The Role of Redox-Sensitive Antioxidants in Oxidative Stress Signalling in *Candida albicans*

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Thesis submitted in accordance with the regulations of Newcastle University for the degree of Doctorate of Philosophy

September 2010

ABSTRACT

Candida albicans is the major systemic fungal pathogen of humans. Consequently, much effort is directed at understanding how this medically relevant fungus evades the antimicrobial mechanisms mounted by the host's immune system. An important defence mechanism employed by phagocytic cells involves the generation of highly toxic reactive oxygen species (ROS). However, although the ability of *C. albicans* to sense and respond to ROS is essential for virulence, the intracellular signalling mechanisms underlying such responses are poorly understood. *C. albicans* mounts a robust oxidative stress response when exposed to H_2O_2 *in vitro* or following phagocytosis, which involves a significant remodelling of the transcriptome and proteome. This is regulated by the Cap1 AP-1 like transcription factor and, to a lesser extent, the Hog1 stress activated protein kinase (SAPK). In addition, exposure of *C. albicans* to H_2O_2 stimulates filamentous growth. However, despite strong relationships between oxidative stress responses, filamentous growth, and virulence, very little is known about the intracellular signalling mechanisms that regulate H_2O_2-responsive signalling pathways in *C. albicans*.

Redox sensitive antioxidant proteins, such as 2-Cys peroxiredoxins and thioredoxins, have recently been demonstrated in model yeasts and mammalian cells to have additional oxidative stress signalling functions. Hence, the aim of this thesis was to investigate the potential roles of thioredoxins and 2-Cys peroxiredoxins in oxidative stress sensing and signalling in *C. albicans*. Thioredoxins are conserved oxidoreductases which regulate the catalytic reduction of diverse proteins including 2-Cys peroxiredoxins which become oxidized upon reducing H_2O_2. Significantly, data is presented which illustrates that Trx1 is the major thioredoxin protein in *C. albicans* and, in addition to functioning as an antioxidant, plays a central role in oxidative stress signalling by regulating distinct pathways. For example, Trx1 is required for the H_2O_2–induced activation of the Hog1 SAPK, as either deletion of *TRX1* or mutation of the catalytic cysteine residues of Trx1 significantly impairs H_2O_2-induced phosphorylation of Hog1. Notably, the sole 2-Cys peroxiredoxin in *C. albicans*, Tsa1, also positively regulates H_2O_2-induced Hog1 activation in *C. albicans*. As Tsa1 is a major substrate of Trx1, this is consistent with a model in which Trx1 regulates Hog1 through regulating the redox status of Tsa1. Evidence is also presented that Trx1 negatively regulates H_2O_2-induced filamentation in a mechanism that is independent of Tsa1 and instead

involves activation of the Rad53 DNA checkpoint kinase. For example, either inactivation of *TRX1*, or treatment of cells with H_2O_2, results in significant hyperphosphorylation of Rad53 and the formation of hyperpolarized buds. In addition, deleting *RAD53* completely abolishes H_2O_2-induced filamentous growth. These findings are consistent with a model in which oxidation and thus inactivation of Trx1 in response to H_2O_2 is a key step in stimulating Rad53 activation and hyperpolarized bud growth. In agreement with its key roles in responses to ROS, cells lacking Trx1 display attenuated virulence in both a macrophage killing model and a murine model of *C. albicans* systemic infection. This may be dependent on its signalling rather than antioxidant functions, as a previous study reported that inactivation of Tsa1 does not attenuate virulence. Collectively, the data presented in this thesis indicates that both Tsa1 and Trx1 have important roles in H_2O_2 signalling and that Trx1 promotes *C. albicans* survival in the host.

DECLARATION

I certify that this thesis contains my own work, except where acknowledged, and that no part of this material has been previously submitted for a degree or any other qualification at this or any other university.

Some of the work in this thesis has been presented in the following publication:

da Silva Dantas, A., Patterson, M.J., Smith, D.A., Maccallum, D.M., Erwig, L.P., Morgan, B.A. and Quinn, J., 2010, Thioredoxin regulates multiple hydrogen peroxide-induced signalling pathways in *Candida albicans*. Mol. Cell Biol., 30 (19), 4550-4563.

ACKNOWLEDGEMENTS

First I would like to thank to my supervisors: Dr. Janet Quinn and Prof. Brian Morgan. Thanks Jan for all the support, constant encouragement and guidance. It`s been a fantastic experience working for you! I would also like to thank Brian Morgan for all his support and advice.

I also want to thank the past and present members of of the Veal (EV), Morgan (BAM), and Whitehall (SKW) labs for all the support and help and for creating such a great working environment. A special thanks to the JQ lab (past and present) for all the help in the lab and for putting up with the Brazilian mood swings. I would like to acknowledge the ORSAS and NIPS Awards and the Institute for Cell and Molecular Biosciences at the Newcastle University for funding this thesis.

A special thanks to my dear friends Alex, Ana, Aza, Erica, Graciela, Helio, Holly, Joana, Kathryn, Marcio, Monika and Renatha for being there during my several existential crises and for reminding me that there is a life outside the lab! Thanks for everything!

Finally I would like to thank my friends and family in Brazil, especially my mother Sonia and my sister Luana, for all the advice, patience and support! Eu amo voces.

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ABBREVIATIONS

1-Cys Prx	1-Cysteine Peroxiredoxin
$^1O_2$	Singlet oxygen
2-Cys Prx	2-Cysteine Peroxiredoxin
A	Adenosine
ADP	Adenosine diphosphate
AhpC	Alkyl hydroperoxide reductase C
AMS	4-acetamido-4'- ((iodoacetyl)amino)stilbene-2,2'-disulfonic acid
AP-1	Activating Protein 1
ARE	Antioxidant responsive elements
As^{3+}	Arsenic
ASK-1	Apoptosis signal-regulating kinase
ATF2	Activating transcription factor 2
ATP	Adenosine triphosphate
BD	Binding domain
bZip	Basic leucine zipper
C. albicans	*Candida albicans*
C. elegans	*Caenorhabditis elegans*
cAMP	Cyclic adenosine monophosphate
Cd^{2+}	Cadmium ion
cDNA	Complementary DNA
CGD	Candida Genome Database
CNC	Cap 'n' collar
C^P	Peroxidatic cysteine
C^R	Resolving cysteine
CRD	Cysteine rich domain
CREB	cAMP response element binding protein
Cu	Copper
Cys	Cysteine
Cys-Se	Selenocysteine
Cys-SH	Reduced cysteine
Cys-SO_2H	Sulphinic acid
Cys-SO_3H	Sulphonic acid
Cys-SOH	Sulphenic acid
DAPI	4',6-diamidino-2-phenylindole
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate

DEM	Diethyl maleate
dGTP	Deoxyguanosine triphosphate
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dNDP	Deoxyribonucleotide diphosphate
DTT	DL-Dithiothreitol
dUTP	Deoxyuridine triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediamine-tetraacetic acid
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
EST	Expressed Sequence Tag
FOXA	Forkhead box subclass A
FOXO	Forkhead box subclass O
g	gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GGLG	Gly-Gly-Leu-Gly
Glu	Glutamine
Gly	Glycine
Gpx	Glutathione peroxidase
Gr	Glutathione reductase
Grx/Glr	Glutaredoxin
GS	Glutathione synthetase
GSH	Reduced glutathione
GSSG	Oxidised glutathione dimer
GST	Glutathione *S*-transferase
H	Hydrogen
H_2O	Water
H_2O_2	Hydrogen peroxide
HIF-1α	Hypoxia-inducible factor 1α
HMW	High molecular weight
HSF	Heat shock transcription factor
HSP	Heat shock protein
IGF-1	Insulin-like growth factor 1
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JNK	Jun N-terminal kinase
kDa	kDalton

l	liter
LB	Luria broth
LMW	Low molecular weight
LOO.	Peroxyl radical
M	Molar
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MeSOX	Methionine sulphoxide
min	Minute(s)
mM	Millimolar
mRNA	Messenger ribonucleic acid
mtDNA	mitochondrial DNA
MW	Molecular weight
N_2O	Nitrite
NADPH	Nicotinamide adening dinucleotide
NES	Nuclear export signal
NF-κB	Nuclear Factor κB
NO	Nitric oxide
NO-	Nitroxyl anion
$NO^{\cdot}$	Nitric acid
NO_2^-	Nitrous oxide
Nrf	Nuclear factor
O_2	Oxygen
$O_2^{\cdot -}$	Superoxide anion radical
$OH^{\cdot}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
orf	open reading frame
p53	Tumour suppressor protein
PDGF	Platelet-derived growth factor
PHGpx	phospholipid hydroperoxide glutathione peroxidase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
p*K*a	Acid dissociation constant
Prx	Peroxiredoxins
PTEN	Phosphatase and tensin homolog
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
RNA	Ribonucleic acid
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
S-S	disulphide bond
S. cerevisiaea	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SAPK	Stress-activated protein kinase
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Ser	Serine
SOD	Superoxide dismutase
Srx	Sulphiredoxin
SUMO	Small Ubiquitin-related Modifier
T	Thymidine
t-BOOH	*tert*-butyl hydroperoxide
TCA	Trichloroacetic acid
Thr	Threonine
TNF-α	Tumour necrosis factor alpha
Tpx	Thiol peroxidase
TRANK	Thioredoxin peroxidase–related activator of NF-κb and JNK
Trr	Thioredoxin reductase
Trx	Thioredoxin
TXNIP	Thioredoxin interacting protein
UV	Ultraviolet
YF	Tyr-Phe
Zn	Zinc
µl	Microliter
µM	Micromolar

Chapter 1. Introduction

1.1. *Candida* species and Candidaemia

Candidaemia is a systemic infection caused by *Candida* species and is associated with the presence of *Candida* yeast cells in the blood, with or without visceral involvement (Odds, 1989). Candidaemia is associated with a high mortality rate of patients that acquire this infection (15-49%), which can increase up to 60% in patients that are in Intensive Care Units (ICU) (Lass-Flörl, 2009 and Rueping *et al.*, 2009). This high incidence of mortality is related to the fact that candidaemia is normally associated with critically ill patients, such as those that have a malignancy, transplant, AIDS, prolonged ICU and hospital stay, neutropenia, or on long term treatments of antibiotics or corticosteroids, or that are subject to the administration of parenteral alimentation. *Candida* species are the fourth most common hospital acquired bloodstream infection (BSI) agent in the United States, also having a high incidence rate in Europe (Pfaller and Diekema, 2007, Richardson and Lass-Flörl, 2008 and Rueping *et al.*, 2009). Although more than 200 *Candida* spp have been catalogued, five species account for approximately 90% of all cases of Candidaemia. The five species are *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis,* and *C. krusei*, of which *C. albicans* accounts for 60% of the cases (Rueping *et al.*, 2009 and Richardson and Lass-Flörl, 2008).

Even though *C. albicans* is the main species found in candidaemia patients, epidemiological studies have shown increased occurrence of non-*C. albicans* systemic infections (Pfaller *et al.*, 2010). The increase number of non-*C. albicans* systemic infections is related to a high tolerance of these species to antifungals (Pfaller and Diekema, 2007). *C. glabrata* and *C. krusei*, for example, are resistant to azoles and other common used antifungals (Pfaller *et al.*, 2009 and Pfaller and Diekema, 2010).

1.1.1. Candida albicans

C. albicans is the most common systemic fungal pathogen in humans, and this high incidence is because this opportunistic pathogen exists as a benign commensal organism in the microflora of the majority of the world's population. The most frequently colonised location in the body is the oral cavity; however *C. albicans* can also colonize the skin and

nails, gastointestinal tract, anus and groin of healthy individuals and also the vaginal canal of healthy women (Odds, 1989).

As an opportunistic pathogen, *C. albicans* can in certain cases cause superficial infections. The most common cases of superficial candidiasis are seen in diabetic individuals, during pregnancy, infancy or old age, and in individuals that use dentures or have occlusive dressings (Hay, 1999). These superficial infections, also called thrush, are extremely common, occurring in the skin and wounds, urogenital tract and vagina (Figure 1.1A and B). For example, 1 in 10 hospital patients will suffer from a superficial *C. albicans* infection, which rises to 80% in HIV infected patients. In addition, 3 out of 4 women will experience an episode of thrush in their lives and of these 20% will suffer from recurrent infections (Ferrer, 2000 and Eschenbach, 2004). As *C. albicans* is the most common *Candida* species isolated from individuals, this likely accounts for the observation that this species is accountable for more than 90% of *Candida*-related superficial infections (Pfaller and Diekema, 2010).

These superficial infections are easily treated and cured in immunocompetent individuals; however if the individual is immunocompromised, systemic candidiasis, can arise (Figure 1.1C-E). Systemic or disseminated candidiasis is characterized by the presence of *Candida* cells throughout the body in organs such as the brain, heart, kidneys, eyes, liver, genital tract and in joints (Odds, 1989). Only when host defences are compromised can these fungal cells disseminate from the gut into the bloodstream. As a consequence of *C. albicans* being the major systemic fungal pathogen of humans, there is much interest in the virulence determinants of this fungus.

1.2. Virulence Determinants of *Candida albicans*

Efficient colonization of the host by *C. albicans* relies on the ability of the fungal cells to; (i) adhere and penetrate mammalian host tissues, (ii) digest complex molecules or cellular structures in order to acquire nutrients, and (iii) protect itself against host defences or other microorganism that might be colonizing the host and invade the mammalian tissues (Figure 1.2). Thus, although many factors are likely to be important for *C. albicans* virulence, the most widely studied include surface adhesion molecules or adhesins, the ability of this fungus to secrete extracellular hydrolytic enzymes and to undergo morphogenetic switching, and also the ability of this fungus to mount robust stress responses. These will be discussed

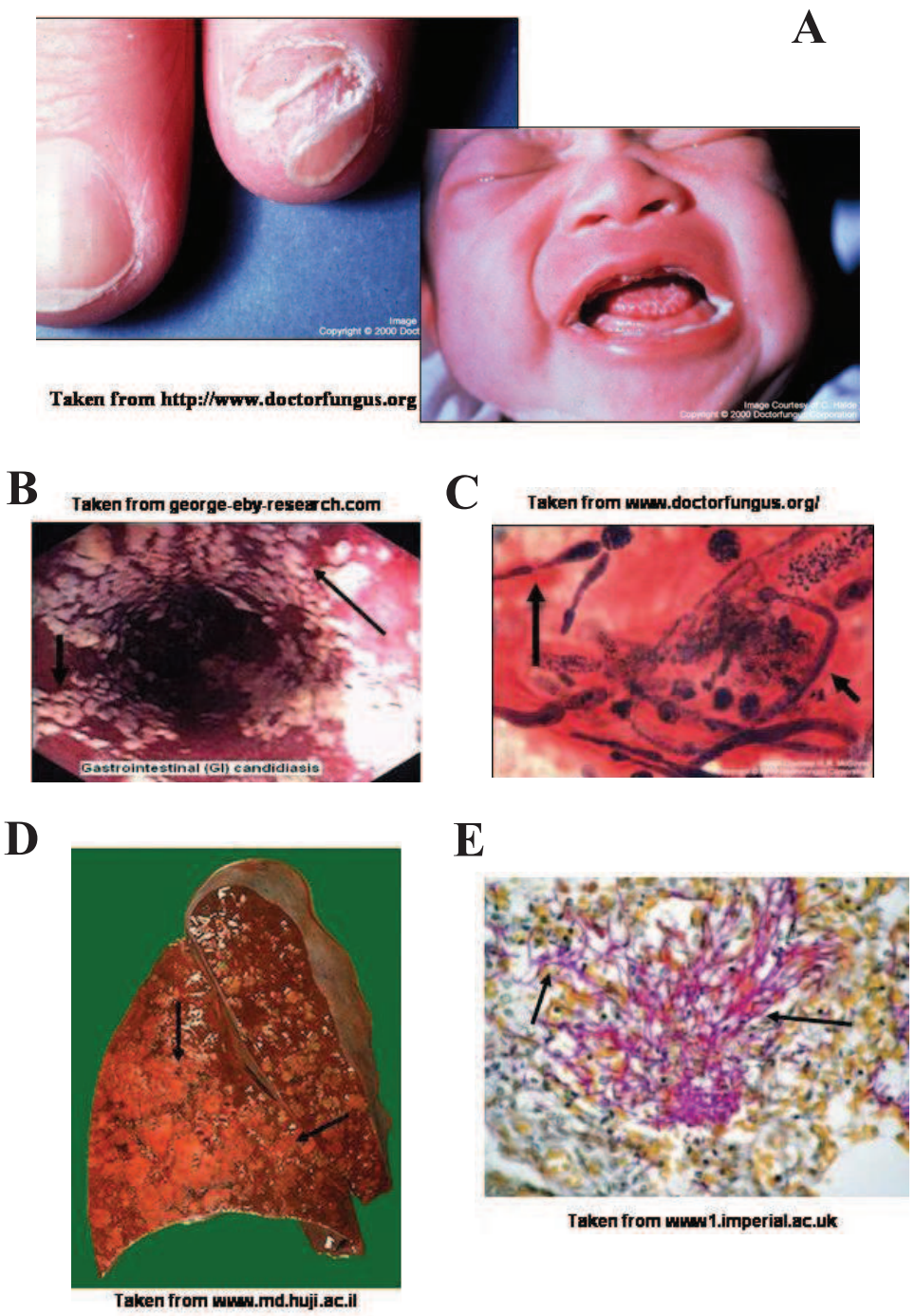


Figure1.1. *C. albicans* superficial and systemic infections.
(A) *C. albicans*-resultant lesions in the nail and mouth (oral candidiasis). (B) *C. albicans* infection in the gastrointestinal tract (C) Microscopic image of *C. albicans* in heart tissue. (D) Systemic candidiasis in the lung. (E) Microscopic image of a bronchus infected with *C. albicans*. Arrows indicate the localization of *C. albicans* in the tissue or organ.

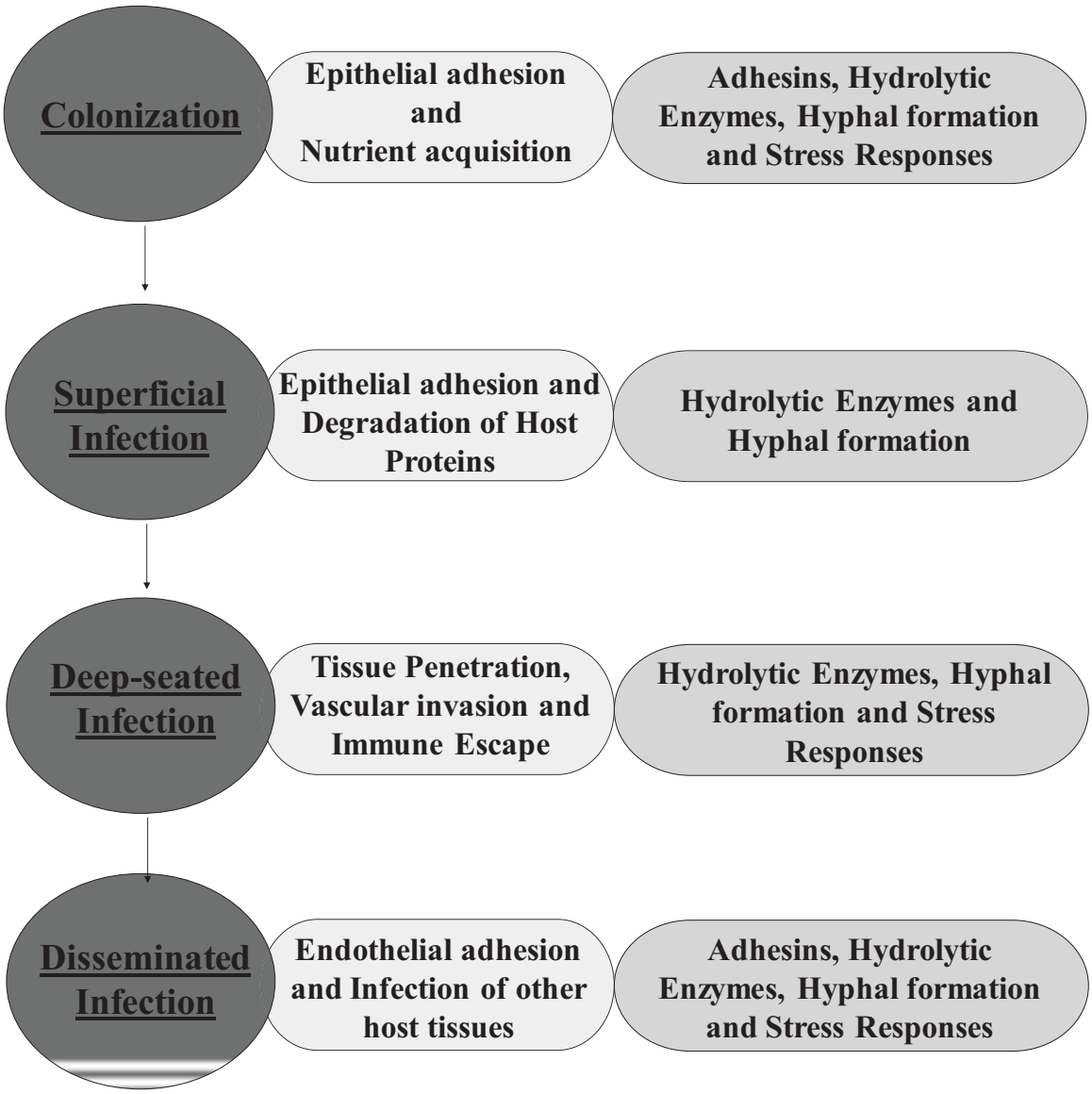


Figure1.2. *C. albicans* infection stages and the virulence determinants associated with them.

Adapted from Naglik *et al.*, 2003

in turn, with particular emphasis on morphogenesis and stress responses as these areas form the basis of the work in this thesis

1.2.1. Adhesins

Adhesion of *C. albicans* cells to host epithelium tissue is the first of many steps that occurs during the infection process. The adherence process is involved in colonization of mucosal surfaces that can later lead to systemic candidiasis or candidaemia (Zhu and Filler, 2010). Thus proteins which promote adhesion of *C. albicans* to host surfaces are important for virulence. These proteins include members of the ALS (agglutinin-like sequence) family which are glycosylphosphatidylinositol (GPI)-anchored cell wall proteins which promote adhesion to both epithelial and endothelial mammalian cells (Hoyer *et al.*, 2008). The two genes most closely related among the *ALS* family, *ALS3* and *ALS1* are up-regulated in both oral and liver *C. albicans* infection models, indicating its role during the infection process (Thewes *et al.*, 2007, Zakikhany *et al.*, 2007, Walker *et al.*, 2009 and Park *et al.*, 2009). Furthermore, deletion of these genes (*ALS1* and *ALS3*) in *C. albicans* results in reduced adhesion and destruction of reconstituted human epithelium tissue (Fu *et al.*, 2002, Kamai *et al.*, 2002, Zhao *et al.*, 2004 and Almeida *et al.*, 2008). Even though all Als proteins have been shown to be important for adhesion and virulence, direct comparison of two null mutants (*als1*Δ and *als3*Δ) in adhesion assays indicates that Als3 is the stronger adhesin (Zhao *et al.*, 2004).

In addition, the GPI-anchored hyphal wall protein (Hwp1), is another adhesin specifically expressed on the surface of *C. albicans* hyphal cells, and plays a role in germ tube adherence to epithelial cells by acting as a substrate for the mammalian transglutaminase enzymes (Staab *et al.*, 1999). The epithelial transglutaminase TGase 1 covalently cross-links the N-terminal region of Hwp1 to host surface proteins, resulting in the adherence of the growing germ tube to the host epithelial cells (Staab *et al.*, 2004 and Ponniah *et al.*, 2007). Deletion of *HWP1* resulted in reduced injury to endothelial cells and impaired virulence in the mouse model (Tsuchimori *et al.*, 2000). The reduced adherence to epithelial and endothelial cells, and the reduced virulence of *C. albicans* cells lacking adhesins, indicates that this class of proteins is involved in colonization, initiation and maintenance of *C. albicans* infection in the host.

1.2.2. Secreted Hydrolases

The ability of *C. albicans* to secrete enzymes that allows for nutrient acquisition and tissue invasion is a well established virulence determinant. The secreted aspartyl proteinases (SAP) family in *C. albicans* is composed of 10 distinct Sap proteins (Sap1-10), which contain characteristic motifs of aspartyl proteinases (Magee *et al.*, 1993 and Monod *et al.*, 1994). The members of the *Candida SAP* family have been shown to degrade a wide range of protein substrates that can be found in epithelial cells, macrophages and even components of the clotting cascade (Naglik *et al.*, 2003). Furthermore, different *SAP* genes are differentially expressed during different conditions of growth, such as yeast (*SAP2*) or hyphal inducing conditions (*SAP4-6*) (Hube *et al.*, 1994). Consistent with this, different *SAP* genes are differentially up-regulated during different phases of infection. *SAP1* and *SAP3*, for example, are expressed as soon as the first lesions start to appear in a model for oral candidiasis, and *SAP6* expression coincides with the increase of lesions in the same model and the appearance of the first hyphae (Schaller *et al.*, 2001). Interestingly, however, recent studies using oral human reconstituted epithelia showed no difference in the expression levels of *SAP1-3* during the course of infection, and only *SAP5* was induced after mucosal contact (Naglik *et al.*, 2008). Furthermore, while previous studies have shown that deletion of members of the SAP family (*SAP1-6*) resulted in strains that cause a decrease in epithelial injury, and also reduced virulence, when compared to wild-type cells, in a recent study the triple mutant *sap4-6*Δ showed no epithelial damage, whereas the triple mutant *sap1-3*Δ showed little epithelial damage (Naglik *et al.*, 2008). Therefore, it was proposed that the differences seen between these studies can be explained by the different infection models used and different experimental approaches used. For example, while in a study by Schaller and colleagues (1999) histological analysis was the only method used to measure epithelial damage (Schaller *et al.*, 1999), recent studies have used both histology and lactate dehydrogenase release as markers for epithelial damage (Naglik *et al.*, 2008).

Just like the SAP family, Phospholipase B (PLB) also plays a key role in *C. albicans* invasive process by breaking down phospholipids present in the membrane of the mammalian host cells. These enzymes hydrolyze the ester linkage in glycerophospholipids present in the cell membrane of host cells, and thus promote tissue invasion (Ghannoum, 2000). Deletion of *PLB*1, results in cells that show reduced invasiveness and virulence in a

mouse model of systemic disease, indicating its role in *C. albicans* pathogenesis (Leidich *et al.*, 1998 and Mukherjee *et al.*, 2001).

1.2.3. Morphogenesis

Another characteristic of *C. albicans*, which is important during the infection process, is its ability to undergo morphogenesis. Morphogenesis is the ability of *C. albicans* to switch between budding and filamentous forms (Whiteway and Oberholzer, 2004). The importance of the morphogenetic switch for the full virulence of this fungus is illustrated by the attenuated virulence exhibited by strains that are unable to switch between yeast and hyphal forms (see section 1.2.3.3).

1.2.3.1. C. albicans Morphological forms

The best studied morphological forms of *C. albicans* include budding yeast cells and filamentous forms that are either pseudohyphae or true hyphae (Figure 1.3A). These distinct morphological forms have both common and specific features. The yeast cells are small, oval cells that can originate either pseudohyphae or hyphae (Figure 1.3A and B – Berman and Sudbery, 2002). When yeast cells are budding, the nucleus migrates to the bud neck, where the septum forms between mother and daughter cells. Subsequently, the replicated nucleus divides into two and one is retained in the mother cell and the second moves into the daughter cell (Sudbery, 2001 and Warenda and Konopka, 2001).

C. albicans true hyphae originate from unbudded yeast cells with the formation of a germ tube (Figure 1.3C), that later forms an extremely narrow filament (~ 2.0 μm width) (Figure 1.3D) (Sudbery, 2001). The localization of hyphal septa and nuclear division is different to that seen in yeast and pseudohyphal cells. For example, in contrast to both yeast and pseudohyphae there is no constriction at the neck of the mother cell, and unlike pseudohyphae there is no constriction at septal junctions. The dividing nucleus is transferred to the extending germ tube and is divided across the septum that is located in the germ tube. Subsequently, one nucleus migrates back to the mother cell, while the remaining nucleus moves to the tip of the elongating daughter cell (Sudbery, 2001 and Warenda and Konopka, 2001).

Pseudohyphae also originate from yeast cells. These are highly branched structures that are less uniform in structure than hyphae and can in fact either physically resemble hyphal

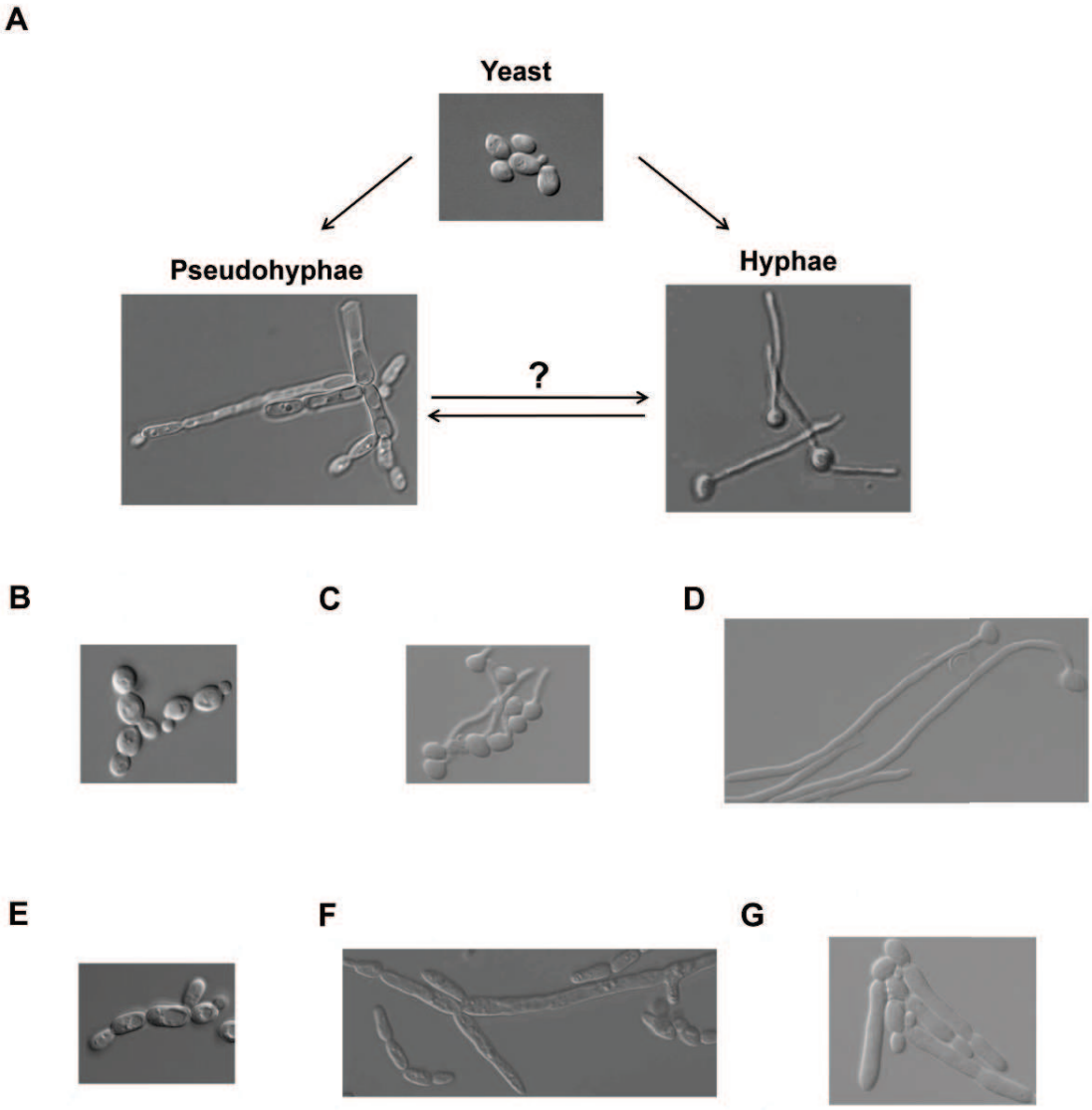


Figure1.3. *C. albicans* morphological forms.
(A) Yeast cells can either form pseudohyphae or hyphae. Microscopic images of *C. albicans* (B) yeast cells, (C) germ tubes, (D) hyphal cells, (E-F) pseudohyphal cells and (G) hyperpolarized buds.

(Figure 1.3F) or yeast cells (Figure 1.3E) (Merson-Davies and Odds, 1989). Similar to yeast cells, they show a constriction at the septum separating mother and daughter cells and nuclear division is similar to that seen in yeast cells (Figure 1.3E and F). However, unlike the budding yeast, pseudohyphal mother and daughter cells remained attached after the septum is formed and the nuclei divided, giving the branched phenotype characteristic of this type of growth (Figure 1.3E and F) (Sudbery, 2001 and Warenda and Konopka, 2001). In addition to hyphae and pseudohyphae, a third distinct polarised growth form known as hyperpolarised buds has more recently been characterised (Bachewich *et al.*, 2003). This morphology is characterised by nuclear migration from mother to daughter cells similar to hyphae cells, yet has a clear constriction at the septum separating the mother cell from the polarised bud as exhibited by pseudohyphal cells (Figure 1.3G) (Whiteway and Bachewich, 2007).

One unifying characteristic of the distinct filamentous forms is a continuous polarized growth (Staebell and Soll, 1985 and Anderson and Soll, 1986). This continuous polarized growth is characterized by the polarization of the actin cytoskeleton at the tip of the *C. albicans* cell. In yeast cells, after the daughter cell reaches 66% of the size of the mother cell, there is a switch from polarized to isotropic growth, characterized by actin polarization throughout the cell (Sudbery *et al.*, 2004). In contrast, hyphae grow only in a polarized way, generating the narrow fragments that characterise these cells (Staebell and Soll, 1985), whereas pseudohyphae and hyperpolarized buds, have a longer period of polarized growth that can later switch to isotropic growth (Bachewich *et al.*, 2005). The importance of polarized growth in hyphal formation has been showed by decreasing the expression of genes involved in polarity regulation, including the main polarity regulator the GTPase *CDC42*, which results in decreased hyphal growth (Bassilana *et al.*, 2003 and Bassilana *et al.*, 2005). One of the main differences between hyphae and pseudohyphae is related to their polarized growth. Whilst in hyphal cells polarized growth at the tip occurs throughout the cell cycle, pseudohyphal buds only show polarized growth during the first part of the cell cycle (Soll *et al.*, 1985 and Crampin *et al.*, 2005). In addition, while hyphal polarized growth depends on a hyphal-specific structure called the Spitzenkörper, pseudohyphal polarized growth is driven by the polarisome (Harris *et al.*, 2005; Virag and Harris, 2006 and Crampin *et al.*, 2005).

There are significant differences in the cell cycles of pseudohyphae and hyphae. The sequence of cell cycle events of pseudohyphae is more similar to events seen in yeast cells than hyphal cells, with the exceptions that pseudohyphae fail to complete cytokinesis

between mother and daughter cells and have an extended G2 phase (Figure 1.4). The first elongated pseudohyphal cell appears in the beginning of the first cell cycle (G1 to S phase), with the formation of a septin ring, initiation of DNA replication, spindle body duplication and appearance of the bud. By the end of the cell cycle (M phase) the septum is formed and constriction between the two dividing cells is observed, with no separation after cytokinesis (Figure 1.4) (Sudbery *et al.*, 2004 and Berman, 2006). In contrast, during hypha formation, the germ tube is formed before the end of the first cell cycle of an unbudded yeast cell, beginning to form in G1 with the formation of a basal septin band between the mother cell and the growing germ tube. Once the first cell cycle (G1 → S phase) has begun, the initiation of DNA replication, spindle body duplication and formation of a septin ring in the growing germ tube occurs. By the end of the cell cycle (M phase) the septum is formed (without any constriction between the septin rings) and the divided DNA migrates back to the mother cell (Figure 1.4 - Sudbery *et al.*, 2004). Hyperpolarized buds result from cell cycle perturbations that result in activation of DNA checkpoints, which results in cell cycle arrest (Shi *et al.*, 2007). Cells have DNA checkpoints in three points of the cell cycle: G1/S, G2/M and S phase (Gottifredi and Prives, 2005), and hyperpolarized growth can arise due to an intra-S checkpoint or a G2/M arrest (Shi *et al.*, 2007). However, the exact cell cycle events occurring in this type of filamentous growth are not fully understood (Whiteway and Bachewich, 2007).

Clearly the cell cycle is intimately linked with the induction of filamentous growth, since deletion of cell cycle regulators or agents that cause cell cycle delay or arrest induce different types of filamentous growth (Berman, 2006). It has been shown that the *C. albicans* G1 cyclins, Ccn1, Hgc1, and Cln3, play key roles in hyphal morphogenesis (Loeb *et al.*, 1999; Zheng *et al.*, 2004; Chapa y Lazo *et al.*, 2005 and Bachewich and Whiteway, 2005). Hgc1 is a cyclin that is not cell-cycle regulated. Instead, *HGC1* is induced in response to hyphae stimulating conditions and significantly, deletion of *HGC1* abolishes hyphal growth under hyphae-inducing conditions (Zheng *et al.*, 2004). Furthermore, *hgc1*Δ cells showed normal expression of hyphal specific genes, indicating that the signalling pathways responsible for hyphal induction are activated and the Hgc1 effect is independent of these pathways (Zheng *et al.*, 2004). Hgc1 was also shown to be important for maintaining phosphorylation of the septin Cdc11, which is essential for sustained polarized growth. Therefore, in the absence of Hgc1, Cdc11 phosphorylation is lost and hyphal growth is aborted (Sinha *et al.*, 2007). On

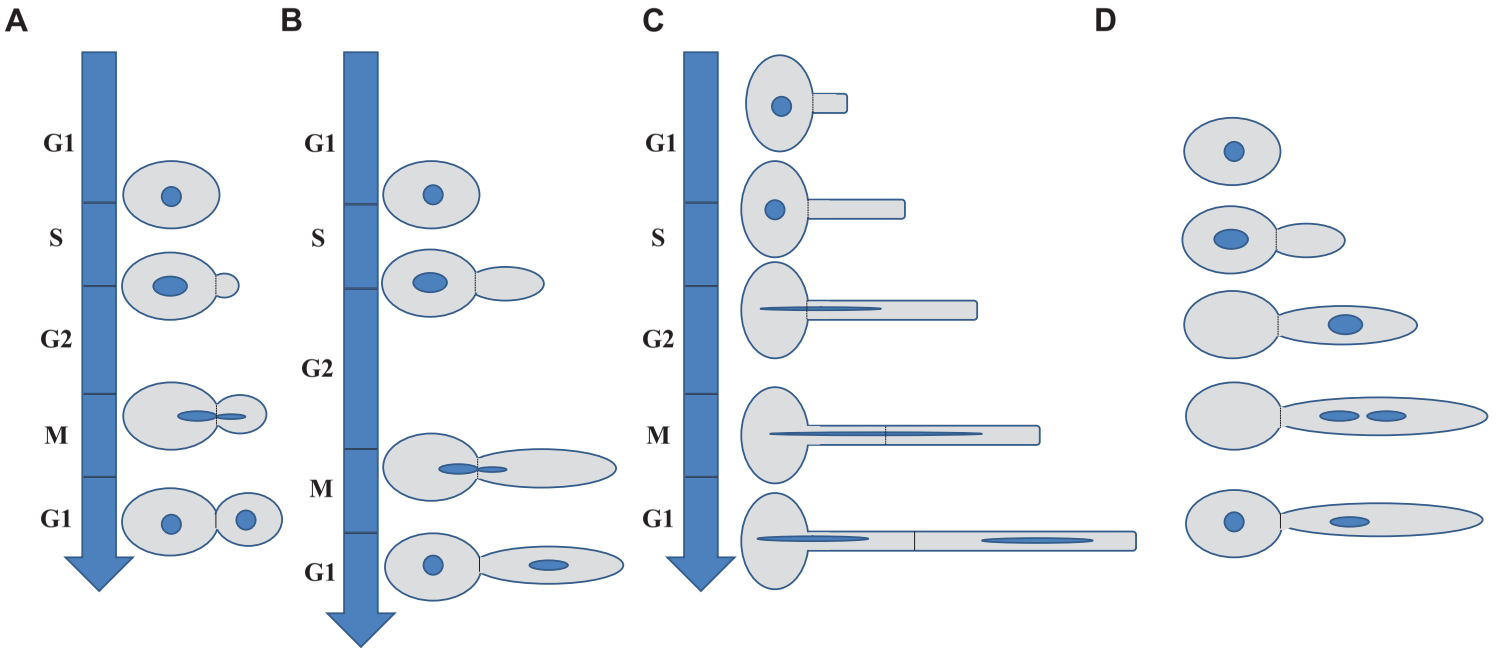


Figure1.4. *C. albicans* cell cycle in the different morphological forms.
Schematic representation of cell cycle in (A) yeast, (B) pseudohyphal and (C) hyphal cells. (D) Schematic representation of nuclear division in hyperpolarized buds. Adapted from Sudbery *et al.*, 2004.

the other hand, other *C. albicans* cyclins are implicated in the inhibition of filamentous growth. Absence of the G1-mitotic cyclin Cln3, for example, induced filamentous growth (hyphae and pseudohyphae) under non-inducing conditions (Chapa y Lazo *et al.*, 2005 and Bachewich and Whiteway, 2005), while depletion of the mitotic cyclins Cln4 and Cln2 results in a constitutive pseudohyphal or hyperpolarized bud phenotype respectively (Bensen *et al.*, 2005).

In addition, the constitutive formation of hyperpolarised buds is seen upon deletion of the polo kinase Cdc5 (Bachewich *et al.*, 2003). Cdc5, based on studies of the orthologous protein in *S. cerevisiae*, is predicted to be one the major regulators of the mitotic exit network, and the hyperpolarized bud phenotype seen in *C. albicans cdc5*Δ cells was associated with blockage of early nuclear division and spindle formation (Bachewich *et al.*, 2003). In addition, genetic mutations that activate the DNA damage cell cycle checkpoint such as depletion of ribonucleotide reductase (RNR) stimulate the formation of hyperpolarised buds (Shi *et al.*, 2007).

1.2.3.2. Regulation of Morphogenetic Switching

Morphogenesis can be induced *in vitro* by several different stimuli. For example, cultures grown at low temperature and pH are predominantly in the yeast form, while nitrogen-limited media or media with high phosphate content induce pseudohyphae formation. In addition, several environmental cues, such as serum, high temperature, neutral pH (Lee`s media), starvation, N-acetylglucosamine (GlcNAc), CO_2, and adherence, can trigger the yeast→hypha transition through the activation of different pathways (Sudbery *et al.*, 2004 and Biswas *et al.*, 2007). In contrast, treatment of yeast *C. albicans* cells with DNA damaging agents, such as hydroxyurea (HU), UV or methyl methanesulfonate (MMS) induces the formation of hyperpolarised buds (Bachewich *et al.*, 2003 and 2005 and Shi *et al.*, 2007).

1.2.3.2.1. Positive regulators of morphogenetic switching

Morphological switching is controlled by a complex network of different signalling pathways and transcription factors which act as either activators or inhibitors of the morphogenetic switch (Figure 1.5) (Brown and Gow, 1999). Positive regulation of hyphal development is mediated by signalling pathways, including the cAMP-dependent PKA

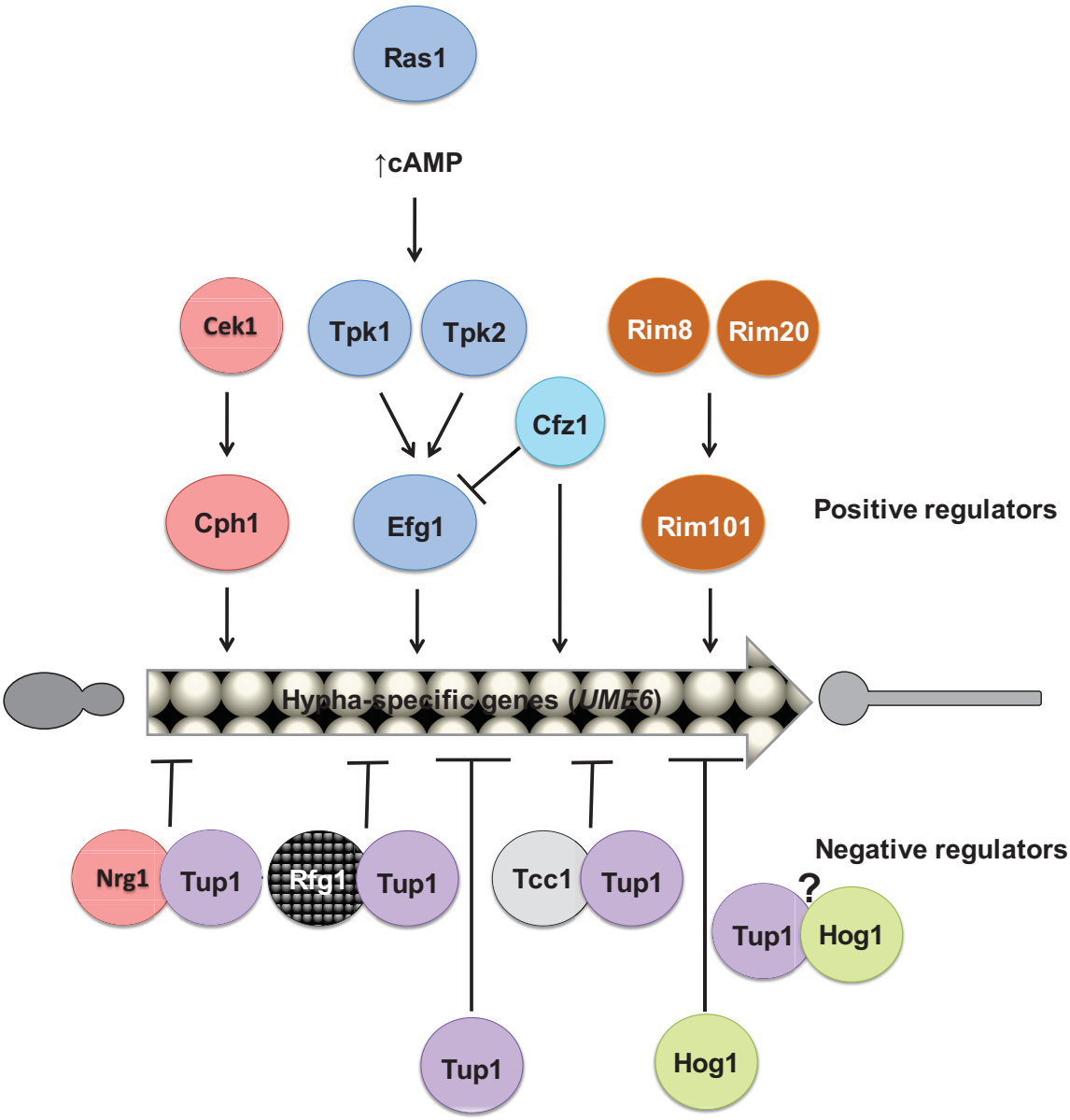


Figure1.5. Postive and negative regulators involved in the yeast→hyphae morphogenetic switch in *C. albicans*.
Adapted from Biswas *et al.*, 2007.

pathway, the Cek1 MAPK and the Rim101 pH signalling pathways (Brown and Gow, 1999 and Ernst, 2000).

The cAMP-dependent protein kinase A (PKA) pathway plays a crucial role in filamentation induced by starvation and serum in *C. albicans* (Feng *et al.*, 1999). In this pathway the Ras protein and its receptor-coupled G proteins act upstream of the adenylate cyclases, stimulating cAMP synthesis and activation of PKA (Maidan *et al.*, 2005). The two *C. albicans* PKAs, Tpk1 and Tpk2, seem to have specific roles in morphogenesis, depending on the environmental cues given to the cells. While Tpk1 seems to play a key role on solid media, Tpk2 is more important for hyphal formation in liquid media (Bockmühl *et al.*, 2001). The Efg1 transcription factor is a downstream target of the cAMP-PKA pathway, and over-expression of Efg1 can bypass the morphogenetic defect of *tpk*2Δ cells (Sonneborn *et al.*, 2000).

Efg1 is a basic helix-loop-helix (bHLH) transcription factor, and its major role in morphogenesis comes from studies showing that that deletion of *EFG1* results in strains that are unable to form hyphae and are defective in inducing hyphal-specific genes (Lo *et al.*, 1997 and Sohn *et al.*, 2003). In addition to its role in the yeast to hyphae morphogenetic switch, Efg1 seem to play a role in the induction of other filamentous forms, such as pseudohyphae as over-expression or Efg1 results in pseudohyphal cell growth (Stoldt *et al.*, 1997). Furthermore, it has been shown that the different subdomains of Efg1 are required for the different morphologies of *C. albicans*. Efg1 contains the APSES domain. The APSES domain is highly conserved and contains a bHLH motif, which is responsible for binding to MCB and/or E-box sequences in the promoter sequence of genes. A study by Noffz and colleagues (2008) has shown the importance of these different subdomains of Efg1 for morphogenesis in *C. albicans* (Noffz *et al.*, 2008).

Mitogen-activated protein kinase (MAPK) pathways are also involved in filamentous growth in *C. albicans*. For example, the Cek1 MAPK pathway is a positive regulator of morphogenesis in response to nutritional starvation, with Cek1 activating the transcription factor Cph1 (Lo *et al.*, 1997, Csank *et al.*, 1998 and Lane *et al.*, 2001).

The Cek1 regulated transcription factor Cph1 was, together with Efg1, one the first regulators of the yeast to hyphae switch to be identified (Liu *et al.*, 1994, Braun and Johnson, 1997 and Lo *et al.*, 1997). Deletion of *CPH1* resulted in strains that exhibited delayed hyphal formation under hyphal-inducing conditions and over-expression of Cph1 resulted in strains

that exhibited an enhanced filamentous growth under yeast-inducing conditions (Liu *et al.*, 1994 and Huang *et al.*, 2008). Furthermore, although Cph1 is not essential for the expression of filament-specific genes, this transcription factor seems to be important for achieving the high levels of such genes seen during filamentous growth (Huang *et al.*, 2008).

Although Efg1 has a central role by itself in inducing the transcription of many genes during the yeast to hyphae morphogenesis, other transcription factors can act alongside Efg1 in this process. For example, Efg1 interacts with Czf1, a transcription factor that regulates hyphal morphogenesis under embedded conditions in which Efg1 and Cph1 are not necessary. This interaction between Czf1 and Efg1 requires regions outside of the Efg1 APSES domain (Noffz *et al.*, 2008). Furthermore, Czf1 seems to repress its own expression, by inhibition of Efg1-mediated *CZF1* up-regulation. This would contribute to the modulation of Czf1 activity during morphogenesis (Brown *et al.*, 1999, Vinces *et al.*, 2006 and Noffz *et al.*, 2008). In addition, Cph1 also seems to act alongside Efg1 to regulate some differentially expressed genes, such as the *SAP* genes (Lane *et al.*, 2001 and Sohn *et al.*, 2003). Although other transcription factors can act alongside Efg1, Efg1 seem to have a prominent role in hyphal morphogenesis. For example, deletion of *EFG1* has a great impact on filamentous induction compared to strains lacking *CPH1*. Nonetheless, double *efg1*Δ*/cph1*Δ mutant show enhanced filamentation defects compared to either single mutant (*efg1*Δ or *cph1*Δ) (Lo *et al.*, 1997 and Braun and Johnson, 2000).

An important target of the Efg1/Cph1 transcription factors which is induced in response to hyphal-inducing conditions is the zinc-finger DNA binding protein Ume6. Under conditions such as starvation (spider media), neutral pH (6.8) or serum at 37°C, *UME6* transcription is induced (Kadosh and Johnson, 2005 and Zeidler *et al.*, 2009). Furthermore, Ume6 itself is required to activate certain hyphal specific genes and consistent with this *ume6*Δ strains display defects in hyphal growth (Banerjee *et al*., 2008). Intriguingly, a recent paper by Carlisle and colleagues (2009) showed that *UME6* expression levels can determine whether *C. albicans* grows in either pseudohyphal or hyphal forms by activating a different subset of genes. Low Ume6 levels induces a group of filament-specific genes that results in pseudohyphal growth, while higher Ume6 levels increases the expression of distinct genes which promotes hyphal growth. These results indicate that *UME6* expression levels are sufficient to determine *C. albicans* morphogenetic state (Carlisle *et al.*, 2009). Furthermore, the *UME6* dose-dependent effects reported, indicates that pseudohyphal cells are an

intermediate form between yeast and hyphal cells, which contrasts to the widely accepted dogma that pseudohyphal and hyphal cells are distinct unrelated forms (Sudbery *et al.*, 2004).

The Rim101 pathway controls pH-induced yeast-hyphae morphogenesis in *C. albicans*. In order to be activated, the transcription factor Rim101 needs to be cleaved (Li and Mitchel, 1997). Proteolysis of Rim101 is activated by the upstream regulators, such as Rim8 and Rim20 (Porta *et al.*, 1999 and Davis *et al.*, 2000). Under alkaline conditions Rim101 is activated and it positively regulates a subset of genes, such as *PHR1* under alkaline conditions (Davis *et al.*, 2000 and Ramón and Fonzi, 2003). It is expected that Rim101 target genes, such as the GPI-anchored glycosidase *PHR1*, would be required for hyphal cell growth under neutral-alkaline pH (7.3-8) (Yesland and Fonzi, 2000).

Much less is known regarding the regulation of the formation of hyperpolarised buds, although this mode of growth is independent of the main yeast to hyphae transcriptional regulators Efg1/Cph1 (Shi *et al.*, 2007). However, a recent study revealed that the Rad53 DNA damage checkpoint kinase is a main regulator of this genotoxic stress-induced filamentous growth. Deletion of *RAD53* in *C. albicans* resulted in yeast-like slow growing cells that are hypersensitive to drugs that are known to activate DNA checkpoints, such as HU and MMS. Significantly, deletion of Rad53 prevents hyperpolarized cell growth in response to HU, MMS and UV (Shi *et al.*, 2007). In addition, both DNA damage and replication checkpoints converge on Rad53 to stimulate hyperpolarized buds. For example, deletion of the *C. albicans* adaptor protein *MRC1* in *C. albicans*, which is responsible for relaying replication stress signals to Rad53 in the model yeast *S. cerevisiae*, results in rendering Rad53 constitutively activation and in cells that grow as hyperpolarized buds, under yeast inducing conditions. On the other hand, *RAD9*, which is required for Rad53 activation in response to DNA damage, results in cells where Rad53 is not constitutively activated and that display yeast cell morphology. In addition, conditions which require Rad9 for activation of the DNA checkpoint, such as treatment with the DNA damaging agent MMS, cannot activate Rad53 nor induce hyperpolarized bud formation in the *rad9*Δ strain. On the other hand conditions which do not require Rad9 for Rad53 activation (HU treatment), can induce both Rad53 activation and hyperpolarized bud formation even in cells that do not express *RAD9*. All these results indicate that that the activation of Rad53 is the signal necessary for the induction of the filamentous form know as hyperpolarized bud, and that induction of checkpoints is required for hyperpolarized bud formation (Shi *et al.*, 2007).

1.2.3.2.2. Negative regulators of morphogenetic switching

With regard to negative regulators of morphogenesis, the Tup1 transcriptional repressor is a major repressor of the yeast→hyphae switch in *C. albicans*, and *tup1*Δ mutants are constitutively filamentous under yeast inducing conditions (Braun and Johnson, 1997). Pioneering studies in the model yeast *S. cerevisiae* revealed that several transcriptional regulators target Tup1 in order to repress specific subsets of genes (Smith and Johnson, 2000). In *C. albicans*, the DNA-binding repressor proteins Nrg1 and Rfg1 act specifically to target the repression of hyphal-development and hyphal-specific genes via Tup1 (Murad *et al.*, 2001a and b). Although *tup1*Δ, *nrg1*Δ and *rfg1*Δ mutant cells exhibit a filamentous phenotype, there is a difference in the capacity to form true hyphae under inducing conditions, with the *tup1* mutant being unable to form true hyphae and *nrg1* null cells being able to form true hyphae in certain conditions (pH, starvation and serum). Furthermore, the phenotype of the double *nrg1*Δ*rfg1*Δ mutant was not identical to the filamentous phenotype seen in *tup1*Δ cells grown and *tup1*Δ cells had higher levels of filamentous cells (100%) when compared with the double *nrg1*Δ*rfg1*Δ mutant. Hence, although Tup1-mediated repression is driven by Nrg1 and Rfg1, it seems that there are other mechanisms independent of these co-repressors acting to regulate Tup1-mediated gene expression (Murad *et al.*, 2001a and Braun *et al.*, 2001).

Indeed, the transcriptional co-repressor *SSN6* contributes to some extent to Tup1 mediated repression of genes, although the majority of Tup1 repressed genes are regulated in an Ssn6-independent manner (García-Sánchez *et al.*, 2005). These results are in agreement with data showing that the morphological defects seen in *ssn6*Δ cells are different from those ones seen in *tup1*Δ cells. For example, while *tup1*Δ cells show a constitutively pseudohyphal phenotype resulting in cells that cannot form true hyphae, *ssn6*Δ have a very unstable phenotype, switching between different morphologies and show wild-type capacity to form serum induced hyphae in liquid media (García-Sánchez *et al.*, 2005 and Hwang *et al.*, 2003). In addition to Ssn6, another transcription co-repressor of Tup1 was identified recently. Tcc1 (Tup1-complex component), was shown to interact with Tup1 and deletion of *TCC1* results in a strain that exhibited a filamentous phenotype (Kaneko *et al.*, 2006). However, the *tcc1*Δ filamentous phenotype was different from the one seen in *tup1*Δ strains, while deletion of *TCC1* resulted in elongated cells, characteristic of filamentous growth, the length of these cells was reduced when compared with cells that do not express *TUP1*. Furthermore, loss of

TCC1 results in the derepression of some Tup1 repressed transcripts. Comparing the levels of de-repression of hyphal specific genes from *tup1*Δ mutants and both *tcc1*Δ and *ssn6*Δ mutants, it was found that *TCC1*-dependent and *SSN6*-dependent derepression was weaker than the one seen in *tup1*Δ strain. Therefore, some may speculate that the level of repression of hyphal specific genes might be associated with different filamentous phenotypes (Kaneko *et al.*, 2006).

Another negative regulator of the yeast→hyphal process is the Hog1 MAPK pathway. Deletion of *HOG1* or its upstream regulators results in strains that are either filamentous under yeast inducing conditions (Enjalbert *et al.*, 2006 and Cheetham *et al.*, 2007) or show accelerated morphogenetic switching compared to wild-type cells (Alonso-Monge *et al.*, 1999, Arana *et al.*, 2005 and Eiseman *et al.*, 2006) The role of Hog1 in regulating morphogenesis seems to be independent of the transcription factors Cph1 and Efg1, and of possible cross-talk between the Hog1 and Cek1 MAPK pathways (Eiseman *et al.*, 2006). Notably, however, several Tup1 regulated genes are derepressed in *hog1*Δ cells, indicating a possible link between Hog1 and the repressive function of Tup1 (Enjalbert *et al*., 2006).

1.2.3.3. Morphogenesis and Virulence

The specific role that each of the *C. albicans* morphological forms plays during the infection process is unknown since both budding and filamentous forms are found at most sites of infection (Odds, 1989). It has been suggested that the invasive growth characteristics of both pseudohyphae and hyphae may help the invasion and colonisation of organs; whereas the yeast form may allow for efficient dissemination through the bloodstream (Whiteway and Oberholzer, 2004 and Kumamoto and Vinces, 2005).

Consistent with this, deletion of positive and negative regulators of the yeast to hyphae morphogenetic switch results in strains that are avirulent in a mice model (Lo *et al.*, 1997, Bendel *et al.*, 2003 and Murad *et al.*, 2001). However, it could be argued that the virulence defect observed in such strains may arise from a lack of induction of hyphal specific virulence determinants such as adhesins and SAP proteins, rather than the physical change in shape. However, evidence against this was provided by a study which revealed that deletion of the hyphal regulated cyclin *HGC1* resulted in avirulent cells that are unable to form hyphae, yet express many hyphal specific genes. This study separates the two processes (induction of hyphal growth and induction of hyphal specific genes) and indicates that the

physical filamention of the cell is indeed a major attribute of *C. albicans* virulence (Zheng *et al.*, 2004).

Furthermore, recent papers have reported strategies to modulate the expression of key morphogenetic regulators, such as *NRG1* and *UME6* during the infection process, thereby allowing direct assessment of the role of the different morphological forms during disease establishment (Saville *et al.*, 2003 and Carlisle *et al.*, 2009). For example, stimulation of filamention *in vivo* (either down regulation of *NRG1* or over-expression of *UME6*) resulted in increased virulence, whereas inhibition of filamentation *in vivo* (overexpression of *NRG1*) resulted in avirulence (Saville *et al.*, 2003 and Carlisle *et al.*, 2009). Furthermore, over-expression of *NRG1* results in *C. albicans* dissemination to the kidney although no significant tissue damage was seen (Saville *et al.*, 2003). These results support a model in which *C. albicans* yeast cells are important for dissemination, yet filamentous forms are important for disease establishment.

1.2.4. Stress Responses

Unicellular microbes, such as *C. albicans*, are particularly susceptible to sudden fluctuations in their growth environment. Adaptation and survival to such environmental changes requires a rapid reprogramming of the microbes transcriptome in which genes that encode stress protective or repair functions are induced (Estruch, 2000). During the course of infection *C. albicans* cells are predicted to encounter a range of environmental insults and there is growing evidence that the ability of this fungus to respond to various stresses is imperative for survival in the host (Figure 1.6 - Kumamoto, 2008).

C. albicans for example is exposed to oxidative stress and nitrosative stress when exposed to cells of the innate immune system, such as macrophages and neutrophils, and epithelial cells (Enjalbert *et al.*, 2007 and Hromatka *et al*., 2005). In addition, this pathogen is predicted to be exposed to osmotic stress in the kidney and pH stress in the gastrointestinal tract (Figure 1.6). Therefore, it is perhaps to be expected that inactivation of proteins that protect cells against environmental stresses results in *C. albicans* strains that show decreased ability to adapt and survive in the host. For example, inactivation of stress-protective enzymes involved in nitrosative stress (Yhb1), oxidative stress (Cta1, Sod1 and Sod5), heat shock (Hsp90), nitrogen and iron starvation (Ilv1 and Ftr1), all result in attenuated virulence of *C. albicans* in the mouse model of systemic candidiasis (Wysong *et al*., 1998; Ramanan

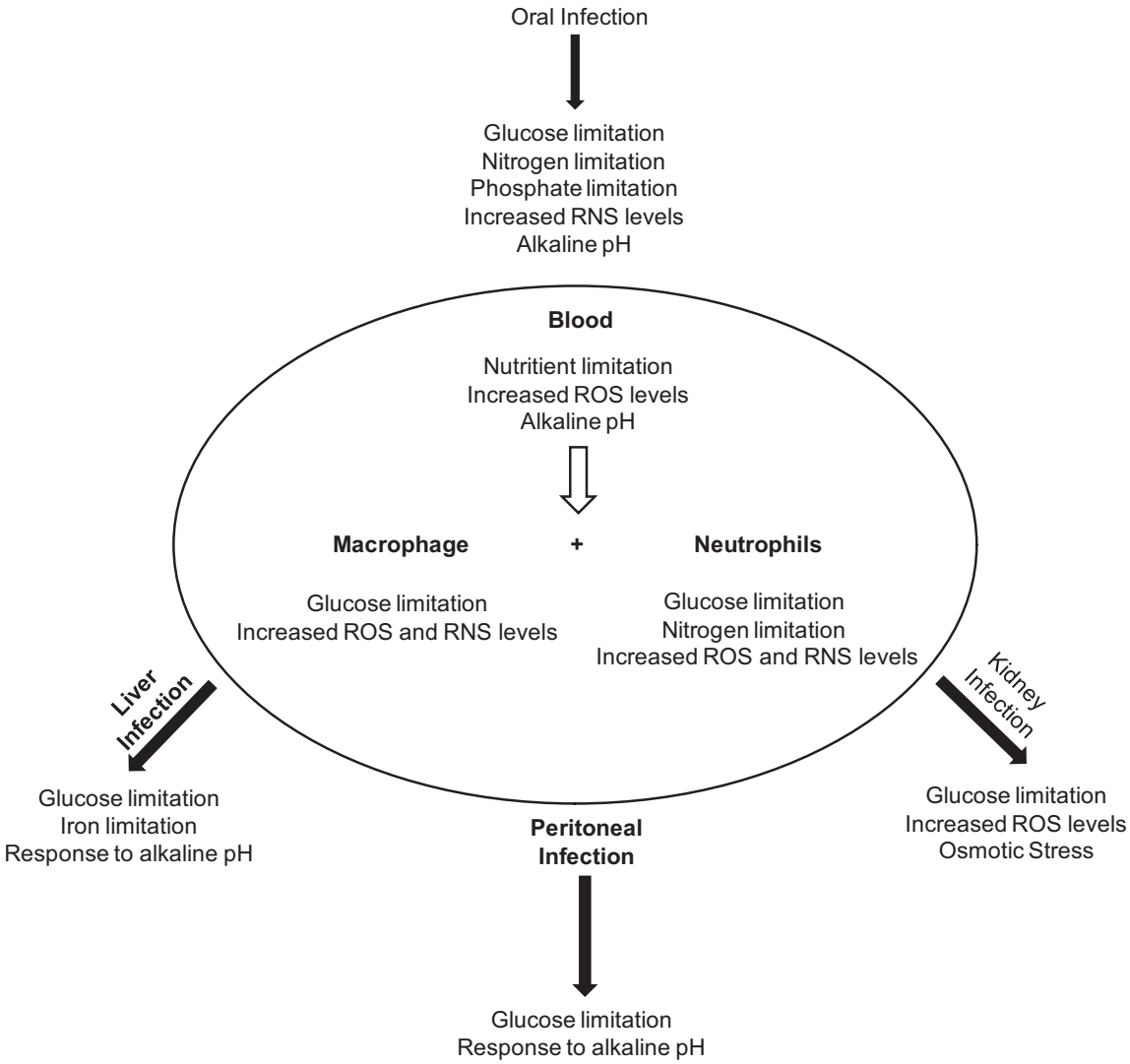


Figure1.6. Stress conditions likely to be encountered in host enviroments by *C. albicans* cells.

Such stresses conditions are supported by the transcription profile of *C. albicans* upon exposure to the above environments.

and Wang, 2000; Hwang *et al*., 2002; Van Dijck *et al*., 2002; Martchenko *et al*., 2004; Hromatka *et al*., 2005; Shapiro *et al.*, 2009 and Kingsbury and McCusker, 2010). Similar findings have also been reported with strains carrying deletions in genes encoding stress regulatory proteins such as the Hog1 stress-activated protein kinase (SAPK), the Ssk1 response regulator, and the transcription factor Cta4 (Alonso-Monge *et al*., 1999 and Chiranand *et al.*, 2008).

1.2.4.1. Stress responses in vitro

Several groups have examined the transcriptional response of *C. albicans* to artificial stresses imposed *in vitro*. Although the complex microenvironment of the host will clearly differ from such single stresses imposed *in vitro*, such investigations are worthwhile as they generate a transcriptional 'signature' response for specific stresses which provides useful insight when attempting to uncover the stresses encountered by *C. albicans* during infection.

A number of studies indicate that stress responses have diverged in order to generate more robust responses in *C. albicans* when compared to those in the model yeasts, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (reviewed in Quinn and Brown, 2007). For example, *C. albicans* is more resistant to oxidative and osmotic stresses than *S. cerevisiae* and *S. pombe* (Jamieson *et al.*, 1996 and Nikolaou *et al.*, 2009). Nonetheless, all of these fungi induce similar functional categories of genes in response to different types of stresses, such as oxidative, osmotic or heavy metal stress (Enjalbert *et al*., 2003 and Enjalbert *et al*., 2006), Furthermore, the stress-responsive regulatory proteins that regulate transcriptional responses to stress are conserved between *C. albicans* and the model yeasts *S. cerevisiae* and *S. pombe* (Nikolaou *et al.*, 2009). Intriguingly, however, there is mounting evidence that the role and regulation of such regulatory proteins has diverged in *C. albicans* when compared with these model yeasts (reviewed in Quinn and Brown, 2007). In the section below, the key elements of specific stress response 'signatures' defined in *C. albicans* will be described, and the similarities and differences in their regulation to that documented in *S. cerevisiae* and *S. pombe* highlighted.

1.2.4.1.1. Heat shock Response

The heat shock response is responsible for protecting cells against changes in temperature. In the model yeasts *S. cerevisiae* and *S. pombe*, induction of chaperones, such as

Heat Shock Proteins (HSPs), indicates that a heat shock response (25º-37ºC) is characterized by protein unfolding (Gasch *et al.*, 2000 and Chen *et al.*, 2003). Similarly, induction of several genes involved in protein folding and refolding was seen in *C. albicans* cells exposed to heat shock (23º-37ºC, 30-42°C and 30-45°C), including the HSPs (*HSP12*, *HSP70*, *HSP78*, *HSP90* and *HSP104*) (Enjalbert *et al.*, 2003 and Nicholls *et al.*, 2009). In addition, repression of genes involved in ribosome biogenesis and RNA processing functions were seen in both *C. albicans* and *S. cerevisiae* cells, indicating that the induction of protein refolding was accompanied by a decrease in RNA processing (Gasch *et al.*, 2000 and Nicholls *et al.*, 2009).

The heat shock response is mainly regulated by the transcription factor (Hsf1) in both *S. cerevisiae* and *C. albicans*. Hsf1 regulates gene expression by regulating heat shock elements (HSEs) that are present in the promoters of target genes (Hahn *et al.*, 2004 and Nicholls *et al.*, 2009). However, while in *S. cerevisiae* the Hsf1-HSE regulon responds to different stresses, such as oxidative stress, it is only induced in response to heat shock in *C. albicans* (Liu and Thiele, 1996 and Nicholls *et al.*, 2009). In addition, while in *S. cerevisiae* the Hsf1-HSE act in combination with the transcription factors Msn2/4 to regulate transcriptional responses to heat shock, in *C. albicans* Hsf1 seems to be acting independently of the *S. cerevisiae* Msn2/4 homologues CaMsn2 and CaMln1, since deletion of these genes does not result in heat shock sensitivity and that *MSN2* and *MLN1* do not play a role in regulating gene transcription in response to heat shock stress (Boy-Marcotte *et al.*, 1999, Nicholls *et al.*, 2004 and Nicholls *et al.*, 2009).

C. albicans is an obligatory animal saprophyte and in contrast with the model yeasts is not expected to encounter great variations in temperature. However, the Hsf1-HSE regulon also plays a key role in adaptation during *C. albicans* normal cell growth by activating the expression of chaperones that are required for survival of this organism in any condition of growth, including when colonizing the human host (Swoboda *et al.*, 1995 and Nicholls *et al.*, 2009). Recent results also indicate that, in addition to its role in responses to heat stress, the Hsf1 regulated gene *HSP90* also plays a key role in *C. albicans* resistance to antifungals (azoles and echinocandins) and the process of morphogenesis. Such findings indicate that components of the heat stress response have vital roles in responding to other environmetal triggers that contribute to *C. albicans* pathogenicity (Cowen *et al.*, 2009, Singh *et al.*, 2009 and Shapiro *et al.*, 2009).

1.2.4.1.2. Osmotic Stress Response

Exposure of cells to high osmolarity leads to dehydration, disruption of ion gradients and decreased viability. Osmotic stress responses in yeasts have been studied by exposing cells to either salt (NaCl or KCl) or sorbitol, which results in hyperosmotic stress. Hyperosmotic stress is characterized by rapid loss of water and turgor pressure, which results in cell shrinkage. In the model yeasts, *S. cerevisiae* and *S. pombe,* hyperosmotic stress results in the induction of genes that encode proteins that synthesise osmolytes such as glycerol and trehalose to retrieve water from the environment, and various sugar and cation transporters (Gash *et al.*, 2000 and Chen *et al.*, 2003). Similar to *S. cerevisiae* and *S. pombe*, exposure of *C. albicans* to hyperosmotic stress also induced a similar group of genes, such as predicted sugar transporters (*STL1*), cation transporters (*ENA22, ENA21*) and genes involved in glycerol accumulation (*GPD2* and *RHR2*) (Enjalbert *et al.*, 2003 and Enjalbert *et al.*, 2006). Proteomic studies in *C. albicans* and *S. cerevisiae* also showed that proteins involved in carbohydrate metabolism, and protein folding and degradation such as members of Hsp70 chaperone family (Pdr13, Ssa1 and Ssb1), were up-regulated in response to hyperosmotic stress (Norbeck and Blomberg, 1996 and Yin *et al.*, 2009). In summary therefore, comparison of the functional categories of genes induced in response to hyperosmotic stress between model yeasts and *C. albicans* reveals that the osmotic stress response is highly conserved between these evolutionarily divergent fungi (Enjalbert *et al.*, 2006 and Yin *et al.*, 2009).

In *S. cerevisiae* the high-osmolality glycerol (Hog1) SAPK plays a key role in the protection against osmotic stress, and activation of this pathway in response to osmotic stress has been studied in detail (reviewed by Hohmann, 2002). *C. albicans* Hog1 is also activated in response to osmotic stress and Hog1 plays a key role in the regulation of osmotic stress induced genes in this fungal pathogen (Enjalbert *et al*., 2006). Intriguingly, the mechanisms underlying Hog1 activation in response to osmotic stress, and other stresses, have diverged from that characterised in *S. cerevisiae* (reviewed in Smith *et al.*, 2010), and this will be described in more detail in section 1.4.3.2.

1.2.4.1.3. Oxidative Stress Response

All aerobically growing cells are exposed to Reactive Oxygen Species (ROS) that are generated as a byproduct of oxidative metabolism. Pathogenic fungi must cope with these

toxic metabolites and also the oxidative burst generated by phagocytic cells of the host immune system (see Section 1.3.1.4). Consequently there is much interest in *C. albicans* responses to oxidative stress, and the transcriptional responses of *C. albicans* to exogenous oxidative stresses have been defined *in vitro* by a number of microarray studies (Enjalbert *et al.*, 2003, Enjalbert *et al.*, 2006, Wang *et al.*, 2006 and Znaidi *et al.*, 2009) and this has been supplemented more recently by complementary proteomic analyses (Kusch *et al.*, 2007 and Yin *et al.*, 2009).

In general, *C. albicans* appears to display similar transcriptional responses to oxidative stresses as do budding and fission yeasts (Enjalbert *et al.*, 2003 and Enjalbert *et al.*, 2006). For example, all three yeasts respond to ROS by activating the synthesis of detoxification mechanisms that include catalase (*CTA1*), superoxide dismutase, and components of the thioredoxin (*TRR1* and *TRX1*) and glutaredoxin systems (*GPX1* and *GST3*) (Gash *et al.*, 2000, Chen *et al.*, 2003, Enjalbert *et al.*, 2003 and Enjalbert *et al.*, 2006). In addition, proteomic studies analyzing the response of *C. albicans* to peroxide stress showed that in addition to antioxidant proteins (Ahp1, Cip1, and Oye32), several proteins involved in protein folding and degradation were also up-regulated (Hsp12, Hsp78, Kar2, orf19.5830, orf19.251, Pdr13, Ssa2, Ssb1, Sse1,Cdc48, Pre9 and Pre10) (Kusch *et al.*, 2007 and Yin *et al.*, 2009).

When comparing stress responses of *S. pombe* and *C. albicans* cells to low and high levels of oxidative stresses, it is apparent that dose-dependent responses exist. For example, previous work in the fission yeast *S. pombe* revealed that different genes are expressed depending on the level of H_2O_2, and different signalling pathways operate in response to weak and strong oxidative stresses (Quinn *et al.*, 2002 and Vivancos *et al*. 2004). Similarly, *C. albicans* also displays different transcriptional responses to low (0.4 mM) and high (5 mM) H_2O_2 concentrations (Enjalbert *et al.*, 2003 and Enjalbert *et al.*, 2006). For example, genes involved in carbohydrate metabolism (*ICL1, MLS1, GPM2, PCK1, GSY1* and *NTH1*) were only induced in response to high levels of peroxide stress, whereas genes involved in the DNA-damage response were up-regulated only in response to low levels of H_2O_2 (*HNT2* and *RGA2*). Also, genes involved in chromatin silencing and epigenetic regulation were specifically repressed in response to high H_2O_2 concentrations (*DOT4, DOT6, ISW2* and *SAS10*) (Enjalbert *et al.*, 2006). However, the molecular basis underlying dose-dependent transcription of H_2O_2-induced genes in *C. albicans* is unclear.

Despite the similarities in the transcriptional responses to oxidative stress shared between *C. albicans, S. pombe* and *S. cerevisiae*, *C. albicans* is much more resistant to oxidative stresses than the model yeasts. For example, 0.4 mM H_2O_2 is relatively toxic to *S. cerevisiae*, whereas *C. albicans* is relatively resistant to such a dose (Jamieson *et al.*, 1996). In addition, *C. albicans* cells are more resistant to growth in plates containing H_2O_2 than both *S. pombe* and *S. cerevisiae* (Nikolaou *et al.*, 2009). Although the molecular mechanisms underlying such differences are not clear, fungal pathogens have likely evolved mechanisms to allow them to mount robust oxidative stress responses to allow survival against the high levels of ROS generated by phagocytic cells. For example, *C. albicans* appears to have evolved extracellular mechanisms for protection against ROS, by targeting antioxidants such as the superoxide dismutase Sod5 and the thiol-specific antioxidant Tsa1 to the cell surface (Fradin *et al.*, 2005 and Urban *et al.*, 2005). The fact that *C. albicans* cells have evolved to express such antioxidants at the cell surface may allow instant detoxification of ROS produced by the host and thus greater tolerance to oxidative stress inducing agents than that seen in *S. cerevisiae* and *S. pombe*.

The transcriptional response to oxidative stress in *S. cerevisiae*, *S. pombe* and *C. albicans* is regulated largely by the AP-1-like transcription factors Yap1, Pap1 and Cap1 respectively, and Skn7-related response regulator transcription factors (Toda *et al.*, 1991, Morgan *et al.*, 1997, Toone *et al.*, 1998 and Wang *et al.*, 2006). In addition, the SAPKs in both *S. pombe* (Sty1) and *C. albicans* (Hog1) play important roles in the oxidative stress response. The role and regulation of these oxidative stress-responsive regulatory proteins is considered in depth in sections 1.4.3.1 and 1.4.3.2. of this chapter.

1.2.4.1.4. Nitrosative stress Response

In addition to ROS, cells of the innate immune system also generate nitric oxide (NO) that can in turn generate Reactive Nitrogen Species (RNS) as a mechanism of defense (See section 1.3.1.4). Transcription profiling of *S. cerevisiae* and *C. albicans* cells treated with compounds that generate nitrosative stress results in the induction of the flavohemoglobin gene *YHB1* which plays a key role in the detoxification of nitric oxide, which is confirmed by the observation that *ybp1*Δ cells are extremely sensitive to NO (Horan *et al.*, 2006, Hromatka *et al*., 2005 and Chiranand *et al.*, 2008). In addition, antioxidant genes (glutathione conjugating and modifying enzymes, NADPH oxidoreductases/dehydrogenases and

peroxidases), genes involved in iron uptake, sulphur assimilation and drugs or heavy metals transporters are also upregulated, whereas genes encoding mitochondrial electron transport chain proteins and ribosomal proteins are downregulated (Sarver and DeRisi, 2005, Horan *et al.*, 2006, Hromatka *et al*., 2005 and Chiranand *et al.*, 2008). Such responses indicate that in response to nitrosative stress these fungi defends themselves by increasing antioxidant defenses, blocking translation, and reducing production of intracellular ROS by decreasing levels of transcription of the mitochondrial electron transport chain components (Horan *et al.*, 2006 and Hromatka *et al*., 2005).

In *S. cerevisiae*, the transcription factor Fzf1 is responsible for activation of the flavohemoglobin Yhb1, which play key role in resistance to nitrosative stress (Sarver and DeRisi, 2005). Interestingly, in *C. albicans* nitrosative stress response is not regulated by an Fzf1 homologue, but by the transcription factor Cta4. Cells lacking Cta4 are extremely sensitive to nitrosative stress and fail to increase *YHB1* transcription in response to NO (Chiranand *et al.*, 2008). Furthermore, comparison of the transcription factors involved in nitrosative stress response in the model yeast *S. cerevisiae* and the pathogenic *C. albicans* showed that they belong to different families of zinc finger proteins (Chiranand *et al.*, 2008). While, *S. cerevisiae* Fzf1p have 5 Cys2His2 zinc fingers in its amino acid sequence, *C. albicans* Cta4 has only one 2-Cys6 zinc finger in its sequence. This 2-Cys6 transcription factor family is unique to fungi and bind to DNA due to its Cys residues that coordinate two zinc atoms (MacPherson et al., 2006 and Chiranand *et al.*, 2008)

1.2.4.1.5. Heavy Metal Stress Response

The heavy metal cadmium (Cd) is a common environmental contaminant, which can react with thiol groups on proteins resulting in diverse consequences. A primary mechanism employed by fungi to control the intracellular levels of heavy metal ions such as Cd is through sequestration with the antioxidant glutathione (gamma-l-glutamyl-l-cysteinyl-glycine). Hence, exposure to heavy metals also generates oxidative stress either through the depletion of intracellular glutathione levels and/or the displacement of Zn^{2+} and Fe^{2+} from proteins, which leads to the generation of highly reactive hydroxyl radicals ($OH^{\bullet}$; reviewed in Halliwell and Gutteridge, 2007 and Stohs and Bagchi, 1995).

Consistent with this, in *C. albicans*, treatment with the heavy metal Cd^{2+} results in induction of glutathione metabolism, which includes up-regulation of genes involved in

sulfate assimilation (*AGP3* and *SUL1*), sulphur-containing amino acid (*MET15*, *MET1*, *MET10, MET2, ECM17* and *CYS3*) and glutathione synthesis (*GSH1* and *GSH2*). In addition up-regulation of heat shock proteins (*SSA4*, *HSP78*, *SIS1*, *HSP90* and *HSP60*) and chaperones (*CPR6*, *SBA1* and *YDJ1*) also indicates that Cd^{2+} treatment results in protein unfolding (Enjalbert *et al.*, 2006). Up-regulation of sulphur-containing amino acid and glutathione synthesis is also seen in *S. cerevisiae* treated with Cd^{2+} (Thorsen *et al.*, 2009) whereas in *S. pombe* scavenging of glutathione from the external environment rather than glutathione synthesis appears to be the primary response (Chen *et al.,* 2003).

Proteomic analysis of *C. albicans* cells treated Cd^{2+} similarly indicated up-regulation of proteins involved in biosynthesis of sulphur-containing amino acids, redox regulation and with chaperone activities (Yin *et al.*, 2009). In addition, the proteomic approach also showed that proteins involved in carbohydrate metabolism (glycolysis, pentose phosphate pathway, fermentation pathway, the tricarboxylic acid (TCA) cycle and amino acid biosynthesis) were highly up-regulated in response to heavy metal stress by Cd^{2+}. The increase seen in proteins involved in both glycolysis and the pentose phosphate pathway can be explained by the requirement of NAPDH required for the reduction of antioxidants such as glutathione (Yin *et al.*, 2009).

In *S. cerevisiae* the AP-1 trancription factor Yap1 is responsible for activation of genes necessary for heavy metal stress, including the ABC transporter *YCF1* and *GSH1*, and is required for cadmium resistance in this organism (Serero *et al.*, 2008). Similarly, the Yap1 homologue in *C. albicans*, Cap1, is also required for activation of cadmium induced proteins such as Ycf1 and Cip1 (Wang *et al.*, 2006). Furthermore, Cap1 is necessary for cadmium resistance and *cap1*Δ strains are unable to grow in the presence of Cd^{2+} (Alarco and Raymond, 1999). In *S. pombe*, the Sty1 SAPK plays a major role in the regulation of the Cd-induced transcriptome (Chen *et al*., 2003). Similarly, transcript profiling revealed that the Hog1 SAPK in *C. albicans*, which is activated in response to Cd^{2+} treatment, also plays a major contribution in regulating Cd^{2+}-induced gene expression (Enjalbert *et al*., 2006). As Hog1 and Cap1 are also major regulators of the oxidative stress response, these proteins will be discussed in more detail in Sections 1.4.3 and 1.4.3.2.

1.2.4.1.6. General or Core Stress Response

In addition to stress responses that are specific to individual stresses, studies in the model yeasts *S. cerevisiae* and *S. pombe* revealed that each mounts a significant general or core stress response following treatment with diverse stresses (Gasch *et al.,* 2000, Causton *et al.,* 2001 and Chen *et al*., 2003). A core stress response is characterised by the presence of genes that respond in a stereotypical manner to diverse stress conditions. This response explains the previously characterised phenomenon of stress-cross protection in which *S. cerevisiae* or *S. pombe* cells exposed to a non-lethal dose of one stress, can survive subsequent treatment with a lethal dose of an unrelated stress (Lewis *et al.*, 1995 and Chen *et al.*, 2003). These responses regulate the expression of several hundred genes, which can be broadly separated into 4 groups; (i) down-regulation of genes involved in DNA mediated processes, and upregulation of genes involved in (ii) protecting against protein denaturation (heat shock proteins or chaperones), (iii) repairing oxidative damage and redox balance (catalases, thioredoxins, glutaredoxins), and (iv) carbohydrate metabolism (Gasch *et al*. 2000, Causton *et al*., 2001 and Chen *et al*., 2003).

Initial studies suggested that there is no core stress response in *C. albicans* (Enjalbert *et al.*, 2003), as no *C. albicans* genes were commonly up-regulated in response to heat (23-37°C), osmotic (0.3M NaCl) and oxidative stresses (0.4 mM H_2O_2). Nonetheless, stress cross protection was observed if *C. albicans* cells were exposed to stresses that activate the Hog1 SAPK (Smith *et al*., 2004). Subsequently, transcript profiling experiments that examined the global responses of *C. albicans* to stresses that activate Hog1 revealed a small subset of genes that were commonly induced by all three of the stress conditions examined (0.3M NaCl, 5 mM H_2O_2 and 0.5 mM $CdSO_4$ - Enjalbert *et al*., 2006). 24 genes were up-regulated in the core stress response (osmotic, oxidative and heavy metal stress) including those implicated in oxidative stress (*CTA1*), carbohydrate metabolism (*HXT61, HXT5, GLK1, GPD2* and *IPF20104*), and protein folding and degradation (*RPN4* and *IPF17186*) (Enjalbert *et al.*, 2006). In addition, genes involved in protein synthesis and RNA processing (*IPF966, IPF3709, NOP4, NMD3, MRPL3* and *RCL1*), transport (*NMD5* and *PHO84*) and transcription (*RRN3, RPO41* and *IPF16752*) were down-regulated in the core stress response in *C. albicans*. Detailed bioinformatic comparisons of equivalent transcript profiling data sets from *C. albicans, S. cerevisiae* and *S. pombe* strongly suggested that the core stress response in *C. albicans* is significantly smaller than the corresponding responses in the model yeasts;

however, some groups of the core stress genes were the same (Enjalbert *et al.*, 2006). Similarly, analysis of the stress induced proteome in *C. albicans* also uncovered a small core stress response; notably, however, the core stress proteins identified in the proteomic studies were not replicated in the transcript profiling analysis (Yin *et al.*, 2009).

