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Loss of *SOD1* and *LYS7* Sensitizes *Saccharomyces cerevisiae* to Hydroxyurea and DNA Damage Agents and Downregulates *MEC1* Pathway Effectors†

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Aerobic metabolism produces reactive oxygen species, including superoxide anions, which cause DNA damage unless removed by scavengers such as superoxide dismutases. We show that loss of the Cu,Zn-dependent superoxide dismutase, *SOD1*, or its copper chaperone, *LYS7*, confers oxygen-dependent sensitivity to replication arrest and DNA damage in *Saccharomyces cerevisiae*. We also find that *sod1*Δ strains, and to a lesser extent *lys7*Δ strains, when arrested with hydroxyurea (HU) show reduced induction of the *MEC1* pathway effector Rnr3p and of Hug1p. The HU sensitivity of *sod1*Δ and *lys7*Δ strains is suppressed by overexpression of *TKL1*, a transketolase that generates NADPH, which balances redox in the cell and is required for ribonucleotide reductase activity. Our results suggest that the *MEC1* pathway in *sod1*Δ mutant strains is sensitive to the altered cellular redox state due to increased superoxide anions and establish a new relationship between *SOD1*, *LYS7*, and the *MEC1*-mediated checkpoint response to replication arrest and DNA damage in *S. cerevisiae*.

Reactive oxygen species (ROS), which include superoxide anions (O_2^-) and hydroxyl free radicals (OH^-), are by-products of aerobic life (36). Both nonenzymatic and enzymatic mechanisms can remove ROS and maintain a reducing environment. Nonenzymatic defenses include production of glutathione, glutaredoxins, thioredoxins, and NADPH (14). Enzymatic defenses include superoxide dismutases (SOD), which are responsible for O_2^- scavenging and dismutation to hydrogen peroxide (H_2O_2) and O_2, and peroxidases, including thiol-dependent peroxiredoxins and thiol-independent catalases, which dissociate H_2O_2 to water and O_2 (4). Failure to detoxify ROS leads directly or indirectly to DNA damage, oxidation of protein and lipids, increased mutation rates, aneuploidy, and, ultimately, cell death (47, 60). In mammalian cells, accumulation of ROS induced by ionizing radiation or exogenous H_2O_2 activates the *ATM*/*ATR* pathway, resulting in increased expression of the tumor suppressor p53 followed by cell cycle arrest and/or apoptosis (4, 35, 62). Patients with defective DNA damage signaling pathway syndromes, such as ataxia telangiectasia, or DNA repair deficiency syndromes, such as Fanconi's anemia or Bloom's syndrome, are susceptible to DNA damage and increased oxidative stress (55).

As befits their role as the first line of defense against ROS and oxidative stress in the cell, SODs are conserved evolutionarily, although the enzymes vary in their subcellular localization and requirements for metal ion cofactors (14). In humans, mutations in cytosolic *SOD1* have been implicated in 20% of cases of the familial form of the motor neuron disease amyotrophic lateral sclerosis (Lou Gehrig's disease) (43, 44). Decreased affinities for zinc and copper ions have been implicated in the protein dimer destabilization and toxic gain-of-function properties of SOD1 protein (3, 13, 24, 41). In *Saccharomyces cerevisiae*, SOD is found in two forms: Sod1p, which depends on copper and zinc for its activity and localizes to the cytosol, the nuclei, and the inner membrane spaces of mitochondria (51), and Sod2p, which relies on manganese for its activity and localizes exclusively to the mitochondria (40). Sod1p activity is exquisitely responsive to intracellular copper levels and depends on the copper chaperone, Lys7p, for copper loading (16). Strains lacking *SOD1* and *LYS7* exhibit a variety of oxygen-dependent phenotypes, including slow growth, sensitivity to hyperoxia and superoxide-generating agents such as menadione and paraquat (50), increased spontaneous mutation rates (23), and methionine and lysine auxotrophy (14). The auxotrophies arise due to disruption of biosynthetic pathways of these amino acids by an altered redox environment. Decreased NADPH levels and oxidation of a putative iron-sulfur (4Fe-4S) reactive center of homoaconitase (Lys4p) in *sod1*Δ strains lead to blockage of methionine and lysine biosynthetic pathways, respectively (14, 49, 58).

As little is known about the molecular relationships between accumulation of excess superoxides and DNA damage checkpoint pathways, we used budding yeast as a model system to dissect these relationships. In *S. cerevisiae*, replication blocks and DNA damage generate signals that activate the protein kinase Mec1p, the ortholog of human *ATM*/*ATR*, which transmits these signals through two downstream pathways. In the first pathway, *MEC1*-dependent phosphorylation and activation of Rad53p, the ortholog of human Chk2, leads to phosphorylation and activation of Dun1p, which mediates the tran-

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† Supplemental material for this article may be found at http://mcb.asm.org.

