The roles and regulation of heat shock proteins in yeast

Faye Elizabeth Middleton

Thesis submitted for the degree of Doctor of Philosophy

Institute for Cell and Molecular Biology

Faculty of Medical Sciences

Newcastle University

September 2017

Declaration

I certify that this thesis is my own work, except where acknowledged, and has not been previously submitted for a degree or any other qualification at this or any other university.

Abstract

Heat shock proteins (HSPs) such as the HSP70s Ssa1 and Ssa2 and the HSP40 Ydj1 in *Saccharomyces cerevisiae* are ubiquitous abundant proteins. With well characterised roles as protein chaperones, HSPs function in heat and oxidative stress responses to prevent aggregation and to aid degradation. The functions of HSPs are important in humans to prevent diseases such as cardiovascular disease and Parkinson's disease.

Interestingly, recent studies have also begun to identify key roles for specific HSPs in stress sensing and signal transduction and the regulation of the cell cycle. For example, work in *S. cerevisiae* revealed that Ssa1 and Ydj1 regulate cell cycle commitment in G1 phase by controlling the availability of the Cdc28-Cln3 cyclin dependent protein kinase complex. Moreover, Ssa1 activity and modification regulates the heat/oxidative stress-dependent activation of the conserved Hsf1 transcription factor. However, despite these yeast studies there is still much to learn about the conserved and organism-specific roles and regulation of HSP70 and HSP40 proteins in cellular processes.

Here studies were initiated in the evolutionarily distant fission yeast *Schizosaccharomyces pombe* to investigate the roles and regulation of Ssa1 (HSP70), Ssa2 (HSP70) and Mas5 (HSP40), homologues of *S. cerevisiae* Ssa1, Ssa2 and Ydj1, respectively. Excitingly, these HSPs were found to play key roles in *S. pombe* in conserved processes such as mitosis, meiosis and autophagy. For example, Ssa2 and Mas5, but not Ssa1, were found to localise to the site of new cell growth suggesting roles in the cell cycle in addition to their roles in the regulation of Hsf1. Moreover, microtubule dynamics and cell size were found to be dependent on Ssa1 and Mas5. Interestingly, microtubule dynamics and cell size are also regulated by their homologues Ssa1 and Ydj1 in *S. cerevisiae*, suggesting conserved roles in cell cycle progression.

Investigations were also performed to identify any connections between Ssa1, Ssa2 and Mas5 in the responses of *S. pombe* cells to heat and/or oxidative stresses. Studies in other organisms have revealed the importance of HSP70 and HSP40 as repressors of the activation of Hsf1. Indeed, consistent with these results Mas5 was found to act as a repressor of the heat stress-induced nuclear accumulation of Hsf1. Previous work revealed that different signalling pathways/transcription factors determine the response of *S. pombe* cells to increasing concentrations of H_2O_2. For example, the thioredoxin system regulated AP-1-like transcription factor Pap1 is important for the response to low but not high concentrations of H_2O_2. Moreover, the oxidation status of thioredoxin system proteins determines the timing of oxidation, activation and nuclear accumulation of Pap1.

Excitingly, the studies described here revealed novel insights into the roles and regulation of Hsf1 in response to H_2O_2 in *S. pombe*. For example, Hsf1 was found to accumulate in the nucleus in response to H_2O_2 in a concentration dependent manner like Pap1. Significantly, although this accumulation was found, like Pap1, to be linked to the thioredoxin system, Hsf1 accumulation appears to be regulated in a different manner to Pap1. Results also revealed that Ssa1 acts as an activator of Hsf1 in the H_2O_2 response while Mas5 acts as a repressor of Hsf1. In *S. cerevisiae* the potential relationship between Hsf1 and Yap1, the Pap1 homologue, in the response to oxidative stress have not been studied. Excitingly, the results presented here suggest that Mas5 is a new regulator of Pap1 activity, influencing Pap1 through the thioredoxin system. Moreover, data suggests cross talk between Hsf1/Pap1 transcriptional responses with Mas5 acting as a previously unidentified key regulator of both responses.

A model is proposed where Mas5 regulation of Hsf1 influences Pap1 activation by regulating the expression of the *tpx1*$^+$ gene encoding the 2-Cys peroxiredoxin Tpx1, a known regulator of

Pap1 that acts in the thioredoxin system. These results are intriguing given recent studies in *S. cerevisiae* suggesting that the thioredoxin system protein Tsa1, the homologue of Tpx1, replaces Ydj1 (Mas5 homologue) as a co-chaperone for HSP70 roles in aggregate recognition and clearance in response to oxidative stress and not heat stress. In conclusion, the studies presented in this thesis provide new insights into the regulation of Hsf1 and the roles of HSP70 and HSP40 HSPs in eukaryotes. Given their relationships with common human diseases it is important to understand the organism-specific and conserved roles of these proteins to allow the development of future medical interventions.

Acknowledgements

First and foremost, I would like to thank my supervisor Professor Brian Morgan. Throughout the PhD he has offered his unwavering support and encouragement. I can never thank him enough for the guidance he has given me over the last 4 years. I must also thank him for offering me the PhD as it brought me to Newcastle where I met my future husband, Craig Wood. Craig has been with me through every step of the PhD, every high and every low. I often felt I bombarded him with the trials of the PhD but he never faltered in his ability to let me see it was all worthwhile.

I would also like to thank my fellow PhD students in the yeast labs for their friendship and advice. I feel truly lucky to have worked in such a happy environment, being able to laugh together made the experience a joy to share. In particular, I want to thank Pippa Harvey for her honesty, her humour and always being ready for a tea break. I'd be lost without her. I also want to thank Johnathan Winter for providing sarcasm and support in a perfect ratio.

When science has come first my family have been patient and kind. They have all provided a shoulder to lean on when times were hard. They smiled politely while I talked about science, they took pride in my accomplishments. The PhD has made me realise how thankful I am to have these people in my life.

There is so much to accomplish during the PhD and it could not have been achieved without the advice of the other supervisors in the yeast lab including Dr Simon Whitehall, Dr Elizabeth Veal and Professor Janet Quinn. As intimidating as lab meeting could be their input helped me to improve. Additionally, I would like to thank Michelle Wray for providing technical support that allowed me to focus on my project.

Finally, the project provided novel insights in to yeast which were facilitated by my personal and professional development thanks to the BBSRC funding. I hope the BBSRC continues enabling scientists to experience the joy of scientific discoveries like I have.

Table of Contents

Abbreviations

1°	Primary
2°	Secondary
4-HNE	4-hydroxynonenal
Δ	Gene deletion
°C	Degrees Celsius
µl	Microlitre
2-cys	2-Cysteine
ADP	Adenosine diphosphate
AMS	4-acetamido-4'-maleimidylstilbene-2,2'-disulphonic acid
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
bp	Base pairs
BSA	Bovine serum albumin
bZIP	Basic leucine zipper domain
C	Cysteine
C. albicans	Candida albicans
$CaCl_2.2H_2O$	Calcium chloride
CDC	Cell division cycle
CDK	Cyclin dependent kinase
CESR	Core environmental stress response
Cgmp	Cyclic guanosine monophosphate
ChIP	Chromatin immunoprecipitation
Cln	Cyclin dependent kinase

CuZnSOD	Copper/Zinc superoxide dismutase
CVD	Cardiovascular disease
CXXC	Dithiol motif cysteine-any-any-cysteine
DAPI	Diamino-2-phenylindole
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DSB	Double strand break
dTTP	Deoxythymidine triphosphate
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EMM	Edinburgh minimal medium
EMM½G	Edinburgh minimal medium with half glutamate
EMM-N	Edinburgh minimal medium lacking a nitrogen source
$FeCl_3.6H_2O$	Iron(III) chloride hexahydrate
FeSOD	Iron superoxide dismutase
Fig	Figure
FITC	Fluorescein isothiocyanate
G418	Geneticin
GC	Guanine cytosine
gDNA	Genomic DNA

GFP	Green fluorescent protein
Grx	Glutaredoxin
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione S-transferase
GTP	Guanosine-5'-triphosphate
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus
H.M.W.	High molecular weight
HPD	Histidine proline aspartic acid
HR-A	Hydrophobic repeat-A
HR-B	Hydrophobic repeat-B
HR-C	Hydrophobic repeat-C
HSE	Heat shock element
HSF	Heat shock factor
Hsf1	Heat shock transcription factor Hsf1
HSP	Heat shock protein
HSP40	Heat shock protein 40 kilodaltons
HSP70	Heat shock protein 70 kilodaltons
HSP90	Heat shock protein 90 kilodaltons
HU	Hydroxyurea
IAA	Iodoacetamide
IgG	Immunoglobulin G
*kanMX*6	Kanamycin resistance selector
KCl	Potassium chloride

KDa	Kilodalton
LB	Luria broth
Liac	Lithium acetate
MDA	Malondialdehyde
M Phase	Mitotic phase
MAPK	Mitogen activated protein kinase
$MgCl_2$	Magnesium chloride
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
$MgSO_4$	Magnesium sulfate
Ml	Millilitre
mM	Millimolar
MMS	Methyl methanesulfonate
$MnSO_4$	Manganese(II) sulfate
MnSOD	Manganese superoxide dismutase
n/a	Not applicable
Na_2HPO_4	Sodium phosphate dibasic
Na_2SO_4	Sodium sulfate
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluoride
NaOH	Sodium hydroxide
$NaPO_4$	Sodium phosphate
NBD	Nucleotide binding domain
NEF	Nucleotide exchange factor

NEM	N-Ethylmaleimide
NES	Nuclear export signal
NETO	New end take off
Ng	Nanograms
NH_4Cl	Ammonium chloride
nGAAn	Amino acid sequence any-glycine-alanine-alanine-any
nH_2O	Deionised water
NH_4Cl	Ammonium chloride
NiSOD	Nickel superoxide dismutase
nM	Nanomolar
NO	Nitric oxide
NOS	Nitric oxide synthase
NRLLLTG	Asparagine-arginine-leucine-leucine-leucine-threonine-glycine
OD	Optical density
OH^-	Hydroxyl radical
ONOO	Peroxynitrite
OS	Oxidative stress
OSR	Oxidative stress response
Pap1	*S. pombe* AP-1 like transcription factor
PCR	Polymerase chain reaction
PDSM	Proline-aspartate-serine-methionine
PEG	Polyethene glycol
PEM	PIPES, EDTA, magnesium sulphate
PEMBAL	PIPES, EDTA, magnesium sulphate, BSA, sodium azide, lysine-monochloride

PEMS	PIPES, EDTA, magnesium sulphate, sorbitol
pH	Potential of hydrogen
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PMSF	Phenylmethylsulfonyl fluoride
Prx	Peroxiredoxin
PTP	Protein tyrosine phosphatase
RNA	Ribonucleic acid
RNR	Ribonucleoside reductase
RNS	Reactive nitrogen species
ROS	Reeactive oxygen species
rpm	Revolutions per minute
SAPK	Stress activated protein kinase
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SAC	Spindle assembly checkpoint
SBD	Substrate binding domain
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
Srx	Sulfiredoxin
SPB	Spindle pole body
SSB	Single strand break
ssDNA	Samon sperm DNA
SSPE	Saline, sodium phosphate, EDTA
TAD	Transactivation domain
tBOOH	T-butyl hydroperoxide

TBS	Tris buffered saline
TBST	Tris buffered saline, tween 20
TBZ	Tiabendazole
TCA	Tricholoracetic acid
TE	Tris, EDTA buffer
Tpx	Thioredoxin peroxidase
Trr	Thioredoxin reductase
Tris	Trisaminomethane
Tris-HCl	Tris hydrochloride
Trx	Thioredoxin
URS	Unique restriction site
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
WT	Wild type
Ye5S	Yeast extract medium with 5 supplements
YFP	Yellow fluorescent protein
$ZnSO_4.7H_2O$	Zinc sulfate heptahydrate
α-P^{32}	Radioactive-labelled alpha phosphate group with 32-Phosphorus

List of Figures

List of Tables

CHAPTER 1

Introduction

1.1 Heat and oxidative stress

Cells are exposed to a range of stress conditions which originate from within the cell and from the environment. For example, reactive oxygen species (ROS) are highly reactive molecules of oxygen which arise from aerobic respiration but also from environmental sources such as the diet. High levels of ROS can cause oxidative stress (OS) defined as an imbalance between the presence of free radicals and the cells ability to detoxify them. OS can result in damaged cellular components such as DNA, protein and lipids.

In addition, all cells experience temperature ranges and large or rapid changes to optimal growth conditions. Heat stress can be detrimental to the cell causing protein denaturation and/or aggregation. This thesis will focus primarily on heat and oxidative stress. However, cells are exposed to other stress conditions (see reviews of cellular stress for further details Gasch *et al*., 2002; Kültz, 2005) which are not within the scope of the present study.

1.1.1 Oxidative stress and disease

Failure to degrade proteins has been linked to several diseases. The amount of damage induced by oxidative and heat stress has been linked to the accumulation of modified molecules that can potentially impair cellular function (Bohr and Anson, 1995; Bokov *et al*., 2004). Hence, not surprisingly these stresses have been linked to many diseases and the ageing process. It has been shown that there is decreased ubiquitination of oxidatively damaged proteins. This has

been linked to ageing due to accumulation of damaged molecules not being degraded (Shang and Taylor, 1995). There is ongoing research to understand the accumulation of stress-induced damage linked to aging and disease (Bokov *et al.*, 2004). The cytotoxicity of oxidative damage has been correlated to a wide variety of age-related diseases. The most notable is cancer and tumour development from chromosomal aberrations (see reviews for further details Reuter *et al*., 2010). The markers of oxidative damage on DNA are shown to accumulate in tissue with age due to persistent oxidative stress (Toyokuni *et al*., 1995).

Cardiovascular diseases (CVD) are a widespread problem of the western world caused by damage to vascular tissue. Oxidative stress may be a key risk factor for developing atherosclerosis (Madamanchi *et al*., 2005). The role of ROS in CVD has led to ROS being considered a major risk factor within the medical field (Madamanchi *et al*., 2005).

Oxidative damage to neurons has been linked with various diseases such as Alzheimer's, Parkinson's and Huntington's disease. However, it is not conclusive if the damage is a cause or an effect of the disease formation (Nunomura *et al*., 2001). Furthermore, it is still unclear if the amyloid plaques (protein aggregates), which are indicative of Alzheimer's disease, are the source of pathology. Similarly, Parkinson's disease shows there is a strong correlation between oxidative stress and the disease but whether the oxidative stress is cause or effect remains unclear (Jenner, 2003).

1.1.1.1 Sources of ROS

ROS are present in the external environment and arise from internal metabolic processes (Haddad, 2004). ROS can have differential effects on the cell depending on the concentration of ROS and the type (Fig. 1. 1). An accumulation of damage to cellular components can occur

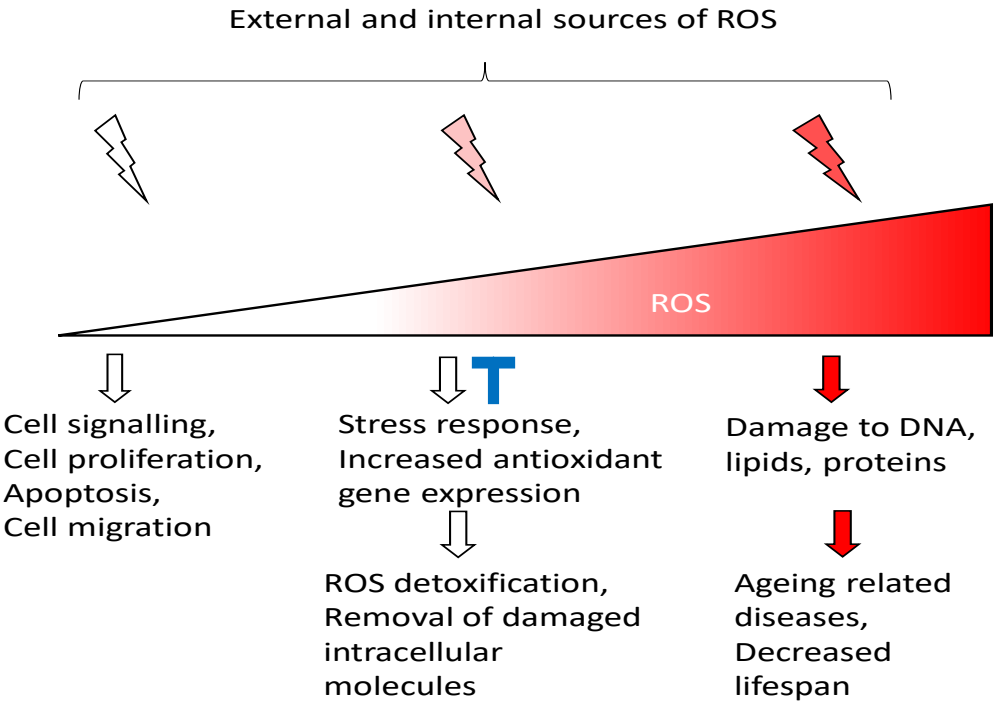


Fig. 1. 1. The concentration-dependent effects of ROS within cells. Cells are exposed to ROS from internal and external sources of varying concentration and reactivity. A low concentration of ROS can be important to the cell for example, as a cell signalling molecule. A higher concentration of ROS can be detrimental to the cell and trigger stress response pathways. This leads to detoxification of the ROS and prevents permanent cellular damage. However, above a certain threshold of ROS concentration there is widespread damage to cellular components that cannot be detoxified.

due to an imbalance of ROS metabolite detoxification/redox buffering systems within cells. This leads to a further increase in ROS present in the cell which causes OS (Bokov *et al*., 2004; Sies, 1997). ROS are oxygen containing radicals, examples being hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH^-). These radicals are highly reactive and thus have a short biological half-life. The greatest source of ROS within the cell is generated during production of ATP by the electron transport chain via respiration (Cash *et al.*, 2007; Costa and Moradas-Ferreira, 2001). The metabolism of oxygen to make ATP can produce charged ROS such as superoxide (Costa and Moradas-Ferreira, 2001). However, superoxide is not a strong oxidising agent and therefore is not highly damaging to the cell (Cash *et al.*, 2007; Haddad, 2004). The major danger posed by superoxide is that it can lead to increased levels of hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-). The latter have a high electron potential and are the strongest oxidising agents found within the cell and thus can induce greater levels of damage to cell components (Sigler *et al.*, 1999).

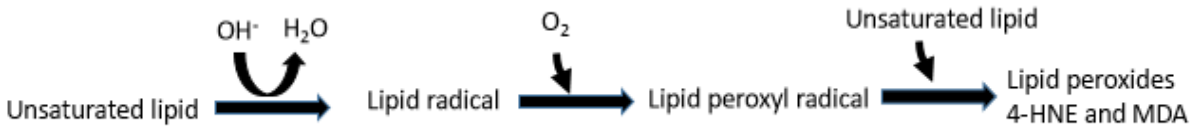
Transition metals are key to many biological molecules and their functions rely on redox cycling metal free radicals in response to oxidative stress (Halliwell and Gutteridge, 1999; Sigler *et al.*, 1999; Veal *et al.*, 2007). For example, copper containing superoxide dismutase degrades superoxide to water and H_2O_2. The copper acts as a reductant by gaining an electron from superoxide reducing the degree of oxidative stress within the cell (Cash *et al.*, 2007). However, the transition metal is another source of oxidative stress to the cell if the redox state becomes imbalanced as it allows the metal radical to damage proteins, DNA and lipids (Bokov *et al.*, 2004; Cash *et al.*, 2007).

H_2O_2 in comparison to hydroxyl radicals is relatively unreactive. However, H_2O_2 can react with metal ions, and of importance is the H_2O_2-induced reduction of iron ions (Fe(II) to Fe(III))

(Abreu and Cabelli, 2010). The oxidation of Fe(II) leads to the formation of hydroxyl radicals, this process is termed the Fenton reaction (Bouayed and Bohn, 2010; Valko *et al*., 2006). In biological systems iron often plays an important role, for example the iron cluster in mitochondrial aconitase; disruption of the aconitase iron cluster can result in Fenton reactions occurring, increasing ROS production (Flint *et al*., 1993; Yan *et al*., 1997). The hydroxyl radicals react with most cell components which requires detoxification by antioxidants or it can lead to oxidative stress (Abreu and Cabelli, 2010; Bokov *et al*., 2004).

1.1.1.2 Oxidative stress-induced damage

Nearly all components of the cell have been identified as being susceptible to oxidative stress-induced damage. For example, lipids can be oxidised by agents such as ROS, this process is termed lipid peroxidation (Bouayed and Bohn, 2010; Vasilaki and McMillan, 2017). Lipid peroxidation can be initiated by a hydroxyl radical removing a hydrogen from a hydrogen-carbon bond resulting in a fatty acid radical. Fatty acid radicals can be further oxidised by molecular oxygen to form a peroxyl fatty acid radical. Peroxyl fatty acids can react with other fatty acids propagating the issue and producing a lipid peroxide. Lipid peroxidation leads to the formation of highly reactive aldehydes malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (Gonzalez *et al*., 2001; Vasilaki and McMillan, 2017). The reaction is represented below:



Lipid peroxidation can affect lipid membrane fluidity as well as being reactive with amino acids leading to protein damage. For example, linoleic acids located in the phospholipid cell membranes can be oxidised relatively easy and results in loss of function and increased presence

of protein aggregates. Lipid membrane peroxidation has been linked to several cancers due to the reactivity of MDA and 4-HNE with DNA to form DNA adducts (Vasilaki and McMillan, 2017).

Alongside damage to lipids, ROS can also modify proteins to induce damage. Protein oxidation by ROS is a post-translational modification that is not always reversible (Berlett and Stadtman, 1997). Amino acids and their residues can become oxidised which can affect the functionality of the protein. Some amino acids such as cysteine and methionine are more susceptible to ROS attack than others (Berlett and Stadtman, 1997; Bokov *et al*., 2004). The protein backbone can also become oxidised by ROS which can lead to fragmentation of the protein. Oxidation of amino acids residues or the peptide backbone can lead to irreversible damage to the protein leading to peptide degradation (Berlett and Stadtman, 1997). Additionally, proteins can form carbonyl derivatives of amino acid groups, several studies have shown that the presence of carbonyl groups on proteins increases within age-related diseased tissue (Garinis *et al.,* 2008; Peyrot and duCrocq, 2008).

Proteins damaged by oxidation are targets for degradation by the proteasome. This increases the turnover of the proteome to maintain the balance of functional proteins. However, it has been shown that over the course of a cell's lifespan it accumulates oxidatively damaged proteins. There are large amounts of evidence showing an accumulation of the presence of oxidised proteins with age (Reeg and Grune, 2015). Oxidised proteins can form stable aggregates; protein aggregates are stable which can propagate and are not able to be degraded by the proteasome. Protein turnover can therefore be indicative of the level of damage occurring as greater degradation and renewal is required; this can be skewed by protein aggregates impeding the catabolism (Grune *et al.,* 2004). If repair is not possible, the ubiquitin-proteasome pathways

mediate the process of protein degradation (See reviews for more details Martinez-Vicente *et al.,* 2005).

In addition to damage of lipids and proteins, oxidative stress has been shown to produce single and double strand breaks in DNA. Over 20 different forms of DNA lesions have been identified (Cooke *et al*., 2003). A well-studied lesion is 8-oxo-2′deoxyguanosine (8-oxo-dG) (Bokov *et al*., 2004; Cooke *et al*., 2003). This guanine analogue is found in higher quantities as the degree of oxidative stress the cell is exposed to increases. However, a basal level of 8-oxo-dG is present from normal cellular respiration. A positive correlation has also been found between an organism's basal metabolic rate and the presence of 8-oxo-dG (Cheng *et al*., 1992). A correlation has been made identified between age and accumulation of DNA damage (including 8-oxo-dG) however the causative link has yet to be made.

Detection of DNA damage can trigger cell cycle checkpoints to prevent replication of aberrant DNA by stalling the cell division cycle. Accumulation of DNA damage such as strand breaks from UV exposure or oxidising agents can lead to phenotypes such as cancer or cell death (Gonzalez *et al.,* 2002). This is biologically significant as these breaks cause genomic instability and are more difficult for the cell to repair than other types of DNA lesions (Khanna and Jackson, 2001). UV exposure produces ionizing radiation which can generate hydroxyl radicals capable of removing a hydrogen ion from a carbon in the sugar of a nucleotide making it a radical. Oxidation of the nucleotide radical ultimately results in cleavage of the DNA strand causing a single strand break. A single DSB within the cell can potentially result in cell death if it results in significant genomic instability (Rich *et. al*., 2000). Numerous mechanisms are employed within cells to repair single and double strand breaks to maintain genome integrity. The

importance of these mechanisms is demonstrated by the many human diseases associated with mutations to DNA damage response pathways (see reviews for details Zhou *et al*., 2000).

To summarise, there are a wide variety of sources of ROS and their capacity to damage cellular components can vary considerably. A Table is presented below of ROS relevant to this study and how they can potentially cause cellular damage.

Table 1.1 Relevant ROS to this study – their reactivity and effect upon cellular components.

ROS	Half life	Source	Mode of Action
Singlet oxygen ($^1O_2$)	1 – 4 μs	Membranes, mitochondria	Oxidises protein residues, reacts with DNA G residues
Superoxide radical (O_2^-)	1 – 4 μs	Membranes, mitochondria	Reacts/disrupts iron-centre proteins
Hydroxyl radical (OH^-)	1 μs	Membranes, mitochondria	Extremely reactive with all cell components
Hydrogen peroxide (H_2O_2)	1 ms	Membranes, mitochondria, peroxisomes	Oxidises proteins and reacts to form OH^- via O_2^-

1.1.2 Heat stress and disease

Cells in their natural environment can experience a range of stresses including heat. Cells contain response mechanisms to alleviate the effects of these temperature changes. For example, exposure to heat stress activates response pathways to maintain the biome including protection of proteins, DNA and lipids. The most well characterised response by cells to heat stress is increased synthesis of heat shock proteins (HSPs) orchestrated by the heat shock transcription

factors (HSFs). HSPs can aid protein chaperoning, refolding events and degradation. If the heat stress response is not sufficient to maintain proteostasis it can lead to pathology such as permanent organelle damage, cell lysis or activate cell death pathways. HSF has been shown to be essential for numerous types of cancer (Anckar *et al*., 2011).

The failure of HSPs to detoxify damaged proteins can lead to misfolded proteins aggregating which can propagate to cause cell damage. The effect of these protein aggregates on cells are exemplified by the protein aggregates (plaques) characteristic of Alzheimer's disease. This highlights the importance of HSPs roles in maintaining the proteome under normal and stress conditions. In aging cells, there is reduced induction of many HSP genes in response to stress which has been hypothesised to be a factor in age related diseases such as Alzheimer's disease (For a review see Najarzadegan *et al*., 2016; Wilhelmus *et al*., 2006). However, whether proteotoxicity linked disease and reduced expression of HSPs is cause or effect has yet to be uncovered.

1.1.2.1 Temperature-induced damage

In nature, cells are subject to daily fluctuations in temperatures but can also be exposed to an extreme range of temperatures beyond their niche habitat. All organisms have adapted to their niche environment and grow at optimal growth temperatures. However, depending on the type and location of the cell they can be exposed to relatively large increases and decreases in temperature termed heat stress. The levels of damaged proteins increase following exposure to heat stress.

Nascent peptides are usually chaperoned to prevent interactions to aid the correct protein conformation/location within the cell. However, under heat stress proteins are more prone to

misfold/unfold or generate "incorrect" interactions leading to their lack of function. In unstressed cells, these non-functional proteins are targeted for refolding or degradation. However, heat stress can significantly increase the levels of misfolded protein/incorrect interactions which can affect cell viability. For example, under heat stress the level of denatured proteins and aberrant interactions significantly increases (Bokov *et al*., 2004; Glover and Lindquist, 1998). Exposure of cells to heat stress can also lead to protein aggregation. These aggregates consist of non-functional proteins that form a non-degradable complex which can be extremely detrimental to the cell as they cannot be removed by protein degradation pathways. Formation and propagation of protein aggregates is linked to heat stress which adds to the degree of proteotoxicity within the cell (Glover and Lindquist, 1998).

1.2 Cellular defences to oxidative stress and heat stress

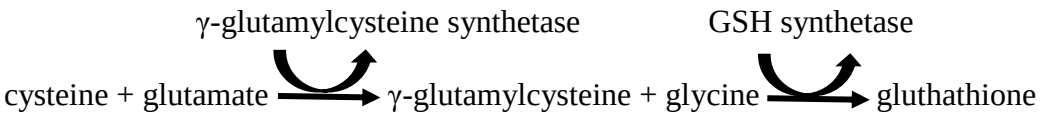
To dampen the damage induced by oxidative and heat stress, cells have numerous response pathways to defend cellular components. To defend against oxidative stress cells can utilise innate and exogenous antioxidants. Whereas, to defend against heat stress cells primarily increase the levels of HSPs. The defences provided by these systems aim to maintain the homeostasis within the cell. The relevant systems to this work will now be expanded upon.

1.2.1 Responses to oxidative stress

Sulfhydryl groups of proteins are a major target for oxidative modification, the redox change can be responded to by various methods. The endogenous response systems of the cell broadly fall in to 2 categories – enzymatic and non-enzymatic. For this work the glutaredoxin and thioredoxin systems will be discussed.

1.2.1.1 Glutathione/glutaredoxin system

A key endogenous non-enzymatic antioxidant is glutathione (GSH). GSH is conserved from prokaryotes to human cells and maintains its role as a radical scavenger; this highlights its efficacy that it has been conserved between the most divergent of systems. GSH is highly abundant and found throughout the cell in regions particularly associated with oxidative stress such as the cytosol, the vacuole, the mitochondria and the peroxisome (Anderson, 1998). GSH is composed of only 3 amino acids (glutamic acid-cysteine-glycine), a highly reactive cysteine allows it to act as a universal nucleophile (Anderson, 1998; Valko *et al*., 2006). It is synthesised by 2 enzymes consecutively; cysteine and glutamate are substrates for γ-glutamylcysteine synthetase to form γ-glutamylcysteine see reaction below. This is the rate limiting step due to cysteine availability. GSH is then synthesised from γ-glutamylcysteine by the addition of glycine by GSH synthetase (Anderson, 1998).



During oxidative stress the sulfhydryl group on the cysteine of GSH can donate an electron resulting in an oxidised GSH molecule. Oxidised GSH forms an oxidised homodimer termed GSSG (Anderson, 1998; Arthur, 2001). The oxidised homodimer is returned to the reduced monomeric GSH by GSH reductase (Arthur, 2001; Valko *et al*., 2006). GSH regulates ROS levels by directly reacting with free radicals. GSH acts as a cofactor for enzymatic antioxidants such as glutathione peroxidase, glutathione reductase and glutathione-S-transferase (discussed later in this section) (Anderson, 1998). The level and redox state of GSH therefore plays a critical role in preventing damage to DNA, protein and lipids from oxidative stress (Anderson, 1998; Noctor and Foyer, 1998).

GSH levels in the cell are tightly regulated. For example, *E. coli* γ-glutamylcysteine synthetase activity was shown to be inhibited by increasing levels of GSH (Anderson, 1998). GSH can be depleted by oxidative stress and by creating mutants that block GSH synthesis. The pathological effects can be severe. For example, in rodents' treated with a selective inhibitor (L-buthionine-*SR*-sulfoximine) of γ-glutamylcysteine synthetase caused a significant decrease in GSH levels and this was associated with increased sensitivity to radiation, cadmium ions and two alkylating agents melphalan and cyclophosphamide (Anderson, 1998; Meister, 1991). It should be noted that cadmium is not itself redox active, however it is highly reactive with thiols and thus can affect the redox balance of the cell. A key example is the reaction of free cadmium ions with GSH resulting in a reduction of the GSH pool thus leading towards an oxidative environment (Cuypers *et al*., 2010).

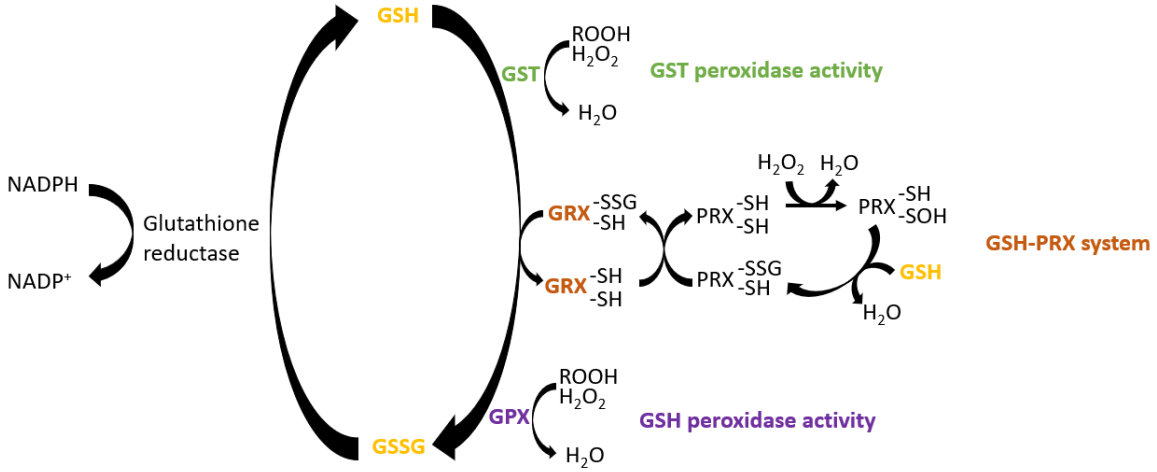
Glutaredoxins (Grxs) are small soluble antioxidant proteins that are GSH-specific thiol reductases. Grxs have been identified in various organisms and viruses. Grxs act as oxidoreductases in the cell by thiol-disulphide redox cycling. Grxs are categorised by the number of catalytic cysteines they contain; dithiols have 2 catalytic cysteines and monothiols have 1 catalytic cysteine (Rodríguez-Manzaneque *et al*., 1999). *S. pombe* and *S. cerevisiae* have 2 dithiols Grx1 and Grx2. *S. cerevisiae* has 3 characterised monothiols Grx3, 4, 5 whereas *S. pombe* has 3 putative monothiols Grx3, 4, 5 (Chung *et al*., 2005). Grxs rely on reduced GSH as an electron donor to reduce disulphide bonds in concert with GSH reductase and NADPH. Dithiols have a conserved motif of Cys-Pro-Tyr-Cys and site-directed mutagenesis has demonstrated that both cysteines are required for effective reduction of mixed protein disulphide bonds (Chung *et al*., 2004). In *S. cerevisiae,* the dithiol Grxs localise to the cytosol, with Grx2 accounting for the majority of the GSH-dependent oxidoreductase activity in the cell. Grx have been shown to be critical for protection against ROS (Luikenhuis *et al*., 1998).

The Grxs also have differential roles in ROS responses, for example in *S. cerevisiae* and *S. pombe* Grx1 is critical in the response to H_2O_2 whereas Grx2 is important for resistance to menadione stress (Luikenhuis *et al*., 1998). In *S. pombe* deletion of *grx2*$^+$ causes cells to become highly sensitive to paraquat (Chung *et al*., 2004). In *S. cerevisiae*, it has been shown that the absence of the monothiol Grx5 leads to sensitivity to menadione and H_2O_2 (Rodríguez-Manzaneque *et al*., 1999). Interestingly, the monothiol Grx4 is important to regulate cellular iron levels of both *S. cerevisiae* and *S. pombe*. Iron scarcity can inhibit transcription of genes encoding iron-dependent proteins. In *S. cerevisiae* Grx3 and Grx4 translocate a critical iron incorporation transcription factor Aft1 to the cytoplasm preventing transcription of genes encoding iron-utilizing proteins in conditions of low iron (Ojeda *et al*., 2006; Pujol-Carrion *et al*., 2006). A similar mechanism was recently published suggesting that repression of transcription in the absence of iron was dependent on Grx4 in *S. pombe* (Vachon *et al*., 2012). During the Fenton reaction iron reacts with H_2O_2 to form a hydroxyl radical, the iron is then reduced by an additional H_2O_2 molecule and produces a hydroperoxyl radical (Gonzalez *et al*., 2002). These studies highlight the importance of Grxs in regulating iron levels as it can impact on ROS levels within the cell.

GSH is also involved in the oxidative stress response via the action of glutathione-S-transferases (GSTs). GSTs conjugate reduced GSH to electrophilic substrates that may potentially be genotoxic or cytotoxic and then the substrates are removed from the cell by GSH conjugate pumps (Sherratt and Hayes, 2001). GSTs are found in many prokaryotes and most eukaryotes and represent a large percentage of the proteome highlighting their importance. GSTs exist as dimers that each contain catalytically active cysteines (Eaton and Bammler, 1999). The substrate binding domain of GSTs has relatively little specificity which is believed to

account for the wide variety of substrates. In addition, there are numerous isoforms of GSTs which further increase the range of xenobiotics that can be detoxified (Eaton and Bammler, 1999). In *S. cerevisiae*, the glutaredoxins Grx1 and Grx2 and the GSTs Gtt1 and Gtt2 have GST activity. There is functional overlap of these proteins but surprisingly it appears that Grx1 and Grx2 account for the majority of the GST activity in *S. cerevisiae* cells (Collinson and Grant, 2003). In *S. pombe*, there are 3 identified *gst*$^{+}$ genes the transcription which are induced in by exposure to H_2O_2 (Veal *et al.*, 2002).

To summarise, the redox reactions dependent on GSH and GSH-enzymes are represented below.



1.2.1.2 Thioredoxin system

The thioredoxin system is conserved amongst all organisms (Holmgren and Lu, 2010). The thioredoxin system consists of peroxiredoxins (Prx), Thioredoxins (Trx) and thioredoxin reductases (Trr) with NADPH to maintain the redox balance within the cell (Fig. 1. 2.). Thioredoxins (Trxs), like Grxs, are small soluble proteins and they contain 2 catalytically active cysteines. In conjunction with the glutaredoxin system, the Trxs function to reduce oxidised proteins by cysteine-thiol disulphide exchange. Trxs are found in prokaryotes and eukaryotes

with much research on human Trxs role in OSR. Humans contain 3 Trx proteins, Trx, Trx2 and spTrx (Nordberg and Arner, 2001). Loss of function of either protein Trx or Trx2 results in loss of viability of an embryo (Nonn *et al*., 2003; Matsui *et al*., 1996). In *S. cerevisiae*, there are 3 Trxs Trx1-3; Trx1 and 2 are cytoplasmic while Trx3 is mitochondrial (Toledano *et al*., 2013). In *S. pombe* there are 3 Trx proteins Trx1, Trx2 and thioredoxin-like Txl1; Trx1 is cytosolic while Trx2 is mitochondrial and Txl1 is cytoplasmic and nuclear (Jiménez *et al*., 2007).

The importance of Trx proteins in oxidative stress is demonstrated by deletion of Trx-encoding genes. In *S. cerevisiae* deletion of *TRX1* or *TRX2* increases sensitivity to H_2O_2 (Mouaheb *et al*., 1998). In wild type *S. cerevisiae* cells *TRX2* is expressed at higher levels than *TRX1* in response to H_2O_2 suggesting it has a greater role in H_2O_2 detoxification (Garrido and Grant, 2002). The importance of Trx proteins in OSR is in reduction of target proteins primarily to reduce cysteine residues and disulphide bonds. Trx contain a dithiol motif (CXXC) composed of a catalytic cysteine and a nearby resolving cysteine. Initially, the catalytically active cysteine attacks the target sulfhydryl group to donate an electron to the oxidised cysteine substrate. Within the Trx1 an intramolecular disulphide bond forms between the 2 cysteines in the dithiol motif. The formation of the Trx internal disulphide results in an oxidised Trx molecule and a reduced cysteine substrate (Toledano *et al*., 2013).

Thioredoxins are returned to a reduced state by thioredoxin reductases (Trrs). Like Trxs, Trrs are conserved from prokaryotes to eukaryotes. Trrs of high eukaryotes have a broad specificity of substrates compared to Trr of other organisms. In addition, Trr from higher eukaryotes such as humans have a different catalytic mechanism compared to Trr from bacteria and fungi. Human Trr contain the dithiol catalytic motif CxxxxC, in comparison fungal species of Trr have the motif CXXC which is shared with the Grx system (Hirt *et al*., 2002). In *S. cerevisiae*, there

is 1 cytoplasmic Trr and 1 mitochondrial, Trr1 and Trr2 respectively. Trr1 is critical for the reduction of cytoplasmic Trx1 and Trx2. Deletion of *TRR1* results in cells with significant sensitivity to H_2O_2 (Machado *et al*., 1997). In *S. pombe*, a single thioredoxin reductase has been identified, Trr1 is responsible for reducing the thioredoxins Trx1 and Txl1 (See Fig. 1. 2.) (Brown *et al*., 2013; Garcia-Santamarina *et al*., 2013). Trr function as homodimers to reduce the target substrate, NADPH acts as the final electron donor.

Eukaryotic peroxiredoxins (Prxs) directly reduce peroxide radicals and play a critical role in detoxifying oxidative stress. Peroxiredoxins require an electron donor to reduce H_2O_2 to water. Within Prxs are a family of thioredoxin peroxidases (Tpx) named because thioredoxin acts as the electron donor. There are 2 types of Prx in the cell which are categorised by the number of cysteines involved in the catalysis of H_2O_2; these are categorised as 1-Cys or 2-Cys Prx enzymes. Both types of Prx contain a peroxidatic cysteine that can be oxidised to a sulfenic acid by peroxide radicals. 1-Cys Prx can be directly reduced to return to its original redox state but have also been shown to use vitamin C (ascorbic acid) as an electron donor (Monteiro *et al*., 2006). In contrast, 2-Cys Prx also contain a resolving cysteine which forms an intermolecular disulphide with the peroxidatic cysteine with an alternate Prx leading to the formation of a homodimer (See Fig. 1. 2.). The disulphide bond can be resolved by thioredoxin and replenish the pool of reduced monomeric Prx (Monteiro *et al*., 2006).

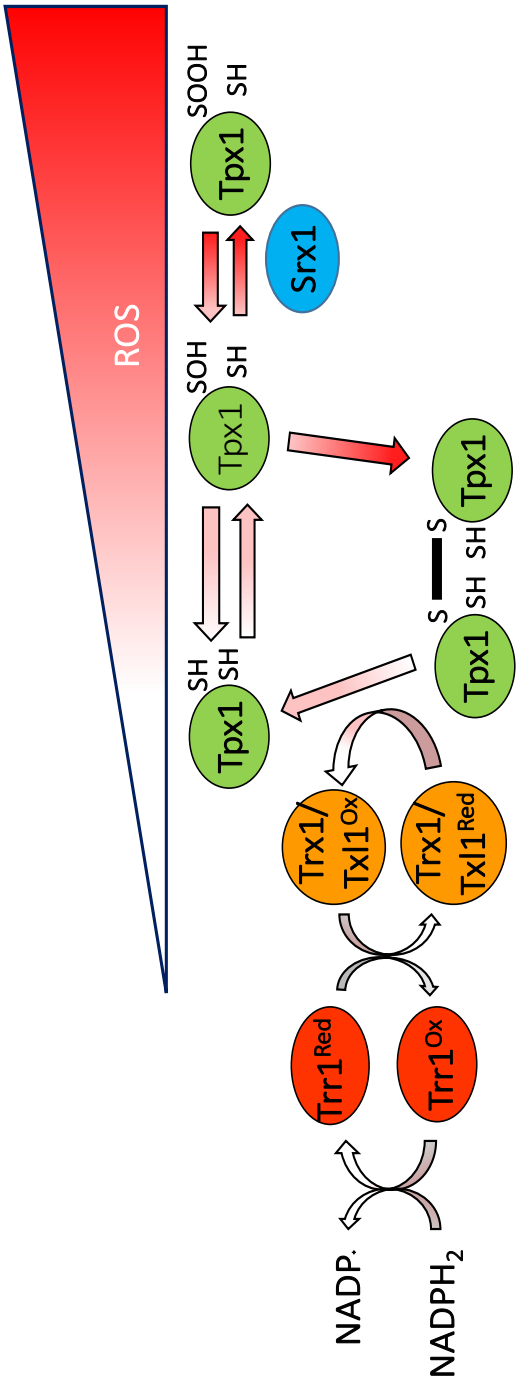


Fig. 1. 2. Model of redox cycling of peroxiredoxin Tpx1 in *S. pombe*. Low concentrations of H_2O_2 (0.2 mM) oxidises the large pool of Tpx1 and completely oxidises Trx1/Txl1. After exposure to high concentrations of H_2O_2 (≥1 mM) the peroxidatic cysteine of Tpx1 becomes hyperoxidised to a sulfinic acid. The sulfinic cysteine of Tpx1 is not a substrate for Trx1/Txl1. Hyperoxidised Tpx1 is a substrate for sulfiredoxin Srx1, returning the sulfinic cysteine of Tpx1 to a sulfenic acid and therefore a substrate for Trx1/Txl1. The pool of Trx1/Txl1 is recycled from an oxidised state via Trr1 and NADPH.

In yeast, all identified Prxs are Tpxs and thus the term can be used interchangeably (Rhee *et al*., 2001). In *S. cerevisiae*, there are 5 known Tpxs of which Tsa1 and Tsa2 show the most significant role in peroxide detoxification (Jang *et al*., 2004). Deletion of *TSA1* results in increased sensitivity to H_2O_2 but *tsa2*Δ cells actually display increased resistance to H_2O_2 relative to wild type cells (Demasi *et al*., 2001; Munhoz and Netto, 2004). These data suggest that the Tpxs have specific non-redundant roles in oxidative stress. In *S. pombe* Tpx1 is the ortholog of Tsa1 and its function as peroxide scavenger has been well characterised (Veal *et al*., 2004). Deletion of *tpx1*$^+$ results in increased sensitivity to acute H_2O_2 stress thus highlighting the importance of Prx activity in OSR (Veal *et al*., 2004). The role of Prx in the oxidative stress response appears to be highly dependent on the degree of oxidative stress, see section 1.2.1.2.1.

1.2.1.2.1 Functions of Peroxiredoxins

Work is ongoing to understand the mechanism of how cells can determine the appropriate response after exposure to different concentrations of H_2O_2. 2-cys Prx Tpx1 and the thioredoxin system have a clear role transducing the oxidative stress signals to regulate transcriptional activity of *S. pombe*.

Tpx1 is a highly abundant Prx that removes peroxides in the cell however it is also required for activation of oxidative stress induced gene expression that is dependent on the transcription factors Pap1 and Atf1. The catalytic activity of Tpx1 is determined by 2 vicinal cysteine residues, the peroxidatic and the resolving cysteine. Scavenging of peroxides oxidises the peroxidatic cysteine in Tpx1 to a sulfenic acid followed by formation of a disulphide bond with the resolving cysteine of an alternate Tpx1 monomer (Day *et al*., 2012). The Tpx1 dimer is no longer able to reduce peroxides. Restoration of a Tpx1 dimer to reduced monomers is performed

by thioredoxins Trx1 and Txl1 (See Fig. 1. 2.; Brown *et al*., 2013). Trx1 is a broad specificity oxidoreductase and the major substrate is Tpx1. oxidation of Trx1 inhibits reduction of other Trx1 substrates. Txl1 reduces Tpx1 but is also believed to maintain Pap1 in a reduced state (Brown *et al*., 2013). Reduction of the thioredoxins is performed by thioredoxin reductase Trr1, there is relatively little Trr1 in the cell in comparison to the levels of Trx1 and Txl1. Trr1 activity is the limiting step in reduction of the thioredoxin system. Low concentrations of H_2O_2 (0.2 mM) oxidises the large pool of Tpx1 and completely oxidises Trx1/Txl1 pool thus Pap1 becomes oxidised and activates Pap1-dependent gene expression.

At high concentrations of H_2O_2 (≥1 mM) the transcriptional response needs to prevent the damage of increased oxidative stress. Tpx1 is believed to be key to relaying/signalling this requirement in the cell. After exposure to high concentrations of H_2O_2 the peroxidatic cysteine of Tpx1 becomes hyperoxidised to a sulfinic acid (Bozonet *et al*., 2005). The sulfinic cysteine of Tpx1 is not a substrate for Trx1/Txl1 which explains why Pap1 remains reduced and inactive at high concentrations of H_2O_2 (See Fig. 1. 2.). The hyperoxidised form of Tpx1 is important to activate the Sty1 MAPK pathway leading to Atf1-dependent gene expression (Veal *et al*., 2002). Hyperoxidised Tpx1 is a substrate for sulfiredoxin Srx1, returning the sulfinic cysteine of Tpx1 to a sulfenic acid and therefore a substrate for Trx1/Txl1. The sulfiredoxin gene *srx1*$^+$ is transcribed by Atf1 at high concentrations of H_2O_2 and thereby creates a coordinated link between high concentration stress response and low OSR via Tpx1 oxidation. Deletion of the *srx1*$^+$ or *tpx1*$^+$ results in severe sensitivity of cells to H_2O_2 this has been shown to be through an inability to regulate the OS transcriptional response (Bozonet *et al*., 2005; Veal *et al*., 2004).

Eukaryotic 2-Cys Prx have another proposed role which is linked to the oxidation status of the catalytic cysteine. Following exposure to high OS (6 mM H_2O_2), eukaryotic 2-Cys Prx are

highly sensitive to hyperoxidation which inactivates the peroxidase activity. The Prx is then believed to shift towards functionality as a molecular chaperone (Jang *et al*., 2004). In *S. pombe*, hyperoxidation of Tpx1 allows Trx1 to reduce alternate substrates which has been shown to increase cell survival (Day *et al*., 2012). Following exposure to high OS, Tpx1 forms a bond with Sty1 and promotes Sty1 phosphorylation which does not require thioredoxin peroxidase activity, this leads to Atf1-dependent gene expression (Veal *et al*., 2004). This suggests that Prx Tpx1 can act as redox sensor to transduce the signal to activate SAPK Sty1 presumably through chaperone activity.

There is considerable conservation of the Prx and thioredoxin system between *S. pombe* and *S. cerevisiae*. The *S. cerevisiae* homolog of Tpx1 is identified as being 2-Cys Prx Tsa1. Tsa1 accounts for the vast majority of the peroxidase activity found within *S. cerevisiae* cells (Ghaemmaghhami *et al*., 2003). Tsa1 shows thioredoxin peroxidase activity for H_2O_2 and peroxides when the thioredoxin system is active. Tsa1 is required for the induction of H_2O_2-dependent gene expression (Ross *et al*., 2000). Like a *tpx1*Δ strain, a *tsa1* mutant is highly sensitive to H_2O_2 (Park *et al*., 2000). Upon exposure to low OS, Tsa1 becomes oxidised to form an intermolecular disulphide resulting in a homodimer as was described for 2-Cys Prx previously. The thioredoxins Trx1 and Trx2 are cofactors for Tsa1 thioredoxin peroxidase activity and depend on the thioredoxin reductase Trr1 for Trx reduction (Garrido and Grant, 2002). Tsa1 plays a critical role in sensing oxidative stress to transduce the signal to activate OSR TFs. Oxidation of the OS-induced transcription factor Yap1, the *S. cerevisiae* homolog of Pap1, is dependent on oxidation of Tsa1 and the thioredoxin system. This shows conservation of the OS pathways between yeasts, however the role of Tsa1 in Yap1 regulation appears to be organism and strain specific. This will be discussed within the context of Yap1 regulation in section 1.3.2.

Like Tpx1, Tsa1 appears to have dual functionality as a thioredoxin peroxidase and a molecular chaperone. Tsa1, similarly to Tpx1 and other eukaryotic 2-cys Prx, is sensitive to hyperoxidation of the peroxidatic cysteine. Hyperoxidised Tsa1 appears to lose thioredoxin peroxidase activity and retain chaperone functionality. Cells lacking Tsa1 protein leads to ribosomal protein aggregates and are sensitive to heat shock, highlighting the importance of this protein as a molecular chaperone in normal and stress conditions (Rand and Grant, 2006).

1.2.1.3 Endogenous antioxidants

In addition to the antioxidant activity of the systems described above, redox balance is also supported by catalase and superoxide dismutase (SOD) which will now both be covered briefly. Catalase functions to dismutate H_2O_2 to water and oxygen. Catalase has a high catalytic rate allowing it to rapidly lower the concentration of H_2O_2 in the cell (Valko *et al.*, 2006). The regions of H_2O_2 concentrations in eukaryotic cells, the peroxisome and the mitochondria, are the regions catalase localises (Chaudiere and Ferrari-Iliou, 1999). A single catalase-encoding gene has been identified in *S. pombe*, *ctt1*$^+$. Disruption of this gene results in severe sensitivity to high concentrations of H_2O_2 (Mutah *et al.*, 1999).

SOD dismutates superoxide to H_2O_2 and oxygen. SODs are classified based on their metal cofactor - iron SOD, manganese SOD, copper-zinc SOD, and nickel SOD (Abreu and Cabelli, 2010). In eukaryotic cells there are typically 2 SODs, Copper/zinc SOD localises to the cytoplasm while manganese SOD is found in the mitochondria to protect the cell from superoxide (Abreu and Cabelli, 2010). For example, *S. pombe* encodes 2 SODs, a mitochondrial manganese SOD and a cytoplasmic copper/zinc SOD. The manganese SOD was found to be

inducible in response to menadione however, copper/zinc SOD is not inducible by menadione but had been found to be regulated by the transcription factor Pap1 (Lee *et al.*, 2002).

1.2.1.4 Exogenous antioxidants

Alongside the endogenous antioxidants, the cellular redox balance can be supported by externally provided molecules. The most notable is α-tocopherol which is the major free radical scavenger of vitamin E, being fat soluble facilitates its passage in to the cell (Halliwell and Gutteridge, 1994). Ascorbic acid, also known as vitamin C is another proven source of antioxidant activity that can be provided as supplement to cells. Further exogenous molecules known to provide antioxidant benefits are flavonoids and carotenoids. For a full review of cellular antioxidants see reviews (Abreu and Cabelli, 2010; Bouayed and Bohn, 2010; Herrero *et al*., 2008; Pham-Huy *et al*., 2008).

1.2.2 Response to heat stress – heat shock proteins

All organisms have developed molecular mechanisms to reduce proteotoxicity induced by stress from an increase in unfolded proteins, incorrectly folding peptides and protein aggregates. Various stresses can affect the proteome leading to an increase in proteotoxicity such as heat and oxidative stress. This requires sensing of the physiological conditions to be relayed for an appropriate response. Defence against oxidative and heat stress requires increased protein chaperone activity (Benjamin and McMillan, 1998). The chaperone functions of Heat Shock Proteins **(**HSPs) play a central role in these stress responses for example, by preventing protein aggregation, aiding folding and degradation (Mayer, 2010). HSPs are ubiquitous and highly conserved amongst eukaryotes and loss of HSPs render cells sensitive to a range of stresses including oxidative and heat stress (Craig and Marszalek, 2011; Kalmar and Greensmith, 2009). HSPs were originally characterised for their significant induction when cells are exposed to

heat stress to mediate proteotoxicity by binding unfolded peptides (Feder and Hofmann, 1999). However, HSPs have also been shown to have chaperone functions and housekeeping roles even under normal physiological conditions (Hartl *et al*., 2011). The roles of HSPs as molecular chaperones and the roles they potentially play in sensing/signalling of proteotoxic stress are not yet fully understood.

An individual HSP often does not act alone and is typically a concerted effort between several HSPs of different families to perform a role within the cell. The distinction between different HSP families is important to understand as it is linked to their functionality during normal and stress conditions.

1.2.2.1 HSP70 and HSP40- classification, structure and HSP70 client protein interaction

HSPs are classified broadly in to families by their molecular weight and sequence identity such as the HSP70s (70KDa mass) and HSP40s (40kDa mass). The HSP70 family is one of the most highly conserved HSP families amongst all organisms. For example, the *S. pombe* HSP70 proteins Ssa1 and Ssa2 show 81% sequence identity to their homologs in the distantly related yeast *S. cerevisiae* Ssa1/Ssa2 (See Table 1.2). To go further, the *S. cerevisiae* homologs Ssa1 and Ssa2 show over 70% sequence identity to their presumed mammalian homologs (See Table 1.3). HSPs are generally induced by heat stress but their roles vary widely amongst the different protein families (Kalmar and Greensmith, 2009). However, although HSPs have similarity for other family members this does not necessarily help to identify its precise role. It is poorly defined how sequence contributes to protein function. For example, in *S. pombe* the HSP70 Ssa family has 4 proteins Ssa1, Ssa2 Ssa3 and Ssa4. Significantly, although Ssa1 shares over 90% identity with Ssa2 they each play individual roles in the cell (Vjestica *et al*., 2013). Furthermore, *S. cerevisiae* Ssa1 and Ssa2 show over 96% nucleotide sequence identity to each other and yet

research is uncovering roles that are specific to each of these proteins (Matsumoto *et al*., 2006; Truman *et al*., 2012).

The *S. cerevisiae* HSP40 Ydj1 is considered a homolog of the *S. pombe* HSP40 Mas5 while only sharing 53% sequence identity yet research suggests these proteins are utilised in some conserved functions between the distantly related yeasts (See Table 1.2; Verges *et al*., 2007; Vjestica *et al*., 2013). Therefore, it is important to consider the domain structure of HSPs alongside their interactions with other HSPs as well as their sequence to better understand their functions. HSP topology analyses has become an aspect that has only recently been explored following the advent of protein modelling software together with crystallography. The importance of key HSP domains/motifs that are linked to functions will now be discussed.

Table 1.2 Homology comparison of *S. cerevisiae* HSP70 Ssa1, Ssa2 and HSP40 Ydj1 to the *S. pombe* proteome (972h-).

Organism	Protein	Top result in *S. pombe*	Identity (%)
S. cerevisiae	Ssa1	Ssa1/Ssa2	81
S. cerevisiae	Ssa2	Ssa1/Ssa2	81
S. cerevisiae	Ydj1	Mas5	53

Table 1.3 Homology comparison of *S. cerevisiae* HSP70 Ssa1, Ssa2 and HSP40 Ydj1 to the *H. sapiens* proteome.

Organism	Protein	Top result in *H. sapiens*	Identity (%)
S. cerevisiae	Ssa1	HSP70-1t	74
S. cerevisiae	Ssa2	Hsc70	77
S. cerevisiae	Ydj1	DNAJ homolog subfamily A member 1 isoform 1	46

Typical HSP70s have a nucleotide binding domain (NBD) followed by a linker region attached to a substrate binding domain (SBD) which allows peptide client binding to be linked to ATP hydrolysis (See Fig. 1. 3. A; Mayer, 2010). The SBD contains a β sandwich subdomain and an extended α subdomain (Mayer and Bakau, 2005; Zhu *et al*., 1996).

Peptide library studies showed that a cleft formed in the β sandwich subdomain recognises extended 7 amino acid sequences with a preference for hydrophobic residues (Mayer, 2010). ATP hydrolysis by the NBD drives a conformational change that increases the affinity of HSP70 for the peptide sequence by the extended α subdomain closing over the peptide in the β sandwich subdomain like a lid (Mayer, 2010). An atomic structural analysis of *E. coli* HSP70 DnaK and human HSP70 show a 7 amino acid sequence can be bound in the cleft of the SBD. The first sequence to be atomically analysed within a HSP70 was the peptide model NRLLLTG (Clerico *et al*., 2015; Zhu *et al*., 1996). The cleft region has a central point with a deep groove that could accommodate an aliphatic hydrophobic residue (Clerico *et al*., 2015). Leucine residues have typically been detected around this point of interaction for example, within *E. coli* DnaK. Around this central binding point there is more permissive binding of residues along with hydrogen bonds, potentially explaining the variation in sequence binding (Clerico *et al*., 2015). Interestingly, studies with the *E. coli* HSP70 HscA first demonstrated that the peptide sequence can be bound to the cleft region in reverse orientation (Tapley *et al*., 2005; Tapley *et al*., 2006). The significance of HSP70 structure allowing reverse orientation of peptide binding is unclear.

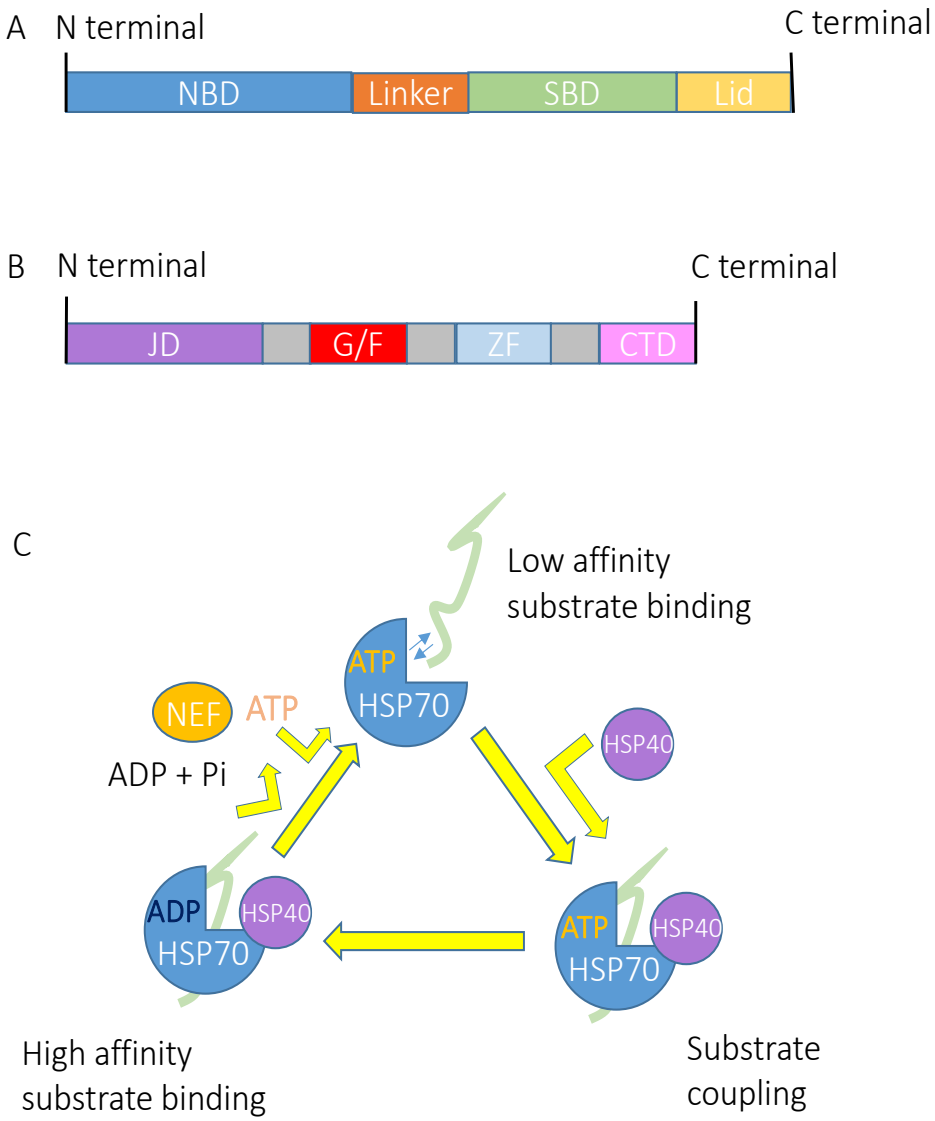


Fig. 1. 3. Representation of generic regions of HSP70 proteins and HSP40 proteins required for substrate regulation. (A) represents HSP70 N-terminal region is identified as the nucleotide binding domain (NBD) followed by a linker sequence to the substrate binding domain (SBD) and the canonical lid sequence. (B) represents a type of HSP40 containing all possible motifs. At the N-terminal is the J domain (JD), downstream is the glycine/phenlyalaline motif, distal to this is a zinc finger (ZF) and terminating in the C-terminal domain (CTD). (C) A representation of HSP70 cyclical binding of substrates. HSP70 in its ATP bound form has low affinity for a substrate and will have a high exchange rate. HSP40 increases the ATPase activity of the HSP70 to convert ATP to ADP. The ADP bound conformation of HSP70 is more compact and stabilises the substrate binding. HSP70 interaction with a NEF exchanges the ADP for ATP and results in substrate release.

The HSP40 family are found throughout all eukaryotes and function by interaction with specific HSP70 partners. HSP40 proteins are also called J proteins after the first HSP40 discovered in *E. coli* named DnaJ (Liberek *et al.*, 1991). In-between HSP40 proteins there is significantly less homology than identified within the HSP70s. For example, the *S. cerevisiae* HSP40 Ydj1 shares only 53% with the *S. pombe* homolog HSP40 Mas5 (see Table 1.2.) This lack of homology amongst HSP40s is partly due the wide range of domains HSP40s may have, the specific functionality of how these domains determine the HSP40 role in the cell is still being uncovered.

Significantly, the J domain structure is functionally conserved amongst HSP40 such as Ydj1 in *S. cerevisiae* and Mas5 the *S. pombe* homolog of Ydj1 (Lu and Cyr, 1998). The J domain architecture is composed of 3 antiparallel helices, a highly conserved sequence of histidine-proline-aspartic acid (HPD), a glycine/phenylalanine rich region and a zinc finger-like region (ZnFR) (Genevaux *et al.*, 2002; Kelley, 1998; Lu and Cyr, 1998). Although the exact function of each of these regions has yet to be fully discovered, mutation of any of the HPD residues impairs the function of the J domain to bind to HSP70 and stimulate ATPase activity (Genevaux *et al.*, 2002). Therefore, it is not surprising that the HPD residues are highly conserved amongst all known J domains but the exact mechanism of action is unknown (Kelly, 1998).

While all HSP40s contain a J domain the numbers of other motifs such as the ZnFR and glycine/phenylalanine rich region varies (See Fig. 1. 3. B; Genevaux *et al.*, 2002; Lu and Cyr, 1998). The presence of these motifs/domains appears to be linked to the specific role of each HSP40. For example, the ZnFR domain is important for binding to HSP70s and is found in DnaJ and Mas5 but not the human homolog of Mas5 Hdj1, although Hdj1 still stimulates HSP70 function (Genevaux *et al.*, 2002; King *et al.*, 1997). The exact function of these additional

motifs has yet to be fully understood. For example, partial deletion of the ZnFR from Ydj1 in *S. cerevisiae* prevented the interaction with the HSP70 Ssa1 based on ATPase activity, although the partial protein still stimulated Ssa1 binding to the client protein (Lu and Cyr, 1998). All HSP40 proteins contain a less well conserved C-terminal domain which is proposed to be involved in substrate binding to deliver client proteins to HSP70s (Kelley, 1998). However, sequence alignment of the HSP40 C-terminal domains shows very little homology amongst the family (Kelly, 1998). The roles of these sequence variations are not known but could reflect diversification of function.

The affinity of a HSP70 for the client protein is allosterically regulated by ATP binding in the NBD alongside co-chaperone activity by a HSP40 (See Fig. 1. 3. C). Structural analysis of *E. coli* HSP70 DnaK shows ATP bound HSP70 domains have very little interface (Zhu *et al*., 1996). However, in the ADP bound form there is an increase in domain interface. The ADP bound form has a high affinity for the client protein. In contrast, the ATP bound form has reduced client binding (Kalmar and Greensmith, 2009; Mayer, 2010; Clerico *et al*., 2015). Defects in nucleotide cycling can be crucial to proteostasis. For example, in *S. cerevisiae*, deletion of both the nucleotide exchange factors (NEF) encoding genes *SSE1* and *SSE2* in some strain backgrounds is lethal and in others results in slow growth (Hideyuki *et a*l., 1993; Trott *et al*., 2005). In human cells the NEF SIL1 is functional for HSP70 BiP and 9 mutations of SIL1 have been found in individuals suffering from Marinesco-Sjögren syndrome (Senderek *et al*., 2005). This suggests that in lower and higher eukaryotes the importance of nucleotide cycling in HSP70 is critical to the normal function of the cell.

HSP70-ADP complex can bind a peptide to the SBD cleft. Although extensive peptide models have been employed to identify HSP70 binding motifs, no native peptide motifs have been

clearly identified. From the studies, predictive models have generated potential HSP70 binding sites. Therefore, it remains to be understood exactly how HSP70s gain physiological client interaction specificity. Interestingly, interaction of the client with the HSP70s does not determine the end fate of the client (i.e chaperone activity versus degradation). Hence the current model is that HSP70 interacts with HSP40 family proteins determines the role of the interaction (Craig and Marszalek, 2011; Kelley, 1998; Sahi *et al*., 2013).

HSP70s can be given specificity of function towards client proteins by an interaction with cochaperone HSP40s (Kelley, 1998). HSP40s are characterised by having an N-terminal J domain that physically interacts with HSP70 to increase ATPase activity (Craig and Marszalek, 2011; Sahi *et al.*, 2013). The HSP40 interaction modifies the chaperone activity of HSP70 resulting in a specific HSP70 function. Indeed, certain HSP70-HSP40 complexes appear to be involved in regulating the cell cycle in response to stress, this will be discussed in detail in Chapter 3 (Truman *et al.*, 2013; Vjestica *et al.*, 2013; Wang *et al.*, 2012). The regulation of the client interaction with the HSP complex is not understood. Nor is it clearly defined how HSP70 is altered by HSP40 J domain interaction. HSP70 client interaction is dependent on the role of HSP40 therefore understanding the function of HSP40 domains will be critical for determining the role in providing HSP70 binding specificity.

1.2.2.2 Functions of HSP70/40

Eukaryotic genomes encode numerous HSPs which are essential for maintaining cellular homeostasis. Interestingly, in *S. cerevisiae* simultaneous deletion of all the HSP70 *ssa* family genes in a single cell is lethal (Daugaard *et al*., 2007). HSP70s can interact with a wide range of client proteins and the functions of these interactions can vary. HSP70s are known to bind peptides during nascent peptide formation, folding events, and tackling protein aggregates. For

example, a HSP70 client may be a nascent chain extending from the ribosome and in this case HSP70 SBD binding prevents the nascent peptide from misfolding (Craig and Marszalek, 2011). An important role of chaperones is to prevent inappropriate protein interactions (Truman *et al*., 2012). When cells are exposed to oxidative or heat stress there is increased levels of denatured proteins. Under these stress conditions HSP70s can mediate stress responses by preventing abnormal protein interactions. If the chaperones cannot assist correct folding the protein will be targeted for degradation (Kalmar and Greensmith, 2009; Mayer, 2010).

Oxidative damage can also lead to non-functional proteins that can be targeted for degradation by HSP interaction (Kalmar and Greensmith, 1998; Lindquist, 1986). Some HSPs overlap in their function to detoxify damaged peptides. This can be seen by multiple HSP genes being induced by various stresses. For example, in *S. pombe* expression of the HSP70 encoding gene *ssa1*$^{+}$ is induced nearly 3-fold in response to heat stress and nearly 4-fold in response to cadmium (Chen *et al*., 2003). HSP stress-induced gene expression has been attributed to the increased requirement of HSPs as chaperones to denatured proteins and to chaperone the increase in stress-induced nascent peptides that are being synthesised (Morimoto, 1998).

Stress response pathways maintain the proteome, however irreparable protein damage can occur. The proteostasis of the cell is maintained in normal and stress conditions by the ATP-dependent proteosomal degradation pathway. Exposure to heat and oxidative stress show increased activity in the proteosomal degradation pathway. Cytoplasmic, short lived and damaged proteins are typically modified by the addition of ubiquitin molecules and targeted for degradation by the ATP-dependent protein degradation pathway (see reviews for ubiquitination pathways, Ciechanover, 1994; Ciechanover, 1998). Misfolded or damaged proteins can be ubiquitinated by ligases, for example the budding yeast San1 or Ltn1, to target proteins for

degradation. Alternatively, ubiquitin ligases are dependent on chaperone activity by HSP to recognise substrates for ubiquitination, such as Ubr1. Ubr1 is suggested to bind directly to a HSP70-HSP40 complex to present the substrate for ubiquitination and acting with the HSP complex to maintain substrate solubility for its degradation (Summers *et al.*, 2013). Interestingly, in mammalian cells a protein CHIP has been identified that links the HSP protein interaction and targeting for proteosomal degradation. Through yeast 2-hybrid screens it was found that CHIP interacts with both Hsc70 and HSP70, and HSP90 C-terminal. In vitro analysis suggested that CHIP interaction with Hsc70 inhibits ATP hydrolysis leading to substrate release and subsequent ubiquitination. The function of the CHIP interaction appears to lead the HSP client protein towards the proteosomal degradation pathway (McDonough and Patterson, 2003).

Excitingly, new research is uncovering a role for HSP70/HSP40 activity in cell cycle regulation. In budding yeast an important event in the initiation to commit to the next cell cycle occurs at Start in late G1 phase (Nurse, 1994). Cyclin and cyclin-dependent kinases (CDKs) are key proteins in regulating progression through the cell cycle and function at checkpoints including Start. Cyclins function to induce CDK kinase activity and can bind to different CDK depending on the phase of the cell cycle. CDKs have little kinase activity of their own, in complex with a cyclin the CDK gains increased phosphorylation activity (Andrews and Measday, 1998; Mendenhall and Hodge, 1998). Thus, CDK activity is tightly controlled by cyclin regulation including protein levels and localisation throughout the cell cycle (Andrews and Measday, 1998; Mendenhall and Hodge, 1998).