In *S. cerevisiae* the core stress response is mainly regulated by the transcription factors Msn2 and Msn4 (Causton *et al.*, 2001). However, whilst *C. albicans* has a single Msn2/4 homologue, CaMsn4, the function of this protein has diverged (Ramsdale *et al.*, 2008). For example, *C. albicans msn4*Δ cells do not show increased sensitivity to heat shock, osmotic or oxidative stress and play no role in the transcriptional responses to these stresses (Nicholls *et al*., 2004). In contrast, *S. pombe* regulates its core stress response through the Sty1 SAPK and the Sty1 regulated transcription factor, Atf1 (Chen *et al.*, 2003). Similar to the Sty1 SAPK in *S. pombe*, the *C. albicans* Hog1 SAPK is activated in response to diverse stresses, such as oxidative, osmotic and heavy metal stress, and expression of *HOG1* can complement the stress sensitive phenotypes of *S. pombe sty1*$^{-}$ cells (Smith *et al.*, 2004). Furthermore, the presence of Hog1 is essential for the observed stress cross-protection in *C. albicans* (Smith *et al.*, 2004). However, despite these observations, the contribution of Hog1 in regulating the core stress responses in *C. albicans* is not as significant as the role of Sty1 in regulating the analogous response in *S. pombe*. For example, whilst Hog1 is important for the regulation of core stress genes in response to osmotic and heavy metal stress in *C. albicans*, Hog1 is dispensable for the oxidative stress induced expression of such genes (Enjalbert *et al.*, 2006). The reason underlying this is unclear as Hog1 is robustly phosphorylated and accumulates in the nucleus following oxidative stress (Smith *et al*., 2004). Furthermore, deletion of *C. albicans HOG1* results in strains which are more sensitive to oxidative stress (Alonso-Monge *et al.*, 2003 and Smith *et al.*, 2004). The role of Hog1 in the oxidative stress response in *C. albicans* is discussed in more detail in section 1.4.3.2 of this chapter.

1.2.4.2.Stress Responses in the Host

Significant insight into the exact nature of the various microenvironments that *C. albicans* occupies in its mammalian host, and hence the nature of the stresses this microbe encounters *in vivo*, has been provided by microarray experiments which has determined the transcript profiles of *C. albicans* in various *in vivo* and *ex vivo* infection models.

The route of infection used by *C. albicans* cells to disseminate to other organs in the host is via the bloodstream, and hence the transcript profile of *C. albicans* following exposure to whole human blood has been reported (Fradin *et al.*, 2003). Exposure of *C. albicans* cells to blood results in the induction or repression of more than 200 genes within the first 10 minutes, indicating that *C. albicans* responds quickly to this environmental change. With regard to stress responses, genes that are involved in the oxidative stress (*SOD1, CAT1, IPF2431* and *TRR1*), heat shock (*SSA4* and *SSB1*), alkaline (*PRA1* and *PHR1*) and nutritional stress responses (glyoxylate and glycolytic enzymes) were among the genes that were significantly up-regulated in response to blood (Fradin *et al.*, 2003). A subsequent study, which examined the *C. albicans* transcriptional response to different blood fractions, demonstrated that the neutrophil sub-fraction was largely responsible for the initiation of such stress responses upon exposure to blood (Fradin *et al*., 2005).

Consistent with these findings, independent studies which examined the transcriptional response of *C. albicans* following phagocytosis by either macrophages or neutrophils, also found that genes involved in nutritional stress and oxidative stress are induced (Lorenz *et al.*, 2004 and Rubin-Bejerano *et al*., 2003). However, there were some notable differences between the stress responses activated by these distinct cells of the innate immune system. For example, while macrophages only induced a carbohydrate starvation response; neutrophils induced a carbohydrate and amino acid starvation response (Lorenz *et al.*, 2004 and Rubin-Bejerano *et al*., 2003). For example, genes induced in *C. albicans* cells ingested by neutrophils were also induced in cells grown in medium without amino acids. Amino acid starvation response is characterized by induction of genes involved in the methionine and arginine biosynthetic pathways, such as *MET1, MET3, MET10, MET14, MET15, ARG1* and *ARG5* (Rubin-Bejerano *et al*., 2003). During the carbohydrate starvation response there was a switch from glycolysis to gluconeogenesis with repression of the glycolytic enzymes (*PFK1* and *PFK2*) and induction of the glyoxylate cycle enzymes (*ICL1, ACO1* and *MLS1*) that are responsible for synthesizing the gluconeogenic precursor oxaloacetate that allows the fungal pathogen to grow on acetate or fatty acids as the sole carbon sources (Lorenz *et al.*, 2004, Rubin-Bejerano *et al*., 2003 and Fernández-Arenas *et al*., 2007).

In addition, consistent with the notion that neutrophils generate a larger respiratory burst than macrophages (reviewed in Forman and Torres, 2001 and Dale *et al.*, 2008**)**, a larger number of antioxidant genes were induced following phagocytosis by neutrophils than

macrophages. For example, the oxidative stress responsive genes *CAP1, CTA1, GPX1, GST3, TRR1* and *TRX1* were induced in response to neutrophils (Rubin-Bejerano *et al*., 2003), whereas *C. albicans* exposed to macrophages resulted in the increased expression of fewer antioxidants (*TRR1*, *GST1* and *GPX1*); however, several DNA repair genes were induced in *C. albicans* cells exposed to macrophages (Lorenz *et al.*, 2004; Fradin *et al.*, 2005 and Fernández-Arenas *et al.*, 2007). These results indicate that cells are exposed to more oxidative stress upon phagocytosis by neutrophils than macrophages, and also indicate that phagocytosis can trigger an oxidative stress response similar that seen by treating *C. albicans* cells with H_2O_2 *in vitro* (Enjalbert *et al.*, 2003 and Enjalbert *et al.*, 2006). For example, treatment of *C. albicans in vitro* with different H_2O_2 concentrations induces the up-regulation of different sets of genes (See Section 1.2.4.1.2; Enjalbert *et al.*, 2003 and Enjalbert *et al.*, 2006), and consistent with macrophages generating low levels of oxidative stress, the induction of DNA repair genes was observed following macrophage exposure or treatment with low levels of H_2O_2 (Lorenz *et al.*, 2004 and Enjalbert *et al*., 2003). Furthermore, single cell profiling experiments of *C. albicans* cells exposed to different phagocytic cells also indicated that phagocytosis by neutrophils but not macrophages induced the expression of the antioxidant reporter genes *CTA1, TRX1* and *GRX2* (Enjalbert *et al.*, 2007).

In addition to examining the transcript profiles of *C. albicans* cells in response to blood exposure or following phagocytosis, experiments have been performed to examine the transcriptional response of this fungus in different infection models of candidiasis. For example, using a model of mucosal infection there was evidence of increased expression of genes involved in the adaptive response to carbon and nitrogen deprivation, nitrosative stress and to alkaline pH (Zakikhany *et al.*, 2007). For example, marker genes of the nitrosative stress response *in vitro*, such as *YHB1, YHB5* and *SSU1* (Hromatka *et al*., 2005) were also up-regulated when *C. albicans* cells were exposed to epithelial cells (Zakikhany *et al.*, 2007). In addition to nitrosative stress, prolonged contact with epithelial cells also results in up-regulation of *C. albicans* alkaline pH-responsive genes (*PHR1*, *PRA1*) indicating that an increase in pH is also seen in the late phases of epithelial infection (Zakikhany *et al.*, 2007).

Transcription profiling of *C. albicans*, during peritoneal infection or subsequent invasion of the liver, also indicated that the ability of *C. albicans* to sense and respond to pH is also important for this fungus to survive and invade the tissues under these experimental conditions (Thewes *et al.*, 2007). Stress responses were also important in the course of liver

and peritoneal infection, in that heat-shock proteins such as, *HSP78, HSP90, HSP104, HSP12* or *SSA4*, and the stress related protein, *DDR48* were upregulated during the infection process (Thewes *et al.*, 2007). In addition, during tissue penetration, cells increased the expression of iron acquisition genes (Thewes *et al.*, 2007). In a separate study, in which the transcript profiles of *C. albicans* were determined during kidney infection, the stress adaptation genes (*CTA1* and *ENA22*) were also up-regulated (Walker *et al.*, 2009). Notably, whilst different niches clearly evoke different transcriptional responses in *C. albicans*, a starvation response appears to be common at various stages of infection. For example, upon kidney invasion, peritoneal invasion, contact with human mucosal cells, or phagocytosis the up-regulation of genes involved in alternative carbon metabolism pathways and fatty acid degradation, indicative of a starvation response, is seen (Zakikhany *et al.*, 2007, Thewes *et al.*, 2007 and Walker *et al.*, 2009).

In summary, data from several transcript profiling experiments have illustrated that stress responses are evoked during infection. Moreover, it is apparent that distinct stress responses are stimulated depending on the particular infection site occupied by *C. albicans*. For example, an oxidative stress response is only robustly activated following exposure to blood or cells of the innate immune system. This, together with the attenuated virulence exhibited by several key antioxidant enzymes and oxidative stress signalling proteins (see Section 1.2.4), reinforces the concept that the oxidative stress response is essential for *C. albicans* virulence (Brown *et al.,* 2009). The aim of this thesis is to investigate the mechanisms by which *C. albicans* responds to ROS and hence the topics of oxidative stress and oxidative stress responses will be considered in more detail below.

1.3. The Oxidative Stress Response

1.3.1. Reactive Oxygen Species and Oxidative Stress

During aerobic respiration in eukaryotic cells, oxygen (O_2) is used by the mitochondrial electron transport chain to increase the yield of ATP production. Despite the beneficial effects of using O_2 in ATP production, incomplete reduction of O_2 can lead to the formation of ROS that are highly reactive chemical molecules (Halliwel and Gutteridge, 2007). For example, 1-4% of the O_2 consumed is not completely reduced by the electron transport chain resulting in the generation of the superoxide anion ($O_2^{\bullet -}$), which in turn can form either

hydrogen peroxide (H_2O_2) or the highly reactive hydroxyl radical ($HO^{\bullet}$) (Fridovich, 1999 and Halliwel and Gutteridge, 2007). When concentrations of ROS exceed the antioxidant defences in a cell, this causes an imbalance in the cellular redox state resulting in oxidative stress. Oxidative stress can produce irreversible damage to the major cellular constituents, proteins, lipids and DNA, and ultimately lead to cell death (Halliwell and Gutteridge, 2007).

1.3.1.1. The Superoxide Anion

The superoxide anion ($O_2^{\bullet -}$) is formed in virtually every type of aerobic cell and is the major ROS generated by the mitochondria. $O_2^{\bullet -}$ is formed as a result of a one electron reduction of O_2, by the components of the electron transporter chain during ATP synthesis (Murphy, 2009).

$$\mathbf{O_2 + e^- \rightarrow O_2^{\bullet -}}$$

$O_2^{\bullet -}$ is extremely unstable and at physiological pH can undergo a number of reactions to form highly reactive intermediates, such as $HO^{\bullet}$ (see below). In addition, it reduces metallic ions in proteins, therefore increasing the availability of metallic ions that can participate in the Fenton reaction (see below). Furthermore, it can also react with nitric oxide (NO) to generate the potent oxidant peroxynitrite, a major cytotoxic agent generated within the phagosome (See Section 1.3.1.4).

1.3.1.2. Hydrogen Peroxide

As described above $O_2^{\bullet -}$ is very reactive and quickly dismutates to form hydrogen peroxide (H_2O_2) and molecular O_2. This occurs more efficiently at low pH and can be catalysed by superoxide dismutase (SOD) enzymes (Fridovich, 1998 and Halliwell and Gutteridge, 2007):

$$\mathbf{2O_2^{\bullet -} + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2}$$

In addition to superoxide dismutase H_2O_2 is also frequently produced by several oxidases *in vivo*, such as NADPH, xanthine and glucose oxidase (Brown *et al.*, 1988 and Gow *et al.*, 1999). H_2O_2 is a membrane permeable ROS and can act as a signalling molecule (Halliwell

and Gutteridge, 2007 and Forman, 2007) and its role in signal transduction will be considered in more detail in section 1.4.4.

1.3.1.3. The Hydroxyl Radical

The hydroxyl radical ($HO^{\bullet}$) is one of the most reactive ROS. This can be generated via a number of distinct reactions including the Fenton reaction involving H_2O_2 and metal ions, the Haber-Weiss reaction involving H_2O_2 and $O_2^{\bullet -}$, or via UV-induced breakage of H_2O_2 or by exposure of water to ionizing radiation (IR) (Halliwel and Gutteridge, 2007):

$$\mathbf{Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + HO^{\bullet}}$$

Fenton reaction

$$\mathbf{2\ O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + OH^- + HO^{\bullet}}$$

Haber-Weiss reaction

$$\mathbf{H_2O_2 \xrightarrow{UV} 2OH^{\bullet}}$$

$$\mathbf{H_2O \xrightarrow{IR} H^{\bullet} + OH^{\bullet}}$$

Furthermore, the hydroxyl radical is a very unstable compound and although has a short half live it can rapidly oxidize cellular compounds like proteins, DNA and lipids (see section 1.3.2) (Czapski, 1984 and Halliwell and Gutteridge, 2007).

1.3.1.4. Generation of ROS by Phagocytes

One of the major mechanisms used by the host`s immune system to eliminate an infectious agent is to produce highly toxic compounds, such as ROS, that can lead to irreversible damage and eventually death of the pathogen (Bogdan *et al.*, 2000 and Nathan and Shiloh, 2000). Macrophages, neutrophils and other phagocytic cells, for example, can eliminate *C. albicans* by using oxidative mechanisms and such cells play a key role in protecting the host against *C. albicans* systemic infections (Fulurija *et al.*, 1996, Qian *et al.*, 1994, Jensen *et al.*, 1994 and Vonk *et al.*, 2006).

An overview of the ROS, and ROS-derived toxic compounds, generated within the phagosome, is illustrated in Figure 1.7. The respiratory burst of such phagocytic cells generates $O_2^{\bullet -}$, by the action of the NADPH oxidase. Under normal pH spontaneous

decomposition of $O_2^{\bullet -}$ can occur, which results in the formation of H_2O_2 and O_2. This reaction occurs through the formation of the radical intermediate hydroperoxyl ($HO_2^{\bullet}$) and occurs faster in acidic environments (Halliwel and Gutteridge, 2007).

$$\mathbf{O_2^{\bullet -} + H^+ \rightarrow HO_2^{\bullet}}$$
$$\mathbf{2\ HO_2^{\bullet} \rightarrow H_2O_2 + O_2}$$

The receptors involved in the induction of the respiratory burst of phagocytic cells are not known, however in response to fungi the ß-glucan receptor, dectin-1, can induce ROS production by NADPH oxidase (reviewed by Netea *et al.*, 2008).

As described in Section 1.3.1.3, both $O_2^{\bullet -}$ and H_2O_2 can subsequently react to form the highly toxic hydroxyl radical $OH^{\bullet}$ (Figure 1.7). Other toxic compounds can be generated in phagocytic cells from ROS such as hypochlorous acid (HOCl) from H_2O_2 by the action of myeloperoxidases (MPO) (reviewed by Dale *et al.*, 2008 and Rada and Leto, 2009):

$$\mathbf{Cl^- + H_2O_2 \xrightarrow{MPO} HOCl + HO^-}$$

In addition, superoxide can react with nitric oxide ($NO^{\bullet}$), which is generated in the phagosome via the action of the enzyme nitric oxide synthase (NOS2), to form the potent cytotoxic agent peroxynitrate ($ONOO^-$; Vázquez-Torres and Balish, 1997):

$$\mathbf{L\text{-arginine} + 1.5NADPH + 1.5H^+ + 2O_2 \rightarrow L\text{-citrulline} + 1.5NADP^+ + 2H_2O + NO^{\bullet}}$$
$$\mathbf{NO^{\bullet} + O_2^{\bullet -} \xrightarrow{NOS2} ONOO^-}$$

Phagocytosis of microorganisms by phagocytic cells, such as neutrophils, results in the formation of a phagosome. These phagosomes are formed when microorganisms bind to phagocytic cells and are engulfed by them. Furthermore, the formation of a phagosome results in increased oxygen consumption and activation of membrane-associated NADPH oxidases complexes that will generate ROS (see reactions above in this section) (reviewed by Dale *et al.*, 2008 and Rada and Leto, 2009). Hence, upon stimulation, phagocytic cells generate around 5-10 mmol/L of $O_2^{\bullet -}$ (Hampton *et al*., 1998).

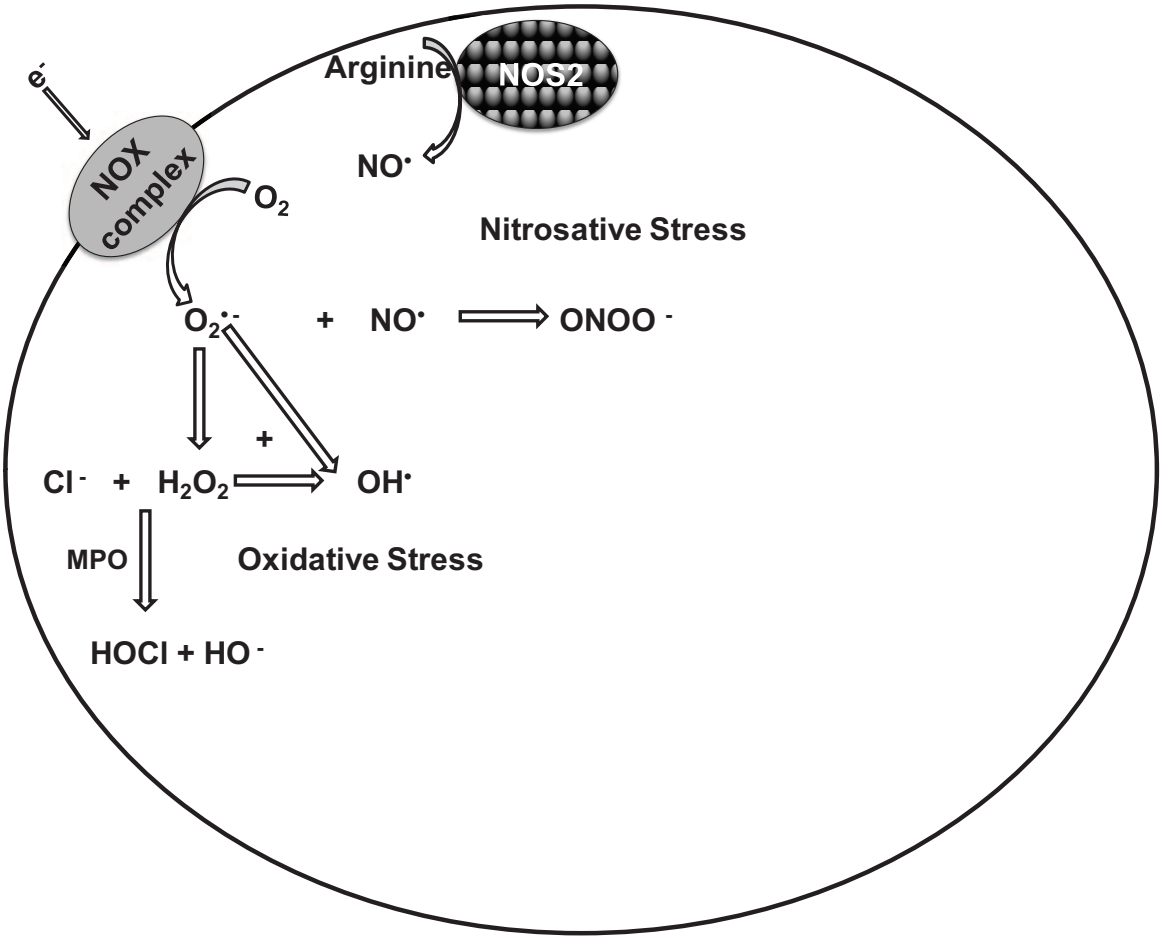


Figure1.7. Generation of ROS and RNS inside the phagosome. Adapted from Brown *et al.*, 2009

Although both neutrophils and macrophages produce ROS, studies have suggested that neutrophils have a stronger candidacidal activity than other phagocytic cells (Cutler and Poor, 1981, Peterson *et al.*, 1986 and Fradin *et al.*, 2005). For example, it was shown that *C. albicans* wild-type cells have higher survival rates when exposed to monocytes when compared with *C. albicans* cells exposed to neutrophils. Furthermore, *C. albicans* strains lacking antioxidants, such as *SOD5* and *CAP1*, have reduced survival in the presence of neutrophils, but not in the presence of monocytes and macrophages (Fradin *et al.*, 2005 and Martchenko *et al*., 2004). A possible hypothesis for the higher mortality rate seen in neutrophils when compared with other phagocytic cells is that higher levels of ROS are generated by neutrophils than macrophages. For example, a stronger transcriptional response to oxidative is evoked in *C. albicans* upon neutrophil exposure compared to macrophages (see Section 1.2.4.2). Nonetheless, there is strong evidence that ROS production by various cell types of the innate immune system is central to the candicidal mechanisms employed by the host. For example, inhibition of the NADPH oxidase enzyme, by inhibition of an essential NADPH subunit required for ROS production, in macrophages and dendritic cells resulted in increased survival of *C. albicans* cells following phagocytosis (Frohner *et al.*, 2009). A similar situation is seen in patients with Chronic Granulomatous Disease which have congenital defects in the NADPH oxidase complex, and are more susceptible to microbial infections, including candidiasis (reviewed in Rada and Leto, 2008). Conversely, inactivation of the GPI-anchored extracellular Sod4 and Sod5 enzymes in *C. albicans*, which have been shown to actively lower the levels of superoxide within the phagosome, severely impairs survival of this fungal pathogen following phagocytosis (Frohner *et al.*, 2009).

1.3.2. Oxidative damage

ROS can cause damage to several cellular structures and organelles, by reacting with nucleic acids, lipids and proteins and this will be described in more detail below.

1.3.2.1. Lipids

Damage to lipids due to lipid peroxidation can modify membrane fluidity, so that cells lose control of membrane permeability, and inactivation of proteins that are bound to membranes may result (Halliwell and Gutteridge, 2007). The initiation of lipid peroxidation

occurs by removal of a hydrogen atom from a methylene group (CH_3OH or RCH_2R) of the lipid, resulting in the formation of a carbonyl radical ($C^{\bullet}$).

$$C{=}C + OH^{\bullet} \rightarrow C^{\bullet}\text{-}C(OH)$$

$$CH_2 + OH^{\bullet} \rightarrow CH^{\bullet} + H_2O$$

The main lipid members found in the membranes of living organisms are polyunsaturated fatty acids (PUFAs) and phospholipids, which are readily oxidized when exposed to ROS. However, PUFA hydroperoxides have been shown to be more toxic than phospholipid hydroperoxides (Kaneko *et al.*, 1996). Once PUFAs are oxidized resulting in the formation of a carbonyl radical ($R^{\bullet}$), this reacts with molecular O_2 resulting in the production of the extremely reactive fatty acid hydroperoxyl radical ($ROO^{\bullet}$). This abstracts hydrogen from adjacent fatty acid groups, resulting in the formation of fatty acid hydroperoxide (ROOH), and thus self-perpetuating lipid peroxidation:

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + CH \rightarrow ROOH + C^{\bullet}$$

In this way, formation of a single fatty acid hydroperoxyl radical can subsequently generate many fatty acid hydroperoxide molecules in a chain reaction, resulting in extensive damage to the cell membrane (Halliwell and Gutteridge, 2007 and Kaneko *et al.*, 1996).

1.3.2.2. Proteins

Proteins can be oxidized either directly by reaction with ROS or by a reaction involving the products of lipid peroxidation. Protein oxidation can occur either in the polypeptide backbone which can result in fragmentation of the polypeptide or in the amino acid side chains, which can result in inactivation of the protein.

In particular, thiol groups are easily oxidized by ROS. The sulphur groups of cysteine residues can be progressively oxidized resulting in a range of cysteine thiol oxidation states, to sulphenic, sulphinic and even sulphonic forms:

$$RSH \rightarrow e^- + RSH^- \text{ (thiolate anion)}$$

$$RSH^- + H_2O_2 \rightarrow OH^- + RSOH^- \text{ (sulphenic acid)}$$

$$RSOH^- + RSH \rightarrow RSSR + H_2O \text{ (dissulphide bond)}$$

$$RSOH^- + H_2O_2 \rightarrow RSOOH + H_2O \text{ (sulphinic acid)}$$

$$RSOOH^- + H_2O_2 \rightarrow RSOOOH + H_2O \text{ (sulphonic acid)}$$

The oxidation of cysteine residues to their sulphenic form can promote inter- or intra-molecular disulphide bonds, and although damaging, some are exploited as a form of redox regulation of protein function. Oxidation of catalytically active cysteine residues can also lead to the inactivation of some proteins such as peroxiredoxins (Prxs) and protein-tyrosine phosphatases (PTPs); although again in some instances this is exploited by proteins involved in the regulation of oxidative stress signaling (Imlay, 2003, Costa *et al.*, 2007, Veal *et al.*, 2007 and Forman *et al.*, 2010). The role of cysteine oxidation states in oxidative stress signaling will be discussed in more detail in section 1.4.4.1 and 1.4.4.2.

ROS can cause damage to several other amino acid residues. For example, tryptophan residues react with the $OH^{\bullet}$ radical forming several products, such as N-formylkynurenine, which can be found in sites where ROS are constitutively produced such as the mitochondria (Halliwell and Gutteridge, 2007). In addition carbonyl derivatives are formed following the oxidation of residues such as lysine, arginine, proline and threonine. Whilst some classes of oxidized protein can be repaired, for example proteins carrying cysteine sulphinic and sulphenic derivatives (Section 1.4.2.4), if amino acid side chains are carbonylated the protein has to be degraded and replaced (Halliwell and Gutteridge, 2007). ROS can also attack the active sites of metal containing enzymes. For example, the superoxide radical, $O_2^{\bullet-}$ oxidizes 4Fe-4S clusters in enzymes, such as mitochondrial aconitase, resulting in inactivation of the protein and thus inhibition of the tricarboxylic acid pathway (Imlay, 2003 and Cabiscol *et al.*, 2000). In addition, the free iron released during this reaction promotes the formation of hydroxyl radicals (via the Fenton reaction) that can cause further molecular damage.

$$[4Fe\text{-}4S]^{+2} + O_2^{\bullet-} + 2H^+ \rightarrow [4Fe\text{-}4S]^{+3} + H_2O_2$$

$$[4Fe\text{-}4S]^{+3} \rightarrow [3Fe\text{-}4S]^{+1} + Fe^{+2}$$

1.3.2.3. DNA

In addition to lipids and proteins, nucleic acids are also susceptible to ROS-induced damage. Base modification, damage to deoxyriboses and abasic sites can be induced by ROS, resulting in base transversions and single or double strand breaks, which are mutagenic and can cause replication errors, genomic instability and cell death (Sigler *et al.*, 1999). While the $OH^{\bullet}$ radical can react directly with either bases or sugars in DNA, forming a range of lesions (Barzilai and Yamamoto, 2004); H_2O_2 does not seem to interact directly with DNA. Instead, H_2O_2-induced DNA damage is thought to be indirect via the $OH^{\bullet}$ radical, that is produced by H_2O_2 and metal ions through the Fenton Reaction (see reaction in section 1.3.1.3 above) (Halliwell and Gutteridge, 2007). Significantly, metal ions localize along the phosphodiester backbone and thus are readily available to generate $OH^{\bullet}$ from H_2O_2.

One of the most studied base damage adducts is 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxoG), which results from the reaction of $OH^{\bullet}$ with the C8 of purine bases. 8-oxoG bases are mainly found in highly transcribed regions in the DNA and results in GC→TA transversions, due to mispair of 8-oxoG with adenine instead of cytosine (Kasai, 2002). This mispair generates point mutations and thus can result in a mistranslated protein (Kasai, 2002, Imlay, 2003 and Evans *et al.*, 2003).

1.4. Protective mechanisms against ROS

The oxidative stress response comprises of non-enzymatic defences, which involves free radical scavengers that can bind and detoxify ROS, and enzymatic defences, which can remove ROS by catalytic mechanisms. Free radical scavengers that provide non-enzymatic defences include ascorbate, glutathione and tocopherols, and enzymatic defences involve the function of enzymes such as catalase, superoxide dismutase, glutathione peroxidases and thioredoxin peroxidases. Both these antioxidant defences will be considered below (Sigler *et al.*, 1999).

1.4.1. Non-enzymatic defences

Non-enzymatic defences are provided by small molecules that can act as ROS scavengers and proteins that sequester metallic ions that participate in ROS generation (Sigler *et al.*,

1999). In the next section, the function of these components in oxidative stress defence is briefly explained.

1.4.1.1. Glutathione

One of the best studied non-enzymatic antioxidants is the tripeptide glutathione (γ-GluCysGly). This tripeptide thiol-containing molecule is highly abundant in cells and can act as an antioxidant by directly reacting with ROS, protecting other proteins against irreversible oxidation (see protein glutathionylation below) or by acting as a reducing agent during the reduction of antioxidant enzymes (see sections 1.4.2.3 and 1.4.2.4) (Grant *et al.*, 2001 and López-Mirabal and Winther, 2008). Glutathione can be found in the reduced (GSH) or oxidized form (GSSG). GSH can react with ROS, such as H_2O_2, which results in the formation of GSSG that in turn is reduced to GSH by the enzyme glutathione reductase (GR) at the expense of NADPH.

$$\mathbf{2GSH + H_2O_2 \rightarrow GSSG + 2H_2O}$$

$$\mathbf{NADPH + H^+ + GSSG \xrightarrow{GR} 2GSH + NADP^+}$$

The importance of GSH in the protection of cells against oxidative stress is seen *in vivo*. For example, studies have shown that in the model yeast *S. cerevisiae*, H_2O_2-induced oxidative stress results in a reduction of GSH levels that results in a decrease in the GSH/GSSG ratio (Grant *et al.*, 1998 and Grant, 2001). In addition, inhibition of *S. cerevisiae* gamma-glutamylcysteine synthetase (Gsh1), which synthesises GSH, resulted in increased H_2O_2 sensitivity in both liquid and solid media, as do cells lacking glutathione reductase (Izawa *et al.*, 1995, Muller *et al.*, 1996, Spector *et al.*, 2001 and Trotter and Grant, 2003). In addition, high levels of GSH are required for survival in response to treatment with lethal peroxide concentrations (Spector *et al.*, 2001).

GSH is also used as a reductant of antioxidants such as glutaredoxins, glutathione peroxidases and 1-Cys peroxiredoxins (Grant, 2001, and Greetham and Grant, 2009). In the model yeast *S. cerevisiae*, glutaredoxins (Grxs) can act as peroxidases reacting directly with H_2O_2 at the expense of NADP and GSH (Collinson *et al.*, 2002).

$$\mathbf{Grx_{ox} + 2GSH + NADP^+ \rightarrow Grx_{red} + GSSG + NADPH + H^+}$$

In addition, the redox state of total Grx is determined by the GSSG/GSH ratio. This is illustrated by experiments that show that Grxs are found mainly in an oxidized state in *S. cerevisiae glr1*Δ cells, which is consistent with the increased GSSG/GSH ratio found in these cells and the role of reduced GSH in the reduction of oxidized Grx (Trotter and Grant, 2003).

GSH can also be found in a conjugated form with proteins (GSSP). GSSP are formed as a result of the disulphide bond formed between proteins and glutathione. This mechanism, called protein glutathionylation, is used by cells to protect cysteine residues from irreversible oxidation that can result in inactivation and degradation of proteins (Shenton and Grant, 2003). For example, *S. cerevisiae* uses glutathionylation to protect glycolytic enzymes, such as Tdh3, enolase and alcohol dehydrogenase, from irreversible oxidation in response to H_2O_2 treatment (Grant *et al.*, 1999 and Shenton and Grant, 2003). Even though the enzymatic activity of these glycolytic enzymes is inhibited in response to H_2O_2, activity is restored when oxidative stress was relieved. Furthermore, little or no restoration of enzymatic activity was seen in *glr1*Δ cells indicating that GSH plays a key role in this process (Grant *et al.*, 1999 and Shenton and Grant, 2003).

GSH is predicted to be an important component of the *C. albicans* oxidative stress response, as *C. albicans GCS1* which encodes gamma-glutamylcysteine synthetase (an enzyme involved in GSH synthesis) is induced in response to H_2O_2 and in cells that are co-cultured with neutrophils (Fradin *et al.*, 2005 and Enjalbert *et al.*, 2006). Furthermore, deletion of *GCS1* or depletion of glutathione (by treatment with diallyl disulphide - DADs) results in ROS accumulation and induction of apoptosis, confirming the protective effect of GSH against oxidative stress and cell death in this fungal pathogen (Baek *et al.*, 2004 and Lemar *et al.*, 2007). Furthermore, deletion of *C. albicans* putative glutaredoxin *GRX2*, which is also induced in response to H_2O_2 and in the presence of neutrophils, results in cells with impaired oxidative stress resistance and increased susceptibility to killing by neutrophils (Chaves *et al.*, 2007).

1.4.1.2. Other non-enzymatic defences

Other low molecular mass compounds can also act as antioxidants. For example, α-tocopherol is an antioxidant molecule that inhibits lipid peroxidation by directly reacting with $ROO^{\bullet}$. In *C. albicans* α-tocopherol can detoxify ROS produced by the mitochondrial respiratory chain (Deveau *et al.*, 2010). In addition, ascorbic acid or vitamin C can also act as

antioxidants. Ascorbic acid generates ascorbate at physiological pH, which in turn can either react directly with ROS, such as $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ (sections 1.3.1.1, 1.3.1.2 and 1.3.1.3), HOCl (section 1.3.1.4) and $ROO^{\bullet}$ (section 1.3.2.1), or react with other antioxidants, such as α-tocopherol (Halliwell and Gutteridge, 2007). Ascorbate acts as an antioxidant cells *in vivo* as illustrated by the decrease in ROS seen in *C. albicans* treated with ascorbic acid plus substances that increase intracellular ROS levels (farnesol and 10% serum at 37ºC) or with H_2O_2 itself (Nasution *et al.*, 2008 and Deveau *et al.*, 2010). In addition, some microorganisms produce D-erythroascorbic acid instead of ascorbic acid, and this five carbon analog of ascorbic acid seem to have similar biological properties as ascorbic acid, including antioxidant functions (Huh *et al.*, 2008). Hence, inactivation of a key enzyme involved in the synthesis of d-erythroascorbic acid in *C. albicans*, results in increased oxidative stress sensitivity in this organism (Huh *et al.*, 2001). Therefore, these other low molecular mass compounds are also involved in the antioxidant defence of *C. albicans* cells.

In addition, the dissaccharide trehalose can also protect cells against a range of stresses in both *S. cerevisiae* and *C. albicans* (Zaragoza *et al.*, 1998 and Benaroudj *et al.*, 2001). In *C. albicans*, for example, accumulation of trehalose protects the cells against oxidative stress and deletion of enzymes involved in trehalose biosynthesis (*TPS1* and *TPS2*) resulted in increased sensitivity to oxidative stress (Zaragoza *et al.*, 1998, Alvarez-Peral *et al.*, 2002 and Martinez-Esparza *et al.*, 2009). Trehalose antioxidant properties can be explained by either direct reaction with ROS or by regulation of ROS generation by decreasing activity of the mitochondrial respiratory chain (Cao *et al*., 2008).

In addition to low molecular mass compounds, small cysteine-rich proteins such as metallothioneins also act as antioxidants. These proteins bind free metal ions, such as Cu^{+} and Fe^{+2}, decreasing availability of these metals for the Fenton reaction (section 1.3.1.3, Jamieson, 1998). Metallothionein genes (*CUP1* and *CRD2*) have been identified in *C. albicans* (http://www.candidagenome.org/) however their role in oxidative stress protection in this organism remains to be clarified.

1.4.2. Enzymatic defences

In addition to non-enzymatic defences, cells also have an arsenal of antioxidant enzymes that can detoxify ROS. Superoxide dismutases, catalases, glutathione peroxidases, peroxiredoxins and thioredoxin/thioredoxin reductase compose this enzymatic arsenal

(Jamieson, 1998). In the following section, their role in detoxifying ROS and how they are regulated in response to oxidative stress will be discussed.

1.4.2.1. Superoxide dismutases

Superoxide dismutases (SODs) catalyze the dismutation of $O_2^{\bullet-}$. This reaction requires a redox active metal ion and results in the production of H_2O_2 (Fridovich, 1998). Cells usually expresses two distinct SODs, a cytoplasmatic SOD (CuZnSOD) that use copper and zinc as cofactors in its active site and a mitochondrial SOD (MnSOD) that uses manganese instead.

$$\textbf{Enzyme-Cu}^{+2} + O_2^{\bullet-} \rightarrow \textbf{Enzyme-Cu}^{+} + O_2$$
$$\textbf{Enzyme-Cu}^{+} + O_2^{\bullet-} + 2H^{+} \rightarrow \textbf{Enzyme-Cu}^{+2} + H_2O_2$$
$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^{+} \rightarrow H_2O_2 + O_2 \textbf{ (net reaction)}$$

In *S. cerevisiae*, the CuZnSOD, Sod1, is localized both in the cytosol and in the mitochondrial intermembrane space, while the MnSOD, Sod2, is localized in the mitochondria matrix. The key role of SODs in protecting cells against oxidative stress in this model yeast was characterized by deletion of SOD genes. Deletion of *SOD1* in the model yeast *S. cerevisiae* results in strains that are hypersensitive to oxidants, such as H_2O_2 and $O_2^{\bullet-}$ generating drugs (paraquat and menadione) (Gralla and Valentine, 1991, Outten *et al.*, 2005 and Auesukaree *et al.*, 2009). Furthermore, in *S. cerevisiae* both Sod1 and Sod2 play a key role in the defence against intracellular $O_2^{\bullet-}$ generated in the mitochondria. For example, *sod1*Δ and *sod2*Δ null mutants cannot grow under aerobic conditions and are extremely sensitive to growth under hyperoxia conditions (Longo *et al.*, 1996 and Outten *et al.*, 2005). In addition, deletion of *SOD1* results in lower NADPH levels under aerobic conditions, which results in an inability to grow under aerobic conditions and to maintain the redox state of cells (Slekar *et al.*, 1996). Furthermore, *S. cerevisiae* GS129 strain, in which the mitochondrial RNA polymerase is mutated, displays abnormal respiration under nutrient-limited conditions that results in increased ROS production and cell death. Interestingly, overexpression of *SOD2* rescues GS129 defective phenotypes, indicating that this mitochondrial enzyme is associated with detoxification of $O_2^{\bullet-}$ generated in the mitochondria (Bonawitz *et al.*, 2006).

Remarkably, *C. albicans* has 6 SOD isoforms: the cytosolic enzymes Sod1 (CuZn) and Sod3 (Mn), the mitochondrial Sod2 (Mn) and the extracellular enzymes Sod4-6 (CuZn).

Similarly to model yeast, deletion of *C. albicans SOD1* also generates strains that show a slow growth phenotype and increased sensitivity to the superoxide generator, menadione (Hwang *et al*., 2002) and deletion of the mitochondrial *SOD2* results in cells that are more sensitive to growth under hyperoxia conditions and to oxidative stress (menadione and paraquat) (Hwang *et al*., 2003). In addition, *C. albicans* also expresses a third intracellular SOD, Sod3, which is induced during stationary phase. This increased expression of Sod3 coincides with repression of *SOD1* gene expression, and could indicate that this protein is involved in both ROS protection and control of the harmful effect of copper (Lamarre *et al.*, 2001). In addition to the three intracellular SODs, *C. albicans* also express three CuZn GPI-anchored SODs (Sod4-6). Conditions which increase ROS, such as treatment with $O_2^{\bullet-}$ generators (menadione and riboflavin), also induced expression of *SOD5* and deletion of this gene resulted in decreased growth in nutrient limiting media plus H_2O_2 (Martchenko *et al.*, 2004). The presence of an arsenal of SODs in *C. albicans* can be explained by the necessity of this organism to detoxify the $O_2^{\bullet-}$ generated by the NADPH oxidases of phagocytic cells. Indeed as said in topic 1.3.1.4, Frohner and collaborators showed that these extracellular SODs, in particular Sod4 and Sod5, contribute to the defense of *C. albicans* against the oxidative burst generated by phagocytic cells. Deletion of these genes results in increased macrophage killing only when phagocytic cells are expressing the Nox enzymes (Frohner *et al.*, 2009). Furthermore, *sod5*Δ cells also display reduced virulence in the mouse model of systemic infection, suggesting that its role in protecting *C. albicans* cells against oxidative stress generated by phagocytic cells is important during the course of a systemic infection (Martchenko *et al.*, 2004, Fradin *et al.*, 2005 and Frohner *et al.*, 2009).

1.4.2.2. Catalases

Catalases are homotetramic, heme-containing enzymes that reduce H_2O_2 resulting in O_2 and H_2O. Catalase has one heme (iron) group embedded in the catalytic site of each catalase monomer and the presence of iron in these heme groups allows the enzyme to react directly with H_2O_2. The proposed reaction is believed to happen in two stages and is described below.

$$\textbf{Catalase-Fe}^{+3} + \textbf{H}_2\textbf{O}_2 \rightarrow \textbf{Catalase-Fe}^{+4} + \textbf{H}_2\textbf{O}$$

$$\textbf{Catalase-Fe}^{+4} + \textbf{H}_2\textbf{O}_2 \rightarrow \textbf{Catalase-Fe}^{+3} + \textbf{H}_2\textbf{O} + \textbf{O}_2$$

$$\textbf{(Net reaction) } 2\textbf{H}_2\textbf{O}_2 \rightarrow \textbf{O}_2 + 2\textbf{H}_2\textbf{O}$$

The model yeast *S. cerevisiae* expresses two catalases, Cta1 that is expressed in the mitochondria and peroxisome and Ctt1 that is expressed in the cytosol (Huh *et al.*, 2003). Both catalases are important for H_2O_2 detoxification and deletion of *S. cerevisiae CTA1* and/or *CTT1* results in strains that are more sensitive to oxidative stress (H_2O_2 and PUFAs) than wild-type cells (Izawa *et al.*, 1996 and Cipak *et al.*, 2008). This increased mortality can be a result of protein oxidation caused by ROS, since catalase protects proteins from oxidation under oxidative metabolism conditions in *S. cerevisiae* (Lushchak and Gospodaryov, 2005).

In addition to model yeasts, the role of catalase has also been assessed in pathogenic fungi such as *Cryptococcus neoformans*, *Aspergillus fumigatus* and *C. glabrata*. *C. neoformans* has four catalase homologues; however only Cat1 activity was detected *in vitro*, since in a *cat1*Δ strain no catalase activity was detected. Furthermore, neither the *cat1*Δ strain nor the strain that the four catalase genes were deleted (*cat1/cat2/cat3/cat4*Δ) display an oxidative stress sensitivity phenotype (Giles *et al.*, 2006). The lack of an oxidative stress phenotype in these *C. neoformans* strains suggests that other antioxidants defences might be acting alongside catalase against oxidative stress and might be compensate for the lack of catalases in this organism. *A. fumigatus* has 3 catalases and they are differentially expressed depending of its morphological form. While CatA is expressed when this fungus is growing as conidia, Cat1 and Cat2 are expressed in *A. fumigatus* cells grown as mycelia. Deletion of CatA or Cat1 and Cat2 resulted in strains that are more sensitive to H_2O_2, indicating that, in contrast to that seen in *C. neoformans*, the catalases are important for detoxifying H_2O_2 in *A. fumigatus* (Paris *et al.*, 2003).

On the contrary, two of the most common *Candida* species, *C. albicans* and *C. glabrata*, have only one catalase gene, *CAT1* and *CTA1*, respectively. Deletion of *C. glabrata CTA1* results in cells that are extremely sensitive to H_2O_2; however the *cta1*Δ strain is still virulent in the mouse model of systemic infection (Cuéllar-Cruz *et al.*, 2008). Similarly, *C. albicans cat1*Δ cells also showed increased sensitivity to peroxide treatment (Wysong *et al.,* 1998 and Nakagawa *et al.*, 2003). Furthermore, treatment of wild-type and *cat1*Δ cells with a dye that binds to ROS and therefore allows the quantification of ROS in cells, indicates that deletion of *CAT1* resulted in cells that show decreased capacity to detoxify ROS (Nasution *et al.*, 2008). In addition, *CAT1* expression is increased when *C. albicans* cells are phagocytised by neutrophils (Enjalbert *et al.*, 2007) and this could explain why *cat1*Δ cells are less virulent in

a mouse model for systemic candidiasis (Wysong *et al.,* 1998 and Nakagawa *et al.*, 2003). In addition to its role in protecting cells against oxidative stress, catalase is also important for other processes in *C. albicans*, such as filamentation (Nasution *et al.*, 2008).

1.4.2.3. Glutathione Peroxidases

Glutathione peroxidases (GPxs) reduce H_2O_2 to water by using GSH as a reducing agent to regenerate oxidized Gpx back to reduced GPx (Herrero *et al.*, 2008). Mammalian GPxs usually contain a selenocysteine residue in its active site that becomes oxidized during the detoxification of H_2O_2. Selenocysteines have a similar structure to cysteine; however they have a selenium atom in the place of the sulphur amino acid present in cysteine. The advantage of selenocysteine is related to its lower pKa, which results in a higher reduction potential compared to that of cysteine residues. In addition to H_2O_2, GPxs also reduce a range of other organic peroxides, such as unsaturated fatty acid hydroperoxides or alkyl hydroperoxides (Gaber *et al.*, 2001).

$$\mathbf{H_2O_2 + 2GSH \xrightarrow{Gpx} GSSG + 2H_2O}$$

The model yeast *S. cerevisiae* expresses three glutathione peroxidases (Gpx1, Gpx2 and Gpx3). However, in contrast to the mammalian enzymes, all Gpxs in this yeast have cysteine in their active sites instead of selenocysteine. Only deletion of *GPX3* results in hypersensitivity to peroxide stress (H_2O_2 and tert-butyl hydroperoxide). This can possibly be explained by enzymatic activity assays that indicated that Gpx3 provides the major contribution to the glutathione peroxidase activity in *S. cerevisiae* (Inoue *et al.*, 1999). However, as discussed in Section 1.4.3.1.1, Gpx3 has additional roles in the oxidative stress response, as a sensor and signal transducer of oxidative stress (Inoue *et al.*, 1999 and Delaunay *et al.*, 2002). In order to perform their functions in the oxidative stress response, Gpxs have to be in the active reduced form. In *S. cerevisiae*, the redox status of all Gpxs *in vivo* is mainly regulated by the thioredoxin system, which is composed of thioredoxin and thioredoxin reductase (See sections 1.4.2.4 and 1.4.2.4.2; Delaunay *et al.*, 2002, Tanaka *et al.*, 2005 and Ohdate *et al.*, 2010). Even though thioredoxin instead of GSH is required to reduce *S. cerevisiae* GPxs *in vivo*, GSH can also serve as an electron donor for this class of proteins in the model yeast. For example, GSH can reduce Gpx2 *in vitro*, even thought the efficiency of this reaction is lower than the thioredoxin-dependent reduction of Gpx2

(Tanaka *et al.*, 2005). Furthermore, Gpx1 is reduced by GSH *in vitro* in a reaction as efficient as the one catalyzed by thioredoxin (Ohdate *et al.*, 2010). Gpx3 can also be reduced by GSH. However this only seems to happen when GPx3 catalyzes reduction of other peroxides, such as *t*-BOOH and phospholipid hydroperoxides (Avery and Avery, 2001).

In addition to its roles in the oxidative stress response, Gpx3 is also involved in regulating protein fate in the model yeast *S. cerevisiae*. For example, Gpx3 interacts with the methionine sulfoxide reductase Mxr1, which reduces methionine sulfoxide residues to methionine in proteins, therefore exhibiting a protein repair activity. Mxr1 activity is reduced in a *gpx3*Δ strain, and overexpression of *GPX3* results in decreased protein oxidation similar to that seen upon overexpressing *MXR1* (Kho *et al.*, 2006). Gpx3 was also found to protect glutamine synthetase (GS) from proteolysis in response to H_2O_2 (Lee *et al.*, 2007). The *S. cerevisiae* Gpx2 and Gpx3 enzymes also exhibit phospholipid hydroperoxide glutathione peroxidase activity, which is responsible for reducing lipid peroxidation in biomembranes. Deletion of *GPX2* and/or *GPX3* resulted in strains that showed decreased phospholipid hydroperoxide glutathione peroxidase activity and high mortality when cells were treated with drugs that specifically induced lipid peroxidation (Avery and Avery, 2001).

C. albicans has four ORFs that have been annotated as Gpx-like proteins (Candida Genome Database - www.candidagenome.org). Although no phenotypic analysis of strains lacking these potential Gpx-like proteins has been reported, *GPX1* transcripts and Gpx1 protein levels have been shown to increase in response to H_2O_2 and following macrophage interaction (Enjalbert *et al.*, 2006, Kusch *et al.*, 2007, Fernández-Arenas *et al.*, 2007 and Yin *et al.*, 2009).

1.4.2.4. The peroxiredoxin-thioredoxin system

Peroxiredoxins (Prx) are ubiquitous peroxidases which are involved in the removal of peroxides at the expense of thiols. With all peroxiredoxin enzymes, the first step involves reaction of the conserved peroxidatic cysteine (Cys-SH) with the peroxide substrate (ROOH). This reaction results in the oxidation of the peroxidatic cysteine to a cysteine sulfenic acid (Cys-SOH).

Prx-SH + ROOH → Prx-SOH + ROH

Different mechanisms are in place to recycle the oxidized peroxidatic cysteine of peroxiredoxins, which depends on the type of Prx. Prxs can be classified either as 1-Cys or 2-Cys peroxiredoxins, depending on the number of catalytic cysteines used in the reduction of peroxides. Reduction of the oxidized peroxidatic cysteine is by direct reaction with the thioredoxin or glutaredoxin systems, glutathione or even ascorbic acid (Manevich *et al.*, 2004 and Monteiro *et al.*, 2006). For example, the mammalian 1-Cys Prx, Prdx6, is reduced by a process that requires glutathionylation of the peroxidatic cysteine by the enzyme GSH transferase (πGST) (Manevich *et al.*, 2004). In *S. cerevisiae* a similar process seems to occur, where GSH seems to act as the electron donor alongside thioredoxin reductase (Trr2) to reduce the 1-Cys peroxiredoxin Prx1 in the mitochondria (Greetham and Grant, 2009).

Prx-SH + ROOH → Prx-SOH + ROH

Prx-SOH + GSH → Prx-SSG + H_2O (Protein glutathionylation)

Prx-SSG + $Trr(SH)_2$ → Prx-SSTrr(SH) + GSH

Prx-SSTrr(SH) + 2GSH → Prx-SH + $Trr(SH)_2$ + GSSG

On the other hand, 2-Cys peroxiredoxins have two cysteines residues, the N-terminal peroxidatic cysteine, and a resolving cysteine that can form a disulphide bond with the peroxidatic cysteine. 2-Cys peroxiredoxins are further classified as typical and atypical. A major difference is that whilst the position of the C-terminal resolving cysteine residue is conserved in typical 2-Cys peroxiredoxins, in atypical 2-Cys peroxiredoxins the position of this residue displays significant variation. During the catalytic breakdown of peroxide, the peroxidatic cysteine of typical 2-Cys peroxiredoxins is oxidized by peroxide to a sulphenic acid as before (ii - Figure 1.8). This however, subsequently reacts with a resolving cysteine on a separate 2-Cys peroxiredoxin protein to form a disulphide bridge (iii - Figure 1.8; Ellis and Poole, 1997).

$Prx(SH)_2$ + $Prx(SH)_2$ + ROOH → (HS)PrxS-SPrx(SH) + ROH + H_2O

On the contrary, in atypical 2-Cys peroxiredoxins, the reaction between peroxidatic and resolving cysteines can occur in the same 2-Cys peroxiredoxin molecule (Hall *et al.*, 2009 and Rhee *et al.*, 2005). 2-Cys peroxiredoxins are reduced by the thioredoxin system at the expense of NADPH (Rhee *et al.*, 2005 - Figure 1.8). The thioredoxin system is comprised of thioredoxin (Trx) and thioredoxin reductase (Trr). During reduction of oxidised 2-Cys

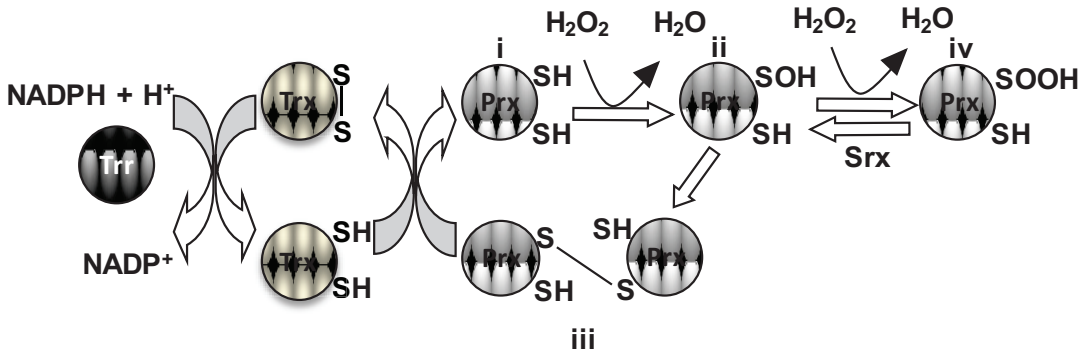


Figure 1.8. H_2O_2 detoxification by the Trx1/Prx system.

During the catalytic reduction of H_2O_2 the peroxidatic cysteine residue of reduced 2-cys peroxiredoxins (Prx) (i) becomes oxidized to sulfenic acid (SOH; ii), then forms a disulfide bond with the resolving cysteine residue on a partner protein forming a homodimer (iii). The oxidized dimer is then recycled by the sequential oxidation and reduction of thioredoxin (Trx) and thioredoxin reductase (Trr) using NADPH. At higher levels of H_2O_2 the peroxidatic cysteine residue of Prx is sensitive to further oxidation to the sulfinic acid (SOOH) derivative (iv) which can be reduced by either sulfiredoxin (Srx) or Sestrin.

peroxiredoxins thioredoxin becomes oxidized. The oxidized thioredoxin is then reduced by thioredoxin reductase at the expense of NADPH (Figure 1.8 - Arnér and Holmgren, 2006). The conserved active site of thioredoxin is composed by the amino acids Gly and Pro, flanked by two Cys residues (Cys-Gly-Pro-Cys). The presence of Cys residues in the active site of thioredoxin, allow these proteins to act as the immediate hydrogen donor during the thioredoxin-mediated reduction of a target protein. For example, thioredoxin reaction with peroxiredoxins is initiated by a nucleophilic attack by the N-terminal Cys thiolate residue (Cys^N-S^-) of thioredoxin to form a mixed disulfide with one of the Cys residues of its substrate (TrxS-SPrx). This mixed disulfide is then reduced by the thioredoxin C-terminal Cys, resulting in the reduced substrate and a disulfide bond in the active site of thioredoxin (Tao, 2008). Oxidized thioredoxin is then reduced by the reaction of thioredoxin reductase and NADPH (Berndt *et al.*, 2008).

$$\mathbf{(HS\text{-}Cys^C)Trx\text{-}(Cys^N\text{-}SH) + Prx\text{-}S_2 \rightarrow (HS\text{-}Cys^C)\text{-}TrxS\text{-}SPrx\text{-}(Cys^R\text{-}SH)}$$

$$\mathbf{(HS\text{-}Cys^C)\text{-}TrxS\text{-}SPrx\text{-}(Cys^R\text{-}SH) \rightarrow Trx\text{-}S_2 + Prx\text{-}(SH)_2}$$

$$\mathbf{Trx\text{-}S_2 + NADPH + H^+ \xrightarrow{Trr} Trx\text{-}(SH)_2 + NADP^+}$$

If the peroxidatic cysteine of the 2-Cys peroxiredoxin is not reduced by thioredoxin this sulphenic acid can be further oxidized by peroxide, generating the sulphinic (SO_2H) and sulphonic (SO_3H) inactive forms (iv - Figure 1.8 - Wood *et al.*, 2003). However, differently from the irreversible oxidation to the sulphonic inactive form, the inactivation of sulphenic acid to the sulphinic form is not irreversible, because the enzyme sulphiredoxin (Srx1) catalyzes the reduction of the sulphinic acid derivative of 2-Cys peroxiredoxins (Figure 1.8 - Biteau *et al.*, 2003, Woo *et al.*, 2005 and Lim *et al.*, 2008). Recent studies have shown that in order to reduce the SO_2H form of the peroxidatic cysteine, sulphiredoxin requires this residue to be phosphorylated forming a phosphoryl sulfinic intermediate, which can then be attacked by the catalytic cysteine of sulphiredoxin to form the PrxSO-SSrx intermediate. Once the Prx-SO_2H is reduced by sulphiredoxin to Prx-SOH, Srx becomes oxidized and is then reduced by thioredoxin (Roussel *et al.*, 2008 and Roussel *et al.*, 2009). The proposed mechanism of peroxiredoxin (SO_2H) reduction by sulphiredoxin and reduction of sulphiredoxin by thioredoxin is presented below:

$$(HS\text{-}Cys^{R})Prx(Cys^{P}\text{-}SO_2H) + ATP \rightarrow (HS\text{-}Cys^{R})Prx\text{-}(Cys^{P}\text{-}SO_2PO_3^{2-}) + ADP$$

$$(HS)Prx(Cys^{P}\text{-}SO_2PO_3^{2-}) + (HS\text{-}Cys^{48})Srx(Cys^{84}\text{-}SH) \rightarrow (HS\text{-}Cys^{R})PrxS\text{-}SSrx(Cys^{48}SH) + Pi$$

$$(HS\text{-}Cys^{R})PrxS\text{-}SSrx(Cys^{48}SH) \rightarrow (HS\text{-}Cys^{R})Prx(Cys^{P}\text{-}SOH) + Srx(S)_2$$

Reduction of sulphinic Prxs

$$Srx(S)_2 + (^{-}S\text{-}Cys^{N})Trx(Cys^{C}\text{-}SH) \rightarrow (^{-}S\text{-}Cys^{C})TrxS\text{-}SSrx(Cys^{84}SH)$$

$$(^{-}S\text{-}Cys^{C})TrxS\text{-}SSrx(Cys^{84}SH) \rightarrow Trx(S)_2 + Srx(SH)_2$$

Recycling of Srx

1.4.2.4.1. Peroxiredoxins

One of the first 2-Cys peroxiredoxin proteins characterised in *S. cerevisiae* was Tsa1, and cells lacking this protein display impaired growth under aerobic conditions and in the presence of oxidants when compared to wild-type cells (Chae *et al.*, 1993). In addition to Tsa1 which is cytoplasmatic, *S. cerevisiae* contains 4 other peroxiredoxin homologues in its genome, the cytoplasmic Tsa2 and Ahp1, the mitochondrial Prx1, and the nuclear Dot5 (Park *et al.*, 2000). Tsa1 and Tsa2 are typical 2-Cys peroxiredoxins, while Ahp1 and Dot5 are atypical 2-Cys peroxiredoxins and Prx1 is a 1-Cys peroxiredoxin. The presence of a range of peroxiredoxins in *S. cerevisiae* suggests that peroxiredoxins co-operate with one another to protect cells against oxidative stress. This is indicated by experiments in which deletion of both *TSA1* and *TSA2* results in cells that are more sensitive to peroxide treatment than the single *tsa1*Δ or *tsa2*Δ deletion strains (Wong *et al.*, 2002). Furthermore, deletion of all peroxiredoxins (*TSA1, TSA2, AHP1, PRX1* and *DOT5*) resulted in strains that under both non-stress conditions and after H_2O_2 treatment displayed increased ROS levels and increased sensitivity when compared with cells that only express only one peroxiredoxin, such as the *tsa1*Δ*/tsa2*Δ*/dot5*Δ*/prx1*Δ strain (Wong *et al.*, 2004).

Even though all 5 peroxiredoxin homologues exhibited thioredoxin-dependent peroxidase activity *in vitro*, this peroxidase activity varied between different homologues. For example, while Tsa1 decomposed H_2O_2 more efficiently than all the other peroxiredoxins, Ahp1 was the most efficient peroxiredoxin in the decomposition of organic hydroperoxides (*t*-BOOH and cumene hydroperoxide). In addition, Tsa2, Prx1 and Dot5 were less efficient than Tsa1

and Ahp1, when decomposing both the H_2O_2 and organic hydroperoxides (Park *et al.*, 2000). Consistant with the higher activity of Tsa1 when compared to other peroxiredoxins, *tsa1*Δ cells are more sensitive to H_2O_2 than the other peroxiredoxin null mutants (*tsa2*Δ*, ahp1*Δ*, prx1*Δ and *dot5*Δ) (Park *et al.*, 2000 and Wong *et al.*, 2002). In addition to peroxides, *S. cerevisiae* peroxiredoxins are also involved in detoxifying peroxynitrite and deletion of *TSA1* resulted in strains that are sensitive to peroxynitrite. Furthermore, the double *tsa1*Δ*tsa2*Δ strain displayed an increased sensitivity to peroxynitrite when compared with *tsa1*Δ cells, indicating that similar to what is seen in the peroxide detoxification, these two proteins act synergistically to detoxify peroxynitrite (Wong *et al.*, 2002).

In addition to its role as a peroxidase, *S. cerevisiae* Tsa1 can also function as a molecular chaperone and protect ribosomal proteins against aggregation (Jang *et al.*, 2004 and Trotter *et al.*, 2008). When the peroxidatic cysteine of Tsa1 is oxidized to sulphonic (SO_3H) acid, Tsa1 forms high molecular weight (HMW) oligomeric complexes that can act as effective and stable molecular chaperones *in vivo* (Jang *et al.*, 2004 and Lim *et al.*, 2008). Under normal or low oxidative stress conditions, Tsa1 forms low molecular weight (LMW) complexes and oligomeric protein structures, that can maintain their peroxidase activity and allows them to bind to ribosomal proteins, such as the translation termination factor Sup35 (eRF3), therefore protecting cells against oxidative damage and maintaining protein synthesis (Sideri *et al.*, 2010). However, when the conditions switch to an acute oxidative stress, the peroxidatic cysteine of Tsa1 becomes overoxidized, which promotes a structural change that induces formation of HMW complexes and activates the chaperone activity of Tsa1, which possibly protects proteins against oxidative-stress induced protein denaturation (Jang *et al.*, 2004 and Lim *et al.*, 2008).

In the other model yeast *S. pombe* there is only one 2-Cys peroxiredoxin, named Tpx1. Similar to *S. cerevisiae*, Tpx1 participates in the defence of oxidative stress insult, with cells lacking Tpx1 being extremely sensitive to peroxide, having an additional slow growth phenotype when grown in aerobic conditions, which could be explained by higher ROS levels in this strain, since *tpx1*$^-$ cells exhibited higher protein carbonylation content than wild-type cells grown under anaerobic conditions (Veal *et al.*, 2004, Bozonet *et al.*, 2005, Vivancos *et al.*, 2005 and Jara *et al.*, 2007). Similar to other typical 2-Cys peroxiredoxins, Tpx1 has an N-terminal peroxidatic cysteine residue (Cys48) and a C-terminal resolving cysteine residue (Cys169), and the peroxidatic cysteine residue becomes hyperoxidized in the

presence of H_2O_2 in a process that only occurred when the thioredoxin system (Trx1, Trr1 and NADPH) were present (Jara *et al.*, 2007). Furthermore, treatment of *S. pombe* cells with lower H_2O_2 concentrations (0.2mM) results in formation of a Tpx1 dimer (Jara *et al.*, 2007), which is consistent with Tpx1 peroxidase activity seen in cells treated with this lower concentration (Bozonet *et al.*, 2005 and Vivancos *et al.*, 2005). On the contrary, treatment of *S. pombe* cells with higher H_2O_2 concentrations (5 mM) resulted in the accumulation of a hyperoxidised inactive Tpx1 monomer (Jara *et al.*, 2007); however formation of the hyperoxidized monomer seems to require prior formation of the hyperoxidized Tpx1 dimer (Jara *et al.*, 2007). Interestingly, *S. pombe* cells expressing Tpx1 resolving cysteine mutated to a serine (Cys169Ser) are more resistant to peroxide stress than both cells that express the Tpx1 peroxidatic cysteine mutated to a serine (Cys48Ser) and *tpx1*$^-$ cells (Jara *et al.*, 2007). However, strains expressing Tpx1^{Cys169Ser} are still sensitive to peroxide stress when compared to wild-type cells, indicating that under higher oxidative stress conditions Tpx1 resolving cysteine is still required (Jara *et al.*, 2007). This increased sensitivity might be related to the central role of Tpx1 is oxidative stress signalling in this organism which is discussed in section 1.4.1. In addition, the peroxidatic cysteine of Tpx1 is susceptible to overoxidiation to the sulphinic (SO_2H) form and the sulphiredoxin in *S. pombe*, Srx1, can reduce SO_2H to SOH (Bozonet *et al.*, 2005 and Vivancos *et al.*, 2005). In addition to Tpx1, *S. pombe* also expresses another 2 peroxiredoxins the 1-Cys peroxiredoxins Dot5, also known as Bcp1, and Pmp20. Both Tpx1 and Bcp1 display a thioredoxin-dependent peroxidase activity, and can detoxify both H_2O_2 and organic peroxides, with the peroxiredoxins Tpx1 and Bmp20 also showing a chaperone activity (Kim *et al.*, 2010). Consistent to its role as a peroxidase, ectopic expression of Bcp1 in *S. pombe* increases the resistance of this cells and also result in increased levels of GSH when compared with GSSG when cells treated with H_2O_2 (Kang *et al.*, 2009).

Similarly to *S. pombe*, *C. albicans* contains a single typical 2-Cys peroxiredoxin, Tsa1. A *C. albicans tsa1*Δ mutant has been shown to be sensitive to oxidative stress, which is consistent with the predicted function of 2-Cys peroxiredoxins in the removal of H_2O_2 (Shin *et al*., 2005 and Urban *et al*., 2005). Tsa1 is localized both in the cytoplasm and nucleus of both untreated and H_2O_2-treated cells (Urban *et al*., 2005), however conflicting studies showed different localization of Tsa1 in response to hyphal inducing conditions, while a study using imunocytochemistry showed that Tsa1 translocated to the nucleus during

transition to hyphae (Shin *et al*., 2005), while a study using a Tsa-GFP construct illustrated that Tsa1 translocated to the nucleus and cell wall under the same conditions (Urban *et al*., 2005). In addition to Tsa1, *C. albicans* also contains the other peroxiredoxins, designated as Ahp1, Ahp2, Prx1, Dot5 and Trp99 (Table 1.1). However, to date, only Tsa1 has been characterised.

1.4.2.4.2. Thioredoxin System

The thioredoxin system is composed of thioredoxin and thioredoxin reductase (Section 1.4.2.4. – reviewed in Arnér and Holmgren, 2006) and has also been extensively studied in *S. cerevisiae*. This yeast contains both a cytoplasmatic thioredoxin system (Trx1, Trx2 and Trr1) and a distinct mitochondrial one (Trx3 and Trr2) (Trotter and Grant, 2005). The importance of thioredoxin in the oxidative stress response is highlighted by the up-regulation of thioredoxin genes in response to H_2O_2 and by the increased sensitivity of strains lacking the cytoplasmatic thioredoxins, Trx1 and Trx2, to peroxide treatment in *S. cerevisiae* (Gash *et al.*, 2000 and Garrido and Grant, 2002).

In the model yeast *S. cerevisiae* the cytoplasmatic thioredoxins, Trx1 and Trx2, have overlapping roles and only deletion of both cytoplasmatic thioredoxins (*trx1*Δ*/trx2*Δ) affects several cellular processes. For example deletion of both thioredoxins in *S. cerevisiae* results in a defective cell cycle, which presents a prolonged S phase and shortened G1 (Muller, 1991) and in defective sulphate assimilation (Muller, 1991 and Draculic *et al.*, 2000). Collectively, these results indicate that Trx1 and Trx2 have overlapping roles in regulation of key enzymes that control this process, such as Ribonucleotide Reductase (RNR) (Camier *et al.*, 2007) and 3′-phosphoadenosine 5′-phosphosulphate (PAPS) reductase, which converts 3′-phosphoadenosine 5′-phosphosulphate (PAPS) to sulphite (Muller, 1991 and Vignols *et al.*, 2005).

In addition to acting as hydrogen donors for RNR (Camier *et al.*, 2007) and PAPS reductase (Schwenn *et al.*, 1988) in the model yeast *S. cerevisiae*, the thioredoxins also act as hydrogen donors for proteins involved in the oxidative stress response such as thioredoxin peroxidases (Park *et al.*, 2000 - see Section 1.4.2.4.), glutathione peroxidases (Delaunay *et al.*, 2002) and the Yap1 transcription factor (Izawa *et al.*, 1999 – see Section 1.4.4.2.2.). Consistent with thioredoxin`s role in the regulation of enzymes involved in the oxidative stress responses, deletion of both *TRX1* and *TRX2* resulted in exponential growing cells that

Table1.1. Peroxiredoxins in *C. albicans* and its homologues in *S. cerevisiae* and *S. pombe*.

Gene	ORF No	Type of Peroxiredoxin	Up-Regulated in Response to XS	*S.cerevisiae* Homologue	*S.pombe* Homologue
DOT5	***orf19.5417***	**1-Cys (Cys^{88})**	**No**	**Dot5**	**Dot5/Bcp**
TRP99	***orf19.424***	**1-Cys (Cys^{69})**	**No**	**Ahp1**	**Pmp20**
PRX1	***orf19.5180***	**Mitochondrial 1-Cys (Cys^{69})**	**No**	**Prx1**	
AHP2	***orf19.6470***	**Atypical 2-Cys (Cys^{33}/Cys^{69})**	**No**	**Ahp1**	**Bmp20**
AHP1	***orf19.2762***	**Atypical 2-Cys (Cys^{27}/Cys^{57})**	**Yes**	**Ahp1**	**Bmp20**
TSA1	***orf19.7417*** ***orf19.7398.1***	**Typical 2-Cys (Cys^{48}/Cys^{169})**	**Yes**	**Tsa1**	**Tpx1**

were sensitive to the peroxide treatment (H_2O_2 and *t*-BOOH) (Garrido and Grant, 2002). Deletion of the cytoplasmatic thioredoxins also resulted in high levels of protein glutathionylation, GSSP (see in section 1.4.1.1.), in untreated cells and H_2O_2 treatment results in reduced levels of GSSP, and may indicate that the mobilization of glutathione from a protein-bound to a free form can serve an antioxidant function (Garrido and Grant, 2002). This increased GSSP levels can be a result of the role of thioredoxins in catalyzing deglutathionylation since thioredoxins catalyze deglutathionylation *in vitro*. In addition, overexpression of components of the thioredoxin system reduced the levels of protein glutathionylation, while protein glutathionylation was increased in *trx1*Δ*/trx2*Δ strain (Greetham *et al.*, 2010). In addition to its role in oxidative stress detoxification, thioredoxins are also involved in oxidative stress signalling, but this will be discussed in more detail in section 4.3.1.

S. cerevisiae also expresses a mitochondrial thioredoxin, Trx3, however deletion of the mitochondrial thioredoxin system (composed by Trx3 and the thioredoxin reductase Trr2) does not result in an imbalance redox state of the cytoplasmatic thioredoxin system (composed by Trx1 and Trx2 and Trr1) and vice-versa (Trotter and Grant, 2005). The cytoplasmatic and mitochondrial thioredoxins seem to have different roles in the *S. cerevisiae* cells since deletion of *TRX3* does not result in a slow growth phenotype and increased sensitivity to peroxide treatment (Trotter and Grant, 2005), similar to the one seen in double *trx1*Δ*/trx2*Δ strains suggesting that the cytoplasmatic thioredoxins are the key enzymes regulating oxidative stress resistance in this model yeast. Furthermore, deletion of the cytoplasmatic thioredoxins (*trx1*Δ*trx2*Δ) or of the enzyme that is responsible for reducing oxidized Trx1/Trx2, thioredoxin reductase (*trr1*Δ) (Trotter and Grant, 2005), resulted in increased GSSG (oxidized GSH) levels when compared to wild-type cells (Garrido and Grant, 2002 and Trotter and Grant, 2003). The increased levels of GSSG could indicate a more oxidized environment in *S. cerevisiae* cells lacking the cytoplasmatic thioredoxin system. On the other hand, deletion of the mitochondrial thioredoxin system, composed by Trx3 and the thioredoxin reductase Trr2, does not alter the GSSG levels when compared to wild-type cells (Trotter and Grant, 2005). Even thought the mitochondrial thioredoxin doesn`t seem to play a role in oxidative stress defence (Trotter and Grant, 2005), deletion of *TRR2* resulted in strains sensitive to an acute H_2O_2 treatment (Pedrajas *et al.*, 1999). Furthermore, Trr2 seems to be important during aerobic growth and in response to a

prolongued exposure of H_2O_2 when the cytoplasmic thioredoxin system is absent (Trotter and Grant, 2005), since the double *trr1*Δ*/trr2*Δ displayed increased sensitivity to oxidative stress and displayed slower growth under aerobic conditions, when compared to the *trr1*Δ or *trr2*Δ single mutants (Trotter and Grant, 2005). These phenotypes seen in the double *trr1*Δ*/trr2*Δ seem to be independent of Trr2 role in reducing oxidized Trx3, since the triple *trx1*Δ*trx2*Δ*trx3*Δ does not exhibit increased oxidative stress sensitivity neither exacerbate the slow growth phenotype of the *trx1*Δ*trx2*Δ strain (Trotter and Grant, 2005). One possibility of the increased oxidative stress sensitivity seen in double *S. cerevisiae trr1*Δ*/trr2*Δ could be related to the role of of Trr2 in reducing the mitochondrial 1-Cys peroxiredoxin Prx1, since Prx1 is constitutively oxidized in *trr2*Δ cells (Greetham and Grant, 2009).

The other model yeast *S. pombe* expresses a cytoplasmatic thioredoxin, $trx1^+$, and a mitochondrial thioredoxin, $trx2^+$. Similarly to what is seen in *S. cerevisiae*, $trx1^-$ cells displayed sensitivity to H_2O_2, while deletion of $trx2^+$ did not result in H_2O_2 sensitivity (Song and Roe, 2008). $trx1^-$ cells also exhibited higher basal levels of Pap1-regulated genes, such as $ctt1^+$ and $trr1^+$, which might indicate a role for Trxl in reducing oxidized and active Papl (Song and Roe, 2008 – see section 1.4.4.2.1 for Pap1 regulation and section 1.4.4.2.2 for Trx1 regulation of Yap1). Furthermore, deletion of $trx1^+$ also resulted in cells that were defective in sulphate assimilation, indicating its possible role as an electron donor for the enzyme PAPS redutase (Song and Roe, 2008). In addition to Trx1 and Trx2, *S. pombe* also possesses a thioredoxin-like 1 (Txl1) protein, which has an N-terminal thioredoxin domain followed by a C-terminal domain that is thought to have a regulatory role (Jiménez *et al.*, 2006). In *S. pombe*, Txl1 exhibited an oxireductase activity, which was thioredoxin reductase dependent, and $txl1^-$ cells displayed sensitivity to the organic hydroperoxide (*t*-BOOH), indicating its role in oxidative stress protection (Jiménez *et al.*, 2007 and Wiseman *et al.*, 2009).

C. albicans has three ORFs that have been annotated as thioredoxin-like proteins, Trx1, Trx2 and Txl1, and a single thioredoxin reductase, Trr1. *TRX1* and *TRR1* transcription increase in *C. albicans* cells treated with H_2O_2 and following exposure to neutrophils, indicating its role in ROS detoxification (Enjalbert *et al.*, 2006 and Fradin *et al.*, 2005). However, such thioredoxin and thioredoxin reductase proteins have not been characterised in this organism.

1.4.3. Regulation of the Oxidative Stress Response

As described in section 1.2.4.1.2, exposure of fungal cells to reactive oxygen species, immediately stimulates a significant transcriptional response resulting in the rapid increase in transcripts encoding antioxidant proteins. The molecular mechanisms involved in the control of the transcriptional response to oxidative stress are well documented in the model yeasts *S. cerevisiae* and *S. pombe* and have provided a framework for the study of the oxidative stress response mechanisms in *C. albicans* (reviewed in Brown *et al.*, 2009). In this section the regulatory proteins involved in the oxidative stress response in the model yeasts *S. cerevisiae* and *S. pombe* and the mechanisms used by the cells to respond to oxidative stress are discussed.

1.4.3.1. Transcriptional regulators

1.4.3.1.1. AP-1 transcription factors

Activating protein 1 (AP-1) transcription factors are members of the bZIP family of transcription factors. This family of proteins is characterized by a region rich in basic amino acids (positively charged) responsible for interaction with the negatively charged DNA molecule. This is located adjacent to a leucine repeat sequence (leucine zipper) that is required for dimerisation of these transcription factors (Miller, 2009). AP-1 transcription factors are involved in oxidative stress response and when exposed to oxidative stress, they translocate to the nucleus and activate the transcription of their target genes via the AP-1 Response Element (YRE: TKACTAA) (Kyriakis and Avruch, 2001 and Kuge and Jones, 1994). In mammalian cells, the AP-1 complex transcription factors forms dimers that can be composed of the families of transcription factors Fos, Jun or activating transcription factor (ATF) (Karin *et al.*, 1997). The activity of these mammalian AP-1 transcription factors is regulated by both phosphorylation and changes in the expression of the different subunits (Karin *et al*, 1997 and Kyriakis and Avruch, 2001).

AP-1-like transcription factors are present in both model yeasts, such as *S. cerevisiae* Yap1 and *S. pombe* Pap1, and pathogenic yeasts, such as *C. albicans* Cap1 and are highly conserved throughtout the fungal kingdom (Nikolau *et al.*, 2009). Initial studies on Yap1 function in *S. cerevisiae*, showed that deletion of *YAP1* resulted in strains that are hypersensitive to peroxide stress (H_2O_2 and *t*-BOOH), while over-expression of *YAP1* had

the opposite effect, increasing the resistance to oxidative stress (Kuge and Jones, 1994). The role of Yap1 in the transcriptional response of *S. cerevisiae* is exemplified by the lack of oxidative stress induced expression in *yap1*Δ cells, of antioxidant encoding genes that have the AP-1 Response Element in their promoter such as *GSH1*, *GLR1*, *AHP1*, *TSA2*, *TRX2* and *TRR1* (Kuge and Jones, 1994, Stephen *et al.*, 1995, Grant *et al.*, 1996 and Fernandes *et al.*, 1997). Yap1 has a basic leucine zipper DNA binding domain (bZIP), has two distinct cysteine rich domains, the N-terminal cysteine-rich domain (N-CRD - Asn279 to Arg313), which contains the nuclear localization sequence (NLS), and a C-terminal cysteine-rich domain (C-CRD - Asn565 to Asn650), which contains the nuclear exporting sequence (NES) (Wood *et al.*, 2004).

The molecular mechanism underlying Yap1 activation in response to oxidative stress is well characterised and is centred on the cellular localisation of the protein. Under non-stress conditions Yap1 is mainly localized in the cytoplasm, whilst in cells treated with agents that cause oxidative stress Yap1 is mainly localized in the nucleus (Kuge *et al.*, 1997). Yap1 localization is regulated by changes in the oxidation state of the protein. When Yap1 is in its reduced form the NES present in its C-terminal region is exposed, Yap1 can subsequently interact with the Crm1 nuclear export factor and thus be transported to the cytoplasm. However, when Yap1 becomes oxidized the NES is masked; Yap1 no longer interacts with Crm1, and thus accumulates in the nucleus (Delaunay *et al.*, 2000). The oxidation state of Yap1 is regulated by formation of disulphide bonds between two distinct cysteine rich domains, the N-terminal cysteine-rich domain (N-CRD) and a C-terminal cysteine-rich domain (C-CRD) (Wood *et al.*, 2003 and Wood *et al.*, 2004). In response to oxidative stress generated by H_2O_2, changes in the redox status of Yap1 two cysteine-rich domains (n-CRD and c-CRD), results in the formation of an intramolecular disulphide bond between N-terminal Cys303 and C-terminal Cys598 and between N-terminal Cys310 and C-teminal Cys629. This disulphide bond changes the molecular conformation of Yap1, masking the nuclear export signal (NES) (Delaunay *et al.*, 2000). Furthermore, a study has recently shown that all the six cysteine residues in Yap1 are important for its role in H_2O_2 sensing and signalling (Okazaki *et al.*, 2007). A model was proposed where H_2O_2 treatment immediately induces a disulfide bond formation between the N-terminal cysteines (Cys310 and Cys315) of Yap1, which is then followed by the formation of the formation of a disulphide bond

between N-terminal and C-terminal cysteine residues (Cys303/Cys598 and Cys310/Cys629) (Okazaki *et al.*, 2007).

This formation of disulphide bonds between the C-CRD and N-CRD is specific to H_2O_2 treatment, since oxidative stress imposed by diamide, which oxidizes GSH, only requires the N-CRD of Yap1 (Kuge *et al.*, 1997). This was supported by experiments that showed that deletion of the C-CRD of *YAP1* resulted in increased diamide resistance, but still resulted in decreased sensitivity to H_2O_2 (Coleman *et al.*, 1999) In addition, while diamide treatment produced disulphide bonds in either C-CRD or N-CRD regions of Yap1, H_2O_2 treatment induced formation of disulphide bonds between the C-CRD and N-CRD (Kuge *et al*., 2001 and Delaunay *et al.*, 2000). For example, mutations in the C-CRD of Yap1 (Cys629Ala) resulted in constitutive nuclear accumulation of Yap1, and hyper-resistance to diamide yet decreased H_2O_2 resistance (Kuge *et al.*, 1997 and Coleman *et al.*, 1999). Interestingly, while induction of *GSH1* in response to H_2O_2 only requires masking of the NES that results in Yap1 trapped in the nucleus (mutation of Cys629), induction of *GSH1* in response to H_2O_2 requires both nuclear localization and correct folding by formation of the disulphide bonds between C-CRD and N-CRD of Yap1 (Gulshan *et al.*, 2005). The different regulation of Yap1 seen in response to diamide or H_2O_2 mirrors the fact that different oxidants stimulate stress specific responses with the induction of different antioxidant genes (Gash *et al.*, 2000). Yap1 localization is regulated by the nuclear export factor Crm1, which binds to Yap1 *in vitro* in a reaction that is dependent on Yap1 oxidation state (Yan *et al.*, 1998). A model is proposed where in untreated cells Yap1 NES regions interact with Crm1, resulting in Yap1 translocation from the nucleus to the cytoplasm; however when cells are treated with H_2O_2, the NES is masked, resulting in inhibition of Crm1 interaction with Yap1 and induced Yap1 nuclear accumulation (Yan *et al.*, 1998 and Delaunay *et al.*, 2000).

Yap1 is not directly oxidised by H_2O_2. Instead, H_2O_2-induced disulphide bond formation is dependent on the thiol peroxidase Gpx3, and on the Yap1-binding protein, Ybp1. Following peroxide treatment, the C-terminal cysteine (Cys36) of Gpx3, also called Oxidant Receptor Peroxidase 1 (Orp1), is oxidized to a sulfenic acid (see section 1.3.2.2), which then forms a disulphide with Cys598 of Yap1 (Delaunay *et al.*, 2002, Ma *et al.*, 2007 and Paulsen and Carroll, 2009). Subsequent formation of Yap1 Cys598S-SCys303 disulphide formation results in Yap1 activation (Delaunay *et al.*, 2002). Gpx3-mediated oxidation of Yap1, and thus nuclear accumulation and Yap1-dependent gene expression, is also dependent on Ybp1,

which as its name suggests, interacts with Yap1. Consequently, deletion of *YBP1* phenocopies the peroxide sensitivity exhibited by *gpx3*Δ cells, and a *gpx3*Δ*ybp1* double mutant is no more sensitive than the single mutants. These results support the model in which Ybp1 acts alongside Gpx3 to regulate Yap1 in response to H_2O_2 (Veal *et al.*, 2003). Interestingly, the commonly used laboratory *S. cerevisiae strain* W303-1 harbours a mutant defective allele of YBP1, namely ybp1-1. In such cells, some oxidation of Yap1 is evident; however this is dependent on the 2-Cys peroxiredoxin Tsa1 and independent of Gpx3. As is mentioned below, and detailed in Section, 1.4.4.2.1., this is more reminiscent of the mechanism employed by *S. pombe* to regulate oxidation of the analogous transcription factor Pap1. However, irrespective of the mechanism of oxidation, once ROS have reached homeostatic levels the Yap1 intramolecular disulphide bonds are reduced. The reduction of Yap1 disulphide bonds is catalyzed by the thioredoxin system, and this is also considered in more detail in Section 1.4.4.1.2.

Like Yap1 in *S. cerevisiae*, Pap1 in *S. pombe* is also a major regulator of oxidative stress induced expression of genes, such as $trr1^+$, $trx2^+$ and $ctt1^+$. In addition, activation of Pap1 in response to oxidative stress also requires inhibition of translocation from the nucleus to the cytoplasm (Toone *et al.*, 1998). The domain structure of Pap1 is similar to Yap1, and mutation of the C-CRD Cys532 or inactivation of $crm1^+$, results in constitutive Pap1 nuclear accumulation (Kudo *et al.*, 1999). Furthermore, in response to oxidative stress an intramolecular disulphide bond between n-CRD Cys278 and c-CRD Cys501 is formed (Castillo *et al.*, 2002 and Vivancos *et al.*, 2004). However, in contrast to *S. cerevisiae* Yap1, Pap1 activation is inhibited in response to increasing H_2O_2 levels (Quinn *et al.*, 2002). Pap1 oxidation, as mentioned above, is mediated directly by the 2-Cys peroxiredoxin Tpx1, and the Sty1 SAPK is also required for Pap1 oxidation albeit indirectly. Furthermore, inactivation of the Gpx3 homologue in *S. pombe* has no effect on Pap1 oxidation, and there is no clear Ybp1-like homologue in the *S. pombe* data base (Bozonet *et al.*, 2005 and Vivancos *et al.*, 2005). The mechanism by which Tpx1 and Sty1 regulate Pap1 oxidation is discussed in more detail in section 1.4.4.1.1.

