Characterization of Surface-Exposed Reactive Cysteine Residues in *Saccharomyces cerevisiae*[†]

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ABSTRACT: Numerous cellular processes are subject to redox regulation, and thiol-dependent redox control, acting through reactive cysteine (Cys) residues, is among the major mechanisms of redox regulation. However, information on the sets of proteins that provide thiol-based redox regulation or are affected by it is limited. Here, we describe proteomic approaches to characterize proteins that contain reactive thiols and methods to identify redox Cys in these proteins. Using *Saccharomyces cerevisiae* as a eukaryotic model organism, we identified 284 proteins with exposed reactive Cys and determined the identities of 185 of these residues. We then characterized subsets of these proteins as *in vitro* targets of major cellular thiol oxidoreductases, thioredoxin and glutaredoxin, and found that these enzymes can control the redox state of a significant number of thiols in target proteins. We further examined common features of exposed reactive Cys and compared them with an unbiased control set of Cys using computational approaches. This analysis (i) validated the efficacy of targeting exposed Cys in proteins in their native, folded state, (ii) quantified the proportion of targets that can be redox regulated via thiol oxidoreductase systems, and (iii) revealed the theoretical range of the experimental approach with regard to protein abundance and physicochemical properties of reactive Cys. From these analyses, we estimate that approximately one-fourth of exposed Cys in the yeast proteome can be regarded as functional sites, either subject to regulation by thiol oxidoreductases or involved in structural disulfides and metal binding.

Cysteine (Cys)[1] is a thiol-containing amino acid that can be relatively easily oxidized under conditions of oxidative stress (*1, 2*). This oxidation may lead to the formation of oxidized Cys-based products, including disulfides, sulfenic, sulfinic, and sulfonic acids, or other oxidation products (*3−8*). An additional Cys modification that has been linked to a variety of cellular processes and recently attracted much attention is S-nitrosylation (*9*). Besides changing properties of the modified Cys, S-nitrosylation may affect protein molecular surfaces via perturbation of electrostatic properties (*10*). Several Cys modifications emerged as paradigmatic cases that explain how a single event of Cys modification can regulate protein function at various levels (e.g., enzyme activity, protein−protein interactions). In recent years, an increasing number of proteins that contain modifiable Cys have been reported that, rather than being damaged by oxidative stress, act as cellular protectors or regulators (*11−19*).

In many other cases, the unwanted oxidation of Cys may result in oxidative damage, thereby modifying protein function.

Although many examples of both functional and unwanted oxidation of Cys residues are known, the overall contribution of oxidative modifications to regulation of protein functions is not clear. In part, this deficiency is due to the difficulty of identifying proteins containing reactive Cys and distinguishing true redox-regulated proteins from proteins that can be oxidized under harsh *in vitro* conditions. Under conditions of mild oxidative stress, oxidative Cys modifications can be repaired by thiol oxidoreductases, such as thioredoxin (*20*) and glutaredoxin (*21*), the two major systems for thiol reduction in eukaryotic cells. However, insufficient information exists on what specific targets of these reductants are.

Over the past decade, different proteomic approaches have been developed to determine the identity of proteins that undergo thiol modifications *in vivo*. In many cases, these methods were directed to answer specific questions, for instance, identify disulfide-bonded or glutathionylated proteins under oxidative stress conditions (*22, 23*), thioredoxin-targeted proteins in chloroplasts and *Escherichia coli* (*24−26*), or target proteins of periplasmic thiol−disulfide oxidoreductases (*27, 28*). These initial methods, however, did not address more general goals, e.g., define the global view of reactive redox thiols in proteins and analyze the fraction of these groups among all Cys residues.

Identification of proteins containing redox-active Cys is challenging. For example, typical strategies involve biochemical analyses and characterization of a limited set of proteins, known to have highly conserved Cys residues, previously selected on the

[1]Abbreviations: Cys, cysteine; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; DTT, dithiothreitol; IAM, iodoacetamide; ICAT, isotope-coded affinity tag; BIAM, biotin-conjugated iodoacetamide (*N*-(biotinoyl)-*N*'-(iodoacetyl)ethylenediamine); TRX1, thioredoxin 1; TRR1, thioredoxin reductase 1; TTR1, glutaredoxin 1; GSH, glutathione; MS/MS, tandem mass spectrometry; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; PDB, Protein Data Bank.

basis of genetic experiments (*8*). More recently, given an increased interest in redox regulation and cellular responses to oxidative stress, a variety of trapping techniques have been developed, which allowed rapid and more extensive analyses of proteins with reactive thiols (*28–31*). However, while these methods are capable of detecting and quantifying redox-regulated Cys residues in various cellular conditions, they lack an ability to directly identify the specific proteins and their redox-regulated Cys residues. Some methods work under native conditions but do not use efficient labeling techniques or did not identify specific redox-regulated Cys (*55–57*). Other recent methods, such as the OxICAT, while efficiently supporting both identification and quantification of reactive Cys, cannot work with proteins in their native conditions (*32*).

In this report, we describe identification of proteins containing redox-active Cys as well as identification of these Cys residues. An additional feature of our proteomic approach is that it was carried out for proteins in native conditions. Accordingly, we could explore the effects of relevant natural reductases, including thioredoxin and glutaredoxin, on the redox state of Cys; at the experimental conditions described in our approach, the enzymatic systems tested fully retained their activity. Altogether, our proteomic approach was capable of identifying the representative set of BIAM-reactive Cys in a eukaryotic model organism (*Saccharomyces cerevisiae*) while at the same time being able to classify them based on the response to different reductants.

Using this proteomic approach, we report 284 yeast proteins and identity 185 peptides containing at least one exposed reactive Cys. In addition, an extensive bioinformatic analysis was carried out to assess features of reactive Cys residues (e.g., dependence on exposure, hydrophobicity, abundanc). Comparing reactive Cys with an unbiased set of control Cys, we estimated the proportion of reactive and redox-regulated Cys residing on protein molecular surfaces. Apparently, approximately one-fourth of exposed Cys in the yeast proteome can be regarded as functional sites, either subject to regulation by thiol oxidoreductases or involved in structural disulfides and metal binding.

EXPERIMENTAL PROCEDURES

Materials. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), iodoacetamide (IAM), trypsin, and dithiothreitol (DTT) were purchased from Sigma; biotin-conjugated iodoacet-amide (*N*-(biotinoyl)-*N'*-(iodoacetyl)ethylenediamine (BIAM)) was from Molecular Probes, complete protease inhibitor cocktail from Roche, and monomeric avidin column kit from Pierce; and the isotope-coded affinity tag ICAT avidin affinity column and ICAT avidin affinity buffer pack were from Applied Biosystems.

Modification of TCEP-Reducible Cys in Yeast Proteins with BIAM. Wild-type (BY4741) yeast cells were grown in 500 mL of yeast extract peptone dextrose (YPD) media at 30 °C until the A_{600} reached a value of 1.2. Cells were collected by centrifugation and suspended in 10 mL of 0.2 M ammonium bicarbonate (ABC), pH of 7.9, containing a quarter of a protease inhibitor cocktail tablet; cells were then broken by beads beater and centrifuged at 15000 rpm for 1 h. The supernatant was treated with 2 mM TCEP at room temperature for 20 min, 0.1 mM BIAM was added, and the sample was incubated at room temperature in the dark for 30 min. The BIAM alkylation reaction was stopped by addition of 10 mM DTT, and the sample was further incubated at room temperature for 45 min. The excess DTT was removed by treating the mixture with 20 mM iodoacetamide (IAM) at room temperature in the dark for 30 min, and the sample was dialyzed

overnight at 4 °C against 150 mM sodium chloride and 50 mM sodium phosphate, pH 7.4 (PBS).

Modification of Exposed Cys in Yeast Proteins. Cells were prepared as described above and broken with beads beater in the dark in the presence of 0.1 mM BIAM, followed by incubation of the cell lysate in the dark at room temperature for 30 min. The insoluble fraction was removed by centrifugation at 15000 rpm for 1 h, and the supernatant was treated sequentially with (i) DTT and (ii) IAM and (iii) dialyzed as described before.

Modification of Thioredoxin 1- (TRX1-) Reducible Cys in Yeast Proteins. Yeast cells were grown in 500 mL of YPD until the A_{600} of 1, 2 mM diamide was added, and after 2 h, cells were collected by centrifugation and suspended in 15 mL of 0.2 M ABC containing 5 mM IAM, and a quarter of the protease inhibitor cocktail tablet was added. Cells were broken with beads beater in the dark, and the lysate was further incubated at room temperature in the dark for 30 min to complete the modification of accessible Cys residues with IAM. After centrifugation at 15000 rpm for 1 h, the supernatant was dialyzed against PBS and divided into two equal samples, which were diluted to 5 mL each with PBS. The first sample was incubated in the presence of 0.5 mM nicotinamide adenine dinucleotide phosphate (NADPH), 0.5 μM TRX1, and 0.1 μM thioredoxin reductase 1 (TRR1). The second sample (control) was incubated with 0.5 mM NADPH and 0.1 μM TRR1. Both samples were incubated at room temperature for 30 min and then treated with 0.1 mM BIAM for 30 min in the dark at room temperature. The excess of BIAM was removed by overnight dialysis against 200 mM ABC as described above.

Modification of Glutaredoxin 1- (TTR1-) Reducible Cys. The TTR1 samples were prepared as described for the TRX1 experiment, except that the first sample was treated with 0.5 mM glutathione (GSH) and 0.5 μM TTR1 and the second sample (control) with 0.5 mM GSH alone.

