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Role of glucose repression in the oxidative stress response of *Schizosaccharomyces pombe*: analysis of transcript levels of *fbp1, hxk2, sod1* and *ctt1* genes in *sty1, atf1* and *pap1* knock-out mutants

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Abstract: Previously we reported that the glucose sensing/signaling pathway affected the oxidative stress response of *Schizosaccharomyces pombe*, based on studies of glucose-repression and oxidative-stress-resistant mutants including *ird5*. *sty1*, encoding a protein kinase, and *aft1* and *pap1*, encoding transcription factors, are important components of the oxidative stress response in *S. pombe*. To analyze the relationship between the glucose sensing/signaling pathway and the oxidative stress response, we generated *sty1*, *aft1*, and *pap1* knock-out mutants in *ird5* and wild-type backgrounds. We evaluated the survival rates of the *ird5* double mutants (5A, 5P, and 5S) and wild-type single mutants (9A, 9P, and 9S) under mild oxidative stress. In addition, we analyzed the transcript levels of genes related to oxidative stress (*sod1*, encoding superoxide dismutase; *ctt1*, encoding catalase) and glucose metabolism (*fbp1*, encoding fructose-1, 6-bisphosphatase; *hxk2*, encoding hexokinase). All deletion mutants showed very low survival rates under oxidative stress (<7%). It was shown that transcript levels of *sod1* drastically increased in single mutants, while those of *ctt1* slightly increased in double mutants. Our results indicate that these mutants are highly sensitive to oxidative stress, and that *atf1*, *pap1*, and *sty1*, as well as *sod1* and *ctt1*, are very important in the oxidative stress response of *S. pombe*.

Key words: Oxidative stress response, glucose repression, Atf1, Pap1, Sty1, knock-out deletion, *Schizosacharomyces pombe*

1. Introduction

Glucose is the preferred carbon and energy source for most organisms. Glucose also plays a key role as a signaling molecule in the regulation of many biological processes. In the fission yeast *Schizosaccharomyces pombe*, extracellular glucose is detected by Git3 G-protein coupled receptor (GPCR), which generates an intracellular signal via the cAMP-dependent protein kinase A, Pka1. This signal is transmitted to downstream pathways such as hexose transport, glycolysis, the utilization of alternative carbon sources, the oxidative stress response, respiration, sporulation, and gluconeogenesis (Maeda et al., 1994; Fernandez et al., 1997; Carlson, 1999; Welton and Hoffman, 2000; Hoffman, 2005). Pka1 is very important in glucose signaling. For example, it regulates both sexual development and gluconeogenesis through the differential phosphorylation of the transcription factor Rst2, an important component in the glucose repression response (Higuchi et al., 2002).

In *S. pombe*, the stress response follows the activation of the mitogen activated protein kinase (MAPK) pathway, which occurs under various adverse environmental conditions. The MAPK pathway includes a set of three conserved protein kinases, the most important of which is the stress-activated kinase, Sty1. Sty1 plays a key role in the stress responses to high temperature, osmotic stress, oxidative stress, nutrient limitation, and heavy metal toxicity (Shiozaki and Russell, 1995; Degols et al., 1996; Shiozaki and Russell, 1996; Degols and Russell, 1997; Samejima et al., 1997). The transcription factors Atf1 (with or without Pcr1) and Pap1 are triggered by Sty1. Atf1 is responsible for cell division under osmotic stress, but not under oxidative or ultraviolet stress (Shiozaki and Russell 1996; Reiter et al., 2008). Pap1 is responsible for survival under oxidative stress, but not under osmotic stress or nutrient limitation (Kumada et al., 1996; Nguyen and Shiozaki, 2002). These transcription factors trigger the expression of stress response genes such as *apt1*, *ctt1*, *sod1*, *pmd1*, *trr1*, and *trx2* (Toone et al., 1998; Mutoh et al., 2002; Madrid et al., 2004). Moreover, the transcription of *fbp1*, which encodes the gluconeogenesis enzyme fructose-1, 6-bisphosphatase (Fbp1), is regulated via MAPK, Sty1, and cAMP (Neely and Hoffman, 2000). The activation of the Pka1-Rst2 pathway inhibits the expression of *fbp1*,

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whereas the activation of Sty1 stimulates its transcription (Higuchi et al., 2002).

In our previous studies we showed that the oxidative stress response was affected by the glucose sensing/signaling pathway in glucose-repression-resistant mutants (*ird5*, *ird11*, *ird13*, and *ird14*) of *S. pombe* (Kig et al., 2005; Palabiyik et al., 2012; Palabiyik et al., 2013). The *ird5* mutant is resistant to oxidative stress induced by H_2O_2. Although *ird5* shows the strongest glucose repression (close to that of the wild type) among these mutants, its elevated expression of *fbp1* (Palabiyik et al., 2013) might be due to the reduced efficiency of glucose consumption (Kig et al., 2005). Glucose depletion results in resistance to oxidative stress in *S. pombe* (Madrid et al., 2004; Roux et al., 2009). Therefore, to explore the relationship between glucose sensing/signaling and the oxidative stress response, we generated mutants of *sty1*, *atf1*, and *pap1*, all of which encode important components of the oxidative stress response in *ird5* and wild-type backgrounds. Deletion analysis is a useful and reliable way to study the functions of genes in *S. pombe*. We used a PCR-based method that enabled the straight forward direct deletion of target genes from the genomes of *S. pombe ird5* mutant and wild-type cells. We constructed three cassettes containing the selectable marker kanMX6 and deleted sequences of the *aft1*, *pap1*, and *sty1* genes. These cassettes were similar to the modular kanMX6 cassette system described by Bähler et al. (1998) and Hentges et al. (2005), except that they contained long sequences (200-600 bp) flanking the target genes. We created new *S. pombe* mutants with target gene deletions (*972atf1*$^-$, *972pap1*$^-$, *972sty1*$^-$, *ird5atf1*$^-$, *ird5pap1*$^-$, and *ird5sty1*$^-$), and then generated the *972atf pap*$^-$ double mutant and the *ird5at1*$^-$*pap*$^-$ triple mutant by genetic crossing. Subsequently, we conducted quantitative real-time PCR analyses to determine the transcript levels of *fbp1* and *hkx2*, which are involved in glucose repression, and *sod1* and *ctt1*, which are involved in the oxidative stress response pathway in the deletion mutants.