The mechanism of regulation of the cell cycle is timed expression of genes. In *S. cerevisiae*, a transcriptional wave that progresses the cell through Start moving the cell from G1 to S phase is triggered by the Cln3-Cdc28 cyclin-CDK complex (Mendenhall and Hodge, 1998; Verges *et*

al., 2007). As the complex Cln3-Cdc28 is the most upstream complex to initiate replication it requires careful regulation to prevent aberrant cell cycle progression. Recent work showed that a chaperone HSP70 Ssa1 binds Cln3-Cdc28 in the cytoplasm preventing Cln3-Cdc28 entry to the nucleus (Truman *et al*., 2012) (Fig. 1. 4.). Therefore, Cln3-Cdc28 is not available to initiate phosphorylation events leading to S phase. It was determined that regulation of the interaction between Ssa1 and the Cln3-Cdc28 complex was dependent on modification of the NBD of Ssa1 and the effect on J domain binding (Verges *et al*., 2007; Truman *et al*., 2012).

Cyclin Cln3 has a J domain which lacks the conserved HPD motif, this is termed the Ji domain (Verges *et al*., 2007; Lowndes, 2007). Cyclin Cln3 Ji domain renders it non-functional to induce HSP70 Ssa1 ATPase activity while causing Ssa1 to maintain a bound conformation to Cln3 in the NBD (Truman *et al*., 2012; Verges *et al*., 2007). Ydj1 is a HSP40 cochaperone of Ssa1 with an active J domain containing the highly conserved HPD motif. The Truman *et al.* (2012) study showed nitrogen starvation leads to phosphorylation of threonine (T36) in the NBD of Ssa1 and increase in Ydj1 levels lead to Ydj1 displacing Cln3. Release of Cln3-Cdc28 allows the complex to move to the nucleus and drive cell cycle progression. However, how this Ssa1 client protein interaction is regulated is not fully understood other than Ydj1 accumulation and phosphorylation within Ssa1 NBD being critical. The role of the closely related protein Ssa2 is not well characterised, the Truman *et al*. (2012) study identifies HSPs as being critical for controlling cell cycle entry it is noted that the role proposed for Ssa1 could be performed by Ssa2.

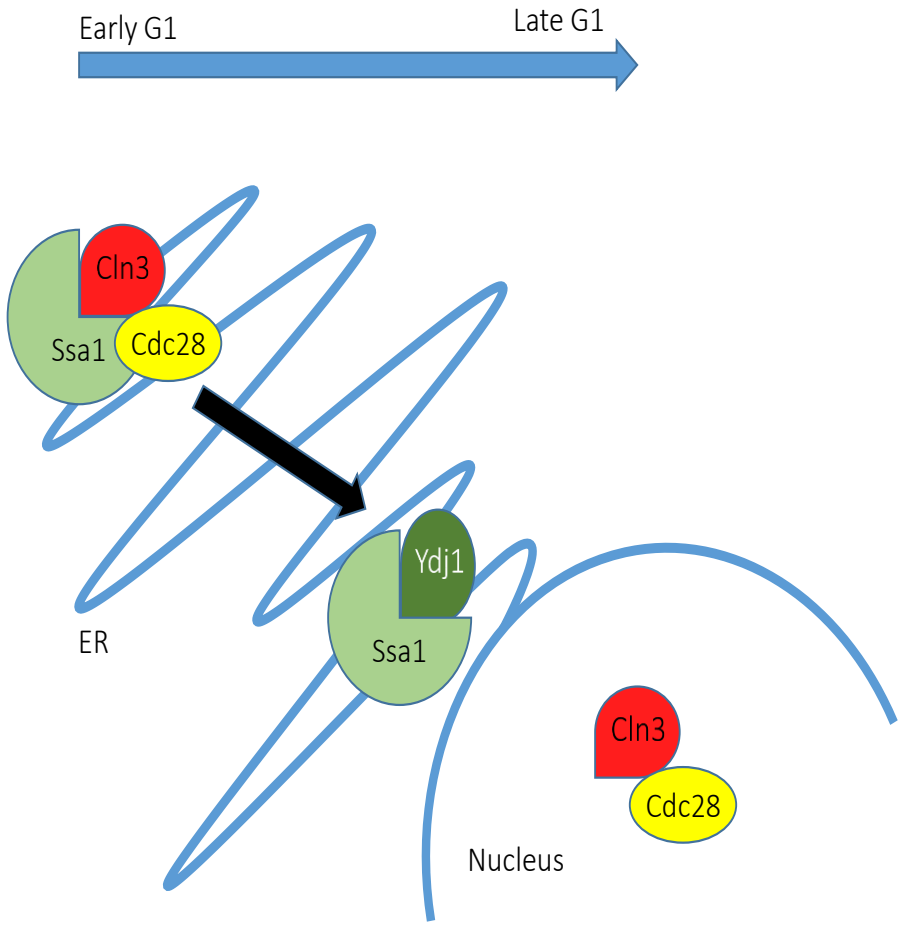


Fig. 1. 4. Model proposed of Ssa1 and Ydj1 regulation of Cln3 during G1 phase of *S. cerevisiae* cells. During early G1 HSP70 Ssa1 is associated with the ER in the cytoplasm. Cln3 of the Cln3-Cdc28 complex is bound to Ssa1 SBD thus preventing Cln3-Cdc28 nuclear activity. Following this, Cln3 is replaced by Ydj1 in the Ssa1 SBD. This results in Cln3-Cdc28 being free to move to the nucleus to activate transcription factors for the next phase of the cell cycle.

Importantly, the study also showed that the role of phosphorylated Ssa1 was conserved in human cells. *S. cerevisiae* Cln3 is homologous to human cyclin D1 while the yeast Ssa1 is homologous to human Hsc70 and these human homologs were shown to functionally interact (Truman *et al*., 2012). The human homolog Hsc70 showed phosphorylation of T38 by the mitotic CDK Cdk1 again showing similarity to Ssa1 regulation. The phosphorylation of Hsc70 by Cdk1 correlated with reduced cyclin D1 levels and would appear to suggest a conservation of HSP70 cycling binding leading to degradation to regulate the G1/S phase transition (Truman *et al*., 2012). This suggests that at least between budding yeast and human cells there is a conservation of phosphorylation of HSP70 by CDK that effects stability/binding of cyclin Cln3/D1 to provide a mechanism of regulating cell cycle progression.

Novel data are also beginning to show that HSP40 have a specific role in regulating the connection between cell size and progression through the cell cycle. The activation of Start in budding yeast as discussed above is regulated by a complex network of proteins. It was believed cells reach a "critical size threshold" before allowing cell cycle progression through Start however this does not explain the wide variation of cell sizes at Start that can be observed within a population (Ferrezuelo *et al*., 2012). There is an inherent link between the size of the cell and the cell cycle, cell size mutant screens highlighted genes involved in cell cycle regulation but also ribosome biogenesis and protein translocation including the HSP40-encoding *YDJ1* gene; deletion of the *YDJ1* gene results in a large cell phenotype highlighting a role in cell growth regulation (Jorgensen *et al*., 2002; Verges *et al*., 2007).

Mathematical modelling of *S. cerevisiae* indicates that cell growth during G1 determines the cell size at Start with Ydj1 acting as the key regulator. It was determined that the critical role in regulating cell size at Start is the rate of growth during G1 is performed by Ydj1 (Ferrezuelo

et al., 2012). Ydj1's role in co-ordinating the growth rate to the initiation of Start was postulated to be due to its role in cell wall growth and ER protein translocation but the exact mechanism was not discovered (Caplan *et al*., 1992; Ferrezuelo *et al*., 2012). Excitingly this suggests that HSP40 proteins, particularly Ydj1 can play a vital role in regulating cell growth and cell cycle progression in normal conditions.

1.3 Activation of gene expression in yeast in response to oxidative stress and heat stress

All aerobic organisms are subject to heat and oxidative stress. Therefore, these cells contain numerous systems to detect the stress and activate the signalling mechanism to activate appropriate responses. A common feature of these responses to stress is increased transcription of genes whose products repair damage and restore homeostasis. Stress inducible transcription factors and the resultant gene expression is discussed below.

When cells are exposed to either heat or oxidative stress an important aspect of the response involves the remodelling of gene transcription. From microarray analysis of cells following heat or oxidative stress over 900 genes are regulated by these stresses. Of these 900 genes, there is an enrichment for upregulation of genes encoding antioxidants and HSPs are upregulated presumably aiding detoxification and supporting pathways for DNA and protein repair (Bonner *et al*., 2000; Liu and Thiele, 1996). This is also accompanied by the repression of transcription of around 600 genes encoding proteins involved in nucleotide or protein synthesis leading to a reduction in total protein levels (Gasch *et al*., 2000; Causton *et al*., 2001). The activation and the attenuation of transcription is tightly regulated to allow the cell to respond to the stress until the conditions improve.

1.3.1 Msn2/4 and stress in *S. cerevisiae*

Activation of cytoprotective gene expression is dependent on several transcription factors including Msn2/4. The zinc finger transcription factors Msn2/4 are found in *S. cerevisiae* are largely functionally redundant. They are activated by a range of environmental stresses including heat and oxidative stress. Msn2/4 bind the stress response element, over 180 genes have been identified as being under Msn2/4 regulation during OSR. Msn2/4 activates transcription of 27 identified H_2O_2-inducible genes resulting in their significant up-regulation (Gasch *et al*., 2000; Hasan *et al*., 2002). The double mutant *msn2*Δ*msn4*Δ is sensitive to H_2O_2 but can mount an adaptive response (Hasan *et al*., 2002). This highlights the importance of the transcription factors in OSR.

Msn2/4 activity is regulated by sub-cellular localisation. In unstressed cells Msn2/4 is mostly absent from the nucleus but when the cell is exposed to stress conditions there is a nuclear accumulation which is accompanied by an increase in Msn2/4-dependent gene expression (Gorner *et al*., 1998). Msn2/4 are targeted for nuclear export by the nutrient sensing/signalling Ras-CaMP-PKA pathway through direct targeted phosphorylation of Msn2/4 in a negative manner (Gorner *et al*., 1998; Hasan *et al*., 2002). Additionally, high glucose conditions have been shown to activate the nutrient sensing/signalling TOR pathway which also negatively regulates Msn2/4 activity (Medvedik *et al*., 2007).

1.3.2 Yap1 and Pap1

Cells utilise several transcription factors to respond to specific stress conditions in an appropriate manner. In yeast a subfamily of AP-1-like proteins (yAP-1) including Yap1 in *S.*

cerevisiae and the homolog in *S. pombe* Pap1 are important in activating the transcriptional response to cellular stress, particularly oxidative stress. yAP-1 proteins contain a bZip DNA binding domain motif that shares homology with the human AP-1 factor protein c-Jun suggesting a level of conservation amongst eukaryotes OSR (Moye-Rowley *et al*., 1989). Overexpression of Yap1 or Pap1 leads to increased resistance from several oxidants including cadmium (Toda *et al*., 1991; Wu *et al*., 1993). Conversely, inactivation of Yap1 or Pap1 results in an increased sensitivity to many oxidative stresses including H_2O_2, diamide and cadmium (Kuge and Jones, 1994; Toda *et al*., 1991). Given these phenotypes it is not surprising that Yap1 and Pap1 regulate the transcription of numerous OSR-encoding genes such as those encoding catalases. Although these transcription factors are homologous it is important to consider their conserved and organism specific roles and regulations in stress response.

1.3.2.1 Yap1 and Yap1 regulation

The transcription factor Yap1 in *S. cerevisiae* is activated in response to various stimuli including several oxidants, metals, and infrared (Kuge and Jones, 1994; Molin *et al*., 2007; Vido *et al*., 2001). For example, in response to H_2O_2 Yap1 changes the transcription of over 70 genes including *TSA1*, *TRX2*, *GSH1*, *CTT1* and *SOD1* which all encode proteins critical in the OSR (Gasch *et al*., 2000; Moye-Rowley, 2003). The genes under Yap1 regulation typically contain a Yap1 regulatory element in the promoter region. Yapl is known to bind the consensus sequence TT/GAC/GTTA such as in the promoter of *GSH1*. However, there are genes known to be under Yap1 regulation that do not contain this motif. Alternate binding sequences for Yap1 are currently unknown (He and Fassler, 2005; Toone and Jones, 1999).

Yap1 is activated in response to oxidants by modification of its structure. In non-stressed cells Yap1 is shuttled out of the nucleus rapidly due to a nuclear export sequence (NES) in the C-

terminal cysteine rich domain (c-CRD) interacting with the conserved nuclear exportin Crm1 (Kuge *et al*., 1998). Yap1 also contains a N-terminal cysteine rich domain (n-CRD). Yap1 accumulates in the nucleus due to modification, directly or indirectly, of its structure which leads to masking of the NES (Delaunay *et al*., 2000; Kuge *et al*., 1997). An increase in yAP-1 transcriptional activity is correlated with an increase in nuclear accumulation. The mechanism of this was identified through analysis of the NES sequence in response to oxidising agents. It was found that redox sensitive cysteines in n-CRD and c-CRD can become oxidised in response to certain oxidants (Kuge *et al*., 1997) These data suggest oxidation of these cysteines results in formation of an intramolecular disulphide, this mask the nuclear export sequence leading to a nuclear accumulation and transcriptional activity (Delaunay *et al*., 2002; Kuge *et al*., 2001).

Yap1 responds to oxidants by 2 mechanisms, either both CRDs are involved or solely the c-CRD depending on the oxidising agent. Both mechanisms mask the NES to result in Yap1 nuclear accumulation and activation. Upon exposure to low H_2O_2 (0.4 mM) a mixed disulphide can be detected between Yap1 and a 20kDa protein that has been identified as glutathione peroxidase-like Gpx3 which then resolves to a Yap1 intramolecular disulphide (Delaunay *et al*., 2002). Additionally, *gpx3*Δ cells do not show the wild type nuclear accumulation or Yap1-dependent gene transcription following H_2O_2 stress. The model proposed is that the Yap1-Gpx3 disulphide is then converted to a Yap1 intramolecular disulphide which masks the NES leading to Yap1 nuclear accumulation and activation. Yap1 resolves from this complex by forming 2 disulphide bonds which involves a cysteine located in the NES and a cysteine within the n-CRD (Delaunay *et al*., 2002). Gpx3 is reduced by thioredoxin to continue ROS scavenging. Data suggests Gpx3 acts as a sensor and transducer for H_2O_2 stress to activate Yap1.

Interestingly, our lab has uncovered another protein, Ybp1 that is required for Yap1 nuclear accumulation via an alternative mechanism (Veal *et al*., 2003). It was found that Ybp1 is cytosolic and oxidised in a Gpx3-dependent manner. The mechanism is less clear but it is known that the protein Ybp1 is required for Gpx3 to form a complex with Yap1 in response to H_2O_2 but not diamide (Veal *et al*., 2003). The function of Ybp1 has only recently been uncovered. H_2O_2 oxidises the thiol peroxidase Orp1 which relays the oxidative status to Yap1 resulting in the latter's activation (Bersweiler *et al*., 2017). It was discovered that Ybp1 functions to generate a complex between oxidised Orp1 and Yap1, it is suggested that this scaffolding function allows the oxidised Orp1 residue to relay its oxidation to one of the critical Yap1 cysteine residues to lead to Yap1 activation.

Interestingly in an alternate *S. cerevisiae* background (W303) Ybp1 is truncated and Yap1 activation of gene expression is found to be Gpx3-independent. However, in this background, the Prx Tsa1 is critical for Yap1 activation. Reversal of Yap1 oxidation is dependent on Trx2. Interestingly, *TRX2* transcription is regulated by Yap1, as are other proteins in the Trx system suggesting that Yap1 has a negative feedback loop that is regulated by Yap1 itself.

1.3.2.2 Pap1 and Pap1 regulation

Pap1 is the homologue of *S. cerevisiae* Yap1 found in the fission yeast *S. pombe*. Considering how distantly the organisms are related, there are many similarities between Yap1 and Pap1. Like Yap1, Pap1 activity is known to be regulated by the redox status of the protein. Also, Pap1 mostly regulates transcription of OSR genes encoding proteins involved in clearance of low level OS. Furthermore, Pap1 is transcriptionally active at 0.2 mM H_2O_2 and over 70 genes under its regulation have been identified (Chen *et al*., 2003). Mutation of key Pap1 residues (cysteine 278 and cysteine 285) results in no significant transcription following mild H_2O_2 stress of OSR

genes encoding proteins such as thioredoxin peroxidase Tpx1 *tpx1*$^{+}$, thioredoxin reductase Trr1 *trr1*$^{+}$, sulfiredoxin Srx1 *srx1*$^{+}$ and GST Gst2 *gst2*$^{+}$ (Calvo *et al*., 2013). *pap1*Δ cells are highly sensitive to numerous oxidising agents including H_2O_2, cadmium and menadione (Toda *et al*., 1991; Toone *et al*., 1998). Deletion of Pap1-dependent genes results in ROS sensitivity. Cells with deletion of genes encoding proteins in thioredoxin pathway either *tpx1*$^{+}$, *trr1*$^{+}$ or *srx1*$^{+}$ are acutely sensitive to H_2O_2 (Bozonet *et al*., 2005; Casso and Beach, 1996; Veal *et al*., 2004). These data indicate Pap1 plays a critical role in oxidative stress induced transcription. However, there is overlap of Pap1 transcriptional targets with other OS-induced transcription factors including Atf1 (see section 1.3.3).

Pap1 contains a highly similar DNA binding domain to that found in *S. cerevisiae* Yap1 and mammalian AP-1. Additionally, Pap1 can bind to and activate transcription from an AP-1 binding site. Pap1 has been shown to bind the consensus sequence TTACGTAA (Fujii *et al*., 2000). Within Pap1 is the typical bZIP motif seen in the AP-1 proteins, this is located proximal to the N terminus. Within Pap1 are 2 CRDs, one in the central region and the other located in the C-terminal encompassing the NES. Pap-1 shuttle from the cytosol to the nucleus, the C-terminal NES is recognised by exportin Crm1 to shuttle the protein back to the cytosol (See Fig. 1. 5.).

Reversible oxidation of residues in response to oxidative stress has been implicated as a stress signalling mechanism. In *S. pombe* low H_2O_2 (0.2 mM) stress induces temporary oxidation of cysteines in Pap1 (particularly C278 and C501) to form a temporary intramolecular disulphide bond. The altered conformation of the protein prevents Pap1 from nuclear export by Crm1 by masking the NES inducing nuclear accumulation and upregulation of Pap1-dependent antioxidant encoding genes that detoxify low H_2O_2 (Calvo *et al*., 2013; Quinn *et al*., 2002).

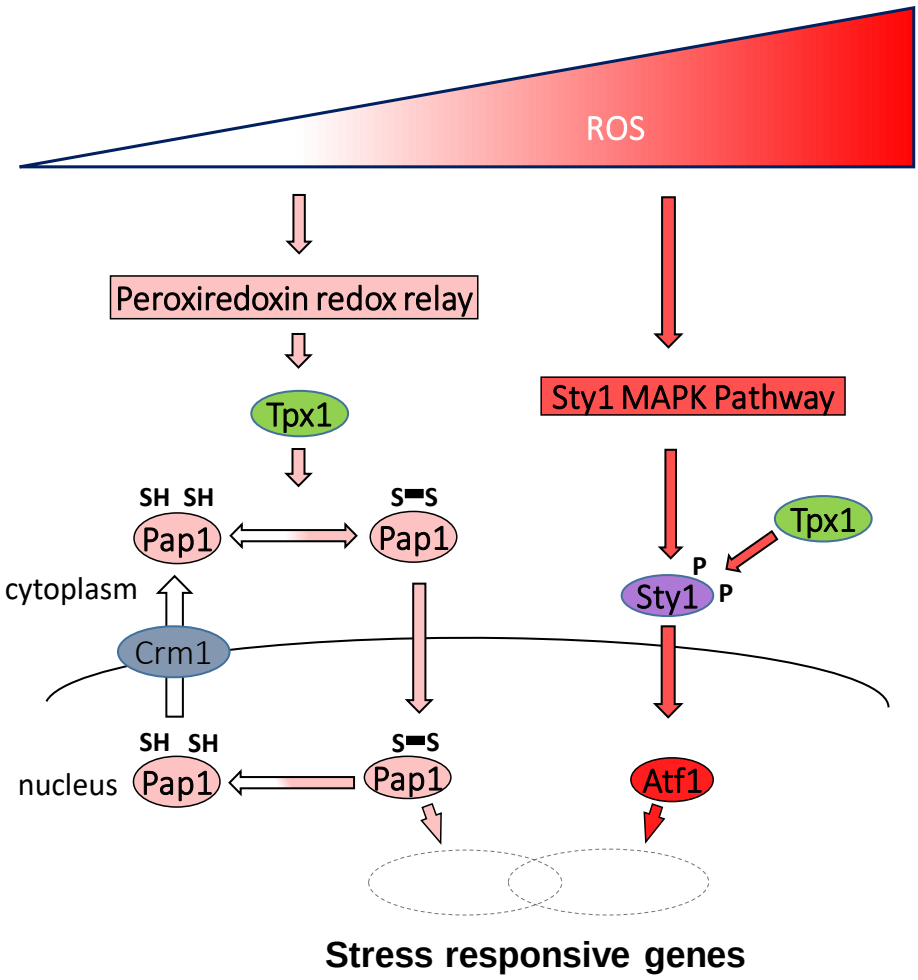


Fig. 1. 5. *S. pombe* H_2O_2 concentration-dependent OSR activates alternate transcription factors. Exposure of cells to low levels of OS leads to oxidation within the Tpx1 pathway resulting in Pap1 oxidation, in this form Pap1 cannot shuttle between the cytoplasm and the nucleus via Crm1. Pap1 nuclear accumulation leads to its transcriptional activity. Higher levels of OS do not activate the Tpx1 pathway in the same manner, higher OS activates the Sty1 pathway with Tpx1 interaction. The phosphorelay of the Sty1 pathway leads to phosphorylation of the transcription factor Atf1 and it becomes transcriptionally active. Pap1 transcribes genes required for detoxification of low levels of OS while Atf1 is responsible for transcription of the genes encoding high OSR proteins however, there are several genes that are known to be transcriptionally regulated by both TFs.

Similar localisation in response to oxidative stress has been noted for *S. cerevisiae* Yap1 discussed previously (see section 1.3.2.1). Deletion of the C-terminal region of Yap1 and Pap1 results in their constitutive nuclear localisation but no induction under H_2O_2 stress (Coleman *et al*., 1999; Kudo *et al*., 1999). The reduction of Pap1 allows Crm1 to export the transcription factor back to the cytosol curtailing the transcriptional response of Pap1 (Toone *et al*., 1998). The current literature accepts that Pap1 is regulated through oxidation in response to various types of oxidative stress in a concentration-dependent manner unlike Yap1. The oxidation and nuclear accumulation of Pap1 provide mechanisms of inducing Pap1-dependent gene transcription to increase expression of OSR proteins.

The molecular mechanism of how oxidation of Pap1 occurs is not well understood. Interestingly, at higher concentrations of H_2O_2 ($\geq$ 1 mM) Pap1 oxidation and nuclear accumulation is delayed. Work is ongoing to understand the mechanism of how cells can determine the appropriate response after exposure to different concentrations of H_2O_2. The thioredoxin system, particularly thioredoxin peroxidase Tpx1, is believed to play a role in relaying the oxidation status of the cell to activate the appropriate transcriptional factor (Veal *et al*., 2014). Tpx1 is a highly abundant peroxiredoxin that removes peroxides in the cell however it is also required for activation of Pap1-dependent gene expression. The catalytic activity of Tpx1 is determined by 2 cysteine residues, the peroxidatic and the resolving cysteine as discussed previously (see section 1.2.1.2.1). Low concentrations of H_2O_2 oxidises the large pool of Tpx1 and completely oxidises Trx1/Txl1 thus Pap1 becomes oxidised and activates Pap1 dependent gene expression (Brown *et al*., 2013; Day *et al*., 2012; Veal *et al*., 2014). However, at high concentrations of H_2O_2 the transcriptional response needs to prevent the damage of increased oxidative stress. Tpx1 is believed to be a key to relaying this requirement in the cell. After exposure to high concentrations of H_2O_2 the peroxidatic cysteine of Tpx1 becomes hyperoxidised to a sulfinic

acid (Bozonet *et al*., 2005). The sulfinic cysteine of Tpx1 is not a substrate for Trx1/Txl1 which leads to Pap1 remaining in a reduced form that is inactive at high concentrations of H_2O_2 (Day *et al*., 2012). Hyperoxidised Tpx1 is a substrate for sulfiredoxin Srx1, returning the sulfinic cysteine of Tpx1 to a sulfenic acid and therefore a substrate for Trx1/Txl1 (Bozonet *et al*., 2005; Vivancos *et al*., 2005).

The mechanism of Tpx1 redox relay to Pap1 activity in *S. pombe* has some similarities to *S. cerevisiae* Yap1 regulation. In the budding yeast Yap1 forms an intermolecular disulphide bond with Gpx3 and is supported by Tsa1 to lead to Yap1 oxidation and activation. Tsa1 in *S. cerevisiae* is the ortholog of Tpx1 in *S. pombe* however, no direct interaction can be found in *S. pombe* between Tpx1 and Pap1 unlike Tsa1 and Yap1 in *S. cerevisiae*. Additionally, in *S. pombe* Pap1 activation is not dependent the ortholog of Gpx3, Gpx1 (Bozonet *et al*., 2005; Vivancos *et al*., 2005).

1.3.3 Sty1/Atf1 and Atf1 regulation

Sensing increased oxidative stress must be relayed by stress-signalling pathways. One such pathway is the highly conserved mitogen activated protein kinase (MAPK) pathway. MAPK relay the stimuli through a 3-tiered cascade of kinases. When the MAPK transduces stress-sensing and signalling it is often termed a stress activated protein kinase (SAPK) pathway. SAPK pathways transduce a range of environmental stresses to effector proteins of gene expression leading to protection of the cell. Sensing and signalling mechanisms for the detected stimulus activate the SAPK pathway for the appropriate response. Initially, phosphorelay from a MAPK kinase kinase (MAPKKK) to a MAPK kinase (MAPKK) culminates in phosphorylation and activation of a MAPK (Shieh *et al*., 1998; Toone and Jones, 1998). The

MAPK targets effector proteins, such as transcription factors, in response to the environmental stress (Toone and Jones, 1998).

MAPK pathway proteins are highly conserved amongst eukaryotes having been characterized in yeast and mammals. In *S. pombe*, activation of the transcription factor Atf1 in response to high H_2O_2 stress (≥ 1 mM) is via MAPK Sty1 (See Fig. 1. 5.; Veal *et al*., 2004). Interestingly, *S. pombe* Sty1 shows significant homology to the mammalian p38 and JNK MAPK pathway suggesting a level of conservation (Toone and Jones, 1998). The components of the Sty1 SAPK pathway have mostly been genetically identified but the roles of each are still under investigation. The MAPK Sty1 is phosphorylated by the MAPKK Wis1 on conserved threonine and tyrosine residues (Toone and Jones, 1998). Wis1 is activated by phosphorylation from a MAPKKK, either Wak1 or Win1 which are functionally redundant (Shieh *et al*., 1997; Shieh *et al*., 1998). In *S. pombe*, the SAPK pathway of Sty1 has been shown to be activated by various environmental stresses including osmotic stress and high oxidative stress. These specific stimuli are sensed and relayed by a system that shows homology to the bacterial two component sensor transcriptional activators. A response regulator termed Mcs4 phosphorylates the MAPKKK Wak1/Win1 (Shieh *et al*., 1997). A phosphotransmitter Spy1 relays the sensory histidine kinase phosphorylation from Mak1, 2, 3 to Mcs4. Mak1,2,3 act as the sensors for various environmental stresses including heat, osmotic and H_2O_2 in high concentration (≥ 1 mM) (Aoyama *et al*., 2000; Aoyama *et al*., 2001).

Atf1 is activated via Sty1 and the SAPK pathway in response to the sensed stress to result in an appropriate transcriptional response. A core set of genes have been identified that are regulated by the transcription factor Atf1 in response to certain environmental stresses. Importantly, Atf1 is critical for the cell to be able to respond to oxidative stress above 1 mM H_2O_2 to activate the

transcriptional response required. Stress activation of Sty1 increases phosphorylation of Atf1 leading to transcriptional activity as well as increased stability of the Atf1 protein (Day and Veal, 2010; Veal *et al*., 2004). *atf1*Δ cells and *sty1*Δ cells are hypersensitive to H_2O_2 as well as salt stress (Takeda *et al*., 1995; Wilkinson *et al*., 1996). Mutations effecting the MAPK relay pathway, particularly Sty1, have a significant effect on Atf1 stability and transcriptional activity in response to H_2O_2.

Atf1 is a bZIP transcription factor that shares homology to mammalian activating transcription factor ATF2 (Wilkinson *et al*., 1996). Evidence suggests Atf1 binds as a heterodimer with another bZIP transcription factor Pcr1 (Watanabe and Yamamoto, 1996). Pcr1 is a homolog of mammalian CREB. The Atf1 heterodimer has been shown to bind the consensus sequence TGACGT (Steiner and Smith, 2005). The Atf1 heterodimer transcribes genes encoding many antioxidants required for high levels of oxidative stress including catalase Ctt1, sulfiredoxin Srx1, and GSTs particularly Gst1 (Quinn *et al*., 2002; Vivancos *et al*., 2006; Veal *et al*., 2002). It has been demonstrated that 1 mM H_2O_2 delays Pap1 transcriptional activity while it activates Atf1 (Veal *et al*., 2007; Vivancos *et al*., 2005). This highlights the significance of Atf1's role and the Sty1 pathway in OSR to high concentrations of H_2O_2. Additionally, it shows the importance of sensing mechanisms relaying the degree of oxidative stress such as Prx Tpx1 and the thioredoxin system activating Pap1 or Atf1.

1.3.4 Hsf1 in *S. cerevisiae* and *S. pombe*

When cells are exposed to heat stress there is an increase in proteotoxicity and this places greater need on HSP functionality for repair or degradation. Transcription of HSP-encoding genes is activated predominantly by the evolutionarily conserved heat shock factors (HSFs) in response to heat stress. In unicellular eukaryotes such as *S. cerevisiae* and *S. pombe* there is a

single HSF (Hsf1) while multicellular eukaryotes contain 4 identified HSF (Hsf1-4) (Bonner *et al*., 2000; Gallo *et al*., 1991). Hsf1 from yeast and humans have been shown to be functional counterparts. The mechanisms of signal transduction regulating HSFs are not well understood. Hsf1 is the principle transcription factor transcribing HSP genes with a significant induction of Hsf1 dependent genes under heat stress (Kregel, 2002). In yeast since there is only one identified HSF which simplifies investigation in these model organisms for HSF function and regulation.

Hsf1 function is determined by its structure. The domain architecture of human Hsf1 is the best characterised so will be used to discuss eukaryotic Hsf1. At the N-terminal is a DNA binding domain (DBD) which shows the highest level of sequence conservation amongst Hsf1 proteins (Fig. 1. 6. A; Anckar and Sistonen, 2011). The DNA binding domain contains a helix-turn-helix structure that is believed to stabilise Hsf1 on the DNA. The region next to the DBD is believed to be an extended –helical containing heptad repeats (HR-A and HR-B), this forms the HR-A/B domain (Fig. 1. 6. A; Anckar and Sistonen, 2011). Hsf1 is functional as a homotrimer and during trimerization the HR-A/B are believed to form a triple stranded coil by interaction of hydrophobic residues (Anckar and Sistonen, 2011; Peteranderl *et al*., 1999).

Hsf1 is prevented from trimerization by a HR-C domain in the C-terminal region. The absence of the HR-C domain in *S. cerevisiae* Hsf1 is believed to be the reason why the protein is constitutively trimerized unlike in *S. pombe* or mammalian cells (Fig. 1. 6. A; Chen *et al*., 1993). At the C-terminal are the transactivation domains (TAD1 and TAD2) which are key regulators of transcriptional activity and the fold induction of the gene. TAD1 and TAD2 are required to initiate transcription and elongation (Anckar and Sistonen, 2011). The activity of the TAD region is repressed by the regulatory domain which is located between the HR-A/B region and

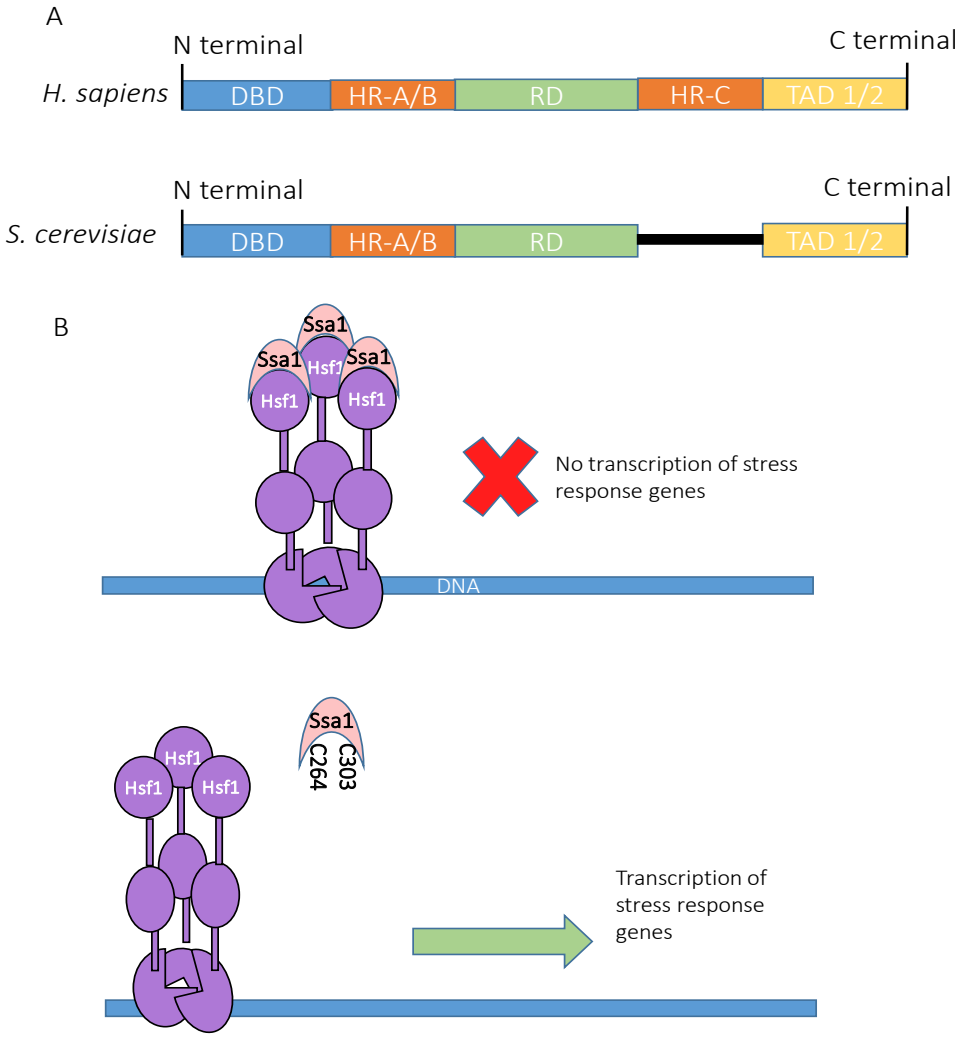


Fig. 1. 6. A representation of Hsf1 domain structure and substrate binding. (A) Representation of Hsf1 domain structure for Mammalian and budding yeast. DNA binding domain (DBD) followed by heptad repeats A/B (HR-A/B), the regulatory domain (RD), another heptad repeat (HR-C) and the transactivation domain (TAD). (B) Model of Ssa1 acting as a sensor for Hsf1 to H_2O_2 stress in *S. cerevisiae***.** Within *S. cerevisiae* cells Hsf1 seems to be constitutively nuclear and associated with DNA. In non-stress conditions Hsf1 is bound to Ssa1 preventing Hsf1 dependent gene expression. Treatment with oxidising agents modified residues in Ssa1 NBD preventing interaction with Hsf1 leading to transcription of stress response genes. Image adapted from Wang *et al.* (2012).

the HR-C region. In the absence of the regulatory domain the TAD domains induce a strong transcriptional response. The regulatory domain exerts negative regulation over the TADs, it is the target of numerous post-translational modifications, it is hypothesised the function of regulatory domain is modulated by the modifications (Anckar and Sistonen, 2011; Green *et al*., 1995). The autoregulation of Hsf1 and other mechanisms are key to how Hsf1 senses a specific stress and results in an appropriate response.

1.3.4.1 Hsf1 regulation

Control of the HSF-dependent transcription of HSP-encoding genes provides an important aspect of stress response. HSFs are proposed to be regulated in several different ways which include altered localisation, modification and modulation. However, these regulatory mechanisms are not conserved amongst all eukaryotes. For example, Hsf1 in *S. cerevisiae* is constitutively nuclear and associated with chromosomal DNA whereas Hsf1 in *S. pombe* and human cells display nucleo-cytoplasmic shuttling of the transcription factor (Anckar and Sistonen, 2011; Gallo *et al*., 1991). Interestingly, *S. pombe* and mammalian cells appears to show greater similarity in their regulatory mechanisms of Hsf1 making *S. pombe* a useful model for studies of Hsf1 (Gallo *et al*., 1991). The mechanism and role of Hsf1 nucleo-cytoplasmic shuttling is unclear but it does indicate Hsf1 nuclear export is important during normal cell conditions for some organisms.

In some cells, Hsf1 shows evidence of auto-regulation by internal domains which repress activity/trimerization. As described above trimerization is inhibited by the HR-C domain which prevents HR-A/B forming a triple coil structure. Interestingly, the regulatory domain has been shown to be a direct heat sensor for the TAD (Newton *et al*., 1996). Hsf1 auto-regulation in

response to stress has been shown to occur. For example, detectable trimerization and DNA binding was shown in vitro in response to heat stress. Interestingly, aromatic amino acids (W37, Y60 and F104) and 2 cysteines (C36 and C104) in the DBD have been proposed to link human Hsf1 monomers following heat stress to form a stable trimer (Lu *et al*., 2009). The aromatic residues in human Hsf1 are conserved in budding and fission yeast Hsf1 yet budding and fission yeast Hsf1 lack the identified cysteine residues. However, yeast Hsf1 are known to trimerize and this suggests that yeast Hsf1s may have alternate mechanisms regulating Hsf1 trimerization.

Hsf1 regulates the transcription of HSP genes by binding to heat shock elements (HSEs) located in the promoter region of each gene. In eukaryotes, the HSE motif is highly conserved consisting of multiple nGAAn, inverted and repeated within the promoter region (Anckar and Sistonen, 2011; Gallo *et al*., 1991). However, trimerization and DNA binding by Hsf1 is not sufficient to result in active transcription. The ability of Hsf1 to activate transcription has been linked for several decades to its phosphorylation status. Hsf1 is known to be hyper-phosphorylated in unstressed conditions while phosphorylation of specific serine residues appear to be stress-inducible (Anckar and Sistonen, 2011). However, the function of specific phosphorylations have yet to be fully defined partly because Hsf1 is relatively serine rich. Furthermore, some serine phosphorylation is inhibitory. Thus, while human Hsf1 serine 230 phosphorylation was linked to increased activity and was induced by heat shock (Holmberg *et al*., 2001), serine 121 phosphorylation inhibited Hsf1 activity (Wang *et al*., 2006).

In *S. pombe*, Hsf1 is phosphorylated upon exposure of cells to heat stress, binds HSEs and increases expression of HSP-encoding genes (Gallo *et al*., 1991). *S. cerevisiae* also demonstrates heat-inducible phosphorylation of Hsf1 that enhances activity. Thus, there are both positive and inhibitory phosphorylation events which effect Hsf1 activity. Interestingly, in

yeast and mammalian cells the phosphorylation status of Hsf1 does not actually affect the binding capacity of Hsf1 to HSEs, suggesting that phosphorylation events effect transcriptional efficiency. Thus, while it is accepted that constitutive and stress-inducible phosphorylation of Hsf1 is important for activation of transcription, all the relevant residues and the mechanistic consequences of phosphorylation have yet to be fully understood.

The regulatory domain is targeted by numerous posttranslational modifications that suggest a mechanism of specific activation due to the particular modification of the regulatory domain. Several of the residues within the regulatory domain have been shown to be phosphorylation targets which have an inhibitory effect on Hsf1 activity in unstressed conditions (Green *et al*., 1995). The presence of inhibitory phosphorylation events suggested to be part of a motif for phosphorylation-dependent sumoylation of Hsf1. The motif for this event, PDSM, was initially characterised in the mammalian Hsf1 but has since been found in other transcription regulators (Anckar and Sistonen, 2011; Hietakangas *et al*., 2006). Sumoylation of proteins is a common inhibitory mechanism of transcription regulators. It has since been shown that sumoylation of Hsf1 occurs within the regulatory domain region where inhibitory phosphorylation was characterised. Interestingly, cells treated with severe heat shock show a temporary reduction in sumoylation which is indicative of sumoylation being an inhibitory mechanism of Hsf1 activity in unstressed conditions (Hietakangas *et al*., 2006). However, the mechanism of sumoylation of Hsf1 and the upstream phosphorylation events has yet to be uncovered.

To add to the complexity of Hsf1 regulation, it was found in response to heat that acetylation is another regulatory mechanism. Acetylation appears to play an inhibitory role in Hsf1 regulation that is linked to phosphorylation of the transcription factor. In response to heat shock, acetylation of Hsf1 is seen to increase as the heat shock response becomes attenuated. One of

the key residues identified was K80 within the DNA binding domain. Acetylation of this residue (lysine 80) reduced the degree of Hsf1 DNA binding capacity and consequently a defective heat shock response (Littlefield *et al*., 1999).

In addition to the mechanisms described above, it has been proposed that Hsf1 is also regulated by direct interactions with HSPs possibly as part of the nucleo-cytoplasmic shuttling mechanism. In human cells, monomeric cytoplasmic Hsf1 interacts with a HSP90 and following proteotoxic stress this interaction is interrupted (Zou *et al*., 1998). Indeed, application of HSP90 inhibitors causes Hsf1 to become trimeric and DNA bound suggesting HSP90 complex with Hsf1 is an important mechanism of Hsf1 repression in unstressed conditions (Ali *et al*., 1998; Anckar and Sistonen, 2011; Zou *et al*., 1998). However, the HSP-Hsf1 complex regulation appears to be multifaceted. For example, in alternate eukaryotic models, mouse and *Xenopus laevis,* HSP90 has also been detected as part of a complex with Hsf1 in heat shocked cells suggesting that HSP90 is not simply acting as a repressor of Hsf1 in unstressed cells (Ali *et al*., 1998; Bharadwaj *et al*., 1999).

HSP70s have also been shown to interact with Hsf1 in human and yeast cells (Anckar and Sistonen, 2011; Wang *et al*., 2012). However, HSP70 interaction with human Hsf1 does not reduce following heat stress but actually increases alongside the increase in HSP70 levels (Abravaya *et al*., 1992; Shi *et al*., 1998). Furthermore, the interaction appears to have a role in repression of the TAD as HSP70-Hsf1 was detected to be DNA bound (Shi *et al*., 1998). The mechanisms underlying HSP70 regulation of Hsf1 is unknown.

Interestingly, prokaryotic RNA polymerase factor σ^{32} is also regulated by HSP70 (DnaK) and HSP40 (DnaJ). σ^{32} is repressed by DnaK and DnaJ until cells are exposed to heat stress, when

the chaperone complex releases σ^{32} allowing activation of HSP-encoding gene expression. The increase in chaperone level inhibits σ^{32} activity providing a negative feedback loop (Straus *et al*., 1990). Strains with non-functional DnaJ or DnaK have increased levels of HSP synthesis and cells cannot attenuate the heat shock response following acute heat stress (Straus *et al*., 1990). This was linked to a derepression of σ^{32} through increased stability and synthesis in the absence of either DnaJ or DnaK. Excitingly, this suggests an ancient conserved mechanism of regulation of transcription factors that respond to heat by HSPs.

It should be considered that of the mechanisms postulated to regulate Hsf1 it is possible that some or all the models are accurate depending on the organism and the stress conditions. It is also possible that, within a cell, fractions of Hsf1 undergo different regulatory mechanisms. Organism-specific mechanisms of Hsf1 regulation have been identified, the structure of *S. pombe* Hsf1 closely resembles that of *Drosophila melanogaster* and human Hsf1. Replacement of the *S. pombe hsf1*$^{+}$ gene with *D. melanogaster* gene allows activation of heat shock transcripts and cells retain viability (Gallo *et al*., 1993). This suggests that there is functional conservation of Hsf1-dependent regulations to heat shock amongst these eukaryotes.

Although the transcription of HSP-encoding genes is extensively Hsf1-dependent, Hsf1 has other target genes such as those encoding transcription factors, translational factors and cell cycle components. The diversity of target genes suggest Hsf1 is important for generating a balance of core biological systems of stressed and unstressed cells (Le Masson *et al*., 2011; Mendillo *et al*., 2012; Vihervaara and Sistonen, 2014). However, a recent paper showed experimentally that a mere 18 genes are Hsf1 targets with all but one being chaperone-encoding genes (Solis *et al*., 2016). In yeast Hsf1 is essential and this is consistent with Hsf1 being required in normal cell homoeostasis. Recently a study in budding yeast found that artificially

induced nuclear export of Hsf1 resulted in proteotoxicity even in unstressed conditions (Solis *et al*., 2016). Furthermore, the study found that if HSP70/90 expression was rescued then proteotoxicity could be prevented. This indicates that the main function of Hsf1 in yeast is to activate transcription of a small set of key chaperones to maintain proteostasis in unstressed and stress conditions.

It could also support the idea that fractions of Hsf1 are performing different roles/being differentially regulated at any given time. The reason for multiple regulatory mechanisms of Hsf1 is likely to allow the transcription factor to sense a wide variety of stress conditions and initiate a specific transcriptional response.

Hsf1 has been well characterised in transcribing HSP-encoding genes under stress conditions, this leads to an increase in HSP levels to prevent proteotoxicity. Hsf1 of many model systems has been shown to be the main transcriptional activator of the heat stress response, however the mechanisms of sensing and signalling the stress leading to Hsf1 activation are not clear.

Novel mechanisms of regulation of Hsf1 have been implicated for response to oxidative stress via HSPs. Recent work has suggested that the HSP70 Ssa1 regulates Hsf1 activity (Wang *et al*., 2012). In *S. cerevisiae* Ssa1 was shown to bind Hsf1 under normal conditions preventing transcription of stress response genes. However, when the cell was exposed to certain oxidising agents, Ssa1 becomes oxidised at cysteines 264 and 303 in the NBD. This oxidation appears to prevent the interaction with Hsf1 and therefore Hsf1 can bind and transcribe stress response genes (Fig. 1. 6. B). The work suggested that Ssa1 acts as a sensor for oxidative stress to regulate transcriptional response of Hsf1. This is the first report of Ssa1 acting as a sensor for oxidation modifying its client interaction as a chaperone. It would be of interest to establish the specificity

of Ssa1 oxidation to different oxidising agents as this was not investigated within the Wang *et al*. (2012) study. The paper did not investigate whether other Ssa family proteins, such as Ssa2, are redundant to each other in Hsf1 oxidation regulation. It is not known if the HSP40 cochaperone Ydj1 is required for the Ssa1 to interact with Hsf1. Furthermore, the study was conducted *in S. cerevisiae*, whether Hsf1 regulation by Ssa1 is organism specific has yet to be examined. Ssa1 acting as a sensor for Hsf1 puts Ssa1 function in the cell cycle in to a new context. How oxidation of Ssa1 effects its binding to Cln3-Cdc28 (see section 1.2.2.2) has not been studied. Given the role of Ssa1 in cell cycle and stress response the coordination of these functions has yet to be explored.

Stress responses are often coordinated with regulation of the cell cycle to prevent damage being propagated to new cells. Furthermore, HSFs have key roles in cell proliferation. Indeed, Hsf1 is essential in both *S. cerevisiae* and *S. pombe*. Recent work has begun to suggest Hsf1 has significant roles in developmental processes. For example, mice lacking Hsf1 (*hsf1*$^{-}$/*hsf1*$^{-}$) display female infertility and growth retardation (Christians *et al*., 2000). Interestingly, although mouse embryonic fibroblasts stressed with heat accumulated at G2/M in the cell cycle and could gain thermotolerance, cells lacking Hsf1 failed to survive heat stress or gain thermotolerance did not accumulate at G2/M (Luft *et al*., 2001). These data indicate HSF (specifically Hsf1) activity is important for viability and stress responses and reveal links to regulation of the cell cycle, Interestingly, expression of *D. melanogaster* Hsf1 in *S. pombe* results in reduced growth and altered cell morphology (Gallo *et al*., 1993). This suggests that Hsf1 has a conserved role in regulating cell proliferation in normal conditions.

1.4 Aims and objectives

HSPs such as the HSP70s Ssa1 and Ssa2 and the HSP40 Ydj1 in *Saccharomyces cerevisiae* are ubiquitous abundant proteins with well characterised roles as protein chaperones functioning in heat and oxidative stress to prevent protein aggregation and aid degradation. These functions are important in eukaryotes to prevent diseases such as cardiovascular disease and Parkinson's disease. Interestingly recent studies have also begun to identify key roles for specific HSPs in stress sensing and signal transduction and regulation of the cell cycle.

Much work has focused on Hsf1 in *S. cerevisiae* during heat stress response. Previous work on Tpx1/Tsa1/Yap1/Pap1 comparing roles and regulation revealed benefits of studying in *S. cerevisiae* and very distantly related *S. pombe* to reveal both organism specific and conserved responses (Quinn *et al*., 2002; Veal *et al*., 2004). Hence the main aim of this thesis was to take advantage of ease of *S. pombe* studies and evolutionary divergence from *S. cerevisiae* to characterise roles and regulation of the HSP70/HSP40 homologues in *S. pombe* to hopefully reveal organism and conserved aspects of function/regulation of Ssa1/Ssa2/Ydj1/Mas5/Hsf1. Specific objectives:

1. Characterise roles of Ssa1, Ssa2 and Mas5 in normal growth as a baseline for studies on stress responses.
2. Investigate potential connections between Ssa1, Ssa2 and Mas5 with oxidative stress responses focusing on examining any relationships with Pap1 and Tpx1.
3. Investigate potential roles and regulation of Hsf1 in responses to oxidative and heat stress and connections to Ssa1, Ssa2 and Mas5 in any detected roles/regulation.

CHAPTER 2

Materials and Methods

2.1 Bacterial and Molecular Biology Techniques

2.1.1 Bacterial growth media

To grow *E. coli* LB media with Bacto-tryptone (2% w/v), Bacto yeast extract (1% w/v), NaCl (1% w/v), pH 7.2 was used (Sambrook *et al*., 1989). To produce solid media Bacto-agar (2% w/v) was included. For selective growth of *E. coli* with plasmids 0.1 mg/ml ampicillin (Sigma-Aldrich) was included in liquid and solid LB media.

2.1.2 Bacterial transformation

Competent *E. coli* cells (SURE strain) (Agilent Technologies) (*e74-(mcrA-)Δ(mcrCB-hsdSMR-mrr)171endA1 SupE44 thi-1 gyrA96 relA lac recB recJ shcJ umuC::Tn5(Kanr) uvrC[F'proAB laclqZ Δmis Tn10 (Tetr)]*) were transformed with plasmid DNA (up to 5 µg) by the calcium chloride method (Sambrook *et al*., 1989). Transformed cells were plated on to solid LB media with ampicillin (see section 2.1.1) and incubated at 37 °C overnight.

2.1.3 Plasmid isolation

Plasmids were extracted from *E. coli* using the Sigma GenElute Plasmid Miniprep Kit following the manufacturer's instructions.

2.1.4 Polymerase chain reaction (PCR)

PCR reactions for sequencing or ligations were performed with the Phusion PCR system (New England Biolabs). Each reaction contained 20 µl Phusion GC Buffer, 1.5 mM $MgCl_2$, 10 µl 100% DMSO, 200 µM of each dNTP (dATP, dCTP, dGTP, dTTP), 1 µg template DNA, 0.5 µM of each oligonucleotide primer (see Table 2.1.) 0.5 µl Phusion DNA polymerase with nH_2O to a final volume of 50 µl. PCR reactions were performed in a T3 Thermocycler (Biometra) using the conditions as outlined:

Step 1: 98 °C for 2 minutes

Step 2: 98 °C for 30 seconds

* Step 3: *x* °C for 1 minute

Step 4: 72 °C for 1 minute per kilobase

Steps 2 to 4 repeated 35 times

Step 5: 72 °C for 10 minutes

Step 6: 4 °C pause indefinitely

* See Table 2.1. for the annealing temperature (step 3) required for the reaction.

For PCR reactions used in diagnostics Simply Red Taq Polymerase (Thermo Scientific) was used. Each reaction contained 30 µl Taq Buffer, 3 mM $MgCl_2$, 10 µl 100% DMSO, 200 µM of each dNTP (dATP, dCTP, dGTP, dTTP), 1 µg template DNA, 0.5 µM of each oligonucleotide primer (see Table 2. 1.), 5 µl Simply Red Taq polymerase with nH_2O to a final volume of 50 µl. PCR reactions were performed in a T3 Thermocycler (Biometra) in the conditions as outlined:

Step 1: 94 °C for 2 minutes

Step 2: 94 °C for 30 seconds

* Step 3: *x* °C for 1 minute

Step 4: 72 °C for 1 minute per kilobase

Steps 2 to 4 repeated 35 times

Step 5: 72 °C for 10 minutes

Step 6: 4 °C pause indefinitely

* See Table 2. 1. for the annealing temperature (step 3) required for the reaction.

DNA was analysed by agarose (1 % w/v) gel electrophoresis with 10 µg/ml SafeView nucleic acid stain (NBS Biologicals) in TAE buffer (40 mM Tris acetate, 1 mM EDTA [pH 8]). Comparison of DNA size was determined by inclusion of a DNA 1 Kb Ladder (Promega, catalogue number PR-G5711). DNA was extracted from agarose gels using Qiaquick Gel Extraction kit (QIAGEN) following the manufacturer's instructions.

2.1.5 Restriction endonuclease digestions and ligation reactions

Restriction endonuclease digestions were performed using restriction enzymes provided by Fermentas and New England BioLabs using the manufacturer's instructions. T4 DNA ligase was provided by Promega. Ligations were performed as described by Maniatis *et al*. (1989). DNA was analysed by agarose (1 % w/v) gel electrophoresis in TAE buffer (40 mM Tris acetate, 1 mM EDTA [pH 8]). The required product for ligation was cut from the agarose gel and extracted using a Qiaquick Gel Extraction kit (Qiagen). Ligations were incubated at 15 °C overnight.

2.1.6 Construction of pRIP42PkC integrating plasmids

Plasmids used in this study are listed in Table 2.2 and plasmid constructions in this study are described below.

To express a protein tagged with 3 Pk epitopes at the C terminus from the normal chromosomal locus the integrating plasmid pRIP42PkC was used as a vector (Maundrell, 1993). Approximately the last 500 to 700 bp at the 3' end of the target gene sequence were amplified from yeast genomic DNA by PCR. The oligonucleotides used in the PCR introduced a BamHI and PstI restriction site at the 5' and 3' end respectively and excluded the stop codon. The PCR product and pRIP42PkC were digested with BamHI plus PstI and purified by agarose gel electrophoresis (1 % w/v) with 10 µg/ml SafeView nucleic acid stain (NBS Biologicals) in TAE buffer (40 mM Tris acetate, 1 mM EDTA [pH 8]). The digested products were extracted from agarose gels using Qiaquick Gel Extraction kit (QIAGEN) following the manufacturer's instructions. The digested and purified PCR product and pRIP42PkC plasmid were ligated together following the manufacturer's instructions. *E. coli* competent cells (Agilent Technologies) were transformed with ligation mix (up to 5 µg) by the calcium chloride method (Sambrook *et al*., 1989). Transformed cells were plated on to solid LB media with ampicillin (see section 2.1.1) and incubated at 37 °C. Plasmids were extracted from *E. coli* using the Sigma GenElute Plasmid Miniprep Kit following the manufacturer's instructions. DNA sequencing of plasmids was completed by GATC Biotech.

2.2 *S. pombe* Techniques

2.2.1 Strains and growth conditions

S. pombe strains used in this study are listed in Table 2.3. Strains were grown at 30 °C unless indicated otherwise. Strains containing the *cdc25-22* allele were grown at 25 °C. *S. pombe* strains were grown in YE5S (yeast extract (0.5 % w/v), glucose (3 % w/v), 225 mg/L supplements (adenine hemisulphate, L-histidine, uracil, L-lysine, and L-leucine) or EMM (3 g/L potassium hydrogen phthalate, 2.2 g/L Na_2HPO_4, 5 g/L NH_4Cl, glucose (2 % w/v), 20 ml/L salt solution (50 x stock: 52.5 g/L $MgCl_2.6H_2O$, 50 g/L KCl, 2 g/L Na_2SO_4, 0.725 g/L $CaCl_2.2H_2O$), 1 ml/L vitamins solution (1000 x stock: 10 g/L nicotinic acid, 10 g/L inositol, 10 g/L biotin, 1 g/L pantothenic acid), 0.1 ml/L minerals (10,000 x stock: 20 g/L citric acid, 5 g/L boric acid, 4 g/L $MnSO_4$, 4 g/L $ZnSO_4.7H_2O$, 2 g/L $FeCl_3.6H_2O$, 1 g/L KI, 0.4 g/L molybdic acid, 0.4 g/L $CuSO_4.5H_2O$) with supplementation of adenine, histidine, leucine and uracil as previously described (Moreno *et al*., 1991). EMM minimal glutamate (EMM½G) was used for induction of mating via conjugation and sporulation. This media contains 1g/L sodium glutamate instead of NH_4Cl. EMM lacking any nitrogen source (EMM-N) by exclusion of NH_4Cl**,** adenine, histidine, leucine and uracil was used for nitrogen starvation assays as described previously (Watanabe *et al*., 2007). Selection of cells expressing the *kanMX4* cassette was performed using 100 µg/ml G418 disulphate (Melford Laboratories Ltd.) in YE5S media. To produce solid media (YE5S and EMM) Bacto-agar (2 % w/v) was added.

Cell growth was followed using a UV-1601 Shimadzu Spectrophotometer, cell density was measured at OD_{595nm} (0.5 = $1x10^7$ cells/ml).

2.2.2 Strain construction

2.2.2.1 Mating and sporulation

S. pombe strains of the opposite mating types were grown on YE5S solid media at 30 °C overnight. Strains to be mated were then inoculated together in nH_2O and 5µl of the mixture were plated on to EMM½G media. Plates were incubated at 25 °C for 2 to 3 days until asci were observed. Asci were suspended in nH_2O and 15 µl were inoculated in a line on YE5S solid media. Asci were picked from the inoculum using a Singer MSM dissection microscope and incubated at 37 °C for 5 hours. Spores were separated using the dissector and plates were incubated at 30 °C until colonies had grown. Genotypes were determined using appropriate selection media and diagnostic PCR analyses.

2.2.2.2 LiAc transformation

S. pombe cells were transformed with DNA by the LiAc method described previously (Moreno *et al*., 1991). Cultures were grown at 30 °C to exponential phase (OD_{595nm} 0.4 - 0.5) in YE5S then cells were harvested by centrifugation at 3000 rpm (Mistral 2000, MSE) for 1 minute. Cells were washed in nH_2O and resuspended in 1 ml 1 x LiAc/TE (0.1 M LiAc pH 7.5, 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0). 1 µg of transforming DNA and 20 µg salmon sperm DNA (Stratagene) were added to the resuspended cells and incubated at room temperature for 10 minutes. 260 µl LiAc/TE/PEG (0.1 M LiAc pH 7.5, 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, PEG (50 % w/v)) were added and the solution was incubated at 30 °C for 30 minutes. 43 µl 100% DMSO were added (final concentration 10% v/v) and the solution was incubated at 42 °C for 5 minutes. Cells were pelleted at 7000 rpm (Hawk 15/05 Sanyo) for 1 minute, washed with 1 ml nH_2O then plated on to selective EMM or YE5S solid media. Plates were incubated at 30 °C for a maximum of 10 days.

pRIP42PkC based constructs (Table 2. 2) were linearised in the PCR region to target the relevant gene locus (see Table 2. 2 for details) and transformed in to *S. pombe* cells. Cells were plated on to EMM-ura solid media to select for the *ura4*$^{+}$ marker of pRIP42PkC. Integration of the construct in to the *S. pombe* genome was confirmed by PCR and sequencing (GATC Biotech).

2.2.3 Cell size analysis

Yeast cells were grown to exponential phase (OD_{595nm} 0.4-0.5) in YE5S media and fixed to microscope slides using poly-lysine followed by addition of VECTASHIELD (Fisher Scientific) containing diamino-2-phenylindole (DAPI). Cell size was measured using an Axioscope (Zeiss) with 63 x oil immersion objective and Axiovision digital imaging software (Release 4.8).

2.2.4 Cell synchronisation assays

2.2.4.1 *cdc25-22* block and release

Exponentially growing cells (OD_{595nm} 0.4 - 0.5) containing *cdc25-22* were grown at 25 °C then rapidly shifted to 37 °C for 4 hours to block the cells at the G2/M phase boundary. The cultures were then moved back to 25 °C via an ice bath to rapidly cool the culture to 25 °C. Samples were collected every 20 minutes over a time-course for protein extraction (see section 2.2.9.2). The synchronisation of the culture was analysed by calculation of the septation index.

2.2.4.2 Hydroxyurea block and release

Exponentially growing cells (OD_{595nm} 0.4 - 0.5) were synchronised by adding 11 mM hydroxyurea (HU) (final concentration) (Sigma Aldrich), previously described method (Baum *et al*., 1997). After 3 hours incubation, cells were filtered from the liquid media using a 8 µm filter (Millipore). Cells were washed with 2 x the original volume of fresh media and resuspended in fresh liquid media. Samples were collected every 20 minutes over a time-course for protein extraction (see section 2.2.9.2). The synchronisation of the culture was analysed by calculation of the septation index.

2.2.5 Induction of autophagy

Exponentially growing cells (OD_{595nm} 0.4 - 0.5) in EMM were divided in to 3 cultures then harvested by centrifugation at 2000 rpm (Mistral 2000, MSE) for 2 minutes. The cells in culture 1 were resuspended in EMM. The cells in culture 2 and 3 were washed 3 times in EMM-N and resuspended in the same volume of EMM-N as the original culture. Additionally, 1 mM PMSF (final concentration) was added to culture 3. All cultures were incubated at 30 °C from 20 hours up to 21 days. Cells incubated for 21 days were replenished with fresh EMM-N media every 72 hours for a maximum of 21 days. Cells were analysed by indirect immunofluorescence microscopy (see section 2.2.10.1) or sensitivity tests (see section 2.2.6). In addition, protein was extracted (see section 2.2.9.2) and analysed by western blotting (see section 2.2.9.3).

2.2.6 Sensitivity tests

Exponentially growing cells (OD_{595nm} 0.4 - 0.5) in YE5S were diluted to OD_{595nm} 0.1 followed by serial 1:10 dilutions in YE5S. Serial dilutions were then spotted onto YE5S agar using a

Sigma Aldrich 96 well replica plater and exposed to the indicated stress conditions. Plates were incubated at 30 °C unless otherwise stated for 2 to 6 days before analyses.

To determine sensitivity to acute oxidative stress, strains were grown to exponential phase (OD_{595nm} 0.4 - 0.5) in liquid YE5S media followed by serial 1:10 dilutions in YE5S. Approximately 100 cells were plated on to YE5S media in triplicate. Following this an acute stress of 25 mM H_2O_2 was added to the original exponential phase culture and cells were plated as described every hour for 4 hours. YE5S media plates were then incubated at 30 °C until colonies were formed. The percentage survival for each time point was calculated by the average number of colonies grown divided by the average number of colonies formed before stress.

2.2.7 *S. pombe* gDNA extraction

S. pombe genomic DNA was extracted as described previously (Moreno *et al*., 1991). Cultures were grown in YE5S to stationary phase (OD_{595nm} > 1.5). Cells were pelleted at 3000 rpm (Mistral 2000, MSE) for 2 minutes and resuspended in 200 µl chromosomal DNA breakage buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, (1 % w/v) SDS, (2 % v/v) Triton X-100, 200 µl glass beads (400 - 600 µm; Biospec product) and 200µl phenol/chloroform/isoamyl alcohol (25:24:1; BDH Laboratory supplies). To lyse the cells a Biospec mini-bead beater was used for 1 minute. An additional 500 µl of DNA breakage buffer were added and then briefly vortexed. Following centrifugation at 9000 rpm (Hawk 15/05 Sanyo) for 5 minutes the supernatant was transferred to a new Eppendorf tube with 0.1 x volume of 3 M sodium acetate and 2 x volume of 100% ethanol. The Eppendorf tube was incubated at -20 °C for a minimum of 30 minutes, the DNA was pelleted by centrifugation at 13000 rpm (Hawk 15/05 Sanyo) for 15 minutes, and the pellet was washed with 70% ethanol. After

allowing the DNA pellet to air-dry it was resuspended in nH_2O to a final concentration of 100 ng/µl.

2.2.8 *S. pombe* RNA analysis

2.2.8.1 RNA extraction

Exponentially growing cells (OD_{595nm} 0.4 - 0.5) were harvested by centrifugation and washed with dH_2O. Cells pellets were resuspended in 200 µl ice cold RNA buffer (100 mM EDTA pH 8.0, 100 mM NaCl, 50 mM Tris-HCl pH 8.0), 10 µl SDS (10 % w/v), 200 µl phenol: chloroform (25:24:1; BDH Laboratories Supplies) and 500 µl oven-baked glass beads. Cells were disrupted by using a Biospec mini-bead beater for 30 seconds then an additional 500 µl RNA buffer were added and briefly vortexed. After centrifugation at 3000 rpm (Hawk 15/05 Sanyo) for 5 minutes, the supernatant was transferred to a fresh Eppendorf tube. An equal volume of phenol: chloroform solution was added and briefly vortexed. Following centrifugation at 13000 rpm (Hawk 15/05 Sanyo) for 1 minute the supernatant was transferred to a fresh Eppendorf tube containing an equal volume of phenol: chloroform solution. After centrifugation at 13000 rpm (Hawk 15/05 Sanyo) for 1 minute, the supernatant was removed to a final Eppendorf tube containing 0.6 x volume of isopropanol and RNA was precipitated at -80 °C overnight. After centrifugation at 13000 rpm (Hawk 15/05 Sanyo) for 15 minutes, the pellet was washed with 70% ethanol. Following centrifugation at 13000 rpm (Hawk 15/05 Sanyo) for 1 minute, the pellet was air-dried then resuspended in 30 µl nH_2O. RNA concentrations were determined by measuring absorbance on a Nanodrop spectrophotometer (Thermo Scientific) and samples were stored at -80 °C.

2.2.8.2 Northern blotting

12.1 µl RNA denaturation buffer (2.5 µl 6.6 M glyoxal, 8 µl DMSO (100 %), 1.6 µl 0.1 M $NaPO_4$ pH 6.5) were added to samples containing equal amounts of RNA and incubated at 50 °C for 15 minutes. RNA was analysed by gel electrophoresis (agarose gel (1.2 % w/v), 15 mM $NaPO_4$ pH 6.5) in 15 mM $NaPO_4$ pH 6.5 running buffer. The gel was run at 80 V for 3 hours with buffer recirculation every 30 minutes. RNA was transferred to a Genescreen hybridisation membrane in 25 mM $NaPO_4$ pH 6.5 overnight. To crosslink the RNA to the membrane a UV Stratalinker 2400 (Stratagene) was used. To generate a gene-specific probe from *S. pombe* genomic DNA, PCR-amplified gene segments (33 ng/µl) was denatured at 100°C for 5 minutes, then put onto ice immediately. To the reaction was then added 10 µl 5 x labelling buffer (supplied with Prime-a-Gene labelling kit), 500 µM each Prime-a-Gene dNTP (dATP, dGTP and dTTP), 2 µl BSA, 1 µl DNA polymerase I Large (Klenow) fragment. The DNA was then labelled with 2 µl µCi (α-P^{32})-dCTP (Amersham) using the Prime-A-Gene (Promega) labelling kit following manufacturer's instructions. Probes were labelled at 37°C for 1 hour. After incubating the membrane at 68 °C for 20 minutes with 5 ml QuickHyb (Agilent) following manufacturer's instructions, the gene-specific probe and 200 µl salmon sperm DNA (10 mg/ml) were added and the membrane was incubated at 68 °C for 90 minutes. The membrane was then washed with 2x SSPE (42.3 mM NaCl, 20 mM $NaH_2PO_4.H_2O$, 2.5 mM EDTA, pH 7.4) at 60 °C for 20 minutes and exposed to a phosphoimager screen (Fujifilm) (Maniatis *et al*., 1989). Data was analysed using a Typhoon FLA 3500 and quantified using ImageQuant TL software.

2.2.9 *S. pombe* protein analysis

2.2.9.1 Soluble protein extraction

Exponential phase growing cells (OD_{595nm} 0.4 - 0.5) were harvested by centrifugation at 3000 rpm (Mistral 2000, MSE) for 1 minute, washed with 1 ml nH_2O and transferred to an Eppendorf tube. Cells were pelleted at 7000 rpm (Hawk 15/05 Sanyo) for 1 minute, resuspended in 200 µl ice cold *S. pombe* lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM imidazole, (0.5 % v/v) Nonidet P-40, (5 % v/v) NaF, (1 % v/v) PMSF, (1 % v/v) aprotinin, (0.2 % v/v) Na_3VO_4, (0.1 % v/v) leupeptin, (0.1 % v/v) pepstatin A) and transferred to a ribolyser tube containing 1 ml ice cold glass beads. Cells were lysed by using a Biospec mini-bead beater for 30 seconds, 1 minute on ice followed by another 30 seconds. An additional 500 µl lysis buffer were added to each sample. The base of the ribolyser tube was pierced with a 19-gauge needle and then placed in to an Eppendorf tube. After centrifugation at 2000 rpm (Harrier 18/8 Sanyo) for 1 minute the Eppendorf tube containing the protein lysate was placed in a centrifuge at 13000 rpm (Hawk 15/05 Sanyo) for 10 minutes at 4 °C. The protein supernatant was transferred to a fresh Eppendorf tube and protein concentrations were determined using Coomassie Protein Assay Reagent (Pierce) following the manufacturer's instructions.

2.2.9.2 Total protein extraction

To investigate insoluble and soluble proteins or the redox status of cysteine residues, proteins were extracted by the TCA lysis method as described previously (Delaunay *et al*., 2000). A volume of cells was collected and mixed with an equal volume of TCA (20 % w/v). After centrifugation at 2000 rpm (Mistral 2000, MSE) for 2 minutes, the pellet was resuspended in 200 µl ice cold TCA (10 % w/v) and transferred to a ribolyser tube containing 1 ml ice cold glass beads. Cells were lysed using a Biospec mini-bead beater for 1 minute followed by 1

minute on ice repeated 3 times. An additional 500 µl TCA (10 % w/v) were added to each sample. The base of the ribolyser tube was pierced with a 19-gauge needle and placed in to an Eppendorf tube. After centrifugation at 2000 rpm (Harrier 18/8 Sanyo) for 1 minute the Eppendorf tube containing the protein lysate was placed in a centrifuge at 13000 rpm (Hawk 15/05 Sanyo) for 10 minutes at 4 °C. The pellet was washed 3 times with 200 µl ice cold acetone (100%) and allowed to air-dry. The pellet was resuspended in 30 µl TCA buffer (100 mM Tris-HCl pH 8.0, 20 mM NEM, (1 % w/v) SDS). To investigate the oxidation of Pap1 the protein pellet was resuspended in 30 µl TCA buffer containing 75 mM IAA and lacking 20 mM NEM. To investigate the oxidation of Tpx1, Trx1 and Txl1 the protein pellet was resuspended in 30 µl TCA buffer containing AMS and lacking 20 mM NEM. The proteins were incubated at 25 °C for 30 minutes followed by 37 °C for 5 minutes. After centrifugation at 13000 rpm (Hawk 15/05 Sanyo) for 3 minutes, the supernatant was moved to a fresh Eppendorf tube. When investigating oxidation of Pap1 the samples was treated with Lambda protein phosphatase (New England Biolabs) following the manufacturer's instructions. Protein concentrations were determined using the Pierce BCA Protein Assay kit (Thermo Scientific) following the manufacturer's instructions.

2.2.9.3 Western blotting

Protein samples were separated on 8-10% SDS polyacrylamide gels and blotted to nitrocellulose membrane as described previously (Towbin *et al*., 1979). The membrane was then blocked for non-specific binding by incubation in (10 % w/v) BSA in 1x TBST (10 x TBS (150 mM NaCl, 10 mM Tris-HCl pH 8.0, (0.1 % v/v) Tween) for 30 minutes then left overnight at 4 °C in primary antibody solution (diluted in 1 x TBST, (0.1% w/v) sodium azide, see Table 2. 4.). The membrane was washed 6 times for 5 minutes in 1x TBST then the secondary antibody solution (diluted in 1 x TBST, see Table 2. 4.) was incubated with the membrane for

1 hour at room temperature. After washing as before the blot was then visualised with ECL Chemiluminescent kit (GE Healthcare) on X-ray film or by Typhoon fluorescence FLA 9500.