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
BY4741	*mat***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics (Huntsville, AL)
BY4741 (*sod1*Δ)	*mat***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sod1*Δ::*KANMX4*	Research Genetics (Huntsville, AL)
BY4741 (*lys7*Δ)	*mat***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 lys7*Δ::*KANMX4*	Research Genetics (Huntsville, AL)
BY4741 (*skn7*Δ)	*mat***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 skn7*Δ::*KANMX4*	Research Genetics (Huntsville, AL)
BY4741 (*tsa1*Δ)	*mat***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 tsa1*Δ::*KANMX4*	Research Genetics (Huntsville, AL)
BY4741 (*yap1*Δ)	*mat***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 yap1*Δ:*KANMX4*	Research Genetics (Huntsville, AL)
W1588-4A	*mat***a** *leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5*	64
U952-3C	*mat***a** *leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 sml1*Δ::*HIS3*	64
U953-61D	*mat***a** *leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 mec1*Δ::*TRP1 sml1*Δ::*HIS3*	64
U960-5C	*mat*α *leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 rad53*Δ::*HIS3 sml1-1*	R. Rothstein
EG103	*mat*α *leu2-3,112 his3Δ1 trp1-289a ura3-52*	24
KS101	*mat*α *leu2-3,112 his3Δ1 trp1-289a ura3-52 sod1*Δ::*LEU2*	17
KS103	*mat*α *leu2-3,112 his3Δ1 trp1-289a ura3-52 lys7*Δ::*LEU2*	V. Culotta
1783	*mat***a** *leu2-3,112 his4 trp1-1 ura3-52 can1r*	51
KS105	*mat***a** *leu2-3,112 his4 trp1-1 ura3-52 can1r sod1*Δ::*TRP1*	51
KS113	*mat***a** *leu2-3,112 his4 trp1-1 ura3-52 can1r zwf1*Δ::*URA3*	51
KS117	*mat***a** *leu2-3,112 his4 trp1-1 ura3-52 can1r sod1*Δ::*TRP1 zwf1*Δ::*URA3*	51
YMB3233	*mat***a** *leu2 ura3 his3*	This study
YMB3234	*mat***a** *leu2 ura3 his3 trp1 sml1*Δ::*HIS3*	This study
YMB3235	*mat***a** *leu2 ura3 his3 sod1*Δ::*KANMX4*	This study
YMB3236	*mat***a** *leu2 ura3 his3 trp1 smll1*Δ::*HIS3 sod1*Δ::*KANMX4*	This study

scriptional induction of gene products involved in DNA synthesis and repair, including the subunits of ribonucleotide reductase (RNR), and Hug1p (6, 18). In the second pathway, Mec1p activation results in the Rad53p-independent activation of Chk1p and Pds1p (11, 21). Both pathways mediate a cell cycle arrest allowing sufficient time for the cell to repair the replication block or DNA damage (21, 45).

In this paper, we investigate the roles of *SOD1* and *LYS7* in the *MEC1*-dependent DNA damage checkpoint response and the oxygen-dependent phenotypes of *sod1*Δ and *lys7*Δ strains. We demonstrate that, in the absence of *SOD1* and *LYS7*, induction of Rnr3p and Hug1 in response to replication arrest or DNA damage is dramatically reduced in the presence of oxygen. These null strains also exhibit oxygen-dependent sensitivity to hydroxyurea (HU) and DNA-damaging agents, suggesting that increased oxidative stress due to elevated levels of superoxide anions compromises the *MEC1*-dependent response to replication arrest and DNA damage. The oxygen-dependent HU sensitivity of these strains was suppressed by addition of *N*-acetyl-L-cysteine (NAC), an antioxidant that scavenges ROS (2). These data led us to screen for high-copy-number suppressors of the oxygen-dependent HU sensitivity of *sod1*Δ strains. In this screen we identified *TKL1*, a transketolase of the nonoxidative branch of the pentose phosphate pathway, which restores NADPH levels and a cellular reducing environment. We have also demonstrated that *TKL1* partially restores Rnr3p and Hug1p induction in *sod1*Δ strains treated with HU under normoxic conditions. These results suggest that certain aspects of the *MEC1* pathway are sensitive to the altered cellular redox state created by excess superoxide anions and point to a functional relationship between *SOD1* and *LYS7* and the *MEC1*-mediated checkpoint response to replication arrest and DNA damage in *S. cerevisiae*.

