Role of the Schizosaccharomyces pombe Enzyme Thioredoxin Peroxidase in Oxidative Stress Resistance

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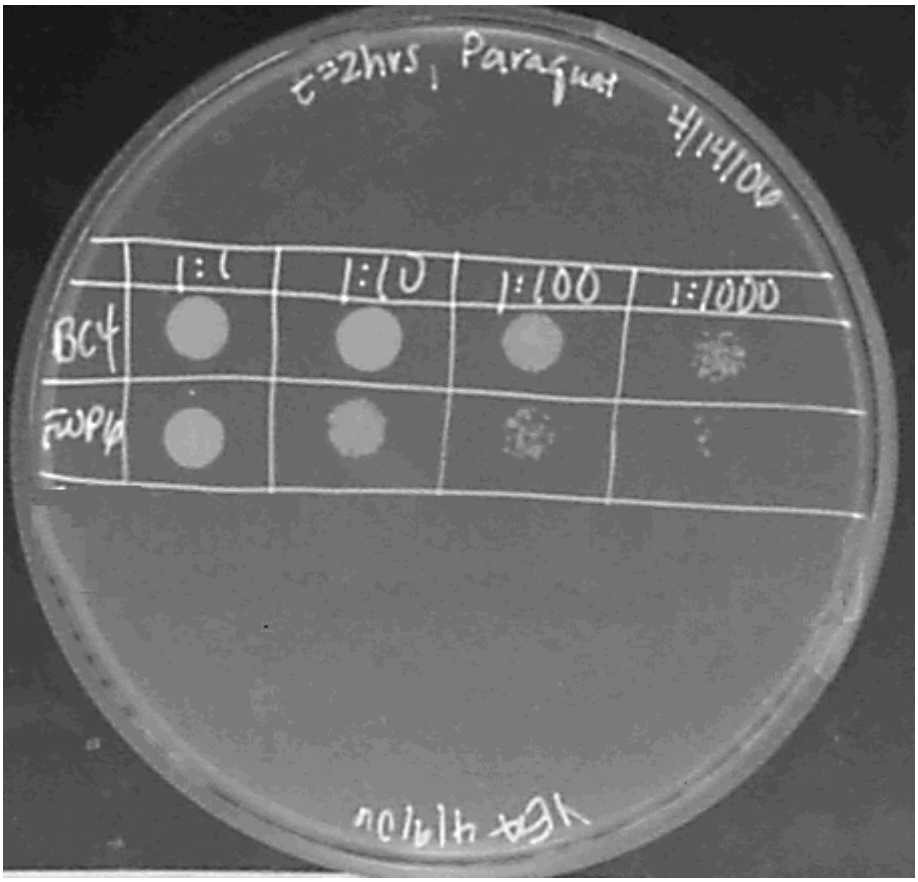

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ABSTRACT

Within cells, reactive oxygen species (ROS) are synthesized naturally and in response to environmental stimuli. However, ROS have deleterious effects on a wide range of cellular molecules. Oxidative stress, caused by the ROS generated by the partial reduction of oxygen, is a major cause of cell damage linked to the initiation and progression of numerous diseases. Thioredoxin peroxidase (Tpx1) plays important roles in cellular defense against ROS. Although homologous genes and their functions have been identified in other eukaryotes, the level of activity as well as the necessity of this protective enzyme in *S. pombe* exposed to oxidative stress has yet to be fully elucidated. To explore the role of the Tpx1 protein in oxidative stress resistance, novel strains were constructed in which the *tpx1* gene was overexpressed. The polymerase chain reaction was used to amplify *txp1*, and the amplified sequence was cloned into the yeast overexpression plasmid, pNMT41, which allows overexpression under the control of the powerful promoter. DNA sequencing was used to determine that the sequences had been properly inserted into the vector. The plasmids were transformed into two *leu-* yeast strains: FWP6 and TP108-3C. Production of the Tpx1 protein was ensured using Western Blot techniques. Experimentation to test the responses of the *tpx1* strain to oxidative stress will employ a variety of reactive oxygen generators, including hydrogen n peroxide, menadione, *tert*-butyl hydroperoxide, and paraquat. The results generally supported the proposed role of Tpx1 to confer additional resistance against the oxidative stress.

In a complementary line of investigation, knockout strains are being constructed to reduce the levels of the Tpx1 in *S. pombe*. Gene deletion cassettes were constructed for *tpx1*. Currently, the strains are being analyzed for the successful replacement of the endogenous *tpx1* gene by homologous recombination. If the absence of the protein results in decreased cell viability, the role of Tpx1 indicated by the overexpression experiments could be supported.

Chapter 1: INTRODUCTION

Within cells, reactive oxygen species (ROS) are synthesized naturally and in response to environmental stimuli. However, ROS have deleterious effects on a wide range of cellular molecules including lipids, DNA, and proteins. Oxidative stress, caused by the ROS generated by the partial reduction of oxygen, is a major cause of cell damage linked to the initiation and progression of numerous diseases, including cardiovascular, autoimmune, and neurodegenerative diseases. Specific diseases include Parkinson's disease, atherosclerosis, and Alzheimer's disease. Oxidative stress has also been implicated in diabetes, cancer, and the aging process. ROS, including superoxide anions ($O_2^{\cdot-}$), hydroxyl radicals ($HO^{\cdot}$), and hydrogen peroxide (H_2O_2), are generated by the chemical reduction of oxygen by a variety of cellular enzymes, by exposure to UV or other environmental agents, and by incomplete reduction of oxygen to water in the mitochondrial respiratory chain (Quinn et al., 2002). Cells have developed a wide range of antioxidant systems to protect themselves from the deleterious affects of ROS through inactivation. Oxidative stress may occur, however, when the balance between ROS production and antioxidant defense is disrupted. Under these conditions, oxidative stress response mechanisms activate repair systems, leading to the expression of genes such at *tpx1*, encoding antioxidant enzymes such as the peroxiredoxins.

Peroxiredoxins regulate the level of ROS by catalyzing the destruction of peroxides. One example is the 2-Cys peroxiredoxin (Prx), which is a highly conserved, extremely abundant antioxidant enzyme that catalyzes the breakdown of peroxides to protect cells from oxidative stress. Thioredoxin peroxidase (Tpx1), the single 2-Cys Prx in *Schizosaccharomyces pombe*, reduces peroxides to water, taking substances with the formula ROOH and reducing them to ROH + H_2O by donating an electron. (Veal, et al., 2004) Peroxiredoxins play an important role

in the reduction of hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH), and peroxynitrite (ONOO-) in eukaryotes. (Nguyên-nhu, et al., 2003) The oxidized state of the Tpx1 enzyme resulting from the reduction of the peroxide is returned to its active form due to activity of the other members of the thioredoxin system (Figure 1). The thioredoxin system, a major antioxidant pathway by which cells detoxify peroxides, consists of thioredoxin peroxidase, thioredoxin, and thioredoxin reductase. In this system, thioredoxin, a ubiquitous, small protein (12 kD) acts as a reducing agent by employing the thiol groups in its active site. It also acts as a scavenger of oxygen free radicals. The production of thioredoxin is increased in response to oxidative stress. Thioredoxin plays an important role in the functionality of thioredoxin peroxidase, as thioredoxin peroxidase uses thioredoxin as an immediate electron donor. The function of thioredoxin is restored when the resulting disulfide bridge (-S-S-) in the active site of thioredoxin is reduced by thioredoxin reductase. (New Science Press Ltd, 2004) Tpx, in the presence of thioredoxin, thioredoxin reductase, and NADPH, provides one of the major pathways to remove toxic peroxides in cells.

Schizosaccharomyces pombe uses a combination of stress-responsive regulatory proteins to monitor oxidative stress and activate the appropriate transcriptional response to increasing concentrations of peroxides. The specific role of one protein, Tpx1, has been investigated through a variety of experiments. The Tpx1 protein has been characterized to have a very high affinity to peroxides and to act as both an antioxidant and a signal transducer. Tpx1 was proven to be an important antioxidant in *S. pombe* through peroxide sensitivity studies of Δ*tpx1* mutant cells. In addition, overexpression of the *tpx1* gene was shown to protect cells against peroxides. (Veal, et al., 2004). Furthermore, it has been discovered that inactivation of the Tpx1 enzyme occurs under certain physiological conditions. A concentration of H_2O_2 greater than 75 μM

completely inactivated the Tpx1 in the *S. pombe* cells of one experiment, demonstrating that H_2O_2 inactivation occurs both *in vivo* and *in vitro*. These results also indicated that, in physiological conditions, Tpx is specialized for removing very low concentrations of H_2O_2. (Koo, et al., 2002) Investigations into the role of Tpx in the cellular response to hydrogen peroxide have demonstrated its importance of Tpx as an antioxidant, although little has been done to determine the implications of Tpx as an antioxidant in the response to other ROS generating chemicals.

In *S. pombe*, one signaling pathway, the Sty1 stress-activated mitogen-activated protein kinase (MAPK) pathway, is required for the response to oxidative stress. The activation of the Sty1 pathway by H_2O_2 occurs in a dose-dependent method. At low levels of H_2O_2 (<1.0mM), the MAPK Sty1, plays a role in the expression of Pap1, a transcription factor that induces gene expression of *tpx1*. (Quinn, et al., 2002) The resulting protein, Tpx1, is critical for the enzymatic degradation of H_2O_2. Through exploration of this regulatory pathway, Tpx1 has been shown to be a key redox-sensing protein specifically required for the activation of the Sty1 pathway in response to H_2O_2. Investigations indicate that Tpx1 regulates Sty1 activation through a direct mechanism involving the formation of peroxide-induced disulfide (-S-S-) complex between Tpx1 and Sty1. (Veal, et al., 2004) In a separate line of experiments to determine the role of Tpx1 in the cellular response to oxidative stress, it was discovered that Tpx1 is a key redox sensor that acts as a molecular switch controlling the transcriptional response to H_2O_2, as Tpx1 is required for the peroxide-induced activation, oxidation, and nuclear accumulation of Pap1 (Bozonet, et al., 2005). A positive feedback mechanism appears to occur in the cells: Pap1 activates the transcription of the *tpx1* gene, and the resulting Tpx1 protein then activates Pap1 at low levels of H_2O_2, leading to additional transcription of the *tpx1* gene. Increasing peroxide

concentrations, however, leads to the inhibition of Pap1 activation as a result of the increased oxidation of Tpx1 and the resulting inactivation of thiordoxin peroxidase activity.

The catalytic mechanism of thioredoxin peroxidase involves two conserved cysteine residues, the peroxidatic cysteine (-S_PH) and the resolving cysteine (-S_RH). Peroxiredoxins all share the same basic catalytic mechanism to breakdown peroxides, in which an active-site peroxidatic cysteine is oxidized to a sulfenic acid (-SOH) by the peroxide substrate. As seen in Figure 1, the reduction of the enzyme to its original, active redox state occurs by a mechanism involving thioredoxin and the resolving cysteine. (Bozonet, et al., 2005). Both Tpx1 catalytic cysteine residues are necessary for the activation of Pap1.

Peroxiredoxins are highly conserved and have been identified in yeast, plant and animal cells, as well as eubacteria and archaea. The antioxidant nature of peroxiredoxin has also been examined in other organisms such as *Saccharomyces cerevisiae*, mammals and even humans. In *S. cerevisiae*, a genetic screen was performed to identify mechanisms important for the transcriptional activation of genes encoding antioxidant proteins. Thioredoxin peroxidase (TPx) was found to be essential for the transcriptional induction of other components of the thioredoxin system, TRX2 (thioredoxin) and TRR1 (thioredoxin reductase), in response to H_2O_2. (Ross, et al., 2000). In a separate line of experiments with *S. cerevisiae*, the protective role of TPx in the cellular defense against heat shock was explored using a wild-type strain and a strain in which the gene encoding TPx has been disrupted by homologous recombination. The two strains demonstrated a distinct difference in growth kinetics and viability after heat shock, which is suspected to increase oxidative stress in cells and cause the formation of harmful ROS. The wild-type strain was more resistant to heat shock than the mutant strain. These results indicate that the lack of TPx expression may be responsible for the thermosensitivity of the mutant cells

and suggest that TPx may play a direct role in cellular defense against heat shock by functioning as an antioxidant protein. (Lee SM, et al., 1998)

The importance of TPx in the aging process has been elucidated using *S. cerevisiae*. The role of Tpx1 in aging yeast cultures was examined using a wild-type strain and a mutant yeast strain in which the gene that encoding TPx was disrupted by homologous recombination. Comparison of 5-day-old (young) stationary cultures of S. cerevisiae and cultures aged for 3 months (old) revealed decreased viability, increased generation of reactive oxygen species, modulation of cellular redox status, and increased cellular oxidative damage reflected by increased protein carbonyl content and lipid peroxidation in both strains, however, the magnitude of this stress was augmented in mutant strain lacking TPx. (Lee JH, et al., 2004). Additional experiments with *S. cerevisiae* have demonstrated that TPx may also play an important protective role against the cell damage caused by singlet oxygen, a highly reactive form of molecular oxygen suspected to harm living systems by oxidizing critical cellular macromolecules. (Kim, et al., 2002).

The role of mammalian 2-Cys peroxidredoxin (Prdx1) has also been studied. After generating mice with the targeted inactivation of Prdx1, the lifespan of the Prdx1-null mice was compared to normal mice. Although the mice lacking Prdx are viable and fertile, they have a shortened lifespan as a result of development of severe haemolytic anaemia and several malignant cancers beginning at the age of 9 months. These findings suggest that the Prdx1 protein functions as a tumor suppressor. *Prdx1*-null mice have abnormalities in numbers, phenotype and function of natural killer cells, implicating Prdx1 as an important defense against oxidants in aging mice. (Neumann, et al., 2003)

Human peroxiredoxin 5 has also been studied. A mitochondrial, peroxisomal, and cytosolic mammalian thioredoxin peroxidase, peroxidredoxin 5, have been discovered. Peroxiredoxin 5 is ubiquitously expressed in tissues and is able to reduce hydrogen peroxide and alkyl hydroperoxides. Overexpression of peroxiredoxin 5 in either the cytosolic, mitochondrial, or nuclear compartment resulted in a significant reduction of cell death; more effective protection was the result of overexpression in the mitochondria, confirming that this organelle is a major target of peroxides. (Banmeyer, et al., 2003) Further exploration of these enzymes have elucidated the role of mitochondrial and cytosolic human peroxiredoxin 5, which protects yeast cells from cytotoxicity and lipid peroxidation induced by paraquat, a ROS generating chemical. Additionally, it has been discovered that mitochondrial human peroxiredoxin 5 protects mitochondrial DNA (mtDNA) from oxidative attacks caused by exogenously added hydrogen peroxide and plasmid DNA from damages induced by metal-catalyzed generation of ROS. (Banmeyer, et al., 2005).

Within the last ten years, the importance of thioredoxin peroxidase has been explored and realized in a variety of organisms. Although much has been elucidated in *S. pombe*, most studies have examined the response to hydrogen peroxide. The response of cells to oxidative stress can be explored in a model organism such as *S. pombe* in order to examine the response of higher eukaryotes, as antioxidant enzymes found in these simpler organisms have homologues in more complex organisms that display various pathological conditions and aging. Yeast is a common model organism; one of the main advantages of using *S. pombe* is that its entire genome has been sequenced, which allows for research to focus on specific genes. The goal of this project was to extend the experimentation in the fission yeast *S. pombe* to include other stressors, including menadione, *tert*-Butyl hydroperoxide, paraquat, and hydrogen peroxide at higher concentrations

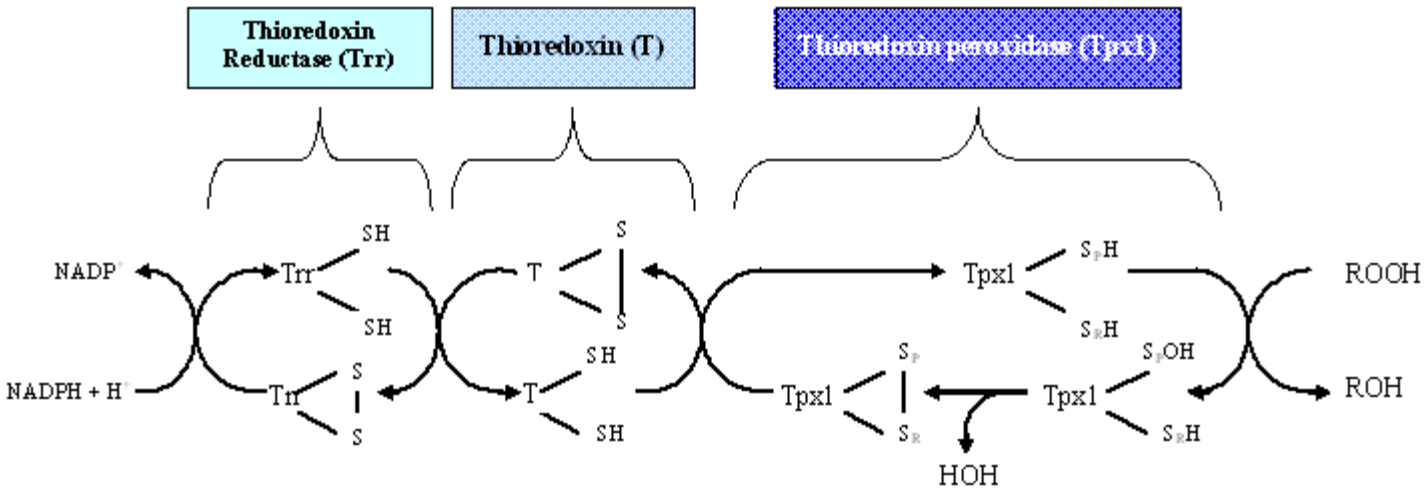
to novel strains overexpressing *tpx1*. The chemical *tert*-Butyl hydroperoxide $(CH_3)_3COOH$ is a free radical initiator that induces oxidative stress. The chemical menadione ($C_{11}H_8O_2$) has demonstrated cytotoxic activity against a variety of cell culture lines and can induce apoptosis of cultured cells via elevation of peroxide and superoxide radical levels. Paraquat is a redox active drug; its toxic effect is thought to be mediated by ROS produced by the enzymatic one-electron reduction of paraquat at the expense of NADPH or NADH, followed by one-electron transfer of dioxygen (O_2), generating superoxide anion ($O_2^{\cdot-}$). The ROS generated by paraquat can damage a wide variety of cellular constituents including DNA, RNA, proteins, sugars and lipids and thereby compromise cell viability. Hydrogen peroxide, a powerful corrosive and oxidizing agent, is naturally present in tissues as a result of cellular metabolism; a solution of this chemical was added to determine the result of additional ROS. The result of these stressors on wildtype and overexpression strains will be compared by increasing enzyme levels above the normal physiological level to determine if additional antioxidant protein increases oxidative stress resistance.

In order to explore the capabilities of the protein encoded by the *tpx1* gene to withstand oxidative stress imposed by reactive oxygen species, a novel strain was first constructed in which the gene was overexpressed. After ensuring production of the protein and enzyme concentration, experiments were conducted to determine the response of the *tpx1* overexpression strains to oxidative stress. Four reactive oxygen species (ROS) were examined: hydrogen peroxide, menadione, *tert*-Butyl hydroperoxide, and paraquat. The concentration of each stressor was finely calibrated in order to retain some viability of the strains. Experiments revealed that increasing Tpx1 enzyme levels above the normal physiological level initially (at 2 hours) increased the resistance to oxidative stress caused by menadione, tBH and paraquat. At 6 hours,

the overexpression of Tpx1 increased the viability of cells exposed to meandione and tBH. At 24 hours, the overexpression of Tpx1 only appeared to increase the viability of cells exposed to tBH.

In a complementary line of investigation to reduce the levels of the protective enzymes in the organism, potential knockouts were constructed to explore the consequences of the complete absence of the *tpx1* gene and the resulting deficiency of the protective enzyme. In an attempt to construct these strains, a method of homologous recombination which relies on natural cellular DNA maintenance and repair mechanisms present in all living organisms was employed. The creation of the knockout strain was never confirmed; as a result, the necessary ROS tests could not be conducted.

Figure 1. Thioredoxin System



The thioredoxin system, a major antioxidant pathway by which cells detoxify peroxides, consists of thioredoxin peroxidase, thioredoxin, and thioredoxin reductase. The oxidized state of the Tpx1 enzyme resulting from the reduction of the peroxide is returned to its active form due to activity of the other members of the thioredoxin system.