2. Materials and methods

2.1. Fission yeast strains and media

Table 1 shows the *S. pombe* strains used in the present study. The mutant cells of *S. pombe ird5* were cultured on selection medium [0.5% (w/v) yeast extract, 3% (w/v) sucrose, and 400 µg/mL 2-deoxy-D-glucose (2-DOG)], while the wild-type cells were cultured in standard rich medium (YE). Synthetic sporulation medium (SPA) was used to induce sporulation (Gutz et al., 1974; Moreno et al., 1991). Cells expressing the deleted-gene cassettes were selected on solid YE plates containing 100 µg/mL G418 (Roche Molecular Biochemicals, Mannheim, Germany).

2.2. Bacterial strain

The host strain used in this study was *Escherichia coli* DH5a. The pFA6a-kanMX6 plasmid harboring bacterial genes, conferring resistance to G418 (Roche), were added to a final concentration of 100 mg/L to 5 mL of LB medium (in w/v: 1% tryptone, 0.5% yeast extract, and 1% NaCl). The cells were grown overnight at 37 °C with shaking at 150 rpm.

2.3. Genomic DNA isolation from *S. pombe*

Genomic DNA was isolated from *S. pombe* cells as described by Bähler et al. (1998). Wild-type *S. pombe* cells were grown overnight to log phase in 5 mL of YE medium at 30 °C with

Table 1. List of strains used in this study.

Strain	Strain symbol	Genotype
972		*h*$^-$ Wild type
975		*h*$^+$ Wild type
ird5		*h*$^-$ Resistance of glucose repression (Kig et al., 2005)
972atf1$^-$	9A	*h*$^-$*atf1*::kanMX6
972pap1	9P	*h*$^-$*pap1*::kanMX6
972sty1$^-$	9S	*h*$^-$*sty1*::kanMX6
ird5atf1$^-$	5A	*h*$^-$*atf1*::kanMX6
ird5pap1$^-$	5P	*h*$^-$*pap1*::kanMX6
ird5sty1$^-$	5S	*h*$^-$*sty1*::kanMX6
972atf1$^-$*pap1*$^-$	9AP	*h*$^-$*atf1*::kanMX6*pap1*::kanMX6
ird5atf1$^-$*pap1*$^-$	5AP	*h*$^-$*atf1*::kanMX6*pap1*::kanMX6

shaking at 180 rpm, and then harvested by centrifugation at 4000 × *g*. The cell precipitate was suspended in 0.2 mL of lysis buffer [2% (v/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris, 1 mM EDTA; pH 8.0]. Next, 0.2 mL of phenol: chloroform: isoamyl alcohol (25: 24: 1) and 0.3 g of acid-washed glass beads were added, and the cells were disrupted using a homogenizer (Sartorius Instruments, Belmont, Surrey, UK) for 4 min (1 min homogenizing and 1 min on ice, twice). Lastly, 1 mL of ethanol was added to the mixture and the mixture was centrifuged at 20,000 × *g*. The pellet was dissolved in 50 mL of water and stored at –20 °C.

2.4. Construction of deletion cassettes

The deletion cassettes were constructed using two-rounds of PCR amplifications and two sets of primers for each gene. In the first round, the upstream and downstream sequences of each gene were amplified in two separate PCR reactions using ExTaq polymerase (Takara, Otsu, Japan). For these reactions, genomic DNA was used as the template, and gene-specific primers were used to amplify the upstream and downstream sequences. For example, for the *pap1* gene, the upstream primer pair was 5′-CGAAACGAACGCGTTTTCAGG-3′ (F), and ggggatccgtcgacTTCACAAAGCAATGCAAGATGAAGG-3′ (R), and the downstream primer pair was 5′-atgaatcggccaacgcgcggCCTTTTTGTTTTTCTTCGCCTCGTG-3′ (F), 5′- GCCTTTGGTCGACG AATATTAGCATC-3′ (R) (primer sequences for kanamycin resistance gene are shown in lower case). The amplified sequences were approximately 285–606 bp away from the target genes. The primer sequences for all target genes are shown in Table 2. The amounts of enzyme and buffer were as recommended by the suppliers and PCR reactions were performed in 0.5-mL tubes. Each PCR mixture (total volume, 25 µL) contained 2.5 µL of 10X buffer, 0.5 mM of each dNTP, 2 µM of each primer, and 1 µL (~ 100 ng) of genomic DNA from the wild type as the template. The thermal cycling conditions were as follows: 45 cycles at 95 °C for 45 s, 55 °C for 1 min, and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min.

For the second round of PCR, the products from first-round PCR (upstream and downstream sequences of each gene) and the pFA6a-kanMX6 plasmid were used as the template, and gene-specific sequences were used as primers (in the case of *pap1*; 5′-CGAAACGAACGCGTTTTCAGG-3′ (F), 5′ -GCCTTTGGTCGACGAATATTAGCATC- 3′ (R)). The deletion cassettes were amplified using the Long Template PCR System (Roche, Indianapolis, IN, USA) (Table 3). The amounts of enzyme and buffer were as recommended by the suppliers. The PCR reactions were performed in 0.5-mL tubes. Each PCR mixture (total volume, 25 µL) contained 5 µL of 5X buffer (containing 1.75 mM $MgCl_2$), 0.4 mM of each dNTP, 2 µM of each primer, and the template (first-round PCR products and pFA6a-kanMX6 plasmid) adjusted to a final concentration of 500 ng. The cycling conditions were as follows: 10 cycles at 94 °C for 10 s, 59 °C for 30 s, and 68 °C for 1 min; 25 cycles at 94 °C for 15 s, 59 °C for 30 s, and 68 °C for 1 min, and then a final extension at 68 °C for 7 min. In each cassette, the KanMX module product was joined together

Table 2. Primers for knock-out cassettes. Primer sequences for the kanamycin resistance gene are shown in bold lower case letters.

Primer	Sequence (5′-3′)
Atf**1**	ACGCACACGGGAAGAGAACA
Atf**2**-kan	**ttaacccggggatccgtcgac**TGACGTTAAAAATCACACACGC
kan-Atf**3**	**atgaatcggccaacgcgcgg**TTCCCTCCGTTGAGGGTTGT
Atf**4**	TCCCACAATACACTAAGACTTGTTGA
Pap**1**	CGAAACGAACGCGTTTTCAGG
Pap**2**-kan	**ttaacccggggatccgtcgac**TTCACAAAGCAATGCAAGATGAAGG
kan-Pap**3**	**atgaatcggccaacgcgcgg**CCTTTTTGTTTTTCTTCGCCTCGTG
Pap**4**	GCCTTTGGTCGACGAATATTAGCATC
Sty**1**	CCGGCGGAACTTCATTCTTTG
Sty**2**-kan	**ttaacccggggatccgtcgac**AACGCTGCGAAGTGCCAAGC
kan-Sty**3**	**atgaatcggccaacgcgcgg**GGTCATCGGGTTTTTAATCCTCCG
Sty**4**	TCAAGCACGTCTGTTTTGAGCCC

with two fragments (in the case of *pap1*; 285 bp and 606 bp fragments corresponding to the 5′ and 3′ flanking region of the target ORF respectively).