Isolation of BIAM-Labeled Proteins. Each BIAM-labeled sample, prepared as described above, was diluted to the volume of the avidin column (2–5 mL) and loaded on the column, followed by incubation for 20 min at room temperature. The column was washed with 1–4 volumes of PBS, flow-through fractions were collected, and the column was further washed with PBS until the absorbance at 280 nm returned to the baseline. Four bed volumes of the elution buffer (2 mM D-biotin, 150 mM sodium chloride, 50 sodium phosphate, pH 7.2) were then applied to elute the BIAM-modified proteins. Each sample was then concentrated using Centricon microconcentrators (Millipore), and the isolated proteins were visualized on SDS–PAGE gels by Coomassie Blue staining. The avidin column was regenerated with 6 column volumes of the low pH regeneration buffer (Pierce).

In-Gel Digestion and Protein Identification. The in-gel digestion procedure was performed as described (*33*), with minor modifications. All visible Coomassie Blue stained bands from the BIAM-modified samples were cut from gels, sliced into 1 mm pieces, destained in 50% acetonitrile and 25 mM ammonium bicarbonate, and SpeedVac-dried. An aliquot of 20 μL of the trypsin solution was added to each sample, excess of trypsin removed after 10 min, and 50 μL of 25 mM ABC added. After overnight digestion at 37 °C, the samples were spun down and supernatants analyzed by tandem mass spectrometry (MS/MS).

Isolation and Identification of BIAM-Labeled Peptides. The BIAM-labeled protein samples were dialyzed against 0.2 M ABC, the trypsin solution was added at the ratio of 1 to 50 of the total protein in the sample, and the digestion was performed overnight at 37 °C. The ICAT avidin affinity column was used to
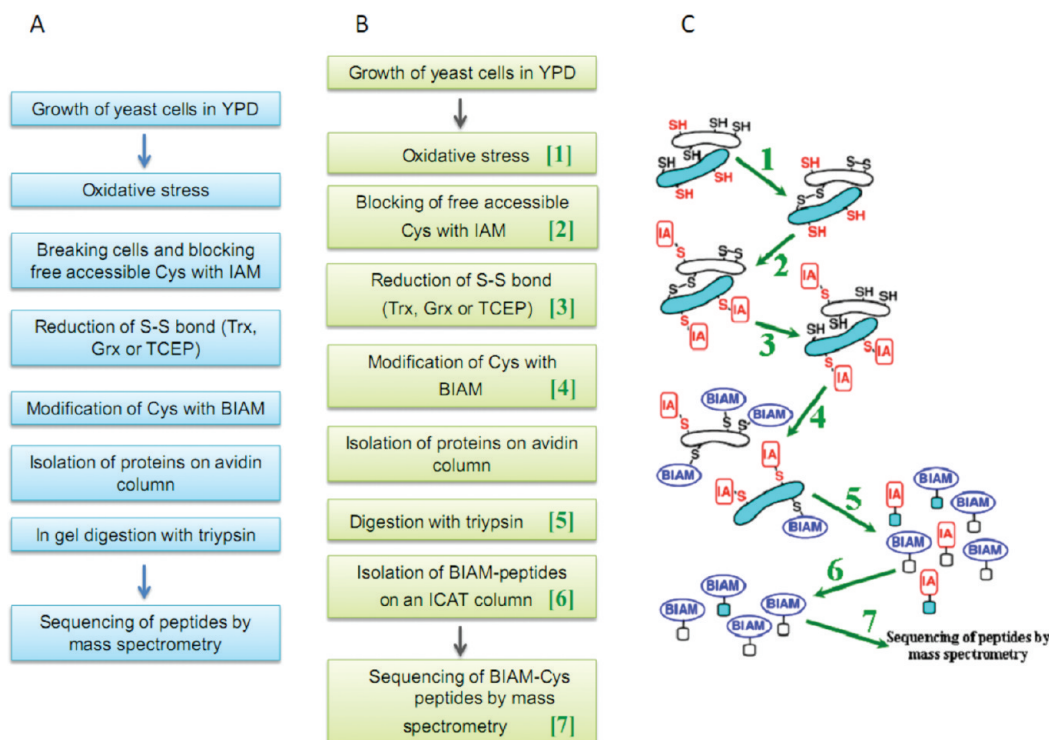
FIGURE 1: Proteomic approaches employed in the current study. Key steps in the overall procedure are shown for the detection of yeast proteins containing surface-exposed reactive Cys residues. (A) An in-gel approach. (B) An LC-MS/MS approach. (C) An alternative representation of the LC-MS/MS approach. In (B) and (C), green number labels refer to different steps in the LC-MS/MS method wherein a number in (B) corresponds to the number in (C).

purify BIAM-labeled peptides. The BIAM-modified peptides were eluted with a 120 min gradient from 10% to 90% of buffer B (0.3% formic acid, 95% acetonitrile, and 5% water) at a flow rate of 0.2 μL/min. Buffer A contained 0.3% formic acid, 95% water, and 5% acetonitrile. The eluate was collected and concentrated to 50 μL on a SpeedVac, and the isolated BIAM-labeled peptides were further analyzed by liquid chromatography−tandem mass spectrometry (LC-MS/MS) using a MicroMass Q-TOF mass spectrometer system (Waters) and/or QSTAR XL hybrid LC/MS/MS system (Applied Biosystems). The MS spectra were analyzed by Mascot (Matrix Science) against the nonredundant NCBI database, with no enzyme restriction and allowing BIAM labeling and carboxamidomethylation of Cys and sulfoxidation of methionines as variables. The other search parameters included peptide mass tolerance of ±0.15 Da, fragment mass tolerance of ±0.15 Da, and the maximum number of missed cleavages as 1. All spectra, which corresponded to possible BIAM-labeled Cys-containing peptides, were then checked manually by an experienced expert.

Detection of BIAM-labeled peptides was maximized by preparing duplicated samples that were analyzed separately by liquid chromatography−tandem mass spectrometry (LC-MS/MS) and performing several LC-MS/MS runs for each sample (typically, until no more additional BIAM-labeled peptides could be detected). Thus, four to six LC-MS/MS runs were performed for each experiment. The BIAM-labeled Cys-containing peptide sequences were further searched against the yeast genome database using BLASTP (http://blast.ncbi.nlm.nih.gov/).

Bioinformatic Analyses of Identified Cys-Containing Proteins. All analyses were made using in-house Python scripts (v2.5). After identification of proteins, we retrieved sequences with an associated experimental structure by browsing the PDB repository (http://www.rcsb.org/). When experimental models were not

available, proteins were modeled with standard homology modeling approaches using Swiss Model. As a control set of proteins, all nonredundant (percent identity between each structure lower than 70%) crystal structures for *S. cerevisiae* proteins were downloaded from the PDB repository. In total, 505 unique protein structures were analyzed and compared with reactive Cys-containing proteins detected by the proteomic approach. Exposure calculations were made with Surface 4.0 (http://www.pharmacy.umich.edu/tsodikovlab/), and hydrophobicity was calculated by implementing the Kyte−Doolittle scale of hydrophobicity. All calculations referred to exposure, composition (both at sequence and structure levels), hydrophobicity, and net charge balance were made with in-house Python scripts running in Linux environment. Abundance data for all yeast open reading frames (ORFs) were retrieved from ref *34*.

RESULTS

Targeting Accessible Cys Residues in Native Proteins by Tagged Alkylating Agents. Identification of accessible reactive Cys residues is a necessary step in understanding the mechanisms of redox regulation of cellular processes. In this work, we initially tested alkylating reagents containing affinity tags (ICAT reagent and BIAM) and found that the use of a biotinylated iodoacetamide, BIAM, resulted in both high coverage of Cys residues and sufficient affinity for further isolation of modified proteins. BIAM rapidly (<30 min) labeled reactive Cys at room temperature, whereas the ICAT reagent was less useful in that it required longer incubation times and higher temperatures (which caused partial protein precipitation and denaturation). In addition, ICAT reagent is a bulkier compound (compared to BIAM), which made fewer Cys accessible for alkylation by this reagent in native proteins.

We employed two approaches to target Cys. The first (Figure 1A) was based on the isolation of BIAM-labeled proteins on avidin
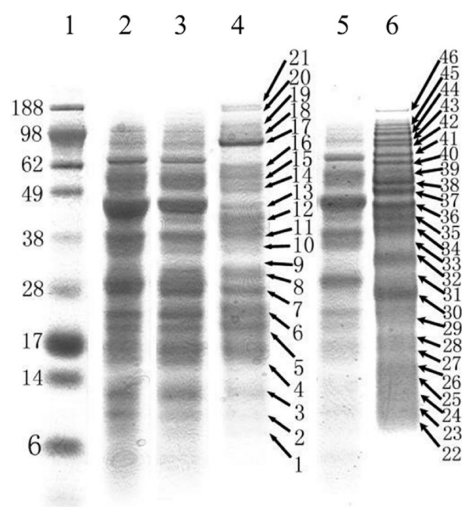
FIGURE 2: BIAM-reactive and TCEP-reducible proteins. In the TCEP-reducible sample, proteins were reduced with TCEP and then labeled with BIAM. In the sample without addition of reducing agents, cells were broken in the presence of BIAM, which reacted with exposed Cys. After isolation on an avidin column, proteins were analyzed by SDS−PAGE. Coomassie Blue stained bands were cut and identified by in-gel tryptic digestion and MS/MS. Lane 1, markers; lane 2, initial cell lysate; lane 3, unbound fraction (not biotinylated proteins); lane 4, enriched BIAM-labeled proteins after elution from the avidin column; lane 5, flow-through from the avidin column of the BIAM-labeled sample; lane 6, BIAM-labeled proteins from the avidin column.

affinity columns, followed by SDS−PAGE, in-gel tryptic digestion, and protein identification by MS/MS. Below, we refer, for simplicity, to this method as the in-gel-based approach. The second approach (Figure 1B,C) involved isolation of BIAM-labeled tryptic peptides and their identification by LC-MS/MS. This method is therefore referred to as the LC-MS/MS approach. Not only did it identify proteins containing reactive Cys but it also determined identities of these Cys residues (by identifying BIAM-modified Cys). We applied these two approaches in several variations to identify proteins containing modifiable and redox-active Cys residues in *S. cerevisiae*, here used as a model organism. Altogether, 284 proteins and 185 Cys residues were identified in the yeast proteome in this work.

