Distinct roles for glutathione S-transferases in the oxidative stress response in *Schizosaccharomyces pombe*

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Running title: GSTs are involved in oxidative stress responses in *S. pombe*
SUMMARY

We have identified three genes, \( \text{gst1}^+ \), \( \text{gst2}^+ \) and \( \text{gst3}^+ \), encoding theta class glutathione S-transferases (GSTs) in *Schizosaccharomyces pombe*. The \( \text{gst1}^+ \) and \( \text{gst2}^+ \) genes encode closely related proteins (79% identical). Our analysis suggests that Gst1, Gst2 and Gst3 all have GST activity with the substrate 1-chloro-2,4-dinitrobenzene (CDNB) and that Gst3 has glutathione peroxidase activity. Although Gst1 and Gst2 have no detectable peroxidase activity, all three \( \text{gst} \) genes are required for the normal cellular resistance to peroxides. In contrast, each mutant is more resistant to diamide than wild-type cells. The \( \text{gst1}\Delta, \text{gst2}\Delta \) and \( \text{gst3}\Delta \) mutants are also more sensitive to fluconazole, suggesting that GSTs may be involved in anti-fungal drug detoxification.

Both \( \text{gst2}^+ \) and \( \text{gst3}^+ \) mRNA levels increase in stationary phase and all three \( \text{gst} \) genes are induced by hydrogen peroxide. Indeed, \( \text{gst1}^+ \), \( \text{gst2}^+ \) and \( \text{gst3}^+ \) are regulated by the Stress-activated protein kinase (SAPK) Sty1.

The Gst1 and Gst2 proteins are distributed throughout the cell and can form homodimers and Gst1-Gst2 heterodimers. In contrast, Gst3 is excluded from the nucleus and forms homodimers, but not complexes with either Gst1 or Gst2. Collectively, our data suggest that GSTs have separate and overlapping roles in oxidative stress and drug responses in fission yeast.
INTRODUCTION

All organisms have evolved protective mechanisms and programmed responses to limit cellular damage from exposure to toxic compounds in their environment. Glutathione S-transferases (GSTs) are evolutionarily conserved enzymes that are important in the detoxification of many xenobiotic compounds. These enzymes catalyse the conjugation of glutathione to electrophilic substrates producing compounds that are generally less reactive and more soluble. This facilitates their removal from the cell via membrane-based glutathione conjugate pumps. The broad substrate specificity of GSTs allows them to protect cells against a range of toxic chemicals (1). However, GST activity can sometimes be deleterious to the cell. For example, dihaloalkanes are bioactivated by conjugation with glutathione, generating more genotoxic metabolites (2).

In mammalian cells, amino acid sequence analysis has identified several subgroups of soluble GSTs, which appear to have evolved from the theta (θ) prototype found in vertebrates, insects, plants and bacteria (1,3). Mammalian cells also contain a distinct microsomal GST family of enzymes but these ER membrane associated enzymes have evolved separately from the soluble GSTs (4).

The prevalence of GSTs, together with the evidence suggesting their importance in bioactivation and detoxification of cytotoxic and genotoxic compounds, have stimulated many investigations into the potential role of these enzymes in disease. For example, epidemiological studies have established the absence of certain GST isozymes as an indicator of susceptibility to specific cancers (5) and GSTπ null mice are more susceptible to carcinogen-induced tumourigenesis (6). There is also much interest in the regulation of cellular GST activity as increased expression of GSTs in tumour cells is frequently associated with multidrug resistance (1).

As a consequence of aerobic growth, organisms are exposed to damaging reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals and hydrogen peroxide. GSTs have long been suspected to be important in protecting cells from oxidative stress by detoxifying some of the secondary ROS produced when ROS react with cellular constituents. For example, GSTs are able to conjugate GSH to the toxic reactive compounds 4-hydroxynonenal and cholesterol α-oxide which are generated during the oxidation of membranes (7,8). The glutathione peroxidase
activity of some GST proteins also suggests that they may be important in the
detoxification of organic hydroperoxides \((9,10,11)\). However, although several
studies have correlated high GST levels with increased resistance to oxidative stress,
there is little direct evidence establishing that GSTs are important for the protection of
cells from oxidative stress \((12)\).

Cells respond to oxidative stress by inducing the expression of genes whose
products protect the cell, for example, by repairing stress-related damage or by
inactivating ROS. In the fission yeast \textit{Schizosaccharomyces pombe} the regulation of
oxidative stress responsive genes is mediated by the Sty1 stress-activated protein
kinase through its downstream target, the Atf1 transcription factor, and by the redox-
sensitive Pap1 transcription factor \((13)\). Interestingly, recent work has established that
GSTs may have a wider role in the response to cellular stress, beyond their enzymatic
activity. In particular, GSTs have been shown to act as stress-sensitive inhibitors of
the mammalian stress-activated protein kinase JNK which help maintain JNK in an
inactive form in unstressed cells \((14)\).

In this report we have identified three GSTs, Gst1, Gst2 and Gst3, in \textit{S. pombe}. We show that each of these proteins has associated GST activity. We also
demonstrate that the expression of the \textit{gst1}+, \textit{gst2}+ and \textit{gst3}+ genes is induced by
hydrogen peroxide \((\text{H}_2\text{O}_2)\), whilst the expression of \textit{gst2}+ and \textit{gst3}+ is strongly
induced in stationary phase. All three GSTs are important for cellular resistance to
oxidative stress, however, they have different roles in response to different types of
oxidative stress.
EXPERIMENTAL PROCEDURES

Strains, media and growth conditions

The *S. pombe* strains used in this study are shown in Table 1. Yeast were grown at 30°C in rich medium (YE5S) or in synthetic minimal medium (EMM2) as described previously (15,16). Experiments were carried out using exponentially growing (mid-log) cells (OD<sub>600</sub>=0.2-0.5) unless otherwise stated. Cells were grown for 3 days after reaching mid-log to achieve stationary phase.

