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Research Article

# Modulation of the specific glutathionylation of mitochondrial proteins in the yeast *Saccharomyces cerevisiae* under basal and stress conditions

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The potential biological consequences of oxidative stress and changes in glutathione levels include the oxidation of susceptible protein thiols and reversible covalent binding of glutathione to the –SH groups of proteins by S-glutathionylation. Mitochondria are central to the response to oxidative stress and redox signaling. It is therefore crucial to explore the adaptive response to changes in thiol-dependent redox status in these organelles. We optimized the purification protocol of glutathionylated proteins in the yeast *Saccharomyces cerevisiae* and present a detailed proteomic analysis of the targets of protein glutathionylation in cells undergoing constitutive metabolism and after exposure to various stress conditions. This work establishes the physiological importance of the glutathionylation process in *S. cerevisiae* under basal conditions and provides evidence for an atypical and unexpected cellular distribution of the process between the cytosol and mitochondria. In addition, our data indicate that each oxidative condition (diamide, GSSG, H<sub>2</sub>O<sub>2</sub>, or the presence of iron) elicits an adaptive metabolic response affecting specific mitochondrial metabolic pathways, mainly involved in the energetic maintenance of the cells. The correlation of protein modifications with intracellular glutathione levels suggests that protein deglutathionylation may play a role in protecting mitochondria from oxidative stress. This work provides further insights into the diversity of proteins undergoing glutathionylation and the role of this post-translational modification as a regulatory process in the adaptive response of the cell.

## Introduction

The reversible redox modification of protein thiols is an important response to changes in the cellular redox environment. Maintaining the cellular thiol redox state between oxidative and reducing conditions is essential for the normal function and survival of cells. The tripeptide glutathione plays a key role as the principal cellular antioxidant and protects protein thiol groups from oxidation. Indeed, cysteine residues can exist in different states of oxidation such as protein disulfide bridges, S-thiolation, or nitrosylation that are reversible, and oxidation into sulfinic and sulfonic acids that are irreversible. Glutathione, which is present in a reduced (GSH) and oxidized (GSSG) form, can also form a mixed-disulfide bridge with an accessible-free thiol group on a protein, a process called S-glutathionylation [1–4]. This may occur in response to an increase in reactive oxygen species (ROS) or, alternatively, oxidized GSSG may react with protein –SH groups, the reverse reaction being mainly catalyzed by glutaredoxins [5,6]. Glutathionylation can occur both under normal physiological conditions and after exposure to oxidative stress, and protects –SH groups against irreversible oxidation, but

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can also result in protein-specific functional changes that play an important role in the response of cells to oxidative damage and in redox signaling [1,2,7–11]. Interest in protein glutathionylation has recently emerged in the literature as a signaling and regulatory mechanism in all organisms, with a possible implication in human diseases [2,3,9,12–14].

Several methods have been developed to detect glutathionylated proteins and hundreds of targets of glutathionylation involved in various cell processes have been identified in animals and photosynthetic organisms [1,9,10,14–19]. Protein glutathionylation was initially studied using <sup>35</sup>S-cysteine labeling of the intracellular GSH pool in the presence of protein synthesis inhibitors [20–23]. This technique was further adapted for large-scale identification using 2D gels and peptide mass fingerprinting after induction of oxidative stress in response to diamide and hydrogen peroxide [24–26]. This method works well to identify abundant proteins, but <sup>35</sup>S-labeling is not sufficiently sensitive to identify glutathionylated proteins that are not present in high-copy numbers and does not categorically reveal the nature of the oxidative modification. Moreover, treatment with protein synthesis inhibitors surely perturbs cell physiology and does not allow studies under physiological conditions.

The introduction of biotinylated glutathione ethyl-ester to cells with subsequent affinity purification of biotin-glutathionylated proteins and specific detection by western blotting has also led to the discovery of protein targets after the induction of glutathionylation with oxidants [27–33]. The major drawback of this method is that the presence of the bulky biotin tag on the glutathione molecule might perturb the function of proteins interacting with glutathione, and especially those controlling glutathionylation. For example, we have observed that some proteins glutathionylated with biotinylated glutathione could not be deglutathionylated by Grxs (Lemaire, unpublished results). Altogether, neither <sup>35</sup>S-labeling nor biotinylated glutathione has the capacity to provide an image of cell glutathionylation without interfering with the physiological processes of the cell.

Another method used to detect glutathionylated proteins is the use of an anti-glutathione antibody (usually mouse monoclonal). It primarily allows the detection of abundant proteins, because the antibody exhibits low sensitivity, and requires the use of fractions enriched for the proteins of interest. In addition, specificity is low because glutathione is a flexible molecule. However, several glutathionylated proteins have been identified using this antibody, including by our group [34–36].

Biotin-based strategies of affinity purification are more effective than other strategies because they allow the analysis of a mixture of glutathionylated proteins rather than total extracts, permitting the detection of significantly less abundant proteins. A method that can be used for isolating glutathionylated proteins from cells without interfering with cell functions has been developed [37,38]. This procedure utilizes a mutant glutaredoxin to specifically deglutathionylate cellular proteins after blocking thiols with the membrane permeable highly thiol-reactive *N*-ethylmaleimide (NEM), followed by NEM-biotin treatment to tag the free sulfhydryl groups. This allows affinity purification and characterization via an avidin–Sepharose column and identification by mass spectrometry analysis. Over 40 novel glutathionylated proteins were discovered, both in stressed cells and in those undergoing constitutive metabolism, using this protocol [37,38]. This ‘biotin–Grx’-switch procedure was later adapted and improved in several studies to characterize target proteins, including by our group [34,37,39,40] (for a review, see ref. [15]). The limit of the procedure may be the specificity of the Grx used to deglutathionylate the proteins, but the major advantage is that it allows the detection of proteins under more physiological conditions because no pretreatment is required. In addition, it can be applied to large-scale studies. However, as the modification is labile and does not necessarily affect the most abundant proteins, its detection in complex mixtures *in vitro*, in the absence of stress induction, remains challenging.

Surprisingly, there are few studies on glutathionylated proteins in the yeast *Saccharomyces cerevisiae* and none under physiological conditions. S-glutathionylation and inhibition of GAPDH, enolase, and alcohol dehydrogenase have been shown after the treatment of cells with hydrogen peroxide [41]. Moreover, *S. cerevisiae* proteasome was found to be sensitive to redox modifications and glutathionylation [42,43]. A more general redox proteomic study has been previously published in ref. [44]. In 2012, our group showed evidence for the glutathionylation of key mitochondrial proteins in wild-type (WT) cells of *S. cerevisiae* and a yeast model of the neurodegenerative disease Friedreich’s ataxia [34].

The aim of this work is to present, using innovative proteomic techniques, a detailed analysis of the protein glutathionylation targets in the yeast *S. cerevisiae* in cells undergoing constitutive metabolism and after cultivation of the cells with various oxidants known to modify the cellular thiol-dependent redox state. We explore the response of the cells upon treatment with diamide, oxidized glutathione GSSG, hydrogen peroxide, or iron excess and analyze the redox proteome after adaptation of the cells to these growth conditions. To perform this study, we had to significantly improve each step of the ‘biotin–Grx’-switch procedure, developed elsewhere, to

strictly limit the analysis to proteins actually undergoing glutathionylation and to characterize the specific glutathionylated peptides. Our results provide evidence for a strong role of protein glutathionylation under basal conditions. Moreover, each stress condition leads to a specific original adaptive response. This work provides further insights into the diversity of proteins undergoing glutathionylation and the role of this post-translational modification as a regulatory process in the adaptive response of the *S. cerevisiae* cell.

## Materials and methods

### Yeast strains, media, and growth conditions

The *S. cerevisiae* strain used was S150-2B (MATa, his3- $\Delta$ 1, leu2-3112, trp1-289, ura3-52). To study the adaptive response of *S. cerevisiae* cells to various stress conditions, cells were cultured in YNR medium (yeast nitrogen base, 2% raffinose, and 0.1% glucose supplemented with the necessary amino acids) in the presence of 1 mM diamide, 100  $\mu$ M iron citrate, 1 mM H<sub>2</sub>O<sub>2</sub> or 5 mM GSSG until reaching the stationary phase. Cells were then collected and mitochondrial purification was performed as described below.

### Isolation of yeast mitochondria

Yeast cells were cultured overnight at 30°C and harvested by centrifugation at 4000 $\times$ g for 10 min at 4°C, washed with 50 ml of ice-cold water, and resuspended in 0.6 M sorbitol, 50 mM Tris-HCl (pH 7.5), supplemented with the complete protease inhibitor mixture (Sigma). We added 0.45 mm diameter sterile glass beads to each tube, and the cells were lysed by vortexing six times for 30 s each, at 2 min intervals, on ice. All subsequent steps were carried out at 4°C. Glass beads and unbroken cells were removed by centrifugation at 4000 $\times$ g for 10 min, supernatants were centrifuged (14 000 $\times$ g, 10 min, 4°C), and the resulting pellets were resuspended in 0.6 M sorbitol and 50 mM Tris-HCl (pH 7.5) for the preparation of mitochondrial protein extracts, the supernatants comprising the cytosolic fraction. Crude mitochondrial fractions were immediately frozen at -80°C. Under our conditions, mitochondrial proteins accounted for 8% of the total cellular proteins.