2.5. Recovery of PCR products

After electrophoretic analysis of DNA fragments on a 1.5% (w/v) agarose gel, the desired DNA fragments (knock-out cassettes) were recovered from the gel in ultra-pure form using a gel DNA Recovery Kit (Roche). The losses during recovery were minimal. This procedure was performed according to the manufacturer's instructions.

2.6. Transformation of cassettes into *S. pombe*

Cells of *S. pombe* were transformed using the lithium acetate method developed by Bähler et al. (1998). For each transformation reaction, the lithium acetate-treated cells (~ 2×10^7 cells) were mixed with a 10 µg knockout cassette, and then spread on YE plates and incubated for 24 h at 30 °C. Replica plates (YE medium containing G418 for selection) were also prepared. Drug-resistant colonies appeared after 4–5 days of incubation.

2.7. Colony PCR

Correct integration of the cassettes was verified by the colony PCR method. Ten colonies of *S. pombe* growing on selection medium were picked with a toothpick and diluted in distilled water. This cell suspension was directly used as the template for PCR, with the following forward primers (for *atf1*: 5′-TGGGATCTATGTGGTGGCAA-3′; for *pap1*: 5′-TGGAGGCTAGACTCAGCGCA- 3′, and for *sty1*: 5′-GAAATCAAAACGCGGACGCT-3′) and a reverse primer corresponding to the kanamycin gene (5′- ttgcccgacattatcgcgag- 3′). The PCR reactions included ExTaq polymerase and enzymes, and buffer at the concentrations recommended by the suppliers. The PCR reactions were conducted in 0.5-mL tubes. Each mixture (total volume, 25 µL) contained 2.5 µL of buffer with 0.5 mM each dNTP, 2 µM each primer, and 1 µL of cell suspension (approximately 10^5 cells) as the template. The thermal cycling conditions were as follows: 45 cycles at 95 °C for 45 s, 55 °C for 1 min, and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min.

2.8. Generation of triple mutants

The mating types of all mutants (*ird5atf1*$^-$, *ird5pap1*$^-$, *ird5sty1*$^-$, *972atf1*$^-$, *972pap1*$^-$, and *972sty1*$^-$) obtained in this study were determined with 975h$^+$ and 972h$^-$ as tester strains. All crosses were performed on SPA medium and were incubated at 25 °C for 3 days. Tetrads were isolated using a de Fonbrune-type micromanipulator. The spore clones were grown on YEA master plates and then replicated on MMA plants containing 2-DOG and G418. The genotypes of ascospores were determined by amplifying relevant genes by colony PCR.

2.9. Induction of oxidative stress

In the present study, deletion-carrying *ird5* and wild-type cells were grown to the log phase in rich medium (YEL) at 30 °C with shaking at 180 rpm. Each culture was divided into two groups: a control group and a test group. For the test group, H_2O_2 (2 mM) was added to create mild oxidative stress (Queen et al., 2002), and the cells were incubated for 1 h. The test and control cultures were diluted to yield approximately 10^2–10^3 cells/plate, and then cultured in YEA medium. The colonies on each plate were then counted to obtain the ratio of survival between the test group and control group as described by Nur et al. (2014). These values are shown as viability percentages.

2.10. RNA isolation and cDNA synthesis

Total RNA was extracted from cells using a High Pure RNA Isolation Kit (Roche), and cDNAs were synthesized using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the manufacturer's instructions.

2.11. Quantitative real-time polymerase chain reaction

To analyze the transcript profiles of various genes in cells grown under these conditions, SYBR green-based quantitative real-time qRt-PCR was performed using Stratagene technology (Stratagene, La Jolla, CA, USA). The *actin* gene from *S. pombe* served as the reference gene

Table 3. Creation of knock-out cassettes. The kanamycin resistance gene harbored by pFA6a-kanMX6 plasmid was 1.5 kb long.

Gene	1st PCR		1st PCR product (bp)	2nd PCR		2nd PCR product cassette (bp)
	Template	Primer		Template	Primer	
Atf1	g DNA	Atf**1**, Atf**2**-kan	765	1st PCR products, pFA6a-kanMX6	Atf**1** Atf**4**	1989
	g DNA	kan-Atf**3**, Atf**4**	444			
Pap1	g DNA	Pap**1**, Pap**2**-kan	606	1st PCR products, pFA6a-kanMX6	Pap**1** Pap**4**	2027
	g DNA	kan-Pap**3**, Pap**4**	285			
Sty1	g DNA	Sty**1**, Sty**2**-kan	218	1st PCR products, pFA6a-kanMX6	Sty**1** Sty**4**	1697
	g DNA	kan-Sty**3**, Sty**4**	699			

to normalize qRt-PCR results (Xue-Franzen et al., 2006). Changes in gene transcript levels were calculated using MxPro software (Stratagene). The primers used in this study, specific to the target genes, were designed in our previous study using the "Primer 3" program (Palabiyik et al., 2012). All of the primers are shown in Table 4.

The qRt-PCRs were performed using the Green FastStart SYBR Master Kit (Roche). The conditions for qRt-PCR varied according to the primers' annealing temperatures. Reactions were conducted in 0.5-mL tubes. Each PCR mixture (total volume, 25 µL) contained 2.5 µL of 10X buffer, 0.5 mM of each dNTP, 2 µM of each primer, and 1 µL (~ 100 ng) of cDNA as the template. The thermal cycling conditions for the melting curve analysis were as follows: preincubation at 95 °C for 1 min; 45 cycles at 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s; and then 1 cycle at 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s.

2.12. Statistical analysis

Data are given as mean values ± standard deviation for two or three experiments. Statistical comparisons were made using the one-way ANOVA module of GraphPad Prism 5. Statistical significance was evaluated by the Tukey test. $P < 0.05$ was considered significant.

