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Cu, Zn Superoxide Dismutase and NADP(H) Homeostasis Are Required for Tolerance of Endoplasmic Reticulum Stress in *Saccharomyces cerevisiae*

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Genome-wide screening for sensitivity to chronic endoplasmic reticulum (ER) stress induced by dithiothreitol and tunicamycin (TM) identified mutants deleted for Cu, Zn superoxide dismutase (SOD) function (*SOD1*, *CCS1*) or affected in NADPH generation via the pentose phosphate pathway (*TKL1*, *RPE1*). TM-induced ER stress led to an increase in cellular superoxide accumulation and an increase in *SOD1* expression and Sod1p activity. Prior adaptation of the *hac1* mutant deficient in the unfolded protein response (UPR) to the superoxide-generating agent paraquat reduced cell death under ER stress. Overexpression of the ER oxidoreductase Ero1p known to generate hydrogen peroxide in vitro, did not lead to increased superoxide levels in cells subjected to ER stress. The mutants lacking *SOD1*, *TKL1*, or *RPE1* exhibited decreased UPR induction under ER stress. Sensitivity of the *sod1* mutant to ER stress and decreased UPR induction was partially rescued by overexpression of *TKL1* encoding transketolase. These data indicate an important role for SOD and cellular NADP(H) in cell survival during ER stress, and it is proposed that accumulation of superoxide affects NADP(H) homeostasis, leading to reduced UPR induction during ER stress.

INTRODUCTION

Aerobic organisms have a range of mechanisms to cope with reactive oxygen species (ROS) derived from endogenous and exogenous sources. ROS can affect cellular components and elicit damage including lipid peroxidation, nucleic acid damage, and oxidation of proteins (Temple *et al.*, 2005). Overwhelming production of ROS, termed oxidative stress, ultimately leads to cell death. Cells have many enzymatic and nonenzymatic mechanisms to remove or detoxify ROS to prevent oxidative stress. Nonenzymatic defenses include small molecules such as the tripeptide glutathione and the dithiol-containing thioredoxins. Enzymatic mechanisms include superoxide dismutases (SODs) that convert superoxide anion to hydrogen peroxide and oxygen, whereas the glutathione dependent peroxidases and catalase convert hydrogen peroxide to water and oxygen (Temple *et al.*, 2005; Perrone *et al.*, 2008).

SODs are highly conserved, although the cellular localization and requirements for metal ion cofactor(s) differ between organisms. Mutations in the human *SOD1*, encoding the Cu, Zn SOD, have been implicated in ~20% of the familial motor neuron disease amyotrophic lateral sclerosis (ALS; Rosen *et al.*, 1993). In *Saccharomyces cerevisiae*, the Cu, Zn SOD, Sod1p, localizes mainly in the cytosol but is also found in the mitochondrial intermembrane space (Sturtz *et al.*, 2001). Sod1p activity is dependent on the copper chaperone of Sod1p (Ccs1p) for copper loading and activation (Culotta *et al.*, 1997). Deletion of *SOD1* or *CCS1* leads to a number of oxygen-dependent phenotypes, including oxygen sensitivity, slow growth, hypersensitivity to superoxide-generating agents such as paraquat, accelerated aging, and auxotrophy for methionine and lysine (Culotta, 2000). Lysine auxotrophy was attributed to oxidation of the iron sulfur center of homoaconitase, whereas methionine auxotrophy was proposed to result from depletion of cellular NADPH by superoxide anion (Slekar *et al.*, 1996).

Cellular NADPH is important for tolerance to ROS. Strains deleted for genes involved in the nonoxidative branch of the pentose phosphate pathway that are important for NADPH generation, such as *RPE1* (encoding ribose-5 phosphate epimerase) or *TKL1* (encoding an isoform of transketolase) are sensitive to H_2O_2 (Juhnke *et al.*, 1996; Thorpe *et al.*, 2004) and also defective in adaptation to H_2O_2 (Ng *et al.*, 2008). NADPH is the cofactor for glutathione reductase and thioredoxin reductase, which are required to reduce oxidized glutathione and thioredoxin, respectively. This highlights the importance of NADPH for defense against ROS and maintenance of cellular redox homeostasis (Holmgren, 1989; Grant *et al.*, 1996a; Muller, 1996; Temple *et al.*, 2005; Ng *et al.*, 2008).

The endoplasmic reticulum (ER) contributes to cellular redox balance since formation of disulphide bonds in proteins that traverse the secretory pathway occurs in this organelle. The ER contains chaperones, ER oxidoreductases, protein disulphide isomerases, and glycosylation enzymes to support optimal protein folding. However, a large influx of protein into the ER, resulting in accumulation of aberrantly folded protein, leads to ER stress (Travers *et al.*, 2000; Liu and Kaufman, 2003; Ron and Walter, 2007). The unfolded protein response (UPR) is a highly conserved cellular

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Abbreviations used: DTT, dithiothreitol; ER, endoplasmic reticulum; ROS, reactive oxygen species; TM, tunicamycin; UPR, unfolded protein response.

pathway that alleviates ER stress (Liu and Kaufman, 2003; Ron and Walter, 2007). In *S. cerevisiae*, the UPR requires the ER transmembrane kinase Ire1p and its downstream transcription factor Hac1p. On accumulation of misfolded/unfolded proteins, Ire1p dimerizes, autophosphorylates, and splices the intron of *HAC1* mRNA. The translated Hac1p then binds to genes whose promoter contains the unfolded protein response element (Cox *et al.*, 1993; Cox and Walter, 1996; Mori *et al.*, 1996; Kawahara *et al.*, 1997). Activation of the UPR alleviates ER stress by up-regulating genes including those required for ER expansion, general secretory pathway function, and protein folding and degradation (Travers *et al.*, 2000). The Ire1p-Hac1p pathway functions in a linear manner because strains deleted for either *HAC1* or *IRE1* have an indistinguishable phenotype and a similar gene expression profile (Travers *et al.*, 2000), and no alternative Ire1p substrates have been identified (Niwa *et al.*, 2005).

ER stress in yeast has been shown to be associated with ROS generation and cell death in a programmed cell death-like manner (Haynes *et al.*, 2004; Hauptmann *et al.*, 2006), but the species of ROS and cellular mechanisms that detoxify them under ER stress have not been fully elucidated. The role of ROS in yeast programmed cell death has been reviewed (Perrone *et al.*, 2008). The ER is a potential source of ROS formation. It has been proposed that 25% of cellular ROS originates from the ER (Tu and Weissman, 2004). The use of oxygen as the terminal electron acceptor by the essential ER oxidoreductase Ero1p during oxidative protein folding can result in ROS production (Tu and Weissman, 2004). Ero1p generates H_2O_2 in vitro (Gross *et al.*, 2006), but the ROS generated by Ero1p in vivo remains unknown.

Here, we screened the *S. cerevisiae* deletion collection (Winzeler *et al.*, 1999) for mutants sensitive to the glycosylation inhibitor tunicamycin (TM) and reductant dithiothreitol (DTT) to identify cellular processes important for ER stress tolerance or for countering ROS generated in this stress. Exposure of cells to DTT leads to accumulation of unfolded proteins in the ER (Jamsa *et al.*, 1994), whereas TM disrupts *N*-linked glycosylation through inhibition of the dolichyl phosphate–dependent *N*-acetylglucosamine-1-phosphate transferase, Alg7p (Barnes *et al.*, 1984; Kukuruzinska and Lennon, 1995). These compounds cause ER stress and activate the UPR (Travers *et al.*, 2000).

Among the mutants that were sensitive, we identified those affected in superoxide detoxification (*sod1*, *ccs1*) and NADPH production (*tkl1*, *rpe1*) to be sensitive to both ER-stressing agents. This prompted us to further investigate their role in ER stress tolerance.

MATERIALS AND METHODS

Yeast, Strains, and Plasmids

The strains used (Table 1) include those derived from BY4743 (Open Biosystems, Huntsville, AL) or from CY4 as described previously (Grant *et al.*, 1996b). Single-gene deletion strains in the CY4 background were generated using PCR to amplify the disrupted allele and flanking region (~200–400 base pairs) from the strains available from the *S. cerevisiae* Genome Deletion Project (Winzeler *et al.*, 1999). The amplified DNA product was transformed into CY4 with selection on YEPD medium containing 220 μg/ml geneticin (Invitrogen, Invitrogen, Carlsbad, CA). Transformation was performed using the lithium acetate–based method (Ito *et al.*, 1983). Each deletant was confirmed by PCR analysis on genomic DNA isolated from the respective strains. Double mutants were generated by mating haploid single mutants and dissecting spores from the resulting heterozygous diploid. The *hac1::NatMX* allele was generated by transforming EcoRI-digested p4339 (Tong *et al.*, 2001) containing the *NatMX* cassette into ST003 (*hac1::KanMX*) with selection on YEPD medium containing 100 μg/ml nourseothricin (Sigma, St. Louis, MO) to generate ST004.

The centromeric *ERO1::lacZ* fusion construct containing 951 base pairs of upstream untranslated region and 42 base pairs of the coding region of *ERO1* was generated by ligating the BglII-digested PCR fragment (amplified using the specific oligonucleotides GCAAAGATCTGTGAGGCACAGTGTGGCAATGGC and GAAGAGATCTTACCTTAATTCTTGCTGAAAAATAC) to the BamHI-digested PYLZ-6 (Hermann *et al.*, 1992) to generate pST3 (*CEN*, *URA3*).

Table 1. *S. cerevisaie* strains used in this study

Strains	Genotype	Source
CY4	*MAT***a** *ura3-52 leu2-3,112 trp1-1 ade2-1 his3-11*	Chris M. Grant
CY4 (MATα)	As of CY4 but *MAT*α	Chris M. Grant
ST002	*sod1::KanMX* in CY4	This study
ST003	*hac1::KanMX* in CY4	This study
ST004	*hac1::NatMX* in CY4	This study
ST007	*sod1::KanMX hac1::NatMX* in CY4	This study
CY7	*glr1::TRP1* in CY4	Grant *et al*. (1996)
JL3	*gsh1::LEU2* in CY4	Grant *et al*. (1996)
CY226	*ctt1::URA3 cta1::URA3* in CY4	Grant *et al*. (1998)
CY237	*gsh1::LEU2 ctt1::URA3 cta1::URA3* in CY4	Grant *et al*. (1998)
ST012	*glr1::TRP1/GLR1, SOD1/sod1::KanMX* in CY4 *MAT***a**/*MAT*α	This study
1783	*MAT***a** *ura3–52 leu2–3,112 trp1-1 his4 can1*^{r}	Slekar *et al*. (1996)
KS105	*sod1::TRP1* in 1783	Slekar *et al*. (1996)
KS113	*zwf1::URA3* in 1783	Slekar *et al*. (1996)
KS117	*sod1::TRP1 zwf1::URA3* in 1783	Slekar *et al*. (1996)
BY4743	*MAT***a**/*MAT*α *his3*Δ*1/his3*Δ*1 leu2*Δ*0/leu2*Δ*0 met15*Δ*0/MET15 LYS2/lys*Δ*0 ura3*Δ*0/ura3*Δ*0*	EUROSCARF
BY4743 (*hac1*)	*hac1::KanMX* in BY4743	EUROSCARF
BY4743 (*sod1*)	*sod1::KanMX* in BY4743	EUROSCARF
BY4743 (*ccs1*)	*ccs1::KanMX* in BY4743	EUROSCARF
BY4743 (*sod2*)	*sod2::KanMX* in BY4743	EUROSCARF
BY4743 (*tkl1*)	*tkl1::KanMX* in BY4743	EUROSCARF
BY4743 (*rpe1*)	*rpe1::KanMX* in BY4743	EUROSCARF

