SUPPLEMENTARY INFORMATIONS

SUPPLEMENTARY METHODS

1. Other yeast strains

CM3260 (*MATα, trp1-63, leu2-3, 112 gcn4-101, his3-609*), and isogenic Y18 (aft1::TRP1) and Y18*aft2Δ* (*aft1::TRP1*, *aft2:: kanMX4*) were described (Blaiseau et al, 2001); CKY263 strain and isogenic derivative *ero1-1* (CKY598) are gift from Chris Kaiser (Massachusetts, USA) (Frand & Kaiser, 1998); *fet3Δ*, *grx3Δ* and *grx4Δ* are from the EUROSCARF collection; *grx3Δgrx4Δ* was constructed by integrating *URA3* at the *GRX4* locus of *grx3Δ*; *gsh1Δgrx3Δgrx4Δ* was constructed by integrating *LEU2* at the *GSH1* locus of *grx3Δgrx4Δ.*

2. Growth conditions for the microarray analyses

The response to toxic GSH levels was established by comparing the mRNA profiles of HGT1 cells grown in the presence of 50 μM GSH during 5 min, 30 min, and 4 hrs to the same cells grown without GSH. GSH was added to the culture media when cells were in the exponential growth phase (OD_{600} ~ 0.3). The response to GSH depletion was established by comparing the mRNA profiles of *gsh1Δ* cells grown in SD medium lacking GSH to the same cells grown in the presence of 1 μM GSH, which corresponds to the minimum GSH concentration supporting a wild-type growth of *gsh1Δ* cells (not shown). To achieve GSH depletion, *gsh1Δ* cells were grown in YPD overnight, re-inoculated at an OD_{600} = 0.1 in SD medium containing 1 μM GSH and grown up to the stationary phase. Cells were then washed, re-inoculated at an OD_{600} = 0.1 in SD medium containing (reference sample) or not (experimental sample) 1μM GSH and grown for either 3 or 6 divisions.

3. Microarray statistical analyses

Raw data sets from the 5 experimental conditions were combined and normalized by the Lowess algorithm method. Principal Component Analysis (PCA) and Condition Tree based on Hierarchical clustering were performed using the unfiltered data sets. Average log ratios were calculated, since each condition had four (2 replicate x 2 dye swaps) slides. Data from the three and six division GSH depletion samples were highly similar (supplementary fig S2, A and B) and were therefore treated as replicate samples of the same condition. Genes were considered statistically differentially expressed when flagged as present in 2 out of the 20 arrays (that is present in at least one replicate), with statistically significant differences established by the 1-way test ANOVA, family-wise error rate of 0.05 and multiple testing corrections (Bonferroni-Holm, step-down method). These two criteria identified 961 genes in the 4 conditions (one for the *gsh1*Δ cells, three for HGT1 cells), out of which those with an expression change of ≥ 2 fold were considered induced or repressed. A lower cutoff of ≥ 1.7 fold was used for the 5 min HGT1 condition.

4. Primers used for quantitative RT-PCR

FET3 (TCAGCATGCCTTCATTCCTACCG, ACCGGCAAAGCAGGAGAATGTC), *KAR2* (TGATAACTTTGAAACCGCCATTG, GTAATTGGATAAGCGACCTTGGA), *ACT1* (CTATTGGTAACGAAAGATTCAG, CCTTACGGACATCGACATCA).

5. Calculation of the GSH redox potential

E_{GSH} was calculated by the Nernst equation at 30 °C: $E_{GSH} = E^{0'}_{GSH}$ - 60.1 mV/2 log $[GSH]^2/[GSSG]$, using the recorded GSH and GSSG concentrations in mM/cell, and the standard redox potential ($E^{0'}_{GSH}$) of GSH at pH 7.0 (-240 mV) (Schafer & Buettner, 2001).