3. Results

3.1. Construction of deletion mutants

Two sequential PCR amplifications were conducted to obtain the a*tf1, pap1,* and s*ty1* knock-out cassettes. The kanamycin resistance gene (flanked by the P_{TEF} promoter and T_{TEF} terminator sequences) was located in the middle of each cassette, between sequences from the flanking regions of each gene. After transferring the cassettes into *S. pombe*, the long regions encompassing the target genes were those at which homologous recombination occurred in the host genome (Figure 1). These cassettes were transformed into the *ird5* mutant using kanMX6, yielding a*tf1*, *pap1*, and s*ty1* disruptions via homologous recombination. Transformants were selected based on their resistance to G418 and 2-DOG. Colony PCR analyses confirmed the gene deletions (Figure 2).

3.2. Viability of *S. pombe* mutants under oxidative stress

To explore the link between the oxidative stress response and glucose repression in *S. pombe*, we deleted some of the stress-response elements from the glucose repression-resistant mutant *ird5* and the wild type. We compared the survival rates of the resulting *ird5* double mutants (*ird5atf1*$^-$: 5A, *ird5pap1*$^-$: 5P, and *ird5sty1*$^-$: 5S) and wild-type single mutants (*972atf1*$^-$: 9A, *972pap1*$^-$: 9P, *972sty1*$^-$: 9S) under mild oxidative stress (Figure 3). All of the mutants in both backgrounds were sensitive to oxidative stress; the mutants of *pap1* were the most sensitive (survival rates of <1.5%). The fact that both *id5* double mutants and wild-type single mutants showed decreased resistance to oxidative stress and confirmed that each of the oxidative stress response elements are very important in the stress resistance response of *S. pombe*.

3.3. Generation of triple mutants

We constructed a *ird5atf1*$^-$*pap1*$^-$ triple mutant and *972atf pap1*$^-$ double mutants from crosses between the *ird5atf1*$^-$ *h*$^-$ and *972pap1*$^-$ *h*$^+$ after analysis of relevant tetrads. We were not able to analyze the transcript profiles of genes related to the stress response in glucose repression in these mutants because their survival rates were too low.

3.4. Transcript levels of genes related to glucose metabolism in *S. pombe* mutants

To determine whether glucose repression was maintained and how glucose was used in the *ird5* double mutants, we determined the transcript levels of *fbp1,* a marker for

Table 4. Sequences of gene-specific qRt-PCR primers.

Gene	Primer	Sequence
hxk2	hxk2L (F)	5′-CAACAAGGACTTTGCCCAAT-3′
	hxk2R (R)	5′-AAGGTGTCGCTCTCCTTTGA-3′
ctt1	ctt1L (F)	5′-ATCCTCAATCCGACCACTTG-3′
	ctt1R (R)	5′-AACGTCGGTAATTTCGTCCA-3′
sod1	sod1L (F)	5′-ATTGGCCGTACCATTGTCAT-3′
	sod1R (R)	5′-GACACCACAAGCGTTACGTG-3′
act1	act1L (F)	5′-AGATTCTCATGGAGCGTGGT-3′
	act1R (R)	5′-TCAAAGTCCAAAGCGACGTA-3′
fbp1	fbp1L (F)	5′-GTATGGTGCTTCGGCTCATT-3′
	fbp1R (R)	5′-TTCATGTTTCGATGGGTCAA-3′

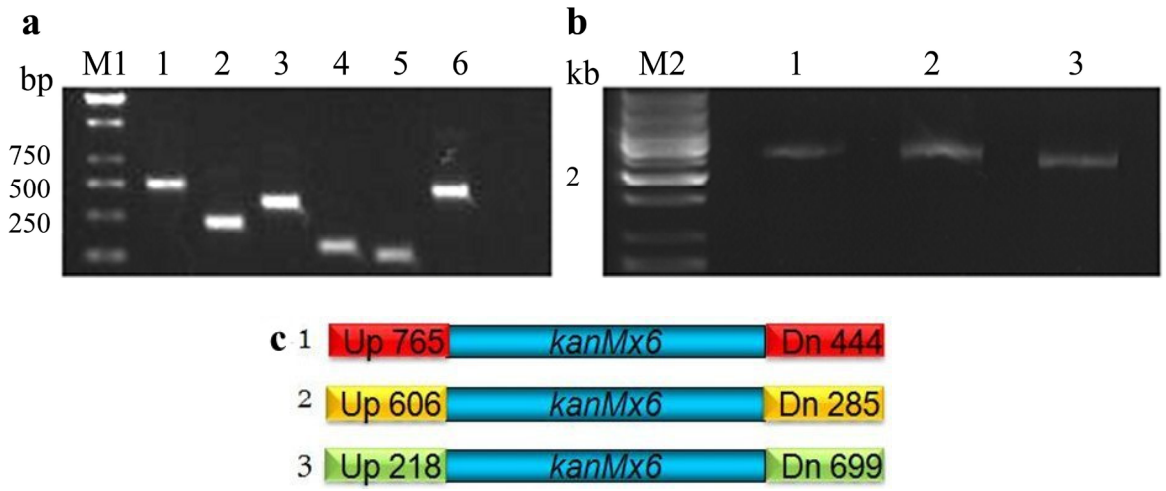

Figure 1. a) First-round PCR products: amplified upstream and downstream fragments of *atf1*, *pap1*, and *sty1* genes. M: DNA marker (Fermentas, 1 Kb); 1: 765 bp DNA PCR product amplified from *atf1*; 2: 444 bp DNA PCR product amplified from *atf1*; 3: 606 bp DNA PCR product amplified from *pap1*; 4: 285 bp DNA PCR product amplified from *pap1*; 5: 218 bp DNA PCR product amplified from *sty1*; 6: 699 bp DNA PCR product amplified from *sty1*.
b) Second-round PCR products of deletion cassettes. M: DNA marker (Fermentas, 1 Kb); 1: *atf1* cassette; 2: *pap1* cassette; 3: *sty1* cassette.
c) Schematic representation of deletion cassettes of Atf1 (1), Pap1 (2), and Sty1 (3).