A centromeric vector containing the *GAL1*-promoter, multiple-cloning site and *CYC1* terminator was generated by digesting JMB671 DNA (a kind gift from Joe Mymryk, University of Western Ontario, London, Ontario, Canada) with ScaI and NgoMIV. The resulting 1.4-kbp fragment was ligated to pRS413 digested with the same enzymes to generate p413GAL1 (*CEN*, *HIS3*). The galactose-inducible *ERO1* plasmid was generated by ligating a BamHI-XhoI–digested PCR fragment amplified from genomic DNA using oligonucleotides GTATTGGATCCATGAGATTAACAACCGCC and CATATAAGATAACTCGAGTTGCTC to vector p413GAL1 digested with BamHI and SalI generating pST1 (*GAL1-ERO1*, *CEN*, *HIS3*). Amplifying ERO1 using ATCGGGATCCATGAGATTAAGAACCGCCATTGC and CTGAGATGAGTTTTTGTTCTTGTATATCTAGCTTATAGGAAAT, followed by a second round of PCR amplification using ATCGGGATCCATGAGATTAGAACCGCCATTGC and CTACTCGAGCTACAGATCCTCTTCTGAGATGAGTTTTTGTTC, generated addition of a *myc* tag to the carboxyl-terminal of *ERO1*. The resulting 1.7-kbp PCR fragment was digested with BamHI and XhoI and ligated to p413GAL1 digested with BamHI and SalI to generate pST2 (*GAL1-ERO1myc*, *CEN*, *HIS3*). Plasmids were verified by sequencing.

pAC595 (*CUP1*-CPY*, *CEN*, *URA3*; Haynes *et al.*, 2004) and pGEV-TRP (*CEN*, *TRP1*), a variant of pGEV-HIS (Gao and Pinkham, 2000) was a kind gift from Antony Cooper (Garvan Medical Research Institute, Sydney, Australia). The UPRE::*lacZ* (*CEN*, *LEU2*) fusion construct was provided by Peter Walter (University of California, San Francisco, San Francisco, CA). The pTKL1 (2μ, *LEU2*; Slekar *et al.*, 1996) was a kind gift from Munira Basari (National Institutes of Health, Bethesda, MD). pES67 (*GAL1*-CPY*HA, *CEN*, *LEU2*; Spear and Ng, 2003) was kindly provided by Davis Ng (Temasek Life Sciences, Singapore). pAF112 (*GAL1-ERO1myc*, *CEN*, *URA3*), pCS452 (*GAL1-ero1*-C150A-C295A-*myc*, *CEN*, *URA3*) and pCS504 (*GAL1-ero1*-C150A-C295A-C100A-C105A-*myc*, *CEN*, *URA3*) described by Sevier *et al*. (2007) was kindly provided by Carolyn Sevier and Chris Kaiser (both from the Massachusetts Institute of Technology, Cambridge, MA).

Growth Conditions and Tolerance Analysis

Yeast strains were grown in YEPD medium containing 1% (wt/vol) yeast extract, 2% (wt/vol) peptone, and 2% (wt/vol) glucose or synthetic complete

medium (SC) containing 0.17% (wt/vol) yeast nitrogen base without amino acid and ammonium sulfate, 0.5% (wt/vol) ammonium sulfate, and 2% (wt/vol) glucose. Auxotrophic supplements were added as specified by the manufacturer's instructions (Sigma) with appropriate amino acid omitted for auxotrophic selection. For anaerobic growth conditions, media were supplemented with 0.1% (vol/vol) Tween 80 and 20 mg/l ergosterol, and plates were maintained in an anaerobic jar using an anaerobic pack and oxygen indicator to verify anaerobiosis (Oxoid, Adelaide, Australia). For tolerance of strains to various compounds, strains were grown in cultures for 2 d, the OD_{600} was adjusted to 1.0, and the culture was serially diluted 10-fold before spotting on agar plates containing, paraquat, TM, or DTT. Agar was cooled to 55°C before addition of the indicated compounds. Agar plates containing the compound(s) were prepared fresh before use. Growth curves were generated by sampling cultures in a shake flask using a conventional spectrophotometer or growth of cultures in the automated plate reader Bioscreen C (Oy Growth Curves Ab) with constant temperature (30°C) and continuous shaking ("high" speed) and OD_{600} read every 30 min.

Genome-Wide Screen for Mutant Sensitive to TM and DTT

Cells were grown for 3 d in 96-well plates containing 200 μl of YEPD before replicating into 200 μl of YEPD containing 6 μg/ml TM or 20 mM DTT in 96-well plates using a 96-pin replicator. Cells were incubated for 3 d at 30°C without agitation. Cultures were resuspended using a 96-well plate shaker before determination of cell density using a 96-well plate reader. Strains that showed complete inhibition of growth in both TM- and DTT-containing media were identified as sensitive. The strains sensitive to these compounds were rearrayed and retested for verification.

SOD Assay

The in-gel SOD activity assay was performed as described (White *et al.*, 1993; Huang *et al.*, 2002; Sanchez *et al.*, 2005). Yeast strains were grown to exponential phase, treated with TM for the indicated time, and harvested, and cells were disrupted using acid-washed glass beads and a mini-bead beater (Biospec Scientific, Bartlesville, OK; 1 min at high speed, 4°C) in 0.1 M Tris-HCl (pH 8) containing 20% (vol/vol) glycerol and 2 mM phenylmethanesulphonyl fluoride (PMSF). The lysate was clarified by centrifugation (5 min 10,000 × *g* at 4°C), and 10 μg was run in a 10% (wt/vol) Tris-acrylamide gel. Gels were stained for 10 min in 36 mM potassium phosphate buffer (pH 8) containing 34.5 μM riboflavin, 0.19 mM nitroblue tetrazolium, and 175 μl tetramethylethylenediamine. Gels were exposed to light for 10 min or until good contrast was achieved.

Western Blot Analysis

Cells were grown as stated, and ~40 ml of cells was harvested and lysed in lysis buffer (0.1 M Tris-HCl, pH 8, 20% [vol/vol] glycerol, 2 mM PMSF, 1% SDS). Protein content of cell lysate was determined and adjusted to 2 μg/μl in sample loading buffer (5% [vol/vol] β-mercaptoethanol, 40% [vol/vol] glycerol, Tris-HCl, pH 6.8). Samples were boiled for 10 min, and 30 μg of each sample was loaded onto 14% (wt/vol) polyacralymide Tris-SDS gel for determination of Sod1p or a 4–12% NuPAGE gel (Invitrogen) for determination of Ero1*myc*p, CPY*HAp or Pgk1p. Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Sod1p and anti-Pgk1p were diluted to 1:1000 and 1:5000, respectively. Anti-hemagglutinin (HA) and anti-*myc* were diluted to 1:1000 and 1:500, respectively. The blots were quantitated using Fuji image gauge software (Tokyo, Japan). Intensity of the Sod1p band was normalized relative to that of Pgk1p and expressed as a fold change relative to untreated cells at zero time.

Dihydroethidium and Propidium Iodide Staining and FACS Analysis

Dihydroethidium (DHE) and propidium iodide (PI) staining of cells were performed essentially as described (Deere *et al.*, 1998; Madeo *et al.*, 1999). For DHE staining, cells (500 μl) were stained with 5 μg/ml final concentration of DHE for 10 min and viewed under a fluorescence microscope without further manipulation. For PI staining, cells (500 μl) were harvested (1 min 13,000 × *g*), resuspended in an equal volume of ice-cold phosphate-buffered saline (PBS) and stained with 3 μg/ml final concentration of PI for 20 min. Approximately 500 cells were counted in duplicate in duplicate experiments. For FACS analysis of DHE stained cells, cells were washed in PBS, stained as above and analyzed using a FACSAria (BD Bioscience, NSW, Australia). For each sample, 20,000 cells were analyzed.

β-Galactosidase Assay

Cells were grown to exponential phase (OD_{600} ~0.5), harvested and analyzed for β-galactosidase activity as described (Rose and Botstein, 1983). β-galactosidase activity was expressed as units (U) of ONPG (*O*-nitrophenyl-4β4-D-galactopyranoside) hydrolyzed (nmol) per min over total protein (mg) and the extent of induction was the ratio of the β-galactosidase activity of the treated sample to that of the untreated sample. For each strain, three independent transformants were assayed.

Multicopy-suppressor Screen

The *sod1* strain from BY4743 background was transformed with a YEp13-based yeast chromosomal gene library (2μ, *LEU2*) and incubated at 30°C on SC media without leucine (SC-LEU). The resulting ~2000 transformants were replica plated onto SC-LEU medium containing 1.5 μg/ml TM. Plasmids from colonies resistant to TM were isolated individually, transformed into the *sod1* mutant of CY4 background and verified as conferring TM resistance. Only plasmids that conferred resistance upon verification were sequenced. A 5.2-kb insert containing *ALG7* was identified in two of the positive plasmids and a 5.1 kb insert containing *TKL1* was identified in one positive plasmid.

Determination of Intracellular Glutathione Levels

Reduced (GSH) and oxidized glutathione (GSSG) were estimated as described previously (Vandeputte *et al.*, 1994; Grant *et al.*, 1996b). Cells were grown in SC medium (40 ml) to an OD_{600} of 1 (~2×10^7 cells/ml), treated or untreated with TM (1 μg/ml) for 4 h and harvested by centrifugation (10,000 × *g*; 20 s), washed in 3 ml of ice-cold PBS buffer, resuspended in 350 μl of ice-cold 8 mM HCl containing 3% (wt/vol) 5-sulfosalicilic acid and broken using a mini-bead beater (1 min high speed, 4°C). The lysate was clarified by centrifugation (5 min, 10,000 × *g*; 4°C) and the supernatant taken to determine total glutathione. GSSG was determined by reacting GSH with 2-vinylpyridine before the assay. Levels of GSH and GSSG were calculated as described in (Vandeputte *et al.*, 1994).

Estimation of Intracellular Redox Environment

Intracellular values of the GSSG/2GSH half-cell redox potential (glutathione E_h) were determined as an indicator of the cellular redox environment (Schafer and Buettner, 2001). These were calculated from intracellular concentrations of GSH and GSSG using the Nernst equation: $E_h' = E_0 - 2.303(RT/nF)\log_{10}[(GSH)^2/(GSSG)]$. Glutathione E_h' values are expressed in mV; E_0 is the standard potential for reduced glutathione (−0.24 mV) at pH 7, R is gas constant (8.31 J mol^{-1} K^{-1}), T is the absolute temperature (303K), n is the number of electrons transferred (2), and F is the Faraday constant (96,406 J V^{-1} mol^{-1}). To estimate intracellular glutathione concentrations cell volumes were estimated from cell major (a) and minor (b) axes using the formula (Wheals, 1982): $V = \pi a^2 b/6$. Cell dimensions were determined microscopically at 100× magnification using a graticule calibrated against a slide micrometer grating. For each strain at least 100 cells were measured, and the average cell volume was determined.