Chapter 2. MATERIALS AND METHODS

Materials

All reagent grade chemicals and the acid-washed glass beads (425-600 microns) were purchased from Sigma Chemical Company (St. Louis, MO). Yeast extract and casamino acids were purchased from Difco (Sparks, MD). Cosmid c576 was obtained from the Sanger Center. Culture plates, tubes, and pipet tips were purchased from Fisher (Pittsburg, PA). Oligonucleotides were purchased from MWG Biotech (High Point, NC). PCR reagents were purchased from Perkin-Elmer (Branchburg, NJ) and from Promega (Madison, WI). Qiagen Spin Miniprep and Midiprep Kits were purchased from Qiagen (Valencia, CA). Restriction enzymes and buffers were purchased from New England Biolabs (Beverly, MA) and from Promega. Salmon sperm DNA was purchased from Pharmacia, Molecular Biology Division (Peapack, NJ). SeaKem GTG Agarose for electrophoresis gels was purchased from Cambrex Bio Science Rockland, Inc. (Rockland, ME). Molecular weight standards, the DIG-Probe Southern Kit, and nylon membranes were purchased from Roche Molecular Biochemicals (Indianapolis, IN). SYBRsafe Green was purchased from Molecular Probes (Eugene, OR). All electrophoresis equipment was purchased from Bio-Rad (Hercules, CA). The PVDF membranes were purchased from Millipore (Bedford, MA). The TOPO TA Cloning Kit was purchased from Invitrogen Corporation (Carlesbad, CA). The secondary antibody used for immunoblotting, goat anti-mouse, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the LumiGLO Chemiluminescent Substrate System was purchased from KPL (Gaithersburg, MD). Film was purchased from Eastman Kodak Company (Rochester, NY). Powdered EMM growth media were purchased from BIO 101 Systems (Irvine, CA).

Strains

The strains of *Schizosacchromyces pombe* employed in the various protocols were 968, 975, FWP6, TP108-3C, TP367, BC4, and BC5. The strains and their corresponding genotypes are listed in Table 1.

Strain	Genotype
968	wild type
975	wild type
FWP6	*leu1*
TP108-3C	*leu1 ura4 pap1::ura4+* Δ*pap*
TP367	*ura4 aft1::ura+ leu1 ade6-M216* Δ*atf*
BC4 (transformed from FWP6)	*leu1* plasmid: *tpx* with *nmt*1 promoter
BC5 (transformed from TP108-3C)	*leu1 ura4 pap1::ura4+* Δ*pap* plamsid: *tpx* with *nmt*1 promoter

Growth Media

Escherichia coli was grown in liquid cultures and on solid media. The media was prepared as indicated below by dissolving in 900 mL distilled water, then bringing the volume up to 1 L with additional distilled water. The solutions were then sterilized for forty minutes in the autoclave; all autoclaving was performed at 250°F and 22 psi. The composition of the media and preparatory notes are given below.

Luria Broth liquid media: 25g/L of Luria Broth

Luria Broth liquid media with ampicillin: After sterilization in the autoclave, the Luria Broth solution was cooled. A 50 µL volume of ampicillin (from a stock solution with a concentration of 50mg/mL) was added to 50 mL of the LB liquid to achieve a concentration of 50 µg/mL.

Luria Broth solid media with ampicillin: 25g/L Luria Broth, 20g/L agar (for 2% agar plates)

After sterilization in the autoclave, the solution was cooled. A 1 mL volume of ampicillin (from a stock solution with a concentration of 50mg/mL) was added to the solution to achieve a final concentration of 50 μg/mL. The plates were stored at 4°C.

Yeast cultures were grown in a variety of sterilized liquid and solid media. The media was prepared as indicated below by dissolving in 900 mL distilled water, then bringing the volume up to 1L with additional distilled water. The solutions were then sterilized for forty minutes in the autoclave. The composition of the media and preparatory notes are given below.

YEA liquid media: 2g/L casamino acids, 5g/L yeast extract, 30g/L D-glucose

YEA solid media: YEA liquid media with 20g/L agar (for 2% agar plates)

YES liquid media: 5g/L yeast extract, 30g/L D-glucose, 225 mg/mL lysine, 225 mg/mL adenine, 225 mg/mL glutamic acid, 225 mg/mL histidine, 225 mg/mL leucine

PM complete liquid media: 3.0 g/L phthallic acid, 3.4 g/L sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$), 5.0 g/L ammonium chloride (NH_4Cl), 30.0 g/L D-glucose, 20.0 mL PM salts (50X), 75 mg/L L-histidine, 75 mg/L uracil, 75 mg/L adenine, 150 mg/L leucine

The solution was brought to pH 5.6 with 1 M potassium hydroxide (KOH). After sterilization in the autoclave, the solution was cooled. Next, 1.0 mL of PM vitamins (1000X) and 0.1 mL of PM minerals (10,000X) were added.

PM complete solid media: 3.0 g/L phthallic acid, 3.4 g/L $Na_2HPO_4 \cdot 7H_2O$, 5.0 g/L NH_4Cl, 30.0 g/L D-glucose, 20.0 mL PM salts (50X), 75 mgLL L-histidine, 75 mg/mL uracil, 75 mg/mL adenine, 150 mg/L leucine, 20 g/L agar (for 2% agar plates)

The solution was brought to pH 5.6 with 1 M KOH. After sterilization in the autoclave, the solution was cooled. Next, 1.0 mL of PM vitamins (1000X) and 0.1 mL of PM minerals (10,000X) were added. Plates were poured and stored at 4°C.

PM –leu liquid media: 3.0 g/L phthallic acid, 3.4 g/L sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$), 5.0 g/L ammonium chloride (NH_4Cl), 30.0 g/L D-glucose, 20.0 mL PM salts (50X), 75 mg/L L-histidine, 75 mg/L uracil, 75 mg/L adenine

The solution was brought to pH 5.6 with 1 M KOH. After sterilization in the autoclave, the solution was cooled. Next, 1.0 mL of PM vitamins (1000X) and 0.1 mL of PM minerals (10,000X) were added.

PM –leu solid media: 3.0 g/L phthallic acid, 3.4 g/L sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$), 5.0 g/L ammonium chloride (NH_4Cl), 30.0 g/L D-glucose, 20.0 mL PM salts (50X), 75 mg/L L-histidine, 75 mg/mL uracil, 75 mg/mL adenine

The solution was brought to pH 5.6 with 1 M potassium hydroxide (KOH). After sterilization in the autoclave, the solution was cooled. Next, 1.0 mL of PM vitamins (1000X) and 0.1 mL of PM minerals (10,000X) were added. Plates were poured and stored at 4°C.

50X PM Salts: 53.5 g/L magnesium chloride ($MgCl_2$), 0.75 g/L calcium chloride ($CaCl_2$), 50g/L potassium chloride (KCl), 2.0 g/L sodium sulfate (Na_2SO_4). The solution was filter-sterilized and stored at 4°C.

1000X PM vitamins: 1.0 g/L pantothenic acid, 10.0 g/L nicotinic acid, 10.0 g/L inositol, 10.0 mg/L biotin. The solution was filter-sterilized and stored 4°C.

10,000X PM minerals: 5.0 g/L boric acid, 4.0 g/L manganese sulfate ($MnSO_4 \cdot H_2O$), 4.0 g/L zinc sulfate ($ZnSO_4$), 2.0 g/L ferrous chloride ($FeCl_2$), 1.6 g/L molybdic acid, 1.0 g/L potassium iodide (KI), 0.4 g/L copper sulfate (Cu_2SO_4), 1.0 g/L citric acid. The solution was filter-sterilized and stored 4°C.

EMM complete liquid medi : 32g/L EMM powder, 75 mg/L L-histidine, 75 mg/L uracil, 75 mg/L adenine, 150 mg/L leucine

EMM complete solid media: 32g/L EMM powder, 75 mg/L L-histidine, 75 mg/L uracil, 75 mg/L adenine, 150 mg/L leucine, 20 g/L agar (for 2% agar plates)

After sterilization in the autoclave, the solution was cooled. Plates were poured and stored at 4°C.

EMM –leu liquid media: 32g/L EMM powder, 75 mg/L L-histidine, 75 mg/L uracil, 75 mg/L adenine

EMM –leu solid media: 32g/L EMM powder, 75 mg/L L-histidine, 75 mg/L uracil, 75 mg/L adenine, 20 g/L agar (for 2% agar plates)

After sterilization in the autoclave, the solution was cooled. Plates were poured and stored at 4°C.

GENERAL PROTOCOLS:

Cosmids

The cosmid containing the *tpx* gene, c576, was obtained from the Sanger Center. Initially, the cosmid was streaked on Luria Broth (LB) plated containing kanamycin (at a concentration of 50 μg/mL) using sterile toothpicks. The plates were grown at 37°C overnight. A colony from the plate was used to inoculate a 3 mL liquid starter culture of liquid LB/Kanamycin (at a concentration of 50 μg/mL) solution. The cultures were grown overnight at 37°C with shaking at 225 rotations per minute (rpm). Cosmids were isolated from the cultures using the Qiagen Mini Prep kit and instructions; no modifications were made to the protocol. The isolated cosmid DNA was run on a 1% agarose gel to estimate the concentration and purity of the DNA. To perform a Midi Prep, a colony from the LB/Kanamycin plate was used to inoculate a 5 mL liquid starter culture of liquid LB/Kanamycin (at a concentration of 50 μg/mL)

solution. The cultures were grown overnight at 37°C with shaking at 225 rpm. The 5 mL overnight starter culture was then added to 95 mL of liquid LB/Kanamycin (at a concentration of 50 μg/mL) in a 500 mL flask. The solution was incubated overnight at 37°C with shaking at 225 rpm. Cosmids were isolated from the cultures using the Qiagen Midi Prep kit and instructions; no modifications were made to the protocol. The isolated cosmid DNA was run on a 1% agarose gel to estimate the concentration and purity of the DNA.

Yeast Culture

Small aliquots of the yeast strains were taken from frozen stocks stored at -80°C by scraping the solid with a sterile toothpick. The frozen stock was then streaked on solid YEA media and incubated at 30°C for three days. Single colonies from the plate were used to inoculate 2 mL starter cultures of YEA liquid media. Cultures were then grown at 30°C for at least eight hours with shaking at 200 rpm. An 8 mL volume of YEA liquid media was then added to the starter culture. These cultures were then grown at 30°C overnight with shaking at 200 rpm. The cells in each culture were examined under a microscope and counted using a hemacytometer in order to determine the cell density. The cultures were then diluted (usually to 2×10^6 cells/mL) to allow the cultures to grow to the desired concentration based on a two hour doubling time.

DNA Gel Electrophoresis

Gel electrophoresis was used to examine the size and integrity of DNA. Small agarose gels were made at a 1% concentration by mixing 0.3 grams of SeaKem GTG agarose with 30 mL 0.5X TBE buffer [400 mL of 5X stock solution diluted to a final volume of 4 L in dH_2O; 5X stock TBE solution: 54 g/L Tris base, 27.5 mL boric acid, 20 mL 0.5 M EDTA (pH 8.0)] or 30 mL SYBRsafe solution. Large agarose gels were made at a 1% concentration by mixing 0.8

grams of SeaKem GTG agarose with 80 mL 1X TAE buffer or 80 mL SYBRsafe solution. The agarose solution was heated until the solution just began to boil by microwaving in 20 second increments. The gel mixture was allowed to cool slightly and then poured into the gel box; a comb was inserted at this point to create the necessary wells. After solidifying for at least 30 minutes, the gel was submerged in 0.5X TBE. Samples were loaded and run for approximately one hour at 100-110 volts. The gel was then transferred to a small Tupperware container and submerged in dH_2O. A 10 μL (for small gels) or a 15 μL (for large gels) volume aliquot of ethidium bromide was added. The gel was incubated in the solution for one hour with constant shaking. A digital picture was taken using a Kodak DC40 digital camera and uploaded onto the computer using Kodak 1D software.

Glycerol Frozen Stock Preparation

Glycerol frozen stocks were prepared to preserve novel strains for future use. Initially, a single colony was used to make a small patch on the appropriate plate; the plate was incubated overnight. The next day, a sterile stick was used to streak through the patch multiple times until cells covered the entire surface of the plate; the plate was incubated for an additional 2-3 days. A sterile P1000 pipet tip was formed into a small scoop by pressing the tip on the inside of the Petri plate lid. The scoop was used to gently scrape the medium and collect the cells from the plate. The cells were then dispersed in a cryovial containing 15% glyercol. After vortexing to evenly suspend the cells, the stocks were frozen at -80°C. Glycerol frozen stocks of bacteria cells containing plasmid with inserted *tpx* gene were prepared by mixing 850 μL of an overnight culture with 150 μL of sterile glycerol in a microfuge tube. After vortexing to evenly suspend the cells, the stocks were frozen at -80°C.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was employed to amplify a portion of genomic DNA. The PCR solution was prepared by combining 12.5 µL of Promega PCR master mix, 1 µL upstream primer (at 100 µM final concentration), 1 µL downstream primer (at 100 µM final concentration), 1 µL DNA template, and 9.5 µL Nuclease free water. The 25 µL PCR reaction was run according to the following program:

94°C – 2 minutes (denature)

94°C – 30 seconds (denature)
55°C – 30 seconds (anneal)
72°C – 2 minutes (extension)
} **Repeat Cycle 35 times**

72°C – 10 minutes (extension)

To verify amplification of the segment, a small aliquot of the solution was run on a 1% agarose gel; a molecular weight marker was also run to determine the size of the amplified sequence.

Restriction Enzyme Digest

A restriction digest was performed to cut the DNA for various reasons, including verification of the presence of an insert in a vector and linearization of a plasmid. In a restriction digest, the enzyme concentration should be adjusted to 5-10 U/µg of DNA. After determining the concentration of the DNA to be digested, the concentration of the enzyme, as recorded by the company's catalog, can be used to determine how much enzyme is needed. Restriction enzymes are usually supplied at concentrations of 10,000 U/mL, or 10 U/µL. In addition, 10X buffers are also included for each restriction enzyme; they are added to the restriction digest solution at a final concentration of 1X. The correct buffer was determined by the New England Biolabs or Promega catalog, which record the ideal buffer for each enzyme. The restriction digest was prepared in a 1.5 mL microcentrofuge tube and incubated in a 37°C water bath; the time of

incubation depended on the protocol. For vector digests, solutions were incubated for 1 hour. However, for the digestion of impure DNA, like that isolated from the "Smash & Grab" method, solutions were incubated overnight. Two enzymes were used for restriction digests: *Eco*RI (12 U/μL) and *Bam*HI (10 U/μL). Restriction digest results were examined on a 1% agarose gel.

PROTOCOLS SPECIFIC TO PROJECT:

Isolation of Genomic DNA

A 10mL culture of yeast was grown overnight. Genomic DNA was isolated according to the "Rapid Method for the Isolation of Yeast Genomic DNA" (Hoffman, et. al). The procedure was modified such that after the pellet was resuspended in TE and the RNase A was added; the solution was incubated at 30°C for a total of 35 minutes. The presence of genomic DNA was verified by running the sample on a 1% agarose gel. Two different concentrations were run for each sample. One sample was run with 5 μL genomic DNA plus 1μL loading buffer; the other sample was run with 2 μL genomic DNA plus 1 μL of loading buffer. Standard III was used as a marker, and was loaded into the gel in a volume of 5μL.

Primer Design

The primers designed and used in the manipulation of *tpx* are seen in Figure 2. Forward and reverse primers were designed to bind to flanking regions of the DNA and allow for amplification of a specific sequence using PCR. The sequence of thioredoxin peroxidase (*tpx1*) was obtained from the National Center for Biotechnology Information website (http://www.ncbi.hlm.nih.gov). Both forward and reverse primers (corresponding to N- and C-terminal sequences) were designed with a target melting point greater than 55°C due to the desired PCR annealing temperature of 55°C. In order to achieve a guanine-cytosine (GC)

content of approximately 50%, both primers were designed as 23-mers; the forward primer had GC content of 39.1% and a melting temperature of 57.1°C and the reverse primer had a GC content of 47.8% and a melting temperature of 60.6°C. The melting temperatures of the two primers were calculated using Oligo Analyzer (www.idtdna.com). The desired primer sequences were synthesized by MWG Biotech: the forward primer was designated SP30 and the reverse primer was designated as SP31.

The sequence of cosmid c576 was used to design primers to amplify the 5' and 3' flanking regions of the *tpx1* gene. The forward primer used to amplify the 5' flanking sequence, SP53, was designed as a 21-mer with a GC content of 57.1% and a melting temperature of 58.2°C. The reverse primer used to amplify the 5' flanking sequence, SP54, was designed as a 20-mer with a GC content of 50.0% and a melting temperature of 53.1°C. The reverse primer used to amplify the 3' flanking sequence, SP55, was designed as a 22-mer with a GC content of 40.9% and a melting temperature of 52.7°C. The forward primer used to amplify the 3' flanking sequence, SP56, was designed as a 20-mer with a GC content of 55.0% and a melting temperature of 56.5°C.

The other primers used were previously designed. The forward primer, SP41, and the reverse primer, SP42, for the *trr* gene were designed for use in a separate experiment. The primers for the TOPO vector, SP34 and SP35, were designed by Invitrogen.

TOPO Cloning

TOPO Cloning procedures were used to create vectors containing the *tpx* gene capable of being transformed into wildtype strains to generate oxexpression strains. The procedures were performed according to Invitrogen's instructions for chemically competent *E. coli* cells. The procedure was modified such that, when setting up the TOPO cloning reaction, 2 μL of PCR

product were used. Also, after adding the TOPO reaction to a vial containing One Shot Cells on ice, the mixture was incubated on ice for 5 minutes. A volume of 25 µL of the transformation was spread onto a Luria Broth (LB) with ampicilin plate warmed to room temperature by first placing 100 µL of LB onto the plate then adding the 25 µL of the TOPO reaction into the 100 µL of LB. Then, 150 µL of the TOPO reaction was spread onto another pre-warmed LB with ampicillin plate. The plates were incubated overnight at 37°C.

Colonies formed on the LB with ampicillin plates were tested to determine which colonies had the insert present in the correct orientation by performing PCR with the SP30 (sense) primer and the URA4 (antisense) primer from the plasmid. To prepare for PCR, a primer mix solution was prepared by combining13 µL of 10 µM SP30, 13 µL of URA4 primer, and 136.5 µL of nuclease free water. A volume of 12.5 µL of Promega PCR Master Mix was aliquoted into each PCR test tube. Twelve colonies from the 150 µL spread plate were picked and labeled. The toothpicks were used to streak a new LB with ampicillin plate and then to transfer remaining cells to the Promega PCR Master Mix solution. The Master Mix plus E. coli colony solution was heated in the Robocycler at 98°C for 5 minutes. Then, 12.5 µL of previously prepared primer mix were added to each of twelve tubes. PCR was performed using 40 cycles. The PCR results were then analyzed on a 1% agarose gel. Overnight cultures were made from the patches of the three *E. coli* colonies containing plasmids with the correct orientation of the gene as determined by PCR analysis.

DNA Sequencing

To isolate plasmid DNA for sequencing, the QIAprep Spin Miniprep Kit Protocol was followed according to QIAGEN's directions on *E. coli* cultures grown overnight. A 10 µL restriction digest was then performed using 1 µL *Eco*RI, 1 µL 10X *Eco*RI Buffer, 5 µL purified

plasmid DNA and 3 µL sterile water. The tubes were then incubated at 37°C for 1 hour. The samples labeled tpx1-1, tpx1-2 and tpx1-3 were then run on a 1% agarose gel, by combining 10 µL plasmid DNA sample with 2 µL sample buffer. A volume of 5 µL Standard III was used as a marker to determine the size as well as the concentration of the plasmids. In order to set up the sequencing reaction, a 10 µL reaction that consisted of 4 µL DTCS Quick Start Master Mix, 1 µL of vector primer (either the forward primer, SP34, or the reverse primer, SP35) (5 µM) and 5 µL of template/water was made. Sample 1-1, 1-2 and 1-3 were added to yield final concentrations of 104.36 fmol, 73.04 fmol, and 52.2 fmol, respectively. The plasmids were diluted with water (2 µL 1-1 with 3 µL water, 2 µL 1-2 with 3 µL water, and 5 µL 1-3 with no water) and heated in the Robocycler for 5 minutes at 86°C. The primer (SP34 or SP35) and DTCS Mix were then added once the plasmid solution had cooled to room temperature. PCR was then performed with the following cycle:

96°C – 20 seconds
50°C – 20 seconds
60°C – 4 minutes
} **Repeat Cycle 33 times**

4°C – overnight hold

The next day, 4 µL of stop solution (equal volumes of 3 M NaOAc, pH 5.2, and 100 mM EDTA, pH 8.0) and 0.5 µL glycogen were added to the PCR reactions. The labeled PCR products were then transferred to a microcentrifuge tube, and 60 µL of cold 95% ethanol (stored at -20°C) were added to each sample. The samples were then vortexed briefly and spun in the cold room at 14,000 rpm at 4°C for 15 minutes. The ethanol was then aspirated from the tube, and the pellets were washed by adding 200 µL of cold 70% ethanol (stored at -20°C). The samples were then spun at 14,000 rpm in the cold room for 4 minutes. The previous step was repeated, and then

after removing the ethanol, the samples were air dried for about 30 to 40 minutes. The samples were then resuspended in 33 μL of sample loading solution. The samples were sequenced with the Beckman Coulter CEQ 2000 system. Each sequence was compared to the vector and *tpx1* sequences using the BLAST algorithm.

Yeast Transformation

To generate overexpression strains using the TOPO + *tpx* vector previously created, transformations were performed following the "High Efficiency Transformation" method (Bahler, et al., 1998). The procedure was modified such that 20 mL of cells were used for each transformation. The transformed cells were plated directly on the selective PM –leu or EMM -leu plates. The cells were centrifuged at room temperature, the salmon DNA was boiled for five minutes then placed on ice before adding the transforming DNA, and then the transformed cells were resuspended in 0.2 mL of water. A volume of 20 μL of resuspended cell solution was mixed with 200 μL of water on the selective plate before spreading the cells. The remaining 180 μL of resuspended cell solution was spread on a separate plate.