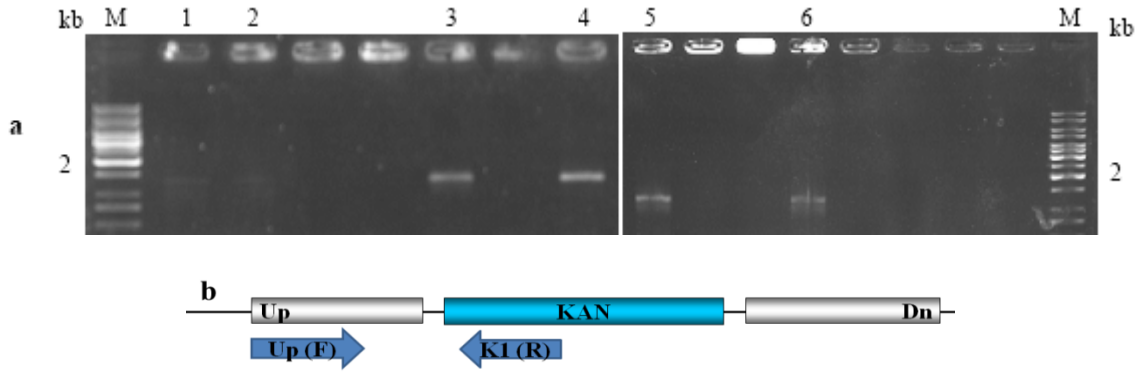

Figure 2. Colony PCR results corresponding to *ird5atf1*: 5A, *ird5pap*: 5P, *ird5sty1*: 5S, *972atf1*: 9A, *972pap1*: 9P, *972sty1*:9S mutants grown on rich medium containing G418.
a) Agarose gel electrophoresis analysis.
b) Schematic representation of regions in cassettes amplified by colony PCR. Up: Upstream sequence of target gene, Dn: Downstream sequence of target gene, KAN: Kanamycin gene sequence amplified from pFA6a-kanMX6 plasmid, Up (F): for *atf1*: 5′ -TGGGATCTATGTGGTGGCAA-3′;for*pap1*:5′-TGGAGGCTAGACTCAGCGCA-3′andfor*sty1*:5′-GAAATCAAAACGCGGACGCT-3′, K1 (R): 5′ -ttgcccgacattatcgcgag- 3′.

glucose repression (Hoffman and Winston, 1989, 1990), and *hxk2*, which is required for glucose metabolism (Petit et al., 1996) in the mutants, and corresponding control lines under both stressed and nonstressed conditions. The transcript profiles of *fbp1* and *hxk2* in the double mutants were roughly similar to those in the single mutants under both stressed and nonstressed conditions (Figure 4). Under nonstressed conditions, the levels of *fbp1* transcripts were slightly higher in all of the mutants than they were in the controls (Figure 4). Under mild oxidative stress, the transcript levels of *fbp1* were slightly lower in all of the mutants than they were in the controls. The up- and downregulated responses of *fbp1* were slightly greater in *sty1* mutants than in *atf1* and *pap1* in both genetic backgrounds.

As expected, all of the mutants showed decreased transcript levels of *hxk2* under both stressed and nonstressed conditions, compared with those in the corresponding control lines (Figure 4). The decreased transcript levels of *hxk2* and *fbp1* in all mutants, under

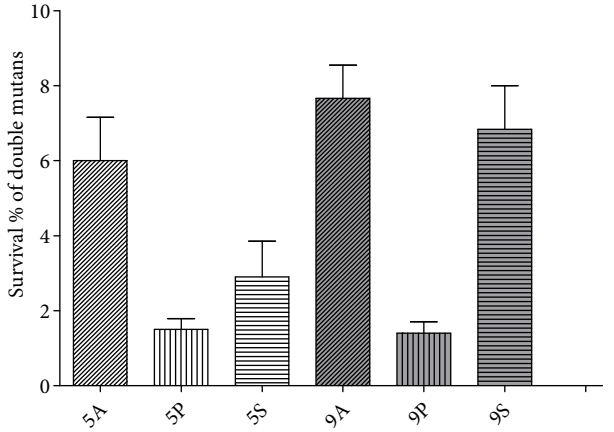

Figure 3. Survival of *ird5* double mutants and wild-type single mutants under mild oxidative stress. Cells were grown to the mid-log phase and then treated with 2 mM H_2O_2 for 1 h. Viability of cells grown on rich medium, treated with (the treated group) and without H_2O_2 (the control), was determined by the colony counting method. Significance was evaluated by the Tukey test. Error bars, ± SD. 5A: *ird5atf1*; 5P: *ird5pap1*; 5S: *ird5sty1*; 9A: *972atf1*; 9P: *972pap1*; 9S: *972sty1*.

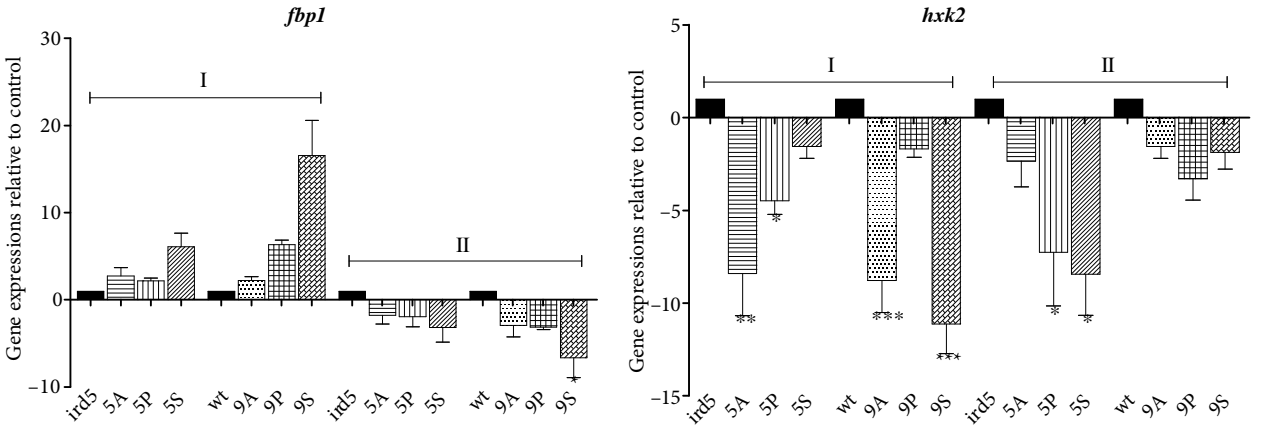

Figure 4. Relative transcript levels of genes involved in glucose metabolism. Transcript levels of *fbp1* and *hxk2* in *ird5* double mutants and wild-type single mutants treated with H_2O_2 (II) or untreated (I), after growth in a rich medium, were analyzed by qRt-PCR. The transcript values are the amount of change compared with that in the corresponding control lines (*ird5* or wt), and all values are normalized to that of *actin*. Significance was evaluated by the Tukey test. ***P<0.0001; **P<0.001; *P<0.05. wt = wild-type 972. Error bars, ± SD. 5A: *ird5atf1*; 5P: *ird5pap1*; 5S: *ird5sty1*; 9A: *972atf1*; 9P: *972pap1*; 9S: *972sty1*.

mild oxidative stress, could have resulted from the very low viabilities of the mutants under these conditions (Figure 3). Together, these results showed that *atf1*, *pap1*, and *sty1* are important components of the glucose metabolic pathway and downstream pathways in *S. pombe*.