Determination of Intracellular NADP(H) Level

Intracellular NADP(H) levels were determined using a 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and phenazine ethosulfate (PES) cycling assay based on that described by Gibson and Larher (1997; Gibon and Larher, 1997). Cultures were grown to exponential phase and subjected to the desired treatment condition, two aliquots of cells (equivalent to 10 OD units) were isolated. For quantification of NADPH or $NADP^+$ cells were lysed in 0.1 M KOH or 0.1 M HCl, respectively. Acid extraction was used to destroy NADPH but to preserve $NADP^+$, while alkaline extraction was used to destroy $NADP^+$ and preserve NADPH (Lowry *et al.*, 1961). Cells were disrupted using glass beads and a mini-bead beater at 4°C. Cell lysates were incubated on ice for 15 min and clarified by centrifugation (13,000 × *g*, 10 min). Acid and alkaline extracted cell lysates were incubated for 10 or 5 min, respectively, at 70°C and chilled on ice for 5 min before initiating the assay. Ten microliters of cell lysate was added to 150 μl of 0.26 M triethanolamine hydrochloride buffer (pH 7.4) containing 2 mM glucose-6-phosphate, PES and MTT, and 4 mM EDTA. Glucose-6-phosphate dehydrogenase (specifically prepared for cycling assays; Sigma) was added to a final concentration of 200 U/ml to each reaction. Color development was monitored at 570 nm over 30 min. The concentration of cellular NADP(H) was determined using the linear range of the standard curve (0.5–5 μM) and normalized to the cell density (OD_{600}) of the harvested culture.

Statistical Analysis

Statistical analyses were performed using paired, two-tailed Student's *t* test.

RESULTS

ROS accumulation has been detected under conditions of sustained ER stress (Haynes *et al.*, 2004; Hauptmann *et al.*, 2006). To study the relationship between ROS generation and ER function, we sought to identify genes that are involved in the cellular response to chronic ER stress. A previous screen for mutants affected in tolerance to transient ER stress using reducing agents (DTT, 2-mercaptoethanol) or

Table 2. Genes whose deletion causes sensitivity to both TM and DTT

Cellular process	Gene
Unfolded protein response	*HAC1 IRE1*
Vaculor H^+ ATPase related	*VMA13 VMA21 VPH2*
Vacuolar protein sorting	*VPS1 VPS20 VPS27 VPS33 VPS34 VPS41 VPS45 VPS54 VPS64 VPS8 VPS9 PEP12 PEP3*
Secretory pathway	*GET1 ERV29 PMR1 LHS1 OST3 SAC1 SPF1 GYP1 YPT7 KCS1 KRE6 MON2*
Lipid homeostasis	*ERG2 ERG6 TCB3 CSG2*
Cell wall integrity pathway	*SLT2 BCK1 RLM1*
Detoxification of superoxide anion	*SOD1 CCS1*
Stress response	*CRZ1 CNB1 HYR1 YAP1*
NADPH generation	*GND1 TKL1 RPE1*
RNA polymerase II related	*PAF1 PGD1 ROX3 BUR2 ELP2 SIN4*
Ribosomal protein subunit	*RPL12A RPL24B RPL37A*
Unknown function	*YBR025C YDR067C YHR100C YKL037W YLR404W YLR426W OPI9 KIN3*
Ungrouped	*AAT2 ADO1 ARO2 CNM67 GTR1 IES2 IES6 LYS9 NUP188 NUP84 REG1 ROT2 XRS2 YAP3 SAC7 SET3 SHP1 SIW14 STE20 STF1 SWI6 UBP14*

TM highlighted the importance of several cellular functions but did not identify mutants involved in cellular responses to ROS (Chen *et al.,* 2005).

Genome-Wide Deletant Screen Identifies Cellular Processes Required for Tolerance to Chronic DTT and Tunicamycin Exposure

To identify genes important for tolerance of chronic ER stress, the *S. cerevisiae* genome-wide deletion collection was screened for mutants exhibiting sensitivity to TM and DTT. The diploid yeast mutant collection was screened for growth after 72 h in YEPD medium containing either 20 mM DTT or 6 μg/ml TM using 96-well plates. These concentrations completely inhibited growth of the *hac1* strain but not of the wild type.

Only those strains sensitive to both compounds were identified as positive to increase the stringency of the screen. As expected, cellular processes previously shown by microarray or mutant analyses to be important for ER stress tolerance, such as the unfolded protein response, cell integrity signaling pathway, vacuolar protein sorting, vacuolar acidification and processes involved in secretory pathway functions (Travers *et al.,* 2000; Bonilla *et al.,* 2002; Bonilla and Cunningham, 2003; Chen *et al.,* 2005; Kimata *et al.,* 2006) were also identified in this screen (Table 2). However, additional processes including those involved in oxidative stress responses (*YAP1*, *HYR1*), NADPH generation (*GND1*, *RPE1*, *TKL1*) and dismutation of the superoxide anion (*SOD1* and *CCS1*) were identified (Table 2). The pentose phosphate pathway is involved in adaptation to, and tolerance of, oxidative stress (Juhnke *et al.,* 1996; Thorpe *et al.,* 2004; Ng *et al.,* 2008). Sod1p detoxifies superoxide anion to H_2O_2 and water and the Cu^{2+}-loading chaperone Ccs1p is required for its activation (Gralla and Valentine, 1991; Culotta *et al.,* 1997). These data highlighted that antioxidant functions are very relevant to cell survival of chronic ER stress. Because the main aim was to study the link between ER stress and ROS-related processes, we investigated further the role of SOD and the NADPH-generating system during ER stress tolerance.

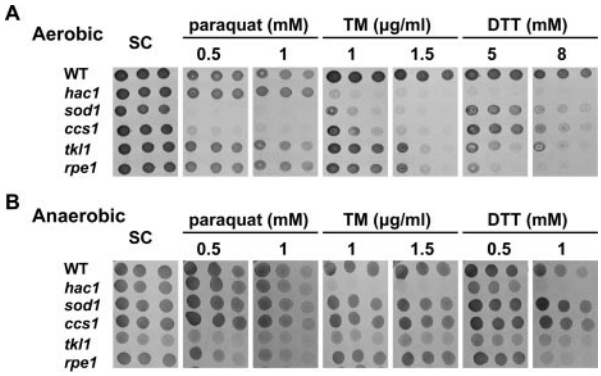

Figure 1. Oxygen-dependent sensitivity of *sod1* and *ccs1* to TM and DTT. The indicated strains (BY4743 background) were grown for 2 d, the cell density was adjusted to OD_{600} 1.0-, 0.1-, and 0.01-, and 5-μl aliquots were spotted on SC solid medium as control and SC containing the indicated concentration of paraquat, TM, and DTT. The plates were incubated for 30°C under (A) aerobic conditions for 2 d or (B) anaerobic condition for 3 d.

Sensitivity of sod1 and ccs1 to Tunicamycin or DTT Is Dependent on Oxygen

Because the sensitivity of the above mutants to agents inducing ER stress might have stemmed from reduced tolerance of ROS generated during ER stress, the sensitivity of each mutant to TM, DTT and paraquat (a superoxide generator) was further assessed on solid SC medium (Figure 1) under aerobic and anaerobic conditions. Hac1p has an established role in the response of cells to ER stress (Cox and Walter, 1996; Travers *et al.,* 2000); therefore, the *hac1* mutant was included in this and subsequent experiments.

The paraquat sensitivity of *sod1* and *ccs1* cells under aerobic conditions (Figure 1A), and the reversal of this phenotype under anaerobic conditions (Figure 1B) were consistent with the importance of Cu, Zn SOD in protection against superoxide anion-induced damage (Gralla and Valentine, 1991; Culotta *et al.,* 1997). Importantly, the TM and DTT sensitivity of the *sod1* and *ccs1* mutants was completely reversed under anaerobic conditions indicating that the sensitivity of these strains to ER-stress generating agents was oxygen-, and most likely ROS-dependent. Anaerobiosis only partially rescued the TM and DTT sensitivity of the *tkl1* mutant, and the DTT sensitivity of the *rpe1* mutant, indicating that maintenance of cellular NADPH during ER stress conditions is important regardless of the availability of oxygen. In contrast the TM and DTT sensitivity of the *hac1* mutant was not influenced by oxygen availability indicating an essential role of the UPR during ER stress under both aerobic and anaerobic conditions. The oxygen-dependent sensitivity of the *sod1* and *ccs1* mutants to ER stress indicates that the requirement of Cu, Zn SOD (Sod1p) for ER stress tolerance may be due to its role in detoxification of superoxide anions.

Sod1p and Its Activity Increase during ER Stress

To examine whether Sod1p and its activity were affected under ER stress, the levels of Sod1p and SOD activities due

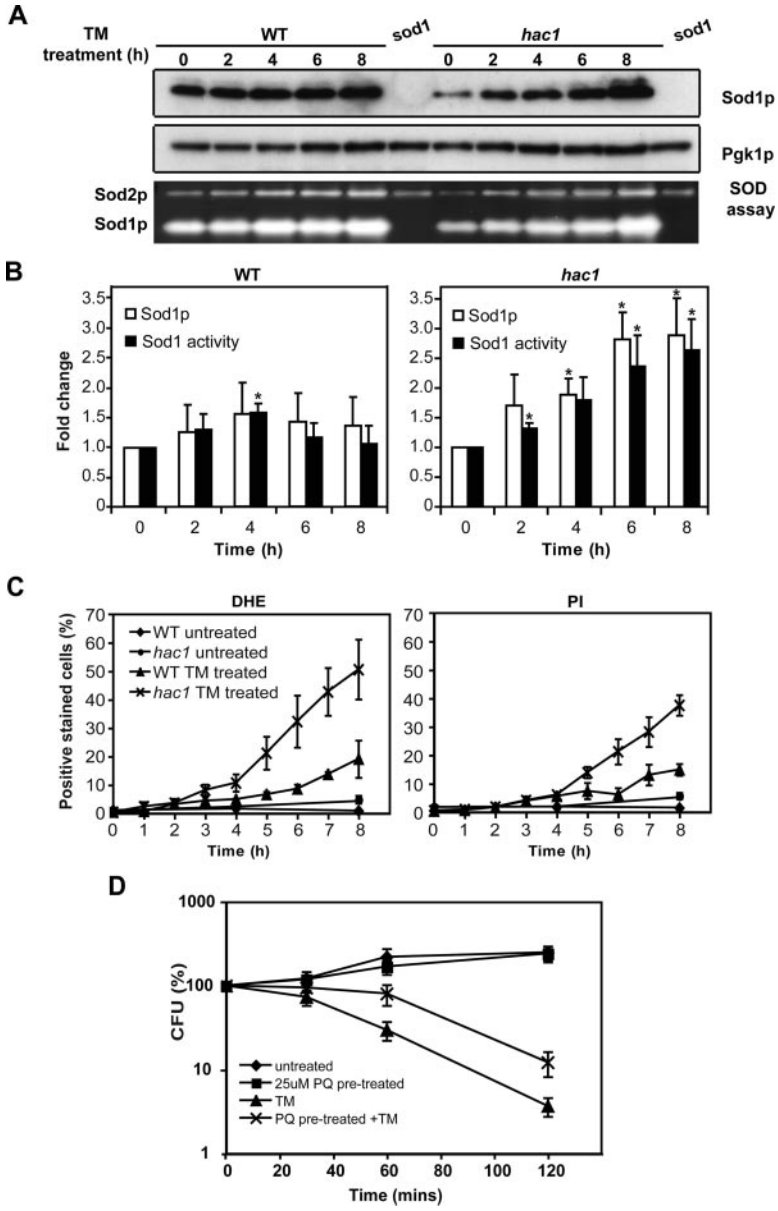

Figure 2. Effect of TM treatment on the level and activity of Sod1p. (A) Induction of Sod1p and SOD activity in wild-type (CY4) and *hac1* (ST003) strains. The strains were grown to exponential phase (OD 0.5), TM was added to 1 μg/ml, and samples collected every 2 h. Superoxide dismutase assays and Western blotting was performed as described in *Materials and Methods.* (B) SOD activity and Sod1p were normalized against Pgk1p loading control and expressed as fold induction relative to untreated cells at zero hour. Error bars, SD of three independent experiments. Significant difference (* $p < 0.05$) when compared with the respective values at zero hour. (C) Cells treated or untreated with TM were stained with DHE or PI and analyzed for percentage of cells positively stained. (D) Colony-forming units (CFU) of *hac1* (ST003) cells pretreated or untreated with 25 μM paraquat for 1 h before treatment with TM. Error bars, SE of four independent experiments.

to Sod1p and Sod2p were quantified in wild-type and *hac1* cells treated with TM over 8 h.

