR reactions were electrophoresced on 1.5% agarose gel. Internal amplicon for *MAC1* were observed in both wild-type and *sef1ΔΔ* genomic DNA however an amplicon for *SEF1* was observed only in the wild-type strain and was absent in the *sef1ΔΔ* mutant, as expected (Clare, 2012).
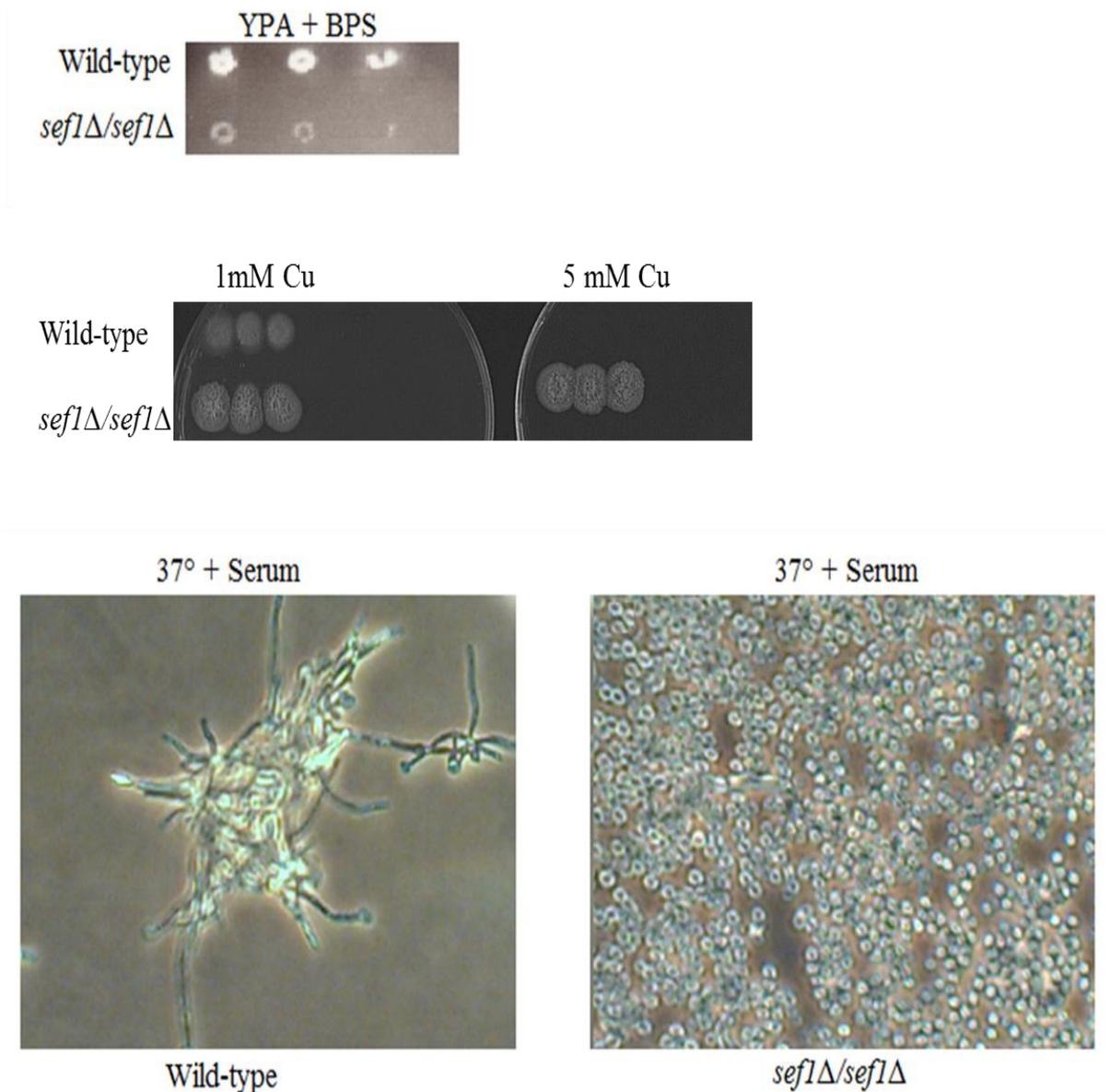
Figure 4. 3: Phenotypic tests carried out on *sef1ΔΔ* in comparison to Wild-type

C. albicans wild-type and *sef1ΔΔ* cells were grown in YPD media for 6 hours and were then (**A.**) serially diluted and spotted onto plates containing various iron and copper ranging conditions, using hedgehog and incubated at 30°C for 3-5 days. The mutant showed reduced growth in iron limited conditions, in the presence of iron chelator BPS but showed better growth in toxic copper conditions. (**B.**) inoculated into fresh YPB with 20% bovine serum. The cells were then grown for a further 3 hours at 37°C before being suspended in YPB and observed under phase contrast microscope at 100x magnifications. The wild-type switched into long, continuous hyphal form whereas the mutant continued to grow as unicellular yeast, at 37°C, in the presence of serum. (Clare, 2012).

for its ability to switch to hyphal form in the presence of serum at 37°C. Under usual circumstances, when *C. albicans* is grown at 37°C, pH 7, in the presence of serum, it switched from its unicellular yeast form into long, continuous hyphae as Haemoglobin has been found to be a trigger for hyphal differentiation (Pendrak & Roberts, 2007). However interestingly when the *sef1∆∆* mutant was grown in the presence of serum and observed under the microscope, it continued to grow in its unicellular yeast form and was found to be incapable of switching to hyphal form (Figure 4.3). This experiment was repeated thrice independently. This indicates that defects in *SEF1* may lead to a defect in the organism's ability to respond to serum which disrupts its capability of switching between its morphological forms. This may in turn affect its virulence.

Effect of iron and copper limitation on growth of *sef1∆/sef1∆* mutant

The *SEF1* gene has shown expression changes in the presence of the iron/copper chelator BPS (Lan CY *et al.*, 2004). It is essential for iron to be converted to its soluble ferrous form before it can be transported via the oxidase/permease complex, into the yeast cell (Dancis A *et al.*, 1990). In *S. cerevisiae*, mutants that had defects in the iron and copper reduction system and hence defects in iron and copper uptake, showed reduced growth in iron and copper restricted conditions (Dancis A *et al.*, 1994). In the human body the pathogen is faced with contrasting iron and copper environmental conditions and has to survive in low-(free)-iron containing blood as well as in high iron containing gut. The *sef1∆∆* mutants were hence tested for its ability to grow in iron and copper limiting as well as excess iron and copper conditions. The mutant and wild-type were initially grown under various conditions of iron and/or copper limitation with/without the iron chelator BPS and/or copper chelator BCS on YPA media.

It was observed that the mutant was found to display a large reduction in growth in iron limited conditions, in the presence of BPS as compared to the wild-type strain. It was interesting to note that the mutant appeared to be able to survive better in toxic copper conditions as opposed to the wild-type strain (Figure 4.3) which indicated that defects in *SEF1* were affecting the copper uptake system in the mutant. Hence it could survive in environmentally toxic copper conditions without it leading to toxic cellular conditions. The effect of iron and copper restriction on the mutant was tested further by comparing their growth with the wild-type as well as reference strain (SN152), in MD media supplemented with the iron and copper chelators BPS and BCS (Figure 4.5).

On comparing the growth of the mutant with the wild-type and reference strain, no difference was observed in copper limiting conditions, in the presence of BCS (data not shown). The mutant showed reduced growth in limiting iron conditions when grown on MD + BPS media, as compared to the wild-type and reference strain. The growth of the mutant increased marginally on addition of copper but the growth defect was almost corrected on addition of iron to the media. The mutant colony also showed a colour difference on the MD + BPS media. The mutant colony appeared pale pink as opposed to the dark red coloured wild-type and reference strain colonies. The chelators used in this study, bind to reduced forms of metals and hence show a colour change. In MD + BPS media supplemented with iron, BPS would bind to iron leading to the dark red colouration. This colour change is also used to measure the rate of cell surface metal reduction. However the difference in colour between the mutant and the reference strains could be indicative of a defect in its ability to reduce extracellular iron.

Sensitivity of the *sef1Δ/sef1Δ* mutant to oxidative stress

It has been reported before that mutants that show defects in iron and copper uptake system, also show increased sensitivity to oxidative stress. In *Saccharomyces cerevisiae,* the presence of hydrogen peroxide leads to mutants showing growth defects due to increased response to oxidative stress (Jungmann J *et al.*, 1993). In certain mutants, the oxidation of enzymes releases single iron ions (highly reactive) that further react with hydrogen peroxide in a Fenton reaction (Figure 1. 4) to form hydroxyl radicals that lead to toxicity in the cell (Mason, 2006b, Halliwell & Gutteridge, 1984). The mutant was tested for its response to oxidative stress by comparing its growth with the reference strains on media in the presence of 0.008% hydrogen peroxide (Figure 4.4, lane 3). In previous studies with *S. cerevisiae* mutants, the mutants showed growth defects at this concentration of hydrogen peroxide (Jungmann J *et al.*, 1993). On comparing the growth of *sef1ΔΔ* mutant with the reference strains SC5314 and SN152, the mutant was found to show a growth defect in the presence of hydrogen peroxide. The addition of copper, had no effect on this growth defect, however on addition of iron to the media, the mutant was able to grow like the reference strains. The results of this experiment indicated that the *sef1ΔΔ* mutant showed increased sensitivity to oxidative stress which could be rescued by addition of iron to the media.

Testing the *sef1Δ/sef1Δ* mutant for respiratory defects

Iron and copper are essential metals and are required by organisms since they are important co-factors and are involved in formation of vital metabolic and respiratory enzymes such as cytochrome c oxidase, succinate dehydrogenase, aconitase *etc*. (De Freitas *et al.*, 2003). As a result, mutants that show defects in iron and copper uptake may also show growth defects and may not be able to consume respiratory carbon
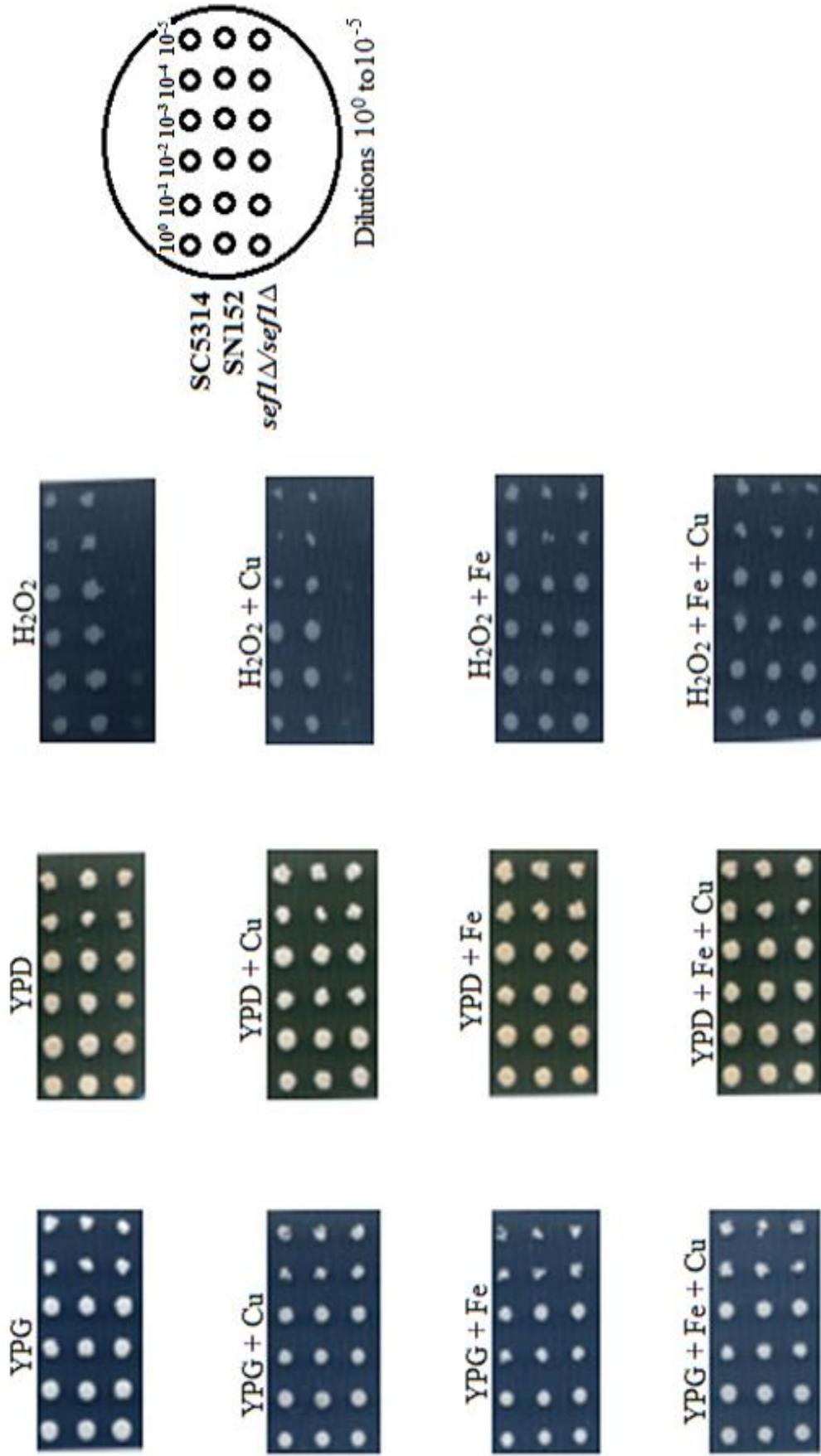
Figure 4. 4: Phenotypic assays to test the growth of *sef1ΔΔ* mutant in comparison to the reference strains under various conditions

Cells were grown at 30°C on a shaker in YPB media overnight in the presence of iron and copper. The next morning they were inoculated into fresh MD media and allowed to grow for 6 hours before being serially diluted and spotted using a hedgehog onto (YPG) 2% glycerol, (YPD) 2% dextrose or 0.008% hydrogen peroxide plates and allowed to grow for 3-5 days at 30°C.

sources such as glycerol and glucose (De Freitas *et al.*, 2003, Jungmann J *et al.*, 1993) due to defects in respiratory proteins. To analyse the respirational competency of our mutant, it was tested for growth on media containing glucose or glycerol as the sole source of carbon and was compared with the growth of the reference strains SC5314 and SN152 (Figure 4.4, lane 1-2). The mutant and the reference strains showed comparable growth and all the strains were able to utilise glycerol and/or glucose as the sole carbon source. These results indicated that the mutant did not show respiratory defects. The only visible difference was that the mutant colony was white in colour as opposed to the reference strains which were brown in colour, when copper was added to the media. This colour change indicated accumulation of copper within the cells during copper replete conditions (Mason, 2006b) and hence the white colour mutant colony could be indicative of a defect in copper uptake as a result of defect in iron uptake.

Sensitivity of the *sef1Δ/sef1Δ* mutant to varying pH

The opportunistic human pathogen *C. albicans* has the ability to respond to and adapt in various niches such as the mouth, gut and vaginal cavity; which have a wide range of pH. The ability of the organism to detect and acclimatise from acidic to neutral to alkaline pH has been found to be controlled by signal transduction pathways which are regulated by transcription factors (Davis, 2003). Hence our mutant was tested for growth on media with varying pH levels from 2.1 − 10.5 and compared with the growth of reference strains SC5314 and SN152 under the same pH conditions (Figure 4.5, lane 2). No visible growth difference was seen between the mutants and the reference strains under acidic or neutral pH conditions however the mutant showed reduced growth at pH 10.5, indicating a growth defect in alkaline conditions. This could be suggesting a

defect in *FRP1*expression, which is an alkaline-induced putative ferric reductase. (Liang *et al.*, 2009).

Sensitivity to very high iron and copper conditions (toxicity)

Defects in iron uptake system have been intrinsically inter-linked with defects in copper uptake system in *C. albicans* (Knight *et al.*, 2005). The previous phenotypic analysis on the mutant, in this study, had indicated a defect in copper uptake in addition to iron uptake and hence the sensitivity of the mutant to iron and copper toxicity was also tested by analysing its ability to grown on media containing very high iron or copper concentration (5mM) and comparing it to the growth of reference strains in the same conditions (Figure 4.5, lane 1.4). No comparable growth difference or growth defects were seen between the strains in this very high iron conditions and all strains were able to grow in presence of high iron (data not shown), indicating it was not toxic to their growth. However interestingly, the mutant appeared to be able to survive better in extreme copper conditions as opposed to the reference strains. The reference strains showed growth defects in very high copper conditions indicating that the high extracellular copper concentration was toxic to their growth. This result indicates a possible defect in copper uptake in the mutant which was preventing the extracellular copper toxicity from leading to intracellular copper toxic conditions within the mutant. Hence this allows the mutant to grow without difficulty in the presence of 5mM $CuCl_2$. This growth difference was even more visible during the preliminary test when the mutant and reference strains were grown on YPA (Yeast peptone dextrose agar) media containing 5mM $CuCl_2$ (Figure 4.3).
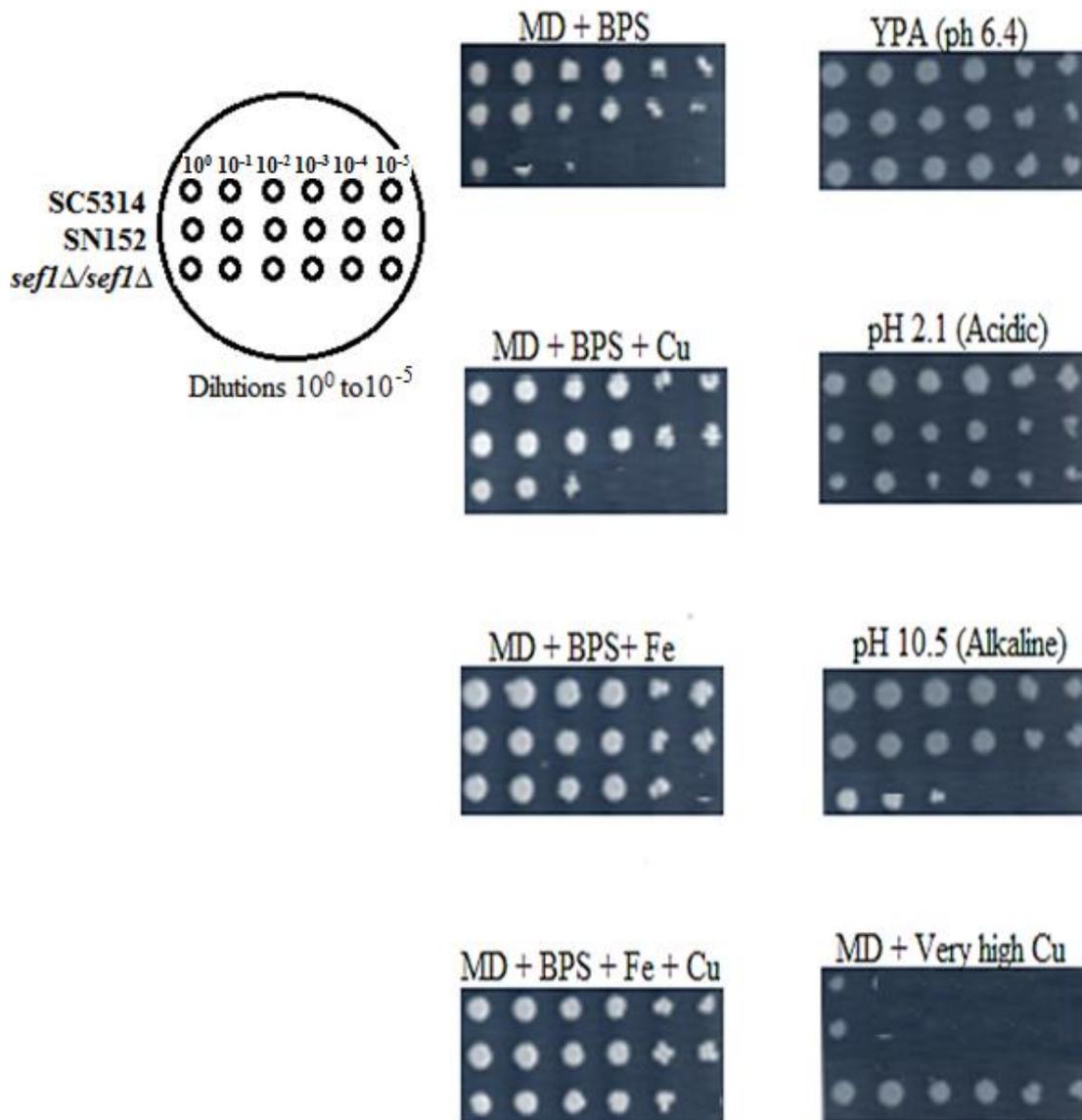
Figure 4.5: Phenotypic assays to test the growth of *sef1ΔΔ* mutant in comparison to the reference strains under various conditions

Cells were grown at 30°C on a shaker in YPB media overnight in the presence of iron and copper. The next morning they were inoculated into fresh MD media and allowed to grow for 6 hours before being serially diluted and spotted using a hedgehog onto MD plates with varying concentration of iron and copper (100μM $FeCl_3$ + 100μM BPS or 100μM $CuCl_2$ + 100μM BPS or 100μM of both metals + 100μM BPS) or MD plates with varying pH (2.1, 6.4 or 10.5). The plate with very high copper contained- 5mM $CuCl_2$ + 100μM $FeCl_3$. The plates were incubated at 30°C for 3-5 days.