Identification of Proteins with Modifiable Cys Residues in S. cerevisiae by In-Gel Analyses. In our procedures, alkylation of protein thiols was carried out at a higher than neutral but still physiologically relevant pH (pH 7.8), which allowed modification of soluble proteins containing accessible Cys. The BIAM-modified proteins were then isolated on affinity columns due to the presence of the biotin tag and analyzed by SDS−PAGE. Initially, we tested this approach by identifying Cys that were present in the reduced form in cellular lysates (Figure 2). As a first experiment, BIAM was included in the cell lysis buffer, without addition of a reducing agent, identifying 42 proteins in 21 bands. The complete list of proteins, with specific band assignment (Figure 2), is shown in Table 1. To extend the analysis to Cys which existed in the oxidized state, in a separate experiment, we pretreated cell lysates with a reducing agent, TCEP (Figure 2), before proceeding with BIAM labeling. Under these conditions, 125 proteins were detected (Table 1).

Protein Targets of Thioredoxin and Glutaredoxin in the In-Gel Analyses. Given the considerable advantage of native conditions for protein targets, we could test the efficacy of natural reductases targeting Cys, such as thioredoxin and glutaredoxin.

Our experimental system allowed exploring differences in sets of targets for these systems by identifying targets for the two major thiol-based reductants in yeast cells, in native conditions. It should be noted that since the experiments with thiol oxidoreductases were done in cell lysates, the proteins identified should be considered *in vitro* targets of these enzymes.

TRX1 is an abundant thiol−disulfide oxidoreductase with broad substrate specificity: it is present in all organisms, and its major function is to reduce disulfide bonds and other forms of oxidized Cys in proteins. To identify proteins that contain Cys reducible by thioredoxin, we (i) induced disulfide bond formation in *S. cerevisiae* by treating yeast cells with diamide, (ii) blocked the remaining reduced Cys with IAM, and (iii) reduced the diamide-oxidized Cys using the yeast thioredoxin system (NADPH, recombinant *S. cerevisiae* thioredoxin reductase, TRR1, and recombinant *S. cerevisiae* TRX1). Finally, the TRX1-reduced Cys were modified with BIAM and isolated using an avidin affinity column. Eighteen TRX1-targeted protein bands were observed on an SDS−PAGE gel (Figure 3), and subsequent mass spectrometry analysis of the in-gel-digested bands identified 60 TRX1 target proteins (Table 1).

Glutaredoxin 1 (Grx1, annotated as TTR1 in the yeast genome, and the latter annotation is used throughout the paper) is another major thiol−disulfide oxidoreductase with broad substrate specificity. However, in contrast to thioredoxin, which primarily reduces disulfide bonds in proteins, glutaredoxin is specific for glutathione-based mixed disulfides (*55, 56*). Although thioredoxin and glutaredoxin are components of different redox systems, they show partially overlapping functions. To identify cellular targets of TTR1, we performed the experiment as described above for TRX1 with the exception that TTR1 was used as a reductant. When the protein extract was treated with TTR1 in the presence of GSH, the SDS−PAGE pattern of affinity-isolated proteins revealed 16 bands (Figure 4). In-gel digestion and sequencing identified 60 proteins (Table 1).

Identification of Modifiable Cys in Yeast Proteins. So far, we presented proteins containing at least one modifiable Cys, but the identity of these Cys residues was not known. The ability to determine the precise position of reactive Cys represents a major advantage of proteomic approaches aimed at detecting reactive thiols in proteins. Indeed, the presence of the biotin tag in BIAM-modified proteins could be used not only to identify proteins but also to determine identities of modifiable Cys residues in these proteins. In the in-gel digestion-based procedure, recovery of BIAM-labeled peptides was low. Therefore, we adopted a different procedure that allowed preparation of tryptic peptides in solution, isolation of BIAM-labeled tryptic peptides, and subsequent identification of these peptides by LC-MS/MS (Figure 1B,C).

Using this technique, we extended the four sets of experiments that detected BIAM-labeled proteins to identify Cys residues in BIAM-modified yeast proteins. In total, 185 peptides containing modifiable Cys were identified. The complete list of proteins, reductants, and identified Cys is shown in Supporting Information, Table S1. Similarly to the in-gel analysis, the LC-MS/MS procedure was also allowed to operate under native conditions and thus to employ different reducing agents. We identified our LC-MS/MS positive targets and subdivided them by nature of the reducing agent controlling their redox state as follows: (i) no reducing agent; (ii) TCEP-treated; (iii) TRX1-treated; and (iv) TTR1-treated.

The LC-MS/MS analysis of the nontreated sample (prepared by including BIAM in the lysis buffer, without addition of

Table 1: List of Proteins Identified by In-Gel Analysis[a]

reducing agent	protein name (band)
none	RPL36A/B(1), RPP2A(1), TRX2(1), RPL33A/B(2), RPS31(2), RPS26A/B(3), PL13A/B(4), TSA(4), RPL12B(5), RPL11(5), AHP1(5), RPS9A/B(6), RPL17A/B(6), RPL18B(6), RPL15A/B(7), RPS5(7), RPS8A(7), RPL7A(7), RPL8A(8), RPS3(8), RPS0A(8), TEF2(8), RPL19(9), RPL10(9), RPL4A(10), RPS6B(10), ADH1(11), FBA1(12), DYS1(12), RPL3(13), ISBYSS(14), SSQ1(14), PDI1(14), TRG1(14), CDC19(15), PUR5(15), EFT2(16), HSP60(17), EFT2(18), ADE3(19), PYC1(20), FAS2(21)
TCEP	RPP1(22), RPL30(22), TRX2(22), GRX1(23), RPL3I(23), RPS10A(23), RPS12(24), MMF1(24), CPR1(25), EGD2(25), YNK1(25), GPX2(26), RPL12(26), RPS18a(26), AHP1(26), HYR1(26), MOG1(27), RPL16(27), RPL6(27), RPS9(27), TSA1(27), RPS5(28), NFU1(28), YJL055W(28), TEF1(28), TPI1(28), GPM1(28), YAL049C(28), RPS0a(29), FUR1(29), SEC53(29), Yjr096w(29), YMR226C(29), BMH1(29), IPP1(29), SPE3(29), ASC1(30), TDH3(30), ADH1(30), RPP0(30), HEM2(30), SGT2(30), SIS1(30), GLN1(31), Ydr051c(31), FBA1(31), URA4A(31), PDB1(31), PSA1(31), ASP1(32), ACT1(32), CYS3(32), ILV5(33), IMD1(33), PGK1(33), ECM33(34), GDH1(34), TEF2(34), TFP1(34), KGD2(35), SHM2(35), Yfr044c(35), GND1(35), PHO3/5/12(35), HXK2(36), LPD1(36), Yhr113w(36), ALD6(36), CDC19(37), ERG13(37), GLK1(37), IMD2/3(37), ALD5(37), ARO8(37), ADE4(38), ALD4(38), CCT2(38), TRP2(38), FRS2(38), LAP4(38), THR4(38), VMA2(39), PDI1(39), EUG1(39), HSP60(39), PDC1(39), DED81(40), SSZ1(40), ADE2(40), SSB2(40), ILV3(40), ASN1/2(40), STI1(40), SSA4(41), ECM10(41), SSA2(41), ILV2(41), TKL1(41), GRS1(41), HSC82(42), Ygl245w(42), ACO1(42), HIS4(42), SEC23(42), LEU1(42), Ynl247w(42), Eft2(43), ADE3(43), ALA1(43), PFK1(43), DLD3(44), GCV2(44), UBA1/2(44), VAS1(44), LYS2(45), ILS1(45), Cdc60(45), PYC1(45), ADE6(46), FAS1(46), FAS2(46)
TRX1	RPL30(1), TRX2(1), RPL31(2), TRX1(2), RPS19A(3), RPS20(3), RPL18(3), RPL20(4), CPR1(4), SOD1(4), AHP1(5), TSA1(5), TSA2(5), GCV3(5), HYR1(5), RPL9(5), GPM1(6), YMR226C(6), RPS3(6), YDL086W(6), PUP2(6), BMH1(7), IPP1(8), BMH28), TDH3(9), ADO1(9), TRR1(9), FBA1(10), URA4(10), ALD4(10), ERG20(10), GLN1(10), BAT2(10), ENO1/2(11), PGK1(11), ADE13(12), ADE4(12), ALD6(12), HSP60(13), ASN1/2(13), ADE2(13), PDC1(13), PGM2(13), PDI1(13), SSB1/2(14), SSA2(14), PYC2(15), SEC23(15), PFK1(15), HIS4(15), EFT2(15), ADE3(16), GCV2(16), MIS1(16), AMS1(17), PHO11(17), Dur1,2(18), GDB1(18), FAS2(18), CRH1(18)
TTR1	RPL30(1), RPP2A(1), RPS22A(2), RPS12(2), RPL22,EGD2(2), RPS20(3), TTR1(3), TSA1(4), TSA2(4), AHP1(4), RPL6(4), RPL16(4), Ymr226c(5), GPM1(5), RPS0(5), LYS7(5), MRT4(5), RPS5(5), ADH1(5), TEF2(5), RPP0(6), BMH2/1(6), SBP1(6), TDH3(7), Ydr051c7), Hem13(7), DYS1(8), Erg20(8), BAT2(8), FBA1(8), GLN1(8), URA4(8), PSA1(8), ALD6(8), CIS3(8), ENO1(9), GND1(9), CDC19(9), OYE2(9), SAH1(9), ALD6(9), PDI1(10), HXK1/2(10), IMD3(11), Glk1(11), PDC1(12), HSP60(12), PGI1(12), ASN1/ASN2(12), SSA1(13), GRS1(13), Eft2(14), ADE3(14), PYC1(14), ACO1(14), PHO3(14), FAS1(15), Dur1,2(15), FAS2(16), Pyc2(17), Fas2(17), FAS1(17), Eft2(17), ENO1(18), TDH3(19)