In wild-type cells, TM treatment led to a small but significant increase in SOD activity (4 h, $p < 0.05$; Figure 2, A and B). In *hac1* cells, the level of Sod1p and SOD activity increased steadily between 2 and 8 h and both Sod1p levels and SOD activity increased approximately threefold after 8 h treatment relative to their respective values before treatment (Figure 2, A and B). Untreated wild-type or *hac1* cells showed no increase in Sod1p or its activity during different growth phase examined (Supplemental Figure S1). The activity of Sod2p also increased upon exposure of cells to TM, however in contrast to *SOD1*, deletion of *SOD2* did not lead to DTT or TM sensitivity on solid media (Supplemental Figure S1), indicating a minor role of Sod2p in ER stress tolerance.

To determine if superoxide was generated during ER stress, dihydroethidium (DHE), a dye relatively specific for this radical (Zhao *et al.*, 2003), was used to stain wild-type and *hac1* cells treated with TM over a period of 8 h, and cell viability was determined using propidium iodide (PI) staining. In untreated wild-type or *hac1* cells there was no increase in DHE or PI staining during the experimental period. For treated wild-type and *hac1* cells the percentage of DHE-stained cells gradually increased, with *hac1* cells showing a higher percentage of staining compared with the wild type after 4 h (Figure 2C). This increase in DHE-stained cells also correlated with an increase in positive PI staining, although the percentage of PI-stained cells was less than those stained with DHE (Figure 2C).

If superoxide anion production contributes to death of cells during ER stress, then prior adaptation of cells to increase their resistance to superoxide anion should lead to increased survival of subsequent ER stress. Exposure of cells to low, sublethal doses of oxidants confers resistance to subsequent treatment with otherwise lethal doses of the reactive oxygen species (Collinson and Dawes, 1992; Jamieson, 1992; Flattery-O'Brien *et al.*, 1993; Alic *et al.*, 2004). To

ensure that any cross-protective effect was not mediated through induction of the UPR, the *hac1* mutant was used to test this hypothesis. Treatment of *hac1* cells with a sublethal dose (25 μM) of paraquat did not lead to a change in colony forming units (CFUs) relative to untreated cells (Figure 2D). Treatment of *hac1* cells (not pretreated with paraquat) with TM for 1 or 2 h led to markedly decreased cell viability (CFUs; Figure 2D). In contrast, pretreatment of *hac1* cells with 25 μM paraquat for 1 h conferred a significant protective effect to a subsequent treatment with the same dose of TM (Figure 2D). Because paraquat pretreatment conferred protection to TM, these data support the hypothesis that superoxide anion accumulation contributes to cell death during ER stress.

Cells simultaneously deleted for genes encoding the cytosolic and peroxisomal catalases (*CTT1* and *CTA1*), and γ-glutamylcysteine synthetase (*GSH1*) exhibit hypersensitivity to exogenous H_2O_2 treatment (Grant *et al.*, 1998). To assess whether H_2O_2 detoxification is important for ER stress tolerance, we tested the sensitivity of *gsh1*, *ctt1 cta1*, and *ctt1 cta1 gsh1* mutants to DTT and TM. These mutants showed wild-type sensitivity to DTT and TM (Supplemental Figure S1), indicating that these processes involved in detoxification of H_2O_2 play a minor role in ER stress tolerance.

The above results demonstrate that induction of ER stress in wild-type cells by a low sub-lethal dose (LD) of TM was associated with a transient increase in Sod1p activity. More severe stress imposed by the same dose of TM but in a *hac1* deletant led to an extensive increase in superoxide anion production as determined by DHE staining, induction of Sod1p, and increase in Sod1p activity (Figure 2). The present data demonstrate that TM-induced ER stress is associated with accumulation of ROS including superoxide anion.

SOD1 Is Required for Wild-type UPR Induction

Given the central role of the unfolded protein response in ER stress tolerance (Travers *et al.*, 2000; Liu and Kaufman, 2003; Ron and Walter, 2007), we sought to determine whether the extent of UPR induction is dependent on the level of Sod1p activity. Expression levels of two *lacZ* reporter constructs were determined in the wild-type and *sod1* and *hac1* cells. The extent of Hac1p activation was determined using a reporter containing the UPR element (UPRE) derived from *KAR2* fused to the *Escherichia coli lacZ* gene (UPRE::*lacZ*; Cox *et al.*, 1993), whereas the other reporter consisted of the 951-base pair upstream sequence of *ERO1* fused to the *lacZ* gene (*ERO1::lacZ*); this construct indicates the expression of *ERO1*, which is regulated by Hac1p and the heat-shock transcription factor Hsf1p (Takemori *et al.*, 2006).

Consistent with previous findings (Cox *et al.*, 1993; Travers *et al.*, 2000; Takemori *et al.*, 2006), there was almost no induction of either *ERO1::lacZ* or UPRE::*lacZ*, respectively, in *hac1* cells upon TM treatment (Figure 3). In the *sod1* strain, the extent of induction of both *ERO1::lacZ* and UPRE::*lacZ* was ~50% of that observed in wild-type cells, indicating that deletion of *SOD1* affects activation of the UPR, and this may contribute to *sod1* sensitivity to ER stress observed in a *hac1* mutant. Deletion of *SOD1* did not affect β-galactosidase activity per se, because the β-galactosidase activity and protein level correlates in the *sod1* mutant (Supplemental Figure S2). However, the requirement for Sod1p to remove superoxide generated during ER stress (Figure 2), and the oxygen-dependent sensitivity of *sod1* to ER stress (Figure 1) must also account for the sensitivity of the *sod1* mutant.

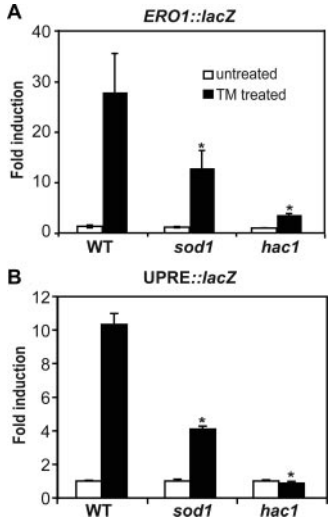

Figure 3. Cells deleted of *SOD1* have reduced UPR induction. Wild-type (CY4), *sod1* (ST002), and *hac1* (ST003) cells were transformed with (A) *ERO1*::*lacZ* or (B) UPRE::*lacZ*. Transformants were grown to exponential phase (OD_{600} ~0.5), and TM was added to 1 μg/ml (TM-treated) or untreated. Cells were harvested after 2 h, and β-galactosidase activity was determined. Values were expressed as fold induction of β-galactosidase activity of TM-treated cells over untreated cells. Error bars, SD of three individual transformants. Significant difference (* $p < 0.05$) when compared with the wild-type cells treated with TM.

ERO1 Does Not Contribute to Superoxide Generated during ER Stress

The essential oxidoreductase Ero1p is required for disulphide bond formation in the ER and was previously shown to generate ROS both in vitro and in vivo (Haynes *et al.*, 2004; Gross *et al.*, 2006). We therefore sought to determine whether Ero1p generates superoxide anions in cells subjected to ER stress.

Carboxypeptidase Y (CPY, encoded by *PRC1*) is a nonspecific peptidase of the vacuolar lumen that undergoes folding in the ER and maturation in the *trans*-Golgi network (Stevens *et al.*, 1982; Valls *et al.*, 1987). Expression of a terminally misfolded form of CPY encoded by *PRC1–1*, denoted CPY*, causes ER stress and activation of the UPR due to accumulation of this protein in the ER (Hiller *et al.*, 1996; Spear and Ng, 2003; Haynes *et al.*, 2004). Therefore, *ERO1* was chronically overexpressed in conjunction with CPY* to induce ER stress.

Because Sod1p levels in wild-type cells vary with carbon source (data not shown), a β-estradiol–inducible system that is compatible with the *GAL1* promoter and does not require a change in carbon source (Gao and Pinkham, 2000) was used to induce CPY*HA and *ERO1myc* from plasmids encoding C-terminally HA-tagged *PRC1–1* (CPY*HA) and a C-terminally *myc*-tagged *ERO1* (*ERO1myc*) fused to the *GAL1* promoter. The Ero1-myc protein was functional because it rescued the temperature-sensitive *ero1–1* mutant at the nonpermissive temperature and increased DTT resistance when overexpressed in wild-type cells (data not shown). Efficacy of the *GAL1*-CPY*HA construct was established previously (Spear and Ng, 2003). Western blot analysis indicated that both proteins could be induced in wild-type and *hac1* cells using this system (Figure 4A). Chronic overexpression of CPY*HA and/or *ERO1myc* had no effect on growth of wild-type cells. However, overexpression of CPY*HA with or without *ERO1myc* coexpression inhibited

