Old Yellow Enzymes, Highly Homologous FMN Oxidoreductases with Modulating Roles in Oxidative Stress and Programmed Cell Death in Yeast

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In a genetic screen to identify modifiers of Bax-dependent lethality in yeast, the C terminus of OYE2 was isolated based on its capacity to restore sensitivity to a Bax-resistant yeast mutant strain. Overexpression of full-length OYE2 suppresses Bax lethality in yeast, lowers endogenous reactive oxygen species (ROS), increases resistance to H2O2-induced programmed cell death (PCD), and significantly lowers ROS levels generated by organic prooxidants. Reciprocally, Δoye2 yeast strains are sensitive to prooxidant-induced PCD. Overexpression and knock-out analysis indicate these OYE2 antioxidant activities are opposed by OYE3, a highly homologous heterodimerizing protein, which functions as a prooxidant promoting H2O2-induced PCD in wild type yeast. To exert its effect OYE3 requires the presence of OYE2. Deletion of the 12 C-terminal amino acids and catalytic inactivation of OYE2 by a Y197F mutation enhance significantly survival upon H2O2-induced PCD in wild type cells, but accelerate PCD in Δoye3 cells, implicating the oye2p-oye3p heterodimer for promoting cell death upon oxidative stress. Unexpectedly, a strain with a double knock-out of these genes (Δoye2 Δoye3) is highly resistant to H2O2-induced PCD, exhibits increased respiratory capacity, and undergoes less cell death during the adaptive response in chronological aging. Simultaneous deletion of OYE2 and other antioxidant genes hyperinduces endogenous levels of ROS, promoting H2O2-induced cell death: in Δoye2Δglr1 yeast high levels of oxidized glutathione elicited gross morphological aberrations involving the actin cytoskeleton and defects in organelle partitioning. Altering the ratio of reduced to oxidized glutathione by exogenous addition of GSH fully reversed these alterations. Based on this work, OYE proteins are firmly placed in the signaling network connecting ROS generation, PCD modulation, and cytoskeletal dynamics in yeast.

Cell suicide responses regulated through programmed cell death (PCD) have been documented not only in higher organisms but also in bacteria and yeast. In nature, unicellular organisms exist as populations in an environment with limited resources (1); thus a conserved suicide program in which older or damaged cells sacrifice themselves and release nutrients to the remaining cells promotes continued group survival (2–4). Cell death with apoptotic features has been reported in yeast treated with low concentrations of acetic acid or hydrogen peroxide, with DNA damage induced by UV radiation treatment, after exposure to high levels of mating pheromone, and upon aging. Recently, it has become clear that a core PCD machinery exists in **Saccharomyces cerevisiae**. For example, yeast with mutations in the **CDC48** gene, an AAA family member involved in the fusion of endoplasmic reticulum-derived vesicles, exhibit characteristic hallmarks of apoptotic cell death including DNA fragmentation, chromatin remodeling, and annexin V staining. Similarly, expression of a mutant form of the mammalian ortholog of **CDC48**, valosin containing protein, induces mammalian cells to undergo apoptosis. Yeast analogs of a number of components of the canonical apoptotic machinery have been described. Yeast homologs for caspase-like proteases, **YCA1** (14); for the **OMI/HtrA2** protease, **NMA111** (15); for apoptosis inducing factor, **YNR074C**; and for apoptosis inducing factor-homologous mitochondrion-associated inducer of death **(AMID)**, **NDI1**, have all been implicated in the regulation of PCD in yeast.

Heterologous expression of mammalian regulators of apoptosis in yeast can influence yeast PCD. Expression of the anti-apoptotic protein Bcl-2 can rescue a superoxide dismutase-deficient yeast strain, whereas expression of the pro-apoptotic counterpart Bax or Bak kills yeast in a manner that resembles PCD induced by these proteins in mammalian cells. Upon expression in yeast, Bax localizes mainly in the mitochondria and promotes mitochondrial membrane hyperpolarization, causing an eventual collapse of ΔΨm and

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**References**

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5. The abbreviations used are: PCD, programmed cell death; ROS, reactive oxygen species; OYE, Old Yellow Enzyme; GSH, γ-glutamylcysteinylglycine; GSSG, glutathione disulfide; CM, complete medium; t-BOOH, tert-buty1 hydroperoxide; CHP, cumene hydroperoxide; HE, hydroethidium; DKO, double knock-out; EMS, ethane methyl sulfonate; mit-GFP, mitochondria targeted green fluorescent protein; HA, hemagglutinin; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; YFP, yellow fluorescent protein.
release of reactive oxygen species (ROS) and cytochrome c. This phenotype has been exploited to isolate proteins inhibiting Bak lethality (20), allowing the identification of BI-1, a highly conserved apoptosis inhibitor (21), enzymes involved in the ROS detoxification such as BI-GST (22) and ascorbate peroxidase (23), and Ku70, an evolutionarily conserved component of the double-stranded DNA repair machinery (24).

Previous work in our laboratory isolated a series of EMS-mutagenized yeast strains that exhibited resistance to Bax-induced PCD (25). To further exploit the effect of Bax and obtain insights on the yeast PCD machinery, we have here utilized one such mutant that failed to target Bax to mitochondria, in a reverse genetic screen to identify yeast proteins that restore sensitivity to Bax. This screen identified the C terminus of the conserved flavin mononucleotide (FMN) oxidoreductase OYE2. Suggestively, the highly related OYE3 protein, which is known to heterodimerize with OYE2 (26), had previously been found to modulate Bax-dependent PCD in yeast (27).

In the current study, we show that full-length OYE2 suppressed Bax lethality in wild type yeast, and is a potent antioxidant protein. This activity contrasts with that of OYE3, which antagonized the protective action of OYE2 in H$_2$O$_2$-induced programmed cell death. The effect of OYE3 requires the presence of OYE2, indicating that it is the oye2p-oye3p heterodimer that facilitates PCD. Surprisingly, the absence of both genes rendered cells hyper-resistant to H$_2$O$_2$-induced PCD by increasing their respiratory efficiency. Deletion of OYE2 with other antioxidant genes elevated endogenous ROS and sensitized cells further to H$_2$O$_2$-induced PCD. In the case of Δoye2 grl1 cells, the cellular redox environment with high levels of oxidized glutathione led to gross morphological aberrations, actin cytoskeleton abnormalities, and defects in organelle partition between mother and daughter cells. Together, these results indicate that OYE2 is a connection point between ROS generation, modulation of PCD, and cytoskeletal regulation.