MATERIALS AND METHODS

Media, strains, plasmids, and general methods. Media and methodology used for yeast growth were as described previously (1, 28, 48), except where indicated. All yeast strains are listed in Table 1. For growth under hypoxic conditions, strains were incubated at 30°C in BBL GasPak anaerobic culture jars (Becton-Dickinson Labware, Lincoln Park, NJ), and oxygen was depleted using a CO_2-generating system with palladium catalyst (50). Normoxic conditions were equivalent to standard growth conditions at 30°C. Plasmids pLJ175 (p*SOD1*/*CEN*/*URA3*), pLS113 (p*LYS7*/*CEN*/*HIS3*) and pKS10 (p*TKL1*/2μm/*LEU2*) were generous gifts of V. Culotta, and pBAD54 (GAP vector) and pBAD790 (GAP-*RNR1*) were generous gifts of S. Elledge. KS103 was engineered with the *sod1*Δ::*TRP1* plasmid pKS3 in strain background FY250 (15). The *sod1*Δ *sml1*Δ strains were constructed by mating strains U952-3C and BY4741/*sod1*Δ, and the meiotic progeny of two independent diploids were analyzed.

Sensitivity to replication arrest and DNA damage. All strains were grown overnight at 30°C under hypoxic conditions and then diluted in fresh medium to obtain a logarithmic phase culture under hypoxic conditions. For growth spotting assays, three microliters of serial dilutions (1:5) of logarithmic phase cultures was spotted on yeast-peptone-dextrose agar (YPD) or YPD containing HU (H8627; Sigma, St. Louis, MO), methane methylsulfonate (MMS) (64294; Fluka Chemika, Switzerland), 4-nitroquinoline-1-oxide (N8141; Sigma, St. Louis, MO) or bleomycin (BLM) (3154-01; Bristol-Myers Squibb Co., Princeton, NJ) at indicated concentrations. For NAC suppression assays, cells were spotted on YPD or YPD containing HU with or without NAC (A7250; Sigma, St. Louis, MO) at indicated concentrations. For viability measurements, logarithmic phase cultures grown under hypoxic conditions were plated in triplicate on YPD or YPD containing 100 mM HU or 0.02% MMS and then incubated under normoxic and hypoxic conditions. For recovery experiments, strains were treated with 100 mM HU for 3, 6 or 9 hours, cells were washed, and appropriate dilutions were plated on YPD. Results (see Fig. 2) are averages from three experiments. Wild-type strain viabilities were taken as 100%.

Cell cycle arrest. Logarithmic phase cultures were treated with 100 mM HU for 3 or 6 hours and then analyzed by flow cytometry as described previously (5), using a Becton-Dickinson FACSort flow cytometer and CellQuest software (BD Biosciences, Boston, MA). For arrest and release experiments, logarithmic phase cultures were treated with 100 mM HU for 6 hours and then washed and resuspended in YPD lacking drug. Aliquots were taken at indicated times and analyzed by flow cytometry as described above. At least 10,000 cells were counted for each sample. Microtubule morphology was examined by tubulin staining,