### Determination of glutathione levels

Glutathione levels were determined using a modified version of the Tietze recycling enzymatic assay as previously described [45,46]. For the estimation of total intracellular glutathione, samples were washed and resuspended in 50 mM potassium phosphate buffer (pH 7.8) containing ice-cold 5% 5-sulfosalicylic acid. Specific glutathione content was calculated from standard curves obtained with various concentrations of GSSG and is expressed in nmol of glutathione/mg of protein. This assay is based on the reduction of each GSSG molecule to give two GSH molecules, and the reading is in GSSG equivalents, because GSSG is used as the standard. The total measured specific glutathione content is thus 0.5 GSH + GSSG. For the quantification of oxidized glutathione (GSSG), samples (including GSSG standards) were treated with 2% (vol./vol.) 4-vinylpyridine for 1 h at room temperature before analysis. The cytosolic and mitochondrial GSH/GSSG ratios were calculated as follows:  $\text{GSH/GSSG} = 2[(\text{total glutathione}) - \text{GSSG}]/\text{GSSG}$ .

### Purification of glutathionylated mitochondrial proteins

Glutathionylated proteins were purified using a modified version of a previously described 'biotin/Grx-switch' procedure [38]. Mitochondrial proteins (~100  $\mu$ g) were treated with 0.05% Triton X-100 for 5 min and then with a mixture of 5 mM NEM and 1 mM S-methyl methanethiosulfonate (MMTS) for 20 min, to block the free -SH groups on proteins. Unreacted NEM and MMTS were removed with a desalting column (Thermo Scientific, Zeba™ Spin Desalting Columns, 7 K MWCO). Samples were then incubated for 45 min with 5  $\mu$ M glutaredoxin (Calbiochem, Glutaredoxin-S2, *Escherichia coli*, Grx1) in the presence of 0.05 mM GSH, 6 U/ml glutathione reductase, and 300  $\mu$ M NADPH to reduce the sulfhydryl groups that had undergone glutathionylation. The samples were then treated with 250  $\mu$ M HPDP-biotin for 30 min to label the newly exposed sulfhydryl groups, and excess HPDP-biotin was removed with a desalting column. Biotinylated and non-biotinylated proteins were then separated using high-affinity streptavidin magnetic beads (Thermo Scientific, Pierce™ Streptavidin Magnetic Beads) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.5; 0.6 M sorbitol), in the presence of protease inhibitor cocktail. After incubation for 1 h, the liquid fractions were collected and the beads were washed with 30  $\mu$ l of water. They were then resuspended in 30  $\mu$ l of 95% formamide and 10 mM EDTA. Biotinylated proteins were dissociated from the streptavidin magnetic beads by heating for 10 min at 90°C.

## Immunodetection experiments

To optimize the alkylation conditions of free thiol groups, isolated mitochondria from WT *S. cerevisiae* cells were treated for 20 min with the following alkylating agents: 20 mM 2-iodoacetamide (IAM), 5 mM NEM, 1 mM MMTS, or a mixture of 1 mM MMTS/5 mM NEM. Samples were then treated for 30 min with 250  $\mu$ M HPDP-biotin, separated on a 4–20% gradient SDS–PAGE gel, transferred to a nitrocellulose membrane, and analyzed by chemiluminescence using an anti-biotin antibody.

To optimize the purification of glutathionylated proteins, 5  $\mu$ g of proteins were separated from high-affinity streptavidin beads were separated on a 4–20% gradient SDS–PAGE gel, transferred to a nitrocellulose membrane, and analyzed by chemiluminescence using an anti-horseradish peroxidase (HRP) streptavidin antibody.

## SDS–PAGE of glutathionylated proteins for mass spectrometry

Samples of 5  $\mu$ g of protein were subjected to a short run (low migration) on a NUPAGE 4–12% acrylamide gel, which was then stained with Coomassie blue (Simply Blue SafeStain, Invitrogen). We digested three bands per condition overnight at 37°C with sequencing-grade trypsin (12.5  $\mu$ g/ml, Promega, Madison, WI, U.S.A.) in 20  $\mu$ l of 25 mmol/l  $\text{NH}_4\text{HCO}_3$ . Trypsin digests from the same lane were then pooled before mass spectrometry analysis.

## LC–MS/MS acquisition

All digests of protein extracts were analyzed using an LTQ Velos Orbitrap equipped with an EASY-Spray nanoelectrospray ion source coupled to an Easy nano-LC Proxeon 1000 system (all devices were purchased from Thermo Fisher Scientific, San Jose, CA, U.S.A.) following chromatographic separation of the peptides. All the experimental conditions for LC–MS/MS acquisitions were recently described [47]. MS/MS data were processed using an in-house Mascot search server (Matrix Science, Boston, MA, U.S.A., version 2.4.1). The mass tolerance was set to 7 ppm for precursor ions and 0.5 Da for fragments. The following variable modifications were allowed for identification of glutathionylated proteins: oxidation (M), phosphorylation (STY), acetylation (K, N-term), and deamidation (N, Q). In a second round, glutathionylated cysteines were identified by taking into account NEM and MMTS modifications and HPDP-biotin derivatization as variable modifications. The maximum number of missed trypsin cleavages was limited to two. MS/MS data were used to search against the Uniprot database restricted to the *S. cerevisiae* taxonomy.

## Quantitative analysis in label-free experiments

The proteins in the various homogenates were subjected to low-migration electrophoresis, stained, and fixed. The bands were subjected to in-gel digestion, in triplicate, overnight at 37°C, with sequencing-grade trypsin (12.5  $\mu$ g/ml, Promega, Madison, WI, U.S.A.) in 20  $\mu$ l of 25 mmol/l  $\text{NH}_4\text{HCO}_3$ . LC–MS/MS acquisition was performed using a 2-h gradient. MS/MS data were processed using a Mascot search server. Label-free quantification was performed on raw data using the Progenesis-QI software version 4.1 (Nonlinear Dynamics Ltd, Newcastle, U.K.) according to ref. [48]. A decoy search was performed using a significance threshold of 0.05. The resulting files were imported into the Progenesis-QI software. Peptides with an ion score below 15 were rejected. Conflicts for the identification of some peptides were resolved manually.

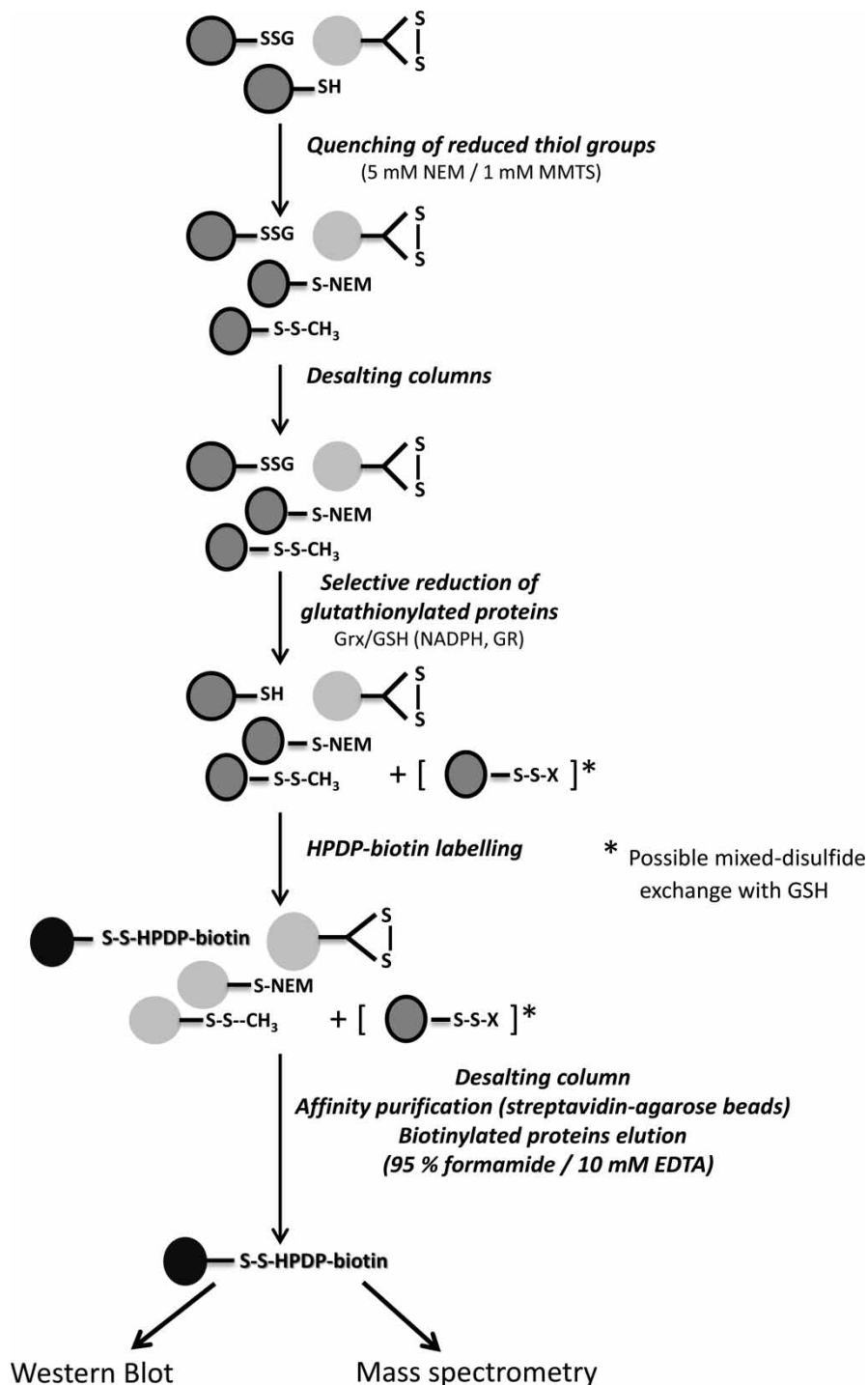
## Statistical analysis

All data points in the figures, tables, and data are the means of at least three independent determinations. The Student's *t*-test was used to identify significant differences.