Protein Extraction

To perform a Western Blot, it was first necessary to isolate the protein from the strains potentially overexpressing *tpx*. Two different protocols were examined to evaluate the efficacy of both. The first, which was later discard, involves the use of trichloroacetic acid (TCA). After growing two separate 10 mL cultures of yeast strains 968 and 975 to a density of approximately 1×10^7 cell/mL, the cultures were centrifuged for five minutes at 1500g at 4°C in the table-top centrifuge. Each pellet was then re-suspended in 500 μL of water and transferred to a 1.5 mL microcentrifuge tube. Next, 50 μL of 1.85M NaOH and 11 μL, 2% β-mercaptoethanol v/v, (BME) were added. The reaction was mixed and then placed on ice for 10 minutes. Next, 50 μL

of 10% TCA was added; the solution was mixed and left on ice for 10 minutes. The solution was centrifuged for five minutes at 12,000g in the microcentrofuge. The supernatant was carefully removed and discarded. Each pellet was then washed with 1 mL of deionized H_2O. The volume of the pellets was estimated and each was re-suspended in an equal volume of 1 M Tris. A volume of 100 µL of the solution was combined with 100 µL of Laemili loading buffer; this mixture was boiled for 10 minutes. The tubes were spun for 1 minute to pellet the wastes; the supernatant contains the protein and was later run on an SDS-PAGE gel.

The second protein extraction protocol examined utilized acid-washed glass beads. To begin, two overnight cultures were created by inoculating a 35 mL volume of YE liquid broth with yeast strain 968 or 975. The solutions were then centrifuged at 1500g for five minutes at 4°C to pellet the cells. Each pellet was washed with 10 mL of cold 1X TE and 100 mM NaCl. The pellets were then re-suspended with 1 mL 1X TE and 100 mM NaCl. Approximately 1 g of 425-600 micron acid-washed glass beads were weighed and added to a 2 mL screw-top centrifuge tube. The solutions were transferred to 2 mL screw-top centrifuge tubes. If air remained at the top of the tube, the space was filled with 1X TE and 100 mM NaCl. The tube was then placed in the microbeater and allowed to beat for 1 minute at 5000 beats per minute. The tube was removed and placed on ice for 1 minute. The tube was beat for 1 minute and then placed on ice for 1 minute a total of five times. A small volume of the cells were examined under the microscope to ensure that at least 60% of the cells appeared to be broken, as noted by their amorphous shape. The solution was then transferred to a new microcentrifuge tube and centrifuged at 10,000g for 2 minutes at 4°C. The supernatant was transferred to a new tube and stored at -20°C until run on an SDS-PAGE gel.

The procedure using the acid-washed glass beads was later repeated for strains BC4, BC5, TP367, TP108-3c, and FWP6. The only modification to the protocol was the volume of the initial overnight culture. Instead of a 35 mL overnight, a 25 mL overnight culture was made for each strain.

Lowry Assay

In order to quantify the proteins present in the extractions, a modified Lowry Assay was performed as described in (Current Protocols in Molecular Biology, Volume 2, 10.1.1). To begin, the necessary reagents were prepared: Reagent A - 0.1N NaOH; 2% Na_2CO_3, Reagent B – 0.5% $CuSO_4 \cdot 5H_2O$; 1.0% sodium citrate (dehydrate salt), Reagent C – Mixed 50 mL of Reagent A with 1.0 mL of Reagent B, which needed to be made fresh, and Reagent E – folin reagent diluted 1:1 with dH_2O. A standard curve was made by using 0 µL, 10 µL, 20 µL, 30 µL, 40 µL and 50 µL of a 1 mg/mL bovine serum albumin (BSA) solution. Next, a 1 mL volume of Reagent C was added to 50 µL of protein solution; 1 mL was also added to each of the tubes containing various volumes of BSA for creation of the standard curve. The tubes were incubated for 10 minutes at room temperature. To these tubes, a 0.1 mL volume of Reagent E was added and the tubes were vortexed immediately. The tubes were then incubated for 30 minutes at room temperature. The standards as well as the protein extraction solutions were read in a UV- visible recording spectrometer at A_{650}.

Western Blot

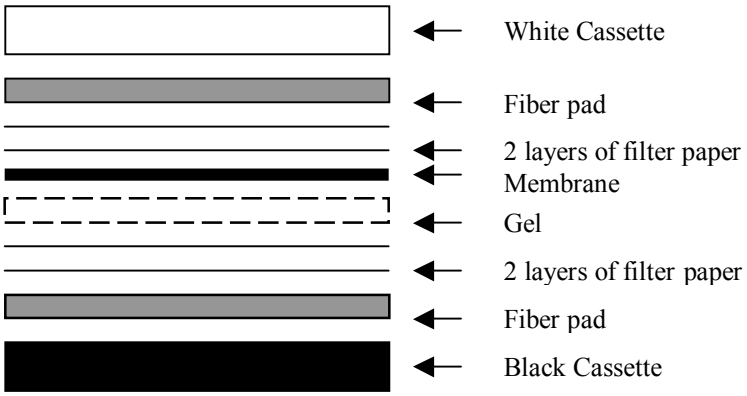
A Western Blot was employed to verify overexpression of the Tpx protein by the novel strains BC4 and BC5. After extracting the protein from the yeast cells, the first step was to run out the samples on a Laemmli protein gel as described in (Current Protocols in Molecular Biology, Volume 2, 10.2.4). A 15% resolving gel was created by combining 3.75 mL of 40%

acrylamide/Bis, 2.5 mL of 1.5 M Tris-HCl pH 8.8, 0.1 mL of 10% SDS, and 3.65 mL of distilled water in a 15 mL conical tube. A 10% stacking gel was created by combining 1.24 mL of 40% acrylamide/Bis, 1.25 mL of 0.5 M Tris-HCl pH 6.8, 0.05 mL of 10% SDS, and 2.46 mL of distilled water in a separate 15 mL conical tube. The casting frame was assembled. A fresh 10% APS solution was made by combining 0.1g of APS with 1.0 mL of water. A 50 µL volume of 10% APS and a 5 µL volume of TEMED were added to the tube containing the resolving gel. After mixing the solution, a transfer pipette was used to pipette the liquid resolving gel in between the two glass plates up to the notch; this was repeated for the second casting frame. Water was gently added to the top of the resolving gel using a new transfer pipette. The gel was allowed to polymerize; the remnants of the 15% resolving gel solution in the conical tube were used to determine when polymerization was complete. The water was poured out. A 50 µL volume of 10% APS and a 5 µL volume of TEMED were added to the tube containing the stacking gel. After mixing the solution, a transfer pipette was used to pipette the liquid resolving gel in between the two glass plates on top of the resolving gel. A comb was added gently into the stacking gel between the two plates to create the lanes. The stacking gel was then allowed to polymerize. After polymerization, the gel cassette sandwich was removed from the casting frame. The gel was placed into the electrode assembly in the Mini Tank. The inner chamber and the outer chamber were filled with 1X running buffer.

The samples were prepared by first making sample buffer. Sample buffer was made by combining 475 µL of Sample Buffer (used in DNA gels) and 25 µL of BME. The protein samples were then diluted 1:2 with sample buffer. These mixtures were boiled for 5 minutes in a water bath and then placed on ice. The samples were loaded into the two gels in duplicate by slowly pipetting into the wells and allowing the samples to settle evenly on the bottom of the

well. A 5 μL volume of Kaleidoscope marker was also added to each gel. The gel was then run at 200V until the markers are all at the bottom of the gel, usually between 30 minutes and 1 hour. One gel was removed and washed three times with water. It was then stained with Simply Blue stain by agitating gently for 1 hour. The stain was discarded. The gel was destained by adding water and agitating the gel for 1 hour. The second gel was used in the Western Blotting procedure.

Initially, 1 L of transfer buffer was prepared with final concentrations of 0.02 M glycine (1.5g), 0.025 M Tris (25 mL), and 10% methanol (100 mL). Next, a piece of PVDF membrane was cut to the size of the gel. The membrane was pre-wet in methanol for approximately 1 minute with rocking. The methanol was discarded and replaced with water. The membrane was incubated in water for 1 minute with rocking. The necessary transfer materials were assembled: four 3 MM Whatman filter papers, two sponges, a transfer cassette, the membrane in water, and a Bio-Rad tank and lid. The filters and sponges were wet in the transfer buffer. The gel was removed from the running apparatus and the bottom right corner was notched for identification purposes. The gel was then equilibrated in transfer buffer. The transfer apparatus was assembled as follows:



The transfer sandwich was properly placed into the transfer apparatus. The entire apparatus was placed into the Bio-Rad tank and the tank was filled with transfer buffer. A stir bar was added to the apparatus. The transfer was conducted overnight by running the apparatus at 20V on a stir plate.

The final portion of the Western Blot was the process of immunoblotting. To begin, 1 L of TBS-T (20 mM Tris, 500 mM NaCl, 0.05% Tween 20) was prepared by combining 20 mL of 1M Tris-HCl, pH 8.0 with 29.22g NaCl and 0.5g Tween 20 (Polyoxyethylenesorbitan Monolaurate). Next, a 5% milk TBS-T solution was made by combining 200 mL of the TBS-T solution previously prepared with 10g of dry milk. The blot was then removed from the transfer apparatus after running overnight and placed in a shallow tray. The position of the molecular weight marker bands were marked on the blot with a pencil. The membrane blot was then equilibrated in TBS-T for 5 minutes with slow rocking. Next, the membrane was blocked in 5% milk TBS-T solution for 1 hour at room temperature. Then, the membrane was washed three times, for 5 minutes each time, with TBS-T at room temperature. The membrane was incubated with 10 mL of primary antibody in a 1:5000 dilution in TBS-T for 1 hour at room temperature with slow rocking. Again, the membrane was washed three times, for 5 minutes each time, with TBS-T at room temperature. Next, the membrane was incubated with 10 mL of secondary antibody in a 1:2000 dilution in TBS-T for 1 hour at room temperature with slow rocking. Then, the membrane was washed three times, for 5 minutes each time, with TBS-T at room temperature. In a 15 mL conical tube, 4 mL of chemiluminescent substrate A was mixed with 4 mL of chemiluminescent substrate B. The 8 mL of reagent were added to the blot, and the blot was hand-rocked for 2 minutes at room temperature. The membrane blot was oriented in the x-ray film cassette, exposed to film and developed in the XOMAT machine.

Deletion Cassette Construction

A deletion cassette was constructed to be used in the deletion of the *tpx* gene via homologous recombination. The deletion cassette construction was performed following the instructions in "Strategies for gene disruptions and plasmid constructions in fission yeast" (Wang, et. al 2004). No modifications were made to the protocol. Primers to amplify the 5' and 3' flanking region of the gene were created using the previously obtained cosmid sequence. The melting temperatures of the four primers were calculated using Oligo Analyzer (www.idtdna.com). The desired primer sequences were synthesized by MWG Biotech and named SP53, SP54, SP55, and SP56; see Figure X for the primer sequences.

After construction of the deletion cassette, the plasmid was linearized using 2 μL of *Bam*HI, 2 μL of DNA, 1 μL of *Bam*HI Buffer, 2 μL 10X BSA, and 13 μL water. To optimize linearlization, the digestion mixture was incubated for various amounts of time at 37°C: 0 hours, 1 hours, 3 hours, and 24 hours. Further linearizations were incubated for approximately 1 hour. Gel electrophoresis was used to analyze the digestions.

Ultra-High Efficiency Methods for Transformation of *S. pombe*

The transformation was performed using the *S. pombe* strain FWP6, following the instructions in "Strategies for gene disruptions and plasmid constructions in fission yeast" (Wang, et. al 2004). No modifications were made to the protocol. The selective media used was PM –leucine or EMM –leucine.

Isolation of Genomic DNA – Repeated on Transformed FWP6

DNA isolation by the "Smash and Grab" method was performed as noted previously with the same modifications to the Hoffman, et al. protocol. Purified DNA was analyzed on a 1%

agarose gel. A 5 µL sample of isolated DNA was combined with 1 µL of Sample Buffer. The gel was run at 100V for 1 hour.

PCR with Gene Specific Primers

In order to determine if homologous recombination occurred, two different PCR reactions were conducted. The first reaction employed the primers SP30 and SP31, amplifying the *tpx1* gene if present. The second reaction employed the primers SP41 and SP42, amplifying the *trr1* gene as a control reaction. The solutions were prepared by combining 12.5 µL of PCR master mix, 1µ of the upstream primer, 1µL of the downstream primer, 1µL of genomic DNA, and 9.5 µL of Nuclease Free water. PCR was performed according to the same times and temperatures listed previously. Results of the PCR reactions were analyzed on a 1% agarose gel.

Southern Blot Analysis with DIG Probes

In order to determine if homologous recombination occurred, a modified Southern Blot procedure was explored. To begin, the integrity of the *tpx* gene-specific primers were examined. PCR was performed by combining 12.5 µL of PCR Master Mix, 1 µL of 10 µM upstream primer SP 30, 1 µL of 10 µM downstream primer SP31, 1 µL DNA from the original cosmid (SPCC576.03c), and 9.5 µL of nuclease-free water for a total volume of 25 µL. PCR was run according to the following settings: 94°C for 2 minutes; 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes (repeat 30 times); and 72°C for 10 minutes. The reaction was run on a 1% agarose gel.

After confirming the integrity of the gene-specific primers, DIG Probes were synthesized using the protocol and reagents provided by Roche with the PCR DIG Probe Synthesis Kit. A 50 µL reaction solution was made by combining 5 µL 10X PCR Buffer (Vial 3), 5 µL PCR DIG Mix (Vial 2), 5 µL of 10 µM upstream primer SP30, 5 µL of 10 µM downstream primer SP31,

0.75 µL Enzyme Mix (Vial 1), 4 µL DNA (TOPO + *tpx* miniprep at a concentration of approximately 20 pg), and 25.25 µL water. PCR was run according to the following settings: 95°C for 2 minutes; 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 40 seconds (repeat 30 times); and 72°C for 7 minutes.

Gel Analysis

Gel analysis was performed by running a 1% agarose gel at 100V for approximately 1 hour. The lanes were loaded as follows: Lane 1 – Blank; Lane 2 – Blank; Lane 3 – 5 µL of 1:100 dilution of labeled probe + 1 µL Sample Buffer; Lane 4 – 5 µL of 1:10 dilution of labeled probe + 1 µL sample buffer; Lane 5 – 5 µL Molecular Weight Standard (included in kit) + 1 µL sample buffer; Lane 6 – 1.46 µL of 50ng/µL plasmid (10^{10} molecules) + 0.3 µL sample buffer; Lane 7 – 1.46 µL of 1:100 dilution of 50ng/µL plasmid (10^{8} molecules) + 0.3 µL sample buffer; Lane 8 – 1.46 µL of 1:10000 dilution of 50ng/µL plasmid (10^{6} molecules) + 0.3 µL sample buffer; Lane 9 – Blank; Lane 10 – Blank.

Transfer of DNA to a Membrane

After running the gel, the DNA was transferred to a membrane for blotting. First, the gel was denatured in 0.5 M NaOH, 1.5 M NaCl for 30 minutes with slow rocking. Next, the gel was neutralized by washing in 1 M Tris, 1.5 M NaCl pH 7.5 for 30 minutes with slow rocking. A 1 L volume of 20X SSC solution was made by first dissolving 175.3g NaCl and 88.2g of sodium citrate in 800 mL of water. The pH of the solution was adjusted to 7.0 using HCl. The volume was then brought up to 1 L. Next, a piece of nylon membrane, cut to the size of the gel, was wet with water; it was then wet in 1X SSC. Two pieces of filter paper were cut to the size of the gel and wet in 10X SSC. The capillary action apparatus was then assembled in a large glass container, filled with 10X SSC, as follows (listed from bottom to top): bottom of pipet box

placed in glass container upside down; large piece of Whatman filter paper that reaches into 10X SSC in glass container; gel; nylon membrane (size of gel); 2 layers of Whatman paper (size of gel); stack of paper towels about 5 inches thick (cut to approximately the size of the gel; glass plate; and weight. The apparatus was left overnight to allow capillary action to transfer DNA to the membrane.

DIG Probe Detection

The apparatus was disassembled and the membrane was placed DNA side up on a piece of filter paper. The DNA was crosslinked to the membrane using the "Auto Crosslink" setting on the UV Stratalinker. Next, buffers were prepared. A 1 L volume of Buffer 1: 100 mM maleic acid, pH 7.5, 150 mM NaCl was prepared. Buffer 2, a solution of 2% blocking reagent, was made by dissolving 1 g of blocking reagent (provided in kit) in 50 mL of Buffer 1; the solution was heated at 42°C for several hours to completely dissolve the solid. Buffer 3 was prepared to contain 100 mM Tris, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$. An anti-DIG solution was prepared by mixing 4 µL anti-DIG antibody with 16 mL of Buffer 1 and 4 mL of Buffer 2. After crosslinking, the membrane blot was washed twice briefly in Buffer 1 with hand-rocking at room temperatures. The membrane blot of was then blocked with Buffer 2 for 30 minutes at room temperature with slow rocking. The buffer was discarded and stored at 4°C. The blot was incubated with the anti-DIG solution at room temperature for 30 minutes with slow rocking. The solution was then discarded and stored at 4°C. The membrane was then washed twice with Buffer 1 at room temperature and with slow rocking, for 15 minutes each time. Next, the membrane was equilibrated for 5 minutes with Buffer 3. The membrane was removed and allowed to dry well. After, the membrane blot was placed on a piece of plastic wrap. Approximately 20 drops of CSPD chemiluminescent detection solution were added to the DNA

side of the membrane. A piece of transparency sheet, cut to the size of the blot, was placed on top of the blot to evenly spread the CSPD over the membrane. Kodak brand x-ray film was exposed to the blot for 1 minute, 5 minutes, and overnight. After exposure, the film was developed using the X-OMAT machine in the dark room.

DIG Probe Hybridization to the Plasmid

In order to verify the ability of the DIG probe to hybridize to the gene of interest, hybridization was tested using the TOPO + *tpx* plasmid which is known to contain the gene. First, the membrane was cut so that only Lanes 6-10 remained. The membrane was then washed twice in a solution of 2X SSC and 0.1% SDS at room temperature for 10 minutes each time. Next, 30 mL of the Easy Hyb Solution, which was included in the kit, was warmed to 42°C in the hybridization oven. The membrane was washed with 25 mL Easy Hyb in a hybridization tube for 15 minutes at 42°C with rotation in the hybridization oven. A 5 µL aliquot of the DIG probe was transferred to a microcentrofuge tube and then denatured by placing in a water bath of boiling water for 5 minutes. The tube was spun down by using the pulse function of the microcentrifuge and then placed on ice. The Easy Hyb Solution from the hybridization tube containing the membrane was decanted into a 50 mL conical tube and stored at -20°C. The denatured probe was then added to the remaining 5 mL of prewarmed Easy Hyb solution; this solution was then added to the membrane. The tube was left in the hybridization oven overnight at 42°C with rotations to allow hybridization. The next day, the solution containing the denatured probe was decanted into a 15 mL tube and saved in -20°C. The membrane was then washed two times, for 5 minutes each time in a solution of 2X SSC and 0.1% SDS at room temperature. Next, the membrane was washed twice in a solution of 0.5X SSC and 0.1% SDS at 65°C for 15 minutes each time. Finally, DIG probe detection was repeated as described above.

Verification of Knockouts

Isolated genomic DNA from the six potential *tpx1* knockouts was digested with *Eco*RI by combining 10 µL of DNA with 1 µL of the New England Biolabs restriction enzyme *Eco*RI and 1 µL of NEB Buffer 2. The solution was incubated in a water bath at 37°C overnight. The next morning, an additional 1 µL of *Eco*RI was added to the reaction; the solution was incubated for approximately three additional hours. The entire volume of the digest was run on a 1% agarose gel. The gel was then stained with ethidium bromide to verify successful digestion. The DNA was then transferred to a PVDF membrane as previously described. Next, hybridization of the probe was performed as noted above. Finally, the protocol described for DIG probe detection was performed. The film was exposed for 1 minute.

Analysis of yeast viability by spot plating

To begin, a 2 mL starter culture of FWP6 was created by inoculating a liquid media solution with a single colony. After approximately 8 hours of growing time, the culture was further diluted. A 10mL culture was grown overnight. The concentration of the culture was determined using a hemacytometer. Next, the solutions were diluted to 2 x 10^6 cells/mL and allowed to enter log phase by incubating at 30°C with shaking for at least 2 hours. The cultures were then re-counted and divided into twenty-one 2 mL cultures diluted to 2 x 10^6 cells/mL. Serial dilutions of 1:10, 1:100, and 1:1000 were made for each culture and spotted on a YEA plate (t = 0 hrs). A range of concentrations of the chemicals was examined by adding the following volume to twenty of the 2 mL cultures:

Hydrogen Peroxide (H_2O_2) - 8.8 M stock	
concentration (mM)	**volume (µL)**
7.5	1.72
5.0	1.15
2.5	(1:10 dilution) 5.75
1.0	(1:10 dilution) 2.3
0.75	(1:10 dilution) 1.7

Table 1. H_2O_2 Concentration Gradient

Menadione - 0.1 M stock	
concentration (mM)	**volume (µL)**
0.1	2.15
0.075	1.5
0.05	1
0.025	(1:10 dilution) 5
0.01	(1:10 dilution) 2.15

Table 2. Menadione Concentration Gradient

tert-*Butyl hydroperoxide (tBH) – 7.77 M stock*	
concentration (mM)	**volume (µL)**
0.075	(1:100 dilution) 1.93
0.05	(1:100 dilution) 1.29
0.025	(1:1000 dilution) 6.4
0.010	(1:1000 dilution) 2.58
0.0075	(1:1000 dilution) 1.93

Table 3. tBH Concentration Gradient

Paraquat – 1 M stock	
concentration (mM)	**volume (µL)**
10	20
7.5	15
5	10
2.5	5
1	2

Table 4. Paraquat Concentration Gradient

A 20 µL volume of dH_2O was added to the remaining 2 mL culture as a control. The cultures were all incubated at 30°C with shaking. Serial dilutions were made and spotted onto YEA plates at 1.5 hours, 4 hours, and 24 hours. It was determined that 1.5 mM of H_2O_2, 0.06 mM of menadione, 0.75 mM of tBH, and 10 mM of paraquat would be used in all future experiments.

