Materials and Methods
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1. Strains and media used

Strains of *Saccharomyces cerevisiae* used in the studies were Y190 (MATa gal 4 gal 80 his 3- Δ 200 trp 1-901 ade 2-101 ura 3-52 leu 2-3,112 URA3::GAL1-LacZ LYS2::GAL4 (UAS)::HIS3 cyhR, wild type) and isogenic Δ Yhb1 (Y190 yhb1::G418R) cells. These are generous gift from Dr. Jonathan Stamler [Liu et al., 2000]. BY4742 (MATα his 3- Δ 1 leu2- Δ0 lys2- Δ0 ura3- Δ0) and isogenic Δ Rpn10, Δ Pre9 strain were generous gift from Dr. S. Sirkar, Assistant Professor, Bose Institute, Kolkata.

The cells were grown in Yeast Peptone Dextrose (YPD) broth [1 % yeast extract (Difco), 2 % bactopeptone (Difco), 2 % dextrose] at 30 °C under shaking condition. For growth in synthetic complete media yeast nitrogen base (0.67% w/v, 2% dextrose with necessary supplements) was used. Yeast carbon base (1.17% w/v without amino acid or ammonium sulphate, 2% dextrose with necessary supplements) was used to grow Y190 cells of *S. cerevisiae*. For isolation and screening of petite mutants, YPG broth [1% yeast extract (Difco), 2% bacto peptone (Difco), 3% glycerol, pH- 7.0] was used. Δ Yhb1 strains were grown in presence of 200 mg/L G418 sulphate (Amresco, Ohio, USA).

2. Measurement of cell growth

For growth measurement experiments, mid-log phase culture was used as inoculum following dilution in fresh media to O.D. 600 nm = 0.07-0.10. Cell growth was monitored tubidimetrically by measuring the absorbance at 600 nm at every two-hour interval.

3. Preparation of S-nitrosoglutathione (GSNO)

1 M aqueous solution of sodium nitrite (NaNO₂) and 1 M solution of reduced glutathione (GSH) in 1 N HCl (1:1, v/v) were mixed placed on ice in the dark to obtain GSNO solution. GSNO concentration was calculated using $\varepsilon_{335\text{ nm}} = 586 \text{ M}^{-1} \text{ cm}^{-1}$ [Hart, 1985].
4. Preparation of peroxynitrite

Peroxynitrite was synthesized from amyl nitrite and hydrogen peroxide [Uppu and Pryor, 1996]. Briefly, 2 M solution of H₂O₂ in 2 M NaOH containing 2 mM EDTA was stirred vigorously with amyl nitrite (Lancaster Chemicals, England) (4:1, v/v) for 3 hours on ice. The aqueous layer was separated and washed thrice with ice-cold hexane. Then the aqueous layer was stirred with activated manganese dioxide (MnO₂) for 30 minutes on ice to remove excess hydrogen peroxide. Finally, it was filtered, aliquotted and stored at –80 °C. Peroxynitrite concentration was calculated using extinction coefficient ε₃₀₂nm = 1670 M⁻¹ cm⁻¹.

5. Preparation of sodium nitroprusside (SNP) stock solution

Sodium nitroprusside (Sigma-Aldrich, St Louis, MO, USA) stock solution (0.5 M) was prepared in MilliQ water in a light protected tube, just prior to use each time.

6. Cell cycle analyses by flow cytometry

Standard flow cytometric methods with propidium iodide for S. cerevisiae were followed. Ethanol fixed cells stored at 4 °C were processed for flow cytometry by taking about 2-3 x 10⁶ cells from each of the samples. The cells were precipitated by centrifugation at room temperature at 3000 rpm for 3 min and the supernatant was discarded. The pellet was re-suspended in 0.5 ml of 50 mM sodium (Na) citrate buffer (pH 8.0) to re-hydrate the cells and again centrifuged to pellet down the cells and the supernatant was discarded. Finally, each pellet was re-suspended in 0.5 ml of 50 mM Na citrate buffer (pH 8.0) containing 0.1 mg/ml RNaseA (Thermo Fisher Scientific, USA) and incubated in a water bath maintained at 37 °C for 2 h. The DNA from the RNaseA treated cells was stained with propidium iodide (MP Biomedicals, USA) keeping a final concentration of 20 μg/ml. Ten thousand cells were analyzed for each sample using the Becton–Dickinson FACSria III and data were analyzed using FACSDiva (version 6.1.3).
7. Growth phase specific protein nitration determination