Deletion of the *gst* genes

The deletion of the *gst1<sup>+</sup>* and *gst2<sup>+</sup>* genes was achieved by replacing the wild-type copy of each gene with the *ura4<sup>+</sup>* gene in CHP429 and CHP428 cells. The *gst1<sup>Δ</sup>gst2Δ* mutant was obtained by mating *gst1Δ* and *gst2Δ* strains.

The *gst3Δ* mutant was obtained by sporulation of a CHP428/429 diploid in which one copy of the *gst3<sup>+</sup>* gene was replaced with *ura4<sup>+</sup>*.

Plasmids

Each *gst1<sup>+</sup>*, *gst2<sup>+</sup>* and *gst3<sup>+</sup>* open reading frame was amplified from genomic DNA by PCR using an N-terminal-specific oligonucleotide primer containing an *NdeI* restriction site in frame with the ATG start codon and a C-terminal-specific oligonucleotide primer containing a *BamHI* restriction site. PCR products were digested with *NdeI* and *BamHI* and ligated into the *NdeI* and *BamHI* sites of pRep1 (17), pRep41Flag (gift of Simon Whitehall) and pRep42HMN (18).

Cell sensitivity assays

Mid-log cells (OD<sub>600nm</sub> 0.2-0.5) were diluted 10, 100 and 1000 fold. 5μl of each dilution and undiluted cells was spotted on to YE5S plates containing different concentrations of oxidising agent. Plates were incubated at 30°C for 2-3 days until sufficient growth had taken place to allow discrimination of any changes in sensitivity.

Preparation of cell lysates
Mid-log cells were harvested and flash frozen. Thawed cell pellets were washed then resuspended in 200µl protein lysis buffer (50mM Tris.Cl pH7.5, 150mM NaCl, 0.5g/100ml Nonidet P40 with 2µg/ml pepstatin A, 2µg/ml leupeptin, 100mg/ml phenylmethylsulphonyl fluoride, 1ml/100ml aprotinin). Cells were lysed by vortexing with 2.5ml glass beads for 45s, cooling on ice, then vortexing for a further 45s. Proteins were washed from the beads with a further 500µl of protein lysis buffer. Cell extracts were clarified by centrifugation at 12000g for 30min. Protein concentrations were estimated using Coomassie Protein reagent (Pierce) and equivalent amounts of protein were used in all experiments.

**GST assays**

GST activity was measured using assays based on the methods of Habig et al. (1974) (19). 25-50µl of freshly prepared cell lysate was mixed in a cuvette with 1ml of GST assay buffer containing either 1mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1mM GSH or 1mM 4-nitrobenzylchloride (4NBC) and 5mM GSH in 0.11M sodium phosphate pH6.5 and incubated at room temperature. The absorbance (CDNB; 340nm and 4NBC;310nm) was measured at timed intervals and the specific GST activity of each lysate was determined from the increase in absorbance, the time between measurements (min) and the amount of protein (µg).

**Glutathione peroxidase assay**

Glutathione peroxidase activity was measured using a modification of the methods of Lawrence and Burk (1976) (20) and Avery et al. (2001) (21). 50µl of freshly prepared cell lysate was mixed in a cuvette with 1ml of glutathione peroxidase assay buffer (50mM potassium phosphate buffer pH7.0, 1mM EDTA, 1mM NaN3, 0.2mM NADPH, 1E.U./ml glutathione reductase (Sigma) and 1mM GSH). After 5 min equilibration the reaction was started by the addition of 20µl of 69mM cumene hydroperoxide. The oxidation of NADPH was monitored by recording the absorbance at 340nm at timed intervals after starting the reaction. The specific glutathione peroxidase activity of each lysate was determined from the decrease in NADPH divided by the time between measurements (min) and the amount of protein (µg). Controls omitting protein were used to correct for the non-enzymatic degradation of NADPH.
RNA analysis

Cells from mid-log and stationary phase cultures in YE5S media were treated as indicated then 25ml harvested at each time point. RNA was prepared by a hot phenol method essentially as described by White et al. (1987) (22). 5µg of total RNA was denatured with glyoxal, separated by agarose gel electrophoresis and transferred to a GeneScreen hybridization membrane (DuPont NEN Research Products). Gene-specific probes were prepared from PCR-generated fragments by labelling with $^{32}$P using a DNA Megaprime labelling kit (Amersham). Hybridization conditions were as described in the GeneScreen protocol. A probe for hmg1$^+$ was used as a loading control.

Immunoprecipitation and western blotting

Flag-tagged proteins were partially purified from cell lysates using anti-Flag (M2) antibody-conjugated agarose (Sigma). Immunoprecipitated proteins were separated by SDS PAGE then transferred to Protran™ nitrocellulose membrane (Schleicher and Schuell). Non-specific interactions were blocked with 10g/100ml milk in TTBS (20mM Tris.Cl pH7.6, 0.1ml/100ml Tween-20) for 30 min at room temperature. The blocked membrane was incubated with monoclonal anti-Myc (9E10) or anti-Flag (M2) mouse antibodies (Sigma), diluted 1/1000 with TTBS containing 5g/100ml bovine serum albumin (BSA), for 2h at room temperature. The membrane was washed with TTBS before incubation with peroxidase-conjugated polyclonal anti-mouse IgG antibodies (Sigma), diluted 1/2000 with TTBS containing 5g/100ml BSA, for 1h at room temperature. Finally, the membrane was washed with TTBS, then incubated with ECL™ (Amersham) chemiluminescent substrates and exposed to X-ray film (Fuji) for an appropriate length of time.