ROS Testing

To determine if the overexpression of the Tpx protein in BC4 affected its viability in the presence of various reactive oxygen species, ROS testing was conducted on both BC4 and FWP6 according to the results of the sensitivity tests. To begin, a 2 mL starter culture of FWP6 and a 2 mL starter culture of BC4 was created by inoculating a liquid media solution with a single colony. After approximately 8 hours of growing time, the culture was further diluted. A 10mL culture was grown overnight. The concentration of the culture was determined using a hemacytometer and then diluted to 2 x 10^6 cells/mL. The cultures were incubated at 30°C with shaking for at least 2 hours to allow entry into log phase. The cultures were then re-counted and each was divided into five 2 mL cultures diluted to 2 x 10^6 cells/mL. Serial dilutions of 1:10,

1:100, and 1:1000 were made for each culture and spotted on YEA plates (t = 0 hrs). Next, the ROS generating chemicals were added. A 1.5 mM concentration of H_2O_2 was added to one 2 mL culture of FWP6 and one 2 mL culture of BC4. A 0.06 mM concentration of menadione was added to one 2 mL culture of FWP6 and one 2 mL culture of BC4. A 0.75 mM concentration of tBH was added to one 2 mL culture of FWP6 and one 2 mL culture of BC4. A 10 mM concentration of paraquat was added to one 2 mL culture of FWP6 and one 2 mL culture of BC4. A 20 µL volume of sterile dH_2O was added to one 2 mL culture of FWP6 and one 2 mL culture of BC4. The cultures were all incubated at 30°C with shaking. Serial dilutions were made and spotted onto YEA plates at 2 hours, 6 hours, and 24 hours.

Figure 2. Primer Sequences

SP30 – *tpx* Forward: nucleotides 4867 to 4899

5' – ATG AGT TTG CAA ATC GGT AAA CC – 3'

SP31 – *tpx* Reverse: nucleotides 5445 to 5423

5' – GTG CTT GGA AAA GTA CTT CTC GG – 3'

SP40 – *trr* Forward: nucleotides 4942, 5242 to 5272

5' – ATG ACT CAC AAC AAG GTT GTT A – 3'

SP41 – *trr* Reverse: nucleotides 6205 to 6182

5' – ATC GGT ATC TTC CAA TTC TTC AAG – 3'

SP34 – PNMT41 Forward

5' – TTT CAA TCT CAT TCT CAC TTT CTG A – 3'

SP35 – PNMT41 Reverse – URA4 reverse priming site

5' – ACA AGG CAT CGA CTT TTT CAA TA – 3'

SP53 – *tpx* 5' arm Forward: nucleotides 4531 to 4551

ACG GGA TCC AGG 5' – CTT ACG AGC AGC ATC CCA CAC – 3'
(*Bam*HI linker)

SP54 – *tpx* 5' arm Reverse: nucleotides 4853 to 4834

5' – GCA AGT GTA GTC AAC GAG AG – 3'

SP55 – *tpx* 3' arm Reverse: nucleotides 5494 to 5515

CCT GGA TCC CGT 5' – TTG ACT AAG CTC ACT TGC TTA C – 3'
(*Bam*HI linker)

SP56 – *tpx* 3' arm Forward: nucleotides 5730 to 5711

5' – GAC AGT TGC TGA GCC CAA AG – 3'

Chapter 3: RESULTS

I. Construction of overexpression TOPO vector

The cloning strategy used for working with the *tpx1* gene is demonstrated in Figure 3. Initially, genomic DNA was isolated from *Schizosacchromyces pombe* using the "Smash and Grab" method. The genomic DNA was used as a template in PCR reactions designed to amplify the sequence of the *tpx1* gene. The primers used to generate this sequence were SP30 and SP31 (shown in Figure 2), generating an amplification product 579 base pairs (bp) in length. Subsequently, the PCR product was cloned into the TOPO41 vector using the TOPO TA cloning kit, creating the TOPO + *tpx* vector. In order to determine if TOPO Cloning was successful, the solution was plated. Formation of colonies on the LB with ampicillin plates verified that the plasmid was taken up by the *E. coli* cells due to the presence of a gene conferring ampicillin resistance in the plasmid. From an abundance of colonies, twelve were selected. Each colony was streaked on a plate with a tooth pick and PCR was performed on the remaining cells using the primers SP35 (URA4 primer) and SP30 to confirm the inserts were in the TOPO vector in the correct orientation. All the streaks grew to high densities indicating that strains were viable. Aliquots of the PCR results were run on a 1% agarose gel; the presence of four bands indicated that four out of the twelve *tpx1* inserts were oriented correctly within the plasmid (Figure 4).

The plasmids with the *tpx1* gene correctly inserted were isolated by MidiPrep procedure using the Qiagen Spin Midiprep Kit. A restriction digest was performed on isolated plasmids with *Eco*RI to verify the presence of the *tpx1*gene. Bands roughly corresponding to the length of the *tpx1* gene were visible in three out of the four lanes of the subsequent gel (Figure 5). Standard III was used as a reference to calculate the concentrations of both the insert and the

plasmid. The calculated concentrations were: *tpx1*-1 230 ng/µL, *tpx1*-2 161 ng/µL and *tpx1*-3 was 46 ng/µL.

The plasmids were then sequenced with the forward primer of the vector, SP34, and the reverse primer for the vector sequence, SP35. The forward sequencing results of tpx1-2, which was used in all subsequent transformations experiments, showed a 12 bp region of homology to plasmid pNMT41 preceding the gene. The absence of added nucleotides between the plasmid nucleotides and the start codon of *tpx1* indicate that no gap was added between the plasmid and the insert during TOPO Cloning. When *tpx1*-2 was sequenced with the reverse primer of the vector, there was an 83 bp region of homology with pNMT immediately before the start of the reverse sequence of the gene, demonstrating the absence of any gap insertion after the gene and before the plasmid. The lack of gap insertions before and after the gene showed that *tpx1*-2 did not acquire any mutations from TOPO cloning and that it was viable for transformation. Similar results were obtained for *tpx1*-1 and *tpx1*-3, but their sequences yielded a higher percentage of uncertain base pair reads. However, they were preserved at -80°C because they were also likely viable candidates for transformation.

Using BLAST, the absence of inserts before or after the gene was verified. Furthermore, BLAST verified the positive identification of the insert as *tpx1*. In order to arrive at these conclusions, three BLAST comparisons were performed. The first was a nucleotide-nucleotide BLAST to compare the nucleotide sequence produced by sequencing to all known sequences in the database; the sequence which had the greatest homology was for *S. pombe* chromosome III, SPCC576.3C, which is the known *tpx1* sequence. Then, a translating BLAST was performed using the nucleotide sequencing results and the protein sequence of *tpx1* obtained from EMBOSS-Transeq, which confirmed the identity of the sequences as well as the lack of a

frameshift mutation in the insert. This tBLASTn search on the determined sequence showed high homology to both the *S. pombe* chromosome III cosmid as well as the sequence of *S. pombe* thioredoxin peroxidase mRNA (Figure 6). Finally, a BLAST to align two sequences was performed in order to compare the nucleotide sequencing results to the nucleotide sequence of the pNMT41 plasmid. Sequencing results demonstrated that no gap had been inserted within the *tpx1* gene.

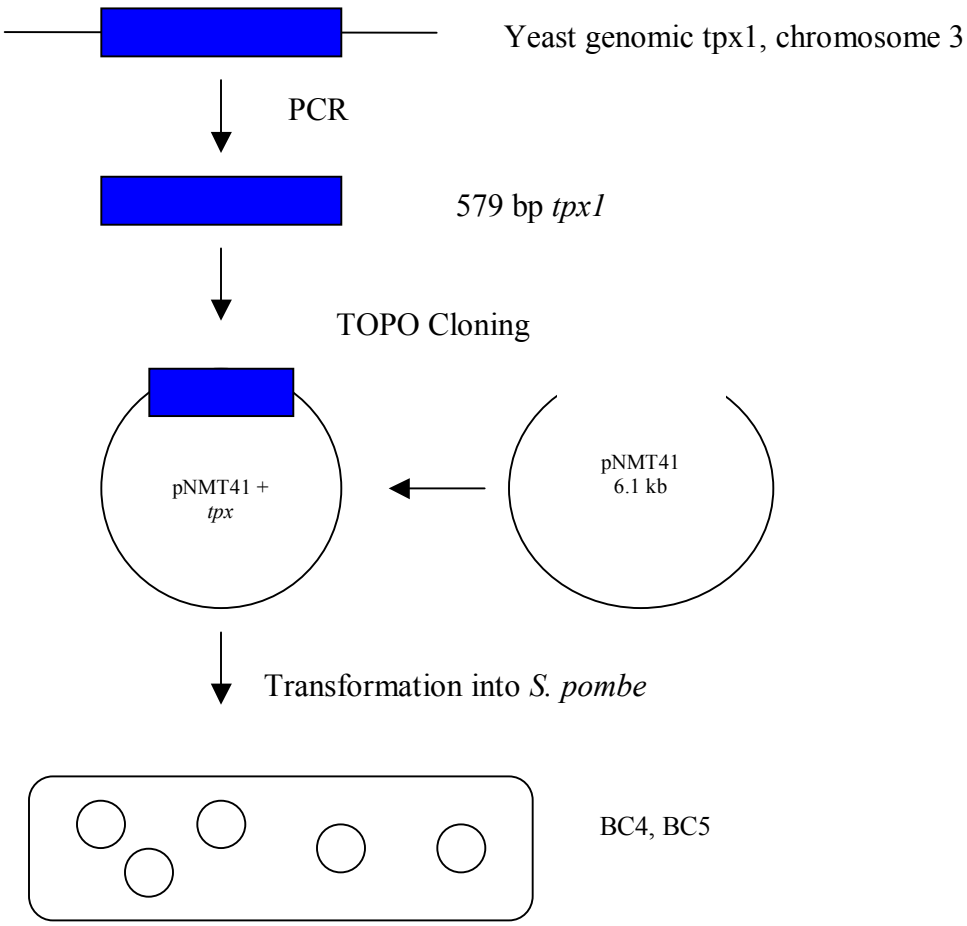


Figure 3. Cloning Strategy used to construct BC4 and BC5. The gene of interest, *tpx1* is inserted into the TOPO-41 vector, and the entire plasmid is subsequently transformed into *S. pombe* strains FWP6 and TP108-3c.

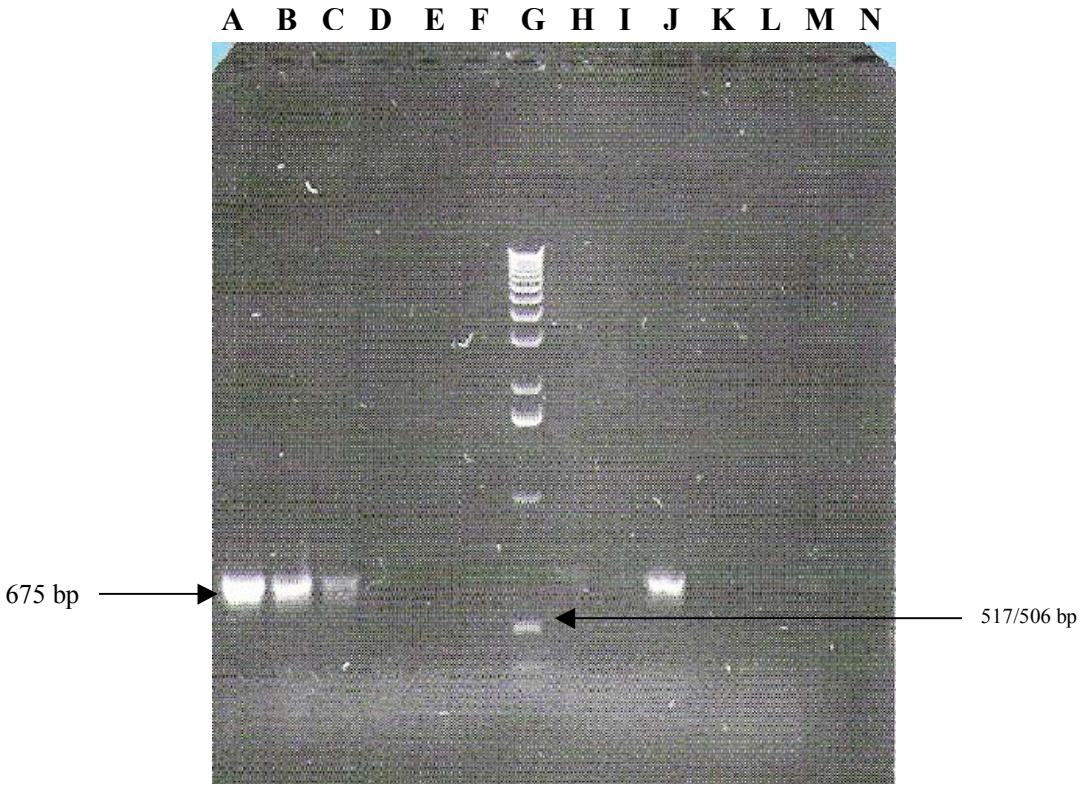


Figure 4. Verification of *tpx1* inserted in proper orientation in TOPO
A 1% agarose gel was used to separate the amplification products from colony PCR. PCR was performed on twelve of the colonies resulting from TOPO cloning; the primers SP35 (URA4 primer) and SP30 were used to confirm the inserts were in the TOPO vector in the correct orientation. In four out of the twelve colonies, *tpx1* inserts were oriented correctly within the plasmid. The expected size of the amplified segment was 675 bp, as the URA4 primer adds an additional 130 bp to the 545 bp *tpx1* gene.

Lane A – F: TOPO + *tpx1* Samples 1 – 6
Lane G: DNA Molecular Weight Marker X
Lane H – M: TOPO + *tpx1* Samples 7 – 12

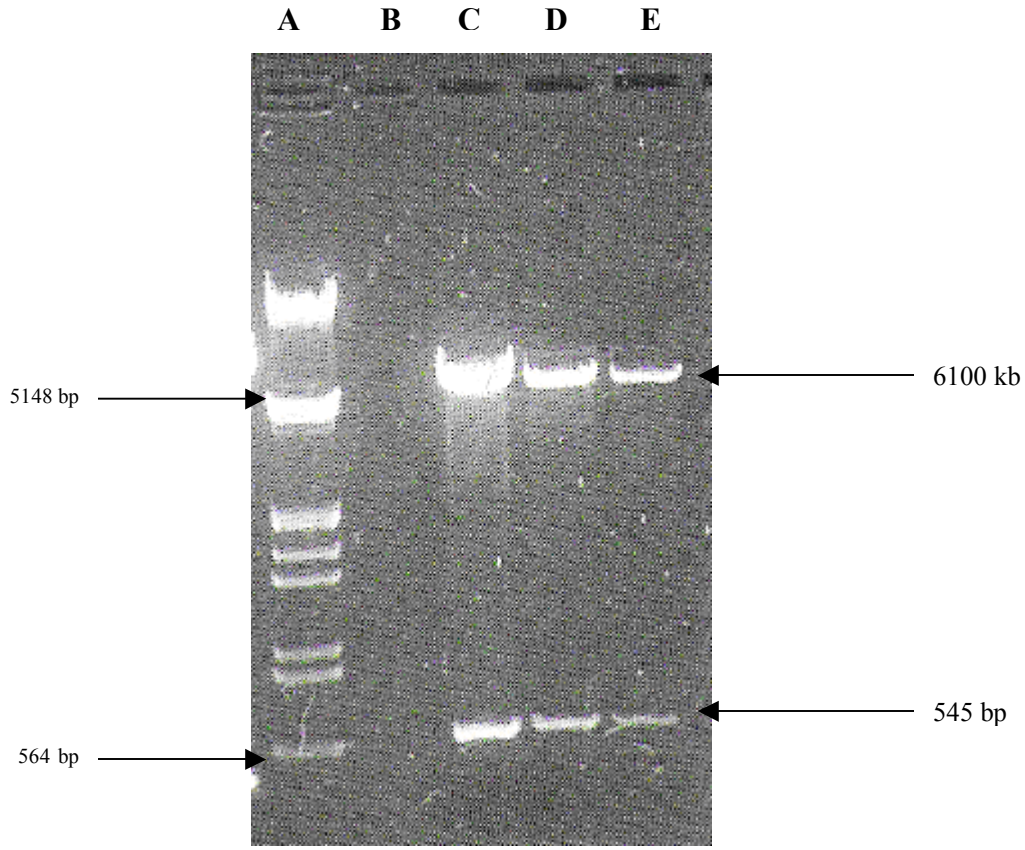
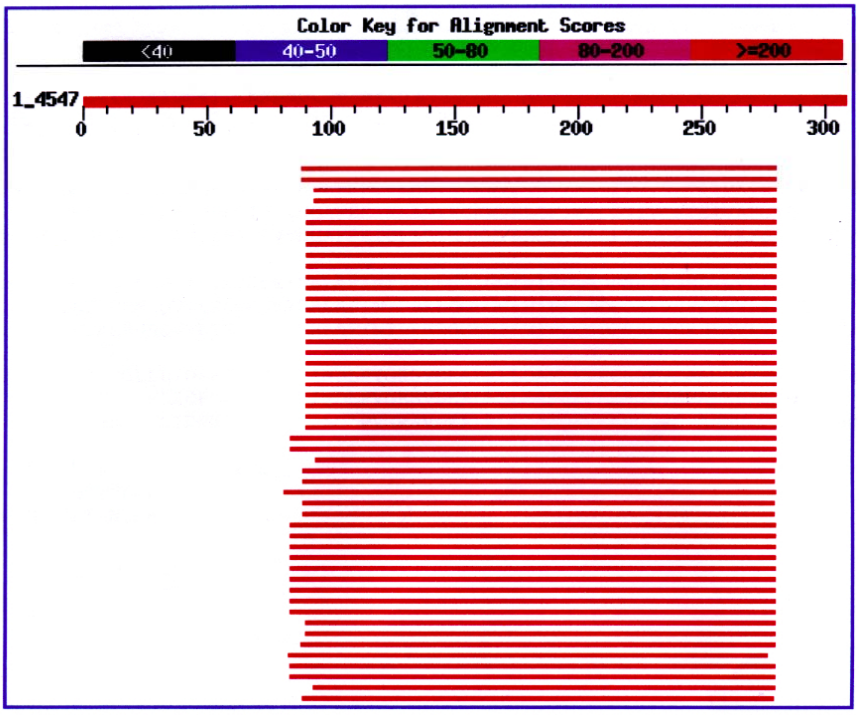


Figure 5. Verification of the presence of *tpx1* in the plasmid isolated by MidiPrep
A 1% agarose gel was used to analyze the results of digesting the plasmid isolated via MidiPrep with the restriction enzymes *Eco*RI. Two bands are expected upon digestion of the TOPO + *tpx1* plasmid with *Eco*RI, due to the presence of two cut sites, one on either side of the insert. The expected size of the TOPO vector is 6.1 kilobases and the expected size of the insert is 545 bp.