Y190 cells of *S. cerevisiae* were grown in different growth media as per the experimental requirement up to log phase of growth. For determining growth phase specific protein nitration, *S. cerevisiae* cells were withdrawn at different phases of growth and cells were subjected to lysate preparation followed by SDS-PAGE analysis and Western blotting experiments with monoclonal anti-3-nitrotyrosine antibody. BY4742 (MATα his 3- Δ 1 leu2- Δ0 lys2- Δ0 ura3- Δ 0) and isogenic Δ Rpn10, Δ Pre9 strains of *S. cerevisiae* were grown in synthetic media to follow the growth of those strains. To initiate protein misfolding 0.1 mM canavanine (Sigma-Aldrich, St Louis, MO, USA) was added at the time of inoculation of BY4742, Δ Rpn10, Δ Pre9 strains of *S. cerevisiae* in synthetic media and the cells were allowed to grow up to ~ 0.5 O.D.

8. Construction of petite mutants

Petite mutants of *S. cerevisiae* were generated according to Goldring et al., 1971. For petite mutant construction, mid log phase cells were incubated in dark at 30 °C with 10 µg/ml concentration of ethidium bromide for 24 hours. It was then plated in YPD agar plates. The colonies unable to grow in presence of glycerol, a non-fermentable carbon source were isolated. It was regrown in YPD medium and re plated in YPD agar.

9. Cell lysis and protein isolation

Single colony of *S. cerevisiae* was grown in YPD medium under shaking condition at 150 rpm for overnight at 30 °C. A very small amount of overnight grown inoculum of both the strains was inoculated in fresh YPD media so that the initial O.D. of the culture was 0.1. The inoculated cultures were grown under shaking condition at 150 rpm for 12 hours. Both the cultures reached at 15 O.D. which was the mid log phase of *S. cerevisiae* growth. Mid log phase cells of Y190 and ΔYHB1 of *S. cerevisiae* were collected following centrifugation at 5000 rpm for 5 minutes. Cells were washed twice in PBS followed by washing in double distilled water and was kept on ice. Temperature was maintained at 4 °C throughout the lysate preparation. Yeast cells were lysed using glass bead lysis method. Cells were suspended in 10 mM Tris-HCl with protease inhibitor
cocktail (Sigma). Acid washed glass beads were added and were subjected to vortex for 1 min followed by immediately keeping the lysate in ice for 1 min. This cycle was followed at least six times until sufficient numbers of cells were found to be lysed. Protein concentrations were measured using Bradford assay [Bradford, 1976]. Samples were lyophilized and stored at -20 °C for further LC/MS analysis.

10. Enrichment of nitroproteins using fluorinated carbon tags

The nitrated peptides were selectively enriched via a recently developed method using fluorinated carbon tags [Kim et al., 2011]. Briefly, digested peptides were acetylated by excess sulfo-NHS-acetate (Thermo Scientific, Rockford, IL, USA) for inhibition of free amine groups. Acetylated peptides were treated with sodium hydrosulfite (Sigma-Aldrich) to convert 3-nitrotyrosine to 3-aminotyrosine. The reduced peptides were dissolved in 250 mM sodium bicarbonate (pH 8.5) and mixed with 250 mM N-succimidyl-3-perfluorobutyl propionate (Fluorous Technologies, Pittsburgh, PA, USA). The resulting peptides were captured by FluoroFlash® silica gel (Fluorous Technologies) and eluted with 100% methanol after extensive washing with 30% methanol in 10 mM ammonium formate.

