RGC1/RGC2 deletions cause increased sensitivity to oxidative stress in Saccharomyces cerevisiae, which can be overcome by constitutive nuclear Yap1 expression

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Thesis

RGC1/RGC2 DELETIONS CAUSE INCREASED SENSITIVITY TO OXIDATIVE STRESS IN SACCHAROMYCES CEREVISIAE, WHICH CAN BE OVERCOME BY CONSTITUTIVE NUCLEAR YAP1 EXPRESSION

by

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B.S., University of California at Irvine, 2010

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RGC1/RGC2 DELETIONS CAUSE INCREASED SENSITIVITY TO OXIDATIVE STRESS IN SACCHAROMYCES CEREVISIAE, WHICH CAN BE OVERCOME BY CONSTITUTIVE NUCLEAR YAP1 EXPRESSION

MICHELLE TSAI

ABSTRACT

Oxidative stress mechanism in yeast presents an innovative pathway to understand in creating the next generation of antifungal drugs. Rgc1 and Rgc2 are paralogous proteins that regulate the Fps1 glycerol channel in hyperosmotic stress. Hyperosmotic conditions lead Hog1 MAP kinase to phosphorylate Rgc2 and cause its dissociation from Fps1, allowing the channel to close and protect the cell from damage. Rgc2 contains pleckstrin homology (PH) domains broken up by long insertions and more phosphorylation sites than targeted by Hog1 in response to hyperosmotic stress. Since none of the other MAP kinases in yeast were seen to phosphorylate Rgc2 during oxidative stress, it is thought that Rgc2 may bind to other proteins. In this study, the sensitivity of a strain deleted for both RGC1 and RGC2 was compared to strains with single deletions in either gene in response to oxidative stress. Having deletions in both RGC1 and RGC2 caused increased sensitivity to hydrogen peroxide whereas strains with deletions in either gene seemed unaffected, correlating with the fact that Rgc1 and Rgc2 are paralogous proteins, able to recover each other’s functions. A second analysis compared mutated Fps1 (fps1Δ-FKSV) and a strain with deletions for both RGC1 and
RGC2 (rgc1/2Delta). The fps1Delta-FKSV strain has four amino acid substitutions in the C-terminal region where Rgc2 binds to Fps1. While both strains grew less than wild-type in hydrogen peroxide, the rgc1/2Delta strain was more sensitive suggesting that Rgc1/2 has an additional role in oxidative stress. To identify the oxidative stress function of Rgc1/2, a genomic overexpression library was transformed into the rgc1/2Delta strain and used for a suppressor screen in the presence of hydrogen peroxide. Although the screen revealed a manageable amount of 49 candidates, only four produced sequences that spanned a protein-encoding region. The candidate plasmids were transformed back into the rgc1/2Delta strain for preparation of a sensitivity assay which showed that the colonies did not survive any better than the starting rgc1/2Delta strain. Without a plausible plasmid candidate, we decided to look into the effect of YAP1 on the rgc1/2Delta strain. Yap1 is a transcription factor known to activate many genes in oxidative stress. Two forms of YAP1 were transformed into rgc1/2Delta: wild-type YAP1 and YAP1-A627E which contains a mutation in the nuclear export signal. Compared to the controls, YAP1-A627E allowed the rgc1/2Delta strain to grow at 1.5mM H2O2 while wild-type YAP1 did not. This result showed that a constitutively nuclear Yap1 can overcome deletions in RGC1 and RGC2. It also suggested that an increased activity in the nucleus was important in hydrogen peroxide resistance and another suppressor screen of rgc1/2Delta was performed looking for spontaneous mutations in the genomic DNA. The screened colonies were tested for their survival on hydrogen peroxide but their resistance appeared to be transient. We have shown Rgc1 and Rgc2 to be important cellular components in oxidative stress in addition to hyperosmotic stress. Further research on Rgc1/2 would provide invaluable knowledge.
on oxidative stress protection in yeast and a better foundation on which to build antifungal drugs.
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LIST OF ABBREVIATIONS

Ask10 Activator of Skn 10
bZip Basic Region Leucine Zipper
Crm1 Chromosome Region Maintenance 1
dd Double-Distilled
EDTA Ethylenediaminetetraacetate
Fps1 Fdp1 Suppressor 1
Gcn4 General Control Nonderepressible 4
Glr1 Glutathione Reductase
Gpd1/2 Glycerol-3-Phosphate Dehydrogenase 1/2
GSH Glutathione
GSSG Glutathione Disulfide
Hog1 High Osmolarity Glycerol 1
KanMX Kanamycin
LB Luria Broth
MAP Mitogen-Activated Protein
MAPK MAP Kinase
MAPKK MAP Kinase Kinase
MAPKKK MAP Kinase Kinase Kinase
MAT Mating-Type
MEK MAP Extracellular Signal-Regulated Kinase
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>MEKK</td>
<td>MAP Extracellular Signal-Regulated Kinase Kinase</td>
</tr>
<tr>
<td>Msn2</td>
<td>Multicopy Suppressor of SNF1 Mutation 2</td>
</tr>
<tr>
<td>Msn4</td>
<td>Multicopy Suppressor of SNF1 Mutation 4</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>Pbs2</td>
<td>Polymyxin B Sensitivity 2</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin Homology</td>
</tr>
<tr>
<td>Rgc1</td>
<td>Regulator of the Glycerol Channel 1</td>
</tr>
<tr>
<td>Rgc2</td>
<td>Regulator of the Glycerol Channel 2</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SD</td>
<td>Synthetic Dextrose</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<tr>
<td>YEPD</td>
<td>Yeast Extract Peptone Dextrose</td>
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<td>Ypr115w</td>
<td>115th ORF on the right arm of chromosome XVI, Watson strand</td>
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INTRODUCTION

Fungal infections affect 300 million people worldwide and while many live commensally with humans, some have the potential to become pathogenic (reviewed in Streinu-Cercel, 2012). They can be acquired through the environment, especially through nosocomial means where Candida is the 4th most common cause of hospital-acquired bloodstream infections (Aittakorpi et al., 2012). This poses a significant problem to people with weakened immune systems, including patients with cancer, organ transplants, or HIV/AIDS (reviewed in Pfaller & Diekema, 2007; Calderone & Clancy, 2012). Fungi are one of the most numerous organisms that inhabit the planet, with 1.5 million identified species to date (reviewed in Benedict & Park, 2014). Despite the large number of fungi and their potential harm toward humans, we only have a limited number of antifungals in which to counter them (reviewed in Georgopapadakou, 1998). Fungi are generally difficult to treat because both fungi and humans are eukaryotic organisms; in order for drugs to be effective against fungi, they need to target something unique to avoid harming the host (reviewed in Georgopapadakou, 1998). This has led to the interest in targeting structures such as the cell wall which is not only unique to fungi, it is also essential for their survival (reviewed in Klis et al., 2006). Current antifungals include Caspofungin which inhibits the enzyme 1,3-β-D-glucan synthase, halting the production of cell wall components (reviewed in Deresinski & Stevens, 2003). Unfortunately, fungi are highly adaptable and treatment against a fungal infection requires patients to take multiple drugs inhibiting different cellular pathways or drugs that target multiple
components of the same pathway (reviewed in Anderson, 2005). The knowledge of additional pathways in yeast, such as their response to oxidative stress, can aid in creating the next generation of antifungal drugs. We work with *Saccharomyces cerevisiae* as our model organism for its simplicity yet ability to reveal cellular mechanisms that are conserved in eukaryotes as well as components unique to fungal species (reviewed in Galagan et al., 2005).