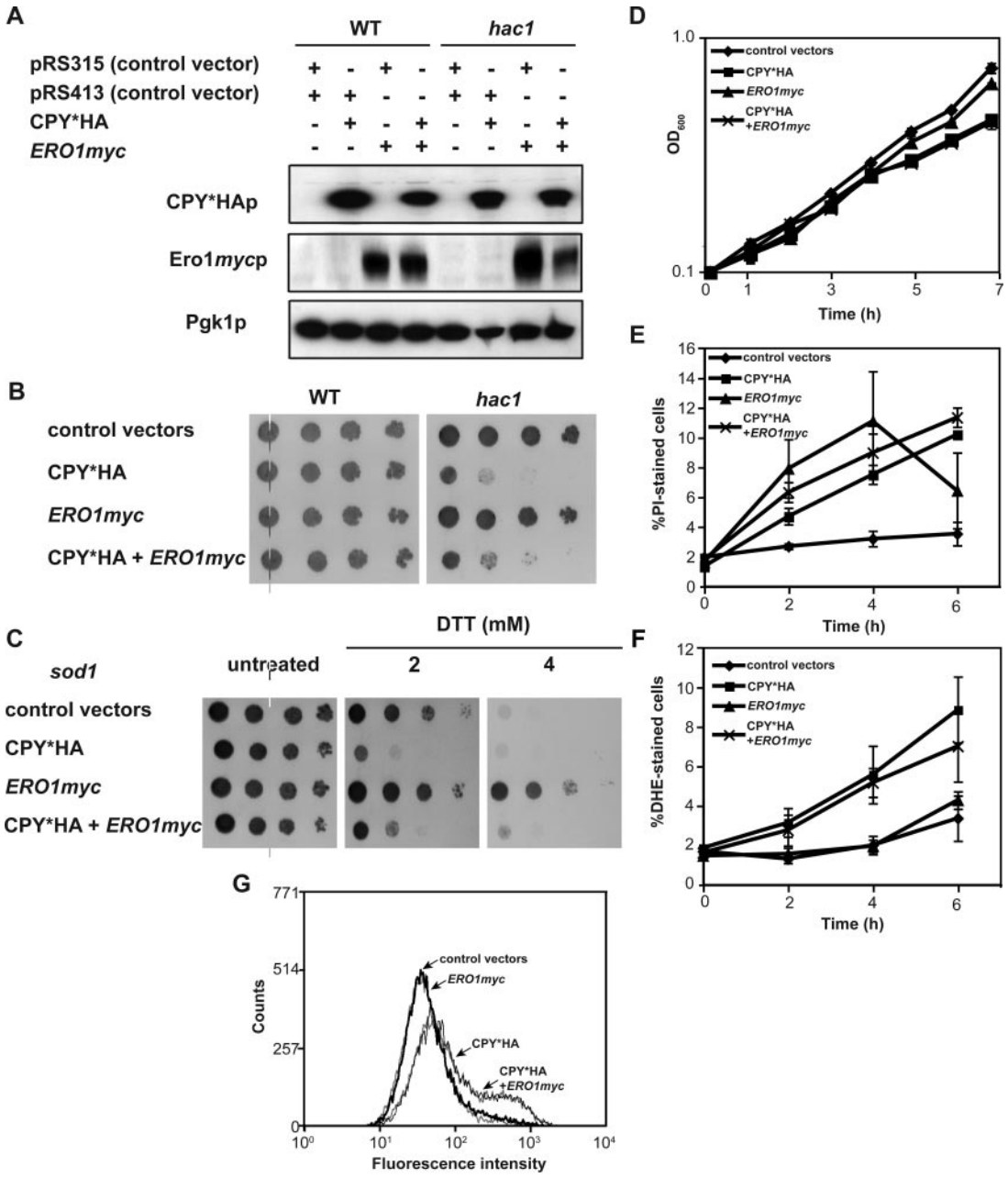

Figure 4. Wild-type Ero1p does not contribute to superoxide production during CPY*-induced ER stress. Wild-type (CY4), *sod1* (ST002), and *hac1* (ST003) cells harboring the pGEV-TRP1 plasmid were transformed with pRS315 or pRS413 and/or pES67 (*GAL1*-CPY*HA) or pST2 (*GAL1-ERO1myc*). (A) Western blot analysis was performed using lysate of cells after 6 h of 5 μM β-estradiol induction in SC-drop out media. (B and C) The OD_{600} of 2-d-old cultures of the indicated strains was adjusted to 1.0, 0.1, and 0.01, and 5-μl aliquots were spotted onto SC dropout medium containing 5 μM β-estradiol, and the indicated concentration of DTT. Plates were incubated for 2 d at 30°C. (D–F) Exponentially growing cultures of *hac1* strains with the indicated plasmid were reinoculated to a final OD_{600} of 0.1 in SC dropout medium containing 5 μM β-estradiol. (D) Cell density was monitored every hour. Error bars, SD of three individual transformants of one experiment. (E) PI staining and (F) DHE staining were performed at the indicated time point. Error bars, SE of three independent experiments. (G) FACS analysis of DHE stained cells was performed 6 h after β-estradiol induction.

growth of UPR-deficient cells (*hac1*), whereas overexpression of *ERO1myc* alone had no obvious effect on growth of either wild-type or *hac1* cells (Figure 4B).

If Ero1p generates appreciable quantities of superoxide in vivo, we surmised that the *sod1* mutant may exhibit sensitivity to Ero1p overexpression. Overexpression of CPY*HA and/or *ERO1myc* had no obvious effect on growth of *sod1* cells (Figure 4C). Overexpression of CPY*HA in *sod1* cells led to growth inhibition under a non-LD of DTT (2 mM) compared with *sod1* cells expressing the control vector alone, supporting the hypothesis that Sod1p is required for wild-type ER stress tolerance. Overexpression of *ERO1myc* alone or in conjunction with CPY*HA did not further increase the sensitivity of *sod1* cells to DTT stress when compared with *sod1* cells overexpressing CPY*HA alone. Furthermore, *ERO1myc* overexpression increased tolerance of

sod1 cells to a concentration of DTT (4 mM) that inhibited the growth of the same cells transformed with the control vectors (Figure 4C). These data indicate that the absence of Sod1p does not render cells more sensitive to increased levels of Ero1p in CPY*HA and/or DTT-induced ER stress or to the effect of increased Ero1p expression in the presence of DTT. It is worth noting that overexpression of *ERO1myc* does not confer increased tolerance of the *hac1* mutant to TM (data not shown), indicating that Ero1p protects cells against ER stress in a manner more related to protein thiol redox status.

To examine further whether Ero1p may generate superoxide under ER stress, CPY*HA and/or *ERO1myc* were acutely overexpressed in *hac1* cells that were stained with DHE over a period of 6 h (Figure 4F). Cell density (Figure 4D) and cell viability using PI staining (Figure 4E) were also monitored. Induction of CPY*HA alone or in conjunction with *ERO1myc* led to a reduction in growth, whereas *ERO1myc* had a lesser effect on *hac1* cells (Figure 4D). Overexpression of CPY*HA led to an increase in DHE-stained cells (Figure 4F), indicating that CPY*HA-induced ER stress also leads to superoxide production as was observed in TM-treated cells (Figure 2, C and D). *ERO1myc* overexpression alone, or in conjunction with CPY*HA, did not lead to an increase in DHE-stained cells compared with cells overexpressing CPY*HA alone. Furthermore, FACS analysis of these cells after 6 h indicated that *hac1* cells overexpressing CPY*HA alone or in conjunction with *ERO1myc* displayed an identical distribution of the DHE fluorescence (Figure 4G). Overexpression of CPY*HA and/or *ERO1myc* results in an increase in PI-stained *hac1* cells, indicating increased cell death when the level of these proteins was increased.

Ero1p can self-regulate its oxidase activity, because of the presence of two cysteine residues (C150 and C295; Sevier *et al.*, 2007). Mutation of these residues to alanine disrupts the ability of Ero1p to regulate its oxidizing activity (Sevier *et al.*, 2007). Overexpression of this hyperactive *ERO1* allele (*ero1*-C150A-C295A) inhibits growth of cells in a manner dependent on the oxidase activity of the protein (Sevier *et al.*, 2007). To determine if cells expressing the hyperactive allele of Ero1p generate superoxide, *ero1*-C150A-295A-*myc* was overexpressed in *hac1* cells using the β-estradiol–inducible system. When *ero1*-C150A-295A-*myc* was overexpressed in *hac1* cells, no obvious difference in growth compared with wild-type *ERO1myc* overexpression in *hac1* cells was observed on solid media using spot test analysis (data not shown). However, a more sensitive approach was used to detect growth differences by monitoring cell density using an automated continuous culture system (Figure 5, A–D). Overexpression of *ero1*-C150A-C295A-*myc* resulted in retarded growth. This retardation of growth was dependent on Ero1p hyperactivity because expression of the control inactive form (*ero1*-C150A-C295A-C100A-C105A-*myc*; Sevier *et al.*, 2007) did not affect growth relative to wild-type *ERO1myc* or the control vector (Figure 5A). Overexpression of *ero1*-C150A-C295A-*myc* increased resistance of *hac1* cells to DTT, similar to that observed for overexpression of wild-type *ERO1myc* (Figure 5B). These data are therefore consistent with the previous observations of Sevier *et al.* (2007).

Overexpression of *ero1*-C150A-295A-*myc* or wild-type *ERO1myc* in conjunction with CPY*HA led to a slight increase in growth of *hac1* relative to cells overexpressing CPY*HA alone (Figure 5C). A similar trend was observed for the above constructs for *hac1* cells treated with DTT (Figure 5D). To determine whether the reduction in growth was associated with increased accumulation of superoxide, the extent of DHE fluorescence in *hac1* cells overexpressing *ERO1myc* or *ero1*-C150A-295A-*myc* in the presence or absence of CPY*HA overexpression was determined. Cells expressing *ero1*-C150A-295A-*myc* yielded a DHE fluorescence profile similar to those expressing *ERO1myc* or the control vectors (Figure 5E). Importantly, expressing CPY*HA alone led to increased DHE-associated fluorescence (Figure 5E), and this was not further increased by coexpression of *ero1*-C150A-295A-*myc* or *ERO1myc*. (Figure 5F). These data indicate that Ero1p does not generate appreciable levels of superoxide anions in cells.

The UPR and Sod1p Protect Cells against ER Stress in an Overlapping Pathway

The requirement of Sod1p for wild-type UPR induction and the increase in Sod1p and its activity in cells treated with TM (Figures 2 and 3) indicated a link between the unfolded protein response and detoxification of superoxide. To determine whether these two processes function in the same or different pathways for ER-stress tolerance, a *sod1 hac1* strain was generated and examined for growth and tolerance to ER stress and the superoxide-generating agent paraquat.

The resulting *sod1 hac1* strain was viable and its growth was compared with the respective single deletants and the wild type (Figure 6A). The four strains showed a similar growth rate in early exponential phase. The *hac1* strain reached a final cell yield similar to wild-type cells, whereas *sod1* and *sod1 hac1* strains had a lower yield than the wild type. The *sod1* and *sod1 hac1* strains showed a reduced growth rate relative to the wild type and *hac1* strains as the cells approached diauxic shift/respiratory phase, with the *sod1 hac1* strain displaying a more pronounced growth retardation. As cells approach the diauxic shift, ROS generation increases, leading to increased *SOD1* transcription (Maris *et al.*, 2001). These data indicate that in the absence of Sod1p, the UPR may also play a role in transition of cells in the diauxic shift.

To further elucidate the relationship between Sod1p and the unfolded protein response to ER stress, chemically (i.e., TM and DTT) and genetically (i.e., CPY*) induced ER stresses were used. Although induction of CPY* under the control of a *CUP1* promoter at 30°C led to inhibition of *hac1* cells, growth of *sod1* cells was unaffected (Figure 6B). This result is consistent with the effect of CPY*HA induction under the control of the *GAL1* promoter using the inducible β-estradiol system (Figure 4C). In contrast, at 37°C (a temperature known to increase unfolded/misfolded proteins), induction of CPY* using 400 or 800 μM $CuSO_4$ resulted in pronounced inhibition of *sod1* cells. This inhibition was not due to the presence of elevated Cu^{2+} because growth of *sod1* cells was similar to that of wild-type cells expressing the empty vector in Cu^{2+}-containing media (Figure 6B). The *sod1 hac1* mutant was also more sensitive to CPY* induction (Figure 6B) at 30 or 37°C relative to the *sod1* and *hac1* single mutants. The ER stress sensitivity of *sod1* cells was therefore correlated with the level of ER stress experienced by cells, because the *sod1* mutant with a defective UPR was more susceptible to ER stress as observed in the double mutant.

Deletion of *SOD1* in *hac1* cells also led to increased sensitivity to either TM or DTT (Figure 6C). Moreover, although growth of the *sod1* strain under ER stress was rescued under anaerobic conditions, that of the *sod1 hac1* double mutant was not (Figure 6C). This indicates that the effects of *SOD1* deletion in the *hac1* mutant were due to accumulation of ROS. The sensitivity of *sod1 hac1* to the superoxide-generating agent paraquat reflected that of the *sod1* deletant, indicating that the UPR plays little role in tolerance of superoxide.