11. Identification of nitrated peptides in Catalase tryptic digest

To identify nitrated peptides, Maxis Impact (Brukers Daltonik, Germany) and software were used to search tandem MS spectra against the NCBI reference database for forward sequences. The tolerance was set to 15 ppm for precursor ions, 1 Da for fragment ions, and one missed trypsin cleavage site was allowed. Oxidation of methionine (+15.99492 Da) and fluorinated carbon tagging of tyrosine (+289.01489 Da) were searched as differential modifications, whereas carbamidomethylation of cysteine (+57.02150 Da) and acetylation of the N-terminus and lysine (+42.01057 Da) were searched as static modifications.
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12. **Western Blot analysis**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [Laemmli, 1970]. Twenty micrograms of protein was separated in 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein was partially transferred to poly vinylidene difluoride (PVDF) membrane (200 mA, 1 hr.) membrane according to standard protocol using wet transfer (Bio-Rad Laboratories Inc., Hercules, CA, USA) apparatus. PVDF membranes were blocked overnight using blocking buffer (0.019 M Tris, 0.136 M NaCl, 0.1 % V/V Tween 20 and 3% W/V nonfat dry milk) at 4 °C. Membranes were then probed with anti-3-nitrotyrosine monoclonal antibody (Abcam, Cambridge, UK) or anti nitrotyrosine monoclonal antibody (Sigma) at 1: 2000 dilutions in TBST (0.019 M Tris, 0.136 M, 0.1 % V/V Tween 20). The membranes were washed three times in TBST for 10 minutes for each wash. After that the membranes were probed with a HRP conjugated goat anti mouse IgG antibody at 1:5,000 dilutions for 30 minutes at room temperature. After that membranes were washed for six times in TBST and six times in TBS (0.019 M Tris, 0.136 M NaCl) for 10 minutes for each wash. Then the immunopositive spots were visualized by using chemilluminiscent reagent (Thermo Scientific Pierce, Rockford, IL, USA) as directed by the manufacturer.

13. **Identification of proteins by mass spectrometry from 2D-DIGE**

Protein spots were excised from the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) gels and digested in-gel according to the manufacturer’s protocol using in-gel tryptic digestion kit, Thermo Scientific, Meridian Road, Rockford, USA. Mass spectra were collected on an MALDI-TOF/TOF mass spectrometer (AB Sciex), and protein identification (ID) was performed using AB Sciex Protein Pilot software, version 3.5, using the MASCOT (Matrix Science, Boston, MA, USA) algorithm and MS DB. Peptide mass fingerprinting (PMF) data obtained from the MS /MS scan served for protein identification. Protein function and localization were identified using Saccharomyces Genome Database (SGD). The identification of a protein was accepted if the peptides (mass tolerance of 100 ppm) covered at least (20–30) % of the complete sequence.
14. Immunofluorescence microscopy

Immunofluorescence microscopy was performed following a modified protocol of Pringle et al., 1989. In brief, 10 ml log phase *S. cerevisiae* culture was taken, washed with 50 mM phosphate buffer, pH 6.5 (solution A). For fixation of the cells, they were resuspended in 5 ml phosphate buffer with 3.7% formaldehyde and kept at room temperature for 1 hour. It was then washed twice with 1.2 M sorbitol in 50 mM phosphate buffer, pH 6.5 (solution B). Permeabilization of the cell wall was done by resuspending the cells in 1 ml solution B and adding Lyticase enzyme (300 U/ml, Sigma) in presence of 2-mercaptoethanol. It was incubated at 37 °C for 1 hour when high proportions of cells were spheroplasts. Immunofluorescence microscopy was performed using anti-3-nitrotyrosine antibody (mouse monoclonal, 1:100 dilutions, Abcam). Alexa Fluor 488 goat anti-mouse IgG (Molecular Probe, Life Technologies) was used as secondary antibody at 1:300 dilutions. For control experiment, primary antibody was preincubated with 5 mM 3- nitrotyrosine (Sigma) for 15 mins and then the mixture was added to spheroplast cells of *S. cerevisiae*. Immunostained cells were double-stained with DAPI (1 mg/ml). Light intensity and exposure times were kept constant for a given set of experiment and collection modalities for Alexa fluor 488 green fluorescence (excitation 495 nm; emission 519 nm).