Indirect immunofluorescence

Fixation and immunofluorescence were carried out using a modification of the methods of Hagan and Ayscough (2000) (23). Mid-log and stationary phase cells were fixed by agitation with 3.7% paraformaldehyde in PEM (100mM PIPES pH7.0, 1.0mM EGTA and 1.0mM MgSO$_4$) for 30 min at 30°C. Fixed cells were washed then resuspended in PEM containing 1.2M sorbitol and 20mg/ml zymolyase and incubated
at 37°C for 70 min to spheroplast. Spheroplasted cells were resuspended in 1.2M sorbitol, 1% Triton X-100 in PEM for 2 min, washed with PEM then blocked with PEMBAL (PEM containing 100mM lysine, 1% BSA and 0.1% sodium azide) for 30 min with rotation at room temperature. The cells were resuspended in monoclonal anti-Myc (9E10) antibodies (Sigma), diluted 1/1000 with PEMBAL and agitated overnight at room temperature. Cells were washed with PEMBAL then incubated for 1h with polyclonal FITC-conjugated anti-mouse IgG antibodies (Sigma) diluted 1/50 in PEMBAL. The immunostained cells were washed with PEM then dried onto a glass microscope slide. The cells were fixed to the slide by immersion in -20°C methanol for 6 min followed by immersion in -20°C acetone for 30s then mounted in Vectashield™ containing 1.5µg/ml DAPI (4′,6-diamidino-2-phenylindole) (Vector labs).

DAPI-stained nuclei and anti-Myc immunofluorescence were visualised by excitation at 365nm (DAPI) and 450-490nm (FITC) under a 63x oil immersion lens using a Zeiss Axioscope fluorescence microscope and digital imaging system (Axiovision).
RESULTS

S. pombe contains three genes encoding potential glutathione S-transferases

Sequence analysis of the recently completed S. pombe genome (24) revealed three open reading frames that encoded proteins with significant homology to human glutathione S-transferase theta (hGSTT1). We have named these genes \(gst1^+\) (SPCC191.09c), \(gst2^+\) (SPCC965.07c) and \(gst3^+\) (SPAC688.04c) (Fig. 1). Notably, \(gst1^+\) and \(gst2^+\) encode very closely related proteins (79% identical).

Comparison of the sequences of these three putative GSTs from S. pombe with the consensus sequence derived for the theta class of GST enzymes ('GSTθ consensus' in Fig.1 and(25)) revealed conservation at 31 and 32 of the 34 amino acid positions in Gst1 and Gst2, respectively. The GSTθ consensus sequence contains 6 amino acids that are only conserved within the theta class of GSTs (shown in red in Fig.1). These 6 amino acids include the serine that is involved in glutathione conjugation in Lucilia Cuprina GST theta (26,27) and probably in other GSTs. The spacing between this serine and the rest of the GST theta consensus sequence is variable (25). Indeed, although Gst1 and Gst2 contain all 6 of the residues that are specific for the theta family of GSTs, the conserved serine and the first 3 hydrophobic residues of the GSTθ consensus sequence are 4 amino acids further from the rest of the 'GSTθ consensus than they are in hGSTT1 (Fig. 1). Gst3 matched the GST consensus at 28 of the 34 amino acid positions and contained 3 of the 6 amino acid residues that are only conserved between theta class GSTs. However, Gst3 has a serine residue at position 46, instead of the asparagine present at this position in all other GST theta enzymes and the residue in Gst3 that is most likely to be involved in glutathione conjugation is unclear.

Overexpression of Gst1, Gst2 or Gst3 increases the GST activity of cell lysate

The similarity of Gst1, Gst2 and Gst3 to GSTs from other organisms suggests that they have GST activity. However, studies of Ure2 from Saccharomyces cerevisiae, which also has extensive homology with other GSTs (28) and Gst1 and Gst2 from S. pombe, have been unable to detect any GST activity [(29) and data not shown]. Hence, to examine whether Gst1, Gst2 or Gst3 had GST activity \(gst1^+\), \(gst2^+\) and \(gst3^+\) were ligated into the pRep1 plasmid to allow high levels of expression in S.
GST activity assays were then performed using lysates prepared from wild-type cells containing these plasmids. Overexpression of Gst1, Gst2 or Gst3 significantly increased the GST activity of cell lysate when assayed with the model GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) (Fig. 2A). However, the same cell lysates did not have increased cellular GST activity towards the substrate 4-nitrobenzyl chloride (4NBC) (Fig. 2A).

Certain theta class GST enzymes, including human GSTT1, have been shown to have glutathione peroxidase activity (26). Hence, we also investigated whether overexpression of Gst1, Gst2 or Gst3 increased the glutathione peroxidase activity of cell lysate. Interestingly, only overexpression of Gst3 caused any substantial increase in the peroxidase activity of cell lysate against cumene hydroperoxide (Fig. 2A). Controls omitting glutathione revealed that this peroxidase activity was specific to glutathione (data not shown).

Taken together, these data suggest that Gst1, Gst2 and Gst3 all have GST activity and that Gst3 has glutathione peroxidase activity.

The contributions of Gst1, Gst2 and Gst3 to cellular GST activity

To assess the contributions of Gst1, Gst2 and Gst3 to cellular GST activity, the GST encoding genes were individually deleted, to give gst1Δ, gst2Δ and gst3Δ strains. The fact that Gst1 and Gst2 are 79% identical suggested that they might have overlapping functions so a gst1Δgst2Δ double mutant was also generated. The GST activities of cell lysates prepared from the gst1Δ, gst2Δ, gst3Δ and gst1Δgst2Δ mutant strains were analysed and compared with the GST activity of wild-type lysate. The lysates prepared from exponentially growing cells were found to have very low GST activity, preventing accurate comparisons, therefore no significant difference between the GST activity of any of the mutant strains and the wild-type could be detected (data not shown). However, consistent with the increased GST expression reported in stationary phase S. cerevisiae cells (29), the GST activity of lysate prepared from stationary phase wild-type S. pombe was 2-3 fold higher than that of lysates from mid-log cells (data not shown). However, the GST activities of stationary phase gst2Δ and gst1Δgst2Δ derived lysates were reproducibly lower than that of the wild-type (Fig. 2B). Preliminary analysis suggests that this is because there is no increase in GST activity in gst2Δ and gst1Δgst2Δ cells as they enter stationary phase.
The GST activity of stationary phase \(gst3\Delta\) cell lysate was slightly reduced compared with the wild-type, whereas the GST activities of \(gst1\Delta\) cell lysates were consistently slightly higher than those of wild-type cell lysates. These data demonstrate that the increase in cellular GST activity in stationary phase is predominately dependent on Gst2.