In *C. albicans*, the key regulator of oxidative-stress induced gene transcription is the Yap1/Pap1 homologue, Cap1 (Alarco and Raymond, 1999 and Wang *et al.*, 2006). Consequently, deletion of *CAP1* results in strains that are hypersensitive to oxidative stress (Alarco and Raymond, 1999) and impaired regulation of transcriptional response to peroxide

stress (Wang *et al*., 2006). Consitent with this, promoter analysis revealed that the YRE [(A/C)T(T/G)A(C/GT(A/C)A] is significantly over-represented in the promoters of genes induced by oxidative stress, compared with other stresses in *C. albicans* (Enjalbert *et al.*, 2006). ChIP on chip experiments have shown that Cap1 binds to the promoter region and regulates the expression of over 80 genes (Znaidi *et al.*, 2009). Among the direct Cap1-targets highlighted in this study were genes with antioxidant functions (such as *CCP1, SOD1, GLR1, GCS1, MDR1, CAT1*, and *TRX1*), hyphal cell wall genes (*SSA2, EBP1, PDC11* and *ADH1*) and genes involved in nitrogen metabolism (*GST1, GST2* and *GST3*) (Znaidi *et al.*, 2009). Furthermore, transcription profiling experiments showed that Cap1 is required for up-regulation of antioxidant (*GLR1, GTT1, TRR1, SOD2* and 3 NADPH dehydrogenases) genes, those involved in energy metabolism (genes involved in pentose phosphate pathway) and also gene involved in protein degradation (proteasome and ubiquitination) (Wang *et al.*, 2006). These studies indicate that in addition to antioxidants, Cap1 also regulates the pentose phosphate pathway, which has a key role in producing the NADPH required for reduction of members of thioredoxin system and glutathione, and the ubiquitin-proteasome system, which is required for removal of damaged proteins (Wang *et al.*, 2006). Even though there was little overlap between the genes that were identified in the ChIP on chip studies and in the transcription profiling studies, transcription of genes representing categories such as antioxidant proteins and energy metabolism was found to be dependent on Cap1 in both studies (Wang *et al.*, 2006 and Znaidi *et al.*, 2009). Proteomic analysis of *C. albicans* cells treated with H_2O_2, showed that 30 proteins are up-regulated in response to H_2O_2 and proteins such as the antioxidant enzymes (Glr1, Trr1p and Cat1) and the putative oxireductases (Ebp1 and Grp4) were up-regulated in a Cap1-dependent manner (Kush *et al.*, 2007). Furthermore, at least 10 Cap1 target proteins were identical to the ones identified in the transcription profiling described above (Wang *et al.*, 2006 and Kush *et al.*, 2007).

Cap1, like Yap1 and Pap1, accumulates in the nucleus in response to H_2O_2 treatment (Zhang *et al.*, 2000). However, in contrast to that seen in *S. pombe*, Cap1 nuclear accumulation is not inhibited in response to higher peroxide concentrations and rapidly accumulates in the nucleus in response to both low (0.4 mM) and high (5 and 10 mM) H_2O_2 concentrations (Smith *et al.*, 2004). However, similar to Yap1 and Pap1, mutation of specific cysteine residues in the c-CRD cysteine in Cap1 (Cys477) resulted in constitutively Cap1 nuclear accumulation and increased expression of a Cap1 target gene (*GLR1*) (Zhang *et al.*,

2002). This mimics the phenotypes of *S. cerevisiae* cells that express the analogous Yap1 mutation (Cys629), such as diamide resistance (Coleman *et al.*, 1999). Although this mutation induced Cap1 activation under non-stressed conditions, a further increase in gene expression in response to oxidative stress was not seen, and cells expressing the Cap1 Cys477Ala mutant did not display wild-type levels of peroxide resistance. In contrast, such cells were hyper-resistant to diamide treatment when compared with wild-type cells (Zhang *et al.*, 2000). The increased expression of proteins mainly involved in glutathione reduction (Trr1, Glr1 and NADPH dehydrogenases) in a *C. albicans* strain that expresses a hyperactivated Cap1 allele could be the reason underlying the hyper-resistance to diamide, but not to H_2O_2 (Alarco *et al.*, 1999 and Kush *et al.*, 2007). These results support a hypothesis that Cap1 is regulated differently in response to different oxidants and that some mechanisms of AP-1 transcription factor activation are conserved between the model yeast *S. cerevisae* and the pathogenic yeast *C. albicans*. However, despite this, nothing has been reported regarding the molecular details underlying Cap1 activation in *C. albicans*.

1.4.3.1.2. Skn7

In addition to AP-1 like factors, Skn7 related transcription factors have also been shown to play a role in the fungal oxidative stress response. The Skn7 transcription factor possesses a conserved N-terminal Hsf1-like DNA binding domain (DBD), which is adjacent to a coiled-coil, a receiver and glutamine-rich domains (Brown *et al.*, 1994). In addition, this receiver domain is associated with two-component regulatory proteins, which are known to be involved in regulating stress responses (Morgan *et al.*, 1997). In *S. cerevisiae*, Skn7 acts alongside Yap1 to induce the transcription of antioxidant genes, such as *TRX2*, *TRR1, TSA1, GPX2, AHP1,* and *CTT1* (Morgan *et al.*, 1997 and He and Fassler, 2005) and its central role in regulating oxidative stress responses is demonstrated by the hypersensitivity of *skn7*Δ mutants to peroxide stress (H_2O_2 and *t*-BOOH) (Lee *et al.*, 1999). In addition, Skn7 together with Hsf1 is also required for induction of heat shock proteins in response to peroxide stress. This result is likely related to the observations that Skn7 has a DNA-binding domain similar to that of Hsf1 and interacts with Hsf1 *in vivo* (Raitt *et al.*, 2000).

In contrast to Yap1, Skn7 is found in the nucleus both before and after oxidative-stress treatment (Raitt *et al.*, 2000), and the exact mechanism(s) by which Skn7 is regulated in response to oxidative stress are not well understood (section 1.4.3.1.1.). However, recently a

study has shown that in contrast with Yap1, Skn7 cysteine residues are not important for its role in oxidative stress response; however Skn7 phosphorylation is required for oxidative stress response in the model yeast *S. cerevisiae*, in a process that does not require any of the six MAPKs of this organism (He *et al.*, 2009). Furthermore, phosphorylated Skn7 is required for recruitment of Yap1 and formation of the Yap1-Skn7 complex that is responsible for activation of gene expression in response to oxidative stress (He *et al.*, 2009). In addition, the residues of Skn7 (Thr437, Ile428 and Val429) required for the complex Skn7-Yap1 in response to oxidative stress were also identified (He *et al.*, 2009). In the model yeast *S. pombe*, the response regulator Prr1 is involved to in stress responses, since *prr1*$^{-}$ cells are sensitive to oxidative stress (H_2O_2) and heavy metal stress (Cd^{+2}) (Ohmiya *et al.*, 1999). Furthermore, oxidative stress-activated genes, such as *trr1*$^{+}$ and *ctt1*$^{+}$, are not fully up-regulated in response to H_2O_2 treatment in *prr1*$^{-}$ cells, indicating that this response regulator is involved in the regulation of these genes in *S. pombe* (Ohmiya *et al.*, 1999). Even thought the mechanism by which Prr1 is regulating oxidative stress responses is not known, *prr1*$^{-}$ cells have an oxidative-stress sensitivity phenotype similar to the one seen in *pap1*$^{-}$ cells, and also are required for the full up-regulation of Pap1 target genes (*trr1*$^{+}$ and *ctt1*$^{+}$) (Ohmiya *et al.*, 1999 and Bozonet *et al.*, 2005), which could indicate that similarly to what is seen in *S. cerevisiae*, Prr1 acts alongside Pap1 to induce gene expression in response to oxidative stress.

Studies in *C. glabrata* have shown that deletion of *SKN7* in this organism resulted in cells that presented increased peroxide sensitivity (H_2O_2 and *t*-BOOH), which could be explained by decreased induction of Skn7 target genes, such as *TRX2, TRR1, TSA1* and *CTA1*, in response to H_2O_2 (Saijo *et al.*, 2010). Furthermore, in *C. glabrata* Skn7 and Yap1 seem to act alongside in response to oxidative stress, since the double *skn7*Δ*/yap1*Δ strain is not more sensitive than the single null strains (*skn7*Δ or *yap1*Δ) (Cuéllar-Cruz *et al.*, 2008). *C. albicans* also expresses a Skn7 homologue and deletion of *SKN7* in this organism also resulted in strains that are sensitive to peroxide stress (H_2O_2 and *t*-BOOH), however its role in regulating gene expression in response to oxidative stress was not assessed (Singh *et al.*, 2004). These results indicate that similar to the model yeast *S. cerevisiae*, the pathogenic fungi, *C. albicans* and *C. glabrata* also require Skn7 for induction of antioxidant genes.

1.4.3.2. Mitogen Activated Protein Kinases Pathways

The Mitogen Activated Protein Kinase (MAPK) pathways are key players in the adaptation and response of eukaryotic cells to environmental change. These pathways contain a MAPK that is activated by phosphorylation by a cascade of kinases; an activated MAPK kinase kinase (MAPKKK) activates a MAPK kinase (MAPKK) by phosphorylation of its N-terminal Ser and Thr residues, which then goes on to activate the MAPK by phosphorylation of conserved Tyr and Thr residues that are located in its kinase domain. Once activated, the MAPK phosphorylates specific serine or threonine residues of its target proteins, which include transcription factors, cell cycle regulators and kinases, that trigger an appropriate cellular response (Figure 1.9 - reviewed in Smith *et al.*, 2010).

Stress activated protein kinases (SAPKs) are members of the MAPK family of proteins that are involved in regulating stress responses. SAPK pathways are conserved from fungi (*S. cerevisiae* Hog1, *S. pombe* Sty1 and *C. albicans* Hog1) to mammals (p38 and JNK) (reviewed in Smith *et al.*, 2010). However, despite the conservation of MAPK pathways in eukaryotes, the inputs and outputs of these pathways differ. This could be expected since different organisms are exposed to, and therefore need to adapt to, different microenvironments. For example, the H_2O_2 levels required to activate *C. albicans* Hog1 are higher than the levels required for activation of *S. pombe* Sty1 (Smith *et al.*, 2004).

The model yeast *S. cerevisiae* High Osmolarity Glycerol (HOG) pathway is possibly the most characterised SAPK pathway and has provided a framework from which to investigate SAPK signalling in other eukaryotic cells (reviewed in Hohmann, 2002 and Hohmann, 2009). It was initially found to be activated under hyperosmotic conditions and to regulate the osmotic stress induced increase in glycerol production (Brewster *et al.*, 1993); however other stresses, such as oxidative stress (Bilsland *et al*., 2004) and heat-shock (Winkler *et al.*, 2002), can also activate this pathway. The Hog1 pathway in this organism is composed of one MAPK and one MAPKK, Hog1 and Pbs2 respectively, and three MAPKKKs, Ssk2, Ssk22 and Ste11 (Brewster *et al*., 1993, Maeda *et al*., 1995 and Posas and Saito, 1997). Hog1 can either be activated by the Sln1 two-component signalling pathway which feeds into the Ssk2/Ssk22 MAPKKKs or by the Sho1 pathway which feeds into the Ste11 MAPKKK. These functionally redundant pathways converge on the MAPKK Pbs2, which subsequently phosphorylates and activates Hog1 (reviewed in Hohmann, 2009).

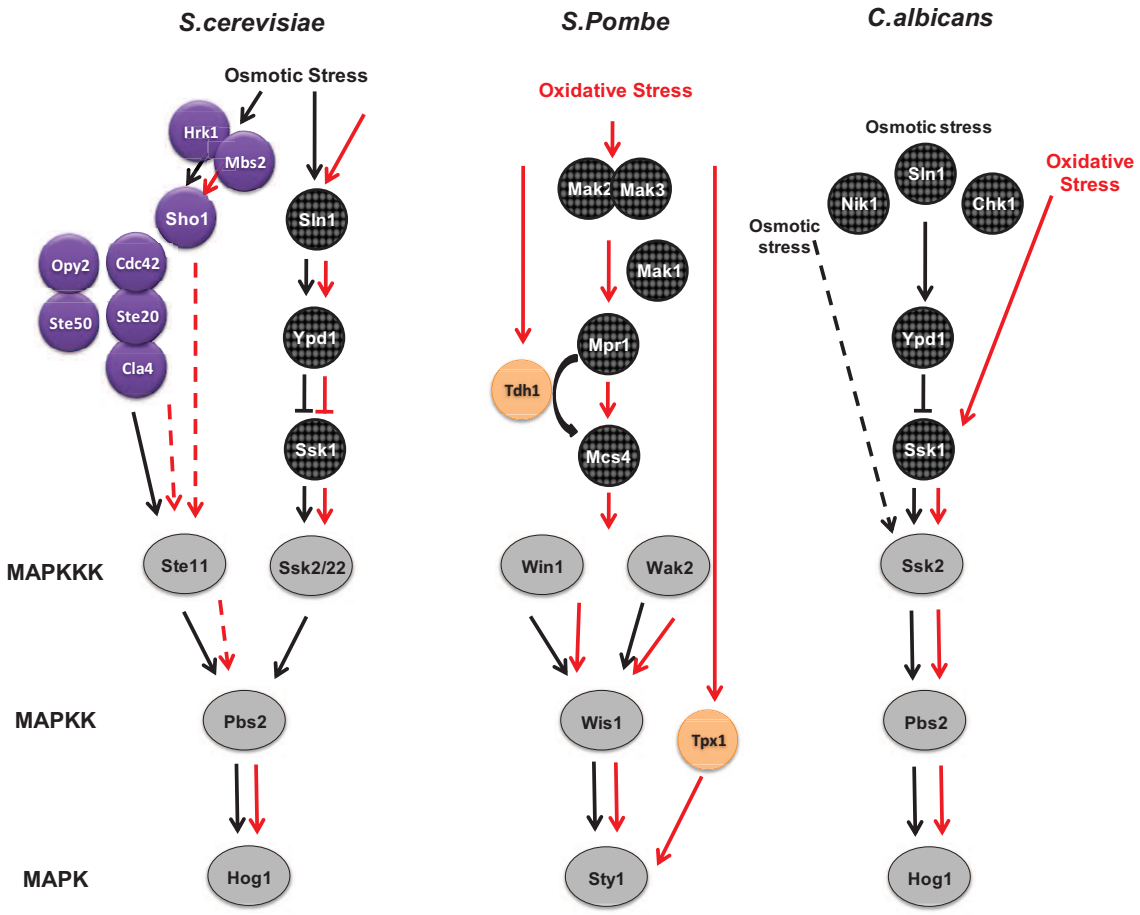


Figure 1.9. Signalling to SAPK pathways in *S. cerevisiae* (Hog1 pathway), *S. pombe* (Sty1 pathway) and *C. albicans* (Hog1 pathway).

The core components of the SAPK pathways are shown in gray, while the two-component signalling proteins are shown in black and purple (Sho1-branch). The uncharacterized, stress-signalling pathways are indicated by a dashed line. Osmotic stress signalling is indicated by black lines, while oxidative stress signalling is indicated by red lines.

The Sln1 pathway which is related to two-component systems commonly found in prokaryotes, actually comprises of three components; the Sln1 histidine kinase, the Ypd1 phosphorelay protein and the Ssk1 response regulator. The Sln1 osmo-sensing histidine kinase, is located in the membrane protein and possesses a cytoplasmic histidine kinase (HK) domain and receiver (Rec) domain. Under normal conditions, the Sln1 histidine kinase is constitutively activated. This results in the auto phosphorylation of a His residue present in the HK domain of Sln1. This phosphate group (His-P) is then transferred to an Asp residue located in the Rec domain of the same protein, Sln1 (For review see Saito and Tatebayashi, 2004). Transfer of the phosphate group from Sln1 to the response regulator Ssk1 occurs via the phosphorelay protein Ypd1. Phosphorylated Ssk1 cannot activate the MAPKKKs of the pathway (Ssk2 and Ssk22), therefore the MAPK Hog1 does not become activated under basal conditions (Posas *et al.*, 1996).

When cells lose turgor pressure (hyperosmolarity conditions) the transmembrane histidine kinase Sln1 is inactivated (Posas *et al.*, 1996) and therefore unphosphorylated Ssk1 accumulates. Furthermore, hyperosmolarity conditions results in a decreased stability of the complex between phosphorylated Ypd1 and Ssk1, resulting in higher levels of dephosphorylated Ssk1 (Kaserer *et al.*, 2009). Dephosphorylated Ssk1 promotes structural changes within Ssk2/22, which releases the interaction between the Ssk2 N-terminal autoinhibitory domain and the kinase catalytic site, resulting in autophosphorylation and activation of Ssk2/22 (Posas and Saito, 1998). Active Ssk2/22 can then phosphorylate and activates the MAPKK Pbs2, which in turn activates Hog1 (Figure 1.9). Hog1 then translocates to the nucleus where it phosphorylates several transcription factors including Hot1 which mediates the up-regulation of genes involved in glycerol synthesis (Alepuz *et al.*, 2003). Once glycerol has been produced by cells, the turgor pressure on the membrane is reduced resulting in restoration of Sln1 activity (Reiser *et al.*, 2003), and the complex between phosphorylated Ypd1 and Ssk1 is stabilized again, resulting in Ssk1 phosphorylation, Hog1 inactivation and decrease in glycerol production (Kaserer *et al.*, 2009). The transcription factor Skn7 (see section 1.4.3.1.2.) can also participate in two component signal transduction, since Skn7 is also a target of Ypd1 phosphorelay protein. Consistent with the Sln1 histidine kinase responding to osmotic stress, two-component mediated phosphorylation of Skn7 regulates the induction of genes with cell wall functions, but has no role in the oxidative stress functions of Skn7 (Li *et al.*, 2002).

In the model proposed for activation of the second osmosensing pathway in *S. cerevisiae*, two transmembrane mucins, Msb2 and Hkr1, interact with Sho1 through their transmembrane domains, in order to generate an intracellular signal that is transduced to the MAPKKK Ste11 (Tatebayashi *et al.*, 2007). The Ste11 MAPKKK activates the MAPKK, Pbs2, in a mechanism that involves activation of the Ste11/Ste50 complex by the Ste20/Cla4 kinases (Tatebayashi *et al.*, 2006 and Smith *et al.*, 2010 - Figure 1.9). Although Hog1 is activated in response to oxidative stress and *hog1*Δ cells are more sensitive that wild-type cells to reactive oxygen species (Singh, 2000 and Bilsland *et al.*, 2004), the mechanisms underlying the relay of oxidative stress signals to Hog1 are unclear. However, *S. cerevisiae sho1* cells are more sensitive to oxidative stress than wild type cells (Singh, 2000), which could be an indication that the Sho1 branch is involved in regulating Hog1 in response to oxidative stress.

Even though Hog1 activation is important for stress responses, constitutive Hog1 activation (or Hog1 hyperactivation) results in arrest of cell growth and cell death (Yaakov *et al.*, 2003 and Wurgler-Murphy *et al.*, 1997). Therefore, in addition to positive regulators, Hog1 is negatively regulated by the protein tyrosine phosphatases (Ptp) Ptp2 or Ptp3. This is shown by deletion of these phosphatase genes, which results in hyperactivation of Hog1 (Maeda *et al.*, 1993), and by ectopic expression of Ptp2 or Ptp3 that results in suppression of the Hog1 hyperactivation (Wurgler-Murphy *et al.*, 1997 and Jacoby *et al.*, 1997). In order to regulate the activation of Hog1 in response to osmotic stress, a negative feedback exists between Hog1 and the protein phosphatases, with phosphorylated Hog1 increasing the activity of Ptp2 (Wurgler-Murphy *et al.*, 1997) and up-regulating *PTP3* gene expression (Jacoby *et al.*, 1997). In addition to Ptps, Hog1 activation is also negatively regulated by Protein phosphatase type 2C (PP2C), which dephosphorylates phospo-threonine and phosphor-serine residues. The *S. cerevisiae* PP2C, Ptc1, negatively regulates Hog1 (Maeda *et al.*, 1993).

Activation of Hog1 leads to regulation of downstream effectors and the targets of Hog1 in *S. cerevisiae*, include the transcription factors Msn2 and Msn4 (Rep *et al.*, 2000), Hot1 (Rep *et al.*, 2000), the bZIP transcription factor Sko1 (Rep *et al.*, 2001) and the protein kinases Rck1 and Rck2 (Bilsland *et al.*, 2004). Rck2, for example, is activated by phosphorylation, and this activation is Hog1 dependent and results in decreased protein

synthesis in response to oxidative stress (Bilsland *et al.*, 2004), by increasing the dissociation of actively-translating ribosomes (Swaminathan *et al.*, 2006).

In *S. pombe,* the equivalent pathway is the Sty1 SAPK pathway, which is rapidly activated and accumulates in the nucleus in response to diverse environmental stresses, including oxidative stress (Millar *et al.*, 1995, Degols *et al.*, 1996, Degols and Russell, 1997, Takatsume *et al.*, 2006 and Rodriguez-Gabriel and Russell, 2005). The Sty1 pathway is composed of two largely redundant MAPKKKs (Wak1 and Win1), the MAPKK Wis1, and the SAPK Sty1 (Figure 1.9 - Millar *et al.*, 1995; Buck *et al.*, 2001 and Shieh *et al.*, 1997). In contrast to *S. cerevisiae*, virtually nothing is known regarding the relay of osmotic stress signals to the Sty1 SAPK. However, two pathways that are required for the relay of oxidative stress signals to Sty1 pathway have been characterised; a two-component related signal transduction pathway (Nyguen *et al*., 2000 and Buck *et al*., 2001), and a pathway containing the 2-Cys peroxiredoxins Tpx1 (section 1.4.4.1. - Veal *et al.*, 2004). While the two-component pathway plays a more prominent role in Sty activation in response to low levels of peroxide stress (Quinn *et al.*, 2002), Tpx1 regulates Sty1 in response to peroxide stress over a range of peroxide concentartions (Veal *et al.*, 2004)

The architecture of the two component signalling pathway in *S. pombe* is similar to the Sln1 pathway in *S. cerevisiae* (reviewed in Smith *et al*., 2010). However *S. pombe* contains three histidine kinases, Mak1, Mak2 and Mak3. Mak2 and Mak3 are highly related and both are required for peroxide induced activation of Sty1. Mak2 and Mak3 relay oxidative stress signals (by an as yet unknown mechanism) to the phosphorelay protein Mpr1, which in turn regulates phosphorelay to Mcs4 (Nguyen *et al.*, 2000; Buck *et al.*, 2001 and Quinn *et al.*, 2002). This phosphorelay system is required for peroxide activation of Sty1, since deletion of *mak2* or *mak3,* or mutation of the two-component phosphorylation sites in Mpr1 and Mcs4, results in impaired activation of Sty1 in response to peroxide stress (Aoyama *et al.*, 2000, Nyguen *et al.*, 2000 and Buck *et al.*, 2001). Furthermore, Mcs4 directly interacts with Wak1 and presumably regulates its activation (Buck *et al.*, 2001), that then activates the Wis1 MAPKK which in turn activates Sty1 by phosphorylation of conserved Thr and Tyr residues. Recently it has been shown that the glycolytic enzyme GAPDH (Tdh1) also participates in the two-component mediated peroxide-induced activation of Sty1. Tdh1 forms a complex with the MAPKKKs Wak1 and Win1, and the Mcs4 response regulator. This enzyme has a redox sensitive cysteine residue (Cys152) which becomes oxidised following oxidative

stress, and mutation of this residue resulted in a decrease in the interaction between Mcs4 and Mpr1 and a decrease in peroxide induced Sty1 activation (Morigasaki *et al.*, 2008). The 2-Cys peroxiredoxin Tpx1 is also required for peroxide induced activation of Sty1, although this is distinct from the two-component pathway and instead involves the formation of a intermolecular disulphide complex between Tpx1 and Sty1. Further details regarding Tpx1 regulation are given in Section 1.4.4.2.1.

In addition to the aforementioned pathways that positively regulate, Sty1, Sty1 is also negatively regulated in response to oxidative stress (Millar *et al.*, 1995 and Shiozaki and Russell, 1995). For example, the phosphatases Pyp1 and Pyp2 dephosphorylate Sty1, and both of these phosphatase genes are up-regulated by Sty1 in response to a range of stresses (Degols *et al.*, 1996, Shiozaki and Russell, 1996 and Wilkinson *et al.*, 1996). This negative feedback mechanism may ensure that Sty1 activation is transient and prevent overstimulation of the Sty1 pathway, which is toxic to the cells (Shiozaki and Russell, 1996).

Sty1 regulation of stress responsive genes occurs through the transcription factor Atf1p (Degols *et al.*, 1996, Shiozaki and Russell, 1996, Wilkinson *et al.*, 1996 and Chen *et al.*, 2003). Atf1 is a bZIP transcription factor that is phosphorylated in response to stress by the active form of Sty1 (Shiozaki and Russell 1996 and Wilkinson *et al.*, 1996). While the Pap1 transcription factor (see Section 1.4.3.1.1) is more important the *S. pombe* response to low H_2O_2 concentrations, Atf1 is required for the response to high H_2O_2 levels (Quinn *et al.*, 2002). Deletion of *atf1*$^+$ results in strains that showed similar oxidative stress sensitivity as the *sty1*$^-$ null strain and this transcription factor induces the expression of several antioxidant genes, such as *ctt1*$^+$, *gpx1*$^+$ and *srx1*$^+$ (Shiozaki and Russell 1996, Wilkinson *et al.*, 1996 and Bozonet *et al.*, 2005). Sty1 regulates Atf1 peroxide-induced activation by two mechanisms; Sty1 mediated phosphorylation of Atf1 stabilises the protein (Lawrence *et al.*, 2007), and *atf1*$^+$ mRNA levels (Day and Veal, 2010). Regarding the stabilization of Atf1 mRNA levels, the formation of an intra-disulphide bond, between Cys 153 and 158 of Sty1 was shown to be required for stabilization of *atf1*$^+$ mRNA, and for *S. pombe* resistance, in response to high levels of peroxide stress (Day and Veal, 2010). These mechanisms underlying Atf1 protein and *atf1*$^+$ mRNA stability act independently, as impairment of both processes results in strains that are more sensitive to peroxide stress than strains that harbour mutations that impair either Atf1 phosphorylation or *atf1*$^+$ mRNA stabilization (Day and Veal, 2010).

In *C. albicans*, the Hog1 SAPK pathway is also involved in the regulation of oxidative stress responses (Alonso-Monge *et al.*, 2003 and Smith *et al*., 2004). As seen with *S. pombe* Sty1, Hog1 is rapidly activated and accumulates in the nucleus in response to oxidative stress (Smith *et al.*, 2004). *C. albicans* Hog1 is regulated by a single MAPKK, Pbs2 (Arana *et al.*, 2005). However, in contrast to the SAPK pathways in *S. cerevisiae* and *S. pombe*, *C. albicans* Hog1 is regulated by a single MAPKKK, Ssk2 (Cheetham *et al.*, 2007). Two-component proteins have also been identified in *C. albicans* although their role in Hog1 regulation remains to be clarified (reviewed in Smith *et al.*, 2010). For example, *C. albicans* has three distinct HKs; Sln1, Chk1 and Nik1. Chk1 is closely related in sequence to the *S. pombe* peroxide-sensing HKs Mak2 and Mak3 (Buck *et al.*, 2001), and deletion of *CHK1* in *C. albicans* results in cells that are sensitive to oxidative stress. However, peroxide induced activation of Hog1 is still seen in *C. albicans chk1*Δ cells and in double *chk1*Δ*sln1*Δ and *chk1*Δ*nik1*Δ mutants (Li *et al.*, 2004 and Román *et al.*, 2005). In addition, homologues of both the phosphorelay protein Ypd1 and the response regulator Ssk1 have been identified (Calera and Calderone, 1999 and Calera *et al.*, 2000). Interestingly, inactivation of the Ssk1 response regulator specifically impairs activation of the Hog1 SAPK in response to oxidative but not osmotic stress (Calera *et al.*, 2000 and Chauhan *et al.*, 2003). Furthermore, *C. albicans ssk1*Δ cells expressing Ssk1 in which the predicted phospho-aspartate residue was mutated (Asp556Asn) resulted in cells that are more sensitive to oxidative stress than *ssk1*Δ cells, even though peroxide-induced Hog1 phosphorylation was unaltered in this strain (Menon *et al.*, 2006). However, Hog1 nuclear accumulation in response to peroxide treatment was impaired as was the expression of certain antioxidant genes (*GSH1, ARH1, GSH2, TSA1* and *CAP1*), which may underlie the increased peroxide sensitivity (Menon *et al.*, 2006). Although Ssk1 is important for peroxide induced activation of Hog1, the upstream regulators of Ssk1 remain elusive as the *C. albicans* HKs appear to be dispensable for Hog1 activation (Román *et al.*, 2005).

Recently the downstream target of Hog1, Sko1, was characterized in *C. albicans*. *C. albicans* Sko1 is phosphorylated in response to osmotic stress in a Hog1-dependent manner (Rauceo *et al.*, 2008). In addition to osmotic stress Sko1 also seems to play a role in oxidative stress responses in *C. albicans*, since *sko1*Δ cells are sensitive to oxidative stress treatments when compared to wild-type cells (Alonso-Monge *et al.*, 2010). Furthermore, *sko1*Δ cells displayed decreased levels of phosphorylated Hog1 in response to oxidative

stress (H_2O_2) when compared with wild type cells, which would indicate that Sko1 is a positive regulator of Hog1 (Alonso-Monge *et al.*, 2010). The double *sko1*Δ*hog1*Δ strain was less sensitivite to H_2O_2 treatment than the *hog1*Δ strain, displaying the same sensitivity as *sko1*Δ strain, which could indicate that Hog1 and Sko1 are acting in the same pathway (Alonso-Monge *et al.*, 2010).

In contrast to the *S. pombe* Sty1 SAPK pathway, the *C. albicans* Hog1 pathway does not play a major role in the up-regulation of H_2O_2-induced genes (Enjalbert *et al.*, 2006), and the role of Hog1 in the *C. albicans* oxidative stress response remains elusive. In addition, while in *S. pombe*, activation of Pap1 is dependent on the Sty1 SAPK (Toone *et al.*, 1998, Quinn *et al.*, 2002, Vivancos *et al.*, 2004 and Bozonet *et al.*, 2005), in *C. albicans* peroxide-induced activation of Cap1 occurs independently of the Hog1 SAPK (Enjalbert *et al.*, 2006). Clearly, the role and regulation of SAPKs in response to oxidative stress has diverged throughout the fungal kingdom. As Hog1 in *C. albicans* is required for pathogenesis (Alonso-Monge *et al.*, 1999), a better understanding of the mechanisms by which Hog1 mediates oxidative stress responses will provide insight into how this pathway promotes survival in the host.

1.4.4. ROS as signalling molecules

Even though ROS are linked with cellular damage (see section 1.3.2), it is now widely appreciated that certain ROS such as H_2O_2 have additional roles in stress signalling (reviewed in Veal *et al.*, 2007). It is known that the redox balance of cells is tightly regulated; therefore small differences in the redox balance of cells can activate signaling pathways (Burhans and Heintz, 2009). H_2O_2, for example, is produced in mammalian cells in response to cytokines, growth factors and hormones, resulting in a signalling cascade that regulates processes such as cell proliferation (Geiszt and Leto, 2004), differentiation (Li *et al.*, 2006), migration (Ushio-Fukai, 2009) and death (Cai, 2005 and Gechev and Hille, 2005). Furthermore, H_2O_2 is a major regulator of signal transduction pathways involved in processes such as mammalian immune cell activation (see section 1.3.1.4) and cellular morphogenesis in fungal pathogens and filamentous fungi (Aguirre *et al.*, 2005, Scott and Eaton, 2008 and Nasution *et al.*, 2008).

1.4.4. 1. Post-translational modifications as a mechanism of regulation of oxidative stress signalling

A major mechanism by which H_2O_2 functions as a signaling molecule is by oxidizing redox sensitive cysteine residues within specific proteins and this is employed as a mechanism of initiation and amplification of the H_2O_2 signal (see section 1.3.2.2.). The role of cysteine modification in the regulation of oxidative stress signalling is discussed in more detail below (section 1.4.5.1.) In addition, oxidative stress can also induce other post-translational modifications of proteins such as gluthationylation and sumoylation. By inducing such modifications in proteins, oxidants can then regulate their signalling function (Burhans and Heintz, 2009). This will be considered in more detail below.

In mammalian cells, glutathionylation of proteins (see section 1.4.1.1.) with signalling functions indicates that this mechanism can also regulate oxidative stress signalling. For example, oxidative stress-induced glutathionylation of a cysteine residue located in the ATP-binding pocket of the mammalian MAPKKK MEKK1, results in inhibition of its kinase activity. Inactivation of MEKK1, which has pro-survival signalling functions, combined with activation of pro-apoptotic pathways, can lead to apoptosis (Cross and Templeton, 2004). In addition, the mammalian tumour suppressor protein p53 is negatively regulated by glutathionylation in response to H_2O_2 treatment, this confers both protection against irreversible oxidation and temporary inhibition of p53 function (Velu *et al.*, 2007). The mammalian transcription factor STAT3 is also regulated by an increase in S-glutathionylation. Oxidative stress increases STAT3 glutathionylation and reduces its phosphorylation, which results in inhibition of STAT3 nuclear accumulation and transcriptional activation (Xie *et al.*, 2009). These are just some examples of how protein glutathionylation can regulate stress signalling in response to oxidative stress.

Sumoylation of proteins is a reversible post-translational modification that is also involved in the oxidative stress responses. This process consists of conjugation of the small ubiquitin-related modifier (SUMO) group, via its C-terminal Gly residue, to the NH_2 group of Lys residues in target proteins (reviewed in Wilkinson and Henley, 2010). The reactions that result in protein sumoylation are catalyzed by a SUMO activating enzyme (E1), a SUMO conjugating enzyme (E2) and SUMO ligases (E3). Conversely, SUMO-specific proteases act to remove and recycle SUMOs from their targets in a process called

desumoylation (reviewed in Bossis and Melchior, 2006). Exposure of either mammalian (Saitoh and Hinchey, 2000) or yeast cells (Zhou *et al.*, 2004) to low H_2O_2 concentrations results in inhibition of global sumoylation, while treatment with higher H_2O_2 levels increased global sumoylation. H_2O_2-induced desumoylation is involved in the regulation of oxidative stress signalling proteins, such as the AP-1 transcription factors, c-Fos and c-Jun. Since sumoylation of c-Fos and c-Jun is known to inhibit their activation, the rapid desumoylation seen after H_2O_2 treatment allows the rapid activation of this transcription factors (Muller *et al*., 2000 and Bossis *et al.*, 2005). In addition, sumoylation can also regulate the activity of other transcription factors, such as ERM and the Hypoxia Inducing Factor (HIF1) (Degerny *et al.*, 2005 and Huang *et al.*, 2009). Significantly, proteins that are directly involved in the sumo/desumoylation reactions are inactivated by oxidation. Increasing H_2O_2 levels can result in either reversible (SH) or irreversible (SO_2H or SO_3H) inactivation of the effectors of sumo/desumoylation, which in turn can be used by cells as a mechanism to sense different levels of ROS (Huang *et al.*, 2009). Therefore, the sumo/desumoylation proteins can act as peroxide regulators, by sensing differences in oxidant levels and subsequently regulating the function of proteins involved in oxidative stress response.

In addition to the modifications discussed above, thiol oxidation of specific signalling proteins is an important regulatory mechanism employed in the oxidative stress response. Furthermore, antioxidant proteins such as peroxiredoxins and thioredoxins have been shown to play a central role in regulating the oxidation status of key signalling proteins, and this will be considered in more detail below

1.4.4.2. Peroxiredoxins and thioredoxins as peroxide sensors and signal transducers

As discussed previously (section 1.3.2.2.), thiol groups of proteins can exist in a range of different oxidation states, which can reflect the amount of ROS, such as peroxide, present in the environment. Furthermore, the H_2O_2-induced inactivation of thiol containing proteins, such as the eukaryotic peroxiredoxins, has been suggested to allow these proteins to function as molecular floodgates. For example, when cells are exposed to low H_2O_2 concentrations the peroxiredoxin enzymes are active and thus function as peroxidases, eliminating the ROS; however, when H_2O_2 concentrations increase the peroxiredoxins enzymes are subjected to oxidative inhibition and they can then act as regulators of peroxide signal transduction

(reviewed in Wood *et al.*, 2003). Consistent with such a model, several groups have shown that redox-sensitive thiol-containing antioxidants, in addition to roles in detoxifying ROS, also play key roles in sensing and regulating oxidative stress responses (reviewed in Veal *et al.*, 2007).

Members of the peroxiredoxins and thioredoxin family are the main examples of redox-sensitive antioxidants which also have oxidative stress signaling functions. Their roles in peroxide detoxification have already been described in this thesis (see sections 1.4.2.4.1. and 1.4.2.4.2.) and their role in oxidative stress signalling will be discussed below.

1.4.4.2.1. 2-Cys Peroxiredoxins and Oxidative Stress Signalling

One of the first studies that identified a role for peroxiredoxins in oxidative stress signalling was performed in *S. cerevisiae*. In this study, it was shown that deletion of the 2-Cys peroxiredoxin *TSA1* resulted in decreased expression of Yap1/Skn7-dependent oxidative stress-induced gene expression (Ross *et al.*, 2000). This result was unexpected as it was predicted that loss of an antioxidant would generate more oxidative stress, and therefore increased gene expression. Following this initial observation, other studies demonstrated that oxidation, and thus activation, of Yap1 is mediated by either the Gpx3/Ybp1 or Tsa1 pathways (Delaunay *et al.*, 2002 and Veal *et al*., 2003; Section 1.4.3.1.1.). Indeed, further analysis of the commonly used wild type laboratory strain W3031-a used in the initial study by Ross *et al* (2000), was subsequently found to harbour a mutant and inactive *ybp1* allele. The residual oxidation of Yap1 seen when the *ybp1*Δ*gpx3*Δ double mutant is treated with H_2O_2, strongly suggests in the absence of a functional Ybp1, Tsa1 can regulate the function of Yap1 (Ross *et al.*, 2000 and Veal *et al.*, 2003). Consistent with this, subsequent studies showed that Tsa1 forms a complex with Yap1 that results in the oxidation of Yap1, in a reaction that requires both the peroxidatic and resolving Cys residues of Tsa1 (Tachibana *et al.*, 2009). However, Yap1 oxidized forms generated by either Tsa1 and Gpx3/Ybp1 are different, which is consistent with the decreased induction seen in a Yap1-target gene (*TRX2*) in W303–1a compared with a distinct wild-type strain expressing a wild-type YBP1 allele (Y17202) (Tachibana *et al.*, 2009). This different oxidation state of Yap1 generated in W303–1a can also explain the increased peroxide sensitivity exhibited by W303–1a compared with wild-type strains that contains a functional Ybp1 (Veal *et al.*, 2003).

More recently, a different 2-Cys peroxiredoxin (Ahp1) in *S. cerevisiae* was identified as a regulator of the Cad1 transcription factor in response to lipid peroxidation induced oxidative stress. Cad1 is a transcription factor similar to Yap1, and Ahp1 was shown to be required for the alkyl hydroperoxide-induction of Cad1-regulated genes, and for the formation of disulfide bonds in Cad1 in response to alkyl hydroperoxides. Therefore, Ahp1 might act as both a peroxidase and regulator of the oxidative stress response induced by lipid peroxidation, since this protein regulates the alkyl hydroperoxide-induced activation of Cad1 and is assumed to have an alkyl hydroperoxide detoxifying activity (Iwai *et al.*, 2010).

Although in *S. cerevisiae*, the 2-Cys peroxiredoxin Tsa1 may have only a minor role in oxidative stress signalling, in *S. pombe* the analogous protein Tpx1, is a central mediator of oxidative stress signal transduction. For example, Tpx1 is required for the peroxide-induced activation of the Sty1 SAPK pathway and oxidation and activation of AP-1-like transcription factor Pap1. In *S. pombe*, Pap1 is rapidly oxidized at lower H_2O_2 concentrations, resulting in Pap1 nuclear accumulation and up-regulation of Pap1-target genes, however Pap1 oxidation is significantly delayed upon exposure to higher levels of H_2O_2 (Bozonet *et al.*, 2005). Pap1 oxidation is mediated entirely by Tpx1 in a reaction that requires Tpx1 peroxidase activity (see Figure 1.8 - section 1.4.2.4. for details in the peroxidase activity of 2-Cys peroxiredoxin). The H_2O_2 concentration dependent regulation of Pap1 is due to the fact that the peroxidatic cysteine of Tpx1 is highly sensitive to overoxidation to sulphinic acid (SO_2). Thus, treatment of *S. pombe* cells with high H_2O_2 concentrations results in overoxidation and inactivation of Tpx1, and hence inhibition of peroxide-induced Pap1 oxidation. The Sty1 SAPK is also required for Pap1 activation (Toone *et al.*, 1998), and this is because at high levels of H_2O_2, Sty1 is activated which leads to the Atf1-dependent transcription of the sulphiredoxin encoding gene *srx1*$^+$ (see Section 1.4.3.2.). Srx1 catalyses the reduction of the inactive sulphinic (SO_2) form of Tpx1 to the sulphenic form, which can then be reduced by the Trx system, to generate active Tpx1 (see section 1.4.2.4. - Figure 1.8) (Bozonet *et al.*, 2005 and Vivancos *et al.*, 2005).

Tpx1 is also essential for the H_2O_2-induced activation of the Sty1 SAPK pathway. Initialy it was shown that ectopic expression of *tpx1*$^+$ in *S. pombe* increased the levels of phosphorylated Sty1 in response to H_2O_2 treatment and that deletion of *tpx1*$^+$ resulted in reduced levels of peroxide-induced Sty1 phosphorylation (Veal *et al.*, 2003). In *S. pombe*, the two-component pathway has a major role in the peroxide-induced activation of Sty1 (Nguyen

et al. 2000 and Buck *et al.* 2001). However, ectopic expression of *tpx1*$^{+}$ in cells that lacked the HK (*mak2*$^{-}$ and *mak3*$^{-}$) and the phosphorelay protein (*mpr1*$^{-}$) still increase the levels of peroxide-induced Sty1 activation. Furthermore, ectopic expression of *tpx1*$^{+}$ in cells that expressed a *mcs4*$^{+}$ form (*mcs4*^{DN}) that no longer can phosphorelay the signal to Sty1 in response to peroxide stress, still results in increased peroxide-induced Sty1 phosphorylation. Collectively, these results indicate that Tpx1 would act either downstream or independently of the two-component pathway in the peroxide-induced activation of Sty1 (Veal *et al.*, 2003). Furthermore, Tpx1 peroxide-induced activation of Sty1 requires the MAPKK Wis1, but not the MAPKKKs (Wak1 and Win1). This was shown by over-expression of *tpx1*$^{+}$ in wild-type cells and cells expressing a mutated form of *wis1*$^{+}$ (*wis1*^{DD}), which results in constitutive activation of Sty1 phosphorylation that is not induced by H_2O_2 (Shiozaki *et al.*, 1998). Ectopic expression of *tpx1*$^{+}$ in *wis1*$^{-}$ cells did not rescue the peroxide-induced Sty1 activation, but ectopic expression of *tpx1*$^{+}$ resulted in increased peroxide-induced activation of Sty1 in cells expressing the of *wis1*^{DD} mutation, indicating that Tpx1 acts downstream of Wak1 and Win1 in order to induce Sty1 phosphorylation in response to peroxide stress (Veal *et al.*, 2003). Even though it is unknown how exactly Tpx1 regulates Sty1 in response to peroxide stress, this regulation occurs independently of its peroxidase activity and involves the formation of a peroxide-induced disulphide complex between the peroxidatic cysteine of Tpx1 and Cys35 of Sty1 (Veal *et al.*, 2003). Therefore, Tpx1 is an essential component of the peroxide signalling in *S. pombe*. Significantly, the different transcriptional responses to low and high levels of stress H_2O_2 documented in *S. pombe* (Quinn *et al.*, 2002 and Chen *et al*, 2003) are mediated by Tpx1 which acts a molecular switch governing Pap1 mediated responses at low levels, and Sty1/Atf1 mediated responses at high levels, of H_2O_2.

The role of peroxiredoxins as molecular sensors and regulators of oxidative stress signalling pathways are conserved in higher eukaryotes. For example, in the model organism *Caenorhabditis elegans* the 2-Cys peroxiredoxin PRDX-2 is required for the arsenite-induced activation of the p38 related SAPK PMK1 (Oláhová *et al.*, 2008). In mammalian cells, the 2-Cys peroxiredoxin, Prx I, also positively regulate the p38 SAPK. Decreased expression of the 2-Cys peroxiredoxin, Prx I, in macrophage-derived foam cells treated with an oxidant (oxLDL) results in decreased p38 MAPK activation, whereas over-expression of this gene resulted in an increase in activation of this SAPK pathway (Conway and Kinter, 2006). In addition, PrxI negatively regulates the JNK SAPK pathway. PrxI suppresses JNK activation

and apoptosis in mammalian cells, by stabilizing the GSTpi-JNK complex and reducing JNK activation, in a reaction that does not require peroxidase activity of PrxI (Kim *et al.*, 2006). Furthermore, PrxI also negatively regulates the upstream regulator of JNK, the MAPKKK Apoptosis signal-regulating kinase 1 (ASK1) (Kim *et al.*, 2008). This study revealed that PrxI interacts with the N-terminal region of ASK1 in cells treated with H_2O_2 in a mechanism that requires both the peroxidatic and resolving cysteines of PrxI. These results indicate that in response to H_2O_2, reduced PrxI forms a complex with ASK1, therefore inhibiting its phosphorylation and its pro-apoptotic activity (Kim *et al.*, 2008).

A recent study, uncovered a novel mechanism by which peroxiredoxins regulate H_2O_2 signalling in response to growth factors in mammalian cells. Activation of growth factor receptors results in H_2O_2 production (by membrane associated-Nox) (Clempus and Griendling, 2006) and activation of the Src kinases (Chiarugi, 2008). In this study, Src kinases were found to phosphorylate membrane-associated PrxI on Tyr 194, leading to inactivation of its peroxidase activity. The phosphorylation-mediated inactivation of PrxI allowed H_2O_2 accumulation locally at the membrane, which subsequently results in the oxidization of critical cysteine residues of effectors such as protein tyrosine phosphatases. Therefore, PrxI phosphorylation allows sustained H_2O_2 accumulation thus permitting peroxide induced signal transduction (Woo *et al.*, 2010).

1.4.4.2.2. Thioredoxin System and Oxidative Stress Signalling

Thioredoxins (Trxs) can regulate oxidative stress signalling by two mechanisms; indirectly by regulating proteins (peroxiredoxins) or processes (deglutathionylation) that are involved in oxidative stress signalling (see sections 1.4.2.4, 1.4.4.1 and 1.4.4.2.1), and directly by regulating the oxidation status of specific stress signalling proteins, such as AP-1 transcription factors and SAPK pathway components, in both model yeasts and mammalian cells (reviewed in Veal *et al.*, 2007).

In *S. cerevisiae*, the cytoplasmic Trxs, Trx1 and Trx2 are involved in regulating the AP-1-like transcription factor Yap1 in response to H_2O_2 (Izawa *et al.*, 1999). As described in Section 1.4.3.1.1, peroxide-induced activation of Yap1 requires oxidation of specific cysteine residues within the n-CRD and c-CRD, which results in Yap1 accumulation in the nucleus and subsequent induction of Yap1-dependent gene expression. Once levels of ROS have reached homeostatic levels, Yap1 disulphide bonds are reduced and Yap1 becomes more

evenly distributed throughout the cell. The finding that in *S. cerevisiae trx1*Δ*trx2*Δ cells Yap1 was constitutively nuclear, coincident with a high basal level of Yap1-dependent gene expression, indicated that Trx1 and Trx2 functioned to inhibit Yap1 activity (Izawa *et al.*, 1999). Subsequently, it was shown that inactivation of *TRX1* and *TRX2* resulted in oxidation of Yap1 under non-stressed conditions (Delaunay *et al.*, 2000). In addition, it was demonstrated that the thioredoxin system can reduce Yap1 disulphide bonds *in vitro* and deletion of *TRR1* which encodes for the cytoplasmic thioredoxin reductase protein, also resulted in constitutive activation of Yap1 (Carmel-Harel *et al.*, 2001 and Kuge *et al.*, 2001). Furthermore, the cytoplasmatic thioredoxins also function to reduce Gpx3 and therefore can regulate Yap1 function, by both reducing Yap1 and by providing reduced Gpx3, which in turn is required for the peroxide-activation of Yap1 (Delaunay *et al.*, 2002). These results are all consistent with a model in which thioredoxin proteins function to reduce the active oxidised form of Yap1. As both *TRX1* and *TRX2* are regulated by Yap1 (Kuge and Jones, 1994 and Morgan *et al.*, 1997), this is also an example of negative feedback regulation. However, it is also significant that peroxide treatment results in the oxidation and inactivation of Trx1 and Trx2, which allows the oxidized active form of Yap1 to be maintained (Kuge *et al.*, 2001). Once the peroxide levels decrease, the reduced active form of Trx1 and Trx2 is restored, consequently resulting in the reduction of oxidized Yap1. Although thioredoxin regulation of the analogous transcription factor Pap1 in *S. pombe* is less well studied, a similar mechanism is likely, as deletion of *trx1*$^{-}$ results in increased transcription of Pap1 regulated genes under non-stressed conditions, and sustained activation of such genes in response to oxidative stress (Song and Roe, 2008).

The role of thioredoxins in regulating the activity of stress-induced transcription factors is conserved in higher eukaryotic cells. For example, mammalian thioredoxin (Trx1) is indirectly involved in induction of AP-1 activation, by regulating the redox state of Ref1, which in turn regulates the redox state of the AP-1 component proteins, c-Jun and c-Fos (DeMorrow *et al.*, 2008). In particular the redox status of Cys252 regulates c-Fos/c-Jun (AP-1) DNA binding activity. Oxidation of Cys252 inhibits AP-1 DNA activity and is reversed by the action of the nuclear redox factor (Ref-1), which can reduce this Cys residue (Xanthoudakis *et al.*, 1992, Xanthoudakis *et al.*, 1994 and Hirota *et al.*, 1997). Trx physically interacts with Ref1, and has been shown to reduce Ref1 both *in vivo* and *in vitro*. Moreover, Trx also interacts with Jab1, which is a known inducer of AP-1 activation. This regulation of

AP-1 component proteins by Jab1 is shown in mammalian cells that overexpressed Jab1, which results in increase AP-1-dependent gene expression. Furthermore, co-expression of thioredoxin in cells overexpressing Jab1 resulted in a partial decrease of AP-1-dependent gene expression (Hwang *et al.*, 2004). Therefore, these results indicate that Trx regulates AP-1 activation indirectly, by regulating the redox state and activity of proteins that are directly involved in AP-1 activation (Ref1 and Jab1). In addition to AP-1, Trx also regulates the transcriptional activity of NF-κβ and p53, likely by similar mechanisms (Seemann and Hainaut, 2005 and Ando *et al.*, 2008). For example, oxidation of p53 results in inhibition of the transcriptional activity of this pro-apoptotic protein. Similarly to that described for AP-1, Trx seems to regulate the oxidation state of p53 and therefore its activity, by regulation of Ref1 redox state (Seemann and Hainaut, 2005).

In addition to its role in oxidative stress signalling through the regulation of transcription factors, Trx1 also directly controls the JNK MAPK pathway in mammals, by regulating the MAPKKK ASK1 (Saitoh *et al.*, 1998 and Liu *et al.*, 2000). In non-stimulated cells, ASK1 can be found as homo-oligomeric structures. These ASK1 oligomeric structures can also associate with other cell proteins to form high-molecular-weight (HMW) complexes which are refered to as the ASK1 signallosome (Tobiume *et al.*, 2002 and Noguchi *et al.*, 2005). In response to peroxide treatment, the ASK1 signallosome can then form an even higher HMW complex, which induces ASK1 phosphorylation (Noguchi *et al.*, 2005). Furthermore, it was shown that the Cys250 residue of ASK1 is essential for the peroxide activation of the MAPK JNK1 (Nadeau *et al.*, 2009). It is known that in response to H_2O_2 treatment, Trx1 positively regulates ASK1, by dissociation from ASK1 Cys250. However, the mechanism by which this dissociation occurs and the exact role of Cys250 in peroxide-induced ASK1 activation is not known (Nadeau *et al.*, 2009). In addition, Trx1 negatively regulates ASK1 by reducing ASK1 disulphide bond multimers. Under resting conditions, reduced Trx1 can reduce these ASK1 multimers, however under oxidative-stress conditions; Trx1 becomes oxidized and can no longer reduce these ASK1 multimers that are required for ASK1 activation (Nadeau *et al.*, 2007). Therefore, Trx1 oxidation state and oxireductase activity is also involved in regulation of MAPK pathways in mammalian cells.

In summary, redox sensitive antioxidants such as 2-Cys peroxiredoxins and thioredoxins have additional roles in the control of oxidative stress responsive signaling proteins in both lower and higher eukaryotes. As oxidative stress responses are vital for the pathogenesis of

the major fungal pathogen of humans, *C. albicans*, the aim of this thesis was to investigate the role of 2-Cys peroxiredoxins and thioredoxin proteins in oxidative stress signalling in this medically relevant fungus.

1.5. Summary and Aims

Different groups have shown that the thioredoxin system plays an important role in the oxidative stress response. The 2-Cys peroxiredoxins play key roles in the regulation of peroxide signalling in the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe,* including the oxidative-stress induced activation of SAPK and the regulation of AP-1 like transcription factors. Furthermore, recent studies of the *C. albicans* 2-Cys peroxiredoxin Tsa1 have revealed that *tsa1*Δ exhibits sensitivity to ROS and also morphogenetic defects. However, the role of Tsa1 in signal transduction and its specific role in morphogenesis in *C. albicans* are unknown. In this context, thioredoxins and sulfiredoxins are required in other yeasts for maintaining the balance between oxidized and reduced Tsa1, and therefore regulating oxidative stress responses. In addition to their role in Tsa1 regulation, thioredoxin and thioredoxin reductase are also involved in the maintenance of the redox homeostasis and in cell cycle control. Therefore the overall objectives of this project are to:

1) Investigate the role of the putative thioredoxin, Trx1, in the pathogenesis of *C. albicans*.
2) Investigate the role of *C. albicans* 2-Cys peroxiredoxin, Tsa1, and thioredoxin, Trx1, in oxidative stress signalling.
3) Investigate the role of the thioredoxin, Trx1, in regulating H_2O_2-induced morphogenesis in *C. albicans.*

Chapter 2. Material and Methods

2.1. Yeast Techniques

2.1.1. Yeast strains and growth conditions

2.1.1.1. C. albicans strains and growth conditions

The *C. albicans* strains used in this study were grown in either Yeast Extract Peptone Dextrose (YPD) media or SD minimal media (Sherman, 1991). YPD contains 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose. SD media contains 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose and appropriate amino acids for selective growth [arginine (300 mg/l), histidine (300 mg/l), leucine (300 mg/l), methionine (300 mg/l), uridine (300 mg/l) and tryptophan (300 mg/l)]. For solid media 10 g/l of agar was added.

In order to promote loss of *URA3,* cells were streaked for single colonies on minimal media containing 5-fluoroorotic acid (5-FOA). 5-FOA minimal medium contains 0.1% 5-FOA, 0.67% (w/v), yeast nitrogen base without amino acids (300 mg/l), 2% (w/v) glucose, uridine (46 mg/l) and 1x URA drop out solution [isoleucine (300 mg/l), valine (1.5 g/l), adenine (200 mg/l), arginine (200 mg/l), histidine (200 mg/l), leucine (1 g/l), lysine (300 mg/l), methionine (200 mg/l), phenylalanine (500 mg/l), threonine (2 g/l), tryptophan (200 mg/l), tyrosine (300 mg/l), uridine (200 mg/l)].

Cells were grown aerobically at 30°C unless indicated otherwise. Growth curves were obtained by diluting stationary phase cells and incubating for 3-4 hours until in mid-log phase (OD_{660}= 0.4-0.6). Cells were then re-diluted to OD_{660}=0.1, and the OD_{660} measured hourly. Data was then plotted in a graph of OD_{660} measurements versus time. The *C. albicans* strains used in this study are listed in Table 2.1 below

Table 2.1 *C. albicans* strains used in this study

Strain	Relevant genotype	Source/Reference
SN148	*arg4*Δ/*arg4*Δ *leu2*Δ/*leu2*Δ *his1*Δ/*his1*Δ *ura3*Δ*::imm434*/*ura3*Δ*::imm434 iro1*Δ*::imm434/ iro1*Δ*::imm434*	Noble *et al.* (2005)
BWP17	*ura3::λ imm434/ura3::λimm434, his1::hisG/his1::hisG, arg4::hisG/ arg4::hisG*	Wilson *et al.* (1999)
JC47	BWP17 *hog1::loxP-ARG4-loxP/hog1::loxP-HIS1-loxP*	Enjalbert *et al.* (2006)
WYS3	BWP17 *rad53::loxP-ARG4-loxP/ rad53:: ura3::λimm434*	Shi *et al.* (2007)
WYS3.1	BWP17 *rad53::loxP-ARG4-loxP/rad53:: ura3::λimm434 RAD53:HIS1*	Shi *et al.* (2007)
HLC54	BWP17 *ura3:: λ imm434/ ura3:: λ imm434/cph::hisG/cph::hisG/efg::hisG/efg::hisG*	Lo *et al*. (1997)
JC842	SN148 *cap1::loxP-ARG4-loxP/cap1::loxP-HIS1-loxP* CIp20:*URA3*	Lab Stock (Miranda Patterson)
JC747	SN148 Cip30:*URA3*	This study
JC894	SN148 *RAD53-MH:URA3*	This study
JC930	SN148 *TRX1-MH:URA3*	This study
JC1014	SN148 pACT:*URA3*	This study
JC1016	SN148 pACT-*RNR1*:*URA3*	This study
JC1160	SN148 pACT-*RNR3*:*URA3*	This study
JC1018	SN148 pACT-*TRX1*:*URA3*	This study
JC1066	SN148 pACT-*TRR1*:*URA3*	This study
JC452	SN148 *trx1::loxP-HIS1-loxP*	This study
JC488	SN148 *trx1::loxP-ARG4-loxP/trx1::loxP-HIS1-loxP*	This study
JC677	JC488 CIp10:*URA3*	This study
JC679	JC488 CIp10-*TRX1:URA3*	This study
JC759	JC488 CIp10-$TRX1^{C30S}$:*URA3*	This study
JC761	JC488 CIp10-$TRX1^{C33S}$:*URA3*	This study
JC763	JC488 CIp10-$TRX1^{C30,33S}$:*URA3*	This study

JC886	JC488 *RAD53-MH:URA3*	This study
JC928	JC488 *TRX1-MH:URA3*	This study
JC1174	JC488 *GFP:URA3*	This study
JC1176	JC488 *TRX1-GFP:URA3*	This study
JC1022	JC488 pACT:*URA3*	This study
JC1024	JC488 pACT-*RNR1*:*URA3*	This study
JC1162	JC488 pACT-*RNR3:URA3*	This study
JC1074	JC488 pACT-*TRX1*:*URA3*	This study
JC1115	JC488 pACT-*TRR1*:*URA3*	This study
JC1113	JC488 pACT-*TSA1*:*URA3*	This study
JC1026	SN148 *tsa1::loxP-ARG4-loxP/tsa1::loxP-HIS1-loxP/tsa1::hisG/ tsa1::hisG/ura3:: λ imm434/ ura3:: λ imm434*	This study
JC1027	JC1026 CIp10:*URA3*	This study
JC1028	JC1026 CIp10-*TSA1*:*URA3*	This study
JC1029	JC1026 CIp10- $TSA1^{C48S}$:*URA3*	This study
JC1030	JC1026 CIp10- $TSA1^{C169S}$:*URA3*	This study
JC1031	JC1026 CIp10- $TSA1^{C48,169S}$:*URA3*	This study
JC499	SN148 *srx1::loxP-ARG4-loxP/srx1::loxP-HIS1-loxP*	This study
JC719	JC499 CIp10:*URA3*	This study
JC722	JC499 CIp10-*SRX1*:*URA3*	This study
JC1274	SN148 *trx2::hisG/ trx2:: ura3:: λ imm434::hisG*	This study
JC1286	SN148 *trx1::loxP-ARG4-loxP/trx1::loxP-HIS1-loxP/trx2::hisG/ trx2:: ura3:: λ imm434::hisG*	This study

2.1.1.2. Schizosaccharomyces pombe strains and growth conditions

S. pombe strains were grown in either YE5S or Edinburgh Minimal Media (EMM) (Moreno *et al.*, 1991). YE5S contains 0.5% (w/v) yeast extract, 3% (w/v) glucose and adenine (55 mg/l), histidine (55 mg/l), leucine (250 mg/l), uracil (55 mg/l) and lysine hydrochloride (55 mg/l). EMM contains 3 g/l potassium hydrogen phthalate, 2.2 g/l Na_2HPO_4, 5 g/l NH_4Cl, 2% glucose, 20 ml/l 50x salt solution (52.5 g/l $MgCl_2.6H_2O$, 0.735 mg/l $CaCl_2.2H_2O$, 50 g/l KCl, 2 g/l Na_2SO_4), 1ml/l 1000x vitamins solution (5 g/l boric acid, 4 g/l $MnSO_4$, 4 g/l $ZnSO_4.7H_2O$, 2 g/l $FeCl_2.6H_2O$, 0.4 g/l molybdic acid, 1 g/l KI, 0.4 g/l $CuSO_4.5H_2O$, 10 g/l citric acid). Appropriate amino acids were added to EMM for selective growth. For solid media 10 g/l of agar was added. Cells were grown aerobically at 30°C unless indicated otherwise. The *S. pombe* strains used in this study are listed in Table 2.2 below

2.1.2. Yeast Strain Construction

All of the oligonucleotide primers used in the strain construction detailed below are listed in Table 2.3

2.1.2.1 Deletion of TRX1, TSA1 and SRX1

In order to create a *C. albicans* homozygous *trx1*Δ strain, the two copies of *TRX1* were deleted by replacing the entire *TRX1* ORF (104 codons) with either *HIS1* or *ARG4* (Dennison *et al.*, 2005). *TRX1* disruption cassettes, comprising either the *ARG4* or the *HIS1* gene flanked by *loxP* sites and 80 nucleotides corresponding to regions 5' and 3' of the *TRX1* open reading frame, were generated by PCR using the oligonucleotide primers TRX1delF and TRX1delR and the plasmid templates pLAL2 or pLHL2 (Dennison *et al*., 2005), respectively. Disruption cassettes were introduced into *C. albicans* strain SN148 (Noble and Johnson, 2005) to sequentially disrupt both alleles of *TRX1* and generate strain JC488 (Figure 2.1).

Table 2.2 *S. pombe* strains used in this study

Strain	*Relevant genotype*	Source/Reference
SB3	*ade6-M216 pap1(3Pk)::ura4 his7-366*	Bozonet *et al.* (2005)
SB4	*ade6 pap1(3Pk)::ura4 tpx1::ura4 his7-366*	Bozonet *et al.* (2005)
SB3-Rep1	*ade6-M216 pap1(3Pk)::ura4 his7-366* Rep1	This study
SB3-Rep1*tpx1*$^{+}$	*ade6-M216 pap1(3Pk)::ura4 his7-366* Rep1-*tpx1*$^{+}$	This study
SB3-Rep1*tsa1*$^{+}$	*ade6-M216 pap1(3Pk)::ura4 his7-366* Rep1-*tsa1*$^{+}$	This study

Table 2.3 Oligonucleotide primers used in this study

Oligonucleotides	Sequence 5' – 3'	Restriction Site
TRX1delF	CTTTTTTTTTCCATATTCCCTTGTTTTTTCACCA ACAAAATAGATATCAATTCTATTCAACCCTTT AACACTTAACACATAGTTATATTTTTTTTCAA AG**CCAGGGTTTTCCCAGTCACG**	
TRX1delR	ACTAGTAAACATCAATTATAGTTATTCCATTC AATTGTAAATATACACCCATCATCATCAATAA TACAATCATCCAAAACTATTATTAGACTATCT TCT**CTCACTAAAGGGAACAAAAGC**	
TRX1CHF	TATTCTCGCAACAGCAACG	
TRX1PromF	GCGC**GGATCC**GAGACTAAATATCGAACACCA CC	*Bam*HI
TRX1TermR	GCGC**GGATCC**ACTTGGAAGTCACTTATTAC	*Bam*HI
TRX1C30SF	GACTTTTTTGCCACTTGG***TCT***GGTCCATGTA	
TRX1C30SR	GCAATCATTTTACATGGACC***AGA***CCAAGTGGC A	
TRX1C33SF	GCCACTTGGTGTGGTCCA***TCT***AAAATGATTG	
TRX1C33SR	TAATGGAGCAATCATTTT***AGA***TGGACCACAC	
TRX1C3033SF	CTTTTTTGCCACTTGG***TCT***GGTCCA***TCT***AAAAT GAT	
TRX1C3033SR	GGAGCAATCATTTT***AGA***TGGACC***AGA***CCAAGT GG	
TRX1MHF	AAAA**CTGCAG**ATGGTTCACGTTGTCACTG	*Pst*I
TRX1MHR	AAAA**CTGCAG**AGCAAGAGAAGCCAAAGC	*Pst*I
TRX1ACTF	GCCC**ATCGAT**ATGGTTCACGTTGTCACTGA	*Cla*I
TRX1ACTR	GCCC**ATCGAT**TAAGCAAGAGAAGCCAAA	*Cla*I
TRX1GFPF	AAAA**CTGCAG**ATGGTTCACGTTGTCACTG	*Pst*I
TRX1GFPR	AAAA**CTGCAG**AGCAAGAGAAGCCAAAGC	*Pst*I
TSA1delF	GCTCTATTGCATTTTATTTCAATCAACTAATTA ATTAGTCACATACATAGATACACTACTACAAA A**CTCACTAAAGGGAACAAAAGC**	
TSA1delR	CTTGTCTAAAATGCAATCTATTTGCTATGAAA CCATAACGAAAAAAAAAACGTACATATATAT CTATAAATCCTGTTTA**CTCACTAAAGGGAAC AAAAGC**	
TSA1URAB1F	CGCG**GGATCC**CGCGCCACCTGCACAGAAGTA CCGG	*Bam*HI
TSA1URAB1R	CGCG**GGATCC**TTTTGTAGTAGTGTATCTATGT	*Bam*HI

	ATG	
TSA1URAB2F	CGCG**GGATCC**CCAGGTGATGAAACCATCAAG CC	*Bam*HI
TSA1URAB2R	CGCG**GGATCC**AACAATGAAAATACTTGGGAG AGAAG	*Bam*HI
TSA1CHF	GAGCTCAATTGTACTTCCAATCTC	
TSA1ORFF	GCGC**GGATCC**ATGGCTCCAGTCGTTCA	*Bam*HI
TSA1ORFR	GCGC**GGATCC**TTATTTGTTGACTTTGTTG	*Bam*HI
TSA1PromF	GCGC**GGATCC**CTCCGTAATCGGTTAATCTGT	*Bam*HI
TSA1TermR	GCGC**GGATCC**AACATTGAAAATACTTGGGAG AGAAG	*Bam*HI
TSA1C48SF	CATTGGCCTTCACATTCGTC***TCC***CCATCAGAA A	
TSA1C48SR	GCAATAATTTCTGATGG***GGA***GACGAATGTG	
TSA1C169SF	CTGAAAAATACGGTGAAGTT***TCC***CCAGCTAAC T	
TSA1C169SR	CCTGGGTGCCAGTTAGCT***GGA***GGAACTTCACC G	
SRX1delF	GTTTTTGAATACAACAATATTAGGTTTACTCA TTCAAAGCATCAACCAGTTCAAGATAGATTAG TATTATAAACGTTCAAA**CCAGGGTTTTCCCA GTCACG**	
SRX1delR	ATTTAATTTTACACAAGAATGCATAGTGGTAA CTGAATTAGTATCACTCCTAAACAGGGTTACA AATAACATCCATTCTG**CTCACTAAAGGGAAC AAAAGC**	
SRX1CHF	CAGATGACTCGATGACTAATGAA	
SRX1PromF	GCGC**GGATCC**ACTCCTTGAAATCAATAAT	*Bam*HI
SRX1TermR	GCGC**GGATCC**CTGATCATCCAATAA	*Bam*HI
RAD53CHF	GGAGACGCGCCAGTTTTTCACCATCTA	
RAD53MHF	AAAA**CTGCAG**TGCTTCGTCCATTGGATAGCG AAAG	*Pst*I
RAD53-MHR	AAAA**CTGCAG**TGAACTTATACTACTTAAACC CGAA	*Pst*I
RNR1CHF	CGTCTTGGCCTATAAATAAAA	
RNR1ACTF	GCCC**ATCGAT**ATGTATGTTTATAAGAGAGAT GGCCG	*Cla*I
RNR1ACTR	GCCC**ATCGAT**CTAACCAGAACACATTGTACA AGATTC	*Cla*I
RNR3CHF	GTCACAAAATGAAAAAAA	
RNR3ACTF	GCCC**ATCGAT**ATGACCAAAATCAAGGTGCTA TCAGG	*Cla*I

RNR3ACTR	GCCC**ATCGAT**CTAGCCAGAGCATGATTGACA	*Cla*I
TRR1CHF	CTAATCTATAATCCCATAA	
TRR1ACTF	GCCC**ATCGAT** ATGGTACACCACAAAGTCAC	*Cla*I
TRR1ACTR	GCCC**ATCGAT**CTAAGCTTCTTGTTCGGAAATG	*Cla*I

Bases correspondent to the sequences recognized by the restriction enzymes used are highlighted in bold, bases correspondent to the *loxP* sites are highlighted in bold and underline and bases correspondent to the point mutations (cysteine → serine) are highlighted in bold and italic.

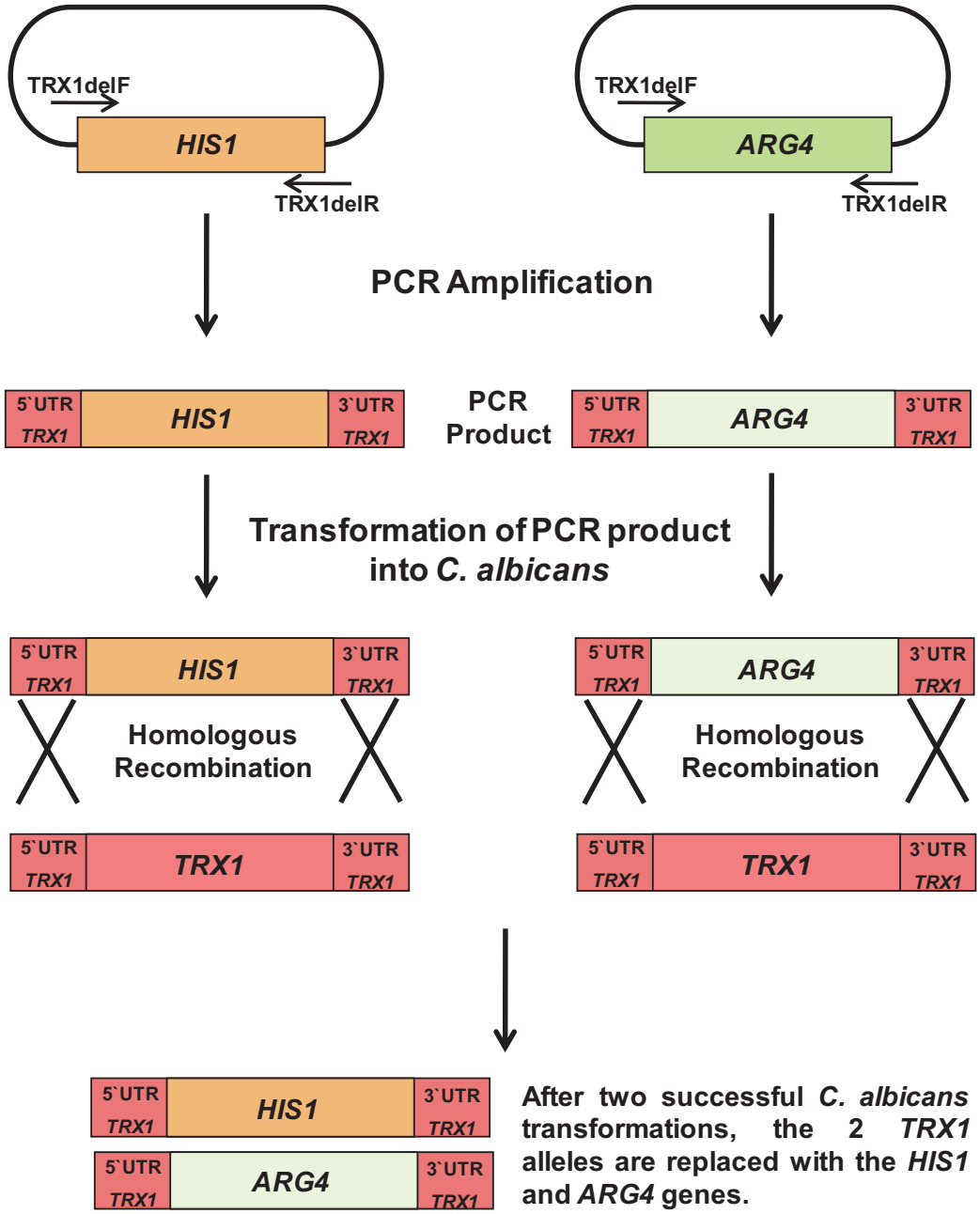


Figure 2.1. Deletion of *C. albicans TRX1*.
To delete *TRX1*, disruption cassettes containing either the *HIS1* or the *ARG4* genes flanked by LoxP sites and 80 bp of the 5` and 3` UTR of *TRX1*. The disruption cassettes were generated by PCR, using the oligonucleotide primers TRX1delF and TRX1delR and the plasmids pLAL and pLHL, which contains either the *ARG4* or the *HIS1* genes. Disruption cassettes were transformed into *C. albicans* SN148 (auxotrophic for arginine, histidine and uridine production) to generate strain JC488 (*trx1*Δ).

The same strategy was used as described above to delete the *SRX1* gene using the oligonucleotide primers SRX1delF and SRX1delR. The resulting *SRX1-ARG4* and *SRX1-HIS1* disruption cassettes replaced the 399bp correspondent to the entire *SRX1* open reading frame to generate strain JC499.

To delete the 4 copies of *TSA1* (*orf19.7417* and *orf19.7398*) in *C. albicans*, two copies were deleted using the strategy described above using the oligonucleotide primers TSA1delF and TSA1delR. The remaining loci were disrupted by Ura-blasting (Fonzi and Irwin, 1993) to generate strain JC1026. The ura-blaster *tsa1::hisG-URA3-hisG* disruption cassette was generated by PCR amplification of the regions flanking *TSA1* using the primer pairs TSA1URAB1F/TSA1URAB1R and TSA1URAB2F/TSA1URAB2R and ligating the resulting products into the *Bgl*II and *Bam*HI sites of p5921, respectively (Gow *et al.*, 1994). The *TSA1-ARG4* and *TSA1-HIS1* disruption cassettes replaced the entire 197 codon *TSA1* open reading frame, and the ura-blaster *tsa1::hisG-URA3-hisG* disruption cassette replaced codons 1-174 of the *TSA1* open reading frame (Figure 2.2). Gene disruptions were confirmed by PCR.

To construct re-integrant control strains the *TRX1* gene plus 1039bp of the promoter region and 299bp of the terminator region were amplified by PCR, using the oligonucleotide primers TRX1PromF and TRX1TermR and genomic DNA as a template. The PCR product was digested with *Bam*HI and subsequently ligated into the *Bam*HI site of CIp10 (Murad *et al.*, 2000), to generate CIp10-*TRX1*. The same strategy was used to generate CIp10-*TSA1* and CIp10-*SRX1* in which the *TSA1* open reading frame plus 701 bp of the promoter region and 204bp of the terminator region were amplified by PCR, using the oligonucleotide primers TSA1PromF and TSA1TermR, and the *SRX1* open reading frame plus 629bp of the promoter region and 204bp of the terminator region were amplified by PCR, using the oligonucleotide primers SRX1PromF and SRX1TermR. The resulting CIp10-*TRX1*, CIp10-*SRX1*, and CIp10-*TSA1* plasmids were digested with *Stu*I and integrated at the *RPS10* locus in the *trx1*Δ, *srx1*Δ, and *tsa1*Δ mutants, respectively, to generate strains JC679, JC722 and JC1028. To generate deletion mutants that were auxotrophically identical to the reconstituted strains, the CIp10 vector was integrated at the *RPS10* locus in the *trx1*Δ, *srx1*Δ and *tsa1*Δ mutants, to generate strains JC677, JC719 and JC1027, respectively. Similarly, to generate a URA^+, ARG^+, HIS^+ wild-type strain, the vector CIp30 (Dennison *et al.*, 2005), a derivative of CIp10, was integrated at the *RPS10* locus in the parental strain SN148 to generate JC747.

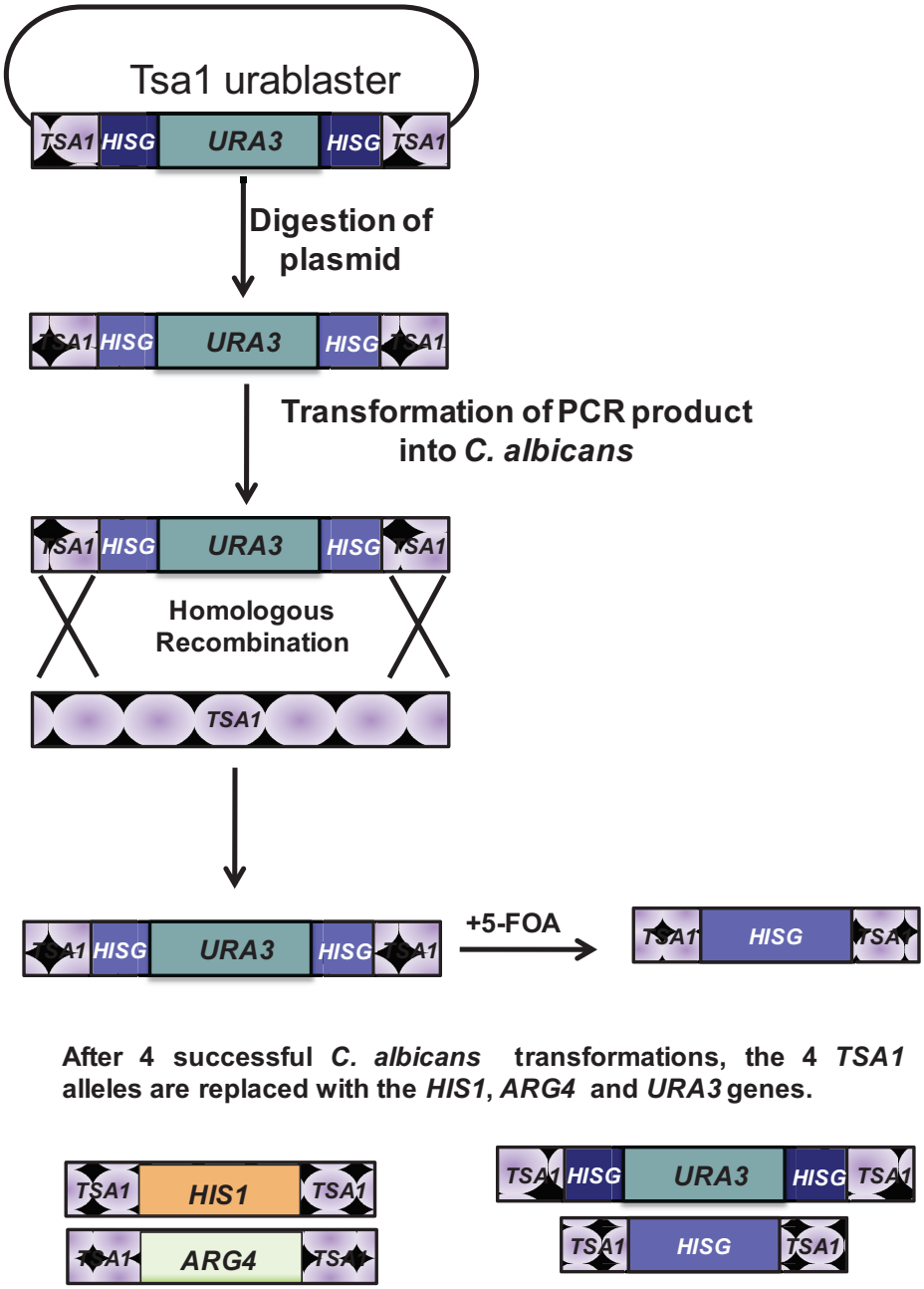


Figure 2.2. Deletion of *C. albicans TSA1*.

To delete *TSA1*, disruption cassettes containing either the *HIS1* or the *ARG4* genes flanked by LoxP sites and 80 bp of the 5` and 3` UTR of *TSA1*. The disruption cassettes were generated by PCR (Figure 2.1), using the oligonucleotide primers CaTsadelF and CaTsadelR and the plasmids pLAL and pLHL as a template. To delete the remaining two copies of Tsa1, the Tsa1 urablaster cassette, which contains the *URA3* gene flanked by hisG sequences was used. After the first transformation with the Tsa1 urablaster, the transformants were streaked in 5-FOA plates in order to select for cells lacking the *URA3* gene, which were subsequently transformed again with the Tsa1 urablaster. Disruption cassettes were transformed into *C. albicans* SN148 (auxotrophic for arginine, histidine and uridine production) to generate strain JC1026 (*tsa1*Δ).

2.1.2.2. Mutagenesis of TRX1 and TSA1

Mutagenesis of *TRX1* to create *trx1*^{C30S} was performed by overlapping PCR (Landt *et al.*, 1990) using the oligonucleotide pairs TRX1C30SF/TRX1TermR and TRX1C30SR/TRX1PromF, with the template CIp10-*TRX1*. The resulting PCR product was used as a template in a subsequent PCR with the primers TRX1PromF and TRX1TermR. The final PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of CIp10. The resulting CIp10-*trx1*^{C30S} plasmid was digested with *Stu*I and integrated at the *RPS10* locus in *trx1*Δ cells (JC488) to generate strain JC759. The same strategy was used to generate strains expressing *trx1*^{C33S} (JC761) and *trx1*^{C30,33S} (JC763), using the oligonucleotide pairs TRX1C33SF/TRX1TermR, TRX1C30SR/TRX1PromF and TRX1C3033SF/TRX1TermR, TRX1C3033SR/TRX1PromF, respectively (Figure 2.3).

Mutagenesis of *TSA1* to create the peroxidatic cysteine mutant *tsa1*^{C48S}, the resolving cysteine mutant *tsa1*^{C169S}, and the double cysteine mutant *tsa1*^{C48,169S} was performed using the same overlapping PCR technique described above. To create CIp10-*tsa1*^{C48S} and CIp10-*tsa1*^{C169S}, overlapping PCRs were performed using the oligonucleotide pairs TSA1C48SF/TSA1TermR and TSA1PromF/TSA1C48SR or TSA1C169SF/TSA1TermR and TSA1PromF/TSA1C169SR, and using CIp10-*TSA1* as a template (Figure 2.4). In order to create CIp10-*tsa1*^{C48,169S}, overlapping PCR was performed using the oligonucleotide pairs TSA1C169SF/TSA1TermR and TSA1PromF/TSA1C169SR and CIp10-tsa1^{C48S} as a template (Figure 2.4). The resulting CIp10-*tsa1*^{C48S}, CIp10-*tsa1*^{C169S}, and CIp10-*tsa1*^{C48,169S} plasmids were digested with *Stu*I and integrated at the *RPS10* locus in the *tsa1*Δ strain (JC1026) to generate strains JC1029, JC1030, and JC1031, respectively. The correct chromosomal insertion of all mutant derivatives of *TRX1* and *TSA1* was confirmed by PCR and DNA sequencing.

2.1.2.3. Tagging of Trx1 and Rad53

To tag Trx1, expressed from the normal chromosomal locus, at the C-terminus with 6-His residues and 2 copies of the myc-epitope, the *TRX1* gene was first amplified by PCR, using the oligonucleotide primers TRX1MHF and TRX1MHR and using CIp10-*TRX1* as a template. The resulting PCR product was digested with *Pst*I and ligated into the *Pst*I site of CIp-C-MH, a derivative of CIp-C-ZZ (Blackwell *et al*., 2003) in which the sequences encoding the TEV cleavage site and Protein A were replaced with those encoding 2 myc

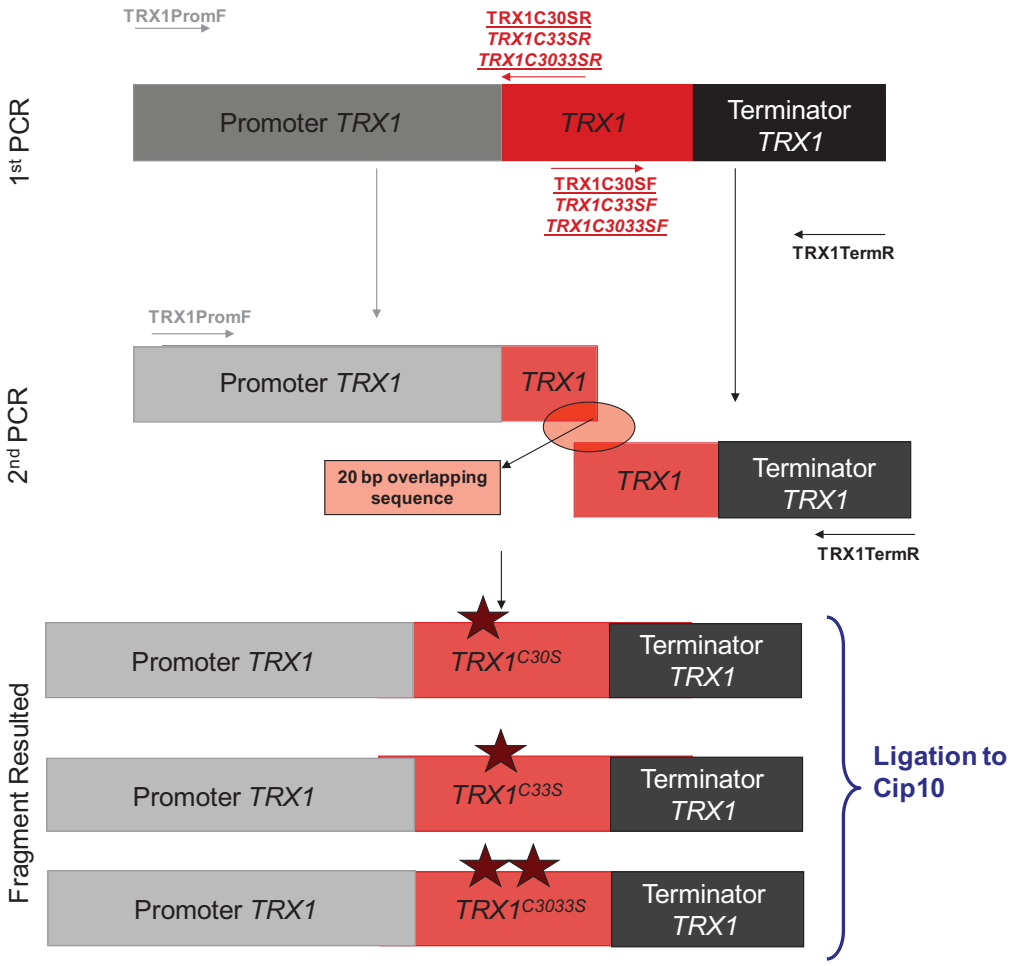


Figure 2.3. Site-directed mutagenesis of *TRX1*.
Mutagenesis of *TRX1* to create the N-terminal cysteine mutant $trx1^{C30S}$, the C-terminal cysteine mutant $trx1^{C33S}$, and the double cysteine mutant $trx1^{C30,33S}$ was performed using the overlapping PCR technique. The first PCRs to construct the cysteine mutants were performed using the oligonucleotide pairs TRX1C30SF(TRX1C33SF or TRX1C3033SF) /TRX1TERMR and TRX1PROMF/TRX1C30SR (TRX1C33SR or TRX1C3033SR) and the plasmid CIp10-*TRX1* as a template. The overlapping PCR was performed using the oligonucleotide pairs TRX1PROMF/TRX1TERMR and used the PCR products obtained in the first PCR reaction, which presented in their sequence a small overlapping region (20bp), as a template. The resulting products of the second PCR reaction were ligated to CIp10 plasmid, resulting in the plasmids CIp10-$trx1^{C30S}$, Clp10-$trx1^{C33S}$ and CIp10-$trx1^{C30,33S}$.