4.3. Rate of growth of *sef1Δ/sef1Δ* mutant in limiting iron conditions

Phenotypic analysis on growth of mutant on solid media, in this study, indicated a growth defect in the mutant when the media was supplemented with iron chelator BPS, due to its incapability to grow in iron restricted conditions (Figure 4.3 - 4.5). Hence the mutant was then tested for growth defects in iron restricted conditions in liquid media and the initial growth rate was analysed in comparison to the reference strains. Each of the experiments were carried out with the background strain SN152 as well as the wild-type strain SC5314 and similar growth results were observed, however for simplicity of data presentation and analysis, only data with the wild-type strains has been depicted. All cells were grown in MD media at 30°C overnight and then 10^4 cells were inoculated in a bioscreen plate containing media with iron or with the appropriate iron limitation. The cells were then grown in the bioscreen at 30°C for 75 hours at an OD of 600 nm and a reading was taken every 15 minutes. A mean of three growth curve repeats is shown (Figure 4.6). The *sef1ΔΔ* mutant strain showed a slow initial growth rate which was even more prominent in iron restricted conditions, when the media was supplemented with BPS. The mutant showed a significant growth defect in iron limited conditions as opposed to the reference strains.
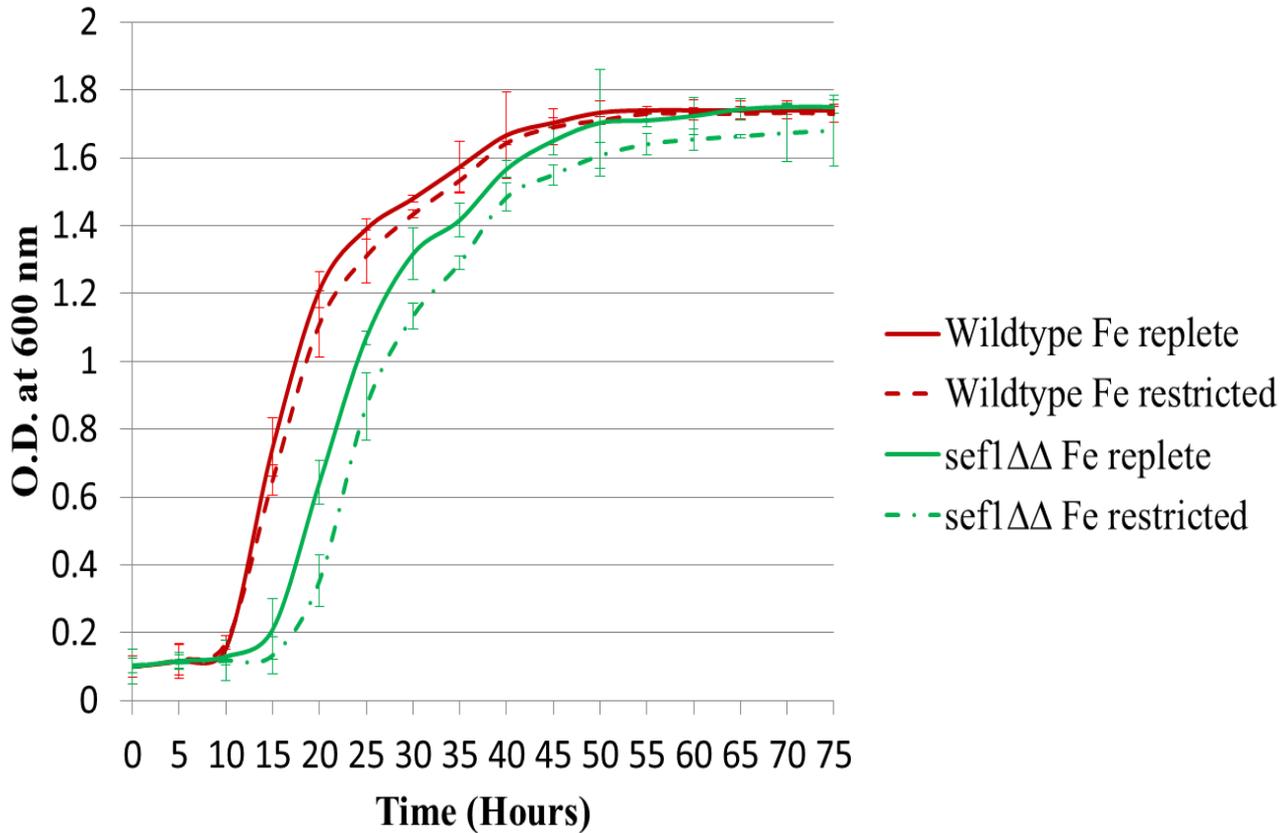
Figure 4. 6: Growth curve analysis of *sef1Δ/sef1Δ* mutant vs. reference strain, in iron restricted conditions

All cells were grown in MD media at 30°C overnight and then 10^4 cells were inoculated in a bioscreen plate containing media with iron or with the appropriate iron limitation. The cells were then grown in the bioscreen at 30°C for 75 hours at an OD of 600 nm and a reading was taken every 15 minutes. A mean of three growth curve repeats is shown and error bars of standard deviation have been represented. The *sef1ΔΔ* showed a slow initial growth rate and a distinct growth defect when media was supplemented with BPS. Media was supplemented with 100μM $FeCl_3$ +100μM $CuCl_2$ to create iron replete conditions and 100μM $CuCl_2$ + 100μM BPS to create iron restricted conditions.

4.4. Testing the *sef1Δ/sef1Δ* mutant's capability to utilise Haemoglobin as a source of iron

C. albicans has the capability to utilise Haemoglobin as a source of iron. Haemoglobin was also found to modify gene expression and acted as a trigger for switch from yeast into hyphal form (Pendrak & Roberts, 2007). When the mutant was tested for its response to serum, it was found that the mutant could not switch into its hyphal form which indicated impairment in its capability to detect serum. It was thought that it would be interesting to also study the mutant's ability to utilise Haemoglobin as a source of iron, and compare with the reference strains. This method was adopted from a technique used by Fadil Bidmos in our laboratory to study meningococcal haemoglobin receptors. The *sef1ΔΔ* mutant and reference strains were grown for 6 hours in the presence of iron and copper in liquid media. The cells were then harvested and spread onto a MD media plate containing 500 μM BPS to create an iron restricted environment. Discs were then saturated with $FeCl_3$, PBS, or Haemoglobin and placed on to the plates that were previously spread with the wildtype or mutant cells, respectively. $FeCl_3$ represent the positive source of iron while saline (PBS) represents the negative control. The plates were incubated for 3-5 days and growth was studied.

The reference strain could utilise $FeCl_3$ and Haemoglobin as a source of iron (as expected) however the mutant showed very slight growth in presence of Haemoglobin (Figure 4.7). This result indicated that deletion of *SEF1* affected the ability of the mutant to use Haemoglobin effectively. The protein Rbt5 has been previously implicated to be involved in utilisation of haem and haemoglobin by *C. albicans* (Weissman & Kornitzer, 2004). Since the *sef1ΔΔ* was found to be unable to utilise Haemoglobin effectively, it might suggest a defect in *RBT5* expression.
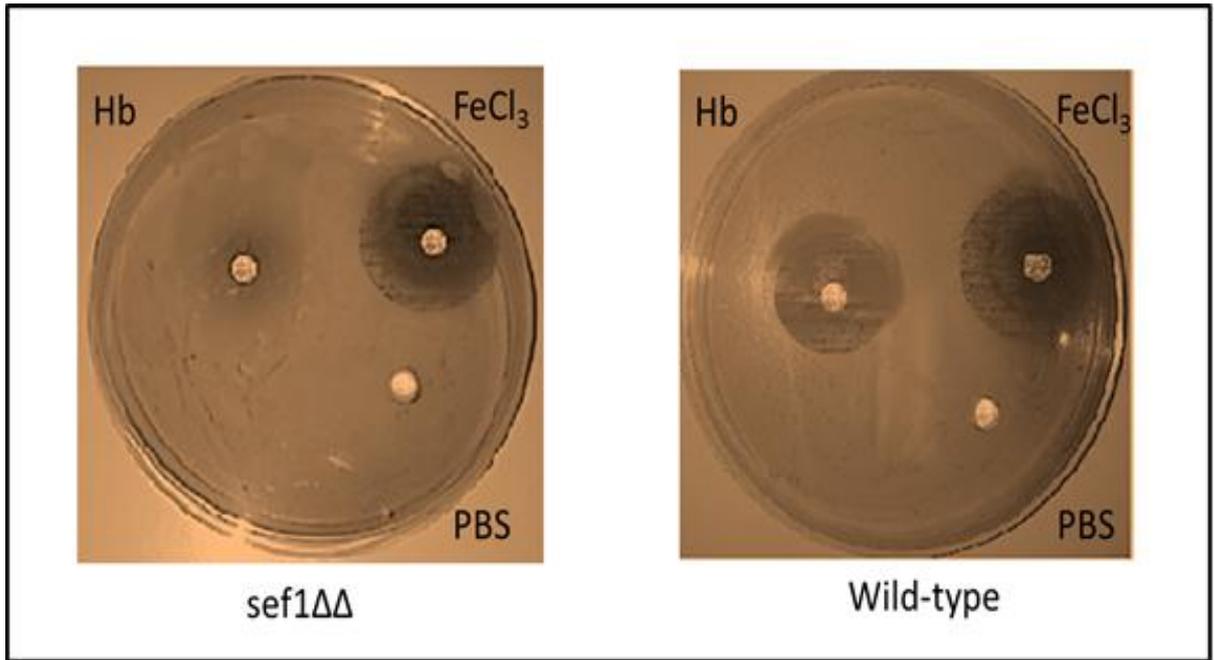
Figure 4. 7: Testing *sef1ΔΔ* for its ability to utilise Haemoglobin as a source of iron

Cells were grown for 6 hours in the presence of iron and copper in liquid media. The cells were then harvested and spread onto a MD media plate containing 500 μM BPS to create an iron restricted environment. Discs were saturated with $FeCl_3$, PBS, or Haemoglobin and placed onto the plates containing the cells. $FeCl_3$ represent the positive source of iron while saline (PBS) represents the negative control. The plates were incubated for 3-5 days and growth was studied. This experiment was repeated thrice independently and similar results were observed.

4.5. Expression of putative ferric reductase genes in *sef1Δ/sef1Δ* mutant

By work done in our laboratory previously, and on sequence comparison and homology to *CaFRE1*, *CaFRE2*, and *ScFRE1*, 17 putative ferric reductase genes had been identified. However transcripts were detected only for *CaFRE1, CaFRE2, CaFRE5, CaFRE10* and *CaFRE12*. *CaFRE10* and *CaFRE12* were found to be the most highly expressed and *CaFRE10* was determined to be regulated by iron levels whereas *CaFRE12* was found to be regulated in response to copper (Mason, 2006b). In a later study in our laboratory, another ferric reductase gene *CaFRE7* was also identified and was found to be significantly responsible for ferric and cupric reductase activity in response to copper levels (Jeeves RE *et al.*, 2011). It was supposed that the other transcripts were highly unstable or expressed at a very low level which made detection and analysis difficult via Northern blotting. Since the *sef1ΔΔ* has shown difficulty in growth in low iron conditions, it was implied that the iron uptake was affected in the mutant which could be a result of defect in iron reduction via the cell surface ferric reductases.

CaFRE7 has been previously been proposed to be regulated by *CaMAC1* (Woodacre, 2007) however in Chapter 3 in this study, our results indicated that Mac1p was not binding directly to *CaFRE7* and hence could be employing a co-regulator to regulate its expression. Hence it was interesting to look at the expression of *CaFRE7* along-with the expression of the other major reductases in the mutant as compared to the reference strain, in low iron conditions (Figure 4.8).

Preliminary Northern blot analysis (Refer 2.8) was carried out to analyse *FRE5*, *FRE7* and *FRE10* expression in the *sef1ΔΔ* mutant since the expression levels of these reductases has not been previously analysed and also because as mentioned before, *CaFRE10* and *CaFRE7* were found to be majorly responsible for the cell surface ferric and cupric reductase activity in *C. albicans* (Jeeves RE *et al.*, 2011). *CaACT1* was used as an endogenous control since it is expressed constitutively in *C. albicans* (Chau *et al.*, 2004). The transcripts were also analysed in iron and copper replete and copper restricted conditions, however maximum difference in expression of the reductase genes between the mutant and reference strain, was observed in iron restricted condition.

The transcripts for *CaFRE10* and *CaFRE7* were most easily observed and the blots were incubated for a week as opposed to an incubation time of 3 weeks for the *CaFRE5* blot. A large reduction in expression was observed for *CaFRE10* between the reference strain and the mutant and addition of copper had no effect on this reduced expression. It was interesting to see the reduced expression of *CaFRE7* in the *sef1ΔΔ* mutant since *CaFRE7* has been previously proposed to be regulated by *CaMAC1*; however these results indicate a significant effect of *SEF1* deletion on its expression. The expression of *CaFRE5* did not alter significantly between the *sef1ΔΔ* and the reference strains. Northern blot results are difficult to quantify with accuracy and hence we decided to look at expression of all the reductases in the *sef1ΔΔ* mutant, using an alternative method of RT-PCR.
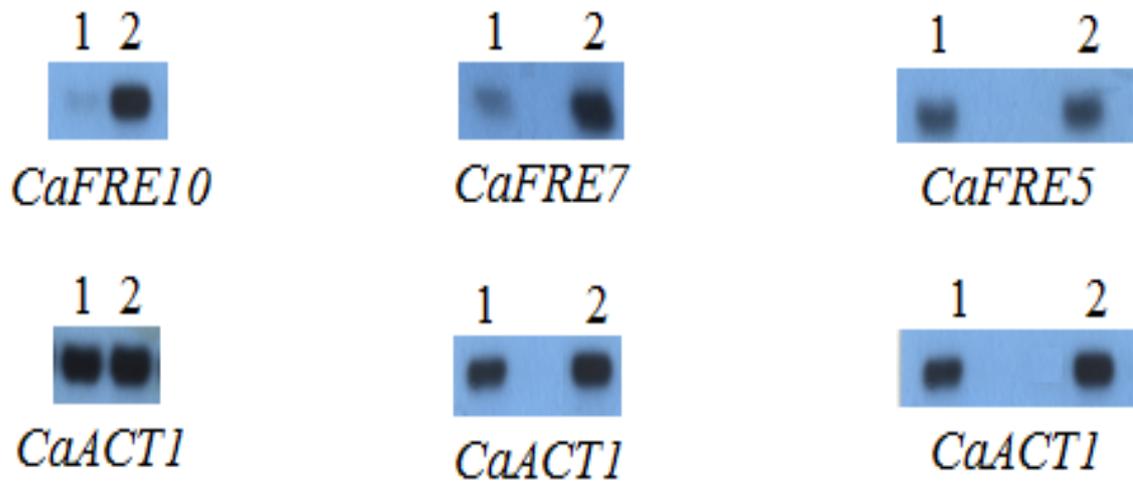
Figure 4.8: Northern blot analysis of *CaFRE10*, *CaFRE7* and *CaFRE5* in *sef1*ΔΔ and reference strains

Cells were grown to mid-log phase and then incubated with 100 μM BPS + 100 μM CuCl$_2$ (iron limited conditions) and then RNA was extracted. RNA was then transferred to a nylon membrane and hybridisation was carried out with radio-labelled (internal) probes for *CaFRE10*, *CaFRE7* and *CaFRE5*. **1.** in every blot indicates the *sef1*ΔΔ strain and **2.** in every blot indicates the reference strain.

4.6. RT-PCR to compare expression levels of potential target genes (FREs, CTR1, FTR1) in the *sef1Δ/sef1Δ* mutant

To identify the potential targets of *SEF1* and to characterise the role it plays in iron or/and copper homeostasis in *C. albicans*, we decided to study the levels of several transcripts in *sef1ΔΔ* under various iron and copper restrictive conditions; and to compare it to transcript levels in wild-type cells. The transcript analysis carried out in this study would shed light on the regulatory mechanism of *SEF1* and would allow us to study the effects of deletion of the gene which would exemplify its functions.

One way to study the expression of various potential targets was to use Northern blotting. However our preliminary Northern blotting data was not significantly quantifiable and hence we decided to get a broader perspective and gather more substantial evidence by using Real-time PCR to carry out expression studies in the *sef1ΔΔ* mutant.

Real-time PCR helps us to detect and measure the amplification of target, as and when it occurs and data is collected throughout the reaction and not just at the end. Cells were grown for 6 hours in MD media containing various iron and copper conditions, until they reached mid-exponential phase and were then washed. RNA was extracted from the cells using the Ribopure Yeast kit protocol (Refer 2.4 and 2.10) and treated with DNase I. The purity and quality of extracted RNA was analysed using the DNA Agilent 2100 Bioanalyzer. The ratio of 28S : 18S rRNA is calculated and the software presents an RNA integrity number (RIN value) for each sample which indicates purity and ideally should lie within $7 - 10$ for a good quality RNA sample. The software then

generates a gel image for the RNA samples and the RIN value for all our samples lied between 8.2 − 8.9 (Figure 4.9) which indicated pure and good quality RNA samples. The concentration of each sample was also determined using a Nanodrop.

RNA was then converted to cDNA using Applied Biosystems High capacity RNA-to-cDNA kit. The cDNA samples were used for RT-PCR experiments with Fast SYBR green master mix and the PCR was performed by Applied Biosystems 7500 Fast Real time PCR machine. An example of the experimental plan is shown in Figure 4.10. A 96 well-plate was used for each experiment and one step RT-PCR reactions were set up. Singleplex PCR was carried out where only one target sequence or endogenous control was amplified per reaction since it required less optimisation. The results were analysed using the Single plate RQ study software. 3 additional replicate reactions were set up for each sample and endogenous control to ensure statistical significance and each experiment was also repeated three times independently. The threshold for each reaction was determined automatically by the SDS software.