Lane A: DNA Molecular Weight Marker III
Lane B – D: TOPO + *tpx1* plasmid digested with *Eco*RI



Sequences producing significant alignments:		Score (bits)	E Value	
gi|3687455|emb|AL031798.1|SPCC576	S.pombe chromosome III co...	391	e-107	
gi|3834572|gb|AF083335.1|	Schizosaccharomyces pombe thiored...	391	e-107	G
gi|12407846|gb|AF305562.1|	Bos taurus peroxiredoxin 2 mRNA,...	275	3e-72	G U
gi|31340884|ref|NM_174763.2|	Bos taurus peroxiredoxin 2 (PR...	275	3e-72	G U
gi|33188450|ref|NM_005809.4|	Homo sapiens peroxiredoxin 2 (...	273	1e-71	G U E
gi|24659878|gb|BC039428.1|	Homo sapiens peroxiredoxin 2, tr...	273	1e-71	G U E
gi|33875462|gb|BC000452.2|	Homo sapiens peroxiredoxin 2, tr...	273	1e-71	G U E
gi|12804326|gb|BC003022.1|	Homo sapiens peroxiredoxin 2, tr...	273	1e-71	G U
gi|50506564|emb|CR625757.1|	full-length cDNA clone CS0DB005...	273	1e-71	U
gi|50504904|emb|CR624097.1|	full-length cDNA clone CS0DB004...	273	1e-71	U
gi|50504648|emb|CR623841.1|	full-length cDNA clone CS0DC021...	273	1e-71	U
gi|50503342|emb|CR622535.1|	full-length cDNA clone CS0DA007...	273	1e-71	U
gi|50503294|emb|CR622487.1|	full-length cDNA clone CS0DC021...	273	1e-71	U
gi|50501774|emb|CR620967.1|	full-length cDNA clone CS0DM013...	273	1e-71	U
gi|50497151|emb|CR616344.1|	full-length cDNA clone CS0DA003...	273	1e-71	U
gi|50495853|emb|CR615046.1|	full-length cDNA clone CS0DC007...	273	1e-71	U
gi|50483357|emb|CR602550.1|	full-length cDNA clone CS0DA001...	273	1e-71	U
gi|50482793|emb|CR601986.1|	full-length cDNA clone CS0DC016...	273	1e-71	U

http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi 12/16/2004

Figure 6. ***Verification of identity of sequence***
A translating BLAST was performed using the nucleotide sequencing results and the protein sequence of Tpx1. The tBLASTn search demonstrated high homology to both the *S. pombe* chromosome III cosmid as well as the sequence of *S. pombe* thioredoxin peroxidase mRNA.

II. Creation of overexpression strains

Using the High Efficiency Transformation protocol, the plasmids containing the *tpx1* gene were transformed into three mutant yeast strains: TP108-3C, TP367, and FWP6. In the final step of this protocol, cell solutions were plated onto PM –leu selective media. Colonies were produced from the transformations with FWP6 and TP108-3C, named BC4 and BC5, respectively, indicating that these cells had successfully taken up the plasmid; no colonies were seen on the plate containing the TP367 cell solution (see Table 5). Successful transformations were patched on a new PM –leu plate and later examined under a microscope to verify that the cells comprising the resulting colonies were actually *S. pombe*. Two of the colonies, one from BC4 and one from BC5, appeared rounder and smaller than *S. pombe*; both were consequently discarded. Frozen stocks of BC4 and BC5 were made for future use. Transformations were repeated using TP367. Only one colony appeared viable on the initial PM –leu plate containing 180 μL of the transformation solution; however, when it was patched onto a new PM –leu plate, there was no growth. This colony was most likely a conglomerate of cells from the initial cell solution, as the volume spread was dense. No successful transformations were produced in TP367 mutant cells.

Strain	Volume Aliquoted onto PM -leu Plate 1 (µL)	Number of Colonies on Plate 1	Colonies on Plate 1 Determined to be S. pombe	Volume Aliquoted onto PM -leu Plate 2 (µL)	Number of Colonies on Plate 2	Colonies on Plate 2 Determined to be S. pombe
BC4	250	3	2	250	4	4
BC5	250	3	3	250	4	3
BC6	180	1	0	20	0	0

Table 5. Results of transformation of TOPO + *tpx* vector into FWP6, TP108-3c and TP367
Following the "High-Efficiency Transformation" protocol, solutions of the given volume were plated onto PM –leu plates. Transformation was successful in the strain FWP6 and TP108-3c; the newly created strains were named BC4 and BC5, respectfully.

III. Western Blot confirmation of Protein Production

The first step in extracting the ovexpressed proteins from the newly created strains BC4 and BC5 was to determine the optimum protocol for protein isolation. Two protein extraction protocols were examined to determine efficiency. The first protocol used acid-washed glass beads and the microbeater apparatus to induce physical disruption (Figure 7), whereas the second protocol used TCA to induce chemical disruption (Figure 8). Protein extraction results from using wild type strains 968 and 975 were compared by staining the Laemili gel with Simply Blue stain. As seen by the presence of the dark banding pattern in every lane of Figure 7, the protein extraction performed by the bead-beater method was successful; the absence of bands in all protein extraction lanes of the gel in Figure 8, corresponding to the use of TCA, indicates that this method was unsuccessful in the isolation of proteins. The bead-beater method was used in all subsequent protein extractions.

After extraction of proteins from the overexpression strains BC4 and BC5, two Laemmli protein gels were run in duplicate. One was stained with Simply Blue to confirm the presence of the proteins and to determine their relative concentrations (Figure 9). As seen by comparing Lanes B and C, the proteins extracted from BC4 were more highly concentrated than the proteins extracted from BC5. The second laemili gel was used in the Western Blotting protocol. The separated proteins were first transferred to a PVDF membrane. Next, immunoblotting, which employed two antibodies, was performed. The primary antibody, the anti-V5 antibody, was included in Invitrogen's TOPO TA Cloning Kit and binds to the V5 epitope present only on proteins produced from the TOPO + *tpx* vector. The secondary antibody, goat anti-mouse, was purchased separately from Santa Cruz Biotechnology and was detected using the LumiGLO Chemiluminscent Substrate System. Illumination was detected using x-ray film. The blot was

exposed to film for 1 minute (Figure 10). Bands visible on the film were compared to the protein standard to determine the size of the proteins present. The Tpx protein has a known weight of 21.2 kilodaltons (kDa). The protein transcribed from the TOPO vector also includes the V5 epitope and the poly-Histidine (6xHis) tag, which adds approximately 2.58 kDa to the predicted molecular weight. The results of the western blot confirmed that both BC4 and BC5 were producing the Tpx protein based on the location of the band; the production of overexpression strains was confirmed.

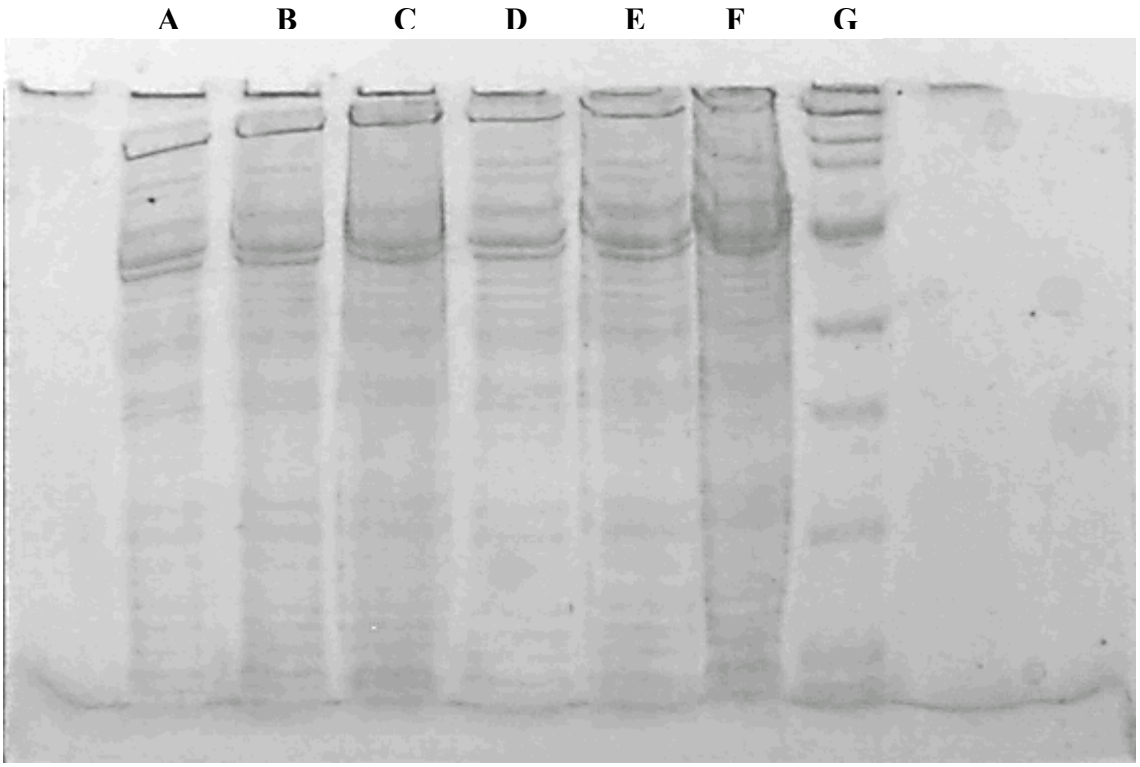


Figure 7. Verification of protein extraction via bead-beater method
A Laemmli protein gel was used to separate the protein extractions performed on *s. pombe* strains 968 and 975 using the bead-beater method. The presence of proteins in Lanes A-F indicates that the procedure was successful.

Lane A: 5 µL 968 protein extract
Lane B: 10 µL 968 protein extract
Lane C: 20 µL 968 protein extract
Lane D: 5 µL 975 protein extract
Lane E: 10 µL 975 protein extract
Lane F: 20 µL 975 protein extract
Lane G: 5 µL Pre-stained SDS-PAGE Protein Standard

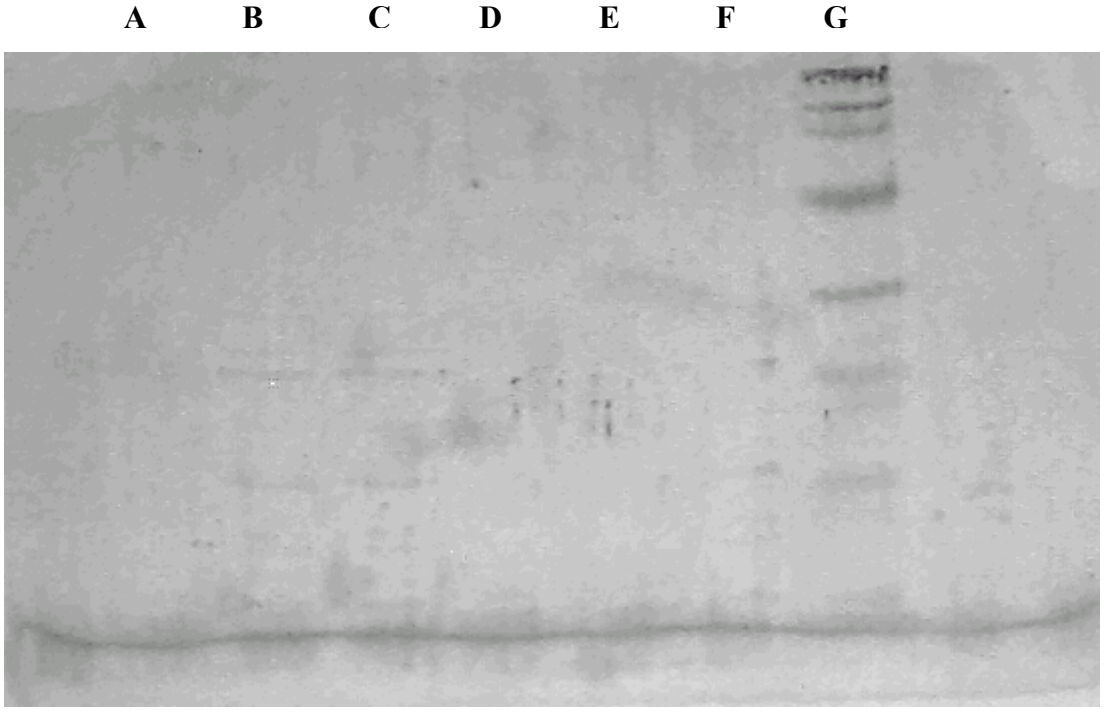


Figure 8. Verification of protein extraction via TCA method
A Laemmli protein gel was used to separate the protein extractions performed on *S. pombe* strains 968 and 975 using the TCA method. The absence of proteins in Lanes A-F indicates that the procedure was unsuccessful.

Lane A: 5 µL 968 protein extract
Lane B: 10 µL 968 protein extract
Lane C: 20 µL 968 protein extract
Lane D: 5 µL 975 protein extract
Lane E: 10 µL 975 protein extract
Lane F: 20 µL 975 protein extract
Lane G: 5 µL Pre-stained SDS-PAGE Protein Standard

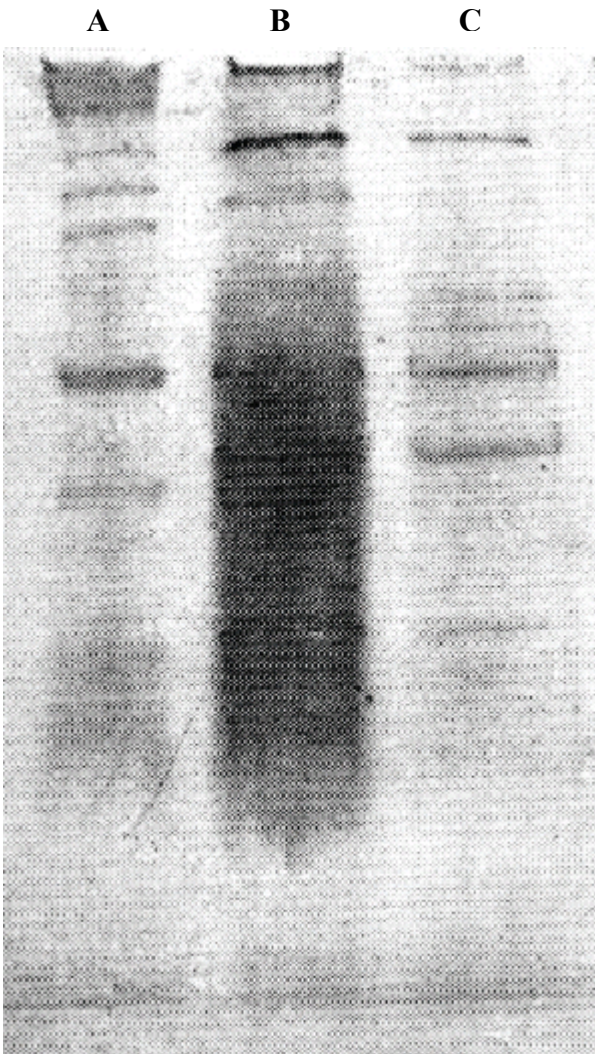


Figure 9. Verification of successful protein isolation
A Laemmli protein gel was used to separate the protein extractions performed on *S. pombe* strains BC4 and BC5 using the bead-beater method. The presence of protein in Lanes B and C indicate that the procedure was successful.

Lane A: Pre-stained SDS-PAGE Protein Standard
Lane B: Protein extract from BC4
Lane C: Protein extract from BC5

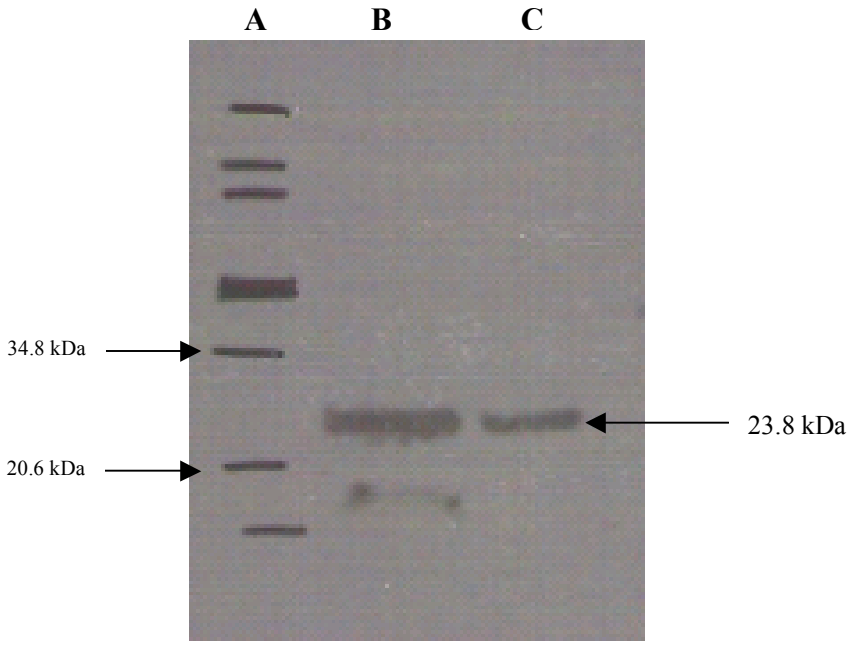


Figure 10. Confirmation of the overexpression of Tpx in BC4 and BC5
A western blot was performed to confirm the overexpression of the Tpx protein in *S. pombe* strains BC4 and BC5 created by transforming FWP6 and TP108-3c, respectively, with the TOPO + *tpx* plasmid. Proteins were detected using the anti-V5 antibody and the goat anti-mouse secondary antibody followed by illumination with chemiluminescence. The expected size of the protein is approximately 23.8 kDa, as the V5 epitope adds approximately 2.6 kDa to the 21.2 kDa of the Tpx1 protein.

Lane A: SDS-PAGE Protein Standard
Lane B: BC4
Lane C: BC5

IV. Construction of deletion cassette for homologous recombination

The protocol for the deletion cassette used to create *tpx1* knockouts employing the process of homologous recombination is outlined in Figure 11. Cosmid c576 (Figure 12) was used as a template in PCR reactions designed to amplify the 5' and 3' flanking sequences for the *tpx1* gene. The 5' flanking sequence corresponded to the nucleotides 4531 to 4853. The primers used to generate this fragments were SP53 and SP54 (shown in Figure 2), generating an amplification product 322 bp in length. The SP53 primer has a unique restriction site for *Bam*HI which was also used to join the two first-round PCR products. The 3' flanking sequence corresponded to the nucleotides 5494 to 5730. The primers used to generate this fragment were SP55 and SP56 (shown in Figure 2), generating an amplification product 236 bp in length. The SP55 primer has a unique restriction site for *Bam*HI which was also used to join the two first-round PCR products. To verify amplification of the 5' and 3' flanking sequence, small aliquots of the PCR reactions were run on a 1% agarose gel and stained with ethidium bromide. The gel results, as seen in Figure 13, verified the correct size of the PCR product for both sequences.

Next, a second round of PCR was performed in order to join the two "first-round" PCR products into a single product through the complementary sequences of the SP53 (5' forward) and SP55 (3' reverse) primers. The second-round PCR product was formed by combining equal amounts of the two first-round PCR products for five-cycles of PCR without additional primers. Then, a 35 cycle PCR reaction was performed with primers SP54 and SP56 in order to amplify the "second-round" PCR product. To verify that the first-round PCR product was joined into a single second-round PCR product, and that this 545 bp product was amplified, a small aliquot of the 35 cycle PCR reaction was run on a 1% agarose gel and stained with ethidium bromide. The gel results, as seen in Figure 14, verified the presence and correct size of the PCR product.

Next, the amplified 545 bp segment created in the second round of PCR was transformed into the TOPO-41 vector. In order to determine if TOPO cloning was successful, the solution was plated on selective media. Formation of colonies on LB with ampicillin plates verified that the plasmid was taken up by the E. coli cells, as the plasmid contains the gene conferring ampicillin resistance. Each colony was derived from a single cell into which a plasmid containing the deletion cassette was transformed. A MidiPrep was subsequently performed to isolate the deletion cassette plasmid. The plasmid was then sequenced with the forward primer of the vector, SP34, and the reverse primer for the vector sequence, SP35. A BLAST to align two sequences was performed in order to compare the nucleotide sequencing results to the nucleotide sequence of the pNMT41 plasmid; regions of homology between the two sequences supported the conclusion that no gap was inserted before the inserted 5' plus 3' flanking region segment of DNA previously created by the sequential PCR reactions. The integrity of the cassette was then examined by digesting with both *Eco*RI and *Bam*HI. As predicted, restriction digest of the deletion cassette with *Eco*RI produced two bands, one the size of the generated 5' plus 3' flanking region segment (545 bp) and one the size of the TOPO vector (6.1 kb). Furthermore, as predicted, the restriction digest performed on the deletion cassette with *Bam*HI created a single, linearized DNA segment, demonstrated as a single band at 6.6 kilobases (Figure 15).