**Oxidative Stress**

In oxidative stress, the natural balance between free radical scavengers and reactive oxygen species (ROS) is disturbed (reviewed in Gate et al., 1999). All organisms are exposed to reactive oxygen species from either endogenous or exogenous sources (reviewed in Halliwell, 2006). Molecular oxygen in its ground state is stable and relatively harmless but can undergo partial reduction to form superoxide anions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and other hydroxyl radicals (reviewed in Morano et al., 2012). Mitochondria are a major source of ROS in eukaryotic cells through the process of oxidative phosphorylation (reviewed in Murphy, 2009). ROS accumulation in the cell generally causes yeast to undergo mitochondria-mediated apoptosis, a process similar to a pathway in mammals (reviewed in Pereira et al., 2008).

Studies have made use of a number of ROS compounds to examine the effects of oxidative stress in yeast since the response will differ depending on the oxidant-specific properties of the compound (reviewed in Temple et al., 2005). The most widely used ROS compound is hydrogen peroxide for its ease of use and stability. It is also naturally
found in cells as a product of aerobic respiration. Other ROS include thiol-reactive compounds, superoxide anions, and heavy metals. If left unchecked, ROS compounds can cause lipid peroxidation, protein oxidation, DNA damage, and apoptosis (reviewed in Morano et al., 2012). To prevent intracellular damage, yeast have evolved mechanisms to combat oxidative stress, such as the production of ROS-degrading enzymes and activation of transcription factors (Martinez-Pastor et al., 1996).

**Cell Defense**

Cell defense against ROS includes protective enzymes and small molecules that act as free radical scavengers. Catalases are the most common enzymes found in cells, converting H$_2$O$_2$ to H$_2$O and O$_2$ (reviewed in Morano et al., 2012). Yeast have two types of catalases, peroxisosomal catalase A for fatty acid β-oxidation and cytosolic catalase T for general use against oxidative stress (Martinez-Pastor et al., 1996; reviewed in Hiltunen et al., 2003). Yeast lacking both types of catalases are surprisingly unaffected by hydrogen peroxide during exponential growth (Izawa et al., 1996). This can be attributed to the redundancy of other enzymes and mechanisms available to protect the cell in oxidative stress. Superoxide dismutases (SODs) convert superoxide anions to hydrogen peroxide which can then be broken down by catalases (reviewed in Culotta et al., 2006). Methionine sulfide reductase protects amino acids, particularly methionine (reviewed in Stadtman et al., 2003), and two thioredoxin systems (cytoplasmic and mitochondrial) protect the cell against general oxidative stress (Gan, 1991; Pedrajas et al., 1999; Schafer & Buettner, 2001; Garrido & Grant, 2002; reviewed in Wood et al., 2003). These
enzymes can be upregulated when the cell is exposed to oxidative stress (reviewed in Morano et al., 2012).

Non-enzymatic changes include compounds acting as free radical scavengers such as glutathione (GSH) and ascorbic acid (reviewed in Morano et al., 2012). Glutathione has many roles in the cell including transportation of amino acid, synthesis of nucleic acid and proteins, enzyme regulation, and metabolism of carcinogens, xenobiotics, and ROS (Schafer & Buettner, 2001). In oxidative stress, glutathione helps keep the cell in a reduced state through conversion to its oxidized glutathione disulfide (GSSG) form (López-Barea et al., 1990). GSSG reverts back to glutathione through glutathione reductase (Glr1) which is maintained by the pentose phosphate pathway (López-Barea et al., 1990). In many eukaryotes, ascorbic acid is known to act as a redox pair with glutathione (reviewed in Winkler et al., 1994).

Activation of Transcription Factors

Reactive oxygen species have dual roles as a means of cell communication and the ability to cause cell damage (reviewed in Lushchak, 2010). The primary transcription factor activated in oxidative stress is Yap1 (Harshman et al., 1988; Moye-Rowley et al., 1989). Yap1 was the second bZip (basic region-leucine zipper)-containing transcription factor identified (after Gcn4) in S. cerevisiae, and is a positive regulator of gene expression (Harshman et al., 1988; Jones et al., 1988). Depending on the oxidative stress agent, Yap1 can undergo one of two conformational changes, both of which involve disulfide bonds and mask the Crm1 nuclear export signal (Yan et al., 1998; Kuge et al.,
2001). As a result, Yap1 is retained in the nucleus where it regulates transcription of over 70 genes (Gulshan et al., 2005; Okazaki et al., 2007; Rowe et al., 2012). Overexpression of YAP1 shows increased tolerance to oxidative stress as well as other metals and drugs (Hertle et al., 1991; Schnell et al., 1991; Haase et al., 1992; Bossier et al., 1993; Wu et al., 1993). Other transcription factors activated in oxidative stress include Skn7, thioredoxin-encoding Trx2, and Msn2/4 (reviewed in Morano et al., 2012).

**Skn7**

Skn7 is a multifunctional protein which has been shown to respond to both hypo-osmotic and oxidative stress (Krems et al., 1996; Morgan et al., 1997; Lee et al., 1999; Li et al., 2002) as well as affect the regulation of cell wall assembly (Brown et al., 1993; Alberts et al., 1998; Ketela et al., 1999; Li et al., 2002). In oxidative stress, Skn7 utilizes a “two-component system,” where the phosphate group delivered by a histidine kinase (e.g., Sln1) is relocated to a conserved aspartate residue of a response regulator, such as Ssk1 or Skn7 (Brown et al., 1994; Krems et al., 1996; Li et al., 1998). Skn7 regulates the transcription of the antioxidant molecule thioredoxin as well as heat shock genes (Morgan et al., 1997; Raitt et al., 2000). Skn7 is important in the regulation of cell cycle, cell wall metabolism, and response to cell wall stress (Krems et al., 1996; Morgan et al., 1997). In a genetic screen looking for activators of Skn7 transcription, a protein from the tenth group was identified and subsequently named Ask10 (Page et al., 1996).
**Ask 10**

Ask10 has many roles. It can act as a transcription factor, regulating RNA polymerase II as a component of the Mediator complex (Liao et al., 1995). It also associates with Srb11, a yeast homolog of mammalian C-type cyclin protein (Cohen et al., 2003). Srb11 normally represses transcription (Hengartner et al., 1998) and requires Ask10 for destruction in oxidative stress which relieves the repression of stress response genes (Cohen et al., 2003). Ask10 has a paralog, Ypr115w, which shares 41% of sequence homology (Yu et al., 2004). Both are large proteins (Ask10 is 127kDa, Ypr115w is 120kDa) that encode pleckstrin homology (PH) domains which include Slm1 and Slm2, redundant proteins essential for cell growth and actin cytoskeleton polarization (Fadri et al., 2005). In a study exploring the phenotype of ask10Δ and ypr115wΔ, an accumulation of glycerol by Gpd1/2 was found which results in cell wall stress and lysis (Beese et al., 2009). Further investigation revealed this was due to a defect in the function of the Fps1 glycerol channel (Beese et al., 2009). *YPR115w* was then given the name *RGC1* (for *Regulator of the Glycerol Channel*) and *ASK10, RGC2* (Beese et al., 2009).