## Results

### Optimization of a purification procedure for the proteomic analysis of glutathionylated proteins in complex samples under physiological conditions

We performed experiments to develop a specific protocol for the purification of glutathionylated proteins for proteomic analyses. We developed the protocol on *S. cerevisiae* isolated mitochondria because changes in the intramitochondrial thiol-dependent redox status are likely to affect essential functions, and the identification of mitochondrial glutathionylation targets is of great interest for many studies using yeast as a cellular model. In the previously published biotin/Grx procedure [38], protein samples are treated with NEM to block the free thiol groups. Protein–glutathione bonds are then reduced using Grx/GSH and derivatized with NEM–biotin,



**Figure 1. Schematic representation of the 'biotin-switch' purification procedure of glutathionylated proteins for proteomic analyses.**

After quenching of the reduced thiol groups using a mixture of 5 mM NEM/1 mM MMTS, the excess alkylating agent was removed, and selective reduction in the mixed glutathione-bound protein was performed using 5  $\mu$ M Grx/0.5 mM GSH (in the presence of NADPH and glutathione reductase). Reduced proteins were then labeled using 250  $\mu$ M HPDP-biotin and purified by affinity chromatography on streptavidin-agarose beads and submitted to immunodetection or mass spectrometry analyses.

resulting in thioether bond formation between the previously glutathionylated cysteine and the biotinylated reagent. The tagged proteins are then purified by biotin–avidin affinity chromatography. We have developed and significantly improved several steps of the biotin/Grx-switch protocol: the alkylation step, the removal of excess alkylating agent and biotin, the choice of the biotin-derivative concentration, and the affinity column elution conditions.

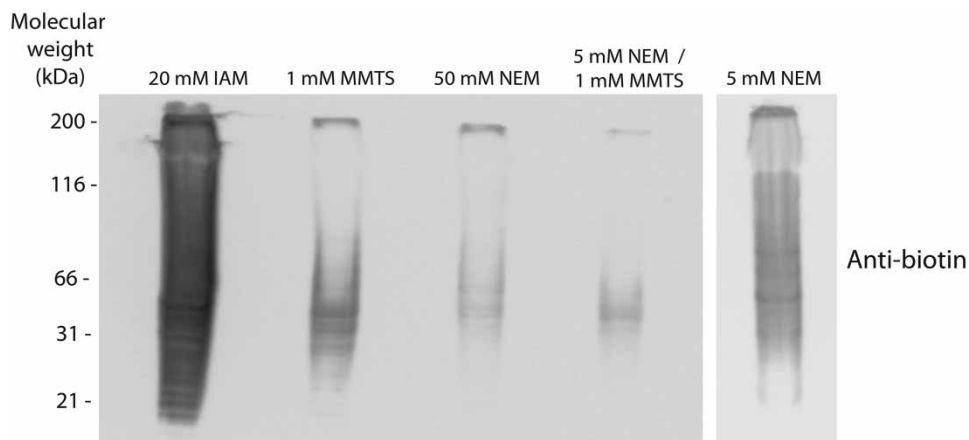
The first crucial step of the reaction is the quenching of free reduced thiol groups (Figure 1). It is crucial to block all free –SH groups to ensure the complete identification of all glutathionylated proteins. NEM is widely used to block the thiol groups because it is efficient at physiological pH, enabling NEM labeling of redox-sensitive cysteines in their native conformation and physiological environment. In addition to NEM, IAM and MMTS are also used as alkylating agents of cellular proteins. We assessed protein alkylation efficiency by treating the mitochondria with varying concentrations of NEM, IAM, or MMTS for 20 min (greater incubation times did not improve the purification process; data not shown). The samples were then incubated with HPDP-biotin which should bind to the remaining free thiol groups, and the proteins were revealed by immunodetection using an anti-biotin antibody. The presence of bands indicates the presence of remaining free thiol groups, which have not reacted with the alkylating agent (Figure 2) and allows evaluation of the efficiency of the alkylating process. In Figure 2, IAM appeared to be a very poor blocking agent, even at a concentration of 20 mM, as many bands were recognized by the anti-biotin antibody, indicating that many free –SH groups were thus not alkylated under these conditions. We found alkylation to be more effective using 50 mM NEM than 5 mM NEM, and, unexpectedly, we observed a very similar pattern when 1 mM MMTS alone was used (Figure 2). We also observed very few visible bands revealed by the anti-biotin antibody when we incubated the mitochondria in a mixture of 5 mM NEM/1 mM MMTS, demonstrating that most of the reduced thiol groups were blocked under these conditions. However, in order to limit the amount of NEM which could interfere in the purification procedure, we thus decided to use both NEM and MMTS to quench the thiol groups. Since the reaction of MMTS with –SH groups leads to a Cys-SSMe disulfide modification that could be reduced in the presence of GSH and give rise to the identification of false positives (see Figure 1), the appropriate proteomic control experiments were performed to check that this unwanted thiol exchange reaction was not interfering with our experimental procedure (data not shown).

During the purification process, the biotin concentration may also affect protein identification, because the hydrophobicity of the biotin tag may diminish the efficiency of the ultimate step of affinity purification. To assess this aspect of the experimental procedure, we purified glutathionylated proteins using the ‘biotin/Grx-switch’ protocol and analyzed them by immunodetection using an HRP-conjugated streptavidin antibody. The use of 250  $\mu$ M HPDP-biotin allowed for better recovery of biotinylated proteins upon elution from the affinity resin compared with 1 mM HPDP-biotin (Figure 3A), a condition where many proteins remained in the supernatant (lane 2). The bands of lane 3 were more intense in the presence of 1 mM biotin, but the counterpart would be to focus on the more abundant proteins and to exclude physiologically interesting glutathionylated proteins from further analysis.

The procedure was also significantly improved by adding two desalting steps. The first desalting column was used after the blocking step to remove the excess of unreacted NEM/MMTS, and strongly improved the recovery from the affinity column and the labeling of glutathionylated proteins by avoiding inhibition of the enzymatic Grx system (Figure 3B). Indeed, an excess of alkylating agent is necessary to ensure blockage of all free thiol groups, but an excess of NEM also diminished the efficiency of the elution step and the further identification of protein targets. The second desalting step was performed after HPDP-biotin labeling to remove any excess biotin before the affinity purification (Figure 3B). This step is crucial to decrease competition between unreacted HPDP-biotin and biotinylated proteins on streptavidin–agarose and thus increase the enrichment efficiency. We also improved the elution step by using 95% formamide/10 mM EDTA to elute the glutathionylated proteins from the affinity resin (Figure 1). Altogether, the experiments presented in Figures 2 and 3 allowed us to optimize some of the critical steps of the purification procedure of glutathionylated proteins and to design the detailed experimental procedure presented in Figure 1.

## **Comparison of the intracellular glutathione-dependent redox status of WT *S. cerevisiae* cells under basal and oxidative stress conditions**

We measured glutathione levels in both cytosol and isolated mitochondria of *S. cerevisiae* cells to correlate potential protein targets of glutathionylation to changes in the intracellular redox status. We compared WT



**Figure 2. Optimization of reduced thiol group alkylation conditions.**

Isolated mitochondria from WT *S. cerevisiae* cells were treated for 20 min with the following alkylating agents: 20 mM IAM, 5 or 50 mM NEM, 1 mM MMTS or a mixture of 1 mM MMTS/5 mM NEM. Samples were then treated for 30 min with 250  $\mu$ M HPDP-biotin, separated on a 4–20% gradient SDS–PAGE gel, transferred to a nitrocellulose membrane, and analyzed by chemiluminescence using an anti-biotin antibody.

cells grown in classic minimal medium with those cultured under conditions known to induce oxidative stress within the cells: 1 mM diamide, 5 mM GSSG, 100  $\mu$ M Fe (as iron citrate), and 1 mM  $H_2O_2$  [34,49,50]. This is an original approach compared with most of the published studies where isolated mitochondria or cell extracts were usually treated with oxidants. In our procedure, the cells were cultivated in the presence of various oxidants until reaching the stationary phase, and we were thus capable of analyzing the redox status of the cells after adaptation to these different oxidative growth conditions. We observed a significant approximately three-fold decrease in total glutathione (GSH + GSSG) levels in the cytosol (Figure 4A, black bars), regardless of the growth conditions, relative to untreated cells. Moreover, there was a difference in the GSH/GSSG ratio between the untreated cells, which was 39 (where glutathione is mostly in the reduced GSH form), and those treated with diamide, GSSG, and  $H_2O_2$ , where the values were 22, 19, and 16, respectively, showing a slight increase in the oxidized form GSSG. The GSH/GSSG ratio remained unchanged in the presence of excess iron in the culture medium.