2.2.10 Localisation of *S. pombe* proteins

2.2.10.1 Indirect immunofluorescence

To visualise *in vivo* protein, cells were prepared using the method described by Hagan and Ayscough (2000). Cells were fixed using (3 % w/v) paraformaldehyde for 20 minutes at 30ºC then washed 5 times in 1x PEM (10 mM PIPES pH 6.9, 1 mM EDTA pH 8.0, 1 mM $MgSO_4$). Cells were resuspended in PEMS (1.2 M sorbitol, 1x PEM) followed by the addition of zymolyase (0.5 mg/ml; ICN Biomedicals) and incubated at 37ºC for 70 minutes. Cells were pelleted at 7000 rpm (Hawk 15/05 Sanyo) for 1 minute and resuspended in PEMS with (1 % v/v) Triton X-100 added. Cells were then pelleted as before, washed 3 times in 1x PEM, resuspended in PEMBAL (0.1 M L-lysine monochloride, (1 % w/v) BSA, (0.1 % w/v) sodium azide, 1 x PEM) and incubated at room temperature for 30 minutes. Cells were incubated with 200 µl primary antibody solution (diluted in PEMBAL, see Table 2. 5.) overnight at room temperature. Cells were washed 3 times in PEMBAL then incubated with 200 µl FITC-conjugated secondary antibody (diluted in PEMBAL, see Table 2. 5.) for 1 hour. Cells were washed 3 times with PEMBAL then fixed to microscope slides as previously described (Hagan and Ayscough, 2000). After VECTASHIELD (Vector Laboratories) containing DAPI was added, cells were observed using an Axioscope (Zeiss) fluorescence microscope with a 63 x oil immersion objective and Axiovision digital imaging software (Release 4.8) (DAPI was detected using Zeiss filter set 2 peak excitation 365 nm; FITC using Zeiss filter set 42 peak excitation at 493 nm; GFP using Zeiss filter set 52 peak excitation 488 nm; RFP using Zeiss filter set 52 peak excitation 488 nm).

2.2.10.2 GFP detection

Visualisation of GFP-epitope tagged proteins in cells followed the same protocol as above excluding antibody additions.

Table 2. 1 Oligonucleotide primers used in this study.

Oligonucleotide name	Oligonucleotide sequence 5' to 3'	Annealing temperature (°C)
GFP.R	CCGGCGGCGGTCACGAACTCCAGC	64
Gst2.RNA.F	TTATACTATTTGGGAATCAGACGCCA	67
Gst2.RNA.R	ATCTTTCGATCCGTATCATACTT	59
Hsf1. RNA.F	TTCAGA CTGCATCGAC AATTTC	64
Hsf1.Chk.F	GACTATCCCAATTCACTTTATCCGGTT	67
Hsf1.RNA.R	ATTCATCTGCTGGATGCGAATAT	65
Hsp104.RNA.F	CTATTCACA TTGCTGCTGC TCTTTT	66
Hsp104.RNA.R	CTCTTCAAATTCACCACGAAATTTG	67
KanMX.R	ATCACGCTAACATTTGATTAAAATAG	60
Leu1.RNA.F	ATGTGTGCAA AGAAAATCGTCGTC	66
Leu1.RNA.R	CCAAACGAGCAATACGAGAAACTT	67
Mas5KoChkF	TCTGAGAAAGTTTTTAAGG	52
Mas5KOChkR	CAAGTATGTTAGATATGCCG	55
Mas5TagChkF	CCGCGATGAAGACCGTTGCAAAGA	66
Mas5TagF	CCATCTGCAGCTGGAATCATTCCCGGTGATG	56
Mas5TagR	CCATGGATCCCTGTTGAGCACATTGAACACC	59
Matingprimer1	AGAAGAGAGAGTAGTTGAAG	55
Matingprimer2	ACGGTAGTCATCGGTCTTCC	55
Matingprimer3	TACGTTCAGTAGACGTAGTG	55

NmtendR	GCAGCTTGAATGGGCTTCC	67
Pap1.RNA.F	ATGTCCGGACAAACTGAGAC	62
Pap1.RNA.R	GCCGTCTTTCATATGAGCAAAATTA	62
Ssa1KoChkF	TTCTTCAAACAGCAATTGTCG	54
Ssa1KoChkR	TAAACAGTACTGATAACATG	54
Ssa1TagChkF	TCCACAAATCGAGGTCACC	62
Ssa1tagF	GATCGTATGGTATCTGCAGCCGAGAAGTACAAGGCTGAAG	63
Ssa1tagR	AAAAAAGGATCCATCCACTTCTTCAACCTCAGGTCC	67
Ssa2KoChkF	CTATTACTTGCATTAATATCAATTAAGGAG	59
Ssa2KoChkR	AAACACTTACATATATTTACAGTCATACTA	60
Ssa2TagF	GGTTCTGCAGGAGCTTTCCGGTATCCCCCC	61
Ssa2TagR	GGTTGGATCCATCAACCTCCTCAACCTCAG	64
Tea1F	AACT TAGTACAGCCACGTCTGAAC	60
Tea1KoChkF	TGAAACCGATATTGTTTATGTTGGATGC	68
Tea1KoChkR	CATTGGTTTGGAACGTGTAGTTATGTTTTA	68
Tpx1.RNA.F	TTACCTTTGTTTGTCCCACTGAGAT	68
Tpx1.RNA.R	ACCCTTGGGGTCGATCAAGAAAAG	68
Trx1.RNA.F	ATGGTGAAACAAGTCTCCGACTCT	62
Trx1.RNA.R	TTAGAGGTTCGCCTTAATAGAA	62
Txl1.RNA.F	GTCAAATTGCCTCCGGGCTTGGCGTCA	68
Txl1.RNA.R	GTTGGGCTGATTGATGTACAATTT	68

Table 2. 2 Plasmids used in this study.

Plasmid Name	**Target gene**	**Unique restriction site**	**Source**
pRIP42PkC	none	~	Laboratory stock *
pFEM1	*ssa1*$^+$	NruI URS in *ssa1*$^+$ sequence	This study
pFEM2	*mas5*$^+$	SalI URS in *mas5*$^+$ sequence	This study
pFEM3	*ssa2*$^+$	XhoI URS in *ssa2*$^+$ sequence	This study

* Originally published by Maundrell (1993).

Table 2. 3 *S. pombe* strains used in this study.

? indicates the mating type is not known. If the adenine allele has not been confirmed it is indicated as *ade6-M21x*, it may be *ade6-M216* or *ade6-M210*.

: indicates an insertion

:: indicates replacement/disruption

Strain name	Genotype	Source
AD81	*ade6-M216, his7-366, leu1-32, ura4-D18, trr1::ura4+*, h-	Brown *et al.* (2013)
AD93	*ade6-M21x, his7-366, leu1-32, ura4-D18, trx1::kanmx4, trx1:Flag:ura4*, h+	Day *et al.* (2012)
AD140	*ade6-M210x, his7-366, leu1-32, ura4-D18, txl1::kanmx4, trx1::kanMX4*, h+	Brown *et al.* (2013)
Bioneer *ssa1*Δ	*ade6-M216, leu1-32, ura4-D18, ssa1::kanMX4*, h+	Kim *et al*. (2010)
Bioneer *ssa2*Δ	*ade6-M216, leu1-32, ura4-D18, ssa2::kanMX4*, h+	Kim *et al*. (2010)
Bioneer *mas5*Δ	*ade6-M216, leu1-32, ura4-D18, mas5::kanMX4*, h+	Kim *et al*. (2010)
CHP428	*ade6-M216, his7-366, leu1-32, ura4-D18*, h+	Laboratory stock
CHP429	*ade6-M210, his7-366, leu1-32, ura4-D18*, h-	Laboratory stock
DY11893	*atg1:YFH:leu1, zhf1:mCherry:kanMX4*, h-	Sun *et al.* (2013)
EV75	*ade6-M216, leu1-32, ura4-D18, txl1::kanMX4*, h+	Bioneer
FM4	*ade6-M216, his7-366, leu1-32, ssa1:3PkC:ura4*, h-	This study
FM5	*ade6-M21x, his7-366, leu1-32, mas5:3PkC:ura4*, h-	This study
FM14	*ade6-M216, his7-366, leu1-32, ura4-D18, ssa1::kanMX4*, h+	This study
FM15	*ade6-M216, his7-366, leu1-32, ura4-D18, mas5::kanMX4*, h+	This study
FM16	*ade6-M21x, his7-366, leu1-32, ura4-D18, ssa2:3PkC:ura4*, h-	This study
FM18	*ade6-M21x, leu1-32, ura4-D18, cdc25-22, ssa1:3PkC:ura4*, h?	This study
FM19	*ade6-M21x, leu1-32, ura4-D18, cdc25-22, mas5:3PkC:ura4*, h?	This study
FM20	*ade6-M21x, leu1-32, ura4-D18, cdc25-22, ssa2:3PkC:ura4*, h?	This study

FM21	*ade6-M216, his7-366, leu1-32, ura4-D18, ssa2::kanMX4*, h?	This study
FM24	*atg8:GFP, ssa1::kanMX4*, h-	This study
FM29	*atg8:GFP, mas5::kanMX4*, h-	This study
FM30	*ade6-M21x, his7-366, leu1-32, txl1:Flag::ura4, mas5::kanMX4*, h?	This study
FM32	*ade6-M21x, his7-366, leu1-32, trx1:Flag:ura4, ssa1::kanMX4*, h?	This study
FM34	*ade6-M21x, his7-366, leu1-32, trx1:Flag:ura4, mas5::kanMX4*, h?	This study
FM35	*ade6-M21x, his7-366, leu1-32, txl1:Flag:ura4, ssa1::kanMX4*, h?	This study
FM36	*ade6-21x, his7-366, ura4-D18, leu1-32, hsf1:GFP:ura4, ssa1::kanMX4*, h?	This study
FM37	*ade6-21x, his7-366, ura4-D18, leu1-32, hsf1:GFP:ura4, mas5::KanMX4*, h?	This study
FM40	*ade6-21x, his7-366, leu1-32, ura4-D18, hsf1:GFP:ura4*, h?	This study
FM42	*ade6-M21x, leu1-32, Tea1:GFP:natR, ssa1::kanMX4*, h?	This study
FM43	*ade6-M21x, leu1-32, Tea1:GFP:natR, mas5::kanMX4*, h?	This study
FM44	*ade6-21x, leu1-32, hsf1:GFP:ura4, pap1::ura4*, h?	This study
FM45	*ade6-21x, leu1-32*,ura4-D18, *hsf1:GFP:ura4, tpx1::ura4*, h?	This study
FM46	*ade6-21x, leu1-32*, ura4-D18, *hsf1:GFP:ura4, trr1::ura4*, h?	This study
FM47	*ade6-21x, leu1-32, ura4-D18, hsf1:GFP:ura4, trx11:: kanMX4, txl1::kanMX4*, h?	This study
FM48	*ade2-M21x, leu1-32, mad2::ura4, mas5::kanMX4*, h?	This study
FM49	*atg1:YFH:leu1, zhf1:mCherry:kanMX4, ssa1::kanMX4*, h-	This study
FM50	*atg1:YFH:leu1, zhf1:mCherry:kanMX4, mas5::kanMX4*, h-	This study
FM52	*ssa1::kanMX4*, h-	This study
FM53	*mas5::kanMX4, h-*	This study
FM54	*ade6-21x, his7-366, leu1-32, ura4-D18, srx1::kanMX4, mas5::kanMX4, h?*	This study

JB30	*ade6-M216*, *his7-366*, *leu1-32*, *ura4-D18*, trx1::kanmx4, h-	Day *et al.* (2012)
JB35	*ade6-M216*, *his7-366*, *leu1-32*, *ura4-D18*, *trx1:Flag:ura4*, *trx1::kanMX4*, h-	Day *et al.* (2012)
JB95	*ade6-21x*, *leu1-32*, *ura4-D18*, *txl1::kanMX4*, *txl1:Flag:ura4*, h+	Brown *et al.* (2013)
JT268	*atg8:GFP*, h-	Kohda *et al. (*2007)
MF13	*atg12::kanMX4*, h-	Flanagan *et al.* (2013)
SB6	*h- ade6-M216 his7-366 leu1-32 ura4-D18 srx1::ura4+*	Bozonet (2006)
SO5672	*ade6-21x*, *leu1-32*, ura4-D18, *hsf1:GFP:ura4*, h-	Vjestica *et al.* (2013)
SO6965	*ade6-21x*, *leu1-32*,ura4-D18, *ssa2::ura4*, h+	Vjestica *et al.* (2013)
SW48	*ade6-M210*, *leu1-32*, *ura4-D18*, *cdc25-22*, h+	Laboratory stock
SW102	ade2-M210, leu1-32, mad2::ura4, h-	He *et al.* (1997)
SW972	h-	Laboratory stock
TP108-3C	*ade6-M21x, leu1-32, ura4-D18, pap1::ura4 h-*	Toda *et al.* (1991)
TK520-1	*ura4-D18*, *leu1-32*, *tea1:GFP:natR*, h?	koyano *et al.* (2015)
VXOO	*ade6-M210*, *leu1-32*, *ura4-D18*, *tpx1::ura4*, h+	Veal *et al.* (2004)

T**able 2. 4** Antibodies used for western blotting in this study.

Antibody	**Source**	**Dilution**	**Secondary antibody**	**Source**	**Dilution**
GFP (rabbit)	Invitrogen	1:1000	Horseradish-conjugated anti-rabbit	Sigma Aldrich	1:2000
M2 Flag (Monoclonal) (rabbit)	Sigma Aldrich	1:1000	Horseradish-conjugated anti-rabbit	Sigma Aldrich	1:2000
Pap1 (Polyclonal) (rabbit)	Eurogentec	1:1000	Horseradish-conjugated anti-rabbit	Sigma Aldrich	1:2000

Pk (mouse)	Sigma Aldrich	1:1000	Anti-mouse IgG	Sigma Aldrich	1:2000
Pp38 (rabbit)	Cell signalling technology	1:1000	Horseradish-conjugated anti-rabbit	Sigma Aldrich	1:2000
Sty1 (rabbit)	Eurogentec	1:1000	Horseradish-conjugated anti-rabbit	Sigma Aldrich	1:2000
Tpx1 (Polyclonal) (rabbit)	Eurogentec	1:1000	Horseradish-conjugated anti-rabbit	Sigma Aldrich	1:2000
Tubulin TAT1 (mouse)	CRUK	1:200	Anti-mouse IgG	Sigma Aldrich	1:2000

All primary antibodies prepared in 1 x TBST with (0.1 % w/v) sodium azide.

All secondary antibodies prepared in 1 x TBST with (10 % w/v) BSA.

Table 2. 5 Antibodies used for indirect immunofluorescence in this study.

Antibody	**Source**	**Dilution**	**Secondary antibody**	**Source**	**Dilution**
Polyclonal Pap1 (rabbit)	Eurogentec	1:200	FITC-conjugated chicken anti-rabbit	Sigma Aldrich	1:1000
Pk (mouse)	Sigma Aldrich	1:200	FITC-conjugated goat anti-mouse IgG	Sigma Aldrich	1:1000
Tubulin TAT1 (mouse)	CRUK	1:200	FITC-conjugated goat anti-mouse IgG	Sigma Aldrich	1:1000

All antibodies prepared in PEMBAL.

CHAPTER 3

The roles of Ssa1, Ssa2 and Mas5 in the growth and division cycle of *S. pombe*

3.1 Introduction

Eukaryotic cells contain several HSP70s and associated HSP40 J domain proteins. These HSP70/HSP40 complexes influence protein folding and protein stability. Specificity of action is dependent both on the identity of the specific HSP70-HSP40 complex and on the identity of the substrate to which they control folding processes. HSP70 proteins also have essential roles in the ubiquitin mediated degradation of certain proteins (see section 1.2.2.1). Hence, HSP70/HSP40 complexes have well studied roles in regulating the folding and stability of cellular proteins in optimal conditions and when cells are exposed to heat stress (see section 1.2.2).

Recent studies have also begun to uncover important roles for HSP70s, and their corresponding HSP40 protein regulators, influencing important cell processes such as cell cycle progression and stress-induced gene expression (see section 1.2.2.2 and 1.3.4). Ssa1, Ssa2 and Ydj1 were found to play keys roles in the regulation of the Cdc28-Cln3 complex which influences the G1 – S phase transition in *S. cerevisiae* (see Introduction section 1.2.2.1).

Excitingly, recent work revealed that direct oxidation of Ssa1 is linked to derepression of Hsf1 in response to thiol oxidising agents in *S. cerevisiae* (see Introduction section 1.3.4). Significantly the conservation of this mechanisms had not been tested in the distantly related *S.*

pombe. Indeed, it was noted by the authors that perhaps *S. cerevisiae* and *S. pombe* may be regulated differently by oxidising agents as Hsf1 in *S. cerevisiae* does not contain any cysteine residues whilst *S. pombe* Hsf1 contains one cysteine. Indeed, work in mammalian cells has shown that direct oxidation of Hsf1 regulates function (Holmberg *et al*., 2001).

Hsf1 and HSPs have been implicated in having a role in cell proliferation. *S. cerevisiae* is termed the "budding" yeast as it produces a daughter cell that appears as a bud from the mother cell. The distantly related yeast *S. pombe* is termed the "fission" yeast due to cell division occurring at a medial septum and producing 2 identical cells. Due to how divergent the yeasts are it is interesting to note how conserved proteins in cell division are between the yeasts. This highlights the importance of considering how homologous proteins may serve conserved or organism specific roles.

In contrast to budding yeast, *S. pombe* divide by fission from the septum and grow at the 'old' end, during G2 phase increases growth at the 'new' end originally created by septation, this is termed new end take off (NETO) (Martin and Chang, 2005; Moser and Russell, 2000). Several factors involved in NETO have been identified from screens of aberrant cell shape mutants. An important factor during NETO is dynamic cytoskeleton rearrangement. For example, actin filaments are polar chains of actin monomers that give mechanical support to cell structure while undergoing rapid redistribution of monomers to allow dynamic response to growth signals. The role of actin in cell structure is conserved amongst eukaryotes but can be utilised in a cell/organism specific manner. In *S. pombe*, the actin filaments are nucleated by formin For3 which regulate formation of actin structures (Feierbach *et al*., 2001; Martin and Chang, 2005). For3 is located at the septum and cell tips. Lack of For3 results in poor cell elongation, this is believed to be due to For3 having a role in the active growing ends of filaments at cell

ends, it definitively shows that actin filaments are an important factor in polarized cell growth (Feierbach *et al*., 2001; Nakano *et al*., 2002).

At cell tips and plus ends of actin filaments is the scaffolding cell end marker Tea1 along with Tea2, Tip1 and Mal3 as part of complex termed the polarisome. The polarisome is a large complex of proteins located primarily at the growing cell end that mediates NETO/cell growth. Some factors are constitutively in the polarisome such as Tea1 while others are recruited. Tea1 seems to be localised within the polarisome to the plus end of filaments which is delivered by Tip1. The delivery of Tip1 and Tea1 to the cell ends is mediated by Tea2 and the latter is regulated by Mal3 (Browning *et al*., 2003; Brunner *et al*., 2000). Tea1 is anchored to the cell tip by interaction with the plasma membrane anchor Mod5 (Snaith and Sawin, 2003). Deletion of Tea1 abolishes NETO and most cells only have monopolar growth. Tea1 and Tea4 directly interact and are essential for For3 recruitment during NETO at the new end. Overexpression of Tea4 delocalises For3 and increases actin cable levels (Martin and Chang, 2005). The exact role of Tea4 is unclear but it interacts with Tea1 and For3 so it could be a scaffold to bring these proteins together in the polarisome (Feierbach *et al*., 2004). Fusion of Tea1 with For3 largely replaces Tea4 function (Martin *et al*., 2005). For3 recruitment to Tea1 and Tea4 at the cell ends to form the polarisome may be important for the initiation of NETO.

The mechanism of regulating cell cycle control to control NETO not clear. Additionally, how NETO is stalled in response to environmental stress is not clear. Although the factors involved are being uncovered there are still significant questions unanswered. Significantly how during G2 the completion of DNA replication and critical cell size initiate a signal cascade to form the polarisome and trigger NETO. Considering the role Hsf1 and HSPs play in the role of normal cell growth it is interesting to consider if they may play a role in *S. pombe* cell division.

Like cell cycle regulation and growth, stress response pathway proteins have been shown to be conserved between the yeasts while others are organism/strain specific. Previous work demonstrated that the 2 Cys Prx Tsa1 regulates the oxidative stress-induced Yap1 transcription factor in the W303 strain background of *S. cerevisiae* (Okazaki *et al*., 2005). Interestingly Yap1 is regulated by Gpx3 and Ybp1 rather than Tsa1 in the BY strain background (Delaunay *et al*., 2002; Veal *et al*., 2003).

To better understand the organism specific and conserved aspects of this regulation of Yap1 the potential role of the single 2 Cys Prx in *S. pombe* Tpx1 in the regulation of the Yap1 homologue Pap1 in response to oxidative stress was explored (Bozonet *et al*., 2005). Excitingly this work revealed that Tpx1, but not Gpx1 (the *S. pombe* homologue of Gpx3), regulates the activation of Pap1 in response to oxidative stress. However, it was also found that Tpx1 regulates the activation of the Sty1/Atf1 pathway by a different mechanism to the regulation of Pap1 (see section 1.3.3). Significantly, work revealed that the regulation of Tpx1 by increasing concentrations of H_2O_2 determined the appropriate transcriptional response to this oxidising agent, Thus, work in *S. cerevisiae* and *S. pombe* revealed both conserved and organism specific role of 2 Cys Prx in the regulation of hydrogen peroxide-induced gene expression

Hence given the success of the approach in studies of the roles and regulation of 2-Cys Prx it was decided to investigate the potential roles of the *S. pombe* homologues of Ssa1, Ssa2 and Ydj1 named Ssa1, Ssa2 and Mas5 respectively in the responses of *S. pombe* to oxidative stress. So, the first step in this investigation was to examine the roles and regulation of Ssa1, Ssa2 and Mas5 in normal growth conditions.

3.2 Results

3.2.1 Cells lacking Ssa1, Ssa2 and Mas5 are viable but display cell cycle defects

Analyses of the *S. pombe* genome database using the sequences of Ssa1, Ssa2 and Ydj1 in *S. cerevisiae* revealed that the proteins Ssa1, Ssa2 and Mas5 respectively were the best homologues of the *S. cerevisiae* proteins (Truman *et al*., 2012, Vjestica *et al*., 2013, see Table 1.3) As a first step to investigate the functions of Ssa1, Ssa2 and Mas5 a *ssa1*Δ strain, *ssa2*Δ strain and *mas5*Δ strains were constructed by obtaining haploid strains from the Bioneer haploid deletion library where the copy of the $ssa1^{+}$ and $mas5^{+}$ gene is deleted individually (Kim *et al*., 2010). These strains were mated to wild type cells several times to reduce the possibility of introducing variability in the genome from the Bioneer genotype. An *ssa2*Δ strain was donated by the Temasek Life Sciences Laboratory, the copy of the $ssa2^{+}$ gene had been deleted, this was crossed to wild type cells like the *ssa1*Δ strain and *mas5*Δ strain were (Vjestica *et al*., 2013).

Importantly, deletion of all three genes individually were obtained and indicated that none of $ssa1^{+}$, $ssa2^{+}$ or $mas5^{+}$ genes are essential. However, the initial generation of the *ssa1*Δ, *ssa2*Δ and *mas5*Δ mutants suggested that these genes are important for cell growth. Following sporulation of the heterozygous diploids it was found that *mas5*Δ and *ssa2*Δ spores formed smaller colonies than wild type control cells and took several more days to grow (Fig. 3. 1. A). In contrast, *ssa1*Δ spores appeared to form colonies of similar size to the wild type control spores (Fig. 3. 1. A).

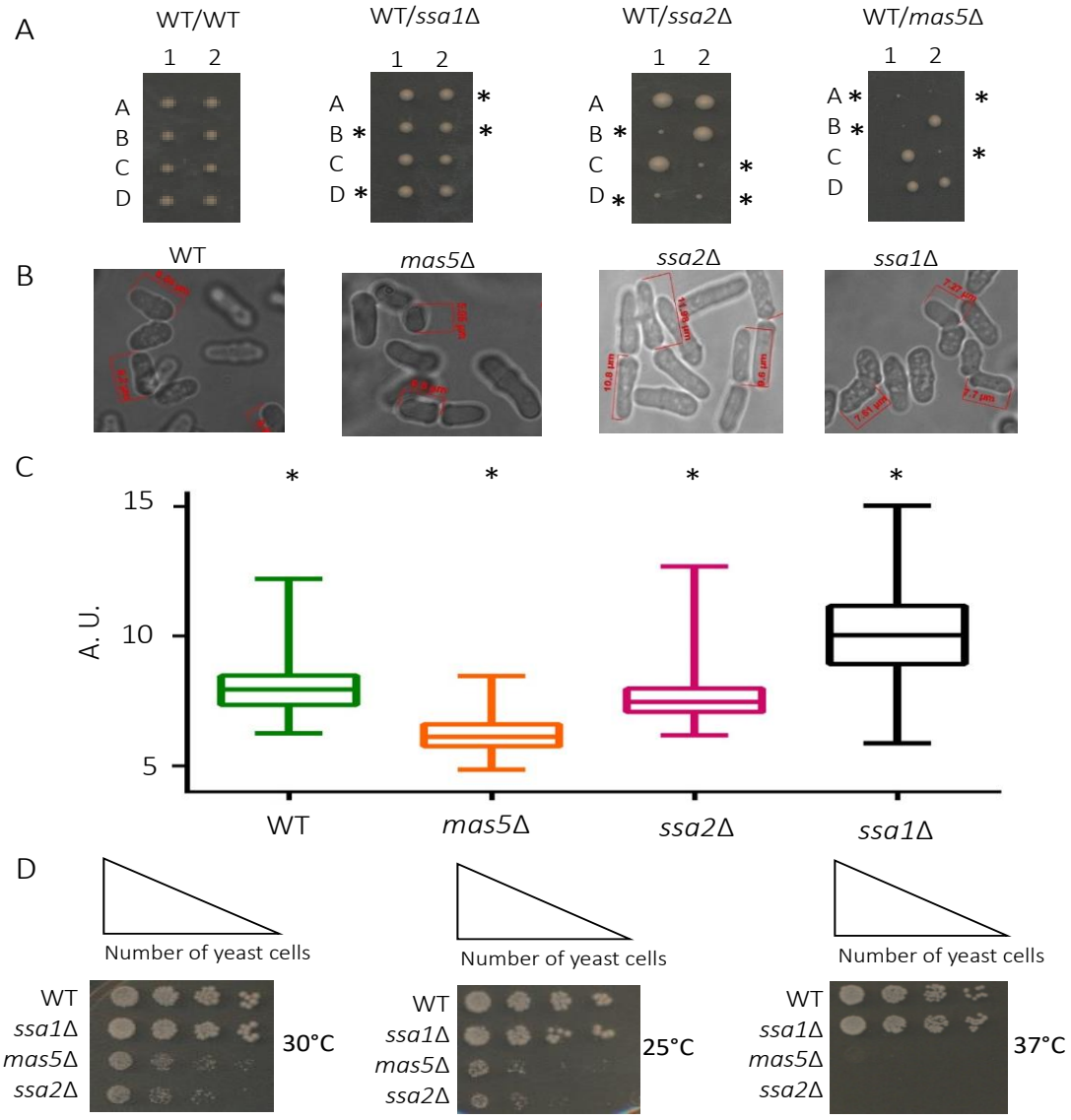


Fig. 3. 1. Cell size is significantly misregulated in the absence of Ssa1, Ssa2 or Mas5. (A) cells with opposite mating types containing wild type (WT) alleles (CHP428 and CHP429), *ssa1*Δ (Bioneer *ssa1*Δ), *mas5*Δ (Bioneer *mas5*Δ) or *ssa2*Δ allele (SO6965) generated asci. 2 representative asci were sporulated and spores separated to form single colonies (A to D) on YE5S solid medium. Colonies were imaged after 5 days at 30 °C. All colonies were replica plated on to YE5S solid medium containing G418 to select for Kanamycin resistance; growth was correlated to the deletion alleles (data not shown). * indicates colonies that could grow in the presence of G418, n = 3. (B) Cells were grown to exponential phase, cells were fixed to microscope slides and imaged using DIC. Cell size was measured using arbitrary units (A. U.) on Axiovision digital imaging software (Release 4.8), n = 3. (C) Cells were visualised by DIC microscopy, measurements of dividing cells from 3 independent replicates (n<60) were used to generate box whisker plot using Prism software (version 7.01). * indicates statistical significance to all other cells (Mann-Whitney U test, $p < 0.01$), n = 3. (D) Cells were grown to exponential phase and serial diluted to the same OD_{595}. Cells were plated to YE5S solid medium and exposed to the temperature indicated. Images shown were taken after 96 hours, n = 3.

Having established that *ssa1*Δ, *ssa2*Δ and *mas5*Δ cells are viable, the growth and morphology of the single mutant strains were next examined. Exponentially growing cells from cultures of wild type, *ssa1*Δ, *ssa2*Δ and *mas5*Δ strains were imaged by differential interference contrast (DIC) microscopy. Unlike *S. cerevisiae* which is a budding yeast, the process of cell growth in the cell cycle of *S. pombe* occurs at the tips of the cell and a medial septum is formed before cytokinesis. Initially, the new cell growth is at one cell tip (monopolar) but then transitions to bipolar growth as the cell enters G2 phase in a process termed new end take off (NETO) (Martin and Chang, 2005). NETO is a highly dynamic process requiring relocalisation, recruitment and modification of proteins (Martin and Chang, 2005, Koyano *et al*., 2010). The length of the *S. pombe* cell is thus a reflection on how fast or slow a cell is dividing.

Analysis of *ssa1*Δ cells revealed that these cells are longer than wild type cells and includes a wider range of cell size (Fig. 3**.** 1. B). In contrast, *ssa2*Δ cells were generally smaller than wild type cells but also showed a wide range in cell size at division (Fig. 3. 1. B). Thus, these data suggest that loss of Ssa1 inhibits cell cycle progression whilst loss of Ssa2 stimulates cell cycle progression. Interestingly, *mas5*Δ cells were consistently much smaller than wild type cells suggesting that Mas5 is also a cell cycle inhibitor (Fig. 3. 1. B). Using a Mann-Whitney U test it was found that the cell size of all deletion mutants were significantly different to the cell size parameters of the wild type control (Fig. 3. 1. C). Hence, taken together this work revealed that Ssa1, Ssa2 and Mas5 regulate cell cycle progression in *S. pombe*.

Given the potential role of Ssa1, Ssa2 and Mas5 as HSPs the growth of the different mutants at a range of temperatures was examined. However, during the analyses of the *ssa2*Δ strain it quickly became apparent that there were problems with consistency of the data. For example, upon initial growth of *ssa2*Δ cultures cells, shortly after obtaining the single mutant, the mutant

cells display a reduced cell size and a decreased rate of growth. However, it was observed that within a few generations these phenotypes disappeared and cultures of *ssa2*Δ cells showed recovery of cell size and growth rates like wild type cells. The underlying basis of this reversion of phenotypes is not known but because of the rapid appearance of recovery it was decided to focus further studies on the *ssa1*Δ and *mas5*Δ mutants which were found to consistently retain their aberrant phenotypes. As can be seen the growth of *mas5*Δ and *ssa2*Δ strains were considerably affected when exposed to sub-optimal temperatures of 25 °C or 37 °C (Fig. 3. 1. D). In comparison, *ssa1*Δ cell growth appears to be unaffected by the temperature range. This leads towards the conclusion that Ssa2 and Mas5 play a major role in temperature response while no clear role for Ssa1 is observed.

In *S. cerevisiae* Ydj1 levels were found to be important for cell cycle progression, with increased levels resulting in stimulation of G1-S phase progression (Ferrezeulo *et al*., 2012; see Introduction section 1.2.2.2). The results presented here revealed that Mas5 may also have a role in cell cycle progression. However, the fact that *mas5*Δ cells are much shorter than normal indicates that in contrast to Ydj1, Mas5 is an inhibitor of cell cycle progression. Intriguingly, the studies of Ssa1 and Ssa2 in *S. cerevisiae* suggested that both these proteins act to inhibit cell cycle progression (Truman *et al*., 2012; Hasin *et al*., 2014; see section 1.2.2.2). However, our analyses of *ssa1*Δ and *ssa2*Δ cells suggest that Ssa1 is an activator of cell cycle progression and that Ssa2, similar to Mas5, acts to inhibit cell cycle progression. This work suggests that like *S. cerevisiae* Ssa1, Ssa2, and Mas5 regulate cell cycle progression. However, they appear to function by a different mechanism(s) in *S. pombe*. This is perhaps not surprising given the very different cell cycles of these very distantly related yeast.

3.2.2 Generation of Ssa1, Ssa2 and Mas5 with epitope tags was successful

To investigate the potential role of Ssa1, Ssa2 and Mas5 in the regulation of the cell cycle and in oxidative stress responses in *S. pombe* it was necessary to construct strains expressing epitope-tagged versions of these proteins. Therefore, a strategy was devised to create strains expressing epitope-tagged versions of Ssa1, Ssa2 and Mas5 from their respective gene loci in *S. pombe* (Fig. 3. 2).

The aim was to create individual strains expressing each protein with 3 copies of the Pk epitope tag (hereafter referred to as the Pk epitope) at the C-terminus and expressed from the normal promoter. To achieve this, first a PCR fragment containing ~500 - 700 nucleotide bases from the 3' of each coding region up to but not including the stop codon was obtained using wild type genomic DNA as template. Next, each gene PCR fragment was ligated with an integrating plasmid encoding the 3 Pk epitope tag and the selectable marker *ura4*$^{+}$(pRIP42PkC) (Fig 3. 2. Step 2). The resulting plasmids, named pFEM1, pFEM2 and pFEM3, contain the coding region for the C-terminal region of Ssa1, Ssa2 and Mas5 respectively fused in frame with the plasmid sequence encoding the Pk epitope (see Table 2. 2).

To create the strains expressing the epitope tagged proteins each plasmid was linearised using a unique restriction site (see Table 2. 2) within the region encoding the C-terminal region of each gene and then transformed in to wild type *S. pombe* cells (CHP429) lacking the *ura4*$^{+}$ marker. The site of linearisation targets the DNA to recombine within the respective wild type gene in the *S. pombe* genome by homologous recombination. Ura^{+} colonies were selected following transformation by growth on media lacking uracil. PCR (Fig 3. 3. A) and DNA sequencing (data not shown) was used to confirm the successful creation of each strain. The

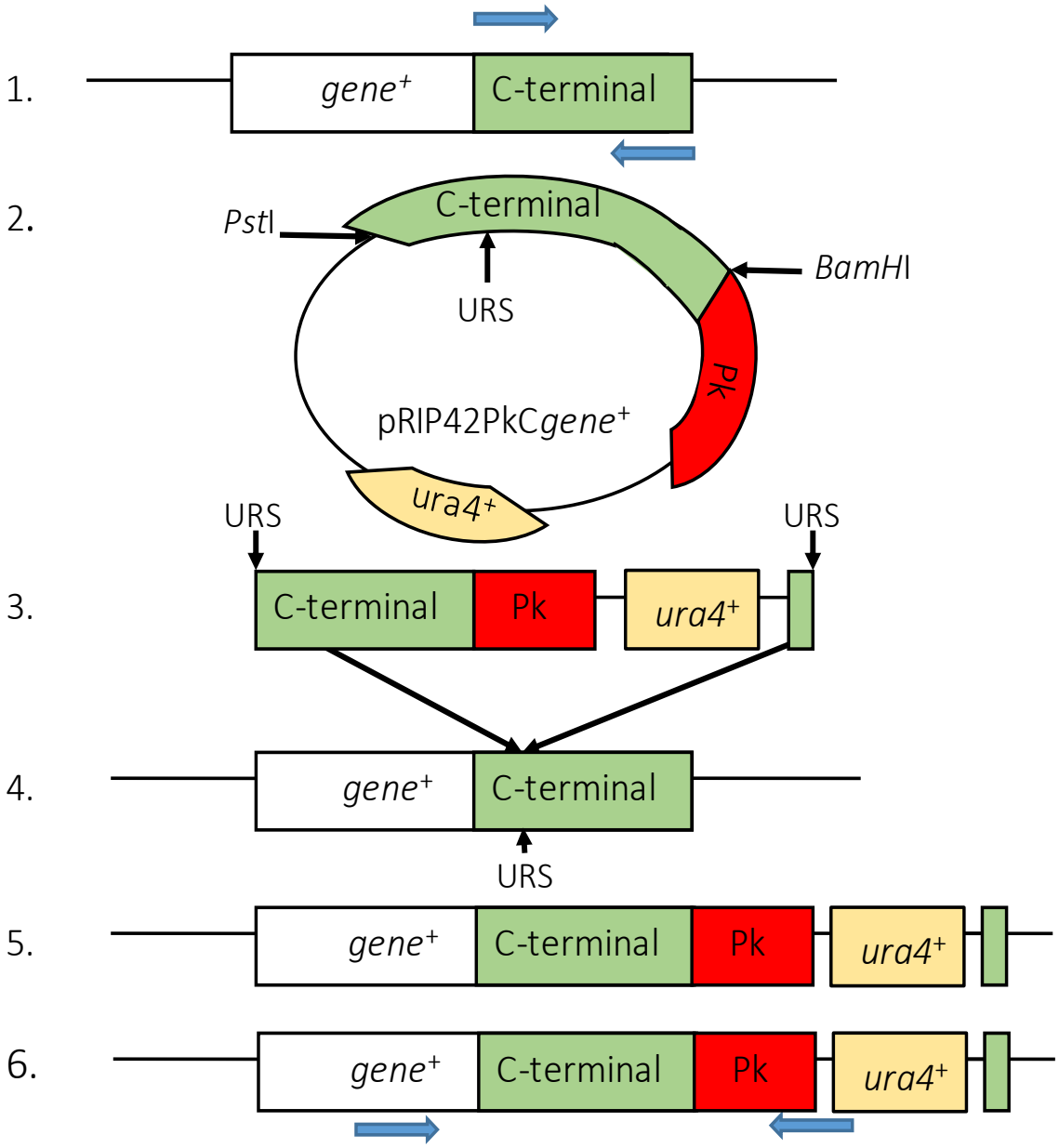
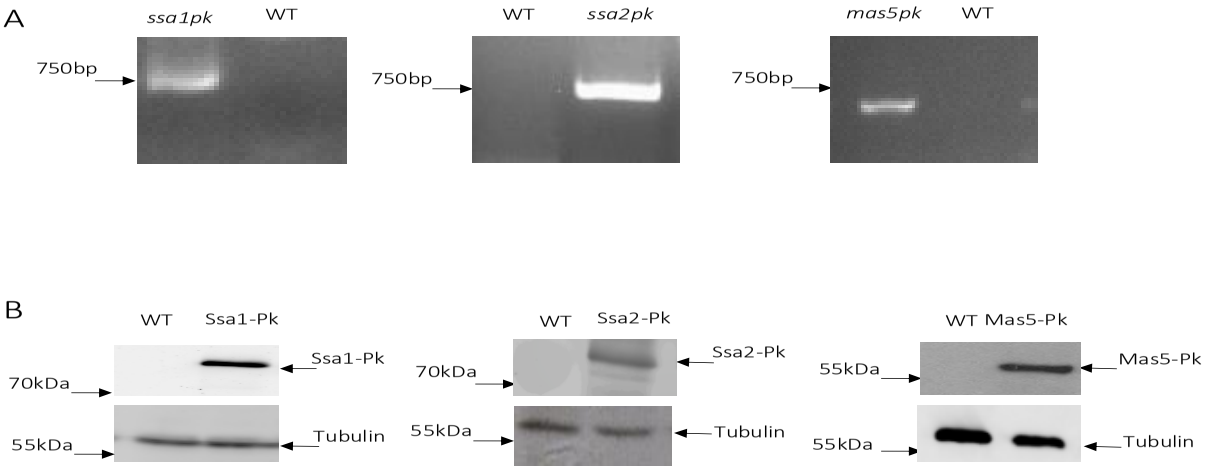


Fig. 3. 2. The strategy used to create *S. pombe* strains expressing a C-terminal Pk epitope tagged Ssa1, Ssa2 and Mas5 from the normal chromosomal locus. Primers were designed to PCR 500 - 750bp of the 3' region of the gene from wild type genomic DNA (*ssa1*$^+$ primers Ssa1TagF and Ssa1TagR; *ssa2*$^+$ primers Ssa2TagF and Ssa2TagR; *mas5*$^+$ primers Mas5TagF and Mas5TagR) introducing restriction sites for PstI and BamHI and removal of stop codon, indicated in green (1). This product is then ligated in frame with sequence encoding the 3 Pk epitope on integrating plasmid pRIP42PkC (2). Following construction of pRIP42PkC-*gene*$^+$, the plasmid was linearised at the unique restriction site (URS) located in the gene sequence pRIP42-*gene*$^+$ (3) to target the PCR cassette to recombine to normal genomic locus in wild type CHP428 cells (4). The presence of the *ura4*$^+$ marker on the plasmid pRIP42PkC allows selection for transformed colonies by growth on media lacking uracil (5). These *ura4*$^+$ colonies were screened with a check PCR (blue arrows indicate primer binding regions) ((*ssa1*$^+$ primers Ssa1TagChkF and NmtendR; *ssa2*$^+$ primers Ssa2TagChkF and NmtendR; *mas5*$^+$ primers Mas5TagChkF and NmtendR) and DNA sequencing to confirm proper integration (6).


A
ssa1pk
WT
750bp
WT
ssa2pk
750bp
mas5pk
WT
750bp
B
WT
Ssa1-Pk
70kDa
Ssa1-Pk
55kDa
Tubulin
WT
Ssa2-Pk
70kDa
Ssa2-Pk
55kDa
Tubulin
WT Mas5-Pk
55kDa
Mas5-Pk
55kDa
Tubulin

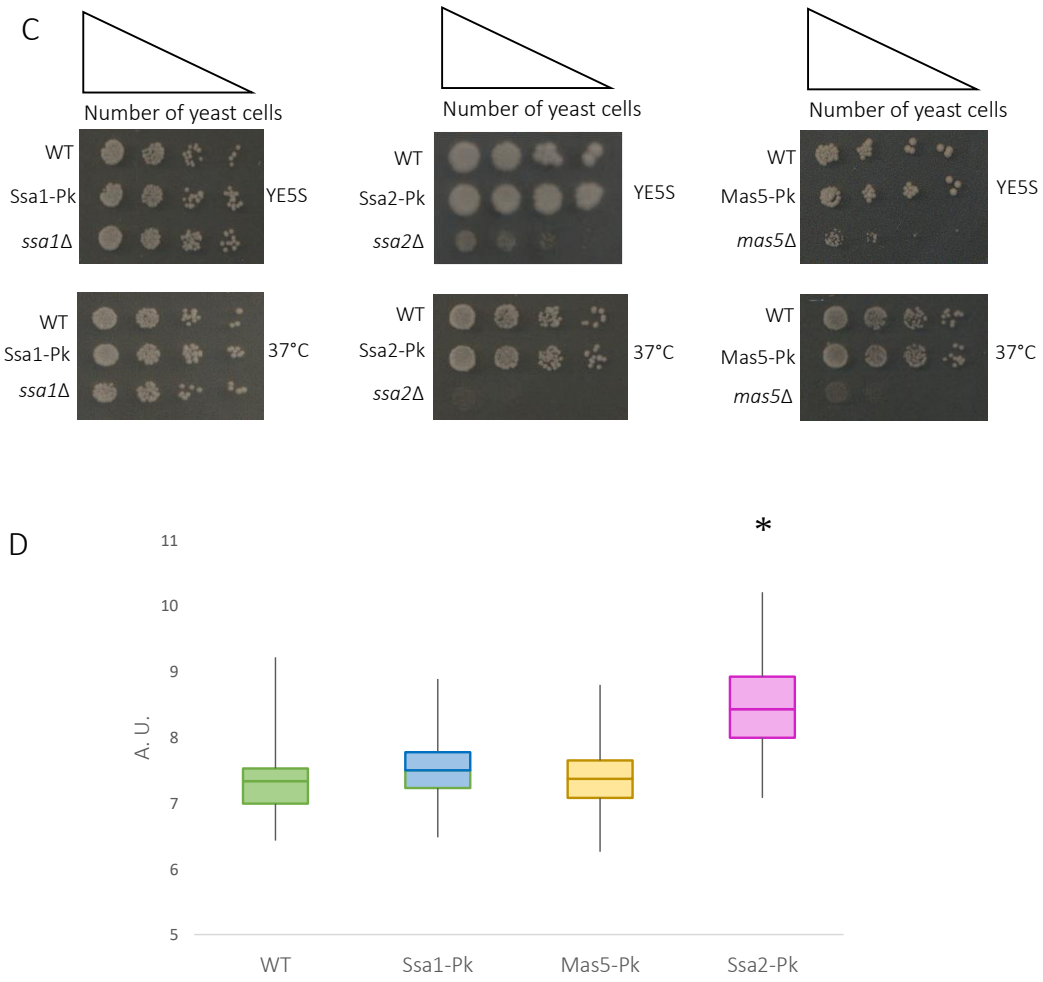


Fig. 3. 3. Successful construction of cells expressing Ssa1, Ssa2 or Mas5 tagged individually with a Pk epitope. (A) DNA from wild type (WT) cells (CHP429) and cells expressing either Ssa1-Pk (FM4), Ssa2-Pk (FM16) or Mas5-Pk (FM5) individually from the normal chromosomal locus were used as templates for a PCR to check the integration of the Pk encoding epitope downstream of the gene using a forward primer recognising the wild type gene sequence (Ssa1TagChkF, Ssa2TagChkF and Mas5TagChkF respectively) and a reverse primer recognising the end of the Pk encoding sequence (NmtendR). Predicted sizes of correct integration are 750 bp, 750 bp and 700bp respectively, n = 3. (B) Proteins were extracted from mid-log growing cultures of wild type (WT) cells and cells expressing Ssa1-Pk, Ssa2-Pk or Mas5-Pk. Samples were extracted by TCA protocol plus NEM and analysed by non-reducing SDS-PAGE and western blotting with anti-Pk antibody and anti-tubulin antibody, n = 3. (C) The same strains in (B) were grown to mid-log phase alongside strains *ssa1*Δ (FM14), *ssa2*Δ (FM21) and *mas5*Δ (FM15) and serially diluted to the same OD_{595}. Cells were plated to YE5S solid medium containing/exposed to the stress indicated. Plates were imaged every 24 hours, images shown were taken after 72 hours, n = 3. (D) Tagged strains expressing Ssa1-Pk (FM4), Mas5-Pk (FM5), Ssa2-Pk (FM16) and wildtype (WT) cells (CHP428) were visualised by DIC microscopy, measurements of dividing cells from 2 independent replicates ($n < 60$) were used to generate box whisker plot using Excel 2013, n = 2. ANOVA test performed to determine significant difference ($p < 0.05$). * indicates significant difference from WT cells.

PCR and sequencing confirmed creation of strains with the correct insertion of the Pk epitope cassette at the *ssa1*$^{+}$, *ssa2*$^{+}$ and *mas5*$^{+}$ gene loci.

To confirm that the tagged proteins were detectable, protein extracts were obtained from the Ssa1-Pk, Ssa2-Pk and Mas5-Pk strains and analysed by western blotting using anti-Pk antibodies (Fig. 3. 3. B). Importantly, a specific band of the correct predicted molecular weight was detected in each of the three protein extracts (Fig, 3. 3. B). Having established that each strain is expressing epitope-tagged Ssa1, Ssa2 and Mas5 the growth of each strain was examined at 30^{o}C and 37^{o}C (Fig. 3. 3. C). In contrast to the analysis of the *ssa1*Δ, *ssa2*Δ and *mas5*Δ cells no obvious growth defect was apparent suggesting that the epitope-tag did not have major effects on protein functions (Fig. 3. 3. C). Cell size was examined of the strains expressing epitope tagged proteins, it was found that Ssa2-Pk expressing cells were significantly larger than wild type cells as indicated by the asterisk (Fig. 3. 3. D). This suggests the epitope is affecting the role of Ssa2 in cell growth of *S. pombe*. This is an issue that could affect the analyses of results but it does lead towards the concept of Ssa2 playing a critical role in cell growth regulation in *S. pombe.*

3.2.3 Ssa1, Ssa2 and Mas5 are not detectably modified during the cell division cycle

In *S. cerevisiae,* it has been shown that phosphorylation of threonine at position 36 (T36) within a conserved sequence found in the HSP70 ATPase domain of Ssa1 correlated to binding of the G1 cyclin Cln3 to Ssa1, preventing the cyclin entering the nucleus, thus inhibiting the cell entering S phase (see section 1.2.2.2). Significantly, the human homolog of Ssa1, Hsc70, is also phosphorylated at the equivalent threonine (T38) and, moreover, this modification influences

cyclin D regulation and consequently cell cycle progression (see Introduction section 1.2.2.2). As described above Ssa1, Ssa2 and Mas5 were all found to play a role in cell cycle progression. Hence the potential phosphorylation of Ssa1, Ssa2 and Mas5 were examined in the cell cycle.

To allow synchronisation of the cultures a temperature sensitive allele of the M phase inducer tyrosine phosphatase *cdc25^{ts}* was introduced by genetic crosses. The presence of the *cdc25*^{ts} allele allows cells to be synchronised in G2 phase by blocking cell cycle progression at 37 °C, the non-permissive temperature. Subsequent growth of the cultures at 25^{o}C allows cell cycle experiments to be performed on synchronised cells. To investigate modification of the epitope tagged proteins during the cell cycle *cdc25*^{ts} cultures expressing Ssa1-Pk, Ssa2-Pk or Mas5-Pk were synchronised at 37 ^{o}C. After release from the block protein extracts were collected every 20 minutes for 160 minutes and were analysed by western blot.

No obvious change in the mobility of Ssa1-Pk, Ssa2-Pk or Mas5-Pk was detected during the cell division cycle (Fig. 3. 4.) It is possible that modification is occurring but is not detectable by this method. However, there is no evidence to suggest that any of these proteins is phosphorylated during the cell cycle of *S. pombe*. It was also observed from the septation index of the Mas5-Pk *cdc25^{ts}* strain a delay in re-entry of cells to the cell cycle occurred. The basis of this delay is unclear but may suggest a relationship between Cdc25 and Mas5 functions. In addition, it also suggests that the epitope tag may affect protein function in this mutant background.

Given the potential role of Ssa1, Ssa2 and Mas5 in response to heat it was possible that the synchronisation protocol used with *cdc25*^{ts} may influence cell cycle effects on modification of

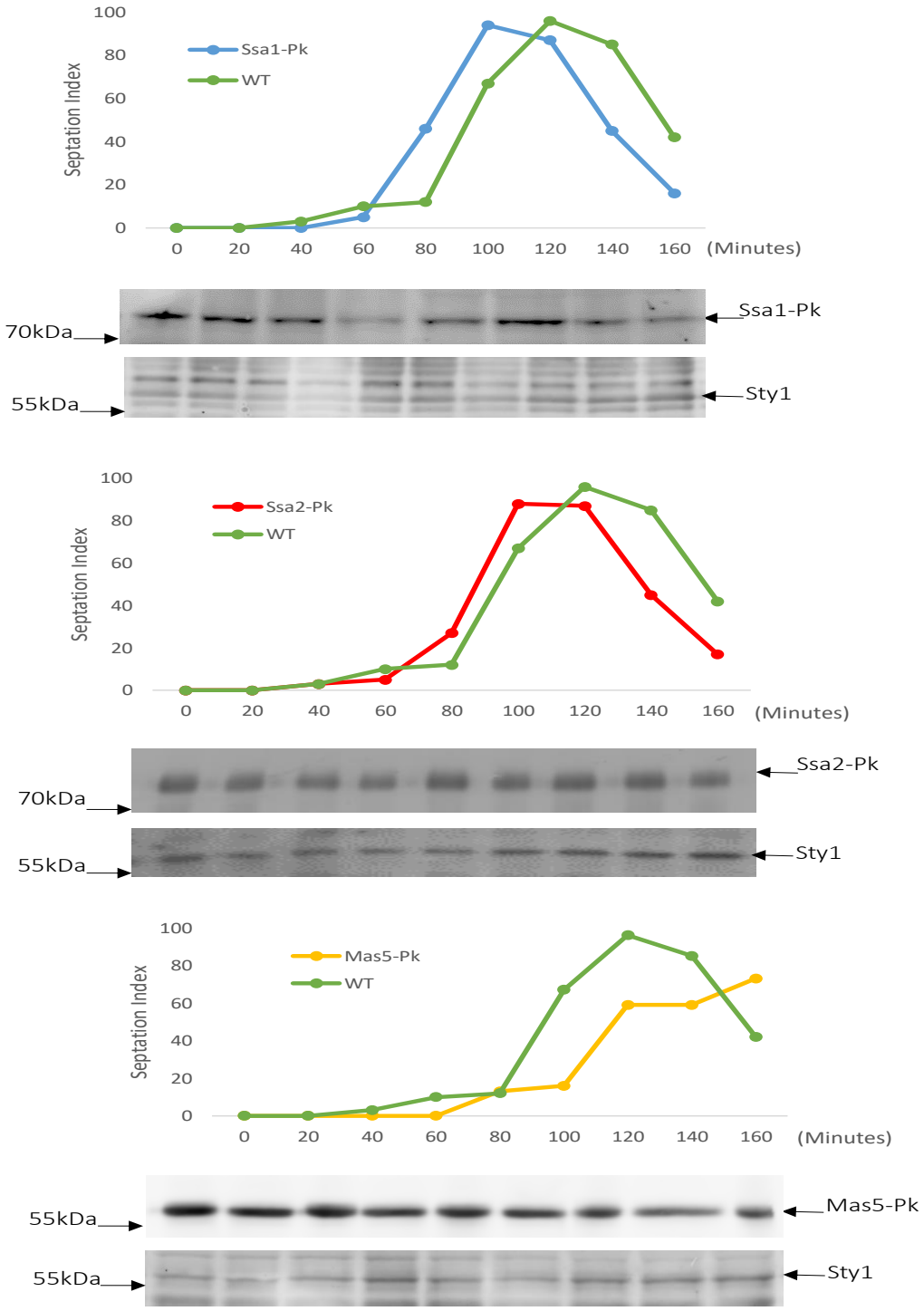


Fig. 3. 4. The mobility of Ssa1-Pk, Ssa2-Pk and Mas5-Pk is not altered during the cell cycle. *cdc25*^{ts} cells (SW48) along with *cdc25*^{ts} cells expressing Ssa1-Pk (FM18), Ssa2-Pk (FM20) and Mas5-Pk (FM19) were synchronised at the G2/M phase boundary by incubation at 37 °C. Following release at 25 °C samples were collected every 20 minutes and analysed by western blot using anti-Pk antibody. Sty1 was used as a loading control. The synchronisation of the cultures was determined by the septation index. The percentage of septated cells was scored in each sample, n = 2.

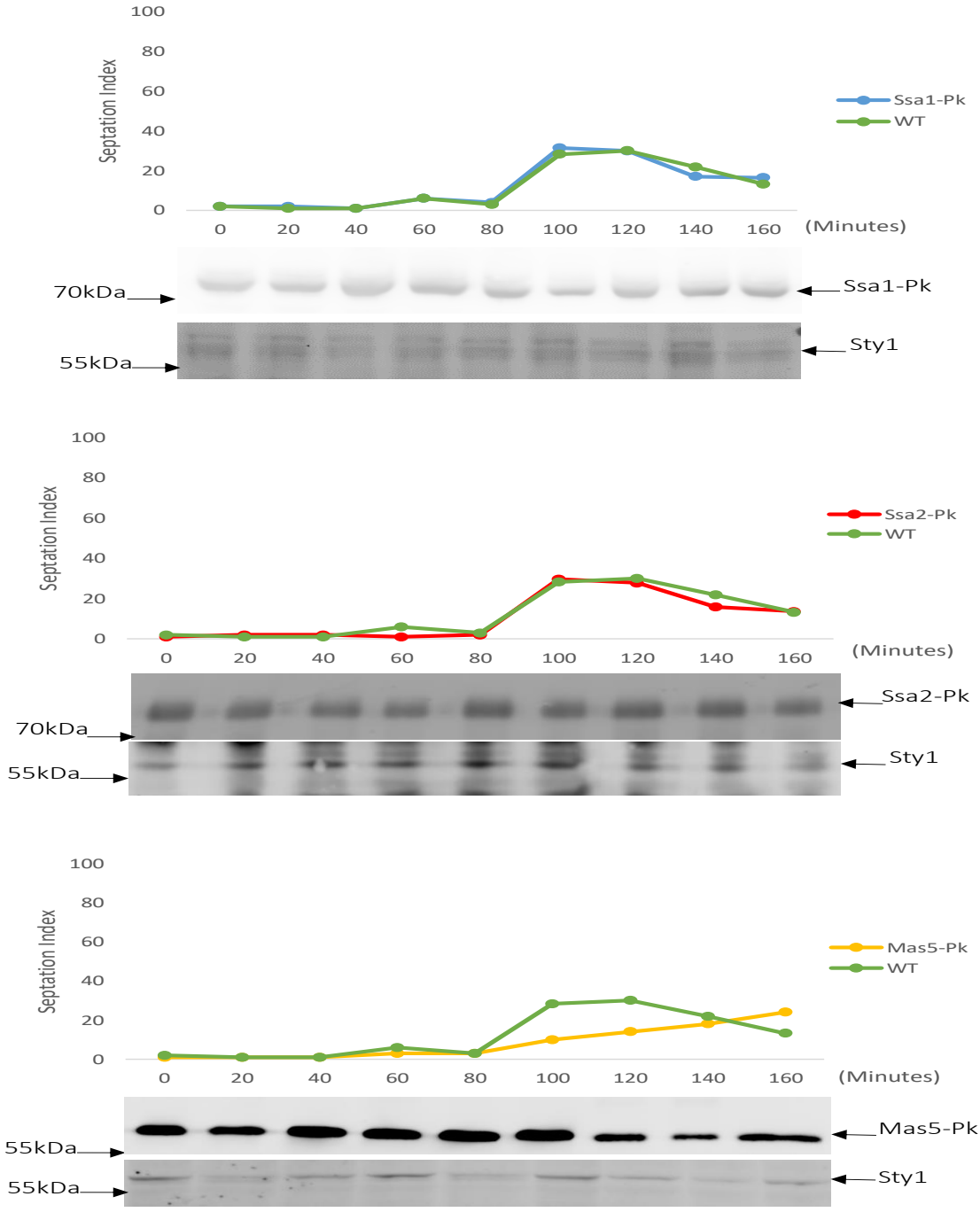


Fig. 3. 5. The mobility of Ssa1-Pk, Ssa2-Pk and Mas5-Pk is not altered during the cell cycle. *cdc25*^{ts} cells CHP429) along with cells expressing Ssa1-Pk (FM18), Ssa2-Pk (FM20) and Mas5-Pk (FM19) were synchronised at the G1/S phase boundary by incubation with HU (11 mM). Following release by removal of HU from the media samples were collected every 20 minutes and analysed by western blot using anti-Pk antibody. Sty1 was used a loading control. The synchronisation of the cultures was determined by the septation index. The percentage of septated cells was scored in each sample, n = 2.

these proteins. Hence an alternative method to synchronise cells using the ribonucleotide reductase inhibitor hydroxyurea (HU), which synchronises cells at S phase, was employed. Exponential phase cells expressing Ssa1-Pk, Ssa2-Pk or Mas5 were synchronised in S phase using HU and, confirmed by microscopy. After release, protein extracts were collected every 20 minutes for 160 minutes and analysed by western blot.

Consistent with the analyses performed using the *cdc25*^{ts} allele, no obvious change in mobility of Ssa1-Pk, Ssa2-Pk or Mas5-Pk was detected during the cell division cycle (Fig. 3. 5.) However, in contrast to the *cdc25*^{ts} studies the septation index of the strains cells peaked at lower levels than those observed using the *cdc25*^{ts} block and release method (compare Fig. 3. 4. and Fig. 3. 5.). Hence, this result suggests more synchrony was achieved in the *cdc25*^{ts} experiments. Interestingly, like
the *cdc25*^{ts} experiments, the strain expressing Mas5-Pk although synchronised appeared to delay progression through the cell cycle compared to wild type cells.

Taken together there is no evidence to support the hypothesis that Ssa1, Ssa2 or Mas5 are modified in a cell cycle-dependent manner. It is interesting to note that T36, the phosphorylated threonine residue in Ssa1 of *S. cerevisiae* is conserved in Ssa1 (and Ssa2) of *S. pombe* (threonine 36 in Ssa1 and threonine 36 in Ssa2). So, it is possible that phosphorylation does occur at one or both sites and/or that one or both sites is phosphorylated under different circumstances. Mutational analysis of these residue would help resolve whether these residues have any cell cycle functions. These data also suggest that it is possible, based on the analysis of synchrony in the *cdc25*^{ts} strain background and following HU treatment, that the Pk epitope tag may affect some aspects of Mas5 function in the cell cycle.

3.2.4 Localisation of Ssa1, Ssa2 and Mas5 suggests different roles for each protein in cell cycle progression

Data presented above (see section 3.2.1.) suggest that Ssa1 is a regulator of cell size whilst Ssa2 and Mas5 act to inhibit the cell cycle. However, despite this connection to cell cycle control no obvious cell cycle dependent modification of any of these proteins could be detected. However, it was possible that the identification of the cellular localisation of these proteins might shed light on their cell cycle functions. Indeed, Ydj1 act on Ssa1/Ssa2 located at the ER to regulate cell cycle progression in *S. cerevisiae* (see section 1.2.2.2). Hence the localisation of Ssa1-Pk, Ssa2-Pk and Mas5-Pk was examined in exponentially growing cells by indirect immunofluorescence.

Analysis of the cells revealed that Ssa1-Pk appeared to localise ubiquitously throughout the cell and this was not affected by cell cycle position (Fig. 3. 6.). However, in contrast both Ssa2-Pk and Mas5-Pk were found to localise to the cell ends and the septum during division (Fig. 3. 6.). Furthermore, both Ssa2-Pk and Mas5-Pk appear at the 'old' end of divided cells and, furthermore, show evidence of involvement at both cell tips upon transition to bipolar growth (Fig. 3. 6.). These results are consistent with the apparent opposing roles of Ssa2 and Mas5 with respect to Ssa1 in cell cycle progression and the localisation of Ssa2 and Mas5 to growing regions of the cell suggest that that they may function together to effect cell growth.

3.2.5 The cell cycle localisation of Tea1 is not affected by loss of either Ssa1 or Mas5

The cellular localisation studies of Ssa1-Pk, Ssa2-Pk and Mas5-Pk suggested that Ssa2 and Mas5 may have important roles at the growing cell tips. As discussed before, NETO determines

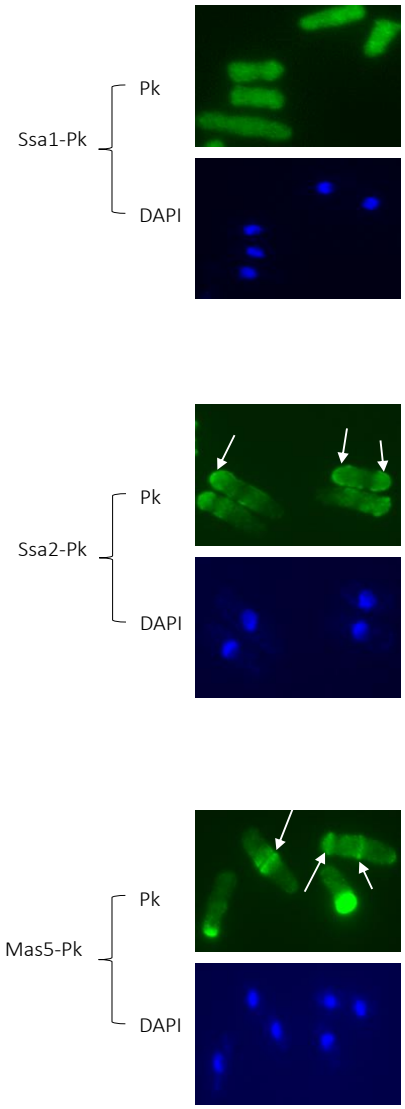
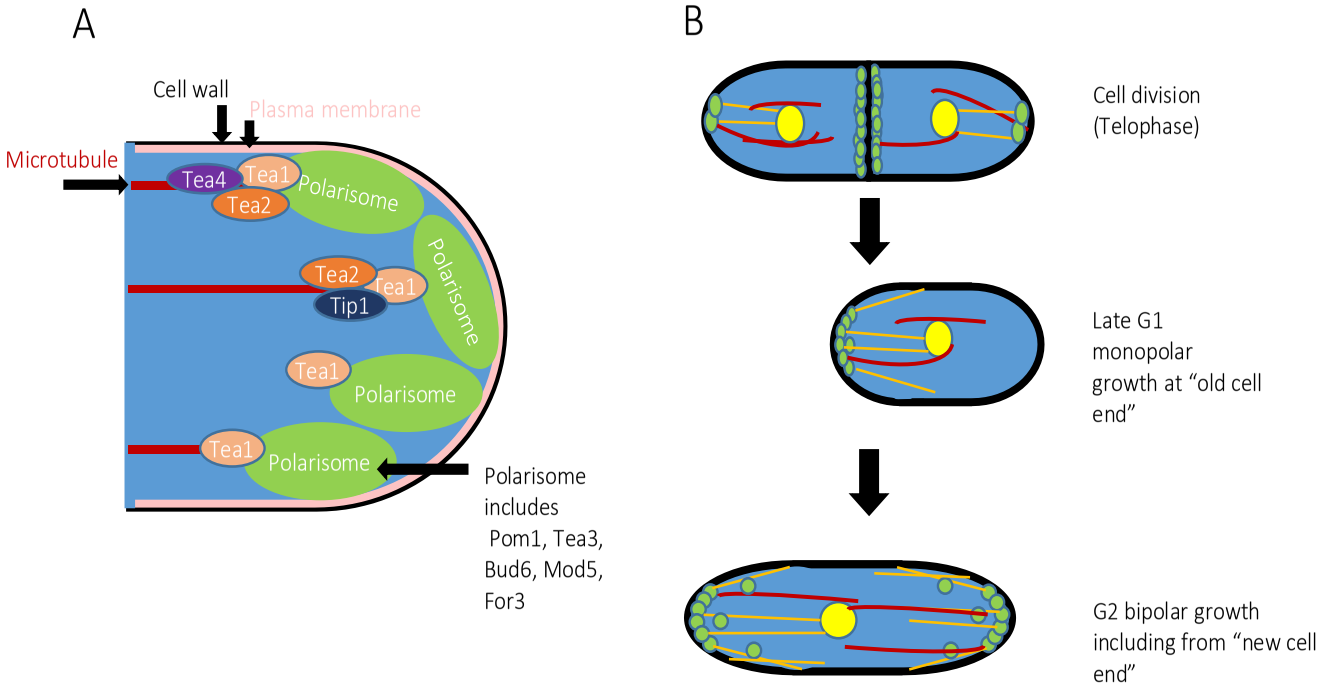


Fig. 3. 6. Localisation of Ssa1-Pk, Ssa2-Pk and Mas5-Pk. FITC (Pk) and DAPI images of representative exponential phase cells expressing Ssa1-Pk (FM4), Ssa2-Pk (FM16) and Mas5-Pk (FM5), fixed using paraformaldehyde. Exposures were kept constant between images. White arrows point to cell localisation features discussed in the text, n = 3.

the linear growth of fission yeast cells and the transition to bipolar growth (see section 3.1). Studies of NETO have revealed that it is regulated by core cell end marker proteins, Tea1 and Pom1 in the polarisome, and that the process is coordinated with microtubule dynamics (Fig. 3. 7. A). Once cells have undergone cytokinesis and divided, growth then continues at the "old cell end" until NETO initiates growth in a bipolar fashion in G2 phase (Fig. 3. 7. B.). Microtubules nucleate from the cell centre until they reach the cell end where signalling initiates depolymerisation of the microtubule back towards the cell end. Failure of cells to elongate microtubules to the cell end can lead to T shaped cells suggesting that cell end identification and microtubules have a feedback mechanism.

How cells identify the new cell end to allow bipolar cell growth has been linked to the localisation of the Tea1 protein. In the absence of Tea1 cells remain monopolar, suggesting it is a key regulator in initiating NETO and identifying the cell end. Hence, it was possible that Ssa2 and/or Mas5 are required for the proper localisation/function of Tea1 and/or microtubules. To examine these possibilities cells were generated that express Tea1 with a C-terminal GFP epitope and contain either the *ssa1*Δ or *mas5*Δ allele. Due to the phenotype reversion issues associated with *ssa2*Δ cells (discussed in section 3.2.1) the localisation of Tea1-GFP in these cells was not examined.

The localisation of Tea1-GFP was examined in exponentially growing wild type, *ssa1*Δ and *mas5*Δ cells by indirect immunofluorescence (Figure 3. 7. C.). As expected, in wild type cells Tea1-GFP localises to the growing cell ends and is absent from "new cell ends" during monopolar growth (Fig. 3. 7. C.). Significantly, Tea1-GFP localisation to the growing cell end appears to be similar to wild type cells in either *ssa1*Δ or *mas5*Δ cells (Fig. 3. 7. C.).


A
Cell wall
Plasma membrane
Microtubule
Tea4
Tea1
Tea2
Polarisome
Polarisome
Tea2
Tea1
Tip1
Tea1
Polarisome
Tea1
Polarisome
Polarisome includes Pom1, Tea3, Bud6, Mod5, For3
B
Cell division (Telophase)
Late G1 monopolar growth at "old cell end"
G2 bipolar growth including from "new cell end"

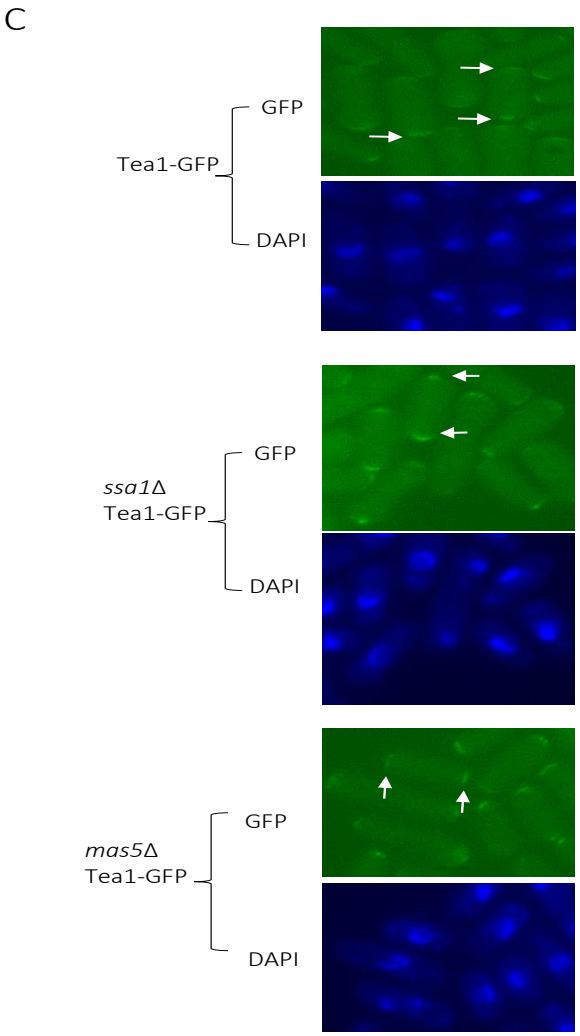


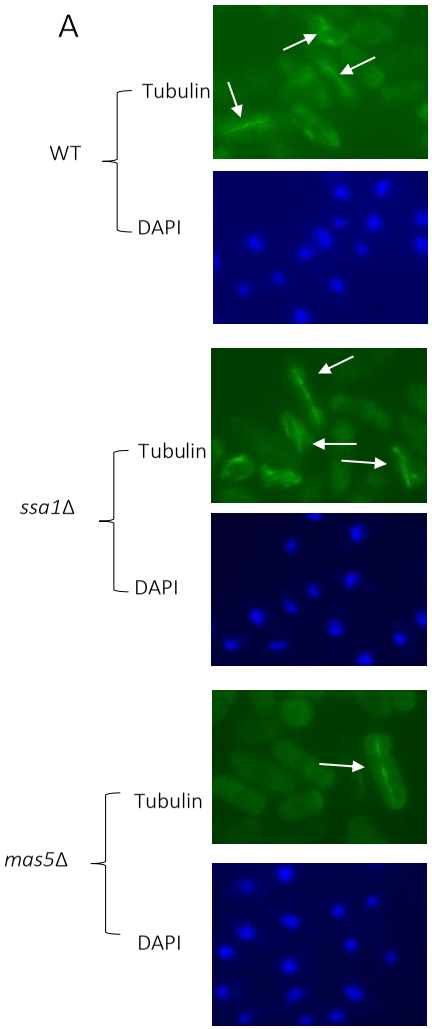
Fig. 3. 7. NETO factors and their regulation. (A) Model of NETO key proteins involved in the process at the cell end. (B) The regulation of cell growth in *S. pombe* during the cell division cycle. (C) Localisation of Tea1 does not appear to be linked to the presence of Ssa1 or Mas5. GFP (fluorescent) and DAPI images of representative exponential phase cells expressing Tea1-GFP (TK520-1) and cells expressing Tea1-GFP lacking the *ssa1*$^{+}$ gene (FM42) or *mas5*$^{+}$ (FM43), fixed using paraformaldehyde. Exposures were kept constant between images. White arrows point to Tea1 localisation features discussed in the text, n = 3.