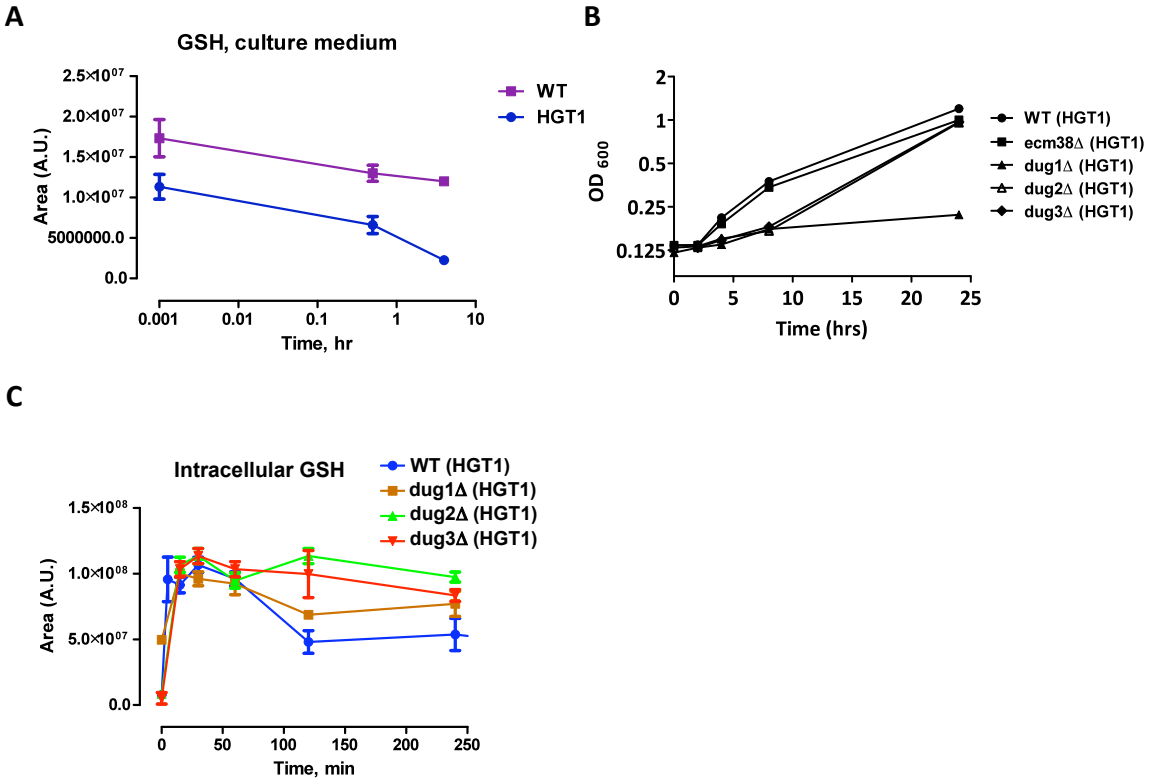
SUPPLEMENTARY TABLES LEGENDS

Table S1. Genes induced/repressed in HGT1 cells after 5 min exposure to 50 μM GSH
Average normalized ratios are in relation to HGT1 cells grown in the presence of GSH (50 μM) during 5 min *versus* HGT1 cells grown in the absence of GSH. Values are averages of 2 biological and technical experiments including dye swaps between experimental (with 50 μM GSH) and reference (without GSH) samples. Genes with average expression ratios ≥ 1.7 were considered up regulated, while those with average expression ratios ≤ 0.59 down regulated.
*Yeast genes induced during ER stress generated by DTT and tunicamycin in either or both of the two microarray-based mRNA profiling studies performed (Kimata et al, 2006; Travers et al, 2000).
‡Genes repressed during ER stress (Kimata et al, 2006).
Aft1p-dependent genes are indicated in bold letters; Aft2p dependent genes are indicated in italics; Aft1p and Aft2p co-regulated genes are underlined (Rutherford et al, 2003).
Genes present in more than one functional category are indicated with grey letters.

Table S2. Genes induced/repressed in HGT1 cells after 30 min exposure to 50 μM GSH
As table S1, except that HGT1 cells grown in the presence of GSH (50 μM) during 30 min, genes with average expression ratios ≥ 2 were considered up regulated, while those with average expression ratios ≤ 0.5 down regulated.