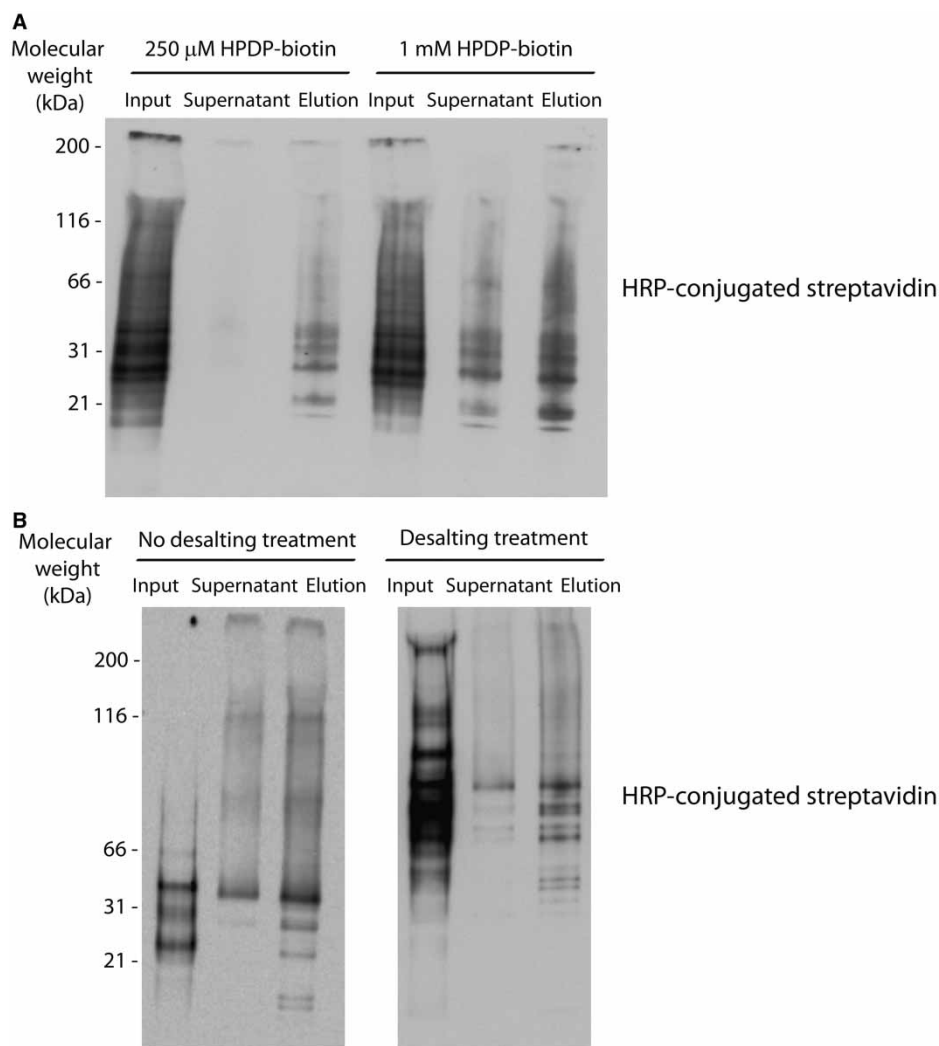
On the contrary, there were no significant changes in total mitochondrial glutathione content (Figure 4B, black bars) between untreated cells and those subjected to stress conditions, with the exception of excess iron. However, oxidized glutathione GSSG increased significantly (Figure 4B, gray bars), especially when cells were grown in the presence of GSSG and  $H_2O_2$ . The intramitochondrial GSH/GSSG ratio ranged from 30 for the untreated cells down to 4 in the presence of GSSG and 5 in the presence of  $H_2O_2$ , reflecting severe glutathione-dependent oxidative stress in the mitochondria. Inversely, total glutathione content increased two-fold in the presence of 100  $\mu$ M excess iron. These data show that mitochondria are more susceptible to culture cell conditions than cytosol because glutathione cannot be synthesized in mitochondria and must be imported from the cytosol. Our data strongly suggest that, under these conditions, glutathione may bind to mitochondrial proteins to adapt to stress conditions and protect the cells from further oxidative modifications.

## Proteomic evidence for strong basal constitutive glutathionylation in WT *S. cerevisiae* cells

We purified glutathionylated proteins from cytosol and mitochondria using the improved biotin/Grx-switch method as described above, and submitted them for mass spectrometry analysis. Differences in the relative levels of peptide modification between two cultures may reflect changes in the level of the modification or in protein abundance. We therefore performed label-free experiments in parallel to assess the relative abundance of proteins in our samples.

The number of identified glutathionylated proteins in cytosol and isolated mitochondria is presented in Figure 5A. The number of cytosolic targets increased approximately two-fold when cells were cultured with

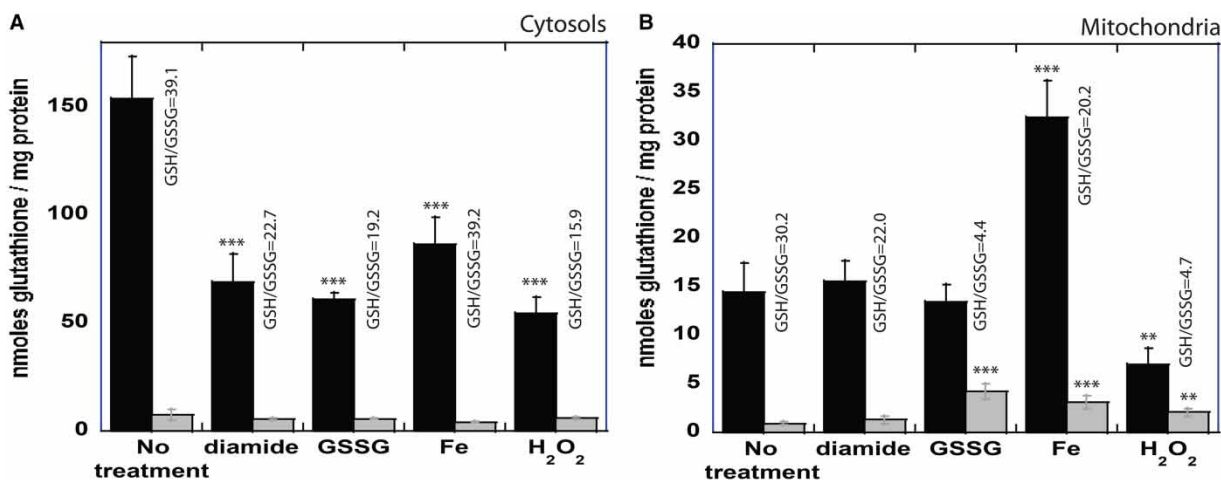




**Figure 3. Optimization of the purification of glutathionylated proteins.**

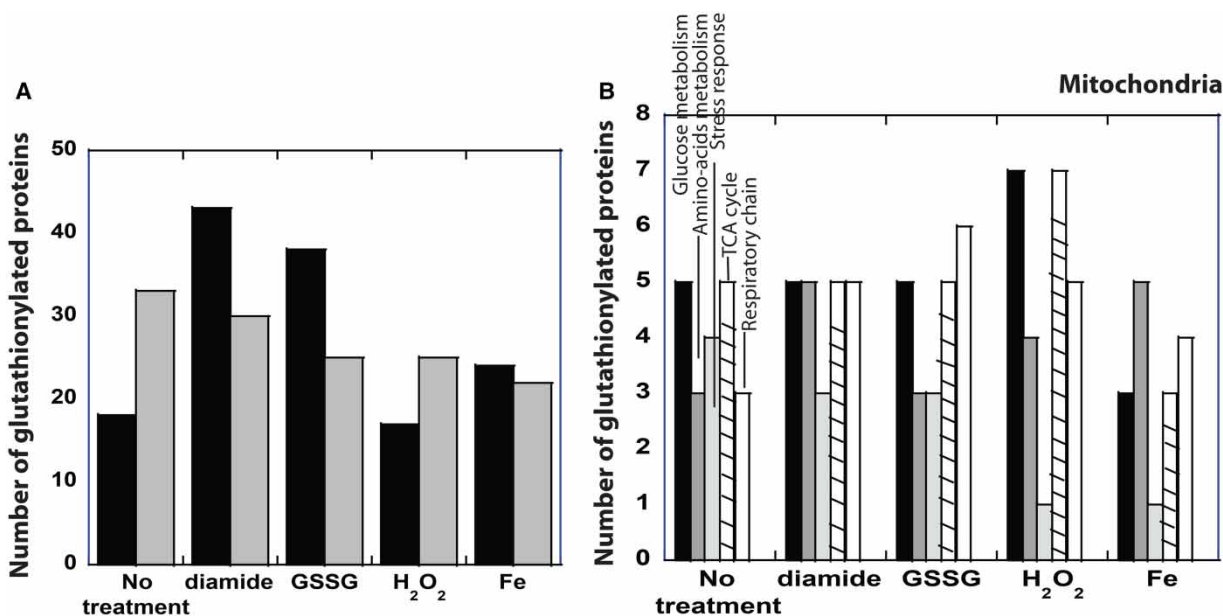
Glutathionylated mitochondrial proteins were purified using the ‘biotin/Grx-switch’ procedure described in the Experimental Procedure and in Figure 1. The proteins (5  $\mu$ g) were separated on a 4–20% gradient SDS–PAGE gel, transferred to a nitrocellulose membrane, and analyzed by chemiluminescence using HRP-conjugated streptavidin. Input: fraction loaded onto the streptavidin–agarose beads; Elution: biotinylated fraction eluted from the streptavidin–agarose beads. (A) A concentration of 250  $\mu$ M HPDP-biotin results in better recovery of biotinylated proteins upon elution of the affinity resin. (B) In the presence of 250  $\mu$ M HPDP-biotin. The addition of desalting steps (Zerba desalting columns) to the purification procedure, to remove excess unreacted alkylating agents and biotin, greatly improved recovery from the affinity column.