**EXPERIMENTAL PROCEDURES**

**Genetic Screen in Yeast**—A previously characterized EMS mutagenized yeast strain R13 (his3 ura3 trpl LexA-operator-LEU2) carrying the pGILDA/Bax plasmid was transformed with a yeast genomic library on the plasmid pJG4-5 (25). Growing colonies were replica plated on glucose/C-Mis,Trp, and galactose-raffinose/C-Mis,Trp. Colonies growing on glucose media but not on galactose, where Bax is expressed, were selected for further characterization. The library plasmids were extracted and then reintroduced into fresh cells and were tested for the reproducibility of the Bax resensitization phenotype. The library plasmids capable of restoring Bax lethality were sequenced.

**Growth Recovery Curves**—Fresh overnight cultures of the various yeast strains grown in Glu/CM media or glucose media lacking the amino acid used as auxotrophic marker were washed with dH$_2$O and resuspended in fresh medium at A$_{600}$ = 0.1. Aliquots were taken at regular intervals and the absorbance was measured. When A$_{600}$ > 1, aliquots were serially diluted and new measurements were taken. To examine the effect of a transient pulse of H$_2$O$_2$ in cultures overexpressing the OYE proteins, fresh cells were resuspended at A$_{600}$ = 0.1 in fresh glucose media and 2 h later, at the end of the lag period, the cultures were supplemented with 1.5 or 1.25 mM H$_2$O$_2$ and incubated with shaking at 30 °C. The ability of the cell populations to recover from the H$_2$O$_2$ insult was assessed by measuring growth at A$_{600}$ at regular intervals. All growth recovery assays were performed independently in triplicate.

**Plasmid Constructs**—The full-lengths of the OYE2 and OYE3 genes were PCR amplified from wild type yeast genomic DNA using primers 5’-OYE2(EcoRI) 5’-GAATTCTAGCTTACATGGTTG-TAAGGACCTTTAGACCC-3’ and 3’-OYE2(XhoI) 5’-CTCGAG-TTAATTTTGTCCAACCAGTTTTAG-3’ for OYE2 and 5’-OYE3 5’-CAATTGTAGCCATTCTAGAAGGGCGCATC-3’ and 3’-OYE3 5’-CTCGAGTCAGTTCTTGTGCTAACCTAATCTACTGC-3’ for OYE3. A fusion of the OYE2 with GFP in the C terminus was prepared using a two-step PCR approach with overlapping primers. In the first step, OYE2 was amplified using primers 5’-OYE2(EcoRI) and 3’-OYE2(GFP), 5’-CTGCCCCTTGCTCAATTTTGTGCC-AACC-3’, and the GFP construct was amplified using the 5’-OYE-GFP 5’-GGTTTGGGACAATATGAGCAAGGGCGAC-3’ and the 3’-GFP(XhoI) 5’-CTCGAGTTACTTGATGCAGGTCGTCATGCC-3’. The amplified products from the first round were gel extracted and purified. A small aliquot of the two fragments was mixed in a new PCR using the 5’-OYE2(EcoRI) and the 3’-GFP(XhoI) external primers. All PCR amplifications were made using Platinum Taq polymerase (Invitrogen). The purified fragments were cloned into the pCR2.1 TOPO TA vector according to the manufacturer’s instructions. All the cloned inserts were subsequently subcloned into the yeast expression vectors: pJG4-6, expressing the proteins under a galactose promoter; and pYX143 and pYX143-HA (hemagglutinin tagged), which are low copy number vectors (ARS/CEN, LEU2) expressing the genes under the control of the constitutive TPI promoter. Expression of the proteins was verified in pYX143-HA and pG4-6 in Western blots using antibodies against the HA tag. BY4741 wild type yeast cells were transformed with pYX143-oye2, pYX143-oye3, or a control empty plasmid. Protein expression was verified indirectly by parallel cloning of the OYE2 and OYE3 cDNAs into the pYX143-HA vector, which expresses the cDNAs fused to a hemagglutinin tag. However, the untagged vectors were used in all subsequent experiments to eliminate the possibility of any interference of the HA tag.

A C-terminal-truncated construct of OYE2 was generated by PCR using the primer 5’-OYE2 and 3’-OYE2 (1-388) 5’-CTCGAGTCAGTTACATGGTTGCTAACCTAATCTACTGC-3’. The product was sequenced and subcloned into the pYX143-HA vector, which expresses the cDNAs fused to a hemagglutinin tag. However, the untagged vectors were used in all subsequent experiments to eliminate the possibility of any interference of the HA tag.
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verify the mutation. The Y197F OYE2 cDNA was subcloned into pYX143, pYX143-HA yeast vectors as above.

Flow Cytometric Studies—Yeast strains growing in glucose complete media, until late logarithmic phase, were washed with PBS and stained. Dihydrothidium (HE; D-1168, Molecular Probes) at 4 μM was used as an indicator of endogenous ROS. Yeast cells harboring plasmids were grown in glucose complete media lacking the amino acid used as the auxotrophic marker. To assess cellular responses to pro-oxidants, aliquots of grown cells were treated for 1 h with 1.5 mM hydrogen peroxide (H2O2) or 1 mM cumene hydroperoxide (CHP). Subsequent to treatment, cells were washed extensively with PBS by repeated centrifugations and finally stained with HE as above. Using FACS, 100,000 cells from each sample were measured. Quantification was performed using the Cytomation software (DAKO).

Microscopy and Fluorescence Measurements—Mitochondrial import and morphology was visualized using plasmid pVT100U-mitGFP or plasmid pYX142-mitGFP (which expresses GFP fused to a mitochondrial matrix targeting sequence), which were introduced in yeast cells (28). The organelles were observed in a single plane so as to observe the relative diameter of the tubules. Actin filaments were stained by fixing with 4% formaldehyde and staining using rhodamine-phalloidin (R-415, Molecular Probes). Nuclei were stained with Hoechst 33342. To mask fluorescence from mitochondrial DNA a low dose of Mitotracker Red CMXRos (M7512, Molecular Probes) was used as a counterstain. The lumen of yeast vacuoles was stained with 50 μM CMAC (Y-7531, Molecular Probes) for 20 min. At the end of incubations cells were washed twice, resuspended in prewarmed PBS, applied on microscope slides and observed under ×1000 magnification in a fluorescent microscope. Necrotic cells with permeabilized outer membranes were measured by Evans blue staining. In addition to FACS analysis, ROS were also measured using a PerkinElmer LS55 Luminescence spectrometer. Fresh cultures of cells were resuspended in PBS at A600 = 0.5 and 1 ml aliquots of cells were stained with Mitotracker Red CMXRos (M-7512, Molecular Probes) for 15 min in the dark and washed once with PBS, at the end of incubation the cells were resuspended in 2 ml and fluorescence was measured over the optimal emission range.