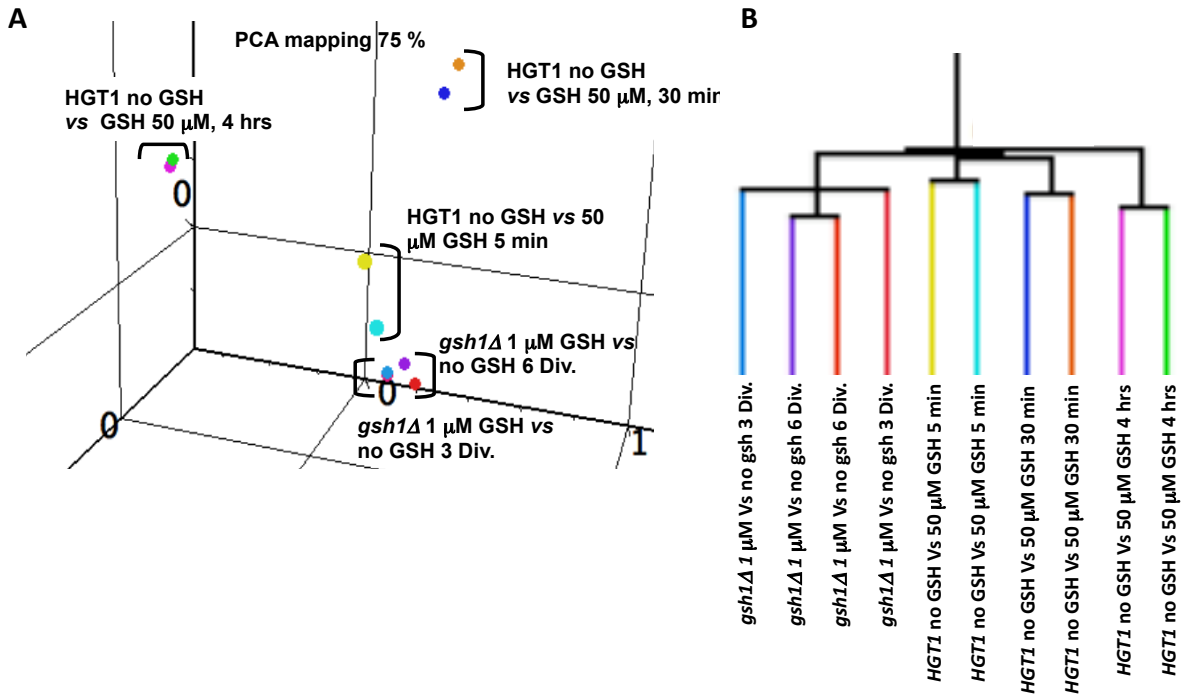
Table S3. Genes induced/repressed upon GSH depletion.
Average normalized ratios are in relation to *gsh1Δ* strain grown in the absence of GSH for 3 and 6 divisions versus *gsh1Δ* grown in the presence of 1μM GSH. The transcript profiles were established using the pooled 3 and 6 division GSH-withdrawn duplicate samples that were highly similar (see supplementary fig. S2). Values are thus averages of 8 biological and technical experiments including dye swaps between experimental (without GSH) and reference (1 μM GSH) samples. Genes with average expression ratios ≥ 2 were considered up regulated, while those with average expression ratios ≤ 0.5 down regulated.
Aft1p-dependent genes are indicated in bold letters; Aft2p dependent genes are indicated in italics; Aft1p and Aft2p co-regulated genes are underlined (Rutherford et al, 2003).
*Aft1p/Aft2p-regulated genes regulated by Yap1p (Wheeler et al, 2003).
Genes that are present in more than one functional category are indicated in grey letters.

