Redox Biology 36 (2020) 101598

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

One cysteine is enough: A monothiol Grx can functionally replace all cytosolic Trx and dithiol Grx

Jannik Zimmermann[a], Julian Oestreicher[a], Steffen Hess[b], Johannes M. Herrmann[b], Marcel Deponte[c,**], Bruce Morgan[a,*]

[a] Institute of Biochemistry, Zentrum für Human- und Molekularbiologie (ZHMB), Saarland University, Saarbrücken, Germany
[b] Cell Biology, University of Kaiserslautern, Kaiserslautern, Germany
[c] Faculty of Chemistry, Department of Biochemistry, University of Kaiserslautern, Kaiserslautern, Germany

ARTICLE INFO

Keywords:
Glutaredoxins
Protein disulfide
Redox catalysis
roGFP2
Thioredoxins

ABSTRACT

Glutaredoxins are small proteins of the thioredoxin superfamily that are present throughout life. Most glutaredoxins fall into two major subfamilies. Class I glutaredoxins are glutathione-dependent thiol-disulfide oxidoreductases whilst class II glutaredoxins coordinate Fe–S clusters. Class I glutaredoxins are typically dithiol enzymes with two active-site cysteine residues, however, some enzymatically active monothiol glutaredoxins are also known. Whilst both monothiol and dithiol class I glutaredoxins mediate protein deglutathionylation, it is widely claimed that only dithiol glutaredoxins are competent to reduce protein disulfide bonds. In this study, using a combination of yeast 'viability rescue', growth, and redox-sensitive GFP-based assays, we show that two different monothiol class I glutaredoxins can each facilitate the reduction of protein disulfide bonds in ribonucleotide reductase, methionine sulfoxide reductase and roGFP2. Our observations thus challenge the generalization of the dithiol mechanism for glutaredoxin catalysis and raise the question of why most class I glutaredoxins have two active-site cysteine residues.

1. Introduction

Glutaredoxins are glutathione-interacting members of the thioredoxin superfamily and are found throughout life. Glutaredoxins can be broadly separated into two highly abundant major subfamilies, termed class I and II glutaredoxins, as well as less prevalent smaller subfamilies [1,2]. Class I glutaredoxins are enzymatically active thiol-disulfide oxidoreductases that use reduced glutathione (GSH) as a co-substrate to facilitate disulfide reduction [3–5]. Class II glutaredoxins bind Fe–S clusters and usually have little or no oxidoreductase activity [6–8]. Class I glutaredoxins, together with thioredoxins, are important for reducing disulfide bonds that are formed as part of the catalytic mechanism of several cytosolic and nuclear enzymes including ribonucleotide reductase [9,10], peroxiredoxins (Prx) [11–15], methionine sulfoxide reductase [16,17], arsenate reductase [18], sulfiredoxin [19] and 3′-phosphoadenosine 5′-phosphosulfate reductase (PAPS reductase) [20,21] (Fig. 1a). Furthermore, class I glutaredoxins can reduce regulatory protein disulfide bonds, for example in the transcription factor OxyR [22] and the collapsin response mediator protein 2 [23,24]. The relative importance of the thioredoxins and glutaredoxins for protein disulfide reduction appears to vary between different substrates and different organisms. Whilst, ribonucleotide reductase from *Escherichia coli* can be reduced by either thioredoxins or the *E. coli* Grx1 [9,10], PAPS reductase in yeast can only be reduced by thioredoxins [25].

Class I glutaredoxins typically contain two active-site cysteine residues, for example in a CPYC motif [1,5]. However, there are examples of class I glutaredoxins that have only one active-site cysteine residue yet are enzymatically active in *in vitro* assays [26–30]. In contrast, class II glutaredoxins usually contain one active-site cysteine residue, for example in a CGFS motif [2]. The terms 'dithiol' and 'monothiol' glutaredoxin have sometimes been used as alternative names for class I and class II glutaredoxins. However, given that examples of both monothiol and dithiol class I glutaredoxins are known, here we use the terms 'monothiol' and 'dithiol' strictly to delineate between glutaredoxins containing either one or two active-site cysteine residues respectively.

Interestingly, several *in vitro* studies have demonstrated that dithiol class I glutaredoxins mutated for their second cysteine residue retain enzymatic activity or become even more active [12,31–35]. This raises the question of why most class I glutaredoxins have two active-site cysteine residues. In general, glutaredoxins can catalyze both protein

* Corresponding author.
** Corresponding author.
E-mail addresses: deponte@chemie.uni-kl.de (M. Deponte), bruce.morgan@uni-saarland.de (B. Morgan).

https://doi.org/10.1016/j.redox.2020.101598
Received 7 April 2020; Received in revised form 20 May 2020; Accepted 26 May 2020
Available online 31 May 2020

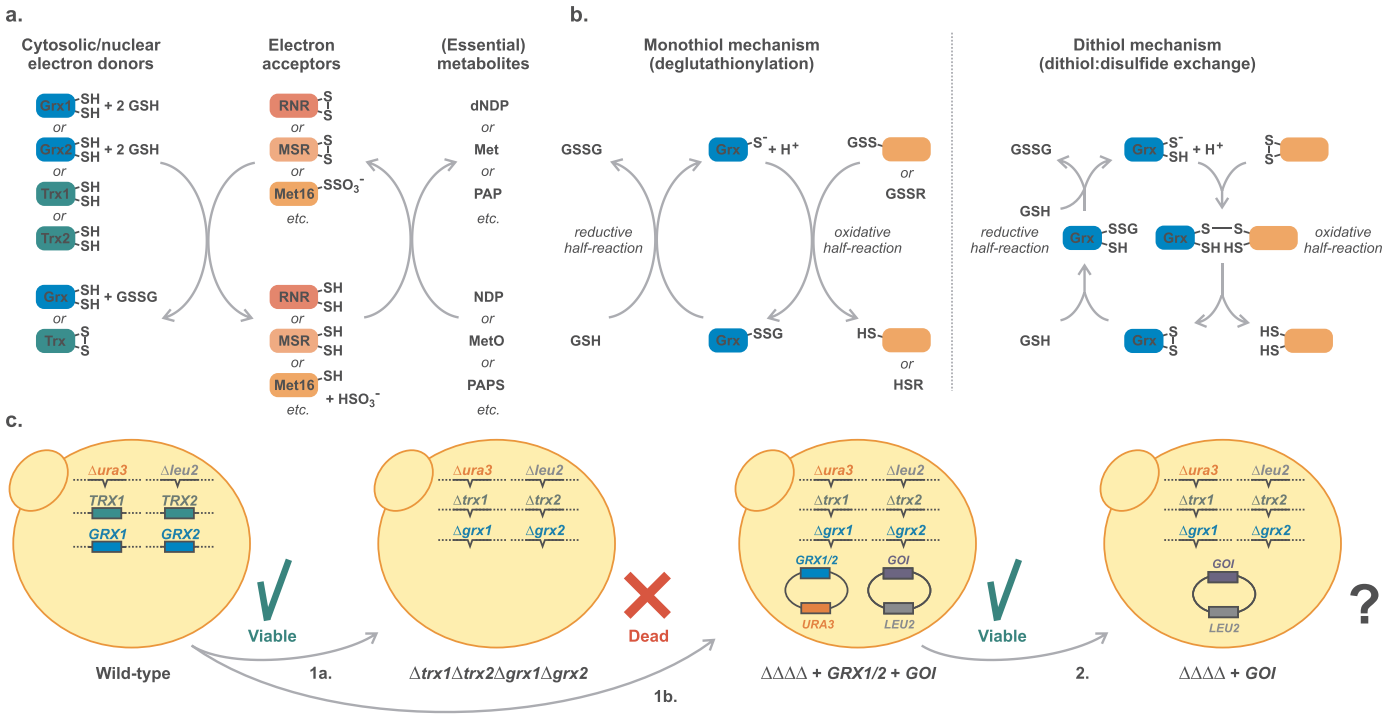


Fig. 1. Relevance and analysis of the cytosolic glutaredoxin and thioredoxin systems in yeast.
a. *Saccharomyces cerevisiae* has two major cytosolic thiol-disulfide oxidoreductase systems consisting of two thioredoxins (Trx1/2) as well as two dithiol class I glutaredoxins (Grx1/2) and reduced glutathione (GSH). Both systems donate electrons to a variety of enzymes such as ribonucleotide reductases (RNR), methionine sulfoxide reductases (MSR) and PAPS reductase (Met16). These and other enzymes catalyze the reduction of nucleoside diphosphates (NDP), methionine sulfoxide (MetO), 3′-phosphoadenosine 5′-phosphosulfate (PAPS) or other metabolites, yielding, for example, essential precursors for DNA or protein synthesis. **b.** One cysteine residue of class I glutaredoxins suffices to catalyze the reduction of glutathionylated substrates (left panel). In contrast, protein disulfide reduction has been claimed to require both active-site cysteine residues of class I glutaredoxins (right panel). **c.** Plasmid-shuffling experiments allow rapid structure-function analyses of a gene of interest (GOI) in yeast. First, relevant genes are knocked out (1a.). To ensure viability and to test the function of the GOI, yeast cells are complemented with an episomal copy of an essential gene as well as the GOI on another plasmid (1b.). After negative selection against the plasmid with the essential gene, cell growth fully relies on the GOI and can only occur if it functionally replaces the essential gene (2.).

deglutathionylation and protein disulfide reduction (Fig. 1b). Protein deglutathionylation can theoretically occur via a dithiol or a monothiol mechanism involving either both cysteine residues or only the more N-terminal of the active-site cysteine residues respectively. However, numerous studies showed that the monothiol reaction is more efficient: The dithiol mechanism is therefore usually considered as a side reaction for deglutathionylation reactions [1,12,33,34,36,37]. In contrast to protein deglutathionylation, protein disulfide reduction was widely claimed to require both active-site cysteine residues and to occur via a dithiol mechanism as reported for *E. coli* ribonucleotide reductase and PAPS reductase [21,38] as well as poplar PrxIIE and human Prx3 [13,39] (Fig. 1b). Nonetheless, mammalian ribonucleotide reductase can be reduced by a monothiol mechanism [32]. Furthermore, recent *in vitro* studies with a metal-binding domain as a protein disulfide substrate also challenged the dithiol mechanism and suggested an alternative monothiol mechanism that includes a GSH-dependent reduction of Grx–SS–protein disulfide bonds [37]. In summary, the reduction of protein disulfide bonds has been postulated to occur via a dithiol mechanism and to necessitate the presence of two active-site cysteine residues in glutaredoxins. However, for eukaryotic glutaredoxins this dogma is predominantly based on *in vitro* studies that were shown to depend upon the recombinant proteins being studied.

The budding yeast, *Saccharomyces cerevisiae*, contains two cytosolic thioredoxins and two enzymatically active cytosolic dithiol class I glutaredoxins [40] (Fig. 1a). A third dithiol glutaredoxin, Grx8, is a poorly active hybrid protein that neither belongs to the class I nor class II glutaredoxins [41]. Yeast also contain three further redox-inactive, monothiol class II glutaredoxins, Grx3, Grx4 and Grx5 [6,8], as well as two enzymatically active monothiol class I glutaredoxins, Grx6 and Grx7, which are targeted to the secretory pathway [26,28]. However, none of these latter five glutaredoxins are relevant for the efficient reduction of cytosolic or nuclear redox proteins.

