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Redox-sensitive YFP sensors monitor dynamic nuclear and cytosolic glutathione redox changes

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Abstract

Intracellular redox homeostasis is crucial for many cellular functions but accurate measurements of cellular compartment-specific redox states remain technically challenging. To better characterize redox control in the nucleus, we targeted a yellow fluorescent protein-based redox sensor (rxYFP) to the nucleus of the yeast *S. cerevisiae*. Parallel analyses of the redox state of nucleus-rxYFP and cytosol-rxYFP allow us to monitor distinctively dynamic glutathione (GSH) redox changes within these two compartments in a given condition. We observed that the nuclear GSH redox environment is highly reducing and similar to the cytosol under steady state conditions. Furthermore, these sensors are able to detect redox variations specific for their respective compartments in glutathione reductase (Glr1) and thioredoxin pathway (Trx1, Trx2) mutants that have altered subcellular redox environments. Our mutant redox data provide *in vivo* evidence that glutathione and the thioredoxin redox system play distinct but overlapping functions in controlling subcellular redox environments. We also monitored the dynamic response of nucleus-rxYFP and cytosol-rxYFP to GSH depletion and to exogenous low and high doses of H₂O₂ bursts. These observations indicate a rapid and almost simultaneous oxidation of both nucleus-rxYFP and cytosol-rxYFP, highlighting the robustness of the rxYFP sensors in measuring real-time compartmental redox changes. Taken together, our data suggest that the highly reduced yeast nuclear and cytosolic redox states are maintained independently to some extent and under distinct but subtle redox regulation. Nucleus- and cytosol- rxYFP register compartment-specific localized redox fluctuations that may involve exchange of reduced and/or oxidized glutathione between these two compartments. Finally, we confirmed that GSH depletion has profound effects on mitochondrial genome stability but little effect on nuclear genome stability, thereby emphasizing that the critical requirement for GSH during growth is linked to a mitochondria-dependent process.

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Keywords

redox-sensitive sensor; redox; glutathione; yeast

Introduction

Redox homeostasis by multiple dynamic equilibrium adjustments and regulation mechanisms is crucial for many cellular functions, including the proper folding of proteins and maintenance of their activity. Numerous studies in recent years have established that defective redox homeostasis and reactive oxygen species (ROS) are implicated in ageing, neurodegenerative diseases and cancer [1]. Eukaryotic cells have two principle thiol redox control pathways that have been most extensively characterized in the yeast *S. cerevisiae*: the glutathione redox system that includes the most abundant non-protein tripeptide glutathione (γ -glutamylcysteinylglycine, GSH), glutaredoxins (Grxs) and glutathione reductase (Glr), and the thioredoxin redox system comprising thioredoxin (Trx) and thioredoxin reductase (Trr) [2,3]. GSH, with its relatively low redox potential (-240 mV) and high intracellular abundance ($1-13$ mM), in equilibrium with its oxidized disulfide form GSSG [4], is often used as a gauge of the cellular redox environment. In *S. cerevisiae*, GSH biosynthesis occurs in the cytosol via two ATP-dependent steps catalyzed by γ -glutamylcysteine synthetase encoded by *GSH1* [5], and glutathione synthetase encoded by *GSH2* [6]. Once synthesized in the cytosol, its distribution, availability and redox states in different organelles, including mitochondria, nucleus, and endoplasmic reticulum, further depend on a poorly understood equilibrium between transport, utilization, relative reduction rates of GSSG by Glr, degradation, and excretion. In addition to the GSH-Grx system, *S. cerevisiae* contains two cytoplasmic Trxs encoded by *TRX1* and *TRX2* [3]. Thioredoxin reductase encoded by *TRR1* directly reduces oxidized Trxs. Although the Trxs and Grxs are similar in structure and have overlapping functions, they are regulated in a different manner [3]. The oxidized, disulfide form of Trx is reduced directly by Trr using NADPH as the electron donor, whereas Grx is reduced by GSH forming glutathione disulfide (GSSG), which is in turn reduced by Glr using electrons donated by NADPH.

Given the importance of thiol redox homeostasis in cellular function, it is useful to monitor the concentration of oxidized and reduced species from a main redox couple such as cellular GSH/GSSG. However, informative measurements of cellular GSH/GSSG redox states remain technically challenging. While the overall cellular GSH and GSSG concentrations can be determined by several conventional methods, their concentrations in an individual compartment remain difficult to estimate. The redox state of GSH as well as the absolute concentration varies from one compartment to another and conventional methods fail to reliably measure these subcellular variations. GSH/GSSG measurements taken via nuclear fractionation essentially disturb cellular as well as organelle integrity resulting in loss of metabolites and undesired changes in redox state, thereby making estimations prone to artefacts. On the other hand, use of redox-active fluorescent dyes has given conflicting results due to a number of factors, including a lack of specificity for the GSH/GSSG couple, irreversibility that prevents measurement of dynamic redox variations, and a lack of compartment specificity [7]. Nevertheless, given the importance of redox pathways operating in the nucleus it is imperative to define the nuclear redox environment and its regulation.

Genetically encoded biosensors may provide an alternative way to overcome the limitations of conventional GSH/GSSG redox measurements [8]. Østergaard and coworkers have developed redox-sensitive yellow fluorescent protein (rxYFP) by inserting an artificial dithiol-disulfide pair in the YFP structure [9]. Both in yeast cells and *in vitro*, the cysteines

in rxYFP specifically equilibrate with GSH and GSSG over a wide range of pH values via rapid disulfide exchange reactions with the cytosolic Grxs. In contrast, the two cytosolic Trxs are unable to undergo thiol-disulfide exchange with this sensor. The ratio of oxidized to reduced rxYFP, that can be assessed via non-reducing SDS-PAGE in which the two forms have different electrophoretic mobilities, can be used to generate an *in vivo* readout of the GSH:GSSG redox state. Their measurements indicated that the *S. cerevisiae* cytosol has a redox potential of -289 mV which is considerably more reducing than whole cell redox measurements (-221 to -236 mV) [10]. More recently, exclusive targeting of rxYFP to the *S. cerevisiae* mitochondrial intermembrane space (IMS) and mitochondrial matrix, respectively, showed that the IMS steady-state GSH/GSSG redox state is considerably more oxidized than the cytosol or matrix, and that IMS GSH/GSSG redox control is maintained independently from the cytosol and matrix [11]. These recent data highlight the importance of examining subcellular compartments separately.