Generation of Double Knock-out Yeast Strains—A yeast strain harboring a deletion of the OYE2 gene was generated by integrating a URA3 cassette originating from the pUG72 plasmid (29). The primers 5′-TCATATAAGCTTAA-TATAAGCCATTGATGTCAAATATG-3′ and 3′-TCATATAAGCTTAA-TATAAGCCATTGATGTCAAATATG-3′ were used to amplify the cassette in a PCR. The amplified DNA fragment was purified and transformed into a Mata strain that originated by repeated backcrosses to a BY4741 background. Proper integration of the URA cassette was verified by PCR using primers 5′-CGCAGCAAAGTTCTTCT-GCTTGATTATTCACATATGT-3′, and 3′-TCATATAAGCTTAA-TATAAGCCATTGATGTCAAATATG-3′, which contain flanking sequences from the 5′ and 3′ of the OYE2 gene, were used to amplify the cassette in a PCR. The amplified DNA fragment was purified and transformed into a Mata strain that originated by repeated backcrosses to a BY4741 background. Proper integration of the URA cassette was verified by PCR using primers 5′-GGGAATTC-TATAAGCCATTGATGTCAAATATG-3′, and 3′-TCATATAAGCTTAA-TATAAGCCATTGATGTCAAATATG-3′. To generate double knockouts the Δoye2::URA3 strain was mated to BY4741 deletion strains Δoye3, Δsod1, Δett1, Δgbr1, Δyca1, and Δgsh1 (Research Genetics), which harbor the G418 antibiotic resistance marker on the deleted gene. Diploid cells were induced to sporulate and the regenerating spores were tested in Glu/CM-ura, +G418 plates. The mating type of the double knock-out haploid strains was subsequently assessed.

ρ0 strains (lacking functional mitochondria) were generated from the ρ0 Δoye2 oye3 strain by growing cells in YPD broth containing 10 μg/ml ethidium bromide for 3 days (30). Dilutions were plated on YPD plates and growing colonies were tested for their capacity to grow on YPGlycerol media.

Induction of PCD with H2O2 and Acetic Acid and Colony Viability Assays—Cells growing in logarithmic phase were used to inoculate at very low density in fresh glucose media. A small aliquot was subsequently removed, serially diluted, and plated on rich YPD plates. This represented the 0 time point and viability of 100%. To the diluted cells, H2O2 was added to 1 mM, and cells were incubated for 2 h at 30°C. Treated cells were enumerated as above and compared with the original count. All experiments were performed independently in triplicate. The presence of necrotic cells was determined by staining with Evans blue and visualization by light microscopy. Acetic acid-induced PCD was performed in accordance to the protocol described by Ludovico et al. (5) using unbuffered Glu/CM media.

Chronological Aging Assays—All strains were grown in 10-ml cultures with unbuffered glucose complete media (2% glucose) until saturation. At the end of the second day of incubation, a small aliquot of cells was removed and the number of live cells was enumerated by serial dilution and plating on YPD plates. This corresponded to the 0 time point. Aliquots of cells were removed regularly and cells were enumerated as above.

Determination of Total Glutathione and Glutathione Disulfide—BY4741, Δoye2, Δoye3, Δoye2 oye3, and Δoye2 gbr1 cells were grown until late log phase in Glu/CM. Total glutathione and glutathione disulfide were measured according to a method described by Griffith (31) and modified by Kampranis et al. (22).

RESULTS

The FMN Oxidoreductase OYE2 Limits Bax-induced Lethality in Yeast—To identify yeast genes that participate in PCD processes we took advantage of the Bax-induced lethal phenotype in yeast. The Bax-resistant EMS-mutant strain R13, which exhibits defects in mitochondrial protein targeting (25), was used in a screen to identify yeast proteins that can restore Bax sensitivity. We transformed cells containing a galactose-inducible Bax expression plasmid with a yeast genomic library cloned into the pJG4-5, galactose-inducible, yeast expression vector. Transformed yeast were initially plated on glucose-selective plates at low density to enable us to pick distinct colonies. Approximately 3,000 colonies were replica-plated on glucose-selective plates at low density to enable us to pick distinct colonies. The FMN Oxidoreductase OYE2 Limits Bax-induced Lethality in Yeast
OYE3, an FMN oxidoreductase homologous to OYE2, attenuated Bax-induced PCD, a significant proportion also showed defects in protein transport to mitochondria, most likely limiting integration of Bax to the outer membrane. Expression of OYE2-(314–400) in R13 cells not only sensitized cells to Bax lethality (Fig. 1A), but also restored the ability of GFP fused to a mitochondrial targeting sequence (mit-GFP) to associate with mitochondria (Fig. 1C), indicating that OYE2 can affect mitochondrial targeting. The size of R13 cells also decreased and resembled wild type appearance.

In wild type yeast, Bax causes changes in the morphology of the mitochondria. Whereas in a percentage of cells the organelles became fragmented, the remaining cells exhibit fewer swollen organelles most likely by fusion as a defense response (Fig. 1B). YFP-Bax localized to mitochondria in wild type cells, whereas in the R13 Bax-resistant mutant the YFP fusion clearly fails to target the organelles (Fig. 1B, bottom row). Co-expression of YFP-Bax with the OYE2-(314–400) C terminus reversed the diffused fluorescence, however, only very weak peripheral fluorescence could be detected (data not shown).

YFP-Bax is lethal to R13 cells, only when co-expressed with the OYE2-(314–400) despite the difficulty in detecting its fluorescence. Recently TOM22, a component of the complex responsible for initial import of mitochondrial targeted proteins was identified as a Bax receptor (32). This could explain the frequent association between Bax resistance and defects in mitochondrial protein translocation in our mutants (25).