SUPPLEMENTARY FIGURES LEGENDS



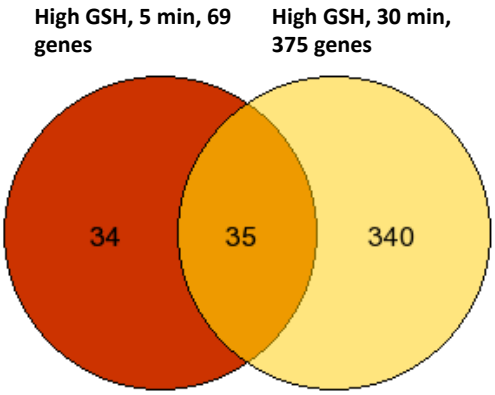
Kumar C. et al. Supplementary fig. S1

Figure S1. GSH is rapidly degraded in HGT1 cells. **(A)** WT and HGT1 cells were grown in SD medium to an OD_{600} = 0.3-0.4 at which time GSH (100 μM) was added. 1 ml culture taken before GSH addition, or 30 or 240 min after, was filtered rapidly through a 0.2μM filter. The filtrate was used for GSH estimation by direct introduction mass spectrometry on a LTQ-orbitrap Discovery (Thermo Fisher Scientific) mass spectrometer, as detailed elsewhere (Godat et al). Values are the mean of three independent samples. Error bars correspond to means ± S.D. The values on the y-axis are arbitrary units (*AU*), and correspond to the surface areas of GSH peaks. **(B)** WT, *dug1Δ*, *dug2Δ dug3Δ* and *ecm38Δ* (γ-glutamyl transpeptidase) overexpressing HGT1 were re-inoculated in fresh SD medium at an OD_{600} ~ 0.1, and grown for 1 hr before adding 50 µM GSH; growth was recorded by measuring the OD_{600} at regular intervals. **(C)** WT, *dug1Δ*, *dug2Δ* and *dug3Δ* were grown in SD medium in the presence of 100 μM GSH, and the concentration of GSH was determined by direct introduction mass spectrometry on a LTQ-Orbitrap (Godat et al). Samples preparation is as described for the GSH estimation by LC-tandem MS. Values are the mean of three independent samples. Error bars correspond to means ± S.D. Y axis values are arbitrary units (*AU*) that correspond to the surface areas of the GSH peaks extracted from mass spectra.



Kumar C. et al. Supplementary fig. S2

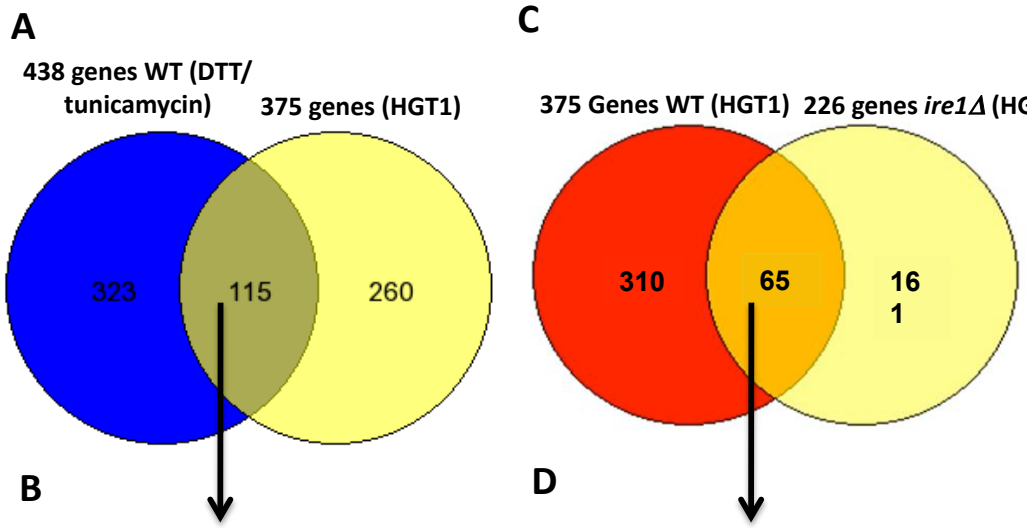
Figure S2. Microarray data replicate analysis by Principal Component analyses (PCA) (A) and Hierarchical Clustering (B). Clustering was performed on the entire unfiltered expression dataset that comprised 6403 x 10 x 2 = 128060 genes from the 5 different experimental conditions, including biological replicates and technical dye swap experiments. The 5 conditions comprised the three time points performed with HGT1 cells grown with 50 µM GSH (5 min, 30 min and 4 hrs) and the 2 time points performed with *gsh1Δ* grown in the absence of GSH (3 and 6 cell divisions). Each colored circle (A) or bar (B) represents average data from a biological replicate and the corresponding technical dye swap array.