In an effort to precisely characterize the nuclear GSH/GSSG redox environment and its regulation, we have targeted the rxYFP sensor to the nucleus of the yeast *S. cerevisiae* and compared the GSH/GSSG redox potential differences with the cytosol. We demonstrate exclusive targeting of nucleus-rxYFP to the nucleus and its dynamic response to an exogenous oxidant and reductant. Redox potential measurements using the nucleus-targeted rxYFP sensor reveal that the nuclear redox environment is highly reducing and similar to the cytosol under steady-state conditions. Furthermore, we tested the specificity and ability of this sensor to register nucleus-specific, localized redox fluctuations using GSH and Trx pathway mutants that have altered subcellular redox environments. Subsequently we evaluated the ability of these probes to sense GSH/GSSG redox environment changes during cellular GSH depletion and in response to mild and acute doses of exogenous H_2O_2 . Finally, we observed and confirmed that GSH depletion has a profound effect on mitochondrial genome stability, while GSH depletion has little effect on nuclear genome stability.

Materials and Methods

Yeast strains, media, and growth conditions

Most strains used in this study are isogenic to the S288c-based parental strain RDKY3615 *MATa*, *ura3-52*, *leu2 Δ 1*, *trp1 Δ 63*, *his3 Δ 200*, *lys2 Δ Bgl*, *hom3-10*, *ade2 Δ 1*, *ade8*, *hxt13::URA3* [12], except those specified, which are W303 derivatives. Gene replacements were made using standard PCR-based homology-directed methods. Double or triple mutations were usually constructed by crossing haploids of opposite mating types that carry the desired mutations followed by dissection of tetrads. Genotypes of spore clones were determined by replica plating, assaying for the markers used to delete the genes. *S. cerevisiae* cells were grown in standard media including yeast extract peptone dextrose medium (YPD) or synthetic complete medium (SC) lacking appropriate amino acids as indicated. Canavanine resistant mutants (Can^r) created by inactivation of the *CAN1* gene were selected on SC-Arginine dropout plates containing 60 mg/l canavanine. The *S. cerevisiae* strains expressing red fluorescent protein (mRFP) -tagged NAB2 was kindly provided by Dr. Palancade [13].

Plasmids

The plasmid carrying NUP49-mCherryFP was kindly provided by Dr. Palancade [14]. The original plasmid pJH208 expressing cytosol-rxYFP was previously described [11]. The plasmid MEHP97 expressing cytosol-rxYFP used in this study was constructed by digesting the plasmid pJH208 with NotI and SacII. The NotI-SacII-digested fragment containing the *PGK1* promoter, the coding sequence of rxYFP, and the *TDH3* terminator was then inserted into the NotI-SacII-cut *LEU2-CEN* vector pRS315 to create MEHP97. The nucleus-rxYFP

plasmid MEHP170 was constructed as follows. The rxYFP coding region was amplified from the plasmid pJH208 with the following primers: 5'-
 CCCGTCATATGTCTAAAGGTGAAGAACTGTTTAC-3', which contains a NdeI site (underlined), and 5'-
 CGGTCACGCGTTTAAACCTTTCTCTTCTTTGGAACCTTTCTCTTCTTTGG
 AACCTTTCTCTTCTTTGGTTTATACAGCTCATGCATGC-3' which contains a MluI site (in bold), a stop codon (in bold and underlined) and three copies of nucleus targeting sequence of SV40 (underlined). The PCR product, fusing three copies of the NLS in frame to the 3' end of rxYFP was then digested with NdeI and MluI, and ligated to the backbone of NdeI-MluI-cut MEHP97 plasmid DNA, thereby changing the rxYFP to nucleus targeting rxYFP, designated MEHP170.

Fluorescence microscopy

The strain transformed with MEHP170 (nucleus-rxYFP) was grown to mid-log phase in selecting SC-Leu medium. For live cell imaging, exponentially growing cells were washed with PBS, resuspended in the mounting solution (75% glycerol in PBS) and mounted on a glass slide covered with polylysine (Sigma). When necessary, 4',6-diamidino-2-phenylindole (DAPI) was added to the mounting solution to a final concentration of 50 µg/ml. Fluorescent and phase contrast images were captured with a Leica microscope (DMRXA) equipped with a cooled CCD camera MicroMAX (Princeton Instruments) under control of the MetaMorph software (Molecular Devices). Images obtained were processed using ImageJ software.

Isolation of nuclei and cytosol from whole yeast cells

The strain transformed with MEHP170 (nucleus-rxYFP) or MEHP97 (cytosol-rxYFP) was grown to mid-log phase in selecting SC-Leu medium. Nuclei containing subcellular fraction was prepared as described in [15] and cytosolic fraction was obtained as described in [16]. Protein concentrations in the fractions were measured by Quant-iT protein assay kit with the Qubit fluorometer (Invitrogen). Each sample in reduced condition was then subjected to electrophoresis on a 12% NuPAGE Novex Bis-Tris gel with MOPS SDS buffer (Invitrogen). After transfer to a nitrocellulose membrane, appropriate portion of membrane was probed with anti-Pgk1 (Invitrogen), anti-Nop1 (Santa Cruz) or anti-GFP (Invitrogen) antibodies.

Redox western blot

Redox Western blot analysis of rxYFP was adapted from previously described methods [11] with slight modifications. In general, 15 ml of cell cultures at mid-logarithmic phase in selecting SC-Leu medium were acid-quenched with 20% trichloroacetic acid (TCA, Sigma) at 4 °C. Cells were harvested and resuspended in 1 ml of 20% TCA. The resuspended cells were pelleted by centrifugation at 4000 g for 10 min at 4°C and the pellet was immediately frozen in liquid nitrogen and then kept at -80°C. For protein extraction, the cell pellet was resuspended in 400 µl of 20% TCA and lysed with glass beads (200 µl) by vortexing for 5 min at 4°C. The lysed cells were centrifuged and the pellet was washed with cold acetone. The dry pellet was resuspended in 90 µl of TES buffer (100 mM Tris Cl pH 8.8, 10 mM EDTA, 1% SDS) containing 50 mM *N*-ethylmaleimide (Sigma). Following a 60-min incubation at 30 °C with agitation in the dark, insoluble protein was removed by centrifugation at 13000 g for 10 min at room temperature. Each sample containing about 40 µg of protein was prepared with NuPAGE LDS sample buffer and was separated on a 12% NuPAGE Novex Bis-Tris gel with MOPS SDS buffer (Invitrogen). After transfer to a nitrocellulose membrane, reduced and oxidized forms of rxYFP were probed with a rabbit anti-GFP antibody (Invitrogen) and a secondary anti-rabbit IgG (IRDye, LI-COR) and then

analyzed by quantitative immunoblot using an Odyssey Infrared Imaging System (LI-COR). The statistical significance was calculated by the Student's t-test.