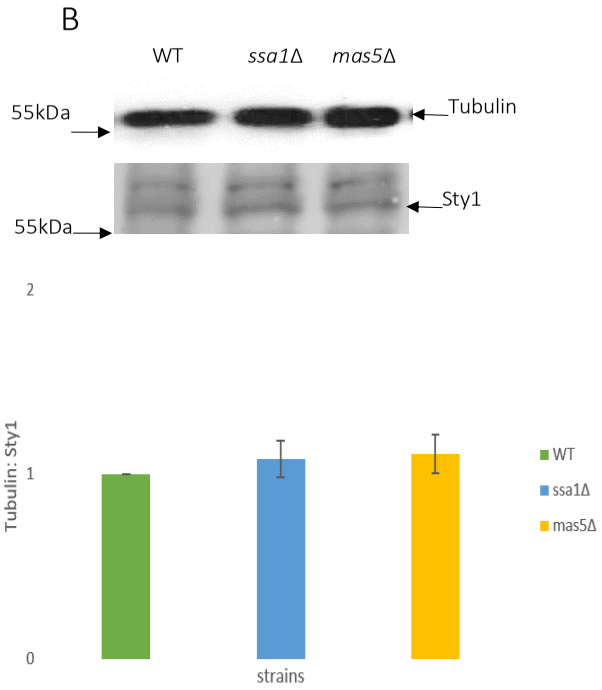
Thus, these data suggest that the cell cycle defects associated with loss of Ssa1 and Mas5 are not linked to effects on the delivery of Tea1 to cell ends.

3.2.6 Microtubule dynamics are regulated by Ssa1 and Mas5

Recent data has revealed the importance of dynamic reorganisation of the cytoskeleton in the regulation of cell polarity (Chang and Martin, 2009; Drummond and Cross, 2000; Snaith *et al*., 2011). For example, microtubules have been linked to cell shape and the delivery of Tea1 to the cell ends (Chang and Martin, 2009). Furthermore, older studies in *S. cerevisiae* revealed that Ssa1 in complex with Ydj1 are required for efficient microtubule dynamics (Oka *et al*., 1998). A temperature sensitive $ssa1^{ts}$ allele resulted in abnormal nuclear migration and abnormal arrays of microtubules that were bent around the cell periphery at the non-permissive temperature (Oka *et al*., 1998). In addition, Ydj1 mutant displayed even more severe microtubule defects (Oka *et al*., 1998). Hence it was possible that the functions of Ssa1, Ssa2, and/or Mas5 in cell cycle progression in *S. pombe* might be linked to a role in microtubule dynamics.

To examine this possibility microtubules were examined in exponentially growing wild type, *ssa1*Δ and *mas5*Δ cells by indirect immunofluorescence (Figure 3. 8. A). In addition, tubulin levels were also examined in these cells by western blot analysis. These data revealed that tubulin levels were similar in wild type, *ssa1*Δ and *mas5*Δ cells (Fig. 3. 8. B). Analyses of microtubules in these different cells revealed that most wild type cells have no observable tubulin structure (Fig. 3. 8. A). Furthermore, when microtubules were detected in wild type cells they appear to be arranged in a single linear structure between two nuclei or as an array of microtubule structures covering the length of the cell (see Fig. 3. 8. D).


A
Tubulin
WT
DAPI
Tubulin
ssa1Δ
DAPI
Tubulin
mas5Δ
DAPI


B
WT
ssa1Δ
mas5Δ
55kDa
Tubulin
55kDa
Sty1
2
1
0
Tubulin: Sty1
strains
WT
ssa1Δ
mas5Δ

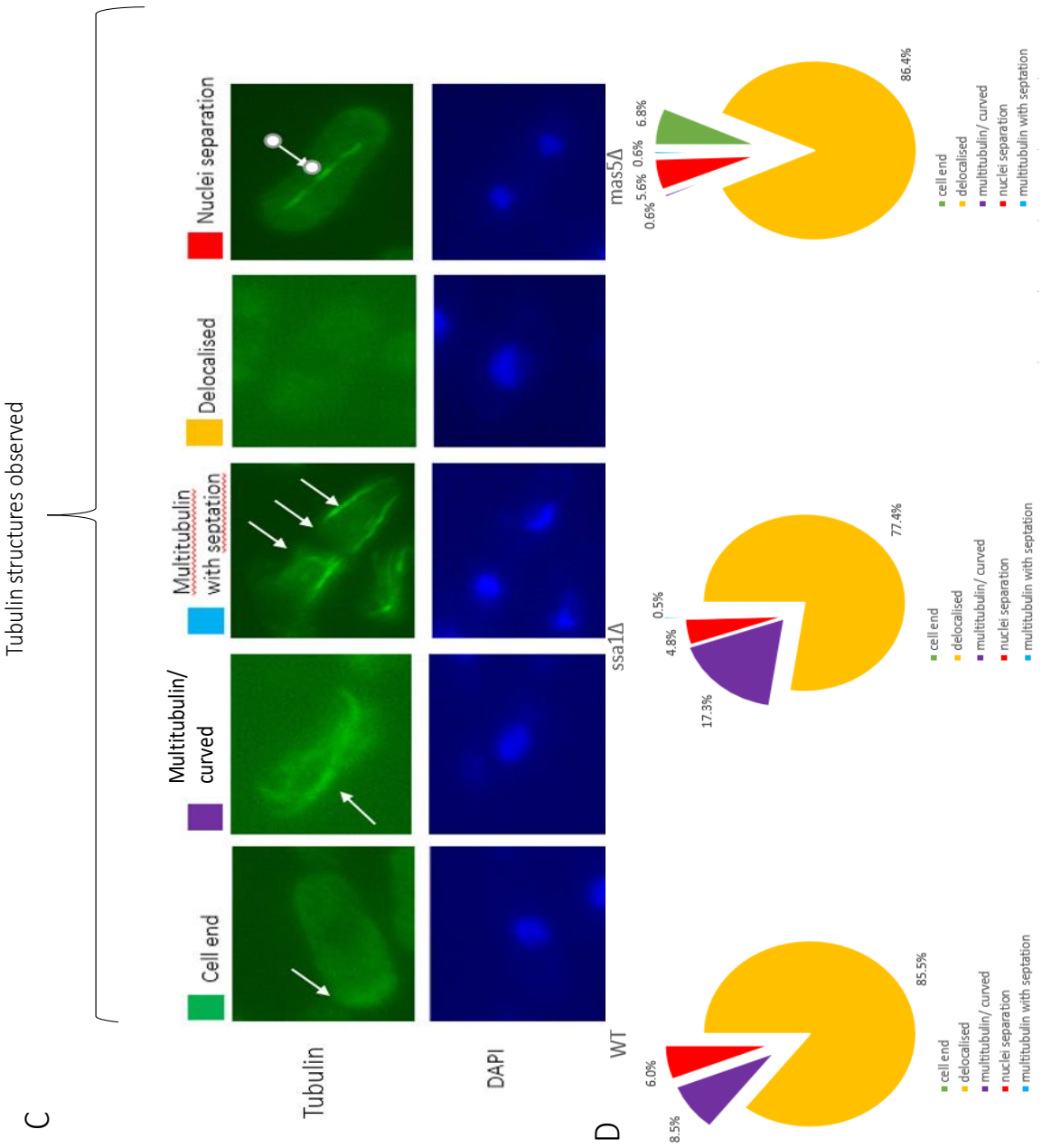


Fig. 3. 8. Tubulin dynamics is misregulated in the absence of Mas5. (A) FITC (fluorescent) and DAPI images of representative exponential phase wild type (WT) cells (CHP429), *ssa1*Δ (FM14) and *mas5*Δ (FM15) cells, fixed using paraformaldehyde. The cells were treated with anti-tubulin antibody and FITC-anti rabbit antibody, exposures remaining constant between images. Images used are representative examples. White arrows point to tubulin dynamics discussed in the text, n = 3. (B) Cells were grown to exponential phase at 30 °C and were collected, protein samples were extracted by TCA protocol plus NEM. Proteins were analysed by non-reducing SDS-PAGE and western blotting with anti-tubulin antibody and Sty1 as a loading control fold change was calculated relative to wild type unstressed sample, ratio set to 1, n = 2. (C) FITC (fluorescent) and DAPI images of representative exponential phase wild type (WT) cells (CHP429), *ssa1*Δ (FM14) and *mas5*Δ (FM15) cells, fixed using paraformaldehyde. White arrows point to tubulin dynamics discussed in the text, n = 500. (D) The tubulin structures observed for each strain were categorised by the types listed and pie-charts generated for each strain (cells > 250).

Interestingly, although the same microtubule phenotypes were observed in *ssa1*Δ cells (Fig. 3. 8 A), no observable tubulin structure was observed in the majority of *mas5*Δ cells, except for either a single linear structure between two nuclei or an aggregation at one cell end (Fig. 3.8 A).

The occurrence of the microtubule phenotypes was categorised and measured (Fig. 3. 8. C and D). Approximately 8% of wild type cells displayed an array of linear tubulin structures that run the length of the cell that can curve at the cell end, while 6% of wild type cells displayed a linear microtubule structure that was associated with the separation of 2 nuclei (Fig. 3. 8. C and D). Significantly, no wild type cells were observed that contained an aggregation of microtubules at the cell end or in an array during septation (Fig. 3. 8. D). Interestingly, the percentage of *ssa1*Δ cells with no tubulin localisation was lower than wild type cells (~77% versus 86%) (Fig. 3. 8. D). Furthermore, *ssa1*Δ cells contained a larger percentage of cells with an array of linear tubulin structures that run the length of the cell that can curve at the cell end (17% versus 8%**)** (Fig. 3. 8. D). However, like wild type cells approximately 5% of *ssa1*Δ cells contained a linear tubulin structure that was associated with the separation of 2 nuclei (Fig. 3. 8. D). Less than 0.5% of *ssa1*Δ cells were observed to contain microtubule aggregation at the cell end or in an array during septation.

These data suggest that Ssa1 may be involved in regulation of microtubule organisation, possibly playing a role in microtubule depolymerisation. Analysis of microtubule structures in *mas5*Δ cells revealed that a similar number of cells to wild type cells showed no sign of tubulin localisation (both wild type and *mas5*Δ ~86%) (Fig. 3. 8. D). Furthermore, there was also a comparable percentage of cells containing linear microtubule structures associated with the separation of 2 nuclei (both wild type and *mas5*Δ ~6%). However, in marked contrast to wild

type cells < 1% of *mas5*Δ cells were observed that contained an array of linear tubulin structures that run the length of the cell that can curve at the cell end (Fig. 3. 8. D). Furthermore, *mas5*Δ cells displayed a unique microtubule localisation of aggregation at the cell end (7%).

These data suggest that Mas5 is required for microtubule dynamics/organisation, perhaps playing roles in the polymerisation and/or stability. Although the molecular roles of Ssa1 and Mas5 are unclear these data support a model where Ssa1 and Mas5 act in opposition to one another to regulate microtubule organisation. Indeed, consistent with this model *mas5*Δ cells and not *ssa1*Δ were found to be much more sensitive than wild type cells to the microtubule depolymerising agent TBZ (Fig. 3. 9.). Interestingly, whilst Ydj1 mutant cells were also found to be hypersensitive to TBZ, in contrast to *ssa1*Δ *S. pombe* cells, *ssa1* mutant *S. cerevisiae* cells were more sensitive to TBZ than wild type cells (Oka *et al*., 1998). Thus, these data suggest that there is conservation in function of Ydj1 and Mas5 in microtubule dynamics but that Ssa1 may play different roles in each organism.

The spindle assembly checkpoint (SAC) ensures the correct orientation of chromosomes along the mitotic spindle before sister chromatids are separated and contains several proteins including Mad2 that are conserved in eukaryotes (see reviews Daniel *et al*., 2006; Musacchio and Salmon, 2007). Many of the components of the spindle assembly checkpoint were identified in genetic screens as causing increased sensitivity to a microtubule depolymerising agent. Hence the observations that *mas5*Δ cells have significant defects in microtubule dynamics/organisation and increased sensitivity to TBZ suggested that perhaps *mas5*Δ mutant cells are dependent on the SAC for viability. To test this possibility the construction of a haploid strain containing both *mas5*Δ and *mad2*Δ was attempted. Significantly, *mas5*Δ*mad2*Δ double

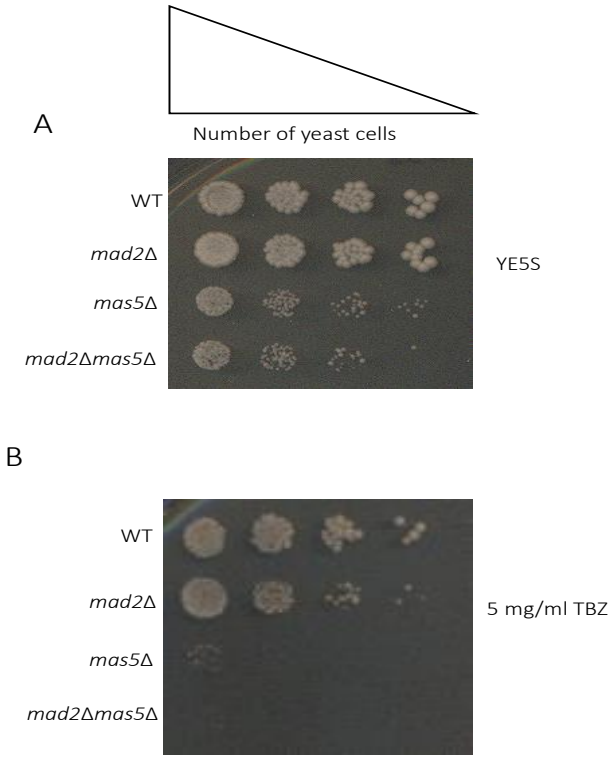


Fig. 3. 9. Cells lacking *mas5*$^{+}$ show increased sensitivity to microtubule depolymerization. Wild type (WT) cells (CHP429), cells with *mas5*Δ allele (FM15), *mad2*Δ (SW102) and *mad2*Δ*mas5*Δ (FM48) were grown to exponential phase and serial diluted to the same OD_{595}. Cells were plated to YE5S solid medium containing/exposed to the stress indicated. Plates were imaged every 24 hours, images shown were taken after 96 hours. (A) Example growth of strains in optimal unstressed conditions. (B) The same strains were grown upon media containing the microtubule depolymerisation agent TBZ (5 mg/ml), images shown were taken after 96 hours, n = 2.

mutant cells could be obtained and furthermore, did not appear to display any growth defects in addition to *mas5*Δ cells in normal growth conditions (Figure 3. 9.).

These data suggest that *mas5*Δ cells do not utilise SAC to retain viability in normal growth conditions. Excitingly, the *mas5*Δ*mad2*Δ double mutant cells appear to show increased sensitivity compared to *mas5*Δ cells when exposed to TBZ. This additive effect suggests that Mad2 is critical for cell survival in the absence of Mas5.

3.2.7 Mas5 has a role in the regulation of meiosis

In eukaryotes, there are numerous proteins and structures, including microtubules, that are required for both the mitotic and meiotic cell cycles. Meiosis differs from mitosis as it reduces a diploid genome to a haploid genome but the process utilises many conserved regulatory factors. The genetics of meiosis are relatively well defined in budding and fission yeast. Furthermore, although the signalling pathway that stimulates meiosis in *S. pombe* is different to humans there are many proteins that regulate DNA recombination and chromosomal segregation that have been conserved.

The movement of chromosomes in mitosis and meiosis is dependent on the attachment of kinetochore to the spindle pole body (SPB). During the long meiotic prophase, there is forceful relocalisation of chromosomes, with the loss of anchoring of the centromere to the SPB region (Ding *et al*., 2004; Fig. 3. 10. A). Importantly, the chromosomes oscillating between cell poles is dependent on microtubule organisation, and this process results in an elongated nucleus (termed the "horsetail stage" in *S. pombe*). Subsequently, the microtubules are induced to change from bundles, such as those observed in interphase, to radial arrays originating from the

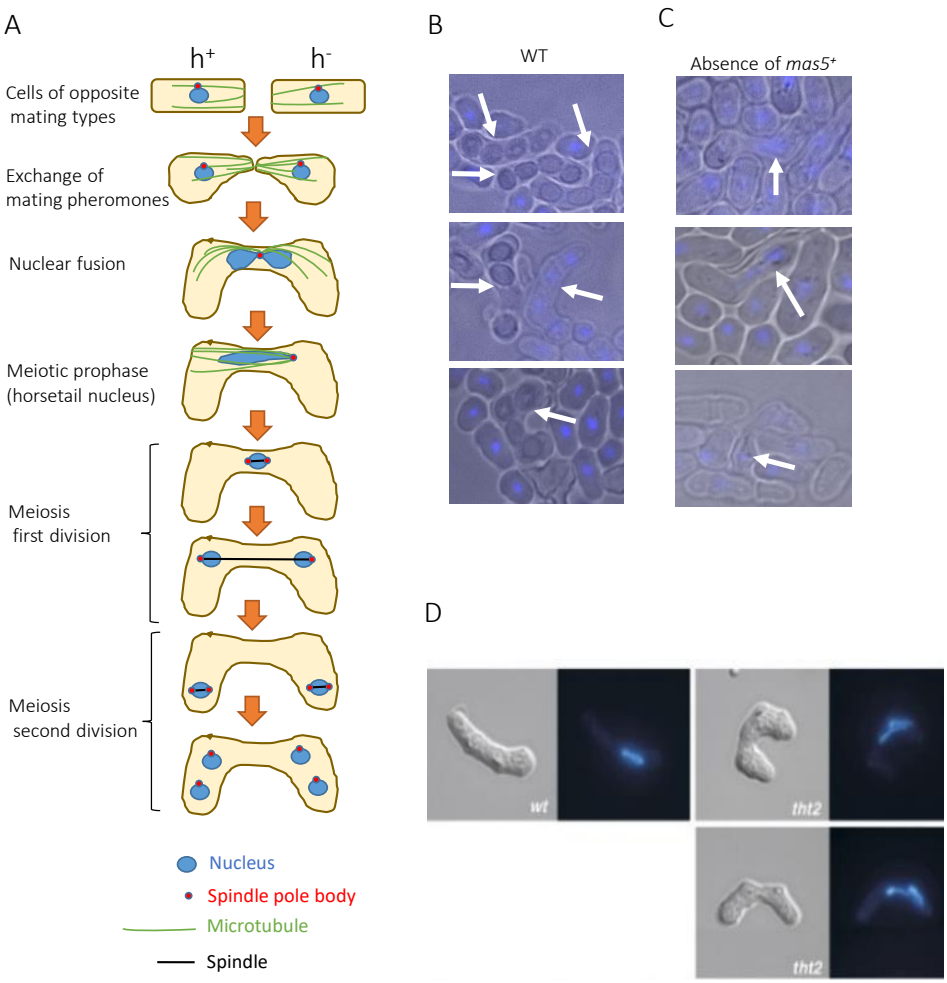


Fig. 3. 10. Cells lacking *mas5*$^{+}$ have defects in meiosis. (A) Schematic representation of the process of meiosis and sporulation in *S. pombe* cells. (B) Wild type (WT) cells (CHP428 and CHP429) of opposite mating type were inoculated together on solid medium with minimal nitrogen source for 3 days to induce mating and sporulation, n = 3. (C) Cells of opposite mating type with *mas5*Δ alleles (FM15) were inoculated together on solid medium with minimal nitrogen source for 3 days to induce mating and sporulation. DIC and DAPI overlay images of representative asci, exposures remaining constant between images. White arrows indicate the asci and highlight DNA localisation based upon DAPI staining. n > 3(D) categorised meiotic defects termed horsetail mutants observed in *S. pombe* mutants from Martin-Castillanos *et al.* (2005).

SPB (Chikashige *et al*., 2014; Fig. 3. 10. A). This arrangement of radial microtubules along with motor protein complexes allows the oscillation of chromosome localisation.

The pheromone signalling concomitantly induces chromosomal reorganisation during which centromeres move away from the SPB while telomeres cluster towards it, this is termed the "bouquet" formation (Chikashige *et al*., 2014; Martin-Castellanos *et al*., 2005; Fig. 3. 10. A). The reorganisation of chromosomes and the "horsetail stage" efficiently pairs chromosomes for meiotic recombination. Once the segregation of the chromosomes begins the centromere re-associates with the SPB location at the poles followed by reductional chromosomal segregation (Ding, 2011). To facilitate this the radial microtubules are interchanged for the mitotic spindle. This highlights the importance of microtubule reorganisation for meiosis as a generalised process but also critically for the fidelity of genetic reductional rearrangement.

During initial investigations it was noted that the presence of the *mas5*Δ allele in heterozygous diploids appeared to lead to slower sporulation and resulted in the formation of increased frequency of abnormal asci (data not shown). Furthermore, when mating and sporulation was attempted using two haploids which both lacked Mas5 it was noted that, although the cells would conjugate leading to activation of the sporulation pathway, the asci that formed were of abnormal morphology compared to wild type asci (compare Fig. 3. 10. B and C). Asci lacking *mas5*$^{+}$ showed no breakdown of the ascus wall and did not result in 4 viable spores. Although DAPI staining of DNA is not highly penetrative of wild type asci, it is possible to observe the internal structure of spores (Fig. 3. 10. B). Intriguingly, the *mas5*Δ asci contained no obvious spores and, moreover, the DNA present appears to be localised to polar cell regions or stretched across the internal region (Fig. 3. 10. C).

Previous genetic analyses have identified mutations in genes that affect different stages of meiosis. Interestingly, comparison of the *mas5*Δ phenotypes to published work revealed that the asci and DNA phenotypes observed in *mas5*Δ cells are most similar to those seen in "twin horsetail" mutants (Fig. 3.10. D) (Martin-Castellanos *et al*., 2005). Mutants in this category, such as deletion of either *tht1*$^{+}$ or *tht2*$^{+}$, form twin horsetail nuclei. The Tht2 mutant was determined to be deficient at karyogamy due to Tht2 being essential for homolog interaction. Whether microtubule organisation is normal in these mutants was not investigated. These data suggest that Mas5 is important for microtubule dynamics during both mitosis and meiosis. Furthermore, the absence of viable spores in *mas5*Δ crosses suggests that Mas5 may be more important for microtubule reorganisation during meiosis than mitosis.

3.2.8 Mas5 may have a regulatory role in autophagy

In *S. pombe* meiosis is induced by nitrogen starvation (Ding, 2011; Kohda *et al*., 2007). Autophagy is a conserved non-specific degradation pathway that is utilised to replenish depleted nutrient pools caused by environmental conditions in eukaryotic cells. Although the autophagy pathway has been well defined in *S. cerevisiae* much less is known about the pathway in *S. pombe* (for reviews see Huang and Klionsky, 2002; Mukaiyama *et al*., 2010).

The process to degrade an organelle requires activation of the Atg1 complex resulting in dynamic rearrangement of the membrane to encapsulate the organelle in a double membraned vesicle (Fig. 3. 11.). This results in the formation of the autophagosome which in mammalian cells fuses with lysosomes or in yeast it fuses with the vacuole. The complex releases vesicles internally containing digestive enzymes in to the autophagosome thus degrading the

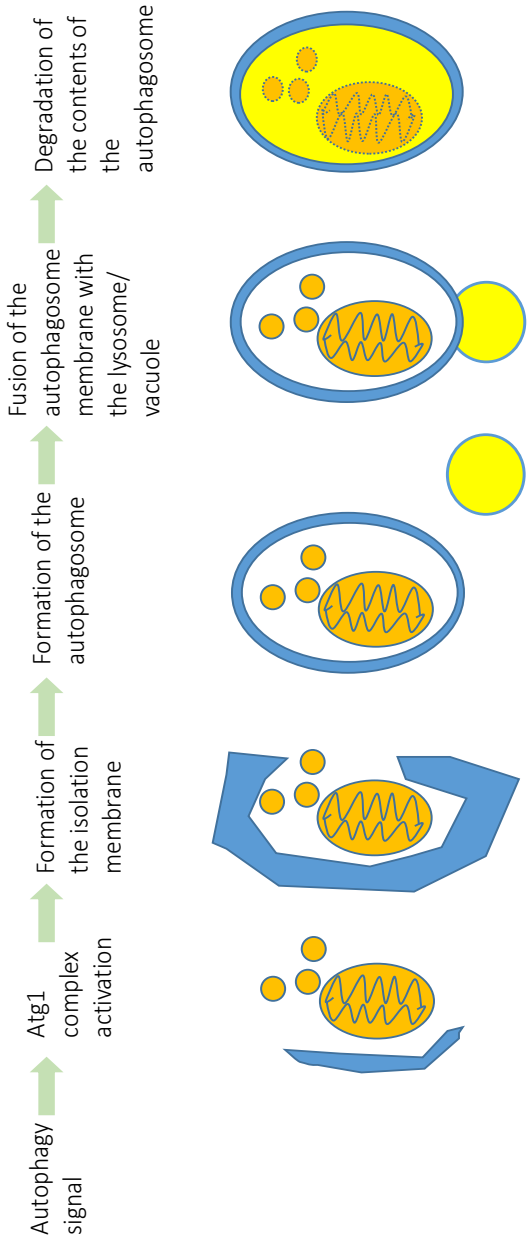


Fig. 3. 11. Model of the key processes occurring in the autophagy pathway. Certain conditions are sensed that trigger the autophagy signal pathway for example starvation. This leads to activation of the Atg1 complex followed by initial formation of a membrane capsule (blue). The membrane capsule isolates organelles and other cellular components from the rest of the cell content. Enclosure of the isolation membrane result in the formation of the autophagosome. Following this, the lysosome (or vacuole) fuses with the autophagosome to release the digestive enzymes in to the content of the autophagosome. The content of the autophagosome is then digested to smaller molecules that can potentially be used to form new cellular components.

organelle. It has been suggested that degradation of redundant proteins by autophagy allows the amino acids to be recycled in the absence of a nitrogen source (Levine and Klionsky, 2004).

Autophagy and meiosis are intimately linked in *S. pombe* cells. For example, a recent study found that certain autophagy deficient mutants (*atg1*Δ, *atg8*Δ, *atg13*Δ) in the absence of nitrogen were unable to mate and sporulate (Kohda *et al*., 2007). However, the mating and sporulation defects could be rescued by providing a suitable nitrogen source (Kohda *et al*., 2007). Given the meiotic defects associated with *mas5*Δ cells when deprived of a nitrogen source it was possible that some or all of the defects might be linked to problems in the autophagy pathway. Previous studies of mutations of autophagy-linked genes such as $atg1^{+}$, $atg8^{+}$ and $atg13^{+}$ revealed that autophagy is vital for long term survival of *S. pombe* cells during nitrogen depletion (Kohda *et al*., 2007). Therefore, to test whether Ssa1 and/or Mas5 played any role in autophagy the long-term survival of wild type, *ssa1*Δ and *mas5*Δ cells to nitrogen starvation was examined.

Firstly, strains were successfully generated with all auxotrophic markers replaced with wild type genes so that no additives were required in the media. Cells were grown to exponential phase in rich liquid media then switched to minimal media lacking a nitrogen source. At different times during the incubation in nitrogen free media the cells were spotted onto rich media to examine viability in the nitrogen free media. After 21 days of nitrogen starvation the ability of cells to grow on a spot test on rich media is an indicator of the functionality of autophagy in the cells. Indeed, as expected *atg12*Δ cells, which blocks a key step in autophagy, lose viability (~100 fold) after 21 days incubation in nitrogen free media (Fig. 3. 12.).

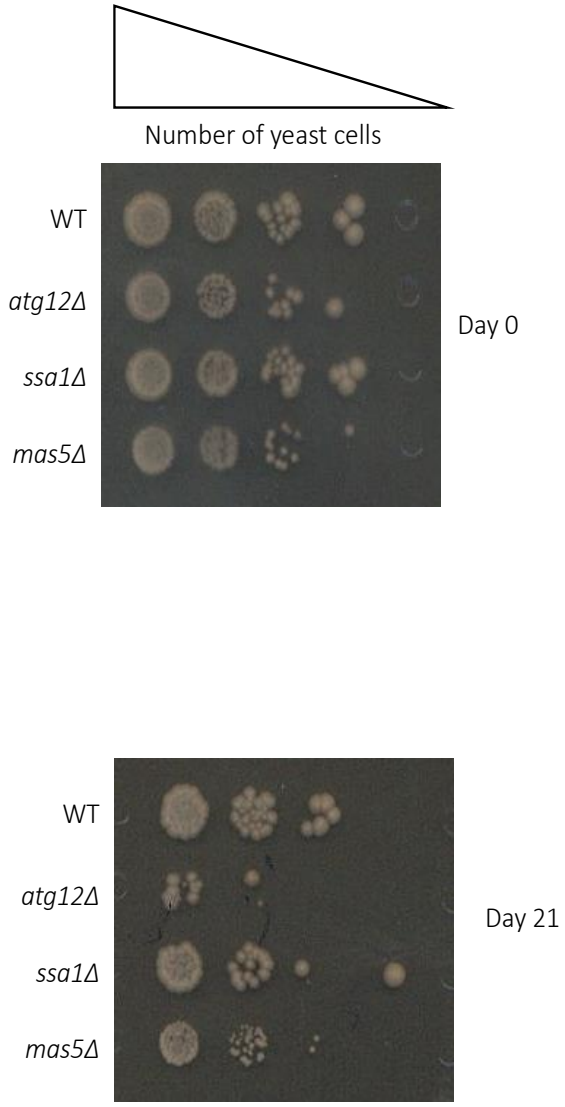


Fig. 3. 12. Mas5 is important for responses of cells to nitrogen starvation. Wild type cells lacking any auxotrophic markers (SW972) and cells with an *atg12*Δ (MF13), *ssa1*Δ (FM52) or *mas5*Δ allele (FM53) were grown to exponential phase in EMM+N media and serial diluted to the same OD_{595}. Cells were plated to YE5S solid medium. The remaining liquid culture of cells were washed with EMM-N 3 times and incubated in EMM-N at 30C for 21 days with EMM-N replenishment every 3 days. Cells were and serially diluted to the same OD_{595} and plated to YE5S solid medium at 21 days, n = 1.

Analysis of *ssa1*Δ cells suggested that they retained similar viability to wild type cells (Figure 3. 12.). In contrast, *mas5*Δ cells possibly showed a small loss of viability in nitrogen free media after 21 days (Figure 3. 12.). Although not conclusive these results at least raised the possibility that Mas5 may have a role in autophagy. It would be useful to observe these strains grown in the presence of nitrogen for the same time to observe their viability as a control.

Previous work established that hydrolysis of an Atg8-GFP fusion protein, detectable by western blot analyses, can be used as an assay of autophagic activity (Klionsky *et al*., 2007). Hence wild type, *ssa1*Δ or *mas5*Δ cells lacking any auxotrophic markers and expressing an Atg8-GFP fusion protein were constructed. The entry of *S. pombe* cells into stationary phase by the lack of nutrients induces autophagy and increased levels of Atg8. To confirm the detection of Atg8-GFP wild type, *ssa1*Δ and *mas5*Δ cells expressing Atg8-GFP were visualised during stationary phase by fluorescence microscopy. Interestingly, small localised GFP signal is detectable in a minority of wild type and *ssa1*Δ cells (Fig. 3. 13.). However, strikingly, a highly detectable Atg8-GFP signal in a punctate ring-like structure was detected in *mas5*Δ cells (Fig. 3. 13.). These data confirm the successful construction of these strains and further supports the hypothesis that Mas5 plays a role in nutrient sensing/autophagy regulation.

Having established that the Atg8-GFP protein could be detected in the strains autophagy in response to nitrogen starvation was examined. The Atg8-GFP assay has been used to study autophagy in response to nitrogen conditions (Flanagan *et al*., 2013; Klionsky *et al*., 2007). In nitrogen replete conditions, which do not stimulate autophagy, Atg8-GFP appears as a full-length protein (Klionsky *et al*., 2007; Mukaiyama *et al*., 2010). However, following exposure of cells to nitrogen free media, the Atg8 protein is conjugated in to the autophagosome complex and the GFP moiety is cleaved (Kohda *et al*., 2007; Mukaiyama *et al*., 2010). The processing

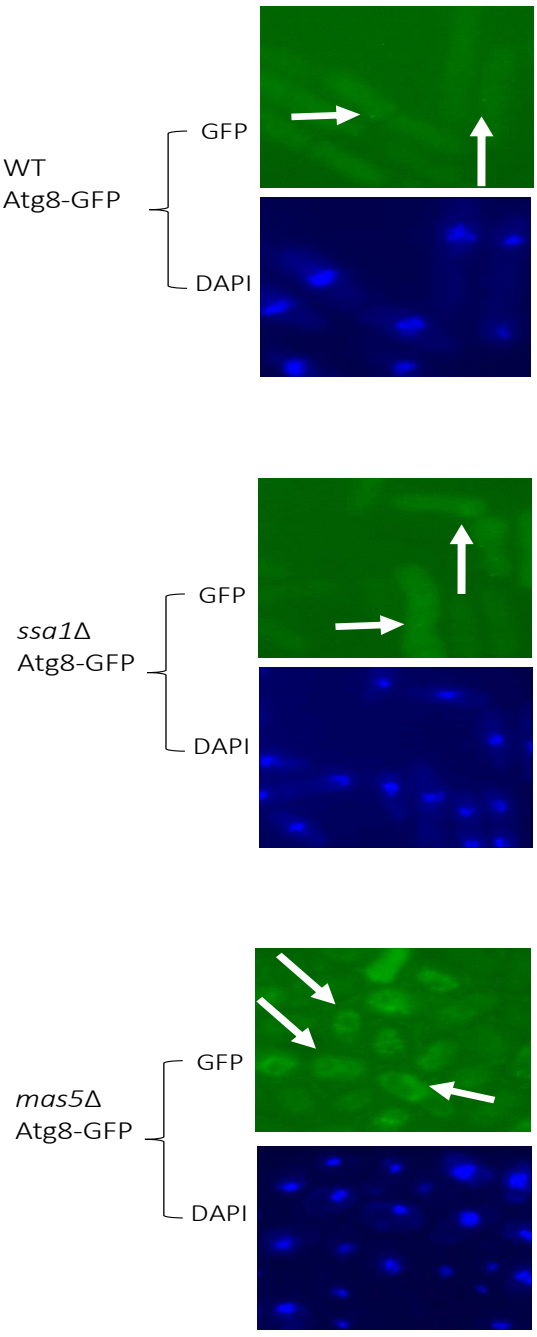


Fig. 3. 13. Atg8-GFP localisation in response to nitrogen starvation in *ssa1*Δ and *mas5*Δ cells. GFP (fluorescent) and DAPI images of representative stationary phase wildtype (WT) (JT268), *ssa1*Δ (FM24) or *mas5*Δ (FM29) cells expressing Atg8-GFP. Cells were taken from YE5S solid medium and fixed using paraformaldehyde, exposures remaining constant between images. White arrows indicate features discussed within the text, n = 3.

and localisation of Atg8-GFP can be visualised in fixed cells by fluorescence microscopy and by western blotting (Kohda *et al*., 2007; Mukaiyama *et al*., 2010). Hence wild type, *ssa1*Δ and *mas5*Δ cells expressing Atg8-GFP were grown to exponential phase in minimal media containing a nitrogen source and then cells collected for fluorescence microscopy and western blot analysis. The cells were then incubated for 20 hours in minimal medium in the absence of a nitrogen source to induce autophagy and into minimal media lacking a nitrogen source and containing the vacuolar protease inhibitor (PMSF) to prevent protein degradation of Atg8-GFP. After 20 hours cells were also collected for fluorescence microscopy and western blot analysis.

Fluorescence microscopy analysis of wild type cells growing in the presence of a nitrogen source (EMM+N) revealed only a low level of Atg8-GFP, indicating that autophagy is not notably active in these conditions (Fig. 3. 14.). However, after shifting the culture to nitrogen deficient media (EMM-N) for 20 hours a strong GFP signal was detected that was organised in a circular formation within cells (Fig. 3. 14.). This result is indicative of an increase in Atg8-GFP, and cleavage of the GFP moiety as Atg8 is conjugated to the autophagosomal membrane (Mukaiyama *et al*., 2010).

In the presence of PMSF there is still an increase in GFP signal but in this case the signal is granular in appearance within the cells (Fig. 3. 14.). This result is consistent with PMSF blocking protease cleavage of the GFP moiety and preventing Atg8-GFP conjugation to the autophagosomal membrane (Mukaiyama *et al*., 2010). Consistent with the viability analysis (see Fig. 3. 12.) the fluorescence signal detected in *ssa1*Δ cells correlate with the fluorescence signals observed in wild type cells (compare relevant panels in Fig. 3. 14.).

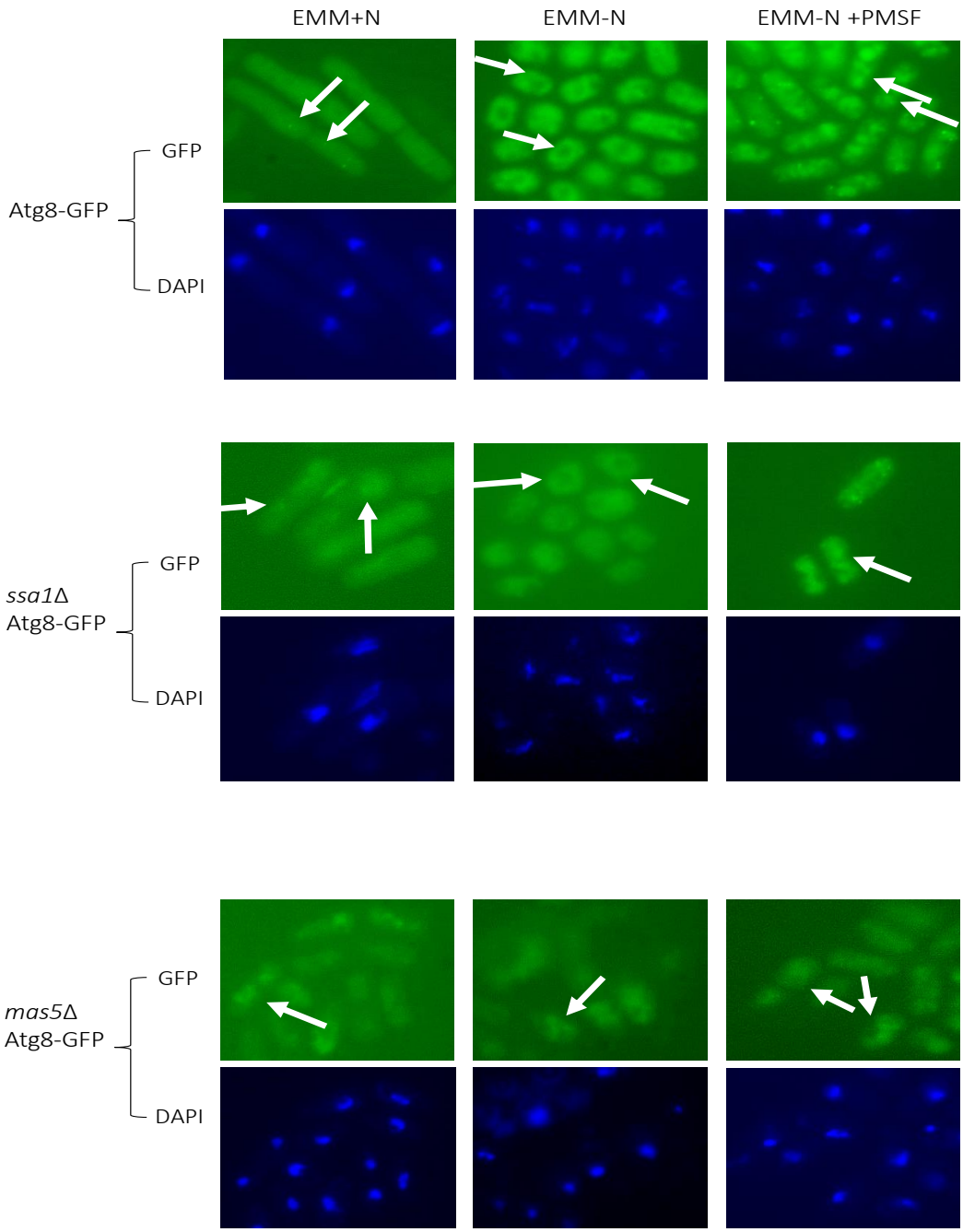


Fig. 3. 14. Atg8-GFP is mislocalised in the absence of Mas5. GFP (fluorescent) and DAPI images of representative exponential phase cells expressing Atg8-GFP cells (JT268) or cells with either *ssa1*Δ (FM24) or *mas5*Δ allele (FM29). Cells were grown in minimal media with a nitrogen source (EMM+N) then shift to minimal media lacking a nitrogen source (EMM-N) for 20 hours with or without a protease inhibitor (EMM-N +PMSF); cells were fixed using paraformaldehyde. Exposures remaining constant between images. White arrows indicate localisation features mentioned in the text, n = 3.

In contrast the fluorescence signal detected in *mas5*Δ cells are very different to that detected in wild type and *ssa1*Δ cells. There is a noticeable increase in Atg8-GFP signal in the *mas5*Δ cells growing in the presence of nitrogen in comparison to the wild type cells (compare relevant panels in Fig. 3. 14.). Moreover, the Atg8-GFP signal detectable in *mas5*Δ cells in these conditions appears to already be in a circular formation suggesting potential conjugation to the autophagosomal membrane (Fig. 3. 14.). Then following nitrogen depletion unlike wild type cells there was no noticeable increase (or decrease) to the signal strength of Atg8-GFP in *mas5*Δ cells and the signal remains in a circular formation (Fig. 3. 14.). Additionally, the presence of PMSF does not appear to have any effect on the localisation or level of Atg8-GFP detected in *mas5*Δ cells (Fig. 3. 14.). Although these data are consistent with the hypothesis that Mas5 plays a role in autophagy the precise function(s) of Mas5 is unclear.

Next, the processing of the Atg8-GFP protein was analysed by western blotting using the samples collected at the same time as cells were fixed for fluorescence microscopy. As expected the full length unprocessed Atg8-GFP protein could be detected in extracts from wild type cells growing in nitrogen replete medium (Fig. 3. 15.). Furthermore, following nitrogen depletion as expected a lower band appears which is attributed to the GFP moiety cleaved from the Atg8-GFP fusion protein due to the activation of autophagy pathway (Fig. 3. 15.). Indeed, treatment of wild type cells in the nitrogen depleted media with the vacuolar protease inhibitor PMSF inhibits the cleavage of Atg8-GFP (Fig. 3. 15.). These results for the wild type control strain are consistent with the observed effects of nitrogen depletion on Atg8-GFP localisation in wild type cells (Fig. 3. 14.) and suggests that autophagy is activated in nitrogen depleted media.

Consistent with the conclusion from the fluorescence microscopic analysis, western blot analysis of the processing of Atg8-GFP protein from *ssa1*Δ cells suggested that autophagy was

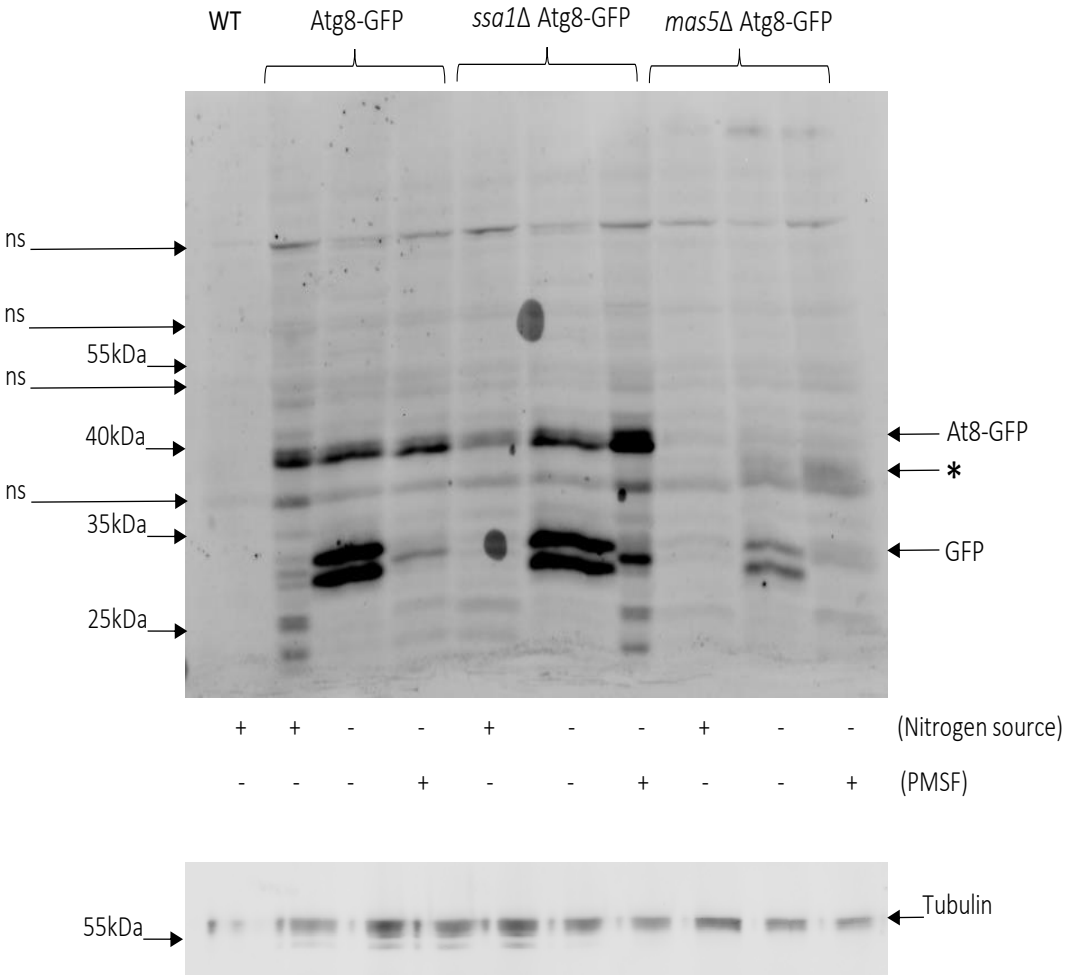


Fig. 3. 15. Mas5 is important for Atg8 maturation. Exponential phase wild type cells (WT) (CHP429) and cells expressing Atg8-GFP (JT268) or cells with either *ssa1*Δ (FM24) or *mas5*Δ allele (FM29) were grown in minimal media with a nitrogen source (EMM+N) then shifted to minimal media lacking a nitrogen source (EMM-N) for 20 hours with or without a protease inhibitor (EMM-N +PMSF). Samples were extracted by the TCA protocol plus NEM. Proteins were analysed by non-reducing SDS-PAGE and western blotting with anti-GFP antibody or anti-tubulin antibody as a loading control, non-specific antibody recognition indicated by ns. Asterisk indicates feature discussed within the text, n = 2.

activated in a similar manner in wild type and *ssa1*Δ cells (Fig. 3. 15.).

Strikingly, western blot analysis of the protein extracts from *mas5*Δ cells revealed very different results. In contrast to wild type and *ssa1*Δ cells, no Atg8-GFP was detectable in nitrogen replete media (Fig. 3. 15.). This contrasts with the fluorescence microscopic analysis of *mas5*Δ cells grown in the same conditions which revealed the presence of a GFP signal, although located in structures not observed in wild type cells (Fig. 3. 14.). This would suggest that the structures observed in the *mas5*Δ cells in these conditions are resistant to analysis with the protein extraction/nonreducing conditions used to investigate the Atg8-GFP protein. Interestingly, following nitrogen depletion, a GFP band can be detected, consistent with processing of Atg8-GFP as this is inhibited by PMSF, although the levels of GFP are much lower than that observed in wild type cells (Fig. 3. 15.).

This data suggests that autophagy is activated in *mas5*Δ cells though perhaps not to the same degree as wild type cells. This result is consistent with the indication that the viability of *mas5*Δ cells may be more sensitive to nitrogen depleted conditions than wild type cells although not to the same extent as *atg12*Δ an autophagy pathway mutant that completely blocks Atg8-GFP processing (Fig. 3. 12.; Flanagan *et al*., 2013). Intriguingly a band can be detected in the *mas5*Δ proteins extracts under nitrogen starvation conditions with a mobility that is slower than GFP but faster than Atg8-GFP (Fig. 3. 15., marked with an asterisk). This may be a non-specific band that appears in *mas5*Δ cells but that is unrelated to Atg8-GFP but it is intriguing to investigate its identity as it may well help to explain the cell localisation and protein analyses.

In conclusion, these data suggest that Mas5, but not Ssa1, is important for autophagy in response to nitrogen starvation, perhaps by playing key roles in chaperoning protein structures that function in this process.

Having identified a connection between Mas5 with Atg8 it was possible that cells lacking Mas5 have problems activating the autophagy pathway in general rather than any specific effect on Atg8. To examine this possibility the effects of loss of Mas5 on other key autophagy proteins was investigated.

Several different components join to form the autophagosome. For example, Atg1 kinase along with its regulators are essential for the formation of the membrane required for autophagy. Indeed, deletion of *atg1*$^{+}$, similar to loss of *atg8*$^{+}$, results in no detectable autophagy (Kohda *et al*., 2007). Significantly, Atg1 is cytosolic in nutrient rich conditions but following nutrient limitation Atg1 kinase activity increases and becomes part of the autophagosome complex (Kim *et al*., 2002; Suzuki *et al*., 2001). The autophagosome then fuses with the vacuole resulting in release of the internal components of the autophagosome in to the vacuole lumen (Fig 3. 11.). Thus, use of a vacuolar membrane marker, such as the zinc transporter Zhf1, can also be useful in establishing the localisation of autophagic components such as Atg1 to the vacuole. Indeed, previous work showed that Atg1 co-localises with Zhf1 in a discreet circular structure in nutrient rich conditions in *S. pombe* cells (Sun *et al*., 2013).

Hence, wild type, *ssa1*Δ and *mas5*Δ strains lacking auxotrophic markers were constructed expressing Atg1-YFP and Zhf1-mCherry. The localisation of Atg1 and Zhf1 were then examined when cells from each strain were grown in nitrogen rich and depleted media. Interestingly, in marked contrast to the fluorescence microscopic analyses of Atg8-GFP, both

the Atg1-YFP and AZhf1-mCherry proteins appeared to be organised in similar circular structures within the cytosol of wild type, *ssa1*Δ and *mas5*Δ cells (compare panels Fig. 3. 16.). Furthermore, when wild type, *ssa1*Δ and *mas5*Δ cells were incubated in nitrogen depleted media Atg1-YFP appears to locate to more condensed foci whilst Zhf1-mCherry appears to be in possibly the same structure (Fig. 3. 16.). This is consistent with the published data on Zhf1 and Atg1 colocalisation (Sun *et al*., 2013, see supplementary data).

Collectively, these data suggest that Atg1-YFP activation within the autophagy pathway is not affected by loss of Ssa1 or Mas5. However, it was noted that the Atg1-YFP signal was consistently brighter in *mas5*Δ cells compared to wild type cells (Fig. 3. 16.). Although this was not examined by western blot analysis it is possible that Atg1 protein levels are affected in the absence of Mas5. Nevertheless, taken together the observation that loss of Mas5 function only appears to affect certain aspects of autophagy suggests that Mas5 may have specific roles in the pathway. It should also be noted that due to the problems with the *ssa2*Δ strain that its role in autophagy has not been investigated. Thus, it will be of interest in the future to determine whether these effects of Mas5 are mediated by a Ssa2/Mas5 complex or whether Mas5 acts with other proteins.

3.3 Discussion

An aim of the study was to explore the potential roles of Ssa1, Ssa2 and Mas5 in the responses of cells to stress. As a first step strains were constructed that lacked either the *ssa1*$^{+}$, *ssa2*$^{+}$ and *mas5*$^{+}$ genes. In addition, strains were constructed that express epitope tagged versions of Ssa1, ssa2 and Mas5 from their respective gene loci. Excitingly, initial characterisation of the strains

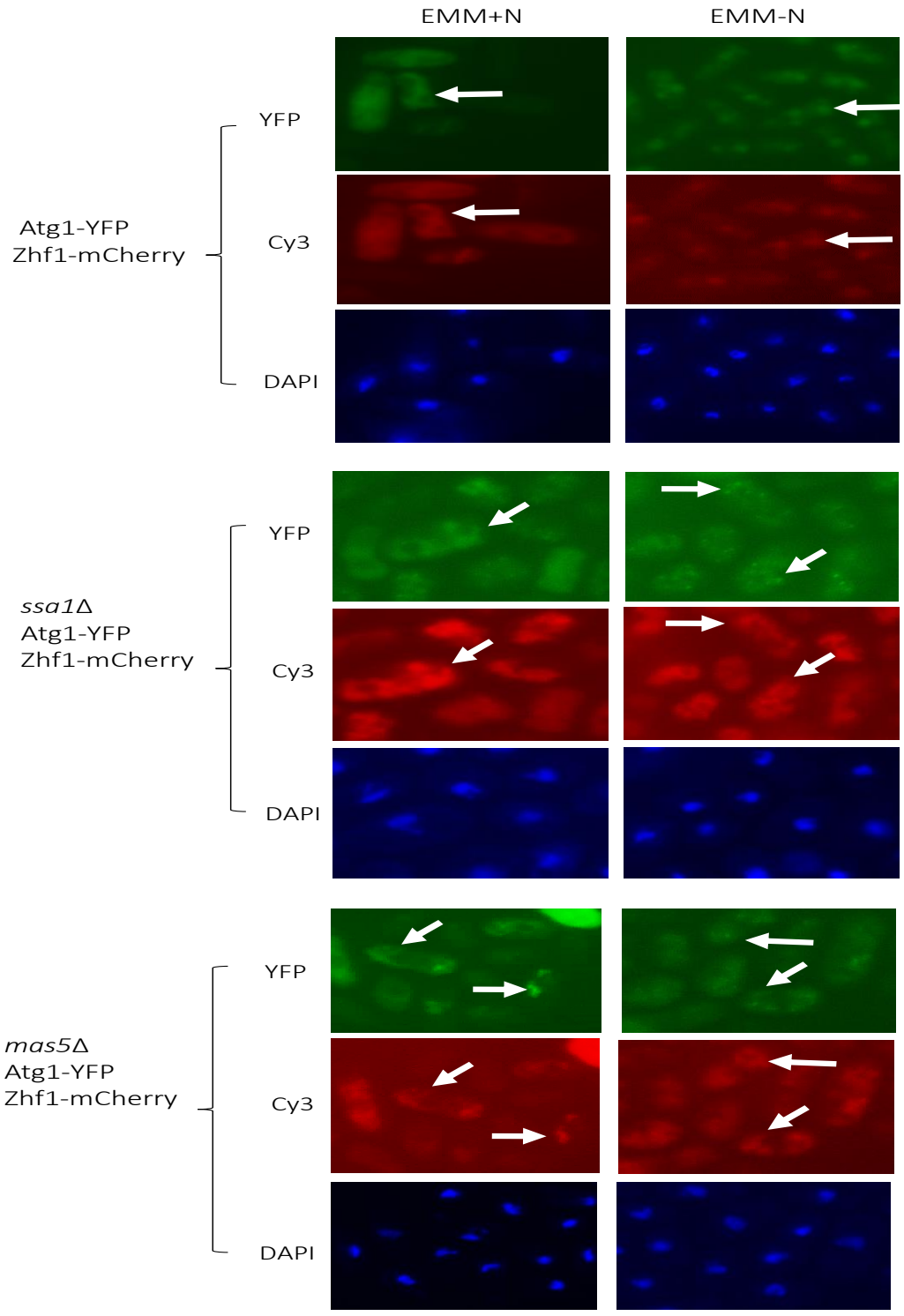


Fig. 3. 16. Co-localisation of vacuolar marker Zhf1-mCherry and Atg1-YFP in *ssa1*Δ and *mas5*Δ cells. GFP (fluorescent) (for YFP), RFP (for mCherry) and DAPI images of representative exponential phase cells expressing Atg1-YFP and Zhf1-mCherry (DY11893) or cells with either *ssa1*Δ (FM49) or *mas5*Δ allele (FM50) were grown in minimal media with a nitrogen source (EMM+N) then shift to minimal media lacking a nitrogen source (EMM-N). Cells were fixed using paraformaldehyde. Exposures remaining constant between images. White arrows discuss localisation features discussed in the text, n = 2.

lacking each of these individual genes revealed new roles for these proteins in the regulation of cell size during the cell division cycle, in the process of meiosis, and in the autophagy pathway. Studies in the distantly related *S. cerevisiae* have shown that the equivalent proteins play important cell cycle roles. Our analyses have revealed both conserved and organism specific aspects of the functions of these proteins in cell cycle regulation in divergent organisms. In addition, novel roles for these proteins in meiosis and autophagy have been uncovered.

Ssa1/Ssa2 and Ydj1 have been linked to the regulation of the cell cycle and stress responses in *S. cerevisiae.* It was worth investigation in the distantly related fission yeast *S. pombe* to understand conserved and organism specific role in stress responses. From homology analysis using Blast software it was clear that *S. cerevisiae* Ssa1, Ssa2 and Ydj1 have the greatest identity to *S. pombe* Ssa1, Ssa2 and Mas5 respectively (Table 1. 1.). Microarray data show that *SSA1* from both organisms is induced (approximately 3-fold) in response to heat, a similar trend is observed for *S. pombe mas5*$^{+}$ and *S. cerevisiae YDJ1* (Caulston *et al*., 2001; Chen *et al*., 2003). However, *S. cerevisiae SSA2* induction is minimal over the time course while *S. pombe ssa2*$^{+}$ showed a 3.6-fold change over the time course (Caulston *et al*., 2001; Chen *et al*., 2003). This highlights that though these homologous proteins are very similar in sequence their regulation has become organism specific.

Thus, to investigate the role of these proteins in *S. pombe*, it was attempted to knock out *ssa1*$^{+}$, *ssa2*$^{+}$ and *mas5*$^{+}$ from *S. pombe* cells. These deletion strains were successfully generated and determined to be viable. However, *ssa2*Δ cells showed phenotypes of severe genetic pressure to revert to wild type and thus made data variable or not possible to collect. Although this reversion to wild type characteristics can't be explained it is possible that it is due to mutation or some adaptation to loss of Ssa2 function. The same behaviour was not observed for *ssa1*Δ

nor *mas5*Δ cells. The focus of the work here and later chapters focused mainly on Ssa1 and Mas5 mutants for this reason.

It was shown that Ssa1, Ssa2 and Ydj1 each play a role in the cell cycle of budding yeast. Ssa1 localises the most upstream complex of Start (Cln3-Cdc28) to the cytosol preventing entry to the next round of DNA replication (Truman *et al*., 2012; Verges *et al*., 2007). In *S. cerevisiae* Ydj1 interaction with phosphorylated Ssa1 promotes cell cycle progression. This is characterised by the *S. cerevisiae ydj1*Δ strain being significantly larger than wild type cells as the *ydj1*Δ is linked to delayed progression through Start. In *S. pombe*, the small *mas5*Δ cells in comparison to the reported size of *S. cerevisiae ydj1*Δ cells suggests that there is a conserved role of this HSP40 in cell size control but it is utilised differently in these distantly related yeasts (Jorgensen *et al*., 2002).

During the cell cycle of *S. pombe* no modification of Ssa1, Ssa2 or Mas5 was observed, this may be reliable but due to the method of detection requiring temperature sensitive mutations or HU treatment it is not conclusive evidence. In *S. cerevisiae*, the transcript levels of *YDJ1*, along with *SSA1* and *SSA2*, do not significantly change during the cell cycle (Spellman *et al*., 1998; Truman *et al*., 2012). A similar trend was observed for the *S. pombe* homologs using elutriation to study the cell cycle (Rustici *et al*., 2004). However, in *S. cerevisiae* a modification of Ssa1 has been linked to progression through Start (Truman *et al*., 2012). The similarities between Ydj1 and Mas5 are striking and suggest that this HSP40 plays a critical role in the cell growth and division of both organisms but is utilised in different ways.

In other eukaryotes, preliminary studies of mammalian homolog of Ssa1 Hsc70 have shown a conserved phosphorylation event, this modification was also correlated to Hsc70 binding of

cyclin D1 (the mammalian homolog of *S. cerevisiae* Cln3). This could excitingly reveal these HSPs to be fundamental for all eukaryotes to adapt to stress conditions and regulate of the cell cycle. In terms of HSP70/HSP40 roles in cell growth it would be interesting to study an organism with well characterised growing end proteins to observe if HSP70/HSP40 function is conserved.

In addition to the cell growth data, there appears to be a conserved role for Ydj1/Mas5 in microtubule reorganisation during mitosis. *S. cerevisiae* Ydj1 appears to be required for efficient microtubule organisation particularly during the budding process (Makhnevych *et al*., 2012; Oka *et al*., 1998). The evidence presented here suggests Mas5 is involved in tubulin dynamics during mitosis, this would suggest conservation of Mas5 role in cell division but also in an organism specific manner. However, the SAC Mad2 data presented here would suggest that Mas5 is not involved in activation of the SAC (at least via Mad2). The SAC can be activated in a Mad2-independent manner so it is possible Mas5 may act on this pathway. Interestingly, in *S. cerevisiae* deletion of *BUB3*, the homolog of *mad2*$^{+}$, is lethal with *ydj1*Δ (Mas5 homolog) (Daniel *et al*., 2006). It would be interesting to see if an *S. pombe bub3*Δ*mas5*Δ strain could be constructed.

In contrast to *S. cerevisiae*, much less is known about the roles and regulation of the homologous proteins Ssa1, Ssa2 and Mas5 (Ydj1 homolog) in *S. pombe.* However, during this investigation a study in *S. pombe* proposed that the heat stress regulation of the cell growth was linked to function of the HSF Hsf1 modulated by Ssa2/Mas5 (Vjestica *et al*., 2013). Their model proposed Ssa2/Mas5 binds Hsf1 and inhibits Hsf1-dependent gene expression in normal conditions. However, upon heat stress Ssa2-Mas5 derepresses Hsf1, triggers Hsf1-dependent transcription, which consequently leads to cell cycle delay. The data presented in the *S. pombe*

study used deletion strains to investigate the roles of Ssa2 and Mas5 in cell division. It has been shown here that Mas5/Ssa2 localises to cell ends and regions of NETO while Ssa1 appears to be ubiquitous throughout the cell. The localisation of these proteins suggests that either Hsf1 modulation requires recruitment of these proteins from NETO regions or Hsf1 is sequestered to these regions as part of the HSP modulation. Analysis of unstressed *ssa2*Δ cells and *mas5*Δ cells showed increased mRNA levels of *hsf1*$^{+}$ dependent genes associated with heat stress (Vjestica *et al*., 2013). Therefore, in the absence of Mas5 it is suggested that Hsf1 is active and leading to monopolar growth.

We have shown here that the key co-ordinating protein of the polarisome Tea1 is not mis-localised in the absence of Ssa1 or Mas5. Mas5 has been shown to interact with Tea1 which corroborates the localisation of Mas5 in this study (Snaith *et al*., 2011). This suggests that there may be more to Hsf1 regulation by Ssa2/Mas5 and the possible link to NETO modulation. However, it raises the question of the role of the interaction between Mas5 and Tea1. It has been proposed that Mas5 in complex with Ssa2 regulates Hsf1 transcription to allow NETO to proceed (Vjestica *et al*., 2013). It is possible that the interaction of NETO factors with Mas5 allows regulation of cell growth signalling to Hsf1 activity. This would lead to the conclusion that in *S. pombe* Mas5 and Ssa2 may have a role in promoting the cell cycle possibly via modulation of Hsf1 and/or NETO. However, no complex between Mas5 or Ssa2 and Hsf1 has been detected.

In the published *S. pombe* study of Hsf1 and NETO, Ssa1 was not investigated beyond heat stress survival analysis of *ssa1*Δ cells. The discovery that Ssa1 is a regulator of cell size parameter suggests that it is involved in cell growth but whether this is linked to Hsf1 and NETO like Mas5/Ssa2 appears to be remains unclear.

The work presented here suggests that Mas5/Ssa2 acts as an inhibitor of cell growth and cell size while Ssa1 may be an activator of the cell size parameter. The published data in *S. pombe* used deletion strains similarly to the work shown here but the method of investigation and the parameters resulted in a very different conclusion of the roles of Ssa1, Ssa2 and Mas5 in the cell cycle (Vjestica *et al*., 2013).

To rationalise the published data with the work presented here there are several different possibilities. Firstly, it is possible that their model of Ssa2/Mas5 complex regulating Hsf1 is correct but the complex has an additional role of chaperoning proteins during cell growth. Secondly, it may be that Ssa2/Mas5 does regulate Hsf1 by localising Hsf1/Ssa2/Mas5 to the cell ends and this acts to signal to gene expression depending on external stimulus sensing. The third option, Ssa2/mas5 binds Hsf1 somewhere unknown in the cell and upon a certain signal Hsf1 is released to the nucleus and Ssa2/Mas5 localise to the growing tip. It is possible that Ssa1 competes with Ssa2 for binding of Mas5 which is part of this Hsf1 release mechanism, it was shown that both Ssa1 and Ssa2 interact with Mas5 (Vjestica *et al*., 2013). In normal conditions, in the cytoplasm Ssa1 and Ssa2 can compete for Mas5 binding which may determine Hsf1 activation. Thus, in an *ssa1*Δ mutant there is no competition for Mas5 binding to Ssa2 so Hsf1 is more repressed in this strain. This implies that that in the absence of Ssa1 that the Ssa2/Mas5 complex does not release Hsf1 so activation of Hsf1 is not optimal in this strain.

Recent work has started to find a co-ordination between the heat shock response and autophagy, primarily that in mammalian cells the activation of HSF or induced expression of HSP70 leads to an attenuation of autophagy (Dokladny *et al*., 2013). Novel results in this thesis suggest in *S. pombe* Mas5 to a role in autophagy. However, whether Mas5 is directly affecting autophagy

proteins or via regulation of factors in the heat shock protein system is not clear. Analysis of Atg8 in *mas5*Δ cells suggests there is a global issue to autophagy regulation. Evidence suggests that in *S. pombe* Hsf1 is regulated by Ssa2/Mas5. Hsf1 is the key regulator of the heat shock response within the cell. Therefore, in the absence of Mas5 it is possible that Hsf1 de-repression is responsible for the misregulation of autophagy in this strain.

S. pombe nutrient sensing can activate meiosis, this makes meiosis and autophagy inherently linked. The fact that there is a defect in autophagy and meiosis in the absence of Mas5 could indicate that these phenotypes are linked. Alternatively, these phenotypes could stem from separate roles Mas5 typically plays within the cell. HSP40 DnaJ proteins have a range of motifs (see Introduction section 1.2.2.1) that have been linked to the functionality of the protein. However, the function of each motif within Mas5 is unknown. It would be useful to generate strains expressing Mas5 with motifs excluded from its sequence and see if any of the phenotypes observed in *mas5*Δ cells occur as it could clarify the extent of the phenotypes observed are through Mas5 directly or through effecting binding of other proteins such as possibly Ssa1, Ssa2 or Hsf1.

In summary, I have constructed several strains including *ssa1*Δ, *ssa2*Δ and *mas5*Δ mutants. Initial investigations were undertaken to characterise and identify novel phenotypes such as localisation of epitope tagged Ssa1, Ssa2 and Mas5 and cell cycle regulation of these proteins. The next step is to investigate the potential roles of Ssa1/Ssa2 and Mas5 in the response of cells to oxidative stress in the next Chapter.

CHAPTER 4

The roles of heat shock proteins in regulating Hsf1 response to heat and H_2O_2

4.1 Introduction

The HSP system is an ancient evolutionarily conserved mechanism of protein chaperoning and degradation which is essential in proteostasis. The main transcription factor regulating HSP transcription is the heat transcription factor (HSF) as discussed previously (see Introduction section 1.3.4). HSFs in eukaryotes have been shown to respond to various cellular stresses including heat and H_2O_2. However, there is little mechanistic evidence of how HSF is regulated to result in an appropriate response to the specific stress condition. Models suggests a role for certain HSPs in Hsf1 activation but the mechanism/function remains unclear. The role of HSP in regulation of HSF in response to heat and H_2O_2 will be the focus of this chapter.

During this project a study was published using *S. pombe* deletion analysis of *ssa2*Δ cells and *mas5*Δ cells that showed Hsf1 was transcriptionally active in unstressed conditions (Vjestica *et al*., 2013). In contrast to *S. cerevisiae*, *S. pombe* divide by fission from the septum and grow at the 'old' end eventually increasing growth at the 'new' end, this is termed new end take off (NETO) (Martin and Chang, 2005; Moser and Russell, 2000). The mechanism of regulating cell cycle control to stall NETO in response to heat stress is not clear. However, recent work proposed that the heat stress regulation of the cell growth was linked to Hsf1/Ssa2/Mas5 function (Vjestica *et al*., 2013). In their model Ssa2/Mas5 binds Hsf1 and inhibits Hsf1-dependent gene expression in normal conditions. However, upon heat stress Ssa2-Mas5 releases

Hsf1, triggers Hsf1-dependent transcription, which consequently leads to cell cycle delay. It is relevant to note however, that the gene expression that contributes to the cell cycle regulation has not been characterised. Furthermore, the model is based on gene deletion analysis and co-immunoprecipitation so the regulation is not fully understood. Nonetheless the data suggest that Hsf1 regulation by Ssa2-Mas5 contributes to cell cycle regulation by heat stress. Consequently, given the studies in *S. cerevisiae* and this recent study in *S. pombe* it would be interesting to investigate further how the conserved proteins HSP70 Ssa2 and HSP40 Mas5 function and are regulated in the stress response of these distantly related organisms.

There were limited data from *S. pombe* cells showing how Hsf1 localisation is affected by stress. *S. pombe* cells are typically cultured between 25 °C and 30 °C. In the published study cells were grown in liquid media at 24 °C which initially raises concerns that cells may sense this as temperature stress (Vjestica *et al*., 2013). If viewed as a temperature stress due to cells being outside their niche range, then it could potentially affect the heat stress response orchestrated by Hsf1. A supplementary image of the study showed that cells grown at 24 °C for 20 hours have nuclear Hsf1-GFP. As it is disputable whether this temperature is a stress on the cells or not the only reliable conclusion that can be made from this data is that Hsf1 is detectable in the nucleus of exponential phase cells at 24 °C. Transfer of *S. pombe* cells to 37 °C for 45 minutes resulted in nuclear exclusion. The number of cells shown for the figure is very limited thus undermining the ability for analysis by the reader. However, it does suggest that localisation of the transcription factor is responsive to heat and that it displays stress inducible nucleo-cytoplasmic shutting. Thus *S. pombe* rather than *S. cerevisiae* may be a more relatable organism to understand Hsf1 regulation in stress response of eukaryotes.

Having established that Ssa1, Ssa2 and Mas5 affect the regulation of several fundamental processes and revealed similarities and differences with *S. cerevisiae* the next step was to investigate Hsf1, Ssa1, Ssa2 and Mas5 in responses to heat and oxidative stress. The main questions of Chapter 4 are: is Hsf1 nuclear localisation and/or activation of gene expression stimulated by heat and/or oxidative stress (H_2O_2). If yes what are the functions/regulation of Ssa1, Ssa2 and Mas5 for Hsf1 localisation/activity? *S. pombe* is an excellent model as Hsf1 nuclear localisation affected by stress unlike *S. cerevisiae*. Also, studying *S. pombe* allows insights into the similarities and differences in Hsf1 regulation by oxidative stress compared to *S. cerevisiae* which will reveal organism-specific regulation and conserved regulation.

4.2 Results

4.2.1 Mas5 may have a role in H_2O_2 stress response

It has previously been shown in this study that Mas5 is required for growth in sub-optimal temperatures (see Chapter 3 section 3.2.1). However, the role of Ssa1, Ssa2 and Mas5 in oxidative stress response has not been investigated in fission yeast. Thus, the initial step was to investigate if Ssa1, Ssa2 or Mas5 play a role in H_2O_2 stress response or heat of *S. pombe*. To begin to test this, the deletion strains used previously, *ssa1*Δ and *mas5*Δ, were exposed to H_2O_2. As discussed previously, it was not possible to investigate the phenotypes of *ssa2*Δ cells (see section 3.2.1). The number of *ssa1*Δ colonies formed upon 1 mM H_2O_2 containing media is similar to wild type colony formation (Fig. 4. 1. A). In contrast, *mas5*Δ colonies appear sensitive to the same H_2O_2 stress as fewer colonies are formed following exposure to 1 mM H_2O_2 in comparison to wild type colony formation (Fig. 4. 1. A.). This suggests that Mas5 has a role in the oxidative stress response pathway during chronic oxidative stress exposure.