Gene	Description
Aft1/ Aft2 targets	
CTH2	mRNA degradation during iron starvation
CAD1	bZIP transcription factor
ARN4	ferric-enterobactin transporter
FRE1	Ferric reductase and cupric reductase
FRE6	Putative ferric reductase
VMR1	ATPase activity
Protein folding/Oxidation	
ERO1	thiol oxidase in the ER
Plasma membrane/ cell wall proteins	
AQR1	multidrug transporter
HXT3	glucose transporter
HXT4	glucose transporter
PHO84	inorganic phosphate transporter
Fe-S cluster-containing proteins	
ACO1	aconitate hydratase
BIO2	biotin synthase
Heme proteins/biosynthesis	
HAP4	Subunit of Hap2p/3p/4p/5p complex
Carbohydrate metabolism	
TOS3	protein serine/threonine kinase
ENO1	phosphopyruvate hydratase
ALD6	aldehyde dehydrogenase
PFK27	6-phosphofructo-2-kinase
UPR-induced unclassified	
COS10	function unknown
FCY22	Putative purine-cytosine permease
Other functions	
POL12	alpha DNA polymerase
POL30	DNA polymerase processivity factor
ACM1	ubiquitin-protein ligase inhibitor
ZAP1	Zinc-regulated transcription factor
VID24	Vacuolar Import and Degradation
HPT1	hypoxanthine phosphoribosyltransferase
ADE17	IMP cyclohydrolase
ARO10	Phenylpyruvate decarboxylase
Function unknown	
YDR222W	function unknown
YAR029W	function unknown
YEL075C	function unknown
YLR462W	function unknown
YEL076C	function unknown
YER189W	function unknown
FMP43	function unknown

Kumar C. et al. Supplementary fig S3

Figure S3. Comparison of the transcriptional response of HGT1 cells exposed to GSH during 5 and 30 min. The list of differentially expressed genes in HGT1 cells exposed to 50 µM GSH during either 5 or 30 min was compared using a Venn diagram. The table displays the 35 commonly regulated genes in the two conditions sorted out into major functional categories. The complete lists of regulated genes, along with average normalized ratios are given in supplementary tables S1 and S2.