Initially a validation experiment was performed to verify that the efficiency of the target gene (say *SEF1*) RT-PCR was the same as the efficiency of the reference gene RT-PCR (*ACT1*). Six random cDNA samples were selected and RT-PCR was carried out for each sample using both primers for the *ACT1* gene and the *SEF1* gene, in different wells in the same plate. Standard curves were plotted by the Applied Biosystems software and efficiency for both primers was calculated. The efficiencies for both PCRs were found to be ≈ 1.98 where an ideal efficiency is of 2 which indicated that the target and reference gene primers were working competently and compatibly and hence could be used for relative quantitation studies. This validation experiment was carried out for all sets of primers used in the RT-PCR study.
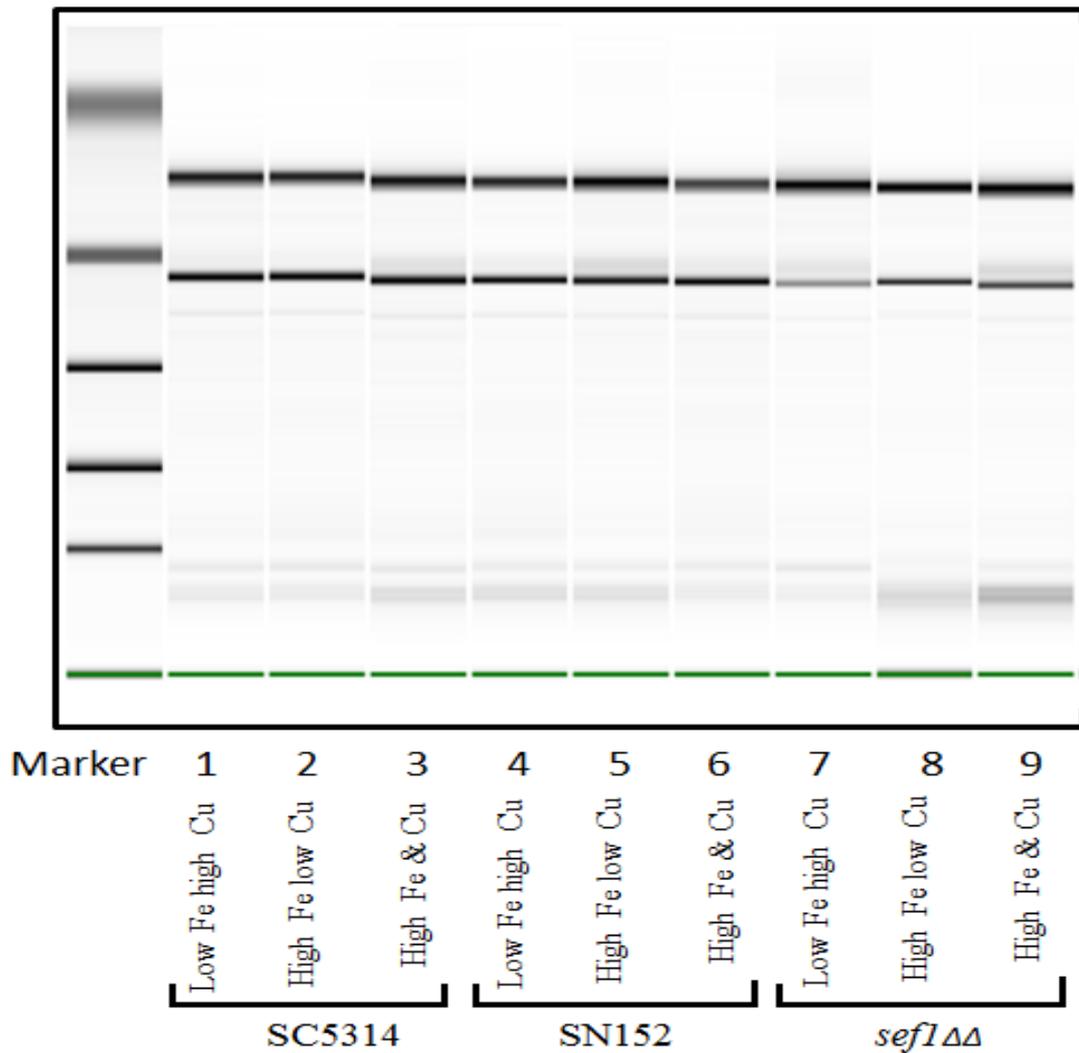
Figure 4.9: Analysis of purity of RNA via Bioanalyser

After extraction and DNAse treatement, RNA samples were run through the Bioanalyser to detect their purity. An Electropherogram was generated by the Agilent Bioanalyzer software, for RNA samples and purity was denoted as RIN values. RIN values from 7 - 10 denotes a good sample and the two fragments (peaks), represent the 28s and 18s rRNA. This picture illustrated the gel image generated by the Agilent Bioanalyzer, depicting the purity and integrity of the RNA samples.

Figure 4.10: A diagrammatic representation of the plate set-up for one of the RT-PCR experiments

Cells were grown for 6 hours in MD media containing various iron and copper conditions, until they reached mid-exponential phase and RNA was extracted. RNA was then converted to cDNA and was used for RT-PCR analysis to monitor change in expression of *SEF1* gene. 96 wells plate was used and four replicates were set up per reaction. *ACT1*, which is a housekeeping gene in *C. albicans*, was used as the reference. The gene names on the R.H.S denote the primer pairs used in each column. The first four columns contained Wild-type cDNA whereas the bottom four columns contained *sef1ΔΔ* cDNA. These plates were then put in Applied Biosystems Fast Real-time 7500 PCR machine where RT-PCR was carried out.

We decided to use relative quantitation technique to carry out the expression analysis in our mutant. "RQ (Relative Quantitation) determines the change in expression of a target in a test sample relative to the same sequence in a calibrator sample." (Applied Biosystem's Relative Quantitation manual). In our study, the Calibrator sample stood for the wild-type SN152 strain (which was used as the background strain to construct the mutant), the reference gene that was chosen was the *ACT1* or *PMA1* gene which are *C. albicans* house-keeping genes (Chau *et al.*, 2004) and hence were used as an endogenous control, the target gene would be the potential targets whose expression was to be analysed (say *CaFRE10*) and the test sample would be the mutant strain *sef1ΔΔ*.

Testing expression of *SEF1* in response to iron and copper

The phenotypic and growth analysis carried out in this study indicated a defect in the *sef1ΔΔ* mutant in iron restricted conditions while it appeared to be able to survive better than the reference strains in copper toxic conditions. Hence the change in expression of *SEF1* was studied in the reference strains in response to various iron and copper (Figure 4.11) conditions. The summarised results of the experiments are depicted in Table 4.1. The expression of *SEF1* was found to increase by 69% in iron restricted conditions and this increase was statistically significant ($p = 0.008$). In very high iron conditions, no significant difference in the level of *SEF1* transcript was detected, which correlates with the phenotypic data we gathered previously in this study. The expression of *SEF1* was found to decrease slightly in copper restricted conditions and might be caused indirectly as a result of other factors. The reference strain was unable to survive in very
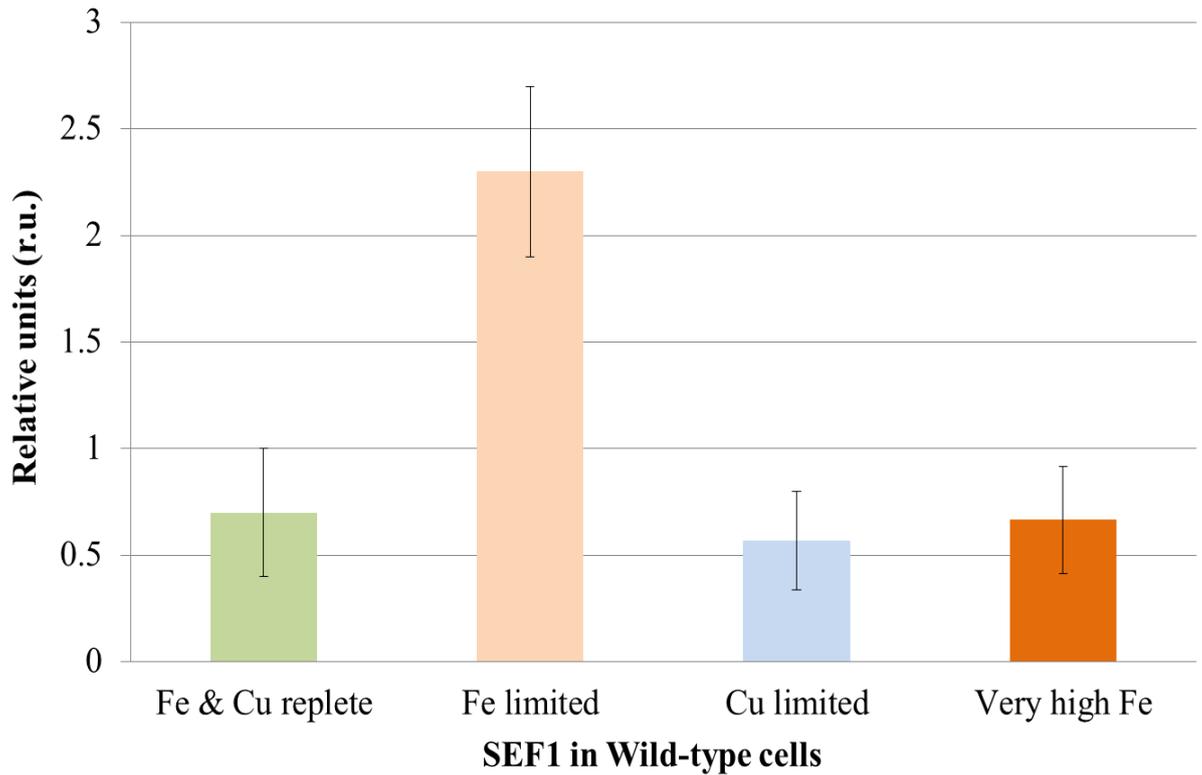
Figure 4.11: Expression o*f SEF1* in response to iron and copper

Cells were grown for 6 hours in MD media containing various iron and copper conditions, until they reached mid-exponential phase and RNA was extracted. Media was supplemented with 100µM FeCl$_3$ +100µM CuCl$_2$ to create iron and replete conditions; 100µM CuCl$_2$ + 100µM BPS to create iron restricted conditions; 100µM FeCl$_3$ +100µM BCS to create copper limited conditions and 5mM FeCl$_3$ +100µM CuCl$_2$ to generate very high iron conditions. RNA was then converted to cDNA and was used for RT-PCR analysis to monitor change in expression of *SEF1* gene. The data is represented in relative units that were generated by the relative quantitation software. Each reading is a mean of four replicates per reaction and three independent experiments. The error bars represent standard deviation.

Condition	Relative units (r.u.)	Difference from Fe & Cu replete (p value)
Iron limited	2.3	0.008
Copper limited	0.57	0.289
Fe & Cu replete	0.7	n/a
Very high Fe	0.67	0.455

Table 4. 1: Expression of *SEF1* in response to iron and copper

Cells were grown for 6 hours in MD media containing various iron and copper conditions, until they reached mid-exponential phase and RNA was extracted and converted to cDNA for RT-PCR analysis to monitor change in expression of *SEF1* gene. Media was supplemented with 100μM $FeCl_3$ +100μM $CuCl_2$ to create iron and replete conditions; 100μM $CuCl_2$ + 100μM BPS to create iron restricted conditions; 100μM $FeCl_3$ +100μM BCS to create copper limited conditions and 5mM $FeCl_3$ +100μM $CuCl_2$ to generate very high iron conditions. The data is represented in relative units. Each reading is a mean of four replicates per reaction and three independent experiments. A results of a one-tailed Student's T-test are shown as p values (α = 0.05). The difference in levels in the metal restricted and excess metal conditions in relation with Fe and Cu replete conditions are shown along-with the p value of this change. The change is deemed to be statistically significant if the p value is less than 0.05.

high copper conditions in the liquid media and hence expression of *SEF1* in extreme copper conditions could not be analysed.

Testing expression of putative ferric reductase genes in *sef1ΔΔ* mutant

The preliminary Northern blot analysis carried out in this study indicated a reduction in expression of the ferric reductase genes *CaFRE7* and *CaFRE10*. However this difference in expression between the mutant and reference strains was not accurately quantifiable. Previously, 17 putative ferric reductase genes have been identified in *C. albicans,* however transcripts have been detected for only 6 of them (*CaFRE1*, *CaFRE2*, *CaFRE5*, *CaFRE7*, *CaFRE10* and *CaFRE12*); while the exact functions of these all these ferric reductases have as yet not been determined. The expression of these six putative ferric reductases was hence analysed in the mutant in comparison to the reference strains in different conditions of iron and copper via RT-PCR. The real time expression of these genes has been represented in relative units as generated by the relative quantitation software and the data for difference in expression in low iron, high copper and high iron, low copper conditions between the strains has been shown in Figure 4.12 and Table 4.2.

In the reference strains, an increase in expression was observed for *CaFRE1*, *CaFRE2* and *CaFRE10* in response to iron restriction, however this increase was most prominent for *CaFRE10* (80%) which has been previously predicted to be the major cell surface ferric and cupric reductase in *C. albicans* (Jeeves RE et al., Mason, 2006b, Woodacre et al., 2008). Contrastingly an increase in expression was observed for *CaFRE7* (71%) and *CaFRE12* (56%) in response to copper as has been previously predicted (Jeeves RE *et al.*, 2011, Mason, 2006b). Out of all the ferric reductase genes, *CaFRE10* showed the highest expression while *CaFRE5* did not show any difference in expression in

response to iron or copper and may not be regulated by these conditions. In the mutant, *CaFRE1, CaFRE2* and *CaFRE10* showed reduced expression and there was no significant increase in expression in response to iron restriction. *CaFRE5* and *CaFRE12* showed similar expression in the mutant as they showed in the reference strains in iron and copper restricted conditions which indicated that deletion of *SEF1* did not have any effect on their expression and they may be regulated by another transcription factor. It is also possible that the conditions tested did not act as a trigger for expression change of *CaFRE5* and *CaFRE12*. Interestingly, in response to copper restriction, the expression of *CaFRE7* was reduced significantly (45% p = 0.004). These results indicated that the mutant was still capable of detecting change in copper levels and increasing expression of *CaFRE7* however its rate of transcription was affected due to absence of *SEF1* which implied that it may be regulated by both CaSef1p and CaMac1p.

Testing expression of other genes involved in iron and copper homeostasis in *sef1ΔΔ* mutant

The data obtained so far in a study indicated that *SEF1* played a role in regulation of not just the ferric and cupric reductase genes but it may also be involved in regulation of other iron and copper homeostasis genes. Due to time constraints and to avoid complications, the expression of seven genes (*CaFRP1*, *CaFTH1*, *CaCCC2*, *CaCTR1*, *CaFTR2*, *CaFET34* and *CaRBT5*) were analysed in various iron and copper conditions in the mutant in comparison to their expression in the reference strains, via RT-PCR. These genes were selected based on their sequence homology to *S. cerevisiae*, their putative functions, their appearance in previous screens in *C. albicans* and the data obtained via the phenotypic and growth studies for *sef1ΔΔ* mutant. *FRP1* is an alkaline-

induced putative ferric reductase, *FTH1* is an uncharacterised transmembrane protein, *CCC2* is a putative copper chaperone protein, *CTR1* is a copper transporter protein, *FTR2* is a putative ferrous iron transmembrane transporter, *CaFET34* is a putative ferroxidase protein and is the functional homologue of *ScFET3*, *and RBT5* is a GPI-anchored cell wall protein involved in haemoglobin utilisation. The real time expression of these genes has been represented in relative units as generated by the relative quantitation software and the data for difference in expression in low iron, high copper and high iron, low copper conditions between the strains has been shown in Figure 4.13 and Table 4.2.

In the reference strains, an increase in expression was observed for *FRP1*, *FTH1*, *FTR2*, *FET34* and *RBT5* in response to iron restriction while *CCC2* and *CTR1* showed an increase in expression in response to copper restriction. These results were in correlation with previous observations for these genes. In the mutant, a significant reduction in expression was seen for *FRP1* (81% p = 0.0021), *FTR2* (62% p = 0.004) and *FET34* (89% p = 0.0009) which indicated that deletion of *SEF1* affected expression of the putative iron transporter, ferroxidase and alkaline-triggered reductase. These results explain the reason for iron uptake being defective in the *sef1ΔΔ* mutant. The expression of *FTH1* and *CCC2* was not significantly altered in the mutant indicating that these genes were not regulated by *SEF1*. Interestingly, in response to copper restriction, the expression of the copper transporter *CaCTR1* was reduced by 33%.

These results indicated that the mutant was still capable of detecting change in copper levels and increasing expression of *CaCTR1* in response to copper restriction however the rate of transcription of *CaCTR1* was affected due to absence of *SEF1* indicating a partial role played by Sef1p in its regulation in association with Mac1p (Marvin *et al.*, 2004). This result explains how the mutant was able to survive in very high copper

conditions. Previously, it was observed that the mutant was incapable of utilising haemoglobin effectively as a source of iron (Section 4.4) and it was predicted that the expression of the haemoglobin utilisation protein Rbt5p could be regulated by *SEF1*. In the mutant, no significant increase in expression of *RBT5* was seen in response to iron restriction (Figure 4.13) which reconfirmed our previous hypothesis. These results indicated that *SEF1* plays a major regulatory role in *C. albicans* and is involved in regulation of various iron as well as copper homeostasis genes. It may regulate some genes in association with other transcription factors.
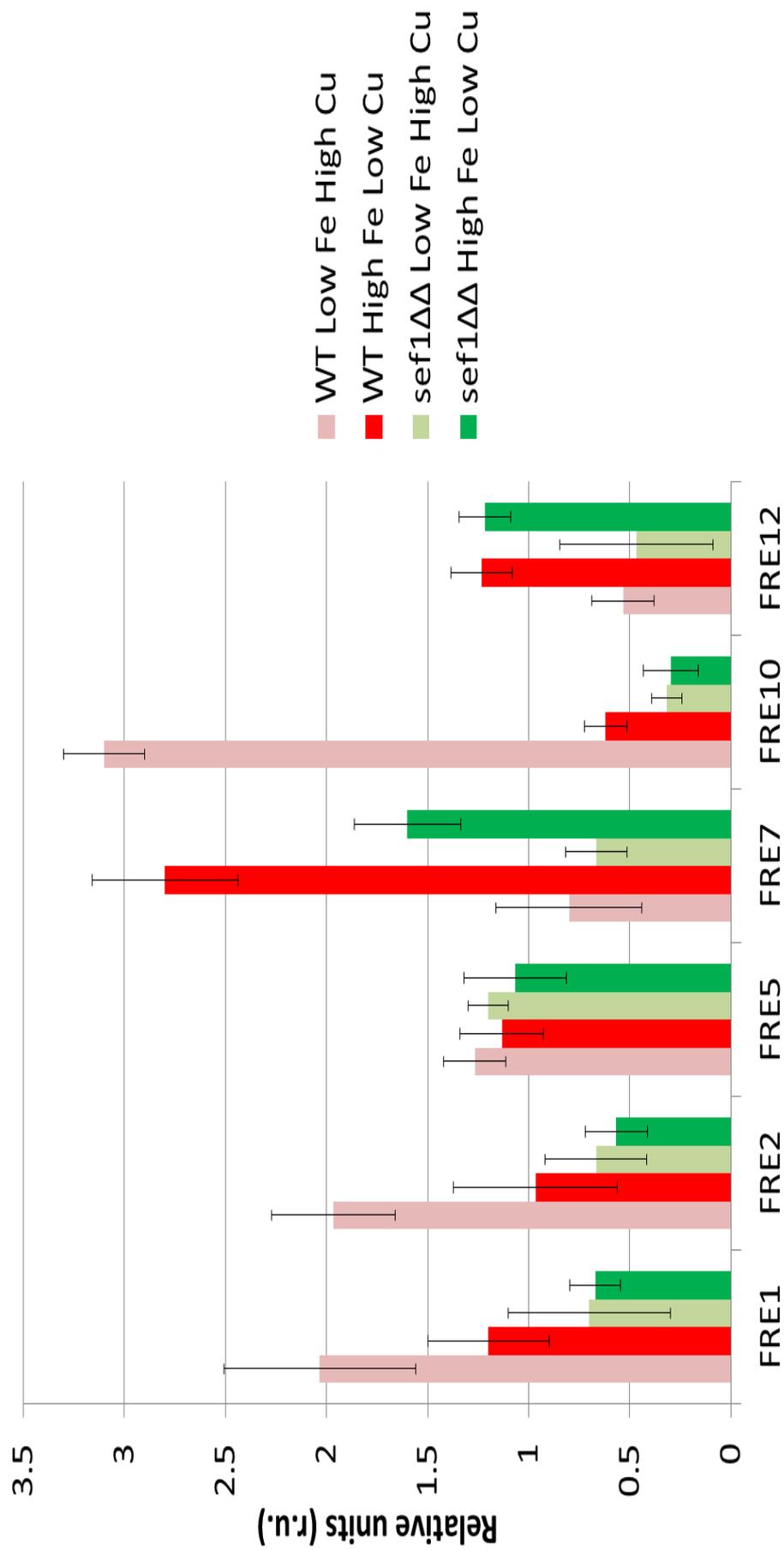
Figure 4. 12: Expression of putative ferric and cupric reductase genes in response to iron and copper in *sef1*ΔΔ mutant

Cells were grown for 6 hours in MD media containing various iron and copper conditions, until they reached mid-exponential phase and RNA was extracted. RNA was then converted to cDNA and was used for RT-PCR analysis to monitor change in expression. The data is represented in relative units that were generated by the relative quantitation software. Each reading is a mean of four replicates per reaction and three independent experiments. The error bars represent standard deviation. Media was supplemented with 100μM CuCl$_2$ + 100μM BPS to create low iron conditions and 100μM FeCl$_3$ +100μM BCS to create high iron conditions.

Figure 4. 13: Expression of genes involved in iron an copper homeostasis in response to iron and copper in *sef1ΔΔ* mutant

Cells were grown for 6 hours in MD media containing various iron and copper conditions, until they reached mid-exponential phase and RNA was extracted. RNA was then converted to cDNA and was used for RT-PCR analysis to monitor change in expression. The data is represented in relative units that were generated by the relative quantitation software. Each reading is a mean of four replicates per reaction and three independent experiments. The error bars represent standard deviation. Media was supplemented with 100μM CuCl$_2$ + 100μM BPS to create low iron conditions and 100μM FeCl$_3$ +100μM BCS to create high iron conditions.