\textit{gst2}^+ and \textit{gst3}^+ mRNA are expressed at higher levels in stationary phase

The observed increase in cellular GST activity in stationary phase wild-type cells prompted us to investigate whether this reflected changes in the expression of the \textit{gst} genes. Hence, RNA was extracted from exponentially growing (mid-log) and stationary phase cells and analysed by northern blotting. This analysis revealed that \textit{gst2}^+ and \textit{gst3}^+ mRNA are both at much higher levels in stationary phase cells (Fig. 3) whereas \textit{gst1}^+ mRNA levels, though difficult to detect, are relatively unchanged (Fig. 3 and data not shown). Together with the analysis of GST activity of stationary phase wild-type and \textit{gst} mutant cells, these data suggest that the increased GST activity in stationary phase cells is due to increased expression of \textit{gst2}^+ and \textit{gst3}^+.

To determine whether the expression of individual \textit{gst} genes was affected by mutation of other \textit{gst} genes, \textit{gst} mRNA levels were examined in wild-type and \textit{gst} mutant cells. This revealed that the changes in GST activity observed in \textit{gst1}\Delta, \textit{gst2}\Delta, \textit{gst1}\Delta\textit{gst2}\Delta and \textit{gst3}\Delta cell lysates (Fig. 2B) are unlikely to be due to indirect effects on the expression of the remaining \textit{gst} genes.

The GST proteins form homodimers and Gst1 and Gst2 proteins form heterodimers \textit{in vivo}

Eukaryotic cytosolic GST enzymes are usually found as homodimers although heterodimers between different GST enzymes have also been reported. Hence we examined whether each of the GST proteins could form homodimers and/or heterodimers with the other GSTs.

Potential \textit{in vivo} protein-protein interactions between the GST proteins were examined by immunoprecipitation experiments. Anti-Flag antibody-conjugated agarose was used to immunopurify Flag-tagged Gst1, Gst2 or Gst3 from wild-type cells co-expressing different combinations of Flag and Myc epitope-tagged GST proteins. These immunoprecipitates were analysed by western blotting with anti-Myc
antibodies to determine whether the co-expressed Myc-tagged GST was present in the immunocomplex with the Flag-tagged GST. These analyses revealed that Gst1 and Gst2 are able to form homodimers and also Gst1-Gst2 heterodimers. However, although Gst3 was able to form homodimers, Gst3 did not form heterodimers with either Gst1 or Gst2 (Fig. 4). The epitope-tagged GST proteins had mobilities consistent with those predicted from their primary amino acid sequences and their expression also increased the GST activities of cell lysates indicating that neither epitope tag affects the function of the protein (data not shown). The identity of the proteins producing the additional minor bands is unknown. It is possible that they are derived from immunoglobulins from the anti-Flag agarose or breakdown products of the Gst proteins.

**Cellular localisation of Gst1, Gst2 and Gst3**

Previous studies have shown that GSTs localise to different parts of the cell. For example, Gtt1 in *S. cerevisiae*, is found associated with the endoplasmic reticulum (29), the mammalian kappa family of soluble GSTs are located in mitochondria (30) and murine GST theta accumulates in the nucleus under certain stress conditions (12). The data described above suggests that Gst1 and Gst2 may act together in the cell, whilst Gst3 has a separate role. Hence, to test this hypothesis further we examined the cellular distribution of each GST protein.

In order to determine the cellular localisation of Gst1, Gst2 and Gst3, wild-type cells containing pRep42-based plasmids expressing each GST fused to an N-terminal Myc epitope, were analysed by indirect immunofluorescence microscopy using anti-Myc antibodies. This analysis revealed that Gst1 and Gst2 were found throughout the cell while, in contrast, Myc-tagged Gst3 was excluded from the nucleus (Fig. 5 and data not shown). Similar localisation patterns were observed in both exponentially growing and stationary phase cells, which suggests that it is unlikely that the increased GST activity in wild-type stationary phase cells reflects a change in the cellular distribution of any of these three proteins. The similar distributions of Gst1 and Gst2 are consistent with them being able to form heterodimers (Fig. 4). These data also support the hypothesis that Gst3 may have a separate role from Gst1 and Gst2.
The expression of \( \text{gst}1^+ \), \( \text{gst}2^+ \) and \( \text{gst}3^+ \) mRNA is induced by \( \text{H}_2\text{O}_2 \)

The expression of some mammalian GSTs increases in response to chemical and oxidative stress (12) and indeed it was also recently reported that the \( S. \text{pombe} \) \( \text{gst}2^+ \) promoter is induced by oxidative stress (31). We have found that different levels of oxidative stress elicit different transcriptional responses in \( S. \text{pombe} \) (32). Therefore, to investigate whether the expression of \( \text{gst}1^+ \), \( \text{gst}2^+ \) and \( \text{gst}3^+ \) was induced in response to different levels of oxidative stress, RNA was extracted from untreated cells and cells treated with 0.1mM or 5.0mM \( \text{H}_2\text{O}_2 \) and the levels of each \( \text{gst} \) mRNA determined by northern blotting. This analysis revealed that the expression of all three \( \text{gst} \) genes was induced by both concentrations of \( \text{H}_2\text{O}_2 \). However, interestingly, \( \text{gst}1^+ \) and \( \text{gst}2^+ \) expression was induced more quickly at the lower concentration of \( \text{H}_2\text{O}_2 \), whereas \( \text{gst}3^+ \) expression was induced more strongly at the higher concentration (Fig. 6).