**Rgc2 and Fps1**

Fps1 functions as a homotetramer (Beese-Sims et al., 2011) and maintains osmolarity by either closing the channel in hyperosmotic conditions or rapidly exporting glycerol in hypo-osmotic conditions, both of which occur within seconds of exposure to the stress (Tamás et al., 1999). The channel is partially controlled by the redundant and positive regulators Rgc1 and Rgc2 (Beese et al., 2009). The C-terminal end of the
channel was found to have a highly conserved region of amino acid residues FKSV (614-617) which is conserved across all fungal Fps1 orthologs (reviewed in Pettersson et al., 2005). Mutations in this region, such as changing the four amino acid residues to alanine (fps1-FKSV), prevented Rgc2 from binding to Fps1 and showed the importance of the C-terminal end for this interaction (Lee et al., 2013). In unstressed conditions, Rgc2 normally binds to the C-terminal end of Fps1, keeping the channel open (Lee et al., 2013). In hyperosmotic stress, Hog1 binds to the N-terminus of Fps1 and phosphorylates Rgc2 multiple times, displacing it from the glycerol channel and allowing the channel to close (Lee et al., 2013) (Figure 1). The interaction between Rgc2 and Fps1 was found to be dependent on a tripartite PH domain on Rgc2 (Lee et al., 2013). PH domains normally bind to phosphatidylinositides, but the PH domains of Rgc1 and Rgc2 are broken up by long insertions, suggesting that they may bind to other ligands or protein-binding domains (Fadri et al., 2005; van Rossum et al., 2005).

**Hog1 MAPK Cascade**

Hog1 is part of a MAPK cascade, a series of phosphorylations aimed to amplify an external signal and quickly mobilize cellular processes (Huang & Ferrell, 1996). There are multiple MAPK pathways in yeast, components of which are even conserved across species including mammals (reviewed in Galagan et al., 2005). Each MAPK pathway contains three levels of protein kinases: a MAP kinase (MAPK), a MAPK kinase (MAPKK) or MEK, and a MAPKK kinase (MAPKKK) or MEKK (reviewed in Hohmann, 2002). The Hog1 pathway is activated under hyperosmotic stress (reviewed in...
Gustin et al., 1998) and consists of three MEKKs (Ssk2, Ssk22, and Ste11), one MEK (Pbs2), and one MAPK (Hog1) (reviewed in Hohmann, 2002). The Hog1-activated MAPK cascade is one of five MAPK signaling pathways that have been identified in yeast which regulate mating, sporulation, invasive growth, and response to high osmolarity and cell wall stress (reviewed in Levin, 2011). Immunoblot analysis of Rgc2 revealed that the unstressed and stressed forms migrate as several distinct bands as a consequence of basal and induced phosphorylation, showing that its regulation is complex (Beese et al., 2009). Mass spectrometry revealed 30 phosphorylation sites on Rgc2, and seven were confirmed to be targeted by Hog1 in response to hyperosmotic stress (Lee et al., 2013). Rgc2 is also phosphorylated under oxidative stress (Cohen et al., 2003), yet none of the four MAP kinases in yeast were found to be involved (Beese et al., 2009).

Cell Wall Integrity

Maintenance of the cell wall in *Saccharomyces cerevisiae* is essential for not only its growth but also survival against various environmental stressors (reviewed in Klis et al., 2006). An estimated 1200 genes in yeast show an altered cell wall-related phenotype when deleted, illustrating its importance (De Groot et al., 2001). The cell wall is made up of two layers, an inner layer consisting of mostly β-1,3-glucan chains (80-90%) with branching through β-1,6 linkages and an outer layer of glycoproteins (reviewed in Levin, 2011). In total, the cell wall accounts for 10-25% of the cell’s mass, depending on growth conditions (Orlean, 1997; reviewed in Smits et al., 1999; Aguilar-Uscanga & François,
2003). It constantly receives information about the environment and adjusts to accommodate different nutrients, temperature, pH, and oxygen levels (Aguilar-Uscanga & François, 2003). Discovering the role of Rgc1 and Rgc2 in oxidative stress could lead to a better understanding of the mechanisms regulating cell wall recovery by yeast and a better foundation on which to build antifungal drugs.

Figure 1. Regulation of the Fps1 glycerol channel by Hog1 and Rgc2. (A) In the unstressed state, Rgc2 normally binds to the C-terminal end of the Fps1 glycerol channel, keeping the channel open. (B) Under hyperosmotic stress, Hog1 is phosphorylated and binds to the N-terminal end of Fps1. (C) Hog1 phosphorylates Rgc2 multiple times. (D) Phosphorylated Rgc2 leaves Fps1, allowing the channel to close and prevent glycerol from flowing through (Original Figure from Lee et al., 2013).
Specific Aims

To investigate the function of Rgc1 and Rgc2 in oxidative stress, we will:

(1) Use techniques in molecular biology to evaluate the relative contributions of Rgc1 and Rgc2 in oxidative stress.

(2) Use additional techniques in genetics to assess candidate genes in a yeast genomic overexpression library that will rescue Rgc1 and Rgc2 in double deletion strains treated with hydrogen peroxide.

We expect these studies to:

(1) Reveal that double mutant rgc1/2Δ strains are more susceptible to oxidative stress than either RGC1 or RGC2 deletion alone.

(2) Identify a number of genes that when overexpressed will allow rgc1/2Δ strains to grow in oxidative stress conditions.
METHODS

Strains and Growth Conditions

*S. cerevisiae* strains (Research Genetics) and plasmids used in this study are listed in Table 1. Deletions in *RGC1* and *RGC2* were formed through recombination using a kanamycin resistance marker (KanMX). Yeast cultures were grown in either YEPD (1% Bacto yeast extract, 2% Bacto Peptone, 2% glucose) or SD (.67% Yeast nitrogen base, 2% glucose) with additional nutrients to select for plasmids.

Table 1. Yeast strains and plasmids used in this study.

<table>
<thead>
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<th>Yeast Strain</th>
<th>Genotype</th>
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<td>DL3169</td>
<td><em>MATa</em> S288c <em>rgc2Δ::KanMX</em></td>
</tr>
<tr>
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<td><em>MATa</em> S288c <em>rgc1Δ::KanMX</em></td>
</tr>
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<td><em>MATa</em> S288c (BY4742) <em>his3Δ leu2Δ ura3Δ lys2Δ</em></td>
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<td>DL4194</td>
<td><em>MATa</em> S288c <em>fps1Δ</em></td>
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<th>Plasmid</th>
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<tr>
<td>p313</td>
<td>His</td>
</tr>
<tr>
<td>p316</td>
<td><em>URA3</em></td>
</tr>
<tr>
<td>p3086</td>
<td><em>URA3</em>, wild-type <em>YAP1</em></td>
</tr>
<tr>
<td>p3089</td>
<td><em>URA3</em>, <em>YAP1-A627E</em> constitutively nuclear mutant</td>
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<td>pRS202</td>
<td>6.3kbp, <em>URA3</em>, 1 HindIII restriction site, high-copy genomic library</td>
</tr>
<tr>
<td>pRS426</td>
<td>5.7kbp, <em>URA3</em>, high-copy</td>
</tr>
<tr>
<td>yEP352</td>
<td>5.2kbp, <em>URA3</em></td>
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**Stress Sensitivity Assay**

Yeast strains were grown in YEPD to mid-log phase (OD=.6) and serially diluted 1:10. The dilutions were spotted onto YEPD plates containing various concentrations of H₂O₂. The plates were incubated for 2 days at 30°C and then photographed.