3.5. Transcript profiles of genes related to the oxidative stress response in *S. pombe* mutants

Next, we analyzed the transcript levels of genes encoding cellular antioxidants in eukaryotic cells from yeast to mammalian (Ekoue and Diamond, 2014). The transcript levels of *sod1* and *ctt1*, encoding superoxide dismutase and catalase, respectively, were analyzed in *ird5* double mutants and *972* wild-type single mutants. Under stressed and nonstressed conditions, the transcript level of *sod1* was 2-, 9- and 10- fold higher in 5A, 5P, and 5S double mutants, respectively, than in the *ird5* control, but was more than 100-fold higher in all of the wild-type single mutants than in the wild-type control (Figure 5). Therefore, the transcript level of *sod1* was much higher (several hundred fold) in single mutants than in double mutants under both stressed and nonstressed conditions.

Under nonstressed conditions, the transcript level of *ctt1* was approximately 3-, 4-, and 6-fold higher in 5A, 5P, and 5S double mutants, respectively, than in the *ird5* control. Among the single mutants, however, only 9P showed a slight decrease in *ctt1* transcript levels under

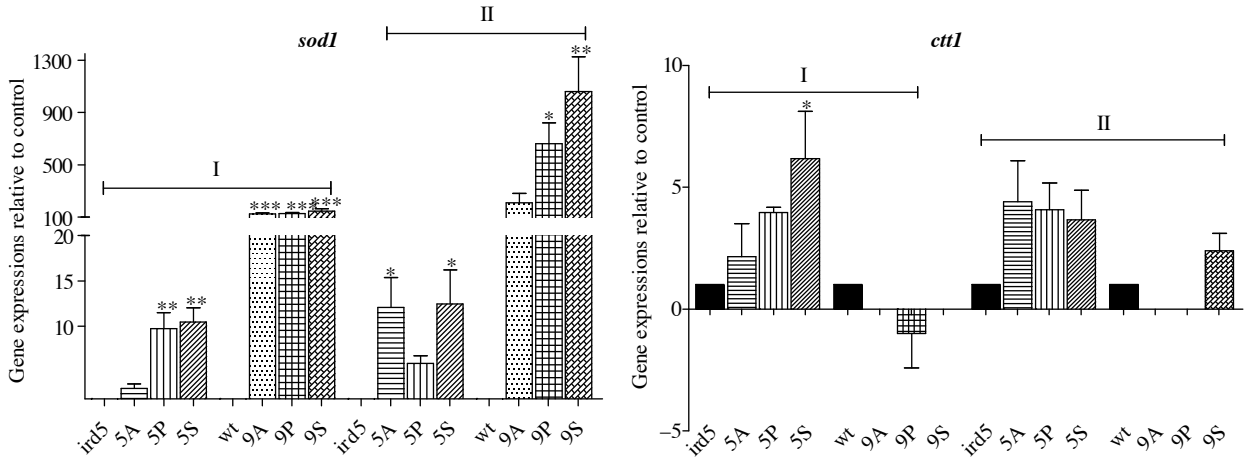

Figure 5. Relative transcript levels of the genes involved in the oxidative stress response pathway. Transcript levels of *sod1* and *ctt1* genes in *ird5* double mutants and wild-type single mutants treated with H_2O_2 (II) or untreated (I), after growth in a rich medium, were analyzed by qRt-PCR. Transcript values are the amount of change compared with that in the corresponding control lines (*ird5* or wt), and all values are normalized to that of *actin*. Statistical significance was evaluated by the Tukey test. ***P < 0.0001; **P < 0.001; *P < 0.05. wt = wild-type 972. Error bars, ± SD. 5A: *ird5atf1*; 5P: *ird5pap1*; **5**S: *ird5sty1*; 9A: *972atf1*; 9P: *972pap1*; 9S: *972sty1*.

nonstressed conditions, compared with that in the wild-type control (Figure 5). However, these differences were not significant.

We also compared transcript levels of *sod1* and *ctt1* in single mutants and double mutants under mild oxidative stress. In the double mutants, there were only slight differences in *sod1* transcript levels between stressed and nonstressed conditions. Specifically, the *sod1* transcript level was drastically increased in 5A and 5S, and unchanged in 5P under mild oxidative stress (Figure 5). For the 9P and 9S single mutants, however, there was a marked increase (several hundred fold) in *sod1* transcript levels under mild oxidative stress, compared with that in unstressed conditions (Figure 5). The *ctt1* transcript levels increased in response to mild oxidative stress in all of the double mutants and only in one of the single mutants (9S). However, these differences were not significant (Figure 5). The transcriptional profiles of *sod1* and *ctt1* in *ird5* double mutants and wild-type single mutants showed that *sod1* and *ctt1* play important roles alongside *atf1*, *pap1*, and *sty1* in glucose metabolism and downstream pathways, as well as in oxidative stress response pathways.

4. Discussion

The results of our previous studies showed that the deficiency of glucose repression in *ird* mutants has led to oxidative stress resistance. We had suggested that in the glucose repression-resistant mutant *ird5*, glucose-depletion conditions led to a low glucose consumption rate and triggered an adaptive response to oxidative stress (Palabiyik et al., 2013).

We deleted genes encoding a key enzyme (Sty1) and transcription factors (Atf1 and Pap1) in the stress response of *S. pombe* to study the role of glucose repression in oxidative stress resistance (Vivancos et al., 2006). Atf1 and Pap1 are involved in regulating cellular defenses against increasing intracellular oxidative stress in *S. pombe* (Gacto et al., 2003). We determined the survival rates of the resulting 5A, 5P, and 5S double mutants and the 9A, 9P, and 9S single mutants under mild oxidative stress. All of the mutants were sensitive to oxidative stress, and especially 5P and 9P mutants (Figure 3). The high sensitivities of 5P and 9P mutants may be due to the oxidative stress response being regulated via the Pap1 transcription factor under mild oxidative stress in *S. pombe* (Quinn et al., 2002). However, previous studies showed that cells lacking Sty1 and Atf1 were sensitive to oxidative stress, but Pap1$^-$ cells were not (Shiozaki and Russell, 1995; Degols and Russell, 1997; Toone et al., 1998; Nguyen and Shiozaki 2002; Kim et al., 2006). In our previous study, *ird5* mutants were more resistant than the wild type to mild oxidative stress (Palabiyik et al., 2013). The fact that all of the deletion mutants in this study were sensitive to oxidative stress demonstrates that Sty1, Atf1, and Pap1 are very important in the oxidative stress resistance of *S. pombe*.