We next overexpressed the full-length open reading frame of OYE2 from the pJG4-4 vector, under the control of a galactose-inducible promoter. In wild type EGY48 cells expressing Bax, overexpression of the full-length OYE2 suppressed Bax lethality, whereas the C terminus OYE2 could not do so (Fig. 2A). OYE2 also reversed to a large extent mitochondrial swelling, as well as excessive mitochondrial fission, both characteristic effects of Bax expression in sensitive yeast strains (Fig. 2B). Full-length OYE2 did not resensitize the R13 mutant cells to Bax (data not shown), suggesting that this action of the OYE2-(314–400) represented a dominant negative effect of the truncated protein. Supporting the OYE2 protective role, Bax expression in Δoye2 was more toxic compared with wild type yeast (data not shown).

OYE2 Localizes to Mitochondria—As OYE2 was isolated based on restoring Bax sensitivity to a strain with deficient localization of Bax to its site of action, the mitochondria, we asked if OYE2 might itself associate with mitochondria. A full-length OYE2-GFP fusion was introduced into EGY48 cells under the control of a galactose-inducible promoter. In wild type EGY48 cells expressing Bax, overexpression of OYE2-(314–400) restored the ability of GFP fused to a mitochondrial targeting sequence (mit-GFP) to associate with mitochondria (Fig. 1C). This indicates that OYE2 localized on the organelle and can thus limit directly the capacity of Bax to insert and oligomerize on the mitochondrial outer membrane. The OYE2-GFP fusion weakly protected wild type cells from Bax lethality and did not act as dominant negative (data not shown).

OYE2 and OYE3 Have Opposing Functions in Regulation of Oxidative Stress and PCD

Reekmans et al. (27) have recently shown that deletion of OYE3, an FMN oxidoreductase homologous to OYE2, attenuated Bax-induced growth arrest, cell death, and caused a decrease in NADPH in yeast. Among the EMS mutant yeasts we previously generated, which are resistant to Bax-induced PCD, the frequent association between Bax resistance and defects in mitochondrial protein translocation in our mutants (25).
stress (33). OYE3 modulates Bax-dependent PCD (27), and heterodimerizes with OYE2 in vivo and in vitro (26). Furthermore, OYE2 and OYE3 share 82% identity at the amino acid level, suggesting a related activity. To elucidate the functional relationship of OYE2, and OYE3, we expressed them at moderate levels in yeast and assessed basal and induced levels of oxidative stress. OYE2 and OYE3 share 82% identity at the amino acid level, suggesting a related activity. To elucidate the functional relationship of OYE2 and OYE3, we expressed them at moderate levels in yeast and assessed basal and induced levels of oxidative stress.

Yeast overexpressing OYE2 or OYE3, or vector-transformed control cells, were treated with 1.5 mM H$_2$O$_2$, with 1 mM of the prooxidant tert-butyl hydroperoxide (t-BOOH), and with 0.2 mM of the prooxidant CHP. After incubation, cells were stained with HE to gauge oxidative stress: Fig. 3A compares FACS-determined HE values for cells overexpressing OYE2 or OYE3 (hatched lines) in reference to cells expressing empty vector (vertical lines) for each condition. In untreated cells, overexpression of OYE2 modestly lowered (-13%), and OYE3 elevated (+24%), endogenous ROS, when compared with wild type BY4741 cells harboring empty vector. These modest changes were reproducible by FACS and experiments measuring ROS using a luminescence spectrometer done in triplicate. OYE2 or OYE3 overexpression did not significantly influence ROS levels following treatment with H$_2$O$_2$ (−3 and +7%, respectively). However, treatment of cells with organic prooxidants generated more dramatic differences in ROS levels dependent on OYE status. Overexpression of OYE2 caused a substantial reduction in ROS levels in both t-BOOH (−13%) and CHP (−44%) treated cells compared with their wild type counterparts, suggesting an antioxidant activity. In contrast, overexpression of OYE3 caused a substantial increase in ROS levels, implying a prooxidant activity (+25% for t-BOOH and +37% for CHP).

To further analyze OYE function, we performed similar experiments, comparing basal or induced ROS in wild type parental BY4741 yeast with ROS in strains deleted for OYE2 or OYE3 (Δoye2 and Δoye3). Both deletion strains showed similar ROS levels under basal conditions, or following H$_2$O$_2$ treatment Δoye2 cells treated with CHP exhibited a small increase in ROS (+12%); whereas Δoye3 cells showed lower ROS levels upon t-BOOH and CHP treatment (−20 and −18%, respectively) (Fig. 3A). These changes in ROS again suggested antioxidant activity for OYE2, and prooxidant activity for OYE3. The ROS changes upon CHP treatment are more dramatic and tend to show higher heterogeneity as a general feature, which was observed in a large series of deletion strains tested (data not shown). To support of these ROS alterations, all experiments with overexpression and deletion strains were also performed using Mitotracker CMXROS staining and detection with a fluorescence spectrometer: identical patterns of variance were seen (data not shown).

To assess the physiological relevance of the changes observed in endogenous ROS levels in our tested strains, we additionally examined Δsod1 and Δctt1 cells harboring deletions in superoxide dismutase 1 and catalase 1, respectively. Δsod1 cells exhibited elevated endogenous ROS (+52%) compared with parental BY4741 WT cells. Treatment with H$_2$O$_2$ led to an additional increase in ROS (+69%). In contrast, no changes in ROS levels were seen in treated or untreated Δctt1 cells (Fig. 3B).

**OYE2 and OYE3 Have Opposing Functions in the Regulation of PCD**—Under standard growth conditions, the growth of yeast overexpressing OYE2 or OYE3 is undistinguishable from that of control cells (not shown). We next asked if OYE status regulated cell viability and sensitivity to PCD induced by different stimuli. H$_2$O$_2$ is a standard inducer of PCD in yeast (6); we examined cellular viability of H$_2$O$_2$-treated yeast cells overexpressing OYE2 or OYE3. Strikingly, cells overexpressing OYE2 recovered more rapidly than did control cells, entering log phase at 12 versus 22 h following H$_2$O$_2$ addition, whereas cells overexpressing OYE3 failed to recover even at 40 h after treatment (Fig. 4A). Given the effect of the C terminus of OYE2 on
heterodimers, OYE3 was overexpressed in Δoye2 cells harboring a deletion for the OYE2 gene. OYE3 homodimers in Δoye2 cells exert a protective function (Fig. 4D). Inversely overexpression of OYE2 in Δoye3 cells maintained its protective function. However, overexpression of OYE2-(1–388) offered no protection in Δoye3 cells, indicating that the protective effect of the C-terminal truncation is exerted upon heterodimerization to OYE3 (Fig. 4E). Additionally, we tested a point mutation of OYE2 in which tyrosine 197 (Tyr196 in Saccharomyces pastorianus OYE) is changed to a phenylalanine, previously shown to cause a dramatic decrease of its oxidative half-reaction but to have little effect on ligand binding and its reductive half-reaction (34). Overexpression of Y197F OYE2 in BY4741 wild type cells caused an important increase in cell viability upon H2O2-induced PCD (Fig. 4G) as in the case of C-terminal truncation. To assess whether the effect of Y197F OYE2 is exerted on the heterodimer with OYE3, we overexpressed the mutant protein in Δoye3 cells (Fig. 4H). Whereas overexpression of OYE2, which are OYE3 homodimers, were protective in H2O2-induced PCD, expression of the Y197F OYE2 in Δoye3 cells was lethal and cells were unable to recover after 30 h incubation. Taken together, our data indicate that formation of oye2p-oye3p heterodimers contributes to the induction of PCD upon oxidative stress, and that obstruction of heterodimer formation in wild type cells by co-expressing OYE2-(1–388) or Y197F OYE2 leads to elevated survival.