A yeast strain deleted for both cytosolic thioredoxins and both cytosolic dithiol class I glutaredoxins, *Δtrx1Δtrx2Δgrx1Δgrx2*, is not viable [40]. However, all four possible triple deletion mutants are viable, demonstrating that retaining any single cytosolic thioredoxin or class I glutaredoxin is enough to maintain yeast viability. We reasoned that the ability of yeast to survive and grow with one single glutaredoxin would allow us to test the capacity of a monothiol class I glutaredoxin to support disulfide reduction of several protein substrates inside living cells (Fig. 1c). We found that both a cytosol-targeted variant of the naturally occurring monothiol glutaredoxin Grx7 as well as the engineered monothiol glutaredoxin, Grx2 C64S, can support the growth of yeast cells deleted for both cytosolic thioredoxins and both cytosolic dithiol class I glutaredoxins. Furthermore, the growth rate of these cells was indistinguishable from that of cells rescued by a dithiol glutaredoxin. These results reveal that a monothiol glutaredoxin must, at minimum, support efficient reduction of yeast ribonucleotide reductase. We furthermore show that monothiol glutaredoxins can mediate reduction of methionine sulfoxide reductase and the non-physiological protein disulfide in redox-sensitive green fluorescent protein 2 (roGFP2). In conclusion, we demonstrate that different enzymatically active monothiol glutaredoxins can support the efficient reduction of multiple different protein disulfides inside living cells. Our observations require a major re-think of current hypotheses on the relevance of the widely conserved active-site second cysteine residue and the dithiol mechanism as well as the physiological functions of monothiol and dithiol class I glutaredoxins in general.

2. Results

2.1. A monothiol class I glutaredoxin can sustain viability of a Δtrx1Δtrx2Δgrx1Δgrx2 strain

To test whether a monothiol class I glutaredoxin can support efficient protein disulfide bond reduction in living yeast cells, we first generated the triple deletion strain Δ*trx1*Δ*trx2*Δ*grx2*. Subsequently, we transformed this strain with a p416TEF plasmid encoding roGFP2-Grx2, a genetic fusion construct between roGFP2 and yeast Grx2. Finally, in this background we deleted *GRX1* to create the quadruple mutant Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* + p416TEF *roGFP2-GRX2*. The quadruple mutant was confirmed by PCR analysis and by growth on agar plates supplemented with appropriate antibiotics or with dropout of specific amino acids and nucleobases (Figs. S1a and b).

We next utilized a plasmid shuffling strategy [42] to test the capacity of different dithiol and monothiol glutaredoxins to maintain cell viability (Fig. 1c). We transformed the Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* + p416TEF *roGFP2-GRX2* strain with p415TEF expression plasmids encoding the yeast dithiol class I glutaredoxins Grx1 and Grx2 as positive controls and the enzymatically inactive human monothiol class II glutaredoxin *Hs*Grx5 or an empty plasmid as negative controls. Finally, we also transformed with a p415TEF plasmid encoding the enzymatically active yeast monothiol class I glutaredoxin Grx7. Grx2 and Grx7 were cloned without their mitochondrial targeting sequence and signal sequence respectively to allow for cytosolic localization. All strains grew well on Hartwell's Complete (HC) agar plates as well as on HC plates lacking leucine and uracil to select for retention of both plasmids (Fig. 2a). We next investigated growth on HC media containing 5-fluoroorotic acid (5-FOA). The p416TEF plasmid contains a *URA3* gene to allow for auxotrophic selection. *URA3* encodes an orotidine 5-phosphate decarboxylase, which can also decarboxylate 5-fluoroorotidine-5′-monophosphate to 5-fluorouridine-5′-monophosphate a toxic metabolite that kills cells [43]. Thus, in the presence of 5-FOA, cells can only grow if they have lost the p416TEF *roGFP2-GRX2* plasmid, which can only occur if the glutaredoxin encoded on the p415TEF plasmid can sustain cell viability. Cells transformed with an empty p415TEF plasmid were unable to grow on HC + 5-FOA media (Fig. 2a). Cells transformed with a p415TEF plasmid encoding class II *Hs*Grx5 were also inviable, whilst cells containing p415TEF plasmids encoding either Grx1 or Grx2 grew well (Fig. 2a). Intriguingly, cells transformed with p415TEF encoding the monothiol class I glutaredoxin, Grx7, also grew well on 5-FOA-containing plates (Fig. 2a).

To demonstrate that our observation is general for enzymatically active monothiol glutaredoxins and not specific to Grx7, we repeated the above experiment with a monothiol mutant of yeast Grx2. We observed that Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells containing a p415TEF plasmid encoding Grx2 with a CPYS active-site motif, i.e. Grx2 C64S, were able to grow in the presence of 5-FOA (Fig. 2b). In contrast, cells containing p415TEF plasmids encoding either Grx2 C61S or Grx2 C61S, C64S mutants were unable to grow in the presence of 5-FOA but grew well on HC plates, thus confirming the essentiality of the N-terminal active-site cysteine residue (Fig. 2b). Furthermore, we observed no difference in growth of Grx7 or Grx2 C64S rescued yeast when, instead of the strong constitutive TEF promoter, we used either the much weaker, constitutive, ADH promoter [44] (Fig. S2a) or the endogenous Grx2 promoter and terminator (Fig. S2b). Thus, the rescue of yeast viability by monothiol class I glutaredoxins is not a peculiarity of 'overexpression' or extreme protein concentrations.

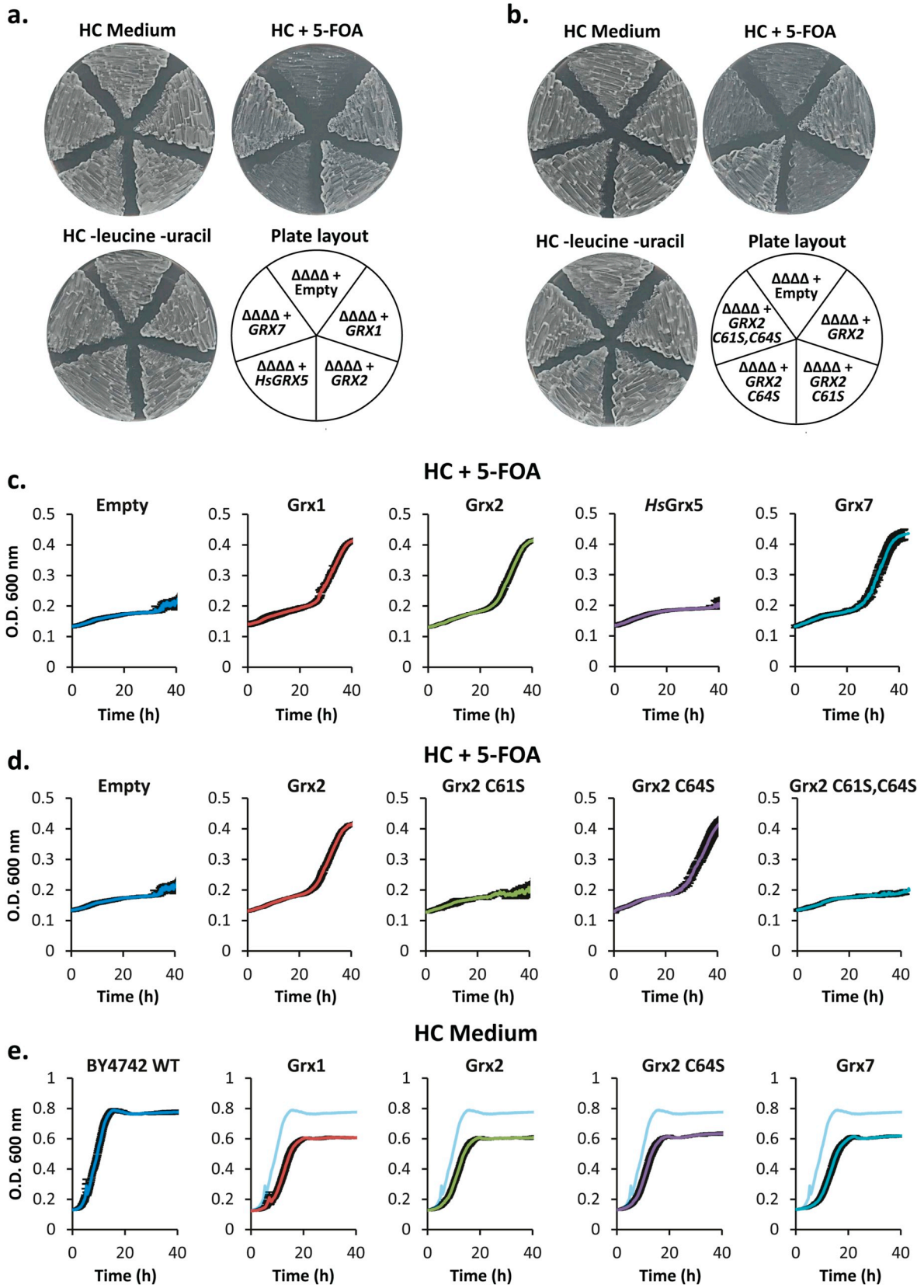
To further validate our results, we performed similar experiments in liquid media. Specifically, we inoculated Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* + p416 TEF *roGFP2-GRX2* strains, which were transformed with p415TEF plasmids encoding Grx1, Grx2, *Hs*Grx5 or Grx7, into HC medium supplemented with 0.1% 5-FOA (Fig. 2c). Consistent with the growth on agar plates, we observed no growth of cells containing a p415TEF empty vector or of cells containing a p415TEF plasmid encoding *Hs*Grx5. Interestingly, an *Hs*Grx5 mutant, which we have recently engineered to be enzymatically active (*Hs*Grx5 Loop + G68P + R97Q) [45], was also unable to restore growth on 5-FOA plates (Figs. S3a and b). On the contrary, Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells containing Grx1, Grx2 or Grx7 grew well (Fig. 2c), whereas cells containing Grx7 C108S, which lacks the active-site cysteine residue, could not grow on either agar plates or in liquid culture (Figs. S3a and b). The long lag phase is due to the selection mechanism of 5-FOA. Only cells that have by chance lost the p416TEF plasmid will be able to grow in the presence of 5-FOA, thus the actual number of cells in the culture that will be able to divide at the beginning of the growth assay is very low. For Grx2 cysteine mutants we also observed the same growth pattern in liquid cultures as we had observed on agar plates. Cells containing Grx2 or Grx2 C64S grew well, whilst cells expressing Grx2 C61S or Grx2 C61S, C64S did not grow (Fig. 2d). Finally, we subjected Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* + p416 TEF *roGFP2-GRX2* cells, transformed with p415TEF plasmids encoding either Grx1, Grx2, Grx2 C64S or Grx7, to two rounds of selection on 5-FOA to ensure complete loss of the p416TEF *roGFP2-GRX2* plasmid. We then grew the resultant Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* + p415TEF *GRX1*, *GRX2*, *GRX2 C64S* or *GRX7* strains on HC media. We observed no difference in growth irrespective of whether the cells contained a monothiol or dithiol class I glutaredoxin (Fig. 2e). Therefore, our observations clearly demonstrate that a monothiol class I glutaredoxin can replace all the essential functions of cytosolic thioredoxins and dithiol class I glutaredoxins. Finally, as a further control, we also tested the ability of thioredoxin cysteine mutants to restore growth. Specifically, we streaked Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* + p416 TEF *roGFP2-GRX2* strains, transformed with p415TEF vectors encoding either Trx1, Trx2, Trx2 C34S or Trx2 C31S,C34S, onto both HC agar plates and HC plates supplemented with 5-FOA. All strains grew well on HC plates, whilst only strains with either Trx1 or Trx2 were able to grow in the presence of 5-FOA confirming the essentiality of both thioredoxin active-site cysteine residues in accordance with a dithiol mechanism (Fig. S3c). We were also interested to test if protein disulfide isomerases (PDIs) could rescue growth of the Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* strain. This is plausible as PDIs can reduce protein disulfide bonds and can themselves be reduced by GSH [46]. However, none of the protein disulfide isomerases Pdi1, Mpd1 or Mpd2 could rescue growth on agar plates (Fig. S3d). In summary, we demonstrate that monothiol class I glutaredoxins can fulfil all essential protein disulfide bond reduction required for sustaining yeast viability and growth.