**Yeast Transformation**

Yeast cultures were transformed following Gietz et al. (1995). Yeast cultures were started overnight in YEPD and placed in a 30°C shaking incubator. The overgrown cells were diluted to an OD=.2 and a volume large enough to distribute 2.5ml of cells over each transformation plate. OD was measured at an absorbance of 600nm. The cells were allowed to regrow to mid-log phase and then centrifuged at high speed (13.5k rpm) for 1 min. The cells were washed with 1x TE-LiAc and resuspended in 50μL TE-LiAc, 2μL of plasmid DNA, and 20μL of ssDNA per 2.5ml of cells. 1x TE-LiAc consists of 100mM TE, 1M LiAc, and ddH₂O (1:1:8). The mixture was allowed to incubate at 30°C for 15 min before adding 300μL of PEG buffer per 2.5ml of cells. PEG buffer consists of 100mM TE, 1M LiAc, and 50% PEG4000 (1:1:8). After an additional incubation at 30°C for 30 min, the cells were heat shocked at 42°C for 20 min. The contents were centrifuged and resuspended with YEPD and allowed to recover at 30°C in a shaking incubator for 1.5 h. The cells were then resuspended in SD-Ura and plated onto dropout SD plates.
Suppressor Screen

Suppressor screens were conducted using \textit{rgc1/2Δ} transformed with pRS202, which contained fragments of yeast genomic overexpression library, and also \textit{rgc1/2Δ} alone. Screens of transformed strains were plated directly on SD-Ura containing various concentrations of hydrogen peroxide. Plasmids were isolated from colonies appearing after 3 days. Screens of \textit{rgc1/2Δ} were performed on YEPD plates containing hydrogen peroxide.

Plasmid Isolation from Yeast

Plasmid DNA in yeast was prepared for isolation following the protocol from Hoffman and Winston (1987). Candidate colonies were grown for two days in SD-Ura in a shaking, 30° incubator. The cultures were centrifuged and supernatant was decanted. 200μL of DNA isolation buffer was added to each pellet. DNA isolation buffer consists of 2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-Cl at pH 8, and 1mM Na$_2$EDTA (100:50:10:5:1). 200μL of phenol-chloroform (1:1) and 0.3g of acid-washed glass beads were added. The cells were lysed by vortex and centrifuged at high speed.

Ethanol precipitation was then performed on each plasmid DNA sample. 3M NaOAc (ten-times diluted) and isopropanol (an amount equivalent to the total volume) was added to each solution. The contents were centrifuged for 20 min at high speed and the supernatant was decanted. Each sample was washed with 500μL of 70% ethanol and centrifuged for 5 min at high speed. The supernatant was decanted and the pellet was allowed to dry before resuspending the plasmid DNA with ddH$_2$O.
**E. coli Transformation**

2µL of isolated DNA was added to 30µL of competent *E. coli* cells. The solutions were left on ice for 1 h to allow adhesion of the DNA. The cells were then heat shocked for 1 min in a 42°C incubator. 1ml of LB was added and the cells were allowed to grow for 1 h in a 37°C shaking incubator. The cells were centrifuged and 900µL of the supernatant was decanted. The cells were resuspended in the remaining liquid, plated onto LB plates containing carbenicillin, and allowed to grow at 37°C overnight. Colonies showing up the following day were picked up and allowed to culture in LB containing carbenicillin for another night in a 37°C shaking incubator.

**Plasmid Isolation from E. coli**

Plasmids amplified in *E. coli* were isolated using a commercial kit for DNA isolation (GeneJET Plasmid Miniprep Kit, Thermo Scientific). The manufacturer’s protocols were followed, except ddH₂O was used to elute the DNA instead of elution buffer:

*E. coli* culture grown overnight was harvested by centrifugation at high speed at room temperature. The cells were resuspended in solution and lysed. The solution was neutralized and centrifuged for another 5 min. The supernatant was transferred to the manufacturer’s spin column and centrifuged for 1 min. The column was washed twice, allowed to incubate, and the DNA was eluted with ddH₂O.
DNA Purity

The concentration of plasmid DNA was performed using a NanoDrop machine (Thermo Scientific).

Restriction Digest and Agarose Gel Electrophoresis

Plasmid DNA isolated from *E. coli* was digested with HindIII. Each digestion of 1μL of plasmid DNA required 1μL 10x buffer, 0.5μL HindIII, and 7.5μL H₂O. The digests were allowed to incubate for 1 h at 37°C.

2μL of a 6x loading dye was added to each DNA sample. Digests were run on a 1% agarose gel at 100V. 10μL of 1kb Plus DNA ladder (100-10,000bp, Fisher Scientific) was run alongside.

DNA Sequencing

DNA samples were sent for sequencing using GeneWiz. For each sample, 2μL of DNA was placed in 7.5μL of ddH₂O. Sequencing was performed from both ends using T3 and T7 primers.

Verification

Candidates were verified by transforming plasmid DNA back into yeast and/or performing a sensitivity assay. Transformation and sensitivity assay procedures were completed using the same procedures described above.
A streak test performed after a suppressor screen consisted of spreading a single colony evenly over a YEPD plate containing the same hydrogen peroxide concentration used in the screen. The starting strain was streaked alongside the candidates for comparison.
RESULTS

rgc1/2, fps1 Sensitivity Assay

Wild-type (DL3187), rgc1/2Δ (DL3207), rgc1Δ (DL3172), and rgc2Δ (DL3169) strains were prepared for a sensitivity assay on YEPD containing 0mM and 3.0mM H₂O₂ (Figure 2). In 3.0mM H₂O₂, rgc1Δ and rgc2Δ grew similarly to wild-type while rgc1/2Δ did not grow as well. This showed that having deletions in both RGC1 and RGC2 cause increased sensitivity to hydrogen peroxide while strains with single deletions in either gene do not. A second sensitivity assay compared three transformed strains: fps1Δ transformed with wild-type FPS1, fps1Δ transformed with an FPS1 allele with a mutation in the C-terminal region (fps1Δ-FKSV) that renders it unable to bind to Rgc1/2, and rgc1/2Δ transformed with vector p313 only (Figure 3). These were also plated on YEPD at 0mM and 3.0mM H₂O₂. At 3.0mM H₂O₂, fps1Δ-FKSV grew nearly as well as wild-type, but rgc1/2Δ-p313 did not. This suggested that Rgc1 and Rgc2 serve an additional function beyond their role in the regulation of Fps1.
Figure 2. Sensitivity assay of \textit{rgc1/2}\textDelta in 3.0mM H$_2$O$_2$. Wild-type (DL3187), \textit{rgc1/2}\textDelta (DL3207), \textit{rgc1}\textDelta (DL3172), \textit{rgc2}\textDelta (DL3169) were prepared for a sensitivity assay on 0mM and 3.0mM H$_2$O$_2$ in YEPD. Individual mutations \textit{rgc1}\textDelta and \textit{rgc2}\textDelta grew similarly to wild-type at 3.0mM H$_2$O$_2$ while \textit{rgc1/2}\textDelta grew less.