In \( S. \text{pombe} \) the stress-induced expression of many genes requires the stress-activated MAP kinase \( \text{Sty}1 \). Hence the expression of \( \text{gst}1^+ \), \( \text{gst}2^+ \) and \( \text{gst}3^+ \) was also examined in a \( \text{sty}1\Delta \) mutant. Although the basal level of \( \text{gst}1^+ \) and \( \text{gst}2^+ \) mRNA was greatly increased in the \( \text{sty}1\Delta \) mutant there was no induction of \( \text{gst}1^+ \), \( \text{gst}2^+ \) or \( \text{gst}3^+ \) expression in response to either low or high levels of \( \text{H}_2\text{O}_2 \) (Fig. 6).

The Pap1 and Atf1 transcription factors are also important for the expression of many stress-induced genes in \( S. \text{pombe} \). For example, some stress-induced genes are regulated by both Pap1 and Atf1 whereas the stress-induced expression of others is dependent on only one of these transcription factors (33,34). Hence, we examined the levels of \( \text{gst}1^+ \) \( \text{gst}2^+ \) and \( \text{gst}3^+ \) mRNA in \( \text{pap}1\Delta \) and \( \text{atf}1\Delta \) mutants, before and after treatment with \( \text{H}_2\text{O}_2 \). The levels of \( \text{gst}1^+ \) and \( \text{gst}2^+ \) mRNA levels were undetectable in the \( \text{pap}1\Delta \) mutant showing that both the basal and the induced expression of these genes is totally dependent on Pap1 (Fig. 6). In contrast, basal and induced \( \text{gst}3^+ \) expression were unaffected by deletion of the \( \text{pap}1^+ \) gene, but there was no \( \text{H}_2\text{O}_2 \)-induced increase in \( \text{gst}3^+ \) mRNA expression in the \( \text{atf}1\Delta \) mutant. These data indicate that the expression of \( \text{gst}1^+ \) and \( \text{gst}2^+ \) mRNA is Pap1-dependent whilst the induction of \( \text{gst}3^+ \) expression is Atf1-dependent (Fig. 6). Interestingly, \( \text{Sty}1 \), \( \text{Atf}1 \) and Pap1 had similar roles in the stationary phase-dependent expression of \( \text{gst}2^+ \) and \( \text{gst}3^+ \) mRNA. (manuscript in preparation).
Gst1, Gst2 and Gst3 protect the cell against oxidative stress

Some GSTs are able to utilise secondary ROS and organic peroxides as substrates suggesting that GSTs may be involved in protecting the cell against damage due to oxidative stress (12). Furthermore, we have shown that Gst3 has peroxidase activity and that the expression of $gst1^+$ $gst2^+$ and $gst3^+$ is induced in response to peroxide stress. Hence, we examined whether the $gst$ mutant strains were more sensitive to oxidative stress than wild-type cells. Sensitivity tests were performed using tetrabutylhydroperoxide (tBOOH), an organic source of peroxide, which is more stable than H$_2$O$_2$ and diamide, a thiol-oxidising agent that causes oxidative stress by multiple mechanisms including the depletion of cellular levels of reduced glutathione.

Consistent with Gst3 being the only GST with significant peroxidase activity, the $gst3\Delta$ single mutant was the most sensitive of the single mutant strains to tBOOH, with approximately 100 fold less growth than the wild-type (Fig. 7A). However, the $gst2\Delta$ mutant also displayed some increased sensitivity. The sequence similarity of Gst1 and Gst2 and the similar induction of their gene expression in response to H$_2$O$_2$, led us to examine whether Gst1 and Gst2 have overlapping roles in oxidative stress resistance. A $gst1\Delta gst2\Delta$ double mutant was much more sensitive than the $gst1\Delta$ and $gst2\Delta$ single mutants suggesting that Gst1 and Gst2 do in fact have overlapping roles in the oxidative stress response (Fig.7A). Similar results were obtained in H$_2$O$_2$ sensitivity tests (Fig. 7B) suggesting that Gst1, Gst2 and Gst3 are important in the general response to peroxide-induced damage.

Intriguingly, the $gst1\Delta$, $gst2\Delta$ and $gst3\Delta$ single mutants were more resistant than wild-type cells to diamide. Furthermore, the $gst1\Delta gst2\Delta$ double mutant was more resistant than either of the $gst1\Delta$ and $gst2\Delta$ single mutants (Fig. 7A). The increased resistance of the mutant strains was not due to increased levels of glutathione within these cells as there were no significant differences between glutathione levels in mutant and wild-type strains (data not shown). These large changes in cellular resistance to tBOOH and diamide following mutation of the $gst$ genes were highly reproducible.

Taken together, these data reveal that these GSTs play different roles in oxidative stress responses in $S.~pombe$ with Gst1 and Gst2 having partially overlapping roles (Fig.7). Interestingly, they also show that altered GST levels have
different effects upon the resistance to oxidative stress depending upon the nature of the stress agent.

The \textit{gst} mutants show increased sensitivity to the antifungal drug fluconazole

Mammalian GSTs are involved in the metabolism of xenobiotic compounds and therapeutic agents. Moreover, the Pap1 transcription factor and the Sty1 SAPK have been shown to be involved in drug responses in fission yeast (33). Hence, the discovery of functional GSTs in \textit{S. pombe}, whose expression is regulated by Sty1, and Pap1, suggested that GST activity might play a part in the detoxification of drugs in yeast. To test this we examined the sensitivity of the \textit{gst1}Δ, \textit{gst2}Δ and \textit{gst3}Δ single mutants and the \textit{gst1}Δ\textit{gst2}Δ double mutant to the anti-fungal drug fluconazole and compared this to the sensitivity of \textit{pap1}Δ, \textit{atf1}Δ and \textit{sty1}Δ mutants. On plates containing 30µg/ml fluconazole, a concentration that does not significantly inhibit the growth of wild-type cells, or indeed the \textit{atf1}Δ or \textit{sty1}Δ mutants, the \textit{gst2}Δ mutant displayed hypersensitivity. In addition the \textit{gst3}Δ and \textit{gst1}Δ\textit{gst2}Δ mutants were reproducibly more sensitive than the \textit{gst2}Δ mutant (Fig. 8). These data suggest that GSTs do play an important role in the detoxification of fluconazole. None of the \textit{gst} mutants were as sensitive to fluconazole as the \textit{pap1}Δ mutant (Fig. 8), however the sensitivity of the \textit{gst2}Δ and \textit{gst1}Δ\textit{gst2}Δ mutant, suggests that a component of the fluconazole sensitivity of the \textit{pap1}Δ mutant may be due to loss of \textit{gst1}+ and \textit{gst2}+ gene expression. The sensitivity of the \textit{gst3}Δ mutant, despite the apparent normal resistance of \textit{atf1}Δ and \textit{sty1}Δ mutants, suggests that the basal levels of Gst3 are required and sufficient for normal cellular resistance.