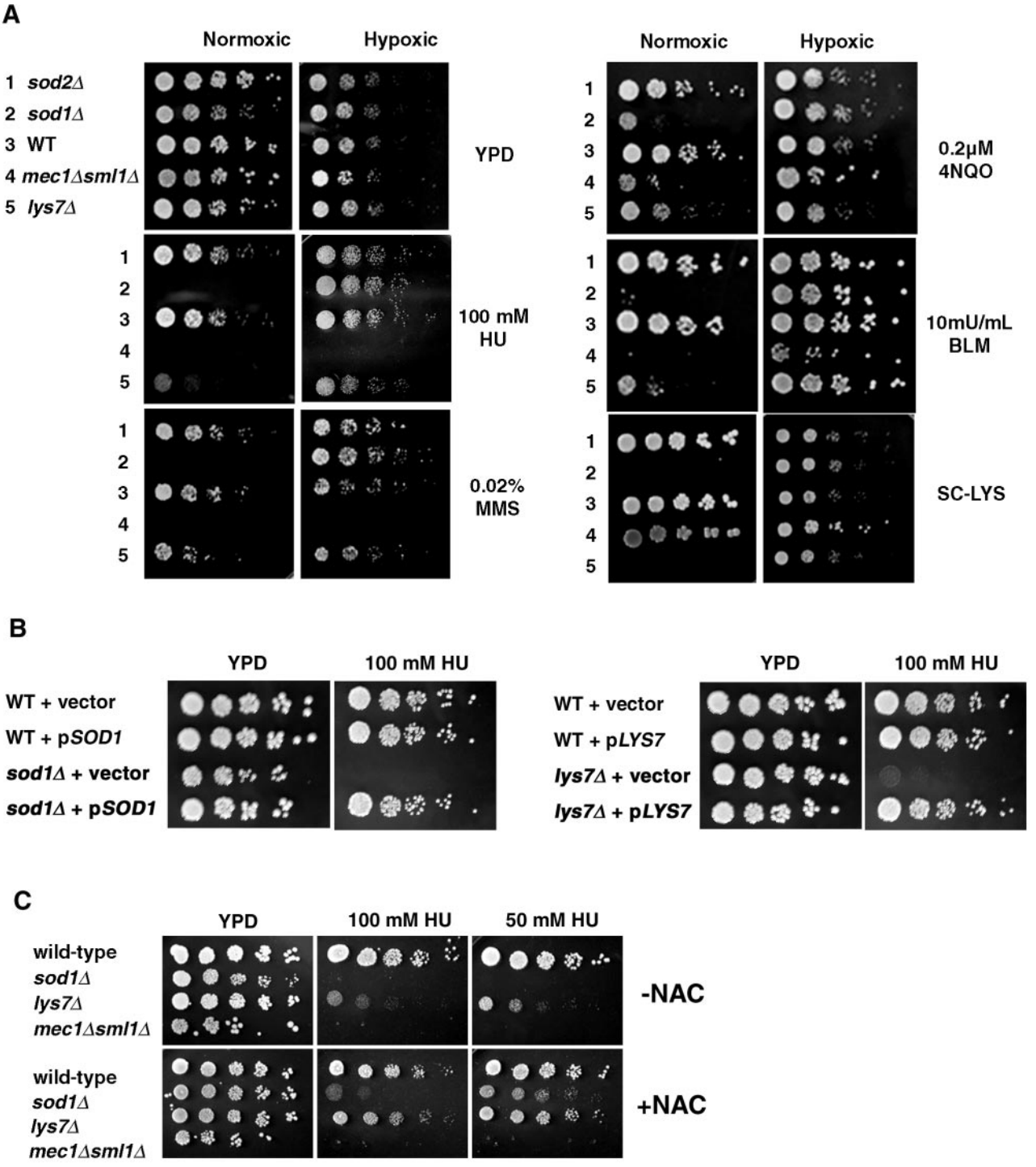
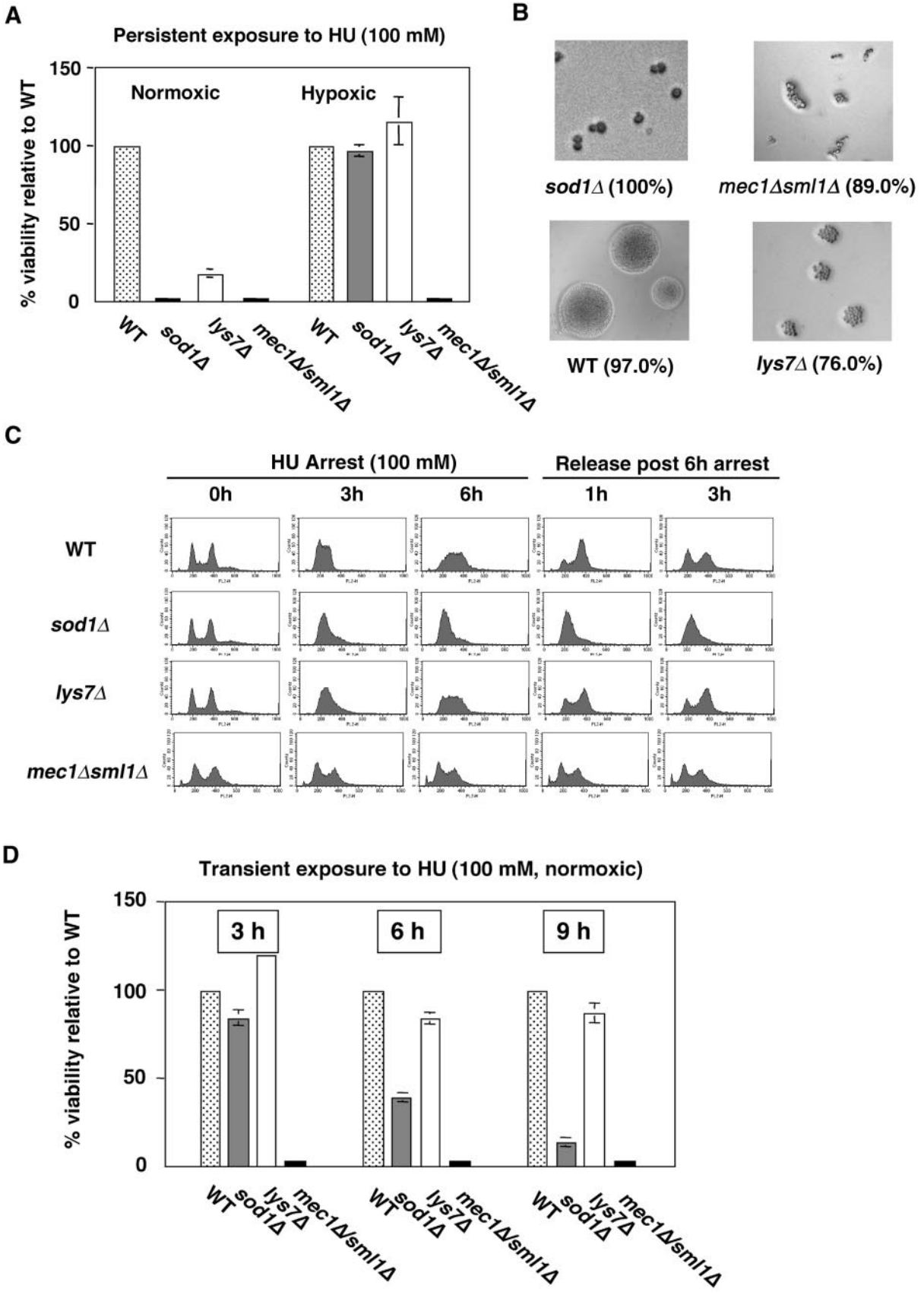