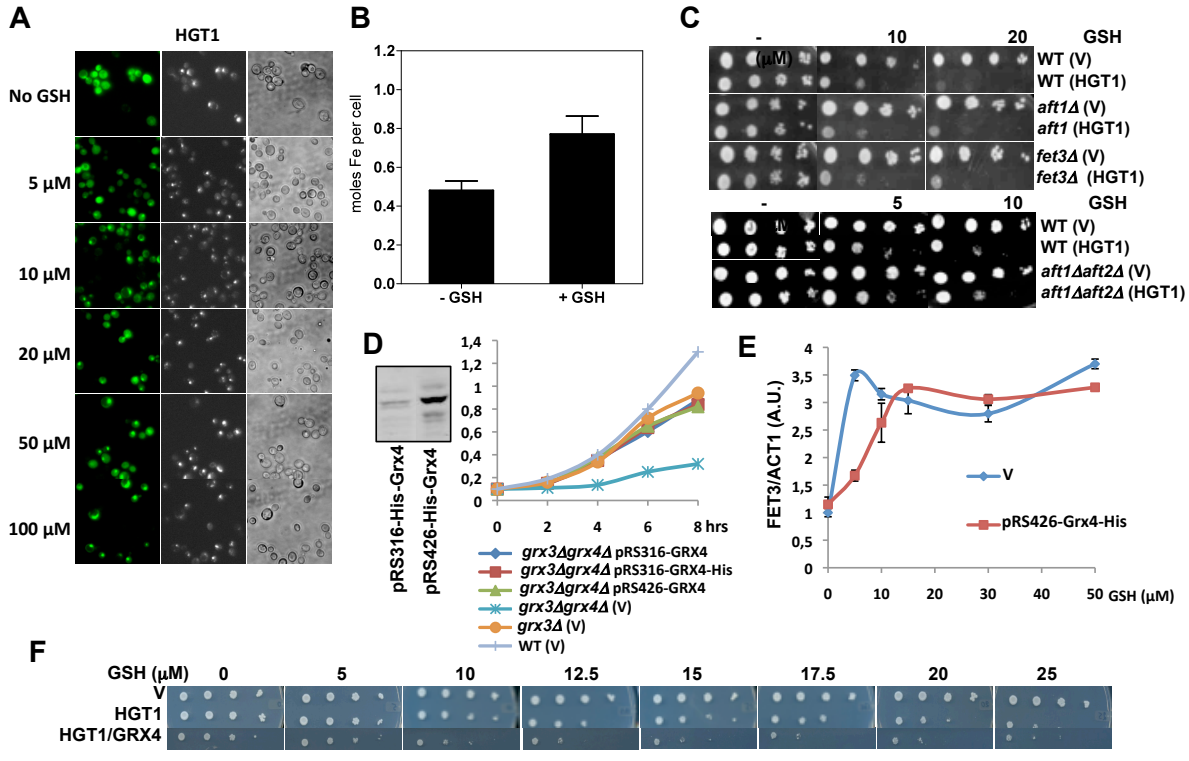


Biological process	Nb genes
Protein folding/oxidation	13
ER translocation/ ER traffic	13
Protein glycosylation	12
Lipid/Phospholipid metabolism	7
ER-associated degradation/ ERAD	8
Plasma membrane/cell wall	8
Aft1/ Aft2 targets	4
Respiration/TCA cycle	2
Amino acid metabolism	2
Other functions	29
Function unknown	19

Biological process	Nb genes
Aft1/Aft2 targets	16
Stress response	12
Heme, Fe-S, Copper proteins/ biosynthesis	7
Carbohydrate metabolism	7
Amino acid/ nitrogen metabolism	4
Mating type response	2
Other functions	12
Function unknown	6

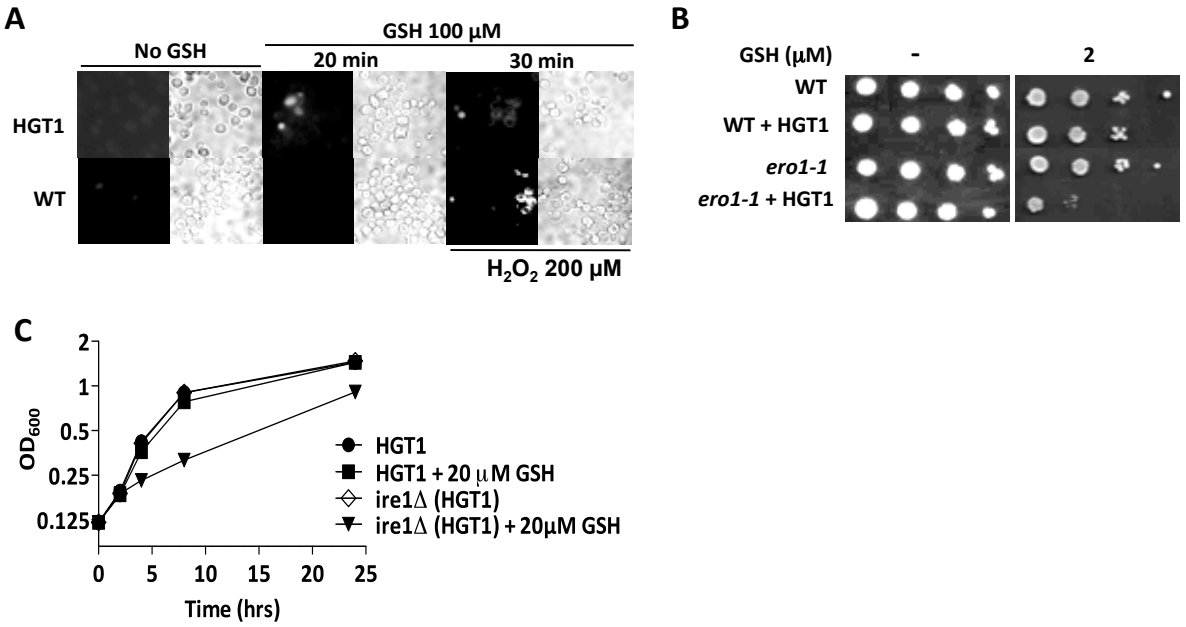
Kumar C. et al. Supplementary fig S4

Figure S4. **The transcriptional response to GSH toxic levels overlaps with the ER stress unfolded protein response.** **(A)** The list of 375 expressed genes after a 30 min exposure of HGT1 cells to 50 µM GSH was compared to the list of the 438 expressed genes in response to DTT or tunicamycin identified elsewhere (Kimata et al, 2006; Travers et al, 2000) by Venn diagram. **(B)** The functional categories enriched within the 115 commonly regulated genes. **(C)** The list of 375 expressed genes after a 30 min exposure of HGT1 cells to 50 µM GSH was compared to the list of the 226 expressed in *ire1Δ* HGT1 cells under the same experimental conditions. **(D)** The functional categories enriched within the 65 commonly regulated genes.