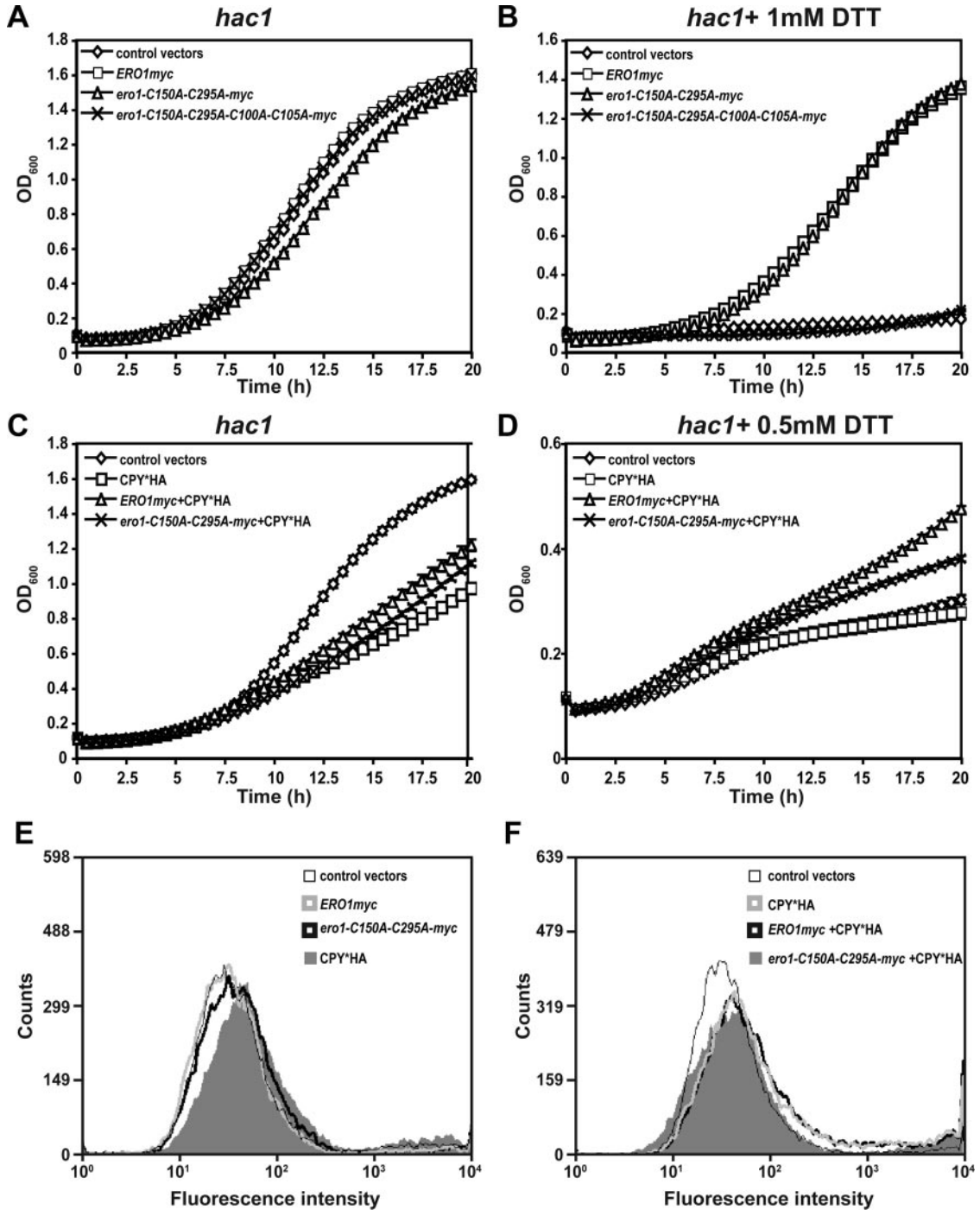

Figure 5. An hyperactive allele of *ERO1* does not increase superoxide anion production. The *hac1* mutant (ST003) harboring the pGEV-TRP1 was transformed with pRS315 or pRS316 (control vectors) and/or pAF112 (*GAL1-ERO1myc*), pCS452 (*GAL1-ero1*-C150A-C295A-*myc*), pCS504 (*GAL1-ero1*-C150A-C295A-C100A-C105A-*myc*), and/or pES67 (*GAL1*-CPY*HA) as indicated. (A) The *hac1* mutant transformed with the indicated plasmid were grown overnight and subcultured to OD_{600} of 0.1 in SC medium containing 5 μM β-estradiol in the absence of DTT or in the presence of (B) 1 mM or (D) 0.5 mM DTT. Growth of cultures was monitored using Bioscreen (C) as described in *Materials and Methods.* Error bars, SD of four replicates. (E and F) *hac1* cells were stained with DHE and analyzed using FACS after 6 h of β-estradiol induction.

These results further support the hypothesis that ER stress leads to ROS production (Harding *et al.*, 2003; Haynes *et al.*, 2004; Hauptmann *et al.*, 2006). The ROS are likely to be superoxide because SOD activity was up-regulated in wild-type and *hac1* cells under ER stress (Figure 2, A and B). Furthermore, deletion of *SOD1* increased sensitivity of wild-type or UPR-deficient (*hac1*) cells to all conditions that generate ER stress (TM, DTT, and CPY*). We therefore rationalized that Sod1p protects cells against ER stress both in the presence and absence of the UPR. Collectively, these results

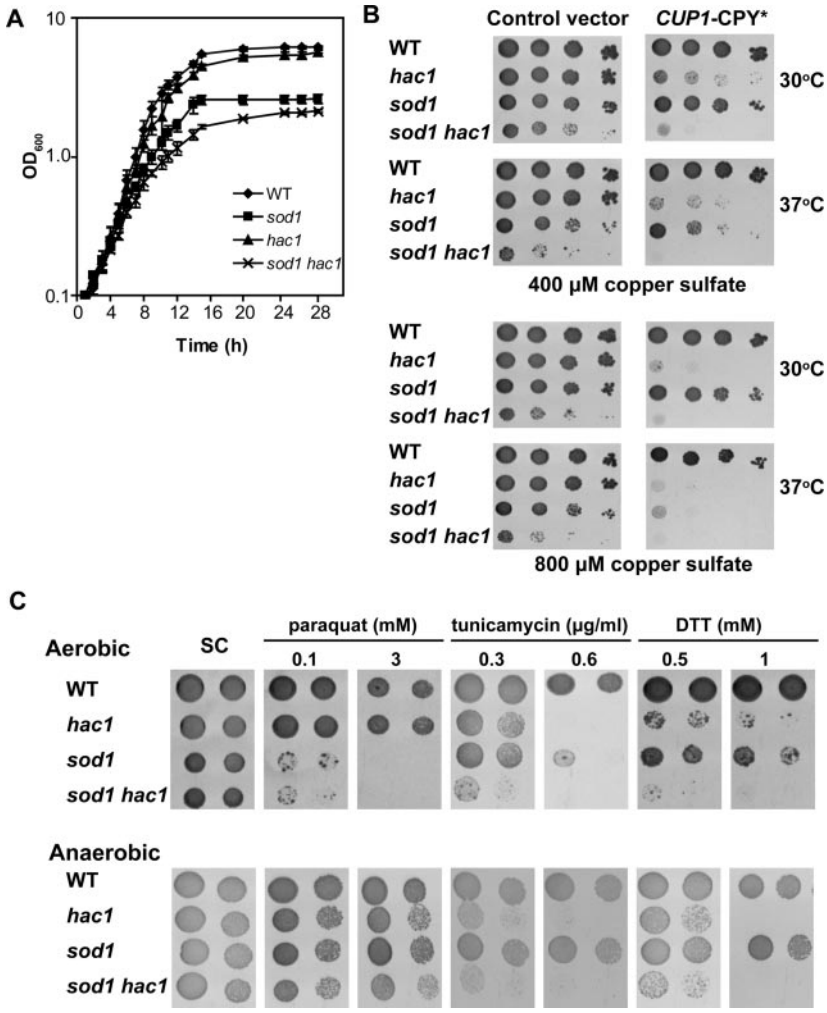

Figure 6. The unfolded protein response and Sod1p protect cells against ER stress in an overlapping manner. (A) Growth of the *sod1 hac1* (ST007) mutant. Indicated strains were grown overnight and reinoculated into fresh medium at a final OD_{600} of 0.1 and incubated at 30°C with agitation. Cell density of each strain was determined over 28 h. Error bars, the SD of three cultures. (B) CPY*-induced ER stress resulted in hypersensitivity of cells deleted of *SOD1* and/or *HAC1*. The strains indicated (CY4 background) were transformed with pRS316 (control vector) or pAC595 (*CUP1*-CPY*). Individual transformants were grown for 2 d, and OD_{600} was adjusted to 1.0, 0.1, 0.01, and 0.001. Five microliters of diluted culture was spotted on to SC-URA media containing 400 or 800 μM copper sulfate and incubated at 30 or 37°C. (C) Deletion of *SOD1* in *hac1* strains increases sensitivity to DTT and TM in an oxygen-dependent manner. The strains were grown to stationary phase, and OD_{600} was adjusted to 1.0 and 0.1. Diluted cultures (5 μl) were spotted on to SC medium without or containing the indicated concentration of compounds and incubated for 2 d (aerobic) or 3 d (anaerobic).

indicate that SOD activity and the unfolded protein response are both required for maximal tolerance of chronic ER stress, and these two processes function in an overlapping manner.

Multiple Copies of TKL1 Suppress the Sensitivity of the sod1 Mutant to TM

To identify additional factors linking Sod1p to resistance to ER stress, a screen for high-copy suppressors of the TM sensitivity of *sod1* cells was performed. The *sod1* mutant was transformed with a multicopy gene library and the resulting ~2000 transformants were replica-plated on to media containing a lethal concentration (LC) of TM that would otherwise inhibited growth of *sod1* cells.

Two plasmids conferred TM resistance on *sod1* cells: the first harbored *ALG7* and the second *TKL1*. *ALG7* encodes dolichyl-P–dependent *N*-acetylglucosamine-1-P transferase, a known target of TM (Barnes *et al.*, 1984; Kukuruzinska and Lennon, 1995), whereas *TKL1* encodes a transketolase isoform that acts in the pentose phosphate pathway that is important for NADPH generation. Overexpression of *TKL1* also rescued the sensitivity of *sod1* cells to DTT (data not shown).

If the rescue of *sod1* to ER stress by *TKL1* overexpression was due to an increase in the generation of NADPH, then this should rely on Zwf1p, the first committed step of the pentose phosphate pathway (Nogae and Johnston, 1990). Overexpressing *TKL1* in the wild-type and *zwf1* and *zwf1 sod1* strains had minimal effect on their tolerance to TM, whereas the *sod1* strain showed increased resistance to this stress (Figure 7A). Similar results were obtained with DTT (data not shown). The effect of *TKL1* overexpression on *sod1* was not a general effect on UPR-deficient cells because *TKL1* overexpression did not suppress the sensitivity of *hac1* to TM (Figure 7B). The data verify that the rescue of *sod1* sensitivity to ER stress by *TKL1* overexpression depends on the NADPH-generating components of the pentose phosphate pathway. From Figure 7A, the *zwf1 sod1* strain was more sensitive to ER stress than the *sod1* strain, indicating that there is some contribution of *ZWF1* to combating ER stress.