[a]Proteins identified in the in-gel analysis: in the "reducing agent" column, the type of reducing agent regulating the redox state of target Cys is reported. "None" in this column refers to ready-to-react thiols (i.e., free Cys with reduced functional groups −SH−, found to react with BIAM without addition of any reducing agents). Standard names (http://www.yeastgenome.org) for each protein are reported, as well as the band number (in parentheses) in which the protein was found. For "none" and "TCEP" rows, band numbering refers to Figure 2, lane 4; for "TRX1" rows, band numbers refer to Figure 3, lane 4; for "TTR1" row, band numbers refer to Figure 4, lanes 4 and 6.
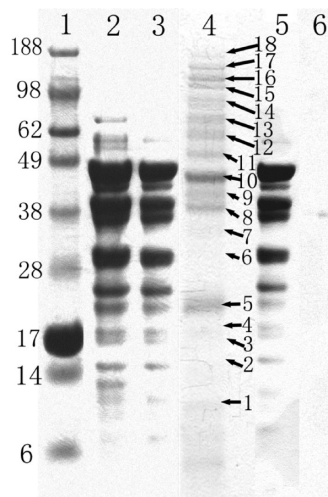
FIGURE 3: TRX1-reducible proteins. Cys residues in the lysate prepared from diamide-treated yeast cells were blocked by IAM (cells were lysed in the presence of this compound). Proteins were reduced by TRX1 and labeled with BIAM, followed by isolation on an avidin column and SDS−PAGE to visualize the target proteins. Coomassie Blue stained protein bands were labeled, cut, and identified by in-gel digestion and mass spectrometry. Lane 1, protein standards (molecular masses in kDa are indicated on the left); lane 2, cell lysate; lane 3, flow-through fraction from the affinity column of the TRX1-reduced sample; lane 4, BIAM-labeled proteins isolated on the affinity column (each number represents one protein band extracted from the gel and analyzed by MS/MS); lane 5, flow-through fraction of the control; lane 6, BIAM-labeled proteins isolated from the control group (i.e., diamide-treated and not subsequently reduced).
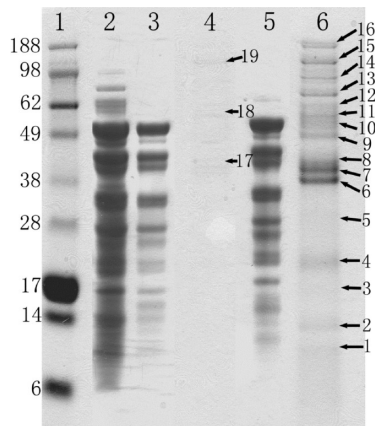

FIGURE 4: TTR1-reducible proteins. Cys residues in the lysate prepared from diamide-treated yeast cells were blocked by IAM (cells were lysed in the presence of this compound). Proteins were reduced by TTR1 and labeled with BIAM, followed by isolation on an avidin column and SDS−PAGE to visualize the target proteins. Protein bands were cut and identified by in-gel tryptic digestion and MS/MS. Lane 1, protein standards (molecular masses in kDa are indicated on the left); lane 2, cell lysate; lane 3, flow-through fraction of the TTR1-reduced sample; lane 4, BIAM-labeled proteins isolated from the control sample that lacked GSH (each number represents one band extracted and analyzed by MS/MS); lane 5, flow-through fraction of the TTR1-reduced sample; lane 6, BIAM-labeled TTR1 target proteins (each number represents one protein band extracted and analyzed by MS/MS).

reducing agents) identified 65 proteins containing BIAM-modified Cys (Table 2, "none" row). Similarly to the in-gel analysis, additional targets could be identified after treatment with TCEP (96 BIAM-labeled proteins, Table 2, "TCEP" row). Finally, when protein-based reducing systems were used, we identified

Table 2: List of Proteins Containing at Least One Redox-Reactive Cys, Identified by the LC-MS/MS Analysis[a]

reducing agent	protein
none	TSA1, MSL5, BNI5, ENO1/2, SSA1, NUP82, TRX1/3, RPL3, PEX19, NPL3, PYC1/2, YOR220W, EFT2, BAT1/2, ZTA1, KREI1, RPS21A/B, YAL049C, AHP1, DAK1, PET191, TPM2, SSB1/SSB2, DYS1, SHE3, CDC55, SHP1, GND1, YLR257W, ADE4, TTR1, FPR1, ALD5, ALD4, YMR031C, RPL4A/B, GCN1, PDI1, YDR365W-A, GCV2, LYS7, YJR119C, RPL34A/B, ZPR1, MSU1, ADH1/ADH2, ERGI10, YKL215C, FBA1, ADE3, PDC1, ALD6, SOD1, CDC19, CUPI-1/2, PRO2, PFK1, ASN1, PSA1, TEF1, RPL37B
TCEP	RPL37B, TRX2, BNI5, GLY1, ENO1/2, RPL8, RPL23A/B, SSA1, IDI1, YMR226C, RPL2, VAS1, FRS2, PEX19, NPL3, TFP1, MAP1, RPS2, RPS31, SHM2, PYC1/2, ARC40, EFT2, PMI40, ILV5, DED81, ECM39, RGR1, AHP1, RPS22A/B, RPL9, AP11, RPL7, RPL22, DYS1, FRT1, YNL247W, RPL12A/B, GND1, YLR257W, ADE4, YBR267W, FAS1, TDH3/1/2, GUA1, FAS2, FPR1, GCV2, ALD4, RPL4A/B, SSB1/SSB2, IDP1, CYR1, HIS1, MPD1, PDI1, IMD2/3, TRX1/3, THR4, EMI2, RPS8A/B, RPLI9, YNL134C, LYS7, ADH3, RPS0A/B, RPL15A, HAP1, POL2, BMH1/2, VPSI5, GRS1, ASC1, MSU1, TRR1, STP1, RPSI17A/B, SAM4, ADH1/ADH2, ERG10, PHOI11/3/5/12, ADE6, FBA1, ADE3, PDC1, ALD6, PDI1, CDC19, CUPI-1/2, PRO2, PFK1, ASN1, PSA1, TEF2, RPSI11A/B, TSA1
TRX1	BNI5, ENO1/2, RPL23A/B, SSA1, ACT1, STI1, PRX1, YSA1, HXK2, RPS8A/B, EDE1, TRX1/3, EPSI, RPS21A/B, AHP1, HXK1/2, ECM33, SSB1/SSB2, ERG20, GLK1, PMI40, TRR1, ADE4, RPS27A, APE3, FAS2, HEM13, CIS3, HYP2, DAPI, YAR1, HIS1, SBP1, TDH3/1/2, THR4, RPL12A/B, PDX3, SSE1, HOM2, FMP12, GASI, DLD3, ADE6, GIS2, ARO4, HYR1, FBA1, ADE3, PDC1, ALD6, PDI1, CDC19, PRO2, PFK1, ASN1, HIS4, TEF2, PSA1, ACO1, PHOI11/3/5/12, TSA1, SOD1
TTR1	DRS1, BNI5, SSA1, ACT1, RPS0A/B, HXK2, TRX1/3, PMI40, RPS21A/B, HXK1/2, SSB1/SSB2, DYS1, ADE4, SOD1, ILV5, TTR1, RPL4A/B, HIS5, RPL10, RPL11, TDH3/1/2, TRX2, GPX2, ADH1/ADH2, PHOI11/3/5/12, ADE6, GIS2, HYR1, FBA1, ADE3, TSA1, ALD6, PDI1, CDC19, PSA1, PFK1, TEF2, ACO1

[a]Proteins identified in the LC-MS/MS analysis: table legend is that of Table 1.