GSH/GSSG assays

Total glutathione (GSH + GSSG), GSH and GSSG in yeast extracts were measured by the 5,5'-dithiobis-2-nitrobenzoic acid (DTNB)-GSSG reductase cycling assay and spectrophotometric analysis of thionitrobenzene (TNB) formation as previously described [17]. The lysate from $\sim 2 \times 10^8$ cells grown in SC medium was used to determine total glutathione and GSSG. For GSSG measurements, cell lysates were treated with 2-vinylpyridine (Sigma) prior to the assay, which covalently reacts with GSH (but not GSSG). GSH in the sample was calculated by subtraction of total glutathione with GSSG. The glutathione quantity (total glutathione, GSH or GSSG) for each cell (Q) is estimated by dividing the measured quantity by cell number used in the assay. To estimate intracellular glutathione concentration, cell volumes (V) were estimated from cell major (*m*) and minor (*n*) axes using the formula: $V = \pi m^2 n / 6$ [18,19]. Cell dimensions were determined microscopically at $\times 100$ magnification using ImageJ. For each strain or condition, at least 100 cells were measured and the average cell volume determined. Total glutathione, GSH or GSSG concentration was obtained by Q/V.

Redox potential calculations

The rxYFP sensor equilibrates with GSH:GSSG pools. Measurement of intracellular concentrations of [GSH] and [GSSG], or the ratio of reduced to oxidized rxYFP have been used to estimate the redox potential using the Nernst equation [9,11]: $E_{\text{GSH}} = E'^{\text{GSH}} - (60.1 \text{ mV}/2) \log[\text{GSH}]^2/[\text{GSSG}] = E'^{\text{rxYFP}} - (60.1 \text{ mV}/2) \log(\text{rxYFP}^{\text{red}})/(\text{rxYFP}^{\text{ox}}) = E_{\text{rxYFP}}$ (Equation 1). Redox potential *E* values are expressed in mV. E'^{GSH} is the standard potential for reduced glutathione, which is -240 mV at pH 7. E'^{rxYFP} is the standard reduction potential of rxYFP which is -265 mV at pH 7. In this equation, [GSH] enters as a squared term, meaning that the reduction potential is dependent on the GSH/GSSG ratio and the absolute concentration of GSH, while E_{rxYFP} is dependent on the ratio of reduced:oxidized rxYFP but not the absolute concentration. The redox potential of GSH and rxYFP at different pH values were calculated according to the equation: $E_{\text{pH}} = E' + [(\text{pH} - 7.0) \times -60.1] \text{ mV}$ (Equation 2)[4]. In this equation, E' is the standard redox potential of GSH or rxYFP at 7.0.

Measurement of mitochondrial DNA mutation and nuclear DNA mutation frequency

The *gsh1* strains were inoculated into SC medium containing $500 \mu\text{M}$ GSH for overnight growth. These preculture cells were diluted to an appropriate density in SC without GSH and cultured for 8 h. The cultures were diluted again and further cultured for another 16 h in SC without GSH. After 24 h GSH depletion, the aliquots of the culture were further diluted and set to inoculate respectively into SC medium without GSH or with indicated concentrations of GSH for additional 24 h. In parallel as a control, one aliquot of preculture was maintained in SC medium containing $500 \mu\text{M}$ GSH. For measurement of mitochondrial DNA mutation, aliquots of culture were removed at times 0, 8, 24 and 48 h after GSH depletion and plated onto YPD plates. The total number of colonies was counted after a 3-day incubation at 30°C and respiratory incompetency (*petite* mutations) was revealed by triphenyl tetrazolium chloride coloration [20]. The frequency of *petite* mutations was calculated as the number of respiratory incompetent white colonies divided by the total number of colony-forming units (white + red) on YPD plates. For measurement of nuclear DNA mutation frequency, cells removed from the defined culture conditions were plated onto YPD or on SC-Arg dropout plates containing canavanine (for Can^r mutations events) to determine the total number of viable cells and the number of mutants, respectively. Can^r mutation frequency was presented as the number of Can^r mutants in 10^6 viable cells.

Results

Targeting rxYFP to the nucleus

A nucleus-targeted version of rxYFP was created by fusing 3 copies of the nuclear targeting signal of SV40 to the C-terminus of rxYFP in the centromeric vector pRS315. Localization and fluorescence signal of the nucleus-rxYFP sensors were analyzed by fluorescence microscopy. Live yeast cells expressing nucleus-rxYFP were stained with DAPI that reveals both mitochondria (string-like structures) and the nucleus (large and round structure). In all the cells inspected, nucleus-rxYFP co-localized with DAPI staining of the nuclear DNA (Figure 1A). To further confirm the nuclear localization of nucleus-YFP, we transformed nucleus-rxYFP in a *NAB2-mRFP* strain in which the gene encoding nuclear abundant poly(A) RNA-binding protein 2 (Nab2) is fused with mRFP [13]. Nab2p localizes to the nucleus at steady state. As shown in Figure 1B, Nab2-mRFP localization was homogeneous throughout the whole nuclear region and nucleus-YFP indeed co-localized with Nab2-RFP. We also transformed a plasmid expressing Nup49-mCherryFP fusion protein into a strain carrying nucleus-rxYFP. Nup49 is part of the nuclear pore complex and Nup49-mCherryFP reveals the nuclear periphery [14], allowing us to monitor the relative position of the nucleus-rxYFP with respect to the nucleus. As shown in Figure 1C, nucleus-rxYFP was exclusively located within the compartment limited by nuclear envelope. These experiments demonstrate that nucleus-YFP sensor is localized in the nucleus and is correctly folded with the expected fluorescence properties. In all the following experiments, the nuclear localisation of nucleus-rxYFP was monitored.

Localization and fluorescence signal of cytosol-rxYFP, expressed from a centromeric vector, were also verified by fluorescence microscopy. This sensor was found to be homogeneously localized in the cytosol as previously reported (Figure 1D) [11], in contrast to the nuclear localization of nucleus-rxYFP (Figure 1A–C).

To confirm the correct localization of the nucleus-rxYFP and compare to cytosol-rxYFP, we conducted a Western blot analysis of isolated nucleus and cytosolic fractions from WT strains transformed with nucleus- or cytosol-rxYFP. As shown in Figure 1E, distribution of cytoplasmic protein Pgk1 and nucleolar protein Nop1 demonstrated the equal loading and high purity of nuclear fractions from two strains expressing respectively nucleus-rxYFP and cytosol-rxYFP (Figure 1E, left panel), and the equal loading and purity of cytosol fractions from these two strains (Figure 1E, right panel). Western blotting using anti-GFP revealed that the nucleus-rxYFP was found predominantly in the nuclei preparation, in contrast to cytosol-rxYFP that was found predominantly in cytosol preparation.

Nucleus-rxYFP senses the nuclear glutathione redox status and responds to oxidant and reductant treatment

In order to separately measure the nuclear and cytosolic GSH/GSSG redox state, rxYFP expressed from nucleus- or cytosol-rxYFP constructs was monitored by redox Western blots. As shown in Figure 2 and Table 1, cytosol-rxYFP was ~19% oxidized under steady-state conditions in wild-type cells (S288c), which is similar to published results [9,11], whereas nucleus-rxYFP was ~12% oxidized. Nucleus-rxYFP is, at most, slightly more reduced than cytosol-rxYFP, but without statistical significance ($P > 0.05$). We tested the reactivity and response of targeted nucleus-rxYFP in comparison to cytosol-rxYFP using the strong thiol-oxidant 4,4'-dithiodipyridine (4-DPS, Sigma) or the disulfide reductant dithiothreitol (DTT, Sigma). Treatment of the cells with 4-DPS (180 μ M final concentration) or DTT (50 mM final concentration) for 20 min shifted the majority of nucleus- or cytosol-rxYFP toward the oxidized or reduced state, respectively (Figure 2).