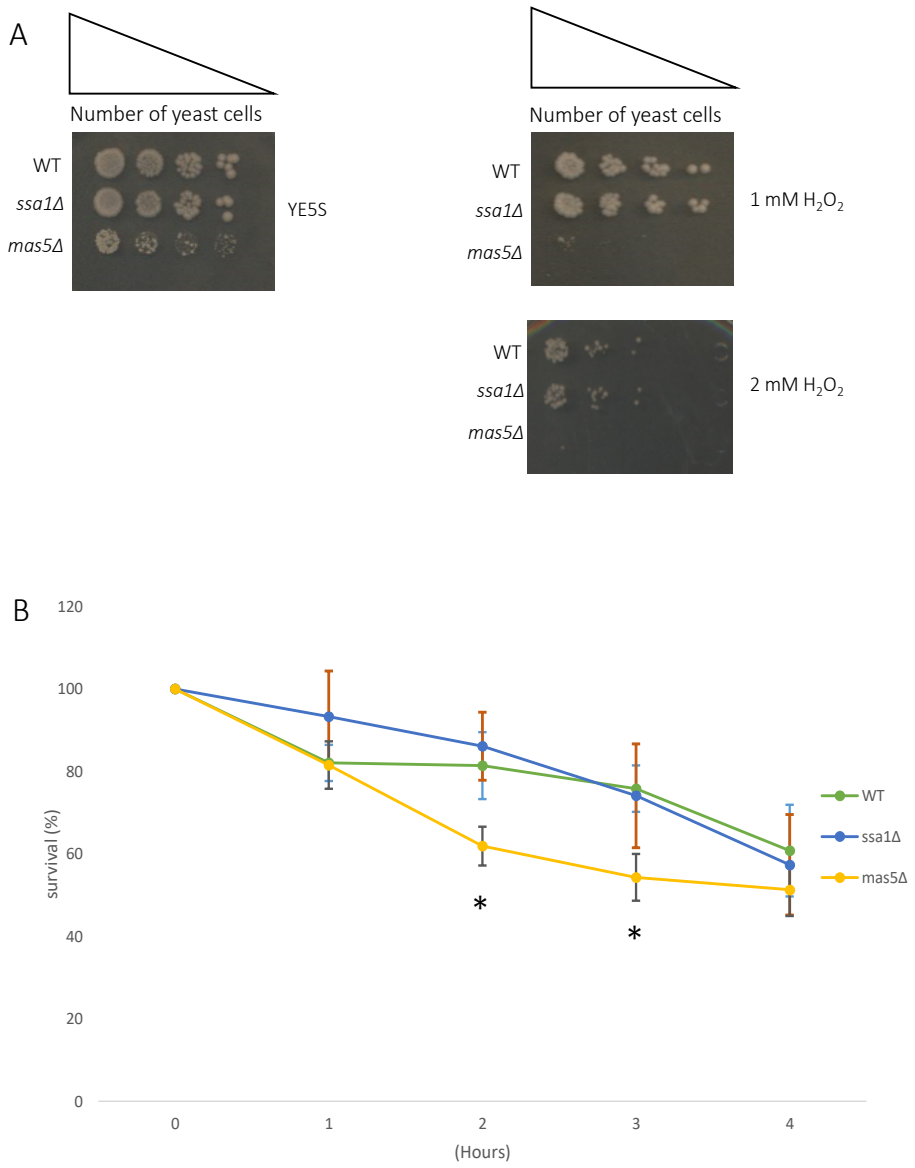


Fig. 4. 1. Viability of *mas5*Δ cells in the presence of H_2O_2 is affected differentially in response to the degree of stress. (A) Wild type (WT) (CHP429), *ssa1*Δ (FM14) and *mas5*Δ (FM15) cells were grown to exponential phase and serial diluted to the same OD_{595}. Cells were plated to YE5S solid medium containing/exposed to 1 mM and 2 mM H_2O_2. Plates were imaged every 24 hours, images shown were taken after 96 hours, n > 3. (B) The same strains were grown to exponential phase and serial diluted to plate 200 cells on YE5S solid medium in triplicate. Cells were exposed to 25 mM H_2O_2 and plated on to YE5S every hour for 4 hours. The YE5S solid medium were incubated for 96 hours and the number of colonies on each plate were recorded to generate the average survival percentage of each strain, n > 3. * indicate statistically significant data points from wildtype survival percentage (Two-way ANOVA, $p < 0.05$).

Cells activate difference stress response pathways to acute and chronic oxidative stress (see section 1.2.1.2.1). To determine if *ssa1*Δ cells or *mas5*Δ cells are sensitive to acute oxidative stress the strains were grown to exponential phase and exposed to an acute stress of 25 mM H_2O_2 during a survival assay (See Chapter 2 section 2.2.6). Using a two-way ANOVA analysis, it was determined that *ssa1*Δ cells survival are not significantly different to wild type cells upon exposure to acute H_2O_2 stress (Fig. 4. 1. B). However, *mas5*Δ cells survival were determined to be significantly lower than wild type cells after exposure to acute H_2O_2 stress after 2 and 3 hours (Fig. 4. 1. B). Taken together these data suggest that Ssa1 does not appear to have a role in cell survival or cell growth in response to H_2O_2 stress. However, it would appear that Mas5 may have a role in H_2O_2 stress sensing. Whether this role of Mas5 is dependent on Hsf1 is unknown.

4.2.2 Strain generation to investigate Hsf1

One of the aims of this project was to investigate if Ssa1, Ssa2 or Mas5 have a role in Hsf1 regulation in *S. pombe*. To investigate Hsf1 in *S. pombe* cells a strain was donated that expresses Hsf1 from the chromosomal locus with a GFP epitope (Vjestica *et al*., 2013). It needed to be confirmed that in this strain the native *hsf1*$^+$ locus was upstream of the GFP sequence. A PCR was set up with an internal forward primer recognising *hsf1*$^+$ sequence and a reverse primer that recognises the GFP sequence. Wild type genomic DNA and the donated strain were used as template DNA for the PCR reaction. PCR generated sequences were separated by gel electrophoresis and visualised with UV radiation. As is seen a PCR product is generated in the donated strain indicating *hsf1*$^+$ is upstream of a GFP sequence (Fig. 4. 2. A) The PCR product was sent for sequencing and confirmed to encode the C-terminal of Hsf1 followed by the GFP sequence (data not shown).

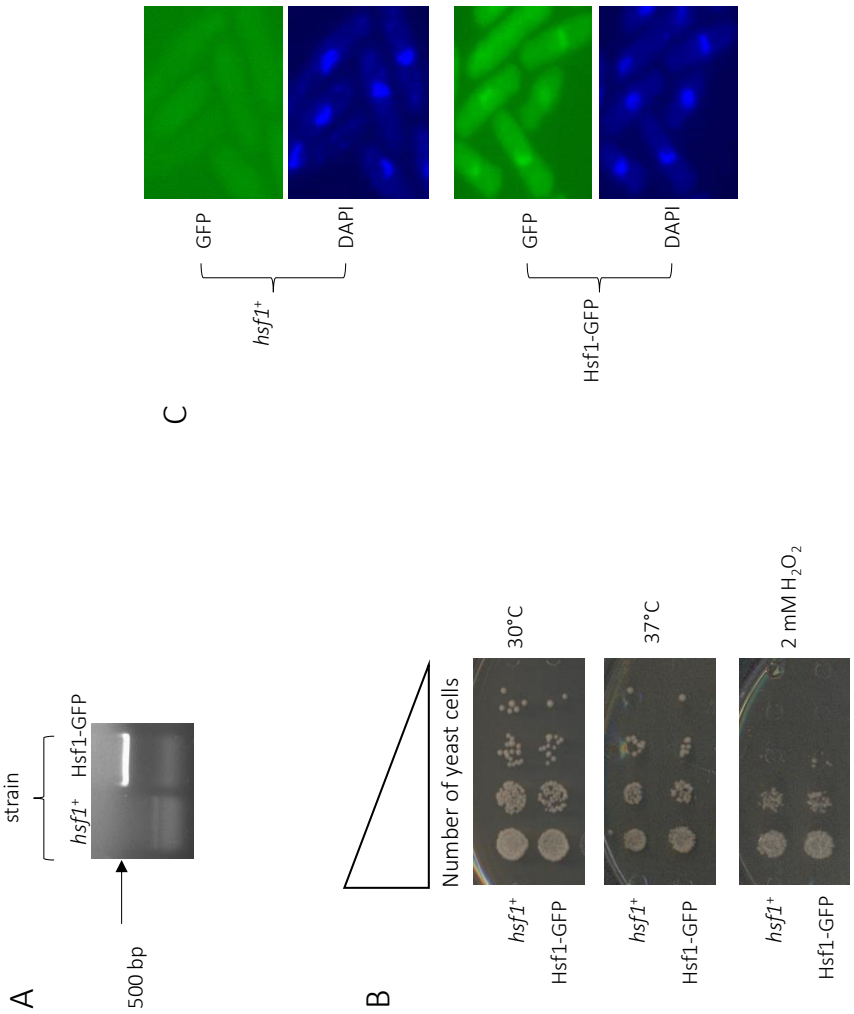


Fig. 4. 2. Strain confirmed to express Hsf1-GFP and no phenotypes detected from the presence of the GFP epitope. (A) A PCR of wild type (CHP428) and Hsf1-GFP (SO5672) encoding genome was set up with an internal forward *hsf1*$^{+}$ primer (Hsf1.Chk.F) and a reverse GFP sequence primer (GFP.R) were used to confirm the presence of the GFP sequence at the C-terminal of the *hsf1*$^{+}$ gene from strain using gel electrophoresis. The Hsf1-GFP strain was crossed to wildtype cells (CHP428), selection plates of *ura*$^{+}$ *his*$^{-}$ colonies were confirmed to contain Hsf1-GFP (FM40) by PCR, n = 2. (B) Strains were grown to midlog and serial diluted to the same OD_{595}. Cells were plated to YE5S solid medium containing/exposed to the stress indicated. Plates were imaged every 24 hours, images shown were taken after 72 hours, n > 3. (C) Microscopy confirmed detection of Hsf1-GFP. GFP (fluorescent) and DAPI images of representative exponential phase cells expressing Hsf1-GFP (FM40). Cells were fixed using paraformaldehyde. Exposures remaining constant between images, n > 3.

The Hsf1-GFP strain was in a different wild type background than has been used in this study so the strain was crossed to wild type cells and sporulated. In the resultant colonies the Hsf1-GFP selection marker (*ura4*$^{+}$) was correlated to *his*$^{-}$ colonies and used to back cross to wild type cells to homogenise the genetic background in case variability had arisen in the original Hsf1-GFP source strain (data not shown). This method allowed the generation of a Hsf1-GFP that could be used to investigate the role of Hsf1 in stress response of *S. pombe* cells in this study.

The study aims to identify the role of Hsf1 *S. pombe* cells. The presence of the GFP epitope needs to be investigated to show it does not affect function. Spot tests as described previously were used to see if colony formation is affected by expression of Hsf1-GFP in cells. In unstressed conditions, the strain expressing Hsf1-GFP formed the same number of colonies as wild type cells (Fig. 4. 2. B). Exposure to 2 mM H_2O_2 results in the same sensitivity as wild type cells in terms of colony formation. This would suggest that if Hsf1 has a role in the oxidative stress response of *S. pombe* cells then the presence of the GFP moiety does not affect the stress response of the cell. This means the strain expressing Hsf1-GFP can be used as a model to investigate the role of Hsf1 in the stress response of fission yeast.

As discussed previously (see Introduction section 1.3.4.1), a known mechanism of Hsf1 regulation is nucleo-cytoplasmic shuttling. The localisation of Hsf1 in *S. pombe* has not been investigated in response to oxidative stress and only preliminarily in response to heat stress. To investigate the localisation of Hsf1 in *S. pombe* cells wild type cells and cells expressing Hsf1-GFP were examined by fluorescence microscopy. In unstressed cells Hsf1-GFP is detectable in dividing cells co-localising with DNA staining but in the majority of cells there is no distinct region of Hsf1-GFP localisation (Fig. 4. 2. C). While confirming the GFP moiety of Hsf1 is detectable in vivo in unstressed cells this also suggests Hsf1 is not constitutively nuclear. This

indicates *S. pombe* cells can shuttle the transcription factor out of the nucleus. This suggests similarity to mammalian Hsf1 localisation whereas budding yeast Hsf1 is constitutively nuclear. The conclusion would be that this initially appears to be a suitable strain and organism to investigate Hsf1 regulation.

4.2.3 Hsf1 regulation following exposure to temperature stress

Typically, *S. pombe* cells are cultured at 30 °C. The localisation of Hsf1 at 30 °C in *S. pombe* cells was not reported in the Vjestica *et al*. study (2013). Thus, having established that generated Hsf1-GFP strain was suitable to use, the next step was to investigate Hsf1 localisation in normal growth conditions followed by cold or heat stress.

At 30 °C the majority of cells show faint nuclear localisation while some cells show no noticeable nuclear localisation as determined by DNA staining (Fig. 4. 3. First column). This suggests that *S. pombe* cells within their niche temperature range do not require Hsf1 to be primarily localised to the nucleus. This is consistent with other published data for *S. pombe* Hsf1 localisation at 30 °C (Matsuyama *et al*., 2006; Ohtsuka *et al*., 2011).

To investigate Hsf1 localisation during temperature stress cells expressing Hsf1-GFP were grown to exponential phase at 30 °C and rapidly transferred to cold stress (25 °C) or heat stress (37 °C). Following rapid transfer to 25 °C there is no major change in Hsf1-GFP localisation until 40 minutes when a strong nuclear localisation is observed in all cells (Fig. 4. 3. A). This localisation is maintained at 60 minutes. In contrast, rapid transfer from 30 °C to 37 °C shows strong Hsf1-GFP nuclear localisation immediately after the temperature shift and is maintained over the time course (Fig. 4. 3. A). This suggests that in wild type cells Hsf1

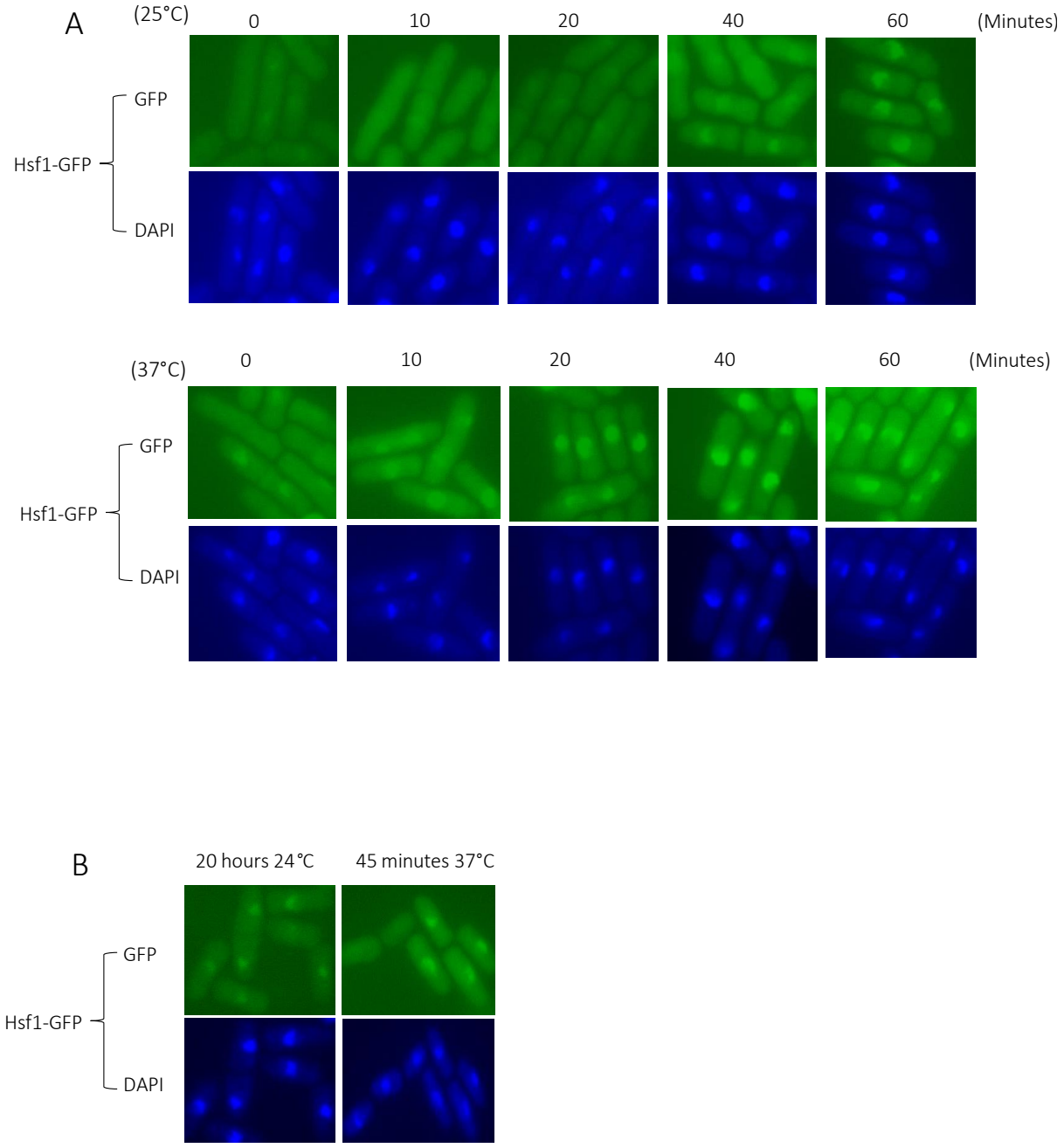


Fig. 4. 3. Hsf1-GFP localisation is sensitive to cold and heat stress. (A) Hsf1-GFP expressing cells (FM40) were grown to exponential phase at 30 °C and then exposed to 25 °C or 37 °C for 10, 20, 40 or 60 minutes, n = 3. (B) Alternately, the same strain was grown for 20 hours at 24 °C followed by an exposure to 37 °C for 45 minutes. GFP (fluorescent) and DAPI images of representative cells. Cells were fixed using paraformaldehyde and exposure remaining constant between images, n = 3.

localisation is regulated by the temperature the cells are exposed to. Interestingly, it suggests there is differential regulation of Hsf1 localisation in response to hot or cold stress with the former resulting in rapid and sustained nuclear accumulation while the latter has a delayed phenotype of nuclear accumulation. It also suggests a requirement for Hsf1 activity beyond the niche temperature range whether that is a reduction or an increase in heat. How the cells sense the temperature shift and the mechanism of Hsf1 shuttling remains to be elucidated.

However, it was found that treatment of *S. pombe* cells with a drug that modifies the exportin Crm1, leptomycin B, resulted in Hsf1 accumulating in the nucleus (Matsuyama *et al*., 2006). This implies Hsf1 cycles between the cytoplasm and the nucleus in unstressed cells.

The published data for *S. pombe* cells Hsf1 localisation in various temperature conditions was limited. To validate the published data and the generation of the Hsf1-GFP strain in this study the experiment of observing Hsf1 localisation was replicated. From the publication, a supplementary image showed that cells grown at 24 °C for 20 hours have nuclear Hsf1-GFP (Vjestica *et al*., 3013). Transfer of cells to 37 °C for 45 minutes resulted in nuclear exclusion. In this study, cells grown at 24 °C for 20 hours have nuclear Hsf1-GFP however there is a minority which do not have a nuclear localisation. Following transfer of cells to 37 °C for 45 minutes the majority of cells have a strong nuclear localisation but a portion of the cells have nuclear exclusion (Fig. 4. 3. B).

The data presented here contradicts the published data by Vjestica *et al*. (2013). However, in the published data only 2 cells are shown as examples of Hsf1 localisation which seems a limited representation. Additionally, the cultures were initially grown at 24 °C which could be considered a cold stress upon *S. pombe* cells. As Hsf1 is a key regulator the heat stress response

then growth of cells under “cold stress” conditions could affect the localisation of Hsf1, as demonstrated in Fig. 4. 3. B. Therefore, the data shown here may be a more accurate representation of Hsf1 localisation/regulation during heat stress response and thus further investigation is required.

The published *S. pombe* study identified Ssa2 and Mas5 as negative regulators of Hsf1 activity that were affected by heat stress (Vjestica *et al*., 2013). In the absence of either Ssa2 or Mas5 Hsf1 was determined to be derepressed, Hsf1 was reported to be constitutively active in these cells. However, the mechanism of Hsf1 repression by these HSPs and how they sense stress remains unclear. Therefore, further experiments are required to elucidate the regulators of Hsf1 and their roles. To understand the regulation of Hsf1 in *S. pombe* cells strains were generated lacking *ssa1*$^{+}$ or *mas5*$^{+}$ and expressing Hsf1-GFP but a strain lacking *ssa2*$^{+}$ could not be reliably generated.

Exponential phase cells grown at 30 °C show Hsf1-GFP localisation to be comparable in wild type and *ssa1*Δ cells. Following temperature shift to 25 °C or 37 °C, Hsf1-GFP localisation in *ssa1*Δ cells displays the same profile as wild type cells (Fig. 4. 4 and Fig. 4. 5). Collectively, these data suggest that Ssa1 is not required to regulate Hsf1 localisation in response to temperature shifts. In the published study Ssa2 was proposed to be a repressor of Hsf1 while Ssa1 was not. The data presented here showed no observable deregulation of Hsf1 localisation in *ssa1*Δ cells which is consistent with the heat sensitivity tests showing no phenotypes (Fig. 3. 1. D), this could be due to redundancy of function within the Ssa family. Interestingly, under all conditions tested cells lacking *mas5*$^{+}$ have constitutively nuclear Hsf1-GFP (Fig. 4. 4 and Fig. 4. 5).

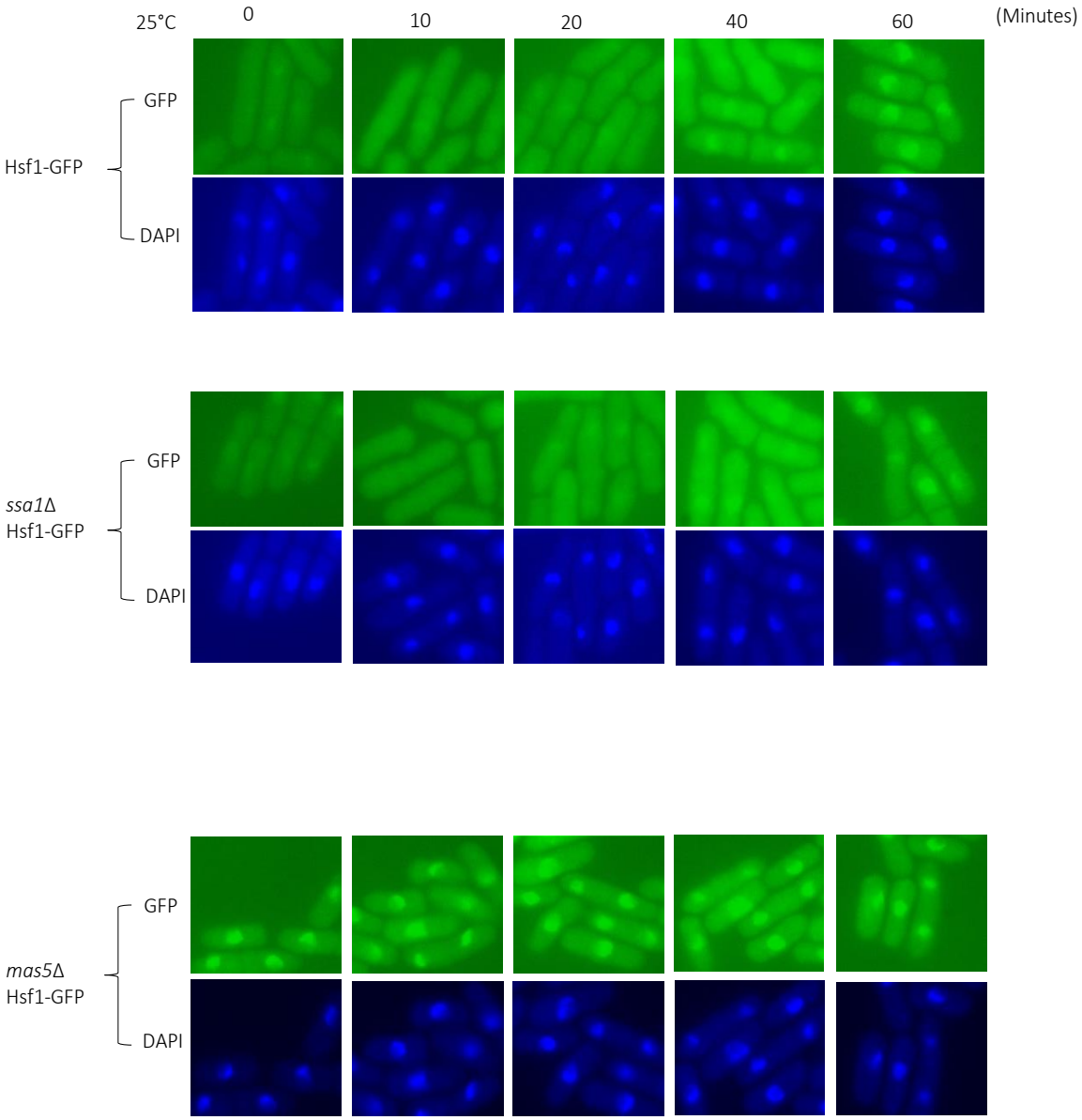


Fig. 4. 4. The absence of Ssa1 or Mas5 does not affect Hsf1-GFP localisation following cold stress. Cells expressing Hsf1-GFP (FM40) with either *ssa1*Δ (FM36) or *mas5*Δ (FM37) were grown to exponential phase at 30 °C then shifted to 25 °C for 10, 20, 40 or 60 minutes. GFP (fluorescent) and DAPI images of representative cells are shown, cells were fixed using paraformaldehyde; exposures remaining constant between images, n = 3.

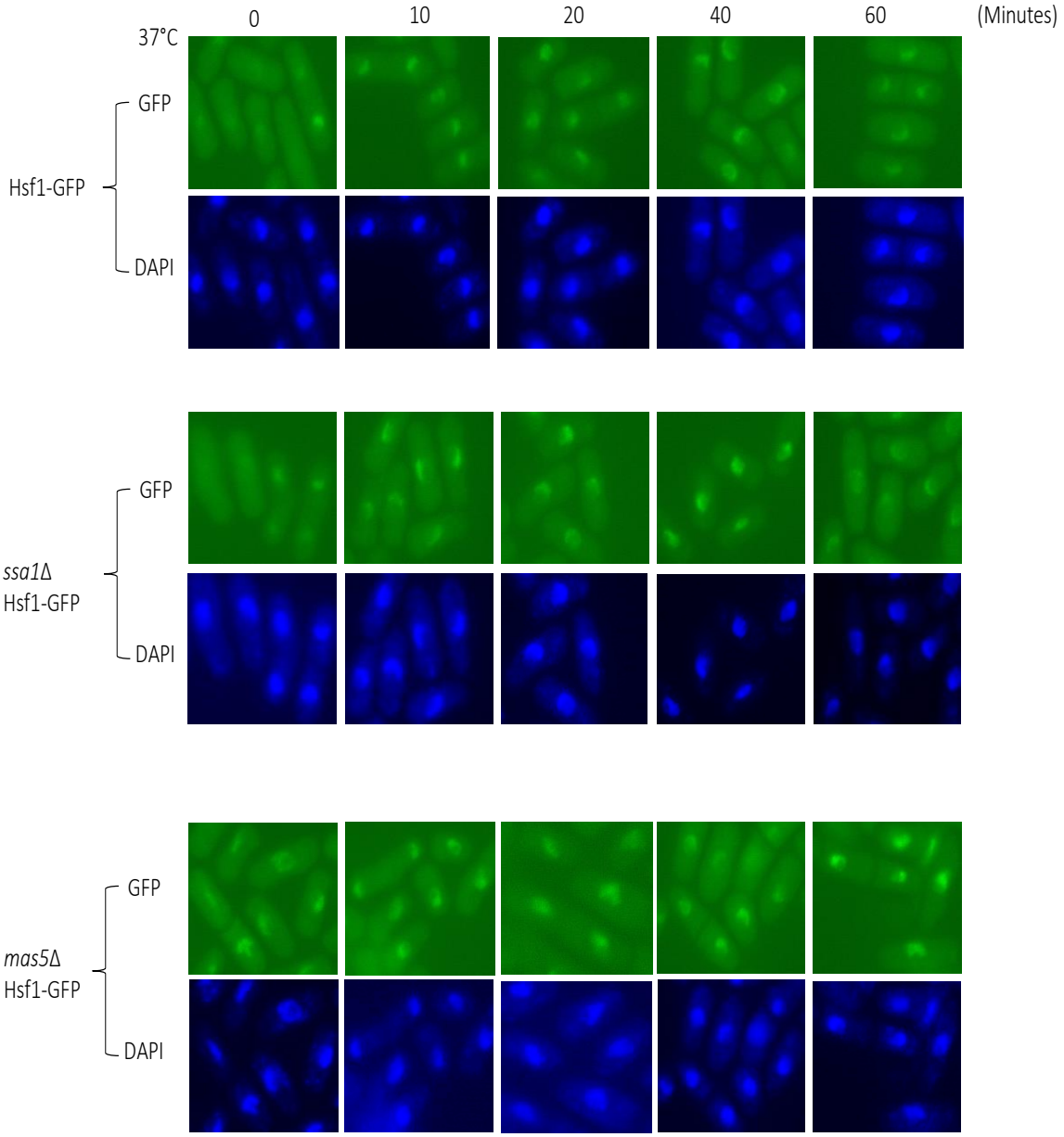


Fig. 4. 5. The absence of Ssa1 or Mas5 does not affect Hsf1-GFP localisation following heat stress. Cells expressing Hsf1-GFP with either *ssa1*$^{+}$ allele (FM36) or *mas5*$^{+}$ allele (FM37) and Hsf1-GFP cells (FM40) were grown to exponential phase at 30 °C and shifted to 37 °C for 10, 20, 40 or 60 minutes. GFP (fluorescent) and DAPI images of representative cells are shown, cells were fixed with paraformaldehyde; exposures remaining constant between images, n = 3.

This suggests that Mas5 is a temperature sensor, in its absence cells have an active heat stress response with nuclear Hsf1. An alternate interpretation, Hsf1 is repressed by Mas5 and in the latter's absence is no longer repressed and Hsf1 is maximally active as proposed in the published study (Vjestica *et al*., 2013). The heat sensitivity tests suggest *mas5*Δ cells are defective in their heat stress response (See Chapter 3 Fig. 3. 1. D), this could be consistent with deregulation of Hsf1 in these cells. However, this temperature sensitivity could be independent of a Hsf1 role. Further analysis of Hsf1 activity in this strain is required to clarify the function of Mas5 in this role. Mas5 as a co-chaperone is believed to exert its role by regulating larger chaperones, Mas5 is known to interact with HSP70s Ssa1 and Ssa2. It is possible that the role of Mas5 as a repressor of Hsf1 nuclear localisation is via regulation of Ssa1 or Ssa2.

4.2.4 Hsf1 localisation is responsive to H_2O_2 stress

Having established Hsf1-GFP nuclear localisation is induced by temperature stress, next was to address the question of whether Hsf1 regulation is important for oxidative stress responses to H_2O_2 like *S. cerevisiae*. In *S. cerevisiae*, researchers can only can look at activation of Hsf1-dependent gene expression as the protein is constitutively localised to the nucleus. However here we can look at both nuclear localisation and activation of gene expression. Previous work showed different transcriptional responses to H_2O_2 at low (0.2 mM) versus high (6 mM) H_2O_2 in *S. pombe* (see section1.2.1.2.1 and 1.3.2) so next it was investigated whether Hsf1 localisation is affected by low (0.2 mM), medium (1 mM) and high (6 mM) concentrations of H_2O_2.

Following exposure of cells to 0.2 mM H_2O_2 stress Hsf1-GFP shows a strong nuclear localisation (Fig. 4. 6. A). The nuclear Hsf1-GFP signal is strongest at 10/20 minutes and decreases after this over the time course. *S. pombe* activation of the transcription factor Pap1

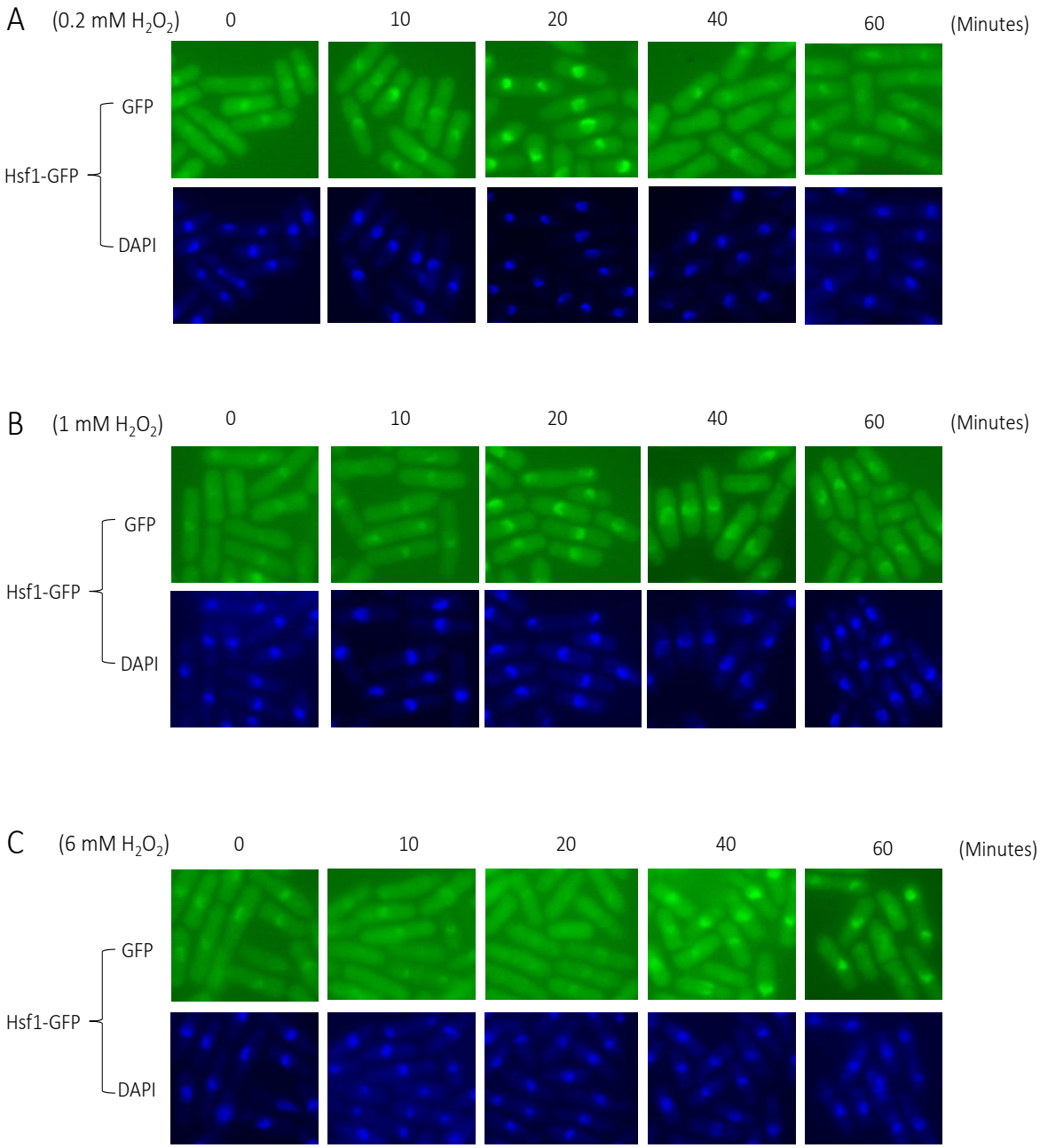


Fig. 4. 6. Hsf1-GFP localisation is sensitive to H_2O_2 stress, Hsf1-GFP localisation to the nucleus is delayed as the H_2O_2 concentration increases. Hsf1-GFP expressing cells (FM40) were grown to exponential phase at 30 °C and treated with 0.2 mM (A), 1 mM (B) or 6 mM (C) H_2O_2 stress for 0, 10, 20, 40 or 60 minutes. GFP (fluorescent) and DAPI images are shown of representative cells. Cells were fixed using paraformaldehyde, exposures remaining constant between images, n = 3.

shows the same nuclear accumulation in response to 0.2 mM H_2O_2 stress (Quinn *et al*., 2002). *S. pombe* has distinct stress response pathways activated depending on the concentration of H_2O_2. When cells are exposed to ≥ 1 mM H_2O_2 stress Pap1 remains inactive and able to exit the nucleus while the stress activated protein kinase regulated transcription factor Atf1 becomes more important. Having shown Hsf1 was responsive to 0.2 mM H_2O_2 the next question was to address if Hsf1-GFP showed a dose dependent localisation to the nucleus such as is observed for Pap1.

When cells expressing Hsf1-GFP were exposed to 1 mM H_2O_2 there is a delay in strong nuclear signal until 20 to 40 minutes (Fig. 4. 6. B). Exposure of the same strain to 6 mM H_2O_2 showed delay of nuclear entry until the final time point 60 minutes (Fig. 4. 6. C). Excitingly, this evidence suggests that Hsf1 localises to the nucleus in response to H_2O_2 stress indicating a sensing mechanism for H_2O_2 stress. However, it should be noted that localisation of Hsf1 to the nucleus does not necessarily indicate that it is transcriptionally active. However, this localisation is increasingly delayed as concentrations of H_2O_2 increase. This indicates that the nuclear accumulation of Hsf1 is regulated by H_2O_2 and that the sensing mechanism is responsive to the concentrations of H_2O_2. This is the same profile observed for Pap1 localisation of cells upon exposure to these concentrations of H_2O_2.

4.2.5 The role of Ssa1 and Mas5 in Hsf1 localisation following exposure to H_2O_2 stress

These data (see section 4.2.4) established that the nuclear localisation of Hsf1 is responsive to H_2O_2 and is concentration dependent. However, it is unknown if Ssa1 or Mas5 are involved in the regulation of Hsf1 localisation. Therefore, the potential roles of Ssa1 and/or Mas5 in this

regulation of nuclear localisation need to be investigated. In *S. cerevisiae* Ssa1 and Ssa2 are redundant for repression of Hsf1 and Ydj1 roles on Hsf1 have not been investigated (see Introduction section 1.3.4; Wang *et al*., 2012). Our data above suggested that loss of Ssa1 had no effect on localisation of Hsf1 by unstressed conditions and after cold and heat stress consistent with redundancy for Hsf1 regulation with Ssa2 in *S. pombe*. Also, we revealed a potential role for Mas5 in the oxidative stress response (see section 4.2.1).

To investigate the role of Ssa1 and Mas5 in Hsf1 localisation, *ssa1*Δ strain and *mas5*Δ strain expressing Hsf1-GFP were exposed to H_2O_2. In *mas5*Δ cells Hsf1-GFP shows a strong nuclear localisation in unstressed conditions, this was not altered by any concentration of H_2O_2 over the time-course (Fig. 4. 7.). This suggests that Mas5 acts as a repressor of Hsf1-GFP localisation to the nucleus in unstressed conditions. This is consistent with the previous *S. pombe* study that also concluded Mas5 is a repressor of Hsf1 (Vjestica *et al*., 2013).

In contrast, *ssa1*Δ cells show no strong nuclear accumulation in unstressed conditions, this appears to be like wild type cells (Fig. 4. 8.). Excitingly, after exposure of *ssa1*Δ cells to 0.2 mM H_2O_2 a strong nuclear accumulation is visible at 40, 60 minutes which is a delay compared to wild type cells (Fig. 4. 8. A). This delay in Hsf1-GFP nuclear accumulation in comparison to wild type cells is consistently seen as the H_2O_2 concentration increases (Fig. 4. 8.).

The study of *S. cerevisiae* Hsf1 response to oxidative stress determined Ssa1 acted as a repressor of Hsf1 in unstressed conditions. The role of Ssa1 in *S. pombe* Hsf1 regulation was not investigated by Vjestica *et al*. (2013). Data shown here suggest that Hsf1 localisation to the nucleus is differentially regulated between heat and oxidative stress. From the work shown here Ssa1 appears to act as a promoter of Hsf1 localisation to the nucleus only under oxidative stress

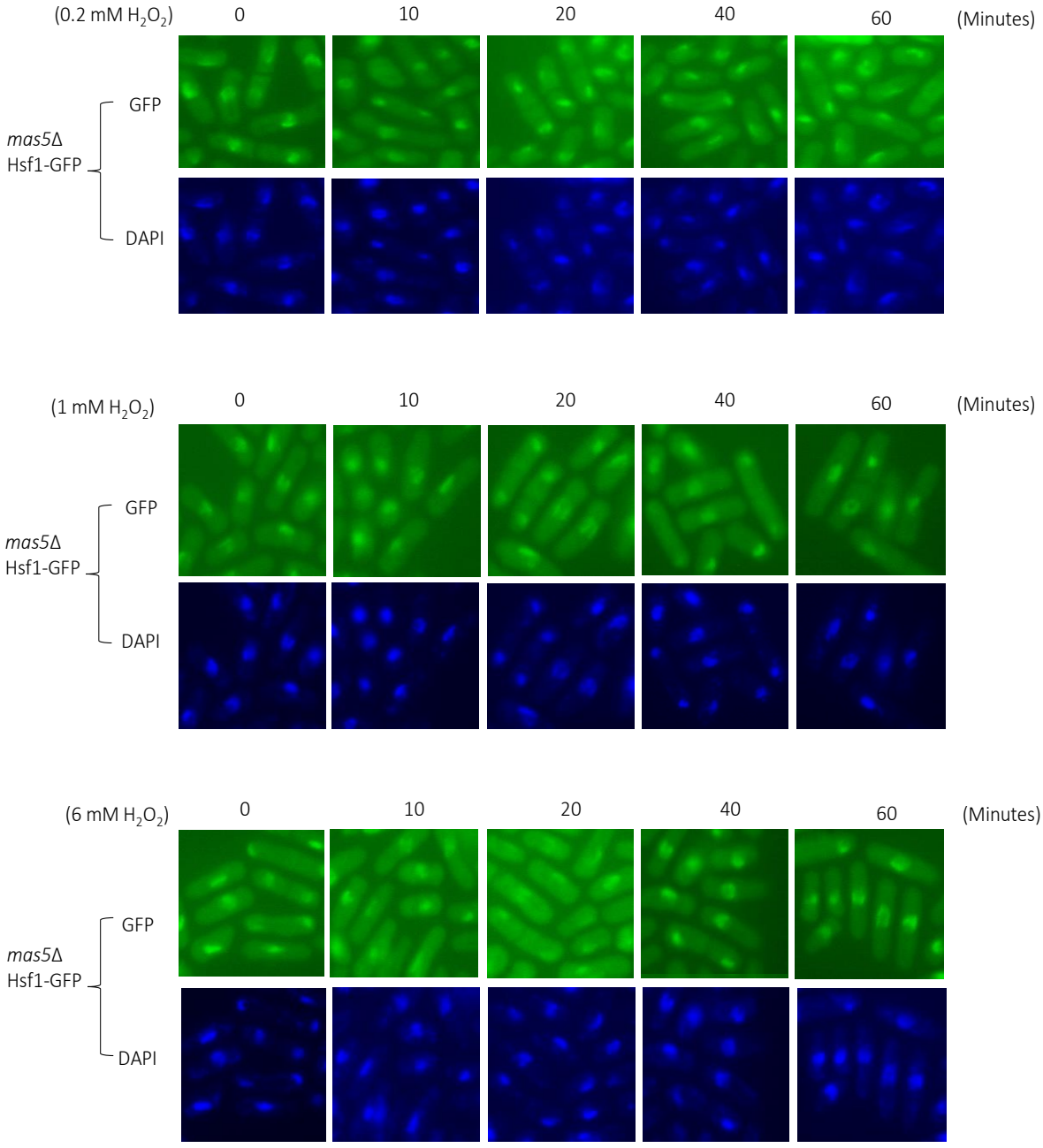
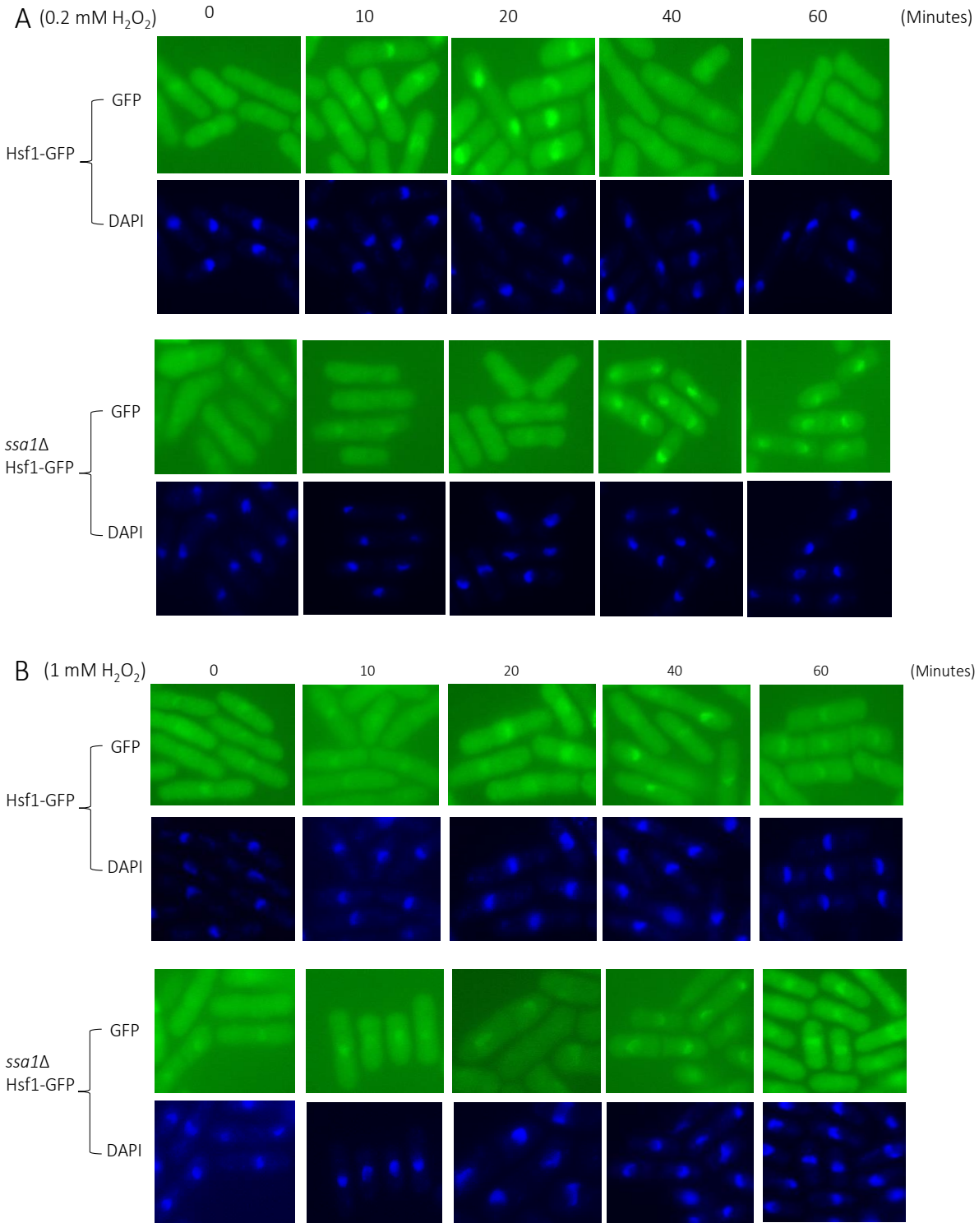


Fig. 4. 7. Hsf1-GFP localisation is unresponsive to H_2O_2 in the absence of Mas5. An *mas5*Δ strain expressing Hsf1-GFP (FM37) was grown to exponential phase at 30 °C and treated with 0.2 mM, 1 mM or 6 mM H_2O_2 stress for 0, 10, 20, 40 or 60 minutes. GFP (fluorescent) and DAPI images are shown of representative cells. Cells were fixed using paraformaldehyde, exposures remaining constant between images, n = 3.


A (0.2 mM H_2O_2)
0
10
20
40
60
(Minutes)
Hsf1-GFP
GFP
DAPI
ssa1Δ
Hsf1-GFP
GFP
DAPI
B (1 mM H_2O_2)
0
10
20
40
60
(Minutes)
Hsf1-GFP
GFP
DAPI
ssa1Δ
Hsf1-GFP
GFP
DAPI

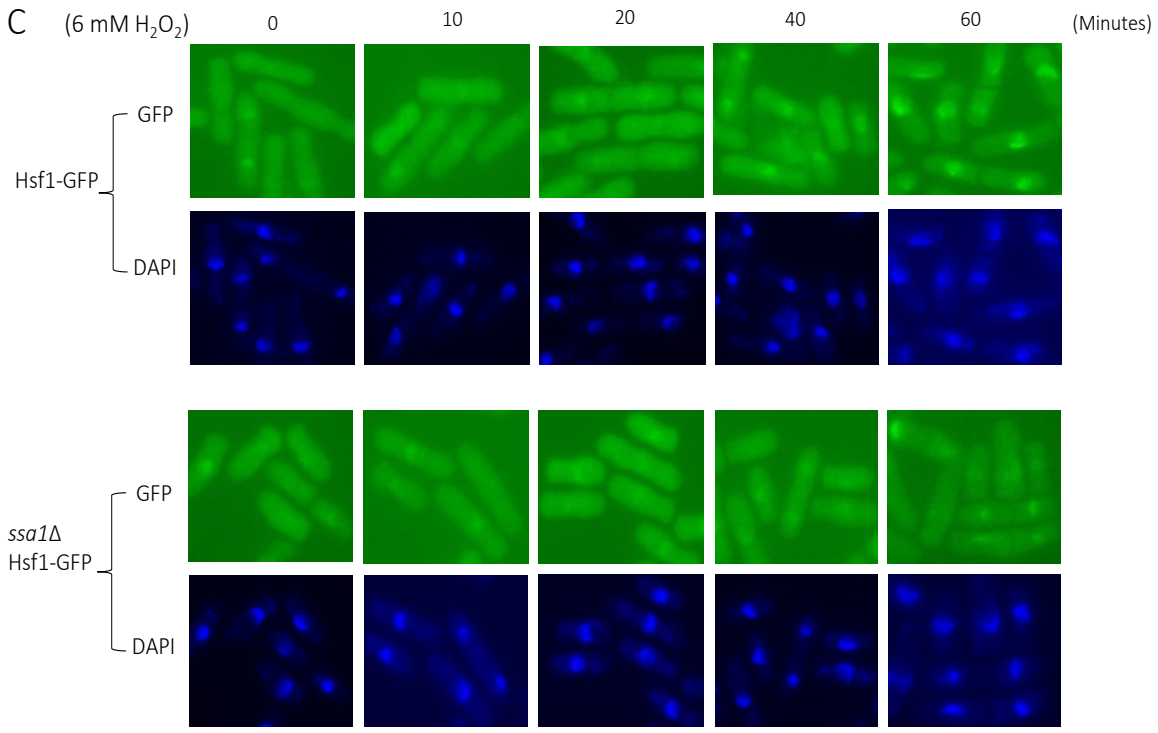


Fig. 4. 8. Hsf1-GFP localisation in response to H_2O_2 stress is delayed in the absence of Ssa1. Strains expressing Hsf1-GFP (FM40) along with *ssa1*Δ allele (FM36) were grown to exponential phase at 30 °C and treated with 0.2 mM (A), 1 mM (B) or 6 mM (C) H_2O_2 stress for 0, 10, 20, 40 or 60 minutes. GFP (fluorescent) and DAPI images are shown of representative cells. Cells were fixed using paraformaldehyde, exposures remaining constant between images, n = 3.

conditions while Mas5 acts as an inhibitor. These data would suggest that unlike cold/heat stress lack of Ssa1 increasingly delays localisation of Hsf1-GFP with increasing concentrations of H_2O_2. This contrasts with *S. cerevisiae* both in the idea that Ssa1 is a repressor in *S. cerevisiae* and redundancy of Ssa1 with Ssa2 in regulating Hsf1 in *S. cerevisiae*. It is worth noting that these data could be interpreted as an indirect effect of *mas5*Δ and/or *ssa1*Δ causing increased H_2O_2/damage but even that argument is not simple as the gene deletions have the opposite effect on Hsf1 localisation.

4.2.6 The role of Ssa1 and Mas5 on Hsf1 activation in response to H_2O_2 stress

Having established the effects of H_2O_2 on Hsf1-GFP localisation and explored the effects of loss of either Ssa1 or Mas5 on H_2O_2-induced localisation of Hsf1 next it was investigated how these effects on localisation translated into effects on Hsf1-regulated gene expression. Work focused on *hsp104*$^+$ levels as this is previously established to be a Hsf1-regulated gene in both budding and fission yeast (Amorós and Estruch, 2001; Vjestica *et al*., 2013). Expression of *hsp104*$^+$ was investigated at 0.2 mM and 6 mM H_2O_2 to indicate Hsf1 activation in response to low and high H_2O_2 stress.

Previous work suggested that large scale analyses of genes induced in *mas5*Δ and *ssa2*Δ cells were genes normally induced by heat but not H_2O_2 (Chen *et al*., 2003; Vjestica *et al*., 2013). However, the Chen *et al*. data (2003) only looked at 0.5 mM H_2O_2 after 60 mins. Subsequently, in the Chen *et al*. (2008) paper they found that heat shock genes tended to be induced following high H_2O_2 stress (6 mM) but repressed at lower concentrations (0.5 mM or low 0.07 mM – clusters 2 and 7 respectively in their analysis – see paper for details Chen *et al*., 2008). *hsp104*$^+$

was one of the genes identified to be repressed/activated in this manner. The hypothesis we propose is that Hsf1 is activated by high (6 mM) concentrations of H_2O_2 and at low levels (0.2 mM) repressed/not activated and that some mechanism represses Hsf1-dependent gene expression.

To test this hypothesis, the levels of *hsp104*$^+$ were investigated in wild type cells and *mas5*Δ and *ssa1*Δ strains. Following exposure to 0.2 mM H_2O_2, wild type and *ssa1*Δ cells show minimal induction of *hsp104*$^+$ over the time course (Fig. 4. 9. A). However, in *mas5*Δ cells the basal level of *hsp104*$^+$ is higher than wild type cells. Additionally, following exposure to 0.2 mM H_2O_2 shows slight induction over the time course. This is consistent with Hsf1 nuclear accumulation of Hsf1 in *mas5*Δ cells but also suggests a possible secondary activation pathway unlinked to the presence of Mas5.

In contrast to 0.2 mM H_2O_2 stress much more induction of *hsp104*$^+$ was observed in wild type and *ssa1*Δ cells following exposure to 6 mM H_2O_2 and again the *ssa1*Δ allele has little impact (Fig. 4. 9. B). In agreement with 0.2 mM H_2O_2 experiments the basal expression of *hsp104*$^+$ in *mas5*Δ cells is higher as expected however, in contrast with 0.2 mM H_2O_2 exposure a large induction is observed following 6 mM H_2O_2 (Fig .4. 9. B). These data imply Mas5 functions to repress Hsp104 but that perhaps Hsf1 is not fully activated until 6 mM H_2O_2 treatment. It suggests that there is another activation mechanism for Hsf1 that is independent of localisation of Hsf1. This could possibly be through other modifications of Hsf1. Alternatively, in *mas5*Δ cells Hsf1 is fully active in basal conditions but another transcription factor is needed for the *hsp104*$^+$ boost observed after 6 mM H_2O_2 stress. This is possible as this experiment can't distinguish between Hsf1-independent and –dependent effects on *hsp104*$^+$ after 6 mM H_2O_2 stress.

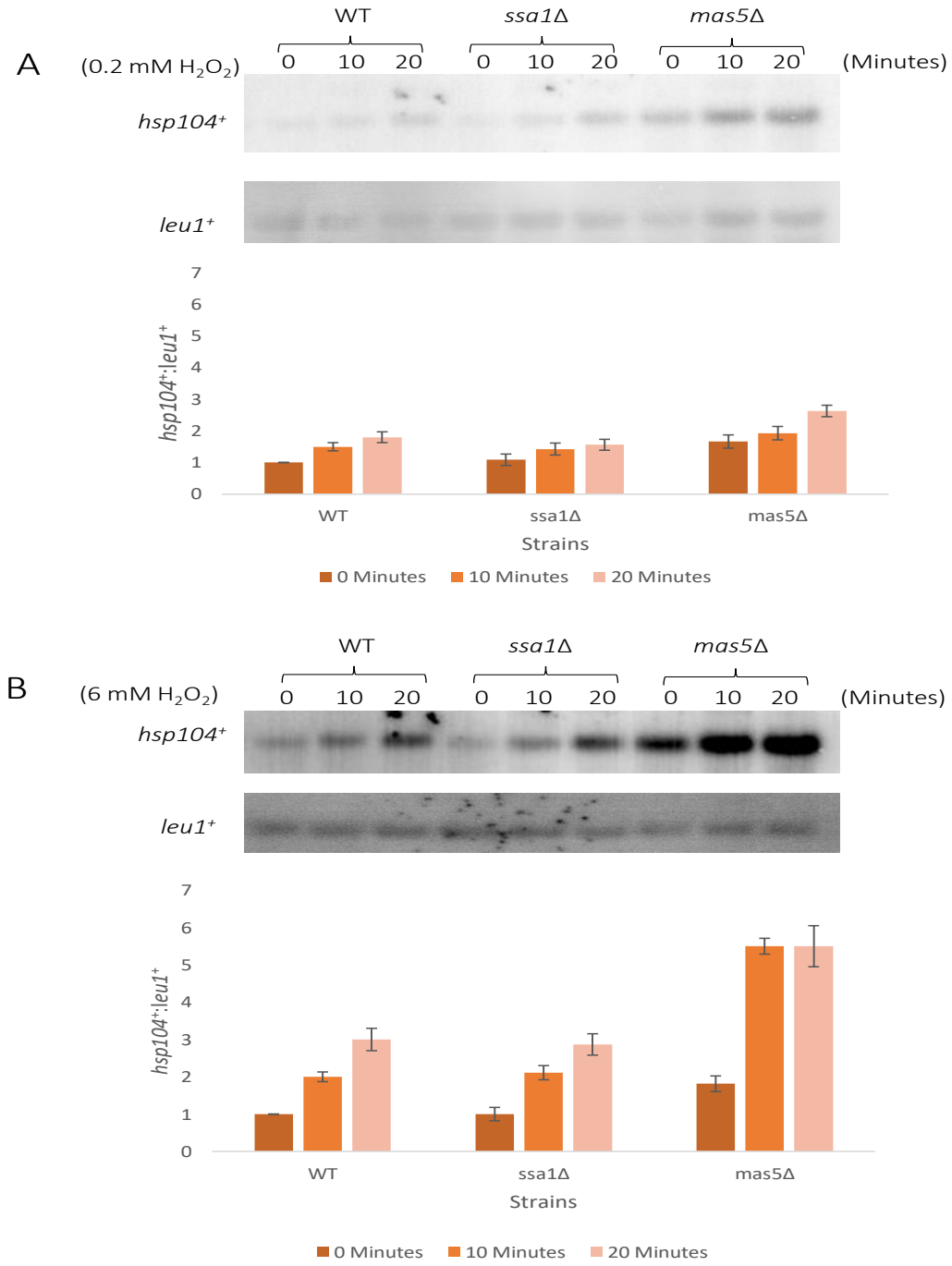
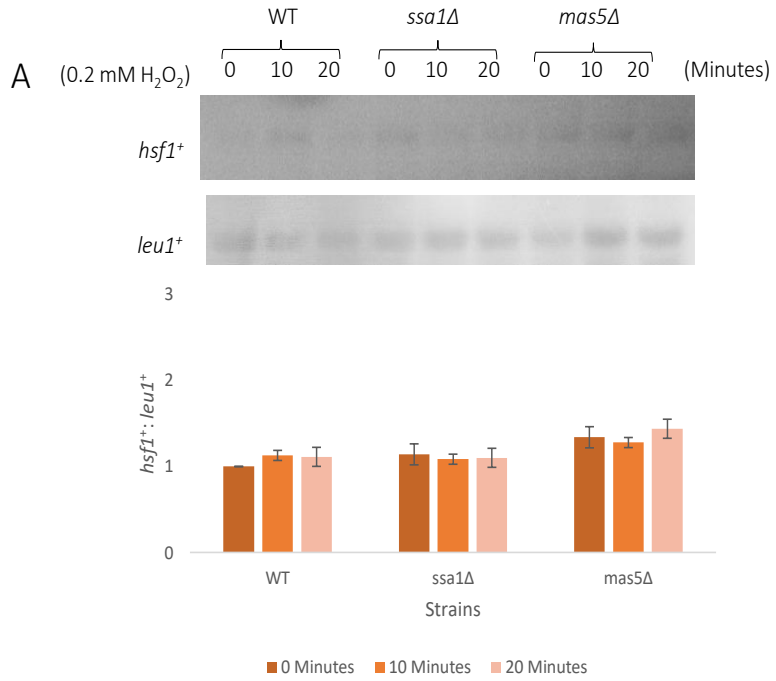


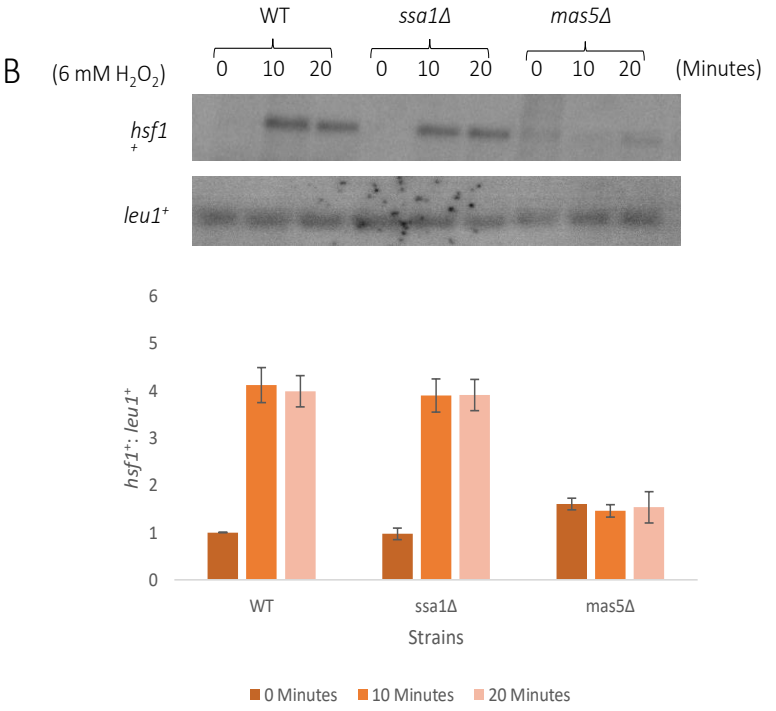
Fig. 4. 9. The effect of deletion of *ssa1*$^{+}$ or *mas5* $^{+}$ from cells upon *hsp104*$^{+}$ RNA levels following low (0.2 mM) and high (6 mM) H_2O_2 stress. Wildtype (WT) cells (CHP429), *ssa1*Δ (FM14) and *mas5*Δ (FM15) cells were grown to exponential phase and RNA was extracted following exposure to 0.2 mM (A) or 6mM (B) M H_2O_2 after 0, 10 or 20 minutes. Equal amounts of RNA were separated by gel electrophoresis and transferred to Genescreen hybridisation membrane. Following crosslinking the membrane was probed with α-P^{32} labelled PCR products of *hsp104*$^{+}$ (primers Hsp104.RNA.F and Hsp104. RNA.R) and *leu1*$^{+}$ (primers Leu1. RNA.F and Leu1.RNA.R). The membrane was exposed to a phosphoimager screen (Fujifilm). Data were analysed using a Typhoon FLA 3500 and quantified using ImageQuant TL software. n = 3, error bars indicate standard deviation.

In agreement with Chen *et al*. (2008) *hsf1*$^{+}$ stress expression data, in wild type cells there is minimal induction of *hsf1*$^{+}$ in response to 0.2 mM H_2O_2 stress but it is induced to a large degree following 6 mM H_2O_2 (Fig. 4. 10. A and B). *ssa1*Δ cells display a wild type *hsf1*$^{+}$ profile under non-stressed conditions and after 0.2 mM and 6 mM H_2O_2 treatment (Fig. 4. 10. A and B). This agrees with *hsp104*$^{+}$ data for the *ssa1*Δ strain (Fig. 4. 9. A and B). In the *mas5*Δ strain like *hsp104*$^{+}$, basal *hsf1*$^{+}$ level is up in both 0.2 mM and 6 mM H_2O_2 experiments before stress (Fig. 4. 10. A and B). This suggests *hsf1*$^{+}$ may regulate its own expression under normal conditions. However, following 0.2 mM and 6 mM H_2O_2 treatment, *mas5*Δ cells have constitutive higher but not inducible *hsf1*$^{+}$ expression (Fig. 4. 10. A and B). This is fundamentally different to *hsp104*$^{+}$ regulation whose basal is increased in *mas5*Δ cells but is inducible after 6 mM H_2O_2 stress (Fig. 4. 9. B). In *mas5*Δ cells *hsp104*$^{+}$ is still inducible at 6 mM H_2O_2 whereas *hsf1*$^{+}$ is constant (Fig. 4. 9. B and Fig. 4. 10. B). These data suggest that the regulation of *hsf1*$^{+}$ expression at high H_2O_2 concentrations may be through Mas5 regulation but that an alternative mechanism is important for *hsp104*$^{+}$.

The data presented here suggest that there is a distinct regulation of Hsf1 at high H_2O_2 concentration and thus it raised the question of whether a modification/induction of Hsf1 is detectable under these conditions. Hsf1 from various organisms is known to have multiple modifications however the location and function of these modifications remains unknown (see section 1.3.4). Therefore, the modification of Hsf1-GFP in unstressed and 6 mM H_2O_2 conditions was investigated by western blotting.

Hsf1-GFP expressing cells showed detectable forms with similar mobility to the 130 kDa molecular weight marker but there are numerous non-specific proteins detectable with the similar mobility (Fig. 4.10. C). Analysis of Hsf1-GFP in response to 6 mM H_2O_2 provides


A
(0.2 mM H_2O_2)
WT
ssa1Δ
mas5Δ
0 10 20 0 10 20 0 10 20
(Minutes)
hsf1+
leu1+
3
2
1
0
hsf1+: leu1+
WT
ssa1Δ
mas5Δ
Strains
0 Minutes
10 Minutes
20 Minutes


B
(6 mM H_2O_2)
WT
ssa1Δ
mas5Δ
0 10 20 0 10 20 0 10 20
(Minutes)
hsf1+
leu1+
6
5
4
3
2
1
0
hsf1+: leu1+
WT
ssa1Δ
mas5Δ
Strains
0 Minutes
10 Minutes
20 Minutes

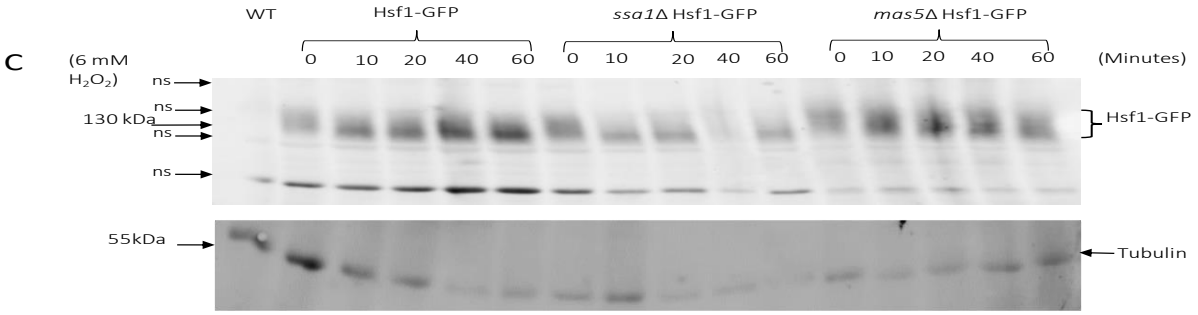


Fig. 4. 10. The effect of deletion of *ssa1*$^+$ or *mas5*$^+$ from cells upon *hsf1*$^+$ RNA levels following low (0.2 mM) and high (6 mM) H_2O_2 stress. Wildtype (WT) cells (CHP429), *ssa1*Δ (FM14) and *mas5*Δ (FM15) cells were grown to exponential phase and RNA was extracted following exposure to 0.2 mM (A) (n = 3) or 6 mM (B) (n = 3) H_2O_2 after 0, 10 or 20 minutes. Equal amounts of RNA were separated by gel electrophoresis and transferred to Genescreen hybridisation membrane. Following crosslinking the membrane was probed with α-P^{32} labelled PCR products of *hsf1*$^+$ (primers Hsf1.RNA.F and Hsf1.RNA.R) and *leu1*$^+$ (primers Leu1.RNA.F and Leu1.RNA.R). The membrane was exposed to a phosphoimager screen (Fujifilm). Data were analysed using a Typhoon FLA 3500 and quantified using ImageQuant TL software, error bars indicate standard deviation. Samples were analysed relative to the wild type unstressed sample, ratio set to 1. (C) Modifications of Hsf1-GFP in response to H_2O_2 stress. Mid-log growing cultures of wild type (WT)(CHP429), cells expressing Hsf1-GFP (FM40), *ssa1*Δ cells expressing Hsf1-GFP (FM36) and *mas5*Δ cells expressing Hsf1-GFP (FM37) were treated with 6 mM H_2O_2 for 10, 20, 40 or 60 minutes. Samples were extracted by TCA protocol plus NEM. Proteins were analysed by non-reducing SDS-PAGE and western blotting with anti-GFP antibody and anti-tubulin antibody.

nothing conclusive. However, in wild type and *ssa1*Δ cells there may be evidence of new gene expression induced following 6 mM H_2O_2 stress being translated to new unmodified Hsf1-GFP. This unmodified form is not detectable in the *mas5*Δ strain before or after 6 mM H_2O_2 stress (Fig. 4. 10. C). There is nothing definitive that can be concluded from this experiment that explains the localisation and/or effects on gene expression shown here.

Collectively the data above suggests that Ssa1 and Mas5 regulate the nuclear localisation of Hsf1 in opposite manner in response to H_2O_2. Also, it seems Hsf1 becomes activated (based on *hsp104*$^+$ expression) by high concentrations of H_2O_2. This could be via another transcription factor and in a manner independent of Mas5. The data also lead to the conclusion that Mas5 regulation affects *hsf1*$^+$ expression at high concentrations.

4.2.7 No modification of Ssa1, Ssa2 or Mas5 can be seen in response to H_2O_2

How H_2O_2 stress is sensed/signalled to regulate Hsf1 localisation/activation is unclear. The literature indicates that Hsf1 regulation can be through modulation by other factors. In *S. cerevisiae*, the data indicate Ssa1 is responsive to H_2O_2 as sensor for Hsf1 while in *S. pombe* cells Hsf1 modulation appears to be by Ssa2/Mas5 but their modification was not reported. Therefore, it raised the question of whether Ssa1, Ssa2 or Mas5 act as H_2O_2 sensors/signalling mechanisms for Hsf1 activity. Thus, the next step was to see if it was possible to detect modification of Ssa1, Ssa2 and/or Mas5 at low or high concentrations of H_2O_2 that might help to explain how Ssa1, Mas5 and possibly Ssa2 regulate Hsf1.