SH* + TCEP targets

ADE3	PYC2	KRE11
ADE4	QNS1	LYS7
ADH1	RPL10	MSL5
ADH2	RPL11	MSU1
ADH3	RPL12A	NPL3
AHP1	RPL12B	NUP82
ALD4	RPL13	PDC1
ALD5	RPL15A	PDI1
ALD6	RPL17	PET191
ASN1	RPL18	PEX19
BAT1	RPL19	PFK1
BAT2	RPL3	PRO2
BNI5	RPL33	PSA1
CDC19	RPL34A	PYC1
CDC55	RPL34B	RPS8B
CUP1-1	RPL36	RPS9
CUP1-2	RPL37B	SHE3
DYS1	RPL40B	SHP1
EFT2	RPL4B	SOD1
ENO1	RPL7	SSA1
ENO2	RPL8	SSB1
ERG10	RPP2	SSB2
FAS2	RPS0A	SSQ1
FBA1	RPS0B	TEF2
FPR1	RPS21A	TFP1
GCN1	RPS21B	TPM2
GCV2	RPS26	RPL40A
GND1	RPS3	IMD2
HSP60	RPS31	IMD3
RPS6	RPS5	ISBYSS

TRX1 targets

PUP2	YAR1	ARO4
PRX1	GAS1	PGM2
RPS19A	PDX3	DAP1
CRH1	EDE1	HYP2
APE3	GCV3	YSA1
AMS1	HOM2	ADO1
RPS27A	SSE1	MIS1
YDL086W	EPS1	ADE13
GDB1	RPL20	FMP12

TRX1 and TTR1 targets

HXK1
ERG20
SBP1
TSA2
GIS2
CIS3
HEM13
DUR1,2

TTR1 targets

SAH1
DRS1
OYE2
HIS5
MRT4

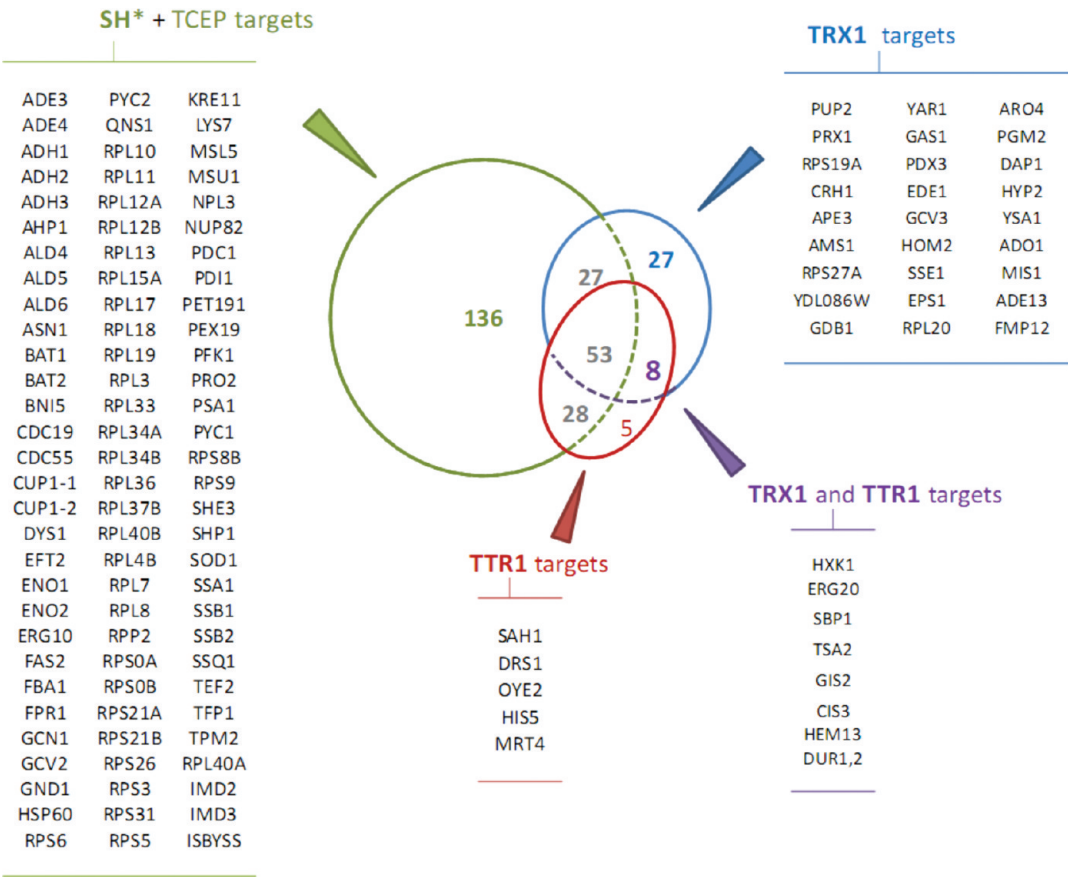Venn diagram values: 136, 27, 27, 53, 8, 28, 5

FIGURE 5: Functional categories of redox target proteins. Proteins identified in all experiments, after removal of redundancy, are shown. They are subdivided according to the reducing agent capable of their reduction; ready-to-react thiols (i.e., targets detected by BIAM in cell lysate without addition of any reducing agent) and TCEP-reducible are labeled as "SH + TCEP targets" in the figure; TRX1-reducible targets are labeled as "TRX1 targets", and TTR1-reducible proteins are shown as "TTR1 targets". The complete lists of proteins targeted exclusively by TRX1 or TTR1 are provided (blue contoured and red contoured tables, respectively). Additionally, among the set of "SH + TCEP targets", the list of proteins containing "ready-to-react" thiols (labeled as "SH", i.e., BIAM-reactive Cys in the absence of reducing agent) is provided.

62 proteins in the TRX1 experiment (Table 2, "TRX1" row) and 34 proteins in the TTR1 experiment (Table 2, "TTR1" row). Detailed information about different thiol oxidoreductase-regulated Cys targets found (e.g., protein names, peptide sequences, response to different reductants) is provided in Supporting Information, Table S1.

Proteins with Redox-Regulated Cys Residues in S. cerevisiae. Combining results from the approaches described so far, we classified each Cys target on the basis of its response to different reducing agents. A schematic representation of these results (after filtering out redundancy) is shown in Figure 5.

Among the targets detected, 90 proteins were found to contain Cys capable of BIAM reactivity in the absence of reducing agent (i.e., these proteins present "ready-to-react" Cys residues, capable of BIAM reactivity without pretreatment with TCEP or TRX1 or TTR1). The identity of each of these proteins is listed in Tables 1 and 2 ("none" rows) and summarized in a graphical form in Figure 5.

Among redox-regulated targets, a considerable number of specific targets for one or both thiol oxidoreductases was found. Altogether, we found 115 targets for TRX1 and 94 targets for TTR1. Therefore, these oxidoreductases could regulate slightly more than half (148 out of 284, or 52.1%) of BIAM-reactive thiols detected. Additionally, considering only redox-sensitive Cys (i.e., BIAM-reactive Cys reducible via any of the reducing agents used, Tables 1 and 2), the proportion increased to 58.5% (as potential targets of either thioredoxin or glutaredoxin).

The majority (78.5%) were not unique targets for TRX1 or TTR1, being reducible with two or more of the sampled reducing agents. However, quite remarkably, in our analysis up to 27 proteins were found to be targeted uniquely by TRX1, while 5 uniquely by TTR1, and 8 additional proteins were targeted by both proteins (but not by TCEP). A complete list of these unique proteins is provided in Figure 5, separated by the reducing agent. Indeed, these numbers are quite high, indicating that a vast number of Cys targets may be subjected to enzymatic regulation of their redox state, and major thiol reductants significantly contribute to maintaining Cys residues in cellular proteins in the reduced state.

Additionally, the broad specificity of both thioredoxin and glutaredoxin systems was confirmed by our analysis. It is likely that the number of redox Cys could increase if additional thiol oxidoreductases (i.e., in addition to TRX1 and TTR1) are employed. In this regard, an important feature of practical use of our proteomic approach is that it allows, without substantial modification of the proteomic approach, to employ a variety of reducing agents (e.g., DTT, TCEP, reductases) and to test them. A critical analysis about the identity and physiological implications of detected targets is provided in the Discussion section, as well as a comparative discussion with relevant literature in the field.