2.2. Monothiol class I glutaredoxins can reduce methionine sulfoxide reductase

The rescue of yeast viability and growth by monothiol class I glutaredoxins demonstrates that, at minimum, these enzymes must facilitate the reduction of ribonucleotide reductase. It is unclear whether reduction of any other cytosolic/nuclear protein disulfide is essential for yeast viability. Nonetheless, there are several protein disulfides that require reduction as part of their normal catalytic mechanism and we were interested to see whether a monothiol class I glutaredoxin could reduce these protein disulfides. One of these proteins is PAPS reductase, which is responsible for reducing activated sulfate to sulfite. This is an important step in the assimilation of inorganic sulfate to produce, for example, methionine and cysteine. Previous studies have shown that yeast deleted for both cytosolic thioredoxins are auxotrophic for methionine and it is therefore assumed that glutaredoxins are unable to reduce yeast PAPS reductase [25]. Our assays supported these observations. BY4742WT, a methionine prototroph, grew well on solid or liquid HC media without methionine and served as a control (Fig. 3a and b). In contrast, BY4742 Δ*trx1*Δ*trx2*, Δ*trx1*Δ*trx2*Δ*grx1* and Δ*trx1*Δ*trx2*Δ*grx2* strains could not grow on either solid or liquid HC media without methionine (Figs. S4a and b). Furthermore, the methionine auxotrophic strain, BY4741, as well as BY4742

Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* strains with p415TEF plasmids encoding either Grx1, Grx2, Grx2 C64S or Grx7 also showed no growth on either solid or liquid HC media lacking methionine (Fig. 3a and b). We conclude that neither a dithiol nor monothiol class I glutaredoxin is capable of supporting sulfate assimilation in the absence of thioredoxins, most likely because glutaredoxins cannot mediate reduction of yeast PAPS reductase.

The methionine auxotrophy of our BY4742 Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2*



(caption on next page)

Fig. 2. A single monothiol class I glutaredoxin can sustain viability of Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* yeast strains.
a. BY4742 Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells containing a p416TEF *roGFP2-GRX2* were transformed with p415TEF plasmids encoding either Grx1, Grx2, *Hs*Grx5 or Grx7. Cells were streaked onto either HC plates, HC + 0.1 g/l 5-FOA or HC without leucine and uracil. Plates were grown for 48 h at 30 °C. **b.** As in (**a.**) except that cells were transformed with p415TEF plasmids encoding either Grx2 or one of the mutants Grx2 C61S, Grx2 C64S, Grx2 C61S, C64S. **c.** and **d.** BY4742 Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells containing a p416TEF *roGFP2-GRX2* plasmid as well as p415TEF plasmids for the expression of the indicated glutaredoxins, as in (**a.**) and (**b.**), were inoculated in HC medium supplemented with 0.1 g/l 5-FOA to an initial OD_{600} = 0.1. Cultures were subsequently grown in a round-bottomed 96-well plate with continuous shaking at 30 °C. **e.** BY4742 Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells containing a p416TEF *roGFP2-GRX2* plasmid and p415TEF plasmids encoding either Grx1, Grx2, Grx2 C64S or Grx7 underwent two rounds of selection on HC plates supplemented with 0.1 g/l 5-FOA to ensure complete loss of the p416TEF plasmid. Subsequently, these cells were inoculated to an initial OD_{600} = 0.1 and were grown in a round-bottomed 96-well plate with continuous shaking at 30 °C. BY4742WT cells were also grown for comparison. The growth curve of the BY4742WT is shown in all other panels in light blue. Error bars in all panels represent the standard error for three experimental repeats. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

strains, due to the absence of a functional thioredoxin, nonetheless afforded us the opportunity to genetically test the reduction of another protein disulfide, namely in methionine sulfoxide reductases. Previous studies have shown that methionine auxotrophic yeast strains with functional methionine sulfoxide reductases can grow using methionine sulfoxide as a source of methionine [47,48]. It was reported that a Δ*trx1*Δ*trx2* yeast strain cannot grow on methionine sulfoxide [47,48]. This is supported by *in vitro* studies on methionine sulfoxide reductases suggesting that 2-Cys methionine sulfoxide reductases, which form an intramolecular disulfide bond, are exclusively reduced by thioredoxins [17]. Nonetheless, in our experiments, BY4742 Δ*trx1*Δ*trx2*, Δ*trx1*Δ*trx2*Δ*grx1* and Δ*trx1*Δ*trx2*Δ*grx2* strains all grew, albeit relatively slowly, on both solid and liquid HC medium supplemented with 0.24 mM methionine sulfoxide in place of methionine (Figs. S4a and c). This observation therefore allowed us to test whether a monothiol class I glutaredoxin can also sustain growth on methionine sulfoxide. To this end, we tested the growth of Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells transformed with p415TEF plasmids encoding either Grx1, Grx2, Grx2 C64S or Grx7

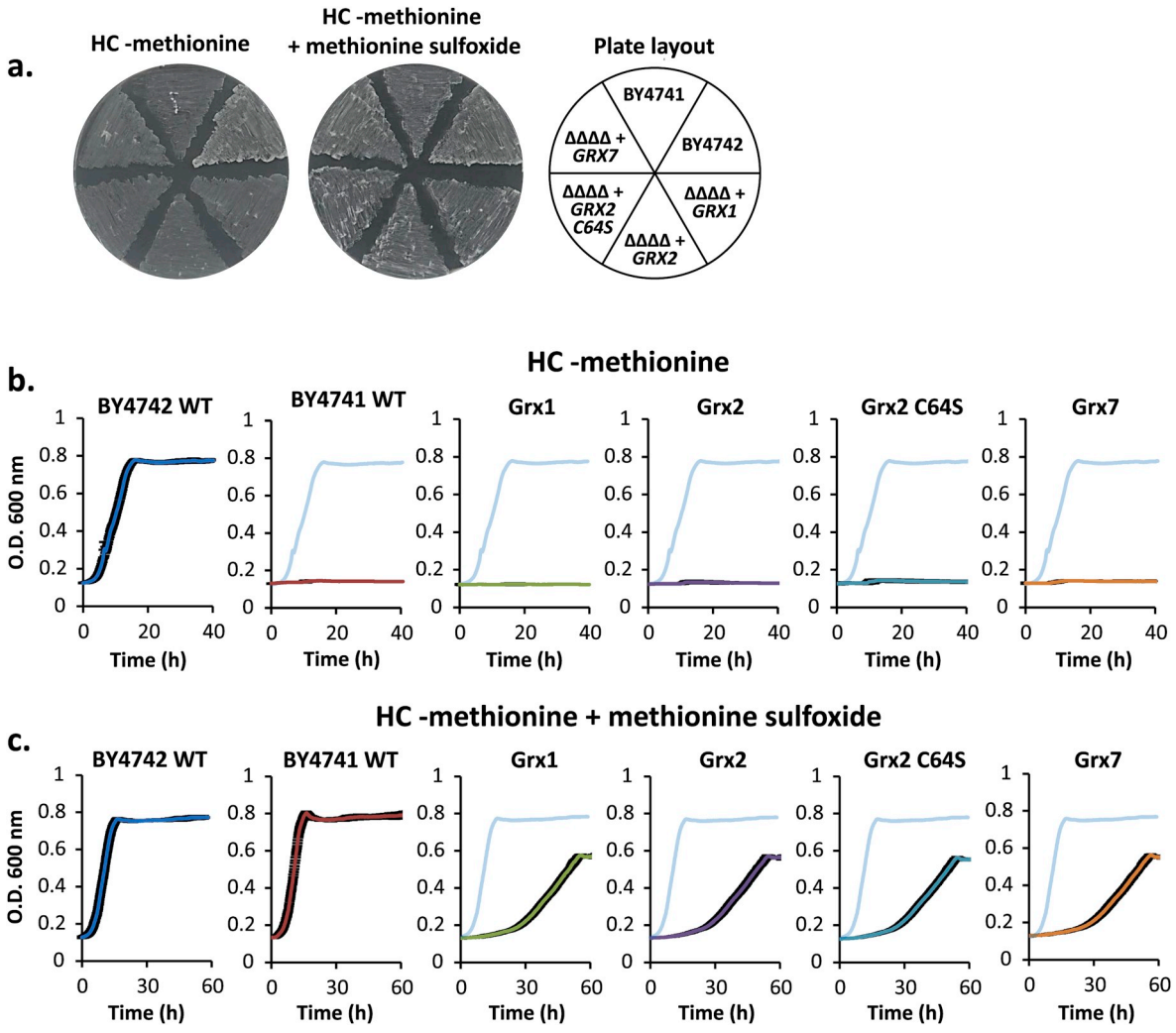


Fig. 3. A monothiol class I glutaredoxin can reduce methionine sulfoxide reductase.
a. BY4742 Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells containing a p415TEF plasmid, for the expression of the indicated glutaredoxins, were streaked either onto HC plates lacking methionine or HC plates supplemented with 0.24 mM L-methionine sulfoxide. Plates were incubated for 48 h at 30 °C. BY4741WT (methionine auxotroph) and BY4742WT (methionine prototroph) cells were included as controls. **b.** and **c.** Cells as used in (**a.**) were inoculated to an initial OD_{600} = 0.1 in either HC medium without methionine or HC medium supplemented with 0.24 mM L-methionine sulfoxide and were grown in a round-bottomed 96-well plate with continuous shaking at 30 °C. The growth curve of BY4742WT cells was reproduced in all panels in light blue. Error bars in all panels represent the standard error for three experimental repeats. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

on both solid and liquid HC media containing 0.24 mM methionine sulfoxide (Fig. 3a,c). Interestingly, all strains grew at a similar rate to the Δ*trx1*Δ*trx2* strain (compare Fig. 2c with Fig. S4c), suggesting that monothiol and dithiol class I glutaredoxins can reduce yeast methionine sulfoxide reductase(s) with equal efficiency. In summary, we demonstrate that monothiol class I glutaredoxins can support growth on methionine sulfoxide, which is strongly consistent with the conclusion that monothiol glutaredoxins can reduce yeast methionine sulfoxide reductase(s).