diamide and GSSG, when compared with untreated cells (Figure 5A, black bars). These results are in agreement with the fact that GSSG and diamide have been shown to promote protein glutathionylation, probably by increasing the intracellular levels of GSSG (Figure 4A) [12,51,52]. However, there was little or no significant change in the level of cytosolic protein glutathionylation when cells were grown in the presence of H<sub>2</sub>O<sub>2</sub> or under conditions of excess iron. We observed very different behavior for the mitochondrial targets. Indeed, we identified twice as many glutathionylated proteins in mitochondria under basal conditions than in cytosol. Moreover, there was a slight decrease in the number of protein targets under conditions of oxidative stress, regardless of the stress conditions used (Figure 5A, gray bars). Altogether, these results suggest that conditions of oxidative stress in the cytosol promote glutathionylation to protect protein thiol groups from further oxidation and may even constitute a storage form of glutathione within the cells. Inversely, oxidative growth



**Figure 4. Cellular distribution of glutathione in WT *S. cerevisiae* cells under basal and oxidative growth conditions.**

WT *S. cerevisiae* cells were cultured in YNB medium or in YNB medium supplemented with 1 mM diamide, 1 mM H<sub>2</sub>O<sub>2</sub>, 5 mM GSSG, or 100 μM iron citrate, and glutathione levels were determined as described in the Materials and Methods section, in (A) cytosol and (B) isolated mitochondria. Total glutathione levels (GSH + GSSG) are shown in black and oxidized glutathione disulfide (GSSG) levels are shown in gray. All data points in the figure are the mean of at least three determinations, normalized to the relative protein content of the samples, and a Student's *t*-test was used to identify significant differences (\*\**P* < 0.01, \*\*\**P* < 0.001 versus untreated cells). In our experimental conditions, mitochondrial proteins accounted for 8% of cellular proteins and cytosolic proteins for 92%.



**Figure 5. Identification of glutathionylated proteins in WT *S. cerevisiae* cells under basal and oxidative stress conditions.**

WT *S. cerevisiae* cells were cultured in YNB medium or in YNB medium supplemented with 1 mM diamide, 1 mM H<sub>2</sub>O<sub>2</sub>, 5 mM GSSG, or 100 μM iron citrate. Glutathionylated proteins from cytosol and isolated mitochondria were purified using the biotin/Grx-switch purification procedure described in Materials and Methods, and subjected to mass spectrometry analyses. (A) Number of identified glutathionylated proteins in cytosol (black bars) and mitochondria (gray bars); (B) Number of identified glutathionylated protein targets in isolated mitochondria related to the primary metabolic pathways: glucose metabolism (black bars), amino acid metabolism (gray bars), and stress response (light gray bars), TCA cycle in striped bars, and respiratory chain in white bars.

conditions lead to severe glutathione-dependent oxidative stress in mitochondria, with lower GSH/GSSG ratios, as shown in [Figure 4B](#), and deglutathionylation, probably associated with glutathione import into the mitochondria. This may help mitochondria to adapt to stress conditions by providing glutathione which they cannot synthesize.

The number of glutathionylated proteins that we identified was lower than the one reported in most of the published studies. We think that this is probably the consequence of the purification and peptide data search tightly restricted to peptides which have actually bound a glutathione molecule. Moreover, in our study, cells were cultured in the presence of the various oxidants until reaching the stationary phase and had time for adaptation. Most of the data in the literature present the acute response of isolated cell extracts or mitochondria to oxidants, whereas our work shows evidence for a different adaptive response in which glutathionylation but also deglutathionylation may play a key role with, as a result, a more restricted number of glutathionylated proteins.

Results of [Figure 4](#) show that the various oxidants used in our study affect in a different manner the glutathione-dependent redox status of both cytosols and mitochondria. However, we decided to focus on mitochondrial targets because changes in the mitochondrial thiol redox state is particularly important due to the high sensitivity of mitochondria to oxidative damage and the key role of redox signaling in this compartment, and such changes may profoundly alter mitochondrial function [9,21]. The glutathionylated cytosolic proteins were mainly those involved in general glucose, amino acid, and lipid metabolism (Supplementary Information S1). Within mitochondria, the level of basal glutathionylation was relatively high in untreated cells, in the absence of stress, with ~40 proteins binding glutathione ([Figure 5](#) and [Table 1](#)), despite a relatively high GSH/GSSG ratio ([Figure 4](#)). The number of glutathionylated proteins in cells undergoing basal metabolism has often been underestimated in the literature because many available detection procedures had major drawbacks that did not allow the identification of glutathionylated proteins without *in vitro* stress induction. Proteins from the principal metabolic pathways were constitutively glutathionylated, such as proteins involved in glucose metabolism (phosphoglycerate kinase and enolase), the TCA cycle [aconitase,  $\alpha$ -ketoglutarate dehydrogenase (KGDH), and pyruvate dehydrogenase (PDH)], or complexes of the mitochondrial respiratory chain ([Table 1](#)). Proteins involved in amino acid metabolism or stress responses were also found to undergo glutathionylation ([Table 1](#) and [Figure 5B](#)). These results show that glutathionylation is not only induced upon oxidative treatment but may also play an important post-translational role under constitutive basal conditions.

## **Proteomic evidence for specific mitochondrial glutathionylation of WT *S. cerevisiae* cells under oxidative conditions**

The number of mitochondrial glutathionylation targets decreased slightly after growing the cells in the presence of diamide, H<sub>2</sub>O<sub>2</sub>, GSSG, or excess iron ([Figure 5A](#)). Interestingly, the patterns of glutathionylation targets differed depending on the growth conditions. Proteins involved in glucose metabolism and TCA cycle were the most abundantly glutathionylated in untreated WT cells and their number decreased markedly when cells were grown in the presence of iron excess, but, inversely, increased upon treatment with hydrogen peroxide ([Figure 5B](#) and [Table 1](#)). Moreover, data from quantitative proteomics experiments ([Table 2](#)) show that this decrease could not be related to a significant decrease in protein abundance. In addition, glutathionylation of proteins involved in stress response decreased markedly when cells were grown under stress conditions, regardless of the growth conditions. We found proteins involved in the energetic pathways of the TCA cycle and mitochondrial respiratory chain to be glutathionylated in all five conditions. However, the relative contribution of the respiratory chain proteins was much higher under conditions of stress ([Figure 5B](#)), with no evident correlation between this effect and changes in protein abundance ([Table 2](#)). The relative importance of glutathionylated proteins from the TCA cycle and respiratory chain was particularly evident after growing the cells in the presence of H<sub>2</sub>O<sub>2</sub> or GSSG, conditions leading to an increase in intracellular GSSG and glutathione-dependent oxidative stress ([Figure 4B](#)), and these changes could not be accounted for by a significant change in protein abundance ([Table 2](#)). No pathway was preferentially glutathionylated when cells were grown in an excess of iron. This result confirms that this specific growth condition leads to a different response than that observed under the other stress conditions, in addition to the import of glutathione into the mitochondria observed in [Figure 4B](#).

Altogether, these data suggest that each growth condition results in a specific response, through the glutathionylation of proteins involved in bioenergetic pathways, especially those of the TCA cycle and respiratory

**Table 1 Summary of proteins identified in the biotinylated fractions of the biotin/Grx-switch assay**

Part 1 of 2

Protein name	Gene <sup>1</sup>	WT mitochondria				
		Not treated	Diamide	GSSG	Fe	H <sub>2</sub> O <sub>2</sub>
TCA cycle	Mascott scores <sup>2</sup>					
Fumarate hydratase	FUM1		460.3			
Dihydrolipoyl dehydrogenase	LPD1	265.0	63.8	51.8	101.9	106.9
Aconitate hydratase, mitochondrial	ACO1	708.9	570.8	691.1	661.3	626.9
2-Oxoglutarate dehydrogenase	KGD1	370.9	486.1	379.7		396.4
Pyruvate dehydrogenase E1 subunit β	PDB1	449.7		234.9		282.7
Succinate dehydrogenase [ubiquinone]	YJL045W					253.9
Succinyl-CoA ligase subunit α	LSC1					139.0
Homocitrate synthase	LYS21	518.87	463.6	450.3	427.4	419.1
Respiratory chain						
Cytochrome <i>b</i> – <i>c</i> 1 complex subunit 7	COX7		388.1	379.3	309.6	360.8
Cytochrome <i>b</i> – <i>c</i> 1 complex subunit 1	COR1		969.4			
Cytochrome <i>b</i> – <i>c</i> 1 complex subunit 2	QCR2	665.9	883.9	1021.9	800.6	1002.3
Cytochrome <i>b</i> 2	CYB2		194.4	224.8	169.5	
Cytochrome <i>c</i> 1	CYT1			352.5		311.1
Cytochrome <i>c</i> oxidase subunit 6	COX6	56.0		51.6		99.3
Cytochrome <i>c</i> oxidase subunit 4	COX4	231.9	388.1	379.3	309.6	143.2
Glucose metabolism						
Phosphoglycerate kinase	PGK1	575.8	460.1	496.2		
Enolase 1	ENO1	472.7				
Enolase 2	ENO2	465.4				
Fructose-bisphosphate aldolase	FBA1	532.6	511.5		441.8	337.2
Trehalose-phosphatase	TPS2	291.3	931.3			
Glycerol-3-phosphate dehydrogenase	GUT2	804.4		800.4	691.4	
D-lactate dehydrogenase [cytochrome] 1	DLD1	277.8		405.8	407.5	395.66
Acetyl-CoA hydrolase	ACH1			408.3		
Amino acid metabolism						
Ketol-acid reductoisomerase	ILV5	701.9	1020.0	1086.4	620.9	896.9
2-Isopropylmalate synthase	LEU4		349.5		192.1	284.8
Acetolactate synthase small subunit	ILV6	159.7	196.0	177.0	132.9	169.4
Aminotransferase	BAT1		301.6		260.8	
S-adenosylmethionine synthase 2	SAM2	701.9	1020.0	376.9	620.9	417.1
Hem biosynthesis						
Ferrochelatase	HEM1	97.5	87.6	178.6	65.5	138.2
Lipid metabolism						
Sterol 24-C-methyltransferase	ERG6	399.2	409.3			
NADPH-cytochrome P450 reductase	NCP1	556.3				563.1
Acetyl-CoA carboxylase	ACC1	1543.8	1451.2		395.5	
Stress						
Flavohemoprotein	YHB1	442.6		503.2		
Heat shock protein 104	HSP104	879.4	856.9			