No modification of Ssa1-Pk, Ssa2-Pk or Mas5-Pk could be detected upon exposure to high or low oxidative stress conditions during the time course (Fig. 4. 11.). Additionally, no change in protein levels could be detected of Ssa1, Ssa2 or Mas5 in response to 0.2 mM or 6 mM H_2O_2

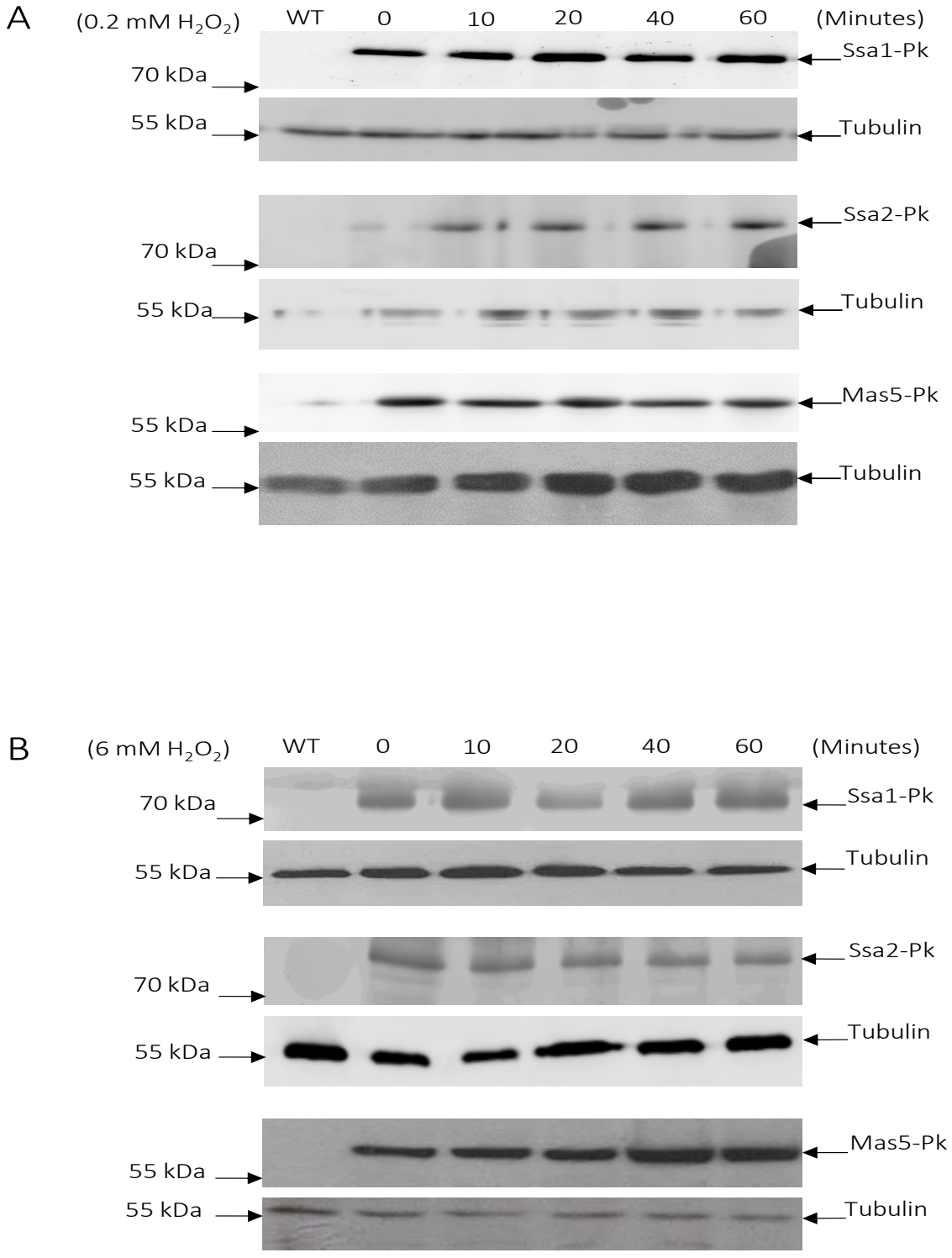


Fig. 4. 11. No modification of Ssa1-Pk, Ssa2-Pk or Mas5-Pk can be detected in response to H_2O_2. Exponential phase cultures of wild type (WT) (CHP429), Ssa1-Pk (FM4), Ssa2-Pk (FM16) or Mas5-Pk (FM5) expressing cells were treated with low (0.2 mM) (A) or high (6 mM) (B) hydrogen peroxide (H_2O_2) stress for 10, 20, 40 or 60 minutes. Samples were extracted by TCA protocol plus NEM. Proteins were analysed by non-reducing SDS-PAGE and western blotting with anti-Pk antibody or anti-tubulin antibody as a loading control, n > 3.

over the time course (Fig. 4. 12.) The lack of change in these HSP modification/protein levels means it remains to be explained how the change in localisation of Hsf1 at low versus high or activation of Hsf1-gene expression at high is regulated.

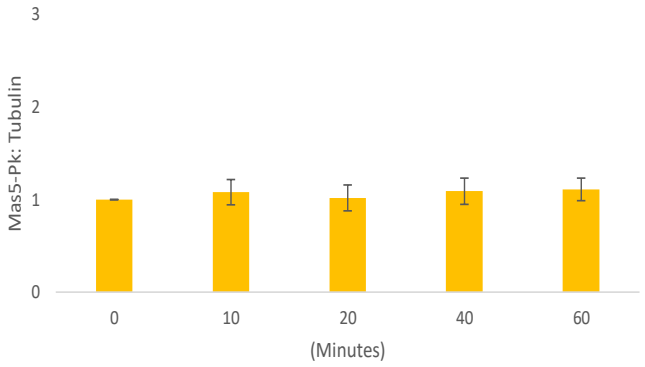
In this chapter, the data has identified a new mechanisms/signal transduction of regulation of responses to increasing concentrations of H_2O_2 in *S. pombe* which involves regulation of Hsf1. Interestingly there appears to be a disconnect between localisation and activation of Hsf1 however the regulation does appear to require the Ssa1 and Mas5 proteins in opposing roles.

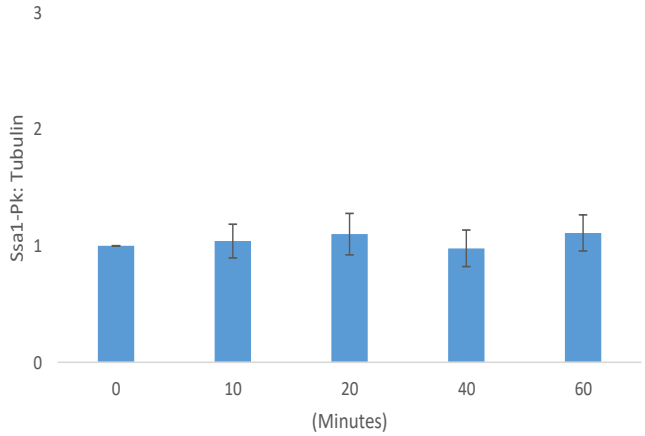
4.3 Discussion

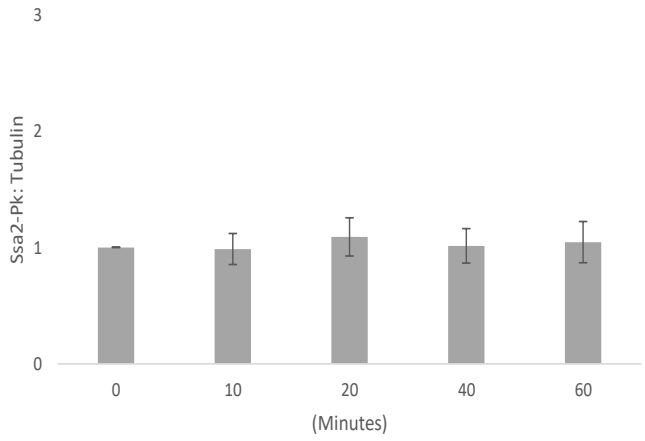
In *S. pombe* characterisation of cold (25 °C) and heat (37 °C) stress showed differential regulation effecting Hsf1 localisation. Nuclear accumulation of Hsf1 following exposure to cold shock was delayed to 40 minutes, in comparison heat stress results in immediate and sustained nuclear localisation. The published *S. pombe* Hsf1 heat stress localisation is limited, cells were grown at 24 °C which further complicates analysis (Vjestica *et al*., 2013). However, an alternate study in *S. pombe* with cells grown at 30°C showed nuclear accumulation of Hsf1 following heat shock at 43 °C for 10 minutes (Vjestica *et al*., 2013). Hsf1 localisation in normal conditions and heat shock presented by Ohtsuka *et al*. (2011) is consistent with the work presented here. Excitingly, nuclear accumulation of Hsf1 upon heat shock has been observed in higher eukaryotes suggesting this is a conserved response (Voellmy, 2004; Vujanac *et al*., 2005).

In addition to this, numerous studies in yeast and higher eukaryotes have identified in response to heat shock increased Hsf1-DNA binding and expression of Hsf1-target genes that can be

A (0.2 mM H_2O_2)


Mas5-Pk: Tubulin
3
2
1
0
0
10
20
40
60
(Minutes)


Ssa1-Pk: Tubulin
3
2
1
0
0
10
20
40
60
(Minutes)


Ssa2-Pk: Tubulin
3
2
1
0
0
10
20
40
60
(Minutes)

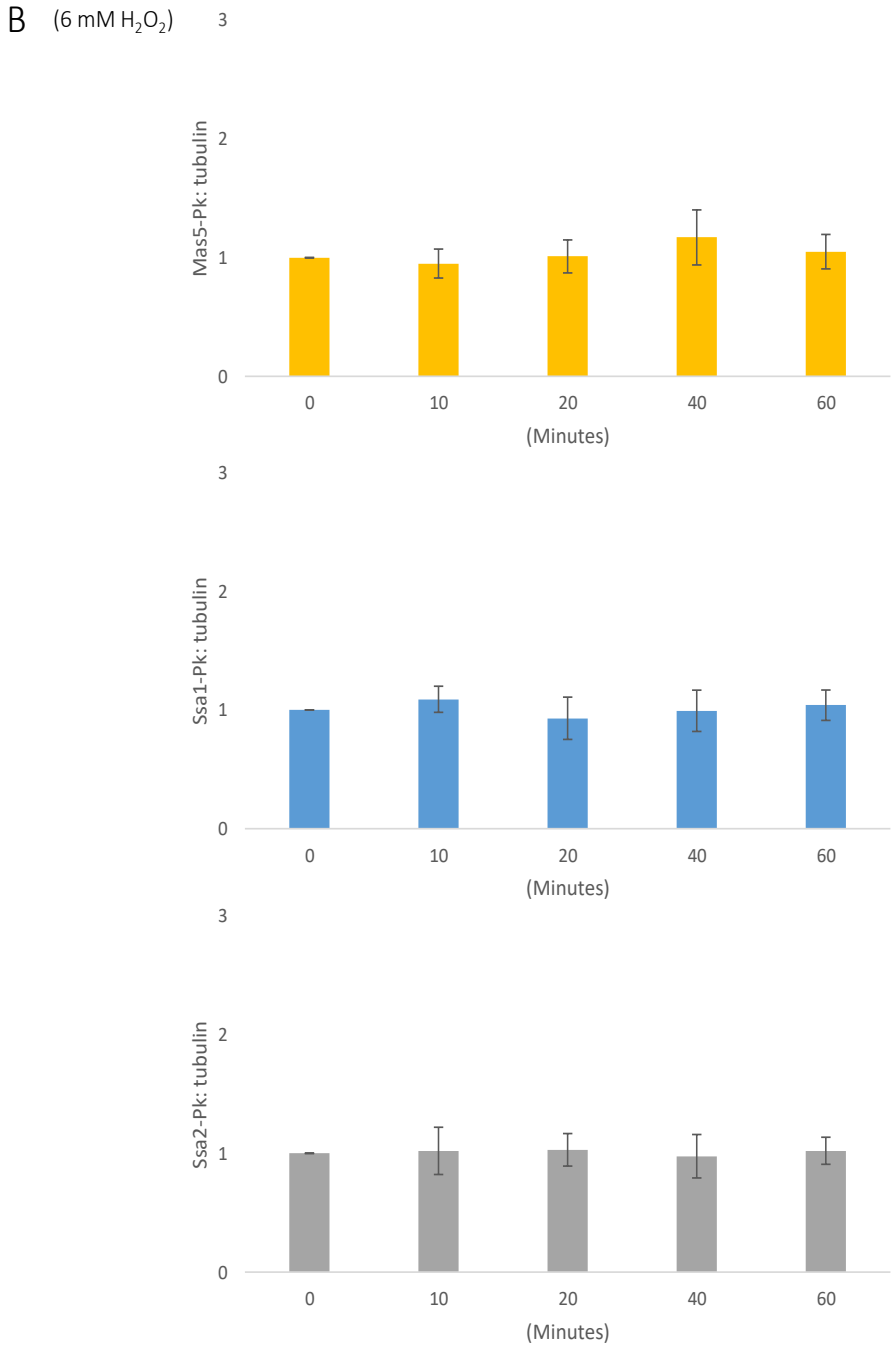


Fig. 4. 12. No change in the level of protein of Ssa1-Pk, Ssa2-Pk or Mas5-Pk can be detected in response to H_2O_2. Exponential phase cultures of wild type (WT)(CHP429), Ssa1-Pk (FM4), Ssa2-Pk (FM16) or Mas5-Pk (FM5) expressing cells were treated with 0.2 mM (A) or 6 mM (B) H_2O_2 stress for 0, 10, 20, 40 or 60 minutes. Samples were extracted by TCA protocol plus NEM. The relative levels of protein samples were quantified using ImageQuant TL software and normalised to the unstressed samples, ratio set to 1, n = 3, error bars indicate standard deviation.

correlated to the increase in nuclear accumulation. The work presented here in *S. pombe* Hsf1 localisation following heat shock suggests that there is a conserved regulation. However, in this study Hsf1 ChIP analysis and oligomerization were not investigated. Therefore in *S. pombe* it cannot be confirmed without further study that Hsf1 nuclear accumulation is correlated to increased trimerization and Hsf1-DNA binding. Until this is investigated it can't be concluded that following heat shock Hsf1 nuclear accumulation correlates to Hsf1 activity.

It has been shown here that Mas5 acts as a repressor of Hsf1 nuclear localisation which agrees with the conclusions of Vjestica *et al*. (2013). It was shown in Chapter 3 that *mas5*Δ cells are sensitive to heat stress again confirming the published data. The heat sensitivity of the *mas5*Δ strain was concluded to be due to derepression of Hsf1 which would support the observed constitutively nuclear localisation of Hsf1 shown here. Expanding upon this, Ssa1 does not appear to have a distinct role in Hsf1 localisation following exposure to sub-optimal temperatures. Vjestica *et al*. (2013) did not test the role of Ssa1 in Hsf1 regulation. The lack of aberrant temperature phenotypes in *ssa1*Δ cells could suggest that Ssa1 has a redundant role within the Ssa family for Hsf1 regulation or alternatively it is not a regulator of Hsf1 during temperature response. Interestingly, the *ssa2*Δ strain was shown here and in the published data to be temperature sensitive (Vjestica *et al*., 2013). This may lead to towards the conclusion that Ssa1 and Ssa2 play distinct roles in heat stress response and thus may have independent roles in Hsf1 regulation.

Until now Hsf1 in *S. pombe* oxidative stress response had not been investigated however the transcription factor has been linked to oxidative stress responses in other organisms (Anckar and Sistonen, 2011; Wang *et al*., 2012). Previous studies in yeast revealed that conserved oxidative stress-induced transcription factors are activated in a concentration dependent manner.

In fission yeast 2 different transcription factors are induced depending on the severity of the H_2O_2 stress to regulate the suitable response (Vivancos *et al*., 2006). In response to low H_2O_2 stress (0.2 mM) the Pap1 transcription factor (homologue of Yap1 in *S. cerevisiae*) accumulates in the nucleus and is activated (Calvo *et al*., 2013; Quinn *et al*., 2002). Pap1 regulation has been discussed in detail (See Introduction section 1.3.2). In summary, Pap1 is not activated by exposure to high concentrations of H_2O_2. It has been demonstrated that 1 mM H_2O_2 induces phosphorylation of the Sty1 MAPK pathway which activates Atf1 transcription factor (Veal *et al*., 2007; Vivancos *et al*., 2006) (Fig. 1. 5.).

Excitingly, the initial studies have identified that Hsf1 localisation in response to different concentrations of H_2O_2 mimics Pap1 localisation. In unstressed cells Hsf1 has no specific localisation suggesting active nucleo-cytoplasmic shuttling. In mammalian cells the same nucleo-cytoplasmic shuttling has been observed, upon environmental stress Hsf1 becomes negatively regulated for nuclear export leading to nuclear accumulation (Vujanac *et al*., 2005). In *S. pombe*, upon 0.2 mM H_2O_2 stress Hsf1 rapidly accumulates in the nucleus, as the concentration of H_2O_2 increases there is an observable delay in nuclear accumulation which is the same regulation observed of Pap1 (Quinn *et al*., 2002). Taken together this suggests that Hsf1 nuclear accumulation in response to stress is conserved amongst eukaryotes but specifically within *S. pombe* Hsf1 and Pap1 share distinctive similarities in dynamics.

The nuclear exportin Crm1 is known to export Pap1, treatment of cells with leptomycin B modifies the exportin to block Pap1 export which mimics 0.2 mM H_2O_2 stress (Toda *et al*., 1991; Toone *et al*., 1998). Additionally, leptomycin B treatment results in Hsf1 nuclear accumulation suggesting that Crm1 is responsible for exporting both transcription factors (Matsuyama *et al*., 2006). It is possible that Hsf1 localisation with H_2O_2 in *S. pombe* is

physically linked to Pap1 localisation as this also cycles and is Crm1-dependent. In other systems Crm1 has been implicated in HSF export but it is not universal so it is possible that in *S. pombe* there is use of a common exportin pathway via Crm1 or the transcription factors localisation is intrinsically linked (Heerklotz *et al*., 2000; Vujanac *et al*., 2005).

Hsf1 transcriptional activity and localisation are linked but has shown complex results. The data presented here for transcription of *hsf1*$^{+}$ and the Hsf1-dependent gene *hsp104*$^{+}$ show significant induction following 6 mM H_2O_2 stress in comparison to 0.2 mM H_2O_2 which agrees with previous large-scale screen analysis (Chen *et al*., 2008). The study by Vjestica *et al*. (2008) did not include analysis of Hsf1 activity in response to high H_2O_2 as they used previous less extensive screen analysis and thus missed a Hsf1 connection to high H_2O_2 response (Chen *et al*., 2003; Chen *et al*., 2008).

It appears that in response to 6 mM H_2O_2 Hsf1 activates gene expression to a significant degree. It was shown here that upon 6 mM H_2O_2 stress strong nuclear accumulation was not observed until approximately 40 minutes while Hsf1-dependent gene expression of *hsp104*$^{+}$ was observable from 10 minutes (Fig. 4. 6. C. and 4. 9. B). This disconnect between activity and localisation of Hsf1 has several possible causes. Within the pool of Hsf1 there are inactive and active forms of which both will/can be localised to the nucleus. Therefore, although Hsf1 is not observed to be accumulating in the nucleus upon 6 mM H_2O_2 stress there can still be an active fraction within the nucleus that is responsible for the observed increase in Hsf1-dependent gene expression.

Due to the similarity in Pap1/Atf1 regulation it is possible that a common regulatory mechanism is sensing the degree of oxidative stress and activating Hsf1. The MAPK Sty1 is

not significantly active under low H_2O_2 stress however at high concentrations becomes activated and localises to the nucleus (see section 1.3.3; Quinn *et al*., 2002). Possibly when active Sty1 reaches the nucleus it activates the inactive Hsf1. To address this, it would be interesting to investigate Hsf1-dependent gene expression in a *sty1*Δ strain.

Hsf1 has been shown to be modulated by HSP70s (see section 1.2.2.2 and 1.3.4.1). In *S. cerevisiae*, Ssa1/Ssa2 were identified as repressors of Hsf1 activity via a direct interaction in unstressed conditions (Wang *et al*., 2012). Exposure to certain oxidising agents oxidised cysteines within Ssa1 that resulted in release of Hsf1 and activation. It is possible Ssa2 also has this function in Hsf1 regulation. HSP40s modulate HSP70 function, in *S. cerevisiae* Ydj1 is known to interact with Ssa1 and Ssa2. It has been proposed that Ydj1 acts as a growth rate sensor for G1 phase but the mechanism was not clear (Ferrezuelo *et al*., 2012).

In *S. pombe* it was reported that the Ydj1 homolog, Mas5 is required for a normal continuous cell growth rate suggesting a conserved function amongst yeast but whether this HSP40 role in cell growth is linked to Hsf1 modulation is not clear (Vjestica *et al*., 2013). However, Mas5/Ssa2 were identified as repressors of Hsf1 activity until heat shock causes depression, Ssa1 was not investigated to establish its role. The role of Ssa1, Ssa2 and Mas5/Ydj1 in Hsf1 regulation under oxidative stress has not been focussed upon.

In this study, in *S. pombe* it was found that *ssa1*Δ allele affects localisation of Hsf1 upon H_2O_2 stress but not Hsf1-dependent gene expression nor $hsf1^+$ levels. This suggests a positive role for Ssa1 in Hsf1 nuclear localisation but not linked to Hsf1 activity on promoters. It must be considered that the disconnect between localisation and activity of Hsf1 could be due to redundancy with Ssa2. Another possibility is that localisation experiments don't distinguish

between inactive versus active protein movement thus appearing to show a disconnect between the localisation and activity of Hsf1. However, if this is a genuine disconnect between localisation/activation data for Hsf1 this contrasts with Pap1 where localisation accumulation and activation are linked.

Although Ssa1 appears to regulate localisation no obvious modification of Ssa1 detected. It is possible that the modification of Ssa1 could not be detected by this method since it may be the oxidation of a single cysteine but is still present as a mechanism of Hsf1 regulation. In contrast to the Ssa1 data, *mas5*Δ cells display constitutively nuclear Hsf1 with an increase in basal *hsp104*$^{+}$ and *hsf1*$^{+}$ which is consistent with the localisation data. However, further activation of *hsp104*$^{+}$ was detected upon 6 mM H_2O_2 stress but not *hsf1*$^{+}$. This suggests a secondary activation of *hsp104*$^{+}$ expression for example another transcription factor activates its expression or an additional modification of Hsf1 occurs in these conditions. Whereas, Mas5 regulation appears to be the only mechanism for *hsf1*$^{+}$ expression as within *mas5*Δ cells *hsf1*$^{+}$ is already maximal and no further induction could be achieved with H_2O_2 treatment.

It was found that Mas5 is oxidised in response to 0.2 mM H_2O_2 after 30 seconds but no further analysis of its oxidation is available (Garcia-santamarina *et al*., 2011). In this study, no modification of Mas5 was observed in response to H_2O_2 concentrations at the time points used. However, this could be due to the method used or that temporal oxidation of Mas5 is quickly resolved. The effect of this oxidation on Hsf1 regulation is unclear, a strain with a form of Mas5 mimicking the oxidised status would be useful to answer this question. Currently how Ssa1 and Mas5 influence localisation of Hsf1 is unclear.

A hypothesis of HSP regulation of Hsf1 is by the availability of the proteins Ssa1, Ssa2 and Mas5. If there is a stress responsive change in protein levels of these HSPs it could result in a competition for Hsf1 and thereby regulate Hsf1 activity. Interestingly, investigation within this study showed the level of Ssa1-Pk is much less than Ssa2-Pk and the level of Ssa2-Pk protein in relation to Mas5-Pk is greater than Mas5-Pk to Ssa1-Pk (data not shown). As Mas5 is a regulatory co-chaperone of both proteins the relative availability of Mas5 to Ssa1 or Ssa2 may be important, whether the oxidation of Mas5 effects interaction with Ssa1 or Ssa2 is unknown. The levels of Ssa1, Ssa2 and Mas5 did not appear to change in response to H_2O_2 (Fig. 4. 12.) so if there is a competition model to regulate Hsf1 it is not dependent on an increase in Ssa1/Ssa2/Mas5 upon H_2O_2 stress.

It is possible that Ssa1/Ssa2/Mas5 act in a chaperone capacity to affect folding/oligomerisation of Hsf1. It is also possible they function to present Hsf1 to the import and/or export machinery for example, Mas5 be required for Crm1 to export Hsf1 from the nucleus by presenting a nuclear export sequence (NES). It is possible that Ssa1/Ssa2/Mas5 affect the function of Crm1 itself. Leptomycin B acts by covalently modifying a conserved cysteine in the NES interaction sequence on Crm1 so it could be speculated that oxidation of the cysteine in Ssa1/Ssa2/Mas5 may be important for a role in Crm1 function. A strain with a mutation of the Crm1 cysteine exists that blocks leptomycin B interaction but leaves Crm1 functional. It would be interesting to use this strain to test whether the mutant Crm1 affects Hsf1 localisation.

What the mechanism is that further activates *hsp104*$^{+}$ in *mas5*Δ strain is not known. However, there are multiple cysteines within mammalian Hsf1 while *S. pombe* Hsf1 has a single cysteine, these have not been investigated for redox sensitivity. Hsf1 could be a target of oxidation to regulate its interaction with Crm1. Interestingly, mammalian and *S. pombe* Hsf1 contain

cysteines and are shown to shuttle between the nucleus and the cytosol. In contrast, *S. cerevisiae* Hsf1 which is constitutively nuclear lacks any cysteines further supporting the theory that oxidation of a Hsf1 cysteine may be important for localisation. It would also be interesting to explore in *S. cerevisiae* Ydj1 roles if any in Hsf1 regulation in response to H_2O_2 to identify if HSP40s are conserved in Hsf1 oxidative stress regulation.

The Chen *et al*. (2008) *S. pombe* data didn't look at *hsp104*$^+$ expression being dependent on transcription factors other than Hsf1 nor Sty1 dependency upon 6 mM H_2O_2 stress. Therefore, the possibility of another transcription factor other than Hsf1 regulating *hsp104*$^+$ expression remains unknown. However, in *S. cerevisiae* Hsf1 acts with transcription factor Skn7 to achieve maximal expression of heat shock genes specifically in response to oxidative stress including *hsp104*$^+$ (Raitt *et al*., 2000) The absence of Skn7 resulted in a different profile of *hsp104*$^+$ induction supporting the model that Hsf1 and Skn7 act in concert for HSP encoding gene-induction following H_2O_2 stress. This further supports the idea that in *S. pombe* another transcription factor works with Hsf1 to induce *hsp104*$^+$ expression in response to oxidative stress. The *S. pombe* homologue of Skn7 is Prr1 and has a HSF-like DNA binding domain and the coiled region that may be relevant for oligomerization. This makes Prr1 a candidate for the increased expression of *hsp104*$^+$ following 6 mM H_2O_2 treatment (Fig. 4. 9. B). It would be interesting to use a *prr1*Δ strain to investigate if this affects *hsp104*$^+$ expression following 6 mM H_2O_2 stress or if it affects Hsf1 localisation. It would be interesting to test if Hsf1 interacts directly with Prr1 or other transcription factors at different concentration of H_2O_2 to allow a concentration dependent activation of gene expression.

The work shown here in *S. pombe* suggests Mas5 acts as a constitutive repressor while Ssa1 appears to promote nuclear accumulation in response to H_2O_2. This leads towards a model that

in unstressed conditions Hsf1 can undergo nucleo-cytoplasmic shuttling via an unknown importin and exported via Crm1 like Pap1 (Fig. 4. 13. A). Exposure to 0.2 mM H_2O_2 does not inhibit nuclear import of Hsf1 or Pap1, however both transcription factors accumulate. Pap1 NES is known to be masked preventing its recognition by Crm1. Mas5 and Ssa2 have been identified as constitutive repressors of Hsf1 (Fig. 4. 13. B). No direct interaction has been detected between Ssa2/Mas5 and Hsf1 nor importin/exportin pathways so their mechanism of Hsf1 regulation can't be speculated. However, the epitope tagged proteins Ssa2 and Mas5 localised to the cell ends and regions of cell growth. In mammalian and budding yeast cells Hsf1 is repressed by HSP70s (Anckar and Sistonen, 2011; Wang *et al*., 2012; Zheng *et al*., 2016). Following stress, derepression of Hsf1 nuclear localisation has been argued to be due HSP70 being targeted to release Hsf1 in favour of protein chaperoning roles due increased protein damage. In conclusion, this chapter has provided new insight into Hsf1 function and regulation in responses to H_2O_2 and identified new mechanisms that suggest organism specific and conserved aspects. Given the connections and similarities between Pap1 and Hsf1 nuclear accumulation in response to increasing concentrations of H_2O_2 in the next Chapter the potential relationships between the regulation of Pap1 with that of Hsf1 will be explored.

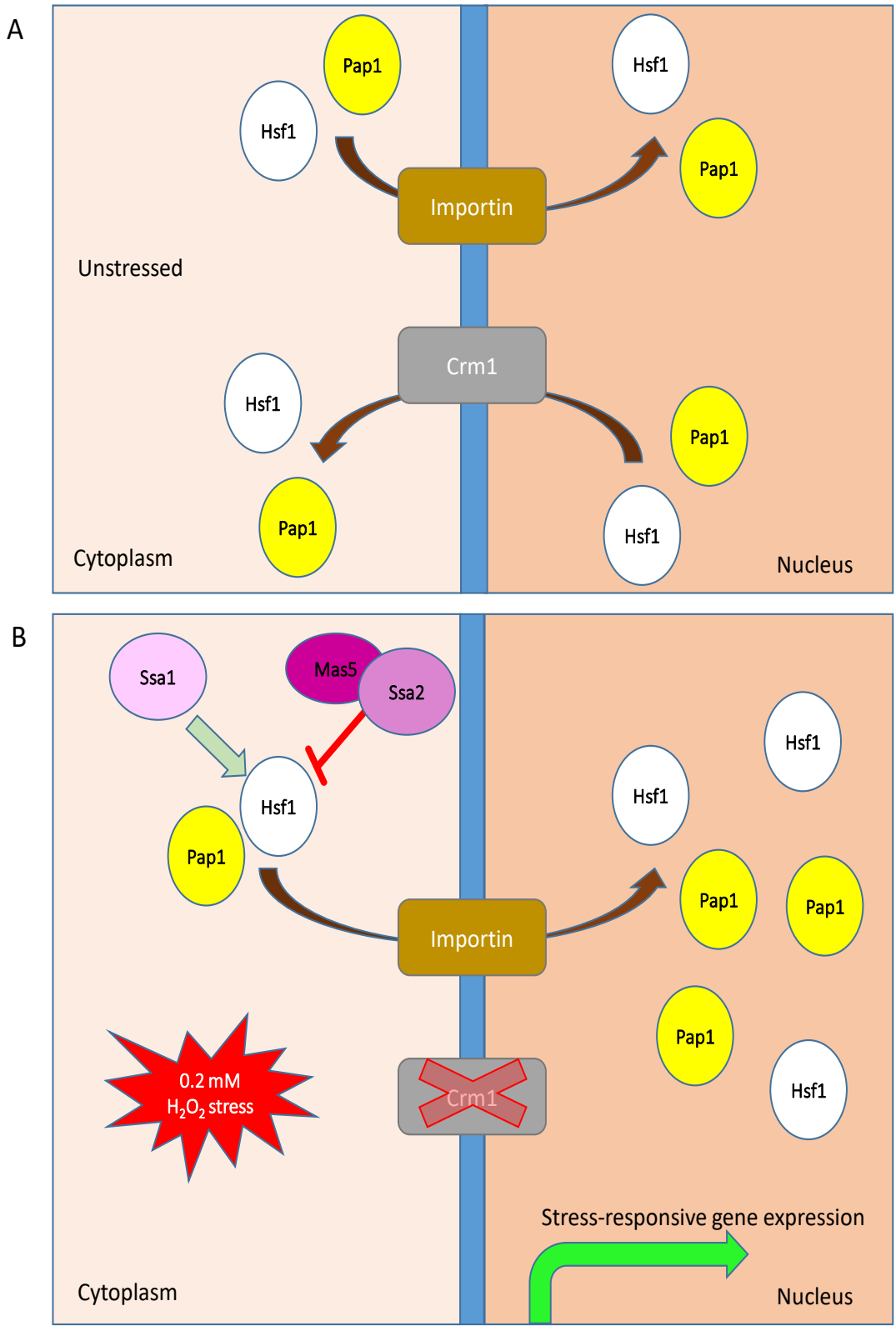


Fig. 4. 13. Model of proposed regulatory factors of Hsf1 in response to H_2O_2 in *S. pombe* cells. (A) In unstressed cells Hsf1 like Pap1 undergoes nucleo-cytoplasmic shuttling involving the exportin Crm1. (B) A theoretical model of how Mas5 and Ssa2 appear to act upon Hsf1 following H_2O_2 stress, while Ssa1 is a specific promoter of Hsf1 nuclear localisation Mas5 seems to act as constitutive repressor possibly with Ssa2.

CHAPTER 5

An investigation of the regulation of Pap1 and Hsf1 by the thioredoxin system

5.1 Introduction

Although HSPs have established roles as chaperones in stress responses recent work in yeast (*S. cerevisiae* and *S. pombe*) have begun to uncover novel roles as sensing/regulators of environmental stress. One of the aims of the project is to investigate the roles and regulation of Ssa1, Ssa2 and Mas5 in *S. pombe* in response to stresses such as oxidative stress.

The work presented in the previous chapter has identified new regulation of Hsf1 in the oxidative stress response that showed similarity to the regulation of Pap1 and Atf1. Additionally, it was established that Mas5 and Ssa1 affect different aspects of the regulation of Hsf1 upon exposure to differing concentrations of H_2O_2. Thus, it was important to investigate the potential relationship between Ssa1 and/or Mas5 and other aspects of the H_2O_2 response such as Pap1. Furthermore, given the similarities in behaviour of Pap1 and Hsf1 nuclear accumulation and Crm1 dependency it was important to investigate whether the regulation of Pap1 had any connections or cross talk with regulation of Hsf1.

In *S. cerevisiae, ssa* mutants have been previously shown to influence *TRX2* expression– a key Yap1-dependent gene but it has not been investigated whether Yap1 is regulated by Ssa proteins (Hasin *et al*., 2014). As discussed in the introduction (see section 1.3.4.1) a study of *S. cerevisiae* identified Ssa1/Ssa2 as a repressor of Hsf1 that was redox sensitive and the gene

expression explored however, the role of Yap1 was not looked at (Wang *et al*., 2012). The work in *S. pombe* suggests that it is worth simultaneous investigation of the transcription factors Hsf1 and the Yap1 homolog Pap1 to find if they share homologous regulation particularly in relation to the role of the HSPs Ssa1 and Mas5. This may elucidate the conservation of sensing/signalling pathways of the oxidative stress response to activate stress-responsive gene expression.

5.2 Results

5.2.1 Mas5 effects Pap1-dependent gene expression

Given the similarity of Hsf1 and Pap1 localisation shown in Chapter 4 and the fact that Yap1-regulated gene expression is affected in *ssa* mutants in *S. cerevisiae* (Hasin *et al*., 2014) it was decided to investigate Pap1 regulation in the absence of *ssa1*$^+$ or *mas5*$^+$. Firstly, it was investigated whether any effect was observed of *ssa1*Δ and/or *mas5*Δ allele on Pap1-regulated gene expression by H_2O_2.

A well characterised Pap1-regulated gene is *gst2*$^+$ encoding glutathione S transferase is induced strongly at low levels in a Pap1-dependent manner and much less induced at higher concentrations (Chen *et al*., 2008; Veal *et al*., 2002). Indeed, as expected *gst2*$^+$ was induced in wild type cells by low levels (0.2 mM) of H_2O_2 and much less induced at high levels (6 mM) (Fig. 5. 1.). Significantly, this result shows similarity to Hsf1-dependent *hsp104*$^+$ gene expression described in Chapter 4. There was no effect of *ssa1*Δ allele on Pap1-dependent gene expression at either low or high concentrations of H_2O_2. However, surprisingly basal *gst2*$^+$ levels appeared higher in *mas5*Δ cells and induced to a greater extent than wild type cells at

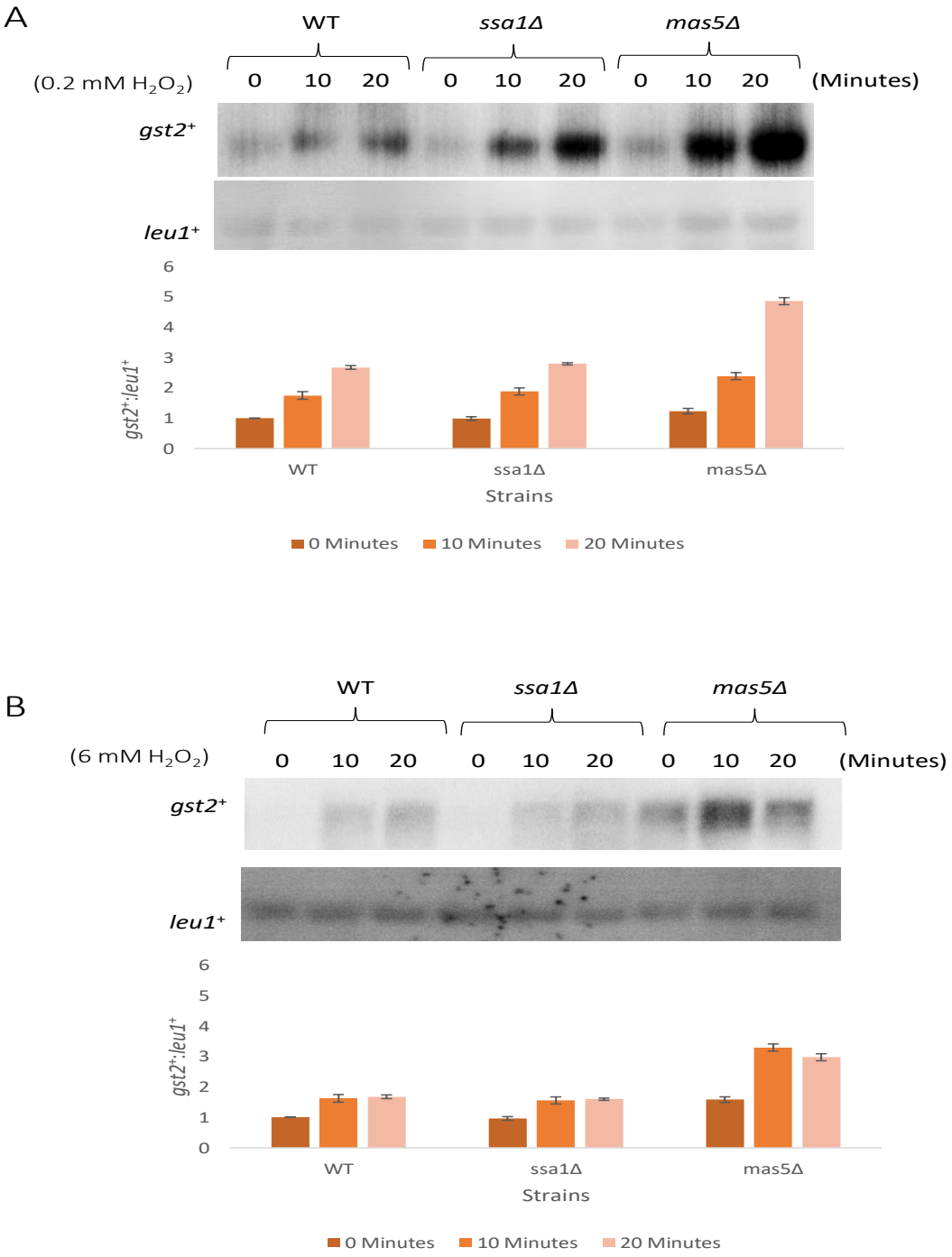


Fig. 5. 1. RNA analysis of Pap1-dependent transcript levels suggests Mas5 is required for Pap1 regulation. Exponentially growing wild type (WT) cells (CHP429), *ssa1*Δ (FM14) and *mas5*Δ (FM15) cells were exposed to 0.2 mM H_2O_2 (A) or 6 mM H_2O_2 (B) and cells were collected after 0, 10, 20 minutes. RNA was extracted, run in a phosphate gel and analysed by northern blotting. Radioactive probes were used to detect Pap1- dependent *gst2*$^+$ transcript levels *leu1*$^+$ as a loading control, the blot was exposed to a phosphoimager screen. Data were analysed using a Typhoon FLA 3500 and quantified using ImageQuant TL software, fold induction was calculated relative to wild type unstressed sample, ratio set to 1. Standard deviation bars included, n = 3.

both low (0.2 mM) and high (6 mM). These data are very similar to the results obtained looking at the effects of *mas5*Δ on *hsp104*$^{+}$ gene expression (Chapter 4 Fig. 4. 9.). Intriguingly, similar results to those obtained with *mas5*Δ cells were obtained in studies of cells lacking specific regulators of Pap1. For example, cells lacking either Trx1 or Txl1 but not both have high basal and higher induction of Pap1-dependent genes (Brown *et al*., 2013) showing similar effects on *gst2*$^{+}$ in response to 0.2 mM H_2O_2. In contrast, cells lacking either Trr1 or both Trx1 and Txl1 show constitutive activation of *gst2*$^{+}$ (Brown *et al.*, 2013). This leads towards a possible theory that *mas5*Δ influences the activity of one or other of thioredoxins Trx1 and Txl1.

Having established the effects of loss of Mas5 but not Ssa1 on *gst2*$^{+}$ expression the next step was to explore the regulation of Pap1 as data suggested that perhaps Pap1 is misregulated in *mas5*Δ cells but not *ssa1*Δ cells. So, next it was investigated how Pap1 localisation in unstressed conditions and in response to H_2O_2 is affected by the presence of *mas5*Δ allele.

5.2.2 Mas5 effects Pap1 activation in response to H_2O_2

In normal conditions Pap1 is predominantly cytosolic but cycles to and from the nucleus but becomes constitutively nuclear when cells are exposed to low concentrations of H_2O_2 (0.2 mM) (see Introduction section 1.3.2.2). It appeared that Mas5 but not Ssa1 was a factor in Pap1-dependent gene expression in normal conditions and in response to H_2O_2. Hence, to investigate Mas5 and regulation of Pap1 the first aspect to examine is the localisation of Pap1 in unstressed conditions and in response to low concentrations of H_2O_2 in wild type cells and *mas5*Δ cells.

As expected in non-stress conditions of wild type cells Pap1 localised primarily to the cytosol (Fig. 5. 2.). In *mas5*Δ cells in non-stress conditions Pap1 was found to be largely cytosolic. Consistent with previous data, after treatment of wild type cells with 0.2 mM H_2O_2 Pap1.

accumulated in the nucleus and remained unchanged over the time-course. However, in contrast in *mas5*Δ cells after treatment with H_2O_2 there was a very rapid accumulation of Pap1 in the nucleus at 10 minutes with a slight decrease by 20 minutes. Hence these data reveal that Mas5 appears to have no observable function in Pap1 localisation in non-stressed conditions, following oxidative stress Mas5 appear to act by inhibiting nuclear accumulation of Pap1 following exposure to low concentrations of H_2O_2.

The localisation of Pap1 and Pap1-dependent gene expression in *mas5*Δ cells does not show similarity to *trr1*Δ cells, *trx1*Δ cells or *trx1*Δ*txl1*Δ cells (Brown *et al*., 2013). Interestingly, the localisation of Pap1 in *mas5*Δ cells shows similarity to wild type cells but more closely to *txl1*Δ cells in that Pap1 is not constitutively nuclear or shows increased nuclear localisation in unstressed conditions. This leads further towards the theory that Mas5 is a regulator of Pap1 or possibly Pap1 pathways.

After examining Pap1-dependent gene expression and localisation the next step was to investigate the oxidation of Pap1. When *S. pombe* cells are exposed to low concentrations of H_2O_2, Pap1 forms intramolecular disulphide bonds that masks the protein's nuclear export sequence leading to a nuclear accumulation (Quinn *et al*., 2002). In contrast to low concentrations of H_2O_2, when cells are exposed to higher concentrations of H_2O_2 Pap1 remains reduced until later time points. Previous work has found that this is due to hyperoxidation of Tpx1 in the Pap1 pathway (Bozonet *et al*., 2005). As Pap1 localisation and Pap1-dependent

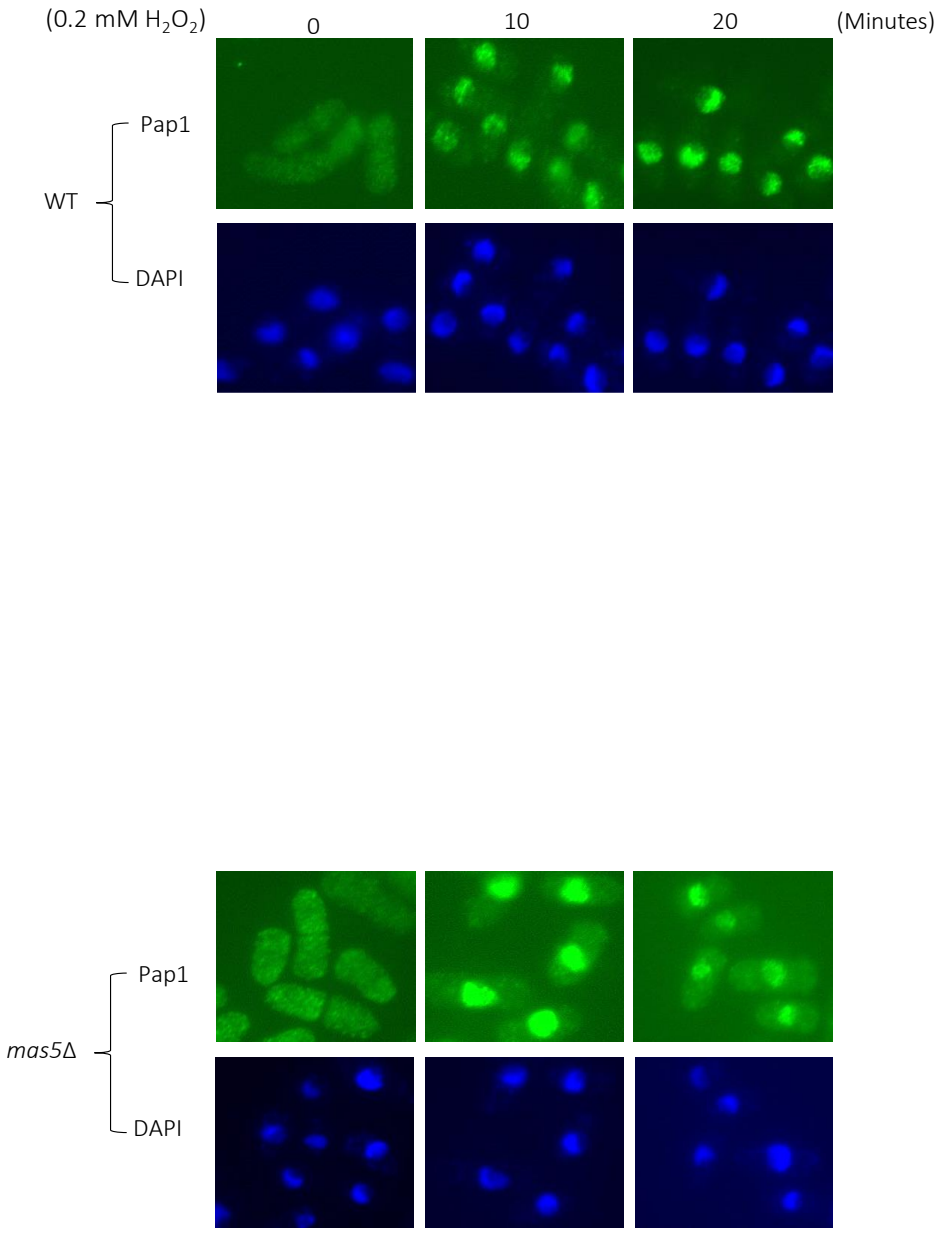


Fig. 5. 2. Misregulation of Pap1 localisation after treatment with 0.2 mM H_2O_2 in *mas5*Δ cells. Pap1 was visualised by indirect immunofluorescence using Pap1 antibodies in exponential phase fixed wild type (WT) (CHP429) and *mas5*Δ (FM15) cells after treatment with 0.2 mM H_2O_2 for 0, 10 or 20 minutes. GFP (fluorescent) and DAPI images are shown of representative cells. Cells were fixed using paraformaldehyde, exposures remaining constant between images, n > 3

gene expression appears misregulated in *mas5*Δ cells the regulation the Pap1 oxidation at low concentrations of H_2O_2 and high concentrations of H_2O_2 was of interest to explore.

Western blot analysis was used to investigate the oxidation of Pap1 upon 0.2 and 6 mM H_2O_2 stress (Fig. 5. 3.). As expected, wild type cells showed no Pap1 oxidation prior to H_2O_2 stress and oxidised rapidly upon 0.2 mM H_2O_2 stress as has been shown in previous work (Brown *et al*., 2013) (Fig. 5. 3. A). In addition, no oxidation of Pap1 was detected after 6 mM H_2O_2 during the time course as expected in wild type cells (Day *et al*., 2012) (Fig. 5. 3. B). Oxidation pattern of Pap1 in wild type cells at 0.2mM and 6 mM H_2O_2 is consistent with the *gst2*$^+$ expression data (Fig. 5. 1.). Furthermore, *ssa1*Δ cells showed very similar oxidation profiles of Pap1 as wild type cells at basal and after 0.2 mM and 6 mM H_2O_2, this is consistent with *gst2*$^+$ expression patterns in the *ssa1*Δ cells. Unstressed *mas5*Δ cells show no evidence of Pap1 oxidation which agrees with the localisation of Pap1 in these cells, this is the same as wild type cells. However, there are more rapid kinetics of oxidation in response to low concentrations of H_2O_2 relative to wild type cells (Fig. 5. 3. A).

The increased rate of oxidation of Pap1 corresponds to more rapid accumulation of Pap1 in the nucleus. These stress data indicate that in *mas5*Δ cells in response to low concentrations of H_2O_2 Pap1 is more rapidly oxidised which is consistent with the nuclear accumulation of Pap1 being more visible. When *mas5*Δ cells are exposed to high concentrations of H_2O_2 Pap1 appears to be oxidised over the time course which is consistent with the gene expression data (Fig. 5. 3. B, Fig. 5. 1.). This contrasts with the response observed in wild type cells.

These data suggest that in *mas5*Δ cells the pathway leading to Pap1 oxidation is not inactivated in response to high concentrations of H_2O_2. Whether this is due to direct misregulation of Pap1

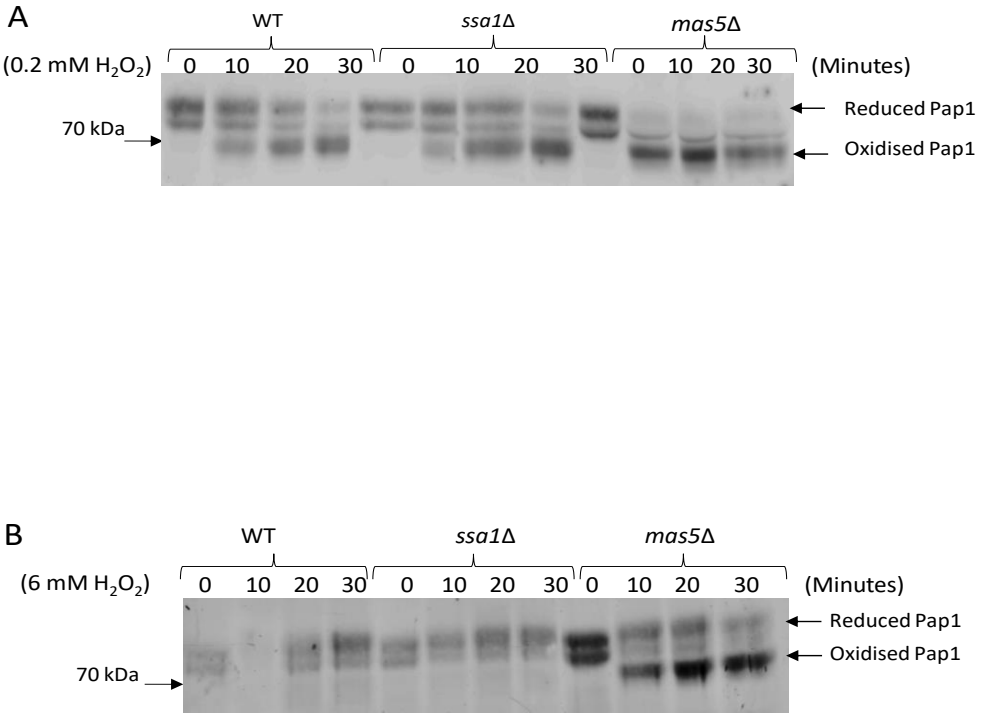


Fig. 5. 3. Oxidation status of Pap1 in response to H_2O_2 of *mas5*Δ cells is misregulated. Exponentially growing wild type (WT) (CHP429), *ssa1*Δ **(**FM14) cells and *mas5*Δ **(**FM15**)** cells were treated with low (0.2 mM) (A) or high (6 mM) (B) H_2O_2 stress for 10, 20 or 30 minutes. Samples were extracted by TCA protocol plus IAA followed by alkaline phosphatase. Proteins were analysed by non-reducing SDS-PAGE and western blotting with anti-Pap1 antibody, n > 3.

or an upstream component in the absence of Mas5 is unclear. In comparison to the published data of Pap1 oxidation in thioredoxin pathway mutants only *txl1*Δ cells show similarity to *mas5*Δ cells in response to 0.2 mM H_2O_2 in terms of Pap1 localisation, oxidation and *gst2*$^+$ expression (Brown *et al*., 2013). However, Pap1 oxidation upon 6 mM H_2O_2 stress in *txl1*Δ cells have not been investigated which would be interesting to examine whether this also shows similarity to *mas5*Δ cells data shown here.

A possible conclusion is that in *mas5*Δ cells Pap1 is being overexpressed which results in the increased visibility of Pap1 nuclear accumulation during H_2O_2 stress, the change to *gst2*$^+$ expression and the visibility of Pap1 protein in western blot analysis. In a recent report overexpression of Pap1 was explored and concluded that higher concentrations of H_2O_2 were required to oxidise Pap1 (i.e. Pap1 oxidation was not observed at 0.2 mM H_2O_2 but was upon 2 mM H_2O_2) (Calvo *et al*., 2013). This is not consistent with the observed Pap1 phenotypes in *mas5*Δ cells which suggests that there is more complexity to this than overexpression of Pap1. Thus, it leads towards the theory Mas5 effecting Pap1 by possibly influencing the regulation of one of the known thioredoxin pathway proteins. Thus, the next step was to look at the RNA and protein expression of known Pap1 regulators Tpx1 (*tpx1*$^+$), Trx1 (*trx1*$^+$) and Txl1 (*txl1*$^+$) in wild type, *ssa1*Δ and *mas5*Δ cells.

5.2.3 RNA and protein levels of thioredoxin pathway factors in *ssa1*Δ and *mas5*Δ cells

Investigation of the thioredoxin pathway factors being regulated by Ssa1 or Mas5 was begun with thioredoxin Trx1. To observe Trx1 protein a strain has been generated previously that expresses Trx1 with a Flag epitope (JB35) (Day *et al*., 2012). This strain was mated to the *ssa1*Δ and *mas5*Δ strains previously used in this work and haploids were selected for the deletion

cassette and Flag marker. Strains were successfully generated with a *ssa1*Δ or *mas5*Δ allele expressing Trx1-Flag.

The level of Trx1-Flag in the Western blot appears to be marginally lower in *ssa1*Δ and *mas5*Δ cells in comparison to wild type cells, however when quantified there was no significant difference (Fig. 5. 4. A).

In wild type cells in normal conditions a low level of *trx1*$^{+}$ is detectable which again is not notably larger than observed in *ssa1*Δ cells (Fig. 5. 4. B). Interestingly, in *mas5*Δ cells there is a clear increase in *trx1*$^{+}$ in relation to wild type cells however this does not appear to translate to a change in protein level. It is important to note that RNA data were collected from strains expressing wild type *trx1*$^{+}$, it is possible that the inclusion of the Flag sequence on the *trx1*$^{+}$ gene is affecting the expression in these strains. However, it can still be stated that the differences in Trx1 (*trx1*$^{+}$) levels in *mas5*Δ cells are not likely to be sufficient to explain the observed misregulation of Pap1 in these cells. Thus, the next step was to investigate the alternate thioredoxin-like protein Txl1 which is directly involved in Pap1 redox cycling.

To observe Txl1 protein a strain has been generated previously that expresses Txl1 with a Flag epitope (JB95) (Brown *et al*., 2013) which was mated to the *ssa1*Δ and *mas5*Δ strains previously used in this work. Strains were successfully generated with a *ssa1*Δ or *mas5*Δ allele expressing Txl1-Flag.

Analysis of Txl1-Flag is complex due to the unusually large number of non-specific reactions of the Flag antibody which has previously been reported (Fig. 5. 5. A; Brown *et al*., 2013). Protein from wild type cells unfortunately has a non-specific band that runs the same as Txl1-

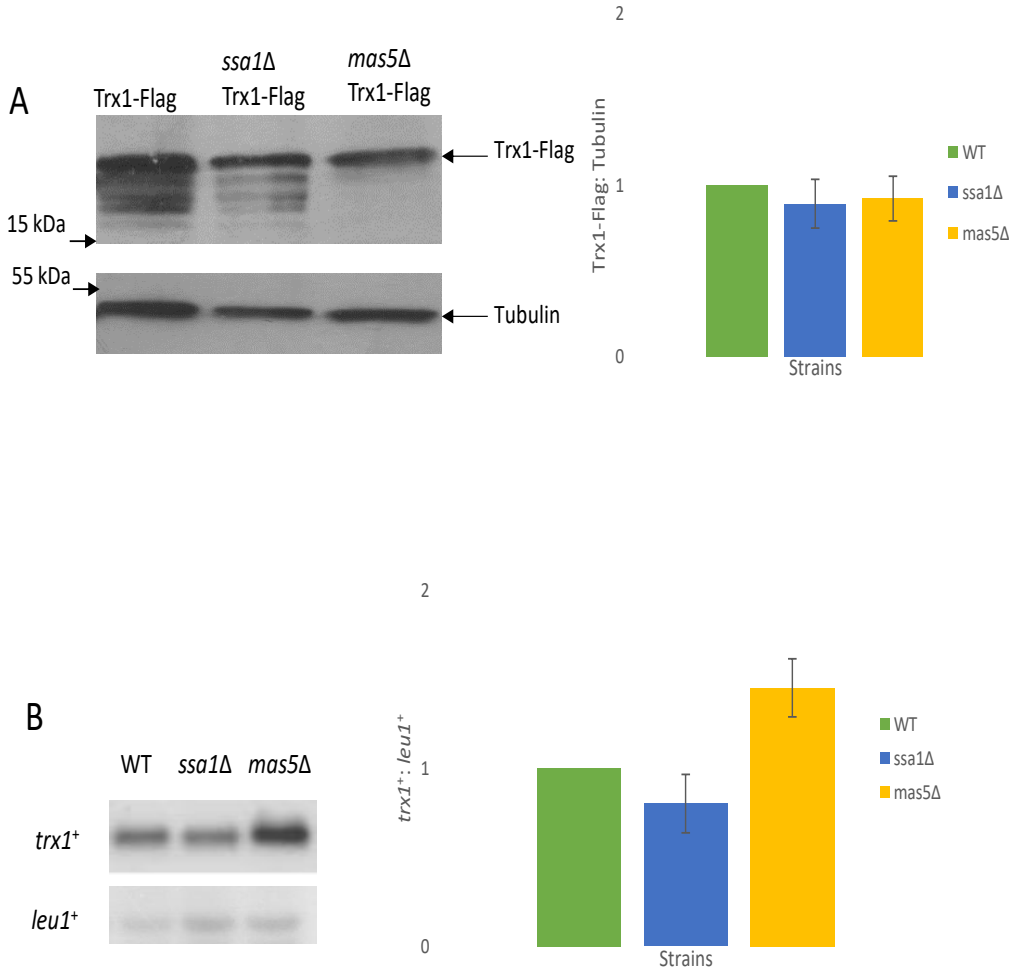


Fig. 5. 4. Investigation of Trx1 protein and t*rx1*$^{+}$ mRNA levels in *ssa1*Δ and *mas5*Δ cells. (A) Exponentially growing Trx1-Flag cells (JB35), with the *ssa1*Δ **(**FM32) allele and *mas5*Δ **(**FM34**)** allele were collected and samples were extracted by TCA protocol. Proteins were analysed by reducing SDS-PAGE and western blotting with anti-Flag antibody, Tubulin was used as a loading control. Levels were calculated relative to wild type unstressed sample, ratio set to 1, n = 3. (B) Wild type (WT) cells (CHP429), *ssa1*Δ (FM14) and *mas5*Δ (FM15) cells were grown to exponential phase and RNA was extracted. Equal amounts of RNA were separated by gel electrophoresis and transferred to Genescreen hybridisation membrane. Following crosslinking the membrane was probed with α-P^{32} labelled PCR products of *trx1*$^{+}$ (primers Trx1.RNA.F and Trx1. RNA.R) and *leu1*$^{+}$ (primers Leu1. RNA.F and Leu1.RNA.R). The membrane was exposed to a phosphoimager screen (Fujifilm). Data were analysed using a Typhoon FLA 3500 and quantified using ImageQuant TL software levels were calculated relative to wild type sample, ratio set to 1. n = 3, error bars indicate standard deviation.

Flag. Therefore, a *txl1*Δ strain is used which does not have the non-specific that aligns to Txl1-Flag. Analysis of Txl1-flag by western blotting remains problematic but the Txl1-Flag can be identified in the presence of these control strains.

The level of Txl1-Flag appears to be unaffected by the presence of the *ssa1*Δ allele but is marginally lower in the *mas5*Δ cells compared to wild type cells (Fig. 5. 5. A). Due to the non-specifics it is hard to qualitatively or quantitatively analyse Txl1-Flag levels in these strains. Therefore, it was necessary to rely on RNA analysis of *txl1*$^+$ levels in these strains to clarify what may be occurring within these cells.

Basal level of *txl1*$^+$ from *ssa1*Δ cells seems similar to wild type cells which is consistent with the Txl1 protein analysis (Fig. 5. 5. B). However, *txl1*$^+$ levels appear to be lower in *mas5*Δ cells than wild type cells which is consistent with lower Txl1 protein levels. Using an ANOVA test, the level of *txl1*$^+$ in *mas5*Δ cells was found to be significantly lower (ANOVA test $p < 0.05$). These data would appear to suggest that Ssa1 is not a factor in regulating *txl1*$^+$ or Txl1 levels. However, it seems that Mas5 is required to maintain a basal transcription of *txl1*$^+$ linked to the basal level of the protein. The analysis of these data has the same caveat as Trx1 (*trx1*$^+$), Txl1 protein has a Flag epitope while the RNA data were produced from the wild type *txl1*$^+$ gene. The presence of the Flag epitope has to be considered of how it may affect the RNA and protein levels of Txl1-Flag.

The next factor in the thioredoxin pathway to be considered is the Prx Tpx1. Tpx1 has been identified as key sensor of oxidative stress and acts as a molecular switch to activate the appropriate transcription factor. Oxidised Tpx1 is reduced by Trx1/Txl1 which rapidly oxidises

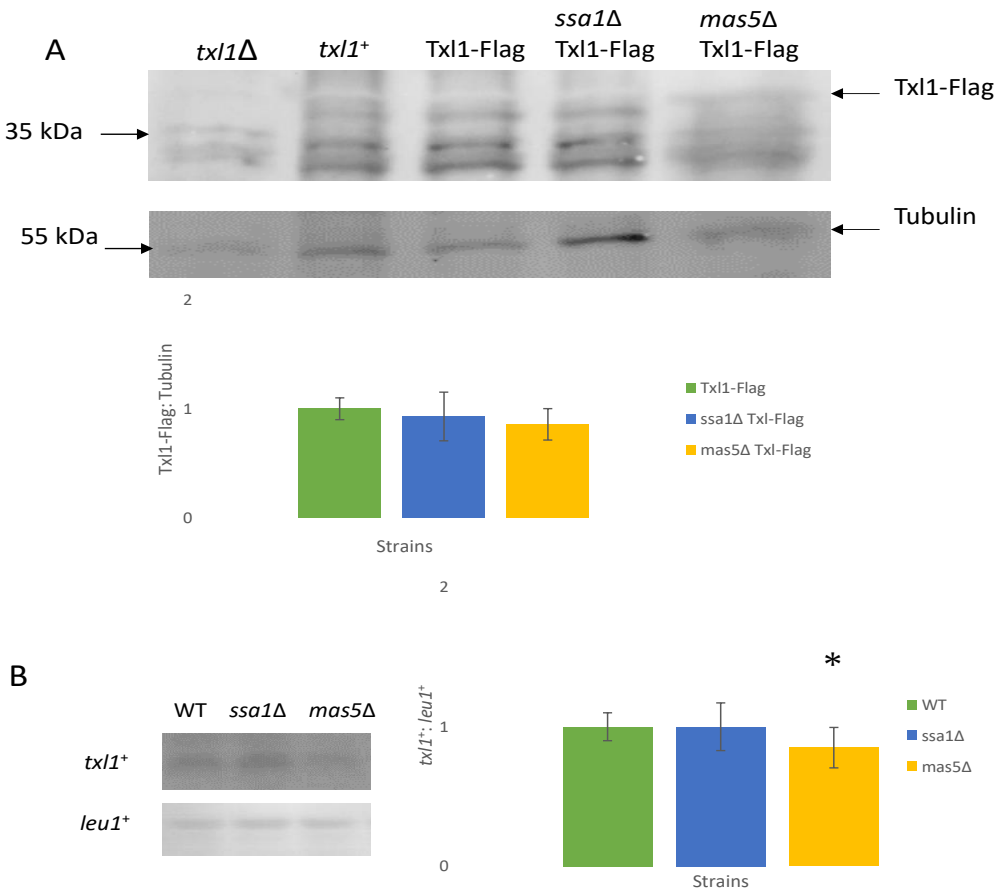


Fig. 5. 5. Investigation of Txl1 protein and *txl1*$^{+}$ mRNA levels in *ssa1*Δ and *mas5*Δ cells. (A) Exponentially growing *txl1*Δ (EV75), *txl1*$^{+}$ (CHP429), Txl1-Flag cells (JB95), with the *ssa1*Δ **(**FM35) allele and *mas5*Δ **(**FM30**)** allele were collected and samples were extracted by TCA protocol. Proteins were analysed by reducing SDS-PAGE and western blotting with anti-Flag antibody, Tubulin was used as a loading control. Levels were calculated relative to wildtype unstressed sample, ratio set to 1, n = 3. (B) Wild type (WT) cells (CHP429), *ssa1*Δ (FM14) and *mas5*Δ (FM15) cells were grown to exponential phase and RNA was extracted. Equal amounts of RNA were separated by gel electrophoresis and transferred to Genescreen hybridisation membrane. Following crosslinking the membrane was probed with α-P^{32} labelled PCR products of *trx1*$^{+}$ (primers Txl1.RNA.F and Txl1. RNA.R) and *leu1*$^{+}$ (primers Leu1. RNA.F and Leu1.RNA.R). The membrane was exposed to a phosphoimager screen (Fujifilm). Data were analysed using a Typhoon FLA 3500 and quantified using ImageQuant TL software, levels were calculated relative to wild type sample, ratio set to 1. n = 3, error bars indicate standard deviation. Statistical significant ($p < 0.05$) was calculated using an ANOVA test, * indicates significant difference from WT.

the pool of thioredoxins leading to oxidation of Pap1. Thus, next the level of Tpx1 (*tpx1*$^{+}$) needed to be investigated in *ssa1*Δ cells and *mas5*Δ cells.

The level of Tpx1in *ssa1*Δ cells appears marginally less than wild type cells, this was confirmed by quantification (Fig. 5. 6. A). In contrast to this, within *mas5*Δ cells there is much larger pool of Tpx1 in comparison to wild type cells (Fig. 5. 6. A). This suggests that Mas5 acts as a promoter of Tpx1 degradation or an inhibitor of *tpx1*$^{+}$ transcription. Therefore, the next step was to establish if Tpx1 levels are increased due to changes in the RNA level or it is due to altered proteostasis of Tpx1.

To investigate this, the transcript levels of *tpx1*$^{+}$ in these strains needed to be observed. Basal level of *tpx1*$^{+}$ appears to be slightly lower in *ssa1*Δ cells than wild type cells which is consistent with lower Tpx1 protein levels (Fig. 5. 6. B). *mas5*Δ cells basal levels of *tpx1*$^{+}$ are higher than wild type cells, this is confirmed by the quantification and consistent with the increased protein level. In summary, the only conclusive data of investigating the thioredoxin pathway factors is that only Tpx1 shows an increase in both protein and gene expression in *mas5*Δ cells.

5.2.4 Pap1 regulation in *mas5*Δ cells shows similarity to overexpression of Tpx1

There is very limited data available of what effects Tpx1 overexpression has upon Pap1 oxidation and gene expression in unstressed or H_2O_2 stress conditions. However, it has previously been shown that overexpression of Tpx1 does not have any major impact on Pap1 in unstressed cells but upon high concentrations of H_2O_2 stress (1 mM) stimulates strong nuclear Pap1 localisation (Bozonet, 2006). This leads towards a theory that the phenotypes of Pap1 regulation in *mas5*Δ cells is due to Tpx1 overexpression. To test this theory the localisation of

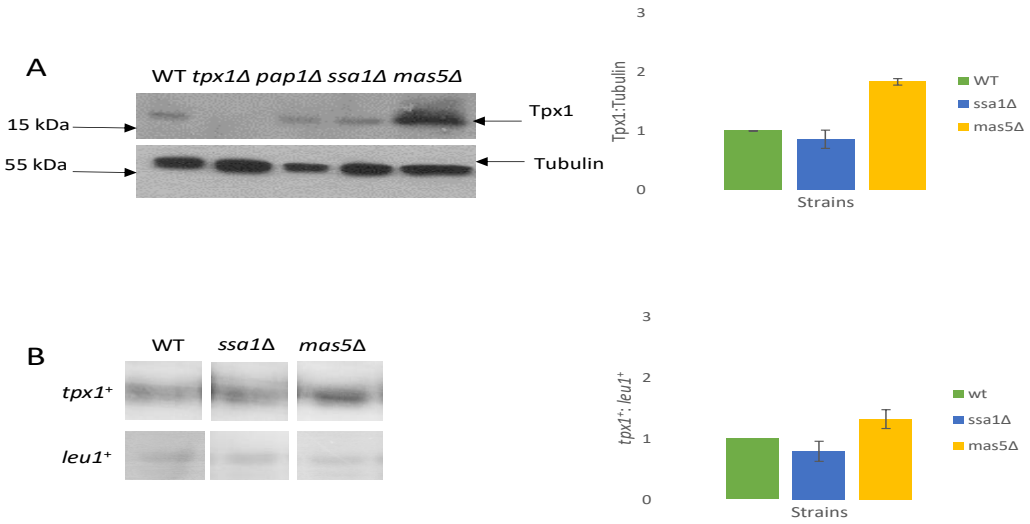


Fig. 5. 6. Investigation ofTpx1 protein and *tpx1*$^{+}$ mRNA levels in *ssa1*Δ and *mas5*Δ cells. (A) Exponentially growing wild type (WT) cells (CHP429), *tpx1*Δ (VXOO), *pap1*Δ (TP108-3C), *ssa1*Δ **(**FM14) and *mas5*Δ **(**FM15**)** cells were collected and samples were extracted by TCA protocol. Proteins were analysed by reducing SDS-PAGE and western blotting with anti-Tpx1 antibody, Tubulin was used as a loading control. Levels were calculated relative to wildtype unstressed sample, ratio set to 1, n = 3. (B) Wild type (WT) cells (CHP429), *ssa1*Δ (FM14) and *mas5*Δ (FM15) cells were grown to exponential phase and RNA was extracted. Equal amounts of RNA were separated by gel electrophoresis and transferred to Genescreen hybridisation membrane. Following crosslinking the membrane was probed with α-P^{32} labelled PCR products of *trx1*$^{+}$ (primers Tpx1.RNA.F and Tpx1. RNA.R) and *leu1*$^{+}$ (primers Leu1. RNA.F and Leu1.RNA.R). The membrane was exposed to a phosphoimager screen (Fujifilm). Samples shown were run in the same northern blot but excess data have been removed from the image. Data were analysed using a Typhoon FLA 3500 and quantified using ImageQuant TL software, fold induction was calculated relative to wild type sample, ratio set to 1. n = 3, error bars indicate standard deviation.

Pap1 in *mas5*Δ cells was investigated in response to 1 mM H_2O_2 to compare it to the localisation observed in these conditions of cells overexpressing Tpx1.

Wild type cells exposed to 1 mM H_2O_2 do not show Pap1 nuclear accumulation until towards the end of the time course (Fig. 5. 7). In contrast, *mas5*Δ cells exposed to 1 mM H_2O_2 show a strong nuclear accumulation of Pap1 across the time course. This is replicative of the data obtained for cells overexpressing Tpx1 and therefore agrees with the model that in *mas5*Δ cells Tpx1 is being overexpressed effecting Pap1 localisation and therefore Pap1-dependent oxidation with Pap1-dependent gene expression.

5.2.5 Oxidation of Thioredoxin pathway proteins in *ssa1*Δ and *mas5*Δ cells

Tpx1 relays the redox state of the cell to Pap1 via Trx1/Txl1. Trx1 and Txl1 have been shown to interact with Tpx1 along with numerous other substrates. The levels of Trx1 and Txl1 are known to be much lower than Tpx1. Thus, there is significant pressure on the availability of Trx1/Txl1 to maintain a pool of reduced Tpx1. The effect of Tpx1 overexpression has not been investigated any further than the effect on Pap1 localisation. Therefore, given previous work showing that Tpx1 regulation of Pap1 involves the oxidation state of Txl1 and Trx1 next it was important to see if there is any difference in oxidation of the Txl1 and Trx1 proteins after H_2O_2 treatment in *mas5*Δ cells. Protein extracts were treated with AMS, a reagent that binds to reduced cysteines of proteins. When analysed by western blot, this usually appears as the reduced protein monomer having reduced mobility due to the AMS moiety in comparison to an oxidised form.