Next, we focused on how glucose repression was maintained and how glucose was used in these mutants under mild oxidative stress. For these analyses, the transcript levels of *fbp1* and *hxk2* genes in the *ird5* double mutants were determined by qRt-PCR. *hxk2* encodes Hxk2 at the start of the glycolytic pathway, and *fbp1* encodes the gluconeogenesis enzyme Fbp1. The transcript profiles of *fbp1* and *hxk2,* in the *ird5* double mutants, were similar to those in the wild-type single mutants under both stressed and nonstressed conditions (Figure

4). The expression of *fbp1* is regulated through Sty1 and cAMP in *S. pombe* (Neely and Hoffman, 2000). Glucose pathways and oxidative stress pathways are linked in *S. pombe* because glucose depletion causes resistance to oxidative stress (Roux et al., 2009; Roux et al., 2010; Zuin et al., 2010). The downregulation of *hxk2* in *S. pombe* mutants can result in upregulation of *fbp1*. Therefore, the decrease in glycolytic flux in the mutants, reflected by their decreased *hxk2* transcript levels, may have led to an increase in gluconeogenesis, as reflected by their increased *fbp1* transcript levels. These results indicate that the stress response elements Sty1, Atf1, and Pap1 play important roles in energy metabolism in *S. pombe*.

In previous studies, under oxidative stress conditions, the expressions of *tpx1* (encoding thioredoxidase), *sod1*, and *ctt1* were quickly induced in a Pap1-dependent manner (Toone et al., 1998; Mutoh et al., 2002), whereas the expression of *gpx1*, encoding glutathione peroxidase, was controlled by Atf1 (Toone et al., 1998). In our previous study, the transcript levels of *pap1* and *sty1* increased in *ird5*, a strain resistant to glucose repression and oxidative stress, under both stressed and nonstressed conditions (Palabiyik et al., 2013). In the present study, we evaluated the transcript levels of *sod1* and *ctt1* in double and single mutants and their respective controls. Although there was no significant difference, with the exception of the 5S double mutant, the transcript level of *ctt1* was higher in the *ird5* double mutants than in the *ird5* control, but lower in the wild-type single mutants than in the wild-type control (Figure 5). In contrast, the transcript level of *sod1* was approximately 2- to 10-fold higher in the *ird5* double mutants than in the *ird5* control, but more than 100-fold higher in all wild-type single mutants than in the wild-type control (Figure 5). These results suggest that under nonstressed conditions, the oxidative stress response in *ird5* double mutants might be via catalase, while that in wild-type single mutants might be via superoxide dismutase. The expression level of *ctt1* is regulated by Pap1 and Sty1, while that of *sod1* is regulated only by Pap1 under oxidative stress (Toone et al., 1998; Mutoh et al., 2002). Furthermore, *ctt1* is expressed in response to oxidative stimuli at a wide range of concentrations, whereas the expression levels of *sod1* and transcription factors (Atf1 and Pap1) depend on the concentration of the oxidative stimulant (Gacto et al., 2003). These results, related to *sod1,* were inconsistent with our previous findings that *sod1* and *ctt1* transcript levels were not significantly different between *ird5* mutants and wild-type cells (Palabiyik et al., 2013). In this context, the deletion of stress response elements such as Atf1, Pap1, and Sty1 may have led to oxidative stress damage in these mutants. The transcript levels of *sod1* changed markedly in the mutants under oxidative stress conditions, while that of *ctt1* altered slightly (Figure 5). The transcript levels increased markedly in 9P and 9S, but not in 9A under mild oxidative stress. The transcript levels increased in the *ctt1* gene in all of the double mutants, but only in one of the single mutants (9S) under oxidative stress were not significantly different. These results indicate that the antioxidant responses in *ird* mutants differ from those in single mutants. That is, under both stressed and nonstressed conditions, the transcript profiles of *sod1* and *ctt1* in double mutants were roughly similar, but those in single mutants were not. Neither *ird5* double mutants nor single mutants in the wild-type background were resistant to oxidative stress (Figure 3). These results suggested that the glucose sensing/signaling pathways are more strongly related to Ctt1 than to Sod1 in the cellular antioxidant defense mechanism of *S. pombe*.

These changes in the transcript levels of *sod1* and *ctt1* genes in *ird5* double mutants and wild-type single mutants show that *sod1* and *ctt1* play important roles alongside *atf1*, *pap1*, and *sty1* in glucose metabolism and downstream pathways, as well as in oxidative stress response pathways in *S. pombe*. The downregulation of *hxk2* and the upregulation of *fbp1* in *S. pombe* mutants can cause a decrease in the glycolytic flux. Therefore, the resulting glucose depletion signal may have led to an adaptive response to oxidative stress in these mutants, even under nonstressed conditions.

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References

Bähler J, Wu JQ, Longtine MS, Shah NG, McKenzie A, Steever AB, Wach A, Philippsen P, Pringle JR (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. Yeast 14: 943-951.

Carlson M (1999). Glucose repression in yeast. Curr Opin Microbiol 2: 202-207.

Degols G, Russell P (1997). Discrete roles of the Spc1 kinase and the Atf1 transcription factor in the UV response of *Schizosaccharomyces pombe*. Mol Cell Biol 17: 3356-3363.

Degols G, Shiozaki K, Russell P (1996). Activation and regulation of the Spc1 stress-activated protein kinase in *Schizosaccharomyces pombe*. Mol Cell Biol 16: 2870-2877.

Ekoue DN, Diamond AM (2014). It takes 2 antioxidants to tango: the interaction between manganese superoxide dismutase and glutathione peroxidase-1. Turk J Biol 38: 748-753.

Fernandez J, Soto T, Vicente-Soler J, Cansado J, Gacto M (1997). Heat-shock response in *Schizosaccharomyces pombe* cells lacking cyclic AMP-dependent phosphorylation. Curr Genet 31: 112-118.

Gacto M, Soto T, Vicente-Soler J, Villa T G, Cansado J (2003). Learning from yeasts: intracellular sensing of stress conditions. Int Microbiol 6: 211-219.