2.3. The cytosolic glutathione pool remains highly reduced in rescued Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* strains

It has previously been demonstrated that the yeast dithiol glutaredoxins, Grx1 or Grx2 can mediate the equilibration between the roGFP2 thiol-disulfide redox couple and the 2GSH/GSSG redox couple under steady-state conditions [49]. Therefore, the degree of roGFP2 oxidation is a quantitative noninvasive real-time measure of the glutathione redox potential provided that suitable glutaredoxins are present to mediate the equilibration [50–52]. We were thus interested to see whether the monothiol glutaredoxins, Grx2 C64S and Grx7 could also equilibrate roGFP2 with the glutathione pool, which would require that they can efficiently oxidize and reduce roGFP2. To address this question, we transformed Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells rescued by p415TEF plasmids encoding either Grx1, Grx2, Grx2 C64S or Grx7 with a p416TEF plasmid encoding roGFP2. We then used a plate-reader assay to monitor the degree of roGFP2 oxidation in these strains (Fig. 4a–d). Interestingly, average roGFP2 oxidation in Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells with Grx1, Grx2 or Grx2 C64S was less than 20% in each case. This result clearly demonstrates that the cytosolic glutathione pool remains highly reduced in these strains, with a glutathione redox potential of ≤ -295 mV. Furthermore, the roGFP2 sensor in all three strains responded very similarly to the exogenous addition of H_2O_2 (Fig. 4a–d), indicating that the monothiol glutaredoxin, Grx2 C64S, is capable of mediating roGFP2 oxidation and reduction, with similar efficiency to dithiol glutaredoxins. Interestingly, the steady-state oxidation of roGFP2 in Grx7 cells, at ~40%, was higher than in the other strains, although the roGFP2 reporter did readily respond to exogenous H_2O_2. This result may either indicate that the cytosolic glutathione redox potential is indeed higher in this strain or could indicate that Grx7 is less efficient in roGFP2 reduction than in roGFP2 oxidation. An imbalance in reduction versus oxidation alone could not change the thermodynamics of roGFP2 oxidation. However, other proteins such as peroxidases, can slowly oxidize roGFP2 and could drive accumulation of oxidized roGFP2 over time [53] if such oxidation could not be efficiently reduced by Grx7. To gain further insight into this question, we expressed genetic fusion constructs between roGFP2 and either Grx2 or Grx7. In such a direct fusion, the effective concentration of glutaredoxin 'perceived' by the roGFP2 reporter will be increased by ~1000-fold and thus may compensate for kinetic limitations [50]. Indeed, in the context of both the roGFP2-Grx2 and roGFP2-Grx7 fusion constructs, the steady-state average roGFP2 oxidation was approximately 10% and both probes responded readily to exogenous H_2O_2 (Fig. 4e and f). Thus, we demonstrate that two different monothiol class I glutaredoxins, Grx2 C64S and Grx7, can mediate rapid oxidation and reduction of roGFP2 inside living cells, although Grx7 seems to be less efficient at reducing roGFP2. Furthermore, our results demonstrate that even in cells lacking both cytosolic thioredoxins and both cytosolic dithiol class I glutaredoxins, the cytosolic glutathione pool remains highly reduced in the presence of a monothiol class I glutaredoxin.

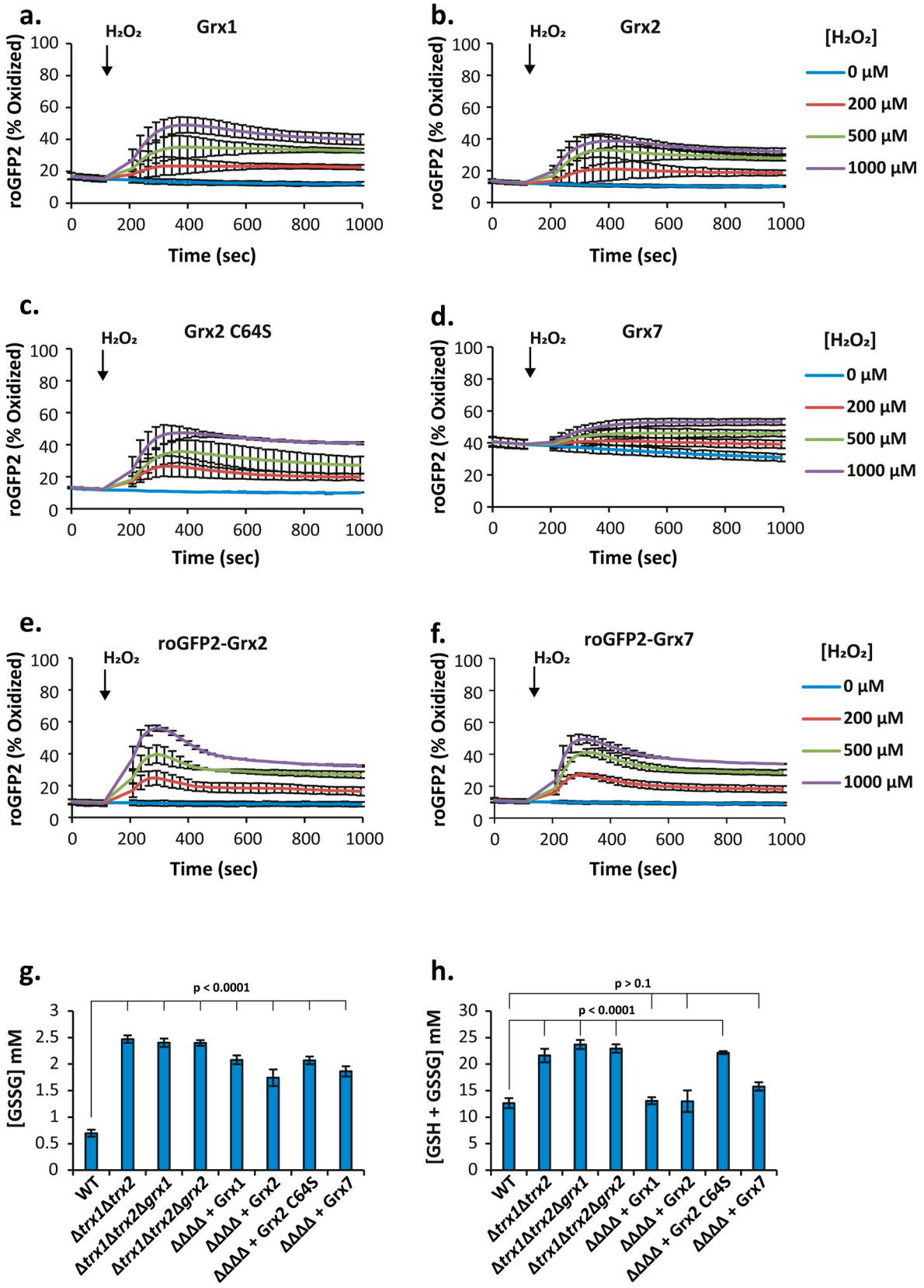
In Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells expressing either Grx2 C64S or Grx7, reduction of every cytosolic disulfide bond presumably oxidizes two molecules of GSH, which serves as the electron donor. We therefore asked whether this led to a detectable increase in cellular GSSG content. In any strain in which the genes encoding both Trx1 and Trx2 were deleted, we did indeed observe a large increase in cellular GSSG content, consistent with GSH/Grx becoming the predominant or even sole source of disulfide reductive power (Fig. 4g). As the roGFP2 reporter indicated that the cytosolic glutathione pool remained highly reduced, the 'extra' GSSG is presumably stored in the vacuole as we have previously shown [49]. We also observed that 'total' cellular glutathione (defined as GSH + GSSG) levels increased in Δ*trx1*Δ*trx2* cells, which is consistent with previous reports [54], as well as in Δ*trx1*Δ*trx2*Δ*grx1* and Δ*trx1*Δ*trx2*Δ*grx2* cells (Fig. 4h). Intriguingly, the presence of either Grx1, Grx2 or Grx7 in Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells restored 'total' cellular glutathione levels to those found in wild-type cells although the reason for this is unclear. This effect was not observed in cells expressing Grx2 C64S (Fig. 4h). In summary, we show that the cytosolic glutathione pool remains highly reduced in cells lacking both cytosolic thioredoxins and a glutaredoxin. The extra 'demand' on the glutathione pool is reflected in increased cellular GSSG, which is presumably stored in the vacuole. Finally, we demonstrate that a monothiol class I glutaredoxin allows efficient roGFP2-based sensing of the glutathione redox potential.

3. Discussion

Why do most class I glutaredoxins have two active-site cysteine residues even though monothiol mutants are usually more active as deglutathionylating enzymes *in vitro*? It is frequently reported that glutaredoxin-mediated protein disulfide reduction requires a dithiol mechanism, and this was indeed shown for *E. coli* ribonucleotide reductase and PAPS reductase [21,38,55,56]. Nonetheless, many other *in vitro* studies with non-glutathione protein disulfide substrates, some of which are frequently cited as examples for a dithiol mechanism, appear to lack the crucial control experiment with GSH and a monothiol class I glutaredoxin to exclude a monothiol mechanism [18,22,23]. In the presence of GSH, a monothiol mechanism might indeed be highly relevant, as demonstrated *in vitro* for the reduction of protein disulfide bonds in mammalian ribonucleotide reductase and the metal-binding domain of the ATPase HMA4 [32,37] as well as the non-glutathione model substrate bis(2-hydroxyethyl)disulfide [45,57,58]. Furthermore, monothiol class I glutaredoxins were recently shown to efficiently oxidize roGFP2 inside living cells and to permit roGFP2-dependent redox measurements [45]. Here we demonstrate that monothiol class I glutaredoxins can also efficiently reduce roGFP2.