To examine the effect of *TKL1* overexpression on NADP(H) homeostasis of *sod1* cells, $NADP^+$ and NADPH levels were determined in the presence and absence of TM treatment (Figure 7C). Overexpression of *TKL1* led to a significant increase in the total pool of NADP(H). Surprisingly, this was due to increased accumulation of $NADP^+$ (Figure 7C). TM treatment of *sod1* cells overexpressing *TKL1* did not affect NADP(H) homeostasis further relative to *TKL1* overexpression alone. This is interesting because exposure of *sod1* cells (without *TKL1* overexpression) to TM led to a ~40% increase in $NADP^+$. These data indicate there is an

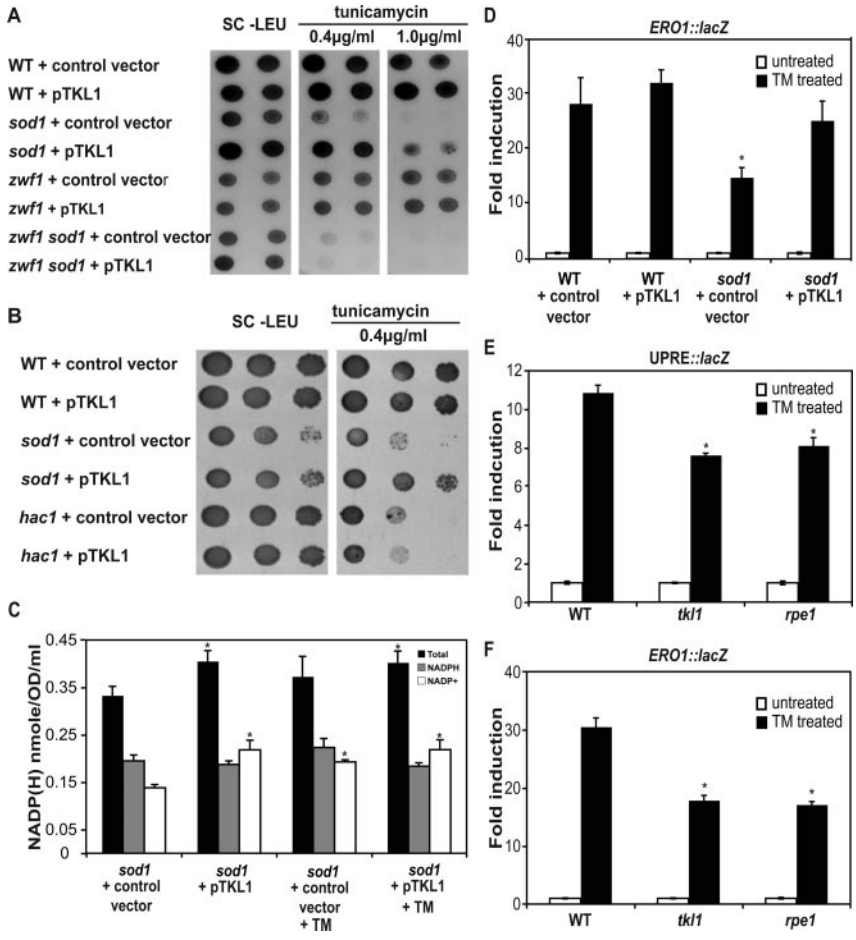

Figure 7. Overexpression of *TKL1* rescues *sod1* from ER stress, affects NADP(H) levels, and restores UPR induction of the *sod1* mutant. (A) Wild-type (1783), *sod1* (KS105), *zwf1* (KS113), and *zwf1 sod1* (KS117) and (B) wild-type (CY4), *sod1* (ST002), and *hac1* (ST003) were transformed with pTKL1 or YEp13 (control vector). Transformants were grown for 2 d, OD_{600} was adjusted to 1.0 and 0.1, and 5 μl of the diluted cultures was spotted on to media with or without the indicated concentration(s) of TM. Cells were incubated for 2 d at 30°C. (C) The *sod1* mutant (ST002) transformed with YEp13 or pTKL1 was cultured to OD_{600} of 0.5, treated or untreated with TM, and assayed for intracellular NADP(H) level. Error bars, SE of six replicates from three independent experiments. Significant difference (* $p < 0.05$) when compared with the untreated control vector of the same species of NADP(H) assayed. Wild-type and *sod1* cells (D) or *tkl1* and *rpe1* cells (E) and (F) were transformed with the indicated plasmids. Transformants were grown to exponential phase and TM was added at a final concentration of 1 μg/ml (TM treated) or untreated. Cells were harvest after 2 h, and β-galactosidase activity was determined. Values were expressed as fold induction of β-galactosidase activity of TM-treated cells over untreated cells. Error bars, SD of three individual transformants. Significant difference (* $p < 0.05$) when compared with the wild-type cells (control vector) treated with TM.

increased burden on NADP(H) homeostasis and on the NADPH-generating systems during ER stress. Because the level of NADPH did not change upon TM treatment and/or after *TKL1* overexpression, one possible explanation of this data is that the protective affect of *TKL1* overexpression on *sod1* ER-stress tolerance (Figure 7A and B) may be due to altered flux through the NADPH-generating pathway.

Mutants Affected in NADPH Synthesis Are Affected in Induction of the UPR

Because *TKL1* overexpression affected the NADP(H) homeostasis of *sod1* cells and conferred increased tolerance of these cells to ER stress (Figure 7, A and B) and because *sod1* cells exhibit reduced UPR induction (Figure 3), we wanted to see whether *TKL1* overexpression rescued the unfolded protein response in the *sod1* mutant.

Overexpression of *TKL1* restored *ERO1::lacZ* induction in *sod1* and had no effect on wild-type cells (Figure 7D). These data indicate the failure of the *sod1* strain to fully induce the UPR can be attributed to disruption of NADP(H) homeostasis. If perturbation of NADP(H) homeostasis leads to reduced UPR induction, we rationalized that mutants affected in NADPH production would also be affected in UPR induction. The extent of TM induction of both *ERO1::lacZ* and UPRE::*lacZ* reporter constructs was significantly lower in *tkl1* and *rpe1* strains (defective in components of the pentose phosphate pathway) when compared with the wild type (Figure 7, E and F). These data indicate that disruption of NADP(H) homeostasis affects the induction of the UPR and provide insight into the effects of cellular NADP(H) on the unfolded protein response.

TM-induced ER Stress Alters Cellular Glutathione Levels

NADPH is important for numerous processes including maintenance of the thioredoxin and the glutathione redox couples. Given the important role of glutathione in regulation of ER oxidation state (Cuozzo and Kaiser, 1999), the impact of secretory pathway dysfunction on cellular glutathione homeostasis (Haynes *et al.*, 2004; Perrone *et al.*, 2005), and the proposal that increased GSH levels induce the UPR (Trotter and Grant, 2002), we sought to examine whether Sod1p is required for maintaining intracellular glutathione homeostasis of cells under ER stress.

TM treatment (4 h) led to increased GSSG and GSH in wild-type cells, as well as *hac1, sod1,* and *sod1 hac1* mutants (Figure 8, A and B). This increase was more marked in the *sod1 hac1* double mutant. In untreated cells of *sod1* and *sod1 hac1,* GSH and GSSG levels were also elevated, and this increased further on TM treatment. Overexpression of *TKL1* had little effect on GSSG or GSH levels in most strains with or without TM, only in the *sod1 hac1* double mutant was there a significant ($p < 0.05$) reduction in GSSG and GSH (Figure 8, A and B). Hence *TKL1* overexpression only affected glutathione levels under extreme ER stress, and loss of Sod1p caused by TM treatment of the *sod1 hac1* mutant.

Because the levels of GSH and GSSG reflect cellular redox state (Schafer and Buettner, 2001; Drakulic *et al.*, 2005) and glutathione is the major redox buffer in most cells (Schafer

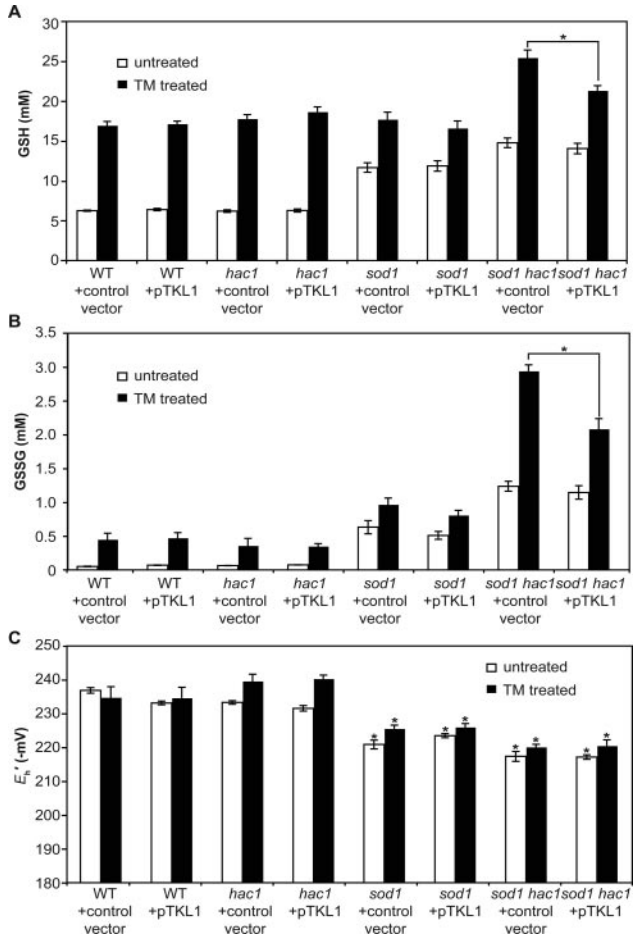

Figure 8. SOD1 is required to maintain cellular redox balance. (A) GSH and (B) GSSG were estimated in cells of the indicated strains untreated, or treated with TM, for 4 h. Error bars, the SE of at least four replicates from three independent experiments. (C) Cellular E_h' of the indicated strains as determined using the glutathione redox couple. Significant difference (* $p < 0.005$) compared with the wild-type strain or as indicated.

and Buettner, 2001), we estimated the cellular E_h' of *sod1*, *hac1,* and *sod1 hac1* strains under TM stress using the GSH/GSSG redox couple (Figure 8C). We also determined whether *TKL1* overexpression had any effect on cellular E_h' in these strains. The *sod1* and *sod1 hac1* strains had a significantly more oxidizing E_h' compared with wild-type or *hac1* cells ($p < 0.005$). Although deletion of *SOD1,* TM-induced ER stress, and *TKL1* overexpression all affected GSH or GSSG levels in some way, only loss of Sod1p caused any substantial change ($p < 0.005$) in cellular glutathione redox potential. Treatment of cells with TM with or without *TKL1* overexpression barely affected cellular redox in all strains tested. This is consistent with the observation that cells can maintain their cellular redox state even when treated with H_2O_2 (Ng *et al.,* 2008).

Because Sod1p is important to maintain ER stress tolerance and cellular glutathione redox potential, we sought to determine whether a change to more oxidizing conditions by deletion of the glutathione reductase gene (*GLR1*) would affect ER stress tolerance further. Intriguingly, we were unable to generate the double *sod1 glr1* mutant under aerobic conditions, but did recover it anaerobically (Supplemental Figure S3). The mutant displayed a severe oxygen-dependent growth defect, which was partially rescued by *TKL1* overexpression because dissection of a heterozygous diploid carrying the overexpression plasmid enabled aerobic isolation of the double mutant. Even this condition led to poor aerobic growth, which precluded use of the strain for analysis of ER stress tolerance.

Interestingly, glutathione may play a less direct role than NADPH during ER stress because deletion of *GLR1* (Muller, 1996; Grant, 2001) or *GSH1* did not result in TM or DTT sensitivity (data not shown).

DISCUSSION

The genome-wide screen for mutants sensitive to chronic TM and DTT stress identified genes encoding functions including the Cu, Zn SOD (Sod1p), the copper chaperone of Sod1p (Ccs1p), and those involved in NADPH generation to be important for chronic ER-stress tolerance. Several observations support the hypothesis that superoxide accumulation affects ER stress tolerance and that ER stress is associated with increased generation of superoxide: 1) the oxygen-dependent sensitivity of *sod1* and *ccs1* to TM and DTT; 2) the increase in Sod1p levels and Sod1p activity after treatment of cells with TM; 3) increased detection of superoxide anions in cells overexpressing CPY* or treated with TM; and 4) preadaptation of cells to superoxide affords protection to ER stress. These observations also demonstrate the importance of Sod1p activity in cell survival during ER stress. Although ROS production upstream of cell death during ER stress has previously been demonstrated (Hauptmann *et al.,* 2006; Haynes *et al.,* 2004), here we show that superoxide is at least one of the species of ROS produced during ER stress. The preadaptation data, together with the specificity of the Sod1p effect (mutants affecting other antioxidant functions do not show sensitivity to ER stress) indicate that there is a link between ER stress and superoxide production, and it is very unlikely that all of the above observations are due to a separate and independent additive effect of loss of Sod1p and of ER stress. However it is possible that some of the ROS detected during ER stress were a consequence of the onset of cell death.