Properties of Reactive Cys-Containing Peptides: Common Features. An open question relevant to proteomic approaches is the coverage of targets identified. Is there bias
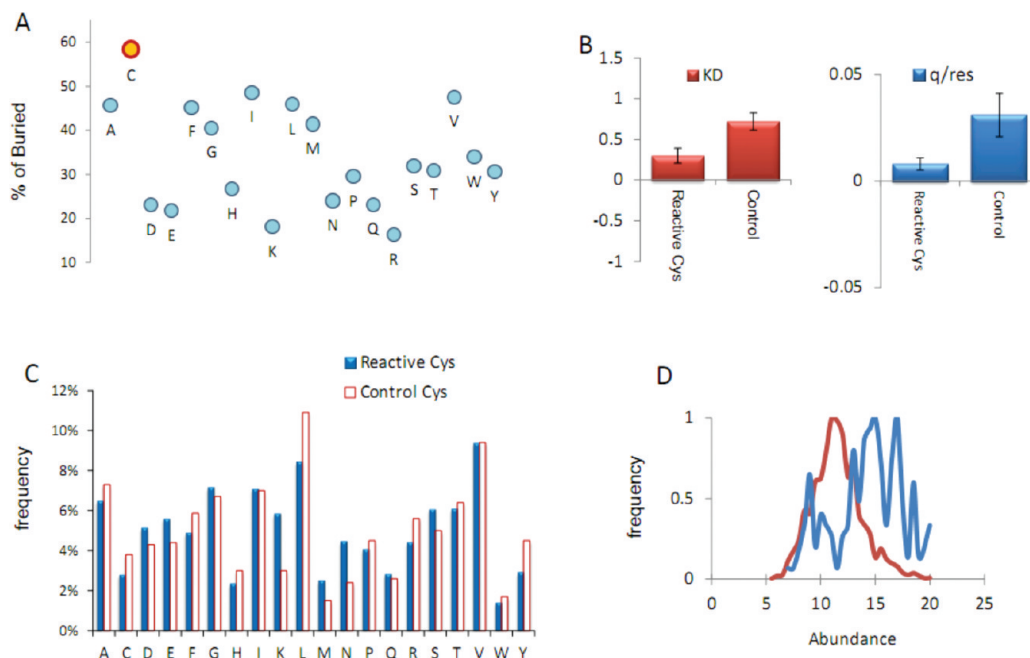
FIGURE 6: Common features of detected Cys-containing peptides. (A) Percentage of buried residues for each amino acid type in a set of 505 yeast protein structures. Cys proved to be a mostly buried residue (~60% of Cys had a whole residue exposure <0.1 Å^2). In contrast, 80% of Cys detected in our analysis were accessible to solvent. (B) Average Kyte−Doolittle (KD) score per residue (red bars, with standard error of mean) and average net charge per residue (blue bars, with standard error of mean) are shown for detected reactive Cys (left red bar and left blue bar, respectively) and control Cys (blue bars). (C) Amino acid composition around reactive Cys (6 Å) (blue bars) compared to control (red contoured bars). Average frequency (from 0 to 1) of each amino acid is shown in the Y-axis. (D) Protein abundance for proteins with reactive Cys residues (blue line) and control proteins (red line). Abundance (X-axis) is in the logarithmic scale (log$_2$); frequency (Y-axis) is normalized to the highest. The average abundance for yeast proteins containing BIAM-reactive Cys was 70736 molecules/cell, while the overall abundance for all yeast ORFs was 12064 molecules/cell, representing an approximately 6-fold increase.

introduced by the method (i.e., restrictions in terms of preferential features of detected peptides)? As a first consideration, due to the intrinsic features of our experimental approach, all peptides share the following sequence-based features: (i) length restriction (detected peptides ranged from 5 to 29 residues, with the vast majority (>98%) being ≤26 residues long) and (ii) positively charged residues (Lys or Arg) for trypsin cleavage, which must be present in proximity to Cys (e.g., at the C-terminus of each peptide). Thus, some reactive Cys lacking a convenient tryptic site would not be detected by our approach. We then conducted bioinformatic analysis based on the common features of our peptides (Supporting Information, Table S1), at both the sequence and structural levels. We retrieved structural information about the proteins containing the identified Cys by PDB searches and homology modeling. In particular, among our peptides, 63 could be assigned to known experimental structures and 51 could be modeled. Overall, 114 structures were analyzed. As a control, we used a set of 505 nonredundant (identity among each pair of proteins in the data set ≤70%) *S. cerevisiae* crystal structures downloaded from the PDB (hence, the control PDB set contains 63 proteins with redox-reactive Cys detected in our experiments). A first feature characterizing BIAM-reactive Cys residues is their accessibility to solvent: 80% of Cys in our peptides were found to be at least partially exposed (>0.1 Å^2). However, in the control set, the majority of Cys were buried (~60%, Figure 6A). Therefore, the enrichment in Cys exposure was considerable in our experimental set. Thus, this analysis indicated that we indeed targeted surface-exposed Cys and worked with proteins in their native state (i.e., for folded proteins, the vast majority of BIAM-reactive residues were expected to be solvent accessible).

We then analyzed structure-based chemical and physical features of reactive Cys: amino acid composition, hydrophobicity, and an average net charge per residue, within 6 Å from reactive Cys. The average amino acid composition around reactive Cys resembled that of the control set of proteins (yet with consistent underrepresentation of aliphatic residues, Figure 6C). In turn, reactive Cys showed lower average hydrophobicity and lower variation in the sample (average values and standard errors are in Figure 6B). At the same time, reactive Cys were characterized by a more strict control of net positive and negative charge balance: many charged residues did not appear to favor Cys reactivity with the slightly hydrophobic BIAM. Altogether, our data depict an ideal BIAM-reactive Cys in native proteins as characterized by a narrower range of both hydrophobicity and net charge, when compared to the control set of Cys. In addition to chemical−physical features, we examined protein abundance of detected targets. For this, we analyzed reactive Cys-containing proteins against a yeast protein abundance data set (34). As expected, our detected peptides were enriched with regard to abundant proteins (Figure 6D), with the majority (>95%) of BIAM-reactive Cys belonging to proteins with abundance higher than 200 molecules/cell (~7.65 in the Z-axis value in Figure 6D). This indicates that targets with very low abundance (e.g., proteins present in fewer than 100 or 200 copies per cell) are likely to be missed by the approach. These data highlight a limitation of most current state-of-the-art proteomic approaches, and it cannot be excluded that some proteins with very low abundance can indeed play a role in cell redox regulation. Therefore, future efforts aimed at further improving the detection limits of proteomic approaches in the field of thiol redox regulation would be desirable.
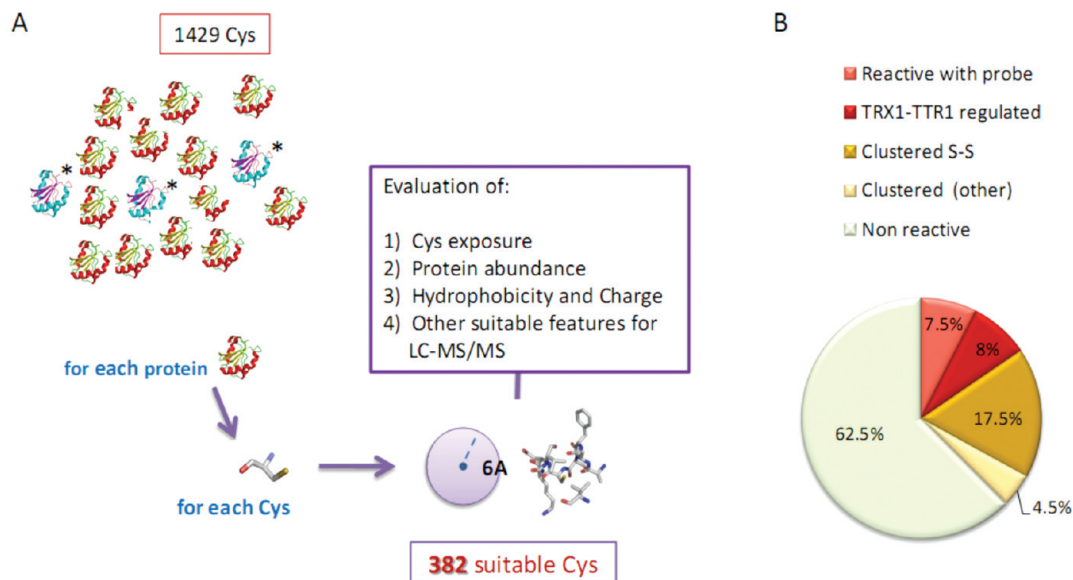
FIGURE 7: Proportion of reactive Cys in the yeast proteome. (A) Theoretical approach that was used to screen an unbiased set of yeast protein structures (depicted in ribbons) to detect reactive thiols, defined as having features that characterize the experimentally detected peptides (see Figure 6 and text). In our structural test case, some proteins had Cys detected by the BIAM-based proteomic approach (marked with an asterisk in the figure), and some were not. By screening the test case for the Cys exposure, hydrophobicity, net charge balance, and abundance, we detected 382 Cys among 1429 Cys residues analyzed. (B) A pie graph showing the proportion of detected BIAM-reactive Cys (reactive with probe, red shaded slices) among all theoretically derived Cys. BIAM-reactive Cys found to be regulated via thioredoxin and/or glutaredoxin systems are shown in dark shaded red. Among other BIAM-reactive Cys (light shaded red slice), the majority (92%) were regulated via TCEP; thus, the fraction of non-redox-regulated BIAM-reactive Cys would be a narrow subslice of ~1% (not shown), within the "reactive with probe" section. Exposed and clustered Cys (yellow shaded slices) are further divided into two groups, where the dark shaded slice represents a theoretical estimation of interacting proximal Cys (disulfides or metal binding Cys; details are provided in the text). The remaining Cys (labeled as "nonreactive") were not identified in our proteomic analysis.

Quantification of the Proportion of Reactive and Redox-Regulated Cys in Yeast Proteins. We combined the features of reactive Cys into a simple sampling algorithm, illustrated in Figure 7A. Our experimental set was screened against an unbiased data set of yeast protein structures (i.e., nonredundant *S. cerevisiae* PDB structures). In this approach, in order to be classified as a potential target, a Cys site needed to satisfy the following criteria: (i) Cys exposure ($> 0.1 \text{ Å}^2$); (ii) hydrophobicity (Kyte−Doolittle score, KD, in the range −1.27 to 1.89, i.e., average KD ± 2 times the standard deviation, SD, in Figure 6B); (iii) net charge balance (net charge per residue in the range −0.052 to +0.068); (iv) a putative Cys in position X should have a Lys or a Arg residue close in the sequence, such that tryptic digest would produce a peptide with an overall length in the range 5−26); (v) the protein containing the Cys should be sufficiently abundant (i.e., ≥200 molecules/cell). All of these parameters, taken separately, describe the vast majority (>95%) of our detected Cys (the cutoff values reported were derived from results shown in Figure 6; the average value ±2 times the standard deviations was used).