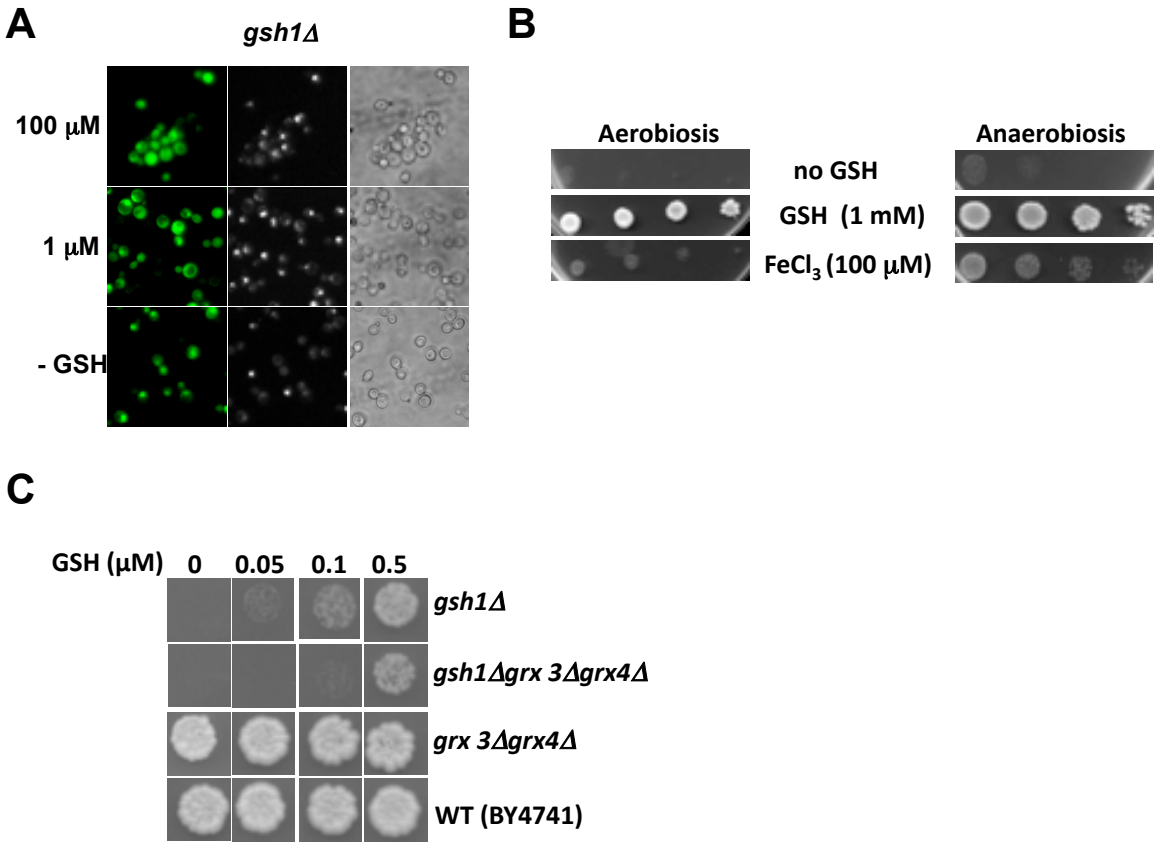
Kumar C. et al. Supplementary fig. S5

Figure S5. (A) GSH toxic levels activate Aft1 in an iron-dependent fashion. WT cells co-expressing pRS314-*TEF-HGT1* and p*TEF-AFT1-GFP* in the exponential phase were incubated with the indicated amount of GSH during 30 min and GFP staining was examined by fluorescence microscopy (left panel). DAPI nuclear staining (middle panel) and visible light image (right panel) are shown. **(B) Excess GSH increases total cellular iron**. WT cells transformed with pRS314-*TEF-HGT1* were inoculated in 250 ml of SD medium without or with GSH (100 µM), grown 1 hr to an OD_{600} = 0.3-0.4, and their iron content determined (moles Fe/cell). Values are the mean of three independent samples. Error bars correspond to means ± S.D. **(C) Iron high affinity uptake inactivation does not change GSH toxicity**. WT, *fet3Δ*, *aft1Δ*, and *aft1Δaft2Δ* transformed with pRS314 (V) or pRS314-*TEF-HGT1* (HGT1) grown to saturation in SD medium, serially diluted and spotted onto SD plates containing the indicated amount of GSH. $FeCl_3$ (100 µM) was added into plates shown in the lower panel to support *aft1Δaft2Δ* growth. **(D)** WT, *grx3Δ*, or *grx3Δgrx4Δ* transformed with either pRS316 (V), pRS316-Grx4-His, pRS426-Grx4-His, pRS316-Grx4 or pRS426-Grx4, as indicated, were processed for western blot with an anti-His antibody (left) (the image derives from a larger one that was trimmed), or monitored for growth in SD medium by turbidity as the indicated time (hrs) to assess transgenes functionality (right). Data are from one experiment. **(E) Grx4 over expression slightly decreases induction of *FET3* by excess GSH**. WT cells transformed with pRS314-*TEF-HGT1* (HGT1) and pRS426 (V) or pRS426-Grx4-His as indicated were incubated 1 hr in SD medium containing the indicated amount of GSH (µM) and processed for *FET3* expression measured by RT-PCR, which is given as *FET3*/*ACT1* signal ratio. Values are the mean of triplicate samples of the same experiment ± S.D. **(F) Grx4 over expression increases GSH toxicity**. WT cells transformed with either pRS314 (V), pRS314-*TEF-HGT1* (HGT1) or pRS426-Grx4-His as indicated, were grown to saturation in SD medium, serially diluted and spotted onto SD plates containing the indicated amount of GSH.