FIG. 1. Sensitivity to replication arrest and DNA damage is oxygen dependent in the absence of *SOD1* or *LYS7*. (A) Three microliters of fivefold serial dilutions of *sod2*Δ, *sod1*Δ, wild-type (BY4741), *mec1*Δ *sml1*Δ (U953-61D), or *lys7*Δ strains was spotted onto YPD or YPD containing 100 mM HU, 0.02% MMS, 0.2 μM 4NQO, or 10 mU/ml BLM or minimal medium lacking lysine (SC-LYS) and incubated under normoxic or hypoxic conditions at 30°C for 3 to 5 days. (B) Wild-type (BY4741), *sod1*Δ, and *lys7*Δ strains were transformed with vector alone, pLJ175 (p*SOD1*/*CEN*/*URA3*), or pLS113 (p*LYS7*/*CEN*/*URA3*). Three microliters of fivefold serial dilutions of each transformant was spotted onto YPD with or without 100 mM HU and incubated under normoxic conditions at 30°C for 2 to 3 days. (C) Wild-type (BY4741), *sod1*Δ, *lys7*Δ, and *mec1*Δ *sml1*Δ (U953-61D) strains were spotted onto YPD or YPD containing 100 mM or 50 mM HU with or without 100 mM NAC and incubated under normoxic conditions at 30°C for 3 to 5 days. WT indicates wild-type strains.


A
Persistent exposure to HU (100 mM)
% viability relative to WT
150
100
50
0
Normoxic
Hypoxic
WT
sod1Δ
lys7Δ
mec1Δsml1Δ
WT
sod1Δ
lys7Δ
mec1Δsml1Δ
B
sod1Δ (100%)
mec1Δsml1Δ (89.0%)
WT (97.0%)
lys7Δ (76.0%)
C
HU Arrest (100 mM)
Release post 6h arrest
0h
3h
6h
1h
3h
WT
sod1Δ
lys7Δ
mec1Δsml1Δ
D
Transient exposure to HU (100 mM, normoxic)
% viability relative to WT
150
100
50
0
3 h
6 h
9 h
WT
sod1Δ
lys7Δ
mec1Δsml1Δ

which was performed as described previously (27), using cells arrested by treatment with 100 mM HU for 3 hours.

Expression analysis by Western blotting. For Western blot analysis of Rad53p, strains were grown to logarithmic phase in hypoxic conditions and then treated as indicated, and whole-cell extracts (WCE) were isolated using a trichloroacetic acid extraction method as follows. Cell pellets were resuspended in 2 N NaOH–1.2 M 2-mercaptoethanol and incubated on ice for 10 min. An equal volume of 50% TCA was added, and samples were further incubated on ice. Samples were then centrifuged (4°C, 1 min, 13,000 rpm), supernatant was discarded, and pellets were washed with ice-cold acetone and recentrifuged. Cell pellets were resuspended in 5% sodium dodecyl sulfate, boiled at 100°C for 3 minutes, and centrifuged, and supernatant was collected as WCE. The Bio-Rad RC/DC protein assay kit (500-0113; Bio-Rad Laboratories, Hercules, CA) was used to determine protein concentration according to the manufacturer's protocol. Forty micrograms of WCE was separated on NuPAGE 4 to 12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA) with MOPS (morpholinepropanesulfonic acid) buffer and transferred to Protran nitrocellulose (pore size, 0.2 μm) (BA83; Schleicher and Schuell, Keene, NH). Membranes were blocked with 5% nonfat milk in 1× Tris-buffered saline containing 0.3% Triton X-100 and then probed using a goat polyclonal antibody (yC-19) raised against the C terminus of Rad53p (1:500) (sc-6749; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by horseradish peroxidase (HRP)-conjugated bovine anti-goat immunoglobulin G secondary antibody (1:5,000) (sc-2350; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For RNR subunit and Hug1p Western blot analyses, indicated strains were grown as described above, and WCE were made by the boil-freeze method as previously described (34). Extracts were separated using 10% Tris-Tricine sodium dodecyl sulfate-polyacrylamide gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% nonfat milk in 1× phosphate-buffered saline (pH 7.0) and then probed using antibodies to Rnr1p (1:10,000), Rnr2p (1:20,000), Rnr3p (1:10,000), Hug1p (1:500), or Tub2p (1:50,000) followed by HRP-conjugated donkey anti-rabbit immunoglobulin G secondary antibody (1:7,500) (NA934V; Amersham Biosciences, Piscataway, NJ). All antibodies were diluted in blocking buffer. Extracts were also tested separately with a rabbit polyclonal antibody raised to full-length Rad53p kindly provided by David Stern (53; data not shown). RNR antibodies were a kind gift of J. Stubbe. The rabbit polyclonal antibody to Tub2p was generously provided by D. Koshland. HRP activity was detected using the Super Signal West Pico chemiluminescent substrate kit (Pierce Biotechnology Inc., Rockford, IL) followed by exposure to BioMax Light film (Eastman Kodak Co., Rochester, NY).