<table>
<thead>
<tr>
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<tr>
<td>Fps1\textDelta</td>
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<td>Fps1\textDelta</td>
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<tr>
<td>Rgc1/2\textDelta</td>
<td>p313</td>
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Figure 3. Sensitivity assay of \textit{fps1-\textit{FKSV} and \textit{rgc1/2}\textDelta in 3.0mM H$_2$O$_2$}. \textit{fps1}\textDelta-\textit{FPS1-wt}, \textit{fps1}\textDelta-\textit{fps1-\textit{FKSV}}, and \textit{rgc1/2}\textDelta-p313 were prepared for a sensitivity assay on YEPD containing 0mM and 3.0mM H$_2$O$_2$. \textit{rgc1/2}\textDelta-p313 did not grow as well as \textit{fps1}\textDelta-\textit{FPS1-wt} at 3.0mM H$_2$O$_2$. 
Plasmid DNA Suppressor Screen

As a first step to identify the oxidative stress function of Rgc1/2, a preliminary experiment transforming \( \text{rgc1/2}\Delta \) (DL3207) and wild-type (DL3187) strains with yEP352 (URA3) was performed at various concentrations of hydrogen peroxide (Figure 4). This was done to determine the concentration of hydrogen peroxide that would prevent background colonies from forming. Approximately 6,000 transformants were spread per plate and a ten-fold reduction in \( \text{rgc1/2}\Delta \) colonies was seen around 1.2mM \( \text{H}_2\text{O}_2 \). Very few colonies grew at 1.8mM \( \text{H}_2\text{O}_2 \) and zero colonies grew at higher hydrogen peroxide concentrations. We initially decided to use 2.0mM and 2.5mM \( \text{H}_2\text{O}_2 \) for the actual experiment using pRS202 (genomic library) but these concentrations turned out to be too strong and no real colonies were seen. The screen was then repeated at lower concentrations of 1.8mM, 1.6mM, 1.5mM, and 1.4mM \( \text{H}_2\text{O}_2 \) and we obtained 49 candidates from about 500,000 colonies.
Figure 4. **Number of yeast colonies seen after transformation at various concentrations of hydrogen peroxide.** Wild-type (DL3187) and \textit{rgc1/2Δ} (DL3187) strains were transformed with yEP352 (\textit{URA3}) and plated on SD-Ura containing hydrogen peroxide between 0mM and 3.0mM. \textit{rgc1/2Δ}-yEP352 did not grow at concentrations above 1.8mM H$_2$O$_2$.

**E. coli Transformation and Agarose Gel Electrophoresis**

Plasmid DNA was isolated from candidates and amplified by \textit{E. coli} transformation. Multiple colonies picked up from the same \textit{E. coli} transformation plate were distinguished from each other by subsequent numbers following a dash. Candidates were digested with HindIII and visualized using agarose gel electrophoresis (Figure 5). All gels were run using the same DNA ladder (100-10,000bp). Visualization often
revealed that the samples did not contain plasmid DNA. Restriction digests showing
either a single heavier band or multiple bands were sent for sequencing (1, 5-2, 7-1, 19,
31, 33-1, 36-2, 39-1, and 42-1, asterisked). Sequencing results for 31, 33-1, 39-1, and 42-1
were positive and are shown in Figure 6. The candidates encoded regions from four
different chromosomes (XII, VII, XV, and XIII, respectively).
Figure 5. Agarose gel of plasmid DNA candidates. Plasmid DNA purified from *E. coli* was digested with HindIII and used for gel electrophoresis. All gels were run using the same DNA ladder (100-10,000bp). Candidates showing the same base number came from the same *E. coli* transformation experiment. Many lanes did not contain DNA. Asterisked candidates were sent for sequencing (1, 5-2, 7-1, 19, 31, 33-1, 36-2, 39-1, and 42-1).
Figure 6. Sequencing results of four plasmid candidates. Sequencing of the plasmid candidates was performed with T3 and T7 primers. Sections A, B, C, and D show sequencing results for candidates 31, 33-1, 39-1, and 42-1, respectively. The plasmid candidates encoded regions that spanned various genes from four different chromosomes (XII, VII, XV, and XIII).
Figure 6. Continued.

Verification of Plasmid Candidates

Plasmid DNA candidates were transformed back into \textit{rgc1/2}\Delta and two samples of each transformant were prepared for a sensitivity assay on YEPD containing 0mM, 2.0mM, 3.0mM, and 4.0mM H\textsubscript{2}O\textsubscript{2} (Figure 7). No colonies were seen at 4.0mM H\textsubscript{2}O\textsubscript{2} for wild-type or \textit{rgc1/2}\Delta. The first sample of \textit{rgc1/2}\Delta-plasmid candidate 31 appeared to grow as well as wild-type transformed with yEP352 (\textit{URA3}), but the second sample did not. It was difficult to see a difference in growth between the samples and their dilutions so we decided to plate the transformants at the original conditions on SD-Ura containing 0mM, 1.8mM, 2.0mM H\textsubscript{2}O\textsubscript{2} (Figure 8). Although contamination was seen on the plates, the transformed plasmid candidates did not appear to grow as well as \textit{rgc1/2}\Delta transformed with yEP352 (\textit{URA3}). Since none of the plasmids were able to confer H\textsubscript{2}O\textsubscript{2} resistance to the \textit{rgc1/2}\Delta strain, this suggested that the observed resistance was caused by other factors.
**Yeast Transformation with YAP1 Plasmids**

Yap1 is one of the primary transcription factors activated in oxidative stress (Harshman et al., 1988; Moye-Rowley et al., 1989) and regulates expression of over 70 genes (Gulshan et al., 2005; Okazaki et al., 2007). We transformed the *rgc1/2Δ* strain with *YAP1* plasmids p3086 (wild-type *YAP1*) and p3089 (constitutively nuclear mutant *YAP1*) using p316 (*URA3*) as a control. These transformants were plated on SD-Ura containing 0mM, 1.0mM, and 1.5mM H$_2$O$_2$ (Figure 9). Wild-type (DL3187) was also transformed with pRS426 (*URA3*) and plated. Thousands of colonies were seen at 0mM H$_2$O$_2$ for all four strains. At 1.0mM H$_2$O$_2$, fewer colonies were seen for *rgc1/2Δ*-p316 compared to the other three transformed strains. At 1.5mM H$_2$O$_2$, hundreds of colonies were seen for wild-type-pRS426 and *rgc1/2Δ*-p3089 while none were seen for *rgc1/2Δ*-p316 or *rgc1/2Δ*-p3086. This showed that constitutively nuclear Yap1 confers resistance to H$_2$O$_2$ in the *rgc1/2Δ* strain.

**Genomic DNA Screening**

A selection for mutants of *rgc1/2Δ* that are resistant to hydrogen peroxide was carried out. A preliminary experiment was performed to determine the concentration of hydrogen peroxide needed for a large enough reduction in *rgc1/2Δ* growth in order to pick up single colonies. Multiple trials revealed that the concentration of hydrogen peroxide needed was dependent on cell density. The presence of more cells allowed for better survival on hydrogen peroxide and 7.0mM H$_2$O$_2$ was determined to be the ideal concentration for these cultures. Single colonies from the starting *rgc1/2Δ* strain were
allowed to grow in YEPD separately so that if the same gene came up from different colonies of the screen, the gene would be instantly verified because the colonies would have mutated independently of each other. 10 colonies from each mating-type (DL3188, MATα and DL3207, MATa) were selected for growth on YEPD plates containing 7.0mM H₂O₂. Approximately 8,000 colonies were seen on each plate and a single colony from each was selected for a streak test on 7.0mM H₂O₂. On the first streak test, many screened candidates did not appear to grow any better than the starting rgc1/2Δ strain so multiple streak tests were done taking only the colonies that grew from the previous test. When it appeared that the candidates were noticeably producing better streaks than the starting rgc1/2Δ strain (Figure 10), they were then grown in culture for a sensitivity assay at 3.0mM H₂O₂ (Figure 11). The screened candidates from MATα and MATa grew similarly and a subset of six candidates from MATα is shown in both Figure 10 and Figure 11. 3.0mM H₂O₂ was chosen for the sensitivity assay as the first experiment comparing rgc1/2Δ and wild-type (DL3187) showed that 3.0mM H₂O₂ was enough to differentiate the ability of a strain to grow in hydrogen peroxide. Also, cultures for the sensitivity assay were grown from single colonies and would not reach as high of a concentration as used in the screen. The sensitivity assay revealed little difference between the starting rgc1/2Δ strain and the screened colonies. The strains used for this experiment were taken from stock some time before and it was thought that they may have acquired mutations that affected their ability to keep their resistance to hydrogen peroxide.
The genomic screen experiment was repeated using fresh colonies of \( rgc1/2\Delta \). 10 individual colonies from each mating-type were screened and prepared for a sensitivity assay on YEPD containing 3.0mM H\(_2\)O\(_2\). A subset of five screened candidates from \( MAT\alpha \) is shown in Figure 12. The candidates grew similarly to the starting \( rgc1/2\Delta \) strain showing that the previous screening result was not due to the age of the colonies.