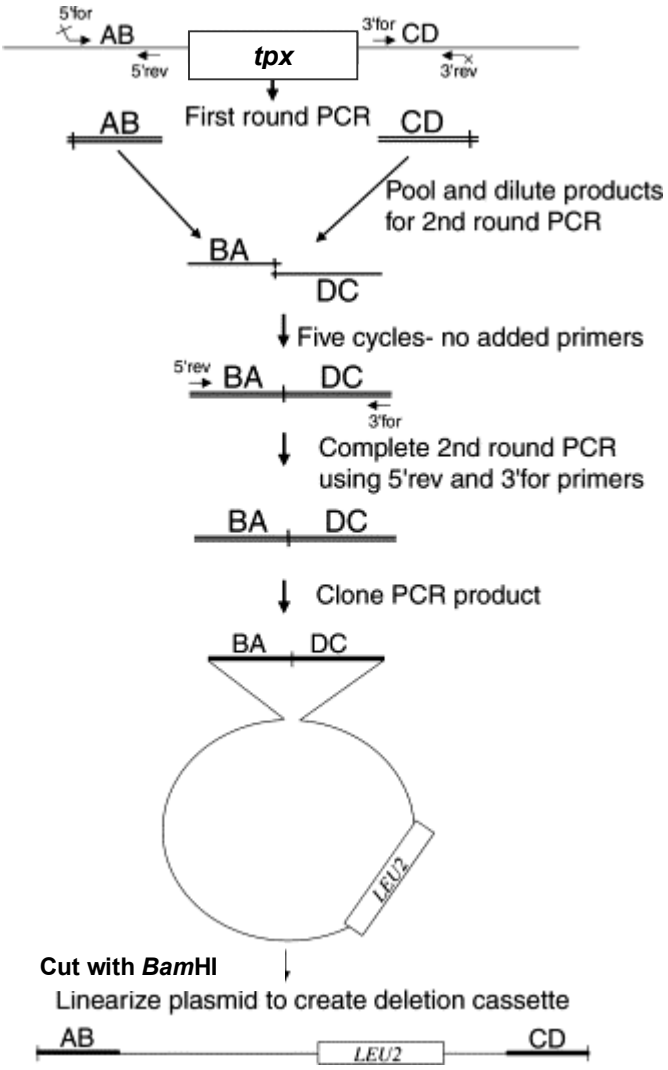
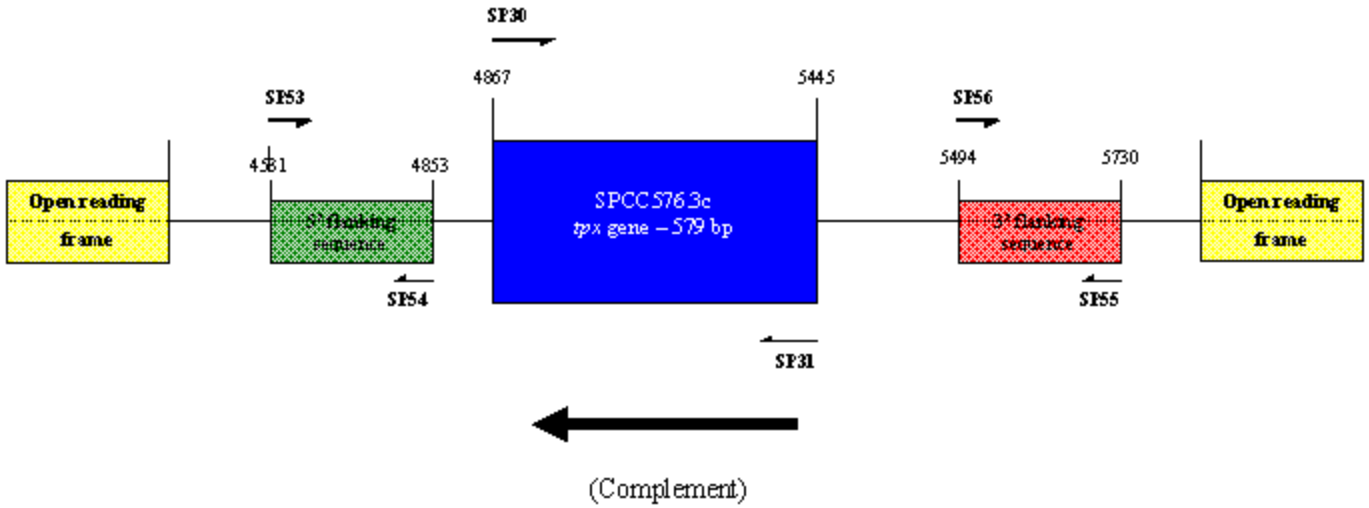


Figure 11. Deletion Cassette Construction
The procedure used to create a deletion cassette and the subsequent linearization of the plasmid which was used in homologous recombination is graphically illustrated. The segment AB corresponds to the 5' flanking arm while the segment CD corresponds to the 3' flanking arm.

Figure 12. Cosmid SPCC576



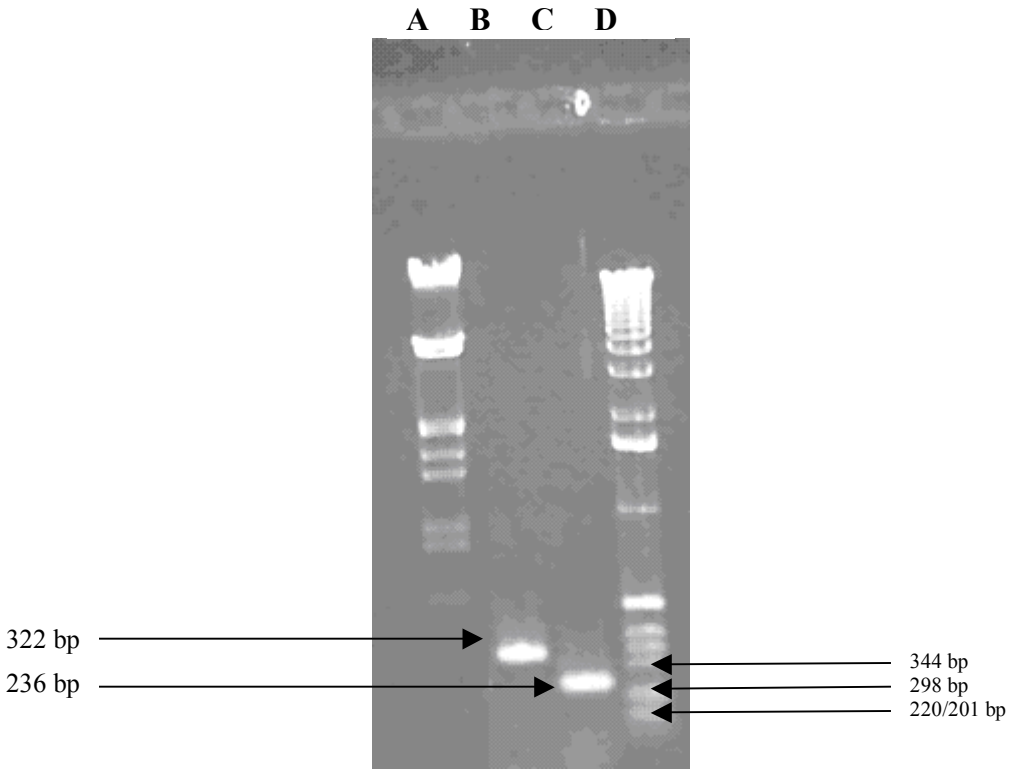


Figure 13. Verification of 5' and 3' flanking sequence amplification
A 1% agarose gel was used to separate the amplification products of the first-round of PCR in the construction of the deletion cassette. The 5' flanking sequence of the *tpx* gene was amplified from cosmid DNA using the primers SP53 and SP54. The expected size of the PCR product is 322 base pairs. The 3' flanking sequence of the *tpx* gene was amplified from cosmid DNA using the primers SP55 and SP56. The expected size of the PCR product is 236 base pairs. Results in Lanes C and D were used by Scott Davis.

Lane A: DNA Molecular Weight Marker III
Lane B: *tpx* 5' arm
Lane C: *tpx* 3' arm
Lane D: DNA Molecular Weight Marker X

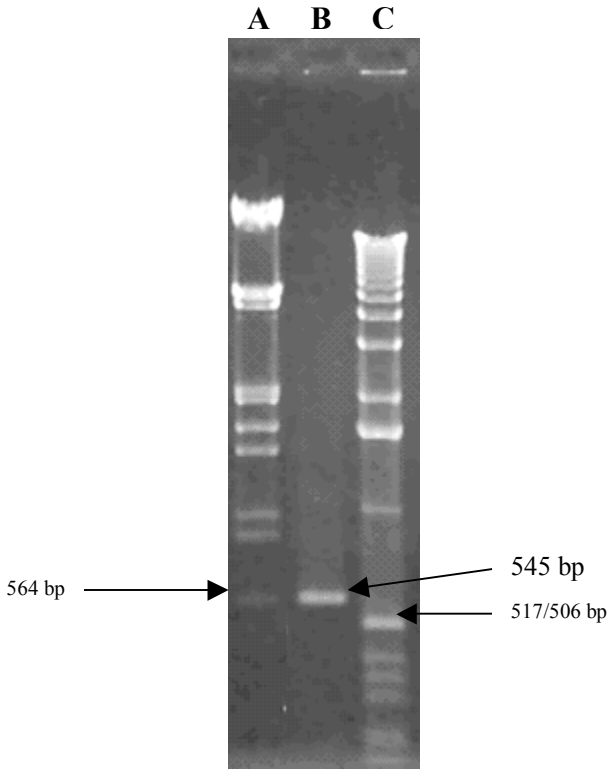


Figure 14. Verification of annealed flanking region segment
A 1% agarose gel was used to separate the amplification products from two rounds of PCR. The first was a five-cycle amplification of the first-round products in the absence of additional primers that allowed the annealing of the lower strand of the 5' flanking region product with the upper strand of the 3' flanking region product. The next round was a standard 35 cycle PCR amplification using the primers SP54 and SP56. The expected size of the amplified tpx product is 545 base pairs.

Lane A: DNA Molecular Weight Marker III
Lane B: *tpx* PCR product
Land C: DNA Molecular Weight Marker X

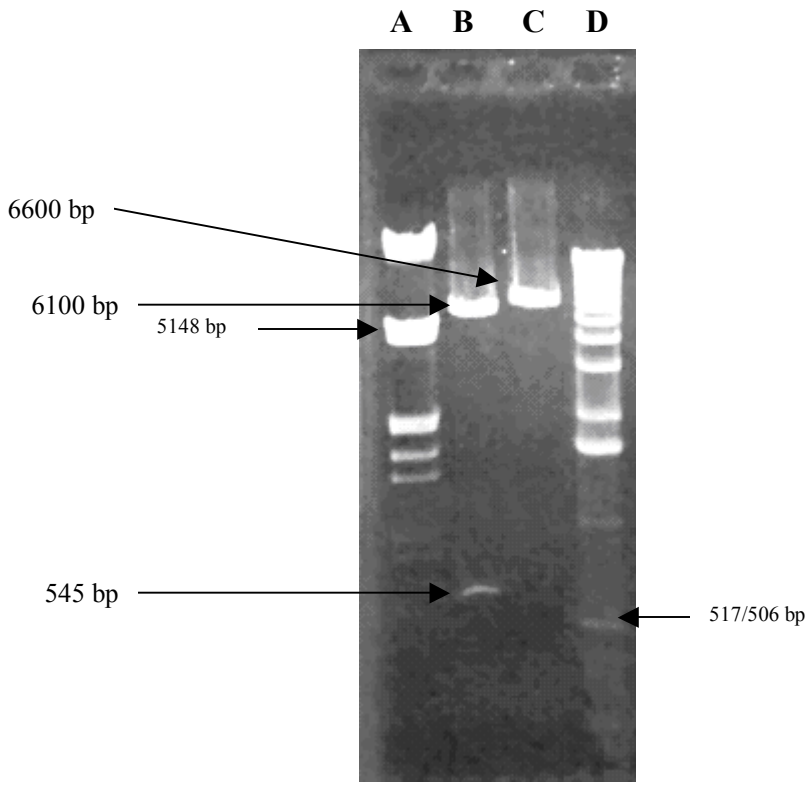


Figure 15. Verification of cassette digestion with *Eco*RI and *Bam*HI
A 1% agarose gel was used to analyze the results of digesting the deletion cassette plasmid with restriction enzymes *Eco*RI and *Bam*HI. Two bands are expected upon digestion of cassette with *Eco*RI, due to the presence of two cut sites, one on either side of the insert. The expected size of the TOPO vector is 6.1 kilobases and the expected size of the insert is 545 base pairs. A single band is expected upon digestion of the cassette with *Bam*HI, due to the presence of the internal cut site located in the middle of the annealed flanking sequence insert. The expected size of the band is 6.6 kilobases.

Lane A: DNA Molecular Weight Marker III
Lane B: TOPO + *txp* annealed flanking region digested with *Eco*RI
Lane C: TOPO + *txp* annealed flanking region digested with *Bam*HI
Lane D: DNA Molecular Weight Marker X

V. Creation of knockout strains

The deletion cassette was linearized with *Bam*HI and then used to induce homologous recombination in FWP6 genomic DNA according to the protocol established for Ultra-High Efficiency Transformation. Successful transformations were indicated by the presence of single colonies on a selective media plate of EMM –leu; six colonies were isolated and used in further experimentation. Colonies were then streaked on a new EMM –leu plate to confirm their ability to grow on the selective media. Subsequently, the "Smash & Grab" method was performed on the six potential knockouts. After confirming that the genomic DNA was successfully isolated for all six samples (Figure 16), two experiments were conducted to determine if homologous recombination occurred and subsequently knocked-out the *tpx* gene.

First, two different PCR experiments were performed on the genomic DNA. One reaction employed the primers for the *tpx* gene, SP30 and SP31. If homologous recombination occurred, no band would be present in lanes corresponding to this reaction. If a band is present at 579 bp, the *tpx* gene remains, and the transformation has resulted in nonhomologous recombination or the uptake of the undigested plasmid. Another reaction, a control experiment, employed the primers for the *trr* gene, SP41 and SP42. If homologous recombination occurred, a band would be visible at 965 bp, as this gene should not be disrupted. As seen in Figure 17, the results were inconclusive. As seen in Lane I and Lane S, PCR successfully amplified the *tpx* and *trr* gene on the cosmid c576 DNA. It appears that PCR was unsuccessful on all genomic DNA isolated via the "Smash and Grab" method, as no bands were seen in Lanes B-H or in Lanes L-R. Although no bands would be expected in Lanes B-H if homologous recombination was successful, as the *tpx* gene would be knocked out, bands corresponding to the *trr* gene should be present in Lanes L-R as this gene should be unaffected by the transformation. The absence of

bands in both lanes suggests that the DNA was impure and therefore the primers in the PCR reaction were unable to access the appropriate sequence and amplify the gene. From the PCR experiments, there was no verification of the creation of knockouts.

In a separate experiment, the genomic DNA was analyzed using Southern Blot procedures in an attempt to verify the creation of knockouts. First, digoxigenin (DIG) probes corresponding to the *tpx* gene were synthesized using the PCR DIG Probe Synthesis kit, the primers SP30 and SP31, and the TOPO + *tpx* plasmid previously constructed. A 1% agarose gel, followed by the transfer of the gel to a nylon membrane and detection using the anti-DIG antibody, chemiluminescence, and exposure to x-ray film for 5 minutes, was used to verify the construction of the plasmid (Figure 18). The expected size of the band was 579 bp. The presence of a band at approximately 579 bp in the lane containing a 1:10 dilution of the labeled probe confirmed the synthesis of the probe. The absence of a band in the 1:1000 dilution of the labeled probe indicates that longer detection times are required for detection. Next, it was necessary to confirm the ability of the newly constructed probe to hybridize with DNA known to contain the *tpx1* gene. The TOPO + *tpx* plasmid was run out on a 1% agarose gel. The hybridization protocol outlined in the DIG Probe kit was followed; hybridization of the DIG probe was detected after transfer to the membrane, incubation with the anti-DIG antibody, treatment with chemiluminescence, and exposure to x-ray film for 30 seconds (Figure 19). The presence of bands in all three lanes indicated that the probe successfully hybridized to the plasmid containing the *tpx* gene and is also sensitive enough to probe for as few as 10^6 molecules.

To determine if the colonies from the transformation with the linearized deletion cassette resulted in homologous recombination and successfully removed the *tpx* gene, the southern blot

procedure was employed. After digesting 10 μL of the potential knockouts with EcoRI overnight, the entire volume was run on a 1% agarose gel. Confirmation of digestion was achieved by staining with ethidium bromide. As seen in Figure 20, digestion was unsuccessful for the genomic DNA isolated by the Smash and Grab method but was successful for the cosmid DNA. Because digestion was unsuccessful for the potential knockouts, the next steps of Southern Blotting were not performed. If the digest was successful, however, the DNA would have been transferred to a nylon membrane. The DIG-probe would be allowed to hybridize to the membrane as described above. If hybridization occurred, the *tpx1* gene remained, and a single band would be visible on the x-ray film after detection with the antibody and chemiluminescence. The absence of hybridization would indicate that the *tpx1* gene had been knocked out by homologous recombination and would be seen by the absence of a band on the film.

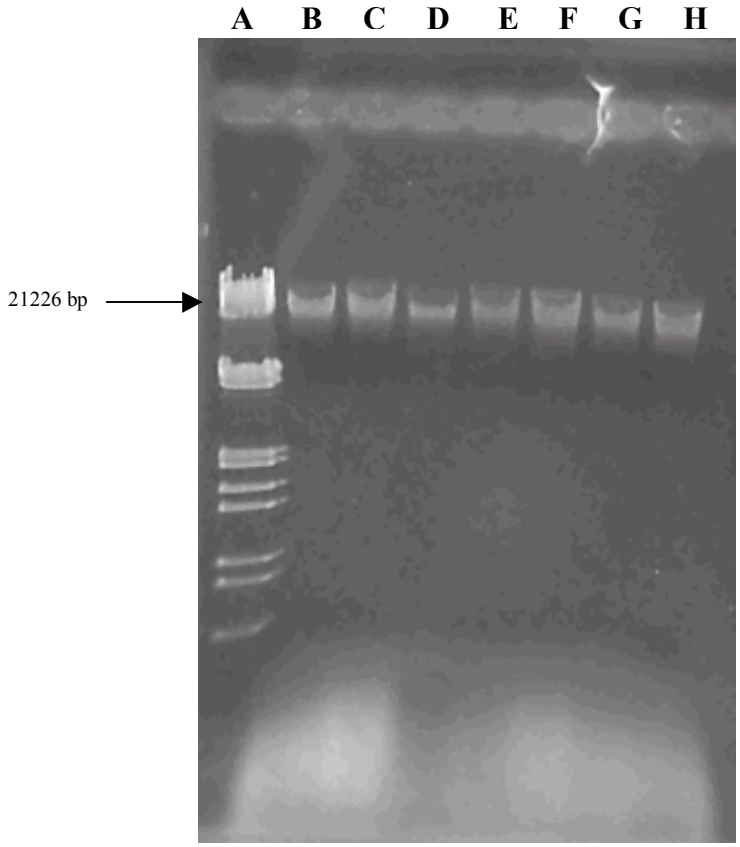


Figure 16. Verification of successful genomic DNA isolation
A 1% agarose gel was used to analyze the results of genomic DNA isolation using the Smash and Grab protoctol. The presence of DNA in every lane indicates that the isolation was successful. The expected size of the band is approximately 22000 bp.

Lane A: DNA Molecular Weight Marker III
Lane B: Smash and Grab DNA of potential knockout #1
Lane C: Smash and Grab DNA of potential knockout #2
Lane D: Smash and Grab DNA of potential knockout #3
Lane E: Smash and Grab DNA of potential knockout #4
Lane F: Smash and Grab DNA of potential knockout #5
Lane G: Smash and Grab DNA of potential knockout #6
Lane H: Smash and Grab DNA of FWP6

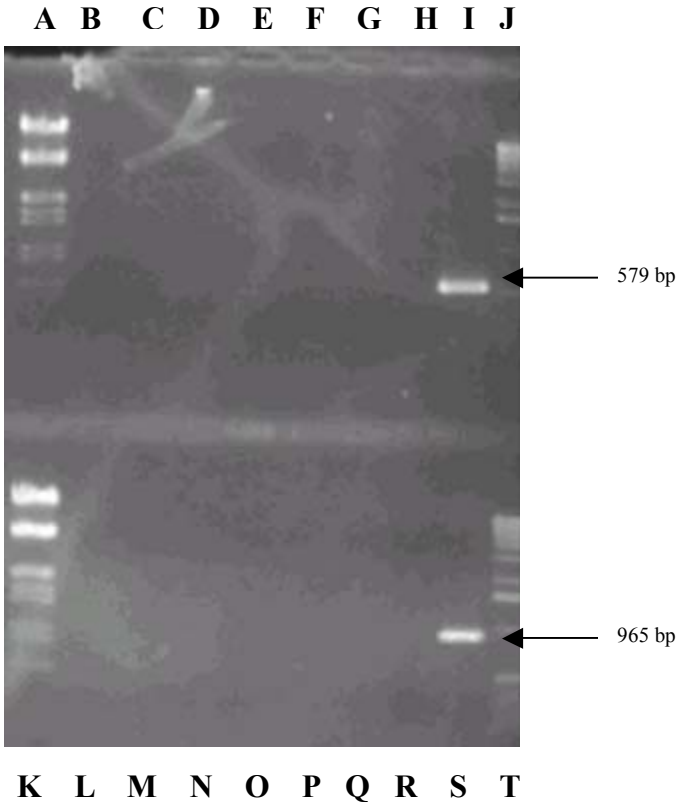


Figure 17. Verification of homologous recombination
To verify that homologous recombination occurred, two different PCR experiments were performed on the genomic DNA from the six potential knockout strains. The reactions corresponding to Lanes B-J employed the primers for the *tpx* gene, SP30 and SP31. The expected size of the *tpx* gene is 579 bp. The reactions corresponding to Lanes L-Q employed the primers for the *trr* gene, SP41 and SP42. The expected size of the *trr* gene is 965 bp. The presence of the appropriate sized bands in Lane I and Lane S indicate that PCR was successful on the cosmid DNA. The absence of bands in Lanes B-J and L-Q indicate that PCR was unsuccessful for the genomic DNA samples isolated by "Smash and Grab".