High-copy-number suppression screen. BY4741/*sod1*Δ was transformed with a pRS202-based library (2μm/*URA3*) (12) and incubated at 30°C under normoxic conditions on medium lacking uracil. Approximately 2,000 transformants were replica plated to YPD containing 100 mM HU, and two colonies were verified as HU resistant. The library plasmids rescued from these strains complemented the HU sensitivity of the BY4741/*sod1*Δ strain. DNA sequencing of the two plasmids indicated that the complementing clones contained a 7.7-kb insert from chromosome XVI (nucleotides 687952 to 695669) containing *TKL1*.

RESULTS

Loss of *SOD1* and *LYS7* sensitizes cells to replication arrest and DNA damage in an oxygen-dependent manner. A genome-wide screen for suppressors of genomic instability by Kolodner and coworkers identified *SOD1* and *LYS7* and three other oxidative stress response genes (*TSA1*, *YAP1*, and *SKN7*) as candidates (25, 26). *SOD1* and *LYS7* were also identified in two separate genome-wide screens of *S. cerevisiae* haploid deletion strains for sensitivity to MMS and HU (10, 38). As both *sod1*Δ and *lys7*Δ strains exhibit several oxygen-dependent phenotypes, including lysine and methionine auxotrophies (50), we tested whether the sensitivity of these strains to replication arrest and DNA damage was also oxygen dependent. We also tested a null strain of the mitochondrial superoxide dismutase, a *SOD2* strain, and a *MEC1* pathway mutant, a *mec1*Δ *sml1*Δ strain. Under normoxic conditions, where oxygen levels are normal, *sod2*Δ, *mec1*Δ *sml1*Δ, and *lys7*Δ strains grew as well as the isogenic wild-type strain on YPD (Fig. 1A), while the *sod1*Δ strain exhibited slightly reduced growth as previously reported (23). On YPD containing the replication arrest agent, HU, or DNA-damaging agent 4-nitroquinoline-*N*-oxide, BLM, or MMS, or on minimal medium lacking lysine, *sod2*Δ strains grew as well as wild-type strains, but *sod1*Δ and *lys7*Δ strains showed growth inhibition (Fig. 1A). These phenotypes of *sod1*Δ and *lys7*Δ are not observed under hypoxic conditions, where levels of oxygen are reduced (Fig. 1A). The *lys7*Δ strain was less sensitive to these agents than the *sod1*Δ strain. We also confirmed that the phenotypes observed for *sod1*Δ and *lys7*Δ were due to loss of *SOD1* and *LYS7*, since plasmid-borne copies of *SOD1* or *LYS7* complement HU sensitivity of *sod1*Δ and *lys7*Δ strains, respectively (Fig. 1B). As the growth inhibition phenotypes were oxygen dependent and thus may depend on increased levels of ROS that occur in the absence of superoxide anion scavenging, we sought to determine whether the antioxidant NAC suppressed HU sensitivity (Fig. 1C). Addition of NAC to the medium containing HU allowed some growth of *sod1*Δ and *lys7*Δ strains in 100 mM HU and significant growth of both strains in 50 mM HU. Addition of NAC, however, did not suppress the HU sensitivity of *mec1*Δ *sml1*Δ strains.