Table 4. 2: Expression of genes involved in iron and copper homeostasis, in *sef1ΔΔ* mutant

Gene	Expression in WT Low Fe (r.u.)	Expression in WT High Fe (r.u.)	Expression in sef1ΔΔ Low Fe (r.u.)	Expression in sef1ΔΔ High Fe (r.u.)	Difference in expression sef1ΔΔ vs WT Low Fe (p value)	Difference in expression sef1ΔΔ vs WT High Fe (p value)
FRE1	2.03	1.2	0.7	0.67	0.0094	0.0697
FRE2	1.96	0.97	0.67	0.56	0.0246	0.0600
FRE5	1.27	1.13	1.2	1.06	0.0917	0.4109
FRE7	0.8	2.8	0.66	1.6	0.3297	0.0045
FRE10	3.1	0.62	0.32	0.29	0.0007	0.0717
FRE12	0.53	1.23	0.47	1.22	0.3456	0.4422
FRP1	3.03	1.2	0.55	0.54	0.0021	0.0562
FTH1	3.53	0.97	3.4	0.87	0.3126	0.4162
CCC2	0.64	2.9	0.62	2.62	0.4884	0.0834
CTR1	0.8	3.13	0.58	2.08	0.1337	0.0854
FTR2	3.24	0.62	1.21	0.3	0.0049	0.07173
FET34	3.5	1.23	0.38	0.32	0.0009	0.01256
RBT5	3.2	0.67	0.59	0.59	0.0014	0.29380

Cells were grown for 6 hours in MD media containing various iron and copper conditions, until they reached mid-exponential phase and RNA was extracted and converted to cDNA for RT-PCR analysis to monitor change in expression. The data is represented in relative units. Each reading is a mean of four replicates per reaction and three independent experiments. A results of a one-tailed Student's T-test are shown as p values (α = 0.05). The difference in expression in the mutant and reference strains is shown along-with the p value of this change. The change is deemed to be statistically significant if the p value is less than 0.05. Media was supplemented with 100µM $CuCl_2$ + 100µM BPS to create low iron conditions and 100µM $FeCl_3$ +100µM BCS to create high iron conditions.

4.7. Measurement of cell surface ferric and cupric reductase activity in the *sef1ΔΔ* mutant

The expression analysis studies carried out in this chapter, via Northern blotting and RT-PCR, showed reduction in expression of many cell surface ferric reductases including *CaFRE10* and *CaFRE7* in *C. albicans sef1ΔΔ* mutant. In a previous study in our laboratory, it was found that *CaFRE10* was the major cell surface ferric and cupric reductase and the ferric and cupric reductase activity was found to decrease by 75% and 50% in a *Cafre10ΔΔ* mutant (Jeeves RE *et al.*, 2011, Mason, 2006b) It was also found that *CaFRE7* was also responsible for cell surface ferric and cupric reductase activity in response to copper (Jeeves RE *et al.,* 2011). In the RT-PCR expression analysis carried out in this study, a distinct reduction in expression of *CaFRE10* and *CaFRE7* was observed in the *sef1ΔΔ* mutant (Figure 4.12) which indicated that the ferric and cupric reductase activity associated with the cell could also be affected. The *sef1ΔΔ* mutant was therefore analysed to study the cell surface ferric and cupric reductase activity (refer 2.12) associated with it, in comparison to the reference strains, in various iron and copper conditions (Figure 4.14 A & B).

In the reference strains, the levels of ferric reductase activity was found to increase in response to restriction of iron and copper in the media, as previously observed in our laboratory (Morrissey *et al.*, 1996). This increase was found to be more conspicuous in response to iron limitation (2.8-fold, $p = 0.005$) as opposed to copper limitation (1.5-fold, $p = 0.037$) as seen in Figure 4.14 A, Table 4.3. In disparity, in the mutant, the basal levels of ferric reductase activity in response to iron decreased by 43% and increase in response to iron limitation was also severely impaired as no significant difference ($p = 0.235$) was seen between the iron limited and iron replete conditions.

A.

B.
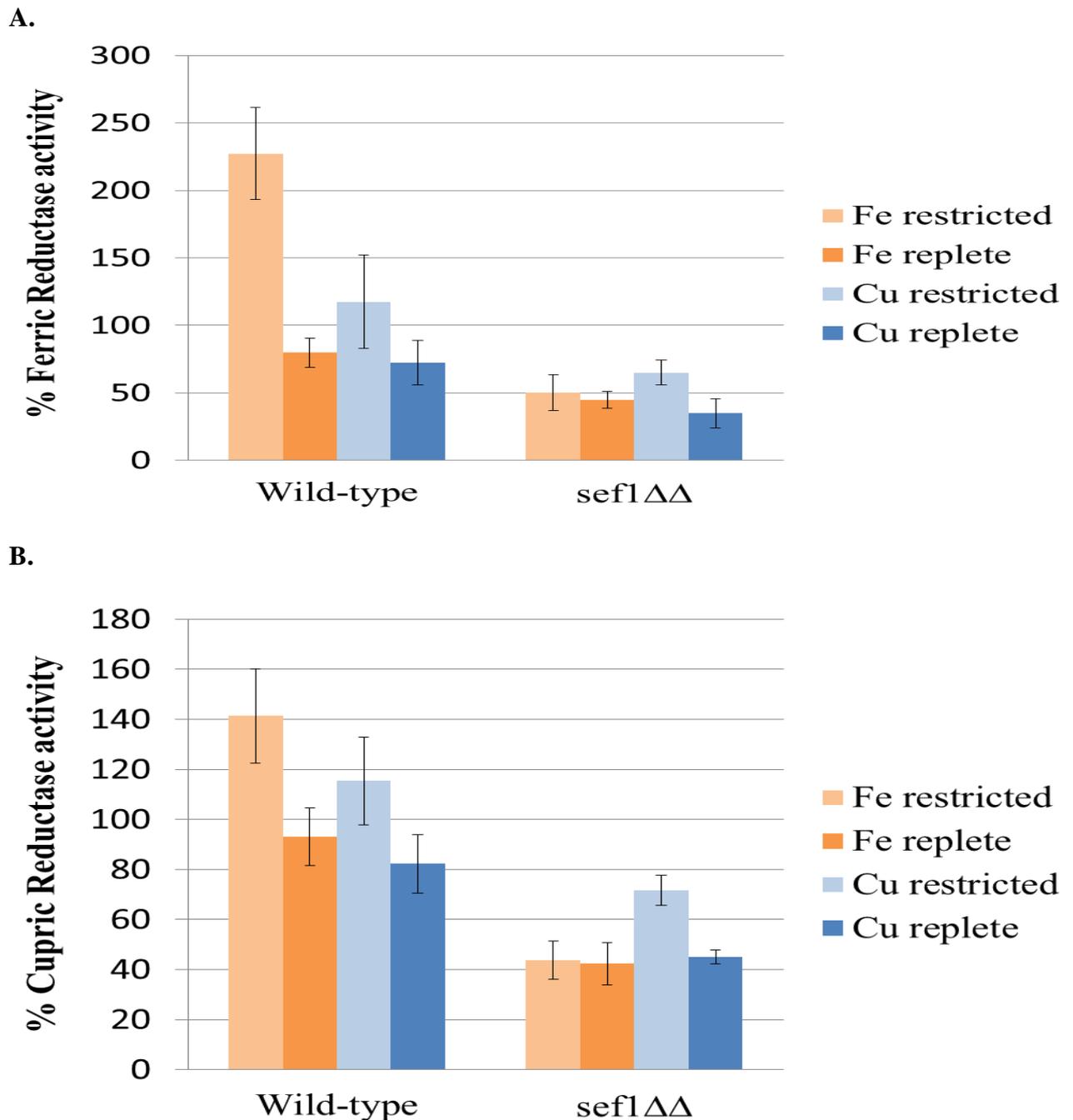
Figure 4. 14: Measurement of cell surface ferric and cupric reductase activity in *sef1ΔΔ* mutant

Cultures were grown up to mid-log phase and the cell surface (**A.**) ferric and (**B.**) cupric reductase activity was calculated, in response to iron or copper limitation. Iron limited media contained 100 μM $CuCl_2$ + 100 μM BPS while the copper limited media contained 100 μM $FeCl_3$ and 100 μM BCS. This graph shows mean of three independent experiments and the error bars represent standard deviation. The *sef1Δ/sef1Δ* mutant's ferric and cupric reductase activity was compared with reference strain activity. Media was supplemented with 100μM $CuCl_2$ + 100μM BPS to create iron restricted conditions and 100μM $FeCl_3$ + 100μM $CuCl_2$ (+/- BPS/ BCS) to create iron and copper replete conditions; 100μM $FeCl_3$ +100μM BCS to create copper restricted conditions.

Strain & Condition	% Cupric redcutase activity			% Ferric redcutase activity		
	Fe restricted	Fe replete	p value	Fe restricted	Fe replete	p value
Wild-type	141.3	91.67	0.041	227.34	79.67	0.005
sef1ΔΔ	43.67	42.4	0.413	50.33	45	0.235
	Cu restricted	Cu replete	p value	Cu restricted	Cu replete	p value
Wild-type	115.3	82.34	0.037	117.34	72.34	0.037
sef1ΔΔ	71.6	45	0.004	65	35	0.024

Table 4. 3: Measurement of cell surface ferric and cupric reductase activity in *sef1ΔΔ* mutant

Cultures were grown up to mid-log phase and the cell surface ferric and cupric reductase activity was calculated, in response to iron or copper limitation. This table shows mean of three independent experiments and the *sef1Δ/sef1Δ* mutant's ferric and cupric reductase activity was compared with reference strain activity. A results of a one-tailed Student's T-test are shown as p values ($\alpha = 0.05$). The difference in levels in the metal restricted and metal replete conditions are shown along-with the p value of this increase. The increase is deemed to be statistically significant if the p value is less than 0.05. Media was supplemented with 100μM $CuCl_2$ + 100μM BPS to create iron restricted conditions and 100μM $FeCl_3$ + 100μM $CuCl_2$ (+/- BPS/ BCS) to create iron and copper replete conditions; 100μM $FeCl_3$ +100μM BCS to create copper restricted conditions.

These results suggested that the mutant's capability to respond to iron was disrupted. Similarly the ferric reductase activity in response to copper, in the mutant, decreased by 51% however a significant increase (p = 0.024) in response to copper limitation was still observed. These results indicated that the mutant's cell surface ferric reductase activity was largely impaired; however it still retained the ability to detect changes in extracellular copper levels but no ability to respond to changes in iron levels.

The levels of cell surface cupric reductase activity associated with the mutant were also established, in comparison to the reference strains (Figure 4.14 B, Table 4.3). In the reference strain, a 1.5-fold (p = 0.041) increase in cupric reductase activity was observed in response to iron and a 1.4-fold (p = 0.037) increase was observed in response to copper. Contrastingly, in the mutant the base levels of cupric reductase activity decreased by 53% (in response to iron) and 45% (in response to copper). These results indicated that the cupric reductase activity associated with the mutant was severely decreased and similar to the ferric reductase activity, there was no significant increase in response to iron (p = 0.413). However the mutant still retained capability of detecting change in copper levels and a small increase in response to copper limitation was observed.

4.8. Measurement of ferric iron uptake in *sef1Δ/Δ* mutant

In previous studies done in *S. cerevisiae*, it was observed that when the cell surface ferric reductase activity was impaired, it lead to reduction in capability of the mutant to carry iron into the cell, as iron first needs to be reduced to its ferrous form before it can be transported within the cell via the oxidase/permease complex (Dancis A *et al.*, 1990,

Dancis A *et al.*, 1994). The rate of iron uptake and accumulation within the cell can be measured by utilising radioactive $FeCl_3$. The amount of ^{55}Fe accumulation within the cell was measured (Refer 2.11) and compared with the radioactive ferric build-up within the reference strains (Figure 4.15). The amount of iron uptake and accumulation in the mutant cell was reduced massively by 82% which indicated that the defects in *SEF1* regulatory pathway, lead to reduction in cell surface ferric reductase activity which in turn severely affected the rate of iron uptake within the cell.
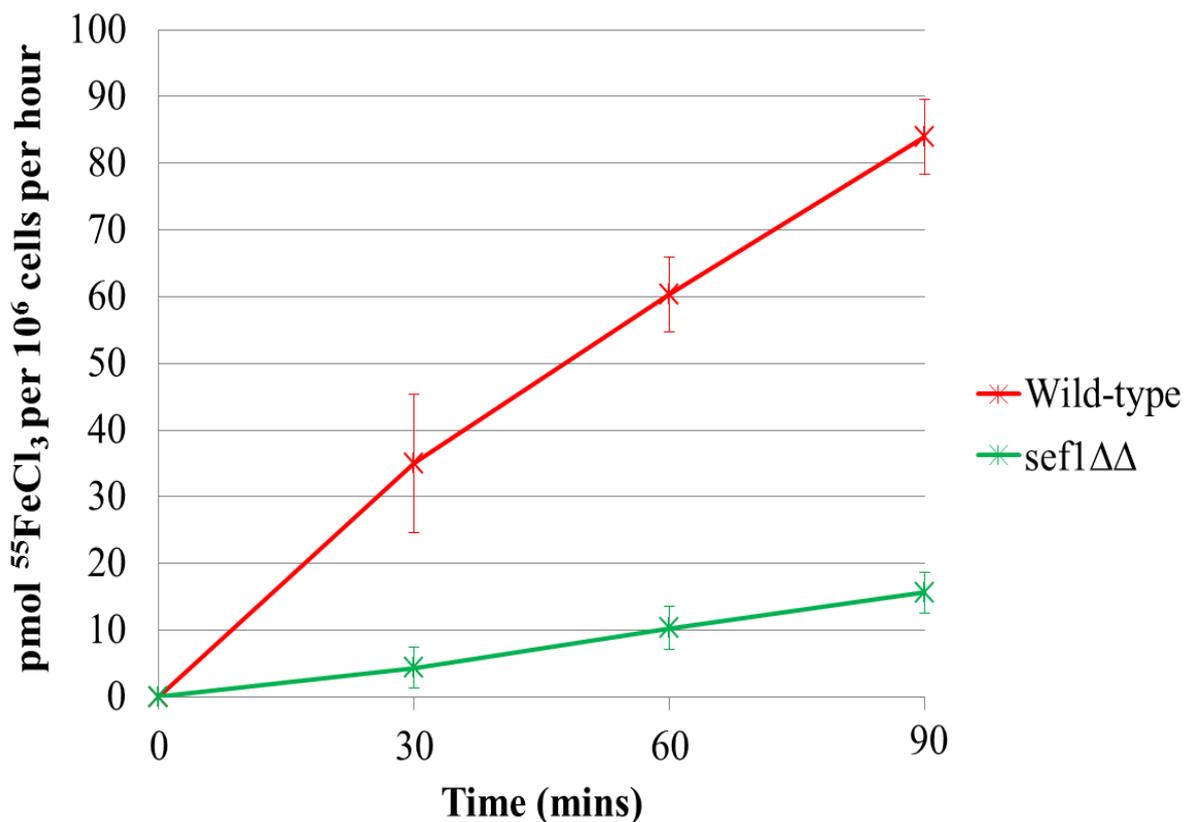
Figure 4. 15: Measurement of ferric iron uptake within the *sef1*ΔΔ mutant

Cells were grown in MD media up to mid-log phase at 30°C under continuous shaking conditions and then incubated with radioactive $^{55}FeCl_3$. Cells were harvested after every 30 minutes when the amount of ^{55}Fe accumulation, within the cell, was determined. This graph shows a mean of three independent experiments. The *sef1*Δ/*sef1*Δ mutant was compared with reference strains to investigate the rate of ferric iron uptake. Refer Methods and materials section 2.11 for a detailed protocol.

4.9. Discussion

The aim of this chapter was to analyse the phenotypic characteristics and growth defects associated with the *sef1ΔΔ* mutant and to also look at the expression levels of various genes proposed to be involved in iron and copper metabolism in the mutant, which would lead to the identification of the role played by *SEF1* in *C. albicans*.

The mutant was tested for its response to serum which showed us that the *sef1ΔΔ* mutant portrayed a defect in detection of haemoglobin and was incapable of switching from the yeast form into its hyphal form. When the mutant was tested for growth in the presence of haemoglobin as the sole source of iron, it was also found to be defective in haem-iron utilisation. These results indicated a defect in regulation of Rbt5 which has been proposed to be involved in haem-iron utilisation and triggering hyphal differentiation in *C. albicans* (Pendrak & Roberts, 2007). The *sef1ΔΔ* mutant was then tested for its response to iron and copper limitation and it was found that the mutant grew poorly in iron restricted media. This result was consistent with what had been observed in other screens (Noble *et al.*, 2010). The mutant was tested for its sensitivity to oxidative stress and *sef1ΔΔ* mutant showed increased sensitivity to hydrogen peroxide which could be rectified by addition of iron to the media. This indicated a defect in the enzymes that contain [4Fe-4S] clusters (De Freitas *et al.*, 2003). The *sef1ΔΔ* mutant did not display any respiratory defects and was able to consume glycerol and glucose as sole sources of carbon.

The *sef1ΔΔ* mutant was tested for its response to varying pH and it showed reduced growth at pH 10.5, indicating a growth defect in alkaline conditions. This suggested a defect in *FRP1*expression, which is an alkaline-induced putative ferric reductase (Liang *et al.*, 2009). The mutant was then tested for its sensitivity to very high iron and very

high copper conditions. The mutant did not show any growth defect in very high iron conditions and grew comparable to the reference strains however it appeared to survive better than the reference strains in very high copper conditions. This result and the pale white coloured colony on glycerol media indicated a defect in copper uptake within the mutant. The extracellular high copper levels were prevented from leading to intracellular copper toxic conditions within the mutant. This result was explained when in response to copper restriction, the expression of the copper transporter *CaCTR1* was reduced by 33% in the mutant. The mutant was found to be capable of detecting changes in copper levels and increasing expression of *CaCTR1* in response to copper restriction however the rate of transcription of *CaCTR1* was affected due to absence of *SEF1*. This result explains how the mutant was able to survive in very high copper conditions since the amount of copper being transported across the cell membrane was reduced as compared to the amount of copper being transported within the cell, in the reference strains. The rate of growth of the mutant was studied and was found to be affected in iron limited conditions.

The expression of *SEF1* was analysed in various iron and copper conditions in the reference strains via RT-PCR and the expression of *SEF1* was found to increase by 69% in iron restrictive conditions which signified a role of *SEF1* in iron uptake and utilisation in *C. albicans*. The expression of various genes involved in iron and copper homeostasis were analysed in the *sef1ΔΔ* mutant in various iron and copper conditions and a reduction in expression of *CaFRE1*, *CaFRE2*, CaFRE10*, CaFRP1*, *CaFTR2*, *CaFET34* and *CaRBT5*. Interestingly, a reduction in expression was also seen for *CaFRE7* and *CaCTR1* which have been previously proposed to be regulated by Mac1p (Marvin *et al.*, 2004, Jeeves RE *et al.*, 2011, Woodacre *et al.*, 2008). The mutant retained the detection of changes in copper levels and was capable of increasing

transcription of *CaFRE7* and *CaCTR1* in response to copper, however the overall levels of transcription were impaired. This indicated a novel regulatory interaction between Sef1p and CaMac1p. These findings were consistent with the growth defects and phenotypic defects observed in *sef1ΔΔ* mutant, in this chapter.

The cell surface associated ferric and cupric reductase activity as well as the iron uptake capacity of the *sef1ΔΔ* mutant was defective. This finding was in co-relation with the other expression analysis data obtained in this study which indicated a defect in iron as well as copper metabolism in *sef1ΔΔ* mutant.

The findings in this chapter re-confirmed some of the findings of other microarray studies carried out on *sef1ΔΔ* mutant (Chen C *et al.*, 2011, Vandeputte P *et al.*, 2011) however it also indicated a novel role of *SEF1* in regulation of copper responsive genes and suggests an association of Sef1p with Mac1p in the regulation of some genes like *CaFRE7* and *CaCTR1* which has not been previously reported.

The results in this chapter indicated that *SEF1* plays an important regulatory role and is involved in regulation of various genes of the iron as well as copper homeostasis system which portrays its significance in virulence causing ability of the organism. The novel finding in this chapter was the indication of involvement of *SEF1* in copper homeostasis and that it may regulate a number of genes indirectly, in association with other regulators such as Sfu1p, Hap43p, Rim101p and specifically Mac1p. This interaction would be looked at in the next chapter to identify the precise manner in which Sef1p interrelates with these other more-studied regulators of *C. albicans*. The role played by *SEF1* in *C. albicans* is unique and unlike its role in *S. cerevisiae* and hence points towards an adaption mechanism in the opportunistic pathogen which helps it to acclimatise and proliferate in the human host system.