15. Sample Preparation for LC

An amount of 200 µg of each protein sample was solubilized in 0.1 % Rapigest (w/v Rapigest in 50 mM ammonium bicarbonate). The solution was concentrated to 100 µl using 3 kDa spin column and heated to 80 °C for 30 min. Samples were reduced with 0.5 mM DTT at 60 °C for 30 min followed by alkylated with 5 µl of 200 mM iodoacetamide for 30 min in dark at room temperature. All the samples were digested with trypsin, where trypsin to protein ratio was maintained at 1:50 for 4-hour incubation at 37 °C and with additional incubation with trypsin at 1:50 ratio for overnight at 37 °C. The sample protein digests were acidified to hydrolyze Rapigest and to stop trypsin activity with 2 µl formic acid (37 % w/v). Tryptic digest was mixed with standard protein digest and used for nano UPLC/ESI QToF HDMS analysis using Synapt G2 HDMS.
16. LC-MS\textsuperscript{E}

For nano HPLC analysis, a NanoACQUITY UPLC/ESI QToF HDMS\textsuperscript{E} with SYNAPT G2 HDMS system configured for conventional 1D chromatography was used. Analytical reversed-phase column used was NanoACQUITY C18, 1.7 µm, 75 µm x 200 mm, ethylene bridged hybrid (BEH), WATERS. For peptide trapping, a trapping column NanoACQUITY UPLC column, Symmetry C18 5 µm, 180 µm x 20 mm, WATERS, was used. Mobile solvents were 0.1% formic acid in water as buffer A and 0.1% formic acid in acetonitrile as buffer B. Injection volume was set at 1 µl. Peptides were eluted from the column in a 60 min linear gradient going from 99% buffer A to 1% buffer B in 60 mins. The flow rate during elution was set at 300 nl/min and the analytical column temperature was set at 37 °C. All samples were analyzed in triplicate. For all measurements, the mass spectrometer was operated in the positive-ion mode with a typical resolving power of at least 10,000 full-width half-maximum. Accurate mass LC-MS data were collected in high definition MSE mode at low energy using low energy of 4 eV and for elevated energy ramping from 15-40 eV, switching every 0.8 sec. The total acquisition time was 60 mins for the mass range 50-2000 m/z. The lock mass and window was set at 785.8426/0.25 Da. Raw data was imported to WATER’S PLGS software and processed using low energy threshold of 100 counts and higher energy threshold of 30 counts. Intensity threshold was set at 500 counts.

17. Data processing, protein identification and quantification

LC-MS data were processed and searched using ProteinLynx Global Server version 2.3 (PLGS 2.3) (Waters). Raw data sets were processed and peak lists generated based on the assignment of precursor ions and fragments based on similar retention times as described previously [Silva et al., 2006; Shen et al., 2009]. S. cerevisiae UNIPROT databank was used to search each triplicate run with the following parameters: peptide tolerance was 10 ppm and 20 ppm for fragment tolerance; trypsin missed cleavages, 1; fixed modification, CAM, Acetyl N-TERM, Oxidation (M), Deamidation (NQ), Nitric oxide NO (CY). Label free quantitation was performed using peak intensity measurements in Waters ExpressionE, which is part of PLGS 2.3. Quantitation is done assuming the intensity response under ESI conditions of the three most intense peptides intensities observed in low collision energy mode in a triplicate set is a function of the molar amount infused in
the mass spectrometer. For protein quantification, data sets were normalized using the PLGS “autonormalization” function and clustering software included in PLGS 2.3. The absolute amount of every identified protein is determined as a ratio of the ‘Hi3’ peptide intensity of the protein of interest to that of the ‘Hi3’ peptide intensity of a spiked internal standard as reference. Only those proteins identified in at least two of three injections was used as significant change in protein abundance. All proteins whose abundances were significantly different between samples were manually assessed by checking the matched peptide and replication level across samples which may be due to highly similar protein isoforms.

18. Statistical analysis

For each quantification, the p-value was calculated for the log2 transformed values by using independent sample t-test. The p-value was calculated based on the normal distribution of the ratios. Proteins with a p-value below 0.05 were considered as statistically significant.

19. Functional annotation

The significantly regulated proteins were analyzed using Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7) functional annotation tool [Huang da et al., 2009; Huang da et al., 2009]. Functionally enriched gene ontology (GO) terms were visualized in semantic space using SimRel functional similarity measure [Schlicker et al., 2006] and the REViGO online visualization tool [Supek et al., 2011] modified with the Cytoscape version 3.1.1. Protein interaction networks were built using the online database resource Search Tool for the Retrieval of Interacting Genes (STRING) [Franceschini et al., 2013] which are visualized by Medusa [Hooper and Bork, 2005], a Java application for visualizing and manipulating graphs of interaction. The interactions include direct (physical) and indirect (functional) associations derived from genomic context, high-throughput experiments, co-expression, and literature mining.
Results and Discussions