**Resistance Test of Candidates**

The ability of the candidates to survive the screen but grow only as well as the starting \( rgc1/2\Delta \) strain on the sensitivity assays suggested that the cells were activating a temporary mechanism to overcome the hydrogen peroxide stress. To test this hypothesis, two candidates from each \( MAT\alpha \) and \( MAT\alpha \) from the previous genomic screen at 3.0mM H\(_2\)O\(_2\) were grown overnight in YEPD containing 3.0mM H\(_2\)O\(_2\) instead of YEPD only. The starting \( rgc1/2\Delta \) strain was allowed to grow overnight in YEPD without hydrogen peroxide. These five strains were prepared for a sensitivity assay on YEPD containing 0mM and 3.0mM H\(_2\)O\(_2\) (Figure 13A). In this test, all of the candidates grew better than the starting strain at 3.0mM H\(_2\)O\(_2\). To verify their resistance, single colonies were taken from the 3.0mM H\(_2\)O\(_2\) plate (Figure 13A, boxed), grown overnight in YEPD, and plated again on 0mM and 3.0mM H\(_2\)O\(_2\) (Figure 13B). The screened colonies then appeared indistinguishable from the starting \( rgc1/2\Delta \) strain, indicating that their resistance to hydrogen peroxide was temporary.
Figure 7. Verification of plasmid DNA candidates by sensitivity assay. Plasmid DNA candidates were transformed back into rge1/2Δ (DL3207) and two samples of each transformant (31, 33-1, 39-1, and 42-1) were prepared for a sensitivity assay on YEPD containing 0mM, 2.0mM, 3.0mM, and 4.0mM H₂O₂. Only the first sample of candidate 31 seemed to grow as well as wild-type (DL3187) transformed with yEP352 (URA3), however, the second sample did not. No colonies grew at 4.0mM H₂O₂.
Figure 8. Verification of plasmid DNA candidates by transformation into \textit{rgc1/2\Delta}. Plasmid candidate transformants (31, 33-1, 39-1, and 42-1) were plated on SD-Ura containing 0mM, 1.8mM, and 2.0mM H$_2$O$_2$. No colonies were seen at 2.0mM H$_2$O$_2$ for candidates 31, 33-1, or 39-1. Fewer colonies were seen for 42-1 than \textit{rgc1/2\Delta}-yEP352.
Figure 9. Transformation of rge1/2Δ with YAP1 plasmids. The rge1/2Δ strain (DL3207) was transformed with plasmids p316 (URA3), p3086 (wild-type YAP1), and p3089 (YAP1-A627E, constitutively nuclear mutant). Wild-type (DL3187) was transformed with pRS426 (URA3). Transformants were plated on SD-Ura containing 0mM, 1.0mM, and 1.5mM H₂O₂. Thousands of colonies were seen for all four transformants at 0mM H₂O₂. At 1.0mM H₂O₂, rge1/2Δ-p316 was more sensitive than the other three strains. At 1.5mM H₂O₂, hundreds of colonies were seen for wild-type-pRS426 and rge1/2Δ-p3089 while rge1/2Δ-p316 and rge1/2Δ-p3086 had none. This shows that constitutively nuclear Yap1 can overcome double deletions in RGC1 and RGC2.
Figure 10. Streak test of \textit{rgc1/2Δ} screened at \textit{7.0mM H}_2\textit{O}_2. Single colonies of \textit{rgc1/2Δ} (DL3207) were screened at 7.0mM H\textsubscript{2}O\textsubscript{2} and re-streaked onto YEPD plates containing 7.0mM H\textsubscript{2}O\textsubscript{2}. Multiple streak tests were performed taking only the colonies that grew from the previous test to see a noticeable difference in growth between the candidates and the starting \textit{rgc1/2Δ} strain. The starting strain was also plated at 7.0mM H\textsubscript{2}O\textsubscript{2}. 
Figure 11. Sensitivity assay of *rgc1/2Δ* screened at 7.0mM H$_2$O$_2$. Single colonies of *rgc1/2Δ* from both mating-types were screened at 7.0mM H$_2$O$_2$ and prepared for a sensitivity assay on YEPD containing 0mM and 3.0mM H$_2$O$_2$. A subset of six candidates from *MATα* are shown. Compared to the starting *rgc1/2Δ* strain (DL3188), no difference was seen in growth at 3.0mM H$_2$O$_2$.

Figure 12. Sensitivity assay of *rgc1/2Δ* screened at 3.0mM H$_2$O$_2$. Fresh colonies of *rgc1/2Δ* were screened at 3.0mM H$_2$O$_2$ and prepared for a sensitivity assay on YEPD containing 0mM and 3.0mM H$_2$O$_2$. A subset of five candidates from *MATα* are shown. No difference was seen between the starting *rgc1/2Δ* strain (DL3188) and the screened candidates.
Figure 13. Sensitivity assay of screened rgc1/2Δ grown in YEPD containing 3.0mM H₂O₂. (A) A subset of four colonies screened at 3.0mM H₂O₂ was grown in YEPD containing 3.0mM H₂O₂ for use in a sensitivity assay. At 3.0mM H₂O₂, the screened colonies grew better than the starting rgc1/2Δ strain. (B) Single colonies taken from boxed area in (A) were then grown in YEPD and plated again at 0mM and 3.0mM H₂O₂. No difference was seen between the candidates and the starting strain. This revealed that the resistance of the screened candidates to hydrogen peroxide was temporary.
DISCUSSION

The purpose of this study was to uncover how Rgc1 and Rgc2 function in oxidative stress protection. It was hypothesized that strains with deletions in both paralogs would be more sensitive to hydrogen peroxide than strains with only a single deletion. It was further hypothesized that a suppressor screen would reveal genes that could rescue the function of Rgc1 and Rgc2, and help characterize their role in oxidative stress. Past findings discovered more phosphorylation sites present on Rgc2 than targeted by Hog1 in hyperosmotic stress and that none of other MAP kinases in yeast were responsible for those phosphorylations during oxidative stress. Combined with the finding that Rgc2 contains pleckstrin homology (PH) domains with long insertions, it is thought that Rgc1 and Rgc2 have the ability to bind to other proteins in addition to Fps1.