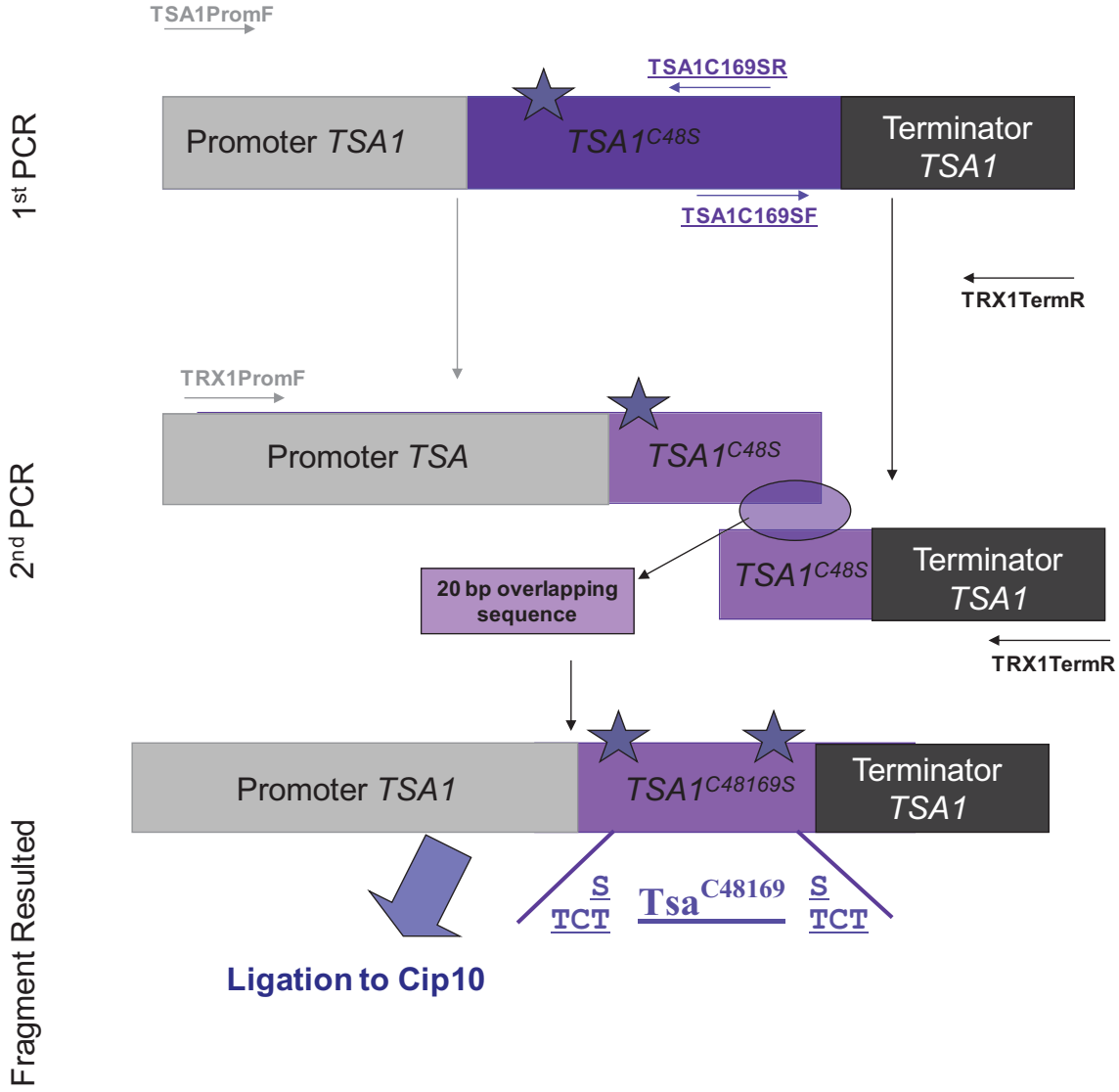


Figure 2.4. Site-directed mutagenesis of *TSA1*.
Mutagenesis of *TSA1* to create the peroxidatic cysteine mutant *tsa1*^{C48S}, the resolving cysteine mutant *tsa1*^{C169S}, and the double cysteine mutant *tsa1*$^{C48/169S}$ was performed using the same overlapping PCR technique described for mutation of Trx1 cysteine residues (Figure 2.3). To create CIp10-*tsa1*^{C48S} and CIp10-*tsa1*^{C169S}, overlapping PCRs were performed using the oligonucleotide pairs TSA1C48SF/TSA1TERMR and TSA1PROMF/TSA1C48SR or TSA1C169SF/TSA1TERMR and TSA1PROMF/TSA1C169SR, using CIp10-*TSA1* as a template. In order to create CIp10-*tsa1*^{C48169S}, overlapping PCR was performed using the oligonucleotide pairs TSA1C169SF/TSA1TERMR and TSA1PROMF/TSA1C169SR and CIp10-*tsa1*^{C48S} as a template. The resulting CIp10-*tsa1*^{C48S}, CIp10-*tsa1*^{C169S}, and CIp10-*tsa1*^{C48,169S} plasmids were digested with *Stu*I and integrated at the *RPS10* locus in the *tsa1*Δ strain (JC1026) to generate strains JC1029, JC1030, and JC1031, respectively.

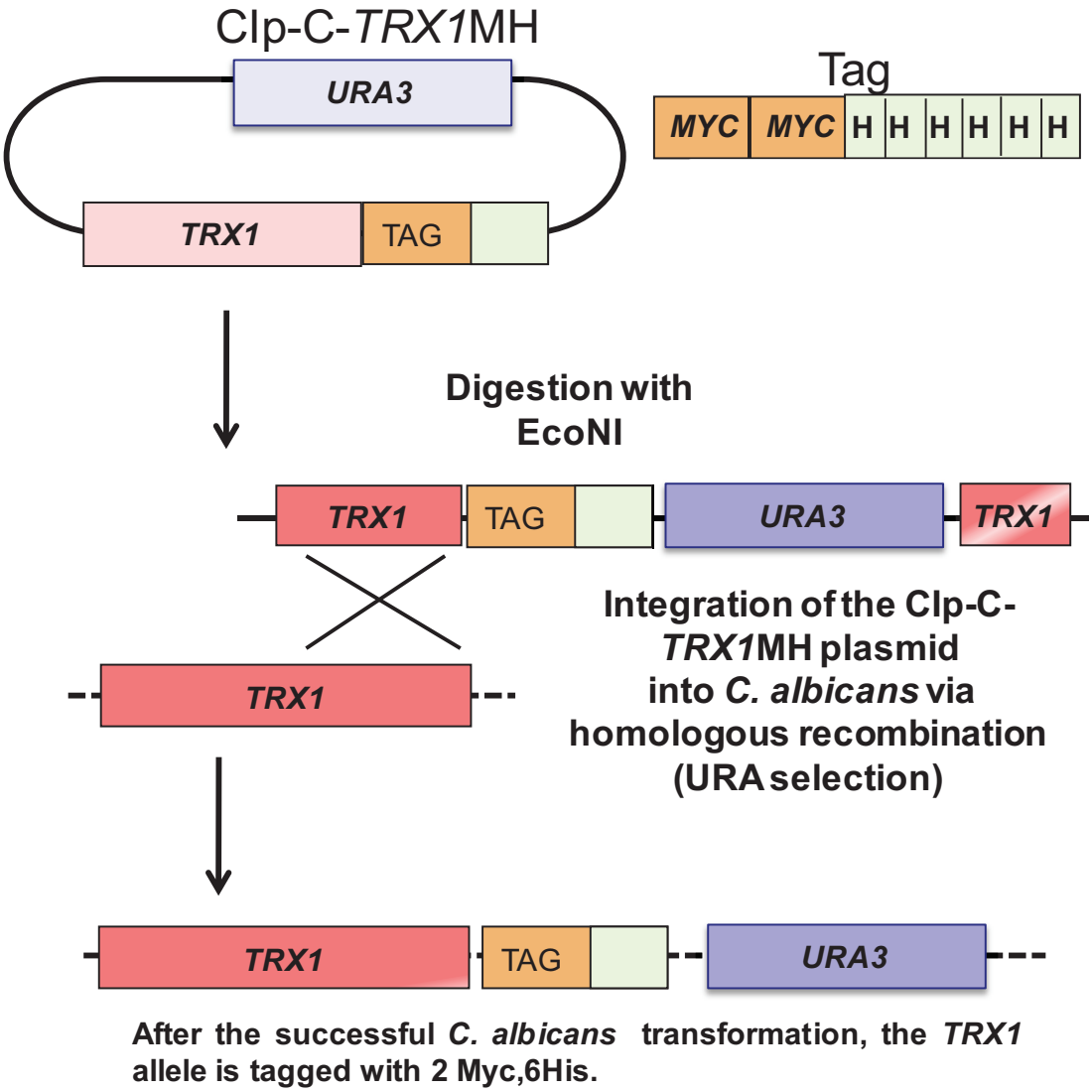


Figure 2.5. Tagging of *TRX1*.

In order to tag Trx1, the *TRX1* gene was cloned into the *Pst*I site of the plasmid CIp-C-MH (D. Smith and J. Quinn, unpublished), resulting in the plasmid CIp-C-*TRX1*MH. The CIp-C-*TRX1*MH plasmid was linearized by digestion with *Eco*NI and transformed into strain JC452. Successful transformants were obtained when the plasmid CIp-C-*TRX1*MH was integrated at the remaining *TRX1* locus in strain JC452 to generate strain JC928.

epitopes and 6 His residues (D. Smith and J. Quinn, unpublished – Figure 2.5). The CIp-C-*TRX1*MH plasmid was then linearized by digestion with *Eco*NI to target chromosomal integration at a *TRX1* locus in SN148 to generate strain JC930, or the remaining *TRX1* locus in JC452 to generate strain JC928. A similar strategy was used to tag Rad53, expressed from their respective chromosomal loci in wild-type and *trx1*Δ strains, using the primer pair RAD53MHF/RAD53MHR. The resulting CIp-C-*RAD53*MH was linearized by digestion with *Hind*III to target chromosomal integration at the respective loci in both wild type cells (SN148) to generate strains JC894, and *trx1*Δ (JC488) cells to generate strain JC886.

To tag Trx1 with GFP, the *TRX1* open reading frame and 888bp of the promoter region was amplified by PCR, using the primer pair TRX1GFPF/TRX1GFPR and genomic DNA as template. The resulting PCR product was subsequently digested with *Pst*I, and ligated into the *Pst*I site of pGFP (Barelle *et al.*, 2004). The resulting pGFP-*TRX1* plasmid was digested with *Stu*I and integrated at the *RPS10* locus in of wild-type (SN148) and *trx1*Δ (JC488) cells generating strains JC1172 and JC1176 respectively. Correct integration at the *RPS10* locus of *TRX1*-GFP was confirmed by PCR and DNA sequencing. To generate control strains, the pGFP vector was also integrated at the *RPS10* locus in the both wild-type and *trx1*Δ cells resulting in strains JC1058 and JC1174.

In all cases strains expressing tagged constructs were checked for protein functionality. For example, phenotypic analysis of heterozygote strains expressing either MH-tagged Trx1 and Rad53 tagged constructs. Similarly, expression of GFP-tagged Trx1 was found to rescue the oxidative stress phenotypes associated with deletion of *TRX1*, respectively (see subsequent results chapters for details).

2.1.2.4. Construction of C. albicans strains ectopically expressing TRR1, TRX1 and RNR1 and 3

To achieve ectopic expression of either *TRR1, TRX1* or *RNR1* or *3* in *C. albicans*, these open reading frames were amplified by PCR from genomic DNA, using the primer pairs TRR1ACTF/TRR1ACTR, TRX1ACTF/TRX1ACTR, RNR1ACTF/RNR1ACTR and RNR3ACTF/RNR3ACTR. The resulting PCR products were digested with *Cla*I and ligated into the *Cla*I site located between the *CaACT1* promoter and the *ScCYC1* terminator in pACT1 (Tripathi *et al*., 2002). The pACT1 vector and the resulting plasmids, pACT-*TRR1*, pACT1-*TRX1*, pACT1-*RNR1* and pACT1-*RNR3* were then digested with *Stu*I and

individually integrated at the *RPS10* locus in SN148 to create strains JC1014, JC1066, JC1018, JC1016 and JC1160 respectively. The same plasmids were similarly integrated into the *trx1*Δ strain (JC488) to create strains JC1022, JC1115, JC1074, JC1024 and JC1162 respectively. Correct integration at the *RPS10* locus, and the DNA sequences of the integrated open reading frames, were confirmed by PCR and DNA sequencing.

2.1.2.5. Expression of TSA1 in S. pombe

To express *C. albicans* Tsa1 in *S. pombe*, the *TSA1* open reading frame was amplified using the oligonucleotide primers TSAORFF and TSAORFR using genomic DNA as template. The resulting PCR fragment was digested with *Bam*HI and cloned into the *Bam*HI site of pRep1 (Basi *et al*., 1993) to generate pRep1-*TSA1*. This plasmid directs the expression of genes under the control of the thiamine repressible $nmt1^{+}$ promoter and also contains the LEU2 auxotrophic marker. pRep1-*TSA1* was transformed into *S. pombe* wild-type (SB3) and $tpx1^{-}$ (SB4) strains.

2.1.3. Yeast Transformation

2.1.3.1. Transformation of C. albicans.

Exogenous DNA was integrated into the *C. albicans* genome by transforming cells using the lithium acetate protocol as described by Burk Braun (http://www.sacs.ucsf.edu/home/johnsonLab/burk/transformation.html). Cells were grown to an OD_{660}=1-3 in rich YPD media, harvested at 2,500 rpm for 2 minutes, and washed in 20 ml of LiAcTE buffer [0.1 M LiAc pH7.5, 1x TE (10 mM Tris-Cl pH 7.5, 1 mM ethylenediaminetetraacetic acid-EDTA- pH 8.0)]. The cells were harvested as above and resuspended in 3 times the cell pellet volume in LiAcTE. To 100µl of the cell suspension, the transforming DNA (up to a volume of 20 µl) and carrier DNA (50 µg Salmon Sperm DNA) was added and mixed well. Subsequently, 750µl of PEG-LiAcTE solution [0.1mM LiAc, 1x TE, 40% (v/v) polyethylene glycol (PEG) 3,350] was added and tubes incubated at 30ºC for at least 3 hours whilst mixing gently. The cells were then heat-shocked at 42ºC for 45 minutes, harvested at 2,000 rpm for 1 minute, resuspended in 200µl of YPD and plated into SD media containing the appropriate amino acids to allow selection of transformants. Plates were then incubated at 30ºC for 2 -5 days.

2.1.3.2 Transformation of S. pombe

S. pombe cells were transformed with plasmid DNA using the lithium acetate protocol as described by Moreno *et al.* (1991). Cells were grown to mid-log in YE5S media (0.5-1 x 10^7 cells/ml), harvested at 3,000 rpm for 3 minutes. Cells were washed in an equal volume of dH_2O, following which were harvested and washed with 1 ml dH_2O followed by 1 ml LiAc/TE solution (100 mM LiAc pH 7.0, 1 x TE (10 mM Tris pH 7.5, 1 mM EDTA pH8)] and resuspended in LiAc/TE at 1 x 10^9 cells/ ml. Plasmid DNA (5-10 µg) and an equal amount of carrier salmon sperm DNA were then added to 100 µl of the cell suspension and incubated at 30°C for 10 min. Subsequently 260 µl of PEG/LiAc/TE (40% PEG 4000, 100 mM LiAc pH 7.0, 1 x TE) was added and the cells incubated at 30°C for 30-60 min. Dimethyl sulphoxide (DMSO) was added (43 µl) and the cells incubated at 42°C for 5 min. Cells were washed in 1 ml dH_2O, resuspended in 200 µl of dH_2O, and plated onto EMM agar plates containing the appropriate amino acids for selective growth. Plates were incubated at 30°C for 3-5 days.

2.1.4 Yeast DNA Extraction

Genomic DNA was extracted from *C. albicans* and *S. pombe* essentially as described by Hoffman and Winston (1987). Single colonies arising from yeast transformation (as described in Section 2.1.3.1) were patched onto SD media containing the appropriate amino acids and grown overnight at 30°C. Cells were collected directly from the agar plate and washed in 1 ml dH_2O, before resuspending in 200 µl of breakage buffer [10 mM Tris-Cl pH 8.0, 2% (v/v) Triton X-100, 1% (w/v) sodium dodecyl sulphate (SDS), 100 mM NaCl, 1 mM EDTA pH 8.0]. The cell suspension was added to screw-capped tubes containing 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and 200 µl of glass beads. The cells were subsequently lysed by bead beating (2 x 15 sec). After bead beating, the cell extracts were clarified by centrifugation at 10,000 rpm for 6 minutes, and the upper aqueous layer transferred to a clean tube. Nucleic acid was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 2-2.5 x the final volume of 100% ethanol, and incubating at -20ºC for 30 min. The nucleic acid was harvested by centrifugation at 13,000 rpm for 10 min, washed with 1 ml of 70% ethanol, and once dry, resuspended in 50 µl of dH_2O.

2.1.5. Yeast Stress Sensitivity Tests

2.1.5.1. Amino acid auxotrophy determination

To test for sulphur containing amino acid auxotrophy, stationary phase cells from an overnight culture were diluted to the same OD_{660}=0.4 and then 5µl of the diluted cultures were spotted onto the edge of SD plates and streaked for single colonies. SD plates containing the following amino acids [arginine (300 mg/l), histidine (300 mg/l), leucine (300 mg/l), uridine (300 mg/l) and tryptophan (300 mg/l)] were prepared with or without the following additives: methionine (200 mg/l), cysteine (200 mg/l), sulfite (2 mM), glutathione (1 mg/l). Plates were then incubated at 30ºC for 24-48h and the ability of strains to grow recorded.

2.1.5.2. Spot tests

Stationary phase cells from overnight cultures were diluted and incubated for 3-4 hours until OD_{660}=0.4-0.6. Cells were then diluted to an OD_{660}=0.2, and serial 10 fold dilutions were spotted onto solid media using a 96-well plate replica platter (Sigma Aldrich), containing a range of concentrations of specific compounds. Plates were then incubated at 30ºC for 24-48 h and the ability of strains to grow recorded.

2.1.5.3. Halo tests

Stationary phase cells from overnight cultures were diluted and incubated for 3-4 hours until OD_{660}=0.4-0.6. 10 µl of cell suspension was pippetted radially from the edge to the centre of YPD plate. A 5mm disk of Whatman microfiber paper (90 mm diameter) soaked in water or 30 % H_2O_2 was placed in the centre of the plate. Plates were then incubated at 30ºC for 24-48 h and the zone of growth inhibition around the central disc assessed.

2.1.5.4. Growth survival in liquid cultures

Stationary phase cells from overnight cultures were diluted and incubated for 3-4 hours until OD_{660}=0.4-0.6. Cells were then treated with the indicated concentration of H_2O_2. Samples were taken at various time points, diluted, and plated into YPD agar plates to

determine surviving cell numbers. Plates were incubated at 30ºC for 24-48h, single colonies were counted, and survival was expressed as a percentage of the time per sample.

2.1.6. Stimulation of morphogenesis in C. albicans

2.1.6.1. Stimulation of hyphal formation.

In order to induce germ-tube and hyphae formation, stationary phase cells from over-night cultures were diluted 1:10 in fresh YPD liquid media (in a final OD_{660}>0.1) containing 10% (v/v) fetal calf serum and incubated at 37ºC for 6h in a rotary incubator (180 rpm).

2.1.6.2. Stimulation of hyperpolarised bud formation

In order to induce hyperpolarized bud growth, stationary phase cells from over-night cultures were diluted to OD_{660}=0.15, and allowed to double twice. Cultures were subsequently treated with indicated concentrations of H_2O_2 and hydroxyurea (HU), and incubated at 30ºC in a rotary incubator (180 rpm) for the indicated times.

2.2 Molecular Biology Techniques

2.2.1 Polymerase Chain Reaction (PCR)

Primers were manufactured either by MWG-Biotech (UK) or Eurogentec (UK) and listed in Table 2.3. PCR reactions were performed using either Phusion® High-Fidelity DNA Polymerase (Finnzymes - Finland) or Taq polymerase enzyme (Cancer Research UK). All PCR reactions were performed in a T3 thermocycler (Biometra, Germany).

PCRs using Phusion® High-Fidelity DNA Polymerase were performed to amplify DNA fragments for cloning, where high proof reading activity was required. PCR reactions were carried out in a final volume of 50 µl and contained 1x Phusion HF Buffer or Phusion GC Buffer (which contains 7.5 mM MgCl), 200 µM dNTP mix, 100 ng template DNA, 5 units of enzyme and 100 pmole of each oligonucleotide primer. The program conditions are described below:

1. 94ºC - 1 min 30 secs (Initial template denaturation)
2. 94ºC - 30 secs (Denaturation)
3. 50-55ºC - 30 secs (Primer annealing)
4. 72ºC - 1 min/ kb (Product extension)

5. Repetition of steps 2-4, 30x

6. 72ºC - 10 mins (Final extension)

PCRs using Taq were performed when proof reading function of the enzyme was unnecessary, such as when screening genomic DNA for correct strain construction. PCR reactions were carried out in a final volume of 50 µl and contained 1x Taq buffer (500mM KCl, 100mM Tris pH 8, 1% TritonX-100, 15mM $MgCl_2$), 200 µM dNTP mix, template DNA, 5 units of enzyme and 100 pmole of each oligonucleotide primer. The program conditions are described below:

1. 94ºC - 2 mins (Initial template denaturation)

2. 94ºC - 30 secs (Denaturation)

3. 45-55ºC - 30 secs (Primer annealing)

4. 72ºC - 1 min/kb (Product extension)

5. Repetition of steps 2-4, 30x

6. 72ºC - 10 mins (Final extension)

To amplify the various *C. albicans* disruption cassettes described in Section 2.2.2, Tag was also used, using the optimised conditions detailed below. PCR reactions were carried out in a final volume of 50 µl and contained 1x Taq buffer (500 mM KCl, 100 mM Tris pH 8, 1 % TritonX-100, 15 mM $MgCl_2$), 200 µM dNTP mix, template DNA, 5 units of enzyme, 3 mM $MgCl_2$ and 250 pmole of each oligonucleotide primer. The program conditions are described below:

1. 94ºC - 2 mins (Initial template denaturation)

2. 94ºC - 30 secs (Denaturation)

3. 50ºC - 30 secs (Primer annealing)

4. 72ºC - 2 min (Product extension)

5. Repetition of steps 2-4, 30x

6. 72ºC - 8 mins (Final extension)

PCR products were analysed on a 1% agarose gel consisting of 100ml of Tris-acetate-EDTA (TAE) and 1g of agarose. Gels were stained with 0.5µg/ml of ethidium bromide. To purify PCR products, the QIAGEN QIAquick™ Gel Extraction Kit was employed following the manufacturer's instructions.

2.2.2. Restriction endonuclease digestion, phosphatase treatment and DNA ligation

Restriction endonuclease digestions were performed at 37ºC for 3 hours (plasmid) or over-night (PCR fragments) using standard techniques (Maniatis *et al.*, 1989). All restriction enzymes used in this study were purchased from Promega (UK).

In order to minimize self ligation of plasmid vectors, linearised vectors were treated with 1 unit of calf intestinal alkaline phosphatase (Roche, Germany) at 37ºC for 15 minutes (Maniatis *et al.*, 1989), prior to gel electrophoresis and purification using the QIAGEN QIAquick™ gel extraction kit.

PCR products were ligated with plasmids using 1µl of T4 DNA ligase (Invitrogen, UK). Ligation reactions were performed at room temperature for 1 hour or at 16ºC overnight. The molar ratio of insert to plasmid was generally 3:1 (Maniatis *et al.*, 1989).

2.2.3. Preparation of competent Escherichia coli cells

Escherichia coli SURE cells (Stratagene) (*e14*$^-$(*mcrA*$^-$) Δ(*mcrCB*$^-$*hsdSMR*$^-$*mrr*)*171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC*::*Tn5* (*Kan*^{r}) *uvrC* [*F'proAB laclZ* Δ*mis Tn10* (*Tet*^{r})]), were used for transformation of the plasmids constructed in this thesis. In order to prepare competent SURE cells for transformation, 5 ml cultures were first grown overnight at 30 °C in 2 XL medium pH 7.0 [20 g/l Bacto Tryptone, 10 g/l Bacto yeast extract, 1 g/l NaCl, 0.2 % (w/v) glucose]. Afterwards 4 ml of the overnight culture was inoculated into 400 ml of pre-warmed medium (2 XL at 30 °C) and incubated at the same temperature (30° C) until cells reached an OD_{600}~ 0.2. At this point $MgCl_2$ (final concentration of 20 mM) was added and cells were grown until reaching an OD_{600}~ 0.5. After the cell culture had reached this OD, the flasks containing the cultures were put in a recipient containing an ice-water mixture, where the cultures were cooled and kept cold for 2 h. Following the incubation in the ice-water mixture, cells were harvested at 3000 rpm for 5 min at 4° C. The cell pellets were gently resuspended in 200 ml of ice cold Ca^{2+}/Mn^{2+} medium (100 mM $CaCl_2$, 70 mM $MnCl_2$, 40 mM NaAc pH 5.5), and this mixture was incubated on ice for 40-45 min before harvesting cells at 2000 rpm for 5 min. The cells were then resuspended on ice in $^1/_{20}$ of the original culture volume, using Ca^{2+}/Mn^{2+} medium plus 15 % (v/v) glycerol. The cells were then aliquoted into 1.5 ml Eppendorf tubes (300 µl), snap frozen in liquid nitrogen and stored at - 80° C.

2.2.4. Bacterial growth media and transformation

Escherichia coli strains were cultured in Luria Broth (LB) media pH 7.2 [2 % (w/v) Bacto tryptone, 1 % (w/v) Bacto yeast extract, 1 % (w/v) NaCl] (Maniatis *et al*., 1989). Competent *E. coli* SURE cells were transformed with plasmids according to a standard $CaCl_2$ method (Maniatis *et al.*, 1989). 100 µl of competent cells were incubated on ice with 1 - 2 µl (5 – 10 µg) plasmid DNA for 30 min and then incubated at 42° C for 2 min. Following this, 1 ml of LB was added and cells were incubated at 37° C for 60 min. 100 µl of media containing the cells was plated onto LB plates (LB media plus 2% of Bacto Agar) containing ampicillin (100µg/ml) for the selection of transformants.

2.2.5. Extraction of plasmids

Plasmids were extracted from *E. coli* using the alkaline lysis protocol (Birnboim and Doly, 1979), exactly as described in Maniatis *et al*., 1989. Plasmids to be sequenced were extracted from *E. coli* using the Genelute Plasmid™ Mini-prep Kit (Sigma, UK), following the manufacturer's instructions.

2.2.6. DNA Sequencing

All plasmids described in this thesis were sequenced by GATC Biotech (UK). In addition, specific regions of genomic DNA from yeast strains generated in this study were PCR-amplified and sequenced by GATC Biotech to confirm the correct integration of tagged or mutant constructs.

2.3. Protein Techniques

2.3.1. Preparation of whole cell extracts

C. albicans and *S. pombe* protein extracts were prepared as described by Quinn *et al.*, 2002. Cultures were grown to mid-log phase and 25 ml of cells were harvested (2,500 rpm for 3 min) and resuspended in 300 µl of cold lysis buffer (20mM Hepes pH 7.3, 350 mM NaCl, 10% glycerol, 0.1% Tween20) containing protease inhibitors [2 µg/ml pepstatinA, 2 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20µg/ml aprotinin) and phosphatase inhibitors (2 mM sodium orthovanadate (Na_3VO_4), 50 mM sodium fluoride (NaF)]. For protein extracts prepared under reducing conditions, the lysis buffer was

supplemented with 10 mM ß–mercaptoethanol. To the cell suspension, 1ml of chilled glass beads was added and the cells placed in a bead beater for 2 x 20 seconds (*C. albicans*) or 2 x 45 seconds (*S. pombe*), with two minutes on ice in between each cycle. An additional 200 µl of cold lysis buffer plus inhibitors was added to the tubes, which were then pierced in the bottom with a hot needle and centrifuged at 2,000 rpm for 1 min at 4ºC to collect the cell lysate. The lysate was then clarified by centrifugation at 13,000 rpm for 10 min at 4ºC. The protein concentration of the supernatant was then determined by the Bradford Assay (Pierce).

2.3.2. Western Blotting

After quantification protein samples were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 8-13% polyacrylamide gels depending on the size of the protein to be detected. Gel recipes were based on the SDS discontinuous buffer system of Laemmli (1970). Following electrophoresis proteins were transferred to a Protran® nitro-cellulose membrane (Whatman) using a BioRad mini-transfer apparatus. Membranes were blocked by incubating with 10% bovine serum albumin (BSA) in TBST (10 mM Tris-HCl pH8, 150 mM NaCl, 0.05% Tween) and probed with the primary (1°) antibody diluted in 5% BSA-TBST for 1 hour to overnight at 4ºC. For specific times and dilutions please see individual experiments detailed below. After incubation with the 1° antibody, the blot was washed 5 x 5 min in TBST, and then incubated at room temperature for 1h with a horse radish peroxidase (HRP)-conjugated secondary antibody, diluted 1:2000, in 5% BSA-TBST. Finally, membranes were washed 3 x 5 min TBST, and proteins detected using the ECL Western blotting detection system (Amershan Pharmacia, Biotech).

2.3.3. Sty1 and Hog1 phosphorylation assays

Sty1 phosphorylation was performed as previously described by Quinn *et al*., (2002). To detect Sty1 phosphorylation, 25 ml of exponentially growing *S. pombe* cells, before and following exposure to H_2O_2, were harvested in 50ml tubes containing 25ml of ice at (2,500 rpm for 3 minutes). Pellets were then immediately frozen in liquid nitrogen and then protein extracts prepared as described in Section 2.3.1. 30 µg of protein extract was subjected to SDS-PAGE on 10% polyacrylamide gels. Phosphorylation of Sty1 was detected by western blotting as described in section 2.3.2, with the exception that phosphatase inhibitors (50 mM NaF and 2 mM Na_3VO_4) were added to the blocking solution and the diluted primary and

secondary antibodies to help preserve phosphorylation of Sty1. Sty1 phosphorylation was detected using a 1:1000 dilution of an anti-phospho-p38 antibody (New England Biolabs) which only recognises the phosphorylated active form of Sty1, which was incubated with the membrane at 4°C overnight. The secondary antibody employed was an HRP-conjugated anti-rabbit IgG antibody (Sigma). After detection of Sty1 phosphorylation, blots were incubated with a stripping solution (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) at 50ºC for 30min. Blots were then reprobed with an anti-Sty1 primary antibody (Day and Veal, 2010), which was used in conjunction with a secondary HRP-conjugated anti-rabbit IgG antibody (Sigma).

To detect Hog1 phosphorylation, cells were harvested and processed as described above. 70 µg of protein extract was subjected to SDS-PAGE on 10% polyacrylamide gels, and phosphorylation of Hog1 was detected exactly as described for Sty1 above. However, to detect total Hog1 levels, an anti-Hog1 antibody was used (y-215, Santa Cruz Biotechnology).

2.3.4. Detection of 2-Cys peroxiredoxins

To detect *C. albicans* Tsa1, protein extracts were prepared as described in Section 2.3.1. 5 µg of protein extracts was separated by electrophoresis on 12.5% SDS-PAGE and following western blotting membranes were probed with a 1:5000 dilution of an anti-prdx3 antibody for 1 h. This antibody was raised against the C-terminal 16 amino acids of *C. elegans* Prdx3 (Oláhová *et al.*, 2008, and was a kind gift of Dr E.A. Veal). The secondary antibody employed was an HRP-conjugated anti-rabbit IgG antibody (Sigma). To detect *S. pombe* Tpx1, 20 µg of protein extracts was resolved on a 12.5% SDS-polyacrylamide gel and probed with a 1:1000 dilution of an anti-tpx1 primary antibody for 1 h. This antibody was raised against the C-terminal 16 amino acids of *S. pombe* Tpx1 (was a kind gift of Dr E.A. Veal).

2.3.5. Detection of myc-tagged proteins

During this project, strains were constructed that expressed Rad53 tagged with 2Myc epitopes and 6His residues (Section 2.1.2.3). Rad53-Myc-His needed to be enriched on Ni^{2+}-Ni^{2+}-NTA agarose prior to detection (see below). A 1:1000 dilution of the 9E10 monoclonal anti-myc antibody (Sigma) was used as the primary antibody, and membranes were

incubated overnight at 4 °C. The secondary antibody employed was an HRP-conjugated anti-mouse IgG antibody (Sigma).

2.3.6. Rad53 Phosphorylation assays

To detect Rad53-Myc-His phosphorylation, 25 ml of exponentially growing *C. albicans* cells expressing Rad53-Myc-His, were harvested (2,500 rpm for 2 min) before and following exposure to 5 mM H_2O_2. Protein extracts were made as described in Section 2.1.1, and 600 µg of protein extract was subsequently incubated with 20µl of Ni^{2+}-NTA agarose beads (Qiagen), pre-equilibrated in lysis buffer, for 1 hr at 4ºC on a rotator. The beads were washed 3 x with lysis buffer and precipitated proteins resolved by SDS-PAGE on long (14 cm) 10 % acrylamide gels. In some cases, Rad53-Myc-His coupled to the Ni^{2+}-beads was subjected to phosphatase treatment. Here, the protein coupled beads were washed in lysis buffer minus phosphatase inhibitors, resuspended in 20µl of the same buffer containing 1 unit of λ phosphatase (New England Biolabs), and incubated for 1 hour at 37ºC. The proteins were resolved on SDS-PAGE as above and Rad53-Myc-His detected as described in Section 2.3.5.

2.3.7. Determination of Protein Oxidation

To examine the oxidation state of several *C. albicans* proteins (Tsa1 and Trx1), proteins were extracted by acid lysis (in order to avoid oxidation during protein extraction). 2 ml of exponential growing cells (untreated and treated) cells plus 2 ml of 20% trichloroacetic acid (TCA) were harvested (2,500 rpm for 1 min) and then snap-frozen. Thawed pellets were resuspended in 200 µl of 10% TCA, bead beaten for 2 x 15 seconds and the protein was precipitated by centrifuging at 13,000rpm for 10 minutes at 4ºC. Pellets were washed three times with acetone (13,000 rpm for 1 min) to remove TCA and resuspended in 20 µl of 100 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% SDS, with or without 25 mM 4-acetamido-4'-[(iodoacetyl)amino]stilbene-2,2'-disulfonic acid (AMS, Invitrogen). AMS is an alkylating agent that binds irreversibly to reduced cysteine residues, increasing the molecular weight of the protein by ~0.5 kDa per AMS-bound cysteine residue. Oxidation of cysteine residues prevents AMS binding and consequently has a lower molecular weight and faster mobility on SDS-PAGE compared to the reduced AMS bound protein. Protein extracts (with or without AMS) were incubated at 25ºC for 30 minutes followed by an incubation at 37ºC for 5 minutes. After incubation, protein extracts were centrifuged at 13,000 rpm for 3 minutes and

5x protein sample loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 1% ß-Mercaptoethanol, 12.5 mM EDTA and 0.02 % Bromophenol Blue) added. Protein concentration was not determined, but the resulting protein extracts plus 5x protein sample loading buffer were divided into two aliquots of equal volume (2 X 12.5 µl) that were used for detection of Trx1 and Tsa1 redox state. Non-reducing protein extracts were resuspended in non-reducing 5x protein sample loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 12.5 mM EDTA and 0.02 % Bromophenol Blue).

In addition, protein oxidation was also determined by using the reducing agent DTT. Proteins were extracted by acid lysis as described above, but the acetone-washed pellets were resuspended in 50 µl of 100 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% SDS plus 50mM DTT and incubated at 37ºC for 1h. Following incubation the protein extractions were then treated with AMS to verify whether the proteins were reduced by DTT. 50 µl of 20% TCA was added to the DTT-treated protein extracts and they were incubated in ice for 30 minutes. Protein was precipitated by centrifuging at 13,000rpm for 15 minutes at 4ºC. Pellets were washed once with acetone to remove TCA and resuspended in 20 µl of 100 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% SDS, with or without 25 mM 4-acetamido-4'-[(iodoacetyl)amino]stilbene-2,2'-disulfonic acid (AMS, Invitrogen) and processed as described above for AMS treatment.

To detect Trx1-Myc-His, ~12.5 µl of protein extraction plus protein sample loading buffer (see above) was resolved by SDS-PAGE on long (14 cm) 13 % acrylamide gels and Trx1-Myc-His detected as described in Section 2.3.5. To detect Tsa1, ~12.5 µl of protein extraction plus protein sample loading buffer was resolved by SDS-PAGE on long (14 cm) 12 % acrylamide gels and Tsa1 detected as described in Section 2.3.4.

2.4. Imaging Techniques

2.4.1. Differential interference contrast (DIC) microscopy

10 ml of cells to be analyzed were fixed in 3% para-formaldehyde [6g para-formaldehyde in 20ml PEM (100 mM piperazine-1 4-bis 2-ethanesulfonic acid-PIPES- pH7.6, 1 mM EGTA, 1 mM $MgSO_4$)] in a rotating plataform for 30 minutes. The samples were then washed 3 times in PEM (2,000 rpm for 2 minutes), resuspended in ~1ml of PEM and 5 µl of sample was spread onto poly-L-lysine-coated slides and allowed to dry. Slides were then

placed in ice-cold methanol (MeOH) for 6 min and transferred to ice-cold acetone for 30s. Differential interference contrast (DIC) images were then captured using a Zeiss Axioscope, with a 63x oil immersion objective, and Axiovision imaging system.

2.4.2 Indirect fluorescence microscopy to detect Pap1 localisation

To examine Pap1 localization, mid-log phase growing *S. pombe* cells (expressing Pk epitope tagged Pap1 – SB3 + Rep1, SB3 + Rep1-*tpx*$^{+1}$ and SB3 + Rep1-*TSA1*), which were treated with 0.2 and 1 mM of H_2O_2 for 10 and 60 minutes, were used. 1.25 ml fresh 15 % formaldehyde solution (6 g para-formaldehyde in 20 ml PEM [100 mM piperazine-1, 4-bis(2-ethanesulfonic acid) (PIPES), 1.0 mM EGTA pH 8.0, 1.0 mM $MgSO_4$] 120 µl 10 M NaOH was added and the mixture incubated at 65 °C, to allow the formaldehyde to dissolve, then cooled) was added to 10 ml of the cultures described above and placed in a rotator plataform at room temperature for 20 – 30 min. Cells were then harvested (2,5000 rpm for 3 min), transferred to Eppendorf tubes and washed three times in 1 ml PEM (3000 rpm for 1 min at room temperature). Cell pellets were then resuspended in 1 ml PEMS (PEM + 1.2 M sorbitol), containing 0.5 mg/ml Zymolyase-20T (200000 U/g from *Arthrobacter luteus*) and incubated at 37 °C for 70 min. The PEMS was removed and cells incubated in 1 ml PEMS with 1 % Triton X100 for 2 min at room temperature. After this step cells were washed three times in PEM and then blocked in 1 ml PEMBAL (PEM + 1 % BSA, 0.1 % sodium azide, 100 mM L-lysine HCl), on a rotator for 30 min at room temperature. After blocking, the PEMBAL was removed and the cells were resuspended in 200 µl PEMBAL, containing a 1/1000 dilution of the specific 1° antibody, and left shaking gently overnight at room temperature. The following day the cells were washed three times in PEMBAL then incubated, with shaking, in 200 µl PEMBAL containing a 1/200 dilution of Alexa Fluor 488 goat anti-mouse 2° antibody. After this incubation, cells were washed twice in PEMBAL, and once in PEM. 5 µl was spread onto poly-L-lysine coated glass microscope slides and allowed to dry. Slides were then placed in ice-cold methanol (MeOH) for 6 min and transferred to ice-cold acetone for 30 s. Once dry, a drop of VECTASHIELD® mounting medium containing DAPI (4'-6-diamidino-2-phenylindole) for nuclear staining was added and a glass cover slip placed on top and sealed.

2.4.3. Fluoresence microscopy to detect Trx1-GFP localisation

Cultures of cells expressing CIp10-*TRX1*-GFP (JC1174) or the empty CIp10-GFP plasmid (JC1176) were either exposed to H_2O_2 or serum (as described in 2.1.6) and processed as described in 2.4.1 for microscopic analysis. GFP staining was visualized by excitation at 440-470nm using a Zeiss Axioscope fluorescence microscope with a 63x oil immersion objective and the Axiovision digital imaging system.

2.4.4. Nuclear staining

To allow co-visualisation of the nucleus when either capturing fluorescence or DIC images, cells were processed as described above and cover slips were mounted onto slides using Vectashield mounting medium containing 1.5mg/ml 4'-6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). DAPI staining was visualized by excitation at 355nm using a Zeiss Axioscope fluorescence microscope with a 63x oil immersion objective and the Axiovision digital imaging system.

2.5. Virulence Analysis

2.5.1. Murine intravenous challenge

The murine intravenous challenge model of *C. albicans* infection (MacCallum *et al.*, 2009 and MacCallum *et al.*, 2010) was employed to determine the impact of deleting *TRX1* on virulence. BALB/c female mice with mean body weight 17.3 ± 0.7 g were housed in groups of 5 with food and water provided *ad libitum*. Wild-type (JC747), *trx1*Δ (JC677) and *trx1*Δ+*TRX1* cells (JC679) were grown overnight on Sabouraud agar (Oxoid) at 30°C. Cells were harvested in sterile saline and cell counts adjusted by hemocytometer to provide a cell suspension estimated to deliver a challenge dose of $3x10^4$ CFU/g body weight. Actual challenge dose was determined from viable counts read 24 h later and was 2.5 x 10^4 CFU/g. Mice were infected intravenously via a lateral tail vein. Body weights were recorded daily. 72 h after challenge the animals were weighed, humanely terminated and kidneys removed aseptically. Fungal burdens were measured by viable counts for two half kidneys per animal; the other half kidneys were fixed, embedded and stained for histopathological examination. Virulence of the challenge strains was assessed by fungal kidney burdens at 72 h, and by percent weight change over 72 h, from which an outcome score was calculated (MacCallum

et al., 2009 and MacCallum *et al.*, 2010). Differences between mean body weight changes and mean kidney burdens were tested statistically by the Mann-Whitney *U* test. All animal experimentation conformed to the requirements of United Kingdom Home Office legislation and of the Ethical Review Committee of the University of Aberdeen.

2.5.2. Macrophage killing assay

The macrophage cell line J774.1 were plated at a density of 5×10^5 in 12-well plates (Nagle Nunc) for 24 h. *C. albicans* wild-type, *trx1*Δ and *trx1*Δ + *TRX1* cells were incubated with J774.1 macrophages at 1:1 (*C. albicans*/macrophage) ratio. Following 3 hrs exposure, wells were washed twice with 1% (wt/vol) sterile phosphate-buffered saline (PBS) to remove excess unbound cells. Macrophage killing was assessed by trypan blue exclusion. A 150-µl sample of trypan blue (Sigma) and 150 µl of 1% PBS were added to cells for 2 min and removed by lightly washing twice with 1% PBS; cells were then fixed with 3% paraformaldehyde (BDH). Cells were then counted under an inverted light microscope (Nikon Eclipse TE2000-U microscope with a ×40 objective) to ascertain the percentage of macrophages killed. Data were obtained in triplicate from at least 3 separate experiments by analyzing at least 200 macrophages per well (McKenzie *et al.*, 2010).

Chapter 3. Construction and analysis of *C. albicans* strains lacking functional Trx1

3.1. Introduction

The thioredoxin system is composed of thioredoxin and thioredoxin reductase. The catalytic site of thioredoxin contains two cysteine residues, which are responsible for forming a disulphide bond with its substrate, resulting in the reduction of the substrate and oxidation of thioredoxin (Eklund *et al.*, 1984). In order to restore its activity, oxidized thioredoxin has to be reduced by thioredoxin reductase at the expense of NADPH (Arnér and Holmgren, 2006 and Fernando *et al.*, 1992 – Section 1.4.2.4).

Thioredoxin genes are induced in response to H_2O_2 in both model yeasts and pathogenic fungi, indicative of a role in oxidative stress protection (Gash *et al.*, 2000; Song and Roe, 2008, Enjalbert *et al.*, 2006 and Missall and Lodge, 2005). Consistent with this deletion of thioredoxin in the model yeasts *S. cerevisiae* and *S. pombe*, and in the pathogenic fungus *C. neoformans,* results in strains that display increased sensitivity to oxidative stress treatment compared to wild-type cells (Garrido and Grant, 2002, Song and Roe, 2008 and Missal and Lodge, 2005). This oxidative stress sensitive phenotype is related to the important cellular function of thioredoxin to regulate the redox state of antioxidants such as peroxiredoxins and glutathione peroxidases (Chae *et al.*, 1994, Pedrajas *et al.*, 2000, Kim *et al.*, 2010, and Ohdate *et al.*, 2010). As described in Chapter 1, Section 1.4.4.2.2, the antioxidant thioredoxin is also implicated in regulating major oxidative stress signalling pathways in both *S. cerevisiae* (the AP-1 transcription factor Yap1; Izawa *et al.*, 1999) and mammals (the p-38 MAPK pathway via regulation of the upstream MAPKKK, ASK1; Saitoh *et al.*, 1998).

In addition to such roles in oxidative stress detoxification and signalling, thioredoxin also reduces protein substrates involved in normal cell metabolism. For example, thioredoxin functions as the main electron donor for Ribonucleotide reductase (RNR), which is responsible for dNTP synthesis (reviewed in Nordlund and Reichard, 2006). Consequently, deletion of thioredoxin in *S. cerevisiae* results in a slow growth phenotype, which is related to impaired DNA synthesis and thus a longer S-phase during the cell cycle (Muller, 1991 and Koc *et al.*, 2006). Thioredoxin is also the electron donor for the enzyme PAPS reductase that plays a major role in sulphur assimilation (Lillig *et al.*, 1999 and Song and Roe, 2008). Consequently, thioredoxin mutants in several fungi including *S. cerevisiae*, *S. pombe* and

Podospora anserina are reported to be auxotrophic for the sulphur containing amino acids, methionine and cysteine (Muller, 1991, Song and Roe, 2008 and Malagnac *et al.*, 2007).

Despite well-documented roles of thioredoxin in the response to oxidative stress and other important cellular processes in both lower and higher eukaryotes, no studies of thioredoxin function have been reported in the medically relevant pathogen *C. albicans*. As oxidative stress responses are intimately linked with *C. albicans* virulence (see Section 1.2.4.2), the aim of the work presented in this chapter was to create and characterise strains lacking thioredoxin function in this fungal pathogen.

3.2. Results

3.2.1. Bioinformatic analysis of potential thioredoxin encoding genes in C. albicans

A BLAST search of the Candida Genome Database (www.candidagenome.org), using the *S. cerevisiae* Trx1 and Trx2 sequences, revealed two open reading frames with significant homology over the length of these proteins, namely *orf19.7611* and *orf19.1976* which are designated *C. albicans* Trx1 and Trx2. *C. albicans* Trx1 displays 55% and 58% identity to *S. cerevisiae* Trx1 and Trx2 respectively, and contains all the conserved motifs and amino acids found in thioredoxin proteins. For example, *C. albicans* Trx1 contains the conserved thioredoxin motif Trp-Cys-Gly-Pro-Cys which contains the catalytic cysteines, and the aspartic acid and lysine residues (Asp24 and Lys54) that are important for the nucleophilic attack of Cys33 on Cys30 that occurs during the reduction of oxidized thioredoxin (Carvalho *et al.*, 2009). Furthermore, in the human Trx1, the lysine, which is thought to be important for the nucleophilic attack of Cys33 on Cys30, is in the position 39 (Carvalho *et al.*, 2009). In contrast, *C. albicans* Trx2 displays less sequence identity with *S. cerevisiae* Trx1 and Trx2 (33% and 31% respectively) and, significantly, the thioredoxin motif in *C. albicans* Trx2 is not conserved (Figure 3.1).

In addition to the bioinformatics analysis that suggests that *C. albicans* Trx1, and not Trx2, is a functional thioredoxin, transcription profiling experiments in *C. albicans* revealed that only *TRX1* is induced after H_2O_2 treatment (Enjalbert *et al.*, 2003 and 2006). Furthermore, a recent study in which the absolute levels of mRNA transcripts encoding various antioxidants enzymes in *C. albicans* were quantified, demonstrated that the

```
CaTrx1        MVHVVTEVNEFQTLL--KENNLVIVDFFATWCGPCKMIAPLLEKFQNEYS--NIKFLKID 56
ScTrx1        MVTQFKTASEFDSAI-AQ-DKLVVVDFYATWCGPCKMIAPMIEKFSEQYP--QADFYKLD 56
ScTrx2        MVTQLKSASEYDSAL-ASGDKLVVVDFFATWCGPCKMIAPMIEKFAEQYS--DAAFYKLD 57
SpTrx1        MVKQVSDSSEFKSIV--CQDKLVVVDFFATWCGPCKAIAPKFEQFSNTYS--DATFIKVD 56
HsTrx1        MVKQIESKTAFQEALDAAGDKLVVVDFSATWCGPCKMIKPFFHSLSEKYS--NVIFLEVD 58
CaTrx2        MLQNIETKQQFSSAL-QNKNDMIVLDFFD-ECSHCSDLNDKLDEFSDMYEAQNIRFYKVN 58
                *:  .     :.  :    :.:::::**    *. *. :   :..: : *   :  * :::

CaTrx1        VDQLGSLAQEYNVSSMPTLILFKNGEEVNRVIGANPAAIKQALASLA------------ 103
ScTrx1        VDELGDVAQKNEVSAMPTLLLFKNGKEVAKVVGANPAAIKQAIAANA------------ 103
ScTrx2        VDEVSDVAQKAEVSSMPTLIFYKGGKEVTRVVGANPAAIKQAIASNV------------ 104
SpTrx1        VDQLSEIAAEAGVHAMPSFFLYKNGEKIEEIVGANPAKLEASIKANL------------ 103
HsTrx1        VDDCQDVASECEVKCMPTFQFFKKGQKVGEFSGANKEKLEATINELV------------ 105
CaTrx2        IEEDRELAEDYKVSSIPTTLFFKKGKVFDKVVGPEPNEIKKVLDKNLMGWSGRPSQSSRT 118
                :::  .:* .  * .:*:  ::* *: . .. *.:   ::  :
```

Figure 3.1. Sequence alignment of thioredoxin proteins from *C. albicans*, *S. cerevisiae*, *S. pombe* and *H. sapiens*.

The amino acid sequences of *C. albicans* Trx1, *S. cerevisiae* Trx1 and Trx2, *S. pombe* Trx1, *H. sapiens* Trx1, and *C. albicans* Trx2 were aligned using ClustalW (Thompson *et al.*, 1994). Dashes indicate single-residue gaps introduced to maximise the alignment. "*" indicates identical residues, ":" indicates substitutions, and "." indicates semi-conserved substitutions. The CGPC motif characteristic of thioredoxin proteins is shown in bold and underlined. Amino acids that have been demonstrated to be important for thioredoxin function are indicated in bold and italic.

ratio of *TRX1*/*TRX2* transcripts is 100 to 1 (Michán and Pueyo, 2009). Based on these findings it was decided to initially investigate the role of Trx1 in *C. albicans*.

3.2.2. Construction of trx1Δ null and catalytic cysteine mutant strains

In order to characterize *TRX1* in *C. albicans* cells, a homozygous null (*trx1*Δ) was generated. The two copies of *TRX1* were deleted by replacing the entire *TRX1* ORF (312 bp) with either the *HIS1* or *ARG4* genes (See Materials and Methods; Dennison *et al.*, 2005). To re-integrate one copy of *TRX1* under the control of its own promoter, the *TRX1* ORF plus 1039bp of its promoter (containing 11 Cap1-Binding Motifs) and 299bp of terminator region were amplified by PCR, and ligated into the CIp10 plasmid (Murad *et al.*, 2000). The resulting CIp10-*TRX1* plasmid was digested and integrated at the *RPS10* locus in the *trx1*Δ mutants to generate strain JC679. To generate deletion mutants that were auxotrophically identical to the reconstituted strains, the empty CIp10 plasmid was integrated at the *RPS10* locus in the *trx1*Δ to generate strain JC677. Similarly, to generate a wild-type strain auxotrophically identical to the null and reconstituted strains, the vector CIp30 (Dennison *et al.*, 2005), a derivative of CIp10 containing the *URA*, *ARG* and *HIS* genes, was integrated at the *RPS10* locus in the parental strain SN148 to generate JC747.

In addition to the creation of *trx1*Δ cells, mutants were generated in which one or both of the catalytic cysteine residues (Cys30, Cys33) were mutated to serine. Serine was chosen due due to its similar side chain size and structure when compared to cysteine. Mutagenesis of *TRX1* was performed by overlapping PCR and the mutant products ligated with the integrating plasmid CIp10 (Murad *et al*., 2000) which was subsequently integrated at the *RPS10* locus in *trx1*Δ cells to generate $trx1^{C30S}$ (JC759), $trx1^{C33S}$ (JC761) and $trx1^{C30,33S}$ (JC763), (See materials and methods for details). A similar strategy was used to generate the reintegrant strain (*trx1*Δ + *TRX1*, JC679), in which the wild-type *TRX1* gene was expressed from the *RPS10* locus (See materials and methods for details). As shown in Figure 3.2, the generation of strains expressing mutant versions of Trx1 was confirmed by DNA sequencing (Cys30→Ser30, Figure 3.2B, Cys33→Ser33, Figure 3.2C and Cys30→Ser30, Cys33→Ser33, Figure 3.2D).

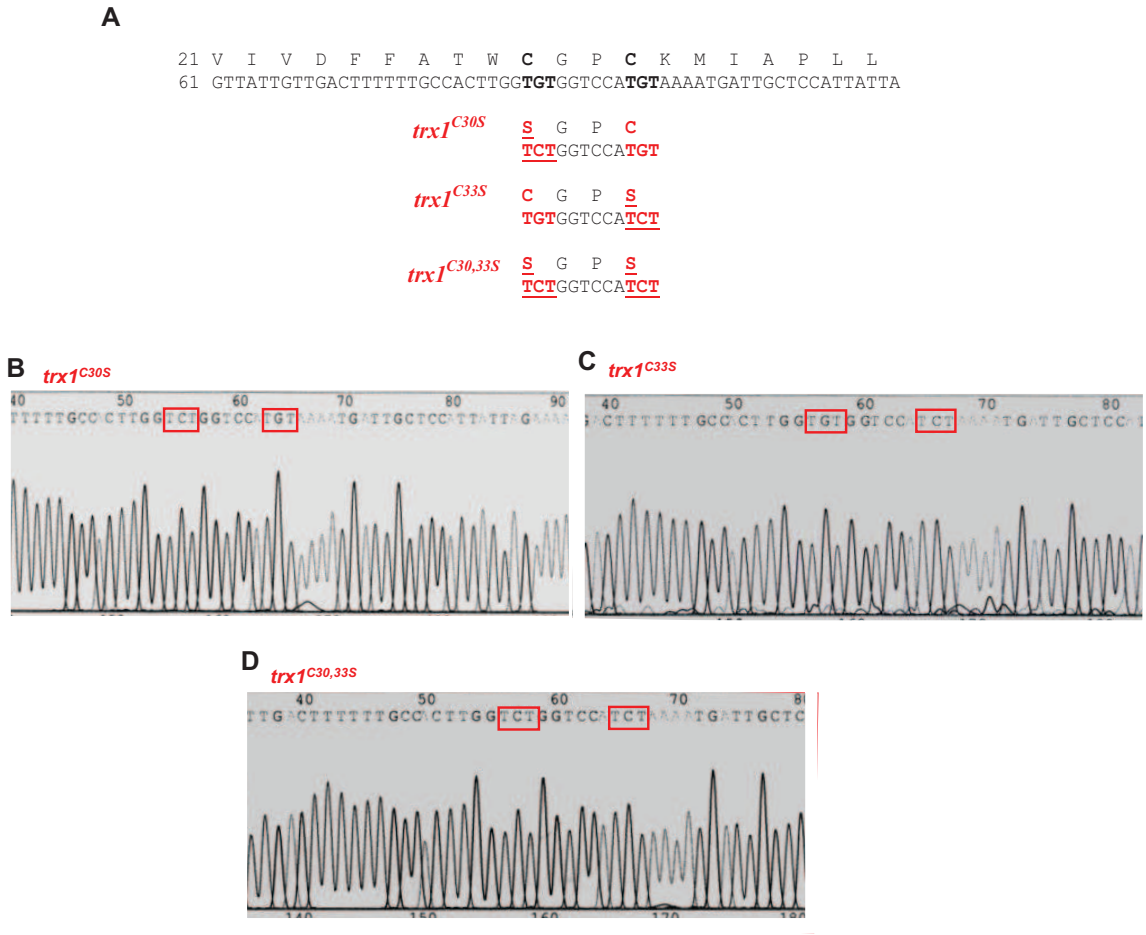


Figure 3.2. DNA sequence analysis of *C. albicans* Trx1 cysteine mutants.
(A) DNA sequence of the region encoding the thioredoxin motif of *C. albicans* Trx1 and the engineered mutations. (B-D) DNA sequences of genomic DNA extracted from: JC759-*trx1*^{C30S} (B), JC761-*trx1*^{C33S} (C), and JC763-*trx1*^{C30,33S} (D). The mutated codons are highlighted in red boxes.

3.2.3. Inactivation of Trx1 results in slow growth

As described above (Section 3.1), *S. cerevisiae* cells lacking *TRX1* and *TRX2* are slow growing, displaying a longer generation time (Muller, 1991). To investigate whether *TRX1* is required for normal cell growth in *C. albicans*, the growth rate of *trxl*Δ cells and cells expressing the catalytic cysteine mutants was assessed. Both, deletion of *TRX1* or mutation of the *trx1* cysteines resulted in strains that were slower growing than wild-type cells in both liquid and solid media (Figure 3.3). Furthermore, reintegration of the wild-type *TRX1* gene (*trx1*Δ + *TRX1*) rescued the slow growth phenotype of *trx1*Δ cells (Figure 3.3). For example, wild-type and reintegrant cells had a doubling time of 60 minutes and this increased to 90 minutes in both the *trx1*Δ and cysteine mutants (Figure 3.3A). In addition, *trxl*Δ cells and the *trx1* catalytic mutants displayed a colony size markedly smaller than the colonies of wild-type and reconstituted strains on solid media (Figure 3.3B). These results indicate that Trx1 is necessary for normal cell growth in *C. albicans* through a mechanism that requires its catalytic activity.

In *S. cerevisiae*, the slow growth phenotype of *trx1*Δ*trx2*Δ cells is attributed to the role of thioredoxin in regulating the activity of RNR (Muller, 1991 and Koc *et al.*, 2006). In order to test the hypothesis that in *C. albicans*, Trx1 would also be playing a role in RNR activity, *trx1*Δ cells were exposed to hydroxyurea (HU), which is a known inhibitor of this enzyme (Sneeden and Loeb, 2004). Figure 3.4 illustrates that deletion of *TRX1*, or expression of the Trx1 cysteine mutants, resulted in increased sensitivity to HU when compared to wild type and reconstituted strains (Figure 3.4A). Unexpectedly, expression of Trx1 ^{C30,33S} (JC763) did not result in sensitivity to HU, instead this strain was more resistant to HU than wild-type (Figure 3.4A). However, the basis for this resistance is not known, since the oxireductase of thioredoxin is known to be important for the activity of RNR, which is inhibited by HU (Sneeden and Loeb, 2004 and Camier *et al.*, 2007). In 1964, thioredoxin was discovered as a dithiol electron donor of *E. coli* RNR. RNR is a tetrameric enzyme composed of two homodimeric subunits, the large subunit α2 (R1) and the small one β2 (R2). The large homodimer contains the substrate binding active site, while the small homodimer contains an oxygen center that generates the tyrosyl radical required for initiation of the dNTP formation reaction. Reaction of the tyrosyl radical (Tyr122) from R2 with a cysteine residue (Cys439) in the large R1 subunit results in the formation of a thiyl radical. This thiyl radical can then remove the 3′ hydrogen atom from NDP, resulting in its reduction to dNDP. During the

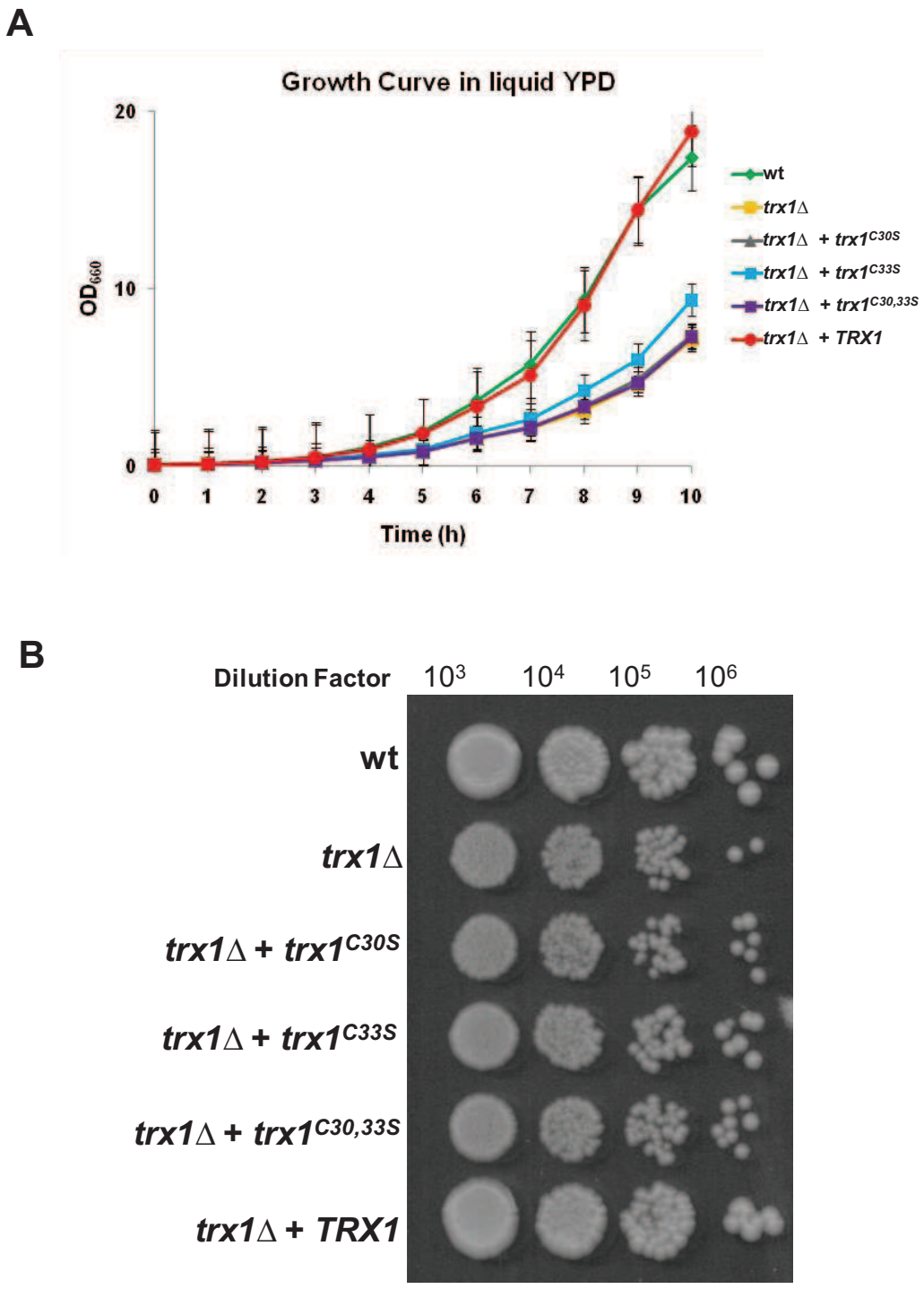


Figure 3.3. Deletion of *TRX1* or mutation of the putative cysteine residues results in a slow growth phenotype.

(A) *C. albicans* wild-type (JC747), *trx1*Δ + Clp10 (JC677), *trx1*Δ + CIp10-*TRX1* (JC679), *trx1*Δ + CIp10-*trx1*^{C30S} (JC759), *trx1*Δ + CIp10- *trx1*^{C33S} (JC761) and *trx1*Δ + CIp10- *trx1*^{C30,33S} (JC763) mid-exponential growing were diluted and grown in YPD for 10 hours and absorbance was measured each hour. (B) Approximately equal numbers of *C. albicans* cells were grown until exponential phase, diluted and spotted in YPD media. Plates were grown for 2 days at 30ºC.

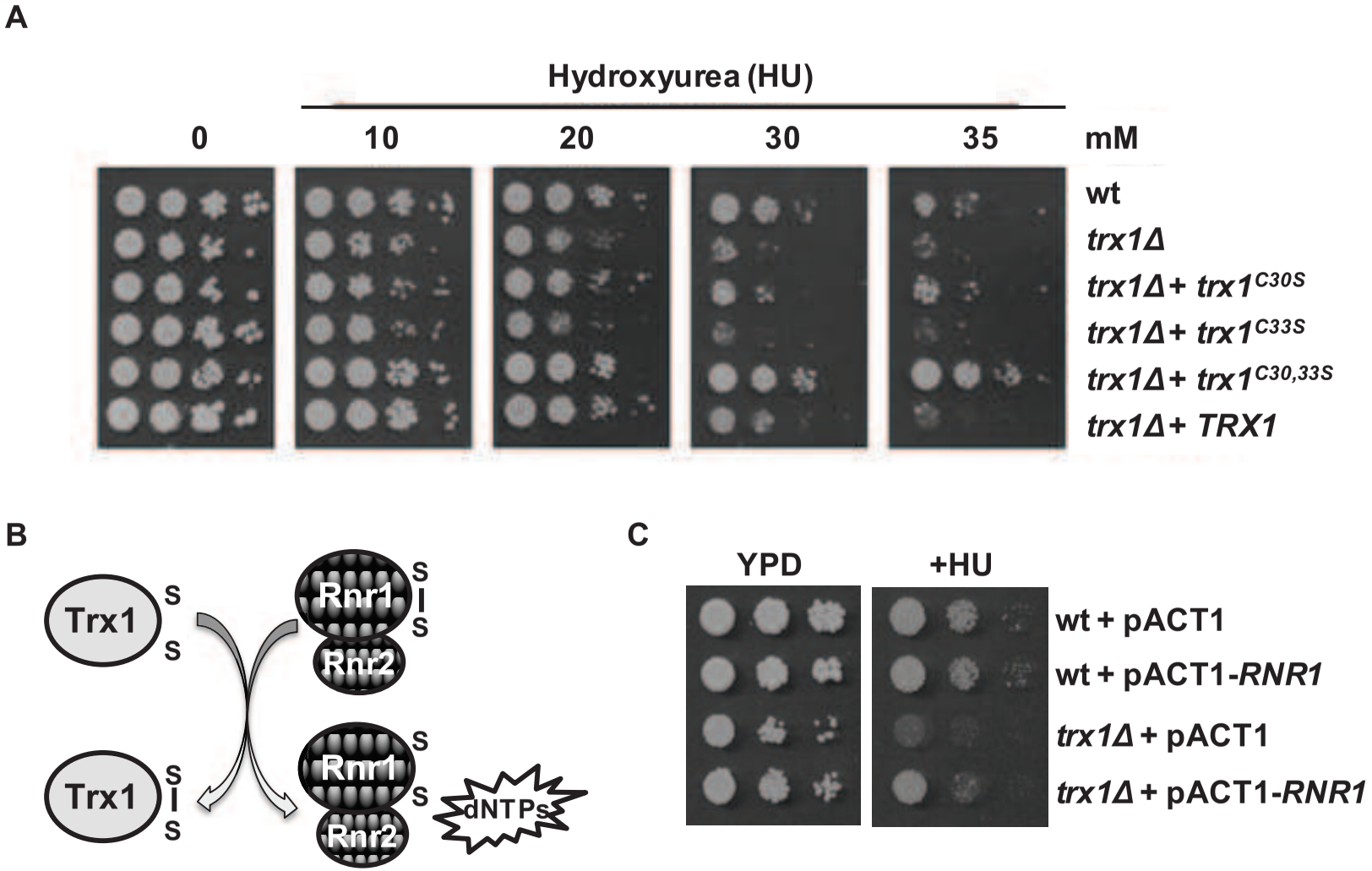


Figure 3.4. Deletion of *TRX1* or mutation of the catalytic cysteine residues of Trx1 results in HU sensitivity which can be rescued by ectopic expression of *RNR1*.

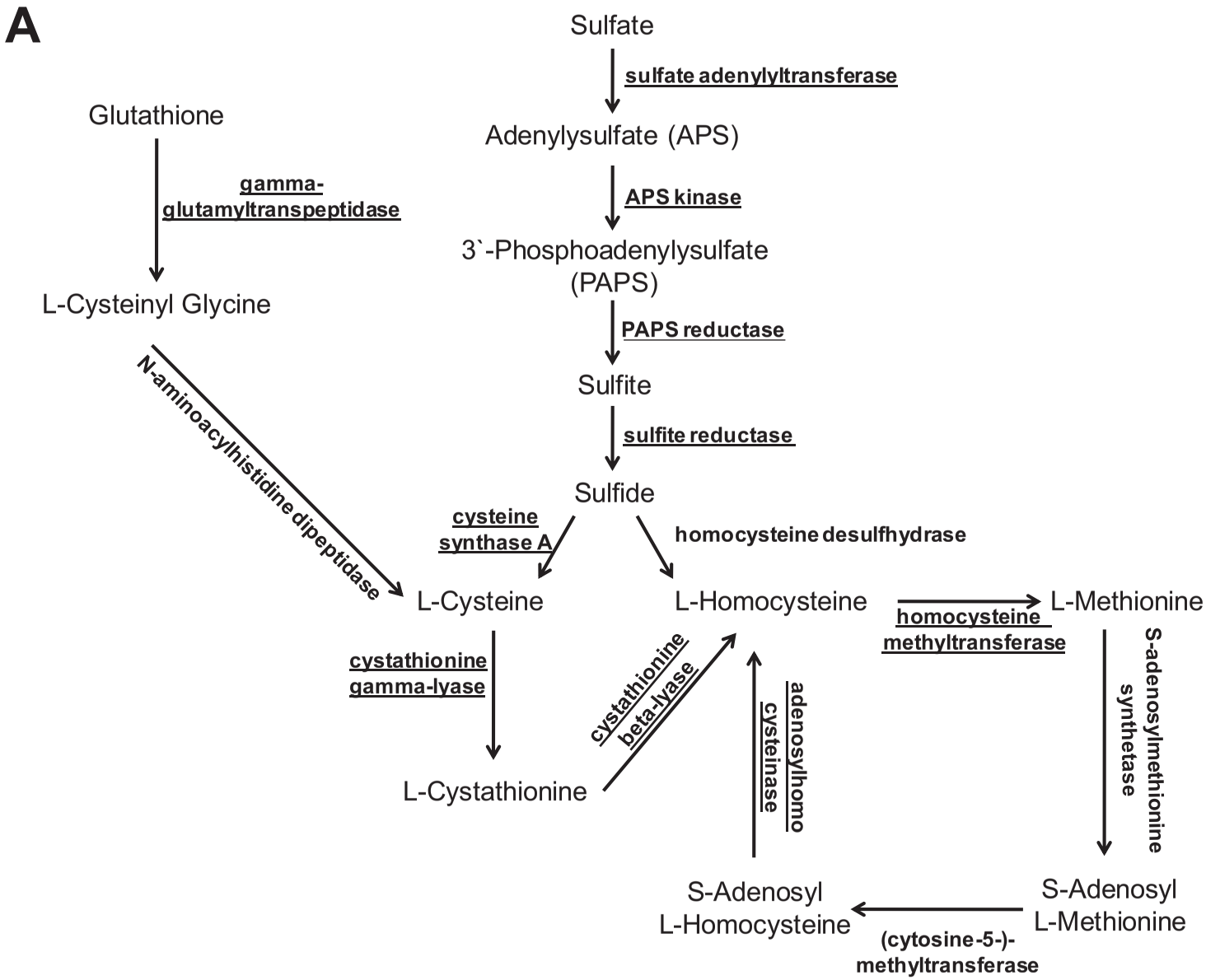
(A) *C. albicans* wild-type (JC747), *trx1*Δ + CIp10 (JC677), *trx1*Δ + CIp10-*TRX1* (JC679), *trx1*Δ + CIp10-*trx1*^{C30S} (JC759), *trx1*Δ + CIp10-*trx1*^{C33S} (JC761) and *trx1*Δ + CIp10-*trx1*^{C30,33S} (JC763) were grown to mid-log phase and then plated in 1/10 serial dilutions on agar plates containing the indicated concentrations of HU. (B) During dNTP synthesis, the large subunit of Rnr becomes oxidized. Oxidized Rnr is reduced by thioredoxin, which in turn becomes oxidized (C) Wild-type and *trx1*Δ cells containing either pACT1 (JC1014 and JC1022, respectively) or pACT1-*RNR1* in which *RNR1* is expressed from the *ACT1* promoter (JC1016 and JC1024, respectively) were spotted onto YPD plates with or without 30 mM HU. Plates were then incubated at 30ºC for 2 days.

reduction reaction of NDPs to dNTPs, two disulfide bonds are formed in the large RNR subunit. Firstly, a disulfide bond is formed between Cys225 and Cys462 which is then transposed to Cys754 and Cys759. Thioredoxin can then act to reduce this last disulfide, allowing RNR to initiate another NDP reduction reaction (Figure 3.4B - Meyer *et al.*, 2009). Furthermore, in the budding yeast *S. cerevisiae* depletion of the cytoplasmatic thioredoxins (Trx1 and Trx2) results in increasing levels of oxidized RNR (large R1 subunit), which results in lower dNTP levels, increased S-phase length and doubling time (Muller, 1991, Koc *et al.*, 2006 and Camier *et al.*, 2007). Therefore, the large subunit of *C. albicans* RNR, *RNR1*, was constitutively expressed by ectopic expression of *RNR1* from the *ACT1* promoter in both wild-type cells and in the *trx1*Δ strain, generating strains JC1016 and JC1024, respectively. The HU sensitivity of *trx1*Δ could be rescued by ectopic expression of *RNR1* from the *ACT1* promoter, which encodes the large subunit of RNR, indicating that the sensitivity to HU seen in *trx1*Δ strain is due to Trx1 regulation of RNR function (Figure 3.4C). Moreover, ectopic expression of *RNR1* seems to also, at least, partially rescue the slow growth phenotype of *trx1*Δ cells (Figure 3.4C). This result would indicate that Trx1 regulation of the large subunit of RNR, Rnr1, is important for normal cell growth; however, further experiments need to be done to confirm this hypothesis.

3.2.4. Cells lacking TRX1 display methionine and cysteine auxotrophy.

As deletion of thioredoxin in the model yeasts *S. cerevisiae* and *S. pombe* results in methionine and/or cysteine auxotrophy (Muller, 1991 and Song and Roe, 2008), we investigated whether *C. albicans trx1* mutants display similar phenotypes. Cells were plated onto minimal agar media with or without sulphur amino acids and growth monitored. As seen in Figure 3.5B, *trx1*Δ cells failed to grow on media lacking methionine and cysteine, while wild type cells and the reconstituted strain grow normally under such conditions. In addition, cells expressing the *trx1* cysteine mutants also failed to grow in media lacking cysteine or methionine (Figure 3.5B), indicating that Trx1 plays a role in the sulphur amino acid biosynthetic pathway and that this role is dependent on its redox activity.

Based on studies in other organisms, such phenotypes are likely related to the role of *C. albicans* thioredoxin in regulating the redox state of PAPS reductase, an enzyme that participates in the sulphur assimilation pathway which is required for the biosynthesis of


A
Sulfate
sulfate adenylyltransferase
Adenylysulfate (APS)
APS kinase
3`-Phosphoadenylysulfate (PAPS)
PAPS reductase
Sulfite
sulfite reductase
Sulfide
Glutathione
gamma-glutamyltranspeptidase
L-Cysteinyl Glycine
N-aminoacylhistidine dipeptidase
cysteine synthase A
homocysteine desulfhydrase
L-Cysteine
L-Homocysteine
homocysteine methyltransferase
L-Methionine
S-adenosylmethionine synthetase
cystathionine gamma-lyase
cystathionine beta-lyase
adenosylhomo cysteinase
L-Cystathionine
S-Adenosyl L-Homocysteine
S-Adenosyl L-Methionine
(cytosine-5-)-methyltransferase

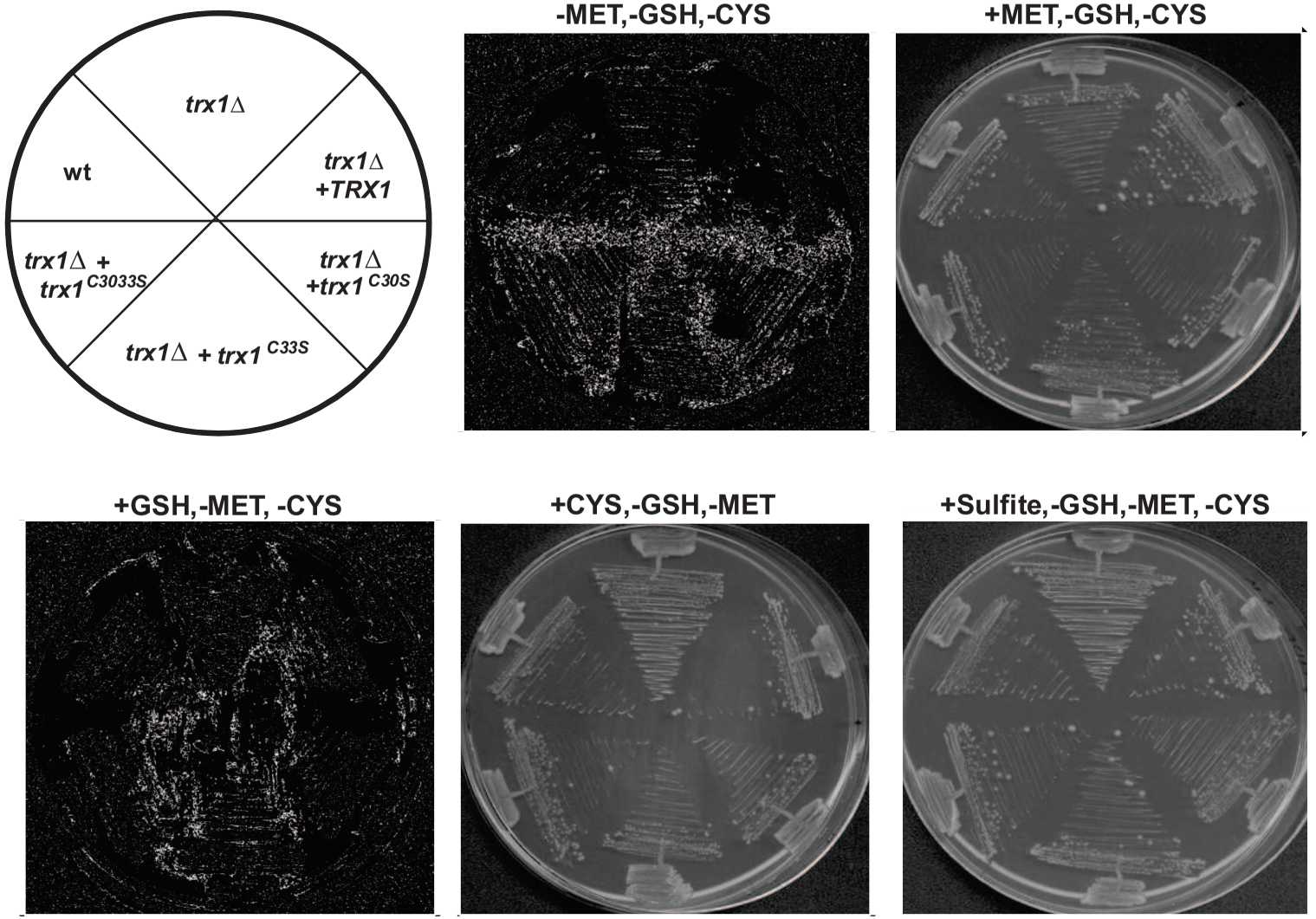


Figure 3.5. Deletion of *TRX1* or mutation of the catalytic cysteine residues of Trx1 results in methionine and cysteine auxotrophy.

(A) Pathway of sulphur containing-amino acid biosynthesis. The enzymes present in *C. albicans* are indicated in bold. (B) *C. albicans* wild-type (JC747), *trx1*Δ + CIp10 (JC677), *trx1*Δ + CIp10-*TRX1* (JC679), *trx1*Δ + CIp10-*trx1*^{C30S} (JC759), *trx1*Δ + CIp10-*trx1*^{C33S} (JC761) and *trx1*Δ + CIp10-*trx1*^{C30,33S} (JC763) were streaked into plates with or without methionine, cysteine, sulfite and glutathione, and grown for 2 days at 30ºC. The position of each strain is shown in the model figure and the presence or absence of the indicated supplement(s) is indicated on top of each plate image.

cysteine and methionine (Chartron *et al.*, 2007 and Carroll *et al.*, 2005 – Figure 3.5A). Consistent with this, *trx1*Δ cells and the cysteine mutants were able to grow in minimal media lacking sulphur amino acids when the product of PAPS reductase, sulfite, was added to the media. Furthermore, addition of GSH to media lacking methionine and cysteine also rescued the defects seen in *trx1*Δ and *trx1* cysteine mutant cells (Figure 3.5B). This is consistent with studies demonstrating that other antioxidants, such as glutaredoxins, can reduce PAPS reductase in a reaction that requires GSH (Tsang, 1981 and Russel *et al.*; 1990). Collectively, these results indicate that the role of *C. albicans* Trx1 in the sulphur amino acid biosynthetic pathway is likely through the regulation of the redox status of the enzyme PAPS reductase.

*3.2.5. trx1*Δ *cells display pleiotropic phenotypes in response to different oxidative stress inducing compounds.*

Thioredoxin is directly involved in maintaining the redox state and activity of peroxiredoxins, which are a major antioxidant protein family (Le Moan *et al.*, 2006). In addition, deletion of thioredoxin in the model yeasts *S. cerevisiae* and *S. pombe* results in defects in oxidative stress resistance (Garrido and Grant, 2002 and Song and Roe, 2008). In order to test whether in *C. albicans* Trx1 is also important for the protection against oxidative stress, *trxl*Δ cells and strains expressing the *trx1* cysteine mutations were exposed to different oxidants.

*C. albicans trxl*Δ cells and strains expressing the *trx1* cysteine mutants displayed equivalent increased sensitivity to H_2O_2 compared to wild-type cells (Figure 3.6). These data illustrate that the catalytic activity of Trx1 is required for wild-type levels of resistance to H_2O_2. Importantly, reconstitution of the wild-type gene *TRX1* into the *trx1*Δ background restored wild-type levels of H_2O_2 resistance (Figure 3.6). In contrast, deletion of *TRX1* or mutation of the cysteine residues increased the cellular resistance to the thiol oxidant diamide when compared to wild-type and reconstituted cells (Figure 3.6). Such phenotypes are similar to that reported for thioredoxin mutants in both *S. cerevisiae* (Garrido and Grant, 2002) and *S. pombe* (Song and Roe, 2008).

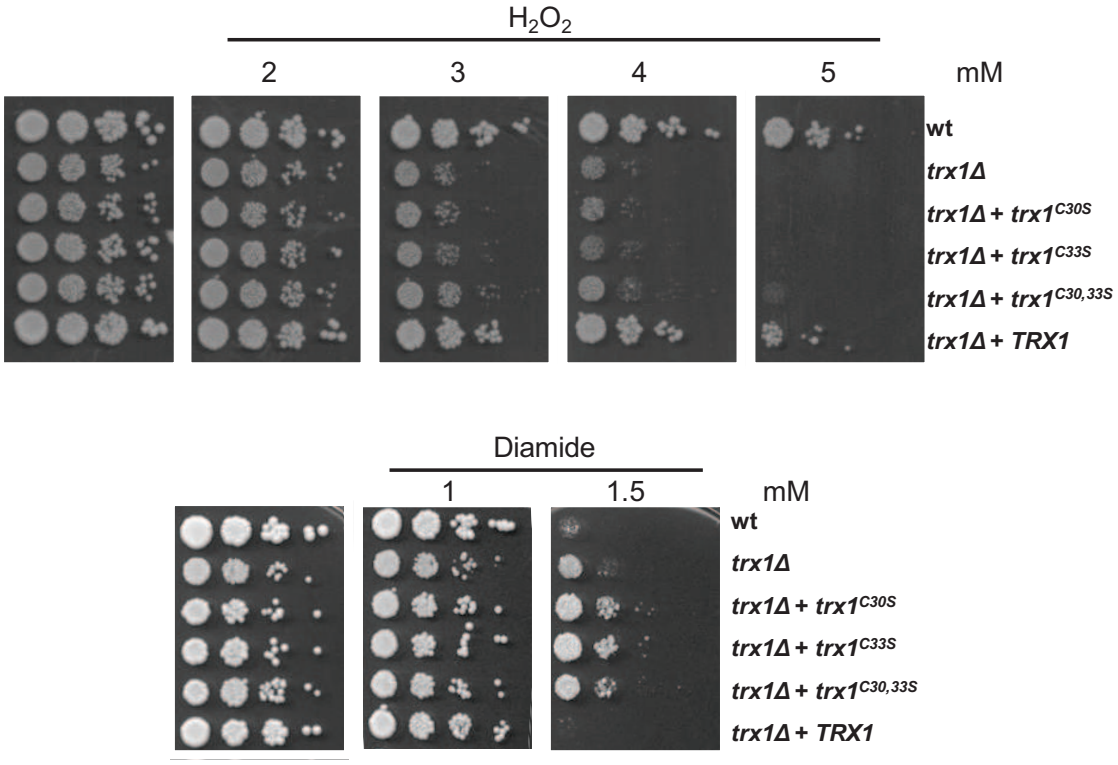


Figure 3.6. Inactivation of Trx1 results in increased sensitivity to H_2O_2 but increased resistance to diamide.