Therefore, nucleus-rxYFP constructs are responsive to redox changes generated by exogenous reagents, similar to cytosol-rxYFP [11].

Total intracellular GSH and GSSG were measured at 4.07 mM and 0.052 mM, respectively (Table 1). Assuming an average intracellular pH value of 7.0 [21], the whole cell redox potential E_{GSH} was calculated from the Nernst equation (Equation 1, see Materials and Methods) by inserting the standard reduction potential of GSH at pH 7.0 ($E^{\circ}_{\text{GSH}} = -240$ mV) along with the absolute concentrations [GSH] and [GSSG]. This yields the value -225 mV, which is similar to a previous report [10]. The ratio of reduced-to-oxidized rxYFP was also used to generate redox potentials of the nucleus and cytosol. The yeast cytosolic pH measurements for glucose-grown cells range from 6.8 to 7.2 [21–24], and to the best of our knowledge, the precise pH of the nucleus is not known. For both *S. cerevisiae* and human cells, most authors assume that the nuclear pH equals that of the cytosol [21,25], so pH 7.0 was used to calculate the redox potential values for both nucleus and cytosol. With the standard reduction potential of rxYFP (E°_{rxYFP}) which is -265 mV at pH 7, and ratio of reduced-to-oxidized rxYFP, the nuclear redox potential calculated from the Equation 1 was -291 mV, whereas the cytosolic redox potential corresponded to -284 mV. Therefore, we conclude that the nuclear GSH redox environment is highly reduced and comparable to the cytosol. It is important to note that thiol-disulfide redox reactions are pH-dependent. If the pH were higher or lower than 7.0, the calculated redox potentials of GSH or rxYFP would accordingly decrease or increase by 6.0 mV for every 0.1 unit change in pH (Equation 2, see Materials and Methods).

Nucleus-rxYFP senses the subcellular redox status in mutants with deficient GSH or Trx redox systems

In order to determine whether nucleus-rxYFP is responsive to *in vivo* redox changes and how the nuclear redox state is affected by deficient GSH or Trx redox systems, we compared the redox state of nucleus- and cytosol-rxYFP in *glr1*, *trx1*, *trx2*, *trx1 trx2*, *trr1* and *trr1 trx1 trx2* mutants. Because a *trr1* deletion is lethal in the S228c background but viable in the W303 background, we chose to perform this set of analyses in W303-based isogenic strains. In the wild-type cells, cytosol-rxYFP was $\sim 8.4\%$ oxidized under steady-state conditions, whereas nucleus-rxYFP was $\sim 5.6\%$ oxidized (Figure 3A, Table 1). Again as in strain S288c, these differences between the cytosol and nucleus redox states in W303 wild type background were also found to be statistically insignificant upon replicate analysis ($P > 0.05$). The redox state varied slightly between the S288c- and W303-based wild-type strains, with W303-based cells exhibiting a slightly more reducing state in both the cytosol and nucleus. Variation of GSH/GSSG redox potentials for several wild-type yeast strains has been previously reported [26].

In *glr1* mutant cells, as previously demonstrated [11], cytosol-rxYFP underwent dramatic redox changes ($\sim 57\%$ oxidized) compared with isogenic wild-type cells ($\sim 8\%$ oxidized) (Figure 3A, Table 1). There was also a similar shift ($\sim 53\%$) in the percent of oxidized nucleus-rxYFP upon deletion of the *GLR1* gene. Glr1, which is responsible for regenerating GSH from GSSG, is localized to the cytosol and mitochondrial matrix, therefore deletion of this gene would have direct consequences for both of these compartments, while the effects on the nucleus are unclear. However, the difference in the oxidation state for nucleus-rxYFP and cytosol-rxYFP in *glr1* cells was not significant ($P > 0.05$). Loss of Trr1 also shifted GSH/GSSG redox balance to a more oxidized state with both cytosol- and nucleus-rxYFP $\sim 30\%$ oxidized (Figure 3A, Table 1). Loss of Trx1 or Trx2 did not remarkably affect the cytosolic and nuclear redox state. Interestingly, a synergistic effect was observed in the double mutant *trx1 trx2* compared with each single mutant, demonstrating that the Trxs have overlapping roles in redox homeostasis (Figure 3A), consistent with previous findings [26]. Furthermore, cytosol-rxYFP is significantly more oxidized in the *trx1 trx2* mutant ($\sim 29\%$)

than nucleus-rxYFP (~19%) ($P < 0.01$). Intriguingly, while cytosol-rxYFP oxidation was similar in *trr1* and *trx1 trx2* (~29%) mutants, the nucleus-rxYFP was significantly less oxidized in the latter mutant (~30% versus ~19% respectively, $P < 0.01$). We reasoned that this could be attributed to the presence of oxidized thioredoxins that would accumulate as a result of *TRR1* inactivation. In this regard we measured the rxYFP redox state in a *trr1 trx1 trx2* triple mutant. Expectedly, the *trr1 trx1 trx2* triple mutant displayed a similar redox profile as the *trx1 trx2* double mutant (Figure 3A), suggesting that indeed the more oxidized rxYFP state observed in the nucleus of *trr1* cells depends on the presence of oxidized Trx1 and Trx2. Further, the differences in the nuclear and cytosolic redox states in *trx1 trx2* and *trr1 trx1 trx2* mutants indicate that the nuclear redox may be regulated independently of the cytoplasm to some extent.

Since the *trr1* single mutant exhibited a severe growth defect, we tested if mutations in *TRX1* and *TRX2* genes could also suppress this growth defect phenotype similar to the restoration of redox state. For this purpose, we analyzed the meiotic products obtained from diploids that were heterozygous for *trr1*, *trx1* and *trx2* deletions. As shown in Figure 3B, additional deletion of *TRX1* or *TRX2* could not improve the growth of *trr1* mutants, as judged by colony size. In contrast, simultaneous deletion of *TRX1* and *TRX2* suppressed the growth defects of *trr1* mutants. Colonies formed by *trr1 trx1 trx2* mutants were of the same size as those formed by the *trx1 trx2* mutants. These observations indicated that the severe growth defect of *trr1* mutants is caused by the presence of oxidized Trx1 and Trx2 that probably leads to the strong redox deregulation in the nucleus as revealed by rxYFP redox analysis.