**Double Deletion of OYE2 and OYE3 Renders Cells Highly Resistant to H2O2-induced PCD**—Extending further on the overexpression results, we analyzed cell death induced by a H2O2 pulse in Δoye2, Δoye3, and Δoye2Δoye3 (double knockout) yeast, versus the BY4741 parental control. In liquid medium, Δoye3 cells recovered more quickly than wild type cells and Δoye2 more slowly than wild type cells (Fig. 5A), reciprocal to the results seen with overexpression (Fig. 4A). Unexpectedly, the Δoye2Δoye3 double knock-out yeast recovered earlier than all the other strains. These recovery results coincide with the independent colony viability assays of cells pulsed with H2O2 for 2 h (Fig. 5B). Δoye2 cells were more susceptible to H2O2-induced PCD than wild type cells, whereas Δoye3 cells were more resistant. Again, Δoye2 Δoye3 cells were highly resistant to H2O2, maintaining >50% viability under conditions where BY4741 cells were only 8% viable. The magnitude of pro-
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Tection of Δoye2 oye3 cells was assessed by comparison to Δyca1 a deletion strain in the apoptotic yeast metacaspase. Cells treated with lower H2O2 dose (0.8 mM) were monitored in growth survival assays. Δyca1 cells at this concentration performed only slightly better than wild type cells, whereas Δoye2 oye3 cells were markedly better off than both strains (Fig. 5C). The extent of survival differences was further enhanced at higher H2O2 concentrations (1.25–1.5 mM) (not shown). Staining of H2O2-treated cells with annexin V/Evans blue showed increased phosphatidylserine externalization, one of the hallmarks of apoptosis (Fig. 5D). Annexin-stained cells did not internalize Evans blue dye, confirming the apoptotic nature of death.

The endogenous ROS levels of Δoye2 oye3 were nearly identical to wild type cells (Fig. 5E). Overexpression of OYE2, OYE3, or OYE2-(1–388) in Δoye2 oye3 cells reduced in all cases survival upon H2O2 treatment (Fig. 5F).

We next compared the relative respiratory capacity of OYE deletion strains, because increases in respiratory rates are frequently escorted with adaptation of the cell antioxidant machinery to cope with increased toxic byproducts of respiration. To assess respiratory capacity, serial dilutions of yeast were plated in parallel on rich medium (YP) with glucose (YPD) or glycerol (YPG) as carbon sources. EGY48 was an efficiently respiring positive control, whereas parental BY4741 cells had a more limited respiratory capacity. Although deletion of single OYE genes had little effect, Δoye2 oye3 cells exhibited growth on glycerol equivalent to the EGY48 cells, and significantly higher than the single knockouts and the BY4741 parental strain (Fig. 5G).

Deletion of both OYE caused a qualitative change in cellular physiology that enhanced respiration, which probably triggered an adaptive response from the antioxidant machinery that effectively kept ROS at low levels.

FIGURE 4. OYE2 and OYE3 modulate H2O2-induced PCD. A, BY4741 WT cells overexpressing OYE2 or OYE3 in moderate levels from a constitutive TPI promoter or harboring an empty vector were resuspended in equal densities in fresh Glu/CM-Leu media, and 2 h later H2O2 was added at 1.5 mM final concentration. The cell cultures were evaluated for their capacity to recover by monitoring A600 at regular intervals. B, BY4741 WT cells overexpressing a C-terminal truncation of OYE2-(1–388) OYE2 recover faster than OYE2 overexpressing cells. C, logarithmically grown cells were resuspended in fresh medium at the same A500. Equal volume aliquots were used to inoculate fresh media at very low density, which were subsequently treated with 1 mM H2O2 for 2 h. Viable cells were enumerated, prior and post-treatment, by plating serial dilutions on YPD plates. D, OYE3 overexpressed in Δoye2 cells is compared with Δoye2 cells harboring empty vector and wild type cells overexpressing OYE3 in the growth recovery assay described above. E, Δoye3 cells overexpressing OYE2, Y197F OYE2, or empty vector were monitored for growth recovery subsequent to H2O2 insult. The OYE2-truncated form exerts no protection in the absence of OYE3. F, BY4741 WT cells overexpressing OYE2, Y197F OYE2, or empty vector were monitored for growth recovery subsequent to H2O2 insult. G, Δoye3 cells overexpressing OYE2, Y197F OYE2, or empty vector were monitored for growth recovery as above. H, Western blot detection of oye2p and its corresponding mutants Y197F and 1–388 C-terminal truncation using anti-HA antibodies.
To examine whether active respiration in \( \Delta \text{oye2 oye3} \) is important for resistance to cell death from oxidative stress, we proceeded to generate \( \Delta \text{r}^0 \) strains (lacking functional respiration) from the isogenic \( \Delta \text{oye2 oye3} \) strain. Cells were treated with ethidium bromide to eliminate mitochondrial DNA (30). Five colonies were tested in parallel to \( \Delta \text{oye2 oye3} \) cells by plating serial dilutions on YPDextrose and YPGlycerol plates to verify the absence of respiration (Fig. 5H). All tested strains were confirmed to be \( \Delta \text{r}^0 \).