Thus, in order to objectively compare how many BIAM-reactive Cys were detected among all potential targets, we first evaluated how many reactive Cys (i.e., true positives) were missed by the algorithm. As noted in the previous section, 63 reactive Cys-containing peptides (70 Cys overall, as some peptides had more than one Cys) were represented by known experimental structures, and 52 of them (which had 60 Cys) were found to be selected by our algorithm (82.5%); 11 Cys-containing peptides were missed.

At the same time, when all 505 protein structures in our control data set of yeast structures were analyzed, 382 Cys (in 355 peptides, as some had more than one Cys) residues were predicted to

be suitable for BIAM modification (i.e., satisfying all above-described criteria).

On the basis of this approach, we estimate that our study detected ~15.5% of Cys (dark and light red shaded slices in Figure 7B) amenable for alkylation by BIAM. Of these residues, the large majority (92.2% of BIAM-reactive Cys) were redox-sensitive (i.e., reducible by one or more reducing agent used), with approximately half of them (52.1% of BIAM-reactive Cys, corresponding to ca. 8% of all exposed Cys, Figure 7) showing thiol oxidoreductase-dependent regulation. At a closer look, this proportion (i.e., 15.5% of reactive Cys) is remarkably high, especially if one considers that a significant number of Cys are probably involved in long-lived disulfides (e.g., structural disulfides) or metal binding; obviously, these Cys thus would not be available for chemical modification with BIAM. Approximately 22% of the remaining exposed Cys (i.e., exposed Cys which were not BIAM-reactive) were found in clusters (defined as Cα to Cα distance for two Cys lower than 8 Å), as shown in Figure 7B (dark and light yellow shaded portions). It is currently believed that the majority of these clustered Cys (up to 80%) would be involved in stable functional interactions with nearby Cys or with metals (*35, 36*).

If a comparable proportion also applies to *S. cerevisiae*, adding up (i) the proportion of thiol oxidoreductase-regulated Cys (dark red shaded portion in Figure 7B) and (ii) the estimated proportion of functionally active clustered Cys (dark yellow shaded portions), approximately one-fourth (~25%, the sum of dark red and dark yellow slices) of all exposed Cys in *S. cerevisiae* ought to be considered functional sites in yeast proteins, as defined by being either reactive and redox-regulated by thiol oxidoreductases in native conditions, or clustered Cys involved in stable functional interactions (structural disulfides and metal binding).

DISCUSSION

Features of the LC-MS/MS-Based Proteomic Method. Many proteomic studies that examine proteins containing reactive and/or redox Cys are gel-based. For example, a common method to investigate target proteins of thiol−disulfide oxidoreductases is to immobilize mutants of this protein class (e.g., SxxC or CxxS active site forms of thioredoxin) on a resin and perform affinity chromatography. The purified proteins could then be visualized on SDS−PAGE or 2D gels, followed by in-gel digestion of protein bands for protein identification (*37*). Alternatively, one can modify reactive Cys with tagged thiol-reactive reagents. These reagents then make the modified protein visible on gels, subsequently allowing its identification (*38*).

However, these methods are labor-intensive as they require extraction, digestion, and handling of multiple protein spots (or bands). Furthermore, false negatives and false positives are inevitable with these methods, because some highly reactive, but low abundance proteins cannot be detected on gels, whereas abundant proteins that lack redox Cys are often detected due to nonspecific binding. Compared with available gel-based methods (including the method described in this article), our LC-MS/MS method showed a better performance and required less expertise in handling the samples. It was also less labor-intensive. The separation of BIAM-labeled molecules from the nonlabeled ones was highly efficient, because two avidin column steps were performed in each experiment. In addition, the HPLC procedure efficiently separated BIAM-labeled from the nonlabeled peptides due to relative hydrophobicity of BIAM.

Samples of BIAM-labeled tryptic peptides could be concentrated (up to 10^4 times, through the entire procedure as shown in Figure 1), allowing detection of proteins with relatively low abundance. However, the key feature of the method was its ability to identify the actual redox Cys. Using this new technique, we could identify protein targets rapidly (∼4 days) and provide exact information about the actual reactive Cys.

Although additional mass spectrometric approaches allowing identification of reactive Cys (as well as quantification of corresponding peptides) were recently developed (*32*), an important feature of the LC-MS/MS method described in the present work is the ability to work with native proteins.

Our approach not only permits the identification of specific redox-reactive thiols but also ensures that the same detected Cys is likely to be reactive in the native protein. Thus, exploitation of targets of different protein-based reductants (e.g., thioredoxin or glutaredoxin) could be carried out, an important feature of our method in the panorama of mass spectrometric methods for the identification and characterization of reactive Cys. Several previous reports described methods capable of dealing with proteins in native conditions (*55−57*). However, our study presents significant differences: (i) the usage of BIAM; (ii) the ability not only to identify the protein but also to specify the identity of BIAM-reactive Cys; (iii) the possibility to classify the reactive Cys on the basis of different reducing agents.

We made use of the above-mentioned features of our proteomic approach to (i) detect and estimate how many BIAM-reactive Cys are present in *S. cerevisiae*, (ii) identify the targets for two major cellular reductants (TRX1 and TTR1), (iii) characterize most relevant biological, chemical, and physical features of detected Cys sites, in order to specifically define potential critical factors limiting the reactivity between some Cys targets and BIAM, and (iv) quantify the proportion of reactive Cys detected by the method and the overall potential Cys targets, using as a test case an unbiased set of yeast protein structures. We found a total of 284 protein targets and identified 185 peptides containing reactive Cys. A considerable fraction of these 284 BIAM-reactive Cys-containing proteins could be targeted in their native conditions by thioredoxin (54 proteins, Figure 5), glutaredoxin (33), or both (61). Altogether, oxidoreductases showed the ability to regulate slightly more than half (52.1%) of all reactive thiols detected, a high proportion which is likely to further increase if other thiol oxidoreductases (besides the two major systems used in this work) are to be employed. Indeed, in this regard an additional key property of the method resides in the ability to potentially employ a wide range of reducing agents, without requiring substantial modification of the proteomic approach (see the Experimental Procedures section for protocols for different reducing agents tested in this work).

Redox-Regulated Proteins and Pathways. An analysis of previously published reports against our data set revealed several redox-regulated proteins targeted by thiol oxidoreductases (the oxidoreductase is indicated in parentheses): TDH3 (TRX1 and TTR1), ENO2 (TRX1 and TTR1), ALD6 (TRX1 and TTR1), FBA1 (TRX1 and TTR1), ADH1 (TTR1), ACO1, EFT2 (TRX1, TTR1), PGM2 (TRX1), THR4 (TRX1), ILV5 (TTR1), PGM2 (TRX1), ACT1 (TRX1), BMH1 (TRX1), HXK1 (TRX1, TTR1), GLN1 (TRX1), and SAH1 (TTR1) (*37, 39−46*). Additionally, many proteins regulating redox homeostasis, such as thioredoxin reductase, thioredoxin, glutaredoxin, protein disulfide isomerases, and superoxide dismutases, were detected. Moreover, for targets for which the exact identity of the redox-regulated Cys was known (e.g., Cys150-XXX-Cys154 in TDH3 or the catalytic Cys in thiol oxidoreductases) we could correctly detect this Cys.

Overall, the most abundant redox-regulated (i.e., via TRX1 and/or TTR1) targets found in our analysis are involved in protein biosynthesis and degradation (33%), including many ribosomal proteins. However, no mitoribosomal proteins were identified, indicating that while cytosolic protein synthesis appears to be extensively redox-regulated, mitochondrial protein synthesis is unlikely to be subject to redox control through thiol−disulfide oxidoreductases. Other pathways represented by the proteins detected in our analysis were (i) the TCA cycle, carbon metabolism, and glycolysis (18%), (ii) response to stress (14%), (iii) amino acid metabolism (12%), (iv) nucleotide biosynthesis (8%), (v) structure assembly and organization (5%), (vi) protein folding (8%), and (vii) tRNA ligase/synthase (4%).

As a further step in the analysis of proteins detected in our study, the ability of the LC-MS/MS approach to identify redox-sensitive Cys allowed predictions to be made in terms of protein function and regulation. In particular, this work revealed a set of proteins for which redox regulation was not previously reported. For example, CIS3 are proteins of the cell wall, which are covalently linked to the wall via specific disulfide bonds (*47*). Previously, it was shown that mutation of Cys214 in CIS3 considerably reduced incorporation of Pir4 in the cell wall (*48*). Interestingly, Cys214 (fully conserved in homologous proteins) was identified in our set as both TRX1- and TTR1-regulated, and therefore, our results suggest a redox-dependent step in the control of yeast cell wall synthesis through a specific modulation of the redox state of a Cys residue in CIS3. Another example is POL2. A previous report revealed that Cys2130 and Cys2133 are essential for its function (*49*). Interestingly, only these two Cys (Supporting Information, Table S1) were identified in our

LC-MS/MS experiments, suggesting redox regulation of POL2, and accordingly the DNA polymerase function, through these Cys residues.