In wild type cells, Trx1-Flag was oxidised rapidly following exposure to 0.2 mM H_2O_2 but not upon 6 mM H_2O_2 over the time course which correlates to when Tpx1 is known to be

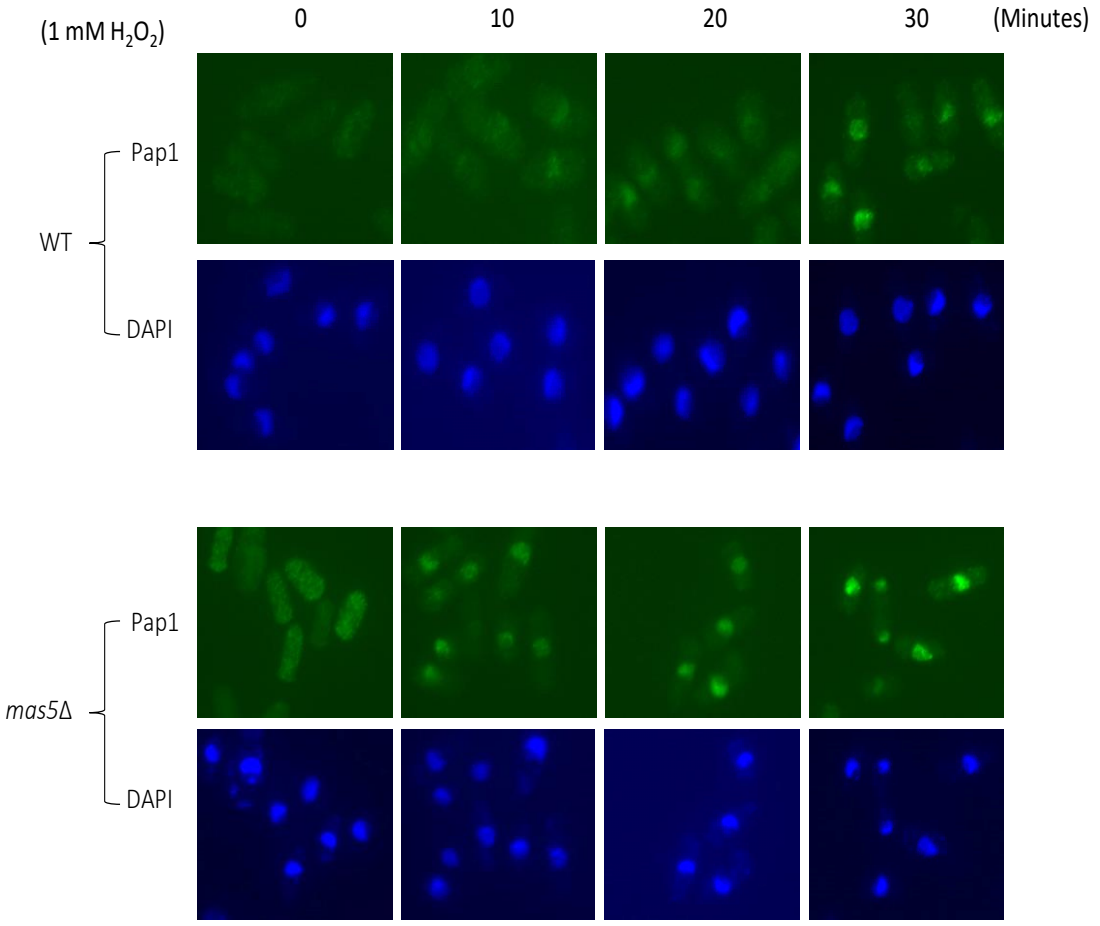


Fig. 5. 7. Misregulation of Pap1 localisation after treatment with 1 mM H_2O_2 in *mas5*Δ cells. Pap1 was visualised by indirect immunofluorescence using Pap1 antibodies in exponential phase fixed wildtype (WT) (CHP429) and *mas5*Δ (FM15) cells after treatment with 1 mM H_2O_2 for 0, 10, 20 or 30 minutes. GFP (fluorescent) and DAPI images are shown of representative cells. Cells were fixed using paraformaldehyde, exposures remaining constant between images, n = 2.

hyperoxidised and thus not a substrate for Trx1 (Fig. 5. 8.) Interestingly, in *ssa1*Δ and *mas5*Δ cells the same profile of Trx1-Flag oxidation is observed as wild type cells in response to H_2O_2 stress. This suggests Pap1 misregulation in *mas5*Δ cells is not linked to the oxidation state of Trx1. Therefore, the next step was to investigate the oxidation of Txl1 in *ssa1*Δ and *mas5*Δ cells.

The importance of Txl1 in Pap1 regulation has previously been discussed (see section 1.3.2.2). Additionally, there are similarities between Pap1 localisation in *txl1*Δ and *mas5*Δ cells (Brown *et al*., 2013). Previous work has showed that Txl1 becomes oxidised upon 0.2 mM H_2O_2 stress but is inhibited from oxidation due to Tpx1 hyperoxidation at 6 mM H_2O_2. Therefore, it raises the question of whether Txl1 can be oxidised at 6 mM H_2O_2 in *mas5*Δ cells. As previously discussed (see section 5.2.3) and noted in published work it is very hard to detect Txl1-Flag and investigation of its oxidation status is even more problematic (Brown *et al*., 2013). It was attempted to analyse oxidation of Txl1-Flag but unfortunately reliable data could not be obtained which means the role of Txl1 in *mas5*Δ cells Pap1 regulation remains unknown and needs more exploration. However, having just shown that Trx1 oxidation in *mas5*Δ cells appears normal. It can't be ruled out that Txl1 oxidation is linked to the *mas5*Δ cells effects on Pap1 activity, localisation, Pap1 dependent gene expression.

Since it has been shown that in *mas5*Δ cells Tpx1 is overexpressed but does not influence Trx1 oxidation it meant it was important to investigate Tpx1 oxidation in these cells in response to 0.2 and 6 mM H_2O_2. Additionally, it may provide insight in to the role of Txl1 since it is involved in Tpx1 oxidation. Investigation of Tpx1 oxidation may also elucidate the effect overexpression of Tpx1 has on the redox status of Tpx1. Wild type, *ssa1*Δ and *mas5*Δ cells were treated with low (0.2 mM) and high (6 mM) H_2O_2 stress and Tpx1 oxidation examined.

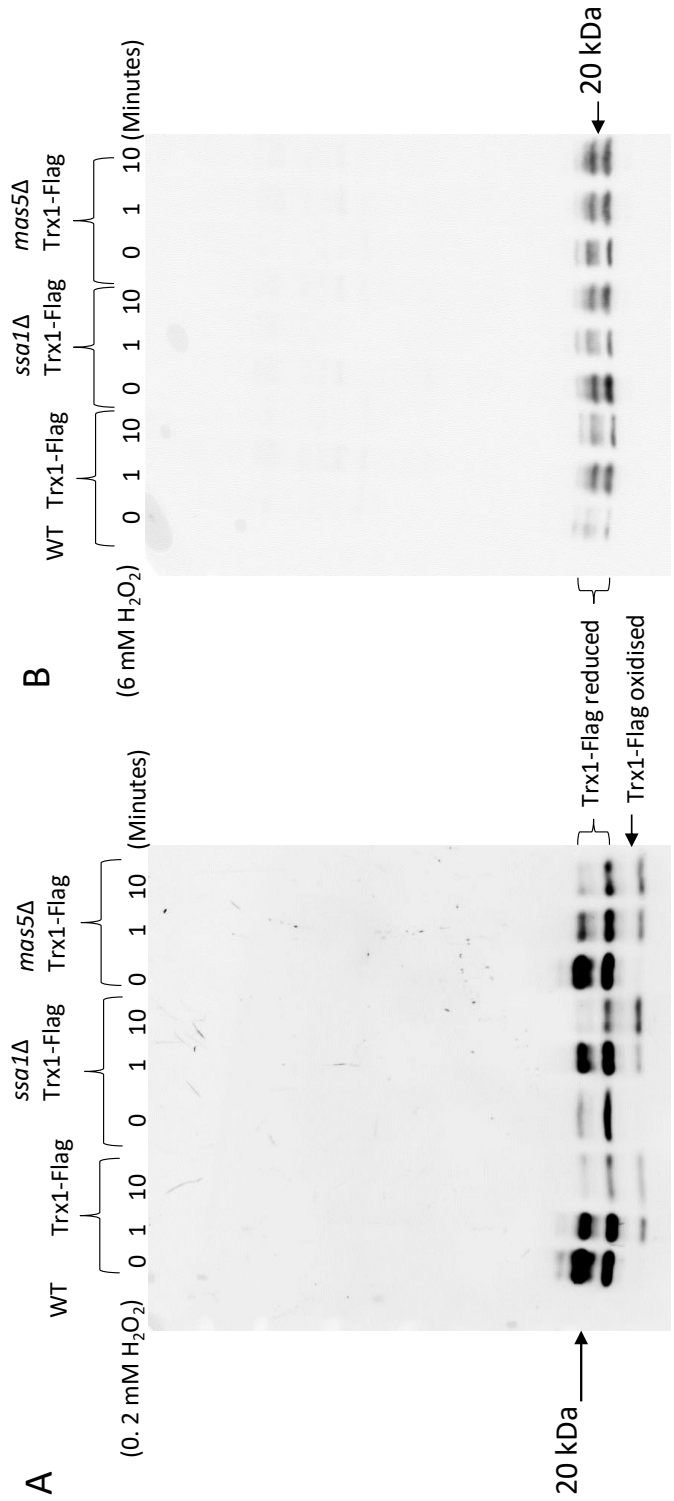


Fig. 5. 8. Oxidation of Trx1-Flag is not altered in *ssa1*Δ and *mas5*Δ cells. Exponentially growing wild type (WT) (CHP429), Trx1-Flag cells (JB35), with the *ssa1*Δ **(**FM32) allele and *mas5*Δ **(**FM34**)** allele were treated with low (0.2 mM) (A) or high (6 mM) (B) H_2O_2 stress for 0, 1 or 10 minutes. Samples were extracted by TCA protocol plus AMS. Proteins were analysed by non-reducing SDS-PAGE and western blotting with anti-Flag antibody, n = 3.

In unstressed conditions Tpx1 is found in the monomeric reduced form (Fig. 5. 9. A). After exposure to low H_2O_2 (0.2 mM) increased levels of the Tpx1 disulphide dimer were detected which remains over the time course in wild type and *ssa1*Δ cells (Fig 5. 9. A). At 10 minutes there is a small detectable amount of hyperoxidised Tpx1 in wild type and *ssa1*Δ cells. Interestingly, in *mas5*Δ cells the level of reduced monomeric Tpx1 appears to be much larger at all time points than wild type cells. Following exposure to low H_2O_2 concentrations there is a small increase in Tpx1 disulphide formation which is recovered over the time-course in *mas5*Δ cells. For each strain the formation of the Tpx1 dimer corresponds to Pap1 oxidation (compare Fig. 5. 3. A and Fig. 5. 9. A). This is due to oxidised Tpx1 completely oxidising the available Trx1/Txl1. Trr1 reduces Trx1/Txl1 and thus Pap1. Competition as substrates for Trr1 results in oxidation of Pap1. Exposure of all 3 strains to a high concentration of H_2O_2 (6 mM) results in a lower mobility form of monomeric Tpx1, this corresponds to hyperoxidised Tpx1 (Fig. 5. 9. B). The hyperoxidation of Tpx1 was sustained over the time course for all the strains when exposed to high H_2O_2 stress. However, in *mas5*Δ cells the hyperoxidation of Tpx1 does not correlate to inhibition of Pap1 oxidation (compare Fig. 5. 3. B and Fig. 5. 9. B).

It is thought that in wild type cells exposed to high H_2O_2 stress Tpx1 is hyperoxidised so there are greater levels of reduced Trx1/Txl1 so Pap1 does not become oxidised. Overexpression of Tpx1 from a plasmid showed very transient oxidation of Trx1 which is not observed in *mas5*Δ cells (Day *et al*., 2012). It was also shown that cells overexpressing Tpx1 exposed to 1 mM H_2O_2 for 10 minutes form both oxidised and hyperoxidised Tpx1 while 6 mM H_2O_2 treatment only resolves hyperoxidised Tpx1. It should be noted that it is unknown how the relative increase in Tpx1 in *mas5*Δ cells compare to the concentration found in cells carrying a high copy plasmid encoding $tpx1^+$. Taken together there are similarities in Tpx1 overexpression from a plasmid and that of *mas5*Δ cells. *mas5*Δ cells in response to H_2O_2 show wild type regulation

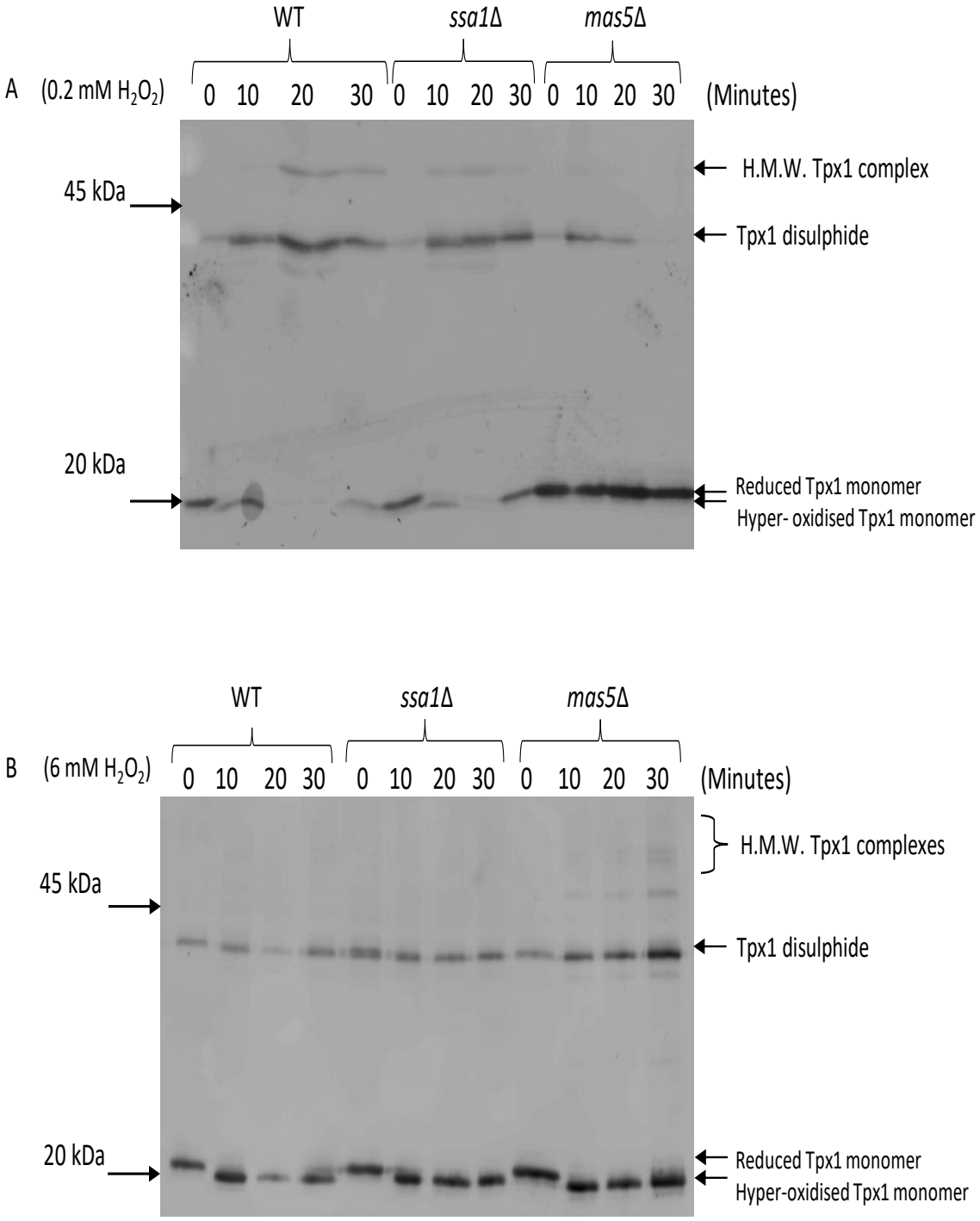


Fig. 5. 9. Mas5 affects the oxidation status of Tpx1 in response to H_2O_2. Exponentially growing wild type (WT) (CHP429), *ssa1*Δ (FM14), *mas5*Δ (FM15) cells were collected after treatment with 0.2 mM (A) or 6 mM (B) H_2O_2 for 0, 10, 20 or 30 minutes. and extracted by the TCA protocol and treated with AMS. Proteins were analysed by non-reducing SDS-PAGE and western blotting with anti-Tpx1 antibody, n > 3.

of Trx1 oxidation but upon 6 mM H_2O_2 show constitutive Pap1 oxidation. The mechanism of how Pap1 is regulated in *mas5*Δ cells could potentially be via a direct interaction between Tpx1 and Pap1 forming a disulphide as has been proposed to exist (Calvo *et al.*, 2013). Alternatively, in *mas5*Δ cells Pap1 oxidation upon high H_2O_2 stress is due to Txl1 oxidation but not Trx1.

5.2.6 Mas5 has a role in Pap1 solubility

Published data of overexpression of Pap1 suggested it lead to inhibition of Pap1 oxidation until higher concentrations of H_2O_2 stress (Calvo *et al*., 2013). It was still possible that Mas5 may have a role in influencing Pap1 by affecting the levels (or activity) of Pap1 Previous work from our lab has showed that Trx1 and Txl1 are both important for maintaining solubility of Pap1. Additionally, it was shown that loss of Trx1 and Txl1 in a *trx1*Δ*txl1*Δ mutant results in constitutively induced *pap1*$^+$ expression (Brown *et al*., 2013). Since there were similarities in *mas5*Δ cells Pap1 localisation and the *trx1*Δ*txl1*Δ mutant but Txl1 oxidation was lacking it raised the question of whether Pap1 solubility was affected in *mas5*Δ cells. Therefore, the next step was to investigate Pap1 levels and solubility. Additionally, it was important to look at *pap1*$^+$ expression in unstressed and 0.2 mM H_2O_2 stress conditions in wild type, *ssa1*Δ and *mas5*Δ cells.

To investigate the total amount of Pap1 present within the cells a total protein extract method can be employed which results in collection of all cellular protein (see section 2.2.9.2 for details). However, to solely investigate the soluble protein fraction a pombe lysis extraction can be performed (see section 2.2.9.1 for details). The latter method is under non-denaturing conditions and thus insoluble proteins are resistant to extraction.

The total amount of Pap1 in wild type cells and *ssa1*Δ cells appear comparable (Fig. 5. 10. A). Interestingly, in *mas5*Δ cells total Pap1 levels appear the same as wild type cells. *ssa1*Δ cells show the same profile of soluble Pap1 as wild type cells (Fig. 5. 10. B). Interestingly, *mas5*Δ cells have a significantly lower level of soluble Pap1 in normal conditions compare to wild type cells as determined by an ANOVA test ($p < 0.01$) (Fig. 5.10. B). This could indicate that Mas5 is required to maintain Pap1 in a soluble state.

It was previously shown that the thioredoxins Trx1/Txl1 are required to maintain Pap1 in a reduced and soluble form (Brown *et al*., 2013). The data presented here suggest Mas5 may be involved in signal transduction to Pap1 either directly or through Trx1/Txl1 regulation. Since Trx1 regulation appears normal in *mas5*Δ cells, this points towards Txl1 as being the important regulator of Pap1 in this strain. In *trx1*Δ*txl1*Δ cells the transcription of *pap1*$^+$ is constitutively induced which is theorised to compensate for the reduction of soluble Pap1 (Brown *et al*., 2013). Thus, it was of interest to see if *pap1*$^+$ levels were affected in the *mas5*Δ strain since the solubility of Pap1 was affected.

*ssa1*Δ cells showed comparable levels to wildtype cells of *pap1*$^+$ (Fig. 5. 10. C). In contrast, *mas5*Δ cells show a larger basal expression of *pap1*$^+$ which is consistent with the data observed in *trx1*Δ*txl1*Δ cells. The constitutive induction of *pap1*$^+$ in *trx1*Δ*txl1*Δ cells is not responsive to 0.2 mM H_2O_2 stress which was believed to be due to de-repression of Pap1 activity (Brown *et al*., 2013). However, in *mas5*Δ cells upon 0.2 mM H_2O_2 stress there is an increase in *pap1*$^+$ level above the large basal (Fig. 5. 10. D). This suggests that in *mas5*Δ cells another level of *pap1*$^+$ regulation is occurring that is still sensitive to H_2O_2 stress. The data lead towards a role for a thioredoxin Trx1/Txl1 in Mas5 regulation of Pap1.

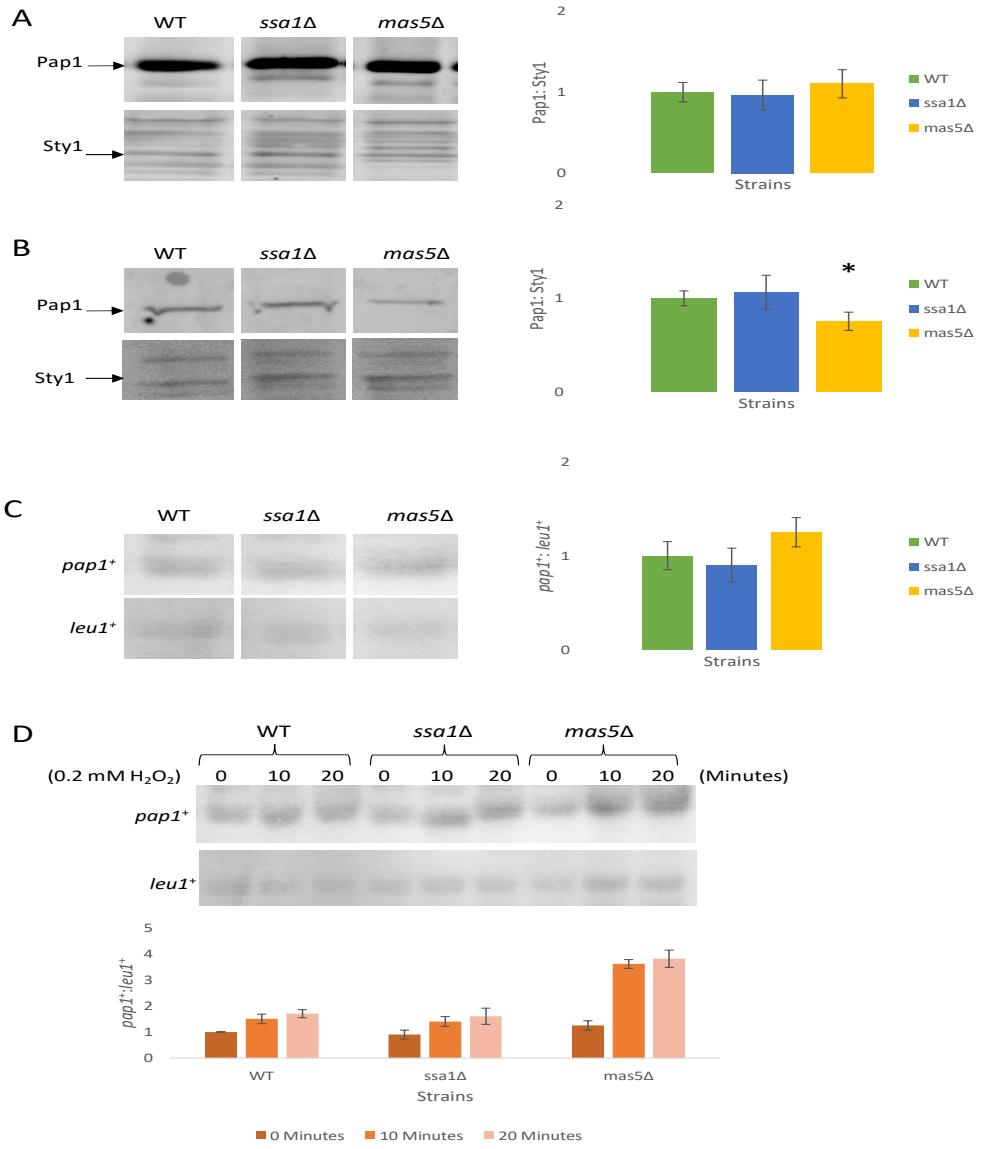


Fig. 5. 10. Mas5 is important for *pap1*$^+$ mRNA transcription and maintaining Pap1 solubility. Exponentially growing wild type (WT) (CHP429), *ssa1*Δ (FM14), *mas5*Δ (FM15) cells were collected and extracted by either the TCA protocol (A) or the pombe lysis method (B) plus NEM, alkaline phosphatase and β-mercaptoethanol. Samples were run in an SDS-PAGE gel and analysed by western blotting for Pap1 or Sty1 levels as a loading control. Samples shown were run in the same western blot but excess data have been removed from the image. Data were analysed using a Typhoon FLA 3500 and quantified using ImageQuant TL software, fold induction was calculated relative to wild type unstressed sample, ratio set to 1. n = 3. Exponentially growing cells were collected (C) or exposed to 0.2 mM H_2O_2 for 0, 10, or 20 minutes (D) then collected and RNA was extracted. RNA was run on a phosphate gel and analysed by northern blotting using radioactive probes for *pap1*$^+$ and *leu1*$^+$ as a loading control, the blot was exposed to a phosphoimager screen. Samples shown were run in the same northern blot but excess data have been removed from the image. Data were analysed using a Typhoon FLA 3500 and quantified using ImageQuant TL software, fold induction was calculated relative to wild type unstressed sample, ratio set to 1. Statistical significance determined by ANOVA test $p < 0.01$, indicated by an asterisk. Samples shown were run in the same western/northern blot but excess data have been removed from the image. n= 3.

5.2.7 Mas5 may have a role in Tpx1 (*tpx1*$^+$) regulation

Despite the above data of Pap1 levels in the *mas5*Δ strain and given the published effect of overexpression Pap1 resulting in increased resistance to H_2O_2 induced oxidation (Calvo *et al*., 2013), there is significant similarity of the effects of Tpx1 overexpression (Bozonet, 2006) and *mas5*Δ cells on Pap1 localisation and activity. Therefore, it was likely that the loss of Mas5 function on Pap1 were due to increased levels of Tpx1. However, it was possible that the effects of loss of Mas5 on Pap1 function were independent of Tpx1. To clarify this issue, the potential connection between Tpx1 and the activation of Pap1 in response to H_2O_2 was further investigated. It was possible that the effect of *mas5*Δ allele on Pap1 activation upon 0.2 mM and 6 mM H_2O_2 stress (i.e. *gst2*$^+$ gene expression Fig. 5. 1. and oxidation of Pap1 Fig. 5. 3.) could have been independent of Tpx1 despite the increased levels of Tpx1 protein in basal conditions. To test this the expression of *tpx1*$^+$ upon 0.2 mM and 6 mM H_2O_2 stress in wild type, *ssa1*Δ and *mas5*Δ cells was investigated.

Wild type cells exposed to 0.2 mM H_2O_2 show an increase in *tpx1*$^+$ levels over the time course which is consistent with the literature since it has been shown that this is a Pap1-dependent gene (Fig. 5. 11. A) (Chen *et al*., 2008). The same profile as wild type cells are observed in *ssa1*Δ cells exposed to 0.2 mM H_2O_2, this is also consistent with the Pap1-dependent *gst2*$^+$ data obtained for the *ssa1*Δ strain (Fig. 5. 1.). Unstressed *mas5*Δ cells transcription of *tpx1*$^+$ is higher than wild type cells as observed before but excitingly show a larger induction in *tpx1*$^+$ levels over the time course. This is also consistent with the *gst2*$^+$ levels observed in the *mas5*Δ strain. Wild type and *ssa1*Δ cells exposed to 6 mM H_2O_2 show a similar increase in *tpx1*$^+$ levels as wild type cells, the induction is lower than observed in response to 0.2 mM H_2O_2 which is consistent with previous reports (Quinn *et al.*, 2002) (Fig. 5. 11. B). Interestingly, *mas5*Δ cells in response to 6 mM H_2O_2 show a much larger induction of *tpx1*$^+$ across the time course than

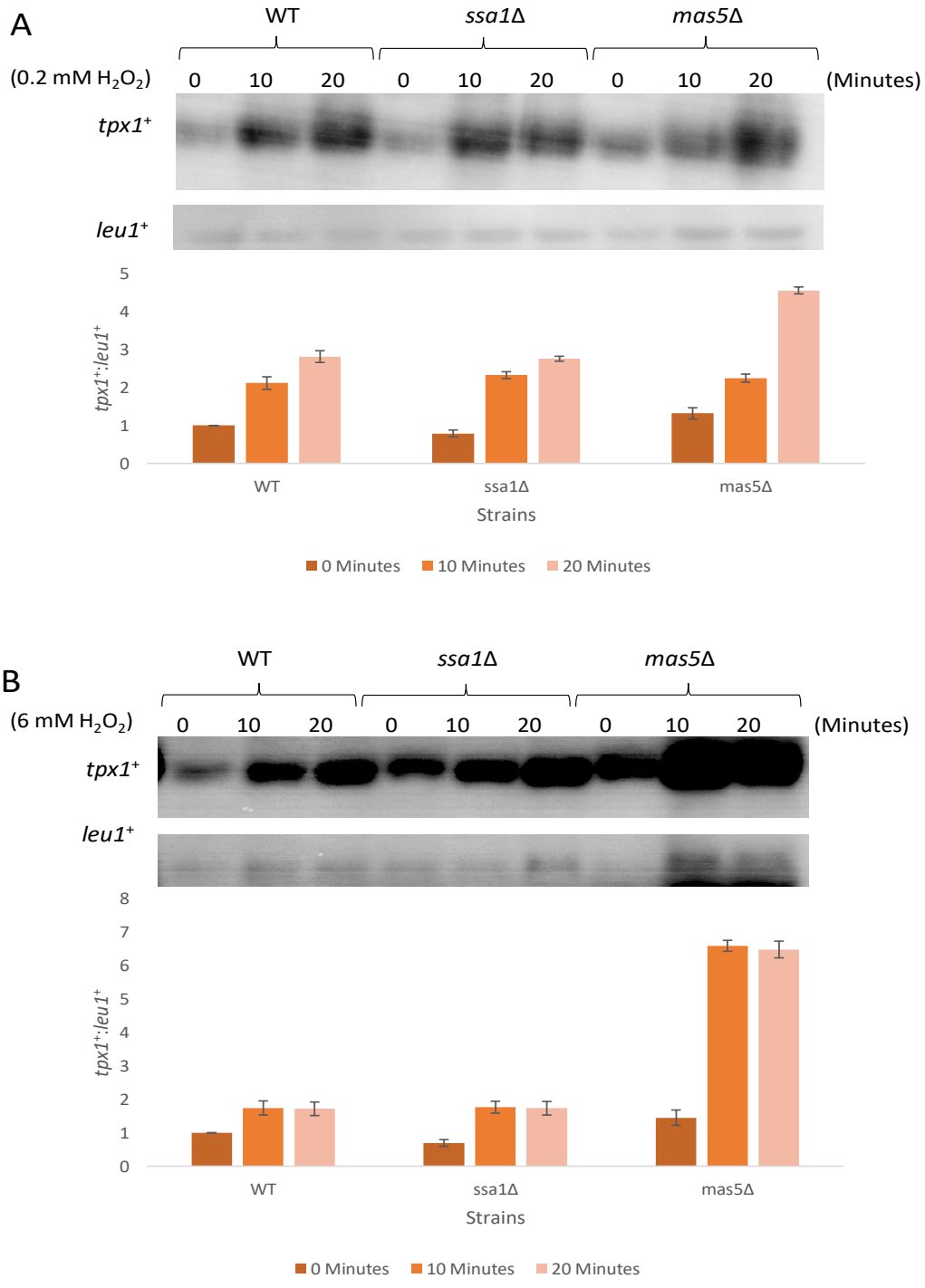


Fig. 5. 11. *tpx1*$^+$ mRNA levels are misregulated in *mas5*Δ cells in unstressed and oxidative stress conditions. Exponentially growing wild type (WT) cells (CHP429), *ssa1*Δ (FM14) and *mas5*Δ (FM15) cells were collected and RNA extracted after exposure to 0.2 mM H_2O_2 (A) or 6 mM H_2O_2 (B) for 0, 10 or 20 minutes. Samples were run in a phosphate gel and analysed by northern blotting. Radioactive probes were used to visualise *tpx1*$^+$ levels and *leu1*$^+$ as a loading control. Samples were quantified using ImageQuant, fold induction was calculated relative to wild type unstressed sample, ratio set to 1, n = 3.

in response to 0.2 mM H_2O_2, this contrasts the *gst2*$^+$ data for this strain which was more greatly induced in response to 0.2 mM H_2O_2. This leads towards the theory that despite both *gst2*$^+$ and *tpx1*$^+$ being Pap1-dependent genes it suggests they may be regulated in a different manner.

There is evidence of this being the case; *gst2*$^+$, *tpx1*$^+$, *trx1*$^+$ and *pap1*$^+$ are all induced by exposure to H_2O_2 and cadmium. However, *tpx1*$^+$, *pap1*$^+$ and *trx1*$^+$ are induced by heat unlike *gst2*$^+$ (Chen *et al*., 2003). The Pap1-depdendent gene *obr1*$^+$ is only induced by H_2O_2. Therefore, if *tpx1*$^+$ expression increase during heat stress was purely due to induced Pap1 levels then *obr1*$^+$ should also increase but *obr1*$^+$ levels are non-inducible in heat stress. This implies that *tpx1*$^+$ induction during heat stress is not simply due to an increase in Pap1 levels, it suggests an alternate heat responsive transcription factor could also regulate *tpx1*$^+$ expression, such as Hsf1.

Tpx1 is known to act as a molecular chaperone independent of its thioredoxin peroxidase activity (Jang *et al*., 2004). This would be consistent with Tpx1 aiding protein folding events in response to heat and thus needs to be heat inducible. Interestingly, in *S. cerevisiae* the Tpx1 homolog Tsa1 has recently been shown to be recruited to protein aggregates during heat and H_2O_2 stress (Hanzen *et al*., 2016). Furthermore, it was established that specifically during H_2O_2 stress Tsa1 is required to recruit Ssa1/Ssa2 to aid in degradation of protein aggregates. Ydj1 was found to be required specifically for disaggregation upon heat stress rather than H_2O_2 stress which was postulated to be due to H_2O_2 induced inactivation of Ydj1.

Collectively, this is interesting to consider how *S. pombe* Mas5 has high basal *tpx1*$^+$ and induced by H_2O_2 stress. This could suggest some level of conservation of Ssa1/2 and Ydj1/Mas5 in stress response to heat and H_2O_2 stress response but may be utilised in an organism specific manner. Therefore, it was attempted to generate a *tpx1*Δ*mas5*Δ strain to test Pap1 activation

and Pap1-dependent gene expression in these cells. However, it was not possible to generate this strain during this project but the reason is unclear. It is possibly a lethal combination of alleles but it may also be due to how many aberrant phenotypes the single mutants contain making it very difficult to generate a double mutant.

To explore the potential role of Tpx1 in *mas5*Δ cells on Pap1 function/regulation another approach was devised. Tpx1 in an oxidised form is a substrate for Trx1 while hyperoxidised Tpx1 can only be reduced by Srx1. Therefore, it would be predicted that upon 6 mM H_2O_2 stress that Srx1 might be more important than at 0.2 mM H_2O_2 to influence the activation of Pap1 in *mas5*Δ cells if the Pap1 regulation was Tpx1-dependent. To test this, a *srx1*Δ*mas5*Δ double mutant strain was generated and used to and analyse oxidation of Pap1 in *mas5*Δ and *srx1*Δ*mas5*Δ cells upon 0.2 mM and 6 mM H_2O_2 stress.

As shown previously, *mas5*Δ cells display strong Pap1 oxidation following exposure to 0.2 mM and 6 mM H_2O_2 stress over the time course (Fig. 5. 12). *srx1*Δ*mas5*Δ cells upon 0.2 mM H_2O_2 stress show the same response as *mas5*Δ cells which is consistent as Tpx1 is not hyperoxidised in response to low H_2O_2 stress (Fig. 5. 12. A). However, *srx1*Δ*mas5*Δ cells treated with 6 mM H_2O_2 there is only transient oxidation of Pap1 over the time course (Fig. 5. 12. B). This shows the loss of Srx1 inhibited Pap1 oxidation in the *srx1*Δ*mas5*Δ double mutant in high H_2O_2 stress conditions. This is consistent with the model that deletion of *mas5*$^+$ induces Tpx1 (*tpx1*$^+$) levels to increase and activates Pap1 at high concentrations of H_2O_2 stress.

In the *srx1*Δ*mas5*Δ strain Pap1-dependent gene expression and Pap1 localisation was not explored which could potentially offer more insight in to the regulation of Tpx1 on Pap1 activity. It is also possible that the *mas5*Δ effects on *tpx1*$^+$ expression could be Hsf1 dependent regulation

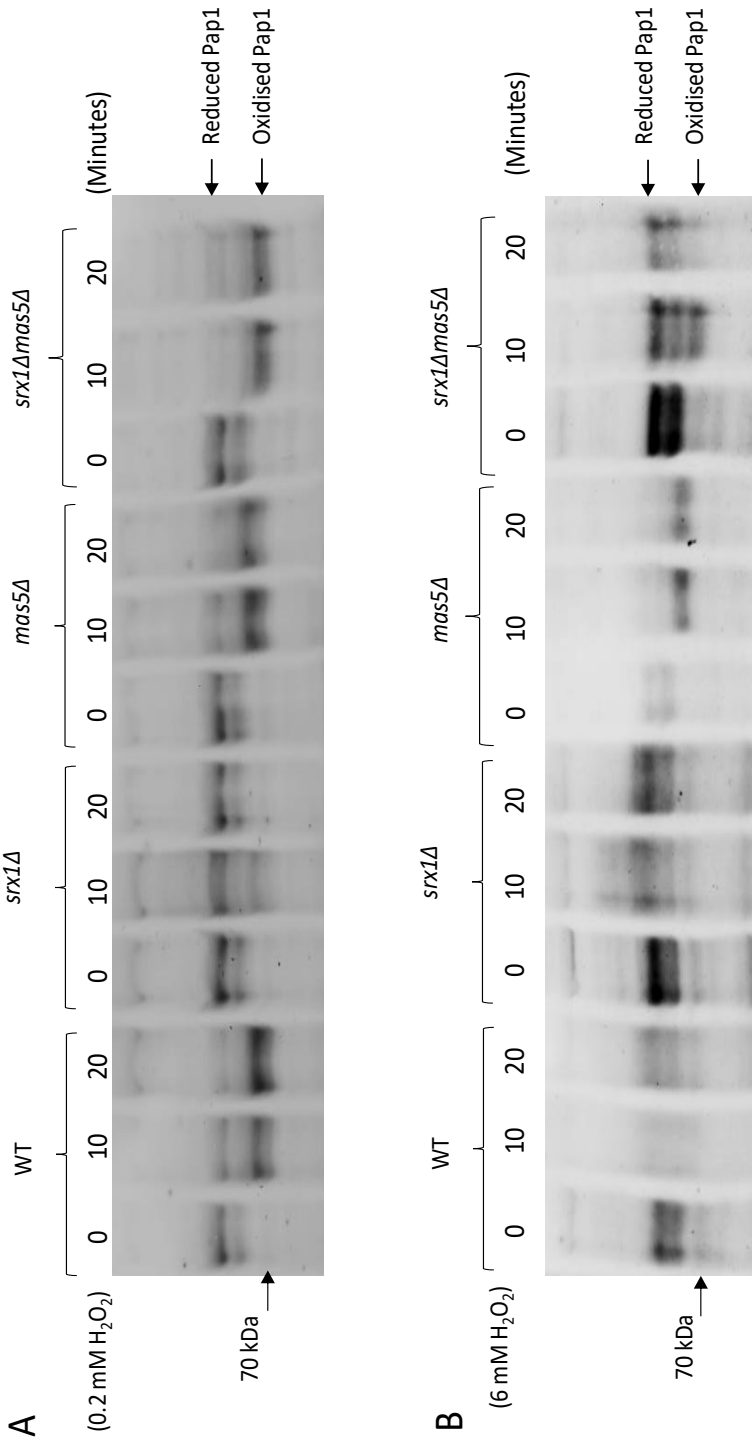


Fig. 5. 12. Deletion of sulfiredoxin has an effect on Pap1 oxidation and *mas5*Δ cells. Exponentially growing wild type (WT) (CHP429), *srx1*Δ (SB6), *mas5*Δ **(**FM15**)** and *srx1*Δ*mas5*Δ (FM54) cells were treated with low (0.2 mM) (A) or high (6 mM) (B) H_2O_2 stress for 0, 10 or 20 minutes. Samples were extracted by TCA protocol plus IAA followed by alkaline phosphatase. Proteins were analysed by non-reducing SDS-PAGE and western blotting with anti-Pap1 antibody, n > 3.

and possibly not due to Pap1 overexpression effects in *mas5*Δ cells, clarification of the elements in the *tpx1*$^{+}$ promoter could help show the transcription factors involved in its regulation. It is intriguing that while Tpx1 levels seem high in *mas5*Δ cells Trx1 oxidation seems normal in response to H_2O_2 stress. It again raises the question of whether *mas5*Δ effect on Pap1 is linked to Txl1 oxidation since it could not be detected. Alternatively, it may be that Tpx1 is directly influencing the oxidation status of Pap1 which has been proposed before and in *mas5*Δ the high Tpx1 levels are affecting this regulation (Calvo *et al.*, 2013).

5.2.8 Thioredoxin mutants affect Hsf1 localisation

The thioredoxin system is central to the regulation of Pap1 activity, localisation and oxidation. It has now been established that there is a connection between Mas5 and Tpx1 regulation of the activity of Pap1. Furthermore, the similarity has been shown of localisation of Hsf1 versus Pap1 in response to increasing concentrations of H_2O_2. Next it was investigated whether the thioredoxin system possibly influences the regulation of Hsf1 localisation.

To test relationships between the thioredoxin system and Hsf1 strains were constructed expressing Hsf1-GFP within either a *trx1*Δ*txl1*Δ, *tpx1*Δ or *trr1*Δ strain. Hsf1-GFP localisation in *trr1*Δ cells was examined in unstressed conditions and after H_2O_2 stress. It has previously been shown that in *trr1*Δ cells Pap1 is constitutively nuclear, oxidised and activating transcription (Brown *et al.*, 2013). In contrast Hsf1 is not constitutively nuclear in unstressed *trr1*Δ cells (Fig. 5. 13.). Furthermore, Hsf1-GFP appears to localise with the same kinetics as observed in wild type cells after 0.2 mM H_2O_2 treatment (Fig. 5. 13. A). Interestingly, 6 mM H_2O_2 treatment of *trr1*Δ cells leads to nuclear localisation of Hsf1-GFP across the time course (Fig. 5. 13. B). This result shows similarity to Pap1 localisation when Tpx1 is overexpressed but also in *mas5*Δ cells in response to 1 mM H_2O_2 stress (Fig. 5. 7). This leads to the conclusion

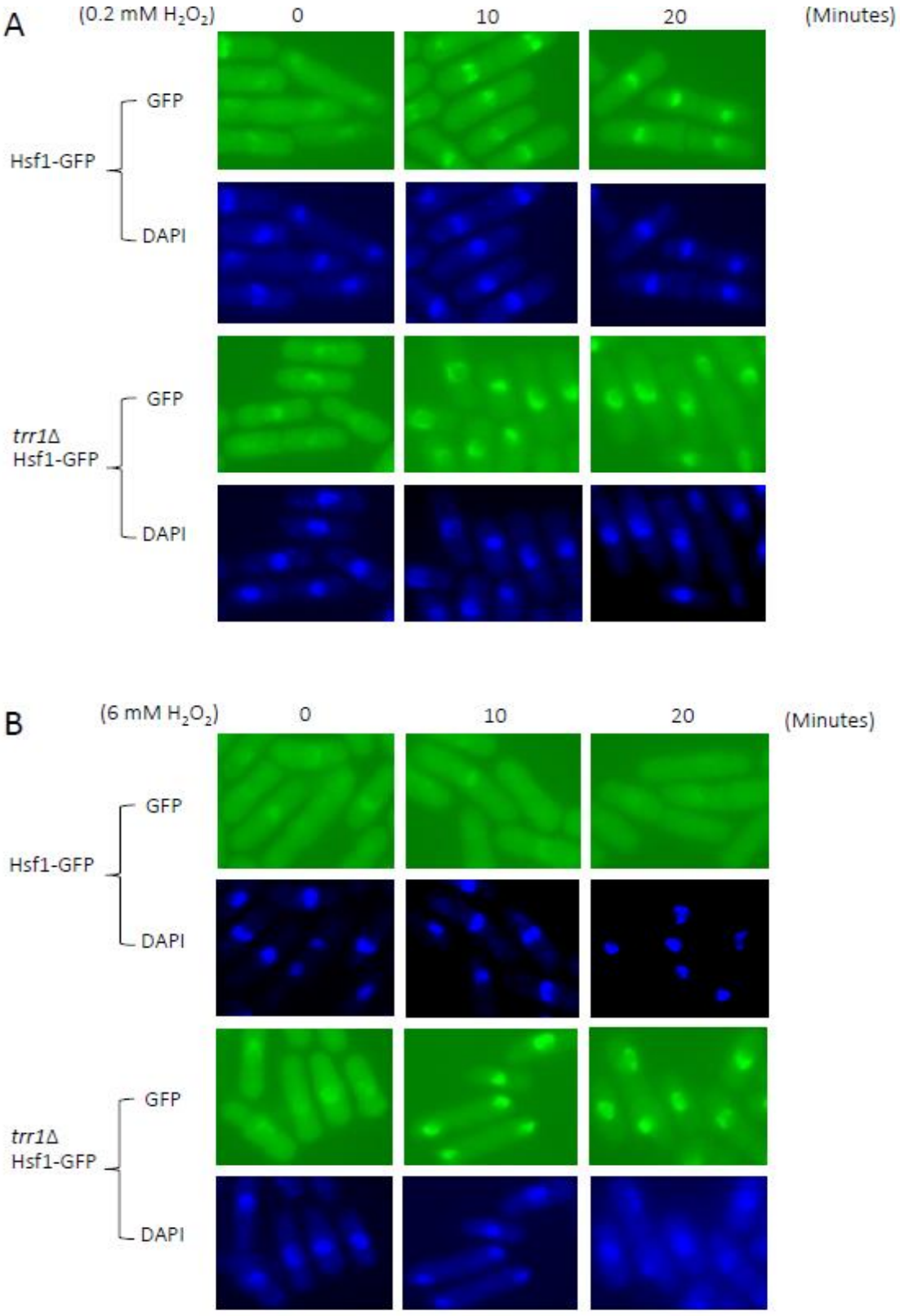


Fig. 5. 13. Hsf1-GFP localisation in *trr1*Δ cells following H_2O_2 stress. Strains expressing Hsf1-GFP with a *trr1*$^+$ (FM40) or *trr1Δ* (FM46) allele were grown to exponential phase at 30 °C and fixed using paraformaldehyde following exposure to 0.2 mM (A) or 6 mM (B) H_2O_2 stress for 0, 10 or 20 minutes. GFP (fluorescent) and DAPI images are shown of representative cells, exposures remaining constant between images, n = 2.

that H_2O_2regulation of Hsf1 is different to Pap1 but both Pap1 and Hsf1 are influenced by Trr1 however in different ways.

Thioredoxins are responsible for maintaining the redox status of many proteins within the cell. 2 thioredoxins have been linked to Pap1 redox status, Trx1 and Txl1. Trr1 is required to redox cycle Trx1 and Txl1. The absence of Trx1 and Txl1 results in Pap1 being largely reduced and no strong nuclear localisation in unstressed cells or following exposure to H_2O_2 which shows similarity to *trr1*Δ cells (Brown *et al*., 2013). Whether Hsf1 is also reliant on Trx1 or Txl1 activity is unknown. Thus, Hsf1-GFP localisation was next investigated in *trx1*Δ*txl1*Δ cells. It would be predicted that *trx1*Δ*txl1*Δ strain would show similar Hsf1 movement as the *trr1*Δ strain in that there is faster nuclear accumulation of Hsf1-GFP in response to high H_2O_2 stress.

The *trx1*Δ*txl1*Δ strain similarly to *trr1*Δ strain did not affect Hsf1-GFP localisation in unstressed cells (Fig. 5. 14). However, in contrast to *trr1*Δ cells, Hsf1-GFP does not show H_2O_2-inducible nuclear accumulation after 0.2 mM or 6 mM H_2O_2 treatment over the time course. This is the opposite result of Hsf1-GFP localisation to treatment of *trr1*Δ cells with H_2O_2 while the effects on Pap1 localisation of *trr1*Δ and *trx1*Δ*txl1*Δ show greater similarity (Brown *et al*., 2013). A hypothesis is that Trx1 and/or Txl1 are required for the nuclear accumulation of Hsf1 following H_2O_2 treatment. Furthermore, considering the *trr1*Δ cells data (Fig. 5. 13.) it could be suggesting that oxidised Trx1 and Txl1 are required to activate Hsf1-GFP localisation. This oxidation of Trx1 and/or Txl1 would normally be delayed after 6 mM H_2O_2 treatment until H_2O_2 levels start to drop. However, in unstressed *trr1*Δ cells there is some oxidation of Trx1 but Txl1 oxidation was not investigated in this strain. The difference may be linked to the to the fact that thioredoxins inhibit Pap1 but may be activating Hsf1-GFP.

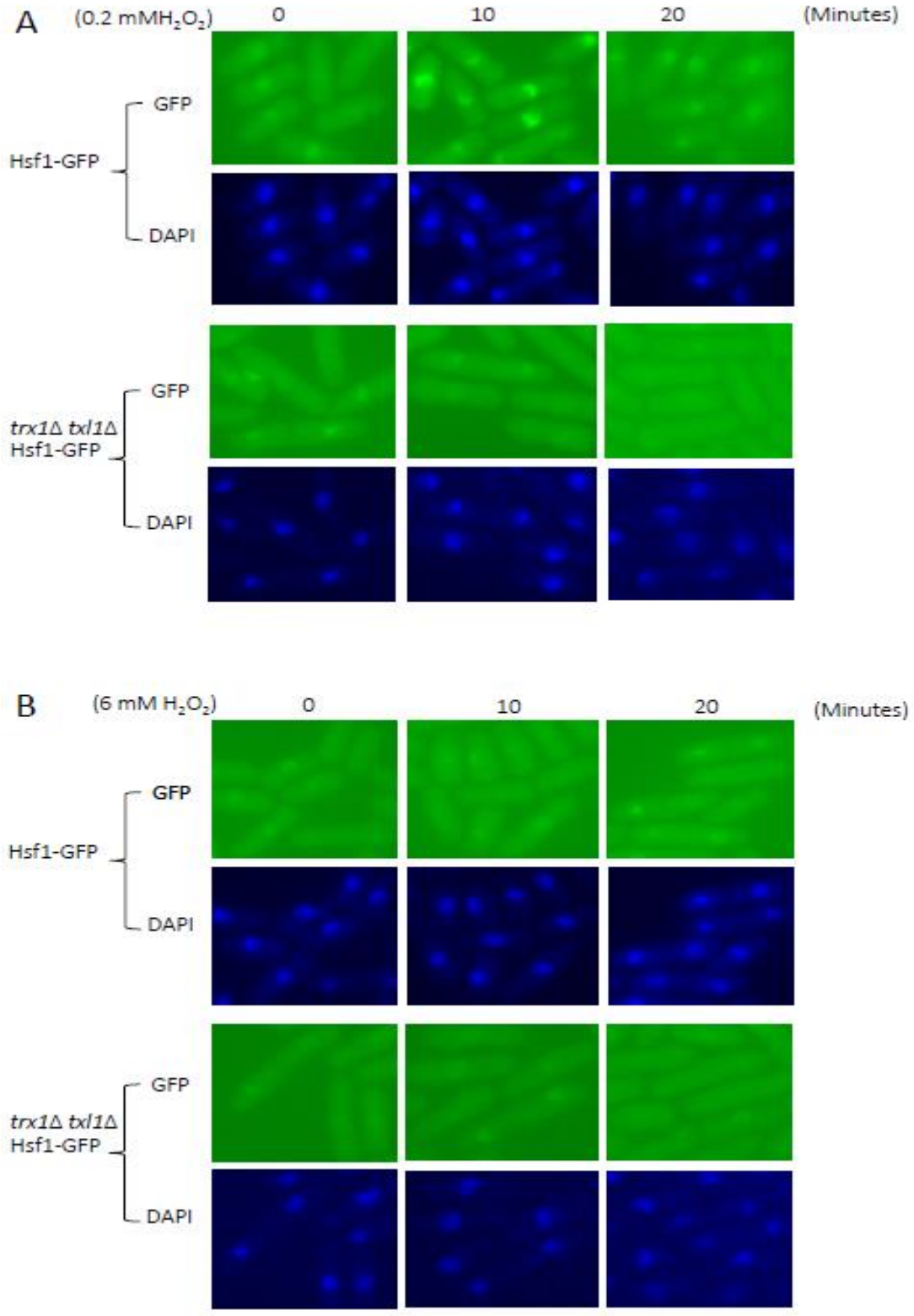


Fig. 5. 14. Hsf1-GFP localisation in *trx1*Δ*txl1*Δ cells following H_2O_2 stress. Strains expressing Hsf1-GFP with a *trx1*$^+$with *trx1*$^+$ (FM40) or *trx1*Δ*txl1*Δ (FM47) allele were grown to exponential phase at 30 °C and fixed using paraformaldehyde following exposure to 0.2 mM (A) or 6 mM (B) H_2O_2 stress for 0, 10 or 20 minutes. GFP (fluorescent) and DAPI images are shown of representative cells, exposures remaining constant between images, n = 2.

Having established a role for Trx1 and/or Txl1 it was next tested whether Tpx1 is important for Hsf1-GFP nuclear accumulation. Tpx1 is essential for the oxidation, activation and nuclear accumulation of Pap1 and an important aspect of this appears to be the oxidation and thus inhibition of Trx1 and Txl1 (Brown *et al.*, 2013). Tpx1 thioredoxin peroxidase activity is required for relay of cellular oxidation status to Pap1. Following exposure to H_2O_2, Tpx1 is proposed to deplete reduced Trx1. This would mean Trx1 is no longer available to maintain Pap1 in a reduced state. In support of this theory, in *tpx1*Δ cells Pap1 is constitutively reduced and cytoplasmic.

Based on the results presented here it would be hypothesised that that reduced Txl1 and/or Trx1 are required for Hsf1-GFP to accumulate in the nucleus in response to stress so it would then be predicted that the *tpx1*Δ allele would not affect Hsf1-GFP localisation. Alternatively, if the oxidation of Trx1 and/or Txl1 are required for H_2O_2 accumulation of Hsf1-GFP then predict that loss of Tpx1 would inhibit nuclear accumulation of Hsf1-GFP.

In *tpx1*Δ cells it was found that Hsf1-GFP was not detectable in the nucleus of any cells (Fig. 5. 15). Following exposure of these cells to 0.2 mM or 6 mM H_2O_2 there is no change to the Hsf1-GFP localisation. This suggests that Tpx1 is required for Hsf1-GFP to move to the nucleus under any conditions. This contrasts the *trx1*Δ*txl1*Δ effects where loss of both thioredoxin proteins only prevented/inhibited stress induced nuclear accumulation of Hsf1-GFP. However, it should be noted that Hsf1-GFP cannot be completely absent from the nucleus since Hsf1 is required for housekeeping roles.

To interpret these data there are several possibilities which are not necessarily exclusive. The first option is Tpx1 influences unstressed cells localisation of Hsf1, this fits with the result that

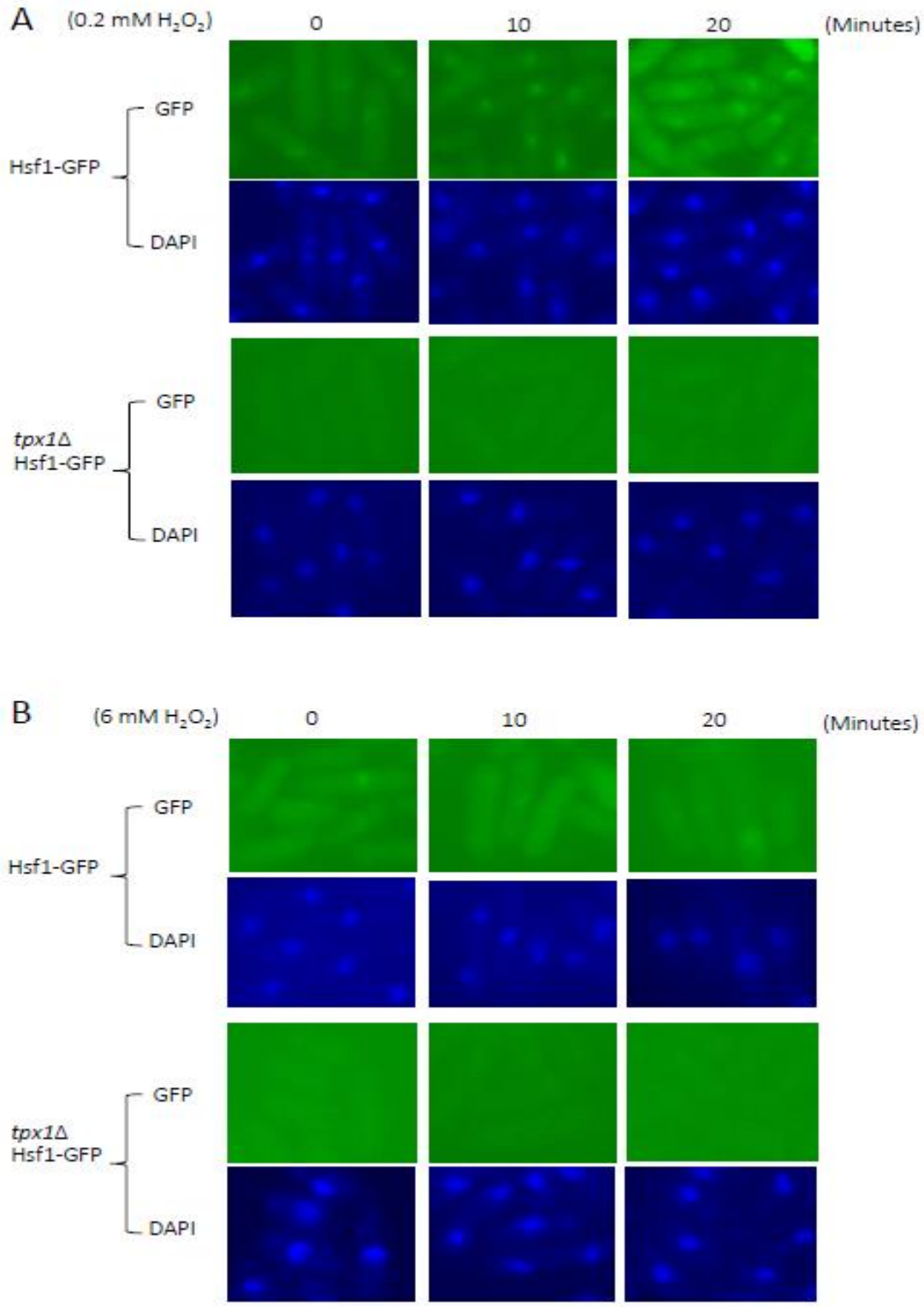


Fig. 5. 15. Hsf1-GFP localisation in *trx1*Δ*txl1*Δ cells following H_2O_2 stress. Strains expressing Hsf1-GFP with a *tpx1*$^+$ (FM40) or *tpx1*Δ (FM45) allele were grown to exponential phase and fixed using paraformaldehyde following exposure to 0.2 mM (A) or 6 mM (B) H_2O_2 stress for 0, 10 or 20 minutes. GFP (fluorescent) and DAPI images are shown of representative cells, exposures remaining constant between images, n = 3.

Tpx1 levels are up in *mas5*Δ unstressed cells as this may also explain high nuclear accumulation of Hsf1 in unstressed *mas5*Δ cells. It is possible that Tpx1 is required for the H_2O_2-induced nuclear accumulation of Hsf1-GFP but this may be a different mechanism that involves oxidation of Trx1/Txl1/Tpx1. A second option is that Tpx1 acts a molecular chaperone so possibly Hsf1 stability/solubility is affected in cells lacking Tpx1.

In conclusion, the data indicate that Pap1 and Hsf1 use the thioredoxin system proteins to sense/signal redox status in the cell but in different ways. There also appears to be an interdependency of regulators of Hsf1 and Pap1 on each other, i.e there is crossover of Pap1 and Hsf1 activity.

5. 3 Conclusion

It was shown in Chapter 4 Hsf1 nuclear accumulation and Hsf1-dependent gene expression are also regulated in H_2O_2 concentration manner involving Mas5. Given the concentration dependent effects of H_2O_2 on regulation of Pap1 and Hsf1 it raised the possibility that there may be cross talk or shared factors between their regulatory pathways.

The data presented in this chapter suggested Mas5 regulates Tpx1 levels through Hsf1 and that this determines the activation of Pap1 in response to H_2O_2. Additionally, the thioredoxin system regulates the nuclear accumulation of Hsf1 in response to H_2O_2 but in a different manner to Pap1. Together these data have revealed new regulatory mechanisms of both Pap1 and Hsf1 and uncovered cross talk between the two transcription factor pathways in response to H_2O_2 stress.

The model proposed from our lab is that in response to low H_2O_2 stress Tpx1 becomes oxidised and oxidises the pool of Trx1 and Txl1. Txl1 is believed to repress Pap1 and that oxidation of Tpx1 leading to oxidation of Txl1 releases the repression of Pap1 (Brown *et al*., 2013). However, there is little available evidence of the effect of overexpressing Tpx1 to further substantiate this model except that it does result in increased Pap1 nuclear localisation following H_2O_2 stress (Bozonet, 2006).

It was shown here that the *mas5*Δ allele has a very similar effect on nuclear accumulation of Pap1 as Tpx1 overexpression. Indeed, it was found that this effect of *mas5*Δ allele results in increased activation of Pap1 that is Tpx1 dependent by using a *srx1*Δ*mas5*Δ strain and that this corresponds to increased activation of Pap1-dependent gene expression. Consistent with this Tpx1 (*tpx1*$^+$) levels were found to be increased in *mas5*Δ cells. However, how the increase in Tpx1 levels is regulated is not clear. Genes encoding the thioredoxin system proteins are inducible in response to H_2O_2 stress but *tpx1*$^+$ is also regulated by heat which leads to the possibility that this gene is also regulated by Hsf1 (Chen *et al*., 2003). Supportive of this theory is that Tpx1 is believed to act as a molecular chaperone, additionally the *S. cerevisiae* homolog Tsa1 shows a molecular chaperone role in heat and H_2O_2 stress that interacts/chaperones Ssa1 specifically in response to H_2O_2 stress (Hanzen *et al*., 2016). It is not known in *S. pombe* if Hsf1 does regulate *tpx1*$^+$ but analysis of the promoter region does identify possible sequences that Hsf1 recognises suggesting it is indeed a Hsf1 target.

It is not known how Tpx1 overexpression effects Pap1 apart from its localisation in 1 mM H_2O_2 which shows similarity to *mas5*Δ cells. No major alteration in Trx1 regulation is observed in the *mas5*Δ strain but Txl1 regulation couldn't be established. It could be speculated that Txl1 is oxidised after 6 mM H_2O_2 treatment which results in the derepression of Pap1 observed in

*mas5*Δ strain. Alternatively, or possibly in addition to, Tpx1 directly oxidises Pap1 at 6 mM H_2O_2 in *mas5*Δ cells. Txl1 needs to be investigated in this strain to clarify the mechanism. Another theory is that the effects observed in the *mas5*Δ strain are due to an effect on Ssa2 activity. Due to the issues with the *ssa2*Δ strain the regulation of Hsf1 and thioredoxin system could not be investigated, it is possible that it may have showed the same phenotypes as the *mas5*Δ strain which would suggest the effects shown here are via the Ssa2/Mas5 complex rather than simply Mas5.

The data presented in this chapter provides evidence of cross talk in activities of the thioredoxin system and heat shock proteins. Study of thioredoxins in *S. pombe* showed that Trx1 and Txl1 act to maintain the solubility of Pap1 and that in absence of them $pap1^+$ gene expression basal is increased to compensate for the amount of available Pap1 (Brown *et al*., 2013). It has been shown here that Mas5 is also required to maintain Pap1 solubility which may be a factor in the increased $pap1^+$ expression in the *mas5*Δ strain. Although the basis of this regulation is unknown it could be linked to Hsf1-regulated gene expression of $pap1^+$ given the coordination of activity described above between Hsf1 and Pap1 in addition to the idea that Pap1 solubility may be linked to Mas5 function of chaperoning the transcription factor. To test if Mas5 and Trx1 with Txl1 are in the same pathway to regulate Pap1 solubility a triple mutant *trx1*Δ *txl1*Δ*mas5*Δ could be generated and analyse if there is an effect of Pap1 solubility in comparison to *trx1*Δ *txl1*Δ strain. It is unlikely that the effect on Pap1-dependent gene expression seen in *mas5*Δ cells is due to increased $pap1^+$ levels since soluble Pap1 levels don't increase and overexpression of Pap1 does not activate oxidative stress regulated genes but $tpx1^+$ was not specifically investigated (Calvo *et al*., 2012). Additionally, overexpression of Pap1 was shown to inhibit Pap1 oxidation in response to H_2O_2 rather than the stimulation of kinetics observed

in *mas5*Δ cells. This leads towards the concept that in *mas5*Δ cells the effects on Pap1 and Pap1-dependent gene expression are through Tpx1 not *pap1*$^{+}$ gene expression regulation.

Investigation of the thioredoxin system effect on Hsf1 nuclear accumulation regulation has revealed some similarity to Pap1 regulation. Deletion of *tpx1*$^{+}$ results in Hsf1 showing no accumulation in any conditions. Excitingly, this is like Pap1 regulation in *tpx1*Δ cells (Brown *et al*., 2013). Deletion of *trr1*$^{+}$ seems to have no effect on Hsf1 kinetics until cells are exposed to 6 mM H_2O_2 which seems to stimulate Hsf1 nuclear accumulation unlike in wild type cells. Hsf1 localisation in unstressed *trr1*Δ cells show similarity to *trx1*Δ*txl1*Δ cells but Hsf1 does not accumulate in the nucleus after H_2O_2 treatment over the time course. This leads towards oxidised Txl1 and/or Trx1 are required for Hsf1 nuclear accumulation. This contrasts the Pap1 data where Trx1/Txl1 seem to act as repressors of Pap1 and oxidation of these thioredoxins results in derepression of Pap1 (Brown *et al*., 2013, see model Fig. 7). It seems that nuclear accumulation of both Hsf1 and Pap1 is affected by H_2O_2 concentration and that this is regulated by the thioredoxin system but differentially.

As shown in the previous chapter Hsf1 localisation and activation is not so tightly linked as Pap1 where oxidation affects both nuclear accumulation and activation of gene expression. For instance, in a *trx1*Δ*txl1*Δ strain Pap1 is constitutively present in the nucleus and there is a pool of reduced and oxidised Pap1 leading to derepression of Pap1-dependent gene expression. Hsf1 appears to require Trx1 and/or Txl1 to be oxidised before it can accumulate in the nucleus but the effect on Hsf1 activity in *trx1*Δ*txl1*Δ strain has not been investigated. It has been show that Crm1 is involved in Hsf1 nuclear export and is dependent on the oxidation status of the conserved cysteine (Matsuyama *et al*., 2006). It is possible that this Hsf1 nuclear accumulation is regulated by the oxidation of Crm1. Mas5 was shown to be oxidised at 0.2 mM H_2O_2 after

30 seconds, it is possible this oxidation is linked to Hsf1 localisation and/or with Crm1 regulation (Garcia-Santamarina *et al*., 2011).

In conclusion, the work presented has revealed new regulation of both Pap1 and Hsf1. This has implications for regulation of AP-1-like transcription factors and Hsf1 in other organisms. The thioredoxin system regulates Pap1 activity and has newly discovered roles in Hsf1 regulation. This study has implication for thioredoxin systems regulation of other pathways that are responsive to H_2O_2 stress and thioredoxin system regulation in other organisms. Thioredoxins are broad specificity oxidoreductases and therefore may have many more targets than Tpx1 and Pap1. Therefore, the careful balance of the oxidation status of Tpx1 and Trx1 with Txl1 provides a potential mechanism to regulate other pathways in response to H_2O_2.

Chapter 6

Discussion

6.1 Summary

Heat shock proteins are highly conserved and function in a diverse range of conditions. Originally characterised for their significant induction upon heat stress, their functions were attributed to protein folding, degradation and chaperoning events (See reviews for details, Craig and Marszalek, 2011; Mayer and Bukau, 2005). However, recent studies have begun to reveal that HSPs play critical roles in regulating processes such as the heat and oxidative stress responses and cell cycle progression. For example, studies in budding yeast revealed that Hsp70s Ssa1 and Ssa2, together with the HSP40 Ydj1, play an important role in cell cycle regulation that also appears to be conserved to mammalian cells (Truman *et al*., 2012; Verges *et al*., 2007).

The conserved heat shock transcription factor Hsf1 is regulated by HSP70s in budding yeast and mammalian cells but display organism specificity such as Hsf1 requiring HSP70 as a repressor in *S. cerevisiae* but as a promoter in *S. pombe* and mammalian cells (Abravaya *et al*., 1992; Wang *et al*., 2012). Interestingly, work in mammalian cells and in budding yeast has uncovered that Hsf1 responds to other stresses such as oxidative stress in addition to heat stress (Wang *et al*., 2012). Although studies in budding yeast have been fruitful, the fact that Hsf1 is constitutively nuclear in contrast to other organisms prevents insight into all aspects of the regulation of this transcription factor. Interestingly, Hsf1 in the distantly related fission yeast *S. pombe* displays regulated nuclear accumulation. Hence, *S. pombe* is an excellent model organism to investigate the regulation of Hsf1 and the relationships with HSP70s/HSP40s

functions. Furthermore, previous studies have already provided excellent insight to many aspects of oxidative stress responses in fission yeast (see section 1.3.2 and 1.3.3).

The aim of this study was to use *S. pombe* to investigate the potential relationships between the HSP70s Ssa1, Ssa2 and the HSP40 Mas5 and the regulation of cell cycle progression, the Hsf1 transcription factor and heat and oxidative stress responses to provide insights into the conserved and organism specific aspects of the functions of these proteins.

6.2 The roles of Ssa1, Ssa2 and Mas5 in the cell division cycle

Previous studies in *S. cerevisiae* had identified HSP70s Ssa1/Ssa2 and HSP40 Ydj1 as critical regulators of Start in the cell cycle by influencing the cellular localisation of the G1 cyclin Cln3 (See Introduction section 1.2.2; Truman *et al*., 2012; Verges *et al*., 2007). Excitingly, studies of the mammalian Hsc70, homolog of Ssa1, suggested that it regulated cyclin D1, a key G1 cyclin in mammalian cells (See Introduction section 1.2.2; Truman *et al*., 2012). Additionally, Ydj1 has been proposed to act as a critical sensor for the growth rate during G1 phase to regulate cell size (Ferrezuelo *et al*., 2004). For example, deletion of *YDJ1* results in significantly larger cells (Jorgensen *et al*., 2002; Verges *et al*., 2007), whereas overexpression of *YDJ1* results in faster passage through G1 phase (Doris, 2008). However, the conserved/organism-specific aspects of these roles of Ydj1 in cell cycle progression were poorly understood at the beginning of the present study. The distantly related model *S. pombe* provided an excellent system to investigate this question further as the cell cycle and growth characteristics of this yeast are well studied (see reviews for details Forsburg and Nurse, 1991).

Initial investigations in this study uncovered cell cycle defects that occurred in the absence of either *ssa1*$^{+}$, *ssa2*$^{+}$ or *mas5*$^{+}$ (see Chapter 3 section 3.2.1). For example, the growth/cell size phenotypes of the *mas5*Δ and *ssa2*Δ strains were similar, although perhaps more severe in the *ssa2*Δ strain. Interestingly, Ssa1 was found to play an opposing role in cell size regulation. These results suggest that Ssa1 and Ssa2 play different opposing roles in the cell cycle and it is tempting to speculate this is linked to competition for interaction with Mas5. However, investigations of the *ssa2*Δ strain were hampered due to the severity of the phenotypes and the rapid appearance of suppressors. Thus, it is important to point out that studies of Ssa2 should be performed in the future to complement studies reported in this thesis of Ssa1 and Mas5 and not Ssa2.

Despite studies here showing links between Ssa1, Ssa2 and Mas5 and cell cycle progression how these proteins are regulated during the cell cycle has been elusive. For example, no changes in the modification status of either epitope tagged Ssa1, Ssa2 or Mas5 proteins was detected during the cell cycle to indicate possible regulatory mechanisms. Interestingly, during this study work established a role for Ssa2/Mas5 in the regulation of Hsf1 and data suggested that this was linked to their cell cycle roles that was tenuously linked to cell growth (Vjestica *et al*., 2013). However, excitingly, it was shown here that epitope tagged Ssa2 and Mas5 localise to regions of new growth in the cell during the cell division cycle while Ssa1 appears to be located throughout the cell (see Fig. 3. 6.). These data are consistent with a model where Ssa1, Ssa2 and Mas5 play distinct roles in the regulation of cell growth in mechanisms independent of Hsf1 function.