C. albicans wild-type (JC747), *trx1*Δ + CIp10 (JC677), *trx1*Δ + CIp10-*TRX1* (JC679), *trx1*Δ + CIp10-*trx1*^{C30S} (JC759), *trx1*Δ + CIp10-*trx1*^{C33S} (JC761) and *trx1*Δ + CIp10-*trx1*^{C30,33S} (JC763) were grown to mid-log phase and then plated in 1/10 serial dilutions on agar plates containing the indicated concentrations of oxidants. Plates were then incubated at 30ºC for 2 days.

3.2.6. The 2-Cys peroxiredoxin Tsa1 is constitutively oxidized in trx1Δ cells.

As described above, the antioxidant function of thioredoxin is attributed to its role in the reduction of peroxiredoxins, which become oxidized and thus inactivated during the catalytic breakdown of H_2O_2 (Chapter 1, Section 1.4.2.4). In *S. cerevisiae*, the 2-Cys peroxiredoxin plays a major role in the detoxification of H_2O_2, and cells lacking *TSA1* are significantly more sensitive to H_2O_2 compared to other peroxiredoxin null strains (Park *et al.*, 2000). To establish whether Trx1 is the sole functional thioredoxin protein in *C. albicans*, the redox state of *C. albicans* Tsa1 in wild-type and *trx1*Δ cells was compared. To facilitate this, an anti-Prdx3 polyclonal antibody raised against the related *C. elegans* 2-Cys peroxiredoxin Prdx-3 was used (Oláhová *et al.*, 2008). Western blot analysis illustrated that the anti-Prdx3 antibody specifically recognises a protein with the predicted molecular mass of Tsa1 (22 kDa) and, significantly, this band is lost upon analysing extracts from *tsa1*Δ cells (Figure 3.7A).

In order to examine the redox state of Tsa1, proteins were extracted by acid lysis to minimize the oxidation of proteins that occurs during cell lysis. Subsequently, protein extracts were treated with AMS (4-acetamido-4'-[(iodoacetyl)amino]stilbene-2,2'-disulfonic acid), an alkylating reagent that reacts specifically with sulfhydral groups of reduced cysteine residues, increasing the size of the protein by 0.5 kDa per reduced cysteine (Nagamori *et al.*, 2002). Oxidation of cysteine residues prevents AMS binding, resulting therefore in increased mobility on SDS-PAGE (Delaunay *et al.*, 2002). AMS-modified proteins were prepared from wild-type and *trx1*Δ cells both before and after H_2O_2 treatment, and analysed by non-reducing SDS-PAGE and western blotting using the anti-Prdx3 antibody (Figure 3.7C). During the detoxification of H_2O_2, the peroxidatic cysteine residue of typical 2-Cys peroxiredoxins becomes oxidised to a sulphenic acid (SOH) (Figure 3.7B – ii), resulting in unfolding of the active site of this proteins and exposing the peroxidatic sulphenic acid, which then forms a disulfide bond with the resolving cysteine residue on a partner protein forming a homodimer (Figure 3.7B – iii, reviewed in Wood *et al.*, 2003 and Aran *et al.*, 2009). The oxidized dimer is then recycled by the sequential oxidation and reduction of thioredoxin and thioredoxin reductase using NADPH. However, in eukaryotic 2-Cys peroxiredoxins, the formation of a second disulfide bond between 2 molecules of 2-Cys peroxiredoxins is slow (Figure 3.7B – iii) and as a result the SOH form of the peroxidatic

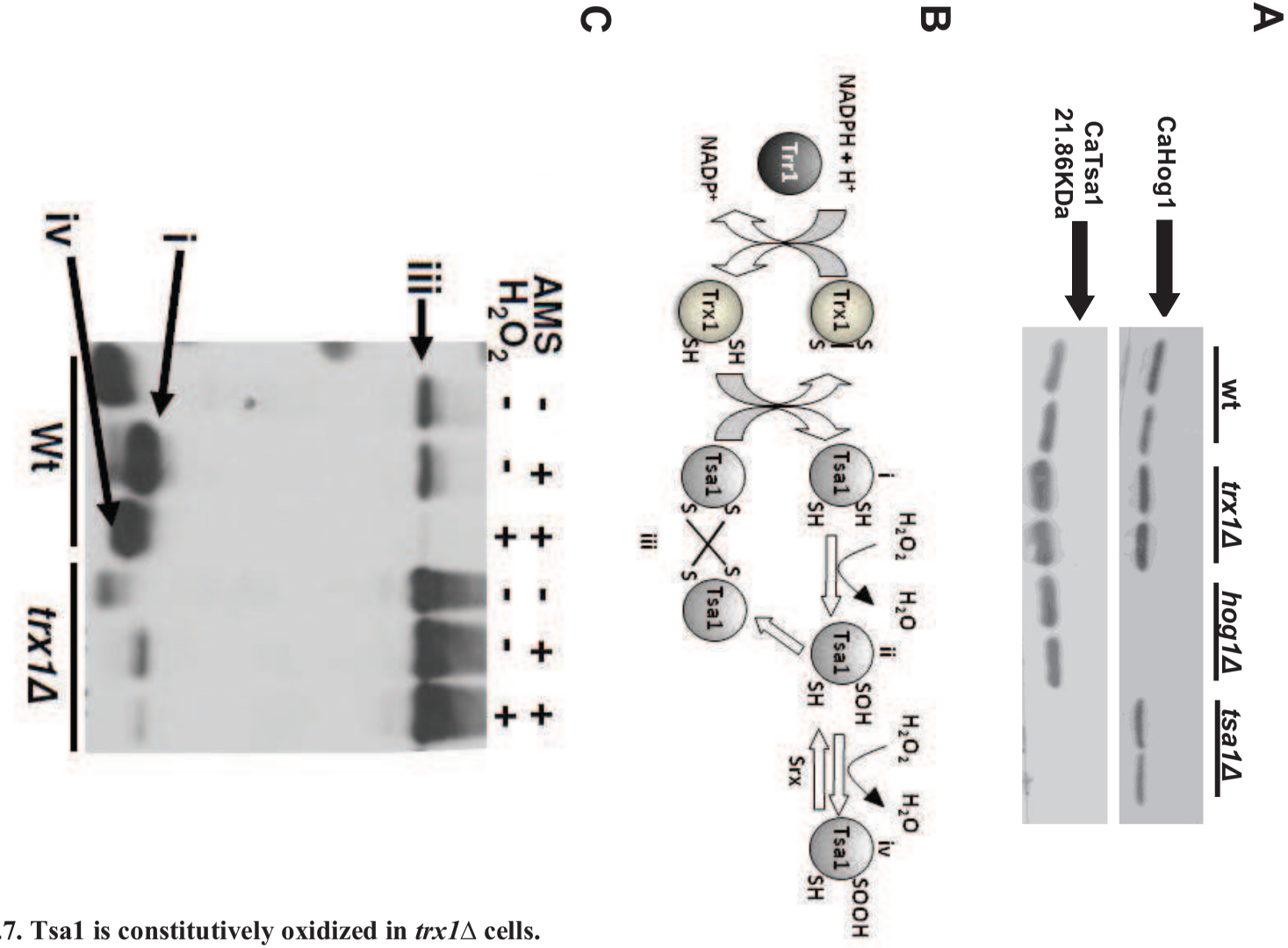


Figure 3.7. Tsa1 is constitutively oxidized in *trx1*Δ cells.

(A) Western blot analysis of whole cell extracts following reducing SDS-PAGE from *C. albicans* strains; wild-type + CIp30 (JC747), *trx1*Δ + CIp10 (JC677), *tsa1*Δ + CIp10 (JC1027) and *hog1*Δ + CIp10 (JC47). The western blot was probed with an anti-prdx3 antibody. (B) A schematic diagram illustrating the different oxidized forms of Tsa1 that are generated upon H_2O_2 detoxification by the Tsa1/Trx system. (C) The oxidation status of Tsa1 was determined by non-reducing SDS-PAGE and western-blotting, using the anti-Prdx3 antibody, of AMS-modified proteins prepared from wild-type (wt-JC747) and *trx1*Δ (JC677) strains exposed to 5 mM H_2O_2 for 30 min. Under non-stressed conditions, Tsa1 is largely present as a reduced monomeric form (i) in wt cells, whereas in *trx1*Δ cells Tsa1 was found predominantly in the oxidized dimeric form (iii).

cysteine residue is sensitive to further oxidation to the sulfinic acid (SOOH) derivative (Jara *et al.*, 2007 - Figure 3.7B – iv).

As expected, in unstressed wild-type cells, Tsa1 was mainly found in a reduced monomeric form (Figure 3.7C) and H_2O_2 treatment resulted in the conversion of the reduced monomeric form of Tsa1 into an AMS-resistant sulphinic monomeric form that has a faster mobility than reduced Tsa1 (Figure 3.7C). In contrast, Tsa1 was found predominantly trapped in the oxidized dimeric form in both untreated and H_2O_2-treated *trx1*Δ cells, and no further oxidation to sulfinic acid (SOOH) is seen *trx1*Δ cells following H_2O_2 treatment(Figure 3.7C). The lack of an overoxidized Tsa1 (SOOH) form is expected since Tsa1 seem to be trapped in the dimeric form and that overoxidation of 2-Cys peroxiredoxins in both mammalian cells and in the model yeast *S. pombe* requires the thioredoxin system (Yang *et al.*, 2002 and Jara *et al.*, 2007). This result illustrates that reduction of the oxidized dimeric form of Tsa1 in *C. albicans* is mediated entirely by Trx1, and therefore that Trx1 is the sole functional thioredoxin in *C. albicans*.

3.2.7. Trx1 is localized in the cytoplasm and nucleus of C. albicans cells.

In *S. cerevisiae*, the functionally redundant thioredoxin proteins Trx1 and Trx2, are located in both cytoplasmic and nuclear compartments in the cell (Huh *et al.*, 2003), whereas a third thioredoxin, Trx3 is located in the mitochondria (Huh *et al.*, 2003). Bioinformatics analysis (Prosite) of the *C. albicans* Trx1 amino acid sequence predicted that this protein had a 60.9% chance to be localized in the cytoplasm and 13.0% chance to be nuclear. Similar bioinformatics analysis of the *S. cerevisiae* Trx1 and Trx2 sequences resulted in similar percentages being obtained.

To determine the cellular localization of *C. albicans* Trx1, strains were constructed in which one copy of *TRX1* was chromosomally tagged with a sequence encoding GFP (see Materials and Methods for details). In brief, the p*TRX1*-GFP plasmid, in which the *TRX1* gene and promoter is placed in frame upstream of *GFP*, was integrated into the *RPS10* locus in the *trx1*Δ strain, to generate the strain JC1176. Expression of Trx1-GFP rescued phenotypes associated with loss of *TRX1*, such as the slow growth and increased sensitivity to H_2O_2 treatment, indicating that the Trx1-GFP fusion protein was functional (Figure 3.8A). Fluorescence microscopy revealed that in untreated cells, and following oxidative stress treatment, Trx1-GFP is localized both in the nucleus and in the cytoplasm (Figures 3.8B and

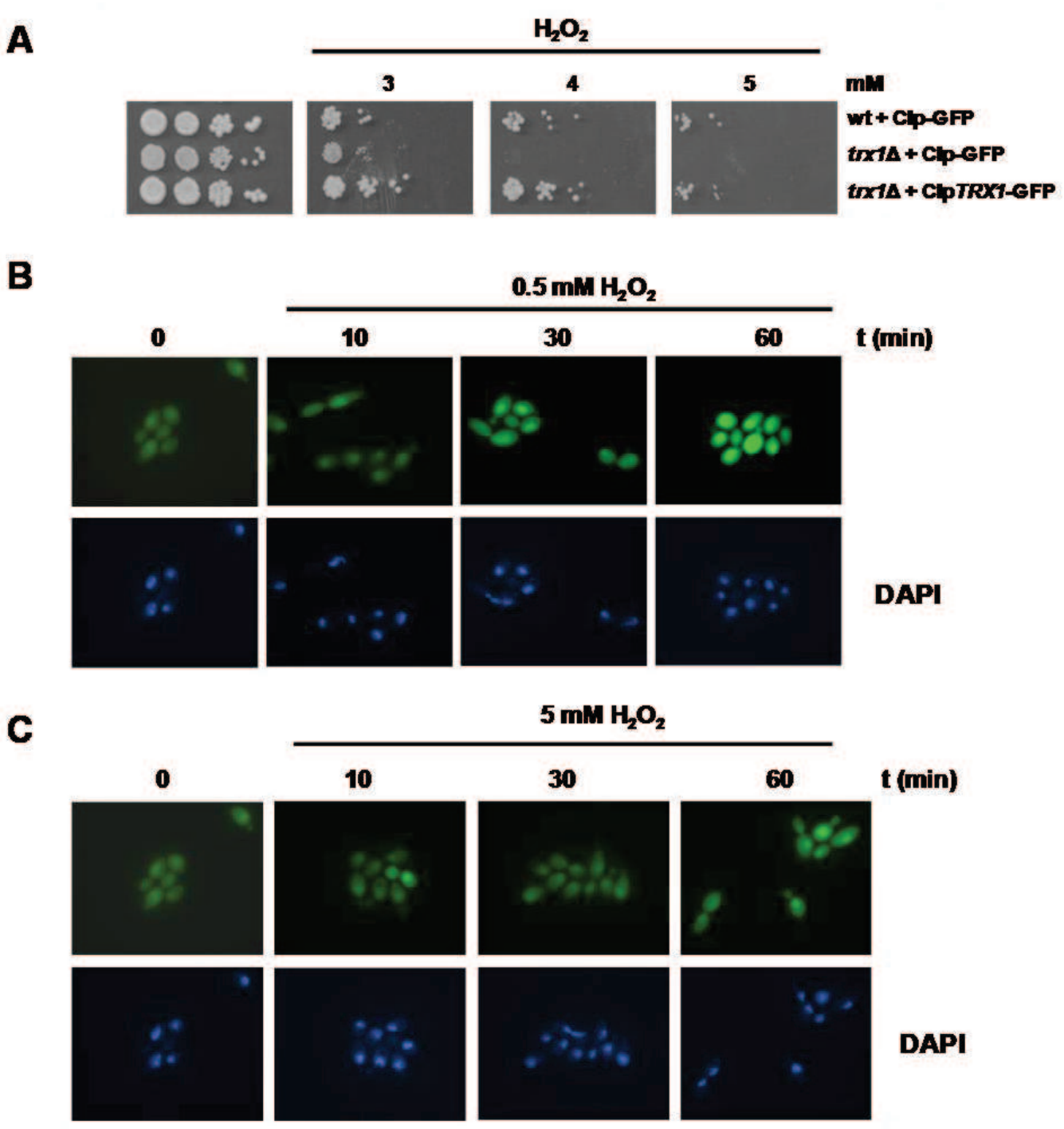


Figure 3.8. Trx1 localizes to both the nucleus and cytoplasm in *C. albicans*.
(A) Wild-type containing pGFP (JC1014) and *trx1*Δ cells containing either pGFP or pGFP-*TRX1* (JC1022 and JC1176, respectively) were spotted onto YPD plates containing increasing H_2O_2 concentrations. Plates were then incubated at 30ºC for 2 days. (B, C) Fluorescence microscopy of cells expressing Trx1-GFP. Exponentially growing cells (JC1176) were untreated or treated with 0.5 mM (B) or 5 mM (C) H_2O_2 for 60 minutes. Nuclei were stained using DAPI.

3.8C). As predicted by microarray experiments which demonstrated that *TRX1* transcripts are highly induced post H_2O_2 treatment (Enjalbert *et al*., 2003 and 2006), treatment of cells with both low (0.5 mM) and high (5 mM) concentrations of H_2O_2 resulted in an increase in Trx1 protein levels as measured by increasing fluorescence (Figures 3.8B and C).

Previous studies reported that the cellular localisation of the Trx1 substrate, Tsa1, changes during the morphogenetic switch from yeast to hyphal cells in *C. albicans*. One study reported that Tsa1 relocated from the cytoplasm to the nucleus in hyphal cells (Shin *et al.*, 2005), whereas a second study reported a redistribution of Tsa1 to the cell wall during hyphae formation (Urban *et al.*, 2005). Hence, in order to address whether the cellular location of Trx1 also changes upon hyphae formation, hyphal growth was induced in cells expressing Trx1-GFP by incubating with 10% serum at 37ºC. As observed previously (Figure 3.8), Trx1-GFP was present in the cytoplasm and nucleus of budding cells (Figure 3.9A). Following germ tube formation and hyphal development the nuclear and cytoplasm localization of Trx1 was maintained (Figure 3.9B). Hence, in contrast to that reported for Tsa1, the cellular localisation of Trx1 does not seem to change during hyphal development.

3.2.8. trx1Δ cells are constitutively filamentous

Morphological analysis of *trx1*Δ cells and strains expressing the *trx1* cysteine mutants revealed that inactivation of *TRX1* results in a filamentous growth phenotype in both solid and liquid yeast inducing media (Figures 3.10A and B). The best characterised filamentous forms of *C. albicans* are true hyphae and pseudohyphae (See Introduction, Section 1.2.3.1). True hyphae are narrower in diameter than pseudohyphae, show no constriction at septa, and the nucleus moves from mother to daughter cell before division. In contrast, pseudohyphae show constriction at septa with nuclear division occurring at the constriction. However, the filamentous cells formed following inactivation of *TRX1* displayed characteristics of both hyphae and pseudohyphae, which is similar to a recently characterised mode of filamentous growth termed hyperpolarised bud. Characteristics of hyperpolarised buds include nuclear movement to daughter cells before nuclear division similar to that seen in hyphae, and constriction at the septa similar to that seen in pseudohyphae (Whiteway and Bachewich, 2007). Similarly, deletion of *TRX1* or inactivation of the cysteine residues, resulted in cells that form elongated buds that contain clear constrictions at septal junctions and, in some cases, nuclear movement from the mother cell into the filament is evident (Figure 3.10B).

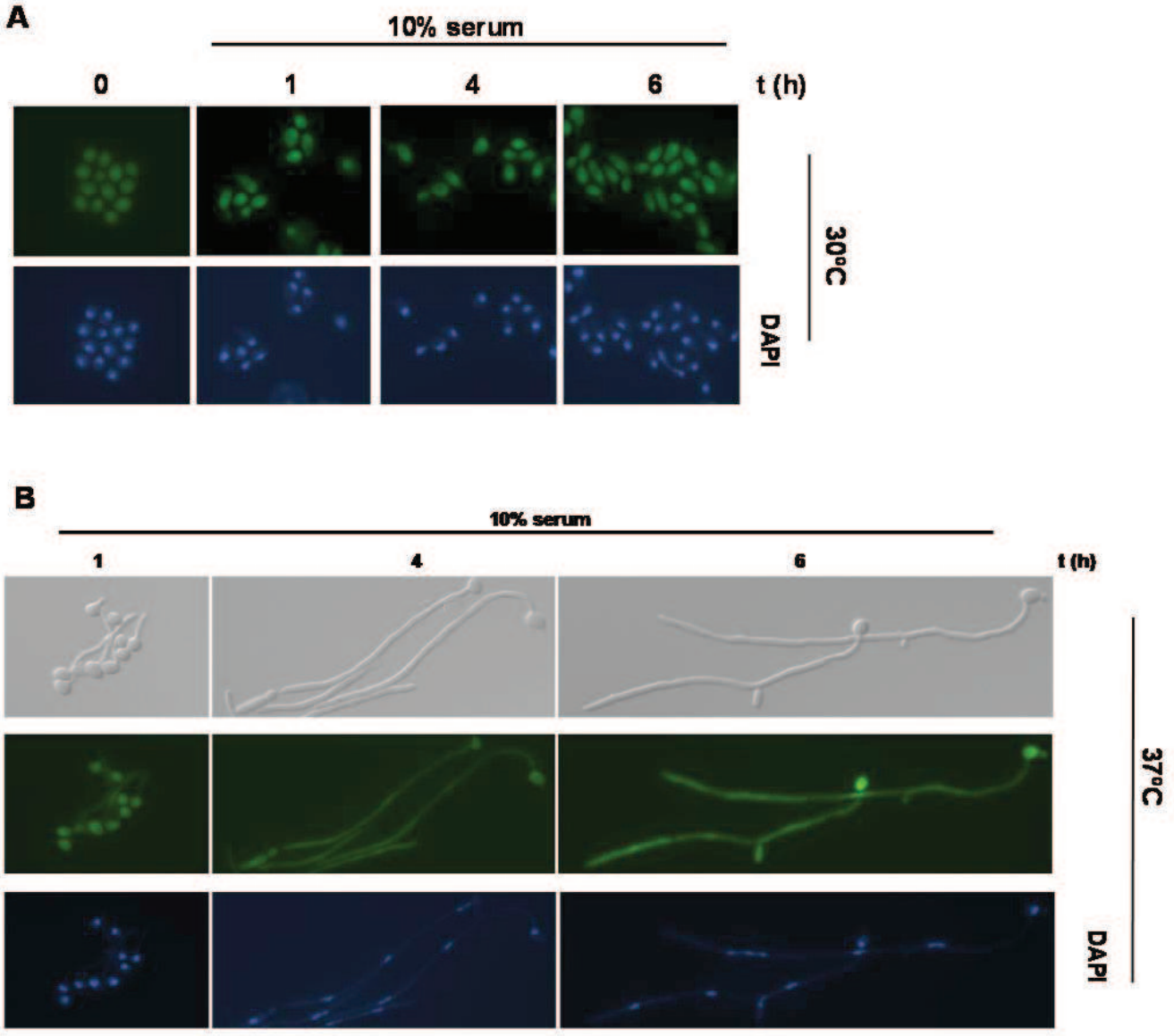


Figure 3.9. The cellular localisation of Trx1 does not change during hyphae formation.
*trx1*Δ cells expressing Trx1-GFP (JC1176) were grown to stationary phase, diluted and grown in YPD + 10% serum. Fluorescene microscopy revealed Trx1 to be located in the nucleus and cytoplasm both in budding cells grown at 30ºC (A) and in hyphal cells (B). Nuclei were stained using DAPI.

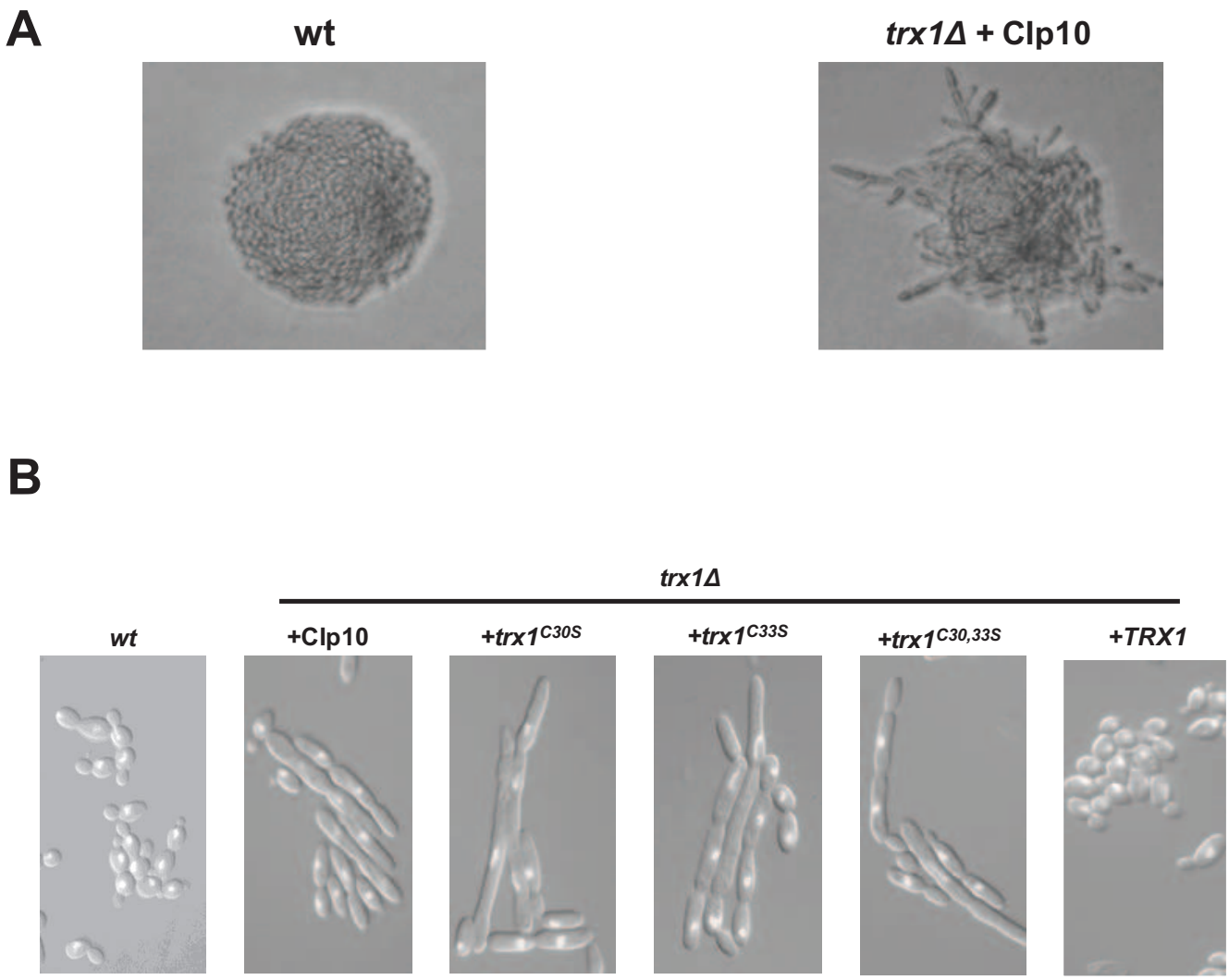


Figure 3.10. Deletion or inactivation of Trx1 results in hyperpolarized buds.
(A) Morphology of wild-type (JC747) and *trx1*Δ (JC677) colonies grown on solid YPD media. (B) Deletion of *TRX1* (JC677) or mutation of the Trx1 catalytic cysteine residues (JC759, JC761 and JC763) results in a filamentous growth in YPD liquid culture similar to hyperpolarized bud growth, whilst reintegration of *TRX1* into *trx1*Δ cells (JC679; *trx1*Δ *+TRX1*) reverses the filamentous growth phenotype. In all cases cells were stained with DAPI to allow both cell morphology and nuclear distribution to be determined by overlaying fluorescence and DIC images.

This filamentous growth characteristic of *trx1*Δ cells could be reversed upon reconstitution of the wild-type *TRX1* gene (Figure 3.10).

3.3. Discussion

Thioredoxins are ubiquitous proteins involved in the regulation of a variety of substrates that participate in a range of cellular processes such as oxidative stress defence, amino acid and dNTP biosynthesis. The catalytic mechanism used by thioredoxin, require its catalytic residues that are responsible for forming a disulphide bond with the substrate, in a reaction that will result in the reduction of the substrate and oxidation of thioredoxin (Arnér and Holmgren, 2006 – Section 1.4.2.4). The aim of this chapter was to investigate the cellular functions of thioredoxin in *C. albicans*. Although two open reading frames are annotated as *TRX1* and *TRX2* in the *Candida* genome database (http://www.candidagenome.org), sequence analysis revealed Trx2 to lack the highly conserved thioredoxin motif (Trp-Cys-Gly-Pro-Cys). In addition, phenotypes exhibited by *C. albicans trx1*Δ cells such as decreased H_2O_2 stress resistance (Figure 3.6), slow growth (Figure 3.3), methionine auxotrophy (Figure 3.5), and impaired reduction of Tsa1 (Figure 3.7), are only observed in *S. cerevisiae* upon deleting the functionally redundant *TRX1* and *TRX2* genes. Hence in contrast to *S. cerevisiae*, these data indicate that Trx1 is the sole functional thioredoxin in *C. albicans*. Interestingly, in further contrast to that seen in both *S. cerevisiae* and *S. pombe* (Pedrajas *et al.*, 1999 and Song *et al.*, 2006), *C. albicans* apparently lacks a mitochondrial thioredoxin. However, *C. albicans* does contain a thioredoxin like (Txl1) protein (*orf19.3319*) that has significant homology to the *S. pombe* Txl1 protein, which has recently been implicated as a co-factor for the ubiquitin-proteasome system (Wiseman *et al.*, 2009).

The oxidoreductase activity of Trx1 is important for growth in *C. albicans*, as cells lacking *TRX1,* or those that express mutations in the catalytic cysteine residues, have a slow growth phenotype (Figure 3.3). Inactivation of *TRX1* increases the sensitivity of *C. albicans* strains to the ribonucleotide reductase inhibitor, hydroxyurea (HU), and ectopic expression of the large subunit of ribonucleotide reductase can rescue the slow growth phenotype seen in *trx1*Δ strains (Figure 3.4). These results suggest that the slow growth phenotype seen in *trx1*Δ cells is due to impaired ribonucleotide reductase activity (Figure 3.4). Indeed, thioredoxin was originally identified as being the electron donor for ribonucleotide reductase in *E. coli* (Laurent *et al*., 1964), and recent studies have illustrated that in *S. cerevisiae* the slow growth

phenotype of *trx1*Δ*trx2*Δ cells is due increased Rnr1 oxidation, which results in decreased dNTP levels (Muller, 1991, Koc *et al.*, 2006, Muller *et al.*, 1995 and Camier *et al.*, 2007).

Similarly, the methionine and cysteine amino acid auxotrophy exhibited by *C. albicans trx1*Δ mutants (Figure 3.5) have been previously reported in thioredoxin mutants in other fungi (Muller, 1991, Song and Roe, 2008 and Malagnac *et al.*, 2007). Thioredoxin has a well established role as an electron donor for 3'-phosphoadenylylsulfate (PAPS) reductase in *E. coli* (Lillig *et al.*, 1999). PAPS reductase catalyses the conversation of 3'-phosphoadenylylsulfate to sulfite, which is an intermediate necessary for the synthesis of cysteine and methionione. Thioredoxin is thought to be required for releasing the resulting product of PAPS reductase, sulphite, from the active site (Carroll *et al.*, 2005). In *E. coli*, this reaction requires the formation of a mixed disulfide between PAPS reductase (Cys239) and the N-terminal cysteine of Trx1 (Cys32), which then is resolved by the C-terminal cysteine (Cys34), resulting in reduced PAPS reductase and oxidized Trx1 (Chartron *et al.*, 2007). Furthermore, in *S. cerevisiae* the cytoplasmatic cysteines (Trx1 and Trx2) interact with the PAPS reductase, MET16, possibly throught the formation of a mixed disulfide between the N-terminal cysteine residue of thioredoxin and Cys245 of Met16 (Vignols *et al.*, 2005). As the methionine and cysteine auxotrophy of *C. albicans trx1*Δ cells can be by-passed by the addition of cysteine, methionine and sulfite, this indicates that Trx1 is also the electron donor for PAPs reductase in this fungus. Furthermore, the fact that glutathione can also rescue the amino acid auxotrophy in *trx1*Δ cells, is consistent with the fact that glutaredoxins can also reduce PAPS reductase, in a reaction that requires GSH (Tsang, 1981 and Russel *et al.*; 1990). However, it is also feasible that glutathione may serve as a precursor for sulphur amino acid biosynthesis, since *C. albicans* possesses the enzymes that can use this tripeptide as a substrate to generate cysteine or methionine (Figure 3.5).

The impaired resistance to H_2O_2 exhibited by *C. albicans trx1*Δ strains is consistent with our observation that Trx1 is needed for the reduction and thus peroxidase activity of the 2-Cys peroxiredoxin Tsa1 (Figure 3.7). Specifically, deletion of *TRX1* results in the formation of oxidised dimeric forms of Tsa1 that cannot be reduced. This result is consistent with that reported in *S. cerevisiae trx1*Δ*trx2*Δ cells (Garrido and Grant, 2002). In contrast, *C. albicans trx1*Δ cells are more resistant to the thiol-depleting agent diamide (Figure 3.6), and similar findings have been reported in the model yeasts *S. cerevisiae* and *S. pombe* upon inactivating thioredoxin (Trotter and Grant, 2002; Song and Roe, 2008). One possible explanation is that

the AP-1 transcription factor Cap1, which plays a major role in *C. albicans* defence against oxidative stress is a target of Trx1 (See Chapter 4). Studies have shown that in *C. albicans* a truncated form of Cap1 confers high levels of diamide resistance compared to the full-length Cap1 (Zhang *et al.*, 2000 and Alarco and Raymond, 1999). Taken together with studies in *S. cerevisiae* that illustrate that thioredoxin negatively regulates the related AP-1 transcription factor Yap1 (Izawa *et al.*, 1999), the resistance of *C. albicans trx1*Δ cells to diamide could be due to Trx1 negatively regulating Cap1 in response to diamide.

In addition to phenotypes described above, which have largely been reported in other fungi upon deletion of thioredoxin function, we unexpectedly found that *C. albicans* Trx1 plays a role in inhibiting filamentous growth since *trx1*Δ cells display characteristics of hyperpolarised bud growth (Figure 3.10). This phenotype does not appear to be related to the role of Trx1 in maintaining active Tsa1, since deletion of *TSA1* does not result in strains that show a filamentous phenotype in non inducing media (Urban *et al.*, 2005 and Shin *et al.*, 2005). Hyperpolarised bud formation is distinct from the well characterised filamentous growth forms of hyphae and pseudohyphae, and has been shown to occur in various *C. albicans* mutants that perturb cell cycle progression or in response to chemicals such as HU or methyl methanesulfonate (MMS) (Bachewich *et al.*, 2003 and Shi *et al.*, 2007) that induce cell cycle arrest. This suggests therefore that the hyperpolarised bud formation in *trx1*Δ cells is related to a cell cycle defect. Moreover, the observation that mutation of either redox sensitive catalytic cysteine residue of Trx1 also resulted in hyperpolarized bud formation under non-stressed conditions (Figure 3.10), suggested that exposure of cells to oxidative stress agents that results in the oxidation of Trx1 may induce polarized cell growth. This hypothesis formed the basis of the experiments presented in Chapter 5.

In conclusion, the data presented in this chapter indicates that Trx1 is the sole functional cytoplasmic/nuclear thioredoxin in *C. albicans.* A list of the phenotypes associated with inactivation of Trx1, and possible targets of Trx1 associated with such phenotypes is summarized in Figure 3.11. The identification of Trx1 as the major thioredoxin subsequently facilitated experiments designed to investigate the role of thioredoxin, and thioredoxin substrates, in oxidative stress signalling in *C. albicans*, which are described in the following chapter.

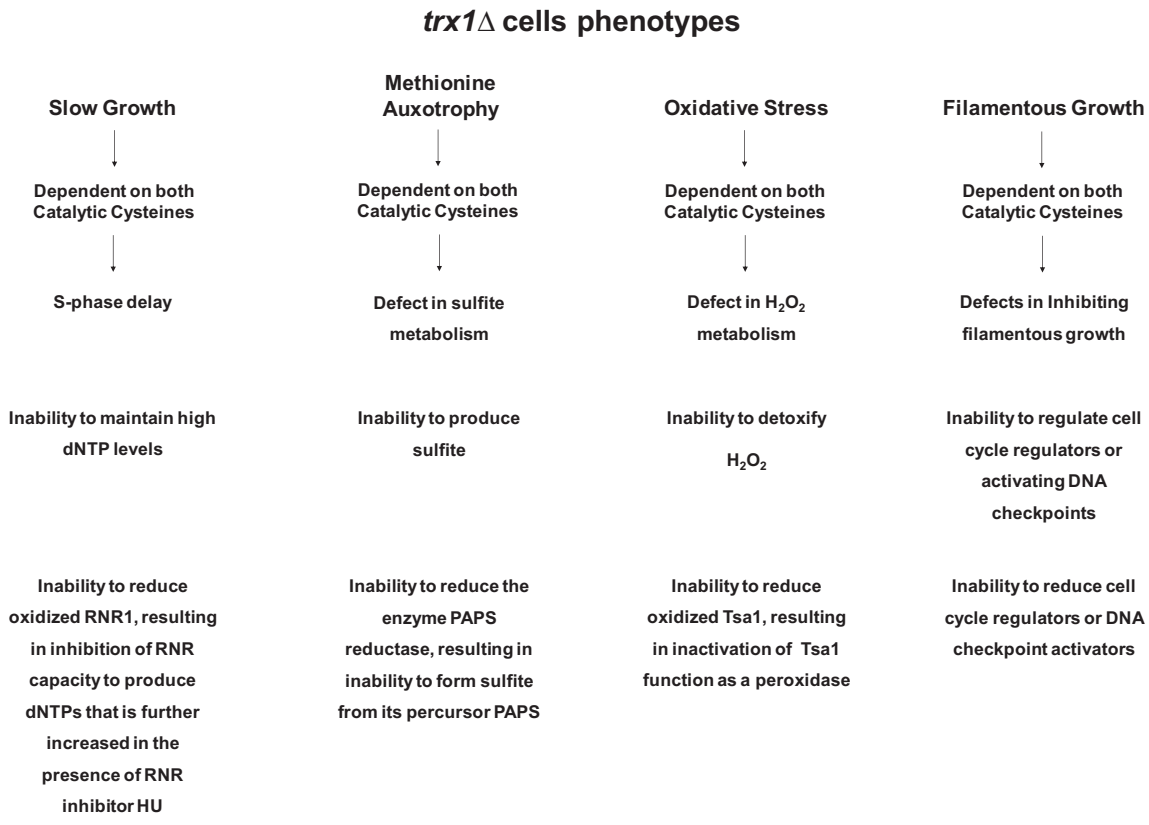


Figure 3.11. Phenotypes associated with *trx1*Δ *C. albicans* cells and the possible causes and proteins associated with them.

Chapter 4. Investigation into the role of the redox sensitive antioxidants, Trx1 and Tsa1, in oxidative stress signalling in *C. albicans*

4.1. Introduction

In order to survive within various hostile environments within the host, *C. albicans* has to be able to mount fast and robust stress responses (Brown *et al.*, 2009). Of particular importance is the oxidative stress response, as high levels of ROS are generated by innate immune cells as an initial microbial defence mechanism (Bogdan *et al.*, 2000, Nathan and Shiloh, 2000 and Vonk *et al.*, 2006). The AP1-like transcription factor Cap1 is the main regulator of oxidative stress-induced gene expression in *C. albicans* (Wang *et al.*, 2006 and Kusch *et al.*, 2007) and deletion of *CAP1* results in increased killing by neutrophils (Fradin *et al.*, 2005). In addition, the Hog1 SAPK is robustly activated in response to oxidative stress (Alonso-Monge *et al.*, 2003 and Smith *et al.*, 2004), and cells lacking *HOG1* display decreased survival following phagocytosis (Arana *et al.*, 2007) and significantly attenuated virulence in a mouse model of systemic candidiasis (Alonso-Monge *et al.*, 1999). However, despite the importance of oxidative stress signalling in promoting *C. albicans* survival in the host, very little is known about the mechanisms involved in regulating the Cap1 and Hog1 signalling pathways (Brown *et al.*, 2009).

There is growing evidence from studies in other systems that redox-sensitive antioxidants, such as 2-Cys peroxiredoxins and thioredoxins, have additional roles in peroxide signalling, acting as both peroxide sensors and regulators of peroxide-induced signalling pathways (Chapter 1, Section 1.4.4.2; Veal *et al.*, 2007). Many such studies have been performed in the model yeasts *S. cerevisiae* and *S. pombe*. For example, in *S. pombe,* the 2-Cys peroxiredoxin Tpx1 is required for both the regulation of the AP-1 like transcription factor Pap1 (Bozonet *et al.*, 2005 and Vivancos *et al.*, 2005) and the peroxide-induced activation of the Sty1 SAPK (Veal *et al*., 2004). Similarly, in *S. cerevisiae*, the 2-Cys peroxiredoxin, Tsa1 has been shown to regulate the activation of the AP-1 like transcription factor Yap1 in certain strain backgrounds (Ross *et al.*, 2000 and Veal *et al.*, 2003). Furthermore, with regard to thioredoxin, in *S. cerevisiae* the thioredoxins Trx1 and Trx2 function to reduce the oxidised active form of the Yap1 transcription factor. Consequently, *trx1*Δ*trx2*Δ double mutants exhibit a constitutive nuclear localization and activation of Yap1 (Izawa *et al.*, 1999 and Carmel-Harel *et al.*, 2001).

Based on such findings in model yeasts implicating both 2-Cys peroxiredoxins and thioredoxins in oxidative stress signalling, the aim of the study presented in this chapter was to investigate the role of the thioredoxin Trx1, and the 2-Cys peroxiredoxin Tsa1, in oxidative stress signalling in *C. albicans*.

4.2. Results

4.2.1. Bioinformatic analysis of 2-Cys peroxiredoxin genes in C. albicans

Analysis of the Candida Genome Database (www.candidagenome.org) revealed that *C. albicans* contains a single typical 2-Cys peroxiredoxin, Tsa1. Previous transcript profiling studies and proteomic analyses reported an increase in *TSA1* mRNA and Tsa1 protein levels respectively, following treatment of *C. albicans* with H_2O_2 or during phagocytosis (Enjalbert *et al*., 2006, Kusch *et al.*, 2007, Yin *et al.*, 2009, Fradin *et al.*, 2005 and Fernandez-Arenas *et al.*, 2007). Such findings are suggestive of a role of Tsa1 in the *C. albicans* oxidative stress response. Alignment of *C. albicans* Tsa1 with the typical 2-Cys peroxiredoxins from the models yeasts *S. cerevisiae* and *S. pombe* illustrates a high level of sequence conservation (Figure 4.1). For example, *C. albicans* Tsa1 displays 73% identity to *S. cerevisiae* Tsa1 and 64% identity to *S. pombe* Tpx1 (Figure 4.1). Of significance, *C. albicans* Tsa1 contains the conserved "peroxidatic" cysteine residue at position 48 (Cys48) and the "resolving" cysteine residue at position 169 (Cys169), that are required for its peroxidase activity, and the proline and threonine residues that are important residues in the surrounding environment of the catalytic site (Hall *et al.*, 2009; Figure 4.1). Eukaryotic 2-Cys peroxiredoxins are much more sensitive to oxidative inactivation than bacterial 2-Cys peroxiredoxins (Wood *et al*., 2003), and this underlies the stress-signalling functions attributed to the eukaryotic enzymes (Veal *et al.*, 2007). The structural basis of this difference in sensitivity is due to the presence of two motifs; a GGLG motif in the middle of the sequence and an YF motif at the C-terminus (Wood *et al.*, 2003). *C. albicans* Tsa1 contains both these motifs (Figure 4.1) indicating that this enzyme will be susceptible to overoxidation and therefore may play a role in redox sensing and signalling in this fungal pathogen.

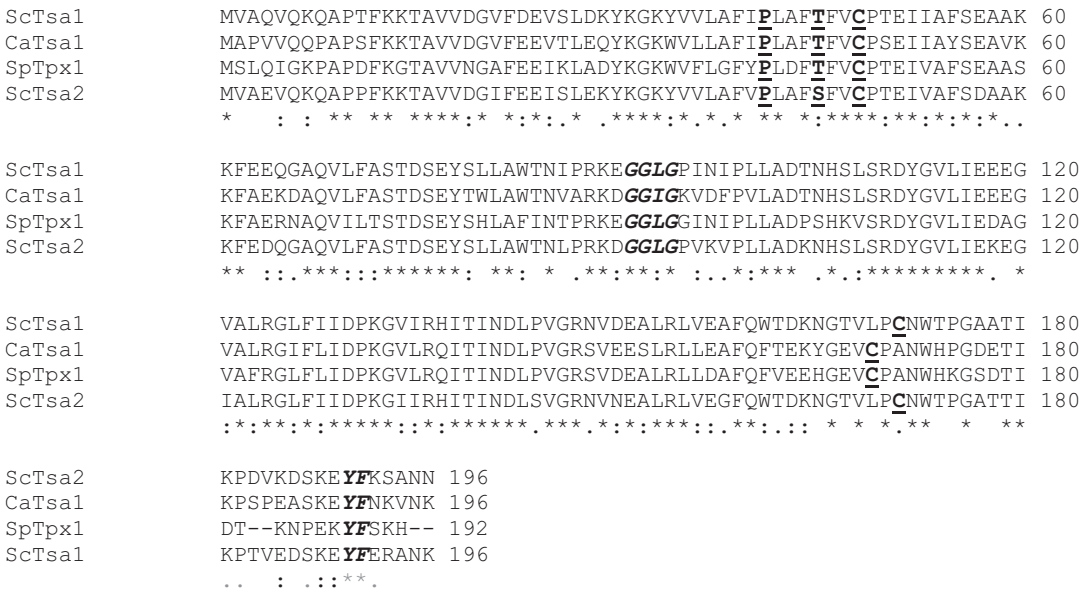


Figure 4.1. Sequence alignment of typical 2-Cys peroxiredoxins from *C. albicans*, *S. cerevisiae* and *S. pombe*.

The amino acid sequences of *S. cerevisiae* Tsa1 and Tsa2, *C. albicans* Tsa1, and *S. pombe* Tpx1 were aligned using ClustalW (Thompson *et al.*, 1994). Dashes indicate single-residue gaps introduced to maximise the alignment. * indicates identical residues, : indicates conserved substitutions, and . indicates semi-conserved substitutions. Conserved amino acids important for the peroxidase activity of 2-Cys peroxiredoxins, including the peroxidatic and resolving catalytic cysteines are represented by bold, underlined residues. The structural elements shown to increase the susceptibility of the eukaryotic 2-Cys peroxiredoxins enzymes to oxidative inactivation (Wood *et al*., 2003), are represented by bold and italicised residues.

4.2.2. Expression of C. albicans Tsa1 complements loss of Tpx1 function in S. pombe tpx1⁻ cells

In the model yeast *S. pombe*, the 2-Cys peroxiredoxin Tpx1 plays a central role in oxidative stress signalling, as it is required for the activation of the two major oxidative stress responsive signalling proteins; the AP-1 like transcription factor Pap1 and the Sty1 SAPK (Veal *et al.*, 2004, Bozonet *et al.*, 2005 and Vivancos *et al.*, 2005). To investigate whether the analogous *C. albicans* protein Tsa1 may also have oxidative stress signalling functions, as a first step the ability of *C. albicans* Tsa1 to complement the defects in oxidative stress responses in *S. pombe tpx1⁻* cells was investigated.

4.2.2.1. Ectopic expression of C. albicans Tsa1 rescues the stress sensitive phenotypes of S. pombe tpx1⁻ cells

To investigate whether *C. albicans* Tsa1 is functional in *S. pombe*, *tpx1⁻* cells were transformed with the plasmid pRep1-*TSA1*, in which *C. albicans TSA1* expression is under the control of the strong thiamine repressible *nmt1⁺* promoter (See Materials and Methods, Basi *et al.*, 1993). In addition, *tpx1⁻* cells were also transformed with pRep1-*tpx1⁺* in which the *S. pombe tpx1⁺* gene is under the control of the *nmt1⁺* promoter, which served as a positive control, and the empty pRep1 vector which provided a negative control. To verify that Tsa1 and Tpx1 were being expressed in *tpx1⁻* strains, protein extracts from *tpx1* +Rep1, *tpx1⁻* +Rep1-*tpx1⁺* and *tpx1⁻* +Rep1-*TSA1* strains were analysed by western blotting using an anti-Prdx3 polyclonal antibody (Chapter 3 - Figure 3.7; Oláhová *et al.*, 2008) and an anti-Tpx1 polyclonal antibody (kind gift from Dr Elizabeth Veal). As illustrated in Figure 4.2, both Tsa1 and Tpx1 are being expressed in *tpx1⁻* cells as indicated by cross-reactive bands of the predicted molecular weight that are absent in extracts prepared from *tpx1⁻* cells (Figure 4.2B). However, whilst the anti-Tpx1 antibody recognised *S. pombe* Tpx1, it failed to recognise *C. albicans* Tsa1. Similarly, the anti-Prdx3 antibody recognised the *C. albicans*, but not the *S. pombe*, 2-Cys peroxiredoxin. This could be explained by the peptide sequences used to generate the anti-Tpx1 and anti-Prdx3 antibodies, as *C. albicans* Tsa1 contains a sequence highly conserved to the peptide used to generate the anti-Prdx3, but not the anti-Tpx1, antibody (Figure 4.2A). Notably, additional smaller bands were reproducibly observed in extracts from cells expressing both Tpx1 and Tsa1. However, different to the C-terminal truncation seen in previous studies (Koo *et al.*, 2002), these forms may

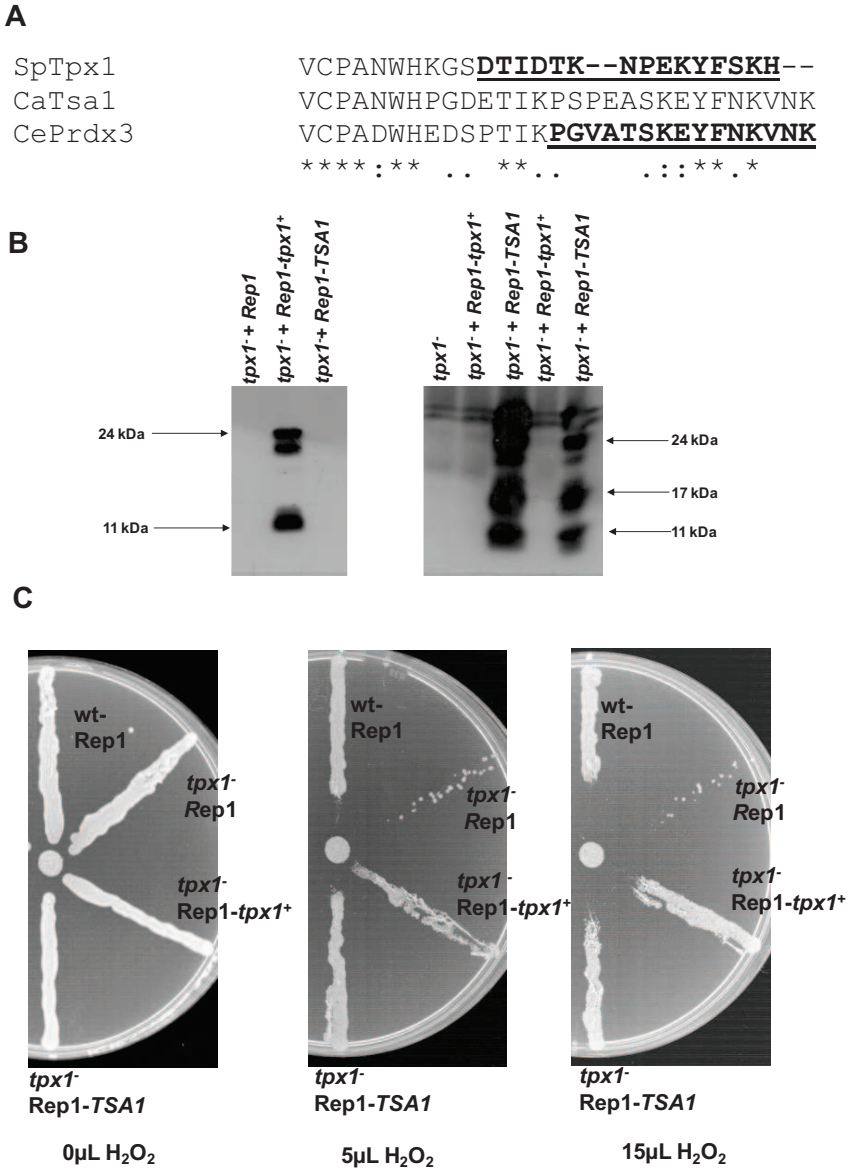


Figure 4.2. Expression of Tpx1 and Tsa1 in *S. pombe* rescues the oxidative stress sensitive phenotype of *tpx1*⁻ cells.

(A) C-terminal amino acid sequences of *S. pombe* Tpx1, *C. albicans* Tsa1 and *C. elegans* Prdx3 were aligned using ClustalW (Thompson *et al.*, 1994), and the peptide sequences used to generate the anti-Tpx1 and anti-Prdx3 antibodies underlined (B) Western blot analysis of whole cell extracts from strains *tpx1*⁻ + pRep1, *tpx1*⁻ + pRep1-*tpx1*⁺ and *tpx1* + pRep1-*TSA1*, using an anti-Tpx1 (left panel) or anti-Prdx3 antibody (right panel). Arrows indicate the specific 2-Cys peroxiredoxin bands. (C) Halo test to measure H_2O_2 resistance of *S. pombe* wild-type cells (NT5 - Zhu *et al.*, 1994), and *tpx1*⁻ cells (VX00 - Veal *et al.*, 2004) transformed with either pRep1, pRep1-*tpx1*⁺ or pRep1-*TSA1*. Cells were grown until stationary phase and 20 µl spread in a single line from the edge to the middle of the plate. Filter paper discs soaked with either water or H_2O_2 were placed at the centre of the plate and cells incubated at 30°C for 72 hours.

be a result of N-terminal truncation(s), since the antibodies used in our study were raised against the C-terminal end of Tpx1 and Prdx3. The physiological significance of these forms is unclear. Furthermore, the observation that different antibodies needed to be used to detect expression of Tpx1 and Tsa1 meant that the levels of these proteins could not be directly compared. However, as both Tsa1 and Tpx1 are being expressed, this allowed an examination as to whether the ectopic expression of these proteins could rescue the H_2O_2-sensitive phenotype of *S. pombe tpx1*$^-$ cells. The halo assays shown in Figure 4.2C illustrates that expression of both *tpx1*$^+$ and *TSA1* restored oxidative stress resistance to *S. pombe tpx1*$^-$ cells. This is suggestive that the *C. albicans* 2-Cys peroxiredoxin Tsa1 can rescue the antioxidant function, and possibly the oxidative stress signalling functions, associated with Tpx1 in *S. pombe tpx1*$^-$ cells.

4.2.2.2. C. albicans TSA1 can restore Pap1 activation in response to H_2O_2 in tpx1$^-$ cells

In *S. pombe*, Tpx1 is essential for the oxidation and thus nuclear accumulation of the AP-1 transcription factor Pap1 in response to H_2O_2 (Bozonet *et al*., 2005 and Vivancos *et al.*, 2005). Since expression of Tsa1 rescues the oxidative stress sensitive phenotype of *tpx1*$^-$ cells, H_2O_2-induced Pap1 activation was assessed in order to investigate if Tsa1 restored Pap1 activity in the *tpx1*$^-$ mutant. To facilitate this, *tpx1*$^-$ cells in which Pap1 was chromosomally tagged with a Pk epitope (Bozonet *et al.*, 2005) were transformed with either pRep1, pRep1-*tpx1*$^+$ or Rep1-*TSA1*. Cells were either untreated or treated with H_2O_2, and Pap1 localisation visualised by indirect immunofluorescence staining using an anti-Pk antibody (Veal *et al.*, 2002 and Bozonet *et al*., 2005). Consistent with previous studies (Bozonet *et al*., 2005 and Vivancos *et al.*, 2005), Pap1 failed to accumulate in the nucleus in *tpx1*$^-$ null strains after H_2O_2 treatment (Figure 4.3). However, expression of either Tpx1 or Tsa1 in *tpx1*$^-$ cells restored Pap1 nuclear accumulation in response to both low (0.2 mM) and high (1 mM) H_2O_2 concentrations (Figure 4.3). Different time points were used to examine Pap1 nuclear accumulation in response to low and high H_2O_2 concentrations as in response to 0.2 mM H_2O_2 concentrations Pap1 is rapidly oxidized and localizes in the nucleus within 10 min of peroxide addition (Quinn *et al.*, 2002 and Bozonet *et al.*, 2005 – section 1.4.4.2.1), whereas Pap1 oxidation is significantly delayed following treatment with 1 mM H_2O_2 as this requires the expression of the Atf1-target gene *srx1*$^+$ (Quinn *et al.*, 2002 and Bozonet *et al.*, 2005 – section 1.4.4.2.1). This result strongly suggests that Tsa1 can

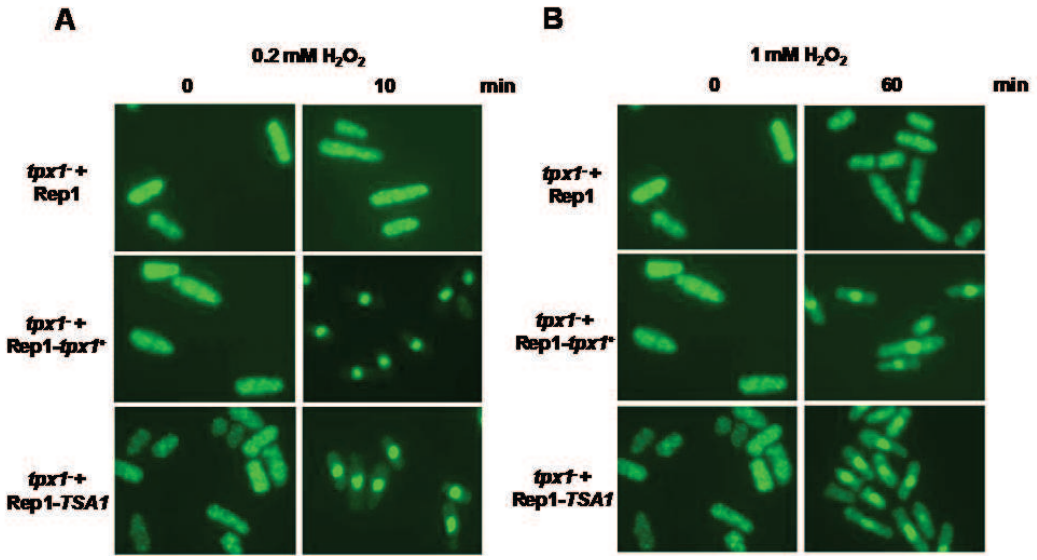


Figure 4.3. Pap1 localization after H_2O_2 treatment.
Pk-tagged Pap1 was visualized by indirect immunofluorescence in *tpx1*$^-$ cells (SB4 - Bozonet *et al.*, 2005) transformed with pRep1, pRep1-*tpx1*$^+$ or pRep1-*TSA1*. Cells were either untreated or treated with 0.2 mM H_2O_2 for 10 min (A) or with 1 mM H_2O_2 for 60 min (B).

rescue Pap1 oxidation and thus nuclear accumulation in response to H_2O_2 in *S. pombe tpx1*$^-$ cells.

4.2.2.3. C. albicans Tsa1 can rescue Sty1 activation in response to H_2O_2 in tpx1$^-$ cells

In addition to its role in regulating peroxide-induced activation and nuclear accumulation of Pap1, Tpx1 has also been shown to be important for H_2O_2-induced activation of the Sty1 SAPK (Veal *et al.*, 2004). In order to investigate whether Tsa1 could replace Tpx1 in the regulation of the H_2O_2-induced activation of Sty1, Sty1 phosphorylation was measured in wild-type cells, *tpx1*$^-$ cells, and *tpx1*$^-$ cells expressing either *tpx1*$^+$ or *TSA1* (Figure 4.4). In order to detect Sty1 phosphorylation, whole cell extracts were prepared from cells before and following H_2O_2 treatment and analysed by SDS-PAGE and western blotting. Sty1 activation was detected using an anti-phospho-p38 antibody that only recognises the phosphorylated, active, form of Sty1 (Millar *et al.*, 1995 and Veal *et al.*, 2004), following which blots were stripped and reprobed using an anti-Sty1 antibody which detects total Sty1 levels (Day and Veal, 2010). Sty1 became rapidly phosphorylated in wild-type cells following a 10 minute exposure to H_2O_2 (Figure 4.4). In contrast, Sty1 activation was virtually abolished in *tpx1*$^-$ cells. Significantly, expression of either *tpx1*$^+$ or *TSA1* in *tpx1*$^-$ cells restores H_2O_2-induced Sty1 phosphorylation to even greater levels than seen in wild-type cells (Figure 4.4). This result illustrates that expression of *TSA1* in *S. pombe* can function to regulate Sty1 activation in response to H_2O_2.

Taken together, these results indicate that the ability of *C. albicans* Tsa1 to rescue the H_2O_2 sensitivity of *S. pombe tpx1*$^-$ cells is likely to be due to a combination of its antioxidant function, and its ability to restore H_2O_2-induced activation of both the Pap1 transcription factor and the Sty1 SAPK.

4.2.3. Creation and phenotypic analysis of C. albicans strains lacking functional Tsa1

As *C. albicans* Tsa1 can restore the oxidative stress signalling functions of Tpx1 in *S. pombe tpx1*$^-$ cells (Section 4.2.2), it was decided to investigate potential peroxide signalling functions of Tsa1 in *C. albicans.*

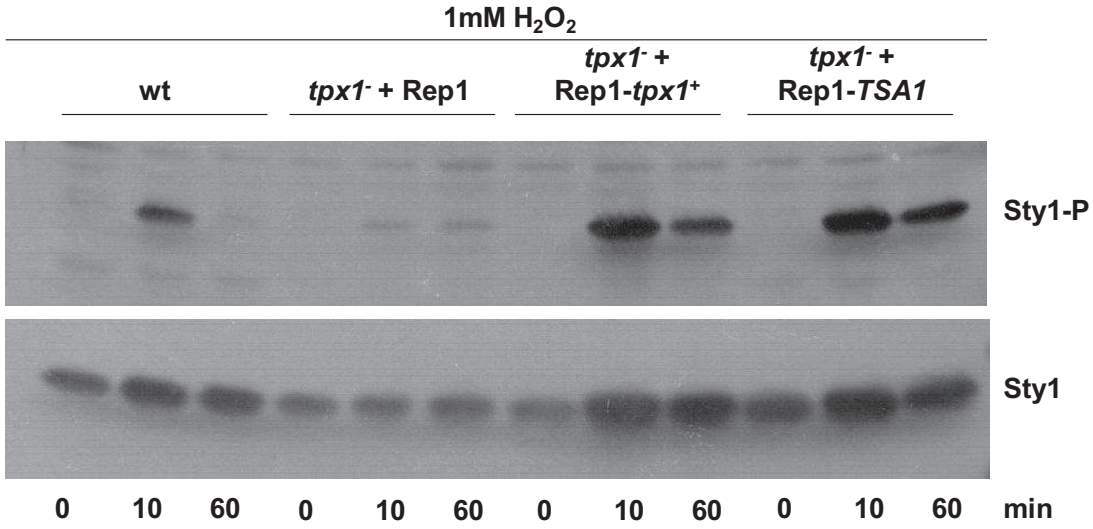


Figure 4.4. Sty1 activation in *tpx1*⁻ cells after treatment with 1 mM H_2O_2.
The levels of phosphorylation of Sty1 (Sty1-P) were determined by western blot analyses of lysates prepared from wild-type cells (Wt - NT5) and *tpx1*⁻ (VX00) cells transformed with pRep1, pRep1-*tpx1*+ or pRep1-*TSA1* following treatment with 1 mM H_2O_2 for 0, 10, or 60 min. Sty-P was detected using an anti-phospho p38 antibody, following which blots were stripped and total levels of Sty1 determined by reprobing with an anti-Sty1 antibody.

4.2.3.1. Construction of tsa1Δ cells and strains expressing Tsa1 cysteine mutants

To analyse Tsa1 function in *C. albicans*, a *tsa1*Δ strain was created. The *TSA1* gene is duplicated in the *C. albicans* genome (*orf19.7417* and *orf19.7398*). In order to delete the 4 copies of *TSA1* and generate strain JC1026, two copies were deleted by replacing the *TSA1* ORF with either the *HIS1* or *ARG4* genes (see Materials and Methods; Dennison *et al.*, 2005), and the remaining two copies were disrupted by the Ura-blaster technique (Fonzi and Irwin, 1993). The *TSA1-ARG4* and *TSA1-HIS1* disruption cassettes replaced the entire 197 codon *TSA1* open reading frame, and the ura blaster *tsa1::hisG-URA3-hisG* disruption cassette replaced codons 1-174 of the *TSA1* open reading frame (see Materials and Methods for details).

In addition to the creation of *tsa1*Δ cells, mutants were generated in which one or both of the catalytic cysteine residues were mutated to serine, since serine has a similar side chain size and structure to cysteine. Mutagenesis of *TSA1* to create the peroxidatic cysteine mutant $tsa1^{C48S}$, the resolving cysteine mutant $tsa1^{C198S}$, and the double cysteine mutant $tsa1^{C48,169S}$ was performed by the same overlapping PCR technique described for creating the *trx1* cysteine mutants (see Materials and Methods for details). Mutant PCR products were ligated with the integrating plasmid CIp10 (Murad *et al*., 2000) which was subsequently targeted to the *RPS10* locus in *tsa1*Δ cells to generate strains $tsa1^{C48S}$ (JC1029), $tsa1^{C169S}$ (JC1030) and $tsa1^{C48,169S}$ (JC1031). A similar strategy was used to generate the reintegrant strain (*tsa1*Δ + *TSA1*, JC1028) in which the wild-type gene was integrated at the *RPS10* locus (see Materials and Methods for details). As shown in Figure 4.5 B-D, the generation of strains expressing mutant versions of Tsa1 was confirmed by DNA sequencing. To confirm that Tsa1 protein levels were similar in strains expressing the reconstituted wild-type and mutant *TSA1* alleles, protein extracts were prepared and analysed by SDS-PAGE and western blotting using the anti-Prdx3 polyclonal antibody (Oláhová *et al.*, 2008). A band corresponding to the molecular weight of Tsa1 was present in extracts from wild-type cells and strains expressing the reconstituted *TSA1* and mutant cysteine alleles, but absent in extracts from *tsa1*Δ cells (Figure 4.5E). Although the levels of wild-type Tsa1, and the Tsa1 cysteine mutants, expressed from the *RPS10* locus appears similar, the overall levels of Tsa1 in wild-type cells is greater (Figure 4.5E). This is likely related to the fact that wild-type cells contain 4 copies of *TSA1* in its genome while the reintegrant strains have only one.

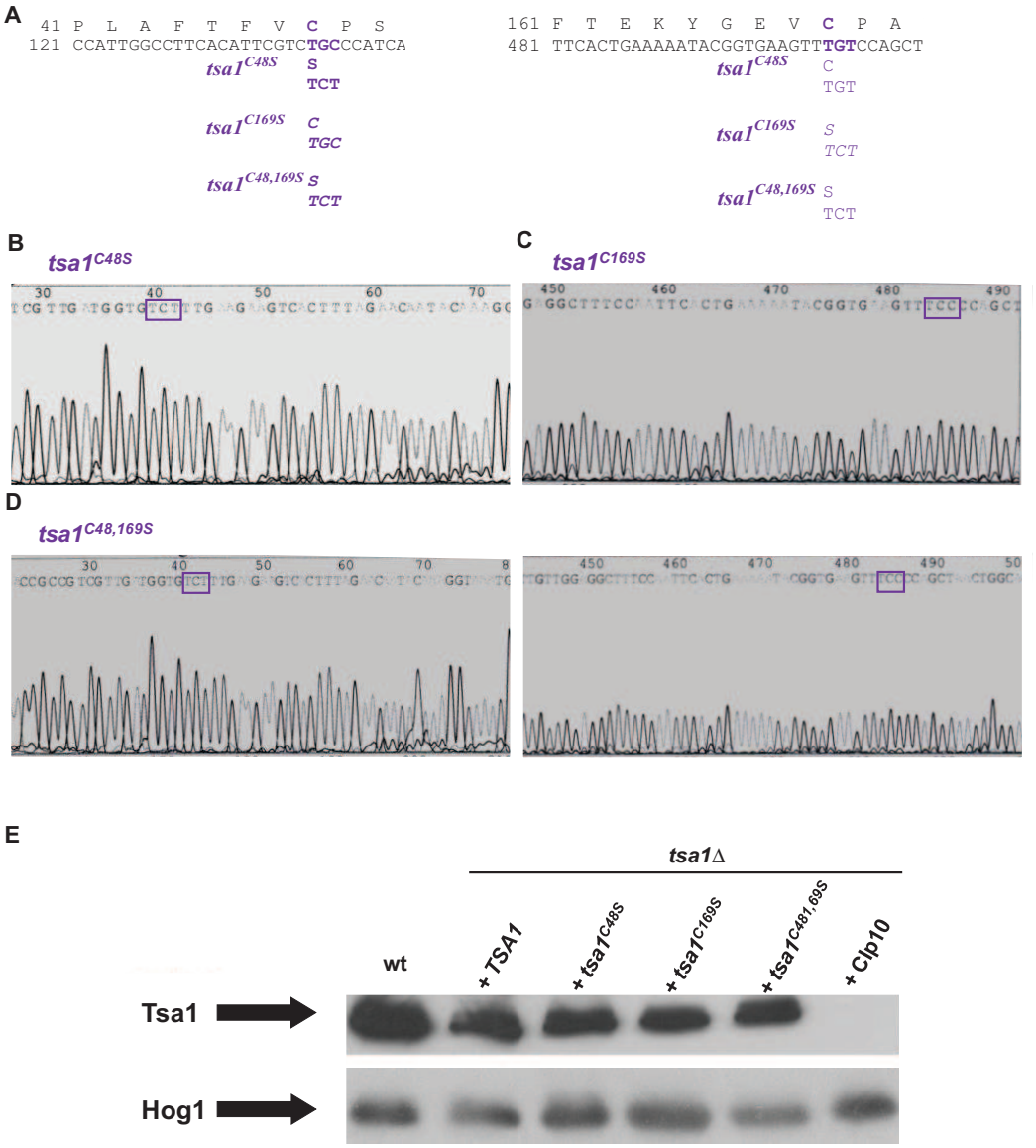


Figure 4.5. DNA sequence analysis and protein level determination of *C. albicans* Tsa1 cysteine mutants.

(A) DNA sequence of the region surrounding the catalytic cysteine residues of *C. albicans* Tsa1 and the engineered mutations. (B-D) DNA sequences of the region surrounding the catalytic cysteine residues from genomic DNA extracted from *tsa1*Δ cells expressing (B) tsa^{C48S} (JC1029), (C) $tsa1^{C169S}$ (JC1030) and (D) $tsa1^{C48,169S}$ (JC1031). The mutated codons are highlighted with purple boxes. (E) Western blot analysis of whole cell extracts from wild-type (JC747), *tsa1*Δ (JC1027) and *tsa1*Δ cells expressing the full-length *TSA1* (JC1028) or the cysteine mutants $tsa1^{C48S}$ (JC1029), $tsa1^{C169S}$ (JC1030), $tsa1^{C48,169S}$ (JC1031), from the *RPS10* locus. Blots were probed with an anti-prdx3 antibody to detect Tsa1 levels, and an anti-Hog1 antibody to control for loading.

4.3.2.2. tsa1Δ cells are sensitive to oxidative stress

Deletion of *TSA1* in *C. albicans* has previously been reported to result in cells that are sensitive to H_2O_2 (Shin *et al.*, 2005 and Urban *et al.*, 2005). In order to confirm this, and test whether the peroxide sensitivity of *C. albicans tsa1*Δ cells is related to its peroxidase activity, cells lacking *TSA1* or expressing the *tsa1* cysteine mutants were spotted onto solid YPD media containing different H_2O_2 concentrations. Consistent with previous reports (Shin *et al.*, 2005 and Urban *et al.*, 2005), deletion of *TSA1* resulted in cells that were more sensitive to H_2O_2 than wild-type cells, and reintegration of *TSA1* rescued this phenotype (Figure 4.6A). Similarly, cells expressing the *tsa1* cysteine mutants displayed increased sensitivity to H_2O_2 compared to the wild-type and reconstituted strains. Strikingly however, cells lacking *TSA1* were reproducibly more sensitive to H_2O_2 than cells expressing the cysteine mutants of Tsa1 (Figure 4.6A). Furthermore, cells lacking *TSA1* were also more sensitive to H_2O_2 than cells lacking the major thioredoxin Trx1 (Figure 4.6B), which maintains the activity of Tsa1 (Chapter 3 - Figure 3.7), whereas cells expressing the catalytic cysteine mutants of Tsa1 phenocopied the peroxide sensitivity exhibited by *trxl*Δ cells (Figure 4.6B). Such results are consistent with the peroxide sensitivity displayed by *trx1*Δ cells being related to its role in regulating the redox status of the catalytic cysteine residues of Tsa1. However, such results also indicate that Tsa1 is required for oxidative stress resistance in *C. albicans* by mechanisms independent of its peroxidase activity.

4.2.3.3. Both Tsa1 and Trx1 are required for the H_2O_2-induced activation of the Hog1 SAPK in C. albicans

In *S. pombe,* Tpx1 regulates the H_2O_2-induced activation of the Sty1 SAPK in a mechanism that involves the formation of a peroxide induced disulphide between Sty1 and Tpx1 (Veal *et al.*, 2004). Ectopic expression of *C. albicans* Tsa1 rescues the defect in peroxide-induced phosphorylation of Sty1 in *S. pombe tpx1*$^-$ cells (Figure 4.4). This, together with previous work illustrating that the *C. albicans* Hog1 SAPK is robustly phosphorylated in response to H_2O_2 (Alonso-Monge *et al.*, 2003 and Smith *et al.*, 2004), prompted an investigation into the role of Tsa1 in regulating peroxide-induced Hog1 activation. In order to examine Hog1 phosphorylation, whole cell extracts were prepared from cells before and after treatment with H_2O_2 and analysed by SDS-PAGE and western blotting. Hog1 phosphorylation was detected using an anti-phospho-p38

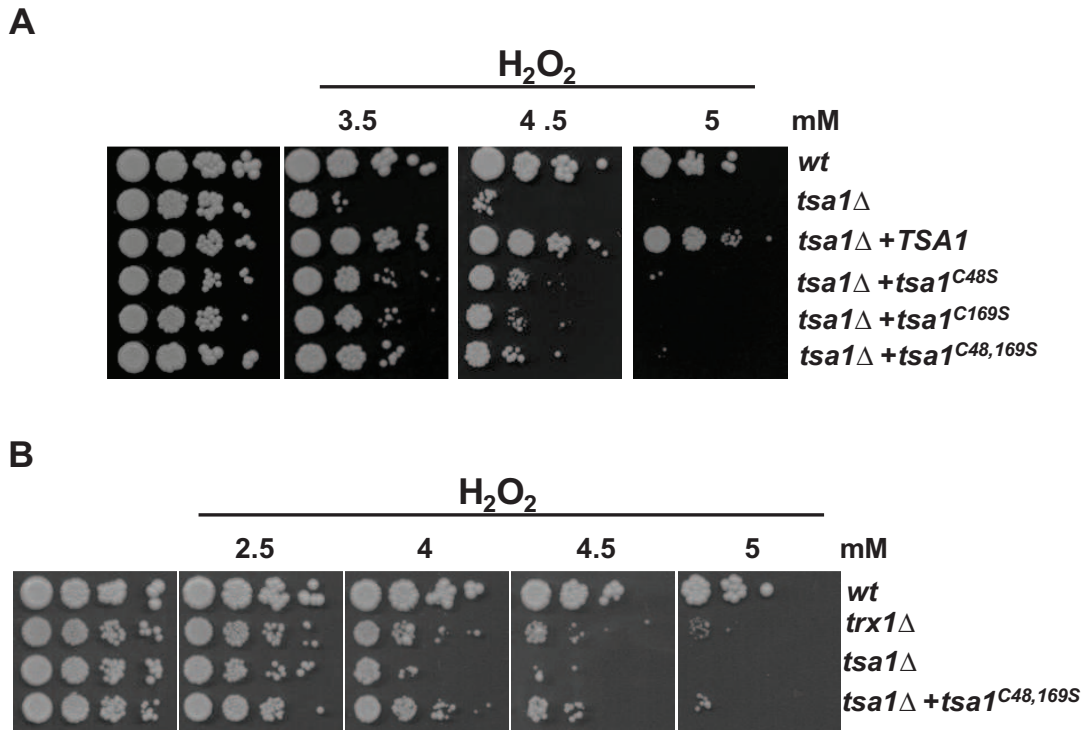


Figure 4.6. Tsa1 is important for oxidative stress resistance in *C. albicans*.
(A) *C. albicans* wild-type (JC747), *tsa1*Δ + CIp10 (JC1027), *tsa1*Δ + CIp10-*TSA1* (JC1028), *tsa1*Δ + CIp10-$tsa1^{C48S}$ (JC1029), *tsa1*Δ + CIp10-$tsa1^{C169S}$ (JC1030) and *tsa1*Δ + CIp10- $tsa1^{C48,169S}$ (JC1031) were grown to mid-log phase and 10-fold serial dilutions plated onto agar plates containing the indicated concentrations of H_2O_2. (B) *C. albicans* wild-type (JC747), *trx1*Δ + CIp10 (JC677), *tsa1*Δ + CIp10 (JC1027) and *tsa1*Δ + CIp10- $tsa1^{C48,169S}$ (JC1031) were grown to mid-log phase and then spotted on agar plates containing the indicated concentrations of H_2O_2 as above. Plates were then incubated at 30ºC for 2 days.

antibody that only recognises the phosphorylated form of Hog1 (Smith *et al.*, 2004), following which blots were stripped and probed with an anti-Hog1 antibody to determine total Hog1 levels. As reported in previous studies, exposure of wild-type cells to H_2O_2 resulted in a rapid and sustained phosphorylation of Hog1 (Figure 4.7A). However, H_2O_2-induced Hog1 phosphorylation was significantly reduced in cells lacking *TSA1* and, importantly, this defect could be rescued upon reintegration of the wild-type copy of the gene (Figure 4.7A).

Although there are no reports of thioredoxin regulating H_2O_2-induced activation of the Sty1 SAPK in *S. pombe*, studies in mammalian cells reported thioredoxin as a negative regulator of the analogous p38/JNK SAPK pathways (Saitoh *et al.*, 1998, Nadeau *et al.*, 2007, Nadeau *et al.*, 2009). Furthermore, our data illustrating that Tsa1 is essential for wild-type levels of Hog1 activation, together with data presented in Chapter 3 (Figure 3.7) illustrating that the thioredoxin Trx1 is required for the activity of Tsa1, prompted an investigation into the role of Trx1 in peroxide signalling to Hog1. Similar to that seen in the *C. albicans tsa1*Δ strain, *trx1*Δ cells treated with H_2O_2 exhibited significantly reduced Hog1 phosphorylation compared with wild-type cells (Figure 4.7B). Furthermore, this defect in Hog1 phosphorylation was rescued upon reintegration of wild type *TRX1* (Figure 4.7B). Hence, both Trx1 and its substrate Tsa1 are important for the relay of oxidative stress signals to the Hog1 SAPK module in *C. albicans*.

Hog1 is phosphorylated in response to a range of stresses (Smith *et al.*, 2004). To determine whether the decreased activation of Hog1 seen in *tsa1*Δ and *trx1*Δ cells was specific to peroxide stress, Hog1 activation was assessed concurrently in wild-type, *tsa1*Δ and *trx1*Δ cells in response to oxidative and osmotic stress. As seen in Figure 4.8, inactivation of either *TSA1* or *TRX1* results in a similar decrease in H_2O_2-induced Hog1 phosphorylation, whereas wild-type levels of Hog1 phosphorylation are seen in *tsa1*Δ and *trx1*Δ strains in response to osmotic stress. This result indicates that the effects of Tsa1 and Trx1 in regulating Hog1 activation are specific to peroxide stress.

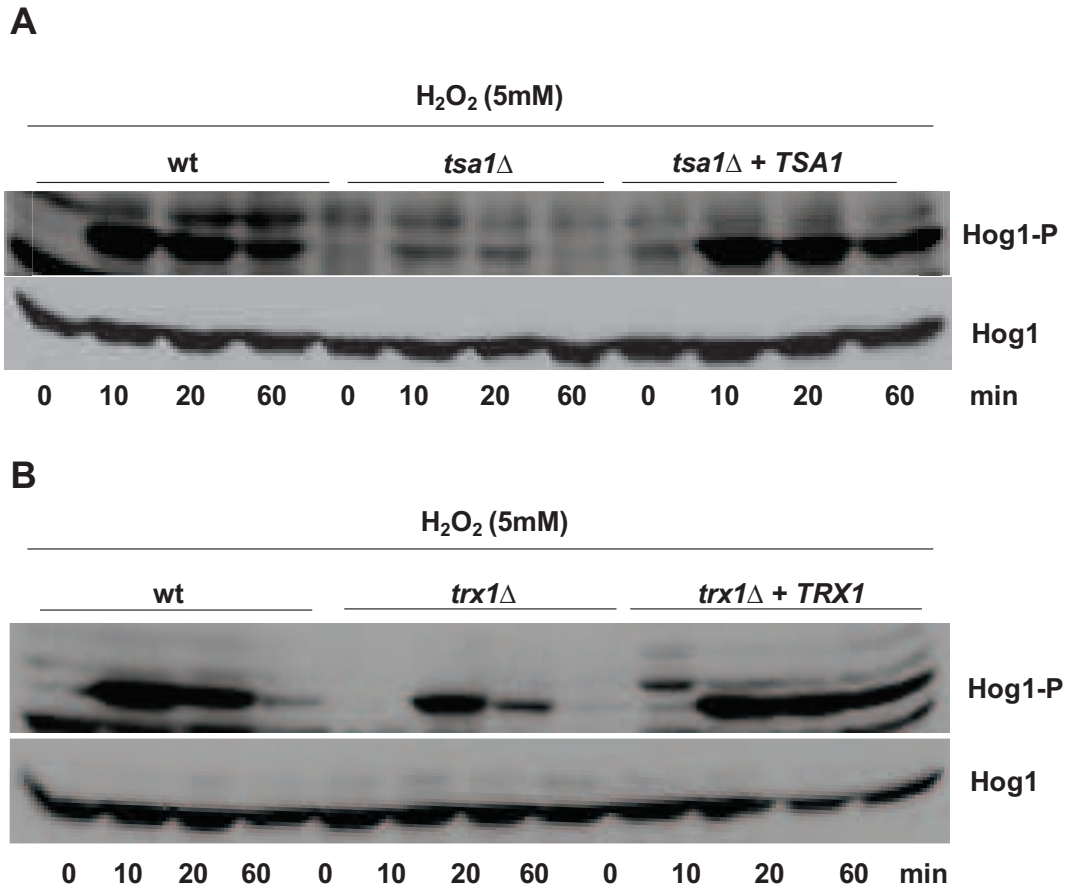


Figure 4.7. Trx1 and Tsa1 are required for H_2O_2-induced activation of the Hog1 SAPK pathway.

The levels of Hog1 phosphorylation (Hog1-P) were determined by western blot analysis of lysates prepared from (A) wild-type (JC747), *tsa1*Δ + CIp10 (JC1027), *tsa1*Δ + CIp10-*TSA1* (JC1028) cells or (B) wild-type (JC747), *trx1*Δ + CIp10 (JC677) and *trx1*Δ + CIp10-*TRX1* (JC679) cells following treatment with 5 mM H_2O_2 for the indicated times. Western blots were probed with an anti-phospho-p38 antibody, to detect the phosphorylated, active form of Hog1 (Hog1-P). Total levels of Hog1 protein were subsequently determined by stripping and reprobing the blot with an anti-Hog1 antibody (Hog1).

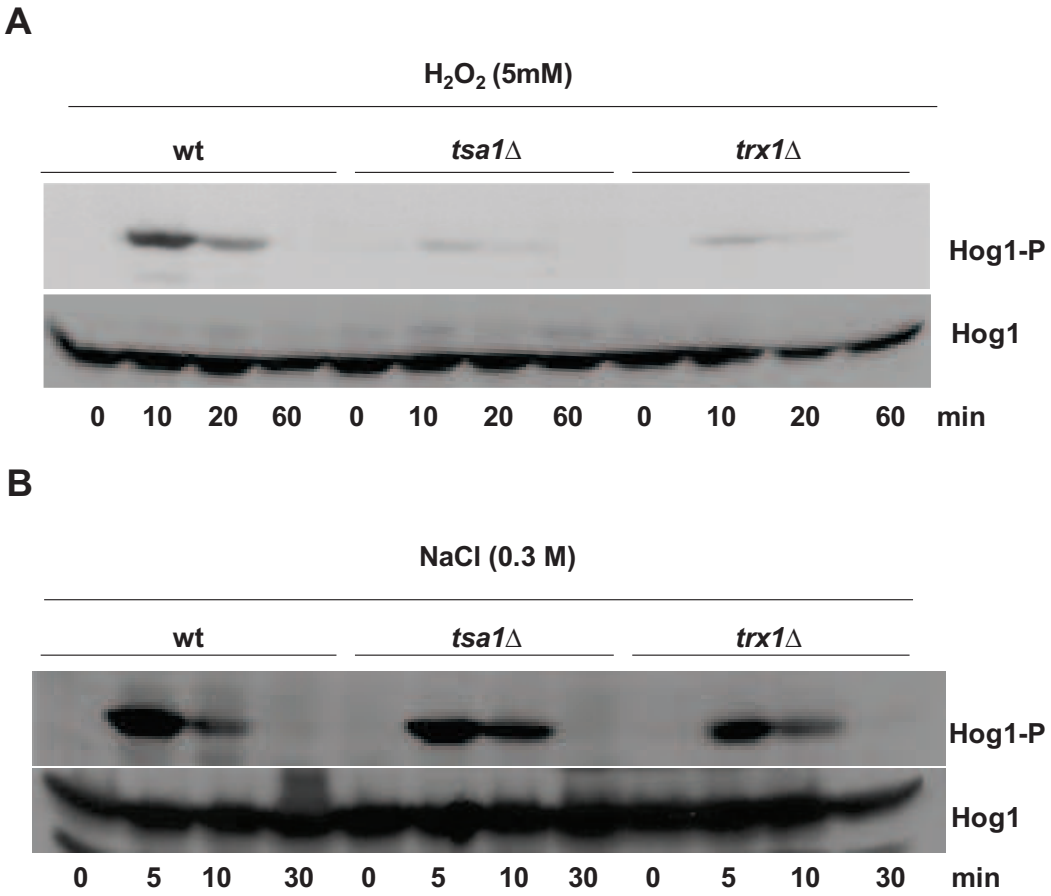


Figure 4.8. Trx1 and Tsa1 are dispensable for osmotic stress-induced activation of the Hog1 SAPK pathway.