A third example involves SOD1 and its molecular metallochaperone LYS7, which were shown to form a transient disulfide bond through Cys57 (SOD1) and Cys229 (LYS7) (*50−52*). Cys57 is initially reduced, while Cys229 binds a copper atom. The transient intermolecular disulfide between the two is a crucial step in SOD1 activation which requires the delivery of copper by LYS7 and the formation of a stable intramolecular disulfide bond Cys57 and Cys146 of SOD1. We found that the participants in this system are susceptible to regulation via thiol oxidoreductases, an intriguing perspective in terms of a specific, enzyme-dependent modulation of this antioxidant enzyme. The reactive Cys in SOD1 can be reduced by either TTR1 or TRX1, while the Cys in LYS7 was only found in the TTR1 data set.

Another example is CPR1, which was previously reported to possess two disulfide pairs (Cys53−Cys170 and Cys128−Cys175), regulated by thioredoxin in *Arabidopsis thaliana* (*53*). In our analysis, we found CPR1 as a TRX1-regulated target in *S. cerevisiae* (Table 1). However, yeast CPR1 lacks both Cys170 and Cys175 (*A. thaliana* numbering), and the only Cys conserved between plant and yeast proteins is Cys53. However, at a closer look on the available crystal structure of yeast CPR1 (PDB code 1IST), Cys53 appears to be isolated (i.e., the only other Cys in the protein, Cys128, is more than 20 Å away). Therefore, our data, while confirming CPR1 as a TRX1-regulated enzyme, suggest a different mechanism of oxidation of Cys53.

Many other interesting candidate redox-regulated proteins emerged from our analysis. For example, GAS1, besides its glucanosyltransferase activity, has recently been assigned a role in transcriptional silencing (*54*). GAS1 was found in our proteomic study to be redox-regulated by TRX1 through Cys265 (Supporting Information, Table S1). To better assess the potential functional implications of the modification, we built a homology model for this protein (the template structure used was yeast GAS4, PDB code 2w61, sequence identity 47%) and found that Cys265 is located within a disulfide bond distance (2.1 Å, sulfur to sulfur distance) to Cys234. This candidate disulfide is solvent accessible (particularly exposed is Cys265) and bridges two unstructured coils connecting two internal β-strands with two external α-helices of the (β/α) barrel of the protein. Reduction of this disulfide would most likely lead to a local destabilization and rearrangement of the relative positioning of these two coils as the region surrounding Cys234 and Cys265 is predominantly negatively charged, and electrostatic repulsion could occur between these two unstructured regions following disulfide reduction. The structural changes could then bring a redistribution of overall properties (e.g., electrostatic potential, solvent-accessible area, and molecular shape) of the molecular surface. In this scenario, it is likely that both protein function and the spectrum of protein−protein interactions would be affected. The latter is potentially relevant with regard to GAS1 role in transcriptional silencing, as it has been shown that GAS1 interacts with Sir2, a member of the sirtuin deacetylase family, which promotes chromatin silencing via direct targeting of Lys residues in histone H4. Thus, on the basis of our results, we can speculate on a mechanism for redox-regulated transcriptional silencing via GAS1, wherein Cys265 acts as a redox sensor, the reduction of the Cys234−Cys265 bond serves as a redox switch promoting or inhibiting the interaction with Sir2, and with other

interacting factors which are involved in chromatin silencing. Similarly, parsing our list of candidates (Tables 1 and 2 and Supporting Information, Table S1) several other interesting cases could be analyzed and critically discussed; however, we leave these speculations to the reader's attention, as they are beyond the present scope of our work.

As a final point of discussion about detected targets, a specific comparison of our data reveals agreement with previous studies by Toledano and co-workers (*58, 59*). These studies are considerably different from the proteomic approach described here (e.g., acid denaturation was involved, TTR1 was not directly tested as reducing agents). Also, they analyzed and compared protein thiol oxidation in yeast wild-type and mutant (for the thioredoxin system) cells. Specifically, 64 proteins with redox-reactive Cys were positively identified (*58*), 40 of which (SOD1, TSA1, AHP1, TSA2, GPX2, PRX1, TRR1, PDI1, SSA2, SSE1, SSB1, STI1, HSP60, HXK2, TPI1, TDH3, ENO1, ENO2, PGK1, FBA1, ALD6, ADH1, PDC1, TKL1, ACO1, SEC53, PSA1, SHM2, LEU1, HOM2, GDH1, ILV5, ADO1, IPP1, TEF1, RPL12A, RPS0A, RPS5, ASC1, CPR1) were also detected in our study.

The 64 proteins reported (*58*) included those found to be highly oxidized in thioredoxin-deficient cells (i.e., proteins found to be overoxidized in the absence of the thioredoxin system): SOD1, CCS1, MXR1, PEP4, PDI1, TSA1, TSA2, AHP1, PRX1, and GPX2. These proteins were suggested as targets of the thioredoxin system under conditions of oxidative stress. SOD11, TSA1, TSA2, AHP1, and PRX1 were also identified in our study as direct targets of the thioredoxin system. Additionally, we found GPX2 to be regulated by TTR1, but not by TRX1 (Tables 1 and 2). Finally, CCS1, PEP4, and MXR1 were not found to be TRX1 targets. Due to major differences in methodology, some variability in the results is expected: this is particularly true in the case of TRX1 targets. In fact, whereas our present work identified potential TRX1 targets by employing an enzyme, previous analysis identified potential TRX1 targets by detecting proteins with overoxidized Cys in the absence, at a genetic level, of the whole thioredoxin system (*58*).

Evaluation of Chemical−Physical Features of a Typical Detected Cys. We designed and conducted bioinformatic analysis of detected peptides to determine which peptides had the salient features characterizing a typical detected Cys. Several criteria, including exposure (Cys needs to be on or near the molecular surface), abundance (target proteins need to be at detectable concentrations, higher than 200 molecules/cell), and overall polarity and net charge, were applied. Analyzing these features, we addressed a more general question: how many reactive Cys are present in the yeast proteome? Using an unbiased set of yeast protein structures as a reference, we estimated the proportion of BIAM-reactive Cys in the set (i.e., Cys belonging to peptides discovered in our proteomic analysis) compared to the overall number of potential targets (i.e., all Cys in the unbiased set of structures having favorable features, as defined by our previous analysis of peptides, for BIAM reactivity in our assay). We found that the percentage of detected Cys among all potential targets was 15.5%. This number is quite high, especially if one accounts for the fact that many other exposed Cys (~20%) cluster in the form of disulfides and metal-coordinating Cys and therefore would not be available for chemical modification with BIAM.

In relation to this work, the most interesting questions would be the following: How many functional Cys sites reside in protein

molecular surfaces? How many of them are redox-regulated in native conditions? In order to address these issues, we performed an evaluation of the proportion of redox-reactive functional Cys in yeast: considering (i) BIAM-reactive Cys found to be specifically redox-regulated via a naturally occurring thiol reductant (i.e., thioredoxin or glutaredoxin, but not TCEP or DTT) and (ii) clustered Cys likely to be involved in structural disulfides. We estimated that approximately one-fourth (25.5%) of all exposed Cys in *S. cerevisiae* ought to be considered functional sites in native conditions, as defined by being either reactive and specifically redox-regulated via a thiol oxidoreductase system or clustered with other Cys and involved in stable functional interactions. Among these functional Cys, ca. one-third are targets of either the thioredoxin or the glutaredoxin systems, or both.

In summary, the proteomic study described in this work represents an improvement over current approaches in the investigation of redox-regulated Cys targets in native conditions: due to its design, a wide range of reducing agents can be tested, and the exact nature of the regulated Cys can be determined. Moreover, allowing the exploration of different protein-based reductants, our approach is fit for high-throughput analysis aiming at determining the specific sets of targets for each thiol oxidoreductase.

However, in spite of the many advantages, several potential limitations of the methodology should be understood and considered while interpreting the results:

(i) The nature of the alkylating agent invariantly introduces bias: some Cys residues may not support its reactivity (e.g., Cys surrounded by many charged residues or solvent-accessible Cys which are not easily accessible to bulky reagents).

(ii) While our work presented an effective approach for testing the susceptibility to different reducing agents and different thiol oxidoreductases, the enzymatic assays were conducted in cell lysates; therefore, at least in some cases, targets may not represent real regulatory sites under *in vivo* conditions (e.g., while they are definitely *in vitro* targets, they might not be *in vivo* regulatory sites, as recently suggested in the case of TRX1 regulation of PRX1 (*60*)).

(iii) Limitations in terms of the ability to detect low abundance proteins (e.g., less than 200 molecules per cell, according to our estimation) with regulatory sites. Therefore, it cannot be excluded that some of such proteins can play important roles in the regulation of cellular redox balance, and thus as also pinpointed in previous similar studies (*58*), future efforts should aim to increase the sensitivity of redox proteomic studies to low abundance protein targets.

Similar limitations are also common to the vast majority of proteomic studies (particularly with regard to bias introduced by alkylating agents and inability to detect low abundance targets). However, they would be much less critical if a simple quantitative analysis of the results is performed, as has been done in this work. Here, we performed computational analysis and theoretical evaluation, with the goal of defining suitable conditions for BIAM-Cys reactivity and for the detection of proteins using the LC-MS/MS approach. We believe that with the *a priori* information (abundance cutoff, chemical–physical features unfavorable for the reaction with the alkylating agent, etc.), many caveats can be accounted for: this should help in the direction of a more objective evaluation and critical discussion of the results obtained with future experiments.

SUPPORTING INFORMATION AVAILABLE

Table S1 as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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