To compare the targeted rxYFP redox potential measurements with whole cell GSH and GSSG levels, total intracellular GSH and GSSG were measured in isogenic W303-based wild-type, *glr1*, *trr1*, *trx1 trx2* and *trr1 trx1 trx2* mutants and GSH redox potentials (E_{GSH}) were determined (Table 1). In *glr1*, *trr1*, *trx1 trx2* and *trr1 trx1 trx2* mutants, whole cell GSSG levels were dramatically elevated (20–55 fold), while GSH levels were similar or only slightly increased compared to the wild-type strain. Whole cell redox potentials (E_{GSH}) were calculated from the Nernst equation (see Materials and Methods) using the absolute concentrations [GSH] and [GSSG] and the standard reduction potential of GSH (–240 mV) [4], giving an E_{GSH} value of –226 mV for W303-based wild-type cells and from –209 mV to –191 mV for the four mutants. The E_{GSH} values were higher in all these mutants, indicating a more oxidizing redox environment compared to wild-type cells. *TRR1* deletion had the most pronounced effect on elevation of both GSH and GSSG and this effect is dependent on the presence of Trx1 and Trx2 (Table 1). Both GSH/GSSG quantification and the rxYFP data (above) suggest that the significantly oxidized nuclear GSH redox environment in the *trr1* mutant is partially due to the presence of the Trx1 and Trx2. The localized cytosolic and nuclear redox potentials (E_{rxYFP}) of wild-type and mutant strains were also calculated by inserting the ratio of reduced-to-oxidized nucleus- and cytosol-rxYFP into the Nernst equation using the standard reduction potential of rxYFP ($E^{\circ'}_{rxYFP} = -265$ mV at pH 7) (Table 1). Globally, the nuclear and cytosolic GSH/GSSG redox environments in these mutants are substantially more oxidized compared to wild-type. Additionally, we note that the nuclear and cytosolic GSH/GSSG redox potential estimated from nucleus- and cytosol-rxYFP (E_{rxYFP}) is considerably more reducing (lower) than the estimated GSH/GSSG redox potential (E_{GSH}) obtained by overall cellular GSH and GSSG measurements (Table 1), demonstrating the utility of targeted, genetically-encoded redox sensors in providing more accurate, compartmentalized redox measurements.

Dynamic and concurrent oxidation of nucleus- and cytosol- rxYFP during GSH depletion

Given that GSH plays key roles in subcellular thiol redox control, ROS detoxification, and iron homeostasis [4,27,28], it is important to determine the compartment-specific redox

changes during GSH depletion. The effects of GSH depletion in the *gsh1* mutant, which is defective in GSH synthesis, on the cytoplasmic, mitochondrial matrix and IMS redox state were recently described using the redox-sensitive green fluorescent protein (roGFP) [29]. However, it is not known whether GSH depletion influences the nuclear redox state. Moreover, a recent study using chemical GSH-depleting agents buthionine sulfoximine (BSO) or diethyl maleate (DEM) on mammalian cells indicated that nuclear GSH pools resist depletion as compared to cytosolic pools, thereby suggesting that nuclear GSH pools might be critical for growth [30]. We decided to address these issues using rxYFP probes and an alternative genetic strategy to deplete GSH. Growth and survival of *gsh1* strains are dependent on availability of exogenous GSH [31], therefore depletion of GSH in *gsh1* strains leads to a growth arrest after a substantial number of cell divisions. We concurrently analyzed cell growth, intracellular GSH and GSSG concentrations, and cytosol- and nucleus-rxYFP redox changes at defined time points (0, 8, 16, 24, 32 and 48 h) during GSH depletion in *gsh1* cells. As shown in Figure 4A, 4B and 4C, in the first 24 h after onset of depletion, *gsh1* cells in medium lacking GSH grow at a similar rate as the culture in medium supplemented with GSH, although intracellular GSH and GSSG decreased drastically. After about ~8–10 divisions in the first 24 h, the growth stalled. At this point, GSH was estimated to be 0.136 mM and GSSG was not detectable in our assay. These limited divisions probably reflect the consumption of GSH accumulated during preculture in GSH-containing medium. As expected, at time 0, the redox state of cytosolic- and nucleus-rxYFP in *gsh1* cells was similar to wild-type cells and remained reduced over the 48h experimental period when grown in medium supplemented with GSH. A modest oxidation of cytosolic- and nucleus-rxYFP was detectable 8 h after onset of depletion (Figure 4D). The oxidized form reached the maximum (60% – 70%) at 24 h, a time point corresponding to the cessation of growth of *gsh1* cells (Figure 4A). Cytosolic- and nucleus-rxYFP remained highly oxidized during the subsequent time points of analysis. The rxYFP oxidation at the 24-h time point likely reflects the detection limit of the probe, hence it is quite possible that both nuclear and cytosol GSH/GSSG redox environments are even more oxidizing than what is being measured in *gsh1* cells at later time points (24 h and after) during GSH depletion. It is also to note that the redox measurements at later time points (24 h and after) might be less reliable due to the fact that the redox probes may be influenced by other redox systems or protein thiols themselves that provide the cellular redox buffering capacity under these conditions [32], and the remaining little GSH may not be in equilibrium with the redox probes. Nevertheless, the fact that the time course of cytosolic and nuclear rxYFP oxidation during the first 16 h of GSH depletion was indistinguishable, suggests dynamic and concomitant changes in cytosolic and nuclear redox environment during GSH depletion. It appears that once the intracellular GSH concentration decreases below a critical threshold and/or the intracellular redox potential increases to a critical level by 24 h after depletion, *gsh1* cells stop growing.

The rxYFP sensors register real-time dynamic subcellular redox changes in response to hydrogen peroxide (H₂O₂) bursts

Exogenous H₂O₂ is largely used as an oxidant to induce intracellular oxidative stress and cell death. To understand the dynamics of cytosolic and nuclear redox changes during an exogenous H₂O₂ burst, a time-course analysis of cytosol- and nucleus-rxYFP, GSH and GSSG concentrations, and corresponding E_{GSH} was performed in wild-type cells expressing cytosol- or nucleus-rxYFP. Cells at mid-exponential phase were treated with a moderate dose (0.4 mM) or high dose (3 mM) of H₂O₂, which resulted in 70% or <5% viability, respectively, after 4 h treatment (data not shown). In the presence of 0.4 mM H₂O₂, both cytosol- and nucleus-rxYFP shifted to increased oxidation and reached the maximum value (~30.0%) by 30 min. Subsequently, the proportion of oxidized form decreased and reached a level more reduced than that observed before adding H₂O₂ (Figure 5A). In the presence of 3

mM H₂O₂, both cytosol- and nucleus-rxYFP became rapidly oxidized, shifting to ~30% oxidized after 5 min and steadily increasing to ~80% oxidized over the 240-min experimental period (Figure 5B). Globally, cytosol- and nucleus-rxYFP responded in a similar manner to H₂O₂ bursts, suggesting dynamic and concomitant changes in the cytosol and nuclear redox environment in response to two different doses of H₂O₂. Interestingly, treatment with 0.4 mM H₂O₂ resulted in higher intracellular GSH concentrations (Figure 5C), consistent with the previous observations that the H₂O₂ stress induces activation of the general environmental stress response cluster genes and the specific H₂O₂ stimulon [33,34]. In contrast, a lethal dose of H₂O₂ resulted in a significant decrease of intracellular GSH (Figure 5C), which is an early hallmark in the progression of cell death in response to a variety of apoptotic stimuli. The rxYFP response and GSH quantification indicate that cells are capable of restoring the reduced cytosolic and nuclear redox environments in response to moderate dose of H₂O₂ and even adapting to the stress situation by establishing a more reducing GSH/GSSG potential compared to non-stressed cells. In contrast, the cytosolic and nuclear redox environments are overwhelmed and irreversibly affected by the lethal dose of H₂O₂.