Continued

**Table 1 Summary of proteins identified in the biotinylated fractions of the biotin/Grx-switch assay**

Part 2 of 2

Protein name	Gene <sup>1</sup>	WT mitochondria				
		Not treated	Diamide	GSSG	Fe	H <sub>2</sub> O <sub>2</sub>
Heat shock protein SSA2	SSA2	1327.5	1054.6	1014.3	964.3	1031.3
Heat shock protein 78	HSP78	646.7	431.0	526.0		
Others						
Elongation factor Tu	TUF1	556.1	439.5			339.8
Outer membrane protein porin 1	POR1	1047.1	1454.8	1061.2	1232.9	1487.0
ATP/ADP carrier 2	PET9	601.7	546.2	491.4	371.8	463.5
Phosphate carrier protein	MIR1	426.1	572.3			
Mitochondrial import receptor subunit	TOM40	297.6			299.7	
Cell division control protein 48	CDC48	179.7	278.5			
Mitochondrial import translocase	TIM44	242.9	224.2	237.7	385.2	220.0

<sup>1</sup>Genes correspond to gene names found in the SGD (*Saccharomyces* Genome Database).

<sup>2</sup>Mascott scores obtained for protein identification using combined MS and MS/MS peak lists (see more details in materials and methods). Only the proteins with Mascott scores  $\geq 50$  were considered.

chain complexes. Moreover, the decrease in glutathionylation targets relative to untreated cells confirms the idea that, under basal conditions, glutathionylation not only protects the –SH groups from further oxidation, but may also constitute a form of glutathione storage. The response to stress then leads to protein deglutathionylation, to compensate for glutathione-dependent oxidative stress.

### Classification on the basis of protein levels provides evidence to support adaptive metabolic strategies

We further explored the metabolic consequences of stress conditions by classifying the differentially expressed proteins using the Autoclass@IJM Bayesian clustering system, in a specific cluster analysis of the label-free data based on direct comparisons of protein abundance between the five sets of conditions (Figure 6) [53]. The classification identified groups of proteins that could not be deduced directly from a classical visual examination of protein abundance profiles. The results of this analysis clearly reflect specific metabolic orientations according to the growth conditions of the cells. Clusters C1 to C3 present proteins which were less abundant when cells were grown in the presence of H<sub>2</sub>O<sub>2</sub> and generally induced in other conditions (except GSSG for cluster C1). These clusters contain proteins from bioenergetic pathways, such as proteins involved in glucose metabolism (hexokinase and phosphoglycerate kinase), the TCA cycle (citrate synthase, SDH, aconitase, and PDH), and the mitochondrial respiratory chain (complex III and ATP synthase subunits). These clusters also contain heat shock proteins and proteins involved in thiol metabolism, such as thioredoxin peroxidase. Clusters C4 and C5 comprise proteins mainly induced after growing the cells in the presence of GSSG: ATP subunits and TCA cycle proteins, such as isocitrate dehydrogenase, malate dehydrogenase, or PDH. Glutathione synthetase was also induced in the presence of GSSG, as well as thioredoxin and peroxiredoxin. A specific response to iron involved complex IV and ATP synthase (cluster C8), whereas the response to H<sub>2</sub>O<sub>2</sub> involved mostly proteins of the TCA cycle, such as fumarate reductase or SDH (cluster C7), and the response to diamide involved the induction of ATP synthase subunits and glutaredoxin (cluster C6). The results of this analysis confirm that *S. cerevisiae* metabolic remodeling, which we found associated with the glutathionylation of specific mitochondrial proteins, is strongly dependent on growth conditions.

### Discussion

Identification of the proteins undergoing glutathionylation in living cells is an important step in defining the molecular targets involved in the regulation of cell function. In *S. cerevisiae*, we investigated the link between changes in glutathione-dependent redox status and the protein targets of protein glutathionylation in cytosols and isolated mitochondria, under basal conditions and after adaptation of the cells to various stress conditions.

**Table 2 List of proteins identified as glutathionylated from proteomic experiments and the mean of their normalized relative abundance in the different conditions, as determined in label-free experiments ( $n = 3$  biological replicates)**

Significances associated with the observed differences between experiments were evaluated with a statistical test (ANOVA  $P$ -value).

Part 1 of 2

Protein name	Gene <sup>1</sup>	WT mitochondria					ANOVA $P$ -value
		Not treated	Diamide	GSSG	Fe	H <sub>2</sub> O <sub>2</sub>	
TCA cycle		Normalized relative abundance ( $n = 3$ biological replicates)					
Fumarate hydratase	FUM1	1.14E+05	6.40E+04	6.31E+04	5.70E+04	1.02E+05	0.43
Dihydrolipoyl dehydrogenase	LPD1	2.23E+05	1.07E+05	1.24E+05	9.01E+04	1.65E+05	0.64
Aconitate hydratase	ACO1	3.83E+05	1.22E+05	1.80E+05	1.31E+05	1.96E+05	0.12
2-Oxoglutarate dehydrogenase	KGD1	3.38E+04	8.90E+04	9.06E+04	8.22E+04	1.47E+05	3.88E–003
Pyruvate dehydrogenase E1	PDB1	2.65E+05	1.92E+05	1.93E+05	1.25E+05	2.63E+05	0.18
Succinate dehydrogenase	YJL045W	1.84E+05	2.89E+05	1.22E+05	4.12E+05	3.71E+05	–
Succinyl-CoA ligase subunit $\alpha$	LSC1	1.23E+05	9.06E+04	6.20E+04	9.08E+04	8.06E+04	0.51
Homocitrate synthase	LYS21	9.41E+04	1.33E+05	4.89E+04	1.33E+05	3.07E+04	0.01
Respiratory chain							
Cytochrome $b$ – $c$ 1 complex sub 7	QCR7	2.62E+05	6.97E+05	2.56E+05	4.47E+05	3.29E+05	0.05
Cytochrome $b$ – $c$ 1 complex sub 11	COR1	8.06E+05	1.08E+06	6.49E+05	7.96E+05	1.08E+06	0.05
Cytochrome $b$ – $c$ 1 complex sub 2	QCR2	7.28E+05	1.90E+06	4.24E+05	1.29E+06	1.62E+06	1.6 E–003
Cytochrome $b$ 2	CYB2	8.92E+04	8.15E+04	1.68E+04	6.83E+04	2.71E+04	0.3
Cytochrome $c$ 1	CYT1	5.33E+05	7.70E+05	7.07E+05	8.35E+05	9.58E+05	3.82E–004
Cytochrome $c$ oxidase subunit 6	COX6	9.24E+04	6.70E+04	7.46E+04	4.93E+04	5.97E+04	0.64
Cytochrome $c$ oxidase subunit 4	COX4	9.90E+04	1.61E+05	8.06E+04	2.03E+05	3.11E+05	1.17E–003
Glucose metabolism							
Phosphoglycerate kinase	PGK1	1.13E+06	5.29E+05	2.66E+05	7.51E+05	5.58E+05	0.24
Enolase 1	ENO1	8.29E+05	8.18E+05	4.62E+05	6.76E+05	7.11E+05	9E–003
Enolase 2	ENO2	2.71E+05	3.27E+05	1.40E+05	2.76E+05	1.97E+05	0.1
Fructose-bisphosphate aldolase	FBA1	5.54E+05	2.48E+05	1.69E+05	1.99E+05	2.58E+05	0.12
Trehalose-phosphatase	TPS2	3.51E+04	5.21E+04	4.23E+04	3.81E+04	5.66E+04	0.75
Glycerol-3-phosphate dehydrogenase	GUT2	2.93E+05	2.22E+05	2.75E+05	2.19E+05	3.67E+05	0.73
D-lactate dehydrogenase	DLD1	5.88E+04	5.30E+04	5.21E+04	5.11E+04	9.92E+04	0.32
Acetyl-CoA hydrolase	ACH1	1.99E+05	9.63E+04	1.30E+05	7.34E+04	1.56E+05	2.00E–02
Amino acid metabolism							
Ketol-acid reductoisomerase	ILV5	4.63E+05	2.89E+05	3.27E+05	1.55E+05	3.57E+05	0.69
2-Isopropylmalate synthase	LEU4	5.04E+04	1.58E+05	1.16E+05	5.69E+04	8.85E+04	6.22E–006
Acetolactate synthase	ILV6	8028.96	1.71E+04	9004.02	1.68E+04	1.36E+04	0.53
Aminotransferase	BAT1	4.47E+04	1.22E+05	7.27E+04	6.02E+04	9.96E+04	2.20E–01
S-adenosylmethionine synthase 2	SAM2	1.40E+05	1.64E+05	1.62E+05	1.02E+05	2.40E+05	0.3
Hem biosynthesis							
Ferrochelatase	HEM1	2.18E+04	4.84E+04	8.00E+04	8.73E+04	4.97E+04	0.44
Lipid metabolism							
Sterol 24-C-methyltransferase	ERG6	1.32E+05	1.21E+05	1.04E+05	1.05E+05	1.78E+05	0.47
Acetyl-CoA carboxylase	ACC1	1.27E+05	9.47E+04	5.81E+04	7.74E+04	1.31E+05	9.38E–004
NADP-cytochrome P450 reductase	NCP1	4.87E+04	3.36E+04	3.98E+04	3.19E+04	8.61E+04	6.36E–03