Rgc1/2 Have a Role in Oxidative Stress Protection

Here we show that a strain containing double deletions in RGC1 and RGC2 is more sensitive to oxidative stress than either deletion alone (Figure 2). The negligible difference between a single deletion in RGC1 or RGC2 and the wild-type strain can be explained by the fact that Rgc1 and Rgc2 are paralogs with 41% sequence homology. This allows them to be functionally redundant and protect the cell from oxidative stress. A second analysis was performed comparing three transformed strains: fps1Δ-FPS1-wt, fps1Δ-fps1-FKSV, and rgc1/2Δ-p313 (Figure 3). Both fps1Δ-fps1-FKSV and rgc1/2Δ-p313 strains had mutations that affected the interaction of Rgc2 and Fps1, yet they
exhibited different degrees of sensitivity. The increased sensitivity of \textit{rgc}\textsubscript{1/2Δ}-p313 to
grow in hydrogen peroxide suggested it has a role in oxidative stress protection in
addition to binding to the Fps1 glycerol channel. This finding led to a genome-wide
search for downstream effectors of Rgc1 and Rgc2.

**Genomic Overexpression Library Screen**

To better characterize the role of Rgc1/2 in oxidative stress, a suppressor screen
was used to search the yeast genome for a gene that when overexpressed could rescue
Rgc1 and Rgc2. The screen was initially performed with \textit{rgc}\textsubscript{1/2Δ} transformed with
\textit{yEP352}, a vector containing the selectable marker \textit{URA3} (Figure 4). This preliminary
experiment revealed that a hydrogen peroxide concentration of 1.2mM resulted in a 10-
fold decrease in background colonies and 1.8mM to be the threshold of colony growth.
For the actual experiment, 2.0mM and 2.5mM H\textsubscript{2}O\textsubscript{2} were chosen to screen \textit{rgc}\textsubscript{1/2Δ}
transformed with \textit{pRS202} (genomic library). These concentrations turned out to be too
strong and no real colonies were seen. The screen was repeated at lower concentrations of
1.8mM, 1.6mM, 1.5mM, and 1.4mM H\textsubscript{2}O\textsubscript{2} and a total of 49 colonies were obtained as
our candidates.

After isolating and amplifying the plasmid DNA, each candidate was digested
with HindIII and visualized using agarose gel electrophoresis (Figure 5). Many lanes
revealed an absence of plasmid DNA which could be due to the \textit{E. coli} transformation
procedure used for amplification. The first attempts at \textit{E. coli} transformation resulted in
seeing at most two to three transformants the following day. Many plates had none.
Although increasing the amount of competent cells used and adhesion time led to hundreds of transformants, the colonies were still relatively smaller than usual. Nevertheless, plates showing more than one transformed colony had multiple colonies picked up in case the original yeast colony was carrying multiple plasmids. The size of pRS202 is 6.3kbp (Addgene) and contains one HindIII restriction site so candidates that showed a single, large band or multiple bands of smaller sizes were sent for sequencing. Out of the nine candidates sent for sequencing, only four (31, 33-1, 39-1, 42-1) came back positive with primers T3 and T7 covering a region of the chromosome (Figure 6). Four different chromosomes were identified (XII, VII, XV, and XIII, respectively) and a variety of genes were located in these regions but we wanted to verify their ability to confer resistance in oxidative stress before pursuing them individually. The plasmid DNA was transformed back into yeast and two samples of each candidate were prepared for a sensitivity assay on YEPD containing 0mM, 2.0mM, 3.0mM, and 4.0mM H$_2$O$_2$ (Figure 7). Only the first sample of candidate 31 seemed to grow as well as wild-type transformed with yEP352 (URA3), but the second sample did not. Although the candidates did not seem to grow any better than $rgc1/2\Delta$-yEP352, it was difficult evaluate differences in growth so we decided to plate the transformants on SD-Ura containing 0mM, 1.8mM, and 2.0mM H$_2$O$_2$ (Figure 8), which were the conditions of the original experiment. We expected the candidates to grow more than $rgc1/2\Delta$-yEP352 (double deletion strain transformed with only a selectable marker), but the candidates actually grew less. There was some contamination seen on the plates which could have affected the results but in general, both verification experiments suggested that there was
something else conferring resistance in \( rgc1/2\Delta \) to hydrogen peroxide than our plasmid candidates.

**Constitutive Nuclear Yap1 Overcomes Deletions in RGC1/2**

Yap1 is one of the primary transcription factors activated in oxidative stress, regulating expression of over 70 genes (Harshman et al., 1988; Moye-Rowley et al., 1989; Gulshan et al., 2005; Okazaki et al., 2007). Without a strong plasmid candidate, we decided to look into the effect of \( YAP1 \) on the \( rgc1/2\Delta \) strain. The \( rgc1/2\Delta \) strain was transformed with p316 (\( URA3 \)), p3086 (wild-type \( Yap1 \)), and p3089 (\( YAP1-A627E \), constitutively nuclear mutant). These transformations were plated on SD-Ura containing 0mM, 1.0mM, and 1.5mM H\(_2\)O\(_2\) (Figure 9). Wild-type (DL3187) was transformed with pRS426 (\( URA3 \)) for comparison. All four strains grew well at 0mM H\(_2\)O\(_2\), showing thousands of colonies. At 1.0mM H\(_2\)O\(_2\), fewer colonies were seen and \( rgc1/2\Delta\)-p316 appeared to be the most sensitive. While \( rgc1/2\Delta\)-p316 had deletions for both \( RGC1 \) and \( RGC2 \), it still contained functional \( YAP1 \) and was able to survive at 1.0mM H\(_2\)O\(_2\). \( rgc1/2\Delta\)-p3086 contained more wild-type \( YAP1 \) and was better able to grow at 1.0mM H\(_2\)O\(_2\) although both strains did not survive at 1.5mM H\(_2\)O\(_2\). At 1.5mM H\(_2\)O\(_2\), only wild-type-pRS426 and \( rgc1/2\Delta\)-p3089 showed colonies. Yap1 is normally localized to the nucleus when the cell is exposed to oxidative stress (Yan et al., 1998; Kuge et al., 2001). The ability of \( rgc1/2\Delta\)-p3089 to grow at 1.5mM H\(_2\)O\(_2\) while \( rgc1/2\Delta\)-p3086 does not, tells us that the localization of Yap1 in the nucleus before the onset of oxidative stress
allowed the cells to better survive and overcome the double deletions in \textit{RGC1} and \textit{RGC2}.

\textbf{Genomic DNA Screen}

To further investigate which chromosomal genes may be activated downstream of Rgc1 and Rgc2, we aimed to create spontaneous mutations through a suppressor screen of \textit{rgc1/2\Delta} using YEPD plates containing hydrogen peroxide. A preliminary experiment was performed to determine the concentration of hydrogen peroxide needed to reduce the growth of a saturated \textit{rgc1/2\Delta} culture to pick up single colonies. This concentration was found to be dependent on cell density and 7.0mM H$_2$O$_2$ was used in the genomic screen. 10 individual colonies were grown separately from each mating-type with the goal of being able to do crosses and genetic studies. These colonies were then used for a streak test on YEPD plates containing 7.0mM H$_2$O$_2$ for verification of their resistance to hydrogen peroxide. The first streak test was inconclusive and surviving colonies were re-streaked until there was a noticeable difference in growth between the screened candidates and the starting \textit{rgc1/2\Delta} strain. The 20 candidates appeared similar and a subset of six candidates from \textit{MAT\alpha} is shown in Figure 10. The screened candidates were also prepared for a sensitivity assay on 3.0mM H$_2$O$_2$. Although the candidates were screened at 7.0mM H$_2$O$_2$, cultures for the sensitivity assay were started from single colonies and would not reach as high of a cell density as used in the screen. The candidates did not appear to grow any better than the starting \textit{rgc1/2\Delta} strain. A subset of six candidates from \textit{MAT\alpha} is shown in Figure 11.
The colonies used in the previous screen were taken from stock some time before and to determine if our results was due to aging of the cells, a fresh stock of rgc1/2Δ was started and the suppressor screen of genomic DNA was repeated at 3.0mM H₂O₂. Single colonies were grown and prepared for a sensitivity assay (Figure 12), but there was no difference in growth between the candidates and starting strain at 3.0mM H₂O₂. This showed that our previous result was not due to the age of the colonies and suggested that the cells were activating a temporary mechanism to overcome the hydrogen peroxide stress.