Three \( \Delta \text{r}^0 \) \( \Delta \text{oye2 oye3} \) strains were selected and pulsed with \( \text{H}_2\text{O}_2 \) for 2 h and colony viability was enumerated by plating on YPD plates (Fig. 5I). All 3 \( \Delta \text{r}^0 \) \( \Delta \text{oye2 oye3} \) strains were sensitive to \( \text{H}_2\text{O}_2 \)-induced PCD. Growth recovery curves, performed in parallel, exhibited identical sensitivities (not shown), but was not the assay of choice due to growth difference between untreated \( \Delta \text{r}^+ \) and \( \Delta \text{r}^0 \) strains. Overall, our data underline the importance of respiration in resistance to PCD from oxidative damage.
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Response of Δo ye2 o ye3 to Aging and Acetic Acid-induced Cell Death—In cultures growing in high glucose levels, an age- and pH-dependent form of programmed cell death occurs, in which the majority of the cells die and eventually an adapted subpopulation emerges in their place (34). To assess this form of PCD wild type BY4741, Δo ye2, Δo ye3, and Δo ye2 o ye3 cells were grown in 10-ml cultures in glucose complete media (2% glucose) until saturation. At the end of the second day of incubation, a small aliquot of cells was removed and the number of live cells was enumerated by serial dilution and plating on YPD plates. This corresponded to the 0 time point. Aliquots of cells were removed regularly and cells were enumerated as above. The Δo ye2 o ye3 cells exhibited reproducibly a substantial decrease in the percentage of dying cells during the adaptive regrowth process (Fig. 5). The reduced cell death during chronological aging could be explained by the physiological changes that have taken place in the DKO strain, as evidenced by their increased respiratory capacity. This induced form of cell death is known to be associated with nutrient, pH, and the redox status of the yeast strain. Although, in the long run (>15 days) the viable cell counts were always lower than wild type cells.

Finally, acetic acid also induces PCD and increases levels of ROS in yeast (5). We therefore proceeded to examine the potential involvement of OYE2 and OYE3 in this type of cell death, using experimental regimens similar to those used for H₂O₂. BY4741, Δo ye2, Δo ye3, and Δo ye2 o ye3 yeast were grown to logarithmic phase, then aliquots of cells were treated with increasing concentrations of acetic acid (10, 20, and 40 mM), and used for colony forming assays (Fig. 5k). The percentage of necrotic cells was estimated by staining treated cells with a non-permeant Evans blue dye, and was found to be comparable with non-treated cells for all cases (1–3%). At the lowest concentration of 10 mM acetic acid, the parental cells exhibited a minimal reduction of cell viability around 20%. The reduction of viability was proportional to the increasing concentrations of acetic acid. The rate of viability loss was equivalent for all strains at each concentration time point, suggesting that acetic acid-induced PCD may utilize a distinct mechanism from H₂O₂-induced PCD.

Double Inactivation of o ye2 with Other Antioxidant Genes Increases ROS and H₂O₂-induced PCD—The preceding data indicate OYE2 regulates intracellular redox conditions. To further explore this idea, we combined deletion of OYE2 with deletion of additional redox control genes. The strains Δsod1, Δctt1, Δyca1, Δglr1 (GLR1) encodes a cytosolic and mitochondrial glutathione oxidoreductase that converts oxidized glutathione to reduced glutathione, a critical cellular antioxidant) (35, 36) and their double knock-out counterparts Δo ye2 sod1, Δo ye2 ctt1, Δo ye2 yca1, and Δo ye2 glr1 were grown to late logarithmic phase in glucose complete media were stained with HE to measure ROS and analyzed by FACS, as above. Additionally, the ability of single and double knock-out strains to recover from H₂O₂ treatment was examined in growth recovery assays (Fig. 6). Comparison of ROS levels between wild type BY4741 cells and the single knockouts Δsod1, Δctt1 (shown in Fig. 3B), Δyca1 and Δglr1 (Fig. 6) showed increased ROS only in the case of Δsod1 (Fig. 3B). When their double knock-out counterparts were compared with the single knockouts, in all cases besides Δo ye2 ctt1 they exhibited substantial increases in ROS levels, confirming further the role of OYE2 as an antioxidant gene because deletion of the gene further exacerbates the endogenous oxidative environment in cells harboring deletions in antioxidant genes. A series of 20 additional single and double mutants to other known antioxidant genes were also tested.
with the same results. Furthermore, examination of the sensitivity to H$_2$O$_2$-induced PCD of single and DKO, $\Delta$sod1 and $\Delta$oye2 sod1 (Fig. 6, top row), $\Delta$ca1 and $\Delta$oye2 yca1 (Fig. 6, third row), $\Delta$glr1 and $\Delta$oye2 glr1 (Fig. 6, bottom row) showed increased sensitivity to H$_2$O$_2$-induced PCD in the double knock-outs compared with the single knock-outs or wild type cells.

Double Inactivation of oye2 with glr1 Increases Morphological Defects—Intriguingly, microscopic observations of the $\Delta$oye2 glr1 strain revealed gross morphological abnormalities of the emerging buds. Examination of the $\Delta$oye2 glr1 mitochondria, by expressing mit-GFP from a pYX142 plasmid, showed thinner than normal organelle mitochondrial tubules, indicating excessive fragmentation (Fig. 6) (37). A role for OYE2 in the protection of actin from oxidative damage was recently proposed (38); based on such a role, defects in actin skeleton dynamics might lead to altered trafficking of Bax to mitochondria, or failure to accurately organize mitochondria.

To assess the role of inactivation of oye2 and glr1 on the actin cytoskeleton and intracellular organelles, we stained wild type, $\Delta$oye2, $\Delta$glr1, and $\Delta$oye2 glr1 strains with rhodamine-conjugated phalloidin to visualize actin, with Hoechst 33342 to detect nuclei, and with CMAC to visualize vacuolar lumens (Fig. 7). $\Delta$oye2 cells resembled the wild type cells in actin stain, although overall they appeared to stain more intensely actin patches (Fig. 7). The same pattern was also observed in the DKO $\Delta$oye2 oye3 cells, although the cells were overall larger in size. Deletion of GLR1 caused a noticeable increase in actin cable staining, but also a decrease in actin patches. In the $\Delta$oye2 glr1 strain there were dramatic aberrations in cell morphology, with cells containing large hyperelongated buds. There was excessive stain of actin cables decorating in a dispersed manner throughout the whole cell. The cells are not pseudohyphal as they are haploid. These suggest a failure in the cells to properly control polarized cell growth. The actin cytoskeleton changes for $\Delta$oye2 cells seen by Haarer and Amberg (38) in the FY23 × 86 genetic background were not observable in BY4741 cells used in the large scale gene deletion project (Research Genetics), as shown in Fig. 7. The appearance of $\Delta$oye2 glr1 cells resembles the extreme phenotype of the actin mutant act1–123 (R68A,E72A).