Kumar C. et al. Supplementary fig. S6

Figure S6. The effect of toxic GSH levels on the secretory pathway (A) Exponentially growing HGT1 and WT cells were incubated 10 min in PBS containing Peroxyfluor-1 (PF1) (10 µM), washed and incubated into PBS containing or not GSH (100 µM) during the indicated time, or supplemented with H_2O_2 (200 µM, 5 min). Cells were analyzed by fluorescence microscopy. Data are typical of three independent experiments. **(B)** WT and cells expressing the temperature sensitive ERO1 allele *ero1-1* carrying or not pRS416-*TEF-HGT1* (HGT1) or vector control (V) were grown to the exponential phase in SD medium lacking GSH, serially diluted and spotted onto SD plates containing the indicated amount of GSH, and incubated at the non-restrictive temperature. **(C)** WT and *ire1Δ* cells carrying pRS416-*TEF-HGT1* (HGT1) were inoculated into SD medium containing the indicated amount of GSH. Growth was monitored by turbidity.



Kumar C. et al. Supplementary fig. S7

Figure S7. (A) GSH depletion activates Aft1. *gsh1Δ* cells carrying p*TEF-AFT1-GFP* were cultured in SD medium containing 100 μM GSH until saturation, collected and re-inoculation in SD media containing the indicated amount of GSH, grown for 3 divisions (6-7 hrs) and analyzed for GFP staining. **(B) Iron rescues GSH auxotrophy**. YPD-grown *gsh1Δ* cells were depleted of GSH by growth for six divisions in SD medium lacking GSH, serially diluted, spotted onto SD plates containing or not $FeCl_3$ (100 μM) or GSH (1 mM), and incubated under aerobic (left) or anaerobic (right) conditions. **(C) *GSH1* and *GRX3*/*GRX4* are synthetic lethal.** WT (S288C background, EUROSCARF (BY4741) and its derived mutants *gsh1Δ*, *grx3Δgrx4Δ* and *gsh1Δgrx3Δgrx4Δ* were depleted of GSH by growth for 8 division in SD medium lacking GSH; 2 10^6 cells of the corresponding cultures were spotted on plates containing the indicated amount of GSH. Data are assembled from different plates.

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