5.   Analysing the regulatory link between iron and copper homeostasis systems in *C. albicans*

5.1. Introduction

Iron and copper are essential for growth and survival of *C. albicans* however regulation of uptake of these metal ions is extremely vital so as to avoid formation of toxic free radicals due to Fenton reactions which would lead to cell damage and eventually cell death (Crichton & Pierre, 2001).

In *S. cerevisiae*, ScAft1p is the major iron responsive regulator and is responsible for regulating transcription of the ferric reductases (*ScFREs*), copper chaperone (*ScATX1*), copper transporter (*ScCCC2*), multi-copper oxidase (*ScFET5*), iron transporter (*ScFTH1*) and siderophore iron transporters (*ScARN1-4*) (Marvin *et al.*, 2004, Yamaguchi-Iwai Y *et al.*, 1995). However it was observed that deletion of the *ScAFT1* homologue- *CaAFT1* in *C. albicans* did not lead to major defects in high affinity iron acquisition and the mutant was capable of growth in low iron media (Marvin *et al.*, 2004, Mason, 2006b). This indicated that although the two organisms had a similar iron and copper uptake system, *C. albicans* had a regulatory mechanism which was quite distinct from the model organism *S. cerevisiae*.

In *C. albicans* a GATA type transcriptional repressor has been previously identified (Lan CY *et al.*, 2004) due its similarities to the GATA-type transcriptional repressors in other fungi such as *URBS1* that regulates siderophore production in *Ustillago maydis* (Voisard et al., 1993)*, SRE* that regulates iron transport in *Neurospora crassa* (Zhou et al., 1998), *SREA* that is involved in siderophore iron uptake and other iron acquisition

in *Aspergillus nidulans* (Oberegger *et al.*, 2001). In a microarray study, it was observed that Sfu1p led to a decrease in expression of *CaFTE34, CaFET35, CaFRE1, CaFRE2, CaFRE5, CaFRE10* and *CaFTH1*and hence was negatively regulating them in response to iron replete conditions (Lan CY *et al.*, 2004). A zinc-finger containing, novel putative positive transcription factor Sef1p was also identified in *C. albicans* (Lan CY et al., 2004). Additionally work carried out in our laboratory revealed an increase in iron uptake and cell surface associated ferric and cupric reductase activity in *sfu1*ΔΔ mutants. Moreover, in *sfu1*ΔΔ mutants reductase activity in response to iron restriction was still detected which indicated the presence of another iron responsive regulator in addition to Sfu1p (Jeeves, 2008). In chapter 4 in this study we saw functional evidence that the potential (positive) regulator Sef1p was leading to an increase in expression of *CaFRE1*, *CaFRE2*, CaFRE10*, CaFRP1*, *CaFTR2*, *CaFET34* and *CaRBT5* in response to iron limitation. A reduction in iron uptake as well as cell-surface-associated ferric and cupric reductase activity was also observed in *sef1*ΔΔ mutants. This indicated that *C. albicans* has both a repressor and an activator that regulates genes involved in iron acquisition and uptake.

Furthermore, work carried out in our laboratory also shows the presence of a copper responsive regulator CaMac1p which has been found to be involved in regulation of copper transporter *CaCTR1*, copper-responsive ferric reductase *CaFRE7* and itself (Marvin *et al.*, 2004, Woodacre, 2007, Woodacre *et al.*, 2008). In chapter 4 we also observed that deletion of *SEF1* led to a reduction in expression of *CaCTR1* and *CaFRE7* which indicated that Sef1p was also interacting with Mac1p in a certain manner to regulate genes.

In summary, in Chapter 4 in this study, we analysed the role of the putative transcription factor Sef1p and characterised its role in high affinity iron uptake in *C.*

albicans. Our results also indicated a role of Sef1p in copper homeostasis. The aim of this part of the study was to analyse the interaction of the three transcription factors CaSfu1p, CaSef1p and CaMac1p and to characterise how they co-ordinated to regulate iron and copper homeostasis in *C. albicans*. *sfu1Δ/ sfu1Δ* (CNA6) and *mac1Δ/mac1Δ* (MEM-m2) mutants constructed in our laboratory for previous studies (Marvin *et al.*, 2004, Woodacre *et al.*, 2008) were used along-with *sef1Δ/sef1Δ* that was used in Chapter 4 in this study (Homann OR *et al.*, 2009); to investigate expression *CaSFU1*, *CaMAC1* and *CaSEF1* regulated by CaSfu1p, CaSef1p and CaMac1p using RT-PCR.

5.2. Analysing growth rate of regulatory mutants in iron and copper deficient environment

This portion of the study was initiated by comparing growth rates of the mutants (*mac1ΔΔ*, *sfu1ΔΔ* and *sef1ΔΔ*) with the wild-type strain. As a part of this study, preliminary growth analyses were carried out on *mac1ΔΔ* and *sef1ΔΔ* mutants by our undergraduate project student, Andreas Damianou. Cells were grown in MD media supplemented with BPS and BCS, to create the appropriate iron and copper restricted environment. Absorbance was measured at 0.D. 600 nm in every 30 minutes, manually. The initial growth rates for the strains were calculated and plotted on a graph (Figure 5.1). The *mac1ΔΔ* and *sef1ΔΔ* mutants showed a slow initial growth rate in iron and copper deficient media. Further growth studies (**A.**) in low copper conditions (supplemented with BCS) in response to variable iron and (**B.**) in low iron conditions (supplemented with BPS) in response to variable copper; also showed that the growth defects observed in *mac1ΔΔ* mutant could be rescued by supplementing the media with copper. The growth defects of *sef1ΔΔ* mutant could be rescued by supplementing the

media with iron, however presence of copper in the media, helped the mutant to grow better. *sfu1ΔΔ* mutant was found to show no visible growth defects in iron or copper deficient conditions (Lan CY *et al.*, 2004)

Following those initial results, in this study the wild-type, *mac1ΔΔ*, *sfu1ΔΔ* and *sef1ΔΔ* mutants were all grown in MD media at 30°C overnight and then 10^4 cells were inoculated in a bioscreen plate containing media with the appropriate iron and copper limitation. The cells were then grown in the bioscreen at 30°C for 75 hours at an OD of 600 nm and a reading was taken every 15 minutes. A mean of three growth curve repeats is shown (Figure 5.3). The *sfu1ΔΔ* mutant did not show any growth defects and grew comparable to the wild-type strain. The *sef1ΔΔ* mutant showed a slower growth rate as compared to the wild-type strain, which could lead to growth defects in metal deficient media. The *mac1ΔΔ* mutant showed the slowest growth rate and showed a significant growth defect as compared to the wild-type strain. It was interesting to compare the rate of growth of the mutants with each other and with the wild-type strain.

A.

Environment of both low iron and low copper conentration

Initial growth rate of WT, *mac1ΔΔ* and *sef1ΔΔ* in low iron & low copper environment.

B.

Growth curve of WT, *mac1ΔΔ* and *sef1ΔΔ* in low iron & low copper environment.
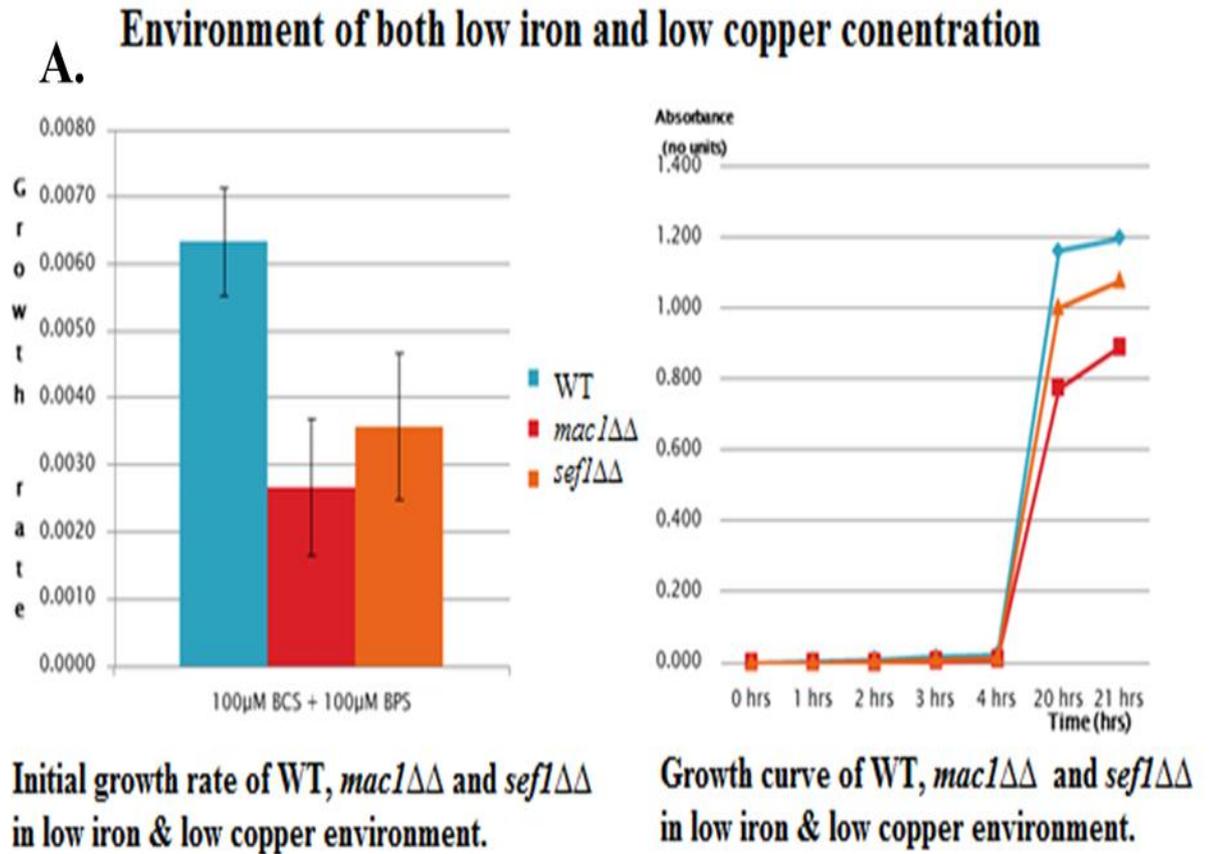
Figure 5. 1: Growth analysis of *mac1ΔΔ, sef1ΔΔ* mutants in comparison to wild-type in iron and copper restricted environment

Cells were grown in MD media supplemented with 100µM BPS and 100µM BCS, to create the appropriate iron and copper restricted environment. Absorbance was measured at 0.D. 600 nm in every 30 minutes, manually. The initial growth rates for the strains were calculated and plotted on a graph (**A.**) while the growth curve over a period of 21 hours was plotted over graph (**B.**) This figure shows the mean results of three separate experiments with error bars of standard deviation (Damianou, 2013).

A. Low Copper and variable iron environment
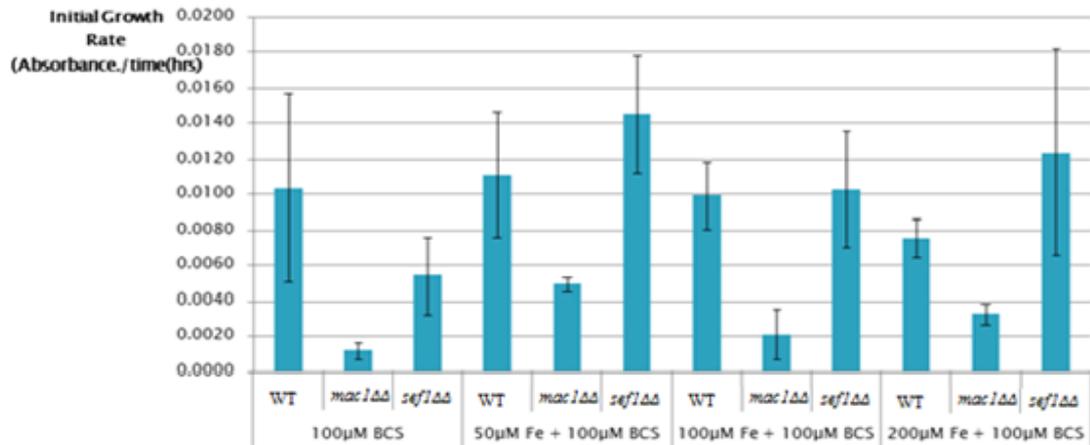
B. Low Iron and variable copper environment
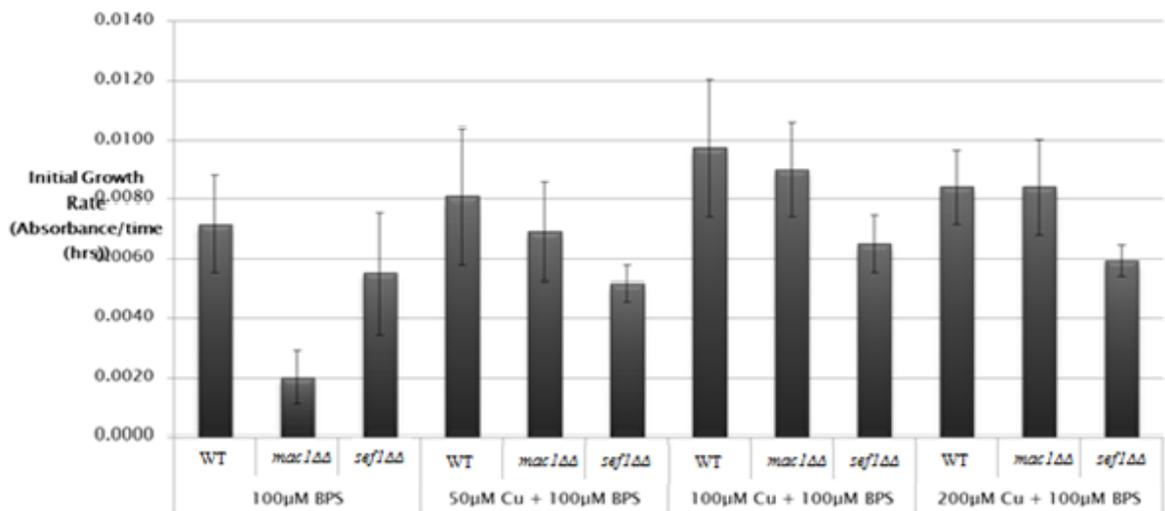
Figure 5. 2: Growth analysis of *mac1ΔΔ, sef1ΔΔ* mutants in comparison to wild-type in response to iron and copper

Cells were grown in MD media supplemented with 100μM BPS or 100μM BCS, to create the appropriate iron or copper restricted environment. Absorbance was measured at 0.D. 600 nm in every 30 minutes, manually. The initial growth rates for the strains were calculated and plotted on a graph (**A.**) in low copper conditions (supplemented with 100μM BCS) in response to variable iron (**B.**) in low iron conditions (supplemented with 100μM BPS) in response to variable copper. This figure shows the mean results of three separate experiments with error bars of standard deviation (Damianou, 2013).
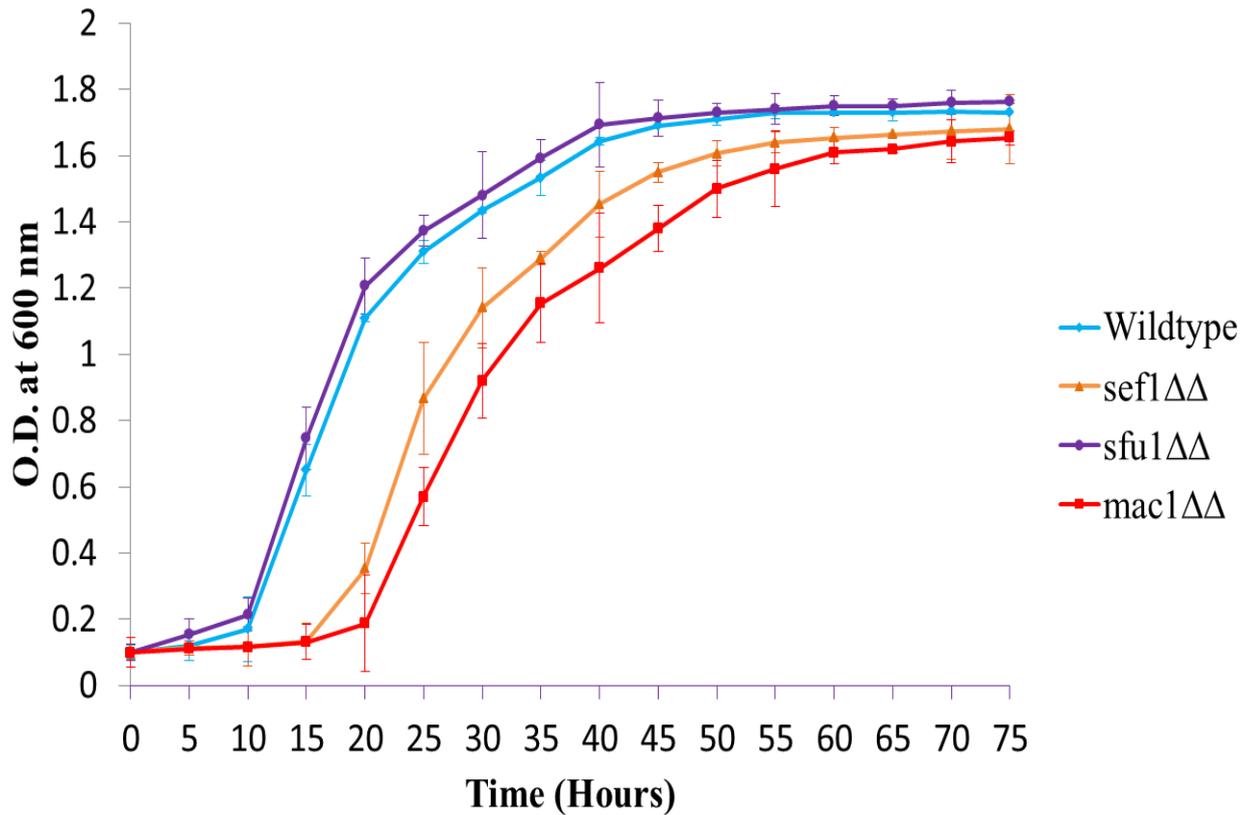
Figure 5. 3: Growth curve analysis of mutants (*mac1ΔΔ, sfu1ΔΔ and sef1ΔΔ*) vs. wild-type strain, in iron and copper restricted conditions

All cells were grown in MD media at 30°C overnight and then 10^4 cells were inoculated in a bioscreen plate containing media with iron and copper limitation, supplemented with 100μM BPS and100μM BCS. The cells were then grown in the bioscreen at 30°C for 75 hours at an OD of 600 nm and a reading was taken every 15 minutes. This figure shows the mean results of three separate experiments with error bars of standard deviation.