Gutz H, Heslot H, Leupold U, Loprieno N (1974). *Schizosaccharomyces pombe*. In: King, RC, editor. Handbook of Genetics. New York, NY, USA: Plenum Press, pp. 395-446.

Hentges P, Van Driessche B, Tafforeau L, Vandenhaute J, Carr AM (2005). Three novel antibiotic marker cassettes for gene disruption and marker switching in *Schizosaccharomyces pombe*. Yeast 22: 1013-1019.

Higuchi T, Watanabe Y, Yamamoto M (2002). Protein kinase A regulates sexual development and gluconeogenesis through phosphorylation of the Zn finger transcriptional activator Rst2p in fission yeast. Mol Cell Biol 22: 1-11.

Hoffman CS (2005). Glucose sensing via the protein kinase A pathway in *Schizosaccharomyces pombe.* Biochem Soc T 33: 257-260.

Hoffman CS, Winston F (1989). A transcriptionally regulated expression vector for the fission yeast, *Schizosaccharomyces pombe*. Gene 84: 473-479.

Hoffman CS, Winston F (1990). Isolation and characterization of mutants constitutive for expression of the *fbp*1 gene of *Schizosaccharomyces pombe*. Genetics 124: 807-816.

Kıg C, Turkel S, Temizkan G (2005). Isolation and characterization of glucose derepressed invertase mutants from *Schizosaccharomyces pombe.* Biosci Biotech Bioch 69: 2475-2478.

Kim SJ, Cho NC, Ryu IW, Kim K, Park EH, Lim CJ (2006). Carbon source-dependent regulation of the *Schizosaccharomyces pombe pbh1* gene. J Microbiol 44: 689-693.

Kumada K, Yanagida M, Toda T (1996). Caffeine-resistance in fission yeast is caused by mutations in a single essential gene,Crm1. Mol Gen Genet 250: 59-68.

Madrid M, Soto T, Franco A, Paredes V, Vicente J, Hidalgo E, Gacto M, Cansado J (2004). A cooperative role for Atf1 and Pap1 in the detoxification of the oxidative stress induced by glucose deprivation in *Schizosaccharomyces pombe.* J Biol Chem 279: 41594-41602.

Maeda T, Wurgler-Murphy SM, Saito HA (1994). Two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature 369: 242-245.

Moreno S, Klar A, Nurse P (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Method Enzymol 194: 795-823.

Mutoh N, Nakagawa CW, Yamada K (2002). Characterization of Cu, Zn-superoxide dismutase-deficient mutant of fission yeast *Schizosaccharomyces pombe*. Curr Genet 41: 82-88.

Nur I, Munna MS, Noor R (2014). Study of exogenous oxidative stress response in *Escherichia coli*, *Pseudomonas* spp., *Bacillus* spp., and *Salmonella* spp. Turk J Biol 38: 502-509.

Neely L, Hoffman CS (2000). Protein kinase A and mitogen-activated protein kinase pathways antagonistically regulate fission yeast *fbp1* transcription by employing different modes of action at two upstream activation sites. Mol Cell Biol 20: 6426-6434.

Nguyen AN, Shiozaki K (2002). MAPping stress survival in yeasts: from the cell surface to the nucleus. In Cell and Molecular Responses to Stress, Storey, K.B. and Storey, J.M., eds., Vol. 3: Sensing, Signaling and Cell Adaptation. Elsevier Science: 75-90.

Palabiyik B, JafariGhods F, OnayUcar E (2013). The effects of glucose sensing/signaling on oxidative stress response in glucose repression mutants of *Schizosaccharomyces pombe*. Genet Mol Res 12: 5046-5056.

Palabiyik B, Kig C, Pekmez M, Dalyan L, Arda N, Temizkan G (2012). Investigation of the relationship between oxidative stress and glucose signaling in *Schizosaccharomyces pombe*. Biochem Genet 50: 336-349.

Petit T, Blázquez MA, Gancedo C (1996). *Schizosaccharomyces pombe* possesses an unusual and a conventional hexokinase: Biochemical and molecular characterization of both hexokinases. FEBS Lett 378: 185-189.

Quinn J, Findlay VJ, Dawson K, Millar JBA, Jones N, Morgan BA, Toone M (2002). Distinct regulatory proteins control the graded transcriptional response to increasing H_2O_2 levels in fission yeast *Schizosaccharomyces pombe.* Mol Biol Cell 3: 805-816.

Reiter W, Watt S, Dawson K, Lawrence CL, Bähler J, Jones N, Wilkinson CR (2008). Fission yeast MAP kinase Sty1 is recruited to stress-inducedgenes. J Biol Chem 283: 9945-9956.

Roux AE, Arseneault G, Chartrand P, Ferbeyre G, Rokeach LA (2010). A screen for genes involved in respiration control and longevity in *Schizosaccharomyces pombe*. Ann NY Acad Sci 1197: 19-27.

Roux AE, Leroux A, Alaamery MA, Hoffman CS, Chartrand P, Ferbeyre G, Rokeach LA (2009). Pro-aging effects of glucose signaling through a G protein-coupled glucose receptor in fission yeast. PLOS Genet 5: e1000408.

Samejima I, Mackie S, Fantes PA (1997). Multiple modes of activation of the stress-responsive MAP kinase pathway in fission yeast. EMBO J 16: 6162-6170.

Shiozaki K, Russell P (1995). Cell cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. Nature 378: 739-743.

Shiozaki K, Russell P (1996). Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. Gene Dev 10: 2276-2288.

Toone WM, Kuge S, Samuels M, Morgan BA, Toda T, 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 Dev 12: 1453-1463.

Vivancos AP, Jara M, Zuin A, Sansó M, Hidalgove E, (2006). Oxidative stress in *Schizosaccharomyces pombe*: Different H_2O_2 levels, different response pathways. Mol Genet Genomics 276: 495-502.

Welton RM, Hoffman CS (2000). Glucose monitoring in fission yeast via the gpa2 Gα, the git5 Gß and the *git3* putative glucose receptor. Genetics 156: 513-521.

Xue-Franzén Y, Kjærulff S, Holmberg C, Wright A, Nielsen O (2006). Genome wide identification of pheromone-targeted transcription in fission yeast. BMC Genomics 7: 303.

Zuin A, Carmona M, Morales-Ivorra I, Gabrielli N, Vivancos AP, Ayté J, Hidalgo E (2010). Lifespan extension by calorie restriction relies on the Sty1 MAP kinase stress pathway. EMBO J 29: 981-991.