S. pombe cell growth is regulated by a dynamic complex termed the polarisome and a key regulator of this complex is the Tea1 protein (for details see reviews; Martin and Chang, 2005;

see section 3.1.). Ssa2 and Mas5 have been identified as interactors of Tea1 and thus raised the question of whether this was linked to their role in cell growth (Snaith *et al*., 2011). Hence, given the connection between Ssa1 and Cln3 localisation in *S. cerevisiae* it was possible that Ssa1, Ssa2 or Mas5 might have role in determining the cellular location of Tea1. However, the cellular localisation of Tea1 was unaffected in the absence of *ssa1*$^{+}$ or *mas5*$^{+}$. Although this suggests that Ssa1 and Mas5 do not regulate the localisation of Tea1 it is still possible that they function redundantly to regulate Tea1 with another unidentified protein and/or may regulate Tea1 in specific stress conditions such as the stall in cell growth that occurs in response to heat stress. It would be interesting in the future to widen the investigation of regulation of cell growth in normal and various stress conditions, including heat and oxidative stresses, by examining the potential relationships between Ssa1, Ssa2 and Mas5 and other polarisome components.

Interestingly, cytoskeleton reorganisation is a key part of cell growth and is orchestrated by microtubule dynamics (Martin and Chang, 2005; Snaith *et al*., 2011). Intriguingly, *mas5*Δ cells display a pronounced misregulation of microtubules during the cell cycle (see Fig. 3. 8.). A similar phenotype has also been observed in *ydj1*Δ mutant *S. cerevisiae* cells suggesting a conservation of function in microtubule organisation (Oka *et al*., 1998). The exact role of Mas5 in microtubule rearrangement is not known but the data presented here suggests that Mas5 plays an important role in regulating cell growth independent of Hsf1. However, defining whether this role is independent/dependent on Ssa1 and/or Ssa2 requires further work.

Like mitosis, internal reorganisation occurs during meiosis and not surprisingly numerous proteins function in both processes. Interestingly, in support of its major role in cell growth and mitosis, it was discovered that at least a single copy of *mas5*$^{+}$ is essential for meiosis to be successful (see section 3.2.7). The basis of this essential meiotic role is unclear but it is tempting

to speculate that this could be linked to the microtubule defects observed during cell growth. Alternatively, Mas5 regulation of Hsf1 may play a vital role in meiosis.

Another intriguing finding that potentially connects Mas5 functions comes from the observation that Mas5 also influences autophagy (see section 3.2.8). A key part of cell growth and meiosis is sensing nutrient availability and internal catabolism to generate nutrients, and autophagy is a process that functions when cells are exposed to limited nutrients (for reviews see Huang and Klionsky, 2002; Mukaiyama *et al*., 2010). Interestingly, recent work has elucidated significant cross talk between the factors involved in the heat shock response and autophagy (Dokladny *et al*., 2013). Hence it is possible that Mas5 acts at an interface of cellular metabolism that coordinates conserved processes such as mitosis, meiosis and autophagy. In this regard Ydj1 also seems to have a role in sensing cell growth and thus it would be interesting to investigate whether this protein has any role in processes such as autophagy in *S. cerevisiae*.

Collectively, these results have revealed novel roles for Ssa1, Ssa2 and Mas5 in cell growth and division, meiosis and autophagy, which show significant elements of conservation with other organisms. Further investigation is needed to provide insight in to the molecular mechanisms by which these HSPs influence these processes.

6.3 Novel insights in to the oxidative stress response in *S. pombe*

Heat shock factor (HSF) is a key transcription factor that regulates the heat shock response in eukaryotes (see section 1.3.4). Since its original discovery there has been extensive work to explore its complex regulation and functions in response to a wide range of stimuli. Heat shock proteins (HSPs) are members of one of the most highly conserved families amongst eukaryotes

and, together with HSFs, are critical for cell survival in normal and stress conditions (for reviews see Anckar and Sistonen, 2011).

Interestingly, the activity of HSFs have been shown to be modulated by HSPs in normal and stress conditions to trigger the appropriate transcriptional response. In *E. coli* the equivalence of eukaryotic HSF is sigma factor σ^{32}. It has been identified as being modulated by the HSP70 homolog DnaK with the HSP40 DnaJ to act as the thermal sensors for σ^{32} activity (Liberek *et al*., 1991). DnaK/DnaJ bind as repressors of σ^{32} to prevent transcription of the heat stress regulon (Arsene *et al*., 2000; Liberek *et al*., 1991). Furthermore, in *Caulobacter crescentus* a link was established between HSP stress response and regulation of the cell cycle. It was found that in this organism reduction of available HSP70 DnaK or proteotoxic stress directly lead to a cell cycle arrest (Jonas *et al*., 2013). Similarly, to *E. coli* they found that depletion of DnaK/DnaJ lead to derepression of σ^{32} activity suggesting this mechanism of HSP70/HSP40 regulation is conserved amongst prokaryotes. It is therefore interesting to see data pointing towards this mechanism being conserved in eukaryotes.

In *S. cerevisiae,* it was discovered that Hsf1 is repressed by Ssa1/2 in unstressed conditions and, upon specific oxidative stress, Ssa1 becomes oxidised leading to de-repression of Hsf1 and transcriptional activity (Wang *et al*., 2012). Interestingly, it was shown that Ssa2 and co-chaperone Mas5 repress Hsf1 activity in *S. pombe* and upon heat stress Ssa2/Mas5 no longer repress Hsf1 leading to activation of Hsf1-dependent gene expression (Vjestica *et al*., 2013). It was found in mammalian cells that HSP70/HSP40 interaction with Hsf1 does occur but did not repress Hsf1 DNA binding rather the complex appears to act to repress Hsf1 trans-activation (Abravaya *et al*., 1992; Shi *et al*., 1998). This suggests that there is conservation of the regulation of HSFs by HSPs in distantly related organisms although whether these regulatory

mechanisms operate identically in all organisms is unclear. Interestingly, recent studies in *S. cerevisiae* revealed that the oxidation status of the Prx Tsa1 affects recruitment of Ssa1 to aid in protein degradation specifically during oxidative stress but not heat stress (Hanzen *et al*., 2016). Thus, given the relationship between Ssa1 and Hsf1 regulation in *S. cerevisiae* an interesting speculation is that Tsa1 may have a role in the regulation of Hsf1.

Here studies were performed in *S. pombe* to gain further insight into the organism specific and conserved aspects of Hsf1 regulation. Interestingly, the work presented here revealed that Ssa1 functions during H_2O_2 stress to promote Hsf1 nuclear accumulation, but curiously this role was not conserved in response to heat stress. This role of Ssa1 in promoting the nuclear accumulation of Hsf1 has not been described previously in eukaryotes. It is worth noting that several studies in this thesis did not include *ssa2*Δ mutant cells due to their heterogeneous growth characteristics and thus it will be important in the future to explore the relationships between Ssa2 and the functions of Ssa1 in nuclear accumulation of Hsf1 in response to H_2O_2 and heat stresses. For example, perhaps Ssa1 and Ssa2 function redundantly to promote Hsf1 nuclear accumulation during heat stress.

Importantly, work published during the course of the thesis study revealed that Ssa2 and Mas5 act as repressors of Hsf1 activity and the data presented here is generally consistent with these published conclusions (Vjestica *et al*., 2013). Although it is not clear if Ssa1 and/or Mas5 are acting directly or indirectly on Hsf1-depdendent transcription, like studies of cell growth described above, the opposing roles of Ssa1 and Mas5 on the regulation of Hsf1 may be linked to competition between Ssa1 and Ssa2 for interaction with Mas5. The *hsp104*$^{+}$ gene has previously been used as a specific reporter of Hsf1 activity (Vjestica *et al*., 2013). However, the analysis presented here of gene expression in *mas5*Δ cells suggest a secondary mechanism

independent of Mas5 can influence *hsp104*$^{+}$ expression in response to H_2O_2 stress. It is possible that this mechanism involves, for example, some other post-translational regulation of Hsf1 such as direct oxidation of the transcription factor. However, it is also possible that this mechanism could be linked to other transcription factors known to be involved in oxidative stress responses such as Prr1. Indeed, Skn7, the homologue of Prr1 in *S. cerevisiae*, has been shown to work synergistically with Hsf1 to achieve an appropriate transcriptional response, including *HSP104* expression, in response to oxidative stress (Raitt *et al*., 2000). Hence, further work is required to elucidate the exact mechanism of *hsp104*$^{+}$ induction in response to H_2O_2 stress in *S. pombe*.

Collectively the data presented here revealed that Hsf1 in *S. pombe* is involved in the transcriptional response to H_2O_2 stress. This connection between Hsf1 and H_2O_2 responses has not previously been reported in *S. pombe*. Furthermore, Hsf1 was found to accumulate in the nucleus in a H_2O_2 concentration dependent manner similar to the Pap1 transcription factor. Additionally, Ssa1 and Mas5 were shown to play opposing roles in the nuclear accumulation of Hsf1 during H_2O_2 stress. These studies have provided new insights into the potential roles and regulation of Hsf1 during oxidative stress and forms the basis of future studies of the relationship between Hsf1 and H_2O_2 responses.

6.4 Evidence of cross talk between Pap1, Hsf1 and the thioredoxin system

As described above the nuclear accumulation of Hsf1 in response to H_2O_2 was found to be regulated in a concentration-dependent manner. Interestingly, previous studies of Pap1 revealed that the nuclear accumulation of the transcription factor in the nucleus in response to H_2O_2 is

also regulated in a concentration-dependent manner (Quinn *et al*., 2002). Furthermore, both Pap1 and Hsf1 nuclear export is dependent on the exportin Crm1 (Toda *et al*., 1992; Matsuyama *et al*., 2006). Taken together with other studies of Pap1 these data indicate that both Hsf1 and Pap1 cycle between the nucleus and the cytoplasm in unstressed cells and display similar nuclear accumulation profiles in response to increasing concentrations of H_2O_2. This suggested that there may be elements of the regulation of these two transcription factors that involve similar mechanisms.

In this study other elements of potential cross talk between Pap1 and Hsf1 were identified. For example, deletion of *mas5*$^+$, in addition to stimulating constitutive nuclear accumulation of Hsf1, also stimulated activation of Pap1, even at concentrations of H_2O_2 where Pap1 activation is normally inhibited (see Chapter 5 section 5.2.2.). Furthermore, this effect of loss of Mas5 function on Pap1 was linked to the increased expression of the Pap1 regulator Tpx1. It would be interesting to investigate whether Tpx1 overexpression was also linked to increased nuclear accumulation of Hsf1 in *mas5*Δ cells. Attempts to construct a *mas5*Δ*tpx1*Δ double mutant to test this hypothesis were unsuccessful in this study. Nevertheless, the nuclear accumulation of Hsf1 was found to be dependent on Tpx1 in response to H_2O_2 supporting the hypothesis that Tpx1 regulates Hsf1 (see Chapter 5 Fig. 5. 15.). Interestingly, although analysis of the effects of other mutations of the thioredoxin system also suggested linkage to regulation of Hsf1, the effects of these thioredoxin system mutations on Pap1 regulation was different, suggesting Hsf1 and Pap1 are regulated by the thioredoxin system by different mechanisms.

The results presented here and previous studies of the regulation of Pap1 suggests that the oxidation status of Trx1 and/or Txl1 are directly linked to the regulation of these two transcription factors. In the case of Pap1, oxidation of Trx1/Txl1 has been proposed to block

the function of these proteins as repressors of the activation of Pap1 (Fig. 6. 1; Brown *et al*., 2013; Day *et al*., 2012). In contrast, the analysis performed in this work suggested a model where oxidation of Txl1 (and possibly Trx1) plays an activation role in stimulating the nuclear accumulation of Hsf1 in response to H_2O_2 (Fig. 6. 1). The molecular detail underlying this positive role for Tx1l and/or Trx1 oxidation are unclear but may be linked to the oxidation of Hsf1 regulators such as Ssa1 and Mas5 and/or oxidation of the sole cysteine residue located in Hsf1. Indeed, Ssa1 has been shown to be an activator of the nuclear accumulation of Hsf1 in response to oxidative stress and it is tempting to speculate that Txl1 and/or Trx1 regulate Ssa1 activity through the oxidation of the protein. It is also possible that Mas5 oxidation status is dependent on Txl1 and/or Trx1 oxidation since it has been shown that Mas5 can be oxidised (Garcia-Santamarina *et al*., 2011). Further experiments will be needed to explore these possibilities.

6.5 Concluding remarks

From prokaryotes to eukaryotes there is evidence of conservation of the regulation of heat shock response orchestrated by a heat shock regulator. Hsf1 appears to be regulated by multiple mechanisms in eukaryotes allowing the protein to be regulated by inputs from a variety of different stress conditions. These different mechanisms of regulation may also allow the protein to respond appropriately by determining specific transcriptional responses for each individual stress condition.

The work here builds on previous studies to demonstrate that heat shock proteins modulate Hsf1 in diverse organisms in response to different stress conditions. Comparison between these studies has revealed both conserved and organism-specific regulatory mechanisms. Furthermore, in addition to its antioxidant role, evidence is accumulating from different

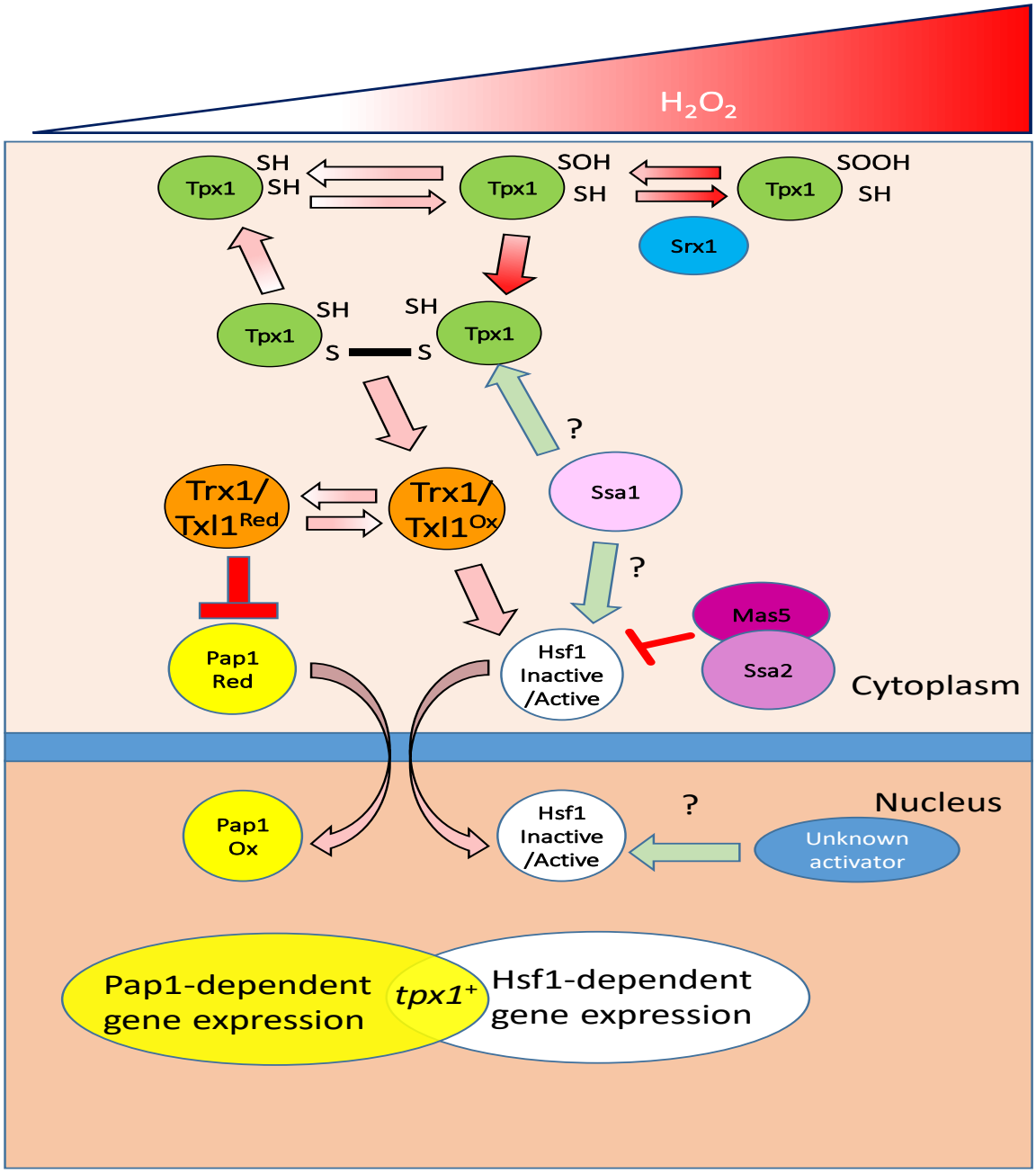


Fig. 6. 1. Model of the regulation of Pap1- and Hsf1-dependent gene expression and possible roles for Ssa1, Ssa2 and Mas5. In unstressed conditions both Pap1 and Hsf1 cycle between the cytoplasm and nucleus. The nuclear accumulation of Pap1 is prevented by maintaining the protein in a reduced state. The nuclear accumulation of Hsf1 is inhibited by the Ssa2/Mas5 complex. In the model both Pap1 and Hsf1 are regulated by the thioredoxin system in response to increasing concentrations of H_2O_2. Oxidation of Tpx1 leads to oxidation of the pool of thioredoxins (Txl1 and Trx1). In the model oxidation and inhibition of Txl1 leads to Pap1 oxidation and nuclear accumulation of active protein. In contrast in the model, oxidation of Txl1 (and/or Trx1) may have a positive role in stimulating nuclear accumulation of Hsf1 through the regulation, for example, of the oxidation status of Ssa1 and/or Mas5. Hsf1 may also be activated by another unknown mechanism.

eukaryotes revealing that the thioredoxin system functions to sense/signal stress conditions and to coordinate different response pathways. This study has revealed new interactions between HSF and the thioredoxin system that regulate stress responses and, given the links between heat shock proteins and antioxidants with numerous human age-related diseases, it will be of significant interest in the future to investigate the conservation of these interactions.

References

Abravaya, K., Myers, M.P., Murphy, S.P. and Morimoto, R.I., 1992. The human heat shock protein HSP70 interacts with HSF, the transcription factor that regulates heat shock gene expression. *Genes and Development, 6*(7), pp.1153-1164.

Abreu, I.A. and Cabelli, D.E., 2010. Superoxide dismutases—a review of the metal-associated mechanistic variations. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, 1804*(2), pp.263-274.

Ali, A., Bharadwaj, S., O'Carroll, R. and Ovsenek, N., 1998. HSP90 interacts with and regulates the activity of heat shock factor 1 in Xenopus oocytes. *Molecular and Cellular Biology, 18*(9), pp.4949-4960.

Amorós, M. and Estruch, F., 2001. Hsf1p and Msn2/4p cooperate in the expression of *Saccharomyces cerevisiae* genes HSP26 and HSP104 in a gene-and stress type-dependent manner. *Molecular Microbiology, 39*(6), pp.1523-1532.

Anckar, J. and Sistonen, L., 2011. Regulation of HSF1 function in the heat stress response: implications in aging and disease. *Annual Review of Biochemistry, 80*, pp.1089-1115.

Anderson, M.E., 1998. Glutathione: an overview of biosynthesis and modulation. *Chemico-Biological Interactions, 111*, pp.1-14.

Andrews, B. and Measday, V., 1998. The cyclin family of budding yeast: abundant use of a good idea. *Trends in Genetics, 14*(2), pp.66-72.

Aoyama, K., Aiba, H. and Mizuno, T., 2001. Genetic analysis of the His-to-Asp phosphorelay implicated in mitotic cell cycle control: involvement of histidine-kinase genes of *Schizosaccharomyces pombe*. *Bioscience, Biotechnology, and Biochemistry, 65*(10), pp.2347-2352.

Aoyama, K., Mitsubayashi, Y., Aiba, H. and Mizuno, T., 2000. Spy1, a histidine-containing phosphotransfer signaling protein, regulates the fission yeast cell cycle through the Mcs4 response regulator. *Journal of Bacteriology, 182*(17), pp.4868-4874.

Arsène, F., Tomoyasu, T. and Bukau, B., 2000. The heat shock response of *Escherichia coli*. *International Journal of Food Microbiology, 55*(1), pp.3-9.

Arthur, J.R., 2001. The glutathione peroxidases. *Cellular and Molecular Life Sciences CMLS, 57*(13-14), pp.1825-1835.

Baum, B., Wuarin, J. and Nurse, P., 1997. Control of S-phase periodic transcription in the fission yeast mitotic cycle. *The European Molecular Biology Organization Journal, 16*(15), pp.4676-4688.

Benjamin, I.J. and McMillan, D.R., 1998. Stress (heat shock) proteins. *Circulation Research, 83*(2), pp.117-132.

Berlett, B.S. and Stadtman, E.R., 1997. Protein oxidation in aging, disease, and oxidative stress. *Journal of Biological Chemistry, 272*(33), pp.20313-20316.

Bersweiler, A., D'Autréaux, B., Mazon, H., Kriznik, A., Belli, G., Delaunay-Moisan, A., Toledano, M.B. and Rahuel-Clermont, S., 2017. A scaffold protein that chaperones a cysteine-sulfenic acid in H_2O_2 signaling. *Nature Chemical Biology, 13*(8), pp.909-915.

Bharadwaj, S., Ali, A. and Ovsenek, N., 1999. Multiple components of the HSP90 chaperone complex function in regulation of heat shock factor 1 in vivo. *Molecular and Cellular Biology, 19*(12), pp.8033-8041.

Bohr, V.A. and Anson, R.M., 1995. DNA damage, mutation and fine structure DNA repair in aging. *Mutation Research/DNAging, 338*(1), pp.25-34.

Bokov, A., Chaudhuri, A. and Richardson, A., 2004. The role of oxidative damage and stress in aging. *Mechanisms of Ageing and Development, 125*(10), pp.811-826.

Bonner, J.J., Carlson, T., Fackenthal, D.L., Paddock, D., Storey, K. and Lea, K., 2000. Complex regulation of the yeast heat shock transcription factor. *Molecular Biology of the Cell, 11*(5), pp.1739-1751.

Bouayed, J. and Bohn, T., 2010. Exogenous antioxidants—double-edged swords in cellular redox state: health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxidative Medicine and Cellular Longevity, 3*(4), pp.228-237.

Bozonet, S.M. (2006) Role of peroxiredoxins in the stress response of *Schizosaccharomyces pombe*. Newcastle University.

Bozonet, S.M., Findlay, V.J., Day, A.M., Cameron, J., Veal, E.A. and Morgan, B.A., 2005. Oxidation of a eukaryotic 2-Cys peroxiredoxin is a molecular switch controlling the transcriptional response to increasing levels of hydrogen peroxide. *Journal of Biological Chemistry, 280*(24), pp.23319-23327.

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C.A., Causton, H.C. and Gaasterland, T., 2001. Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. *Nature Genetics, 29*(4), pp.365-371.

Brown, J.D., Day, A.M., Taylor, S.R., Tomalin, L.E., Morgan, B.A. and Veal, E.A., 2013. A peroxiredoxin promotes H_2O_2 signaling and oxidative stress resistance by oxidizing a thioredoxin family protein. *Cell Reports, 5*(5), pp.1425-1435.

Brunner, D. and Nurse, P., 2000. CLIP170-like tip1p spatially organizes microtubular dynamics in fission yeast. *Cell, 102*(5), pp.695-704.

Calvo, I.A., Ayté, J. and Hidalgo, E., 2013. Reversible thiol oxidation in the H_2O_2-dependent activation of the transcription factor Pap1. *Journal of Cell Science, 126*(10), pp.2279-2284.

Caplan, A.J., Cyr, D.M. and Douglas, M.G., 1992. YDJ1p facilitates polypeptide translocation across different intracellular membranes by a conserved mechanism. *Cell, 71*(7), pp.1143-1155.

Cash, T.P., Pan, Y. and Simon, M.C., 2007. Reactive oxygen species and cellular oxygen sensing. *Free Radical Biology and Medicine, 43*(9), pp.1219-1225.

Casso, D. and Beach, D., 1996. A mutation in a thioredoxin reductase homolog suppresses p53-induced growth inhibition in the fission yeast *Schizosaccharomyces pombe*. *Molecular and General Genetics, 252*(5), pp.518-529.

Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G., Lee, T.I., True, H.L., Lander, E.S. and Young, R.A., 2001. Remodeling of yeast genome expression in response to environmental changes. *Molecular Biology of the Cell, 12*(2), pp.323-337.

Chang, F. and Martin, S.G., 2009. Shaping fission yeast with microtubules. *Cold Spring Harbor Perspectives in Biology, 1*(1), p.a001347.

Chaudiere, J. and Ferrari-Iliou, R., 1999. Intracellular antioxidants: from chemical to biochemical mechanisms. *Food and Chemical Toxicology, 37*(9), pp. 949-962.

Chen, D., Toone, W.M., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N. and Bähler, J., 2003. Global transcriptional responses of fission yeast to environmental stress. *Molecular Biology of the Cell, 14*(1), pp.214-229.

Chen, D., Wilkinson, C.R., Watt, S., Penkett, C.J., Toone, W.M., Jones, N. and Bähler, J., 2008. Multiple pathways differentially regulate global oxidative stress responses in fission yeast. *Molecular Biology of the Cell, 19*(1), pp.308-317.

Chen, Y., Barlev, N.A., Westergaard, O. and Jakobsen, B.K., 1993. Identification of the C-terminal activator domain in yeast heat shock factor: independent control of transient and sustained transcriptional activity. *The European Molecular Biology Organization Journal, 12*(13), p.5007-5018.

Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S. and Loeb, L.A., 1992. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G----T and A----C substitutions. *Journal of Biological Chemistry, 267*(1), pp.166-172.

Chikashige, Y., Yamane, M., Okamasa, K., Mori, C., Fukuta, N., Matsuda, A., Haraguchi, T. and Hiraoka, Y., 2014. Chromosomes rein back the spindle pole body during horsetail movement in fission yeast meiosis. *Cell Structure and Function, 39*(2), pp.93-100.

Christians, E., Davis, A.A., Thomas, S.D. and Benjamin, I.J., 2000. Embryonic development: maternal effect of Hsf1 on reproductive success. *Nature, 407*(6805), p.693.

Chung, W.H., Kim, K.D. and Roe, J.H., 2005. Localization and function of three monothiol glutaredoxins in *Schizosaccharomyces pombe*. *Biochemical and Biophysical Research Communications, 330*(2), pp.604-610.

Chung, W.H., Kim, K.D., Cho, Y.J. and Roe, J.H., 2004. Differential expression and role of two dithiol glutaredoxins Grx1 and Grx2 in *Schizosaccharomyces pombe*. *Biochemical and biophysical research communications, 321*(4), pp.922-929.

Ciechanover, A., 1994. The ubiquitin-proteasome proteolytic pathway. *Cell, 79*(1), pp.13-21.

Ciechanover, A., 1998. The ubiquitin–proteasome pathway: on protein death and cell life. *The European Molecular Biology Organization Journal, 17*(24), pp.7151-7160.

Clerico, E.M., Tilitsky, J.M., Meng, W. and Gierasch, L.M., 2015. How hsp70 molecular machines interact with their substrates to mediate diverse physiological functions. *Journal of Molecular Biology, 427*(7), pp.1575-1588.

Coleman, S.T., Epping, E.A., Steggerda, S.M. and Moye-Rowley, W.S., 1999. Yap1p activates gene transcription in an oxidant-specific fashion. *Molecular and Cellular Biology, 19*(12), pp.8302-8313.

Collinson, E.J. and Grant, C.M., 2003. Role of yeast glutaredoxins as glutathione S-transferases. *Journal of Biological Chemistry, 278*(25), pp.22492-22497.

Cooke, M.S., Evans, M.D., Dizdaroglu, M. and Lunec, J., 2003. Oxidative DNA damage: mechanisms, mutation, and disease. *The Federation of American societies for Experimental Biology Journal, 17*(10), pp.1195-1214.

Costa, V. and Moradas-Ferreira, P., 2001. Oxidative stress and signal transduction in *Saccharomyces cerevisiae*: insights into ageing, apoptosis and diseases. *Molecular Aspects of Medicine, 22*(4), pp.217-246.

Craig, E.A. and Marszalek, J., 2011. *S. cerevisiae* HSP70 chaperones. Encyclopedia of life sciences (ELS).

Cuypers, A., Plusquin, M., Remans, T., Jozefczak, M., Keunen, E., Gielen, H., Opdenakker, K., Nair, A.R., Munters, E., Artois, T.J. and Nawrot, T., 2010. Cadmium stress: an oxidative challenge. *Biometals, 23*(5), pp.927-940.

Daniel, J.A., Keyes, B.E., Ng, Y.P., Freeman, C.O. and Burke, D.J., 2006. Diverse functions of spindle assembly checkpoint genes in *Saccharomyces cerevisiae*. *Genetics, 172*(1), pp.53-65.

Day, A.M. and Veal, E.A., 2010. Hydrogen peroxide-sensitive cysteines in the Sty1 MAPK regulate the transcriptional response to oxidative stress. *Journal of Biological Chemistry, 285*(10), pp.7505-7516.

Day, A.M., Brown, J.D., Taylor, S.R., Rand, J.D., Morgan, B.A. and Veal, E.A., 2012. Inactivation of a peroxiredoxin by hydrogen peroxide is critical for thioredoxin-mediated repair of oxidized proteins and cell survival. *Molecular cell, 45*(3), pp.398-408.

Delaunay, A., Isnard, A. D., Toledano, M. B., 2000. H_2O_2 sensing through oxidation of the Yap1 transcription factor. *European Molecular Biology Organization Journal, 19*(1), pp. 5157-5166.

Delaunay, A., Pflieger, D., Barrault, M.B., Vinh, J. and Toledano, M.B., 2002. A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. *Cell, 111*(4), pp.471-481.

Demasi, A.P., Pereira, G.A. and Netto, L.E., 2001. Cytosolic thioredoxin peroxidase I is essential for the antioxidant defense of yeast with dysfunctional mitochondria. *Federation of European Biochemical Societies letters, 509*(3), pp.430-434.

Ding, D., 2011. A rush hour towards sexual reproduction: The chromosome dynamics during meiosis. *Chinese Science Bulletin, 56*(33), pp.3500-3503.

Ding, D.Q., Yamamoto, A., Haraguchi, T. and Hiraoka, Y., 2004. Dynamics of homologous chromosome pairing during meiotic prophase in fission yeast. *Developmental Cell, 6*(3), pp.329-341.

Dokladny, K., Zuhl, M.N., Mandell, M., Bhattacharya, D., Schneider, S., Deretic, V. and Moseley, P.L., 2013. Regulatory coordination between two major intracellular homeostatic systems heat shock response and autophagy. *Journal of Biological Chemistry, 288*(21), pp.14959-14972.

Doris, K.S., 2008. The regulation of the cell division cycle in response to oxidative stress in *Saccharomyces cerevisiae*. Newcastle University.

Eaton, D.L. and Bammler, T.K., 1999. Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicological Sciences: An Official Journal of the Society of Toxicology, 49*(2), pp.156-164.

Estruch, F., 2000. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *Federation of Microbiological Societies Microbiology Reviews, 24*(4), pp.469-486.

Feder, M.E. and Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology, 61*(1), pp.243-282.

Feierbach, B. and Chang, F., 2001. Roles of the fission yeast formin for3p in cell polarity, actin cable formation and symmetric cell division. *Current Biology, 11*(21), pp.1656-1665.

Feierbach, B., Verde, F. and Chang, F., 2004. Regulation of a formin complex by the microtubule plus end protein tea1p. *The Journal of Cell Biology, 165*(5), pp.697-707.

Ferrezuelo, F., Colomina, N., Palmisano, A., Gari, E., Gallego, C., Csikász-Nagy, A. and Aldea, M., 2012. The critical size is set at a single-cell level by growth rate to attain homeostasis and adaptation. *Nature Communications, 3*, p.1000-1012.

Flanagan, M.D., Whitehall, S.K. and Morgan, B.A., (2013) An Atg10-like E2 enzyme is essential for cell cycle progression but not autophagy in *Schizosaccharomyces pombe*. *Cell Cycle. 12*(2), pp. 271–277.

Flint, D.H., Tuminello, J.F. and Emptage, M.H., 1993. The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *Journal of Biological Chemistry, 268*(30), pp.22369-22376.

Forsburg, S.L. and Nurse, P., 1991. Cell cycle regulation in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *Annual Review of Cell Biology, 7*(1), pp.227-256.

Fujii, Y., Shimizu, T., Toda, T., Yanagida, M. and Hakoshima, T., 2000. Structural basis for the diversity of DNA recognition by bZIP transcription factors. *Nature Structural and Molecular Biology, 7*(10), pp.889-893.

Gallo, G.J., Prentice, H. and Kingston, R.E., 1993. Heat shock factor is required for growth at normal temperatures in the fission yeast *Schizosaccharomyces pombe*. *Molecular and Cellular Biology, 13*(2), pp.749-761.

Gallo, G.J., Schuetz, T.J. and Kingston, R.E., 1991. Regulation of heat shock factor in *Schizosaccharomyces pombe* more closely resembles regulation in mammals than in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology, 11*(1), pp.281-288.

García-Santamarina, S., Boronat, S., Calvo, I.A., Rodríguez-Gabriel, M., Ayté, J., Molina, H. and Hidalgo, E., 2013. Is oxidized thioredoxin a major trigger for cysteine oxidation? Clues from a redox proteomics approach. *Antioxidants and Redox Signaling, 18*(13), pp.1549-1556.

García-Santamarina, S., Boronat, S., Espadas, G., Ayté, J., Molina, H. and Hidalgo, E., 2011. The oxidized thiol proteome in fission yeast—optimization of an ICAT-based method to identify H_2O_2-oxidized proteins. *Journal of Proteomics, 74*(11), pp.2476-2486.

Garinis, G.A., Van der Horst, G.T., Vijg, J. and Hoeijmakers, J.H., 2008. DNA damage and ageing: new-age ideas for an age-old problem. *Nature Cell Biology, 10*(11), pp.1241-1247.

Garrido, E.O. and Grant, C.M., 2002. Role of thioredoxins in the response of *Saccharomyces cerevisiae* to oxidative stress induced by hydroperoxides. *Molecular Microbiology, 43*(4), pp.993-1003.

Gasch, A.P. and Werner-Washburne, M., 2002. The genomics of yeast responses to environmental stress and starvation. *Functional and Integrative Genomics, 2*(4), pp.181-192.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D. and Brown, P.O., 2000. Genomic expression programs in the response of yeast cells to environmental changes. *Molecular Biology of the Cell, 11*(12), pp.4241-4257.

Genevaux, P., Schwager, F., Georgopoulos, C. and Kelley, W.L., 2002. Scanning mutagenesis identifies amino acid residues essential for the in vivo activity of the *Escherichia coli* DnaJ (Hsp40) J-domain. *Genetics, 162*(3), pp.1045-1053.

Ghaemmaghami, S., Huh, W.K., Bower, K. and Howson, R.W., 2003. Global analysis of protein expression in yeast. *Nature, 425*(6959), p.737-741.

Glover, J.R. and Lindquist, S., 1998. Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. *Cell, 94*(1), pp.73-82.

Gonzalez, C., Sanz-Alfayate, G., Agapito, M.T., Gomez-Nino, A., Rocher, A. and Obeso, A., 2002. Significance of ROS in oxygen sensing in cell systems with sensitivity to physiological hypoxia. *Respiratory Physiology and Neurobiology, 132*(1), pp.17-41.

Görner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H. and Schüller, C., 1998. Nuclear localization of the C_2H_2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes and Development, 12*(4), pp.586-597.

Green, M., Schuetz, T.J., Sullivan, E.K. and Kingston, R.E., 1995. A heat shock-responsive domain of human HSF1 that regulates transcription activation domain function. *Molecular and Cellular Biology, 15*(6), pp.3354-3362.

Grune, T., Jung, T., Merker, K. and Davies, K.J., 2004. Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and 'aggresomes' during oxidative stress, aging, and disease. *The International Journal of Biochemistry and Cell Biology, 36*(12), pp.2519-2530.

Haddad, J.J., 2004. On the antioxidant mechanisms of Bcl-2: a retrospective of NF-κB signaling and oxidative stress. *Biochemical and biophysical research communications, 322*(2), pp.355-363.

Hagan, I. and Ayscough, K.R. (2000) Fluorescence microscopy in yeast. Protein Localization by Fluorescence Microscopy: A Practical Approach. Allan V. edit, Oxford University Press, New York, New York, pp. 179-206.

Hahn, J.S., Hu, Z., Thiele, D.J. and Iyer, V.R., 2004. Genome-wide analysis of the biology of stress responses through heat shock transcription factor. *Molecular and Cellular Biology, 24*(12), pp.5249-5256.

Halliwell, B. and Gutteridge, J.M.C., 1999. Oxidative stress and antioxidant protection: some special cases. *Free Radicals in Biology and Medicine*, pp.485-543.

Hanzén, S., Vielfort, K., Yang, J., Roger, F., Andersson, V., Zamarbide-Forés, S., Andersson, R., Malm, L., Palais, G., Biteau, B. and Liu, B., 2016. Lifespan control by redox-dependent recruitment of chaperones to misfolded proteins. *Cell*, *166*(1), pp.140-151.

Hartl, F.U., Bracher, A. and Hayer-Hartl, M., 2011. Molecular chaperones in protein folding and proteostasis. *Nature, 475*(7356), pp.324-332.

Hasan, R., Leroy, C., Isnard, A.D., Labarre, J., Boy-Marcotte, E. and Toledano, M.B., 2002. The control of the yeast H_2O_2 response by the Msn2/4 transcription factors. *Molecular Microbiology, 45*(1), pp.233-241.

Hasin, N., Cusack, S.A., Ali, S.S., Fitzpatrick, D.A. and Jones, G.W., 2014. Global transcript and phenotypic analysis of yeast cells expressing Ssa1, Ssa2, Ssa3 or Ssa4 as sole source of cytosolic Hsp70-Ssa chaperone activity. *Biomedical Central Genomics, 15*(1), pp.194-210.

He, X., Patterson, T. E. and Sazer, S., 1997. The *Schizosaccharomyces pombe* spindle checkpoint protein mad2p blocks anaphase and genetically interacts with the anaphase-promoting complex. *Proceedings of the National Academy of Sciences of the United States of America, 94*(15), pp.7965–7970.

He, X.J. and Fassler, J.S., 2005. Identification of novel Yap1p and Skn7p binding sites involved in the oxidative stress response of *Saccharomyces cerevisiae*. *Molecular Microbiology, 58*(5), pp.1454-1467.

Heerklotz, D., Döring, P., Bonzelius, F., Winkelhaus, S. and Nover, L., 2001. The balance of nuclear import and export determines the intracellular distribution and function of tomato heat stress transcription factor HsfA2. *Molecular and Cellular Biology, 21*(5), pp.1759-1768.

Herrero, E., Ros, J., Bellí, G. and Cabiscol, E., 2008. Redox control and oxidative stress in yeast cells. *Biochimica et Biophysica Acta General Subjects, 1780*(11), pp.1217-1235.

Hideyuki, M., Takayoshi, K., Hozumi, T., Dai, H., Tokichi, M. and Chikako, T., 1993. Isolation and characterization of SSE1 and SSE2, new members of the yeast HSP70 multigene family. *Gene, 132*(1), pp.57-66.

Hietakangas, V., Anckar, J., Blomster, H.A., Fujimoto, M., Palvimo, J.J., Nakai, A. and Sistonen, L., 2006. PDSM, a motif for phosphorylation-dependent SUMO modification. *Proceedings of the National Academy of Sciences of the United States of America, 103*(1), pp.45-50.

Hirt, R.P., Müller, S., Embley, T.M. and Coombs, G.H., 2002. The diversity and evolution of thioredoxin reductase: new perspectives. *Trends in Parasitology, 18*(7), pp.302-308.

Holmberg, C.I., Hietakangas, V., Mikhailov, A., Rantanen, J.O., Kallio, M., Meinander, A., Hellman, J., Morrice, N., MacKintosh, C., Morimoto, R.I. and Eriksson, J.E., 2001. Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. *The European Molecular Biology organisation Journal, 20*(14), pp.3800-3810.

Jang, H.H., Lee, K.O., Chi, Y.H., Jung, B.G., Park, S.K., Park, J.H., Lee, J.R., Lee, S.S., Moon, J.C., Yun, J.W. and Choi, Y.O., 2004. Two enzymes in one: two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. *Cell, 117*(5), pp.625-635.

Jenner, P., 2003. Oxidative stress in Parkinson's disease. *Annals of Neurology*, 53(S3).

Jiménez, A., Mateos, L., Pedrajas, J.R., Miranda-Vizuete, A. and Revuelta, J.L., 2007. The *txl1*$^{+}$ gene from *Schizosaccharomyces pombe* encodes a new thioredoxin-like 1 protein that participates in the antioxidant defence against tert-butyl hydroperoxide. *Yeast, 24*(6), pp.481-490.

Jonas, K., Liu, J., Chien, P. and Laub, M.T., 2013. Proteotoxic stress induces a cell-cycle arrest by stimulating Lon to degrade the replication initiator DnaA. *Cell, 154*(3), pp.623-636.

Jorgensen, P., Nishikawa, J.L., Breitkreutz, B.J. and Tyers, M., 2002. Systematic identification of pathways that couple cell growth and division in yeast. *Science, 297*(5580), pp.395-400.

Kalmar, B. and Greensmith, L., 2009. Induction of heat shock proteins for protection against oxidative stress. *Advanced Drug Delivery Reviews, 61*(4), pp.310-318.

Kelley, W.L., 1998. The J-domain family and the recruitment of chaperone power. *Trends in Biochemical Sciences, 23*(6), pp.222-227.

Khanna, K.K. and Jackson, S.P., 2001. DNA double-strand breaks: signaling, repair and the cancer connection. *Nature genetics, 27*(3), p.247-254.

Kim, D.U., Hayles, J., Kim, D., Wood, V., Park, H.O., Won, M., Yoo, H.S., Duhig, T., Nam, M., Palmer, G. and Han, S., 2010. Analysis of a genome-wide set of gene deletions in the fission yeast Schizosaccharomyces pombe. *Nature Biotechnology, 28*(6), pp.613-617.

King, C., Eisenberg, E. and Greene, L., 1997. Effect of yeast and human DnaJ homologs on clathrin uncoating by 70 kilodalton heat shock protein. *Biochemistry, 36*(14), pp.4067-4073.

Klionsky, D.J., Cuervo, A.M. and Seglen, P.O., 2007. Methods for monitoring autophagy from yeast to human. *Autophagy, 3*(3), pp.181-206.

Kohda, T.A., Tanaka, K., Konomi, M., Sato, M., Osumi, M. and Yamamoto, M., 2007. Fission yeast autophagy induced by nitrogen starvation generates a nitrogen source that drives adaptation processes. *Genes to Cells, 12*(2), pp.155-170.

Kregel, K.C., 2002. Invited review: heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *Journal of Applied Physiology, 92*(5), pp.2177-2186.

Kudo, N., Taoka, H., Toda, T., Yoshida, M. and Horinouchi, S., 1999. A novel nuclear export signal sensitive to oxidative stress in the fission yeast transcription factor Pap1. *Journal of Biological Chemistry, 274*(21), pp.15151-15158.

Kuge, S. and Jones, N., 1994. YAP1 dependent activation of TRX2 is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. *The European Molecular Biology Organisation Journal, 13*(3), pp.649-655.

Kuge, S., Arita, M., Murayama, A., Maeta, K., Izawa, S., Inoue, Y. and Nomoto, A., 2001. Regulation of the yeast Yap1p nuclear export signal is mediated by redox signal-induced reversible disulfide bond formation. *Molecular and Cellular Biology, 21*(18), pp.6139-6150.

Kuge, S., Toda, T., Iizuka, N. and Nomoto, A., 1998. Crm1 (XpoI) dependent nuclear export of the budding yeast transcription factor yAP-1 is sensitive to oxidative stress. *Genes to Cells, 3*(8), pp.521-532.

Kültz, D., 2005. Molecular and evolutionary basis of the cellular stress response. *Annual Review of Physiology, 67*(1), pp.225-257.

Lee, Y.Y., Jung, H.I., Park, E.H., Sa, J.H. and Lim, C.J., 2002. Regulation of Schizosaccharomyces pombe gene encoding copper/zinc superoxide dismutase. *Molecular Cells*, *14*(1), pp. 43-49.

Le Masson, F., Razak, Z., Kaigo, M., Audouard, C., Charry, C., Cooke, H., Westwood, J.T. and Christians, E.S., 2011. Identification of heat shock factor 1 molecular and cellular targets during embryonic and adult female meiosis. *Molecular and Cellular Biology, 31*(16), pp.3410-3423.

Levine, B. and Klionsky, D.J. (2004) Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Developmental Cell, 6*(1), pp. 463–477.

Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C. and Zylicz, M., 1991. *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. *Proceedings of the National Academy of Sciences, 88*(7), pp.2874-2878.

Littlefield, O. and Nelson, H., 1999. A new use for the 'wing' of the 'winged' helix-turn-helix motif in the HSF? DNA cocrystal. *Nature Structural Biology, 6*(5), pp. 464-475.

Liu, X.D. and Thiele, D.J., 1996. Oxidative stress induced heat shock factor phosphorylation and HSF-dependent activation of yeast metallothionein gene transcription. *Genes and Development, 10*(5), pp.592-603.

Lowndes, N.F., 2007. Chaperoning the Cln3 cyclin prevents promiscuous activation of Start. *Molecular Cell, 27*(1), pp.1-2.

Lu, M., Lee, Y.J., Park, S.M., Kang, H.S., Kang, S.W., Kim, S. and Park, J.S., 2009. Aromatic-participant interactions are essential for disulfide-bond-based trimerization in human heat shock transcription factor 1. *Biochemistry, 48*(18), pp.3795-3797.

Lu, Z. and Cyr, D.M., 1998. Protein folding activity of Hsp70 is modified differentially by the hsp40 co-chaperones Sis1 and Ydj1. *Journal of Biological Chemistry, 273*(43), pp.27824-27830.

Luft, J.C., Benjamin, I.J., Mestril, R. and Dix, D.J., 2001. Heat shock factor 1–mediated thermotolerance prevents cell death and results in G2/M cell cycle arrest. *Cell stress and chaperones, 6*(4), pp.326-336.

Luikenhuis, S., Perrone, G., Dawes, I.W. and Grant, C.M., 1998. The yeast *Saccharomyces cerevisiae* contains two glutaredoxin genes that are required for protection against reactive oxygen species. *Molecular Biology of the Cell, 9*(5), pp.1081-1091.

Machado, A.K., Morgan, B.A. and Merrill, G.F., 1997. Thioredoxin reductase-dependent inhibition of MCB cell cycle box activity in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry, 272*(27), pp.17045-17054.

Madamanchi, N.R., Vendrov, A. and Runge, M.S., 2005. Oxidative stress and vascular disease. *Arteriosclerosis, Thrombosis, and Vascular Biology, 25*(1), pp.29-38.

Makhnevych, T., Wong, P., Pogoutse, O., Vizeacoumar, F.J., Greenblatt, J.F., Emili, A. and Houry, W.A., 2012. Hsp110 is required for spindle length control. *Journal of Cell Biology, 198*(4), pp.623-636.

Maniatis, T., Sambrook, J., Fritsch, E. F. (1989) Molecular cloning: a laboratory manual. 2nd Edition, New York: Cold Spring Harbour Laboratory.

Martin, S.G. and Chang, F., 2005. New end take off: Regulating cell polarity during the fission. *Cell Cycle, 4*(8), pp.4046-4049.

Martin, S.G., McDonald, W.H., Yates, J.R. and Chang, F., 2005. Tea4p links microtubule plus ends with the formin for3p in the establishment of cell polarity. *Developmental Cell, 8*(4), pp.479-491.

Martín-Castellanos, C., Blanco, M., Rozalén, A.E., Pérez-Hidalgo, L., García, A.I., Conde, F., Mata, J., Ellermeier, C., Davis, L., San-Segundo, P. and Smith, G.R., 2005. A large-scale screen in *S. pombe* identifies seven novel genes required for critical meiotic events. *Current Biology, 15*(22), pp.2056-2062.

Martinez-Vicente, M., Sovak, G. and Cuervo, A.M., 2005. Protein degradation and aging. *Experimental Gerontology, 40*(8), pp.622-633.

Matsui, M., Oshima, M., Oshima, H., Takaku, K., Maruyama, T., Yodoi, J. and Taketo, M.M., 1996. Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Developmental Biology, 178*(1), pp.179-185.

Matsumoto, R., Rakwal, R., Agrawal, G.K., Jung, Y.H., Jwa, N.S., Yonekura, M., Iwahashi, H. and Akama, K., 2006. Search for novel stress-responsive protein components using a yeast mutant lacking two cytosolic Hsp70 genes, SSA1 and SSA2. *Molecules and Cells (Springer Science and Business Media BV)*, 21(3).

Matsuyama, A., Arai, R., Yashiroda, Y., Shirai, A., Kamata, A., Sekido, S., Kobayashi, Y., Hashimoto, A., Hamamoto, M., Hiraoka, Y. and Horinouchi, S., 2006. ORFeome cloning and global analysis of protein localization in the fission yeast *Schizosaccharomyces pombe*. *Nature Biotechnology, 24(*7), pp.841-848.

Maundrell, K., 1993. Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene, 123*(1), pp.127-130.

Mayer, M.P. and Bukau, B., 2005. Hsp70 chaperones: cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences, 62*(6), pp.670-684.

Mayer, M.P., 2010. Gymnastics of molecular chaperones. *Molecular Cell, 39*(3), pp.321-331.

McDonough, H. and Patterson, C., 2003. CHIP: a link between the chaperone and proteasome systems. *Cell Stress and Chaperones, 8*(4), pp.303-308.

Medvedik, O., Lamming, D.W., Kim, K.D. and Sinclair, D.A., 2007. MSN2 and MSN4 link calorie restriction and TOR to sirtuin-mediated lifespan extension in *Saccharomyces cerevisiae*. *PLoS biology, 5*(10), pp.2330-2341.

Meister, A., 1991. Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy. *Pharmacology and Therapeutics, 51*(2), pp.155-194.

Mendenhall, M.D. and Hodge, A.E., 1998. Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews, 62*(4), pp.1191-1243.

Mendillo, M.L., Santagata, S., Koeva, M., Bell, G.W., Hu, R., Tamimi, R.M., Fraenkel, E., Ince, T.A., Whitesell, L. and Lindquist, S., 2012. HSF1 drives a transcriptional program distinct from heat shock to support highly malignant human cancers. *Cell, 150*(3), pp.549-562.

Molin, M., Renault, J.P., Lagniel, G., Pin, S., Toledano, M. and Labarre, J., 2007. Ionizing radiation induces a Yap1-dependent peroxide stress response in yeast. *Free Radical Biology and Medicine, 43*(1), pp.136-144.

Monteiro, G., Horta, B.B., Pimenta, D.C., Augusto, O. and Netto, L.E., 2007. Reduction of 1-Cys peroxiredoxins by ascorbate changes the thiol-specific antioxidant paradigm, revealing another function of vitamin C. *Proceedings of the National Academy of Sciences, 104*(12), pp.4886-4891.

Moreno, S., Klar, A., Nurse, P. (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods in Enzymology, 194*(1), pp.795-823.

Morimoto, R.I., 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes and Development, 12*(24), pp.3788-3796.

Moser, B.A. and Russell, P., 2000. Cell cycle regulation in *Schizosaccharomyces pombe*. *Current Opinion in Microbiology, 3*(6), pp.631-636.

Mouaheb, N., Thomas, D., Verdoucq, L., Monfort, P. and Meyer, Y., 1998. In vivo functional discrimination between plant thioredoxins by heterologous expression in the yeast *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences, 95*(6), pp.3312-3317.

Moye-Rowley, W.S., 2003. Regulation of the transcriptional response to oxidative stress in fungi: similarities and differences. *Eukaryotic Cell, 2*(3), pp.381-389.

Moye-Rowley, W.S., Harshman, K.D. and Parker, C.S., 1989. Yeast YAP1 encodes a novel form of the jun family of transcriptional activator proteins. *Genes and Development, 3*(3), pp.283-292.

Mukaiyama, H., Nakase, M., Nakamura, T., Kakinuma, Y. and Takegawa, K., 2010. Autophagy in the fission yeast *Schizosaccharomyces pombe*. *FEBS letters, 584*(7), pp.1327-1334.

Munhoz, D.C. and Netto, L.E.S., 2004. Cytosolic thioredoxin peroxidase I and II are important defenses of yeast against organic hydroperoxide nsult catalases and peroxiredoxins cooperate in the decomposition of H_2O_2 by yeast. *Journal of Biological Chemistry, 279*(34), pp.35219-35227.

Musacchio, A. and Salmon, E.D., 2007. The spindle-assembly checkpoint in space and time. *Nature reviews. Molecular Cell Biology, 8*(5), pp.379-385.

Mutah, N., Nakagawa, C.W. and Yamada, K., 1999. The role of catalase in hydrogen peroxide resistance in fission yeast *Schizosaccharomyces pombe*. *The Canadian Journal of Microbiology, 45*(2), pp. 125-129.

Najarzadegan, M.R., Ataei, E. and Akbarzadeh, F., 2016. The Role of Heat Shock Proteins in Alzheimer Disease: A Systematic Review. *Journal of Syndromes*, *3*(1), pp.1-6.

Nakano, K., Imai, J., Arai, R., Toh-e, A., Matsui, Y. and Mabuchi, I., 2002. The small GTPase Rho3 and the diaphanous/formin For3 function in polarized cell growth in fission yeast. *Journal of Cell Science, 115*(23), pp.4629-4639.

Newton, E.M., Knauf, U., Green, M. and Kingston, R.E., 1996. The regulatory domain of human heat shock factor 1 is sufficient to sense heat stress. *Molecular and Cellular Biology, 16*(3), pp.839-846.

Noctor, G. and Foyer, C.H., 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Biology, 49*(1), pp.249-279.

Nonn, L., Williams, R.R., Erickson, R.P. and Powis, G., 2003. The absence of mitochondrial thioredoxin 2 causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice. *Molecular and Cellular Biology, 23*(3), pp.916-922.

Nordberg, J. and Arnér, E.S., 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radical Biology and Medicine, 31*(11), pp.1287-1312.

Nunomura, A., Perry, G., Aliev, G., Hirai, K., Takeda, A., Balraj, E.K., Jones, P.K., Ghanbari, H., Wataya, T., Shimohama, S. and Chiba, S., 2001. Oxidative damage is the earliest event in Alzheimer disease. *Journal of Neuropathology and Experimental Neurology, 60*(8), pp.759-767.

Nurse, P., 1994. Ordering S phase and M phase in the cell cycle. *Cell, 79*(4), pp.547-550.

Ohtsuka, H., Azuma, K., Murakami, H. and Aiba, H., 2011. *hsf1*$^{+}$ extends chronological lifespan through Ecl1 family genes in fission yeast. *Molecular Genetics and Genomics, 285*(1), pp.67-77.

Ojeda, L., Keller, G., Muhlenhoff, U., Rutherford, J.C., Lill, R. and Winge, D.R., 2006. Role of glutaredoxin-3 and glutaredoxin-4 in the iron regulation of the Aft1 transcriptional activator in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry, 281*(26), pp.17661-17669.

Oka, M., Nakai, M., Endo, T., Lim, C.R., Kimata, Y. and Kohno, K., 1998. Loss of Hsp70-Hsp40 chaperone activity causes abnormal nuclear distribution and aberrant microtubule formation in M-phase of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, 273(45), pp.29727-29737.

Okazaki, S., Naganuma, A. and Kuge, S., 2005. Peroxiredoxin-mediated redox regulation of the nuclear localization of Yap1, a transcription factor in budding yeast. *Antioxidants and redox signaling, 7*(3), pp.327-334.

Park, S.G., Cha, M.K., Jeong, W. and Kim, I.H., 2000. Distinct physiological functions of thiol peroxidase isoenzymes in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry, 275*(8), pp.5723-5732.

Peteranderl, R., Rabenstein, M., Shin, Y.K., Liu, C.W., Wemmer, D.E., King, D.S. and Nelson, H.C., 1999. Biochemical and biophysical characterization of the trimerization domain from the heat shock transcription factor. *Biochemistry, 38*(12), pp.3559-3569.

Peyrot, F. and Ducrocq, C., 2008. Potential role of tryptophan derivatives in stress responses characterized by the generation of reactive oxygen and nitrogen species. *Journal of Pineal Research, 45*(3), pp.235-246.

Pham-Huy, L.A., He, H. and Pham-Huy, C., 2008. Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science, 4*(2), pp.89-96.

Pujol-Carrion, N., Belli, G., Herrero, E., Nogues, A. and de la Torre-Ruiz, M.A., 2006. Glutaredoxins Grx3 and Grx4 regulate nuclear localisation of Aft1 and the oxidative stress response in *Saccharomyces cerevisiae*. *Journal of Cell Science, 119*(21), pp.4554-4564.

Quinn, J., Findlay, V.J., Dawson, K., Millar, J.B., Jones, N., Morgan, B.A. and Toone, W.M., 2002. Distinct regulatory proteins control the graded transcriptional response to increasing H_2O_2 levels in fission yeast *Schizosaccharomyces pombe*. *Molecular Biology of the Cell, 13*(3), pp.805-816.

Raitt, D.C., Johnson, A.L., Erkine, A.M., Makino, K., Morgan, B., Gross, D.S. and Johnston, L.H., 2000. The Skn7 response regulator of *Saccharomyces cerevisiae* interacts with Hsf1 in vivo and is required for the induction of heat shock genes by oxidative stress. *Molecular Biology of the Cell, 11*(7), pp.2335-2347.

Reeg, S., and Grune, T. (2015). Protein Oxidation in Aging: Does It Play a Role in Aging Progression? *Antioxidants & Redox Signaling, 23*(3), pp.239–255.

Rand, J.D. and Grant, C.M., 2006. The thioredoxin system protects ribosomes against stress-induced aggregation. *Molecular biology of the cell, 17*(1), pp.387-401.

Reuter, S., Gupta, S.C., Chaturvedi, M.M. and Aggarwal, B.B., 2010. Oxidative stress, inflammation, and cancer: how are they linked? *Free Radical Biology and Medicine, 49*(11), pp.1603-1616.

Rhee, S.G., Kang, S.W., Chang, T.S., Jeong, W. and Kim, K., 2001. Peroxiredoxin, a novel family of peroxidases. *International Union of Biochemistry and Molecular Biology life, 52*(1), pp.35-41.

Rodríguez-Manzaneque, M.T., Ros, J., Cabiscol, E., Sorribas, A. and Herrero, E., 1999. Grx5 Glutaredoxin plays a central role in protection against protein oxidative damage in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology, 19*(12), pp.8180-8190.

Ross, S.J., Findlay, V.J., Malakasi, P. and Morgan, B.A., 2000. Thioredoxin peroxidase is required for the transcriptional response to oxidative stress in budding yeast. *Molecular Biology of the Cell, 11*(8), pp.2631-2642.

Rustici, G., Mata, J., Kivinen, K., Lió, P., Penkett, C.J., Burns, G., Hayles, J., Brazma, A., Nurse, P. and Bähler, J., 2004. Periodic gene expression program of the fission yeast cell cycle. *Nature Genetics, 36*(8), pp.809-817.

Sahi, C., Kominek, J., Ziegelhoffer, T., Yu, H.Y., Baranowski, M., Marszalek, J. and Craig, E.A., 2013. Sequential duplications of an ancient member of the DnaJ-family expanded the functional chaperone network in the eukaryotic cytosol. *Molecular Biology and Evolution, 30*(5), pp.985-998.

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989. *Molecular cloning: a laboratory manual* (No. Ed. 2). Cold spring harbor laboratory press.

Senderek, J., Krieger, M., Stendel, C., Bergmann, C., Moser, M., Breitbach-Faller, N., Rudnik-Schöneborn, S., Blaschek, A., Wolf, N.I., Harting, I. and North, K., 2005. Mutations in SIL1 cause Marinesco-Sjögren syndrome, a cerebellar ataxia with cataract and myopathy. *Nature Genetics, 37*(12), pp.1312-1314.

Shang, F. and Taylor, A., 1995. Oxidative stress and recovery from oxidative stress are associated with altered ubiquitin conjugating and proteolytic activities in bovine lens epithelial cells. *Biochemical Journal, 307*(1), pp.297-303.

Shi, Y., Mosser, D.D. and Morimoto, R.I., 1998. Molecularchaperones as HSF1-specific transcriptional repressors. *Genes and Development, 12*(5), pp.654-666.

Shieh, J.C., Wilkinson, M.G. and Millar, J.B., 1998. The Win1 mitotic regulator is a component of the fission yeast stress-activated Sty1 MAPK pathway. *Molecular Biology of the Cell, 9*(2), pp.311-322.

Shieh, J.C., Wilkinson, M.G., Buck, V., Morgan, B.A., Makino, K. and Millar, J.B., 1997. The Mcs4 response regulator coordinately controls the stress-activated Wak1-Wis1-Sty1 MAP kinase pathway and fission yeast cell cycle. *Genes and Development, 11*(8), pp.1008-1022.

Sies, H., 1997. Oxidative stress: oxidants and antioxidants. *Experimental Physiology, 82*(2), pp.291-295.

Sigler, K., Chaloupka, J., Brozmanova, J., Stadler, N. and Höfer, M., 1999. Oxidative stress in microorganisms—I. *Folia Microbiologica, 44*(6), pp.587-624.

Snaith, H.A. and Sawin, K.E., 2003. Fission yeast mod5p regulates polarized growth through anchoring of tea1p at cell tips. *Nature, 423*(6940), pp.647-651.

Snaith, H.A., Thompson, J., Yates, J.R. and Sawin, K.E., 2011. Characterization of Mug33 reveals complementary roles for actin cable-dependent transport and exocyst regulators in fission yeast exocytosis. *Journal of Cell Science, 124*(13), pp.2187-2199.

Solís, E.J., Pandey, J.P., Zheng, X., Jin, D.X., Gupta, P.B., Airoldi, E.M., Pincus, D. and Denic, V., 2016. Defining the essential function of yeast Hsf1 reveals a compact transcriptional program for maintaining eukaryotic proteostasis. *Molecular Cell*, *63*(1), pp.60-71.

Spellman, P.T., Sherlock, G., Zhang, M.Q., Iyer, V.R., Anders, K., Eisen, M.B., Brown, P.O., Botstein, D. and Futcher, B., 1998. Comprehensive identification of cell cycle–regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Molecular Biology of the Cell*, *9*(12), pp.3273-3297.

Steiner, W.W. and Smith, G.R., 2005. Optimizing the nucleotide sequence of a meiotic recombination hotspot in *Schizosaccharomyces pombe*. *Genetics*, *169*(4), pp.1973-1983.

Summers, D.W., Wolfe, K.J., Ren, H.Y. and Cyr, D.M., 2013. The Type II Hsp40 Sis1 cooperates with Hsp70 and the E3 ligase Ubr1 to promote degradation of terminally misfolded cytosolic protein. *PloS one*, *8*(1), p.e52099.

Sun, L.L., Li, M., Suo, F., Liu, X.M., Shen, E.Z., Yang, B., Dong, M.Q., He, W.Z. and Du, L.L., 2013. Global analysis of fission yeast mating genes reveals new autophagy factors. *PLoS Genetics*, *9*(8), p.e1003715.

Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T. and Ohsumi, Y., 2001. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *The European Molecular Biology Organisation journal*, *20*(21), pp.5971-5981.

Takeda, T., Toda, T., Kominami, K.I., Kohnosu, A., Yanagida, M. and Jones, N., 1995. *Schizosaccharomyces pombe* atf1+ encodes a transcription factor required for sexual development and entry into stationary phase. *The European Molecular Biology Organisation journal*, *14*(24), pp.6193-6208.

Tapley, T.L., Cupp-Vickery, J.R. and Vickery, L.E., 2005. Sequence-dependent peptide binding orientation by the molecular chaperone DnaK. *Biochemistry*, *44*(37), pp.12307-12315.

Tapley, T.L., Cupp-Vickery, J.R. and Vickery, L.E., 2006. Structural determinants of HscA peptide-binding specificity. *Biochemistry*, *45*(26), pp.8058-8066.

Toda, T., Shimanuki, M. and Yanagida, M., 1991. Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases. *Genes and Development*, *5*(1), pp.60-73.

Toda, T., Shimanuki, M., Saka, Y., Yamano, H., Adachi, Y., Shirakawa, M., Kyogoku, Y. and Yanagida, M., 1992. Fission yeast pap1-dependent transcription is negatively regulated by an essential nuclear protein, crm1. *Molecular and Cellular Biology*, *12*(12), pp.5474-5484.

Toledano, M.B., Delaunay-Moisan, A., Outten, C.E. and Igbaria, A., 2013. Functions and cellular compartmentation of the thioredoxin and glutathione pathways in yeast. *Antioxidants and Redox Signaling, 18*(13), pp.1699-1711.

Toone, W.M. and Jones, N., 1998. Stress-activated signalling pathways in yeast. *Genes to Cells, 3*(8), pp.485-498.

Toone, W.M. and Jones, N., 1999. AP-1 transcription factors in yeast. *Current Opinion in Genetics and Development, 9*(1), pp.55-61.

Toone, W.M., Kuge, S., Samuels, M., Morgan, B.A., Toda, T. and Jones, N., 1998. Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (Exportin) and the stress-activated MAP kinase Sty1/Spc1. *Genes and Development, 12*(10), pp.1453-1463.

Towbin, H., Staehelin, T. and Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, 76*(9), pp.4350-4354.

Toyokuni, S., Okamoto, K., Yodoi, J. and Hiai, H., 1995. Persistent oxidative stress in cancer. *Federation of European Biochemical Societies letters, 358*(1), pp.1-3.

Trott, A., Shaner, L. and Morano, K.A., 2005. The molecular chaperone Sse1 and the growth control protein kinase Sch9 collaborate to regulate protein kinase A activity in *Saccharomyces cerevisiae*. *Genetics, 170*(3), pp.1009-1021.

Truman, A.W., Kristjansdottir, K., Wolfgeher, D., Hasin, N., Polier, S., Zhang, H., Perrett, S., Prodromou, C., Jones, G.W. and Kron, S.J., 2012. CDK-dependent Hsp70 Phosphorylation controls G1 cyclin abundance and cell-cycle progression. *Cell, 151*(6), pp.1308-1318.

Vachon, P., Mercier, A., Jbel, M. and Labbé, S., 2012. The monothiol glutaredoxin Grx4 exerts an iron-dependent inhibitory effect on Php4 function. *Eukaryotic cell, 11*(6), pp.806-819.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M. and Mazur, M., 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions, 160*(1), pp. 1-40.

Vasilaki, A.T. and McMillan, D.C., 2017. *Encyclopedia of Cancer*, pp. 2054-2055.

Veal, E.A., Day, A.M. and Morgan, B.A., 2007. Hydrogen peroxide sensing and signaling. *Molecular cell, 26*(1), pp.1-14.

Veal, E.A., Findlay, V.J., Day, A.M., Bozonet, S.M., Evans, J.M., Quinn, J. and Morgan, B.A., 2004. A 2-Cys peroxiredoxin regulates peroxide-induced oxidation and activation of a stress-activated MAP kinase. *Molecular cell, 15*(1), pp.129-139.

Veal, E.A., Ross, S.J., Malakasi, P., Peacock, E. and Morgan, B.A., 2003. Ybp1 is required for the hydrogen peroxide-induced oxidation of the Yap1 transcription factor. *Journal of Biological Chemistry, 278*(33), pp.30896-30904.

Veal, E.A., Tomalin, L.E., Morgan, B.A. and Day, A.M., 2014. The fission yeast *Schizosaccharomyces pombe* as a model to understand how peroxiredoxins influence cell responses to hydrogen peroxide. *Biochemical Society Transactions 42*(4), pp.909-916.

Veal, E.A., Toone, W.M., Jones, N. and Morgan, B.A., 2002. Distinct roles for glutathione S-transferases in the oxidative stress response in *Schizosaccharomyces pombe*. *Journal of Biological Chemistry, 277*(38), pp.35523-35531.

Vergés, E., Colomina, N., Garí, E., Gallego, C. and Aldea, M., 2007. Cyclin Cln3 is retained at the ER and released by the J chaperone Ydj1 in late G1 to trigger cell cycle entry. *Molecular cell, 26*(5), pp.649-662.

Vido, K., Spector, D., Lagniel, G., Lopez, S., Toledano, M.B. and Labarre, J., 2001. A proteome analysis of the cadmium response in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry, 276*(11), pp.8469-8474.

Vihervaara, A. and Sistonen, L., 2014. HSF1 at a glance. *Journal of Cell Science 127*, pp.261-266.

Vivancos, A.P., Castillo, E.A., Biteau, B., Nicot, C., Ayté, J., Toledano, M.B. and Hidalgo, E., 2005. A cysteine-sulfinic acid in peroxiredoxin regulates H_2O_2-sensing by the antioxidant Pap1 pathway. *Proceedings of the National Academy of Sciences of the United States of America, 102*(25), pp.8875-8880.

Vivancos, A.P., Castillo, E.A., Jones, N., Ayté, J. and Hidalgo, E., 2004. Activation of the redox sensor Pap1 by hydrogen peroxide requires modulation of the intracellular oxidant concentration. *Molecular Microbiology, 52*(5), pp.1427-1435.

Vjestica, A., Zhang, D., Liu, J. and Oliferenko, S., 2013. Hsp70-Hsp40 chaperone complex functions in controlling polarized growth by repressing Hsf1-driven heat stress-associated transcription. *PLoS Genetics, 9*(10), p.e1003886.

Voellmy, R., 2004. On mechanisms that control heat shock transcription factor activity in metazoan cells. *Cell Stress and Chaperones, 9*(2), pp.122-133.

Vujanac, M., Fenaroli, A. and Zimarino, V., 2005. Constitutive nuclear import and stress-regulated nucleocytoplasmic shuttling of mammalian heat-shock factor 1. *Traffic, 6*(3), pp.214-229.

Wang, X., Khaleque, M.A., Zhao, M.J., Zhong, R., Gaestel, M. and Calderwood, S.K., 2006. Phosphorylation of HSF1 by MAPK-activated protein kinase 2 on serine 121, inhibits transcriptional activity and promotes HSP90 binding. *Journal of Biological Chemistry, 281*(2), pp.782-791.

Wang, Y., Gibney, P.A., West, J.D. and Morano, K.A., 2012. The yeast Hsp70 Ssa1 is a sensor for activation of the heat shock response by thiol-reactive compounds. *Molecular Biology of the Cell, 23*(17), pp.3290-3298.

Watanabe, Y. and Yamamoto, M., 1996. *Schizosaccharomyces pombe* pcr1+ encodes a CREB/ATF protein involved in regulation of gene expression for sexual development. *Molecular and Cellular Biology, 16*(2), pp.704-711.

Wilhelmus, M.M.M., Otte-Höller, I., Wesseling, P., De Waal, R.M.W., Boelens, W.C. and Verbeek, M.M., 2006. Specific association of small heat shock proteins with the pathological hallmarks of Alzheimer's disease brains. *Neuropathology and Applied Neurobiology, 32*(2), pp.119-130.

Wilkinson, M.G., Samuels, M., Takeda, T., Toone, W.M., Shieh, J.C., Toda, T., Millar, J.B. and Jones, N., 1996. The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast. *Genes and Development, 10*(18), pp.2289-2301.

Wu, A.W.J.A., Wemmie, J.A., Edgington, N.P., Goebl, M., Guevara, J.L. and Moye-Rowley, W.S., 1993. Yeast bZip proteins mediate pleiotropic drug and metal resistance. *Journal of Biological Chemistry, 268*(25), pp.18850-18858.

Yan, L.J., Levine, R.L. and Sohal, R.S., 1997. Oxidative damage during aging targets mitochondrial aconitase. *Proceedings of the National Academy of Sciences, 94*(21), pp.11168-11172.

Zheng, X., Krakowiak, J., Patel, N., Beyzavi, A., Ezike, J., Khalil, A.S. and Pincus, D., 2016. Dynamic control of Hsf1 during heat shock by a chaperone switch and phosphorylation. *Elife, 5*, p.e18638.

Zhou, B.B.S. and Elledge, S.J., 2000. The DNA damage response: putting checkpoints in perspective. *Nature, 408*(6811), pp.433-439.

Zhu, X., Zhao, X., Burkholder, W.F., Gragerov, A., Ogata, C.M., Gottesman, M.E. and Hendrickson, W.A., 1996. Structural analysis of substrate binding by the molecular chaperone DnaK. *Science(Washington), 272*(5268), pp.1606-1614.

Zou, J., Guo, Y., Guettouche, T., Smith, D.F. and Voellmy, R., 1998. Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell, 94*(4), pp.471-480.