5.3. Investigation of regulation of *SEF1* by Sfu1p and CaMac1p using RT-PCR

Previous micro-array studies have shown expression of *SEF1* to be increased in low iron conditions (Homann OR *et al.*, 2009) and it was also to be increased in *sfu1*ΔΔ mutants (Lan CY *et al.*, 2004). In chapter 4 in this study, we observed the expression of *SEF1* was analysed in various iron and copper conditions in the wild-type strain via RT-PCR (Refer 2.10) and the expression of *SEF1* was found to increase by 69% in iron restrictive conditions which signified a role of *SEF1* in iron uptake and utilisation in *C. albicans*. The expression of *SEF1* was analysed in wild-type, *mac1*ΔΔ and *sfu1*ΔΔ in low iron, low copper and iron and copper replete media (Figure 5.4). As was seen previously, the expression of *SEF1* was found to increase mainly in response to iron restriction (69%), in the wild-type strain while interestingly it showed a slight increase (22%) in response to copper restriction as well. The expression of *SEF1* was found to be much higher (74%) in *sfu1*ΔΔ and iron as well as copper sensitive expression was lost in this mutant. Interestingly, the expression of *SEF1* was found to be slightly lower in *mac1*ΔΔ and copper sensitive expression was lost in this mutant however the increase in expression in iron restriction was still retained in *mac1*ΔΔ. These results indicate that Sfu1p supresses *SEF1* in iron and copper replete conditions while Mac1p positively interacts with *SEF1* in copper restricted conditions, however, many more experimental repeats would have to be carried out to determine statistical significance.
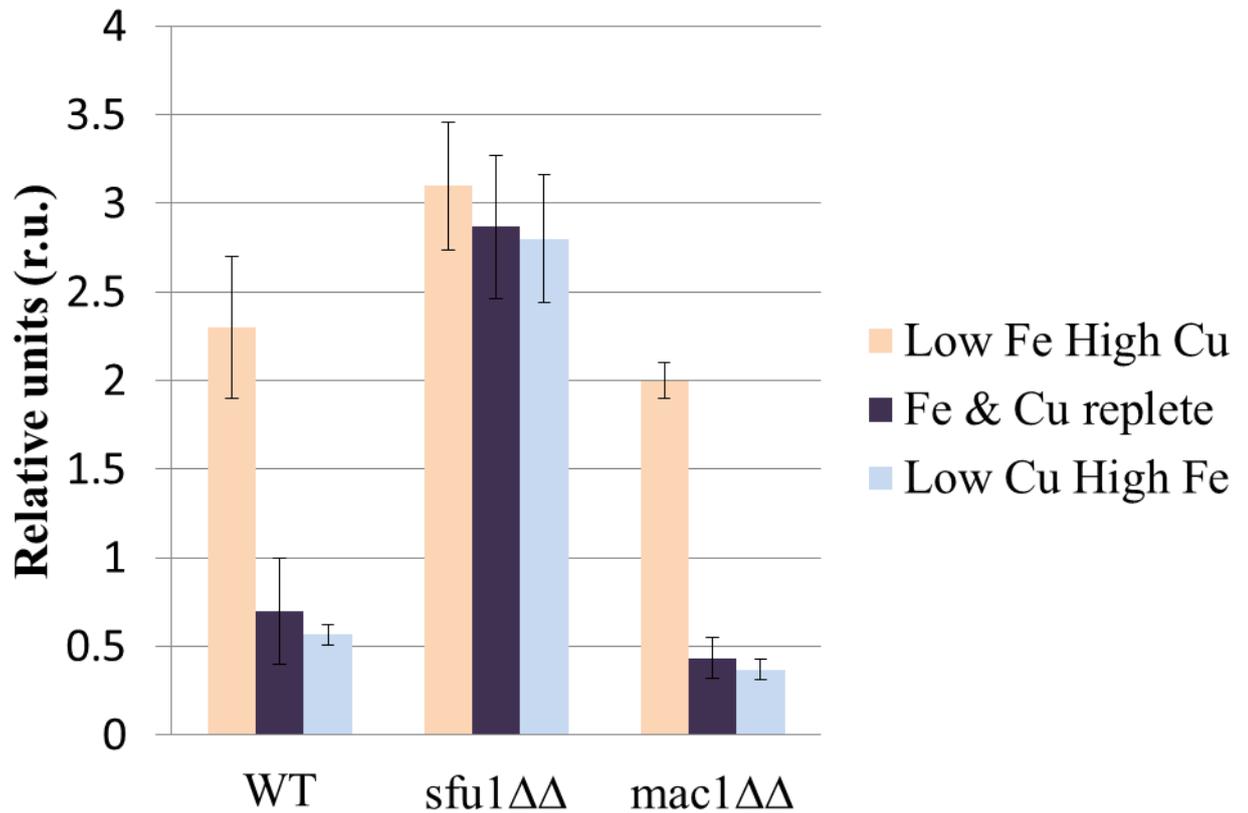
Figure 5. 4: Expression of *SEF1* in wild-type (SC5314), *sfu1*ΔΔ (CNA6) and *mac1*ΔΔ (MEM-m2)

In low iron, low copper and iron + copper replete media, using RT-PCR

Cells were grown for 6 hours in MD media containing low iron, high copper (supplemented with 100μM $CuCl_2$ + 100μM BPS), low copper, high iron (supplemented with 100μM $FeCl_3$ + 100μM BCS) and iron + copper replete media (100μM $FeCl_3$ + 100μM $CuCl_2$); until they reached mid-exponential phase. RNA was then extracted, converted to cDNA and was used for RT-PCR analysis to monitor change in expression. The data is represented in relative units that were generated by the relative quantitation software. Each reading is a mean of four replicates per reaction and three independent experiments. The error bars represent standard deviation.

5.4. Investigation of regulation of *MAC1* by Sfu1p and Sef1p using RT-PCR

Work carried out in our laboratory using β-galactosidase *CaMAC1* -promoter reporter constructs have shown that the expression of *CaMAC1* increases in response to copper limitation and it was also found to be self-regulated as the expression was found to be higher in a *Camac1ΔΔ* mutant (Woodacre *et al.*, 2008). However it was predicted that an alternative regulator to CaMac1p was also regulating transcription of *CaMAC1* since the levels of β-galactosidase activity from the *CaMAC1* –promoter was 15 times more than the basal activity from the *CaCTR1* promoter which is also regulated by CaMac1p. This indicated that transcription of *CaMAC1* was also dependent on another regulator in combination with CaMac1p (Woodacre, 2007). The levels of expression of *CaMAC1* was analysed in wild-type, *sef1ΔΔ* and *sfu1ΔΔ* in low iron, low copper and iron and copper replete media using RT-PCR (Figure 5.5, Refer 2.10).

In the wild-type strain, the expression of *CaMAC1* was found to increase slightly in low iron conditions however a maximal increase in expression was observed in low copper conditions (61% p = 0.03) which is consistent with what was previously observed with the β-galactosidase reporter constructs. In the *sfu1ΔΔ* mutant, the overall expression of *CaMAC1* was higher, especially in response to copper restriction- by 46% (significant, p = 0.022). The significant ≈2-fold increase in expression in response to copper limitation was maintained in the *sfu1ΔΔ* mutant however no significant changes in expression, were observed in response to iron limitation. Interestingly, in the *sef1ΔΔ* mutant the increase in expression of *CaMAC1* in response to low copper was reduced by (33%) and no change in response to iron limitation was detected.
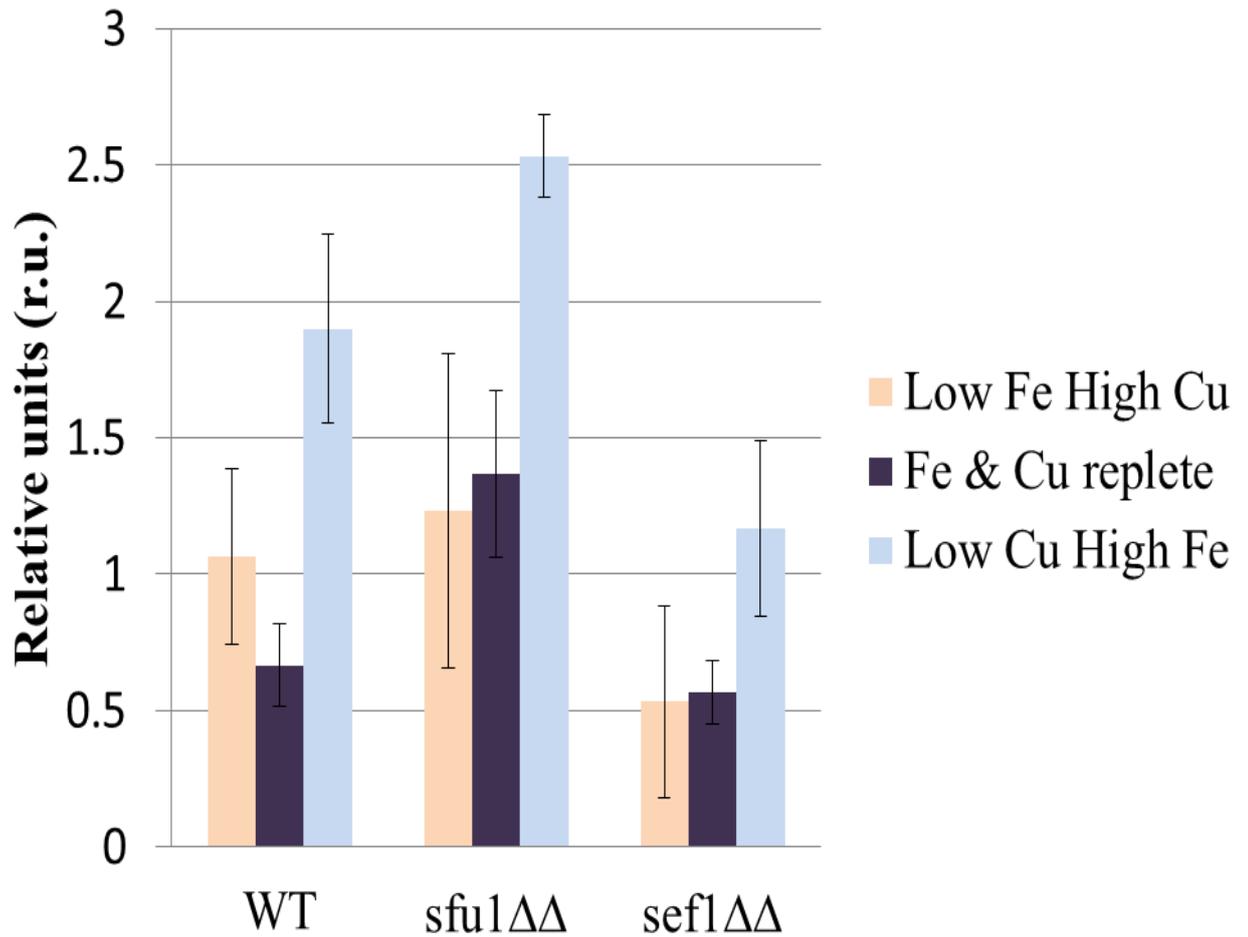
Figure 5. 5: Expression of *MAC*1 in wild-type (SC5314), *sfu1*ΔΔ (CNA6) and *sef1*ΔΔ

In low iron, low copper and iron + copper replete media, using RT-PCR

Cells were grown for 6 hours in MD media containing low iron, high copper (supplemented with 100μM $CuCl_2$ + 100μM BPS), low copper, high iron (supplemented with 100μM $FeCl_3$ + 100μM BCS) and iron + copper replete media (100μM $FeCl_3$ + 100μM $CuCl_2$); until they reached mid-exponential phase. RNA was then extracted, converted to cDNA and was used for RT-PCR analysis to monitor change in expression. The data is represented in relative units that were generated by the relative quantitation software. Each reading is a mean of four replicates per reaction and three independent experiments. The error bars represent standard deviation.

5.5. Investigation of regulation of *SFU1* by Sef1p and CaMac1p using RT-PCR

Northern blot studies on expression of *SFU1* carried out in our laboratory previously have shown that expression of *SFU1* increases in response to iron replete as well as in high copper conditions (Woodacre *et al.*, 2008). However on RT-PCR analysis (Refer 2.10) of *SFU1* expression in wild-type, *sef1ΔΔ* and *mac1ΔΔ* in low iron, low copper and iron and copper replete conditions (Figure 5.6), it was observed that *SFU1* expression significantly increased (36% p = 0.07) in response to copper restriction as compared to iron and copper replete condition.

The increase in expression in response to high iron was re-confirmed and was found to be significant (p = 0.029) as well as consistent with the previous results. The increase in expression of *SFU1* in response to high iron condition was much higher in *sef1ΔΔ* mutant however the copper responsive increase in expression was lost as no significant difference in expression was seen between the iron and copper replete condition and the copper restricted condition. In the *mac1ΔΔ* mutant, the overall expression of *SFU1* was at its maximum and much higher (57% p = 0.0004) than in the wild-type strain, however the increase in expression in response to high iron was still maintained. Interestingly *SFU1* transcription increased by 31% (p =0.02) in high copper conditions, in *mac1ΔΔ* mutant. This regulatory pattern is completely different from what has been observed for Mac1p up to date and indicates that CaMac1p may not only be functioning as a activator but may also act as a repressor of certain genes.
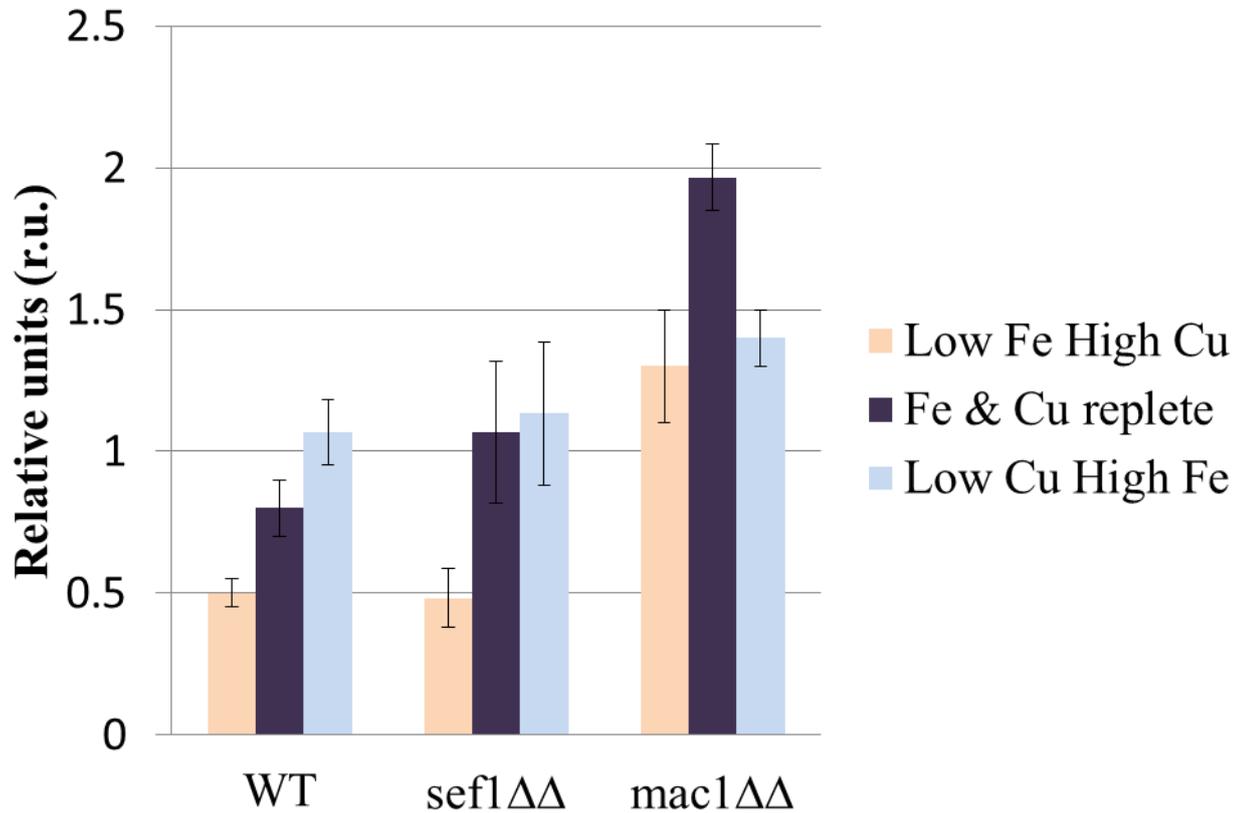
Figure 5. 6: Expression of *SFU1* in wild-type (SC5314), *mac1ΔΔ* (MEM-m2) and *sef1ΔΔ*

In low iron, low copper and iron + copper replete media, using RT-PCR

Cells were grown for 6 hours in MD media containing low iron, high copper (supplemented with 100μM $CuCl_2$ + 100μM BPS), low copper, high iron (supplemented with 100μM $FeCl_3$ + 100μM BCS) and iron + copper replete media (100μM $FeCl_3$ + 100μM $CuCl_2$); until they reached mid-exponential phase. RNA was then extracted, converted to cDNA and was used for RT-PCR analysis to monitor change in expression. The data is represented in relative units that were generated by the relative quantitation software. Each reading is a mean of four replicates per reaction and three independent experiments. The error bars represent standard deviation.

5.6. Analysis of *SFU1* and *SEF1* being directly regulated CaMac1p

As described in Chapter 3, the His-tagged CaMac1 protein was expressed using the pET28α vector (Refer 2.9) and was used in a gel-shift assay (Figure 5.7). The probes for gel-shift assays for *CaMAC1*, *CaSEF1* and *CaSFU1* promoters were amplified using primers MAC1 probe F and MAC1 probe R; SEF1 probe F and SEF1 probe R; SFU1 probe F and SFU1 probe R respectively (Table 2.6) The probe oligonucleotides were gel purified and labelled with DIG. A DIG-labelled PCR probe of the *CaMAC1*, *CaSEF1* or *CaSFU1* promoter was then incubated with purified CaMac1 protein in the presence of either 30 μM BCS (a copper chelator) or 100 μM $CuCl_2$. Samples were run on a native gel, blotted to a membrane and detected with anti-DIG Fab fragments and CSPD. Protein-probe binding was seen as an additional distinctive band on the blot indicative of the retarded mobility on the gel of a protein : DNA complex.

The previously described *CaMAC1* auto-regulation seen in Chapter 3 was detected again as the recombinant CaMac1p bound the *CaMAC1* promoter in copper restrictive conditions. This was used as a positive control for protein - probe binding. A second fragment indicative of CaMac1p binding to *CaSEF1* promoter, was not observed in the gel-shift indicating the CaMac1p does not directly bind to *CaSEF1* and perhaps regulates it post-transcriptionally. Interestingly, CaMac1p - *CaSFU1*-promoter binding was detected in high copper conditions as opposed to the usual copper restriction responsive binding displayed by CaMac1p. This indicated a direct regulatory role played by CaMac1p in expression of *SFU1* however the manner in which CaMac1p regulates *SFU1* appears to be different than its traditional role of a positive regulator.
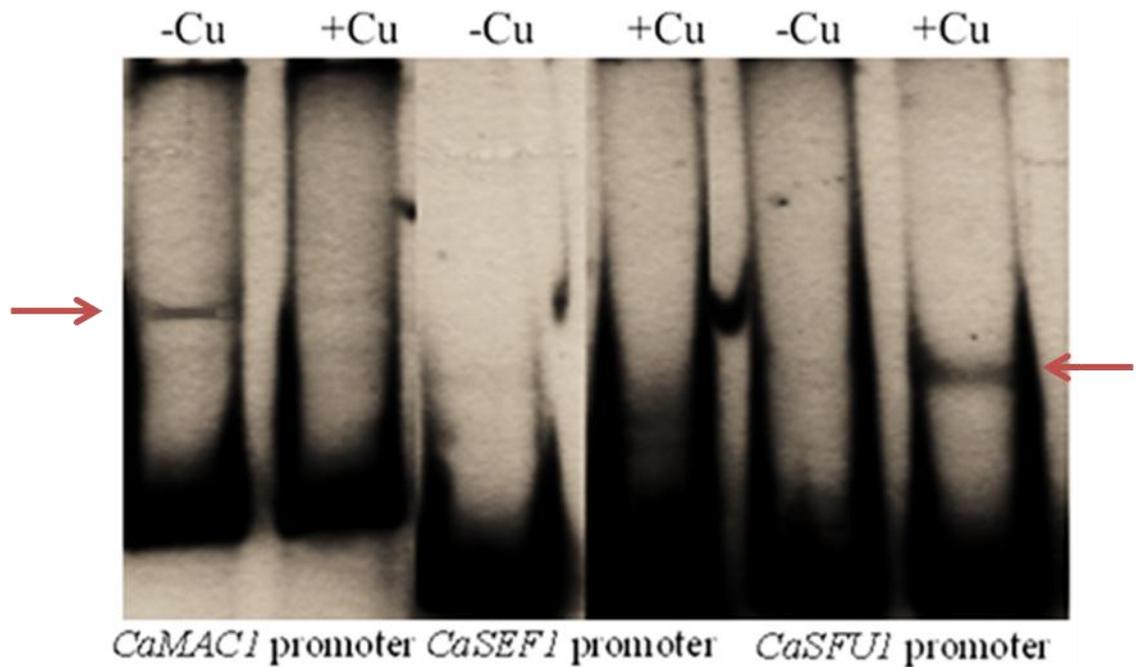
Figure 5. 7: Gel Shifts *of CaMAC1, CaSEF1* and *CaSFU1* Promoters with the CaMac1 Protein

A DIG-labelled PCR probe of the *CaMAC1*, *CaSFU1* or *CaSEF1* promoter was incubated with purified CaMac1 protein in the presence of either 30 μM BCS (a copper chelator) or 100 μM CuCl$_2$. Samples were run on a native gel, blotted to a membrane and detected with anti-DIG Fab fragments and CSPD. Protein-probe binding is seen as an additional distinctive band on the blot which is characteristic of the retarded mobility in the gel of protein : DNA complexes. Refer Methods and materials Section 2.9 for a detailed protocol.

5.7. Discussion

Iron uptake system in *C. albicans* is intricately interlinked with the copper uptake system (Knight SA *et al.*, 2002, Knight SA *et al*., 2005) and hence it was likely that copper responsive regulator CaMac1p would also play a part in regulation of high affinity iron uptake and vice versa. The aim of this part of the study was to analyse the interaction of the three transcription factors CaSfu1p, CaSef1p and CaMac1p and to characterise how they co-ordinated to regulate each other's regulation and in turn regulated iron and copper homeostasis in *C. albicans*.