We reasoned that the HU and DNA damage sensitivity phenotypes of *sod1*Δ and *lys7*Δ strains might be due to presence of excess superoxide anions caused by Sod1p and Lys7p deficiency and not due to an excess of other forms of ROS. We tested strains lacking genes that mediate the oxidative stress response to H_2O_2 (*SKN7*, *TSA1*, and *YAP1*) (26) and determined that these strains were able to grow on medium containing HU or MMS (see Fig. S1 in the supplemental material). Taken together, these results suggest that *SOD1* and

FIG. 2. Strains lacking *SOD1* and *LYS7* arrest, but *sod1*Δ strains fail to produce viable colonies on HU medium. (A) Wild-type (WT; BY4741), *sod1*Δ, *lys7*Δ, and *mec1*Δ *sml1*Δ (U953-61D) strains were plated on YPD containing 100 mM HU, and plates were incubated under hypoxic or normoxic conditions for 3 to 6 days. *lys7*Δ strains showed small colonies only upon prolonged incubation for 8 days at 30°C. (B) Terminal phenotypes of strains from panel A grown on HU-containing medium under normoxic conditions were recorded using a light microscope. The percentage of cells with the depicted morphology is indicated in parentheses for each strain. (C) DNA content analysis by flow cytometry. Wild-type (BY4741), *sod1*Δ, *lys7*Δ, and *mec1*Δ *sml1*Δ (U953-61D) strains were grown under normoxic conditions to logarithmic phase (column 1) and treated with 100 mM HU for 3 hours (column 2) or 6 hours (column 3). Strains arrested with 100 mM HU for 6 hours were released into drug-free medium, and an aliquot of cells was removed every 30 min for up to 4 hours. The DNA contents of cells from 1 (column 4) and 3 hours (column 5) post-HU release are shown. Cells from these samples were analyzed by flow cytometry, and DNA fluorescence units per cell were measured. (D) Wild-type (BY4741), *sod1*Δ, *lys7*Δ, and *mec1*Δ *sml1*Δ (U953-61D) strains were grown under normoxic conditions to logarithmic phase and treated with 100 mM HU for 6 hours. Cells were washed, and appropriate dilutions were plated on YPD, allowed to recover under normoxic conditions, and counted after 2 to 3 days of incubation at 30°C. For panels A and D, at least 2,500 cells per strain were plated per experiment. For each experiment, the percent viability of wild-type strains was considered to be 100%. Plating efficiency for wild-type cells was 36% on YPD containing HU versus YPD alone. Numbers are averages from three experiments, and error bars represent standard deviation.

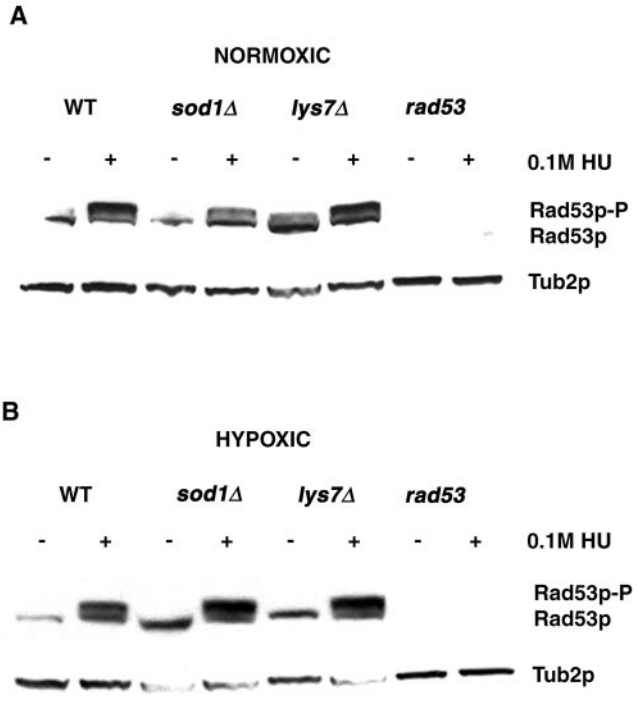


FIG. 3. Rad53p is expressed and phosphorylated following replication arrest and DNA damage. Wild-type (WT; BY4741), *sod1*Δ, *lys7*Δ, and *rad53*Δ *sml1-1* (U960-5C) strains were grown in YPD in the presence or absence of HU (100 mM, 3 hours) under normoxic (A) or hypoxic (B) conditions as indicated. WCE were subjected to Western blotting using a Rad53p C-terminal polyclonal antibody, and a Tub2p polyclonal antibody was used as a loading control.

LYS7 are required for oxygen-dependent resistance to replication arrest and DNA damage and that the sensitivity of these strains to HU and MMS may be due to excess superoxide anions that arise in the absence of these superoxide anion scavengers.