\textbf{DISCUSSION}

We have identified three genes encoding glutathione S-transferases in \textit{S. pombe} that are involved in the oxidative stress response. We demonstrate that all three proteins have GST activity. Furthermore, we show that the expression of these \textit{gst} genes is induced by, and required for, the response to peroxide in \textit{S. pombe}. Although GSTs have long been suspected of protecting cells against secondary ROS, this is the first report unequivocally establishing that glutathione S-transferase genes are required to protect eukaryotic cells against peroxide-induced cell death.
Sequence analysis suggested that \( \text{gst}1^+ \), \( \text{gst}2^+ \) and \( \text{gst}3^+ \) encode GSTs. However, all three proteins, but especially Gst1 and Gst2, also share extensive sequence homology with the Ure2 protein from \( S. \text{cerevisiae} \) (although none possess the N-terminal prion-like domain present in Ure2) for which no GST activity has been detected [(29) and data not shown]. Our data showing that overexpression of Gst1, Gst2, or Gst3 increases the GST activity of cell lysate with the model substrate CDNB (Fig. 2A) strongly suggests that all three proteins have GST activity. Analysis of gene deletion mutants also revealed that Gst2 is required for the GST activity of stationary phase cells (Fig. 2B). Taken together these data suggest that Gst1, Gst2 and Gst3 are indeed functional GSTs.

The \( \text{gst} \) genes in \( S. \text{pombe} \) can be divided into two distinct groups based on sequence homology; \( \text{gst}1^+ \) and \( \text{gst}2^+ \) encode proteins that are 79% identical, whilst the protein encoded by \( \text{gst}3^+ \) is only 14% identical with Gst1 and Gst2. The identification of such a closely related gene pair suggests that the proteins have related functions. Indeed, we show by coimmunoprecipitation studies that, in addition to forming homodimers, Gst1 and Gst2 can form Gst1-Gst2 heterodimers. However, a lack of conservation at certain positions in Gst1 and Gst2 may reflect different roles for these proteins within the cell. For example, Gst2 forms an intermolecular disulphide bond via its only cysteine residue (Fig. 1 and data not shown) but Gst1 contains no cysteine residues. Indeed, our analysis of \( \text{gst}1^+ \) and \( \text{gst}2^+ \) mRNA levels and the GST activity and stress sensitivity of the \( \text{gst}1\Delta \) and \( \text{gst}2\Delta \) mutants all suggest that Gst2 is more abundant, particularly in stationary phase cells and seems to make a larger contribution to cellular GST activity and stress resistance. The third GST, Gst3, shows substantial sequence divergence from Gst1 and Gst2 and furthermore is the only 1 of these GSTs that has associated glutathione peroxidase activity. The observed peroxidase activity of Gst3 together with differences in cellular localisation, protein-protein interactions and in the transcription factor dependency of \( \text{gst}3^+ \) gene induction, suggests that Gst3 may have a distinct cellular role. However, all 3 GST proteins have activity with CDNB, are induced by oxidative stress and are required for peroxide and fluconazole resistance. Moreover, \( \text{gst}3^+ \) mRNA levels, like \( \text{gst}2^+ \) mRNA levels, are higher in stationary phase cells suggesting that there is also overlap between the roles of these GSTs.
We have revealed that Gst3 is excluded from the nucleus. In contrast, Gst1 and Gst2 are both distributed throughout the cell (Fig. 5). The cellular distribution of GST theta in rat liver has been reported to change in response to stress, although the functional significance of this observation is unclear (2). However, we have been unable to observe any change in the cellular distribution of Gst1, Gst2 or Gst3 in response to oxidative stress with H$_2$O$_2$ or nutrient limitation (Fig. 5 and data not shown). The similar cellular distributions of Gst1 and Gst2 also support the immunoprecipitation data showing that they are able to form heterodimers. However, it appears that the GST activity of Gst1 or Gst2 does not require the formation of Gst1-Gst2 heterodimers as overexpression of either Gst1 or Gst2 increases the GST activity of $gst1^{+}gst2^{+}$ mutant cells (data not shown).

The $gst1^{+}$, $gst2^{+}$, and $gst3^{+}$ genes are induced in response to H$_2$O$_2$ in a Sty1 (SAPK)- dependent manner. However, there are differences between the regulation of the expression of $gst1^{+}$, $gst2^{+}$ and of $gst3^{+}$. Basal and induced levels of $gst1^{+}$ and $gst2^{+}$ mRNA are totally dependent on the Pap1 transcription factor whereas both genes are still induced by low levels of H$_2$O$_2$ in an $atf1^{+}$ strain. On the other hand, $gst3^{+}$ gene expression is induced more strongly at higher levels of H$_2$O$_2$, and although this induction does not require Pap1, it is completely dependent on the Atf1 transcription factor. These data are consistent with a recent study describing different roles for Pap1 and Atf1 in regulating the cellular response to H$_2$O$_2$; Pap1 is more important than Atf1 for the activation of target genes in response to low levels of H$_2$O$_2$, while the transcriptional response to high concentrations of H$_2$O$_2$ is dependent on Atf1 (32). The requirement for different GST activities at different levels of stress may reflect the different cellular localisation of these GSTs (Fig. 5) or differences in their substrate specificity.