Effect of GSH depletion on mitochondrial and nuclear genome stability

In the absence of GSH, *gsh1* cells undergo limited divisions before arrest, probably reflecting the consumption of GSH accumulated during preculture in medium with GSH. In the GSH depletion experiment, a steady increase in the proportion of respiratory incompetent cells was observed, measuring 20% by 24 h and 90% by 48 h after onset of depletion (Figure 6A). This dramatic increase between 24 and 48 h suggests that once intracellular GSH concentrations decrease below a critical threshold, irreversible loss of respiratory competency ensues. This high prevalence of respiratory incompetency in the *gsh1* mutant could be completely prevented by supplementation with 5 μ M GSH (data not shown). These data indicate a specific requirement for GSH for cell growth and maintenance of respiratory competence and confirm the recent finding on the critical role of GSH in maintaining mitochondrial genome stability [29].

Since GSH depletion results in a profound instability of mitochondrial DNA (Figure 6A) and oxidation of the nuclear GSH/GSSG redox state (Figure 4), we studied whether GSH depletion also causes nuclear genome instability. The Can^r mutation assay that detects mutations inactivating the *CAN1* gene was performed as described in Materials and Methods. We expected that the potential DNA damage associated with 24 or 48 h GSH depletion would be revealed in this experiment. However, as shown in Figure 6B, GSH depletion did not affect nuclear genome stability, in striking contrast with the profound effect of GSH depletion on the mitochondrial genome.

We reasoned that the redox deregulation- or oxidative stress- induced nuclear DNA damage during GSH depletion could be processed by other antioxidants or by different DNA damage repair pathways, thus masking the effect on GSH depletion in *gsh1* single mutant. We therefore assessed the nuclear genome stability by combining *gsh1* deletion with a peroxiredoxin *tsa1* mutant. Tsa1 plays a key role in suppression of a broad spectrum of mutation phenotypes [35,36]. As shown in Figure 6C, the Can^r frequency of the *gsh1 tsa1* double mutant under GSH depletion conditions was about 2-fold higher than *gsh1 tsa1* in medium supplemented with GSH ($P < 0.01$). This observation suggests that, in the absence of peroxiredoxin Tsa1, GSH depletion and the consequent redox changes have significant effects on nuclear genome stability detectable by the Can^r assay.

Discussion

The lack of tools for measuring the status of defined intracellular redox couples in a given compartment has been a limitation for basic research. Genetically-encoded biosensors promise to overcome the limitations of conventional redox measurements [8]. The cytosolic rxYFP, initially developed by Østergaard and coworkers, specifically registers the intracellular GSH:GSSG redox state via disulfide exchange reactions with cytosolic Grxs in yeast [9]. This reaction has a similar equilibrium constant between pH 6.7 and 7.9 and occurs rapidly in the cytosol. The rxYFP sensor has also been exclusively targeted to the yeast mitochondrial matrix and IMS and shows distinct steady-state redox environments and independent GSH metabolism in these two subcellular compartments [11]. Similar GFP-based sensors have been targeted to other subcellular compartments in eukaryotic cells, such as endoplasmic reticulum, lysosome, plastids, and peroxisomes in order to examine dynamic redox changes in these compartments [37–40]. However, no studies have used nucleus-targeted versions of these sensors to specifically monitor fluctuations in the nuclear GSH:GSSG redox potential in response to genetic and environmental factors. Nevertheless, numerous studies have established that the nuclear redox state and the GSH/GSSG pool in particular play important roles in the physiology of the cell [41,42]. To better reflect the nuclear GSH/GSSG redox environment, we targeted rxYFP to the nucleus in yeast. Correct localization of nucleus-rxYFP was unequivocally confirmed by fluorescence microscopy and by fractionation analysis. Nucleus-rxYFP reacted *in vivo* to addition of an oxidant (4-DPS) or reductant (DTT) and to the elimination of Glr1, Trr1, or simultaneous loss of Trx1 and Trx2. Furthermore, nucleus-rxYFP responded differently to *trr1* deletion, *trx1* and *trx2* double deletion, or *trr1*, *trx1* and *trx2* triple deletion, compared to cytosol-rxYFP. These results demonstrate that nucleus-rxYFP selectively registers redox variations within the nucleus, and together with cytosolic rxYFP and other compartment-targeted rxYFP sensors, it is possible to monitor dynamic redox changes within different subcellular compartments.

In S288c-based wild-type cells, nucleus-rxYFP was ~12% oxidized, which is similar if not slightly more reduced than cytosol-rxYFP, reflecting similar or at most subtle differences in the thiol-disulfide equilibrium between these two compartments. Assuming that the pH of the nucleus equals that of the cytosol that is approximately pH 7.0 [24,25,43], the nuclear redox potential measured with rxYFP is –291 mV. The rxYFP redox state for the cytosol reported here, –284 mV, is similar to that published previously [11]. A previous report on the mitochondrial matrix and IMS rxYFP redox state demonstrates that the redox balance in these mitochondrial compartments is shifted more toward disulfide formation than in the cytosol. Because reduction potential is strongly dependent on pH, the matrix value (–296 ± 5 mV) is actually more reducing than the cytosol despite the higher percentage of oxidized rxYFP in this compartment. Furthermore, the IMS GSH/GSSG redox potential (–255 ± 3 mV) measured by IMS-rxYFP is considerably more oxidizing than the cytosol and mitochondrial matrix [11]. Taken together, the steady-state redox state of rxYFP in several subcellular compartments analyzed in the present study and in Hu et al [11] establishes that the order from most reducing to most oxidizing is mitochondrial matrix > nucleus > cytosol > IMS. A similar trend was observed in mammalian cells measured with direct GSH/GSSG quantification or with the redox sensor roGFP1 [44]. The values of redox potentials of these four compartments are considerably more reducing than the whole cell redox measurements using absolute concentration of [GSH] and [GSSG], reported as –225 mV in this study (Table 1) and –232 mV in a work by Hwang et al [10]. The significant differences between whole cell GSH/GSSG redox measurements and subcellular GSH-specific redox sensor measurements emphasize the importance of examining subcellular compartments separately. It is highly possible that redox compartmentalization is linked to organelle functions and redox signaling. For instance, the nuclear compartment is relatively reducing, perhaps reflecting an environment necessary for normal DNA metabolism, cell growth and

deactivation of a number of redox-regulated transcription factors. Additional investigation of redox regulation and cellular response to oxidative stress in the context of redox compartmentalization should improve understanding of these critical mechanisms in diverse physiological and pathological processes.