Importantly, we found that two different monothiol class I glutaredoxins, Grx7 and Grx2 C64S, with a natural and an engineered CPYS active-site motif respectively, maintained viability of yeast strains lacking all endogenous cytosolic thioredoxins and dithiol class I glutaredoxins. Indeed, the growth rates of yeast strains rescued with monothiol or dithiol class I glutaredoxins were virtually indistinguishable. Considering the subcellular localization of yeast Grx1 and Grx2 [59,60] and of their functional replacements, we conclude that the dithiol mechanism is dispensable for essential reductions of known and unknown cytosolic and nuclear protein disulfide substrates in yeast. Thus, yeast ribonucleotide reductase can be reduced via a monothiol mechanism *in vivo* in accordance with the *in vitro* data for mammalian ribonucleotide reductase [32]. Our data also imply that other protein disulfides can be efficiently reduced by a monothiol class I glutaredoxin. This includes the proteins methionine sulfoxide reductase and roGFP2. However, PAPS reductase cannot be reduced by either a monothiol or dithiol glutaredoxin. Whether the dithiol mechanism is also dispensable in other eukaryotes remains to be shown. Nonetheless, at this stage we cannot fully exclude that the dithiol mechanism might become essential under alternative experimental conditions, for example due to absent compensatory mechanisms or altered glutaredoxin concentrations [61]. For example, according to the 'cysteine resolving model', the second cysteine residue might resolve kinetically trapped glutaredoxin species with unreactive Grx-SSG or Grx–SS–protein conformations that cannot efficiently react with GSH because of steric or

geometric constraints [1,5,41]. Such trapped glutaredoxin species are not restricted to substrates or hypothetical redox signaling partners but might include unspecific Grx–SS–protein disulfides that accumulate under certain conditions, such as observed in pull-down experiments with mutant monothiol glutaredoxins [62–64]. The physiological impact of these trapped species might depend on the total glutaredoxin concentration, although different promoter strengths had no impact on our growth assays under the chosen conditions. In summary, we found that a single monothiol class I glutaredoxin suffices for yeast cell viability and that the dithiol mechanism is dispensable for essential



(caption on next page)

Fig. 4. The glutathione redox potential is highly reduced in Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* yeast cells.
BY4742 Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells containing a p416TEF plasmid encoding roGFP2 and a p415TEF plasmid encoding either **a.** Grx1, **b.** Grx2, **c.** Grx2 C64S or **d.** Grx7 were resuspended to an OD_{600} = 7.5 in 100 mM MES-Tris pH 6 and transferred into wells of a flat-bottomed 96-well plate. H_2O_2 was added at the concentrations indicated and the change in roGFP2 oxidation was monitored with a fluorescence plate-reader-based assay. **e.** BY4742 Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells containing an empty p416TEF plasmid as well as a p415TEF plasmid encoding either, **e.** roGFP2-Grx2 or **f.** roGFP2-Grx7 were treated as described for panels **a–d.** RoGFP2 oxidation was likewise monitored following addition of H_2O_2 at the indicated concentrations. **g.** 'Total' glutathione assay. For each of the strains described in **a–d**, as well as BY4742WT, Δ*trx1*Δ*trx2*, Δ*trx1*Δ*trx2*Δ*grx1* and Δ*trx1*Δ*trx2*Δ*grx2* strains, a DTNB-based assay was used to measure 'total' glutathione (GSH and GSSG; reported as GSH equivalents) in lysates produced from 50 OD_{600} units of these cells, where 1 OD_{600} unit represents 1 ml of culture with an OD_{600} = 1. **h.** As for **g.**, but with an adapted protocol to monitor GSSG levels in cell lysates. P-values were determined by a one-way ANOVA analysis followed by a Tukey's test.

glutaredoxin catalysis. The second active-site cysteine residue in dithiol class I glutaredoxins might have conserved (non-essential) functions other than the reduction of ribonucleotide reductase. One of these alternative functions that remains to be addressed in future studies is a hypothetical role as a resolving cysteine for trapped glutaredoxin species.

How can a monothiol glutaredoxin reduce a protein disulfide substrate? We consider three of several non-exclusive mechanistic models. a) The monothiol class I glutaredoxin itself performs the initial nucleophilic attack on the protein disulfide bond (Fig. 5a). This would result in a transient intermolecular disulfide between the glutaredoxin and the protein substrate. In this scenario GSH reduces the intermolecular Grx–SS–protein disulfide. Theoretically, GSH could either attack (i) the glutaredoxin sulfur atom, yielding a glutathionylated glutaredoxin and the reduced protein substrate, or (ii) the sulfur atom of the protein substrate, yielding a glutathionylated protein, which is subsequently deglutathionylated by the glutaredoxin. Both reaction sequences result in glutathionylated glutaredoxin (step 3 in Fig. 5a). The glutathionylated glutaredoxin subsequently reacts with a second GSH molecule yielding GSSG. A direct reaction between a monothiol class I glutaredoxin and substrate seems very likely because this part of the reaction is identical to the dithiol mechanism. Pathway (i) has previously been suggested for the reduction of HMA4 [37], whereas pathway (ii) takes the reaction geometry into account and is supported by the kinetic patterns with the model substrate bis(2-hydroxyethyl) disulfide [45,57,58]. b) GSH non-enzymatically reacts with the protein disulfide substrate (Fig. 5b). The glutathionylated protein is subsequently deglutathionylated following a regular monothiol mechanism. While the second part of this reaction sequence is in perfect agreement with numerous *in vitro* studies (e.g. Ref. [33,36]), the rate constant for the first part of the reaction sequence depends on the protein disulfide substrate. This rate constant is probably too small for most substrates to be significant [61]. c) In a variation of the latter mechanism, glutaredoxins or a glutathione transferase may deprotonate and activate GSH, to increase its nucleophilicity for the initial attack on the protein disulfide (Fig. 5c). GSH activation is a common principle in the structurally related glutathione transferases, some of which can reduce disulfide bonds or sulfenic acids [5]. A prediction of this mechanism is that the GSH-mediated reduction of protein disulfides, resulting in glutathionylated proteins, could be catalyzed in the presence of a glutaredoxin without a cysteine residue provided that GSH activation can still occur. It will be exciting to test this possibility, perhaps in the context of future *in vitro* studies.

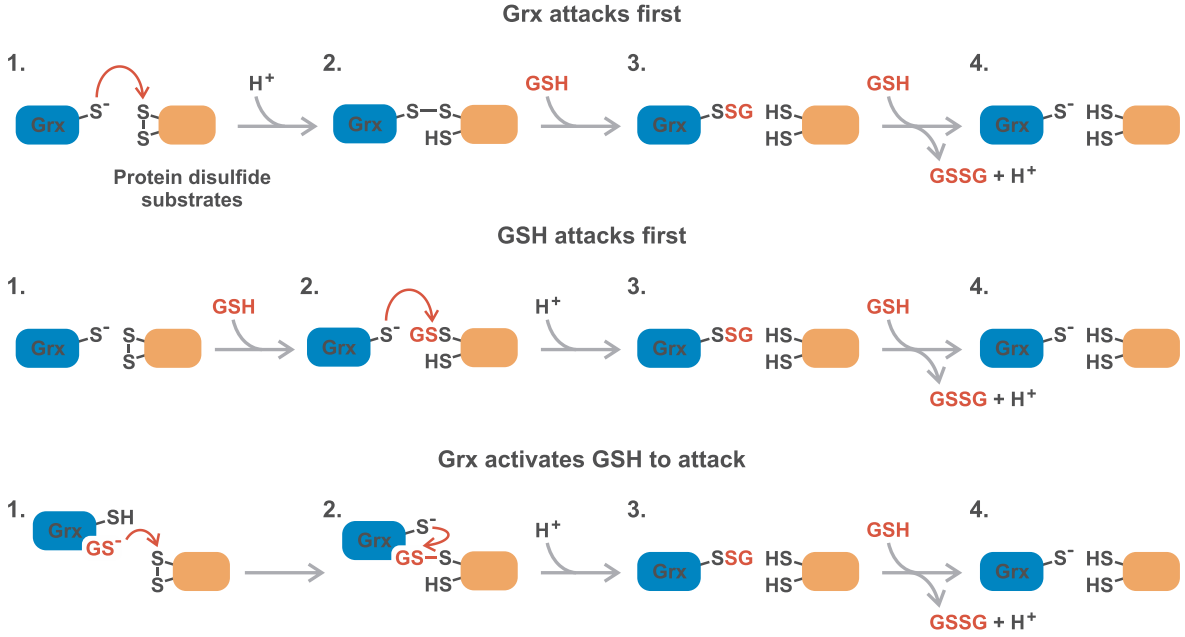


Fig. 5. Plausible mechanisms for reduction of protein disulfides by a monothiol glutaredoxin.
a. Glutaredoxin attacks first. Here the nucleophilic active-site cysteine of the glutaredoxin attacks one of the sulfur atoms of the protein disulfide, leading to the formation of an intermolecular disulfide bond between the protein and the glutaredoxin. GSH would then act to resolve this intermolecular disulfide, thereby directly or indirectly generating a glutathionylated glutaredoxin. This can be reduced by a second molecule of GSH yielding GSSG. **b.** GSH attacks first. In this model, GSH performs an uncatalyzed nucleophilic attack on the protein disulfide resulting in a glutathionylated protein. The active-site, nucleophilic cysteine of a monothiol glutaredoxin can then attack the sulfur atom of the glutathione moiety resulting in the glutathionylation of the glutaredoxin. Finally, another GSH would be required to regenerate reduced glutaredoxin, thereby producing GSSG. **c.** Glutaredoxin (or another protein) activates GSH to attack. Here GSH is activated by glutaredoxin leading to deprotonation of the GSH moiety, possibly by acid-base catalysis. The GS^- anion then serves as an efficient nucleophile to attack one of the sulfur atoms of the target protein disulfide bond. This would lead to the glutathionylation of the protein. The deglutathionylation can then proceed by a classic monothiol glutaredoxin mechanism as described for **a.** and **b.**

4. Materials and methods

4.1. Growth of yeast strains

All yeast strains were generated in a BY4742 (his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) background unless otherwise indicated (Supplementary Table 1.). For all experiments cells were grown in Hartwell (HC) media supplemented with 2% glucose as carbon source. Amino acids were dropped out for plasmid selection and supplements were added as indicated.

4.2. Construction of yeast strains

A standard homologous recombination protocol was used to create gene deletions [65]. Antibiotic resistant cassettes were amplified by PCR using primers designed to have 50–60 base-pairs of homology to the genomic regions immediately up- and downstream of the gene to be deleted. *GRX1* and *GRX2* were deleted in a BY4742 Δ*trx1*Δ*trx2* background [53]. For *GRX1* deletion, the primers S1-GRX1-FWD 5′-AATTA TACAAATAGACAAAACCTCAGAAGGAAAAAAAATGCGTACGCTGCAGG TCGAC-3′ and S2-GRX1-REV 5′-TATAAACCTGTGTGCATGGAAAAAACT TTGTCTGCCCTTAATCGATGAATTCGAGCTCG-3′ were used to amplify a *natNT2* cassette from a pFA6-*natNT2* plasmid. For *GRX2* deletion, the primers S1-GRX2-FWD 5′-ATTAACGGACACTCCAACTACTGTTATATAT TGTTTCATGCGTACGCTGCAGGTCGAC-3′ and S2-GRX2-REV 5′-TGTAA ATATTATGAAGGGGATATTAGCGTAATTTAAACTAATCGATGAATTCGA GCTCG-3′ were used to amplify an *hphNT1* cassette from a pFA6-*hphNT1* plasmid. The PCR products of these reactions were transformed into yeast cells using a standard lithium acetate/polyethyleneglycol-based protocol. Briefly, yeast cells were harvested and resuspended in One-Step-Transformation buffer containing 40% polyethylene glycol, 0.2 M lithium acetate and 0.1 M Dithiothreitol (DTT) followed by addition of 10 μl of salmon testes single-strand DNA and PCR-product/plasmid DNA. Cells were then incubated at 45 °C for 30 min with continuous shaking and subsequently transferred to appropriate YPD plates for selection. Gene deletions were confirmed by PCR on genomic DNA using primers designed to bind to chromosomal regions approximately 400 base pairs up- and downstream of the gene of interest and by plating on HC agar plates supplemented with the appropriate antibiotics.