The $gst1^{+}$, $gst2^{+}$ and $gst3^{+}$ genes were found to be required for normal resistance to tBOOH and H$_2$O$_2$. The $gst1^{+}$ mutant showed similar resistance to wild-type cells (Fig. 7) consistent with it making a minor contribution to cellular GST activity (Fig. 2B). Although the $gst1^{+}gst2^{+}$ mutant had similar levels of GST activity to the $gst2^{+}$ single mutant, the $gst1^{+}gst2^{+}$ mutant was more sensitive to tBOOH and H$_2$O$_2$, suggesting that Gst1 may protect against oxidative stress by a mechanism that is independent of its GST activity. Indeed, this concept is not novel as GSTs have previously been implicated as direct inhibitors of the oxidative stress-regulated
SAPK(14). Alternatively, the induction of \( \text{gst}1^+ \) mRNA by \( \text{H}_2\text{O}_2 \) in wild-type cells, together with these sensitivity data, suggest that Gst1 may make a more important contribution to GST activity and cellular resistance following peroxide treatment.

Amino acid sequence comparisons between Gst1, Gst2 and Gst3 and the GSTs, Gtt1 and Gtt2, from the budding yeast \textit{Saccharomyces cerevisiae} revealed that Gst3 is the only \textit{S. pombe} protein with any significant homology to Gtt1 and that Gst1 and Gst2 are the only potential homologues of Gtt2. Analysis of \( \text{GTT1} \) and \( \text{GTT2} \) revealed that although, like \( \text{gst}2^+ \) and \( \text{gst}3^+ \), \( \text{GTT1} \) expression was increased in stationary phase, neither \( \text{GTT1} \) and \( \text{GTT2} \) was induced by, or required for, cellular resistance to oxidative stress (29). In contrast, we show here that the expression of \( \text{gst}1^+ \), \( \text{gst}2^+ \) and \( \text{gst}3^+ \) is induced by peroxide and moreover, that deletion of the \( \text{gst}1^+ \), \( \text{gst}2^+ \) and \( \text{gst}3^+ \) genes significantly reduces the cellular resistance to peroxide stress.

As the primary amino acid sequences of Gst1, Gst2 and Gst3 are much closer related than Gtt1 and Gtt2 to human GST theta, these studies in \textit{S. pombe} may be more likely to reflect the role of mammalian GSTs.

In apparent contradiction to the importance of Gst1, Gst2 and Gst3 in protecting the cell against peroxide-induced oxidative stress, the \( \text{gst} \) mutants were considerably more resistant to diamide than the wild-type cells. However unlike peroxide, which produces ROS that react with lipids, proteins and DNA to directly damage cellular components, diamide oxidises free thiols causing oxidative stress by direct protein oxidation and also by oxidation and depletion of the cellular antioxidant, glutathione. The diamide resistance of the \( \text{gst} \) deletion mutants is entirely consistent with a recent study demonstrating that GSTpi null mice are more resistant to chemical toxicity induced by high doses of acetaminophen, a drug whose toxicity is also related to the reduction in glutathione levels associated with it (35). The similarities between the diamide resistance of the \( \text{gst} \) mutants in \textit{S. pombe} and the acetaminophen studies in GSTpi null mice suggests that these \textit{S. pombe} \( \text{gst} \) mutants might be developed for use as a pre-screen for toxicity of chemicals/drugs. Furthermore, these studies lend credence to the use of \textit{S. pombe} as a model system for drug metabolism/resistance in mammals.

We also show that Gst1, Gst2 and Gst3 are important for normal cellular resistance to the anti-fungal drug fluconazole (Fig. 8). Fluconazole is an azole-based anti-mycotic agent which targets the production of the predominant component of the fungal cell membrane, ergosterol (36). Azole-based anti-fungal agents are clinically
important for the treatment of fungal infections including the life-threatening systemic infections that are frequently associated with treatments and diseases that debilitate the immune system such as AIDS. The studies presented here reveal a role for GSTs in the resistance of yeast fluconazole. This may be relevant to the treatment of anti-fungal resistant strains and also in the development of new anti-fungal drugs.

In summary, this paper demonstrates, for the first time, that GSTs are required for the cellular resistance to oxidative stress in a eukaryote and also suggests that they have important roles in the response of yeast to anti-mycotic drugs.

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Figure Legends

**Figure 1 Alignment of the predicted protein sequences of S. pombe Gst1, Gst2 and Gst3 with human GST theta (hGSTT1)** The primary amino acid sequences were aligned using CLUSTAL W (37). The sequence order was determined by the alignment programme and reflects the relationships between the sequences. Numbers indicate the position of the adjacent amino acid in the protein sequence. Amino acids which are identical in *S. pombe* Gst1 and Gst2 sequences are shown in bold type. Any of these amino acids that are also found in hGSTT1 and Gst3 are also indicated by bold type. The line of text labelled ‘GSTθ consensus’ shows the consensus residues in theta class GSTs (25) with red type indicating the residues that are only conserved in theta class GSTs and not in other classes of soluble GST. † represents N, D, E or Q, * represents hydrophobic residues and # represents polar amino acid residues.

**Figure 2 GST activity is associated with Gst1, Gst2 and Gst3 [A]** The GST activities, with 3 different substrates; CDNB, 4NBC and cumene hydroperoxide, of lysates prepared from mid-log cultures of wild-type (CHP429) cells containing pRep1
Specific activities shown were calculated as μmoles of product formed (or NAPDPH utilised for cumene hydroperoxide) per min per μg cellular protein. Error bars represent the standard deviation from the mean. [B] The specific GST activities of lysates prepared from wild-type (CHP429) and \(gst\) mutants grown to stationary phase were calculated and then expressed as percentages of the wild-type activity. The figure represents the average data from multiple experiments. Error bars represent the standard error.