Growth analysis of the mutants indicated that the *sfu1*ΔΔ mutant did not show any growth defects and grew comparable to the wild-type strain. The *sef1*ΔΔ mutant showed a slower growth rate as compared to the wild-type strain; while the *mac1*ΔΔ mutant was the most affected and showed the slowest growth rate with a significant growth defect in iron and copper lacking media.

On analysis of regulation of *SEF1* by Sfu1p and CaMac1p (Figure 5.4), it was observed that the expression of *SEF1* was found to be much higher in *sfu1*ΔΔ and iron as well as copper sensitive expression was lost in this mutant. These results in combination with the higher expression of *SFU1* detected in high iron conditions (Figure 5.6) and previous data implicating it as a GATA-type transcriptional repressor (Lan CY *et al.*, 2004) indicate that Sfu1p may be likely to be repressing expression of *SEF1* in high iron conditions.

Interestingly, the expression of *SEF1* was found to be slightly lower in *mac1*ΔΔ mutant (not statistically significant) and copper sensitive expression was lost in this mutant however the increase in expression in iron restriction was still retained in *mac1*ΔΔ.

These results indicate that CaMac1p positively interacts with *SEF1* (directly or indirectly) in copper restricted conditions and leads to enhancement of its transcription in response to low copper. However, during our gel-shift assay, we did not observe CaMac1p binding to *SEF1-* promoter (Figure 5.7), and on analysis of the upstream region of the gene, no CuRE sites were detected indicating that CaMac1p could be employing a co-activator which would activate expression of *SEF1*; or CaMac1p must be regulating it in a novel post-transcriptional manner. In a recent study with Sef1p, it was revealed that a multiple level of post-transcriptional regulation occurs in *C. albicans* such as cytoplasmic localization of proteins, nuclear localization of protein, differential protein stability or phosphorylation of proteins (Chen & Noble, 2012).

An alternative explanation for this is an indirect effect of absence of CaMac1p in *Camac1ΔΔ* mutant which would lead to de-repression of *SFU1* which would explain the decrease in expression of *SEF1* due to suppression by Sfu1p in iron and copper replete conditions in *Camac1ΔΔ* mutant.

On analysis of regulation of *MAC1* by Sfu1p and Sef1p (Figure 5.5), it was observed that the overall expression of *CaMAC1* was higher in *sfu1ΔΔ* mutant while the significant ≈2-fold increase in expression in response to copper limitation was still maintained. These results indicated that Sfu1p was interacting with *CaMAC1* directly or indirectly to regulate its expression in some manner, in response to iron.

A possible explanation for these results is the indirect activation of *CaMAC1* in *sfu1ΔΔ* due to secondary copper starvation conditions being generated within the cell. Evidence from previous studies in this laboratory has shown an increase in iron uptake in a *sfu1ΔΔ* mutant (Jeeves RE *et al.*, 2011); while in a microarray study, it was seen that the expression of multi-copper oxidases *CaFET34* was also higher in *sfu1ΔΔ* mutants (Lan CY *et al.*, 2004). *CaFET34* has been proposed to be the major cell surface iron

oxidase and the functional homologue of *ScFET3* in *C. albicans* (Cheng et al., 2013). Hence increased iron uptake in *sfu1ΔΔ* is brought about by increased expression of *CaFET34*. As iron and copper uptake is linked, multi-copper oxidase requires copper for its functioning which would lead to an increased demand for copper by the cell. This increased demand would be met via an increased uptake of copper by the copper transporter *CaCTR1*. *CaCTR1* is known to be activated by CaMac1p (Marvin *et al.*, 2004)and hence increased expression of *CaCTR1* would be brought about via an increased production of CaMac1p. Hence in s*fu1ΔΔ* mutants an increase in iron uptake would lead to an increase in activity of muti-copper oxidase which would lead to secondary intracellular copper starvation conditions which would ultimately be counteracted by increase in expression of *CaMAC1*.

Interestingly, in the *sef1ΔΔ* mutant the increase in expression of *CaMAC1* in response to low copper was reduced while no change in response to iron limitation was detected. This indicated that Sef1p was perhaps functioning along-with Mac1p to regulate the expression of *CaMAC1*. This co-regulation would explain the previous results where a higher level of basal *CaMAC1* promoter activity was detected in a *Camac1ΔΔ* mutant.

From the results obtained so far, we can hypothesise that Sfu1p which is a GATA-type transcriptional repressor, represses iron acquisition genes in response to high iron conditions and simultaneously also represses the positive regulator *SEF1*.

The transcriptional activator Mac1p appears to be functioning in a manner different from its traditional role as a positive regulator. Mac1p was found to bind to the CuREs present upstream in *SFU1*-promoter region in response to high copper conditions (Figure 5.7). The overall expression of *SFU1* was also found to be higher in *Camac1ΔΔ* and a increment in expression was also observed in high copper conditions in

*Camac1*ΔΔ. These results indicated that CaMac1p was portraying a novel regulatory pattern as a repressor in addition to its well-studied activator role. It is possibly supressing expression of *SFU1* by binding to it in high copper conditions. This would lead to a de-repression of *SEF1* by Sfu1p which would explain the slight increase in expression of *SEF1* in response to high copper. This is consistent with the finding that *SFU1* shows the presence of two CaMac1p binding sites (CuREs) upstream to its start codon. It shows the presence of CuREs in both orientations and the 3'-5' oriented CuRE sequence is present at the -823 site while the 5'-3' oriented CuRE is present at the -576 site. These CuREs are unusually located outside the normal range of transcription factor binding sites (Woodacre *et al.*, 2008), however many genes have cis-acting enhancer elements that may lie thousands of base pairs away from the gene, they are regulating and their binding may augment or repress transcription. It is likely that CaMac1p acts as repressor and regulates *SFU1* in a copper responsive manner.

This study has made it abundantly clear that these regulators are interdependent and their co-regulation is complex (Figure 5.8). They play a role in regulating each other's transcription and in turn co-ordinate to regulate iron and copper acquisition as well as prevention of toxicity within the cell. Further studies involving *sfu1*ΔΔ/*sef1*ΔΔ double mutants or *sef1*ΔΔ/*mac1*ΔΔ double mutants would help shed more light on the exact role played by each of these regulatory proteins however due to time constraints this was not achieved during this project. The results in this chapter have given us a possible hypothesis that links these regulators with each other intricately in a novel manner as a part of a regulatory circuit. This explains how the pathogenic *C. albicans* has an advantage over other non-pathogenic yeasts in being more capable of adapting to the various micro-environments of the mammalian host that have divergent iron and copper levels.
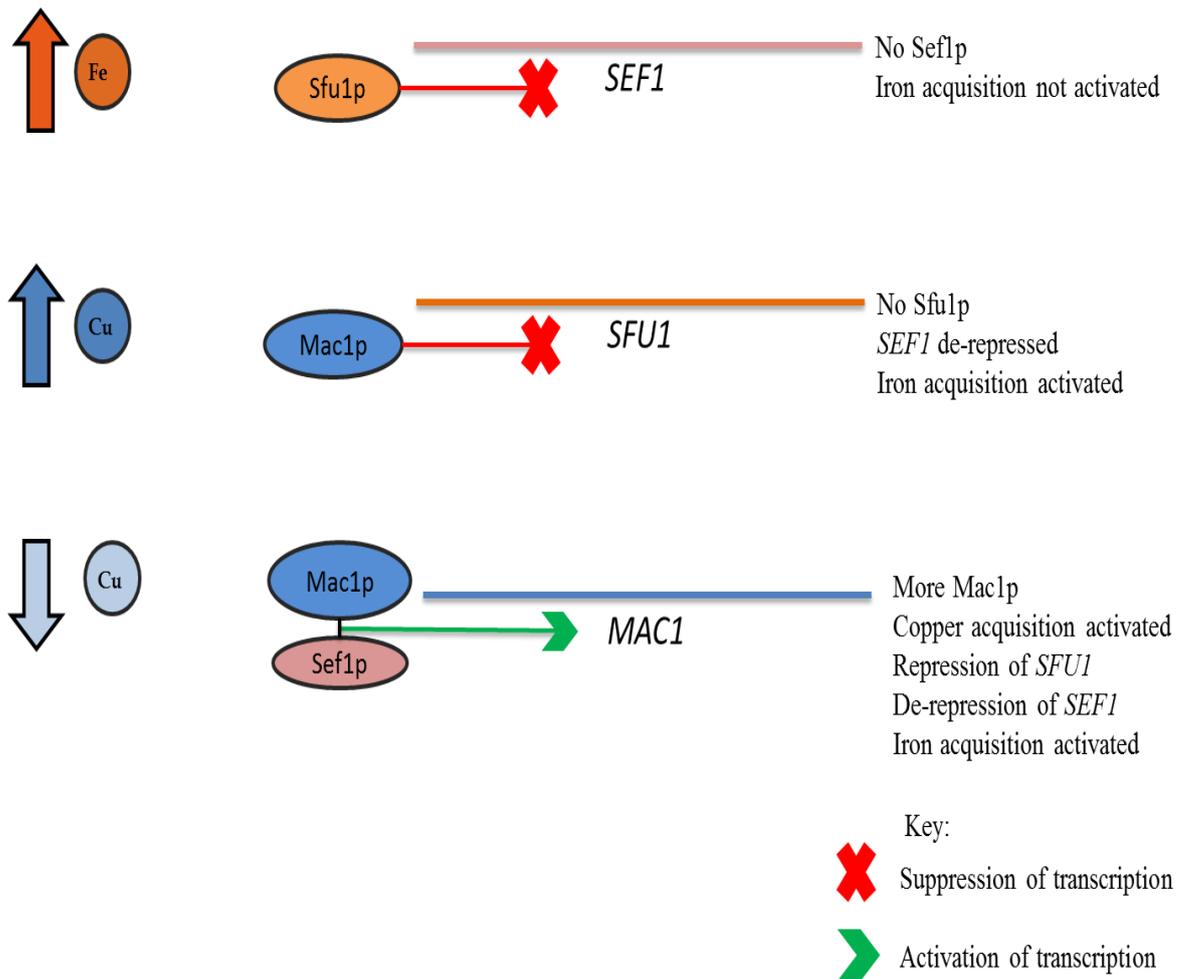
Figure 5. 8: A diagrammatic representation detailing the 'feed forward' regulatory circuit in *C. albicans*

The genes are denoted by lines, the proteins are denoted by ovals and the iron or copper conditions are mentioned by arrows. Activation of transcription is denoted in green while suppression of transcription is denoted in red. This regulatory circuit has been proposed based on the expression studies carried out in this chapter. Sfu1p, Sef1p and Mac1p are all interdependent and play a vital role in regulation of each other's transcription and in turn in regulation of iron and copper acquisition.

6. Conclusion

Candida albicans is an opportunistic pathogen that causes systemic infections in immunocompromised hosts. Factors that assist the organism to change from a commensal to a pathogenic one are resistance to anti-fungal drugs, adherence to surfaces, morphology and iron and copper homeostasis. Iron and copper are essential to the organism and are required for prevention of oxidative stress, for respiratory reactions in the cell and also function as important proteins and as co-factors of enzymes such as cytochrome oxidase, superoxide dismutase, ferrous iron transporters etc. (Hwang CS *et al.*, 2002). In *C. albicans* iron and copper acquisition is intimately interlinked and is essential for virulence as was observed from experiments performed in systemic mouse models of Candidiasis (Marvin *et al.*, 2004, Ramanan N *et al.*, 2000). Iron and copper play an important role in the interactions between *C. albicans* and the human host and therefore help in establishment of the disease (Marvin *et al.*, 2003). However the intracellular concentration of these vital metals has to be strictly maintained below toxic levels that may lead to cell damage. Hence the regulation of iron and copper homeostasis was studied in this research project.

Previous work carried out in our laboratory identified the major copper-responsive transcription factor CaMac1p in *C. albicans* which has been found to activate transcription of genes involved in iron as well as copper homeostasis. Using β-galactosidase (*CaMAC1*-promoter) reporter constructs in a *mac1*ΔΔ mutant, it was demonstrated that *CaMAC1* is transcriptionally autoregulated in response to copper replete conditions in contrast to the *S. cerevisiae MAC1* homologue, which is constitutively transcribed. It was also found that the presence of one binding site for CaMac1p in the promoters of *CaCTR1, CaMAC1* and the ferric/cupric reductase gene

CaFRE7 is adequate for copper-responsive regulation (Woodacre, 2007, Woodacre *et al.*, 2008).

The work described in chapter 3 in this report demonstrated the research strategy used to study the molecular mechanism of action of CaMac1p in *C. albicans* and to identify, as well as highlight the differences between the Mac1 proteins in *C. albicans* and *S. cerevisiae*.

Yeast two hybrid assays were used to detect protein – protein interaction and it was found that in *C. albicans* intermolecular self-association between the entire Mac1p-Mac1p is quite strong (in the presence of copper) and not weak or copper independent as was the case in *S. cerevisiae*. Contrastingly the interaction of complete Mac1 proteins in *S. cerevisiae* had shown a negligible amount of β-galactosidase activity (Serpe M *et al.*, 1999) while the interaction of entire CaMac1 proteins in *C. albicans* showed 15-times more significant amount of β-galactosidase activity which indicated a difference in folding of the quaternary structure of the proteins in the two organisms. This is also supported by the yeast-two hybrid assay which was used to detect intramolecular interactions. CaMac1p did not undergo a copper induced intramolecular interaction, and the N-terminal DNA binding domain of the protein does not associate with the C-terminal transactivation domain which leads to the absence of a heterodimer between the two separate fusion proteins, in copper replete conditions. This was unlike the presence of an intramolecular interaction in the two domains of the Mac1 protein, in *S. cerevisiae* (Joshi A *et al.*, 1999, Serpe M *et al.*, 1999).

It was then seen that on deletion of N-terminal domain the intermolecular protein : protein interaction reduced significantly. This indicated that the presence of the N-terminal domain is beneficial to protein-protein interactions. The lack of repressive

intramolecular interaction between the N-terminal and C-terminal domains allows the protein interacting C2 domain in C-terminus to be better exposed which allows maximum protein interaction to occur, via it. Contrastingly in *S. cerevisiae*, the N-terminal domain was found to be masking the binding domain of the C-terminal and hence the protein interactions via the C-terminal, increased in the absence of the N-terminal domain (Joshi A *et al.*, 1999, Serpe M *et al.*, 1999).

In this study it was found that in *C. albicans*, in copper replete conditions, the CaMac1 protein molecules that were already present do not undergo a copper-induced intramolecular interaction. The lack of N-terminal binding to the C-terminal promotes protein interactions which lead to CaMac1p forming dimers in its unused state. Since N-terminal also seems to play a role in dimerisation, it is likely that the CaMac1p dimers interact with each other in a way different from the ScMac1p self-interactions. Dimerisation in *C. albicans*, increases in copper awash conditions, and possibly the folding of the protein to incorporate dimerisation, leads to the DNA binding domain of the protein as well as the activation domain being concealed, thereby preventing the dimer from forming Mac1-DNA complex with the promoters of target genes. This prevents their transcription and also preventing its own transcription. As a result *CaMAC1* is not constitutively transcribed, and in copper replete conditions, no new protein is produced. In contrast, under copper scarce conditions, the lack of a repressive intermolecular interaction between two CaMac1 proteins, allows the activation domain to be exposed and it can then initiate transcription of target genes.

In contrast, under copper limited conditions, the lack of a repressive protein interaction (dimerisation) between two CaMac1 proteins, allows the activation and DNA binding domains to be exposed and the protein can hence bind to target gene-promoter and initiate transcription of target genes.

Using *CaCTR1*-promoter/*lacZ* fusions, it was found that the promoter containing more than one (two or three) copy of CuRE1 displayed an upsurge in *β*-galactosidase activity levels, as compared to the wild-type promoter. However, this increased expression was small and was likely to be an additive consequence of greater number of optimal sites, for binding and activation by CaMac1; rather than being a result of a synergistic influence caused by coupling of the CuRE sites since the increase was not exponential like it was in, similar experiments in, *S. cerevisiae*.

In *S. cerevisiae*, dissimilar results were observed as there was a massive 8-fold and 13-fold increase in expression when the number of CuREs were increased to two and three, respectively, which indicated that the CuRE sites showed synergism. The non-exponential increase in expression is each case in *C. albicans* (unlike in *S. cerevisiae*), indicated that the activation of target genes in *C. albicans* by CaMac1p was not interdependent on the collective presence of CuRE sites and hence presence of more than one CuRE was not fundamental. This is consistent with our previous results and hypothesis that indeed CaMac1p does not function as a dimer *in vivo* and hence one CuRE is sufficient for wild-type levels of transcription of target genes, in response to copper depletion. Repetitive CuRE elements are not necessary for high affinity DNA binding based on *CaCTR1*-promoter/*lacZ* fusion data.

This data is yet again contrasting to the observations made in *S. cerevisiae* where minimum of two CuRE elements was found to be necessary for high level *in vivo* transcriptional activation and increasing the number of CuRE elements in promoter/*lacZ* fusions from one to four showed "a more than additive effect on *lacZ* expression levels consistent with synergism" (Jensen *et al.*, 1998). The data in this research study suggests that in *C. albicans*, there occurs no synergism or dimerisation

between the CaMac1 molecules bound to multiple sites and hence more than one CuRE is not essential for high level expression of CaMac1p-regulated genes.

EMSA experiments demonstrated that *CaCTR1* was induced in copper restricted conditions due to direct regulatory binding of CaMac1p to its promoter region. It also gave us functional evidence for the previously described *CaMAC1* auto-regulation, as the recombinant CaMac1 protein also bound to the *CaMAC1* promoter in copper restrictive conditions. Interestingly a second retarded fragment indicative of CaMac1p binding to *CaFRE7* promoter, was not observed on the EMSA suggesting that CaMac1p does not directly bind *CaFRE7* promoter. The gel-shift data indicated that it was plausible that an intermediate transcription factor was involved in the regulation of *CaFRE7* via *CaMAC1* or CaMac1p co-regulated *CaFRE7* by employing another regulatory protein and by forming a complex with it. It is also conceivable that CaMac1p regulates *CaFRE7* in a post-translational manner and hence it does not directly bind to *CaFRE7* promoter.

An alternate reasoning for these contrary results could be that the purified CaMac1 protein was of very low concentration and hence the gel-shift was unable to detect the very low level of CaMac1p binding to *CaFRE7* promoter-probe. A number of different techniques and columns were used but CaMac1p continued to show extremely low solubility and the protein yield could not be increased. Therefore the small concentration of functional protein that could be obtained- was used for the gel-shift study in this chapter.

The iron acquisition proteins of *C. albicans* are generally responsive to iron limitation and hence they are only expressed during iron restriction when efficient iron uptake is required. However, this is quite a simplified view as several different pathways can be

employed by the yeast to obtain iron from the host, with some of these intrinsically linked with copper homeostasis. Several transcription factors have been implicated in the iron responsive regulation of these systems including; Aft1p, Rim101p, CBF and Sfu1p.

As mentioned previously in this report, *C. albicans* lacks functional homologues of Aft1p and Aft2p that are the major iron-related gene regulators in *S. cerevisiae*. *Caaft1Δ/Caaft1Δ* mutants displayed phenotypes that were indistinguishable from the wild-type in all of the growth tests performed which suggested that CaAft1p did not perform an identical function in *C. albicans*; to ScAft1p in *S. cerevisiae*. (Mason, 2006b).

There have been reports of numerous iron-responsive proteins being negatively regulated by *SFU1*which is a GATA-type transcriptional repressor (Lan CY *et al.*, 2004) but to date, much remains unknown about *C. albicans* iron-acquisition activation. It was unclear as to what precisely controls the genes involved in high affinity iron uptake in *C. albicans* since Sfu1p has not been able to solely explain iron uptake regulation entirely and there were indications of existence of another regulator.

Recent reports identified the putative transcription factor *SEF1* which was predicted to represent a positive regulator of *C. albicans* iron acquisition (Homann OR *et al.*, 2009) and displayed presence of DNA binding Zinc-finger domain. *sef1Δ/sef1Δ* displayed growth reduction on YEPD with either low iron or alkaline pH when compared to wild type which suggested a role of Sef1p in iron homeostasis. (Lan CY *et al.*, 2004, Homann OR *et al.*, 2009). To gain a better understanding of the role played by *SEF1* in iron acquisition we carried out phenotypic, growth studies and also investigated if

known high affinity iron uptake genes are regulated by *SEF1* using the *sef1ΔΔ* mutant in Chapter 4.

The *sef1Δ/sef1Δ* mutant was tested for its response to serum which showed us that the *sef1ΔΔ* mutant portrayed a defect in detection of serum and was incapable of switching from the yeast form into its hyphal form. When the mutant was tested for growth in the presence of haemoglobin as the sole source of iron, it was also found to be defective in haem-iron utilisation. These results indicated a defect in regulation of Rbt5 which has been proposed to be involved in haem-iron utilisation and triggering hyphal differentiation in *C. albicans* (Pendrak & Roberts, 2007). The expression of *RBT5* was later analysed in the mutant, using RT-PCR and was found to be affected as predicted.