4.3. Cloning and plasmid construction

Genes encoded on p415TEF, p416TEF or pRS315 plasmids as used in this study were PCR-amplified from either pre-existing plasmids or genomic DNA. Genomic DNA was always extracted from BY4742WT cells. Briefly, cells were vortexed in 30 μl 0.2% SDS and boiled at 96 °C for 10 min. Subsequently, cells were vortexed again followed by centrifugation at 18000*g* for 1 min. One μl of supernatant was used as DNA-template in the PCR-reaction-mix.

GRX1 was amplified from genomic DNA using the forward primer 5′-CATGGGATCCACCATGGTATCTCAAGAAACTATCAAGCAC-3′ and reverse primer 5′-CTAGCTCGAGTTAATTTGCAAGAATAGGTTCTAACAA TTC-3′ and subsequently cloned into an empty p415TEF plasmid using BamHI and XhoI restriction sites. *GRX2* was amplified by PCR from p416TEF *roGFP2-GRX2* [49] using the forward primer 5′-CATGTCTAGA ACCATGGTATCCCAGGAAACAGTTGCTCAC-3′ and reverse primer 5′-CTAGCTCGAGGTCGACGGTATCGATAAGCTTCTATTG-3'. Please note that *GRX2* harbors two in-frame start codons for either mitochondrial translocation or cytosolic expression. *GRX2* was amplified without the mitochondrial targeting sequence and cloned into an empty p415TEF plasmid using XbaI and XhoI restriction sites. *HsGRX5* and *HsGRX5* Active (Loop + G68P + R97Q) gene sequences with codons optimized for *Saccharomyces cerevisiae* expression were amplified from either p416TEF *roGFP2-HsGRX5* or p416TEF *roGFP2-HsGRX5* Active (Loop + G68P + R97Q) plasmids [45] using the forward primer 5′-CGTAGTGGGATCCAC CATGGCTGGTTCTGGTGC-3′ and the reverse primer 5′-CAGTGCTCGAGT TATTTTGAATCTTGATCTTTCTTTTCATC-3′ and subcloned into an empty p415TEF plasmid using BamHI and XhoI restriction sites. Please note that all *HsGRX5* constructs lack the mitochondrial pre-sequence and start with a methionine followed by residue Ala32. *GRX7* and *GRX7*C108S sequences were PCR-amplified from either p416TEF *roGFP2-GRX7* or p416TEF *roGFP2-GRX7*C108S plasmids [45] using the forward primer 5′-CGTAGT GGGATCCACCATGGTCAACGAAAGTATTACTACTCACC-3′ and the reverse primer 5′-CAGTGCTCGAGTCAGGCACTCTCAGATTGCG-3′ and subsequently cloned into an empty p415TEF plasmid using BamHI and XhoI restriction sites. *GRX7* was cloned without the secretory pathway-targeting signal-sequence to ensure cytosolic localization. Gene sequences for *roGFP2-GRX2* and *roGFP2-GRX7* were subcloned from p416TEF *roGFP2-GRX2* [49] and p416TEF *roGFP2-GRX7* [45] plasmids into empty p415TEF plasmids using XbaI and XhoI restriction-sites. *TRX1* was amplified from genomic DNA using the forward primer 5′-CATGGGATCCACCATGGTTA CTCAATTCAAAACTG-3′ and reverse primer 5′-CTAGCTCGAGTTAAGCA TTAGCAGCAATGGCTTGC-3'. *TRX2* was amplified from genomic DNA using the forward primer 5′-CATGGGATCCACCATGGTCACTCAATTAAA ATCCG-3′ and reverse primer 5′-CTAGCTCGAGCTATACGTTGGAAGCAA TAGCTTGC-3'. Both genes were cloned into empty p415TEF plasmids using BamHI and XhoI restriction sites. *PDI1*, *MPD1* and *MPD2* were each amplified from genomic DNA. *PDI1* was amplified using the forward primer 5′-CTAGCGGATCCACCATGCAACAAGAGGCTGTGGCCCCTG-3′ and reverse primer 5′-GCATGACTCGAGTTAAATGGCATCTTCTTCGTCAG CCA-3'. *MPD1* was amplified using the forward primer 5′-CAGAATGGAT CCACCATGCAAAACTTTTACGATTCCGATCCTCATATATCAGAG-3′ and reverse primer 5′-CAGTTCCTCGAGTTACTTGTTTCCTGAGGAGGAATGGT TCTT-3'. *MPD2* was amplified using the forward primer 5′-CAGTCTGGA TCCACCATGTACAGTGAAGCTGTCACGATGGTCA-3′ and reverse primer 5′-CAGATCTCGAGTTAACTACTGGTATCTTCTAACTGGTCTTCTATGTG-3'. All sequences were amplified lacking both the N-terminal signal-sequence and the C-terminal HDEL-sequence to ensure cytosolic localization and were cloned into empty p415TEF plasmids using BamHI and XhoI restriction sites.

GRX2 and *GRX2C64S* were subcloned from p415TEF *GRX2* and p415TEF *GRX2 C64S* plasmids into an empty p415ADH vector using XbaI and XhoI restriction sites to generate the p415ADH *GRX2* and p415ADH *GRX2 C64S* plasmids. *GRX7* was subcloned from a p415TEF *GRX7* plasmid to an empty p415 ADH plasmid using BamHI and XhoI restriction sites to generate p415ADH *GRX7*.

For the construction of the pRS315 *GRX2* plasmid, the promotor region 839 bp up-stream of the *GRX2* start codon was PCR-amplified from genomic DNA using the forward primer 5′-CATGTCTAGAGTTGCACAA AGATATCGATAACCCGTTGC-3′ and the reverse primer 5′-CTAGGGATC CGAAACAATATATAACAGTAGTTGGAGTG-3′ and subsequently cloned into an empty pRS315 vector using XbaI and BamHI restriction sites. In a second cloning step *GRX2* with its endogenous terminator was amplified from genomic DNA from the second ATG start codon, i.e. lacking the mitochondrial targeting sequence using the forward primer 5′-CATGGG ATCCACCATGGTATCCCAGGAAACAGTTGCTCAC-3′ and the reverse primer 5′-CTAGAAGCTTCCTCAGACGGAATTTAGCGGGTCTCATTGG-3′ binding 171 bp down-stream of the *GRX2* stop codon. Subsequently, the PCR product was ligated into the previously created pRS315 *GRX2*Promotor vector using BamHI and HindIII restriction sites to generate pRS315 *GRX2* with endogenous promoter and terminator. Site-directed mutagenesis was subsequently performed on the pRS315 *GRX2* plasmid using the GRX2C64S forward primer 5′-CATACTGCCCTTACAG TAAAGCTACTTTG-3'and the GRX2C64S reverse primer 5′-CAAAGTAG CTTTACTGTAAGGGCAGTATG-3′ to generate pRS315 *GRX2 C64S*. All constructs were confirmed by sequencing (Eurofins Genomics).

4.4. Site-directed mutagenesis

The p415TEF *GRX2* plasmid was used as a template for mutation of Cys61 to serine using the *GRX2*C61S forward primer 5′-GCAAAGACA TACTCGCCTTACTGTAAAG-3′ and the *GRX2*C61S reverse primer 5′-CTTTACAGTAAGGCGAGTATGTCTTTGC-3'. Cys64 was mutated to

serine using p415TEF *GRX2* as template together with the *GRX2*C64S forward primer 5′-CATACTGCCCTTACAGTAAAGCTACTTTG-3′ and the *GRX2*C64S reverse primer 5′-CAAAGTAGCTTTACTGTAAGGGCAGT ATG-3'. Subsequently, the newly generated p415TEF *GRX2*C64S was used as template to generate the double cysteine mutant p415TEF *GRX2*C61S, C64S using the forward primer 5′-GCAAAGACATACTCTC CTTACAGTAAAGC-3′ and the reverse primer 5′-GCTTTACTGTAAGGA GAGTATGTCTTTGC-3'. Mutation of either *TRX2* Cys34 alone or both Cys31 and Cys34 to serine was performed using p415TEF *TRX2* as a template in combination with the primers *TRX2*C34S forward 5′-GGT GTGGGCCATCTAAAATGATTGC-3′ and *TRX2*C34S reverse 5′-GCAATC ATTTTAGATGGCCCACACC-3′ or *TRX2*C31S, C34S forward 5′-GCCAC ATGGTCTGGGCCATCTAAAATGATTGC-3′ and *TRX2*C31S, C34S reverse 5′-GCAATCATTTTAGATGGCCCAGACCATGTGGC-3'. All site-directed mutagenesis was performed using a standard PCR-based protocol with an S7 Fusion Polymerase (Biozym). Methylated template DNA was digested by *DpnI* (NEB), followed by transformation into chemically competent *E. coli* Top 10 cells and subsequent plasmid extraction. Mutations were confirmed by sequencing (Eurofins Genomics).

4.5. 5-FOA-based 'plasmid-shuffling' assays

In Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* yeast cells, cell viability was initially maintained by the presence of a p416TEF *roGFP2-GRX2* plasmid. This strain was subsequently transformed with p415TEF plasmids for the expression of different enzymes including glutaredoxins, thioredoxins, protein disulfide isomerases and mutants thereof. Briefly, cells were harvested and resuspended in One-Step-Transformation buffer as described previously. Following the addition of 5 µl salmon testes single-strand-DNA and ~400 ng plasmid DNA, cells were incubated shaking at 45 °C for 30 min and subsequently plated onto appropriate HC plates for plasmid selection. Following transformation of the p415TEF plasmid cells were rigorously selected by two rounds of growth on HC plates without leucine and uracil. Subsequently, cells were streaked onto HC plates supplemented with 0.1% 5-fluoroorotic acid (5-FOA, Zymo Research) and incubated for 48 h at 30 °C. 5-FOA is only converted into toxic 5-flurouracil in cells harboring a functional *URA3*. Therefore, 5-FOA is used as counter-selection for cells expressing a p416TEF plasmid. In other words, growth of Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* cells on 5-FOA containing plates can only occur if the protein encoded on the p415TEF plasmid is capable of supporting cell viability. For further experiments strains growing on 5-FOA plates were re-streaked again and subsequently inoculated in HC medium lacking uracil to ensure all cells lost the p416TEF plasmid. In that way Δ*trx1*Δ*trx2*Δ*grx1*Δ*grx2* + p415TEF *GRX1/GRX2/GRX2C64S/GRX7/roGFP2-GRX2/roGFP2-GRX7* strains were generated.