Our nuclear and cytosolic GSH/GSSG redox measurements in Trx pathway mutants provide independent evidence of functional overlap between the GSH and Trx redox systems and within the Trx system in maintaining redox homeostasis. Previous genetic analyses indicate that the Trx-Trr system shares functional overlap with the glutathione system and that the Trx-Trr system helps prevent overaccumulation of GSSG in cells lacking the glutathione reductase Glr1 [45–48]. Our present study provides new evidence that the Trx-Trr system is specifically involved in controlling nuclear GSH/GSSG redox homeostasis even in the presence of Glr1. Trx1 and Trx2 appear to share overlapping function in this regard. Furthermore, our data suggest that the strong redox deregulation in the nucleus caused by Trr1 mutation is mediated by both oxidized Trx1 and Trx2. Indeed, *trr1 trx1 trx2* triple mutants exhibit better fitness than the *trr1* single mutant in standard YPD medium (Figure 3B), further pointing to the adverse effect of oxidized thioredoxins.

One interesting question is whether cytosolic and nuclear compartments have different redox characteristics and distinct redox regulation. It is generally presumed that nuclear pores should allow diffusion of low molecular weight GSH and GSSG. However, several studies in animal and plant cells provide evidence for the existence of functionally distinct pools of GSH/GSSG in the nucleus that are dynamically regulated during the cell cycle [42,49,50]. Furthermore, studies on 3T3 fibroblasts have shown that addition of the γ -glutamylcysteine synthetase inhibitor BSO significantly decreases the total cellular GSH pool; however, the nuclear GSH pool is much more resistant than the cytosolic pool to BSO-mediated depletion [30]. Little is known regarding the mechanisms for distinct nuclear and cytosolic redox control, as well as the kinetics of GSH/GSSG redox flux and exchange between these two compartments in yeast. In the present study, we observed only subtle, if any, redox differences between these two compartments in wild-type cells. However, stress conditions generated by *trx1 trx2* double deletion or *trr1 trx1 trx2* triple deletion resulted in statistically significant redox differences between the two compartments, suggesting that the nuclear GSH/GSSG redox state could be at least partially regulated independently of the cytoplasm. On the other hand, nucleus- and cytosol-rxYFP registered dynamic and concurrent oxidation during GSH depletion and in response to two doses of H₂O₂ bursts at defined time points, suggesting that these stress conditions have similar effects on the two GSH/GSSG pools. However, one has to exert some caution while analyzing the rxYFP redox measurements in such stress conditions. Transient difference in nuclear and cytosolic GSH/GSSG pools during GSH depletion may not be reflected in the time points analysed in the present study. The redox measurements particularly at later time points (24 h and after) during GSH depletion might be less reliable due to the reasons mentioned above (see Results section). Similarly, exogenous H₂O₂ may rapidly and homogeneously diffuse to the cytosol and nucleus, leading to homogenous oxidation of both cytosolic and nuclear GSH/GSSG pools through the action of glutathione peroxidases. Future studies examining other types of stress conditions will reveal which type of stress specifically influences cytosolic vs. nuclear GSH/GSSG pools. Overall, these data favor the view that the highly reduced yeast nuclear and cytosolic GSH/GSSG redox states are maintained independently and under distinct but subtle redox regulation. Nucleus- and cytosol-rxYFP register compartment-specific localized redox fluctuations that may be largely influenced by exchange of GSH and/or GSSG between these two compartments. Indeed, both active and passive mechanisms of regulation and exchange cannot be excluded.

GSH is involved in numerous processes in cells including redox buffering, detoxification of organic and inorganic hydroperoxides, detoxification of xenobiotics, cellular signaling and iron–sulfur cluster assembly [4,27,51,52]. Here we find that GSH homeostasis plays a vital role in the maintenance of mitochondrial DNA and respiratory competency of cells, consistent with a previous report [29]. Although *gsh1* cells are capable of undergoing ~ 8–10 divisions in medium lacking GSH before growth arrest, the remarkable increase of respiratory incompetence that occurs after 8–10 cell divisions suggests that a threshold concentration is essential for maintenance of mitochondrial DNA. Both iron-dependent and iron-independent mechanisms have been proposed [29]. In contrast with the profound effect of GSH depletion on mitochondrial DNA damage and redox state, nuclear genome stability was not significantly affected in the Can^r assay. It is possible that the efficient antioxidant systems and DNA repair mechanisms may mask the mutagenic effect induced by the absence of GSH. Indeed, the *gsh1 tsa1* double deletion mutant under GSH depletion displayed higher mutation frequency than the *gsh1 tsa1* double mutant supplemented with GSH. Another reason for the greater importance of GSH in the maintenance of mitochondrial DNA stability might be because mitochondria are a substantial source of reactive oxygen species, while the nuclear DNA is present in chromatin, which may provide greater protection from oxidative damage. This also raises the question of whether there is an essential role for the nuclear pool of GSH/GSSG, which is not well defined. Madeo *et al.* reported that accumulation of reactive oxygen species caused by GSH depletion triggered the apoptotic pathway in *S. cerevisiae* and that hypoxia or radical scavenging prevented apoptosis [53]. All together, our results suggest that nuclear genome instability is not a major consequence caused by GSH depletion, although we cannot exclude the possibility that the mutator assay does not reflect all aspects of genome instability.

Conclusion

Intracellular redox homeostasis is crucial for many cellular functions but accurate measurements of cellular compartment-specific redox states remain technically challenging. We targeted a yellow fluorescent protein-based redox sensor rxYFP to the nucleus of the yeast *S. cerevisiae*. Parallel analyses of the redox state of nucleus-rxYFP and cytosol-rxYFP allow us to monitor distinctively dynamic glutathione redox changes within these two compartments in a given condition. These data show robustness of the rxYFP sensors in measuring real-time compartmental redox changes and provide *in vivo* evidence that glutathione and the thioredoxin redox system play distinct but overlapping functions in controlling subcellular redox environments. This is the first report of exclusive targeting of a YFP-based redox sensor to the yeast nucleus to specifically monitor fluctuations in the nuclear glutathione redox potential compared with that in cytosol in response to genetic and environmental factors.