Many organelle segregation events utilize actin cables for polarized transport. Nuclei stained with Hoechst revealed that a large number of the hyperelongated buds are lacking a nucleus in the $\Delta$oye2 glr1 cells, likely caused by failure to partition the organelle. A significant proportion of the hyperelongated buds, during active growth phase, spontaneously died as shown by their permeability to the Evans blue stain. However, vacuoles partitioned successfully between mother and daughter cells, although the daughter vacuoles localized at the very tip of the elongated bud, giving it a very characteristic appearance (Fig. 7).

Addition of Exogenous GSH Restores Cell Morphology in $\Delta$oye2 glr1 Cells—The cytoskeletal and morphological aberrations are specific to the double inactivation of OYE2 and GLR1, because a double knock-out $\Delta$oye2 gsh1 (GSH1) catalyzes the first step in glutathione biosynthesis, although it contains very high levels of endogenous ROS, and is very sensitive to oxidative stress, it does not assume the aberrant cytoskeletal morphology (Fig. 8). This suggests a special role for OYE2 in combination with oye2p in the regulation of actin polymerization. To assess GSH and GSSG levels in late log phase cultures from BY4741 WT cells, $\Delta$oye2, $\Delta$oye3, $\Delta$oye2 oye3, and $\Delta$oye2 glr1 cells were treated as described (22). GSH levels are significantly lower only in $\Delta$oye2 cells ($p < 0.0001$), whereas in GSSG there is

A. Makris, unpublished data.
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FIGURE 8. Supplementation of Δoye2 gsr1 cells with exogenous GSH restores normal cell morphology. Δoye2 gsh1 cells exhibit substantially higher levels of ROS than Δgsh1 cells (vertical lines) (top left). The cells although are unable to synthesize GSH and are very sensitive to stress, maintain normal morphology (top right). Δoye2 gsr1 cells revert back to normal cell morphology when the medium was supplemented with 5 mM GSH (middle row). Δoye2 gsr1 exhibit a dramatic 6-fold increase in GSSG levels (bottom row).

This close association is supported by the recent identification of TOM22, a component of the outer mitochondrial membrane import complex (TOM) as a mitochondrial receptor of Bax (32). In the R13 strain currently examined, Bax failed to localize to mitochondria enabling cells to survive. The presence of the C terminus of OYE2 restored Bax sensitivity and additionally enabled proper mit-GFP targeting. Mutations in the mitochondrial import complex (including TOM22) and actin/cytoskeleton-dependent transport are known to cause disruptions in mitochondrial morphogenesis (39).

The Old Yellow Enzyme of yeast was the first flavoprotein to be discovered, in 1932. OYE was initially purified from Saccharomyces carlsbergensis (oye1p), and later from S. cerevisiae (oye2p and oye3p). Old Yellow Enzymes form homodimers, but can also form heterodimers of 45-kDa subunits with one monovalently bound FMN per subunit. The enzyme is rapidly reduced by NADPH and can be reoxidized by oxygen. Both OYE proteins have been shown to catalyze the NADPH-dependent reduction of quinones and of several α- and β-un satu rated carbonyl compounds (26, 40). To date, few studies have addressed the physiological relation of the two S. cerevisiae OYE.

The use of heterologous proteins Bax and Bak in yeast pointed early on to the involvement of mitochondria and oxidative stress in eliciting cell death (19, 22, 41). Although there have been many doubts raised with regard to the necessity of the presence of a conserved suicide program in unicellular organisms, several studies in the past few years uncovered yeast apoptotic components that are direct counterparts of the standard mammalian apoptosis regulators. In the current study we show that the two OYE proteins modulate oxidative stress and the propensity of cells to undergo H$_2$O$_2$-induced apoptosis. The oye2p and oye3p proteins exert an opposing effect in oxidative stress in wild type cells. The oye2p protein clearly maintains a protective antioxidant role; efficiently removing ROS generated from organic prooxidants, whereas the oye3p protein enhances ROS levels under the same conditions. The study of H$_2$O$_2$-induced PCD in overexpression and deletion strains confirmed these activities and identified the oye3-oye2 heterodimer as being responsible for the enhanced sensitivity to H$_2$O$_2$-induced PCD. The oye2p is an important participant in the antioxidant machinery, as its simultaneous deletion with other antioxidant genes exacerbates oxidative stress (Fig. 6).

OYE2 and OYE3 share extensive homology (82% identity). Construction of the molecular models of OYE2 and OYE3 based on the structure of OYE from S. pastorianus (42) reveals that the vast majority of changes are found on surface-exposed regions of the two proteins and appear to result in significant alterations on the surface charge, which could have a profound effect on the protein interaction specificities of the two isoenzymes. Information compiled in public protein interaction data bases such as Biogrid has identified different protein partners for OYE2 and OYE3 (43–46). In the vicinity of the active site all residues that are found in the crystal structure of S. pastorianus OYE to be interacting with FMN are conserved. Only one substitution is observed in the surface binding cavity. Phe$^{297}$ of OYE2 is substituted with Ser in OYE3. The residues could play an important role in the substrate specificity of the enzymes.

a striking 6-fold increase in the Δoye2 gsr1 cells. To further validate the importance of GSSG, we attempted to reverse the GSH/GSSG ratio in the cells, by exogenously supplementing media with 5 mM GSH. As seen in Fig. 8, supplementation with GSH completely reversed the aberrant morphology of the oye2 gsr1 cells. These results confirm the participation of oxidized glutathione together with the absence of oye2 in eliciting actin cytoskeletal changes.