The levels of Hog1 phosphorylation (Hog1-P) were determined by western blot analyses of lysates prepared from wild-type (JC747), *tsa1*Δ + CIp10 (JC1027) and *trx1*Δ + CIp10 (JC677) cells treated with 5 mM H_2O_2 (A) or 0.3 M NaCl (B) for the indicated times. Western blots were probed with an anti-phospho-p38 antibody, to detect the phosphorylated, active form of Hog1 (Hog1-P). Total levels of Hog1 protein were subsequently determined by stripping and reprobing the blot with an anti-Hog1 antibody (Hog1).

4.2.3.4. The catalytic activity of Tsa1 is dispensable for H_2O_2-induced Hog1 activation

As both Tsa1 and Trx1 are required for the peroxide-induced activation of Hog1 in *C. albicans* (Figures 4.7 and 4.8), this suggested a mechanism whereby Trx1 was required to maintain the reduced active form of Tsa1 to allow Hog1 activation. Indeed, consistent with such a model, the peroxidatic cysteine of Tpx1 in *S. pombe* is required for peroxide-induced activation of Sty1 (Veal *et al.*, 2004). In order to determine whether this mechanism of 2-Cys peroxiredoxin mediated SAPK activation is conserved in *C. albicans*, H_2O_2-induced Hog1 phosphorylation was examined in *tsa1*Δ strains expressing the *tsa1* peroxidatic (*tsa1*^{C48S}) and resolving (*tsa1*^{C169S}) mutations. As previously seen, Hog1 phosphorylation was reduced in *tsa1*Δ cells compared to wild-type cells (Figure 4.9). However, mutation of either the peroxidatic or resolving cysteines of Tsa1 resulted in significant levels of peroxide-induced Hog1 phosphorylation (Figure 4.9). This result indicates that Tsa1 does not need to be catalytically active to stimulate H_2O_2-induced Hog1 activation, and that the mechanism by which 2-Cys peroxiredoxins regulate SAPK activation in response to oxidative stress is not conserved between *S. pombe* and *C. albicans.*

4.2.3.6. The catalytic activity of Trx1 is essential for H_2O_2-induced Hog1 activation

In order to investigate whether the catalytic activity of Trx1 is important for H_2O_2-induced Hog1 activation, Hog1 phosphorylation was examined in cells expressing the catalytic cysteine mutant versions of Trx1 (Chapter 3, section 3.2.2) post H_2O_2 treatment. As previously shown, deletion of *TRX1* resulted in a significant decrease in the levels of peroxide-induced Hog1 phosphorylation. However, in contrast to that seen with the Tsa1 cysteine mutants, significantly lower levels of phosphorylated Hog1 were seen in cells expressing Trx1 cysteine mutants compared to that seen in wild-type cells and the reconstituted strain (Figure 4.10). Collectively, these results indicate that whilst the catalytic cysteine residues of Tsa1 are dispensable for Hog1 activation, the catalytic activity of Trx1 is essential for the effective relay of oxidative stress signals to Hog1.

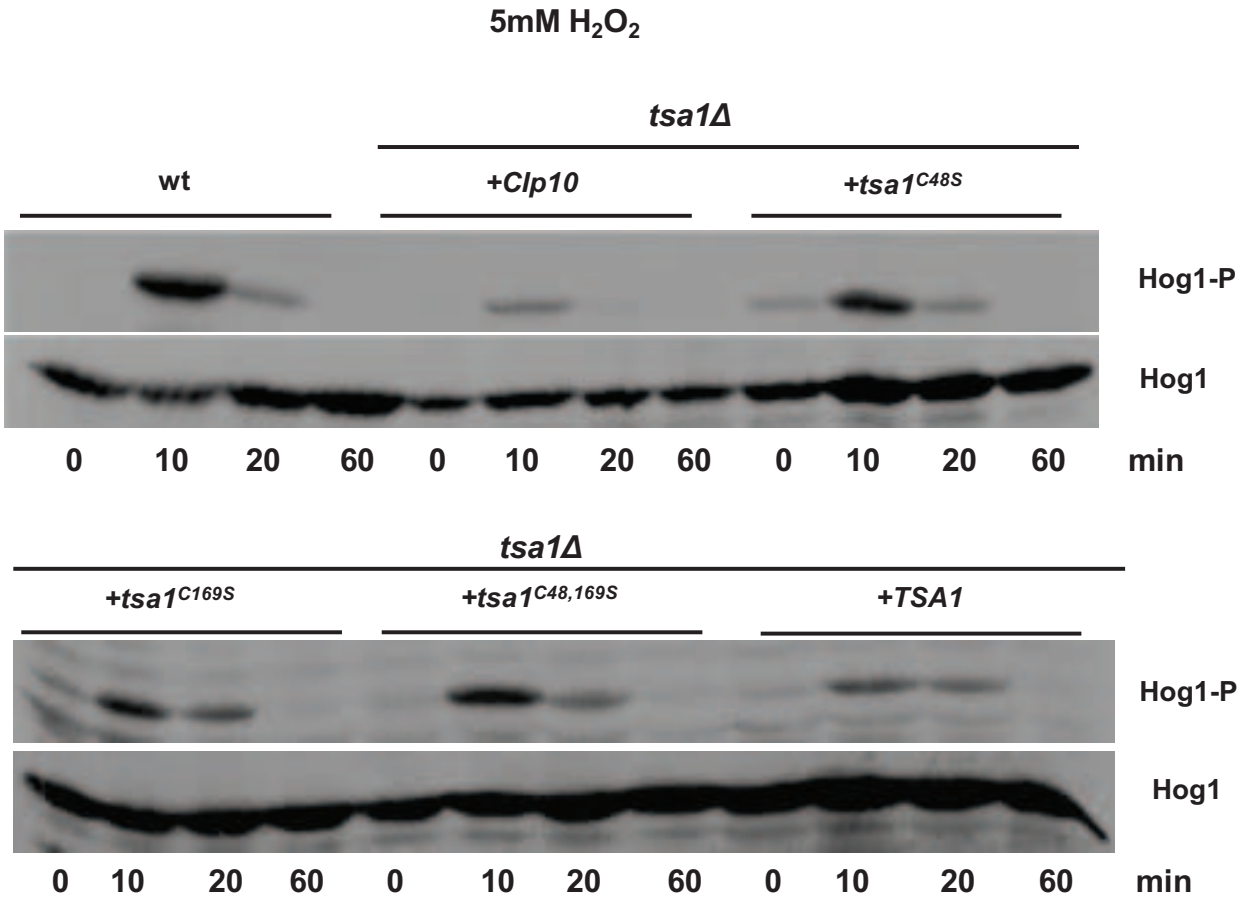


Figure 4.9. The catalytic cysteine residues of Tsa1 are dispensable for H_2O_2-induced Hog1 activation.

Hog1 phosphorylation (Hog1-P) was determined by western blot analyses of lysates prepared from wild-type (JC747), *tsa1*Δ + CIp10 (JC1027), *tsa1*Δ + CIp10-*TSA1* (JC1028) cells, and *tsa1*Δ cells expressing $tsa1^{C48S}$ (JC1029), $tsa1^{C169S}$ (JC1030) and $tsa1^{C48,169S}$ (JC1031) after treatment with 5 mM H_2O_2 for the indicated times. Western blots were probed with an anti-phospho-p38 antibody, to detect the phosphorylated, active form of Hog1 (Hog1-P). Total levels of Hog1 protein were subsequently determined by stripping and reprobing the blot with an anti-Hog1 antibody (Hog1). Total Hog1 levels are indicated.

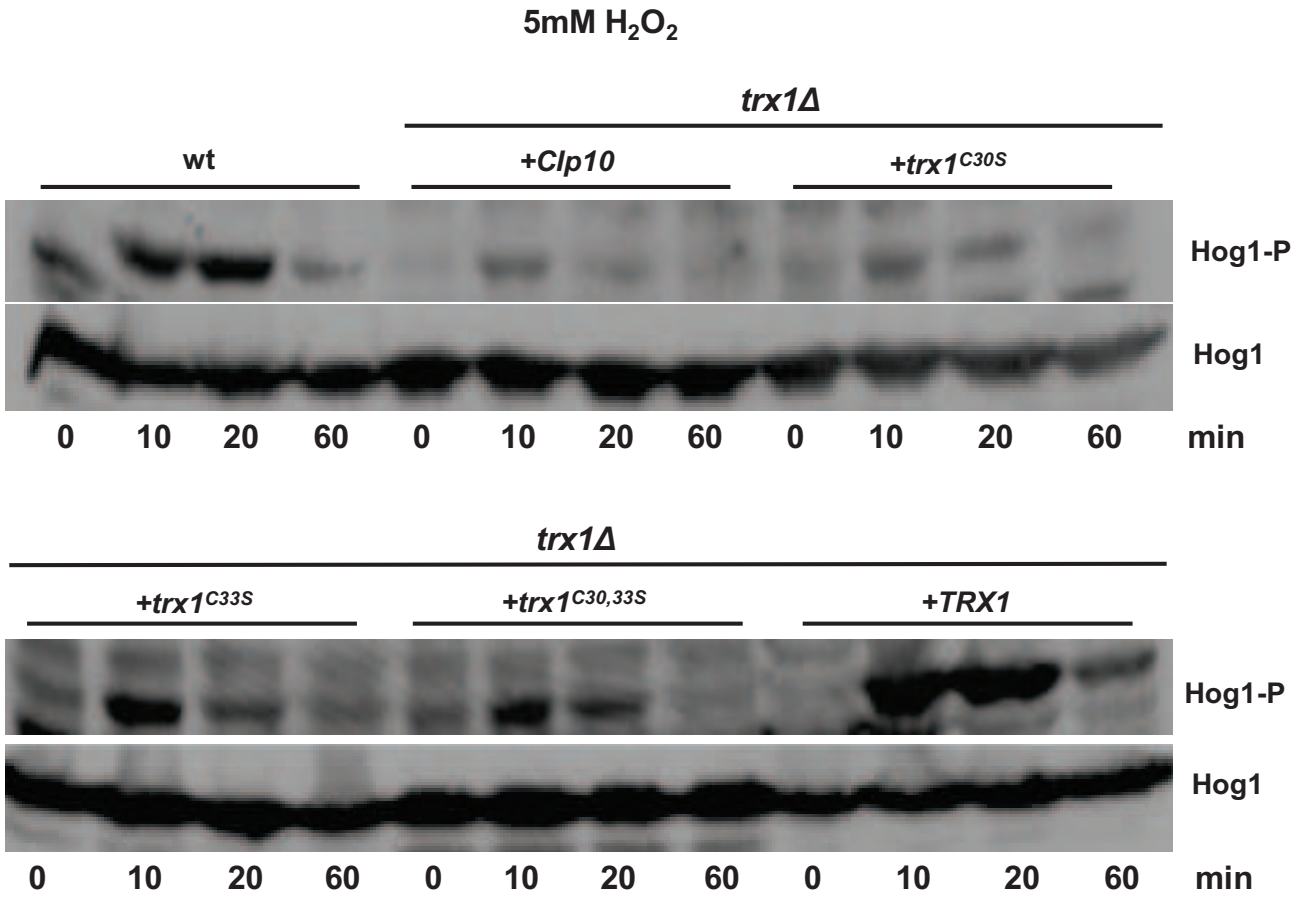


Figure 4.10. The catalytic cysteine residues of Trx1 are required for H_2O_2-induced Hog1 activation. Hog1 phosphorylation (Hog1-P) was determined by western blot analysis of lysates prepared from wild-type (JC747), *trx1*Δ + CIp10 (JC677), *trx1*Δ + CIp10-*TRX1* (JC679), *trx1*Δ + CIp10-*trx1*^{C30S} (JC759), *trx1*Δ + CIp10-*trx1*^{C33S} (JC761) and *trx1*Δ + CIp10-*trx1*^{C30,33S} (JC763) cells after treatment with 5 mM H_2O_2 for the times indicated. Western blots were probed with an anti-phospho-p38 antibody, to detect the phosphorylated, active form of Hog1 (Hog1-P). Total levels of Hog1 protein were subsequently determined by stripping and reprobing the blot with an anti-Hog1 antibody (Hog1).

4.2.3.7. Tsa1 is trapped as a dimer in cells expressing Trx1 cysteine mutants

As the catalytic activity of Trx1, but not Tsa1, is needed for peroxide-induced Hog1 activation it is possible that these antioxidants work in separate pathways to relay stress signals to the SAPK module. However, as shown previously (Figure 3.7 –Chapter 3) Tsa1 is trapped in an oxidised dimeric form in *trx1*Δ cells. Hence, an alternative explanation is that dimeric Tsa1 represses Hog1 activation, or that Tsa1 needs to be in the monomeric form to activate the SAPK. To further investigate this possibility, Tsa1 dimer formation was assessed in wild-type cells as previously described in topic 3.2.6 (Chapter 3) following exposure to a range of H_2O_2 concentrations. Interestingly, exposure of cells to H_2O_2 concentrations that induce dimer formation (0.25-1 mM H_2O_2) does not result in activation of Hog1 (Figure 4.11A); whilst exposure to higher H_2O_2 concentrations (2.5-10 mM) results in monomeric oxidized Tsa1 and peroxide-induced Hog1 activation (Figure 4.11A). This result is consistent with the model in which dimeric Tsa1 could be inhibiting Hog1 activation, or that monomeric Tsa1 is the active form. As peroxide-induced activation of Hog1 is decreased in cells expressing *trx1* cysteine mutants (Figure 4.10), the presence of Tsa1 dimers was also analyzed in these cells. Similar to that seen upon deletion of *TRX1*, expression of the *trx1* cysteine mutants resulted in Tsa1 being trapped in the oxidised dimeric form under both non-stress and oxidative stress conditions (Figure 4.12B). Furthermore, in cells expressing $trx1^{C33S}$ in which the resolving cysteine is mutated, a band with a molecular mass corresponding to a Tsa1-Trx1 heterodimer is seen (Figure 4.12B). Finally, as the catalytic cysteine residues of Tsa1 are dispensable for wild-type levels of Hog1 activation, the presence of Tsa1-dimer was also analysed in these cells. As predicted, mutation of the catalytic cysteines of Tsa1 did not stimulate dimer formation, in contrast to that seen in the *trx1* cysteine mutants (Figure 4.12B). Collectively, these results are consistent with a model that either the dimeric form of Tsa1 prevents Hog1 activation in response to peroxide stress, or that the monomeric form is needed for Hog1 activation.

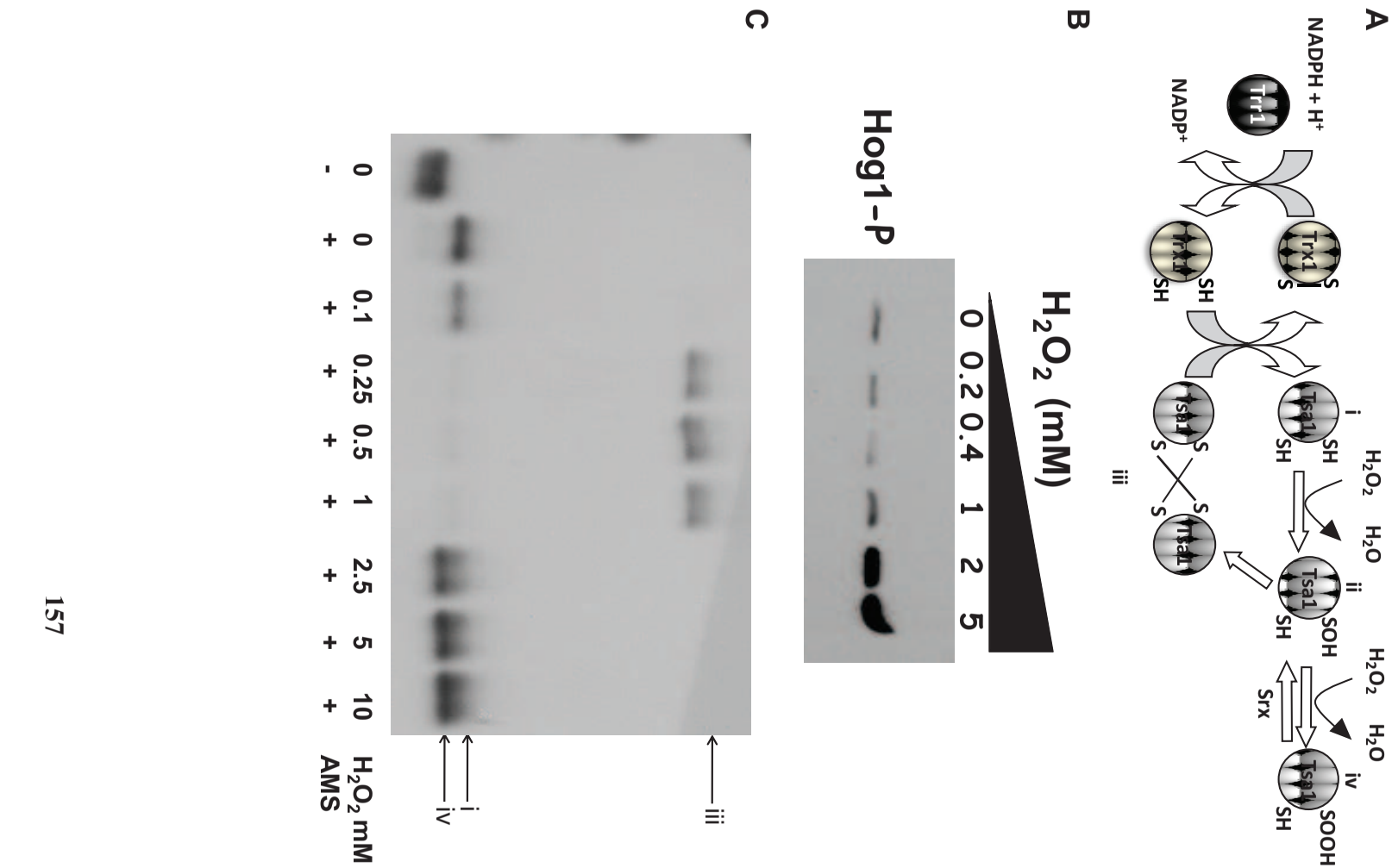


Figure 4.11. Analysis of Hog1 activation and Tsa1 oxidation state in response to increasing levels of H_2O_2.

(A) H_2O_2 detoxification by the Trx1/Tsa1 system. (B) Hog1 is only activated following exposure to high levels of H_2O_2. Figure taken from Smith *et al.*, 2004. Hog1 phosphorylation (Hog1-P) was determined by western blot analysis of lysates prepared from wild-type (JC4) cells after treatment with increasing H_2O_2 concentrations for 10 minutes. Western blots were probed with an anti-phospho-p38 antibody, to detect the phosphorylated, active form of Hog1 (Hog1-P). (C) Dimeric Tsa1 is only formed following exposure to low levels of H_2O_2. The oxidation status of Tsa1 was determined by non-reducing SDS-PAGE and western-blotting of AMS-modified proteins prepared from wild-type cells (JC747) exposed to a range of H_2O_2 concentrations for 10 min. Under non-stressed conditions, Tsa1 was largely present as a reduced form (i). Treatment with lower H_2O_2 concentrations (0.25-1 mM) induced oxidation of Tsa1 and dimer formation (iii), while treatment with higher H_2O_2 concentrations (2.5-10 mM) induced formation of the overoxidized monomeric form (iv).

Figure 4.12. Tsa1 oxidation state in cells expressing *trx1* and *tsa1* cysteine mutants.
(A) H_2O_2 detoxification by the Trx1/Tsa1 system. Western blot analysis of whole cell extracts from *C. albicans* strains before (-) and after (+) peroxide stress. (B) wild-type (JC747), *trx1*Δ + CIp10 (JC677), *trx1*Δ + CIp10-*TRX1* (JC679), *trx1*Δ + CIp10-*trx1*^{C30S} (JC759), *trx1*Δ + CIp10-*trx1*^{C33S} (JC761) and *trx1*Δ + CIp10-*trx1*^{C30,33S} (JC763) (C) wild-type (JC747), *tsa1*Δ + CIp10 (JC1027), *tsa1*Δ + CIp10-*TSA1* (JC1028) cells, and *tsa1*Δ cells expressing *tsa1*^{C48S} (JC1029), *tsa1*^{C169S} (JC1030) and *tsa1*^{C48,169S} (JC1031). The oxidation status of Tsa1 was determined by non-reducing SDS-PAGE and western-blotting of proteins prepared from strains exposed to 5mM H_2O_2 for 10 min. Tsa1 can be visualized in its monomeric reduced form (i), as a monomeric overoxidized form (iv) or as a dimer (iii).

4.2.4. Creation and phenotypic analysis of C. albicans strains lacking SRX1

To further explore the possibility that the formation of the overoxidized monomeric form of Tsa1, generated at higher levels of H_2O_2, is the key signalling event to promote peroxide-induced Hog1 activation (Figure 4.10), *C. albicans* strains lacking the sulfiredoxin Srx1 were generated. In the model yeasts, *S. cerevisiae* and *S. pombe*, the sulfiredoxin, Srx1, has been shown to reduce overoxidized Tsa1/Tpx1 (Biteau *et al.*, 2003 and Bozonet *et al.*, 2005).

4.2.4.1. Construction of srx1Δ cells

A BLAST search of the Candida Genome Database (www.candidagenome.org), against the *S. cerevisiae SRX1* and *S. pombe* $srx1^+$ sequences, revealed a single open reading frame (*orf19.3537*) with significant homology of these proteins (Figure 4.13). *C. albicans* Srx1 displays 53% similarity to *S. pombe* Srx1 and 63% similarity to *S. cerevisiae* Srx1, including the cysteine residue involved in the reduction of 2-Cys peroxiredoxins and adjacent amino acids (Figure 4.13 – Biteau *et al.*, 2003). In order to generate the *srx1*Δ strain (JC499), the two copies of *SRX1* were deleted by replacing the *SRX1* ORF with either the *HIS1* or *ARG4* genes (see Materials and Methods; Dennison *et al.*, 2005). The *SRX1-ARG4* and *SRX1-HIS1* disruption cassettes replaced the entire 399bp open reading frame of *SRX1* (see Materials and Methods for details).

4.2.4.2. srx1Δ cells are sensitive to peroxide stress

To investigate whether *C. albicans* Srx1 is required for wild-type levels of peroxide resistance, *srx1*Δ cells were spotted onto solid YPD media containing different H_2O_2 concentrations. Consistent with previous reports where deletion of *S. cerevisiae SRX1* (Biteau *et al.*, 2003) and $srx1^-$ in *S. pombe* (Bozonet *et al.*, 2005) results in cells that exhibit peroxide sensitivity, *C. albicans srx1*Δ cells were more sensitive to H_2O_2 than wild-type cells, and reintegration of *SRX1* into the *srx1*Δ mutant rescued this phenotype (Figure 4.14). To investigate whether the peroxide sensitivity of *srx1*Δ cells may be related to the role of Srx1 in maintaining the peroxidase activity of Tsa1, the relative peroxide sensitivities of *srx1*Δ, *tsa1*Δ and the $tsa1^{C48,169S}$ cysteine mutant were compared. Previously, it was shown that the *tsa1*Δ null mutant was more sensitive to peroxide stress than strains expressing the catalytic cysteine mutants (Figure 4.6). As illustrated in Figure 4.14, *C. albicans srx1*Δ cells are more resistant to H_2O_2 than *tsa1*Δcells, but show similar sensitivity to cells expressing

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CaSrx1          -MSMYT-SRLATEYVPLSEIKRPIPPVLDYQKIDAMLSTLKG-VPMESATCKVED----I 53
ScSrx1          -MSLQS-NSVKPTEIPLSEIRRPLAPVLDPQKIDAMVATMKG-IPTASKTCSLEQAEAAA 57
SpSrx1          MTSIHTGSNNNIVELDMSELIRPIPPVLDMNKVNSMMETMTGKTPPASCGLTSED----L 56
                  *: : .      : :**: **:.**** :*:::*: *:.*  *  *   . *:

CaSrx1          TAGELPPIDVFKIRENGKNFYFAFGGCHRFQAYDRISKETEKEVM-VKSRILPATRKSLR 112
ScSrx1          SAGELPPVDVLGVRVKGQTLYYAFGGCHRLQAYDRRARETQNAAFPVRCRVLPATPRQIR 117
SpSrx1          EAGELPPVDVLTFKKSGKPYYFAFGGCHRLRAHDEAGRKK------VRCKLVNCSPNTLR 110
                 ******:**: .: .*:  *:*******::*:*. .::.      *:.::: .: . :*

CaSrx1          IYLGASVDALFDSVDSESSS 132
ScSrx1          MYLGSSLDIE---------- 127
SpSrx1          LYLGASANKFLDSD------ 124
                :***:* :
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Figure 4.13. Sequence alignment of Srx1 from *C. albicans*, *S. cerevisiae* and *S. pombe*.
The amino acid sequences of *C. albicans* Srx1, *S. cerevisiae* Srx1, and *S. pombe* Srx1 were aligned using ClustalW (Thompson *et al.*, 1994). Dashes indicate single-residue gaps introduced to maximise the alignment. * indicates identical residues, : indicates conserved substitutions, and . indicates semi-conserved substitutions. The catalytic cysteine involved in the reduction of 2-Cys peroxiredoxins (Biteau *et al.*, 2003) is underlined, and the conserved residues adjacent to the catalytic cysteine are shown in bold.

the *tsa1* cysteine mutant (*tsa1*^{C48,169S}) (Figure 4.14). This is consistent with the peroxide sensitivity seen in *srx1*Δ cells being attributed to a decrease in the peroxidase activity of Tsa1.

4.2.4.3. Tsa1 oxidation is prolonged in srx1Δ cells

Deletion of *SRX1* in the model yeasts results in prolonged oxidation of the 2-Cys peroxiredoxins Tsa1 (Biteau *et al.*, 2003) and Tpx1 (Bozonet *et al.*, 2005), when cells are treated with H_2O_2. In order to establish whether *C. albicans* Srx1 acts as an antioxidant by reducing overoxidized Tsa1, the redox state of *C. albicans* Tsa1 in wild-type and *srx1*Δ cells was compared. Proteins were extracted as previously described in chapter 3 (topic 3.2.6) and analysed by reducing SDS-PAGE and western blotting using the anti-Prdx3 antibody. As expected, in unstressed wild-type cells, Tsa1 was mainly found in reduced form (Figure 4.15), which can bind 2 AMS molecules. Following H_2O_2 treatment, the reduced monomeric form of Tsa1 was initially converted to the overoxidised sulphinic monomeric form (SOOH), in which only one AMS molecule binds(Figure 4.15 - 10 min time point). Subsequently, 30 minutes after H_2O_2 treatment, Tsa1 can be found in both the overoxidised sulphinic form (SOOH), and the oxidised sulphenic form (SOH) which is competent to form a disulphide bond on the resolving cysteine on a partner protein, and thus can no longer bind to AMS (Figure 4.15). 60 minutes post peroxide treatment, Tsa1 is mainly found in the reduced form and in the AMS-resistant sulphenic form. In contrast, in *srx1*Δ cells Tsa1 was trapped in the overoxidized sulphinic form following H_2O_2 treatment (Figure 4.15). This result suggests that similar to that reported in the model yeasts (Biteau *et al.*, 2003 and Bozonet *et al.*, 2005), *C. albicans SRX1* reduces the sulphinic form of Tsa1.

4.2.3.7. Deletion of SRX1 results in prolonged H_2O_2-induced Hog1 activation

As Tsa1 is trapped in the overoxidised sulphinic form in *srx1*Δ cells, Hog1 activation in response to peroxide stress was assessed in this mutant. As illustrated in Figure 4.16, Hog1 activation in wild-type cells in response to H_2O_2 was transient peaking at 10 minutes. In contrast, deletion of *SRX1* resulted in significantly prolonged Hog1 activation in that substantial Hog1 phosphorylation was apparent 60 minutes post H_2O_2-treatment (Figure 4.16). Reconstitution of *SRX1* into *srx1*Δ cells partially reversed the prolonged kinetics of Hog1 activation in the *srx1*Δ mutant. This result is consistent with the hypothesis that the

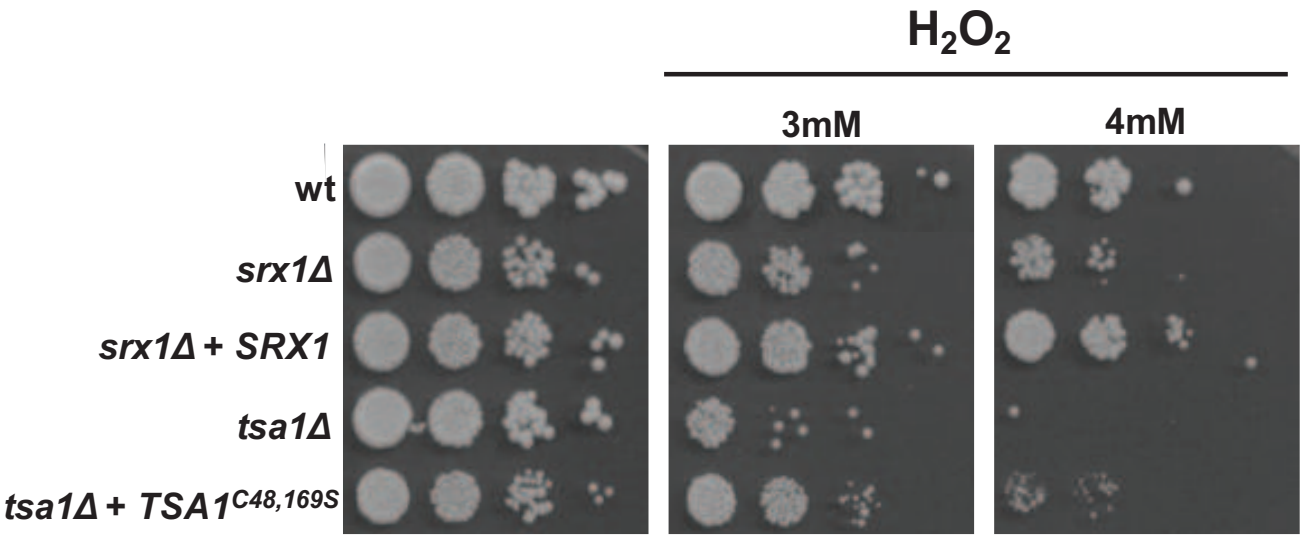

Figure 4.14. Deletion of *SRX1* results in peroxide stress sensitivity in *C. albicans*.
C. albicans wild-type (JC747), *srx1*Δ + CIp10 (JC719), *srx1*Δ + CIp10-*SRX1* (JC721), *tsa1*Δ + CIp10 (JC1027), and *tsa1*Δ + CIp10-*TSA1*^{C48,169S} (JC1031) cells were grown to mid-log phase and then 10-fold serial dilutions plated onto agar plates containing the indicated concentrations of H_2O_2. Plates were then incubated at 30ºC for 2 days.

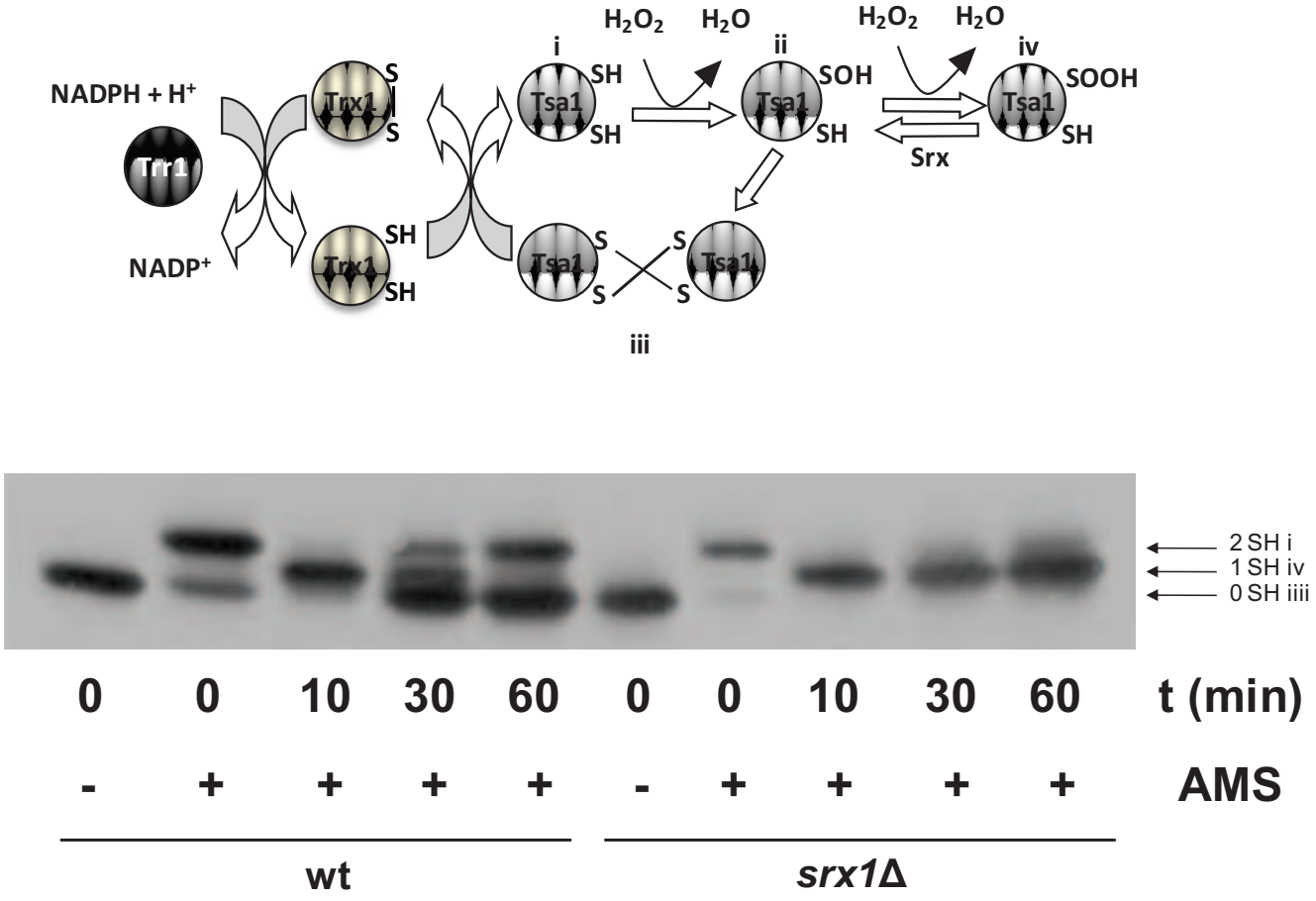

Figure 4.15. Deletion of *SRX1* results in prolonged over-oxidation of Tsa1 in cells treated with H_2O_2.

Western blot analysis of whole cell extracts from *C. albicans* strains wild-type + CIp30 (JC747) and *srx1*Δ + CIp10 (JC719). The oxidation status of Tsa1 was determined by reducing SDS-PAGE and western-blotting of AMS-modified proteins prepared from wt and *srx1*Δ strains exposed to 5 mM H_2O_2 for 60 min. Under non-stressed conditions, Tsa1 was largely present as a reduced form (i). Treatment with H_2O_2 induced oxidation of Tsa1 (ii/iv and iii) in wt cells, as illustrated by faster mobility of Tsa1. In *srx1*Δ cells Tsa1 is mainly trapped in the overoxidised form (iv) following H_2O_2 treatment.

formation of the overoxidized form of Tsa1 is required for Hog1 activation. However, the mechanism underlying the prolonged activation of Hog1 in *srx1*Δ cells may be less direct, as the *srx1*Δ mutant is more sensitive to H_2O_2, and Hog1 activation increases with increasing levels of H_2O_2 (Smith, *et al*., 2004).

4.3. Discussion

The aim of this chapter was to investigate potential roles of the 2-Cys peroxiredoxin Tsa1, and the thioredoxin Trx1, in regulating oxidative stress signalling in *C. albicans*. Significantly, the data presented illustrate that both Tsa1 and Trx1 are required for the H_2O_2-induced activation of the Hog1 SAPK in this fungal pathogen.

2-Cys peroxiredoxins are peroxidases that have two catalytic cysteine residues; an N-terminal peroxidatic cysteine and a C-terminal resolving cysteine (Hall *et al.*, 2009 and Rhee *et al.*, 2005 – Chapter 1, Figure 1.8). During the detoxification of H_2O_2, the peroxidatic cysteine of 2-Cys peroxiredoxins becomes oxidized to a sulphenic acid (-SOH) form which can either be reduced by the thioredoxin system or can be further oxidized to a sulphinic acid (-SOOH) form. This 'over-oxidised' form cannot be reduced by thioredoxin and thus results in an oxidative inactivation of the enzyme (Figure 3.7; reviewed in Veal *et al.*, 2007). Importantly, these two oxidation states are predicted to underlie the peroxide sensing and signalling functions of this group of enzymes (Wood *et al.*, 2003, Veal *et al.*, 2004 and Bozonet *et al.*, 2005). For example, the H_2O_2-concentration dependent overoxidation of the *S. pombe* 2-Cys peroxiredoxin Tpx1, facilitates the molecular switch from Pap1 to Sty1-dependent oxidative stress responses in fission yeast (Bozonet *et al.*, 2005). At low levels of H_2O_2, Tpx1 remains active and the peroxidase activity facilitates the oxidation and activation of the Pap1 transcription factor (Bozonet *et al.*, 2005 and Vivancos *et al.*, 2005). However, upon increasing levels of H_2O_2 Tpx1 is susceptible to oxidative inactivation. Such forms of Tpx1 are unable to stimulate Pap1 oxidation (Bozonet *et al.*, 2005 and Vivancos *et al.*, 2005), yet are competent for promoting activation of the Sty1 SAPK in response to H_2O_2 (Veal *et al.*, 2004). Significantly, sequence analysis of the *C. albicans* 2-Cys peroxiredoxin Tsa1 revealed the presence of the structural elements shown to increase the susceptibility of the eukaryotic 2-Cys peroxiredoxins enzymes to oxidative inactivation (Figure 4.1, Wood *et al.*, 2003). Moreover, data presented in this chapter illustrates that *C. albicans* Tsa1 can form the

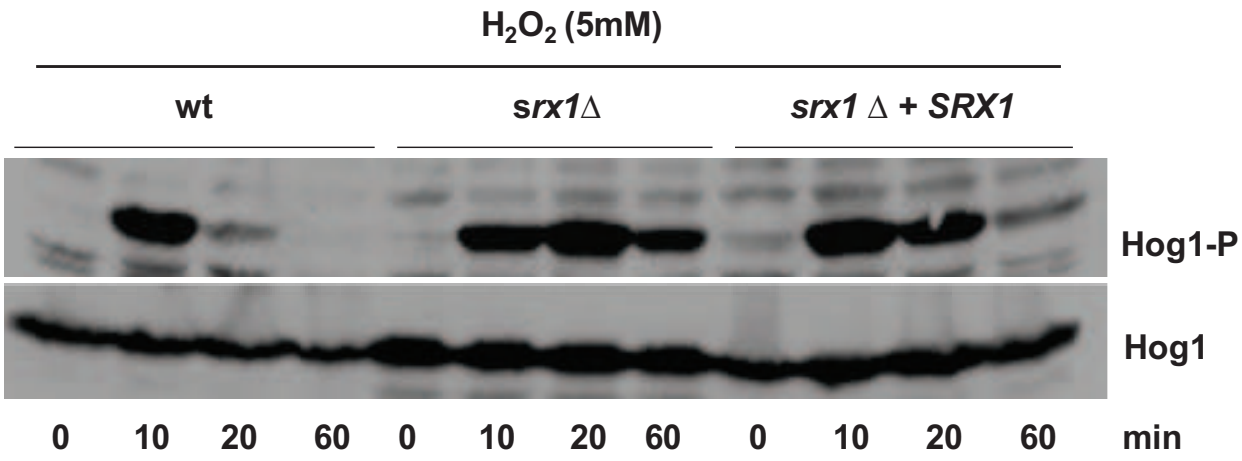

Figure 4.16. Deletion of Srx1 results in prolonged H_2O_2-induced Hog1 activation.
Hog1 phosphorylation (Hog1-P) was determined by western blot analysis of lysates prepared from wild-type (JC747), *srx1*Δ + CIp10 (JC719) and *srx1*Δ + CIp10-*SRX1* (JC721) cells after treatment with 5 mM H_2O_2 for the times indicated. Western blots were probed with an anti-phospho-p38 antibody, to detect the phosphorylated, active form of Hog1 (Hog1-P). Total levels of Hog1 protein were subsequently determined by stripping and reprobing the blot with an anti-Hog1 antibody (Hog1).

over-oxidised sulphinic derivative (Figure 4.15), and therefore may also have roles in peroxide signalling.

Initial evidence in support of this was obtained from expressing *C. albicans TSA1* in *S. pombe tpx1*$^{-}$ cells, upon which Tsa1 convincingly rescued the peroxide-signalling defects of this mutant (Figures 4.2, 4.3 and 4.4). Specifically, the oxidative stress-induced Pap1 nuclear accumulation and Sty1 phosphorylation were restored in *tpx1*$^{-}$ cells upon expression of *C. albicans TSA1*. Subsequently, *C. albicans* cells lacking *TSA1* were generated which facilitated an examination into the role of Tsa1 in the relay of peroxide signals to the Hog1 SAPK module in this fungal pathogen. Previous studies illustrated that Hog1 is phosphorylated in response to H_2O_2 (Alonso-Monge *et al.*, 2003 and Smith *et al.*, 2004), and moreover that treatment of *C. albicans* cells with increasing concentrations of H_2O_2 results in increased levels of Hog1 phosphorylation (Smith *et al.*, 2004). Based on these observations, it may be expected that deletion of an antioxidant such as Tsa1, which results in cells with increased sensitivity to H_2O_2, would result in greater activation of Hog1 at a given peroxide concentration. In contrast, deletion of *TSA1* significantly impairs Hog1 activation specifically in response to H_2O_2 (Figures 4.7 and 4.8). This indicates that Tsa1 acts as a positive regulator of H_2O_2-induced activation of Hog1, and is similar to that seen in *S. pombe* in which Tpx1 is required for the peroxide-induced activation of Sty1 (Veal *et al.*, 2004). However, the precise mechanism of 2-Cys peroxiredoxin mediated activation of *S. pombe* Sty1 and *C. albicans* Hog1 appears different. In *S. pombe* H_2O_2-induced activation of Sty1 requires the peroxidatic cysteine of Tpx1 (Veal *et al.*, 2004), while in *C. albicans* both catalytic cysteine residues are dispensable for the peroxide-induced activation of Hog1 (Figure 4.9). These results indicate that Tsa1 has roles in oxidative stress signalling that are independent of its peroxidase activity. Indeed, this result is consistent with the observation that *C. albicans* cells lacking Tsa1 are more sensitive to H_2O_2 than cells expressing catalytically-inactive forms of Tsa1 (Figure 4.6).

In addition to Tsa1, data presented in this chapter illustrates that the major regulator of the oxidation state, and thus activity of Tsa1, the thioredoxin protein Trx1, is also essential for H_2O_2-induced activation of Hog1 (Figures 4.7 and 4.8). However, in contrast to Tsa1 regulation of the Hog1 SAPK, the two catalytic cysteine residues of Trx1 are crucial for H_2O_2-induced activation of Hog1 (Figure 4.10), suggesting that Trx1 regulates the oxidation status of Hog1 or a protein(s) required for Hog1 regulation. For example, Hog1, or one of the

upstream components of the Hog1 pathway, may be susceptible to oxidative inactivation and Trx1 functions to maintain such a protein in a reduced active state. Indeed, in mammalian cells, the thioredoxin Trx regulates the oxidation state of the MAPKKK Ask1, which regulates peroxide signalling to the downstream p38 and JNK SAPKs (Nadeau *et al.*, 2007 and Nadeau *et al.*, 2009). However, such a mechanism is unlikely to be duplicated in *C. albicans* as Trx1 is a positive regulator of Hog1, whereas in mammalian cells, Trx acts as a negative regulator of Ask1, by inhibiting the formation of the oligomeric active form by reducing an important cysteine residue in Ask1 (Nadeau *et al.*, 2007 and Nadeau *et al.*, 2009). However, in contrast to mammalian MAPKKK, fungal MAPKK appear to be regulated by 2-component-related phosphorelay systems (Santos and Shiozaki, 2001). In *C. albicans*, there are two response regulators, Skn7 and Ssk1, that are predicted to regulate Ssk2, but only Ssk1 seems to be essential for H_2O_2-induced Hog1 activation (Chauhan *et al.*, 2003 and Singh *et al.*, 2003). However, it remains to be established whether Trx1 functions in the same pathway as Ssk1 to relay H_2O_2 signals to Hog1, or whether Trx1 is regulating the MAPKKK (Ssk2), the MAPKK (Pbs2) or Hog1 itself .

An alternative mechanism underlying Trx1 regulation of Hog1, may be related to the role of Trx1 in regulating the oxidation status of Tsa1. Both Trx1 and Tsa1 are required for H_2O_2-induced Hog1 activation and, significantly, Tsa1 is trapped in an oxidized dimeric form in either *trx1*Δ cells or cells expressing the catalytic cysteine mutants of Trx1 (Figure 4.12). Thus, it is possible that either the dimeric form of Tsa1 inhibits Hog1 activation or, alternatively, that a monomeric form of Tsa1 acts as an activator of Hog1. Consistent with these possibilities, H_2O_2 concentrations that induce Tsa1 dimer formation are not competent to stimulate Hog1 phosphorylation, whereas higher H_2O_2 concentrations that result in Tsa1 overoxidation do stimulate Hog1 activation (Figure 4.11). Furthermore, mutation of either catalytic cysteine residue of Tsa1, which prevents dimer formation, does not significantly impair Hog1 activation in response to peroxide stress (Figure 4.12). A precedent for the monomeric enzyme being functional has been recently reported in mammalian cells, in which the monomeric form of the 2-Cys peroxiredoxin PrxI is needed to maintain an interaction with the lipid phosphatase protein PTEN in response to oxidative stress, therefore allowing this phosphatase to be functional under these conditions (Cao *et al.*, 2009). Indeed, preliminary evidence suggests that it is the over-oxidised sulphinic monomeric form of Tsa1 that is optimal for H_2O_2-induced activation of Hog1 in *C. albicans*. For example, inactivation

of Srx1, the sulphiredoxin that reduces sulphinic groups, results in both Tsa1 being trapped in the sulphinic overoxidized form and prolonged Hog1 activation in response to H_2O_2 (Figures 4.15 and 4.16). However, it is important to note that the increased Hog1 activation seen in *srx1*Δ cells may be indirect due to the increased sensitivity of this mutant H_2O_2 (Figure 4.14). It also remains possible that Trx1 and Tsa1 function in distinct pathways to regulate Hog1, and further experimentation is clearly necessary to dissect the precise roles of Trx1 and Tsa1 in the relay of H_2O_2 signals to the Hog1 SAPK.

In the model yeasts, *S. cerevisiae* and *S. pombe*, both 2-Cys peroxiredoxins and thioredoxins have also been implicated in the regulation of the AP-1 like transcription factors. In *S. pombe* oxidation of the Pap1 transcription factor is entirely dependent on the 2-Cys peroxiredoxin Tpx1 (Bozonet *et al.*, 2005 and Vivancos *et al.*, 2005), and in *S. cerevisiae* the 2-Cys peroxiredoxin is required for Yap1 function in strains lacking a functional Ybp1 (Ross *et al*., 2000 and Veal *et al*., 2003). In contrast thioredoxin has been shown to function as a negative regulator of Yap1 function in *S. cerevisiae* by reducing oxidised Yap1 (Izawa *et al.*, 1999, Delaunay *et al.*, 2000 and Delaunay *et al.*, 2002). In a concurrent PhD project in Dr Quinn's laboratory, Miranda Patterson used the *trx1*Δ and *tsa1*Δ strains generated in this study to analyse any potential roles in the regulation of the Cap1 AP-1 like transcription factor in *C. albicans*. No effect on Cap1 activation was seen in cells lacking Tsa1 (not shown), illustrating that, in contrast to Pap1 oxidation in *S. pombe*, Cap1 oxidation occurs independently of 2-Cys peroxiredoxin function in *C. albicans.* However, inactivation of *TRX1* did impact on Cap1 regulation. As illustrated in Figure 4.17, an oxidized form of Cap1 persisted in *trx1*Δ cells compared to wild-type cells following H_2O_2 treatment (Figure 4.17A) and, consistent with this, both H_2O_2-induced Cap1 nuclear accumulation (Figure 4.17B) and H_2O_2-induced expression of the Cap1-dependent gene, *TRR1* (Figure 4.17C), were prolonged in *trx1*Δ cells. Collectively, these data demonstrate that Trx1 does play some role in regulating the reduction and thus inactivation of Cap1. However, there are some differences to that reported in *S. cerevisiae*, in which reduction of the related Yap1 transcription factor is entirely thioredoxin-dependent. Thus, *in S. cerevisiae*, thioredoxin deficiency results in constitutive oxidation, nuclear accumulation, and Yap1-dependent gene expression in the absence of H_2O_2 (Izawa *et al.*, 1999, Delaunay *et al.*, 2000 and Delaunay *et al.*, 2002). However, although Trx1 clearly plays some role in regulating the

reduction of Cap1 following oxidative stress in *C. albicans*, activation of Cap1 in the absence of H_2O_2 was not detected in *trx1*Δ cells.

In summary, data presented in this chapter illustrates that Tsa1 and Trx1 have major peroxide signalling functions in the human fungal pathogen *C. albicans*. To the best of our knowledge this is the first report that thioredoxin regulates stress signalling to fungal SAPK pathways. Furthermore, this work has also uncovered differences in thioredoxin, or 2-Cys peroxiredoxin, dependent SAPK regulation observed in other systems. For example, the finding that Trx1 is critical for the H_2O_2-induced activation of Hog1 is in marked contrast to that reported in mammalian systems in which thioredoxin functions as a repressor of the Hog1-related JNK and p38 SAPK signalling cascades (Saitoh *et al*., 1998). Furthermore, mutation of either catalytic cysteine residue of the *C. albicans* 2-Cys peroxiredoxin, Tsa1, does not significantly impair Hog1 activation in response to peroxide stress. This contrasts with previous work in *S. pombe* where the peroxidatic cysteine residue of the 2-Cys peroxiredoxin Tpx1 was found to be essential for H_2O_2-induced activation of the Hog1-related Sty1 SAPK (Veal *et al*., 2004). Collectively these data add to the growing evidence that whilst SAPKs are one of the most evolutionarily conserved eukaryotic stress signalling proteins (Nikolaou *et al.*, 2009), the mechanisms by which stress signals are transmitted to such SAPK modules differs considerably.

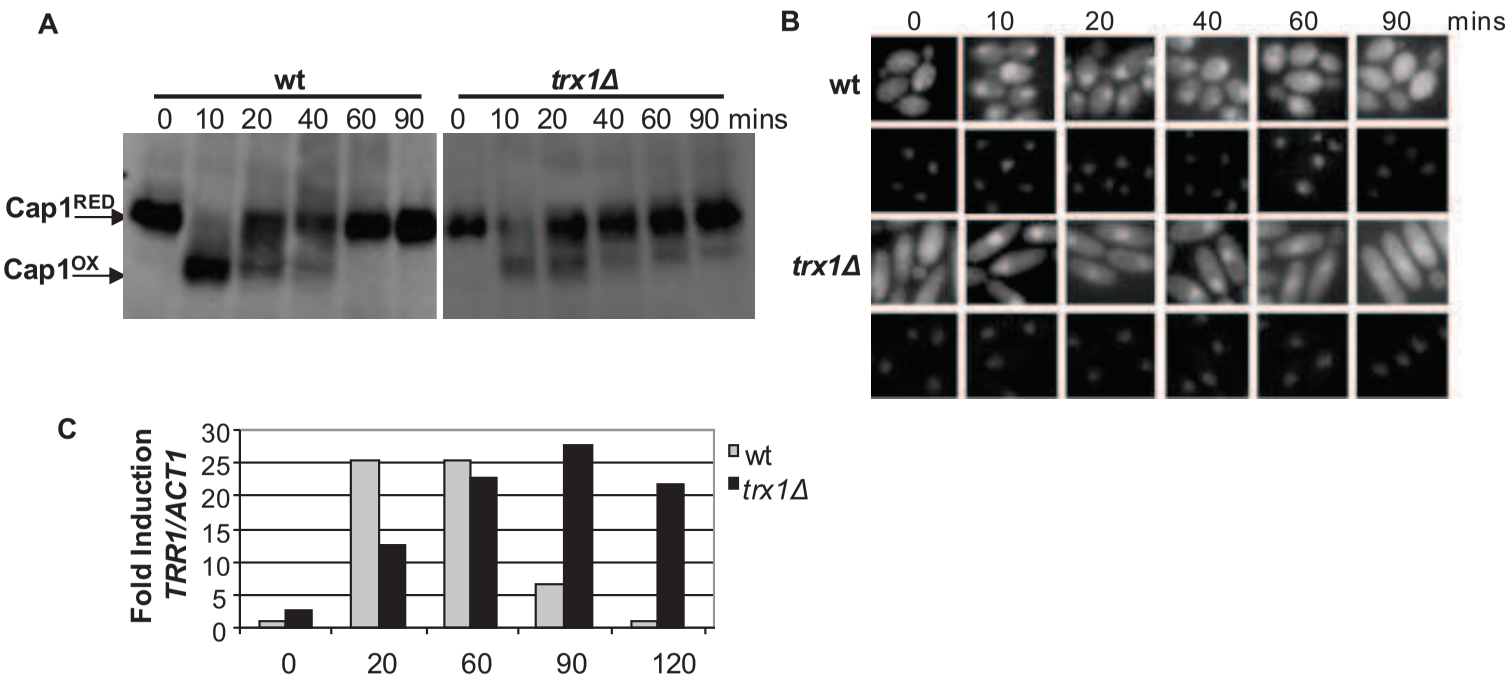

Figure 4.17. Deletion of Trx1 prolongs activation of the Cap1 AP-1-like transcription factor.
(A) Loss of Trx1 prolongs H_2O_2-induced Cap1 oxidation. Cap1 oxidation was analyzed by non-reducing SDS-PAGE and western blotting of AMS modified proteins prepared from wild-type (JC948) and *trx1*Δ (JC983) cells expressing 2myc-6His tagged Cap1, following treatment with 5 mM H_2O_2 for the indicated times. (B) H_2O_2-induced nuclear accumulation of Cap1 is prolonged in *trx1*Δ cells. The localization of Cap1-GFP was determined by fluorescence microscopy in wild-type (JC1060) and *trx1*Δ (JC1052) cells following treatment with 5 mM H_2O_2 for the indicated times. (C) Loss of Trx1 prolongs H_2O_2-induced Cap1-dependent gene expression. Northern blot analysis of RNA isolated from exponentially-growing cultures of wild-type (JC747) and *trx1*_ (JC677) cells following treatment with 5 mM H_2O_2.The level of *TRR1* RNA was quantified relative to the *ACT1* loading control. These experiments were performed by Miranda Patterson.

Chapter 5. Trx1 regulates H_2O_2-stimulated polarized growth in *C. albicans*

5.1. Introduction

In recent years it has been shown that H_2O_2 can act as a signalling molecule in mammalian cells promoting cell differentiation (Li *et al.*, 2006). As described earlier (Chapter 1 - section 1.3.1.4), phagocytic cells produce $O_2^{\bullet-}$ via the activity of NADPH oxidase (NOX) complexes. Interestingly, several fungi, such as *Aspergillus nidulans*, *Botrytis cinerea*, *Epichloë festucae*, *Magnaporthe grisea*, *Neurospora crassa* and *Podospora anserina*, have also been shown to express NADPH oxidases (Lara-Ortiz *et al.*, 2003, Malagnac *et al.*, 2004, Takemoto *et al.*, 2006, Egan *et al.*, 2007, Cano-Dominguez *et al.*, 2008 and Segmüller *et al.*, 2008). Furthermore, deletion of the NOX encoding genes has been associated with pleiotropic cell differentiation defects, which range from abnormal hyphal growth and branching to inhibition of spore germination, resulting in organisms that are unable to colonize the host (Lara-Ortiz *et al.*, 2003, Malagnac *et al.*, 2004, Takemoto *et al.*, 2006, Egan *et al.*, 2007, Cano-Dominguez *et al.*, 2008 and Segmüller *et al.*, 2008). Indeed, staining of hyphae with ROS-reactive dyes has shown that a ROS gradient is formed during polarised growth of *E. festucae* and *A. nidulans* cells (Takemoto *et al.*, 2006, Tanaka *et al.*, 2006 and Semighini and Harris, 2008), indicating that restricted production of ROS at the tip is important in determining hyphal growth. In addition, an increase in ROS levels is seen in *N. crassa* just before formation of aerial hypha and conidia differentiation, indicating that ROS may be a signal that triggers such differentiation events (Lledías *et al.*, 1999, Aguirre *et al.*, 2005 and Hansberg *et al.*, 1993). Furthermore, inactivation of a catalase enzyme in this organism (catalase-3) results in higher ROS levels and increased amounts of aerial hyphae and conidia (Michán *et al.*, 2003).

Interestingly, it has been reported that the levels of intracellular ROS in germinating *C. albicans* cells are up to 3 times higher than that found in yeast cells (Schröter *et al.*, 2000). Furthermore, induction of germ tube formation by serum addition was accompanied by an increase in ROS production, with no increase in ROS production in a strain that was unable to generate hyphae (Schröter *et al.*, 2000). In addition, it has been shown that *C. albicans* cells treated with H_2O_2 display filamentous growth (Phillips *et al.*, 2003 and Nasution *et al.*, 2008). The increase in filamentation appeared to be concentration dependent, since treatment

of cells with higher non-lethal concentrations of H_2O_2 resulted in increased filamentation (Nasution *et al.*, 2008). Deletion of genes encoding key antioxidant enzymes, such as *TSA1* and *CAT1*, also resulted in increased H_2O_2-induced filamentation compared to wild-type cells which was accompanied by higher levels of intracellular H_2O_2 (Nasution *et al.*, 2008). Consistent with a previous study (Schröter *et al.*, 2000), the more recent work by Nasution and colleagues reported that serum addition induced an increase in the intracellular levels of H_2O_2 in *C. albicans*. Furthermore, addition of an antioxidant, such as ascorbic acid, was able to inhibit serum-induced filamentous growth. Collectively these data indicate that, similar to other fungi, ROS seem to be required for *C. albicans* filamentous growth (Nasution *et al.*, 2008). Intriguingly, however, no NOX-like enzymes have been identified in *C. albicans*.

Although previous studies illustrated that exposure to ROS promotes filamentous growth in *C. albicans* (Phillips *et al.*, 2003 and Nasution *et al.*, 2008), the precise morphological characteristics of these filamentous growth forms were not defined. Furthermore, the signaling pathways and targets involved in promoting *C. albicans* H_2O_2-induced filamentous growth are unclear. However, as reported earlier in this thesis (Chapter 3, Figure 3.11), deletion of *TRX1* or mutation of the redox-sensitive catalytic cysteine residues of Trx1, resulted in a filamentous growth phenotype under non-stressed conditions. These data are consistent with a model in which H_2O_2-induced inactivation of Trx1 is a trigger in stimulating H_2O_2-induced filamentous growth. The aim of the work presented in this chapter was to precisely define the morphology of H_2O_2-induced filaments, and to investigate the role of Trx1 in regulating such filamentous growth in *C. albicans.*

5.2. Results

5.2.1. Oxidative stress and filamentous growth

5.2.1.1. H_2O_2 treatment induces hyperpolarized bud growth in C. albicans

In order to characterize the *C. albicans* filaments formed in response to ROS, wild-type cells were treated with 5 mM H_2O_2 and the cell morphology analyzed. A close examination of H_2O_2-induced filamentation revealed that exposure of *C. albicans* cells to H_2O_2 stimulated extensive bud elongation in which nuclear movement from the mother cell to the daughter

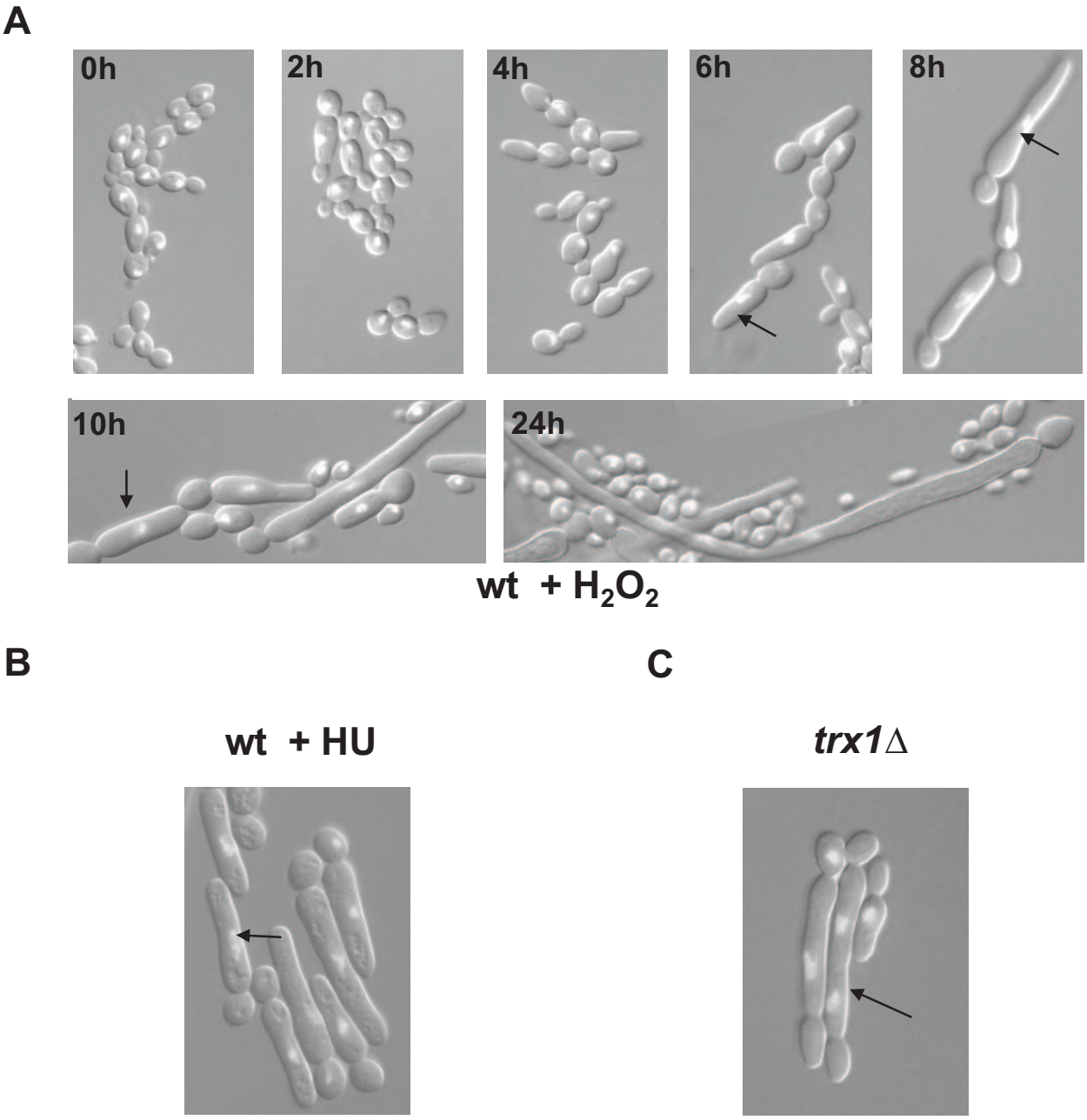

Figure 5.1. Treatment of wild-type cells with H_2O_2 results in hyperpolarised bud growth, similar to wild-type cells treated with HU or upon deletion of *TRX1*.
Morphological analysis of (A) wild-type cells (JC747) treated with 5 mM H_2O_2 for the indicated times, (B) wild-type cells treated with 50mM of HU for 6 hours or (C) mid-exponential phase *trx1*Δ cells (JC677) grown in YPD at 30ºC. In all cases cells were stained with DAPI to allow both cell morphology and nuclear distribution to be determined by overlaying fluorescence and DIC images. Arrows indicate nuclear migration to elongating bud.

bud was evident, as were clear constrictions at the septal junction (Figure 5.1A). Moreover, the majority of cells did not divide further, but rather the buds continued to elongate over time (Figure 5.1A). This H_2O_2-induced hyperpolarized bud growth phenotype is distinct from hyphae since the hyperpolarized buds display a clear constriction at the septum, contrary to hyphal cells. Furthermore, in contrast to pseudohyphae, in which the first nuclear division occurs across the mother bud neck, nuclear movement from the mother cell to the polarized bud is evident. Instead this hyperpolarized bud growth phenotype is very similar to that characterized recently in various *C. albicans* mutants that perturb cell cycle progression, or in response to chemicals such as HU (Figure 5.1B) or methyl methanesulfonate (MMS) that induce cell cycle arrest (Shi *et al.*, 2007) as discussed in Chapter 1, Section 1.2.3.1. Indeed, consistent with links to the cell cycle, exposure of *C. albicans* to H_2O_2 has been reported to result in a G2/M phase arrest (Phillips *et al.*, 2003).

Importantly, cells lacking *TRX1* display a hyper-polarised bud phenotype (Figure 5.1C and Figure 3.10B) strikingly similar to that seen following H_2O_2 and HU treatment of wild-type cells (Figures 5.1A and 5.1B). This prompted an investigation into whether inactivation of Trx1 could trigger H_2O_2 and HU induced polarized cell growth.

5.2.1.2. Inhibition of Trx1 appears to specifically regulate H_2O_2-induced hyperpolarized bud formation

As a first step in investigating the role and specificity of Trx1 in regulating hyperpolarised bud formation, *trx1*Δ cells were treated with either H_2O_2 or HU, and the cell morphology examined. Consistent with the hypothesis that inactivation of Trx1 is a trigger of hyperpolarized bud formation in response to H_2O_2, cells lacking *TRX1* did not elongate further in response to H_2O_2 (Figure 5.2). In contrast, *trx1*Δ cells displayed considerable additional elongation in response to HU (Figure 5.2). These results suggest that inhibition of Trx1 is a key step in H_2O_2-induced, but not HU-induced, polarized cell growth.

5.2.1.3. Prolonged oxidation of Trx1 in response to increasing levels of H_2O_2 correlates with prolonged filamentation

Trx1 contains two redox-active catalytic cysteine residues (Cys30 and Cys33), and mutation of such cysteine residues results in cells that form hyperpolarised buds (Figure 3.10). These findings are consistent with a model in which H_2O_2 induced oxidation of Cys30

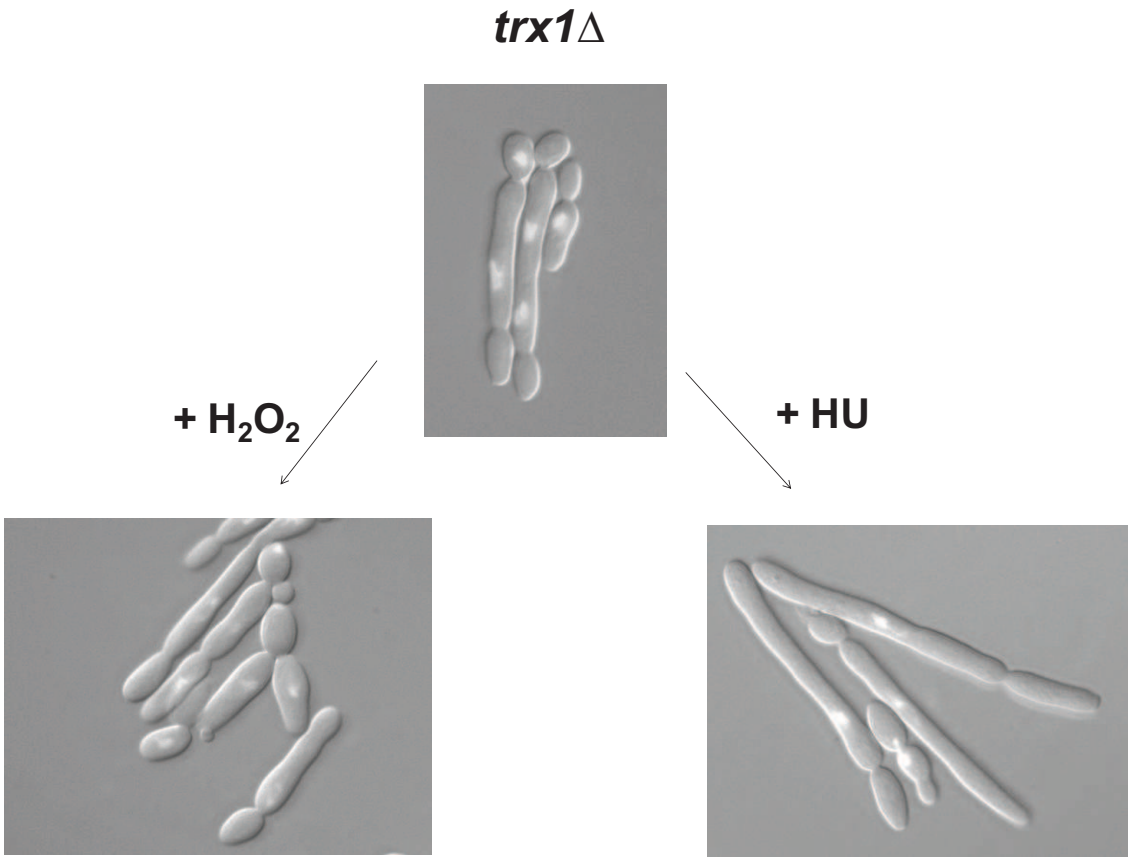

Figure 5.2. Trx1 regulates H_2O_2-induced hyperpolarised bud formation, but not HU-induced hyperpolarized growth.

Morphological analysis of *trx1*Δ cells (JC677) grown in YPD at 30ºC and treated with either 5mM H_2O_2 or 50mM HU for 6 hours. In all cases cells were stained with DAPI to allow both cell morphology and nuclear distribution to be determined by overlaying fluorescence and DIC images.

and Cys33 triggers H_2O_2-induced polarized cell growth. To investigate whether Trx1 oxidation correlates with H_2O_2-induced polarized growth, such events were assessed in wild-type cells in response to low (0.5 mM) and high (5 mM) concentrations of H_2O_2 (Figure 5.3A). In order to examine the oxidation state of Trx1, Trx1 was chromosomally tagged at the C-terminus with 6-His residues and 2 copies of the myc-epitope and H_2O_2-induced oxidation of Trx1 was monitored using the thiol-reducing agent AMS as described in Chapter 2, section 2.3.7. The mobility of Trx1-specific bands increased in response to H_2O_2, compared to those seen in unstressed cells, indicating that Trx1 is rapidly oxidized following H_2O_2 treatment (Figure 5.3B). Based on previous studies which have examined oxidation of the *S. cerevisiae* thioredoxins (Trotter and Grant, 2003) or mammalian Trx1 (Chen *et al.*, 2006), we would expect to see only one band corresponding to the oxidized form, as upon oxidation an intramolecular disulphide is formed between the two cysteine residues of Trx1. However, intriguingly, two AMS-resistant oxidized forms of Trx1 (Trx1-MH^{ox}) are evident. Both forms are lost upon treatment of cell extracts with the reducing agent DTT prior to AMS addition (Figure 5.3C), thus confirming that the bands represent different oxidised forms of Trx1. The molecular basis for this is unknown but could either represent oxidation of one (middle band) or two cysteine residues (lower band), or it may be indicative of another redox-induced modification such as glutathionylation as seen on mammalian Trx (Casagrande *et al.*, 2002). It is also noteworthy that the extent of oxidation of Trx1 displays considerable experiment to experiment variation. For example, complete oxidation (lower band) of Trx1 is seen in Figure 5.3C, but not in Figure 5.3B, following treatment of cells with 5 mM H_2O_2. Nonetheless, whatever the molecular basis for the different oxidized forms of Trx1, Trx1 is rapidly oxidized in response to low and high levels of H_2O_2 and, as expected, oxidized forms of Trx1 persist for longer following exposure to higher H_2O_2 concentrations (Figure 5.3B). Intriguingly, with regard to the kinetics of H_2O_2 induced polarized cell growth, this appears to happen more quickly in response to low levels (0.5 mM H_2O_2) of oxidative stress. However, prolonged filamentation is observed in cells treated with 5 mM H_2O_2 compared to cells treated with 0.5 mM H_2O_2 (Figure 5.3A), and this correlates with the sustained oxidation of Trx1 seen upon treatment with higher H_2O_2 concentrations.

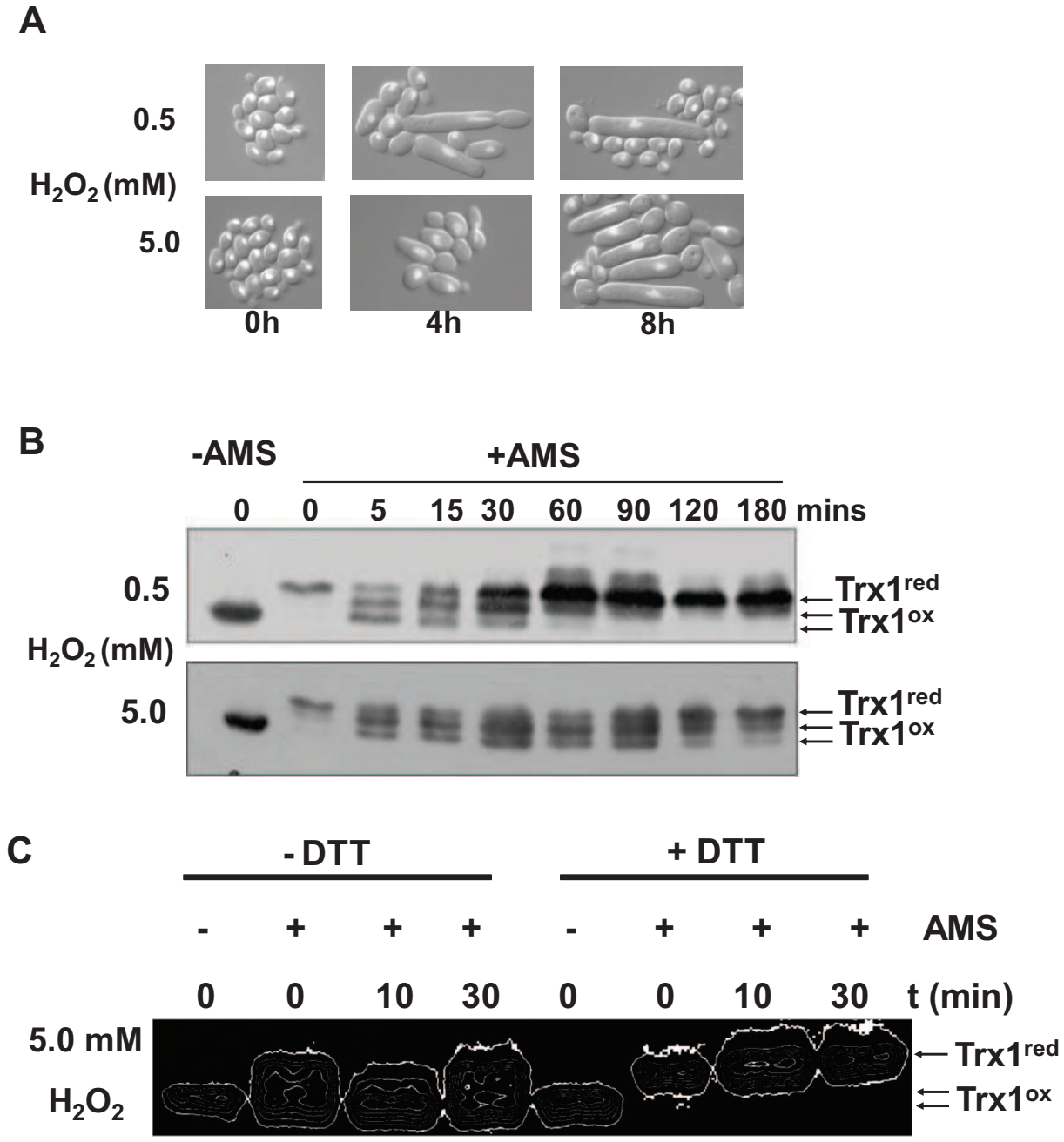

Figure 5.3. Oxidation of Trx1 is important for H_2O_2-induced polarized cell growth.
(A) The kinetics and extent of H_2O_2-induced polarized cell growth is dependent on the level of H_2O_2. Wild-type cells (JC747) were either untreated or treated with low (0.5 mM) or high (5 mM) levels of H_2O_2, for 4 or 8 h and images taken. (B) Oxidation of Trx1 is prolonged following exposure to high levels of H_2O_2. The oxidation of 6His-myc tagged Trx1 (Trx1-MH) was analyzed by non-reducing SDS-PAGE and western blotting of AMS-modified proteins prepared from cells (JC930) exposed to 0.5 and 5mM H_2O_2 for the indicated times. (C) AMS-resistant oxidized forms of tagged Trx1 ($Trx1^{ox}$) was analyzed by non-reducing SDS-PAGE and western blotting proteins prepared from cells (JC930) exposed to 5mM H_2O_2 for the indicated times and treated with the reducing agent DTT prior to AMS addition.

5.2.1.4. Ectopic expression of TRR1 inhibits H_2O_2-induced, but not HU-induced, polarized cell growth

To further investigate whether oxidation of Trx1 is a key step in stimulating H_2O_2-induced hyperpolarized bud growth, *TRR1* which encodes the enzyme thioredoxin reductase that reduces oxidized thioredoxin (Chapter 1 – section 1.4.2.4.2), was ectopically expressed in wild-type cells under the control of the strong *ACT1* promoter (Tripathi *et al.*, 2002). Significantly, ectopic expression of *TRR1* was found to specifically inhibit H_2O_2-induced, but not HU-induced, polarized growth (Figure 5.4A). Furthermore, wild-type cells containing pACT1-*TRR1* did not show increased resistance to H_2O_2 when compared to wild-type cells expressing the empty pACT1 vector (Figure 5.4B), indicating that the Trr1-mediated inhibition of H_2O_2-induced filamentous growth was not due to increased tolerance to oxidative stress. In order to confirm whether the inhibition of H_2O_2-induced filamentous growth seen in wild-type cells expressing *ACT1* promoter driven *TRR1* was dependent on Trx1, *TRR1* was also ectopically expressed in *trx1*Δ cells. As illustrated in figure 5.4C, ectopic expression of *TRR1* in *trx1*Δ cells did not revert the filamentous phenotype of *trx1*Δ cells (Figure 5.4C). This result suggests that the inhibition of H_2O_2-induced filamentous growth seen upon ectopic expression of *TRR1* is dependent on Trx1. Collectively, the results presented in Figures 3.10, 5.3 and 5.4, strongly suggest that inactivation of Trx1 by oxidation stimulates H_2O_2-induced polarized cell growth.

5.2.2. Investigation into the mechanism of hyper-polarised bud formation in response to oxidative stress

The data presented above is consistent with a model in which oxidation of Trx1 stimulates H_2O_2-induced polarized cell growth in *C. albicans*. Previous studies in both yeast and mammals indicate that the primary function of thioredoxin proteins in H_2O_2-signalling is to regulate the oxidation status of key regulatory proteins (Chaper 1, section 1.4.4.2.2). Indeed, data presented in Chapter 4 revealed that Trx1 is central to the regulation of oxidative stress signalling pathways in *C. albicans*, by regulating the function of both the Hog1 SAPK and the Cap1 transcription factor in response to H_2O_2. Hence, in order to identify the targets of Trx1 that regulate H_2O_2-induced filamentous growth, mutant strains in which genes encoding known targets of Trx1 were deleted (*tsa1*Δ, *cap1*Δ and *hog1*Δ) were analyzed for their ability to induce hyperpolarized buds in response to H_2O_2. This approach was comple-

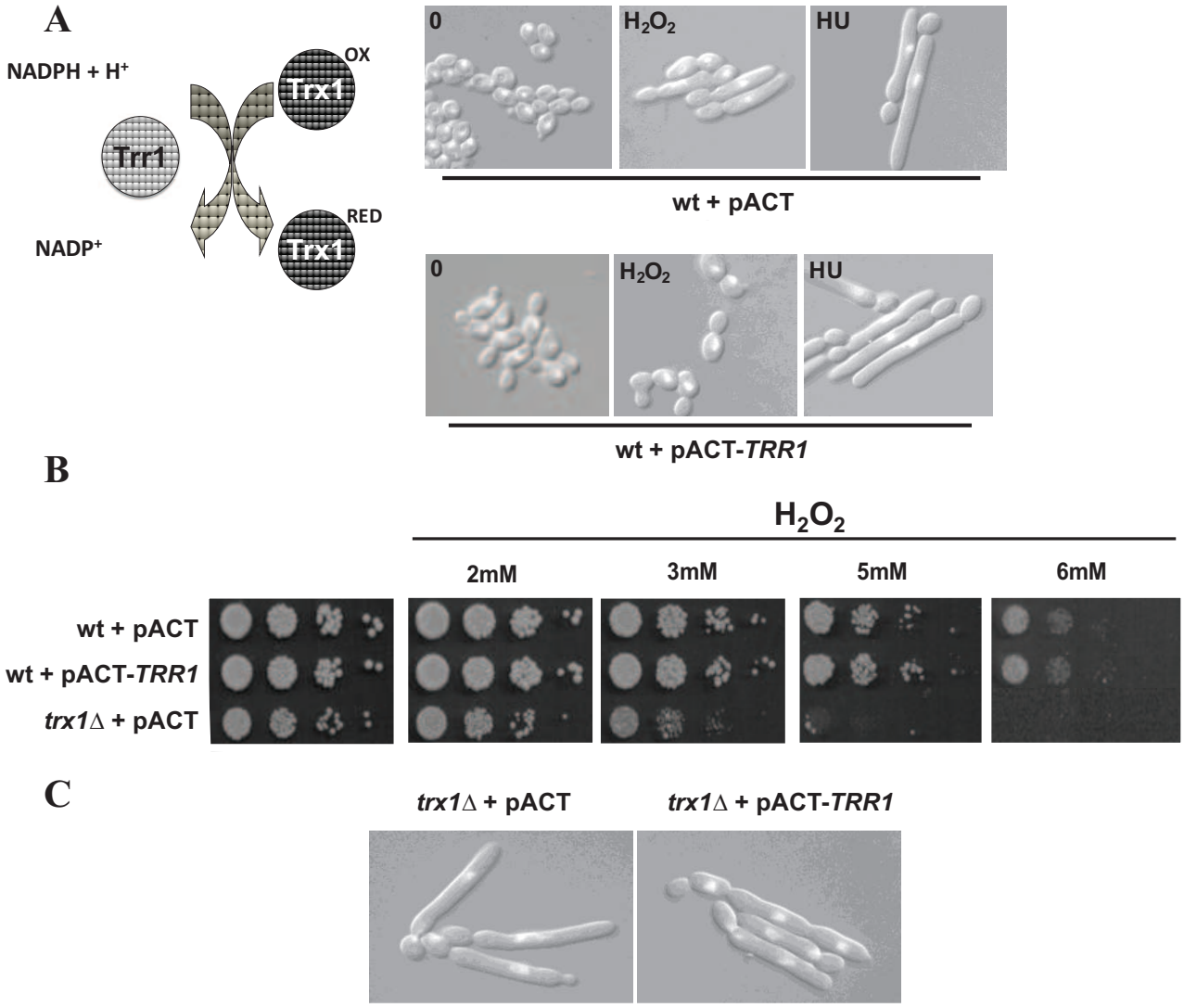

Figure 5.4. Ectopic expression of Trr1 results in specific inhibition of H_2O_2-induced polarized cell growth.

(A) Trr1 reduces oxidized Trx1 at the expense of NADPH (left panel). Ectopic expression of *TRR1* (pACT1-Trr1) inhibits H_2O_2-induced, but not HU-induced, polarized cell growth. Wild-type cells (wt), containing either vector (pACT1, JC1014) or pACT1-*TRR1* (JC1066), in which *TRR1* is expressed from the *ACT1* promoter, were untreated (0) or treated with either 5 mM H_2O_2 or 50 mM HU for 6 h. Images were captured as described in Figure 5.1. (B) Ectopic expression of *TRR1* does not increase the cellular resistance to H_2O_2 . Approximately 10^4 cells, and 10-fold dilutions thereof, of exponentially-growing wild-type (wt) strain in which either the empty vector pACT1 (JC1014) or pACT1-*TRR1* (JC1066) had been integrated at the *RPS10* locus and *trx1*Δ cells containing the pACT1 empty vector (JC1022), were spotted onto YPD plates containing increasing concentrations of H_2O_2 were incubated at 30°C for 24 h. (C) Ectopic expression of *TRR1* does not inhibit the filamentous phenotype exhibited by *trx1*Δ cells. Images illustrate the morphology of exponentially growing *trx1*Δ cells containing either pACT1 (JC1022) or pACT1-*TRR1* (JC1115).

mented by also analysing H_2O_2-induced filamentation in mutant strains carrying deletions in genes previously associated with filamentous growth (*efg1*Δ/*cph1*Δ and *rad53*Δ) (Lo *et al.*, 1997 and Shi *et al.*, 2007).

*5.2.2.1. cap1*Δ*, hog1*Δ *and tsa1*Δ *cells form polarized buds in response to* H_2O_2

Trx1 is a negative regulator of the AP-1 like transcription factor Cap1, acting to partially reduce oxidized Cap1 in response to H_2O_2 treatment (Figure 4.16). Since Trx1 also negatively regulates polarized cell growth, this is consistent with the hypothesis that Cap1 oxidation and activation could be triggering H_2O_2-induced filamentation. In order to test this, *cap1*Δ cells (strain made by Miranda Patterson) were treated with H_2O_2 and examined for hyperpolarized bud formation. As illustrated in Figure 5.5A deletion of *CAP1* did not inhibit H_2O_2-induced polarized cell growth. The filaments seen in *cap1*Δ cells treated with H_2O_2 are similar in length to the ones seen in wild-type cells treated with the same H_2O_2 concentration. This indicates that activation of the Trx1 substrate, Cap1, is not the trigger for H_2O_2-induced polarized cell growth.

However, in addition to Cap1, Trx1 also regulates the activity of the Hog1 SAPK. Furthermore, both Hog1 activation (Smith *et al.*, 2004 – Figure 4.11) and hyperpolarised bud formation increase (Figure 5.3) with increasing concentrations of H_2O_2, indicating that Hog1 activation may be the trigger for peroxide induced polarized cell growth. However, Trx1 acts to inhibit H_2O_2-induced polarized cell growth and yet is required for H_2O_2-induced activation of Hog1. Consistent with these opposing roles of Trx1 regulation of Hog1 and filamentous growth, deletion of *HOG1* did not impair H_2O_2-induced filamentation. For example, morphological analysis of *hog1*Δ cells in response to H_2O_2 treatment, showed that deletion of *HOG1* resulted in wild-type levels of hyperpolarized growth formation in response to H_2O_2 (Figure 5.5B). This result indicates that H_2O_2-induced hyperpolarized bud formation is independent of Trx1 regulation of the Hog1 SAPK.