Continued

**Table 2 List of proteins identified as glutathionylated from proteomic experiments and the mean of their normalized relative abundance in the different conditions, as determined in label-free experiments (n = 3 biological replicates)**

Part 2 of 2

Protein name	Gene <sup>1</sup>	WT mitochondria					ANOVA P-value
		Not treated	Diamide	GSSG	Fe	H <sub>2</sub> O <sub>2</sub>	
Stress							
Flavoheprotein	YHB1	3.53E+05	2.43E+05	3.36E+05	1.87E+05	3.83E+05	0.48
Heat shock protein 104	HSP104	5.31E+06	5.20E+06	8.31E+06	2.59E+06	6.95E+06	0.63
Heat shock protein 78	HSP78	2.69E+04	6.05E+04	3.35E+04	3.88E+04	6.38E+04	0.12
Others							
Elongation factor Tu	TUF1	7.02E+04	6.09E+04	4.72E+04	4.99E+04	9.09E+04	0.57
Outer membrane protein porin 1	POR1	2.54E+06	4.29E+06	2.65E+06	3.36E+06	4.90E+06	0.54
ATP/ADP carrier 2	PET9	1.54E+06	1.57E+06	1.89E+06	1.48E+06	2.56E+06	6.50E−01
Mitochondrial phosphate carrier	MIR1	3.12E+05	7.75E+05	4.76E+05	5.04E+05	1.01E+06	3.10E−01
Mitochondrial import receptor	TOM40	2.58E+05	4.71E+05	2.15E+05	4.05E+05	4.60E+05	3.00E−01
Cell division control protein 48	CDC48	9.70E+04	2.14E+05	4.30E+04	2.07E+05	1.00E+05	2.80E−01
Mitochondrial import translocase	TIM44	1.10E+05	8.84E+04	1.02E+05	8.53E+04	1.29E+05	6.00E−01

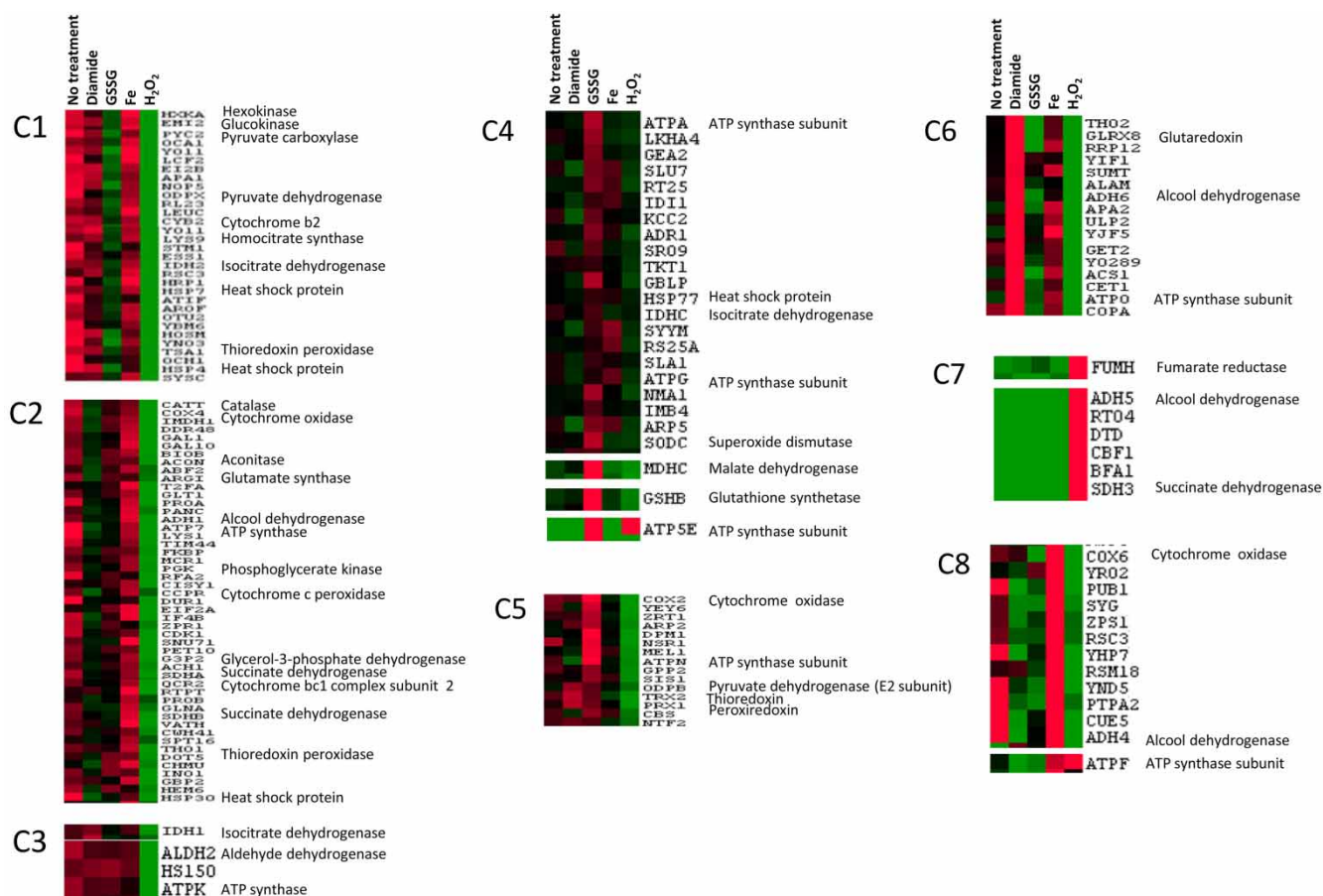
<sup>1</sup>Genes correspond to gene names found in SGD (*Saccharomyces* Genome Database)

We particularly focused on mitochondrial targets since mitochondria are central to oxidative stress and redox signaling [3,54–56].

This study required that we improve some of the crucial steps of the ‘biotin–Grx’-switch procedure initially presented in ref. [38], and we thus developed an optimal protocol for the analysis of *S. cerevisiae* cells (Figures 2 and 3). This approach has several major advantages over current approaches, including the detection of proteins that are glutathionylated under basal conditions, as no pretreatment is required. One of the biggest advantages of this procedure is its specificity due to the strict limitation of the database search of modified tagged HPDP peptides to proteins that have actually undergone glutathionylation. In addition, we were able to rigorously identify the specific oxidized cysteines within each glutathionylated protein and the sequence of each peptide is presented in Supplementary Information. This high specificity may partly explain the relatively low number of protein targets identified in our study relative to that reported in the literature. Another reason is due to the originality of our experimental approach. Indeed, instead of the usual *in vitro* treatment of isolated mitochondria or cell extract with oxidants, cells were cultured in the various oxidizing conditions to stationary phase. Under these conditions, *S. cerevisiae* cells had time to trigger metabolic changes and to adapt to the changes in intracellular redox status through protein glutathionylation and deglutathionylation.

We also explored the intracellular distribution of the glutathionylation process, and the distribution of protein targets was different in cytosol and mitochondria (Figure 5A). Although these changes have been observed in intact cells but in response to non-physiological insults, we can hypothesize that glutathionylation might play different physiological roles in the two cellular compartments. Based on the glutathione measurements shown in Figure 4, there was no significant glutathione-dependent oxidative stress in cytosol, whatever the growth conditions, but stress conditions lead to a little change in GSH/GSSG ratios, which could suggest the binding of glutathione to proteins. In the case of iron, the loss of cytosolic GSH could be due to a stimulated transport of GSH toward the mitochondria, as previously reported by our group [34]. However, the mechanism involved in protein glutathionylation is apparently not due to an increase in GSSG as the GSH/GSSG ratio remained constant under the five conditions tested (with the exception of a small decrease observed in the presence of GSSG and H<sub>2</sub>O<sub>2</sub>) (Figure 4). Inversely, severe oxidative stress was observed in mitochondria, with the GSH/GSSG ratio dropping from 30 to 4, when cells were cultured in the presence of GSSG and H<sub>2</sub>O<sub>2</sub>.