4.6. Intracellular measurements of roGFP2 oxidation

Redox-sensitive GFP2 (roGFP2) contains two cysteine residues on opposing β-strands directly adjacent to the GFP chromophore. These two cysteines residues can form a disulfide bond, which leads to a change in chromophore protonation (predominantly anionic in the reduced roGFP and predominantly neutral in the oxidized roGFP). The anionic chromophore has a fluorescence excitation maximum around 490 nm whilst for the neutral chromophore fluorescence excitation has a maximum around 400 nm. For both chromophores states fluorescence emission is around 510 nm. Thus, the fluorescence excitation ratio directly correlates to the degree of roGFP2 oxidation, which can be determined according to equation (1):

$$OxD_{roGFP2} = \frac{(I400sample*I480red) - (I400red*I480sample)}{(I400sample*I480red - I400sample*I480ox) + (I400ox*I480sample - I400red*I480sample)} \quad (1)$$

All yeast strains in this study express either roGFP2 or genetic fusions between roGFP2 and glutaredoxins from either p415TEF or p416TEF plasmids. RoGFP2 was synthesized with codons optimized for expression in yeast as described previously [49,53], as was *Hs*Grx5. For roGFP2 assays, cells were grown in HC medium lacking uracil and leucine for plasmid selection at 30 °C to an $OD_{600} \approx 3.5$. Subsequently, 1.5 OD_{600} units of cells were harvested and resuspended in 200 µl of 100 mM MES/Tris pH 6 and transferred to the well of a flat-bottomed 96-well plate. The 96-well plates were centrifuged at 15 g for 5 min to form a loose cell pellet at the bottom of each well.

For each strain samples treated with either 20 mM diamide or 100 mM DTT served as fully oxidized and fully reduced roGFP2 controls, respectively. These controls enable the determination of the degree of oxidation (OxD), according to Equation (1). RoGFP2 fluorescence was monitored using a BMG Labtech CLARIOstar fluorescence plate-reader. For dynamics measurements, roGFP2 fluorescence was monitored for ~15 min following the addition of exogenous H_2O_2 at the concentrations indicated [51].

4.7. Whole cell lysate GSH and GSSG measurements

The concentration of GSH and GSSG in yeast cells lysates was performed using a modified version of a 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) assay as described previously [66]. Yeast cultures were grown in HC medium lacking uracil and leucine for plasmid selection until they reached an $OD_{600} \approx 3.5$. At this stage, fifty OD_{600} units of cells (where 1 OD_{600} unit equals 1 ml of culture with an $OD_{600} = 1$) were harvested by centrifugation at 1000*g* for 3 min at 25 °C. Cells were washed once with 10 ml of Milli-Q H_2O, re-harvested by centrifugation and finally resuspended in 250 µl SSA buffer (1.3% sulfosalicylic acid, 8 mM HCl). Cells were lysed by addition of 0.5 mm glass-beads and subsequent shaking using a Disruptor Genie cell homogenizer (Carl Roth GmbH and Co, Karlsruhe, Germany) at 4 °C. A further 100 µl of SSA buffer was added and samples disrupted for a further 5 min. The resultant cell lysate was incubated on ice for 15 min to precipitate proteins followed by centrifugation at 16000*g* for 15 min at 4 °C.

To determine the 'total' glutathione concentration in the lysate (defined as GSH + GSSG, where one molecule of GSSG represents two GSH equivalents), 5 µl of supernatant was mixed with 995 µl ice-cold KPE buffer (100 mM potassium phosphate, 5 mM ethylenediaminetetraacetic acid [EDTA] pH 7.5). To determine GSSG levels, 100 µl supernatant was treated with 2 µl 20% (v/v) 2-vinylpyridine in ethanol and 40 µl 1 M MES/Tris pH 7.0 to raise the pH and alkylate GSH. Samples were incubated for 1 h at 25 °C. Both 'total' glutathione samples and GSSG samples were subsequently measured using a DTNB-recycling assay. For 'total' glutathione samples, 20 µl of the cell lysate was taken for the DTNB assay. For GSSG samples, 2 µl of sample was taken and adjusted to 20 µl with ice-cold KPE buffer. Next, for all samples were transferred to wells of a 96-well plate. To the samples, 120 µl KPE buffer containing 2 mg/ml NADPH and 2 mg/ml DTNB was added. Reactions were started by the addition of 0.16 U glutathione reductase. Absorbance change at 412 nm was measured using a BMG Labtech CLARIOstar plate-reader. 'Total' glutathione and GSSG concentrations were determined according to GSH and GSSG concentration standard curves.

4.8. Growth curves

Precultures for plasmid-shuffling experiments were grown in HC lacking leucine and uracil for plasmid selection, while precultures for all other experiments were grown in HC medium. Precultures were diluted to $OD_{600} = 0.5$ in fresh media and grown for a further 4 h at 30 °C. Subsequently, 1 OD_{600} unit of cells were harvested, washed and resuspended in 1 ml of distilled water. Aliquots of 10 µl of the cell suspension were added to 90 µl of the appropriate HC-medium in a 96-well plate. Cells were incubated with continuous shaking at 30 °C using a BioTek-Microplate Reader. The OD_{600} was automatically recorded

every 10 min for at least 40 h. All growth curves were repeated at least 3 times.

Author contributions

B.M. and M.D. conceived the study, helped design and supervise the experiments, analyzed and interpreted experimental data and wrote the manuscript. J.M.H supervised experiments and helped to write the paper. J.Z. helped design and perform experiments, analyzed data and helped to write the manuscript. J.O. performed the measurements of glutathione content in cell lysates. S.H. performed all growth curve experiments.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

B.M., M.D. and J.M.H. acknowledge generous support from the DFG Priority Program SPP1710 (MO 2774/2-1 and DE 1431/8-2 and HE2803/8-2). We thank Jun.-Prof. Dr. Leticia Prates Roma for helpful comments on this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101598.