Trx1 also regulates the oxidation state of the 2-Cys peroxiredoxin Tsa1, as deletion of *TRX1* or expression of *trx1* cysteine mutants results in the constitutive oxidation of Tsa1 (Figures 3.7 and 4.12). Hence, it was possible that Trx1-regulation of the oxidation state of Tsa1 may be important in stimulating the formation of hyperpolarized buds. However, in contrast to cells lacking *TRX1*, *tsa1*Δ cells and strains expressing *tsa1* cysteine mutants do not display a hyperpolarized bud phenotype in the absence of H_2O_2 (Figure 5.5C). Further-

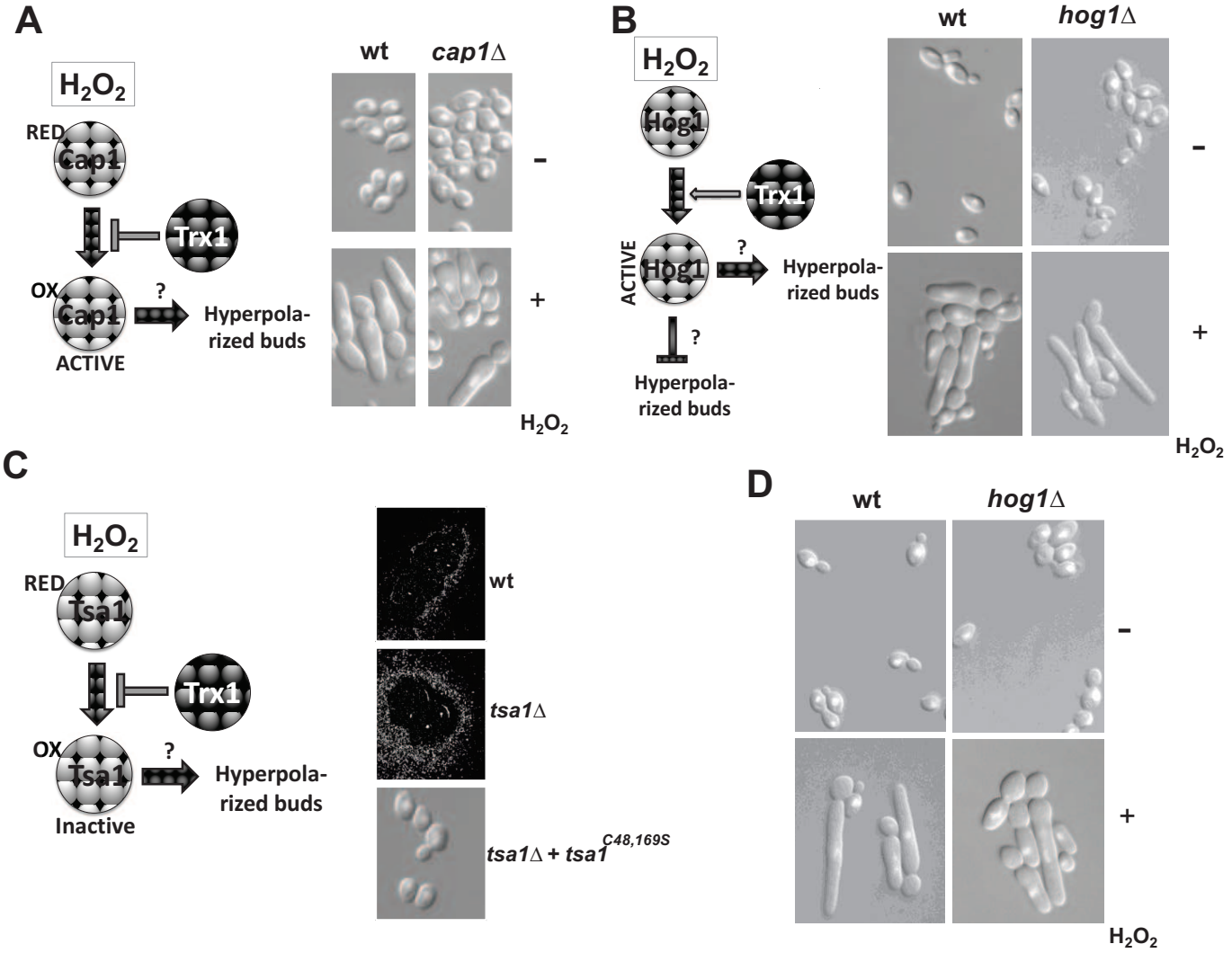

Figure 5.5. ***cap1*Δ, *hog1*Δ *and tsa1*Δ cells can form polarized buds in response to H_2O_2.**
(A) Cap1 becomes oxidized and therefore activated in response to H_2O_2 treatment. Cap1 activation could be the trigger for formation of hyperpolarized growth. Therefore, Trx1 could be inhibiting hyperpolarized growth by inhibiting Cap1 oxidation (left panel). However loss of *CAP1* does not impair H_2O_2-induced polarized cell growth. Wild-type (JC747) and *cap1*Δ (JC842) cells were treated with 1 mM H_2O_2 for 6 h (right panel). (B) Hog1 becomes activated in response to H_2O_2 treatment. Activated Hog1 could be either triggering or inhibiting formation of hyperpolarized buds. Therefore, Trx1 could be inhibiting hyperpolarized growth by activating Hog1 (left panel). However *hog1*Δ cells form hyperpolarized buds in response to H_2O_2. Wild-type (BWP17) and *hog1*Δ (JC47) cells were treated with 5 mM H_2O_2 for 6 h (right panel). (C) Tsa1 becomes oxidized and therefore inactive in response to H_2O_2 treatment, which could trigger formation of hyperpolarized buds (right panel). However, deletion of *TSA1* or expression of $tsa1^{C48,169S}$ mutant does not exhibits hyperpolarized bud formation under non-inducing conditions (left panel). (D) Furthermore, *tsa1*Δ cells can also form hyperpolarized buds in response to H_2O_2. Wild-type (JC747) and *tsa1*Δ (JC1027) cells were treated with 5 mM H_2O_2 for 6 h. All images were captured as described in Figure 5.1.

more, H_2O_2 treatment also induced hyperpolarized bud formation in *tsa1*Δ cells (Figure 5.5D), similar to that seen in wild-type cells, indicating that Tsa1 is not required for H_2O_2-induced filamentous growth.

Taken together, the data present in Figure 5.5, indicates that H_2O_2-induced polarised cell growth occurs independently of the Trx1 substrates Tsa1 and Cap1, and the Trx1 regulated Hog1 signalling pathway.

5.2.2.2. efg1/cph1Δ cells form hyperpolarized buds in response to H_2O_2

Since none of proteins/pathways that are known targets of *C. albicans* Trx1 (Hog1, Cap1 and Tsa1) appear to be involved in H_2O_2-induced filamentous growth, mutants with previously characterised filamentation defects were also tested for defects in H_2O_2-induced hyperpolarised bud formation. For example, the morphology of a mutant lacking the major transcriptional regulators of *C. albicans* hyphal formation, Cph1 and Efg1 (Lo *et al.*, 1997) was analyzed in response to H_2O_2. Deletion of *EFG1* and *CPH1* had no impact on the stimulation of H_2O_2-induced filamentous growth (Figure 5.6A), indicating that the pathways regulating hyphal growth and H_2O_2-induced hyperpolarised buds are distinct. Consistent with with the observation that cells lacking the hyphal regulators Efg1 and Cph1 could form H_2O_2-induced filaments, the filamentous forms seen in *trx1*Δ cells were competent to produce hyphae under inducing conditions (Figure 5.6B).

5.2.2.3. rad53Δ cells do not form polarized buds in response to H_2O_2

A previous study showed that in *C. albicans* the DNA checkpoint kinase Rad53 is a key regulator of hyperpolarized bud formation in response to a range of genotoxic stress conditions, such as HU treatment (Shi *et al.*, 2007). Therefore, it was possible that Rad53 was also regulating hyperpolarized bud formation in response to H_2O_2. To investigate this, *rad53*Δ cells (Shi *et al.*, 2007) were treated with H_2O_2 and their morphology assessed. Significantly, H_2O_2-induced filamentous growth was abolished in *rad53*Δ cells and reintegration of a wild-type copy of *RAD53* rescued the defect in H_2O_2-induced filamentous growth (Figure 5.7A). This indicates that Rad53 is also important in regulating the induction of hyperpolarized growth in response to H_2O_2. The Rad53 kinase is also important for the oxidative stress response in *C. albicans*, as *rad53*Δ cells display increased sensitivity to H_2O_2 compared to the reconstituted strain (Figure 5.7B).

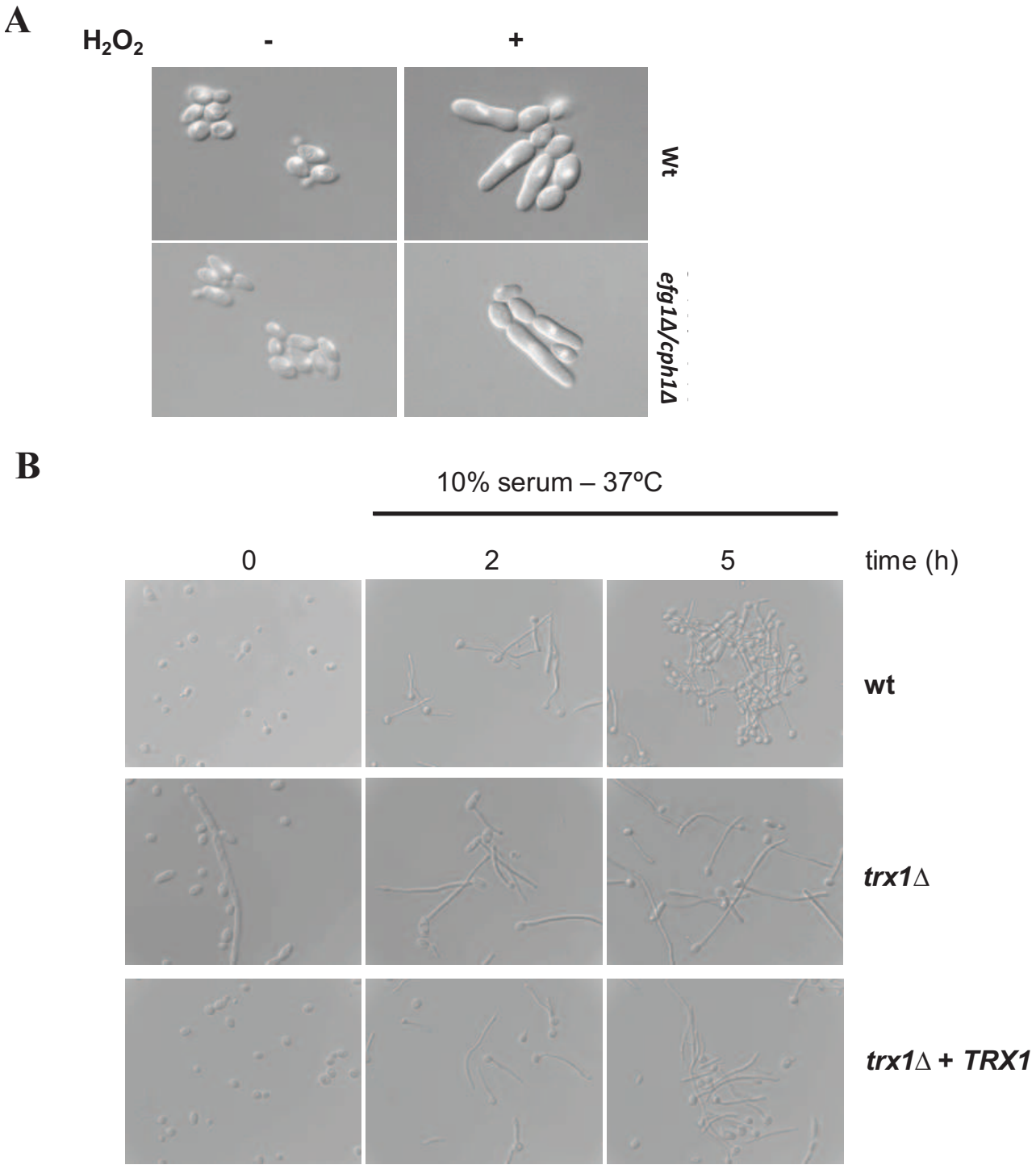

Figure 5.6. H_2O_2-induced hyperpolarized buds formation does not require Efg1 or Cph1.
(A) Cells lacking the major transcriptional regulators of hyphae formation, Efg1 and Cph1, form hyperpolarised buds following treatment with H_2O_2. Wt (CAIA4) and *efg1*Δ/*cph1*Δ cells (HLC69) were treated with 5 mM H_2O_2 for 6 h. (B) *trx1*Δ cells can form hyphae. Stationary phase cells were diluted 1:10 in fresh YPD media supplemented with 10% serum and grown at 37°C. Images were taken prior to and following exposure to serum for 2 and 5h. Images of cells in (A) were images were captured as described in Figure 5.1. Cells in (B) were only captured as DIC images.

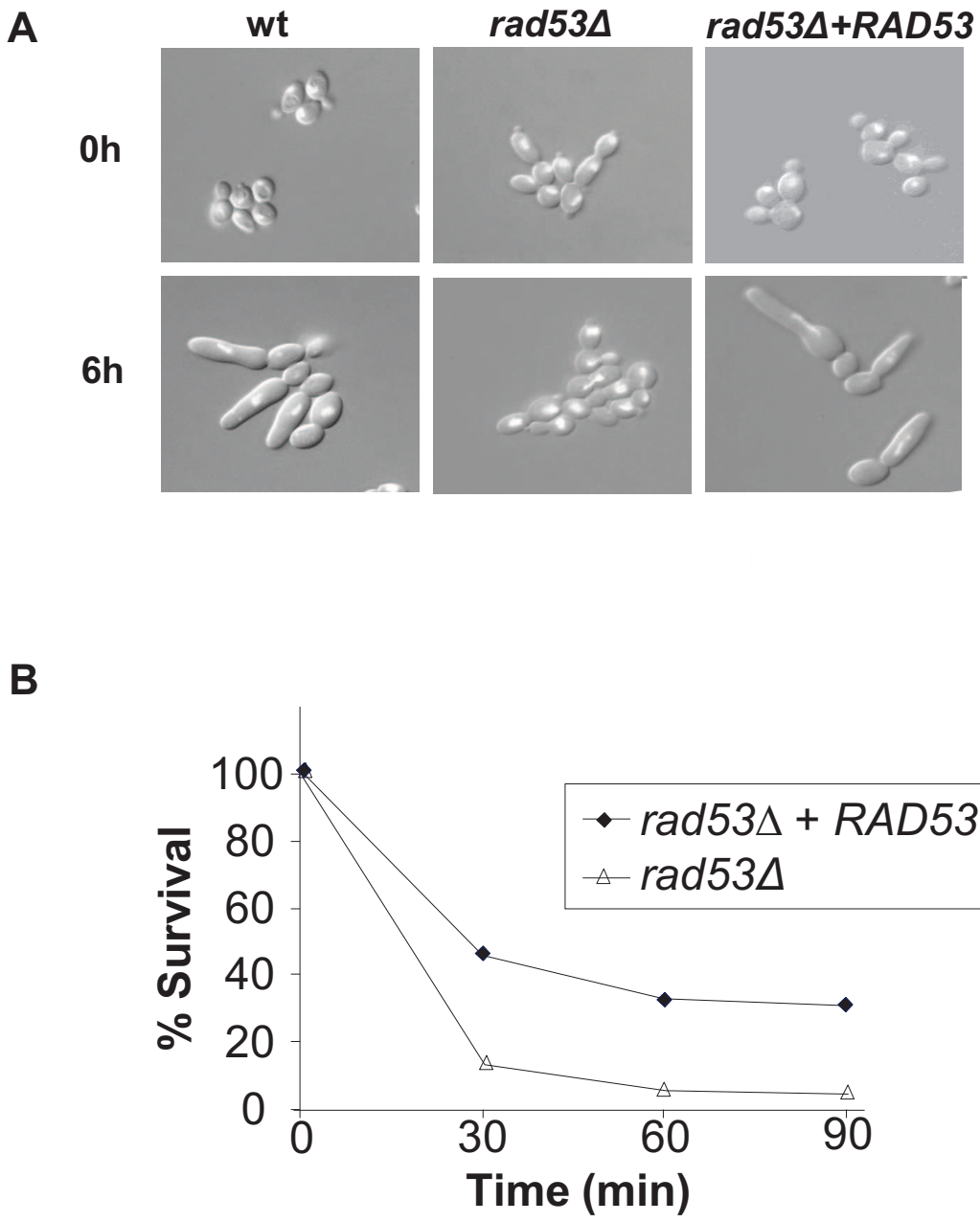

Figure 5.7. The checkpoint kinase Rad53 is required for H_2O_2-induced polarized cell growth and the oxidative stress response.

(A) Loss of Rad53 function blocks H_2O_2-induced polarized cell growth. Wild-type (BWP17), *rad53*Δ (WYS3), and *rad53*Δ+*RAD53* (WYS3.1) cells were treated with 5 mM H_2O_2 for 6 h, and images captured as described in Figure 5.1. (B) Rad53 is essential for oxidative stress resistance. *rad53*Δ (WYS3) cells and the reconstituted strain *rad53*Δ+*RAD53* (WYS3.1) were treated with 5 mM H_2O_2 and cell survival at the indicated times.

5.2.2.4. Rad53 is hyper-phosphorylated in response to H_2O_2, HU and in trx1Δ cells

Exposure of *C. albicans* to various genotoxic stresses, such as HU or MMS, results in hyperphosphorylation, and presumably activation, of the Rad53 kinase (Shi *et al.*, 2007). Therefore it was possible that in response to oxidative stress caused by H_2O_2 treatment Rad53 would also become hyperphosphorylated. In order to detect phosphorylation of Rad53, a strain was generated in which Rad53 was tagged at the C-terminus with 2-Myc epitopes and 6His residues (see Chapter 2 for details). The phosphorylation state of Rad53 was detected by western blot analysis of cell extracts prepared from cells before and following exposure to H_2O_2. As illustrated in Figure 5.8A, Rad53 exhibited a slower mobility on SDS-PAGE following H_2O_2 treatment, and these slower migratory forms were lost upon treatment of cell extracts with λ phosphatase. This indicates that Rad53 also becomes hyperphosphorylated in response to H_2O_2 in wild-type cells. Significantly, Rad53 is also hyperphosphorylated in *trx1*Δ cells, compared with wild-type cells, under non-stressed conditions (Figure 5.8B). Some further H_2O_2-induced phosphorylation of Rad53 is also evident in *trx1*Δ cells (Figure 5.8A), although this does not appear to stimulate filamentation, since *trx1*Δ filaments do not extend further in response to H_2O_2 (Figure 5.2A). Thus, either exposure of cells to H_2O_2, or inactivation of Trx1, activates the Rad53 DNA checkpoint kinase. Collectively, these results strongly suggest that the H_2O_2-induced oxidation, and thus inactivation of Trx1, triggers activation of Rad53, which in turn stimulates polarized cell growth.

5.2.2.5. Ectopic expression of RNR does not prevent HU or H_2O_2-induced hyperpolarized growth in C. albicans

In *C. albicans,* depletion of the large subunit of RNR results in cell cycle arrest and formation of hyperpolarized growth under non-stress conditions (Shi *et al.*, 2007). This result indicates that the DNA-replication stress induced by depletion of *RNR,* or by treatment with the RNR inhibitor HU, induces filamentous growth in *C. albicans* (Shi *et al.*, 2007). In the model yeast *S. cerevisiae,* thioredoxin regulates the oxidation state of large subunit of RNR in response to oxidative stress (Camier *et al.*, 2007, Chapter 3 – section 3.2.6). Therefore, it was possible that the hyperpolarized buds formed in response to H_2O_2 treatment and upon deletion of *TRX1*, could be a result of inactivation of RNR under these conditions. As showed previously, consistent with defects in the DNA-replication stress pathway, *trx1*Δ

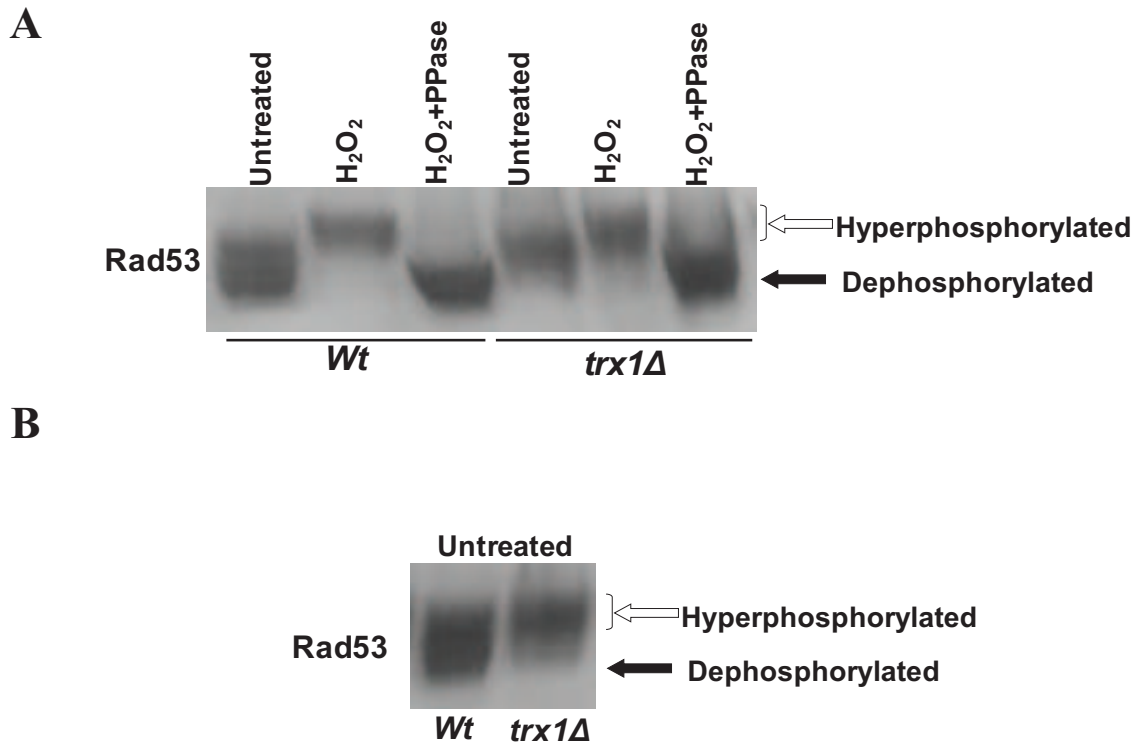

Figure 5.8. The checkpoint kinase Rad53 is activated in *trx1*Δ cells and in wild-type cells following H_2O_2 treatment.
(A) Rad53 is hyperphosphorylated in response to H_2O_2 and in unstressed cells lacking Trx1. Wild-type (Wt, JC894) and *trx1*Δ (JC866) cells expressing myc-6His tagged Rad53 were untreated or treated with 5 mM H_2O_2 for 2 h. Lysates were analyzed by western blot either before or after treatment with λ-phosphatase. Rad53 was detected using an anti-myc antibody. (B) The mobility of Rad53 under non-stressed conditions in wild-type and *trx1*Δ cells was also compared side by side to emphasis the slower mobility of Rad53 in cells lacking *TRX1*.

cells displayed sensitivity to HU treatment when compared to wild-type cells (Figure 3.4). Furthermore, ectopic expression of *RNR1*, which codes for the large subunit of RNR (see chapter 3, topic 3.2.6 for details), in *trx1*Δ cells rescues the HU sensitivity of this mutant (Figure 3.4). However, ectopic expression of *RNR1* in *trx1*Δ cells did not inhibit the filamentous phenotype associated with loss of Trx1 (Figure 5.9B). Similarly, ectopic expression of *RNR1* in wild-type cells did not inhibit HU-induced filamentation (Figure 5.9C). However, in contrast to that seen with *trx1*Δ cells, ectopic expression of *RNR1* in wild-type cells did not increase resistance to this drug (Figure 5.9A). This could be explained by the fact that RNR activity in wild-type cells is not impaired and that a close homologue of *RNR1*, *RNR3,* is up-regulated in wild-type cells in response to HU (Bachewich *et al.*, 2005).

As the *C. albicans* genome contains a second gene that also encodes a large subunit of RNR, *RNR3,* this raised the possibility that Rnr3 could be the target of Trx1 which regulates H_2O_2-induced filamentation. However, in contrast to that seen with *RNR1,* ectopic expression of *RNR3* in *trx1*Δ cells did not rescue the HU sensitivity of this mutant (Figure 5.9A). As expected, therefore, ectopic expression of *RNR3* had no impact on the morphology of *trx1*Δ cells (Figure 5.9B). Furthermore, wild-type cells ectopically expressing *RNR3* did not exhibited decreased filamentation in response to HU (Figure 5.9C). Collectively, these results suggest that peroxide-induced activation of the Rad53 pathway, which is dependent on Trx1 and leads to polarized cell growth, is independent of Trx1 regulation of the large subunit of RNR.

5.3. Discussion

The data presented in this chapter illustrates that the filamentous growth form induced by H_2O_2 treatment in *C. albicans* has all the characteristics of hyperpolarized buds, such as nuclear migration from mother to daughter cells and constriction at the septum separating the mother cell from the polarised bud (Figure 5.1 - Whiteway and Bachewich, 2007). As hyperpolarized bud formation has only been shown previously to be induced in response to chemicals such as HU or MMS, or mutations that perturb cell cycle progression (Bachewich *et al.*, 2003 and Shi *et al.*, 2007), the *in vivo* relevance of this type of growth was unclear. Significantly, the data presented in this chapter illustrate that a physiologically relevant stress, imposed by treatment with the oxidative stress inducing agent H_2O_2, also induces hyperpolarized growth in *C. albicans*. This fungal pathogen undoubtedly encounters ROS

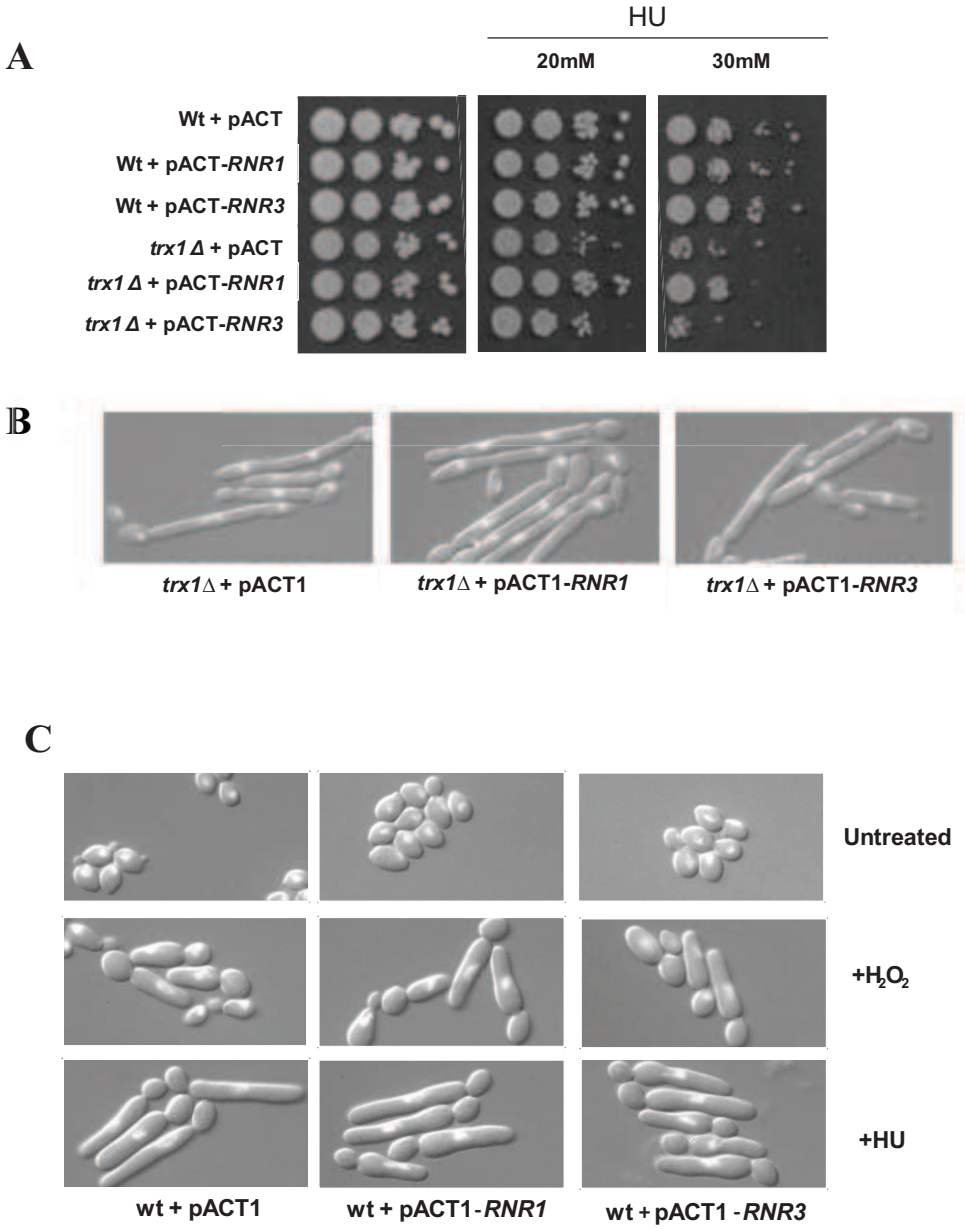

Figure 5.9. Trx1-mediated polarized cell growth is independent of Rnr.
(A) *trx1*Δ cells display increased sensitivity to HU and this can be rescued by ectopic expression of *RNR1*. Approximately 10^3 cells, and 10-fold dilutions thereof, of mid-exponential-growing wild-type (wt) and *trx1*Δ cells containing either pACT1 (JC1014 and JC1022, respectively), pACT1-*RNR1* (JC1016 and JC1024, respectively) or pACT1-*RNR3* (JC1160 and JC1162, respectively) were spotted onto YPD plates with or without the indicated HU concentrations and incubated at 30°C for 24 h. (B) Ectopic expression of either *RNR1* or *RNR3* (B) does not either reverse the polarized cell growth phenotype of *trx1*Δ cells (upper panel) or (C) inhibit H_2O_2- or HU-induced polarized cell growth in wild-type cells (bottom panel). Micrographs of wild-type (wt) cells were taken after treatment with 5 mM H_2O_2 or 50 mM HU for 6 h. Images were captured as described in Figure 5.1.

such as H_2O_2 during infection. Therefore H_2O_2-induced polarized bud growth may allow migration and escape from ROS-rich environments promoting survival in the host. There is also evidence that ROS play some role in the formation of serum-induced hyphae in *C. albicans* (Schröter et al., 2000 and Nasution *et al.*, 2008) in addition to their role in stimulating hyperpolarized bud formation described in this chapter. How such ROS are generated, however, remains elusive as the *C. albicans* genome (www.candidagenome.org) does not contain any apparent homologue of the NOX enzymes found in filamentous fungi (reviewed in Scott and Eaton, 2008). Furthermore, if ROS are key in triggering hyphal growth in *C. albicans*, this would appear to be via a mechanism independent of Trx1 as hyphal formation is not impaired in *trx1*Δ cells (Figure 5.6).

With regard to the mechanism of H_2O_2 induced hyperpolarised growth, the data presented is consistent with a model in which Trx1 functions as an inhibitor of this process, and H_2O_2- induced oxidation and inactivation of Trx1 allows for the induction of H_2O_2 induced filamentation. In addition, it appears that Trx1 is specifically involved in negatively regulating H_2O_2-induced hyperpolarised bud formation, as *trx1*Δ cells are resistant to further filamentation following H_2O_2 treatment yet display considerable elongation in response to HU (Figure 5.2). The model that H_2O_2-induced inactivation of Trx1 is key to hyperpolarised growth is further supported by the findings that deletion of *TRX1* or mutation of *trx1* cysteine residues results in a phenotype similar to the one seen upon treatment with H_2O_2 (Figure 3.10). In addition, the fact that oxidation of Trx1 is required for hyperpolarized growth formation is further evidenced by the prolonged filamentous growth and sustained oxidation of Trx1 induced by treatment of wild-type cells with higher H_2O_2 concentrations (5 mM) compared to lower levels of H_2O_2 (0.5 mM) (Figure 5.1). Significantly, ectopic expression in wild-type cells of *TRR1*, the enzyme that reduces oxidized Trx1, results in the inhibition of H_2O_2-induced, but not HU-induced polarized growth (Figure 5.4).

With regard to the mechanism of Trx1 mediated H_2O_2-stimulated filamentous growth, the data presented in this chapter indicate that it is unlikely to be due to Trx1-dependent regulation of Tsa1, Hog1 or Cap1, as cells lacking these proteins exist largely in the budding yeast form under non-stressed conditions, and all form hyperpolarized buds following H_2O_2 treatment. Another class of proteins that could be a target of Trx1 includes the morphogenetic regulators Efg1 and Cph1, since they are positive regulators of hyphal development (Brown and Gow, 1999 and Ernst, 2000). Therefore, Trx1 could be acting as a

negative regulator of these proteins in *C. albicans.* However, in contrast to the lack of hyphae formation seen in the double *efg1*Δ*/cph1*Δ strain under inducing conditions (Lo *et al.*, 1997 and Braun and Johnson, 2002), H_2O_2 induced hyperpolarized bud formation is not impaired (Figure 5.6A). This is consistent with the observation that the filamentous forms seen upon H_2O_2 treatment are distinct from hyphae, and that *trx1*Δ cells can form hyphae under hyphae-inducing conditions such as 10% serum at 37ºC (Figure 5.6B).

Instead, evidence is provided that Trx1-mediated H_2O_2-induced polarized cell growth involves activation of the Rad53 DNA checkpoint kinase. *C. albicans* Rad53 is activated in response to H_2O_2 treatment, which is similar to that previously reported in the model yeast *S. cerevisiae,* and in mammalian cells, in which the DNA checkpoint kinases (Rad53 and Chk2, respectively) are also activated in response to oxidative stress (Leroy *et al.*, 2001 and Abdelmohsen *et al.*, 2007). Furthermore, ectopic expression of Trx in mammalian cells inhibits the phosphorylation of the *C. albicans* Rad53 homologue Chk2 in response to oxidative stress (Muniyappa *et al.*, 2009). A model by which Rad53 is activated in response to H_2O_2, therefore resulting in formation of hyperpolarized buds in *C. albicans*, is illustrated in Figure 5.10. In this model, under normal conditions reduced Trx1 inhibits activation of the Rad53 DNA checkpoint kinase, either directly or via inhibition of upstream members of the Rad53 pathway. However, when cells are treated with H_2O_2, Trx1 becomes oxidized and thus inactivated, and the inhibitory effects of Trx1 on Rad53 phosphorylation are lifted. This is supported by data in this chapter in which it is shown that Rad53 is hyperphosphorylated in response to H_2O_2 treatment and in unstressed *trx1*Δ cells (Figure 5.8), and by the fact that H_2O_2-induced hyperpolarized bud formation is completely abolished in *rad53*Δ cells (Figure 5.7). In order to directly investigate whether the formation of hyperpolarized buds seen in *trx1*Δ cells is dependent on Rad53, several attempts were made to create a double *trx1*Δ*/rad53*Δ strain. Unfortunately, all such attempts failed which is suggestive of synthetic lethality and that Rad53 function is essential for viability in the event that Trx1 function is lost or *vice versa*.

The findings in *C. albicans* that depletion of RNR stimulated Rad53 activation and hyperpolarized growth (Shi *et al.*, 2007), together with previous work which identified RNR as a substrate for thioredoxin (See Chapter 3, section 3.2.6), suggested that Trx1- and H_2O_2-dependent polarized cell growth may be due to inactivation of RNR. Indeed, *C. albicans trx1*Δ cells are sensitive to the RNR inhibitor HU (Figure 3.4) and ectopic expression of

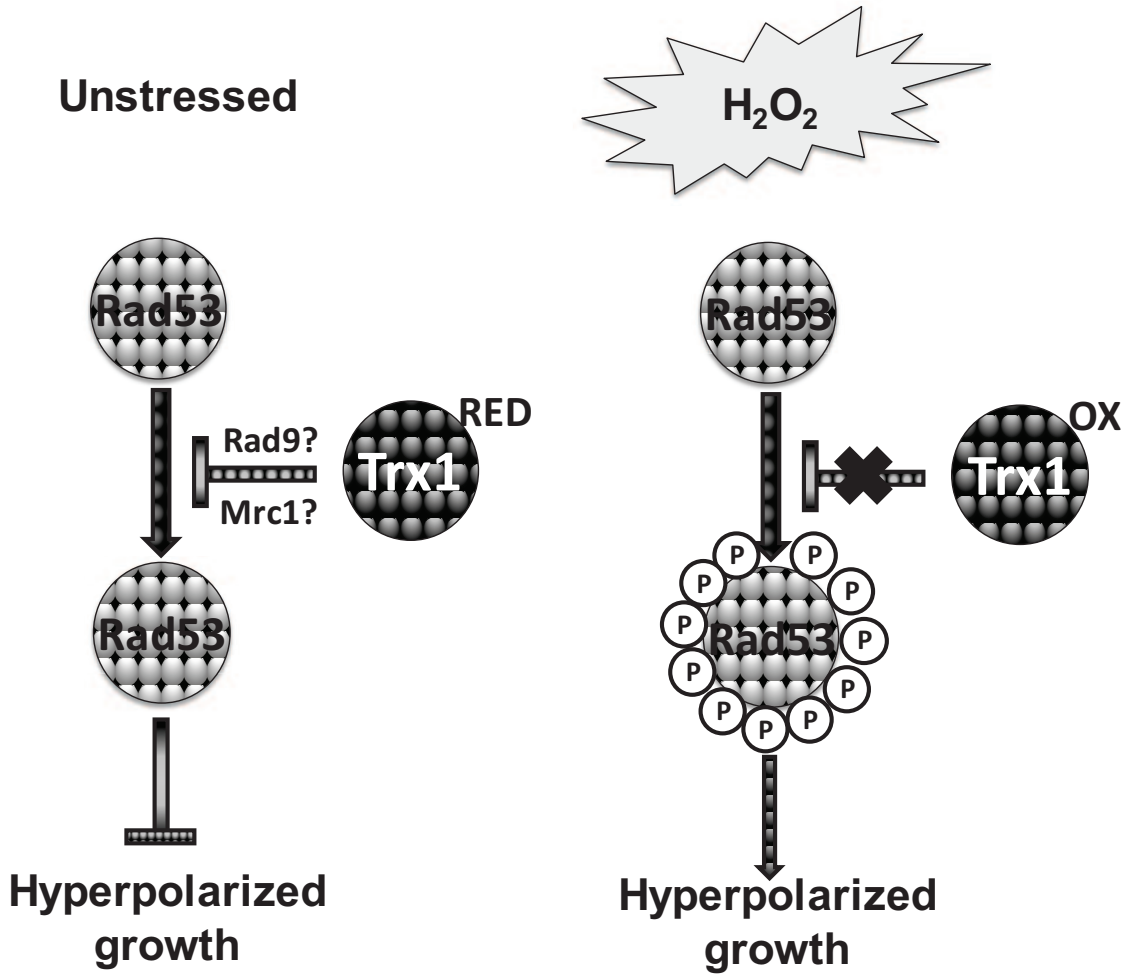

Figure 5.10. Model depicting the possible role of Trx1 in H_2O_2-induced polarized growth in *C. albicans*.

In the model under non-stressed conditions, Trx1 negatively regulates Rad53 activation, therefore inhibiting polarized cell growth. However, oxidation of Trx1 results in hyperphosphorylation and therefore activation of Rad53, resulting in hyperpolarized cells growth.

RNR1 partially rescued the increased sensitivity to HU exhibited by the *trx1*Δ mutant. However, ectopic expression of *RNR1* did not reverse or inhibit the formation of hyperpolarized buds characteristic of *trx1*Δ cells. This indicates that the hyperpolarized buds formed upon inactivation of *TRX1* are not due to a defect in Trx1-dependent regulation of RNR function (Figure 5.9). This is further supported by the observation that *trx1*Δ cells display considerable further elongation in response to the RNR inhibitor HU (Figure 5.4A).

Studies in *S. cerevisiae* have illustrated that phosphorylated Rad53 activates the DNA checkpoint either in response to DNA-damage or incomplete replication, therefore halting cell cycle progression until the DNA damage or replication defect is repaired (reviewed by Branzei and Foiani, 2006 and Pellicioli and Foiani, 2005). In *C. albicans,* the Rad53 DNA checkpoint kinase receives DNA-replication and DNA-damage signals via the signal transducers Mrc1 or Rad9 respectively (Figure 5.10 - Shi *et al.*, 2007). Deletion of *RAD9* resulted in cells that were sensitive to the DNA damaging agent MMS, and failed to arrest the cell cycle at G2/M upon MMS treatment. In contrast, *rad9*Δ cells did not display sensitivity to the replication poison HU, and displayed normal HU mediated cell-cycle arrest. Consistent with these findings, *rad9*Δ cells displayed filamentous growth in response to HU, yet in response to MMS hyperpolarized bud formation was abolished. These findings show that Rad9 is essential for the relay of DNA damage signals to Rad53 (Shi *et al.*, 2007). In contrast, deletion of *C. albicans MRC1* results in cells that were sensitive to both HU and MMS. Consistent with findings in *S. cerevisiae* (Katou *et al.*, 2003), *C. albicans mrc1*Δ cells displayed a slower S phase indicating that Mrc1 is involved in DNA-replication checkpoint activation (Shi *et al.*, 2007). Furthermore, Rad53 is hyperphosphorylated in *mrc1*Δ cells under non-stressed conditions and displays constitutively filamentous growth (Shi *et al.*, 2007).

Although Trx1 mediated regulation of Rad53 appears to be independent of RNR, it is not yet known whether Trx1 is involved in either the DNA damage or DNA replication checkpoints that converge on Rad53. It is noteworthy that deletion of *MRC1* or *TRX1*, both results in hyperphosphorylated Rad53 and hyperpolarized bud growth. With regard to Mrc1, this is suggested to be due to activation of the DNA-damage checkpoint (Shi *et al.*, 2007), as in *S. cerevisiae* DNA-damage-like structures are generated during DNA synthesis in *mrc1*Δ cells (Katou *et al.*, 2003). Furthermore, *C. albicans* cells treated with H_2O_2, displayed a cell cycle arrest (G2/M phase) (Phillips *et al.*, 2003) similar to the one seen in *C. albicans* cells

treated with MMS, which is Rad9 dependent (Shi *et al.*, 2007). Therefore, it is possible that Trx1 inhibition of Rad53 activation in response to H_2O_2 is mediated through the DNA-damage checkpoint, however this hypothesis remains to be investigated. Alternatively, Trx1 could be directly regulating Rad53 itself, although preliminary experiments indicate that Rad53 is not oxidized following H_2O_2 (data not shown). Similar to *S. cerevisiae* Rad53, the analogous protein in *C. albicans* has a central kinase domain that is flanked on each side by an FHA (forkhead-associated) domain, FHA1 and FHA2 (Shi *et al.*, 2007). FHA domains are phosphopeptide recognition domains found in many regulatory proteins that recognise both phosphothreonine and phosphotyrosine-containing epitopes and have been shown to have specific role in activation of this protein in addition to specific roles in the activation of Rad53 downstream effectors (Durocher *et al.*, 1999 and Tam *et al.*, 2008). Interestingly, the two FHA domains of *C. albicans* Rad53 seem to promote interaction with different upstream and downstream targets of Rad53 since mutation of residues in these FHA domains resulted in the inhibition of specific Rad53 functions (Shi *et al.*, 2007). Hence one further possible avenue of investigation would be to combine the *trx1*Δ mutant with specific Rad53 FHA domain mutations, to further dissect the mechanism of Trx1 mediated Rad53 regulation.

In summary, the data presented in this chapter indicate that H_2O_2-mediated activation of the DNA checkpoint pathway leads to filamentous growth, and that H_2O_2-induced inactivation of Trx1 is an important trigger in this process. In this regard it is interesting to note that ROS-stimulated polarized cell growth has been documented in several filamentous fungi (Takemoto *et al.*, 2006, Tanaka *et al.*, 2006 and Semighini and Harris, 2008). Although it is known that many of these filamentous fungi express NADPH oxidases or NOX complexes, which results in the localized production of ROS that act to regulate hyphal growth (reviewed in Scott and Eaton, 2008), the means by which these fungal cells sense and initiate polarized growth in response to ROS is unknown. Significantly, the data presented in this chapter, may provide major insight into this process by revealing that the oxidation of an evolutionarily conserved antioxidant protein, thioredoxin, is a key signalling event in H_2O_2-induced polarized cell growth in *C. albicans*. Excitingly, thioredoxin has also been implicated in cell differentiation in other fungi, since deletion of a thioredoxin homologue in both *A. nidulans* and *P. anserina*, results in defects in cell differentiation that ranges from defects in fruiting body formation to reduced hyphal growth (Thön *et al.*, 2007 and Malagnac

et al., 2007). However, the role of thioredoxin in directly regulating ROS-induced differentiation in filamentous fungi has yet to be addressed.

Chapter 6. Final Discussion

6.1. Summary

The ability of the major systemic fungal pathogen of humans, *C. albicans*, to survive the ROS generated by the immune system of the host is essential for virulence (reviewed in Brown *et al*., 2009). Despite this, little is known regarding the role and regulation of oxidative stress- responsive signalling pathways in this medically relevant fungus. Redox sensitive antioxidant proteins have recently been implicated in oxidative stress signalling in both model yeast and mammalian cells, by regulating the function of stress signalling proteins (reviewed in Veal *et al*., 2007). Hence, the main aim of this thesis was to investigate the potential roles of such redox sensitive antioxidant proteins, the thioredoxins and 2-Cys peroxiredoxins, in oxidative stress sensing and signalling in *C. albicans*. The data presented in this thesis revealed that the thioredoxin protein Trx1 has a multiplicity of roles in the regulation of cellular responses to H_2O_2 in this fungal pathogen. For example, Trx1 functions as an antioxidant by regulating the reduction and thus activity of the 2-Cys peroxiredoxin Tsa1, which detoxifies H_2O_2. In addition, data is shown illustrating that Trx1 also regulates two distinct H_2O_2-responsive signalling proteins in *C. albicans*; the Hog1 SAPK and Rad53 DNA checkpoint kinase. Significantly, evidence is also provided that Trx1-mediated regulation of Rad53 is an important mechanism underlying H_2O_2-induced polarized cell growth in *C. albicans*. A concurrent PhD project in the laboratory, performed by Miranda Patterson, further demonstrated that Trx1 also regulates the Cap1 AP-1-like transcription factor. Although Trx1 regulates three distinct H_2O_2-responsive pathways in *C. albicans*, this is probably by different mechanisms. As illustrated in the model presented in Figure 6.1, Trx1 acts as an inhibitor of both the H_2O_2-induced activation of Rad53 and Cap1, but functions as an activator of Hog1. Data are also presented that the Trx1 substrate, the 2-Cys peroxiredoxin Tsa1, also regulates H_2O_2-induced activation of the Hog1 SAPK. Consistent with these key central roles of Trx1 in oxidative stress responses and the relationship between ROS and pathogenesis, *C. albicans* cells lacking Trx1 displayed significantly attenuated virulence in a mouse model of infection (see Section 6.5).

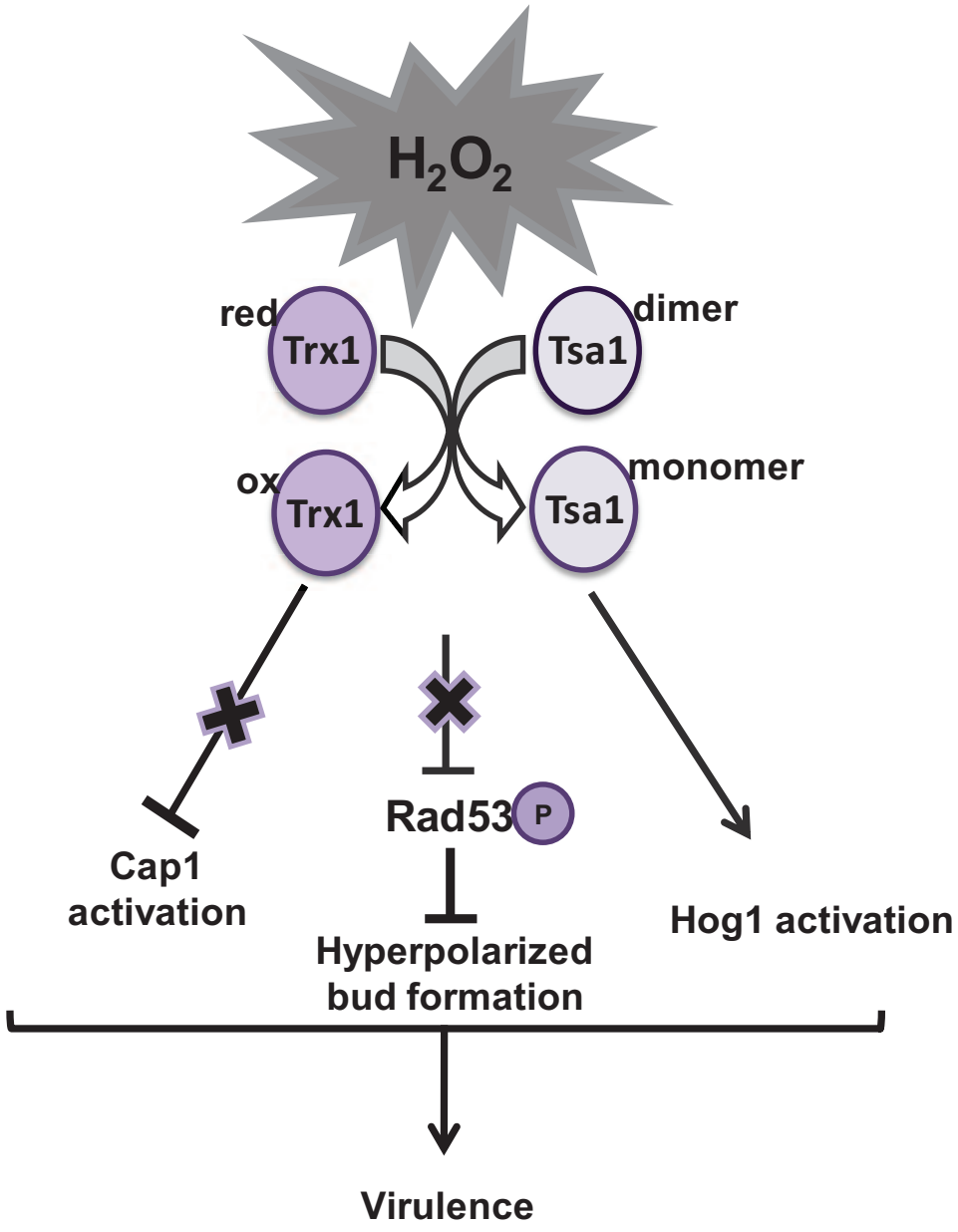

Figure 6.1. Model depicting the multiples roles of Trx1 in H_2O_2-signaling in *C. albicans*.
Trx1 negatively regulates Cap1 activation and polarized cell growth, yet positively regulates the Hog1 SAPK. Upon exposure to H_2O_2, Trx1 becomes oxidized by reducing oxidized substrates such as the 2-Cys peroxiredoxin Tsa1, and no longer can inhibit Cap1 activation and polarized cell growth. However, in contrast, Trx1 and Tsa1 are both required for the activation of Hog1. One model is that Trx1 function acts to prevent Tsa1 becoming trapped in a form (dimeric) that is unable to activate Hog1, although it is also possible that Trx1 and Tsa1 function independently to regulate H_2O_2 induced activation of Hog1. These multiple roles of Trx1 in regulating oxidative stress signaling in *C. albicans* is consistent with the significantly attenuated virulence displayed by *trx1*Δ cells in a mouse model of disease.

6.2. Trx1 is the main thioredoxin in *C. albicans*

Similar to that reported in the model yeast *S. cerevisiae*, the *C. albicans* genome contains two genes, *TRX1* and *TRX2*, encoding putative cytoplasmic/nuclear thioredoxins. However, in contrast to the situation in *S. cerevisiae* in which Trx1 and Trx2 are largely functionally redundant, in *C. albicans* Trx1 appears to provide all of the cytoplasmic/ nuclear thioredoxin function in this fungus. Indeed, deletion of *TRX1* in *C. albicans* results in phenotypes only seen upon deletion of both *TRX1* and *TRX2* in *S. cerevisiae*, such as slow growth, sulphur-containing amino acid auxotrophy, and oxidative stress sensitivity (Garrido and Grant, 2002 and Muller, 1991). This could either be due to the higher levels of Trx1, compared to Trx2, in both unstressed cells and cells treated with H_2O_2 (Enjalbert *et al.*, 2006 and Michán and Pueyo, 2009), or it could result from differences in the biochemical activity of Trx1 and Trx2 in *C. albicans*. Although the oxidoreductase activity of these proteins has not been measured directly, it is noted that Trx2 does not contain the highly conserved thioredoxin motif which surrounds the catalytic cysteine residues (Trp-Cys-Gly-Pro-Cys). Clearly, further investigation would be needed to ascertain whether Trx2 is catalytically active but, nonetheless, this protein does not appear to make a major contribution to thioredoxin function in *C. albicans*. Preliminary data, however, revealed that deletion of *TRX2* in *C. albicans* (Dantas and Quinn- unpublished) results in cells that are slightly more sensitive to H_2O_2 than wild-type cells, yet a double *trx1*Δ*trx2*Δ strain showed only a minimal increase in peroxide sensitivity when compared with the *trx1*Δ cells (Figure 6.2). However, further experimentation is needed with these strains to clarify whether deletion of *TRX2* impacts on any of the other phenotypes described for *trx1*Δ cells.

In *S. cerevisiae*, the slow growth phenotype of *trx1*Δ*trx2*Δ cells is associated with Trx1 and Trx2 regulating the activity of RNR, the enzyme required for dNTP synthesis (Camier *et al.*, 2007), whereas the methionine/cysteine auxotrophy is due to Trx1 and Trx2 regulating the enzyme PAPS reductase which is necessary for sulfite production, a necessary precursor for cysteine biosynthesis (Chartron *et al.*, 2007). Although, it has not been shown directly in this thesis that Trx1 regulates RNR and PAPS reductase in *C. albicans*, evidence is provided that this is likely the case. For example, *trx1*Δ cells are sensitive to HU, which is a known inhibitor of RNR, and ectopic expression of the large subunit of RNR, *RNR1*, rescues the HU sensitivity exhibited by *trx1*Δ cells. In addition, Trx1 likely regulates PAPS reductase, since addition of the product of this enzyme, sulfite, rescues the cysteine/methionine auxotrophy of

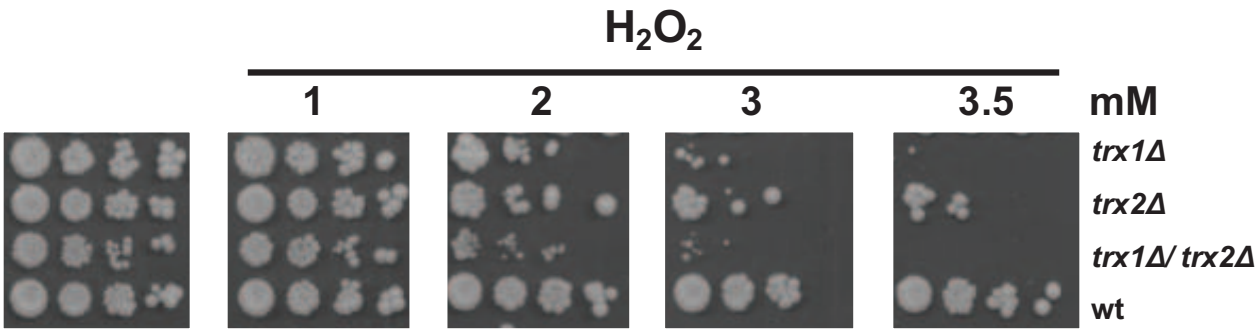

Figure 6.2. *C. albicans trx2*Δ cells are less sensitive to H_2O_2 than *trx1*Δ cells.
*C. albicans trx1*Δ (JC488), *trx2*Δ (JC1274), *trx1*Δ/*trx2*Δ (JC1286) and wild-type (SN148) cells were grown to mid-log phase and 10-fold serial dilutions plated onto agar plates containing the indicated concentrations of H_2O_2. Plates were then incubated at 30ºC for 2 days.

*trx1*Δ cells. However, data is presented in this thesis that *C. albicans* Trx1 directly regulates the reduction and re- activation of the 2-Cys peroxiredoxin Tsa1 and this likely contributes to the oxidative stress sensitivity exhibited by *trx1*Δ cells. For example, Tsa1 is mainly found in the oxidized dimeric state in *trx1*Δ cells and in cells expressing *trx1* cysteine mutants. Furthermore, Tsa1 is clearly an important antioxidant in *C. albicans,* as *TSA1* is highly up-regulated in response to H_2O_2 treatment (Enjalbert *et al.*, 2006), and *tsa1*Δ cells display increased sensitivity to H_2O_2 compared to wild-type cells as previously reported (Urban *et al.*, 2005 and Shin *et al.*, 2005).

Interestingly, bioinformatic analysis indicates that *C. albicans* does not have a mitochondrial thioredoxin system, since neither thioredoxins Trx1 and Trx2, or the thioredoxin reductase Trr1, contain recognisable mitochondrial target sequences. This contrasts to that in both model yeasts *S. cerevisiae* and *S. pombe*, which have a mitochondrial thioredoxin (Pedrajas *et al.*, 1999 and Song *et al.*, 2006). Therefore, it is possible that in *C. albicans*, Trx1 is responsible for maintaining redox homeostasis in the cytosol, while glutathione reductase (Glr1) and the GSH-glutaredoxin system could be responsible for maintaining the redox homeostasis in the mitochondria. Sequence analysis revealed that *C. albicans* has two putative mitochondrial glutaredoxins (*orf19.2782* and *orf19.6059*) and that *GLR1* also has a mitochondrial leader sequence. Therefore it is possible that the glutaredoxin system plays a major role in mitochondrial redox homeostasis in *C. albicans*, although further investigation is required to test this.

In addition to the known or predicted substrates of *C. albicans* Trx1, it is likely that other previously uncharacterized substrates exist, in particular those involved in the oxidative stress-signalling functions of Trx1. Such substrates could be identified by combining "thiol-trapping" and tandem affinity purification (TAP) methodologies. "Thiol-trapping" occurs by mutating the second catalytic cysteine residue of Trx1 (Cys33Ser) which 'traps' Trx1 in an intermediate mixed disulphide with substrate proteins (Vignols *et al.*, 2005). This technique was successfully applied in organisms such as the model yeast *S. cerevisiae* (Vignols *et al.*, 2005), the photosynthetic organism *Chlamydomonas reinhardtii* (Lemaire *et al.*, 2004) and mammalian cells (Nadeau *et al.*, 2007), and allowed for the identification of known targets such as peroxiredoxins, thioredoxin reductases, PAPS reductase and the ASK1 MAPKKK, and novel targets such as adenylate kinase, methionine sulfoxide reductase (MSR), and molecular chaperones (Lemaire *et al.*, 2004, Vignols *et al.*, 2005, Nadeau *et al.*, 2007 and

2009). MSR reduces oxidized methionine residues in proteins in a reaction that results in MSR itself becoming oxidized (Lowther *et al.*, 2002 and Le *et al.*, 2009). The thioredoxin system or the GSH-glutaredoxin system can subsequently reduce the oxidized MSR (Olry *et al.*, 2004 and Tarrago *et al.*, 2009). As oxidation of methionine residues can induce loss and regulation of protein function, this oxidative stress-induced modification could also potentially be exploited in cell signalling (reviewed by Hoshi and Heinemann, 2001 and Emes, 2009). Bioinformatic analysis showed that the *C. albicans* genome contains two *MSR* genes, so it is possible that Trx1 also regulates MSR proteins in this fungus.

With regard to other potential peroxiredoxin substrates for Trx1, bioinformatic analysis indicates that *C. albicans* also expresses the cytoplasmatic 1-Cys peroxiredoxins Dot5 and Trp99, the mitochondrial 1-Cys peroxiredoxin Prx1 and the atypical 2-Cys peroxiredoxins Ahp1 and Ahp2. However, their role in the antioxidant defence of this fungal pathogen and the role of thioredoxin in regulating their function remain to be elucidated. Studies in *S. cerevisiae* have shown that although Tsa1 is the main peroxiredoxin involved in H_2O_2 detoxification, the atypical 2-Cys peroxiredoxin Ahp1 is the main peroxiredoxin involved in detoxifying the hydroperoxide *t*-BOOH (Park *et al.*, 2000). Further characterization of *C. albicans* Ahp1 is warranted as *AHP1* was identified as a core stress gene in this fungal pathogen (Enjalbert *et al*., 2006). In summary, therefore, thiol-trapping could be a useful approach to identify further Trx1 substrates in *C. albicans*, which may provide significant insight into the signalling functions of this protein.

6.3. Trx1 and Tsa1 regulate the peroxide-induced activation of Hog1

Previous work in mammals and the model yeast *S. pombe*, demonstrated that thioredoxin or 2-Cys-peroxiredoxins respectively, regulate the oxidative stress-induced activation of the SAPK pathways in these organisms (Saitoh *et al*., 1998 and Veal *et al*, 2004). Based upon these findings, an investigation into the role of the thioredoxin Trx1, and the 2-Cys peroxiredoxin Tsa1, in H_2O_2-induced activation of the *C. albicans* Hog1 SAPK was performed. As reported in *S. pombe*, where the 2-Cys peroxiredoxin Tpx1 positively regulates the Sty1 SAPK pathway in response to H_2O_2, (Veal *et al*, 2004), Tsa1 also positively regulates H_2O_2-induced Hog1 activation in *C. albicans*. However, the mechanism by which Tsa1 regulates Hog1 may differ from Tpx1-mediated regulation of Sty1. For example, mutation of either catalytic cysteine residue of Tsa1 does not significantly impair

Hog1 activation in response to peroxide stress. This contrasts with work in *S. pombe* that demonstrated that the peroxidatic cysteine residue of Tpx1 was essential for H_2O_2-induced activation of Sty1 (Veal *et al*, 2004). Although the precise mechanism of Tsa1-mediated regulation of Hog1 is not yet known, it is interesting to speculate that this may be related to the H_2O_2-concentration dependent regulation of the redox status of Tsa1. For example, Hog1 is not significantly activated in *C. albicans* until cells are exposed to approximately 2 mM levels of H_2O_2. This correlates with Tsa1 switching from an oxidized dimeric form produced at lower levels of H_2O_2, to an over-oxidised monomeric form which is generated following treatment of cells with >2 mM H_2O_2. This monomeric form of Tsa1 is a result of overoxidation of the peroxidatic cysteine to the sulphinic acid (SO_2H) form. Therefore upon exposure of *C. albicans* to lower H_2O_2 concentrations, Tsa1 is active as a peroxidase, however when exposed to higher H_2O_2 concentrations Tsa1 is inactivated, and it is possible that only this form is competent to activate Hog1. Consistent with this, deletion of the *C. albicans* sulfiredoxin gene, *SRX1*, results in both prolonged overoxidation of Tsa1, and prolonged Hog1 activation, in response to H_2O_2. In this regard it is interesting that overoxidized peroxiredoxins have been demonstrated to function as molecular chaperones (Jang *et al.*, 2004). In response to an acute oxidative stress, the *S. cerevisiae* 2-Cys peroxiredoxin Tsa1 forms high molecular weight (HMW) oligomeric complexes when its peroxidatic cysteine is oxidized to sulphonic (SO_3H) acid. These HMW complexes act as effective and stable molecular chaperones *in vivo* protecting proteins against oxidative-stress induced protein denaturation (Trotter *et al.*, 2008 and Lim *et al.*, 2008). A similar mechanism may also underlie Tsa1 mediated Hog1 activation in *C. albicans* in which Tsa1 could function as a molecular chaperone for Hog1. Alternatively, dimeric Tsa1, which is formed at lower H_2O_2 concentrations, inhibits Hog1 activation and that this inhibition is overcome at higher H_2O_2 due to the formation of over-oxidised monomeric Tsa1. Indeed, it is possible that Tsa1 simply needs to be in a monomeric state, whether over-oxidised or not, to activate Hog1 as significant levels of Hog1 activation were achieved in cells expressing the monomeric cysteine mutants of Tsa1. However, irrespective of the precise mechanism by which Tsa1 regulates Hog1, Tsa1 may act as a molecular switch regulating the H_2O_2 concentration dependency of Hog1 activation. This molecular switch is dependent on the H_2O_2-concentration dependent overoxidation and inactivation of 2-Cys peroxiredoxins. In *S. pombe*, Tpx1 acts as a molecular switch mediating Pap1 activation at low levels of peroxide

stress and Sty1/Atf1 mediated responses at high levels of H_2O_2 and this is due to the dependency of Sty1, but not Pap1, on active Tpx1(Veal *et al.*, 2004, Bozonet *et al.*, 2005 and Vivancos *et al.*, 2005).

In addition to Tsa1 positively regulating the H_2O_2-dependent activation of Hog1, data presented in this thesis provides the first report that thioredoxin is also required for the oxidative stress-induced activation of a fungal SAPK. Furthermore, the result that Trx1 is critical for the H_2O_2-induced activation of Hog1 is in striking contrast to that described in mammalian systems, in which thioredoxin functions as a repressor of the analogous JNK and p38 SAPK pathways (Saitoh *et al.*, 1998). The molecular basis underlying the apparently opposing roles of thioredoxin regulation of *C. albicans* and mammalian SAPK pathways is unclear. The upstream Ask1 MAPKKK in the mammalian SAPK pathways is activated via cysteine oxidation and, moreover, Trx1 negatively regulates this pathway by reducing the oxidized cysteines of Ask1 (Nadeau *et al.*, 2007 and 2009). As Trx1 is a positive regulator of the Hog1 SAPK in *C. albicans* it seems unlikely that a similar mechanism is in place in this fungus. Instead, it is tempting to speculate that, as the Trx1 substrate Tsa1 is also required for Hog1 activation, that Trx1 regulates Hog1 through regulating the redox status of Tsa1. Indeed, Tsa1 is largely trapped in the oxidised dimeric form in cells lacking *TRX1* and, as discussed above, Hog1 does not appear to be activated under conditions in which dimeric Tsa1 is formed. Furthermore, data presented in this thesis indicate that Trx1 function is necessary for the formation of the monomeric overoxidized form of Tsa1, which may be necessary for Hog1 activation. This also indicates that reduction of the Tsa1 dimer is a necessary prerequisite for overoxidation to proceed. Indeed, previous work on the mammalian 2-Cys peroxiredoxins, Prx1 and Prx2, demonstrated that thioredoxin function is required for the monomeric reduced form of these enzymes to become overoxidized (Yang *et al.*, 2002 and Low *et al.*, 2007). To summarise, both Trx1 and Tsa1 are crucial for the H_2O_2-induced activation of the Hog1 SAPK in *C. albicans.* However, whether Trx1 and Tsa1 function in the same pathway, or independently, to relay H_2O_2-signals to Hog1 remains to be determined. It is also not known at which point in the SAPK pathway Tsa1 and Trx1 function. For example, the response regulator Ssk1, which is predicted to regulate the *C. albicans* MAPKKK Ssk2, is essential for H_2O_2-induced activation of Hog1 (Chauhan *et al.*, 2003). However, it remains to be established whether Trx1 functions in the same pathway as Ssk1 to relay H_2O_2 signals to Hog1. Nonetheless, it is important to delineate the oxidative

stress regulation of Hog1 in *C. albicans*, as deletion of *HOG1* in this fungal pathogen results in oxidative stress sensitivity, reduced survival following phagocytosis and reduced virulence (Alonso-Monge *et al.*, 1999 and Arana *et al.*, 2007). Such results are consistent with the oxidative stress-induced activation of Hog1 being important for survival of *C. albicans* in the host. Furthermore, as SAPKs, thioredoxins, and 2-Cys peroxiredoxins are highly conserved in eukaryotes, the delineation of H_2O_2-induced activation of *C. albicans* Hog1 may provide insight into the redox regulation of SAPK pathways in other fungi and possibly other eukaryotic systems.

6.4. Trx1 regulates H_2O_2-induced hyperpolarized bud formation by regulating the Rad53 DNA checkpoint pathway

In this thesis, data is presented that either inactivation of *TRX1*, or treatment of cells with H_2O_2, stimulates the formation of hyperpolarized buds. This is a recently characterized filamentous form in *C. albicans* that is distinct from pseudohyphae and hyphae (reviewed in Whiteway and Bachewich, 2007). Significantly, the fact that H_2O_2 stimulates hyperpolarised bud formation provides the first evidence that a physiologically relevant condition induces this filamentous form of growth in *C. albicans,* as this had only previously been observed in response to either mutations or chemicals that perturb cell cycle progression (Bachewich *et al.*, 2005 and Shi *et al.*, 2007). However, *C. albicans* encounters H_2O_2 during infection, thus H_2O_2-induced polarized bud growth may allow migration and escape from ROS-rich environments promoting survival in the host. Indeed, upon phagocytosis by macrophages *C. albicans* forms filaments that allow it to penetrate and escape from the macrophage (Lorenz *et al.*, 2004). Thus it is possible that ROS-induced polarized cell growth may be triggered upon phagocytosis. However, there is evidence that this is not the case. For example, the filaments formed during phagocytosis resemble true hyphae, and inactivation of the major hyphae-regulators Efg1 and Cph1 abolishes filamentation within the macrophage (Lorenz *et al.*, 2004). In contrast, deletion of *EFG1* and *CPH1* has no impact on H_2O_2-induced hyperpolarized bud formation. Instead, data presented in this work revealed that the oxidation of Trx1 is a key signalling event in H_2O_2-induced polarized cell growth in *C. albicans*. Moreover, this is not due to Trx1-dependent regulation of Tsa1, Hog1 or Cap1, as cells lacking these proteins all form filaments following H_2O_2 treatment. Instead, evidence is provided that Trx1-mediated H_2O_2-induced polarized cell growth involves activation of the

Rad53 checkpoint kinase. For example, treatment of *C. albicans* cells with H_2O_2 results in significant hyper-phosphorylation of Rad53, and deleting *RAD53* completely abolishes H_2O_2-induced filamentous growth. This, together with the findings that deletion of *TRX1* results in both constitutive hyper-phosphorylation of Rad53 and in a hyperpolarized bud morphology. is consistent with a model in which inactivation of Trx1 in response to H_2O_2 is a key step in stimulating Rad53 activation and hyperpolarized bud growth. This is further supported by the observations that mutation of the redox-sensitive catalytic cysteine residues of Trx1 also results in hyperpolarized bud morphology and that ectopic expression of Trr1, which reduces oxidized Trx1, inhibited H_2O_2-induced filamentation.

Significantly, our finding that Trx1 inhibits Rad53 activation under non-stressed conditions may be conserved in higher eukaryotes, as a recent study illustrated that ectopic expression of thioredoxin inhibits the phosphorylation of the analogous DNA damage checkpoint kinase Chk2 in mammalian cells (Muniyappa *et al.*, 2009). The precise mechanism whereby Trx1 regulates the Rad53 DNA-checkpoint pathway is unclear, but data presented in this thesis indicates that it is not through regulation of the Trx1 substrate RNR. This is significant as depletion of this essential enzyme, or treatment of cells with the RNR inhibitor HU, also stimulates hyperpolarized bud formation (Shi *et al*., 2007). Alternatively, as Rad53 has four cysteine residues in one of two regulatory FHA domains (Shi *et al*., 2007), Rad53 may be activated upon the formation of an intramolecular disulphide bond formation, or possibly via the formation of an intermolecular disulphide bond between Rad53 and one of its upstream regulators, such as Rad9 and Mrc1. Therefore, Trx1 could be acting as an inhibitor of this activation by reducing such disulphide bonds. However, an important first-step in delineating Trx1 regulation of Rad53 will involve determining whether it is the DNA-damage or DNA- replication checkpoint pathways, which converge on Rad53 that is regulated by Trx1. In addition, to identify the substrate(s) of Trx1 important for filamentation, thiol-trapping experiments as described in section 6.2, could be employed to identify Trx1-interacting proteins. Alternatively, a genetic screen could be carried out to identify suppressors of the *trx1*Δ filamentous phenotype. In the genetic screen, *trx1*Δ cells, which form wrinkly colonies on solid agar, could be transformed with a *C. albicans* genomic library and colonies which suppressed the wrinkly phenotype could be used for the identification of the genes that are required for the filamentous growth of *trx1*Δ cells.

The physiological importance of H_2O_2-induced Rad53 activation is clear, as *C. albicans* cells lacking *RAD53* are very sensitive to oxidative stress. Therefore, further investigation of Trx1-dependent regulation of Rad53 is warranted. Furthermore, as discussed in Chapter 5, ROS-stimulated polarized cell growth is not limited to *C. albicans.* Nonetheless, there is little, if any, information of the mechanism(s) underlying how fungal cells sense and initiate polarized growth in response to ROS. The data presented in this thesis, that the oxidation of an evolutionarily conserved antioxidant protein, thioredoxin, is a key signalling event in *C. albicans* H_2O_2-induced polarized cell growth, may provide significant insight into ROS-mediated polarized growth in other fungi.

6.5. Deletion of Trx1 attenuates the virulence of *C. albicans.*

As the data presented in this thesis clearly demonstrate that Trx1 plays a central role in the oxidative stress response in *C. albicans*, it was decided to investigate the role of Trx1 in *C. albicans* virulence using two distinct virulence models; a three day murine intravenous challenge model of *C. albicans* infection (MacCallum *et al.*, 2009 and 2010), which was performed by Dr Donna MacCallum (University of Aberdeen), and a macrophage killing assay (McKenzie *et al.*, 2010) which was carried out in the laboratory of Dr Lars Erwig (University of Aberdeen).

In the three day murine intravenous challenge model of *C. albicans* infection, weight loss (day 0-3) correlates with ultimate infection outcome (MacCallum *et al.*, 2009). This three day model to assay fungal virulence has an outcome score based upon two parameters, weight loss and fungal burdens (MacCallum *et al.*, 2010). Therefore, a higher outcome score is indicative of greater weight loss and higher fungal burdens. The weight loss, fungal burdens, and resulting outcome score from mice infected with wild-type cells, the *trx1*Δ mutant, and the reconstituted strain (*trx1*Δ + *TRX1*) are summarised in Table 6.1. The outcome scores obtained, correspond to mean survival times of between 5–10 days (wild-type and reintegrant strain) and 20–25 days (*trx1*Δ mutant). Consistent with this, histopathology analysis of kidney sections taken 3 days post infection clearly illustrated significantly less fungal elements in the kidneys from mice infected with *trx1*Δ cells (Figure 6.3). Taken together, these data illustrate that the *trx1*Δ mutant is clearly attenuated in virulence in comparison with control strains.

Table 6.1 Outcome scores of murine intravenous challenge model of *C. albicans* infection

strains	weight loss (%)	weight gain (%)	kidney fungal burdens (Log10 CFU/g)	Outcome scores
wild-type	15.1±6.1	NA*	5.0±0.7	12.5 +/- 3.7
***trx1*Δ**	NA*	0.7±2.3	3.1±0.4	2.8 +/- 1.2**
trx1*Δ + *TRX1	11.0±5.4	NA	4.8±1.0	10.4 +/- 3.5

* NA = Not applicable

**Statistical analysis (Mann Whitney U non-parametric comparison) revealed that for all parameters, weight loss, kidney fungal burden and outcome score, the difference between *trx1*Δ cells and either wild-type or reintegrant cells was highly significant ($P<0.01$). In contrast there was no statistical difference between the wild-type and reintegrant strain.

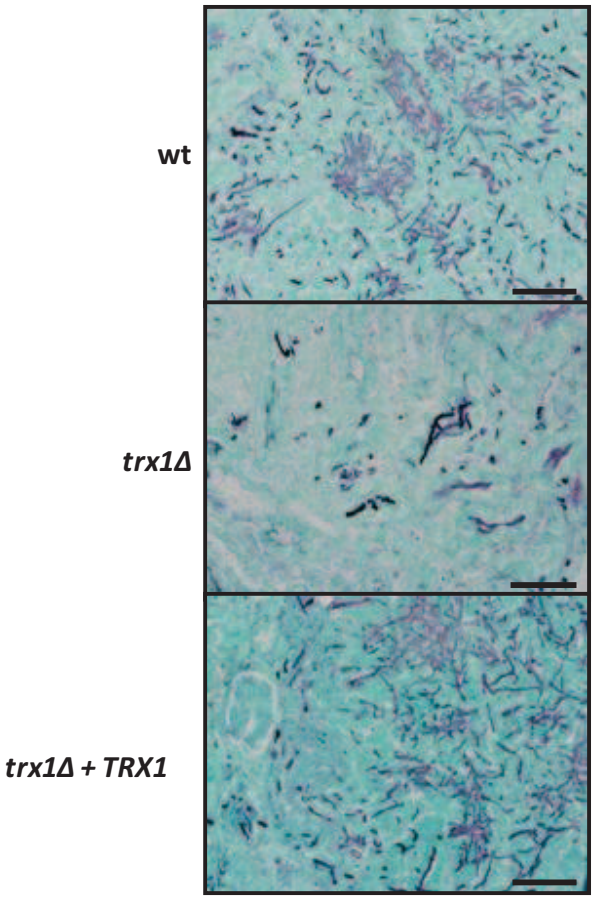

Figure 6.3. Visualization of fungal cells in kidney sections from animals infected with wild-type, *trx1*Δ, and *trx1*Δ + *TRX1* cells.

Kidney from mice infected with either wild-type (JC747), *trx1*Δ (JC677) or *trx1*Δ + *TRX1* (JC679) strains for 3 days. Kidney sections (5 μm) were stained by methenamine silver stain and post-stained with light green. Bar = 50 μm.

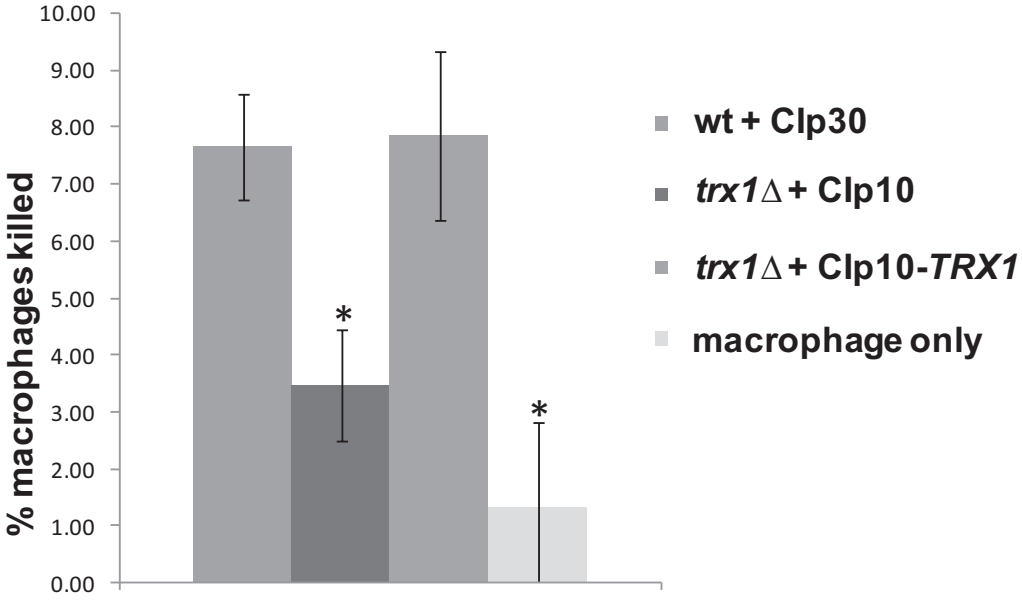

Figure 6.4. Killing of macrophages by *C. albicans trx1*Δ cells.
Percentage of J774 macrophages killed by wild-type (wt + CIp30, JC747) *trx1*Δ or the *trx1*Δ reconstituted strain (*trx1*Δ + *TRX1*, JC679) compared to a control using only macrophages. The ratio of *C. albicans*/macrophage is 1:1 ($n = 3$) and * represent data that were statistically different from wild-type cells ($P<0.05$), using ANOVA and Dunnets test.

In the macrophage killing assay, wild-type, *trx1*Δ and *trx1*Δ + *TRX1* cells were incubated with macrophages at a 1:1 ratio, and survival of macrophages monitored following 3 hrs exposure (Figure 6.4). The uptake of wild-type, *trx1*Δ and *trx1*Δ + *TRX1* cells was the same (data not shown). However, inactivation of *TRX1* inhibited *C. albicans* mediated macrophage killing, and this was rescued upon reintegration of the wild-type gene. Therefore Trx1 function is important for *C. albicans* to effectively kill macrophages.

The fact that deletion of *TRX1* in *C. albicans* results in strains that display reduced virulence in two different models of infection, provides further evidence that the ability of this fungal pathogen to mount robust responses to ROS is vital for survival in the host. Importantly, the role of Trx1 in promoting pathogenesis may be dependent on its signalling rather than antioxidant functions, as inactivation of Tsa1, the major peroxidase substrate of Trx1, does not attenuate virulence in a systemic mouse model of infection (Urban *et al*., 2005). However, further clarification of the precise role of Trx1 in regulating the Hog1 SAPK, the Cap1 transcription factor and the Rad53 kinase, is necessary before the relative contributions of these pathways in Trx1-mediated virulence can be assessed.

6.6. Concluding remarks

Redox-sensitive antioxidant proteins are extremely well-placed to undertake ROS sensing functions as their redox status depends upon the levels of ROS within the environment. Indeed, recent studies from yeast to humans have identified several conserved antioxidant proteins as regulators of signal transduction pathways that respond to ROS (Veal *et al*., 2007). The comprehensive analysis of an antioxidant in oxidative stress signalling presented in this study, extends previous work by demonstrating that Trx1 acts to regulate all of the thus far identified H_2O_2-responsive pathways in *C. albicans*. This indicates that the utilization of antioxidant proteins as regulators of ROS-induced signal transduction pathways may be more extensive than previously thought. Significantly, as robust oxidative stress responses are vital for the virulence of many fungal pathogens (reviewed in Brown *et al*., 2009), this work in *C. albicans* may provide a platform to further investigate antioxidant-mediated signalling and virulence in other fungi.

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Appendix

MOLECULAR AND CELLULAR BIOLOGY, Oct. 2010, p. 4550–4563 Vol. 30, No. 19
0270-7306/10/$12.00 doi:10.1128/MCB.00313-10

Thioredoxin Regulates Multiple Hydrogen Peroxide-Induced Signaling Pathways in *Candida albicans*▽

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Received 18 March 2010/Returned for modification 14 April 2010/Accepted 19 July 2010

The ability of the major systemic fungal pathogen of humans, *Candida albicans*, to sense and respond to reactive oxygen species (ROS), such as H_2O_2 generated by the host immune system, is required for survival in the host. However, the intracellular signaling mechanisms underlying such responses are poorly understood. Here, we show that thioredoxin (Trx1), in addition to its antioxidant activity, plays a central role in coordinating the response of *C. albicans* to ROS by regulating multiple pathways. In particular, Trx1 function is important for H_2O_2-induced phosphorylation of the Hog1 stress-activated protein kinase and to reverse H_2O_2-induced oxidation and activation of the AP-1 like transcription factor Cap1. Furthermore, Trx1 regulates H_2O_2-induced hyperpolarized bud growth in a mechanism that involves activation of the Rad53 checkpoint kinase. Consistent with its key roles in responses to ROS, cells lacking Trx1 displayed significantly attenuated virulence in a murine model of *C. albicans* systemic infection. Collectively, our data indicate that Trx1 has a multifaceted role in H_2O_2 signaling and promotes *C. albicans* survival in the host.

Candida albicans is the leading cause of systemic fungal infections worldwide (40). In many healthy individuals, the fungus exists as a benign commensal. However, in patients with underlying immunological deficiencies or those undergoing chemotherapy or receiving immunosuppressants after organ transplantation, *C. albicans* can overwhelm protective host defense mechanisms and disseminate via the bloodstream (39). The subsequent invasion of internal organs results in deep-seated systemic infections that are often fatal. Significantly, despite advances in antifungal therapy (40), *Candida* species remain the fourth leading cause of hospital-acquired bloodstream infections in the United States, with a mortality rate of 30% (59).

The status of the host immune system clearly influences the ability of *C. albicans* to cause disease. An important defense mechanism employed by innate immune cells involves the activation of the NADPH oxidase (Nox) complex (5), which generates high levels of superoxide within the phagosome that are then rapidly converted to H_2O_2. Patients with congenital defects that affect the Nox complex exhibit enhanced susceptibility to systemic candidiasis (53), confirming the importance of reactive oxygen species (ROS)-based fungicidal mechanisms. Consistent with this, oxidative-stress responses of *C. albicans* are important for survival in the host. For example, *C. albicans* can evade oxidative killing by macrophages (27, 28), and inactivation of oxidative-stress-protective enzymes attenuates virulence (23, 31, 60). In addition, transcript profiling studies have shown that *C. albicans* mounts a significant oxidative-stress response upon exposure to human blood (18), macrophages (28), and neutrophils (17). This response is regulated largely by the AP-1-like transcription factor Cap1 and to a lesser extent by the Hog1 stress-activated protein kinase (SAPK) (15, 57), and cells lacking either Cap1 or Hog1 are more prone to being killed by phagocytes (4, 17). *CAP1* was first identified in a genetic screen to identify *C. albicans* genes that increased the resistance of *Saccharomyces cerevisiae* to the antifungal agent fluconazole (1). Subsequently, however, *cap1*Δ cells were shown to be sensitive to a wide range of ROS, in addition to various drugs (2, 61). Consistent with such phenotypes, a recent study employing genome-wide location profiling detected Cap1 binding to 89 target genes *in vivo*, several of which have well-defined roles in the oxidative-stress response and drug resistance (62). The *C. albicans HOG1* gene was originally cloned by functional complementation of the osmosensitive phenotype associated with the *S. cerevisiae hog1* mutant (44). However, subsequent studies demonstrated that *C. albicans hog1*Δ cells also displayed increased sensitivity to ROS and, moreover, that exposure of *C. albicans* to H_2O_2 stimulates the activation and nuclear accumulation of Hog1 (3, 49). In addition to Cap1- and Hog1-mediated responses to oxidative stress, a recent study demonstrated that exposure of *C. albicans* to H_2O_2 stimulates the fungus to switch from a yeast to a filamentous mode of growth (37, 41). However, despite strong relationships between morphogenetic switching, resistance to ROS, and virulence, little is known about the intracellular signaling mechanisms that regulate H_2O_2-responsive signaling pathways in *C. albicans* (9).