Data of Figure 5 and Table 1 show evidence of the scale of the glutathionylation process under basal conditions, particularly in mitochondria where ~30 proteins were identified in the absence of stress. This is, to our



**Figure 6. Classification of the differentially expressed proteins in WT *S. cerevisiae* cells under basal and oxidative stress conditions.** Proteins with different normalized abundances [analysis of variance (ANOVA) *P*-value lower than 0.05] were sorted by the AutoClass Bayesian clustering system (webserver AutoClass@IJM, <http://ytat2.ijm.univ-paris-diderot.fr/>) [53]. The data used for this classification are the results of three separate biological experiments. All clusters and accessions are presented in Supporting Information S2.

knowledge, the first study in yeast that emphasizes the importance of the glutathionylation process in the absence of oxidative treatment, after adaptation of the cells to new growth conditions. This modification mainly affected proteins involved in glucose metabolism and enzymes of the TCA cycle, as well as those involved in amino acid metabolism and complexes of the respiratory chain (Figure 5B). This reflects that proteins involved in cellular bioenergetics are the most sensitive to redox regulation processes. Since the oxidants were added in the growth medium, we think that the present study is closer to reflect natural events, even though not perfectly of course, than previous studies where oxidants were added to purified mitochondria or cells, and suggests that glutathionylation may play an active role in the regulation of these pathways.

The mechanisms of glutathionylation *in vivo* have not yet been completely elucidated. We know that in the absence of stress, the high intramitochondrial GSH/GSSG ratio of WT cells minimizes protein glutathionylation by thiol disulfide exchange [57]. However, transient glutathionylation of surface cysteines may indeed be an important antioxidative defense, because this process could prevent the formation of higher, irreversible oxidation states [3,58,59]. Under constitutive metabolism, glutathionylation may play a role in the regulation and/or signaling of some of the identified proteins, but may also constitute a form of stored glutathione to help maintain a high GSH/GSSG ratio. Indeed, mitochondria cannot synthesize glutathione and it must be imported from the cytosol.

We grew the cells in the presence of diamide and GSSG, which have been shown to promote glutathionylation *in vitro*, and H<sub>2</sub>O<sub>2</sub> because it has been used in many previous studies to induce oxidative stress. In addition, an increase in glutathionylated proteins has been described after exposure of HeLa cells to a sublethal dose of diamide, reaching >15% of all protein cysteines [50]. We also cultured the cells in the presence of



excess iron because we published that it induces glutathione import into the mitochondria [34]. Our approach was different from published studies because the stress molecules were added to the growth medium and not incubated *in vitro* with isolated mitochondria or cell extracts; thus, we were able to study the adaptive metabolic response of the cells.

Cells exhibit a remarkable ability to cope with high rates of ROS production because of a complex scavenging system that includes either antioxidant molecules or enzymes. We observed strikingly different patterns of glutathionylated proteins for the four sets of oxidative conditions tested in our study. However, there was no direct correlation between the number of glutathionylated targets and significant changes in protein abundances, as shown in the label-free data of Table 2. The number of detected proteins involved in glucose metabolism, such as phosphoglycerate kinase, glycerol-3-phosphate dehydrogenase, or enolase, decreased markedly under all stress conditions tested, relative to untreated cells. In parallel, we observed an increase in identified respiratory chain complex subunits and TCA cycle enzymes when cells were cultured in the presence of GSSG and H<sub>2</sub>O<sub>2</sub>, conditions in which a low GSH/GSSG ratio (~4) was observed. The exact mechanisms leading to glutathionylation *in vivo* remain unclear. The modifications are labile, difficult to control, and may affect only a small proportion of the molecules of a particular protein. However, when the GSH/GSSG ratio is low, high GSSG concentrations have been shown to promote glutathionylation through the formation of mixed disulfides by thiol–disulfide exchange between protein sulfhydryl groups and GSSG [2,6,7,60]. Low GSH/GSSG ratio also increases glutathionylation levels by limiting GSH-dependent deglutathionylation. In the presence of ROS, glutathionylation can also be mediated by a two-electron oxidation of the protein thiol to sulfenic acid followed by reaction with GSH, thereby protecting critical SH groups from irreversible oxidation to sulfinic or sulfonic acids [61]. We also characterized targets of glutathionylation in the presence of diamide or excess iron, where the GSH/GSSG ratio of 20 was slightly lower than the value of 30 for untreated cells. Such small changes in the intracellular GSH/GSSG ratio are unlikely to lead to substantial S-glutathionylation of proteins by thiol–disulfide exchange involving GSSG and protein thiols. Indeed, the reactivity of most cysteine residues is such that the ratio of GSSG versus GSH in cells would need to change considerably to induce S-glutathionylation [39,62]. Other mechanisms may be involved depending on the growth conditions or even the target protein. Several mechanisms of protein glutathionylation may be involved in parallel for each experimental condition.

Specific cysteine thiols of respiratory chain complexes have been identified as ROS targets during oxidative stress, and thiol modifications of respiratory chain complexes have been detected in many studies [21,58,63,64]. We consistently found subunits of complexes III and IV of the mitochondrial respiratory chain in our proteomic analyses, as expected. We exclusively observed subunits 1 and 7 of cytochrome *bcl* (complex III) under stress conditions, which could not be related to changes in protein abundance. Ubiquinol cytochrome *c* oxidoreductase of complex III and cytochrome *c* oxidase (complex IV) activities respond to the oxidative stress status of mitochondria. In human cells, overproduced GSSG may react with the thiol groups of these complexes [24,65]. Moreover, complex III is considered to be a key site of electron leakage and superoxide formation during oxidative phosphorylation in yeast. Taken together, these observations suggest that reversible redox modifications of respiratory chain complexes play the physiological role of protecting them from irreversible oxidative damage caused by elevated mitochondrial ROS production. The TCA cycle enzyme KGDH was found to be glutathionylated under four of the five conditions tested, in agreement with the literature, showing that the enzymatic activity of KGDH can be regulated through glutathionylation of the lipoic acid cofactor covalently bound to the E2 subunit [39,66,67]. Indeed, direct treatment of purified KGDH with GSSG leads to the glutathionylation of all three KGDH subunits. Moreover, the E1 subunit of PDH was glutathionylated under basal conditions and in the presence of diamide and H<sub>2</sub>O<sub>2</sub>, as well as the dihydrolipoamide dehydrogenase E2 subunit shared by KGDH and PDH (Table 1). A previous study has shown the modulation of pyruvate dehydrogenase activity after H<sub>2</sub>O<sub>2</sub> treatment [68]. This emphasizes the importance of glutathionylation in the regulation/protection of pyruvate dehydrogenase/KGDH complexes and its potential role in regulating the TCA cycle activity. Aconitase was also characterized in our study. The [4Fe–4S] iron–sulfur cluster of the aconitase active site is essential for catalytic activity, but also renders aconitase highly susceptible to ROS. Indeed, it has been suggested that aconitase activity is modulated by glutathionylation and that the modification of cysteine residues close to the [4Fe–4S] cluster of aconitase may impair enzyme activity [69,70]. Interestingly, for all these proteins, there was no specific sequence of peptide which could be identified as a signature for protein glutathionylation (for the sequences of all identified biotinylated peptides, see Supplementary Data).

Our study establishes the physiological importance of the glutathionylation process in the yeast *S. cerevisiae* and provides evidence for an atypical cellular distribution of the process, which reflects the different

mechanisms used by the cellular compartments to cope with oxidative stress. Moreover, our results show that each condition leads to a specific adaptive metabolic response. We also propose that protein de-glutathionylation may play an unexpected role in the regulation of the intracellular redox status and in protecting mitochondria from oxidative stress. Indeed, the acute response to a stress, associated with protein glutathionylation, may be followed by an adaptive response, in which de-glutathionylation of selected cysteine residues may play a key role in redox status regulation. Moreover, the increase in the de-glutathionylation process could also be due to an increase in glutaredoxin activity under stress conditions. Indeed, it has been described that activation of human Grx2 (inactive as an iron–sulfur complex in no stress conditions) could take place in conditions of oxidative stress, leading to an increase in the de-glutathionylation activity, and that activation of Grx2 could facilitate the regulation of specific proteins [71].

Our results suggest that the maintenance of the thiol-dependent redox status of the cells, during the adaptive response to new growth conditions, may involve the maintenance of a fragile equilibrium between both the glutathionylation and de-glutathionylation processes. In addition, we show that each growth condition elicits a specific adaptive response, implicating specific proteins involved in specific pathways, which mostly participate in the energetic maintenance of the cells. The next step will be to develop a quantitative approach to establish the level of modification of each oxidized cysteine residue and correlate the data with potential changes in the activities of the enzymes. Indeed, it is crucial to evaluate how the modification might affect the structure or function of the corresponding protein and to assess whether a sufficient proportion of the protein is affected to have a physiological impact in the cell. This would provide new insights to elucidate the importance of mitochondrial ROS production and its consequences on living cells.

### Abbreviations

GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; IAM, 2-iodoacetamide; KGDH,  $\alpha$ -ketoglutarate dehydrogenase; MMTS, S-methyl methanethiosulfonate; NEM, N-ethylmaleimide; PDH, pyruvate dehydrogenase; ROS, reactive oxygen species.

### Author Contribution

F.A. designed and conducted experiments and wrote the manuscript. J.M.C. designed experiments and analyzed proteomic data. R.G. performed experiments. C.G. performed experiments.

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### Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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