ROS have been reported to accumulate during ER stress not only in *S. cerevisiae* (Haynes *et al.,* 2004; Hauptmann *et al.,* 2006) but also in *Caenorhabditis elegans* (Harding *et al.,* 2003). ROS and ER stress play an important role in pathogenesis of many human diseases including diabetes, atherosclerosis, and neurodegenerative diseases (Kincaid and Cooper, 2007; Malhotra and Kaufman, 2007). In each of these cases ROS accumulation was detected, but the species present was not fully elucidated. Despite this, the extent of ROS production was shown to be affected by the level of *ERO1* (Harding *et al.,* 2003; Haynes *et al.,* 2004).

In this study, overexpression of CPY* in *hac1* cells led to increased production of superoxide. However, overexpression of *ERO1* alone, or in conjunction with CPY*, did not lead to any further increase in the level of superoxide. Furthermore, overexpression of Ero1p did not increase sensitivity of *sod1* cells to CPY* and/or DTT exposure. These results indicate that appreciable levels of superoxide anions are unlikely to be generated during Ero1p-dependent oxidative protein folding under the conditions used.

Ero1p generates stoichiometric quantities of H_2O_2 during disulphide bond formation in vitro (Gross *et al.,* 2006). H_2O_2 accumulation during ER stress may lead to particularly serious consequences for the cell if superoxide accumulation occurred simultaneously since reduction of metals ions including Fe^{3+} and Cu^{2+} by superoxide can promote genera-

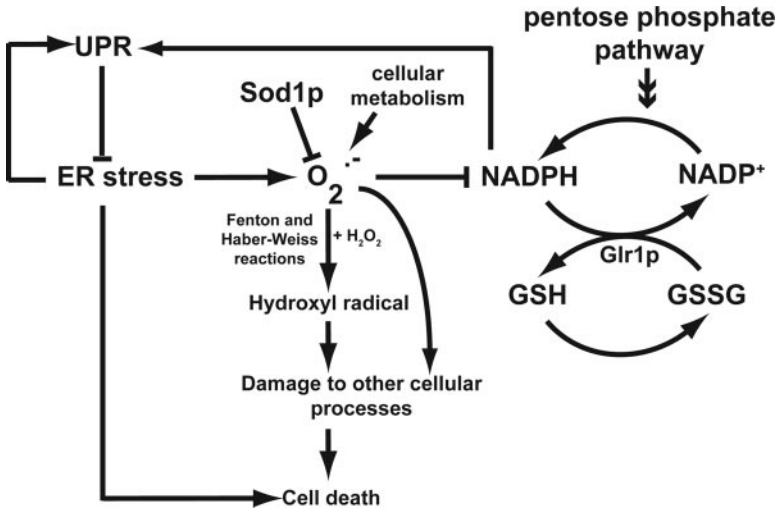

Figure 9. Proposed model for the role of Sod1p under ER stress and its effect on cellular NADP(H), glutathione, and the unfolded protein response.

tion of the highly reactive hydroxyl radical from H_2O_2 via Haber-Weiss and Fenton reactions (Perrone *et al.*, 2008). Although cells possess effective means for selective removal of superoxide and H_2O_2 , this is not the case for hydroxyl radicals (Perrone *et al.*, 2008). It is therefore important for cells to possess a mechanism to modulate Ero1p to minimize excess H_2O_2 production. Sevier *et al.* (2007) have shown that self-regulation of Ero1p oxidase activity is regulated by oxidation of two cysteine residues at positions 150 and 295 of the protein. The authors proposed that these cysteine residues play a role in minimizing excess H_2O_2 production by inhibiting continuous reduction of Ero1p by oxygen, thereby avoiding genesis of an hyper-oxidizing condition in the ER. Because ER stress induces expression of *ERO1* (Travers *et al.*, 2000; Takemori *et al.*, 2006), regulation of Ero1p activity may be important to minimize H_2O_2 production under conditions of elevated superoxide anion accumulation, e.g., during ER stress as demonstrated in this study. Here, however, overexpression of the mutant *ero1*-C150A-295A did not lead to overaccumulation of superoxide, further supporting the proposal that Ero1p is not a source of superoxide in vivo.

Interestingly, although simultaneous deletion of cytosolic and peroxisomal catalases (*CTT1*, *CTA1*) and γ-glutamyl-cysteine synthetase (*GSH1*) leads to extreme sensitivity to exogenous H_2O_2, (Grant *et al.*, 1998), it did not affect tolerance to ER-stressing agents. Overexpression of *ERO1* in the *ctt1 cta1 gsh1* triple mutant did not inhibit growth in the presence or absence of the ER-stressing agent DTT (data not shown). These data indicate that although it is possible that Ero1p generates H_2O_2 in vivo, the levels produced are not sufficient to overwhelm any residual H_2O_2 detoxification activity or damage coping capacity present in the *ctt1 cta1 gsh1* triple mutant. This is in contrast to the reduced tolerance of this mutant to exogenous H_2O_2 when this oxidant was added extracellularly at millimolar concentrations (Grant *et al.*, 1998).

The data above appear to conflict with previous findings by Haynes *et al.* (2004) who demonstrated a role for Ero1p in ROS generation during ER stress in vivo. This difference may be due to the nature of ER stress experienced by cells and to different strain backgrounds. Here, overexpression of CPY* in *hac1* cells led to increased superoxide production, and increasing Ero1p levels did not affect the level of superoxide produced. In the study by Haynes *et al.* (2004), whereas CPY* was used to generate ER stress, ROS production associated with *ERO1* overexpression was demonstrated to occur in *ire1 erv29* cells. The possible contribution of strain background in influencing the outcome is further supported by the demonstration that deletion of *IRE1* in *erv29*, harboring a constitutively active allele of *HSF1*, led to reduced tolerance to TM and CPY* (Liu and Chang, 2008). In contrast, Haynes *et al.* demonstrated that deletion of *IRE1* in the *erv29* mutant reduced sensitivity to CPY* overexpression. Additional genetic differences between the strain backgrounds used to conduct these studies may also have affected observations made. This may include difference in genetic backgrounds leading to altered tolerance of strains to ROS as observed previously (Veal *et al.*, 2003).

Superoxide accumulation in the *sod1* mutant also reduced tolerance to ER stress by dampening activation of the UPR. These changes, at least in part, were mediated through effects of superoxide on NADP(H) homeostasis because overexpression of *TKL1*, reversed sensitivity of *sod1* cells to TM and DTT, and restored UPR induction to wild-type levels. Moreover, mutants disrupted in the nonoxidative branch of NADPH synthesis exhibited a defect in UPR induction that correlated with sensitivity to ER stress. A model summarizing the role of Sod1p and NADP(H) homeostasis in ER stress tolerance is given in Figure 9.

The UPR is essential to counter ER stress (Travers *et al.*, 2000). The decreased tolerance of *sod1* cells to ER stress is at least in part due to the effects of superoxide accumulation on NADP(H) homeostasis, which in turn affects the magnitude of UPR induction (Figure 9). This proposal is supported by the observation that *TKL1* overexpression rescued the ER stress sensitivity and the UPR induction defect of *sod1* cells. However, *TKL1* overexpression did not fully restore the sensitivity of *sod1* cells to DTT or TM in contrast to anaerobiosis, indicating that superoxide accumulation in cells also reduces ER stress tolerance through mechanisms other than UPR induction. Therefore, the sensitivity of *sod1* cells to ER stress is primarily attributed to superoxide accumulation, and this accumulation leads to disruption of both NADP(H) homeostasis and UPR induction (Figure 9). Increased superoxide accumulation in *hac1* cells does not appear to affect ER stress tolerance through altered NADP(H) homeostasis because *TKL1* overexpression did not affect their tolerance to ER stress. Moreover, it is unlikely that superoxide accumulation per se in *hac1* cells during ER stress was a primary cause of death because anaerobiosis did not increase tolerance of these cells to ER stress.

NADPH is required for maintenance of the thioredoxin and glutathione redox systems. The effect of deleting *SOD1*

or affecting the NADPH biosynthesis pathway on induction of the UPR may therefore be due to changes in the glutathione and/or thioredoxin redox couples. It is unlikely that increased accumulation of oxidized thioredoxin led to reduced UPR induction in the above strains, because deletion of thioredoxin reductase (Trr1p) causes oxidized thioredoxin to accumulate (Holmgren, 1989) but leads to hyper-induction of the UPR and increased resistance to TM (Trotter and Grant, 2002). Deletion of *TRR1* also led to a approximately threefold increase in intracellular GSH accumulation, leading to the proposal that the elevated UPR in *trr1* mutants is a consequence of elevated GSH levels, which in turn leads to accumulation of misfolded proteins in the ER (Trotter and Grant, 2002). Overexpression of *GSH1* also leads to a 30% increase in UPR induction (Cuozzo and Kaiser, 1999). However, *sod1* (this study) and *tkl1* and *rpel* (Ng *et al.*, 2008) mutants all accumulate higher levels of GSH relative to wild-type cells, yet exhibit a reduced UPR induction. These data indicate that elevated GSH in *sod1*, *tkl1*, and *rpe1* mutants is unlikely to account for altered UPR induction in these cells. Disruption of *SOD1, TKL1,* or *RPE1* also leads to increased accumulation of oxidized glutathione (GSSG) in cells. Deletion of *GLR1* also leads to increased accumulation of GSSG, but this mutant exhibited a similar induction of the UPR as wild-type cells in response to DTT (Trotter and Grant, 2002). Analysis of the intracellular glutathione redox potential showed that *sod1* cells had a more oxidizing environment (this study), whereas the *tkl1* and *rpe1* mutants display a wild-type redox potential (Ng *et al.*, 2008). Therefore the altered UPR observed in *sod1*, *tkl1*, and *rpe1* cells was more closely correlated with altered NADP(H) homeostasis and was unlikely to have resulted from altered thioredoxin, GSH, GSSG, or the intracellular redox environment.

ER stress caused a change in NADP(H) homeostasis, whereby the level of total NADP(H) and $NADP^+$ increased significantly during this stress, and the absolute level of NADPH in *sod1* cells during ER stress was comparable to that in untreated cells. This indicates that ER stress is unlikely to disrupt NADPH synthesis directly because this would be expected to lead to increased $NADP^+$ at the expense of NADPH. Rather ER stress appears to increase the burden on the NADPH-generating system leading to accumulation of $NADP^+$. Overexpression of *TKL1* therefore may afford protection of *sod1* cells to ER stress, not only through reversal of the UPR defect, but also by increasing flux through the NADPH-generating system, thereby facilitating processes that require NADPH.

This study highlights the vital role of Sod1p and NADP(H) homeostasis in induction of the UPR and survival of cells during ER stress. Accumulation of superoxide in cells may lead to reduced UPR induction and elevated ER stress. Because the role of the UPR is to alleviate ER stress and ER stress is associated with elevated superoxide production, accumulation of this ROS in cells before and/or during ER stress may pose serious consequences for cell survival by promoting a downward spiral toward cell death. H_2O_2 formation by the ER or other processes in the cell may further increase the deleterious consequences of ER stress and superoxide accumulation, by promoting production of the highly reactive hydroxyl radical. These findings illustrate the interconnected nature of ER stress, superoxide anions, and the UPR and NADPH homeostatic systems, which may have important implications for disease processes particularly those associated with ER stress and ROS, including neurodegenerative disease and diabetes.

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