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The abbreviations used are

ROS	reactive oxygen species
GSH	reduced glutathione

Grx	glutaredoxin
Glr	glutathione reductase
Trx	thioredoxin
Trr	thioredoxin reductase
GSSG	glutathione disulfide
rxYFP	redox-sensitive yellow fluorescent protein
IMS	mitochondrial intermembrane space
YPD	yeast extract peptone dextrose medium
SC	synthetic complete medium
Can^r	canavanine resistant
DAPI	4',6-diamidino-2-phenylindole
TCA	trichloroacetic acid
4-DPS	4,4'-dithiodipyridine
DTT	dithiothreitol
H₂O₂	hydrogen peroxide
BSO	buthionine sulfoximine

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Highlights

- A redox-sensitive yellow fluorescent protein (rxYFP) is targeted to the yeast nucleus.
- Nucleus- and cytosol- rxYFP can monitor compartment-specific dynamic redox changes.
- Glutathione and thioredoxin system play overlapping roles in subcellular redox control.
- Nuclear redox may be regulated independently of the cytosol.
- Oxidized nuclear redox environment upon GSH depletion does not affect nuclear genome stability.

Figure 1.

Localization of nucleus-rxYFP. (A) Wild-type yeast cells expressing nucleus-rxYFP and stained by DAPI were analyzed by fluorescence microscopy. Differential interference contrast (DIC) image and overlay image of YFP and DAPI signal (merge) are also shown. (B) *NAB2-mRFP* strain transformed with a nucleus-rxYFP expressing plasmid. (C) Wild-type yeast cells expressing nucleus-rxYFP and Nup49-mCherry were also analyzed for localization of YFP relative to the nuclear marker. (D) Wild-type yeast cells expressing cytosol-rxYFP and stained by DAPI were analyzed by DIC and fluorescence microscopy. (E) Nuclei or cytosol from intact cells were prepared (Materials and Methods) and subjected to Western blot analysis using antibodies directed against Pgk1 (cytosol marker), Nop1 (nucleus marker) and rxYFP. In the right panel, isolated nuclei (40 μ g of protein) from cells expressing nucleus-rxYFP (n) or cytosol-rxYFP (c) was prepared and analyzed. The same blot was cut and blotted for indicated antibody. In the right panel, cytosol portion (40 μ g of protein) from cells expressing nucleus-rxYFP (n) or cytosol-rxYFP (c) was prepared and analyzed. The same blot was cut and blotted for indicated antibody.

Figure 2.

Nucleus-rxYFP senses the nuclear GSH/GSSG redox status and responds to oxidant and reductant treatment. Wild-type yeast cells expressing cytosol- or nucleus-rxYFP were treated with 180 μ M 4-DPS or 50 mM DTT for 20 min and collected for redox Western blot analysis. Reduced (red) and oxidized (ox) forms of rxYFP were quantified using an Odyssey Infrared Imaging System. Two representative blots are shown. The reported values are the mean $\pm$ SD of at least three independent experiments.

Figure 3.

rxYFP redox response in various mutant cells (W303 background) affecting GSH or TRX redox systems and effects of deletions of thioredoxin genes on the growth of *trr1* mutants. (A) Representative redox Western blots of the samples are shown. Percentage of oxidized rxYFP was quantified using an Odyssey Infrared Imaging System. The reported values are the mean $\pm$ SD for 3–7 independent experiments. * $P < 0.05$, ** $P < 0.01$. (B) Tetrads from diploids heterozygous for *trr1*, *trx1* and *trx2* were dissected and analyzed for the presence of selection or auxotrophic markers (*trr1::kanMX4*, *trx1::HIS3*, and *trx2::URA3*). Circles indicate *trr1* single mutants; triangles indicate *trr1 trx1* mutants; squares indicate *trr1 trx2* mutants; diamonds indicate *trr1 trx1 trx2* mutants; hexagons indicate *trx1 trx2* mutants.

Figure 4.

Dynamic subcellular redox changes of *gsh1* cells during GSH depletion. The *gsh1* cells expressing the rxYFP sensors were inoculated into SC–Leu medium containing 500 μ M GSH for overnight growth. These preculture cells were diluted to an appropriate density in SC–Leu with or without 500 μ M GSH. During the 48 h time course, cultures were diluted when necessary to maintain exponential growth. (A) Cell density (OD₆₀₀) of *gsh1* cells was monitored at 0, 8, 24, 32 or 48 h after onset of GSH depletion. (B) and (C) Time course of intracellular GSH (B) and GSSG (C) concentrations during GSH depletion. Samples were removed 0, 8, 24, 32 and 48 h after onset of GSH depletion and prepared for GSH and GSSG quantification. (D) Redox Western blot of the samples removed from two parallel cultures, with or without GSH, at 0, 8, 24, 32 and 48 h after onset of GSH depletion. Percentage of oxidized forms of rxYFP was quantified using an Odyssey Infrared Imaging System. In A–D, the reported values are the means $\pm$ SD for two independent experiments.

Figure 5.

Subcellular redox changes in response to H₂O₂ bursts. Wild-type strains expressing the rxYFP sensors at mid-exponential phase were treated with 0.4 mM H₂O₂ (A) or 3.0 mM H₂O₂ (B). Redox Western blots were performed on samples removed before and 5, 15, 30, 60, 120, 240 min after addition of H₂O₂ (left). Percentage of oxidized forms of rxYFP was quantified using an Odyssey Infrared Imaging System (right). (C) Time course of total intracellular GSH concentration in response to 0.4 mM H₂O₂ and 3.0 mM H₂O₂ bursts. Samples were removed before and 5, 15, 30, 60, 120, 240 min after addition of H₂O₂ and prepared for GSH quantification. The reported values are the mean $\pm$ SD of at least two independent experiments.

Figure 6.

Effect of GSH depletion on mitochondrial and nuclear genome stability. The strains (S288c background) were inoculated into SC medium containing 500 μM GSH for overnight growth before dilution to an appropriate density in SC without GSH. (A) Effect of GSH depletion on respiratory incompetency. The *gsh1* cells were removed at 0, 8, 24, and 48 h after onset of GSH depletion and plated onto YPD plates to form colonies. The frequency of *petite* mutations was calculated as described in Materials and Methods. The reported values are the means ± SD for 2–5 independent experiments. (B) Effect of GSH depletion on nuclear DNA mutation. The *gsh1* cells that underwent 24 h GSH depletion were either inoculated in SC without GSH or supplemented with indicated concentrations of GSH for another 24 h. Cells removed from these defined culture conditions were plated onto YPD and selection plates containing canavanine to determine the total number of viable cells and the number of mutants, respectively. The reported mutation frequency is the median frequency of at least five independent cultures. (C) Effect of GSH depletion on nuclear DNA mutation in *tsal gsh1* mutants. The *tsal gsh1* mutants that underwent 24 h GSH depletion were either inoculated into SC medium containing 500 μM GSH or without GSH for another 24 h. Can⁺ mutation frequencies were calculated as above and is the median frequency of 21 independent cultures. The error bars indicate 95% interval confidence based on order statistics with the formula available at <http://www.math.unb.ca/~knight/utility/MedInt95.htm>. Statistical significance was evaluated by the Mann-Whitney test using programs available at <http://faculty.vassar.edu/lowry/vshome.htm>.

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