The *sef1Δ/sef1Δ* mutant was tested for its sensitivity to oxidative stress and it increased sensitivity to hydrogen peroxide which indicated a defect in the enzymes that contain [4Fe-4S] clusters (De Freitas *et al.*, 2003). The *sef1ΔΔ* mutant did not display any respiratory defects and was able to consume glycerol and glucose as sole sources of carbon.

The *sef1Δ/sef1Δ* mutant was also tested for its response to varying pH and it showed reduced growth at pH 10.5, indicating a growth defect in alkaline conditions that suggested a defect in *FRP1*expression, which is an alkaline-induced putative ferric reductase (Liang *et al.*, 2009). This was later reconfirmed using RT-PCR to carry out expression analysis on *FRP1* in *sef1ΔΔ*.

The expression of *SEF1* was analysed in various iron and copper conditions in the reference strains by means of RT-PCR and the expression of *SEF1* was found to increase in iron restrictive conditions which signified a role of *SEF1* in iron acquisition and utilisation in *C. albicans*. The expression of various genes involved in iron and

copper homeostasis were analysed in the *sef1ΔΔ* mutant in various iron and copper conditions and a reduction in expression of *CaFRE1*, *CaFRE2*, CaFRE10*, CaFRP1*, *CaFTR2*, *CaFET34* and *CaRBT5* was evidenced.

Interestingly, a reduction in expression was also seen for *CaFRE7* and *CaCTR1* which have been previously proposed to be regulated by Mac1p (Marvin *et al.*, 2004, Jeeves RE *et al.*, 2011, Woodacre *et al.*, 2008). The mutant was found to be capable of detecting change in copper levels and increasing expression of *CaCTR1* in response to copper restriction however the rate of transcription of *CaCTR1* was affected due to absence of *SEF1*. Initially during the phenotypic assays, the *sef1ΔΔ* mutant was also found to survive better than the reference strains in very high copper conditions. This indicated a novel regulatory interaction between Sef1p and CaMac1p. The mutant was able to survive in very high copper conditions perhaps because the amount of copper being transported across the cell membrane, in the mutant, was much lesser than the amount of copper being transported within the cell, in the reference strains. It also indicated that either *SEF1* was playing a direct role in regulation of the copper transporter *CaCTR1* or it was affecting its expression indirectly by interacting with the major copper-responsive regulator CaMac1p. This was looked at in Chapter 5 and is discussed further.

The cell surface associated ferric and cupric reductase activity as well as the iron uptake capacity of the *sef1ΔΔ* mutant was defective. This finding indicated a defect in iron as well as copper metabolism in *sef1ΔΔ* mutant which depicts the significance of Sef1p, in virulence causing ability of the organism. The findings in this chapter re-confirmed some of the findings of other microarray studies carried out on *sef1ΔΔ* mutants obtained from *in vivo* mouse models (Chen C *et al.*, 2011, Vandeputte P *et al.*, 2011) however, it also suggested a novel role of *SEF1* in regulation of copper

responsive genes and proposed a co-association of Sef1p with Mac1p in the regulation of some genes like *CaFRE7* and *CaCTR1,* which has not been previously reported.

The unique finding in this chapter was the indication of involvement of *SEF1* in copper homeostasis and that it may regulate a number of genes indirectly, in association with other regulators such as Sfu1p and specifically Mac1p. Reports from previous work carried out in our laboratory also proposed that *SFU1* was also self-regulated and the regulation of the two transcription factors *SFU1* and *MAC1* was intimately linked (Jeeves RE *et al.*, 2011, Woodacre, 2007, Woodacre *et al.*, 2008). This interaction was looked at in Chapter 5 to obtain a clear understanding of how CaMac1p, Sfu1p and Sef1p are inter-related and work together to co-regulate iron and copper homeostasis in *C. albicans*.

Using EMSA experiments, Mac1p was found to bind to the CuREs present upstream in *SFU1*-promoter region in response to high copper conditions. Additionally, on RT-PCR analysis, the overall expression of *SFU1* was detected to be higher in *Camac1Δ/Camac1Δ* and a reduction in expression was also observed in low copper conditions in *Camac1Δ/Camac1Δ*. These results indicated that CaMac1p was portraying a novel regulatory pattern as a repressor in addition to its well-studied activator role. It is likely to be supressing expression of *SFU1* by binding to it in high copper conditions.

Through RT-PCR analysis, the expression of *SEF1* was found to be much higher in *sfu1Δ/sfu1Δ* and higher expression of SFU1was detected in high iron conditions. These results, in addition to the previous data implicating it as a GATA-type transcriptional repressor (Lan CY *et al.*, 2004), indicated that Sfu1p was likely to be repressing expression of *SEF1* in high iron conditions. This was consistent with recent findings in

another study that was conducted to identify role of Sfu1p and Sef1p in virulence and commensalism (Chen C *et al.*, 2011).

Interestingly, the expression of *SEF1* was observed to be much lower in *mac1Δ/mac1Δ* mutant and copper sensitive expression was lost in this mutant which indicated that CaMac1p positively interacts with *SEF1* (directly or indirectly) in copper restricted conditions and leads to enhancement of its transcription in response to low copper. However, during our gel-shift assay, we did not observe CaMac1p binding to *SEF1-* promoter in the conditions tested and on analysis of the upstream region of the gene, no CuRE sites were detected which indicated that CaMac1p could be employing a co-activator which would activate expression of *SEF1* or CaMac1p must be regulating it in a novel post-transcriptional manner. In a recent study with Sef1p, it was revealed that a multiple level of post-transcriptional regulation occurs in *C. albicans* such as cytoplasmic localization of proteins, nuclear localization of protein, differential protein stability or phosphorylation of proteins (Chen & Noble, 2012).

Centred on all the data gathered, an alternative explanation for this result is- an indirect effect of absence of CaMac1p in *Camac1Δ/Camac1Δ* mutant which would lead to de-repression of *SFU1* which also explains the decrease in expression of *SEF1*, as a consequence of suppression by Sfu1p, in iron and copper replete conditions, in *Camac1ΔΔ* mutant.

On analysis of regulation of *MAC1* by Sfu1p and Sef1p, it was observed that the overall expression of *CaMAC1* was higher in *sfu1ΔΔ* mutant which indicated that Sfu1p was interacting with *CaMAC1* directly or indirectly to regulate its expression in some manner, in response to iron.

A plausible reasoning for these results is- the indirect activation of *CaMAC1* in *sfu1Δ/sfu1Δ* due to secondary copper starvation conditions being generated within the cell. In s*fu1Δ/sfu1Δ* mutants an increase in iron uptake would lead to an increase in activity of muti-copper oxidase, resulting in secondary intracellular copper starvation conditions which would ultimately be counteracted by increase in expression of *CaMAC1*, to allow increased copper uptake by means of *CaCTR1* (Marvin *et al.*, 2004).

Interestingly, in the *sef1Δ/sef1Δ* mutant the increase in expression of *CaMAC1* in response to low copper was comparatively lesser, while no change in response to iron limitation was detected. This indicated that Sef1p was perhaps functioning along-with Mac1p to co-regulate the expression of *CaMAC1* itself. This co-regulation explains the previous results where a higher level of basal *CaMAC1* promoter activity was detected in a *Camac1ΔΔ* mutant, in another study in our laboratory (Woodacre *et al*., 2008).

From all the evidence gathered during this research project (Chapter 3), we can summarise that in presence of copper, the lack of intramolecular interactions allows improved exposure of the C2 domain in C-terminus which allows the protein to undergo greater protein-protein interactions via the C-terminus. This allows it to form homodimers, which are the transcriptional inactive forms in *C. albicans*. This protein-protein contact is different from the one observed in *S. cerevisiae* in absence of copper and leads to folding of the proteins which consecutively conceals the DNA binding and activation domains, leading to lack of transcription of *CaMAC1* and target genes via CaMac1p.

It was determined that the C-terminus domain of CaMac1p is majorly involved in protein - protein interactions in *C. albicans* and that CaMac1p is capable of binding to itself and undergoing self-interaction but even though it was the C-terminus that was

involved in protein - protein interactions, the N-terminus domain of CaMac1p was likely aiding to increase binding activity of C-terminus domain which was different than *S. cerevisiae*. Using gel-shift assays CaMac1p was also demonstrated to bind to its own promoter which confirmed the hypothesis that *CaMAC1* is self-regulated and the presence of one CuRE site allows for optimal activation since the sites are not synergistically associated unlike *S. cerevisiae*.

In conclusion, from the data gathered in this research study, through Chapters 3-5, the following model is proposed, for iron and copper-related genes repression/activation in *C. albicans* - Sfu1p which is a GATA-type transcriptional repressor, represses iron acquisition genes in response to high iron conditions and simultaneously also represses the positive regulator *SEF1* while the transcriptional activator Mac1p appears to be functioning in a manner different from its traditional role as a positive regulator. It is possible that CaMac1p acts as repressor and regulates *SFU1* in a copper responsive manner. Sef1p was also likely to be functioning along-with Mac1p to co-regulate the expression of *CaMAC1* itself. In low-iron conditions, Sfu1p may not be able bind to DNA, or may bind to the DNA with a low affinity and as a result the target genes would no longer be repressed. Hence in low iron limited conditions *SEF1* would be expressed which would then act as transcriptional activator of iron uptake genes (which are not bound and suppressed by Sfu1p) as well as *CaMAC1*, consequently leading to increase in transcription and expression of iron and copper acquisition genes (Figure 5.8 and 6.1).

This study has made it abundantly clear that these regulators are interdependent and their co-regulation is part of a complex circuit. They play a role in regulating each other's transcription and in turn co-ordinate, to regulate iron and copper acquisition, as well as to prevent development of toxicity within the cell. This affirms that *C. albicans*

portrays a mechanism referred to as the 'Feed forward loop' where one transcription factor regulates the expression of another and then together they regulate the expression of a common target (Chen C *et al.*, 2011, Mangan & Alon, 2003).

Genome-wide microarray studies that were recently carried out in *sfu1ΔΔ, sef1ΔΔ* mutants *in vivo*, in mouse models, defined an altered role played by Sfu1p and Sef1p transcription factors in commensalism and in pathogenesis (Chen C *et al.*, 2011, Chen & Noble, 2012, Cheng *et al.*, 2013). This added another dimension to the complex relationship between these transcription factors which is consistent with the findings of our research. In the study by Chen *et. al.*, in *C. albicans*, the iron homeostasis regulatory system was studied in two disparate environments in the human host – the gastrointestinal tract (gut) and the blood stream and they interestingly found that Sef1p intercalates itself between the two iron-responsive repressors Hap43p and Sfu1p. Sef1p was found to activate iron-uptake genes and was indispensable for virulence in bloodstream infections in mouse models, however, Sfu1p was found to repress iron-uptake genes, was not essential for virulence but supported commensalism in the gastrointestinal tract. This finding proposed to allow the organism to play a reciprocal role which allows it to survive in the high iron environment in the gut versus the low iron environment in the blood stream and thereby allowing it to switch between its commensal and pathogenic roles (Chen C *et al.*, 2011).

Our *in vitro* analysis data gathered about Sef1p and Sfu1p (Chapter 4 and 5), supports the evidence gathered by the Chen *et. al.* group and is consistent with the theory that Sef1p acts as a positive regulator of iron uptake genes in *C. albicans*. However, in the Chen *et. al.* study, they did not look at the inter-relation of the iron homeostasis regulatory circuit with the copper homeostasis regulatory circuit. Therefore, our study
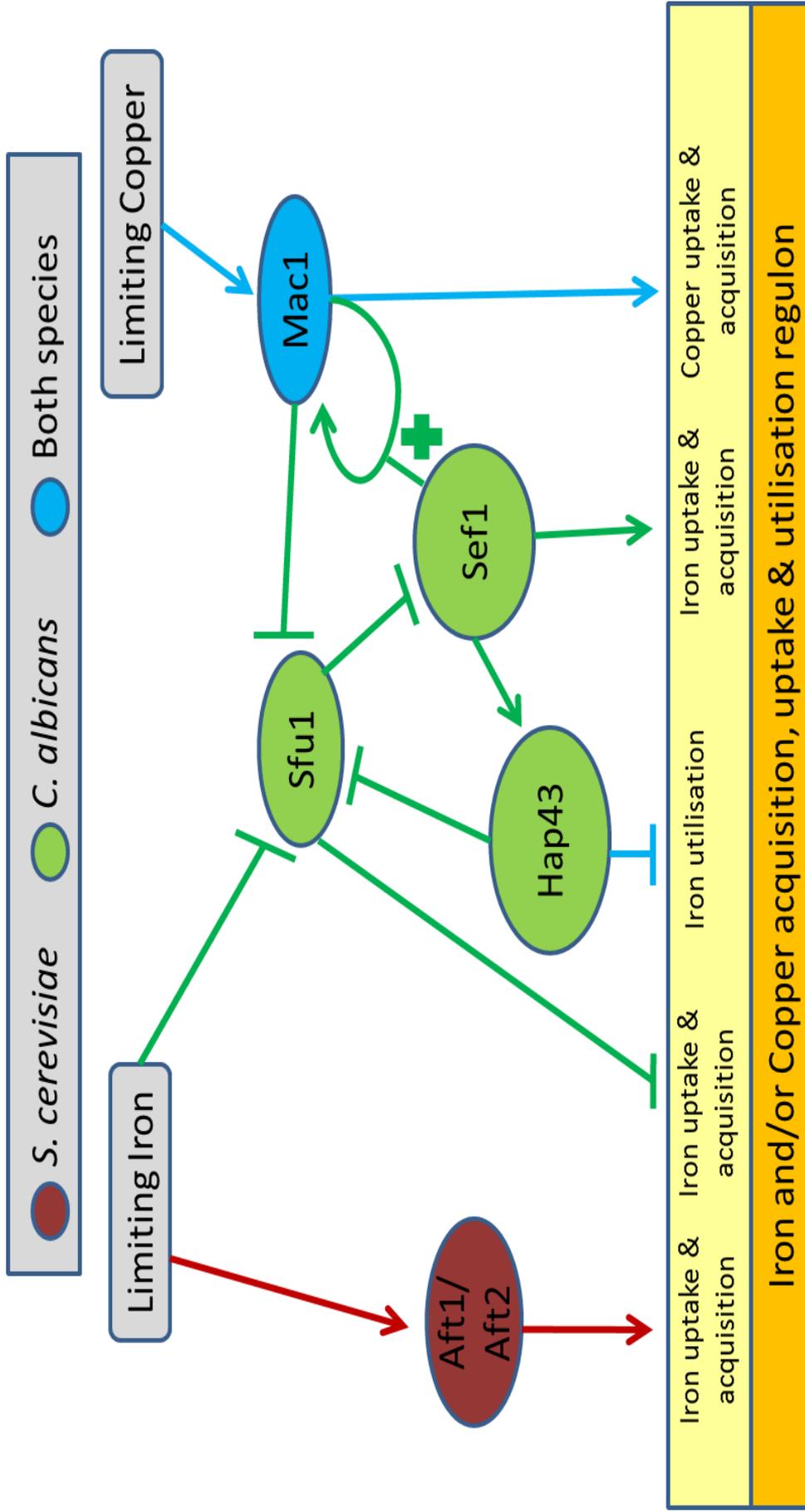
Figure 6. 1: Diagrammatic representation defining the iron and copper homeostasis regulatory circuit in *Saccharomyces cerevisiae* and *Candida albicans*.

This diagram highlights the similarities and differences in between the iron and copper homeostasis system's regulatory pattern, gathering all the information available from this research study as well as other studies, performed in these two organisms. Arrows denote activation of transcription while bars denote suppressions of transcription. The colour red represent the events that occur in *S. cerevisiae* only, green represents the events that occur in *C. albicans* only uwhile blue represents the events that occur in both organisms. These transcription factors regulate the expression of their own genes as well as play a role in regulation of each other's genes in response to iron and copper in the environemt; and hence are interdependent.

adds another dimension to this developing transcriptional circuit by showing the interdependent role played by Mac1p in association with Sef1p and Sfu1p (Figure 6.1).

Further studies involving *sfu1ΔΔ/sef1ΔΔ* homozygous mutants or *sef1ΔΔ/mac1ΔΔ* homozygous mutants need to be carried out and these mutants need to be tested for their ferric iron uptake capability, as well as their cell surface ferric and cupric reductase competence. This would help shed more light on the particular role played by each of these regulatory proteins however due to time constraints it was not achieved during this project. The results in this chapter have given us a potential hypothesis that links these regulators with each other intricately in a novel manner as part of a regulatory circuit (Figure 6.1).

In the future, it would also be interesting to test if the wild-type CaMac1 protein from *C. albicans* can interact and bind with the Mac1 protein from *S. cerevisiae*. If CaMac1p shows binding with Mac1p then it would indicate that the binding domains in the two

homologous proteins are extremely identical and hence the *S. cerevisiae* genomic library (which is easily available) could be used to screen for additional proteins that CaMac1p can interact with. This would shed more light on inter-species protein interactions and provide information on the evolutionary differentiation of Mac1p. It would also lead to identification of additional homologous proteins in the CaMac1p regulon and thus help in increasing our understanding of how CaMac1p regulates the iron and copper homeostasis. However, similar results could be obtained by carrying out genome-wide microarray studies in *Camac1Δ/Camac1* mutants, but this would prove quite expensive.

It would also be interesting to study the localization of all the 17 different putative ferric reductases, found in *C. albicans,* using fluorescence microscopy. Their

localization to different cellular regions in response to various regulators and various conditions would explain the distinct role of each regulator in cytoplasmic, nuclear and vacuolar systems and help distinguish between them.

Finally, due to time constraints, we were unable to test *in vivo*, whether the proposed theory that- the CaMac1p monomer is indeed the transcriptionally active unit while the CaMac1p-CaMac1p homodimer is the transcription hindrance unit. Further studies are being carried out to integrate the truncated CaMac1p (lacking its C2 domain), into a *mac1ΔΔ* mutant. This complementation would allow the organism to produce truncated CaMac1p monomers but would prevent it from producing CaMac1p-CaMac1p homodimers (since it lacks the dimerisation C2 domain). The truncated CaMac1p (lacking C2 domain) would have to be previously fused to another highly efficient activation unit such as VP16 to ensure it is transcriptionally active, however, unable to undergo protein interactions. This complementation would be tested to see whether in the complement, CaMac1p is transcriptionally active and whether the complementation is able to rescue the copper-responsive phenotypes of the original *mac1ΔΔ* mutant. Positive results from this experiment would help prove our hypothesis.

Alternatively, GFP complementation studies could be carried out via which, the homodimer could be tracked *in vivo*. This would allow us to map the route charted by the homodimer in response to copper and would indicate whether it played or did not play a role in transcriptional activation of CaMac1p target genes (Prof. Pete Sudbery, personal communication).

S. cerevisiae has been found to maintain a Sef1p orthologue which shares amino acid sequence similarities with the Sef1p in *C. albicans* however it plays a different role and does not function as a regulator of iron homeostasis. That role is played by the Aft

family of transcription factors in *S. cerevisiae* (Groom *et al.*, 1998). The role played by *SEF1* in *C. albicans* is unique and unlike its role in *S. cerevisiae* and hence points towards an acclimatisation mechanism in the opportunistic pathogen which helps it to persist and proliferate in the human host system.

In conclusion, the work in this thesis demonstrates that in spite of there being sequence similarities between proteins in *S. cerevisiae* and *C. albicans*, their mechanism of action and functioning differs consistently *in vivo*, within the two organisms. The two yeasts also show vast variances in the regulation of their metal acquisition systems. The data presented helps us differentiate between the copper and iron homeostasis systems in *C. albicans* from similar system in the model yeast- *S. cerevisiae*. These dissimilarities possibly enable *C. albicans* to respond more precisely to environmental changes (such as changes in copper or iron levels), conferring a quicker adaption to the human host environment, that leads to an advantage in the disease-causing process.

In all organisms, strict control and regulation of iron and copper is essential as free cellular ions can be toxic for cells. For pathogens, these homeostasis systems are even more important as iron is highly restricted *in vivo* host environment and is often the growth limiting factor. Copper is also restrictive in the host but can become elevated during inflammation. Also, iron restriction is used by many pathogens as a signal that they have entered a host and can express virulence factors. Therefore the study of a pathogens iron and copper homeostasis system and their regulation increases our understanding of the infection progress and their response to the *in vivo* environment. Through this study, by gaining an understanding of the molecular mechanism of action of these regulatory proteins, we increase our understanding of the precise role of iron and copper acquisition in the virulence of *C. albicans* and other pathogenic fungi.

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