There is growing evidence that redox-sensitive antioxidant

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▽ Published ahead of print on 2 August 2010.

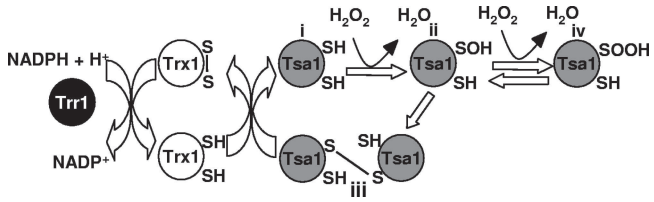

FIG. 1. H_2O_2 detoxification by the Trx1/Tsa1 system. A major antioxidant substrate for thioredoxin is the 2-Cys peroxiredoxin enzyme Tsa1. Tsa1 contains two highly conserved cysteine residues (named the peroxidatic and resolving cysteines) that are involved in the thioredoxin-coupled catalytic reduction of H_2O_2. In the first step, the peroxidatic cysteine residue of reduced Tsa1 (i) becomes oxidized to sulfenic acid (SOH) (ii) and then forms a disulfide bond with the resolving cysteine residue on a partner protein, forming a homodimer (iii). The oxidized dimer is then recycled by the sequential oxidation and reduction of thioredoxin and thioredoxin reductase, using NADPH. However, in eukaryotic 2-Cys peroxiredoxins, disulfide bond formation (iii) is slow, and as a result, the SOH form of the peroxidatic cysteine residue is sensitive to further oxidation to the sulfinic acid (SOOH) derivative (iv), which can be reduced by either sulfiredoxin or sestrin enzyme (54).

proteins with roles in the detoxification of ROS can also act as sensors and regulators of ROS-induced signal transduction pathways (54). One such protein is the highly conserved oxidoreductase thioredoxin, which regulates the catalytic reduction of diverse proteins. During the catalytic cycle of thioredoxin, two conserved cysteine residues become oxidized, and this disulfide form is reduced directly by NADPH and thioredoxin reductase (Fig. 1). Major substrates for thioredoxin include peroxiredoxin enzymes (56), which become oxidized upon the reduction of H_2O_2 and utilize thioredoxin in their catalytic cycles (Fig. 1); ribonucleotide reductase (RNR), required for deoxynucleoside triphosphate (dNTP) synthesis (25); and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) reductase (26), an enzyme involved in sulfate assimilation. Significantly, thioredoxin has also been implicated in the regulation of the redox state of H_2O_2-responsive signaling proteins, such as mammalian apoptosis signal-regulating kinase 1 (Ask1) (43) and Yap1, the orthologue of Cap1, in *S. cerevisiae* (13). However, despite the importance of thioredoxin in oxidative-stress signaling in both lower and higher eukaryotes, no studies of this protein have been reported in the medically relevant pathogen *C. albicans*.

Here, we demonstrate that the thioredoxin Trx1 regulates three distinct regulatory proteins that are activated in response to H_2O_2 stress in *C. albicans*: the Cap1 transcription factor, the Hog1 SAPK, and the Rad53 DNA checkpoint kinase. Furthermore, our data indicate that H_2O_2-induced inactivation of thioredoxin triggers activation of the Rad53 checkpoint pathway, which is essential for H_2O_2-induced filamentation. Consistent with its central role in the *C. albicans* oxidative-stress response, inactivation of Trx1 significantly attenuates the virulence of this fungal pathogen.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. The strains were grown in either YPD medium (2% yeast extract, 1% Bacto peptone, 2% glucose) or SD medium (6.79 g/liter yeast nitrogen base without amino acids, 2% glucose) supplemented with the required nutrients for auxotrophic mutants (47).

Strain construction. All of the oligonucleotide primers used for generating the constructs described below are listed in Table 2.

Deletion of *TRX1*, *CAP1*, and *TSA1*. *TRX1* disruption cassettes, comprising either the *ARG4* or the *HIS1* gene flanked by *loxP* sites and 80 nucleotides corresponding to regions 5′ and 3′ of the *TRX1* open reading frame, were generated by PCR using the oligonucleotide primers TRX1delF and TRX1delR and the plasmid template pLAL2 or pLHL2 (14), respectively. Disruption cassettes were introduced into *C. albicans* strain SN148 (38) to sequentially disrupt both alleles of *TRX1* and generate strain JC488. These *TRX1* disruption cassettes replaced the entire 104-codon open reading frame of *TRX1*.

The same strategy described above was used to delete the *CAP1* gene using the oligonucleotide primers CAP1delF and CAP1delR. The resulting *CAP1-ARG4* and *CAP1-HIS1* disruption cassettes replaced the entire 499-codon *CAP1* open reading frame.

To delete the 4 copies of *TSA1* (*orf19.7417* and *orf19.7398*) in *C. albicans*, two copies were deleted using the strategy described above with the oligonucleotide primers TSA1delF and TSA1delR, and the remaining loci were disrupted by Ura blasting (16) to finally generate strain JC1026. The Ura blaster *tsa1*::*hisG-URA3-hisG* disruption cassette was generated by PCR amplification of the regions flanking *TSA1* using the primer pairs TSA1URAB1F/TSA1URAB1R and TSA1URAB2F/TSA1URAB2R and ligating the resulting

TABLE 1. Strains used in this study

Strain	Relevant genotype	Source or reference
SN148	*arg4*Δ/*arg4*Δ *leu2*Δ/*leu2*Δ *his1*Δ/*his1*Δ *ura3*Δ::*imm*^{434}/*ura3*Δ::*imm*^{434} *iro1*Δ::*imm*^{434}/*iro1*Δ::*imm*^{434}	38
BWP17	*ura3*::λ*imm434*/*ura3*::λ*imm434* *his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG*	58
JC47	BWP17 *hog1*::*loxP-ARG4-loxP*/*hog1*::*loxP-HIS1-loxP*	15
HLC69	*efg1*::*hisG*/*efg1*::*hisG cph1*::*hisG*/*cph1*::*hisG*	27
WYS3	BWP17 *rad53*Δ::*ARG4*/*rad53*Δ::*URA3*	48
WYS3.1	BWP17 *rad53*Δ::*ARG4*/*rad53*Δ::*URA3* *RAD53*:*HIS1*	48
JC747	SN148 CIp30 (*URA3 HIS1 ARG4*)	This work
JC948	SN148 *CAP1-MH–URA3*	This work
JC894	SN148 *RAD53-MH–URA3*	This work
JC896	SN148 *RNR1-MH–URA3*	This work
JC930	SN148 *TRX1-MH–URA3*	This work
JC1014	SN148 pACT1 (*URA3*)	This work
JC1016	SN148 pACT1-RNR1 (*URA3*)	This work
JC1066	SN148 pACT1-TRR1 (*URA3*)	This work
JC1060	SN148 *CAP1-GFP–URA3*	This work
JC842	SN148 *cap1*::*loxP-ARG4-loxP* *cap1*::*loxP-HIS1-loxP* CIp10 (*URA3*)	This work
JC488	SN148 *trx1*::*loxP-ARG4-loxP*/*trx1*::*loxP-HIS1-loxP*	This work
JC677	JC488 CIp10 (*URA3*)	This work
JC679	JC488 CIp10-TRX1 (*URA3*)	This work
JC759	JC488 CIp10-trx1^{C30S} (*URA3*)	This work
JC761	JC488 CIp10-trx1^{C33S}(*URA3*)	This work
JC763	JC488 CIp10-trx1$^{C30/33S}$(*URA3*)	This work
JC886	JC488 *RAD53-MH–URA3*	This work
JC888	JC488 *RNR1-MH–URA3*	This work
JC983	JC488 *CAP1-MH–URA3*	This work
JC1052	JC488 *CAP1-GFP–URA3*	This work
JC1176	JC488 *TRX1-GFP–URA3*	This work
JC1022	JC488 pACT1 (*URA3*)	This work
JC1024	JC488 pACT1-RNR1 (*URA3*)	This work
JC1115	JC488 pACT1-TRR1 (*URA3*)	This work
JC1026	SN148 *tsa1*::*loxP-ARG4-loxP*/*tsa1*::*loxP-HIS1-loxP*/*tsa1*::*hisG*/*tsa1*::*hisG*	This work
JC1027	JC1026 CIp10–*URA3*	This work
JC1028	JC1026 CIp10-TSA1 (*URA3*)	This work
JC1029	JC1026 CIp10-tsa1^{C48S} (*URA3*)	This work
JC1030	JC1026 CIp10-tsa1^{C169S} (*URA3*)	This work
JC1031	JC1026 CIp10-tsa1$^{C48/169S}$ (*URA3*)	This work

TABLE 2. Primers used in this study

Oligonucleotide	Sequence (5′→3′)
TRX1delF	CTTTTTTTTTCCATATTCCCTTGTTTTTTCACCAACAAAATAGATATCAATTCTATTCAACCCTT TAACACTTAACACATAGTTATATTTTTTTTCAAAGCCAGGGTTTTCCCAGTCACG
TRX1delR	ACTAGTAAACATCAATTATAGTTATTCCATTCAATTGTAAATATACACCCATCATCATCAATA ATACAATCATCCAAAACTATTATTAGACTATCTTCTCTCACTAAAGGGAACAAAAGC
TRX1ORFF	GCGCGGATCCATGGTTCACGTTGTCACTGA
TRX1ORFR	GCGCGGATCCTAAGCAAGAGAAGCCAAA
TRX1PromF	GCGCGGATCCGAGACTAAATATCGAACACCACC
TRX1TermR	GCGCGGATCCACTTGGAAGTCACTTATTAC
TRX1C30SF	GACTTTTTTGCCACTTGGTCTGGTCCATGTA
TRX1C30SR	GCAATCATTTTACATGGACCAGACCAAGTGGCA
TRX1C33SF	GCCACTTGGTGTGGTCCATCTAAAATGATTG
TRX1C33SR	TAATGGAGCAATCATTTTAGATGGACCACAC
TRX1C3033SF	CTTTTTTGCCACTTGGTCTGGTCCATCTAAAATGAT
TRX1C3033SR	GGAGCAATCATTTTAGATGGACCAGACCAAGTGG
TRX1MHF	AAAACTGCAGATGGTTCACGTTGTCACTG
TRX1MHR	AAAACTGCAGAGCAAGAGAAGCCAAAGC
TRX1GFPF	AAAACTGCAG ATGGTTCACGTTGTCACTG
TRX1GFPR	AAAACTGCAGAGCAAGAGAAGCCAAAGC
TSA1delF	GCTCTATTGCATTTTATTTCAATCAACTAATTAATTAGTCACATACATAGATACACTACTACAA AACTCACTAAAGGGAACAAAAGC
TSA1delR	CTTGTCTAAAATGCAATCTATTTGCTATGAAACCATAACGAAAAAAAAAAACGTACATATATAT CTATAAATCCTGTTTACTCACTAAAGGGAACAAAAGC
TSA1URAB1F	CGCGGGATCC CGCGCCACCTGCACAGAAGTACCGG
TSA1URAB1R	CGCGGGATCCTTTTGTAGTAGTGTATCTATGTATG
TSA1URAB2F	CGCGGGATCCCCAGGTGATGAAACCATCAAGCC
TSA1URAB2F	CGCGGGATCCAACAATGAAAATACTTGGGAGAGAAG
TSA1PromF	GCGCGGATCCCTCCGTAATCGGTTAATCTGT
TSA1TermR	GCGCGGATCCAACATTGAAAATACTTGGGAGAGAAG
TSA1C48SF	CATTGGCCTTCACATTCGTCTCCCCATCAGAAA
TSA1C48SR	GCAATAATTTCTGATGGGGAGACGAATGTG
TSA1C169SF	CTGAAAAATACGGTGAAGTTTCCCCAGCTAACT
TSA1C169SR	CCTGGGTGCCAGTTAGCTGGAGGAACTTCACCG
RAD53MHF	AAAACTGCAGTGCTTCGTCCATTGGATAGCGAAAG
RAD53MHR	AAAACTGCAGTGAACTTATACTACTTAAACCCGAA
RNR1ACTF	GCCCATCGATATGTATGTTTATAAGAGAGATGGCCG
RNR1ACTR	GCCCATCGATCTAACCAGAACACATTGTACAAGATTC
RNR1MHR	AAAACTGCAGACCAGAACACATTGTACAAGATTCTGG
TRR1ACTF	GCCCATCGAT ATGGTACACCACAAAGTCAC
TRR1ACTR	GCCCATCGATCTAAGCTTCTTGTTCGGAAATG
CAP1delF	CACCCCCCTTTTTTTTCCCATACAAAGAAACCAATTAGTTCAATACATTCTACACCAAGAATTA AACAACCATTTTCAACTATCCTTATTCCCATA AAAACCAGGGTTTTCCCAGTCACG
CAP1delR	CTGGTTAATAACCAAATGTAAATATAAATACAAAAAAATAAAGCCAAATAGATGTCAATTGA AATACCGTAAAATAAAATAAACCCACCACTAACTTGACTCACTAAAGGGAACAAAAGC
CAP1MHPstF	ATTGTCTGCAGGTTCCCCAAGGTGTGCCGGATTTG
CAP1MHPstR	AATGTCTGCAGATGTTTTATACTTCGCTCTAGTAATTG
Cap1promPstIF	AATGTCTGCAGGCTTGGGTTTGCTTTTAGGATCG
Cap1GFPPstIR	AATGTCTGCAGTCATGTTTTATACTTCGCTCTAGTAATAATTG

products into the BglII and BamHI sites of p5921, respectively (20). The *TSA1-ARG4* and *TSA1-HIS1* disruption cassettes replaced the entire 197-codon *TSA1* open reading frame, and the Ura blaster *tsa1*::*hisG-URA3-hisG* disruption cassette replaced codons 1 to 174 of the *TSA1* open reading frame. Gene disruptions were confirmed by PCR.

To construct reintegrant control strains, the *TRX1* gene plus 1,039 bp of the promoter region and 299 bp of the terminator region were amplified by PCR, using the oligonucleotide primers TRX1PromF and TRX1TermR, and ligated into the BamHI site of CIp10 (34). The *TSA1* open reading frame plus 701 bp of the promoter region and 204 bp of the terminator region was amplified by PCR, using the oligonucleotide primers TSA1PromF and TSA1TermR, and ligated into the BamHI site of CIp10. The resulting CIp10-TRX1 and CIp10-TSA1 plasmids were digested with StuI and integrated at the *RPS10* locus in the *trx1*Δ and *tsa1*Δ mutants, respectively, to generate strains JC679 and JC1028. To generate deletion mutants that were auxotrophically identical to the reconstituted strains, the empty CIp vector was integrated at the *RPS10* locus in the *trx1*Δ, *cap1*Δ, and *tsa1*Δ mutants, to generate strains JC677, JC842, and JC1027, respectively. Similarly, to generate a URA^+ ARG^+ HIS^+ wild-type strain, the vector CIp30 (14), a derivative of CIp10, was integrated at the *RPS10* locus in the parental strain SN148 to generate JC747.

Mutagenesis of TRX1 and TSA1. Mutagenesis of *TRX1* to create $trx1^{C30S}$ was performed by overlapping PCR using the oligonucleotide pairs TRX1C30SF/TRX1ORFR and TRX1C30SR/TRX1ORFF, with the template CIp10-TRX1. The resulting PCR product was used as a template in a subsequent PCR with the primers TRX1ORFF and TRX1ORFR. The final PCR product was ligated into the BamHI site of CIp10 and then integrated at the *RPS10* locus in *trx1*Δ cells (JC488), as described above, to generate strain JC759. The same strategy was used to generate strains expressing $trx1^{C33S}$ (JC761) and $trx1^{C30,33S}$ (JC763), using the oligonucleotide pairs TRX1C33SF/TRX1ORFR and TRX1C30SR/TRX1ORFF, and TRX1C3033SF/TRX1ORFR and TRX1C3033SR/TRX1ORFF, respectively. Mutagenesis of the catalytic cysteine residues of Tsa1 was performed in the same way, and the resulting CIp10-$tsa1^{C48S}$, CIp10-$tsa1^{C169S}$, and CIp10-$tsa1^{C48,169S}$ plasmids were integrated at the *RPS10* locus in the *tsa1*Δ strain (JC1026) to generate strains JC1029, JC1030, and JC1031, respectively. The correct chromosomal insertion of all mutant derivatives of *TRX1* and *TSA1* was confirmed by PCR and DNA sequencing. Western blotting experiments showed that proteins of the expected molecular weight were expressed and that the wild-type and mutant versions of the Tsa1 and Trx1 proteins were present at similar levels (our unpublished results).

Tagging of Trx1, Cap1, Rad53, and Rnr1. To tag Trx1 expressed from the normal chromosomal locus at the C terminus with 6 His residues and 2 copies of the myc epitope, the *TRX1* gene was first amplified by PCR using the oligonucleotide primers TRX1MHF and TRX1MHR with CIp10-TRX1 as a template. The resulting PCR product was ligated into the PstI site of CIp-C-MH, a derivative of CIp-C-ZZ (8) in which the sequences encoding the TEV cleavage site and protein A were replaced with those encoding 2 myc epitopes and 6 His residues. The CIp-C-TRX1MH plasmid was then linearized by digestion with EcoNI to target chromosomal integration at the *TRX1* locus in SN148 to generate strain JC930. The same strategy was used to similarly tag Rad53 and Cap1, expressed from their respective chromosomal loci in wild-type and *trx1*Δ strains, using the primer pairs RAD53MHF/RAD53MHR and CAP1MHPstF/CAP1MHPstR, respectively. The resulting CIp-C-RAD53MH and CIp-C-RNR1MH plasmids were linearized by digestion with HindIII to target chromosomal integration at their native loci in both wild-type (SN148) and *trx1*Δ (JC488) strains to generate strains JC894/JC886 and JC896/JC888, respectively. Similarly, CIp-C-CAP1MH was linearized by digestion with SphI to target integration at the *CAP1* locus in both wild-type (SN148) and *trx1*Δ (JC488) strains to generate strains JC948 and JC983, respectively.

To tag Trx1 with green fluorescent protein (GFP), the *TRX1* open reading frame and 888 bp of the promoter region were amplified by PCR, using the primer pair Trx1GFPF/Trx1GFPR and genomic DNA as a template, and ligated into the PstI site of pGFP (7). The resulting pGFP-TRX1 plasmid was digested with StuI and integrated at the *RPS10* locus in *trx1*Δ (JC488) cells to generate the strain JC1176. To tag Cap1 with GFP, the *CAP1* open reading frame and 670 bp of the promoter region was amplified by PCR, using the primer pair CAP1promPstIF/CAP1GFPPstIR and genomic DNA as a template, and ligated into the PstI site of pGFP (7). The resulting pGFP-CAP1 plasmid was digested with StuI and integrated at the *RPS10* locus in SN148 and *trx1*Δ (JC488) cells to generate strains JC1060 and JC1052, respectively. Correct integration at the *RPS10* loci of both Trx1-GFP and Cap1-GFP was confirmed by PCR and DNA sequencing.

In all cases, strains expressing tagged constructs were checked for protein functionality. For example, phenotypic analysis of heterozygote strains expressing either MH-tagged Cap1, Rad53, or Trx1 confirmed the functionality of these tagged constructs. Similarly, expression of GFP-tagged Cap1 and Trx1 was found to rescue the oxidative-stress phenotypes associated with deletion of *CAP1* or *TRX1*, respectively (our unpublished data).

Construction of strains ectopically expressing TRR1 or RNR1. To achieve ectopic expression of either *RNR1* or *TRR1* in *C. albicans*, these open reading frames were amplified by PCR, using the primer pairs RNR1ACTF/RNR1ACTR and TRR1ACTF/TRR1ACTR and genomic DNA as a template, and ligated into the ClaI site between the *C. albicans ACT1* (*CaACT1*) promoter and the *S. cerevisiae CYC1* (*ScCYC1*) terminator in pACT1 (51). The resultant plasmids, pACT1 (vector), pACT1-RNR1, and pACT-TRR1, were then individually integrated at the *RPS10* locus in SN148 to create strains JC1014, JC1016, and JC1066, respectively. pACT1, pACT1-RNR1, and pACT-TRR1 were also integrated at the *RPS10* locus in the *trx1*Δ strain (JC488) to create strains JC1022, JC1024, and JC1115, respectively. Correct integration at the *RPS10* locus and the DNA sequences of the integrated open reading frames were confirmed by PCR and DNA sequencing.

Microscopy. Cells to be analyzed were fixed in 3.7% paraformaldehyde, washed in PEM [100 mM (PIPES)piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 7.6, 1 mM EGTA, 1 mM $MgSO_4$), and spread onto poly-L-lysine-coated slides. Coverslips were mounted onto the slides using Vectashield mounting medium containing 1.5 mg/ml DAPI (4′-6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA). Differential interference contrast (DIC) images and DAPI and GFP fluorescence were captured using a Zeiss Axioscope with a 63× oil immersion objective and the Axiovision imaging system.

Protein oxidation assays. Cells were harvested in 20% trichloroacetic acid (TCA) and then snap-frozen. The thawed pellets were washed with acetone to remove the TCA and resuspended in 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% SDS, 25 mM 4-acetamido-4′-((iodoacetyl)amino)stilbene-2,2′-disulfonic acid (AMS) (Invitrogen). Proteins were separated by SDS-PAGE under nonreducing conditions, the oxidation of Cap1-MH was analyzed by Western blotting using anti-myc antibodies (Sigma), and the oxidation of Tsa1 was determined using an anti-peroxiredoxin antibody (kindly provided by E. Veal, Newcastle University).

Stress sensitivity tests. For stress sensitivity assays, the strains to be tested on solid medium were grown at 30°C to mid-exponential phase. The cells were diluted in YPD medium, and approximately 10^4 cells, and 10-fold dilutions thereof, were spotted in 5 μl onto YPD agar containing the stress agent to be tested at the indicated concentrations. The plates were incubated at 30°C for 24 h. To determine survival in liquid cultures, 5 mM H_2O_2 was added to exponentially growing cells. Cells were taken at various time points, diluted, and then plated on YPD agar to determine the numbers of surviving cells. The plates were incubated at 30°C for 24 to 48 h, and survival was expressed as a percentage of the time-zero sample.

Protein phosphorylation assays. Protein extracts were prepared, and phosphorylated Hog1 was detected by Western blotting with an anti-phospho-p38 antibody (New England Biolabs) as described previously (49). The blots were stripped, and the total levels of Hog1 were determined by probing with an anti-Hog1 antibody (Santa Cruz Biotechnology). Phosphorylation of Rad53-MH was performed as described previously (48), except that an anti-myc antibody (Sigma) was used. Dephosphorylation of Rad53 was performed as described previously (48), and λ phosphatase was purchased from New England Biolabs.

RNA analysis. RNA preparation and Northern blot analyses were performed as described previously (15). Gene-specific probes were amplified by PCR from genomic DNA using oligonucleotide primers specific for *TRR1* and *ACT1* (15).

Virulence analysis. The murine intravenous-challenge model of *C. albicans* infection (29, 30) was employed to determine the impact of deleting *TRX1* on virulence. BALB/c female mice with a mean body weight of 17.3 ± 0.7 g were housed in groups of 5 with food and water provided *ad libitum*. Wild-type (JC747), *trx1*Δ (JC677), and *trx1* plus *TRX1* (JC679) cells were grown overnight on Sabouraud agar (Oxoid) at 30°C. Cells were harvested in sterile saline, and cell counts were adjusted by hemocytometer to provide a cell suspension estimated to deliver a challenge dose of 3×10^4 CFU/g body weight. The actual challenge dose was determined from viable counts read 24 h later and was 2.5×10^4 CFU/g. Mice were infected intravenously via a lateral tail vein. Body weights were recorded daily. Seventy-two hours after challenge, the animals were weighed and humanely terminated, and the kidneys were removed aseptically. Fungal burdens were measured by viable counts for two half kidneys per animal; the other half kidneys were fixed, embedded, and stained for histopathological examination. The virulence of the challenge strains was assessed by kidney fungal burdens at 72 h and by percent weight change over 72 h, from which an outcome score was calculated (29, 30). Differences between mean body weight changes and mean kidney burdens were tested statistically by the Mann-Whitney U test. All animal experimentation conformed to the requirements of United Kingdom Home Office legislation and of the Ethical Review Committee of the University of Aberdeen.

RESULTS

Trx1 regulates H_2O_2-induced filamentation. A recent study reported that exposure of *C. albicans* to H_2O_2 stimulates filamentous cell growth (37), although the morphological characteristics of the filamentous cells were not defined. *C. albicans* can switch between several morphological forms, including yeast, pseudohyphae, and hyphae (50). Here, our close examination of H_2O_2-induced filamentation revealed that exposure of *C. albicans* cells to H_2O_2 stimulated extensive bud elongation in which nuclear movement from the mother cell to the daughter bud was evident, as were clear constrictions at the septal junction (Fig. 2A). Moreover, the majority of cells did not divide further, but rather, the buds continued to elongate over time (Fig. 2A).

The H_2O_2-induced hyperpolarized bud growth phenotype is distinct from both hyphae and pseudohyphae. For example, in contrast to hyphae, the hyperpolarized buds display a clear constriction at the septum. Furthermore, in contrast to pseudohyphae, in which the first nuclear division occurs across the mother bud neck, nuclear movement from the mother cell to the polarized bud is evident. Indeed, cells lacking the major transcriptional regulators of hyphal growth, Efg1 and Cph1 (24), could filament as wild-type cells in response to H_2O_2 (Fig. 2B). This hyperpolarized bud growth phenotype is very similar to that characterized recently in various *C. albicans* mutants that perturb cell cycle progression or in response to chemicals, such as hydroxyurea (HU) or methyl methanesulfonate (MMS), that induce cell cycle arrest (6, 48). Indeed, consistent

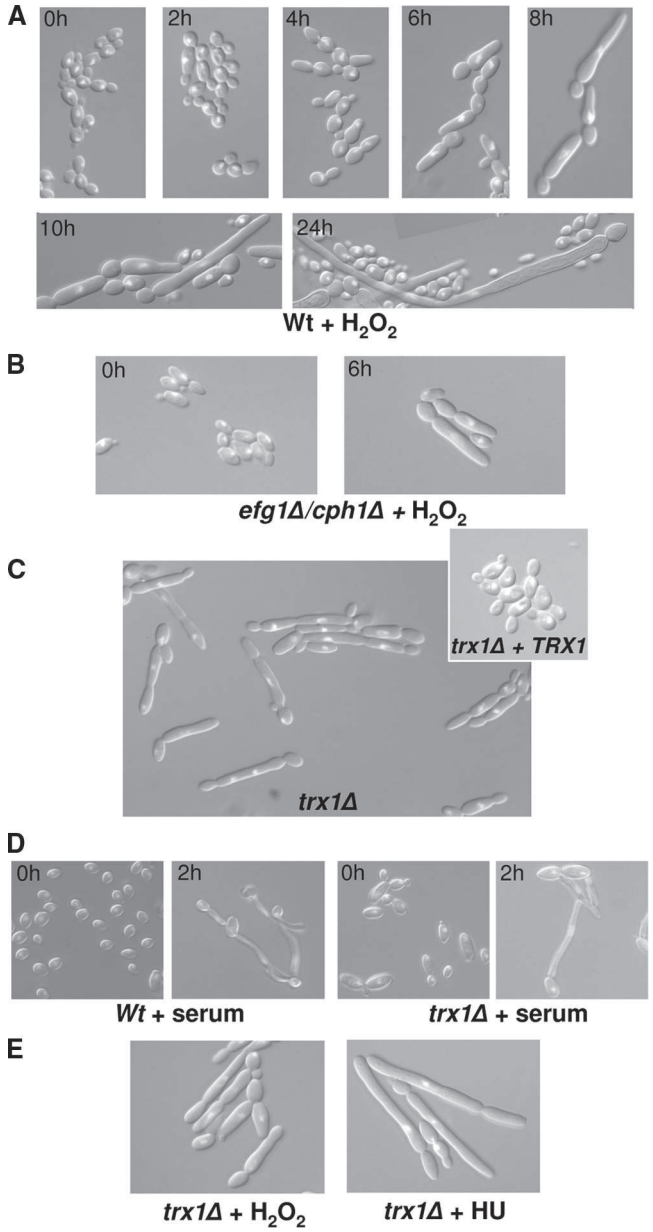

FIG. 2. H_2O_2 treatment of wild-type cells, or deletion of *TRX1*, results in hyperpolarized bud growth that is distinct from hyphae. (A) Treatment of wild-type cells (Wt; JC747) with 5 mM H_2O_2 for the indicated times stimulates hyperpolarized bud growth. (B) Cells lacking the major transcriptional regulators of hypha formation, Efg1 and Cph1, form hyperpolarized buds following treatment with H_2O_2. *efg1*Δ *cph1*Δ cells (HLC69) were treated with 5 mM H_2O_2 for 6 h. (C) Morphological analysis of mid-exponential-phase *trx1*Δ cells (JC677) illustrates that loss of Trx1 results in filamentous growth similar to that exhibited by wild-type cells following H_2O_2 treatment. This filamentous phenotype was reversed upon reconstitution of a wild-type *TRX1* allele (JC679). (D) *trx1*Δ cells can form hyphae. Stationary-phase cells were diluted 1:10 in fresh YPD medium supplemented with 10% serum and grown at 37°C. The images were taken prior to and following exposure to serum for 2 h. (E) HU, but not H_2O_2, stimulates filamentation in *trx1*Δ cells. *trx1*Δ cells (JC677) were treated with H_2O_2 (5 mM) or HU (50 mM) for 6 h. In all cases, the cells were stained with DAPI to allow both cell morphology and nuclear distribution to be determined by overlaying fluorescence and DIC images.

with links to the cell cycle, exposure of *C. albicans* to H_2O_2 has been reported to result in G_2/M phase arrest (41).

To identify potential regulators of H_2O_2-induced filamentation, we analyzed a panel of potential stress regulatory mutants, generated in our laboratory, for defects in ROS-stimulated polarized cell growth. Significantly, *C. albicans* cells lacking the thioredoxin protein Trx1 exhibited a filamentous growth phenotype under nonstressed conditions similar to that observed when cells were treated with H_2O_2 (Fig. 2C). For example, *trx1*Δ cells formed elongated buds that contained clear constrictions at septal junctions, and in some cases, nuclear movement from the mother cell into the filament was evident. This filamentous growth characteristic of *trx1*Δ cells could be reversed upon reconstitution of the wild-type *TRX1* gene (Fig. 2C). Furthermore, consistent with the observation that cells lacking the hyphal regulators Efg1 and Cph1 could form H_2O_2-induced filaments (Fig. 2B), the filamentous forms seen in *trx1*Δ cells were competent to produce hyphae under hypha-inducing conditions (Fig. 2D).

As a range of genotoxic agents, such as HU and MMS, have been shown to stimulate polarized bud growth in *C. albicans* (48), we investigated whether Trx1 specifically regulated such growth in response to H_2O_2. Interestingly, although the polarized buds characteristic of *trx1*Δ cells displayed considerable additional elongation in response to HU, such polarized buds did not elongate further in response to H_2O_2 (Fig. 2E). These data suggest that inhibition of Trx1 is a key step in H_2O_2-induced, but not HU-induced, polarized cell growth.

***TRX1* encodes the major thioredoxin in *C. albicans*.** Sequence alignment of *C. albicans* Trx1 with thioredoxin proteins from *S. cerevisiae* and humans revealed a high level of sequence conservation (Fig. 3A), which included the classical active-site sequence Trp-Cys-Gly-Pro-Cys containing the catalytic cysteines (21). Phenotypic analysis revealed that deletion of *TRX1* resulted in both impaired resistance to H_2O_2 (Fig. 3B) and an inability to grow on medium lacking methionine (Fig. 3C). Importantly, the increased H_2O_2 sensitivity and methionine auxotrophy could be reversed upon reintegration of the wild-type *TRX1* gene, but not by introduction of $TRX1^{C30S}$, $TRX1^{C33S}$, and $TRX1^{C30,33S}$ mutant alleles, in which either or both of the catalytic cysteine residues were mutated to serine (Fig. 3B and data not shown). Such phenotypes are consistent with well-established roles of thioredoxins as cofactors for 2-Cys peroxiredoxins, which reduce peroxides (56), and PAPS reductase (26), an enzyme essential for sulfur assimilation. Furthermore, these data indicate that Trx1 is the sole thioredoxin in *C. albicans*, in contrast to *S. cerevisiae*, which contains two functionally redundant thioredoxins, Trx1 and Trx2 (see below).

To further investigate whether Trx1 is the major thioredoxin in *C. albicans*, the role of Trx1 in regulating the reduction of the 2-Cys peroxiredoxin Tsa1 was examined. Oxidation of Tsa1 was examined by nonreducing SDS-PAGE and Western blotting of AMS-modified proteins prepared from wild-type and *trx1*Δ cells both before and after H_2O_2 treatment (Fig. 3D). AMS is an alkylating agent that reacts specifically with sulfhydryl groups of reduced cysteine residues, increasing the molecular mass of the protein by 0.5 kDa per reduced cysteine. Oxidation of cysteine residues prevents AMS binding and thus results in increased mobility on SDS-PAGE (13). As expected,

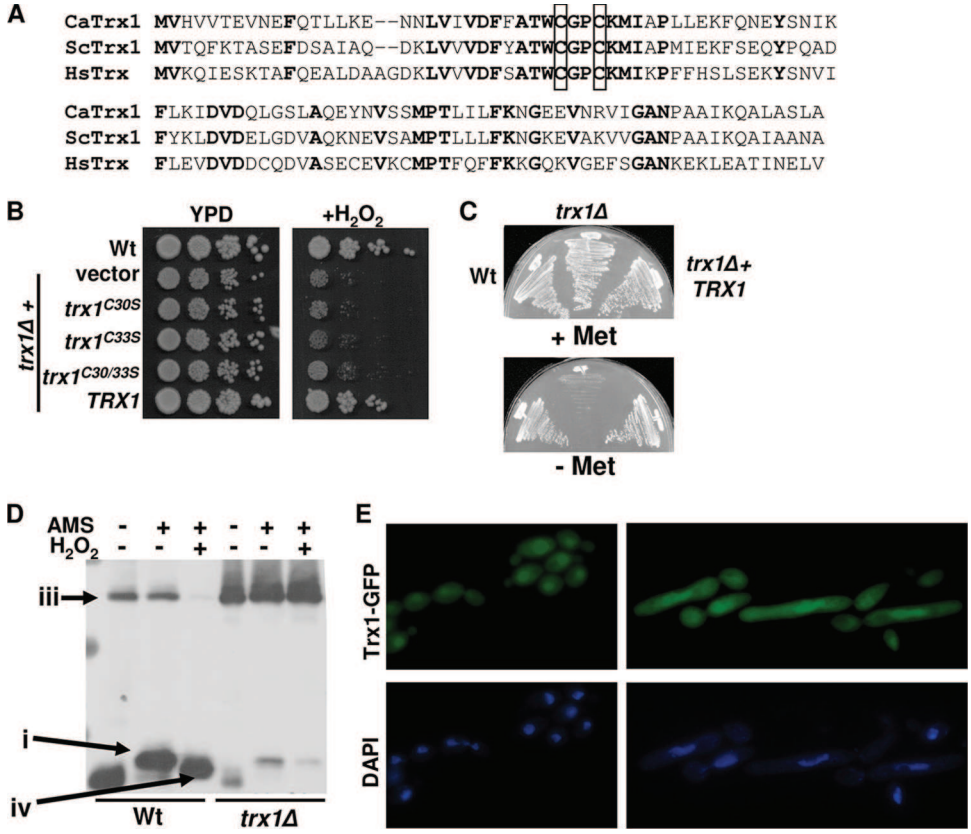

FIG. 3. Characterization of *C. albicans* Trx1. (A) Sequence alignment of thioredoxin proteins from *C. albicans* (Ca), *S. cerevisiae* (Sc), and *Homo sapiens* (Hs). Amino acids conserved in all three proteins are in boldface, and the catalytic cysteine residues in the thioredoxin motif, conserved from yeast to humans, are boxed. (B) Deletion of *TRX1* or mutation of the catalytic cysteine residues (Cys30 and Cys33) to serine results in equally impaired growth on media containing H_2O_2. Approximately 10^4 cells, and 10-fold dilutions thereof, of mid-exponential-phase wild-type cells (JC747), *trx1*Δ cells (JC677), and *trx1*Δ cells expressing either $trx1^{C30S}$ (JC759), $trx1^{C33S}$ (JC761), $trx1^{C30,33S}$ (JC763), or *TRX1* (JC679) were spotted onto YPD plates with or without 3.5 mM H_2O_2 and grown at 30°C for 24 h. (C) Cells lacking *TRX1* display methionine auxotrophy. Wild-type (JC747), *trx1*Δ (JC677), and the reconstituted mutant (*trx1*Δ+*TRX1*; JC679) strains were streaked onto SD medium with (+) or without (−) methionine and grown at 30°C for 24 h. (D) Analysis of the role of Trx1 in regulating the oxidation status of the 2-Cys peroxiredoxin Tsa1. Tsa1 oxidation was analyzed by nonreducing SDS-PAGE and Western blotting of AMS-modified proteins prepared from wild-type (JC747) and *trx1*Δ (JC677) cells exposed to 5 mM H_2O_2 for 0 and 10 min. The bands annotated i, iii, and iv represent the various forms of Tsa1 shown in Fig. 1. (E) Trx1-GFP is present in both the cytoplasm and the nucleus. The localization of GFP-tagged Trx1 was determined by fluorescence microscopy in untreated cells (JC1176) and following exposure to 5 mM H_2O_2 for 6 h. The positions of the nuclei were visualized with DAPI.

in unstressed wild-type cells, Tsa1 was largely present in a reduced monomeric form (Fig. 3D). However, consistent with Trx1 providing the major thioredoxin activity in *C. albicans*, Tsa1 was found predominantly in the oxidized dimeric form in *trx1*Δ cells (Fig. 3D). As expected, after H_2O_2 treatment, the reduced monomeric form of Tsa1 was rapidly converted in wild-type cells to an AMS-resistant hyperoxidized monomeric form that had a faster mobility than reduced Tsa1. However, in contrast, Tsa1 was mainly trapped in the oxidized dimeric form in *trx1*Δ cells (Fig. 3D). Thus, reduction of oxidized Tsa1 is mediated entirely by Trx1 in *C. albicans*, further illustrating that Trx1 is the major thioredoxin.

The model yeast *S. cerevisiae* contains two functionally redundant thioredoxin proteins, Trx1 and Trx2, which are located in both cytoplasmic and nuclear compartments in the cell (22). In addition, *S. cerevisiae* contains a third thioredoxin that is located in the mitochondria. To determine the cellular localization of *C. albicans* Trx1, a strain was constructed in which one copy of *TRX1* was chromosomally tagged with a sequence encoding GFP. Similar to what was reported for Trx1 and Trx2 in *S. cerevisiae* (22), fluorescence microscopy revealed that *C. albicans* Trx1-GFP was present in both cytoplasmic and nuclear compartments (Fig. 3E). Moreover, this distribution of Trx1 did not change following H_2O_2 treatment and the formation of hyperpolarized buds (Fig. 3E).

Phenotypes exhibited by *C. albicans trx1*Δ cells, such as decreased H_2O_2 stress resistance, methionine auxotrophy, and impaired reduction of Tsa1, are observed in *S. cerevisiae* only upon deleting *ScTRX1* and *ScTRX2* (19, 33). Hence in contrast to *S. cerevisiae*, these data illustrate that *C. albicans* contains only one cytoplasmic/nuclear thioredoxin. However, we noted that a second open reading frame (*orf19.1976*) is annotated as Trx2 in the *Candida* genome database (http://www.candidagenome.org). Significantly, however, the active-site sequence characteristic of thioredoxin and present in Trx1 (Trp-Cys-Gly-Pro-Cys) is not con-

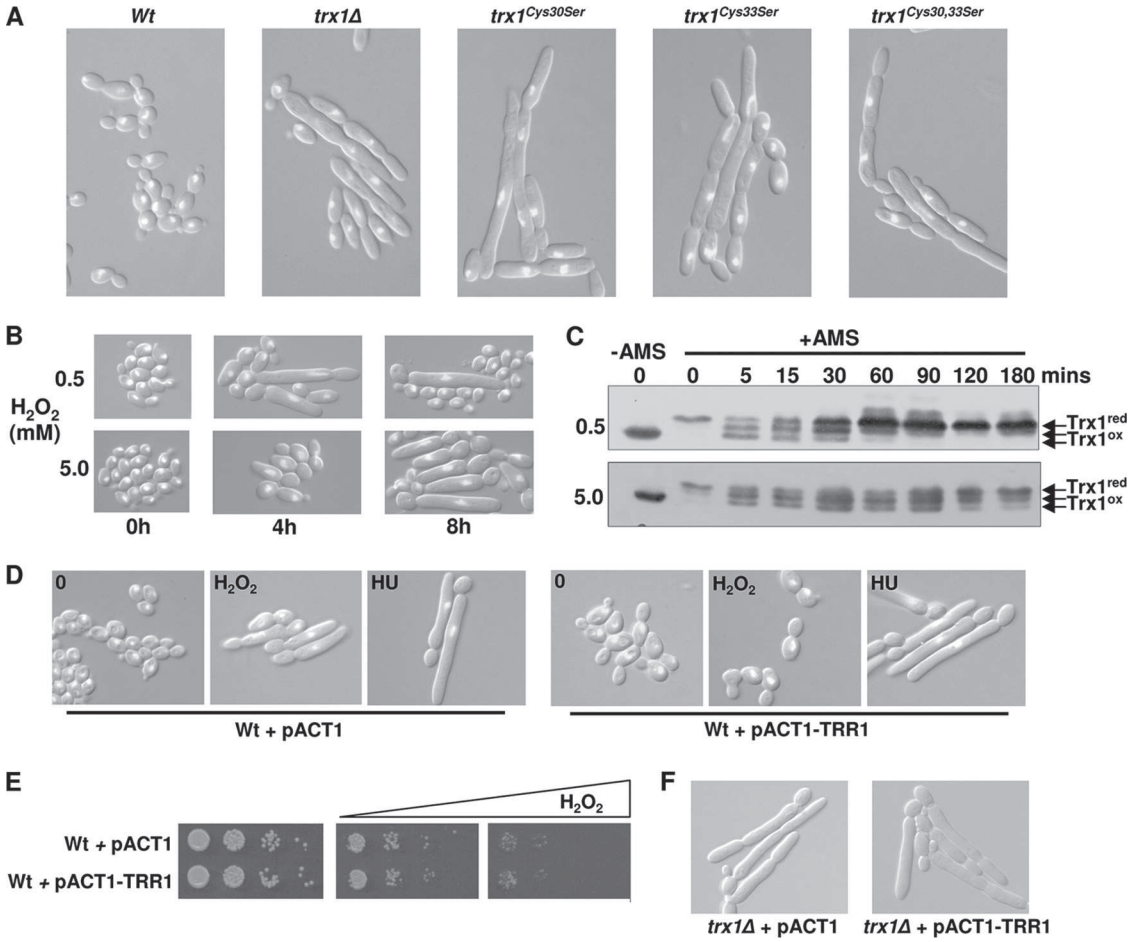

FIG. 4. Oxidation of Trx1 is important for H_2O_2-induced polarized cell growth. (A) Mutation of the redox-sensitive cysteine residues of Trx1 results in filamentous growth. Shown are micrographs mid-exponential-phase wild-type cells (JC747), *trx1*Δ cells (JC677), and *trx1*Δ strains expressing $trx1^{C30S}$ (JC759), $trx1^{C33S}$ (JC761), or $trx1^{C30,33S}$ (JC763). (B) The kinetics and extent of H_2O_2-induced polarized cell growth is dependent on the level of H_2O_2. Wild-type cells (JC747) were either untreated or treated with low (0.5 mM) or high (5 mM) levels of H_2O_2 for 4 or 8 h, and images were taken. Although polarized growth occurs over a range of H_2O_2 concentrations, it occurs more quickly in response to 0.5 mM H_2O_2 yet persists following exposure of cells to 5 mM H_2O_2. (C) Oxidation of Trx1 is prolonged following exposure to high levels of H_2O_2. The oxidation of 6His-myc-tagged Trx1 (Trx1-MH) was analyzed by nonreducing SDS-PAGE and Western blotting of AMS-modified proteins prepared from cells (JC930) exposed to 0.5 and 5 mM H_2O_2 for the indicated times. Unexpectedly, two AMS-resistant oxidized forms of Trx1 ($Trx1\text{-}MH^{ox}$) were evident. Both forms were lost upon treatment of cell extracts with the reducing agent dithiothreitol (DTT) prior to AMS addition (data not shown), thus confirming that the bands represented different oxidized forms of Trx1. The molecular basis for this is unknown, but a redox-induced modification, such as glutathionylation, as reported in human Trx (10), is a possibility. (D) Ectopic expression of *TRR1* (pACT1-Trr1) inhibits H_2O_2-induced, but not HU-induced, polarized cell growth. Wild-type cells, containing either vector (pACT1; JC1014) or pACT1-TRR1 (JC1066), in which *TRR1* is expressed from the *ACT1* promoter, were untreated (0) or treated with either 5 mM H_2O_2 or 50 mM HU for 6 h. The images were captured as described in the legend to Fig. 2. (E) Ectopic expression of *TRR1* does not increase cellular resistance to H_2O_2 Approximately 10^4 cells, and 10-fold dilutions thereof, of exponentially growing wild-type strains in which either the empty vector pACT1 (JC1014) or pACT1-TRR1 (JC1066) had been integrated at the *RPS10* locus were spotted onto YPD plates containing increasing concentrations of H_2O_2 and incubated at 30°C for 24 h. (F) Ectopic expression of *TRR1* does not inhibit the filamentous phenotype exhibited by *trx1*Δ cells. The images illustrate the morphology of exponentially growing *trx1*Δ cells containing either pACT1 (JC1022) or pACT1-TRR1 (JC1115).

served in *orf19.1976*. This is consistent with our data illustrating that *TRX1* encodes all of the cytoplasmic/nuclear thioredoxin functions in *C. albicans*.

Oxidation of Trx1 is important for H_2O_2-induced polarized cell growth. The observation that cells lacking *TRX1* display a filamentous phenotype similar to that seen following H_2O_2 treatment of wild-type cells (Fig. 2) suggested that inactivation of Trx1 by oxidation may be an important trigger for H_2O_2-induced polarized cell growth. Consistent with this, mutation of either redox-sensitive catalytic cysteine residue (Cys30 or Cys33) of Trx1 also produced filamentous growth, similar to that seen upon deletion of *TRX1*, under non-filament-inducing conditions (Fig. 4A). Furthermore, prolonged oxidation of Trx1 was seen following exposure of cells to high (5 mM) compared to low (0.5 mM) levels of H_2O_2, and this correlated with sustained filamentous growth (Fig. 4B and C). Importantly, ectopic expression of *TRR1*, which encodes the enzyme thioredoxin reductase that reduces oxidized thioredoxin (Fig. 1), was found to specifically inhibit H_2O_2-induced, but not HU-induced, polarized cell growth (Fig. 4D). Cells expressing

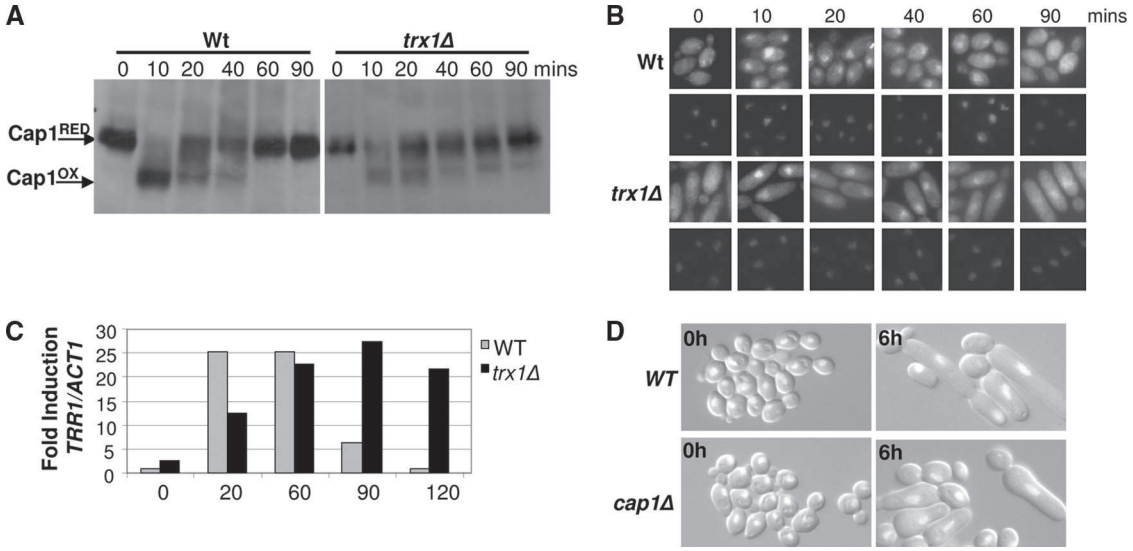

FIG. 5. Inactivation of Trx1 prolongs activation of the Cap1 AP-1-like transcription factor. (A) Loss of Trx1 prolongs H_2O_2-induced Cap1 oxidation. Cap1 oxidation was analyzed by nonreducing SDS-PAGE and Western blotting of AMS-modified proteins prepared from wild-type (JC948) and *trx1*Δ (JC983) cells expressing 2myc-6His-tagged Cap1, following treatment with 5 mM H_2O_2 for the indicated times. (B) H_2O_2-induced nuclear accumulation of Cap1 is prolonged in *trx1*Δ cells. The localization of Cap1-GFP was determined by fluorescence microscopy in wild-type (JC1060) and *trx1*Δ (JC1052) cells following treatment with 5 mM H_2O_2 for the indicated times. (C) Loss of Trx1 prolongs H_2O_2-induced Cap1-dependent gene expression. Shown is Northern blot analysis of RNA isolated from exponentially growing cultures of wild-type (JC747) and *trx1*Δ (JC677) cells following treatment with 5 mM H_2O_2. The level of *TRR1* RNA was quantified relative to the *ACT1* loading control. (D) Loss of Cap1 does not impair H_2O_2-induced polarized cell growth. Wild-type (JC747) and *cap1*Δ (JC842) cells were treated with 1 mM H_2O_2 for 6 h, and images were captured as described for Fig. 2.

Trr1 ectopically were not more resistant to H_2O_2 than wild-type cells (Fig. 4E); thus, the Trr1-mediated inhibition of H_2O_2-induced polarized growth was not due to increased tolerance for oxidative stress. Moreover, ectopic expression of Trr1 had no effect on the filamentous characteristics of *trx1*Δ cells, demonstrating that Trr1 does not act independently of Trx1 (Fig. 4F). Taken together, the results presented in Fig. 4 strongly indicate that inactivation of Trx1 by oxidation stimulates H_2O_2-induced polarized cell growth.

Trx1 regulates the Cap1 transcription factor independently of polarized cell growth. In *C. albicans*, the AP-1-like transcription factor Cap1 is the major regulator of oxidative-stress-induced gene expression (15, 16). In *S. cerevisiae*, the Cap1-related transcription factor Yap1 is activated by H_2O_2 by a mechanism involving the oxidation and nuclear accumulation of the protein (reviewed in reference 32). Significantly, thioredoxin negatively regulates Yap1 activity by acting to reduce oxidized Yap1 (13). Thus, in *S. cerevisiae trx1*Δ *trx2*Δ cells, Yap1 is partially constitutively oxidized and accumulates in the nucleus under nonstressed conditions (13, 24). Hence, it was possible that H_2O_2-induced oxidation, and thus inhibition of Trx1 in *C. albicans*, results in the oxidation and activation of Cap1, which then stimulates polarized cell growth. To test this hypothesis, we first compared the H_2O_2-induced activation of Cap1 in wild-type and *trx1*Δ cells. H_2O_2-induced oxidation of Cap1 was monitored using the thiol-reducing agent AMS as described above (13). The mobility of Cap1-specific bands increased in response to H_2O_2 compared to those seen in unstressed cells, indicating that Cap1 was rapidly oxidized following H_2O_2 treatment (Fig. 5A). Moreover, this oxidation was transient, as the majority of Cap1 was reduced 1 h after H_2O_2 treatment (Fig. 5A). In contrast to Yap1 oxidation in *S. cerevisiae*, oxidation of Cap1 was not detected in unstressed *C. albicans trx1*Δ cells (Fig. 5A), and consequently, no nuclear accumulation of Cap1 (Fig. 5B) or Cap1-dependent gene expression (Fig. 5C) was evident. Notably, however, an oxidized form of Cap1 persisted in *trx1*Δ cells compared to wild-type cells following H_2O_2 treatment (Fig. 5A) and, consistent with this, both H_2O_2-induced Cap1 nuclear accumulation (Fig. 5B) and H_2O_2-induced expression of the Cap1-dependent gene *TRR1* (Fig. 5C) were prolonged in *trx1*Δ cells. Collectively, these data demonstrate that Trx1 does play some role in regulating the reduction, and thus inactivation, of Cap1, although Trx1-independent reduction of Cap1 is also evident. Therefore, Trx1 negatively regulates both Cap1 activation and polarized cell growth, which is consistent with the hypothesis outlined above that activation of Cap1 may be important for H_2O_2-induced filamentation. However, cells expressing a constitutively active mutant version of Cap1 are not filamentous (61), and H_2O_2-induced polarized cell growth was not inhibited in *cap1*Δ cells (Fig. 5D). Thus, these observations illustrate that Trx1 regulates polarized cell growth in a Cap1-independent manner.

Trx1 and Tsa1 regulate H_2O_2-induced activation of the Hog1 SAPK. In addition to activation of Cap1, treatment of *C. albicans* with H_2O_2 stimulates the phosphorylation and activation of the Hog1 SAPK (3, 49). Thus, it was possible that Hog1 activation is required for H_2O_2-induced polarized cell growth. Indeed, the levels of Hog1 phosphorylation increase with increasing concentrations of H_2O_2 (49). Consistent with this possibility, loss of a ROS-scavenging antioxidant, such as Trx1, would be predicted to result in greater activation of Hog1 at a

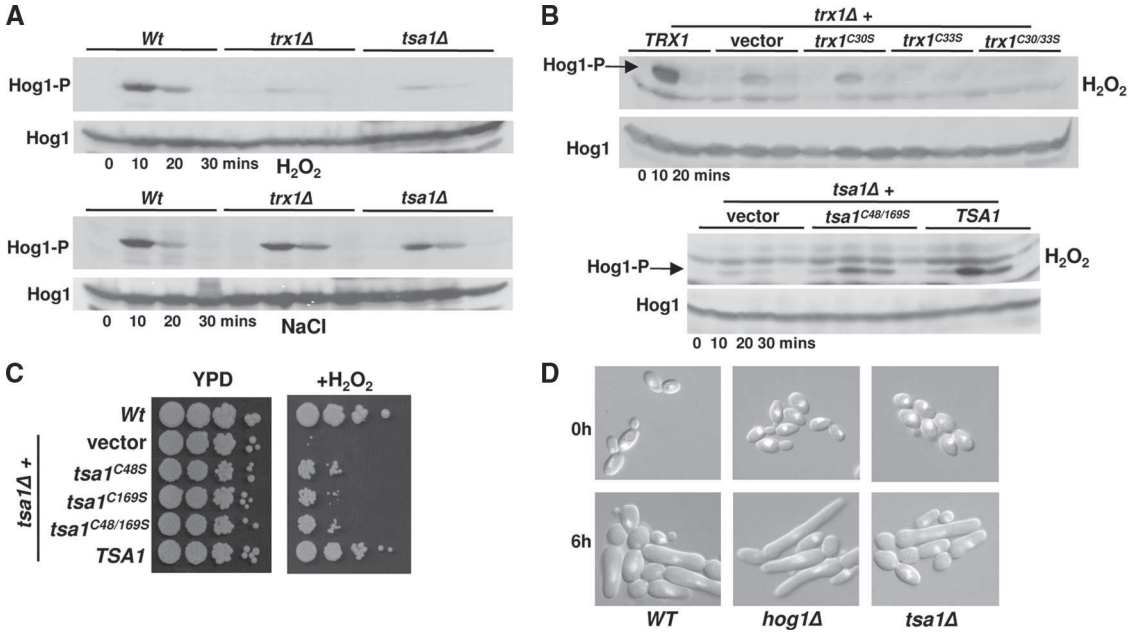

FIG. 6. Trx1 and Tsa1 are required for H_2O_2-induced activation of the Hog1 SAPK pathway. (A and B) Activation of Hog1 in response to H_2O_2 requires Trx1 and Tsa1. Hog1 phosphorylation was detected by Western blot analysis of lysates prepared from wild-type (JC747), *trx1*Δ (JC677), or *tsa1*Δ (JC1027) cells (A); *trx1*Δ cells (JC677) or *trx1*Δ cells, containing vector or expressing either $trx1^{C30S}$ (JC759), $trx1^{C33S}$ (JC761), $trx1^{C30/33S}$ (JC763), or *TRX1* (JC679; reconstituted strain) (B, top); or *tsa1*Δ cells containing vector or expressing either $tsa1^{C48/169S}$ (JC1031) or *TSA1* (JC1028) (B, bottom) following treatment with either 5 mM H_2O_2 or 0.3 M NaCl for the indicated times. The Western blots were probed with an anti-phospho-p38 antibody to detect the phosphorylated, active form of Hog1 (Hog1-P). Total levels of Hog1 protein were subsequently determined by stripping and reprobing the blot with an anti-Hog1 antibody (Hog1). (C) Cells lacking Tsa1 are more sensitive to H_2O_2 than cells expressing catalytically inactive forms of Tsa1. Approximately 10^4 cells, and 10-fold dilutions thereof, of exponentially growing wild-type (JC747) and *tsa1*Δ (JC1027) strains and *tsa1*Δ strains expressing either $tsa1^{C48S}$ (JC1029), $tsa1^{C169S}$ (JC1030), $tsa1^{C48/169S}$ (JC1031), or *TSA1* (JC1028) were spotted onto YPD plates with or without 3.5 mM H_2O_2. The plates were then incubated at 30°C for 24 h. (D) Loss of either Hog1 or Tsa1 does not impair H_2O_2-induced polarized cell growth. Wild-type (BWP17), *hog1*Δ (JC47), and *tsa1*Δ (JC1027) cells were untreated or treated with 5 mM H_2O_2 for 6 h, and images were captured as described for Fig. 2.

given level of H_2O_2 due to disturbed redox balance, perhaps explaining the polarized-growth phenotype associated with *trx1*Δ cells. However, in stark contrast to this prediction, either deletion of *TRX1* or mutation of the catalytic cysteine residues of Trx1 in fact significantly impaired H_2O_2-induced phosphorylation of Hog1 (Fig. 6A and B). Furthermore, this effect was specific for H_2O_2, as wild-type levels of Hog1 phosphorylation were stimulated in *trx1*Δ cells in response to osmotic stress (Fig. 6A). Previous studies indicated that the primary function of thioredoxin in H_2O_2 signaling is to regulate the oxidation status of key regulatory proteins (13, 36). Hence, our data showing that the two catalytic cysteines of Trx1 are important for Hog1 activation strongly suggest that a protein(s) required for Hog1 regulation is susceptible to Trx1-regulated oxidation.

We previously demonstrated that the H_2O_2-induced activation of the Hog1-related Sty1 (also known as Spc1/Phh1) SAPK in the evolutionarily distant yeast *Schizosaccharomyces pombe* is regulated by the 2-Cys peroxiredoxin Tpx1 (55). Hence, given the function of Trx1 in the catalytic cycle of the analogous enzyme, Tsa1, in *C. albicans* (Fig. 1 and 3D), it was possible that Trx1-dependent regulation of Hog1 involves Tsa1 (52). Indeed, as observed in *S. pombe*, deletion of *TSA1* significantly impaired Hog1 phosphorylation in response to H_2O_2, but not osmotic stress (Fig. 6A). In *S. pombe*, Tpx1-dependent activation of StyI is entirely dependent on the peroxidatic cysteine (Cys 48), but not the resolving cysteine (Cys 169), of Tpx1 (55). To investigate whether *C. albicans* Hog1 is similarly regulated by Tsa1, *tsa1* mutants were generated in which either or both of the predicted peroxidatic (Cys48) and resolving (Cys169) cysteine residues were mutated to serine. However, in contrast to Tpx1 regulation of StyI in *S. pombe* (55), activation of Hog1 in response to H_2O_2 could still be detected in cells expressing Tsa1 in which the catalytic cysteine residues were mutated (Fig. 6B). This adds to the growing evidence that 2-Cys peroxiredoxins, such as Tsa1, execute vital signaling roles that are independent of peroxidase activity (54). Indeed, consistent with this, *C. albicans* cells lacking Tsa1 were reproducibly more sensitive to H_2O_2 than cells expressing catalytically inactive forms of Tsa1 (Fig. 6C).

Consistent with our observations that Trx1 acts to inhibit H_2O_2-induced polarized cell growth and yet is required for H_2O_2-induced activation of Hog1, deletion of *HOG1* did not impair H_2O_2-induced filamentation (Fig. 6D). However, our finding that loss of Trx1 function results in the constitutive oxidation of Tsa1 (Fig. 3D) raised the possibility that Trx1-regulated oxidation of Tsa1 by H_2O_2 may be important for both Hog1 activation and polarized cell growth. However, unlike *trx1*Δ cells, *tsa1*Δ cells do not display polarized cell growth in the absence of H_2O_2, and furthermore, H_2O_2 treatment induced polarized cell growth in cells lacking *TSA1* (Fig. 6D). Hence these data suggest that Trx1 regulates polarized cell growth independently of either Tsa1 or Hog1.

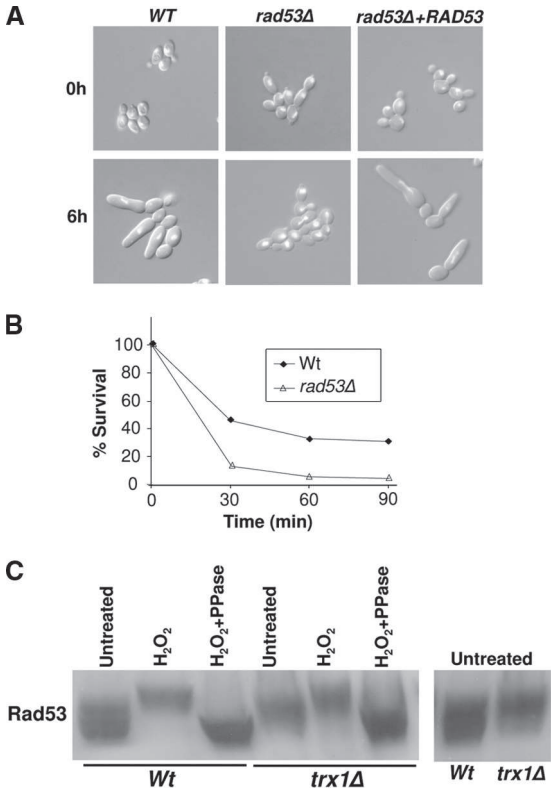

FIG. 7. The checkpoint kinase Rad53 is activated in *trx1*Δ cells and is required for H_2O_2-induced polarized cell growth and the oxidative-stress response. (A) Loss of Rad53 function blocks H_2O_2-induced polarized cell growth. Wild-type (BWP17), *rad53*Δ (WYS3), and *rad53*Δ plus *RAD53* (WYS3.1) cells were treated with 5 mM H_2O_2 for 6 h, and images were captured as described for Fig. 2. (B) Rad53 is essential for oxidative-stress resistance. *rad53*Δ (WYS3) cells and the reconstituted *rad53*Δ plus *RAD53* strain (WYS3.1) were treated with 5 mM H_2O_2, and cell survival at the indicated times was calculated as described in Materials and Methods. (C) Rad53 is hyperphosphorylated in response to H_2O_2 and in unstressed cells lacking Trx1. Wild-type (JC894) and *trx1*Δ (JC866) cells expressing myc-6His-tagged Rad53 were untreated or treated with 5 mM H_2O_2 for 2 h. The lysates were analyzed by Western blotting either before or after treatment with λ-phosphatase. Rad53 was detected using an anti-myc antibody. The mobilities of Rad53 under nonstressed conditions in wild-type and *trx1*Δ cells were also compared side by side to emphasize the slower mobility of Rad53 in cells lacking *TRX1*.

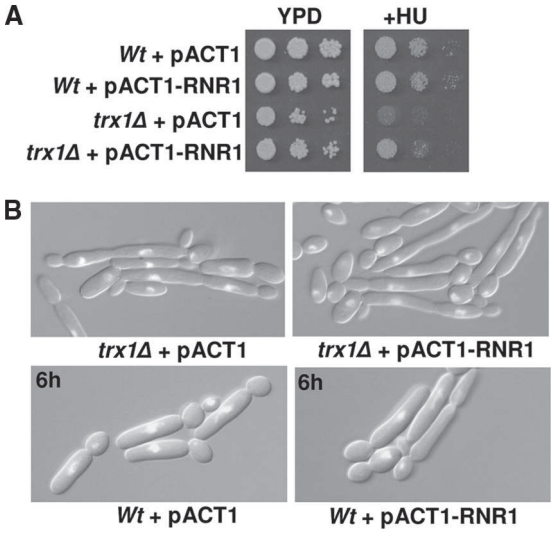

FIG. 8. Trx1-mediated polarized cell growth is independent of Rnr1. (A) *trx1*Δ cells display increased sensitivity to HU, and this can be rescued by ectopic expression of *RNR1*. Approximately 10^3 cells, and 10-fold dilutions thereof, of mid-exponential-phase growing wild-type and *trx1*Δ cells containing either pACT1 (JC1014 and JC1022, respectively) or pACT1-RNR1, in which *RNR1* is expressed from the *ACT1* promoter (JC1016 and JC1024, respectively), were spotted onto YPD plates with or without 30 mM HU and incubated at 30°C for 24 h. (B) Ectopic expression of *RNR1* does not either reverse the polarized cell growth phenotype of *trx1*Δ cells (top) or inhibit H_2O_2-induced polarized cell growth (bottom). Shown are micrographs of *trx1*Δ cells containing either pACT1 (JC1022) or pACT1-RNR1 (JC1024) (top) or wild-type cells containing either pACT1 (JC1014) or pACT1-RNR1 (JC1016) after treatment with 5 mM H_2O_2 for 6 h. The images were captured as described for Fig. 2.

The Rad53 checkpoint kinase is hyperactivated either in response to H_2O_2 or in *trx1*Δ cells and is essential for H_2O_2-induced polarized cell growth. Although Trx1 regulates the major oxidative-stress-responsive Cap1 and Hog1 pathways in *C. albicans*, our data demonstrate that neither pathway is involved in Trx1-regulated polarized cell growth. However, the DNA checkpoint kinase Rad53 was recently shown to play a central role in mediating polarized cell growth in *C. albicans* in response to genotoxic insults, such as the drugs HU and MMS (48). Hence, it was possible that the Rad53 pathway was also important for H_2O_2-induced filamentation. Significantly, H_2O_2-induced polarized cell growth was abolished in *rad53*Δ cells, which remained exclusively in the yeast form (Fig. 7A). Moreover, this defect was corrected by reintegrating a wild-type copy of *RAD53* (Fig. 7A). Rad53 function is clearly important for the cellular response to ROS, as demonstrated by the increased sensitivity of *rad53*Δ cells to H_2O_2 (Fig. 7B). Furthermore, similar to that observed upon activation by HU and MMS (48), Western blot analysis of Rad53 tagged at the C terminus with 2 myc and 6 His residues, revealed that Rad53 becomes hyperphosphorylated in response to H_2O_2 (Fig. 7C). Significantly, Rad53 is also hyperphosphorylated in *trx1*Δ cells under nonstressed conditions (Fig. 7C), although some H_2O_2-induced phosphorylation was also evident. Thus, either exposure of cells to H_2O_2 or inactivation of Trx1 activates the Rad53 DNA checkpoint kinase. The relationship between Rad53 and Trx1 functions was further emphasized by our inability to create a *trx1*Δ *rad53*Δ double mutant, suggesting synthetic lethality. Collectively, these results strongly suggest that the H_2O_2-induced oxidation, and thus inactivation of Trx1, triggers activation of Rad53, which in turn stimulates polarized cell growth.

HU inhibits RNR activity, and notably, RNR is a well-established substrate for thioredoxin (25). Hence, this suggested that Trx1- and H_2O_2-dependent polarized cell growth may be due to inactivation of RNR. Indeed, consistent with Trx1 regulating RNR function, *trx1*Δ cells were more sensitive than wild-type cells to HU, and this sensitivity could be rescued by ectopic expression of *RNR1*, which encodes the large subunit of RNR (Fig. 8A). In contrast, however, ectopic expression of *RNR1* had no obvious impact on Trx1- and H_2O_2-dependent polarized cell growth (Fig. 8B). These data, together with the previous observation that HU treatment further stimulates po-

larized bud growth in *trx1*Δ cells (Fig. 2E), indicate that Trx1-dependent activation of the Rad53 pathway and subsequent polarized cell growth are largely independent of Trx1 regulation of Rnr1.

Deletion of *TRX1* attenuates virulence in *C. albicans*. As Trx1 plays a central role in the oxidative-stress response in *C. albicans*, the role of Trx1 in virulence was assessed using a 3-day murine intravenous-challenge model of *C. albicans* infection (29, 30). Previous data demonstrated that weight loss (days 0 to 3) correlates with the ultimate infection outcome (29). Based upon this finding and increases in fungal burdens occurring during disease progression, a 3-day model to assay fungal virulence was developed, with an outcome score based upon these two parameters (30). A higher outcome score is indicative of greater weight loss and higher fungal burdens. Three days after intravenous challenge, mice infected with wild-type cells had lost an average of 15.1% ± 6.1% of their body weight while the group infected with *trx1*Δ plus *TRX1* reintegrant cells had lost 11.0% ± 5.4%. In contrast, the group infected with *trx1*Δ cells had gained weight, 0.7% ± 2.3%. The corresponding data for kidney fungal burdens, expressed as $\log_{10}$ CFU/g, were 5.0 ± 0.7, 4.8 ± 1.0, and 3.1 ± 0.4, respectively. Consistent with this, histopathology analysis of kidney sections taken 3 days postinfection clearly illustrated a dearth of fungal elements in the kidneys from mice infected with *trx1*Δ cells (Fig. 9). Subsequently, the outcome scores determined for the three strains were as follows: wild type, 12.5 ± 3.7; *trx1*Δ, 2.8 ± 1.2; and *trx1*Δ plus *TRX1* reintegrant, 10.4 ± 3.5. This corresponds to mean survival times of between 5 and 10 days (wild type and reintegrant) and 20 and 25 days (*trx1*Δ mutant) (29, 30). Statistical analysis (Mann-Whitney U nonparametric comparison) revealed that for all parameters—weight loss, kidney fungal burden, and outcome score—the difference between *trx1*Δ cells and either wild-type or reintegrant cells was highly significant ($P < 0.01$). In contrast, there was no statistical difference between the wild-type and reintegrant strains. Taken together, these data illustrate that the *trx1*Δ mutant is clearly attenuated in virulence in comparison with control strains.

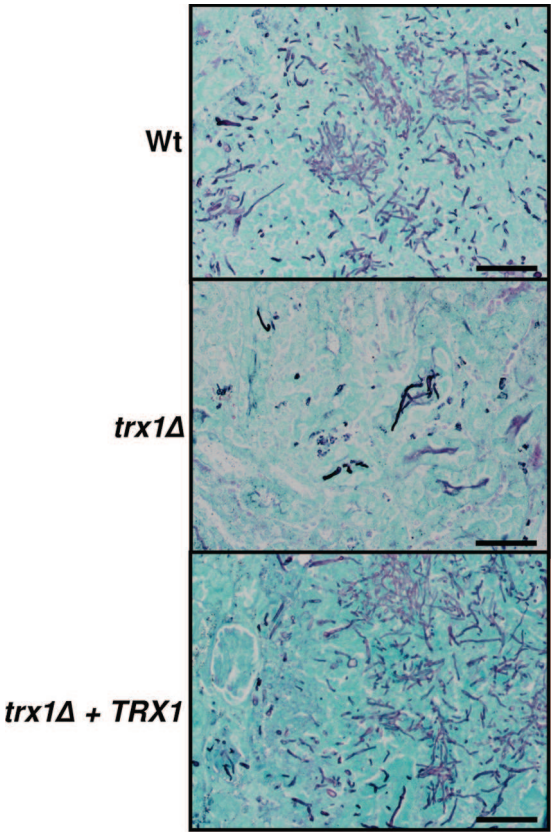

FIG. 9. Visualization of fungal cells in kidney sections from animals infected with wild-type, *trx1*Δ, and *trx1*Δ plus *TRX1* cells for 3 days. Kidney sections (5 μm) were stained with methenamine silver stain and poststained light green. Bars = 50 μm. Significantly greater numbers of fungal cells were evident following infection with the wild-type (JC747) or reconstituted (JC679) strain than with the *trx1*Δ null mutant strain (JC677).

DISCUSSION

The data presented here reveal that the thioredoxin protein Trx1 has a multiplicity of roles in the regulation of cellular responses to H_2O_2 in *C. albicans.* For example, Trx1 functions as an antioxidant by regulating the reduction and thus activity of the 2-Cys peroxiredoxin Tsa1, which detoxifies H_2O_2. Furthermore, our studies have demonstrated that Trx1 also regulates three distinct responses to H_2O_2 stress in *C. albicans*: polarized cell growth, activation of the Hog1 SAPK, and activation of the Cap1 transcription factor (Fig. 10). In agreement with these key central roles of Trx1 in oxidative-stress responses and the relationship between ROS and pathogenesis (19), *C. albicans* cells lacking Trx1 displayed significantly attenuated virulence in a mouse model of infection. Interestingly, the role of Trx1 in promoting pathogenesis may be dependent on its signaling rather than antioxidant functions, as inactivation of Tsa1, the major peroxidase substrate of Trx1, does not attenuate virulence (52). Significantly, as robust oxidative-stress responses are vital for the virulence of many fungal pathogens (9), this work with *C. albicans* provides a platform to further investigate antioxidant-mediated signaling in other fungi.

During the course of this study, a paper reported that H_2O_2 treatment stimulates hyphal growth in *C. albicans*, although the morphological characteristics of the filamentous cells were not defined (37). Here, we show that exposure of *C. albicans* cells to H_2O_2 stimulates the formation of hyperpolarized buds. Although we observed hyperpolarized bud growth in response to a range of H_2O_2 concentrations (Fig. 4B and data not shown), the majority of experiments in this study were performed using 5 mM H_2O_2. The oxidative burst is a major mechanism employed by phagocytes to kill pathogens, and although it is not known what levels of H_2O_2 accumulate within the phagosome, evidence suggests that the concentrations are substantial (42). Indeed, following incubation of wild-type *C. albicans* with neutrophils at a 1:1 ratio for 1 h, only 40% of the fungal cells survived (17). In this study, we chose to use 5 mM H_2O_2, as a similar level of survival was seen following a 1-h exposure of *C. albicans* to this concentration of peroxide stress (Fig. 7B). Furthermore, the Hog1 SAPK is robustly activated only following treatment of cells with >2 mM H_2O_2 (49), and this protein is essential for *C. albicans* to survive phagocytosis (4).

The observation that H_2O_2 stimulates hyperpolarized bud

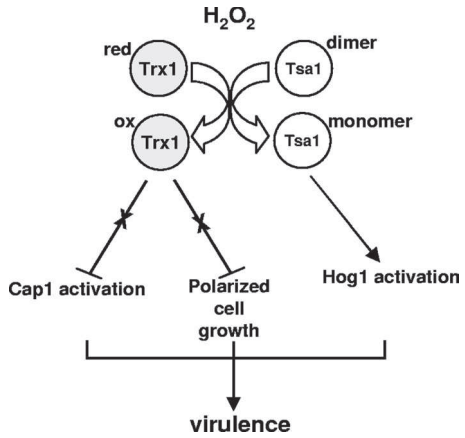

FIG. 10. Model depicting the multiple roles of Trx1 in H_2O_2 signaling in *C. albicans*. Trx1 acts as a repressor of Cap1 activation and polarized cell growth and as an activator of the Hog1 SAPK. Upon exposure to H_2O_2, Trx1 becomes oxidized (ox) by reducing (red) oxidized substrates, such as the 2-Cys peroxiredoxin Tsa1. In the model, oxidation of Trx1 relieves the inhibitory effects of the protein on both Cap1 activation and polarized cell growth. However, in contrast, Trx1 and Tsa1 are both required for the activation of Hog1. One model is that Trx1 function acts to prevent Tsa1 becoming trapped in a form (dimeric) that is unable to activate Hog1, although it is also possible that Trx1 and Tsa1 function independently to regulate H_2O_2-induced activation of Hog1. Trx1 clearly has multiple roles in ROS signaling in *C. albicans*, and consistent with this central role in oxidative-stress responses, *trx1*Δ cells display significantly attenuated virulence in a mouse model of disease.

formation provides the first demonstration that a physiologically relevant stress induces this mode of growth in *C. albicans*. Indeed, the *in vivo* relevance of such polarized bud growth was unclear, as it has previously been observed only in response to either mutations or chemicals that perturb cell cycle progression (4, 39). However, *C. albicans* undoubtedly encounters ROS, such as H_2O_2, during infection, and thus, H_2O_2-induced polarized bud growth may allow migration and escape from ROS-rich environments, promoting survival in the host. Significantly, ROS-stimulated polarized cell growth is not limited to *C. albicans.* For example, several filamentous fungal species contain Nox complexes similar to those in phagocytic cells, and the localized synthesis of ROS by such enzymes is important for regulating polarized hyphal growth (46). However, how fungal cells sense ROS and initiate polarized growth in response to ROS is entirely unknown. Our data may have provided major insight by revealing that the oxidation of an evolutionarily conserved antioxidant protein, thioredoxin, is a key signaling event in H_2O_2-induced polarized cell growth in *C. albicans*. Moreover, it is unlikely that this H_2O_2-stimulated growth is due to Trx1-dependent regulation of Tsa1, Hog1, or Cap1, as cells lacking these proteins all form filaments following H_2O_2 treatment. Instead, we provide evidence that Trx1-mediated H_2O_2-induced polarized cell growth involves activation of the Rad53 checkpoint kinase. For example, we found that exposure of cells to H_2O_2 results in significant hyperphosphorylation of Rad53, and deleting *RAD53* completely abolishes H_2O_2-induced filamentous growth. These results are similar to those of a recent study which reported that exposure of cells to a range of genotoxic stresses simulated Rad53 phosphorylation and hyperpolarized bud formation that was entirely Rad53 dependent (48). Furthermore, our observations that deletion of *TRX1* results in both constitutive hyperphosphorylation of Rad53 and a hyperpolarized bud morphology is entirely consistent with a model in which inactivation of Trx1 in response to H_2O_2 is a key step in stimulating Rad53 activation and hyperpolarized bud growth. This model is further supported by the observations that mutation of the redox-sensitive catalytic cysteine residues of Trx1 also resulted in a hyperpolarized bud morphology and, moreover, that *trx1*Δ-associated filaments cannot elongate further in response to H_2O_2. We also attempted to directly investigate whether the filamentous phenotype of *trx1*Δ cells was abolished upon deletion of *RAD53*. However, numerous attempts to create a *trx1*Δ *rad53*Δ double mutant proved unsuccessful, strongly suggesting that Rad53 function is essential for viability in the event that Trx1 function is lost, or *vice versa*.

Previous work has shown that activation of the Cap1 transcription factor and the Hog1 SAPK pathway play key roles in protecting *C. albicans* cells from ROS (3, 15, 61). However, with the exception of one study showing that the response regulator protein Ssk1 is important for H_2O_2-induced activation of Hog1 (11), the mechanisms underlying the activation of these different pathways in response to ROS are poorly understood. Here, we demonstrate that Trx1 regulates both of these major H_2O_2-responsive signaling pathways, and notably, this is the first report that thioredoxin regulates stress signaling to fungal SAPK pathways. However, our studies have also uncovered significant differences from thioredoxin-dependent regulation observed in other systems. For example, in contrast to Trx1 regulation of Cap1 in *C. albicans*, reduction of the related Yap1 transcription factor in *S. cerevisiae* is entirely thioredoxin dependent. Thus, in *S. cerevisiae*, thioredoxin deficiency results in constitutive oxidation, nuclear accumulation, and Yap1-dependent gene expression in the absence of H_2O_2 (13). However, although Trx1 clearly plays some role in regulating the reduction of Cap1 following oxidative stress, activation of Cap1 in the absence of H_2O_2 was not detected in *trx1*Δ cells. Furthermore, our finding that Trx1 is critical for the H_2O_2-induced activation of Hog1 is in marked contrast to what has been reported in mammalian systems, in which thioredoxin functions as a repressor of the Hog1-related JNK and p38 SAPK signaling cascades (43). The molecular basis underlying the apparently opposing roles of thioredoxin regulation of *C. albicans* and mammalian SAPK pathways is unclear. It is known that the upstream Ask1 mitogen-activated protein kinase kinase kinase (MAPKKK) in the mammalian SAPK pathways is activated via cysteine oxidation and, moreover, that Trx1 negatively regulates this pathway by reducing the oxidized cysteines of Ask1 (35, 36). As Trx1 is a positive regulator of the Hog1 SAPK in *C. albicans*, it seems unlikely that a similar mechanism is in place in this fungus. Instead, we propose a model in which a Hog1 regulatory protein(s) is susceptible to oxidative inactivation and Trx1 functions to maintain this regulator in an active, reduced state (see below). Furthermore, in contrast to Ask1, analogous MAPKKKs in fungi appear to be regulated by 2-component-related phosphorelay systems, which are absent from mammals (45). For example, the response regulator Ssk1, which is predicted to regulate the *C. albicans* MAPKKK Ssk2 (12), is essential for H_2O_2-induced

activation of Hog1 (11). However, it remains to be established whether Trx1 functions in the same pathway as Ssk1 to relay H_2O_2 signals to Hog1.

Although Trx1 influences three major H_2O_2-responsive pathways in *C. albicans*, this is probably done by different regulatory mechanisms (Fig. 10). Indeed, Trx1 acts as an inhibitor of both H_2O_2-induced polarized cell growth and Cap1 activation but acts as an activator of Hog1. Moreover, it is unlikely that Trx1-dependent regulation of Cap1 is indirect via regulation of Tsa1 and/or Hog1, as Cap1 function is not impaired in cells lacking either of these proteins (15, 52). Furthermore, *tsa1*Δ cells are more sensitive to ROS than *trx1*Δ cells (data not shown), suggesting that the sustained activation of Cap1 that occurs in *trx1*Δ cells is not a consequence of decreased resistance to H_2O_2. In different strain backgrounds of *S. cerevisiae*, either Tsa1 or the thiol peroxidase enzyme Gpx3 promotes oxidation of Yap1, and thioredoxin reduces oxidized Tsa1, Gpx3, and Yap1 (13). However, previous work has not identified a role for Tsa1 in the regulation of Cap1 (52). Thus, although elements of Cap1 regulation appear different from Yap1 in *S. cerevisiae*, it is possible that Trx1 directly regulates the oxidation state of Cap1, and possibly a Gpx3-related protein. Similarly, we propose that a regulator(s) of polarized cell growth is oxidized in response to H_2O_2 and that this triggers activation of the Rad53 pathway. In this model, Trx1 acts as an inhibitor of Cap1 and polarized growth by reducing active, oxidized Cap1 and the polarized cell growth regulator(s), respectively. Both Cap1 activation (15) and the extent of polarized cell growth (Fig. 4) are dependent on the level of H_2O_2 stress imposed on the cell. These observations support a model in which the degree of stimulation of these cellular processes is precisely tailored to the levels of peroxide stress experienced by the cell via the H_2O_2 concentration-dependent oxidation and inhibition of Trx1 (Fig. 4). Currently, the identity of the polarized-growth regulator regulated by Trx1 is unknown, but our data suggest that it is not the Trx1 substrate Rnr1. Current experiments are directed at identifying the Trx1 substrate(s) that regulates polarized cell growth. With regard to Trx1 regulation of the Hog1 SAPK, the two catalytic cysteine residues of Trx1 are crucial for H_2O_2-induced activation of Hog1, suggesting that Trx1 influences the oxidation status of a protein(s) required for Hog1 regulation. Furthermore, as described above, the positive role of Trx1 in Hog1 signaling is consistent with a model in which Trx1 functions to maintain a Hog1 regulator in a reduced, active state. Significantly, we demonstrated that the Trx1 substrate, Tsa1, is also required for Hog1 activation, and moreover, Tsa1 is trapped in the oxidized, dimeric form in *trx1*Δ cells. Thus, it is possible that this dimeric form of Tsa1 inhibits Hog1 activation or, alternatively, that the monomeric form of Tsa1 acts as an activator of Hog1. Consistent with these possibilities, mutation of either catalytic cysteine residue of Tsa1, which prevents formation of the oxidized dimeric form, does not significantly impair Hog1 activation in response to peroxide stress. Interestingly, this contrasts with previous work in *S. pombe* where the peroxidatic cysteine residue of the 2-Cys peroxiredoxin Tpx1 was found to be essential for H_2O_2-induced activation of the Hog1-related StyI SAPK (55). However, it is also possible that Trx1 and Tsa1 function in distinct pathways to regulate Hog1. Further experimentation is ongoing to dissect the precise roles of Trx1 and Tsa1 in the relay of H_2O_2 signals to the Hog1 pathway.

High levels of ROS are associated with cellular damage and consequently have been linked to human disease and the aging process. However, it is now evident that ROS also have important roles as signaling molecules. Redox-sensitive antioxidant proteins respond to increased ROS levels and restore redox homeostasis and are thus well placed to undertake ROS-sensing functions. Indeed, recent studies from yeast to humans have identified several conserved antioxidant proteins as regulators of signal transduction pathways that respond to ROS (54). The comprehensive analysis of an antioxidant in oxidative-stress signaling in a single organism, presented in this study, extends previous work by demonstrating that the thioredoxin protein Trx1 acts to regulate all of the known major H_2O_2-responsive pathways in *C. albicans*. Hence, these data suggest that the utilization of antioxidant proteins as regulators of ROS-induced signal transduction pathways may be more extensive than previously thought.

ACKNOWLEDGMENTS

We thank Al Brown, Ken Haynes, Frank Odds, and Elizabeth Veal for helpful discussions and comments on the manuscript. We are also very grateful to Elizabeth Veal (Newcastle University, United Kingdom), and Yue Wang (Institute of Molecular and Cell Biology, Singapore) for reagents.

This work was funded by the Wellcome Trust (J.Q., B.A.M., L.P.E., and D.M.M.), a BBSRC DTG studentship (M.J.P.), and an ORS award from Newcastle University and a Ph.D. studentship from ICAMB (A.D.S.D.).

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