PCR with *tpx* specific primers SP30 and SP31:
Lane A: DNA Molecular Weight Marker III
Lane B - G: Smash and Grab DNA of potential knockout #1-6
Lane H: Smash and Grab DNA of FWP6
Lane I: Cosmid c576 DNA
Lane J: DNA Molecular Weight Marker X
PCR with *trr* specific primers SP41 and SP42:
Lane K: DNA Molecular Weight Marker III
Lane L - Q: Smash and Grab DNA of potential knockout #1-6
Lane R: Smash and Grab DNA of FWP6
Lane S: Cosmid c576 DNA
Lane T: DNA Molecular Weight Marker X

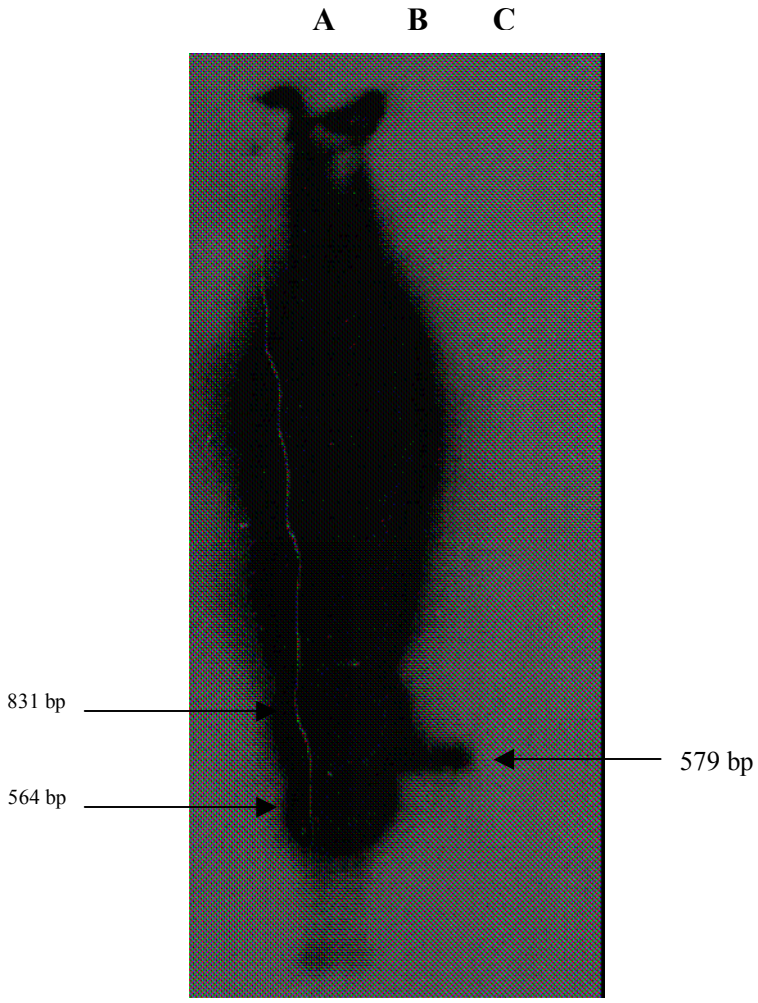


Figure 18. Verification of successfully created DIG-probe
A film was exposed to the membrane containing the DIG probe for 5 minutes to confirm the presence of the probe and to determine the sensitivity of the anti-DIG antibody. The probe was detected using the anti-DIG antibody followed by the CSPD chemiluminscent. The presence of a band in Lane B indicates the presence of the DIG-probe; the absence of a band in Lane C indicates that the probe was diluted below the point of detection. The expected size of the probe was 579 bp.

Lane A: 5 µL DIG-labeled molecular weight marker
Lane B: 5 µL 1:10 dilution of DIG probe
Lane C: 5 µL 1:1000 dilution of DIG probe

Figure 19. Verification of DIG-probe hybridization to plasmid
A film was exposed to the membrane containing the TOPO + *tpx* plasmid after the hybridization of the DIG probe. The 30 second film exposure was performed to confirm the ability of the probe to hybridize to the plasmid and to determine the sensitivity of the anti-DIG antibody. The probe was detected using the anti-DIG antibody followed by the CSPD chemiluminscent. The presence of a bands in Lanes A, B, and C indicates the integrity of the DIG-probe to hybridize to the plasmid which is know to contain the gene that the probe was designed to recognize. The presence of a band in Lane A, which contains 10^6 molecules of plasmid, confirmed the sensitivity indicated by the DIG probe kit.

Lane A: 10^6 molecules of TOPO + *tpx* plasmid
Lane B: 10^8 molecules of TOPO + *tpx* plasmid
Lane C: 10^{10} molecules of TOPO + *tpx* plasmid

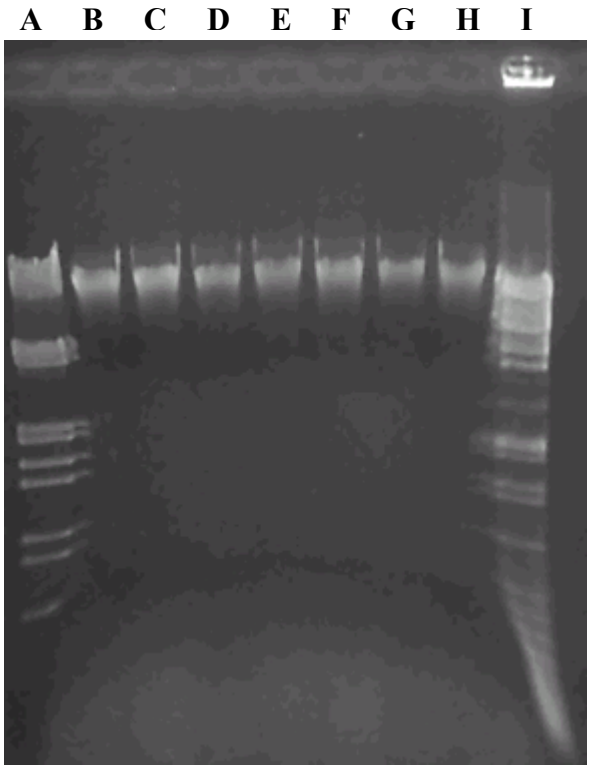


Figure 20. Verification of EcoRI restriction digest
A 1% agarose gel was used to analyze the result of the overnight EcoRI restriction digest on the genomic DNA of potential *tpx1* knockouts #1-6, the genomic DNA of FWP6 and the cosmid DNA C576. Digestion was successful only on the cosmid DNA, as seen by the presence of numerous bands of various sizes in Lane I; the presence of a single band in Lanes B-H indicate that digestion was unsuccessful.

Lane A: DNA Molecular Weight Marker III
Lane B - G: EcoRI digest of potential knock outs #1-6 genomic DNA
Lane H: EcoRI digest of FWP6 genomic DNA of FWP6
Lane I: EcoRI digest of cosmid C576 DNA

VI. Reactive Oxygen Species (ROS) Sensitivity Tests

The novel *S. pombe* strain, BC4, in which the Tpx protein is being overexpressed, as confirmed by Western Blot, was selected for sensitivity tests. Five experiments were run simultaneously. Four chemicals known to generate ROS were examined: hydrogen peroxide (H_2O_2), menadione, *tert*-Butyl hydroperoxide (tBH), and paraquat; water was examined as a point of comparison to determine how BC4 would grow in the absence of a ROS. The wildtype *S. pombe* strain FWP6 was used as the control to test for normal yeast growth under the oxidative stress conditions presented by the additions of the ROS or water to a solution of cells growing in log phase. In order to determine the appropriate concentration of each chemical needed to induce a stress but provide a balance between survival and death, a range of concentrations was tested; optimization was determined by the ability of the strain to form individual colonies on YEA media. For H_2O_2, concentrations from 7.5 mM to 0.75 mM were explored; 1.5 mM was determined to be the best concentration and used in subsequent experiments. For menadione, concentrations from 0.1 mM to 0.01 mM were explored; 0.06 mM was determined to be the best concentration and used in subsequent experiments. For tBH, concentrations from .75 mM to 0.075 mM were explored; 0.75 mM was determined to be the best concentration and used in subsequent experiments. For paraquat, concentrations from 10 mM to 1 mM were explored; 10 mM was determined to be the best concentration and used in subsequent experiments. A 20 µL volume of water was used in the experiments.

Initially, 2 mL cultures of FWP6 and BC4 were created by diluting cells growing in log phase, as confirmed by visible replication under the microscope, to a concentration of 2×10^6 cells/mL. At this point (t = 0 hrs), serial dilutions were made (1:10, 1:100, 1:1000 and sometimes 1:10000) and a 5 µL volume was spotted onto a YEA plate along with a 5 µL volume

of the undiluted culture (Figure 21). After spotting, in order to determine the viability of the BC4 strain in comparison with the wildtype FWP6 strain, the designated concentration of the chemical was added to the 2 mL cultures of both BC4 and FWP6 cells. After two hours (t = 2 hrs) of incubation of these cultures at 30°C and shaking at 200 rotations per minute, another set of serial dilutions were made and spotted (Figure 22). The cells were incubated for an additional four hours (t = 6 hrs); serial dilutions were made and spotted (Figure 23). The cells were then incubated overnight. Twenty-four hours after the initial addition of chemicals (t = 24 hrs), the samples were removed, diluted and spotted (Figure 24). All of the plates were grown for 46 hours at 30°C.

The plates were then analyzed by the number of separate colonies formed at dilutions 1:100 and 1:1000 as well as the concentration of growth at 1:1 and 1:10. For each chemical, the growth of the BC4 strain was compared to that of FWP6 (Table 6). At time t = 0 hours, before the chemicals were added, the concentration of growth at 1:1, 1:10, and 1:100 were consistent between BC4 and FWP6 and for each of the four chemicals as well as water (Figure 21). The number of separate colonies was distinguishable only at the 1:1000 dilution. The number of colonies of BC4 were on the same magnitude as the number of colonies of FWP6 for all four chemicals as well as water, although the number of colonies of FWP6 were fewer than that of BC4 in the cell cultures containing H_2O_2 and menadione; the number of colonies of FWP6 was greater than that of BC4 in the cell culture to contain paraquat. These differences can be attributed to the sample of the original solution initially pipetted, and were not significant in the comparison of cell viability.

Two hours after adding the chemicals, differences in cell viability were visible (Figure 22). Serial dilutions of the cell cultures containing H_2O_2 demonstrated that overexpression of

Tpx1 increases oxidative stress resistance. The concentration of growth at 1:1 and 1:10 was confluent in both BC4 and FWP6. At 1:100, colonies were visible for both strains, but BC4 appeared denser. The difference was visible at the dilution 1:1000, as 13 BC4 colonies were viable but only one, very small FWP6 colony was viable. The serial dilutions of the cell cultures containing menadione demonstrated that overexpression of Tpx1 also increases oxidative stress resistance in the presence of this ROS generator. The concentration of growth at 1:1 was confluent in both BC4 and FWP6. At 1:10, the growth of BC4 was confluent, but separate colonies can be distinguished for FWP6. At 1:100, colonies were visible for both strains; individual BC4 colonies were indistinguishable but 23 FWP6 colonies visible. A significant difference was visible at the dilution 1:1000, as 18 BC4 colonies were viable but only one, very small FWP6 colony was viable. The serial dilutions of the cell cultures containing tBH demonstrated that overexpression of Tpx1 also increases oxidative stress resistance in the presence of this ROS generator. The concentration of growth at 1:1 was confluent in both BC4 and FWP6. At 1:10, the growth of BC4 was confluent, but separate colonies can be distinguished for FWP6. At 1:100, colonies were visible for both strains; BC4 colonies were larger and denser, whereas FPW6 colonies were very small and less dense. A difference was visible at the dilution 1:1000, as 2 large and 2 small BC4 colonies are viable but only 3 very small FWP6 colonies are viable. The serial dilutions of the cell cultures containing paraquat demonstrated that overexpression of Tpx1 also increases oxidative stress resistance in the presence of this ROS generator. The concentration of growth at 1:1 and 1:10 was confluent in both BC4 and FWP6, but less dense for FWP6 at 1:10 as compared to the BC4 spot at 1:10. At 1:100, growth was confluent for BC4, but individual FWP6 colonies were visible. A significant difference was visible at the dilution 1:1000, as there were more than 30 BC4 colonies which

have grown together, whereas only 9 FWP6 colonies were viable. The concentration and number of cells for BC4 and FWP6 were consistent at all serial dilutions for the cell cultures containing water, indicating that differences seen previously are incurred by the presence of the various chemicals.

After six hours of incubation with the various chemicals, fewer differences of viability between BC4 and FWP6 strains were visible (Figure 23). When comparing all of the serial dilutions of the BC4 and FWP6 cell cultures containing H_2O_2, little difference is seen; at 1:1000, 5 large and 3 small BC4 colonies were present whereas 5 large and 2 small FWP6 colonies were present. For the cell cultures containing menadione, the overepxression of Tpx1 has resulted in a difference in viability between BC4 and FWP6. Growth was confluent for both strains at the 1:1 dilution, but at 1:10, the growth of BC4 is confluent but not for FWP6. At 1:100, individual BC4 and FWP6 colonies were visible, but the spot of BC4 was much denser. The difference was quantifiable at 1:1000, as 10 BC4 colonies were viable, whereas only 4 very small FWP6 colonies were viable. For the cell cultures containing tBH at 6 hours, the overepxression of Tpx1 has resulted in a difference in viability between BC4 and FWP6. Growth was confluent for both strains at the 1:1 dilution, but at 1:10, the growth of BC4 was confluent but not for FWP6. At 1:100, individual BC4 and FWP6 colonies were visible, but the spot of BC4 was much denser. The difference was quantifiable at 1:1000, as 4 BC4 colonies were viable, whereas only 1 very small FWP6 colony was viable. Comparison of all of the serial dilutions of the BC4 and FWP6 cell cultures containing paraquat reveals little difference in viability. The FWP6 spot was slightly less dense at 1:100 than that of BCR. At 1:1000, 10 small BC4 colonies were present whereas 4 small FWP6 colonies are present. Again, the concentration and number of cells for

BC4 and FWP6 were consistent at all serial dilutions for the cell cultures containing water, indicating that differences seen previously are incurred by the presence of the various chemicals.

Twenty-four hours after the initial addition of the chemicals, the results were various (Figure 24) . The FWP6 cell culture containing H_2O_2 produced more viable cells than the BC4 cell culture containing H_2O_2. At the 1:1 and 1:10 dilutions, growth was confluent for both strains. At 1:100, growth of FWP6 was confluent; although very dense, growth was not confluent for BC4 at the same dilution. At 1:1000, no individual FWP6 colonies could be distinguished, but large BC4 colonies are visible. At 1:10,000 only 2 BC4 colonies were viable whereas 22 FWP6 colonies were viable. When comparing all of the serial dilutions of the BC4 and FWP6 cell cultures containing menadione, little difference was seen. At 1:1000, both spots were very dense, but not completely confluent; although some individual colonies were distinguishable, colonies have grown together making quantifiable analysis impossible. The overexpression of Tpx1 appears to have increased the resistance of BC4 to oxidative stress caused by tBH when compared to FWP6 at 24 hours. At 1:1 and 1:10 growth was confluent for both strains. At 1:100, growth was confluent for BC4 and also for FWP6, but uneven borders were apparent for FWP6, indicating that the growth was less dense. At 1:1000, BC4 growth was very dense whereas 14 individual FW6 colonies were distinguishable. When comparing all of the serial dilutions of the BC4 and FWP6 cell cultures containing paraqut, little difference was seen; at 1:1000, 1 BC4 colony was present and 2 FWP6 colonies were present. Again, in the cell cultures containing water, the concentration and number of cells for BC4 and FWP6 were consistent at all serial dilutions.

Time (hours)	Strain	Colonies at 1:1000 dilution				
		H_2O_2	Menadione	tBH	Paraquat	Water
0	BC4	10	14	11	5	19
	FWP6	2	5	8	15	15
2	BC4	13	18	2 Lg, 2 Sm	>30	16
	FWP6	1	1	3 Sm	9	6
6	BC4	5 Lg, 3 Sm	10	4	10 Sm	34
	FWP6	5 Lg, 2 Sm	4 Sm	1	4 Sm	19
24	BC4	2*	dense	confluent	1	confluent
	FWP6	22*	dense	14	2	confluent

* Colonies counted at 1:10,000 dilution

Table 6. Reactive oxygen species sensitivity experiment
The number of colonies on each plate were counted at the 1:1000 dilutions and recorded; each plate was a specific time and chemical. The differentiation between large colonies and small colonies was made only when there was an apparent difference. Growth was determined "dense" when individual colonies were not visible, but the border of the spot was rough. Growth was determined "confluent" when individual colonies were not visible and the border of the spot was smooth.

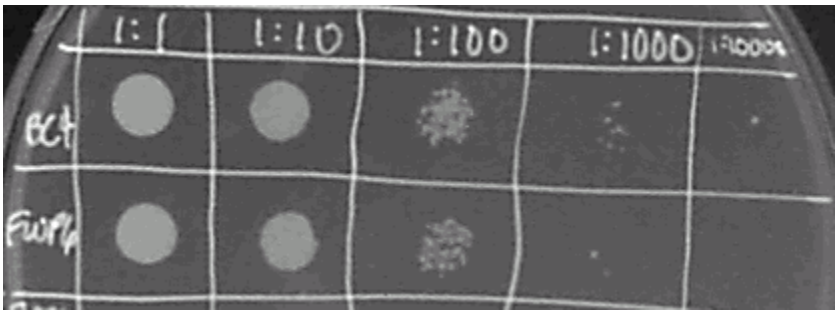


Figure 21a. H_2O_2

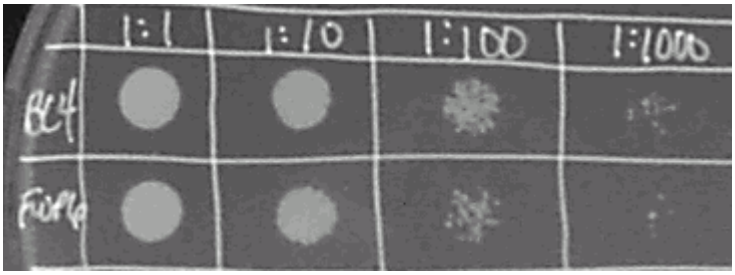


Figure 21b. Menadione

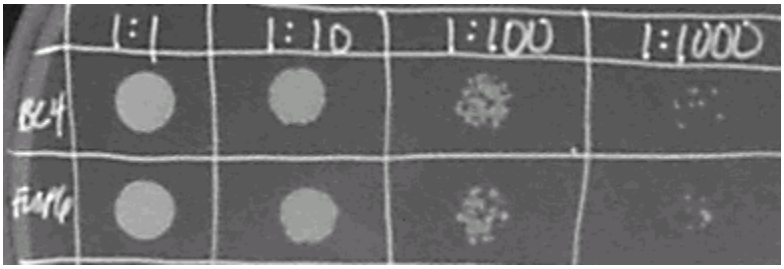


Figure 21c. tBH

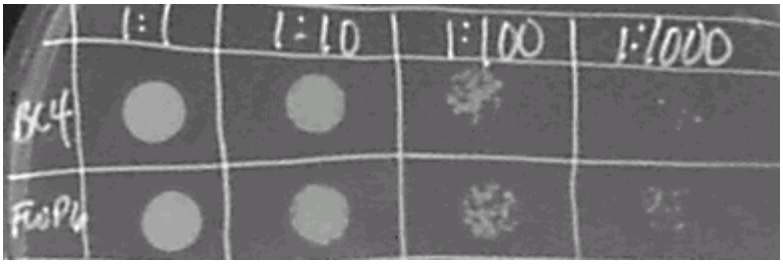


Figure 21d. Paraquat

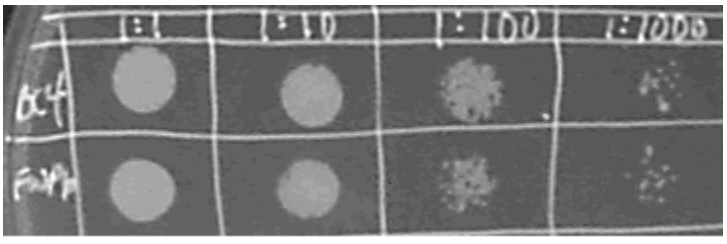


Figure 21e. H_2O

Figure 21. Serial dilutions at t = 0
Before chemicals were added, 5 µL of 1:1, 1:10, 1:100, and 1:1000 serial dilutions of BC4 and FWP6 were plated on YEA plates. Plates were all incubated at 30°C for 46 hours.