Figure 3 \(gst2^+\) and \(gst3^+\) mRNA are expressed at higher levels in stationary phase

RNA extracted from mid-log and stationary phase wild-type (CHP429), \(gst1\Delta\), \(gst2\Delta\), \(gst3\Delta\) and \(gst1\Delta\&gst2\Delta\) cells was analysed by autoradiography of northern blots hybridised with gene-specific radiolabelled probes for \(gst1^+\), \(gst2^+\), \(gst3^+\) and \(hmg1^+\) (loading control) mRNA.

Figure 4 Gst1, Gst2 and Gst3 form homodimers and Gst1-Gst2 heterodimers

Flag-tagged proteins were immunopurified from cell lysates prepared from mid-log cultures of wild-type (CHP429) cells containing the Flag vector control (-) or plasmids expressing the indicated epitope-tagged proteins. Immunoprecipitated proteins were separated by SDS PAGE and analysed by western blotting with anti-Myc antibodies to determine coimmunoprecipitated proteins. The membranes were stripped and reprobed with anti-Flag antibodies to confirm that approximately equal amounts of Flag-tagged protein were present in the immunoprecipitates.

Figure 5 Gst1 and Gst2 proteins are found throughout the cell whereas Gst3 appears to be excluded from the nucleus.

The cellular distribution of 6xHisMyc-tagged GST proteins, expressed from a pRep42HMN plasmid in wild-type (CHP429) cells, was examined by indirect immunofluorescence with anti-Myc antibodies on fixed mid-log (exponentially growing) and stationary phase cells. The cell nuclei were visualised using DAPI. More than 100 cells of each sample were examined and representative images are shown.
Figure 6 The expression of \( \text{gst}1^+ \), \( \text{gst}2^+ \) and \( \text{gst}3^+ \) mRNA is regulated by \( \text{H}_2\text{O}_2 \). RNA extracted from wild-type (972), DMsty1, DMpap1 and DMatf1 cells treated with 0.1mM or 5.0mM \( \text{H}_2\text{O}_2 \) for 0, 15 or 60 min was analysed by autoradiography of northern blots hybridised with gene-specific radiolabelled probes for \( \text{gst}1^+ \), \( \text{gst}2^+ \), \( \text{gst}3^+ \) and \( \text{hmg}1^+ \) (loading control) mRNA.

Figure 7 The \( \text{gst} \Delta \) mutants are more sensitive to \( \text{tBOOH} \) and \( \text{H}_2\text{O}_2 \) but more resistant to diamide The ability of neat (-) and 10-fold serial dilutions of mid-log cultures of wild-type and \( \text{gst} \) mutant strains to grow on YE5S plates containing [A] \( \text{tBOOH} \), diamide or [B] \( \text{H}_2\text{O}_2 \) are shown. These sensitivity experiments were carried out 4 times and a representative experiment is shown.

Figure 8 Gst1, Gst2 and Gst3 have roles in fluconazole resistance The ability of neat(-) and 10-fold serial dilutions of mid-log cultures of wild-type, \( \text{gst}1\Delta \), \( \text{gst}2\Delta \), \( \text{gst}3\Delta \), \( \text{gst}1\Delta \text{gst}2\Delta \), \( \text{pap}1\Delta \), \( \text{atf}1\Delta \) and \( \text{sty}1\Delta \) mutant strains to grow on YE5S plates containing 30\( \mu \text{g} / \text{ml} \) fluconazole are shown. This sensitivity experiment was carried out twice and a representative experiment is shown.

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| Strain   | Genotype                                | Source          |
|----------|-----------------------------------------|-----------------|
| CHP428   | $h^+ leu1-32 ura4-D18 his7-366 ade6-210$ | lab stock       |
| CHP429   | $h^+ leu1-32 ura4-D18 his7-366 ade6-216$ | lab stock       |
| EV22     | $h^+ leu1-32 ura4-D18 his7-366 ade6-210$ | this study      |
| EV23     | $h^+ leu1-32 ura4-D18 his7-366 ade6-210$ | this study      |
| EV26     | $h^+ leu1-32 ura4-D18 his7-366 ade6-216$ | this study      |
| EV25     | $h^+ leu1-32 ura4-D18 his7-366 ade6-216$ | this study      |
| EV26     | $h^+ leu1-32 ura4-D18 his7-366 ade6-216$ | this study      |
| 972      | $h^+$                                    | Dongrong Chen Murchie |
| DMMatf1  | $h^+ afl1::ura4^+$                       | Dongrong Chen Murchie |
| DMPap1   | $h^+ pap1::ura4^+$                       | Dongrong Chen Murchie |
| DMSyl1   | $h^+ sty1::ura4^+$                       | Dongrong Chen Murchie |
**Figure 1**
Figure 2
Figure 3

- **gst1**
- **gst2**
- **gst3**
- **hmg1**

The figure shows protein expression levels in different strains under mid-log and stationary phases.
Figure 4
Figure 5

Exponential

| anti-my c (FITC) | Nuclei (DAPI) |
|------------------|---------------|
| **Gst1**         |               |
| **Gst2**         |               |
| **Gst3**         |               |

Stationary phase

| anti-my c (FITC) | Nuclei (DAPI) |
|------------------|---------------|
|                  |               |
|               | wild-type | sty1Δ   | pap1Δ   | atf1Δ   |
|---------------|-----------|---------|---------|---------|
| **H₂O₂ (mM)**| 0.1 5.0  | 0.1 5.0 | 0.1 5.0 | 0.1 5.0 |
| **time (min)**| 0 15 60  | 0 15 60 | 0 15 60 | 0 15 60 |

- **gst1**
- **gst2**
- **gst3**
- **hmg1**

**Figure 6**
Figure 7
Figure 8: The figure shows the growth of yeast cells under control conditions and in the presence of 30μg/ml fluconazole. The cells are tested at different dilutions: 10^-1, 10^-2, 10^-3. The strains tested include wild-type, gst1Δ, gst2Δ, gst3Δ, gst1Δgst2Δ, pap1Δ, atf1Δ, and sty1Δ.
Distinct roles for glutathione S-transferases in the oxidative stress response in Schizosaccharomyces pombe

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