References

[1] L. Liedgens, M. Deponte, The catalytic mechanism of glutaredoxins, in: Leopold Flohé (Ed.), Glutathione, CRC Press, 2019, pp. 251–261 ISBN 9780815365327.
[2] J. Couturier, J.P. Jacquot, N. Rouhier, Evolution and diversity of glutaredoxins in photosynthetic organisms, Cell. Mol. Life Sci. 66 (2009) 2539–2557.
[3] C.H. Lillig, C. Berndt, A. Holmgren, Glutaredoxin systems, Biochim. Biophys. Acta 1780 (2008) 1304–1317.
[4] J.J. Mieyal, M.M. Gallogly, S. Qanungo, E.A. Sabens, M.D. Shelton, Molecular mechanisms and clinical implications of reversible protein S-glutathionylation, Antioxidants Redox Signal. 10 (2008) 1941–1988.
[5] M. Deponte, Glutathione catalysis and the reaction mechanisms of glutathione-dependent enzymes, Biochim. Biophys. Acta 1830 (2013) 3217–3266.
[6] E. Herrero, M.A. de la Torre-Ruiz, Monothiol glutaredoxins: a common domain for multiple functions, Cell. Mol. Life Sci. 64 (2007) 1518–1530.
[7] J. Couturier, J. Przybyla-Toscano, T. Roret, C. Didierjean, N. Rouhier, The roles of glutaredoxins ligating Fe-S clusters: sensing, transfer or repair functions? Biochim. Biophys. Acta 1853 (2015) 1513–1527.
[8] C.E. Outten, A.N. Albetel, Iron sensing and regulation in Saccharomyces cerevisiae: ironing out the mechanistic details, Curr. Opin. Microbiol. 16 (2013) 662–668.
[9] A. Holmgren, Glutathione-dependent synthesis of deoxyribonucleotides. Purification and characterization of glutaredoxin from Escherichia coli, J. Biol. Chem. 254 (1979) 3664–3671.
[10] A. Holmgren, F. Aslund, Glutaredoxin, Methods Enzymol. 252 (1995) 283–292.
[11] A.V. Peskin, P.E. Pace, J.B. Behring, L.N. Paton, M. Soethoudt, M.M. Bachschmid, C.C. Winterbourn, Glutathionylation of the active site cysteines of peroxiredoxin 2 and recycling by glutaredoxin, J. Biol. Chem. 291 (2016) 3053–3062.
[12] C.F. Djuika, S. Fiedler, M. Schnolzer, C. Sanchez, M. Lanzer, M. Deponte, Plasmodium falciparum antioxidant protein as a model enzyme for a special class of glutaredoxin/glutathione-dependent peroxiredoxins, Biochim. Biophys. Acta 1830 (2013) 4073–4090.
[13] E.M. Hanschmann, M.E. Lonn, L.D. Schutte, M. Funke, J.R. Godoy, S. Eitner, C. Hudemann, C.H. Lillig, Both thioredoxin 2 and glutaredoxin 2 contribute to the reduction of the mitochondrial 2-Cys peroxiredoxin Prx3, J. Biol. Chem. 285 (2010) 40699–40705.
[14] F. Pauwels, B. Vergauwen, F. Vanrobaeys, B. Devreese, J.J. Van Beeumen, Purification and characterization of a chimeric enzyme from Haemophilus influenzae Rd that exhibits glutathione-dependent peroxidase activity, J. Biol. Chem. 278 (2003) 16658–16666.
[15] N. Rouhier, E. Gelhaye, P.E. Sautiere, A. Brun, P. Laurent, D. Tagu, J. Gerard, E. de Fay, Y. Meyer, J.P. Jacquot, Isolation and characterization of a new peroxiredoxin from poplar sieve tubes that uses either glutaredoxin or thioredoxin as a proton donor, Plant Physiol. 127 (2001) 1299–1309.
[16] H.Y. Kim, Glutaredoxin serves as a reductant for methionine sulfoxide reductases with or without resolving cysteine, Acta Biochim. Biophys. Sin. 44 (2012) 623–627.
[17] L. Tarrago, E. Laugier, M. Zaffagnini, C. Marchand, P. Le Marechal, N. Rouhier, S.D. Lemaire, P. Rey, Regeneration mechanisms of Arabidopsis thaliana methionine sulfoxide reductases B by glutaredoxins and thioredoxins, J. Biol. Chem. 284 (2009) 18963–18971.
[18] L. Lopez-Maury, A.M. Sanchez-Riego, J.C. Reyes, F.J. Florencio, The glutathione/glutaredoxin system is essential for arsenate reduction in Synechocystis sp. strain PCC 6803, J. Bacteriol. 191 (2009) 3534–3543.
[19] S. Boukhenouna, H. Mazon, G. Branlant, C. Jacob, M.B. Toledano, S. Rahuel-Clermont, Evidence that glutathione and the glutathione system efficiently recycle 1-cys sulfiredoxin in vivo, Antioxidants Redox Signal. 22 (2015) 731–743.
[20] C. Berndt, J.D. Schwenn, C.H. Lillig, The specificity of thioredoxins and glutaredoxins is determined by electrostatic and geometric complementarity, Chem. Sci. 6 (2015) 7049–7058.
[21] C.H. Lillig, A. Prior, J.D. Schwenn, F. Aslund, D. Ritz, A. Vlamis-Gardikas, A. Holmgren, New thioredoxins and glutaredoxins as electron donors of 3'-phosphoadenylylsulfate reductase, J. Biol. Chem. 274 (1999) 7695–7698.
[22] M. Zheng, F. Aslund, G. Storz, Activation of the OxyR transcription factor by reversible disulfide bond formation, Science 279 (1998) 1718–1721.
[23] M. Gellert, S. Venz, J. Mitlohner, C. Cott, E.M. Hanschmann, C.H. Lillig, Identification of a dithiol-disulfide switch in collapsin response mediator protein 2 (CRMP2) that is toggled in a model of neuronal differentiation, J. Biol. Chem. 288 (2013) 35117–35125.
[24] L. Brautigam, L.D. Schutte, J.R. Godoy, T. Prozorovski, M. Gellert, G. Hauptmann, A. Holmgren, C.H. Lillig, C. Berndt, Vertebrate-specific glutaredoxin is essential for brain development, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 20532–20537.
[25] E.G. Muller, Thioredoxin deficiency in yeast prolongs S phase and shortens the G1 interval of the cell cycle, J. Biol. Chem. 266 (1991) 9194–9202.
[26] N. Mesecke, A. Spang, M. Deponte, J.M. Herrmann, A novel group of glutaredoxins in the cis-Golgi critical for oxidative stress resistance, Mol. Biol. Cell 19 (2008) 2673–2680.
[27] N. Mesecke, S. Mittler, E. Eckers, J.M. Herrmann, M. Deponte, Two novel monothiol glutaredoxins from Saccharomyces cerevisiae provide further insight into iron-sulfur cluster binding, oligomerization, and enzymatic activity of glutaredoxins, Biochemistry 47 (2008) 1452–1463.
[28] A. Izquierdo, C. Casas, U. Muhlenhoff, C.H. Lillig, E. Herrero, Saccharomyces cerevisiae Grx6 and Grx7 are monothiol glutaredoxins associated with the early secretory pathway, Eukaryot. Cell 7 (2008) 1415–1426.
[29] J. Couturier, C.S. Koh, M. Zaffagnini, A.M. Winger, J.M. Gualberto, C. Corbier, P. Decottignies, J.P. Jacquot, S.D. Lemaire, C. Didierjean, et al., Structure-function relationship of the chloroplastic glutaredoxin S12 with an atypical WCSYS active site, J. Biol. Chem. 284 (2009) 9299–9310.
[30] M. Zaffagnini, M. Bedhomme, C.H. Marchand, J.R. Couturier, X.H. Gao, N. Rouhier, P. Trost, S.P. Lemaire, Glutaredoxin s12: unique properties for redox signaling, Antioxidants Redox Signal. 16 (2012) 17–32.
[31] O. Bjornberg, H. Ostergaard, J.R. Winther, Mechanistic insight provided by glutaredoxin within a fusion to redox-sensitive yellow fluorescent protein, Biochemistry 45 (2006) 2362–2371.
[32] F. Zahedi Avval, A. Holmgren, Molecular mechanisms of thioredoxin and glutaredoxin as hydrogen donors for Mammalian s phase ribonucleotide reductase, J. Biol. Chem. 284 (2009) 8233–8240.
[33] M.M. Gallogly, D.W. Starke, A.K. Leonberg, S.M. Ospina, J.J. Mieyal, Kinetic and mechanistic characterization and versatile catalytic properties of mammalian glutaredoxin 2: implications for intracellular roles, Biochemistry 47 (2008) 11144–11157.
[34] Y. Yang, S. Jao, S. Nanduri, D.W. Starke, J.J. Mieyal, J. Qin, Reactivity of the human thioltransferase (glutaredoxin) C7S, C25S, C78S, C82S mutant and NMR solution structure of its glutathionyl mixed disulfide intermediate reflect catalytic specificity, Biochemistry 37 (1998) 17145–17156.
[35] Y.F. Yang, W.W. Wells, Identification and characterization of the functional amino acids at the active center of pig liver thioltransferase by site-directed mutagenesis, J. Biol. Chem. 266 (1991) 12759–12765.
[36] S.A. Gravina, J.J. Mieyal, Thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase, Biochemistry 32 (1993) 3368–3376.
[37] A.A. Ukuwela, A.I. Bush, A.G. Wedd, Z. Xiao, Glutaredoxins employ parallel monothiol-dithiol mechanisms to catalyze thiol-disulfide exchanges with protein disulfides, Chem. Sci. 9 (2018) 1173–1183.
[38] J.H. Bushweller, F. Aslund, K. Wuthrich, A. Holmgren, Structural and functional characterization of the mutant Escherichia coli glutaredoxin (C14——S) and its mixed disulfide with glutathione, Biochemistry 31 (1992) 9288–9293.
[39] F. Gama, C. Brehelin, E. Gelhaye, Y. Meyer, J.P. Jacquot, P. Rey, N. Rouhier, Functional analysis and expression characteristics of chloroplastic Prx IIE, Physiol. Plantarum 133 (2008) 599–610.
[40] T. Draculic, I.W. Dawes, C.M. Grant, A single glutaredoxin or thioredoxin gene is essential for viability in the yeast Saccharomyces cerevisiae, Mol. Microbiol. 36 (2000) 1167–1174.
[41] E. Eckers, M. Bien, V. Stroobant, J.M. Herrmann, M. Deponte, Biochemical characterization of dithiol glutaredoxin 8 from Saccharomyces cerevisiae: the catalytic redox mechanism redux, Biochemistry 48 (2009) 1410–1423.
[42] R.S. Sikorski, J.D. Boeke, In vitro mutagenesis and plasmid shuffling: from cloned gene to mutant yeast, Methods Enzymol. 194 (1991) 302–318.
[43] J.D. Boeke, F. LaCroute, G.R. Fink, A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance, Mol. Gen. Genet. 197 (1984) 345–346.
[44] D. Mumberg, R. Muller, M. Funk, Yeast vectors for the controlled expression of

heterologous proteins in different genetic backgrounds, Gene 156 (1995) 119–122.
[45] L. Liedgens, J. Zimmermann, L. Wäschenbach, F. Geissel, H. Laporte, H. Gohlke, B. Morgan, M. Deponte, Quantitative assessment of the determinant structural differences between redox-active and inactive glutaredoxins, Nat. Commun. 11 (2020) 1725.
[46] N.J. Bulleid, L. Ellgaard, Multiple ways to make disulfides, Trends Biochem. Sci. 36 (2011) 485–492.
[47] F. Vignols, C. Brehelin, Y. Surdin-Kerjan, D. Thomas, Y. Meyer, A yeast two-hybrid knockout strain to explore thioredoxin-interacting proteins in vivo, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 16729–16734.
[48] N. Mouaheb, D. Thomas, L. Verdoucq, P. Monfort, Y. Meyer, In vivo functional discrimination between plant thioredoxins by heterologous expression in the yeast Saccharomyces cerevisiae, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 3312–3317.
[49] B. Morgan, D. Ezerina, T.N. Amoako, J. Riemer, M. Seedorf, T.P. Dick, Multiple glutathione disulfide removal pathways mediate cytosolic redox homeostasis, Nat. Chem. Biol. 9 (2013) 119–125.
[50] A.J. Meyer, T.P. Dick, Fluorescent protein-based redox probes, Antioxidants Redox Signal. 13 (2010) 621–650.
[51] B. Morgan, M.C. Sobotta, T.P. Dick, Measuring E(GSH) and H2O2 with roGFP2-based redox probes, Free Radic. Biol. Med. 51 (2011) 1943–1951.
[52] M. Schwarzlander, T.P. Dick, A.J. Meyer, B. Morgan, Dissecting redox biology using fluorescent protein sensors, Antioxidants Redox Signal. 24 (2016) 680–712.
[53] B. Morgan, K. Van Laer, T.N. Owusu, D. Ezerina, D. Pastor-Flores, P.S. Amponsah, A. Tursch, T.P. Dick, Real-time monitoring of basal H2O2 levels with peroxiredoxin-based probes, Nat. Chem. Biol. 12 (2016) 437–443.
[54] E.G. Muller, A glutathione reductase mutant of yeast accumulates high levels of oxidized glutathione and requires thioredoxin for growth, Mol. Biol. Cell 7 (1996) 1805–1813.
[55] A. Vlamis-Gardikas, A. Holmgren, Thioredoxin and glutaredoxin isoforms, Methods Enzymol. 347 (2002) 286–296.
[56] C.H. Lillig, C. Berndt, Glutaredoxins in thiol/disulfide exchange, Antioxidants Redox Signal. 18 (2013) 1654–1665.
[57] P. Begas, L. Liedgens, A. Moseler, A.J. Meyer, M. Deponte, Glutaredoxin catalysis requires two distinct glutathione interaction sites, Nat. Commun. 8 (2017) 14835.
[58] P. Begas, V. Staudacher, M. Deponte, Systematic re-evaluation of the bis(2-hydroxyethyl)disulfide (HEDS) assay reveals an alternative mechanism and activity of glutaredoxins, Chem. Sci. 6 (2015) 3788–3796.
[59] J.R. Pedrajas, P. Porras, E. Martinez-Galisteo, C.A. Padilla, A. Miranda-Vizuete, J.A. Barcena, Two isoforms of Saccharomyces cerevisiae glutaredoxin 2 are expressed in vivo and localize to different subcellular compartments, Biochem. J. 364 (2002) 617–623.
[60] K. Kojer, V. Peleh, G. Calabrese, J.M. Herrmann, J. Riemer, Kinetic control by limiting glutaredoxin amounts enables thiol oxidation in the reducing mitochondrial intermembrane space, Mol. Biol. Cell 26 (2015) 195–204.
[61] M. Deponte, The incomplete glutathione puzzle: just guessing at numbers and figures? Antioxidants Redox Signal. 27 (2017) 1130–1161.
[62] L.D. Schutte, S. Baumeister, B. Weis, C. Hudemann, E.M. Hanschmann, C.H. Lillig, Identification of potential protein dithiol-disulfide substrates of mammalian Grx2, Biochim. Biophys. Acta 1830 (2013) 4999–5005.
[63] N. Rouhier, A. Villarejo, M. Srivastava, E. Gelhaye, O. Keech, M. Droux, I. Finkemeier, G. Samuelsson, K.J. Dietz, J.P. Jacquot, et al., Identification of plant glutaredoxin targets, Antioxidants Redox Signal. 7 (2005) 919–929.
[64] N. Sturm, E. Jortzik, B.M. Mailu, S. Koncarevic, M. Deponte, K. Forchhammer, S. Rahlfs, K. Becker, Identification of proteins targeted by the thioredoxin superfamily in Plasmodium falciparum, PLoS Pathog. 5 (2009) e1000383.
[65] C. Janke, M.M. Magiera, N. Rathfelder, C. Taxis, S. Reber, H. Maekawa, A. Moreno-Borchart, G. Doenges, E. Schwob, E. Schiebel, et al., A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes, Yeast 21 (2004) 947–962.
[66] I. Rahman, A. Kode, S.K. Biswas, Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method, Nat. Protoc. 1 (2006) 3159–3165.