**Temporary Resistance of Screened Genomic Candidates**

To test whether the screened colonies were activating a temporary mechanism in response to hydrogen peroxide, a subset of four candidates from the genomic screen at 3.0mM H₂O₂ were grown in YEPD also containing 3.0mM H₂O₂. The starting rgc1/2Δ strain was allowed to grow in YEPD only. These five strains were plated for a sensitivity assay on YEPD containing 0mM and 3.0mM H₂O₂ (Figure 13A). This time, all of the candidates grew better than the rgc1/2Δ strain. It was surprising that the starting strain only had one spot of colonies at 3.0mM H₂O₂, but this can be attributed to a lower concentration of cells used; only four dilutions are seen at 0mM H₂O₂ when there should be at least five. To verify their resistance to hydrogen peroxide, single colonies were taken from the 3.0mM H₂O₂ plate (Figure 13A, boxed) and grown overnight in YEPD for a follow-up sensitivity assay (Figure 13B). The same candidates that appeared to be resistant to hydrogen peroxide in the first assay now grew no better than the starting
rgc1/2∆ strain. We realize this method of inducing spontaneous mutations in the genomic DNA may not work and that the cells are simply activating transient mechanisms to overcome the hydrogen peroxide.

Cellular response to oxidative stress is important for survival in *S. cerevisiae* since an accumulation of ROS leads to apoptosis (reviewed in Perrone et al., 2008). While de novo synthesis is required for resistance to ROS (Collinson & Dawes, 1992), post-translational modifications of cellular components is the faster, more common response in oxidative stress (reviewed in Biswas et al., 2006). This may be the reason for our difficulty in creating spontaneous mutations during the genomic suppressor screen. Metabolic responses currently known to change in oxidative stress include carbohydrate metabolism and the use of the pentose phosphate pathway to create a reducing environment (Ralser et al., 2007; Chechik et al., 2008). Yeast will also arrest cell cycle in G2, allowing time for repair and preventing the accumulation of mutations (reviewed in Shackelford et al., 2000). In our screen, it is also possible that the strains were mutated but then repaired themselves before the mutations could be passed onto the daughter cells.

**Conclusion**

Our results support the published literature that Rgc1 and Rgc2 are paralogs, able to cover each other’s function in oxidative stress. This study revealed that double deletions in *RGC1* and *RGC2* make *S. cerevisiae* more sensitive to oxidative stress than either deletion on its own. We also showed that *rgc1/2Δ* is more sensitive than *fps1-*
FKSV (Fps1 with four amino acid substitutions in the binding site of Rgc2) in oxidative stress even though both mutations affect binding of Rgc2 to Fps1, keeping the glycerol channel constitutively closed. These findings suggest Rgc1 and Rgc2 have a role in oxidative stress in addition to regulating the Fps1 glycerol channel during osmotic stress. Constitutively nuclear Yap1 (YAPI-A627E) was found to allow rgc1/2Δ to survive at 3.0mM H2O2 when wild-type YAPI did not which suggests a dependency on the nucleus and genomic DNA to protect the cell from oxidative stress. Finally, simple exposure to hydrogen peroxide is not the best way to create spontaneous mutations in the genomic DNA as cells will likely activate transient changes to counter the stress. Future research could be done on finding other proteins that interact with Rgc1/2 including upstream kinases that activate it in oxidative stress. Further analysis of the genes activated by Yap1 in oxidative stress that rescues rgc1/2Δ could also be performed. We believe understanding the role of Rgc1 and Rgc2 in oxidative stress is useful and should be continued. Knowing its mechanism of action could provide a better foundation on which to build the next generation of antifungal drugs.
LIST OF JOURNAL ABBREVIATIONS

Antioxid. Redox Sign. Antioxidants & Redox Signaling
Biochem. J. Biochemical Journal
Biochem. Pharmacol. Biochemical Pharmacology
Biochemistry–Moscow+ Biochemistry–Moscow
Biochim. Biophys. Acta Biochimica et Biophysica Acta
Biol. Cell Biology of the Cell
Clin. Infect. Dis. Clinical Infectious Diseases
Comp. Funct. Genom. Comparative and Functional Genomics
Curr. Opin. Microbiol. Current Opinion in Microbiology
EMBO J. EMBO Journal
Emerg. Infect. Dis. Emerging Infectious Diseases
Eukaryot. Cell Eukaryotic Cell
FEMS Microbiol. Rev. FEMS Microbiology Reviews
Gene. Dev. Genes & Development
Genome Res. Genome Research
| J. Biol. | Journal of Biology |
| J. Biol. Chem. | Journal of Biological Chemistry |
| Mech. Ageing Dev. | Mechanism of Ageing and Development |
| Microbiol. Mol. Biol. R. | Microbiology and Molecular Biology Reviews |
| Mol. Biol. Cell | Molecular Biology of the Cell |
| Mol. Cell | Molecular Cell |
| Mol. Microbiol. | Molecular Microbiology |
| Plant Physiol. | Plant Physiology |
| Trends Biochem. Sci. | Trends in Biochemical Sciences |
| Trends Cell Biol. | Trends in Cell Biology |
REFERENCES


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NORTHWOOD HIGH SCHOOL
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RESEARCH EXPERIENCE

BOSTON UNIVERSITY
Graduate Research Student – Molecular and Cell Biology
• Studied the function of yeast Rgc1/Rgc2 in oxidative stress

THE UNIVERSITY OF CALIFORNIA, IRVINE
Undergraduate Research Student – Biology Education
• Managed over 700 exams and experimental assignments per experiment
• Developed assignments based on findings to be used in the biology class

THE UNIVERSITY OF CALIFORNIA, IRVINE
Undergraduate Research Student – Cardiology
• Studied correlation between heart attack and life expectancy in Drosophila
• Worked on additional topics that included female fertility over time
CENTER FOR EDUCATIONAL PARTNERSHIPS  IRVINE, CA

- Enriched elementary schools in underserved communities
- Taught students concepts about kinetic, potential, and conservation of energy
- Encouraged participation in pretest and posttest

MEDICALLY RELAVENT EXPERIENCE

HOAG MEMORIAL HOSPITAL PRESBYTERIAN  NEWPORT BEACH, CA

- Registered patients in the emergency room
- Acquired customer service skills
- Collected demographics and insurance information
- Learned to multi-task and work in a fast-pace environment

ORANGE COAST COLLEGE  COSTA MESA, CA

EMT Intern  Aug. 2010 – May 2011
- Responded to 911 calls and played a greater role in patient care
- Learned how to perform patient assessments, take vital signs, and administer a select group of medications, such as glucose, aspirin, and nitroglycerin
- Observed extreme cases: sinus tachycardia, 5150 psychiatry hold, tracheostomy patient with bilateral leg amputations

WESTERN MEDICAL CENTER  SANTA ANA, CA

- Shadowed doctors, nurses, and specialists
- Logged over 500 hours in the ER department
- Trained 55 volunteers to work in the ER
- Served as collegiate president and membership chair
- Observed medical procedures: IV, EKG, CT scan, physical examinations, patient counseling, orthopedic casting, incisions, sutures, endoscopy