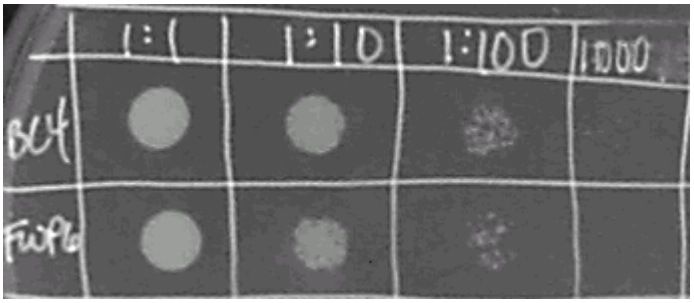


Figure 22a. H_2O_2

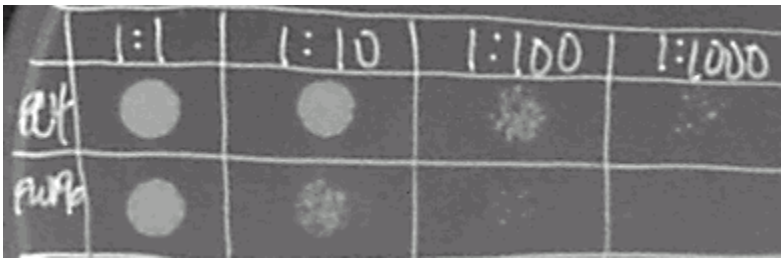


Figure 22b. Menadione

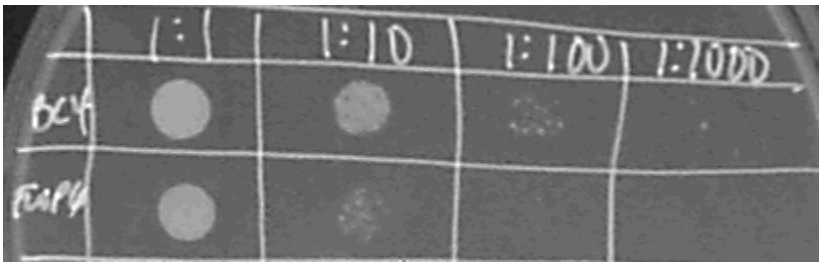


Figure 22c. tBH

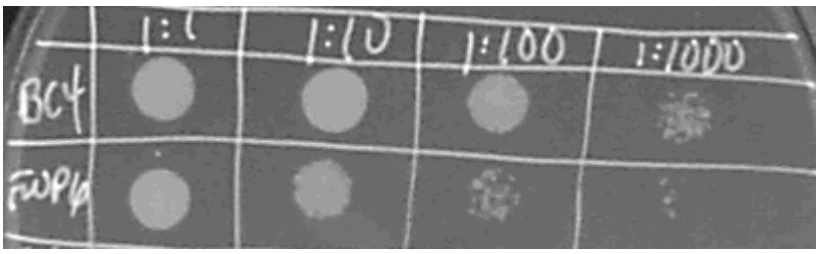


Figure 22d. Paraquat

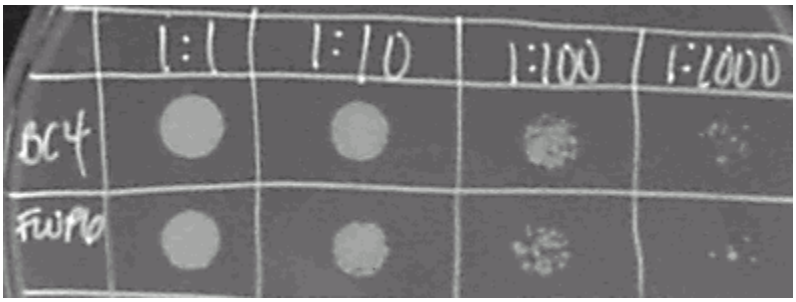


Figure 22e. H_2O

Figure 22. Serial dilutions at t = 2
After 2 hours of incubation in the presence of the various chemicals, 5 µL of 1:1, 1:10, 1:100, and 1:1000 serial dilutions of the BC4 and FWP6 solutions were plated on YEA plates. Plates were all incubated at 30°C for 46 hours.

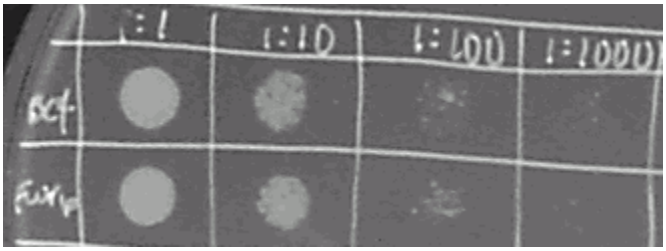


Figure 23a. H_2O_2

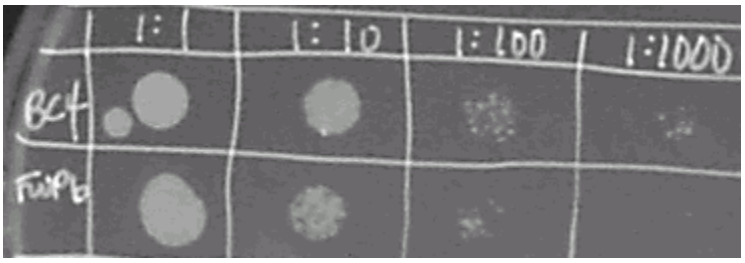


Figure 23b. Menadione

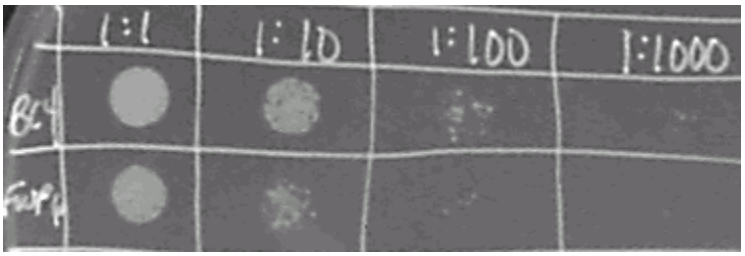


Figure 23c. tBH

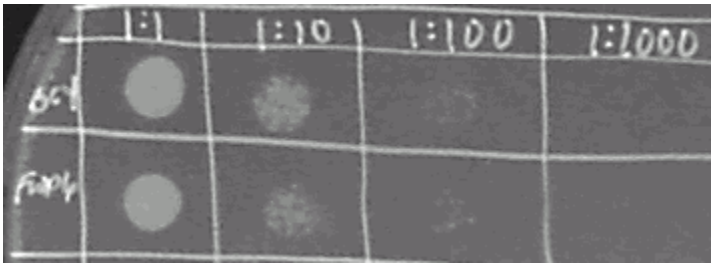


Figure 23d. Paraquat

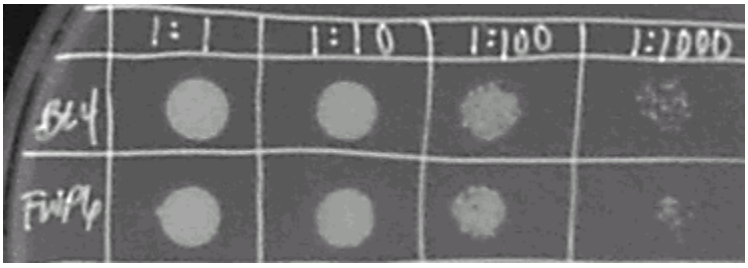


Figure 23e. H_2O

Figure 23. Serial dilutions at t = 6
After 6 hours of incubation in the presence of the various chemicals, 5 µL of 1:1, 1:10, 1:100, and 1:1000 serial dilutions of BC4 and FWP6 were plated on YEA plates. Plates were all incubated at 30°C for 46 hours.

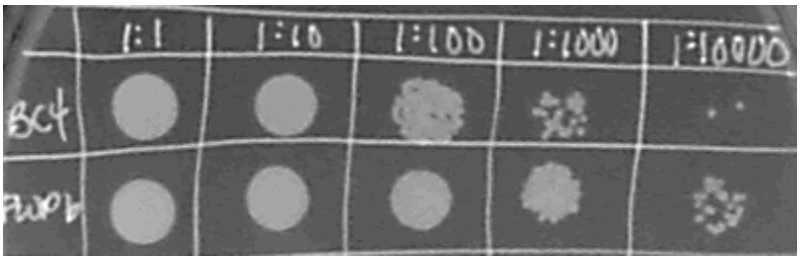


Figure 24a. H_2O_2

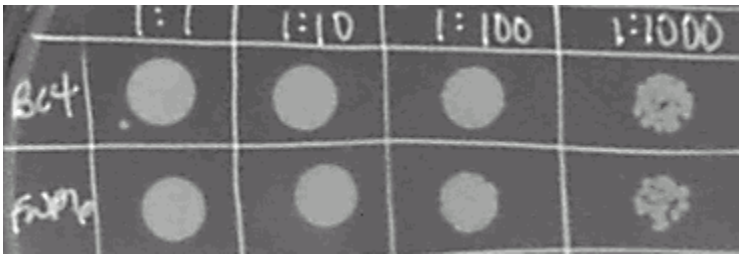


Figure 24b. Menadione

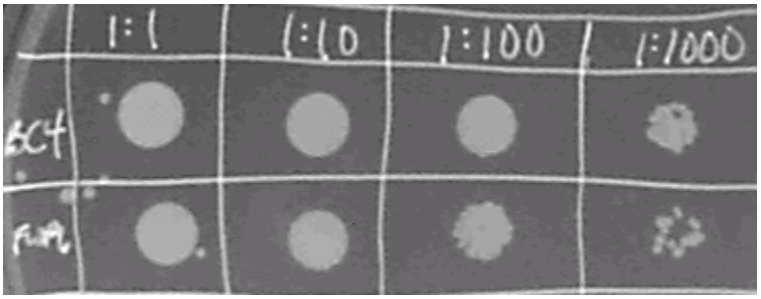


Figure 24c. tBH

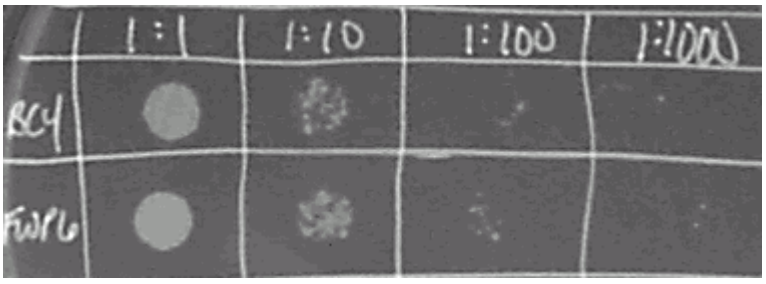


Figure 24d. Paraquat

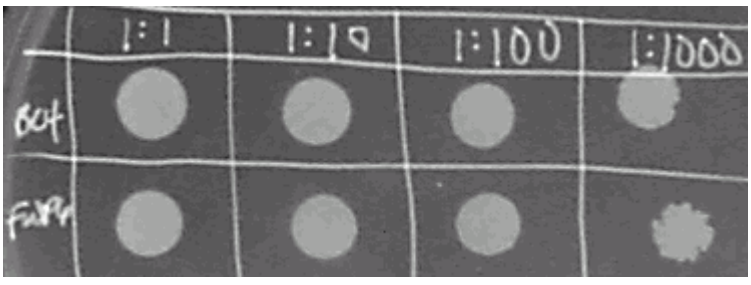


Figure 24e. H_2O

Figure 24. Serial dilutions at t = 24
After 24 hours of incubation in the presence of the various chemicals, 5 µL of 1:1, 1:10, 1:100, and 1:1000 serial dilutions of BC4 and FWP6 were plated on YEA plates. Plates were all incubated at 30°C for approximately 50 hours.

Chapter 4: Discussion

Overexpression of Tpx1

In order to determine if increasing enzyme levels above the normal physiological level increased oxidative stress resistance in cells overexpressing Tpx1, new genotypes were first constructed. After calibrating the concentration of ROS generators necessary to stress the cells, the wildtype FWP6 strain and the novel overepression strain BC4 were exposed to H_2O_2, menadione, tBH, and paraquat. Cell viability of the two strains after exposure to the various chemicals was examined at 2, 6, and 24 hours. By comparing the two strains at time t = 0 hours (Figure 21), it is apparent that a comparable concentration of BC4 and FWP6 were exposed to the same concentration of chemcial, as seen through similar growth at all dilutions. Differences in cell viability were apparent in the cultures exposed to all four chemicals, as the cells overexpressing Tpx1 were more viable than the wildtype cells expressing the physiological level of the same enzyme. The overexpression of Tpx1 conferred the highest resistance to oxidative stress in the cells exposed to menadione and paraquat.

Six hours after the initial addition of the four chemicals, the wildtype and mutant cell viability was again examined. Fewer differences between the viability of BC4 and FWP6 strains were visible at this time (Figure 22). The abundance of Tpx1 enzyme in the BC4 cultures exposed to H_2O_2 and paraquat did not seem to confer an additional advantage beyond that of the normal level of Tpx1 enzyme in the FWP6 cultures. Some difference is seen at 6 hours in the concentration of cells growing in the BC4 sample exposed to tBH. The greatest difference between BC4 and FWP6 at t = 6 hours was seen in the culture to which menadione was added; at the 1:10, 1:100 and 1:1000 dilutions, variations are visible. At a 1:1000 dilution, the

difference between 10 large BC4 colonies and 4 small FWP6 colonies suggests that Tpx1 plays a role in protecting against the elevated levels of peroxide and superoxide created by menadione.

After twenty-four hours of incubation with H_2O_2, menadione, tBH, and paraquat, examination of cell viability indicated varied results. The FWP6 cell culture containing H_2O_2 produced more viable cells than the BC4 cell culture containing H_2O_2. In a separate experiment conducted to explore the possible protective activity of overexpressed Tpx1 enzyme in *S. pombe* cells, it was discovered that H_2O_2 concentrations greater than 75 μM completely inactivated thioredoxin peroxidase (Koo, et al., 2002). In this experiment, if the FWP6 and BC4 cells had a similar viability, then it could be assumed that the Tpx1 enzymes were inactivated in both cultures; these results were not seen, as the wildtype FWP6 cells were more viable than the BC4 cells at 24 hours (Figure 24a). Although there are several different pathways to deal with peroxide-induced oxidative stress in cells, the results seen here indicate that the overexpression of Tpx1 may have interfered with normal protective pathways that could counteract the oxidative stress imposed by the 1.5 mM concentration of H_2O_2 added to this cell culture.

The FWP6 and BC4 cultures containing menadione as well as the FWP6 and BC4 cultures containing paraquat showed few differences in cell viability. In the presence of both chemicals, the concentration of FWP6 cells and the concentration of BC4 cells were very similar. Although overexpression of Tpx1 in the BC4 strain initially increased the viability of the BC4 cells as compared to the wildtype FWP6, at 24 hours the overexpression of Tpx1 seems to make no difference (Figure 24b and 24d). One possible explanation for the comparable viability can be attributed to the theory of natural selection. After 24 hours, the cells have undergone several divisions; at this point, the cells that confer a higher resistance might remain, replicating and surviving the oxidative stress imposed by these chemicals. Alternatively, chronic exposure to

oxidants may cause upregulation of other pathways that reduce the impact of damaging oxygen species.

The overexpression of Tpx1 appears to have increased the resistance of BC4 to oxidative stress caused by tBH when compared to FWP6 at 24 hours of incubation in the presence of tBH, a free radical generator. The difference between the two strains is seen in the 1:1000 dilution, as the growth of the BC4 cells was very dense whereas only 14 individual FWP6 colonies were distinguishable (Figure 24c). The increased viability of the cells overexpressing Tpx1 supports the role of this enzyme as an antioxidant in the presence of tBH.

Further exploration of the enzymatic action of Tpx1 in BC4 is still necessary. First, other chemicals known to cause oxidative stress in the cell should be examined. Also, especially in the case of H_2O_2, in which it has been previously discovered that a high concentration of the chemical inactivates the protein, various concentrations of chemicals could be explored. In the experiments just proposed, as well as the ROS sensitivity tests performed, a Tpx activity assay could be performed to determine if inactivation of the enzyme has occurred and to compare the activity of the enzyme in response to the various chemicals. Additionally, glutathione levels should be examined. Thioredoxin and glutathione work together as a buffer system to react with the ROS. The levels of glutathione would indicate the buffering capacity of the cells to withstand the oxidative stress.

The sensitivity experiments conducted in this experiment to determine the response of BC4 to the four ROS generating chemicals also needs to be repeated for the strain BC5. The strain BC5 is a novel strain in which Tpx1 is being overexpressed in the *S. pombe* strain TP108-3c. Initially, the wildtype strain TP108-3c would need to be calibrated with the four chemicals. Next, the same experiments as performed for BC4 would be performed, including time points

taken and spot testing done at zero, two, six, and twenty-four hours. The differences, if any, in the results of BC4 and BC5 could then be examined.

Creation of a Tpx1 knockout

In an attempt to examine the necessity of Tpx1 *S. pombe* viability and the consequences of the complete absence of the *tpx1* and *trr1* genes and the resulting deficiency of the protective enzymes, the construction of knockouts was initiated. Methods employing homologous recombination, which relies on natural cellular DNA maintenance and repair mechanisms present in all living organisms, provide the means to create such strains. Using a protocol developed to utilize homologous recombination, a deletion cassette was constructed and its integrity verified by DNA sequencing. After linearization of the plasmid, a transformation was performed and the culture was incubated for 48 hours to allow for homologous recombination to occur before plating on selective media of EMM -leu. The formation of colonies indicated the presence of the gene for leucine, which is a part of the deletion cassette construct; the colonies represented individual cells into which the deletion cassette has entered. Three outcomes are possible reasons for the ability of the transformed cells to survive on the selective media: homologous recombination, nonhomologous recombination, or uptake of the unlinearlized deletion cassette plasmid. To select for homologous recombination two different experiments were utilized: PCR and Southern Blotting.

After isolating the six potential knockout colonies, genomic DNA was isolated. The initial gel of the isolation confirmed the presence of DNA, as seen through a single band at approximately 22 kilobases. The purity of the DNA was subsequently questioned as both the PCR experiments and restriction digest were unsuccessful; if using impure DNA, the primers of the PCR experiment, as well as the restriction enzyme EcoRI would have difficulty accessing the

DNA and working properly. The absence of amplified bands from PCR as well as the lack of proper digestion by EcoRI supported this conclusion. Before further PCR experiments or the Southern Blot protocol can be conducted, pure genomic DNA is required. Although the isolation of genomic DNA was repeated and an additional phenol extraction was added in an attempt to increase purity, results remained inconclusive.

To continue this project, another method of genomic DNA extraction could be investigated. Once pure DNA is obtained, PCR experiments need to be performed as discussed previously in Chapter 2: Materials and Methods. The absence of bands in the PCR experiments conducted with the primers specific to the sequence of the *tpx1* gene indicate that homologous recombination has occurred. These results should be compared to a control PCR experiment, in which a gene sequence, such as *trr1*, produces amplification of the *trr1* gene. Additionally, to determine if the resistance to the selective media plate is a result of recombination or the uptake of plasmid, a third reaction employing the primers for the TOPO plasmid, SP35 and SP36, could be used. If the plasmid is not disrupted when digested with BamHI, the resulting band would be 725 bp. However, if the plasmid was linearized upon digestion with BamHI, no band will be present in these lanes. Inconclusive results could be further examined using Southern Blotting techniques. If knockouts are not produced, alternative methods of construction should be investigated.

If confirmation of a knockout strain is achieved, a number of experiments should be conducted to elucidate the role of Tpx1 *S. pombe*. ROS tests will be conducted as previously performed for the overexpression strains using the same concentrations of H_2O_2, menadione, tBH, and paraquat. The next step will be rescue experiments, in which overexpression constructs are reintroduced into the genome of the knockout. Results of the rescue with the overexpression

vector indicate whether or not the absence of the gene in the knockout is the reason for the observed phenotypes. The knockouts will also indicate if the enzyme is required for cell viability. As discussed for the overexpression strains, the levels of glutathione in the knockout will be examined to analyze the buffering capacity of the cells to withstand the oxidative stress.

Conclusions

The original hypothesis, that the overexpression of Tpx1 would confer additional resistance to oxidative stress caused by reactive oxygen species, has been examined. Novel strains were constructed and the overexpression of the Tpx1 protein was confirmed by western blotting. Experimentation with ROS generating chemicals, namely hydrogen peroxide, menadione, *tert*-Butyl hydroperoxide, and paraquat, tested the ability of the strain BC4 to resist oxidative stress. The initial response (t = 2 hrs) demonstrated differences in cell viability in the cultures exposed to all four chemicals, as the BC4 strain cells overexpressing Tpx1 were more viable than the wildtype FWP6 cells expressing the physiological level of the same enzyme. At six hours, differences in cell viability were apparent in the cultures exposed to menadione or tBH, as the BC4 strain cells overexpressing Tpx1 were more viable than the wildtype FWP6 cells expressing the physiological level of the same enzyme in these cultures. The prolonged response to the chemical, evaluated at 24 hours, showed a noticeable difference in cell viability in the cultures exposed to tBH, as the BC4 strain cells overexpressing Tpx1 were somewhat more viable than the wildtype cells. These results generally support the proposed role of Tpx1 to confer additional resistance against the stress imposed by ROS.

In order to further support this conclusion, experiments with Δ*tpx1* cells need to be performed. If the absence of the gene and subsequently, the absence of the protein, results in a decreased ability to confer resistance to oxidative stress, the conclusions made about Tpx1 in the

overexpression experiments could be supported. Frozen stocks of the six potential knockouts have been made and labeled "tpx pKO #1-6"; they are stored in the -80°C freezer in Higgins 444. After successful genomic extraction, the absence of the *tpx1* gene needs to be evaluated before ROS sensitivity experiments can be conducted.

Although little research has been conducted on the thioredoxin peroxidase gene and its protein products in *S. pombe*, especially in the presence of such a wide range of stressors, this research is important. The understanding of the implication of ROS may have significant implication connected to pathological conditions such as several human diseases and the aging process. In order to fully determine this role, many possibilities of further experiments into the function of this antioxidant should be conducted in order to fully understand and define these cellular processes.

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