DISCUSSION

A significant proportion of the EMS-mutant yeast strains that are resistant to Bax lethality exhibit additional aberrations in mitochondrial morphology and defects in proper targeting of a mit-GFP to the mitochondrial matrix (25% of strains) (25).
and should be addressed in future experiments (supplemental Figs. S1 and S2). Experimental examination of catalytic stereospecificity of OYE2 and OYE3 using α,β-unsaturated carbonyl compounds identified differences between them (47). In vivo assays of yeast cells exposed to the toxic α,β-unsaturated carbonyl acrolein, a product of lipid peroxidation in biological systems, identified OYE2 but not OYE3 for its contribution to acrolein tolerance (48). Our data show that in addition to catalytic specialization and differential protein binding, the stoichiometry of the oye2p-oye3p heterodimer to homodimers is important for the propensity of the cells to undergo cell death upon oxidative trigger. The catalytic activity of OYE2 is important for the function of the complex, as seen by the inactivating mutation at Tyr197 of OYE2. The C terminus of OYE2 is also shown to participate in PCD modulation. A 12-amino acid truncation at the C terminus of OYE2 substantially elevated the protection levels conferred in H2O2-induced apoptosis. A larger fragment of the C terminus restored Bak sensitivity and proper mitochondrial targeting in the R13 mutants. Interestingly the C terminus of OYE2 was also identified as the site of interaction with actin. Mutation of the 3 terminal amino acids in OYE2 abolished this binding (38). Overexpression of the Y197F OYE2 mutant exhibited the same protective response as the truncated OYE2 protein in wild type cells but was lethal in H2O2-insulted cells lacking OYE3 indicating that the protective effects of the altered proteins were due to obstruction of the oye2p-oye3p heterodimer. The integrity of the core apoptotic machinery appears important for the death promoting effects of oye3p-oye2p because deletion in apoptosis inducers abolished the induction of cell death caused by OYE3 overexpression (data not shown).

Transcriptional control of the OYE genes may play a key role in determining the formation of homodimers versus heterodimers in the cells. Data from microarray studies indicate that the two OYE genes are not co-regulated. Computational approaches for inferring sets of regulatory modules (sets of co-regulated genes that are controlled by a shared regulatory program) assigns the two genes in different modules. OYE3 clusters into a mitochondrial module that is under the control of BCY1, the cAMP-dependent protein kinase regulatory subunit, whereas OYE2 clusters into a diverse group enriched in enzymes (includes GSH2, GLR1, and TRR1) under the control of UME1, the negative regulator of meiosis. OYE2 expression is substantially reduced when cells enter stationary phase, but no such drastic change is seen for OYE3 (49, 50). Thus, differential regulation of the two genes could modulate sensitivity to PCD at various stages in their life cycle.

Deletion of both OYE genes brought about a qualitative change in the cells, elevating respiratory activity, which led to their increased resistance to PCD as well as the reduction of cell death during senescence prior to adaptive regrowth. Incapacitation of respiration in the Δoye2 oye3 cells diminished their resistance to cell death from oxidative damage. The importance of functional mitochondria in resistance to oxidative stress has been known for some time (51). Apoptosis caused by expression of the yeast AMID homologue NDI1 can be repressed by increased respiration on glucose-limited media (17). The changes in resistance to H2O2 seen in Δoye2 oye3 cells are not caused by elevated ROS levels upon increased respiration, because those are identical to the parental wild type cells. Grant and co-workers (51) postulated that the role of mitochondrial function in resistance may depend in some energy requiring process, which remains to be identified.

A first biological function of OYE2 was recently uncovered by Haarer and Amberg (38). Flexibility in the C terminus of yeast actin, and the red blood cell actin, allows two cysteine residues (Cys374 and Cys285) to come into proximity, and in a sufficiently oxidizing environment form a disulfide bond. In human cells, actin undergoes glutathionylation of Cys374 during cell adhesion, and impairment of actin glutathionylation inhibits the disassembly of the actinomyosin complex (52). The oye2 protein, but not the oye3 protein, was shown to interact with actin possibly in the proximity of the Cys285-Cys374 disulfide bond (38). A nearly complete knockdown (37–38%) of OYE2 crossed to oye3Δ strain was used by Haarer and Amberg (38) to show defects in cytoskeletal organization, with excessive quantities of actin cables and actin cortical patches, as well as morphological aberrations.

The oye2 and double knock-out oye2 oye3 strains used in the current study were in the BY4741 genetic background. Cells exhibited very mild cytoskeletal changes showing more intensely stained cortical patches, but not increased actin cables. However, the Δoye2 glr1 strain exhibited an exacerbated phenotype that was very similar to the act1Δ-23 mutant, not only confirming the participation of the oye2p protein in actin polymerization, but also underscoring the importance of additional players participating, such as oxidized glutathione (Fig. 8). Actin cables are the tracks directing polarized cell secretion and organellar segregation. The actin filament bundles are anchored at one end to discrete regions in the cortex and radiate toward the rest of the cell (53). During cell division, in the process of nuclear positioning and segregation, actin was found to play a role in mitotic spindle orientation, as revealed in studies of actin gene mutations causing disruptions in nuclear orientation (54). The vacuole is also associated with actin cables, and specific alleles of the actin gene reduce the efficiency of vacuole inheritance (55).

Increased levels of glutathionylated proteins have been found in many human diseases such as Fredreichs ataxia, hyperlipidemia, and diabetes mellitus (56, 57). S-Glutathionylation offers the cell the advantage of a reversible mechanism that prevents the protein Cys thiol group from irreversible oxidation. The effects seen in the case of the Δoye2 glr1 cells clearly suggest the presence of an enzymatic mechanism that mediates glutathionylation of actin in the presence of high GSSG levels. In mammalian cells, a similar role in the catalysis of reversible protein thiol glutathionylation was recently assigned for glutaredoxin 2 (58).

The specific cooperative phenotype caused by the simultaneous deletion of oye2 and glr1 can be explained by the absence of the protective function of the oye2p on actin that renders cells with high levels of GSSG highly susceptible to actin glutathionylation, altering the polymerization dynamics. The effect seen is dependent on the presence of high levels of GSSG (Fig. 8), because the presence of increased ROS levels does not by itself cause the dramatic cytoskeletal rearrangements. The inactiva-
tion of gsh1 with oye2, although it led to high ROS levels and extreme sensitivity to oxidative stress, does not cause the characteristic morphology. In a previous study, we observed that when yeast cells were treated with the prooxidant CHP the total glutathione levels rose substantially compared with untreated cells. This was not the case for H₂O₂ treatment. Moreover, this GSH increase was dependent on the presence of the transcriptional activator Yap1 (59). Taken together, our data show that increased ROS do not automatically translate into depleted GSH levels, and that the presence of high GSSG is important on its own right.

Evidence from several studies suggests cross-talk between the dynamics of actin cytoskeleton, the release of ROS by mitochondrial, and RAS signaling, in controlling PCD in eukaryotic cells (60, 61). The results shown further reinforce this model when yeast cells were treated with the prooxidant CHP the total glutathione levels, and that the presence of high GSSG is important on its own right.

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