Strains lacking *SOD1* arrest but fail to produce viable colonies on HU-containing medium. Our results have shown that *sod1*Δ and to a lesser extent *lys7*Δ strains exhibit sensitivity to growth on HU-containing medium similar to the checkpoint mutant (*mec1*Δ *sml1*Δ). To determine whether these strains were similar to or distinct from *mec1*Δ *sml1*Δ in terms of growth phenotype, we analyzed these strains for (i) viability and terminal phenotype in the persistent presence of HU, (ii) ability to arrest the cell cycle in response to HU treatment, and (iii) cell cycle progression and viability upon release from transient HU treatment. Quantitative analyses showed that on HU-containing medium *sod1*Δ strains formed no visible colonies under normoxic conditions and a small percentage (12.5%) of *lys7*Δ cells formed very small colonies only after prolonged incubation (8 days) under normoxic conditions (Fig. 2A). This effect was oxygen dependent as the viability of *sod1*Δ strains was only slightly reduced and that of *lys7*Δ strains was unaffected on HU-containing medium under hypoxic conditions. As expected, the control strain *mec1*Δ *sml1*Δ produced no viable colonies under both normoxic and hypoxic conditions. To determine whether the terminal phenotypes of the *sod1*Δ and *lys7*Δ strains on HU-containing medium under normoxic conditions were similar to *mec1*Δ *sml1*Δ strains, we assessed the cell morphology of the *sod1*Δ and *lys7*Δ strains in the persistent presence of HU. On HU-containing medium, wild-type strains arrest in S phase of the cell cycle and then adapt and resume growth to form colonies. A *mec1*Δ *sml1*Δ strain, however, does not arrest and instead continues to divide for two to three divisions, giving rise to abnormally shaped microcolonies of cells (59). Our results showed that on HU-containing medium, 100% of the cells plated from the *sod1*Δ strains failed to grow beyond the large-budded stage, and a large portion (76%) of the *lys7*Δ cells that failed to produce a visible colony gave rise to microcolonies of about 25 to 30 normally shaped cells (Fig. 2B). As expected, almost all (97%) of the cells from the wild-type strain produced colonies of normal size and shape whereas a majority (89%) of cells from the *mec1*Δ *sml1*Δ strain gave rise to clumps of less than 15 irregularly shaped cells. These results showed that while *sod1*Δ, *lys7*Δ, and *mec1*Δ *sml1*Δ strains are all sensitive to growth on HU-containing medium, the terminal growth phenotypes of *sod1*Δ and *lys7*Δ strains are distinct from those of both the wild-type and *mec1*Δ *sml1*Δ strains.

We next investigated the cell cycle arrest phenotypes by examining the nuclear DNA content of the strains treated with HU (100 mM for 3 and 6 hours) and upon release from a 6-hour HU treatment. Unlike wild-type cells that arrest in G_1/S phase with a short spindle, *mec1*Δ *sml1*Δ strains fail to arrest, and cells show an elongated spindle after 3 hours of HU treatment (19, 30, 59). Flow cytometry analysis showed that similar to wild-type strains, *sod1*Δ and *lys7*Δ strains arrested in response to HU treatment (3 hours) under normoxic conditions (Fig. 2C, column 2). In addition, DAPI (4′,6′-diamidino-2-phenylindole) and tubulin staining showed that *sod1*Δ and *lys7*Δ strains with large-bud arrest morphology maintained short spindles similar to wild-type strains when treated with HU (100 mM for 3 hours) (data not shown). Consistent with the terminal phenotypes that we observed on HU-containing medium, the *sod1*Δ strains maintained G_1/S-phase arrest after 6 hours (Fig. 2C, column 3) and 9 hours (data not shown) of treatment with HU. The wild-type and *lys7*Δ strains exhibited broader S-phase peaks and some cells with 2N DNA content (Fig. 2C, column 3) after 6 hours in HU, consistent with progression into the cell cycle. The checkpoint mutant *mec1*Δ *sml1*Δ failed to arrest in response to HU treatment as evidenced by both S-phase and 2N peaks after 3, 6 (Fig. 2C, column 2), and 9 hours (data not shown) in HU. To determine whether *sod1*Δ and *lys7*Δ strains arrested with HU progress into the cell cycle when released from that arrest, we performed an arrest release experiment by resuspending HU-treated cells (100 mM, 6 hours) in drug-free medium and analyzing aliquots at 30-minute intervals for up to 4 hours. Wild-type and *lys7*Δ strains progressed through the cell cycle starting at 30 minutes (data not shown) and as depicted in Fig. 2C for representative time points of 1 (column 4) and 3 (column 5) hours post-HU release. In contrast, *sod1*Δ strains progressed only slightly through the cell cycle following release from HU treatment. Examination of the flow cytometry samples of the *sod1*Δ strain under the microscope from 1 and 3 hours post-HU release showed that all the cells displayed the morphology seen in Fig. 2B. The *mec1*Δ *sml1*Δ strain failed to arrest in response to HU treatment, resulting in catastrophic divisions for a few generations with multibudded cells. These

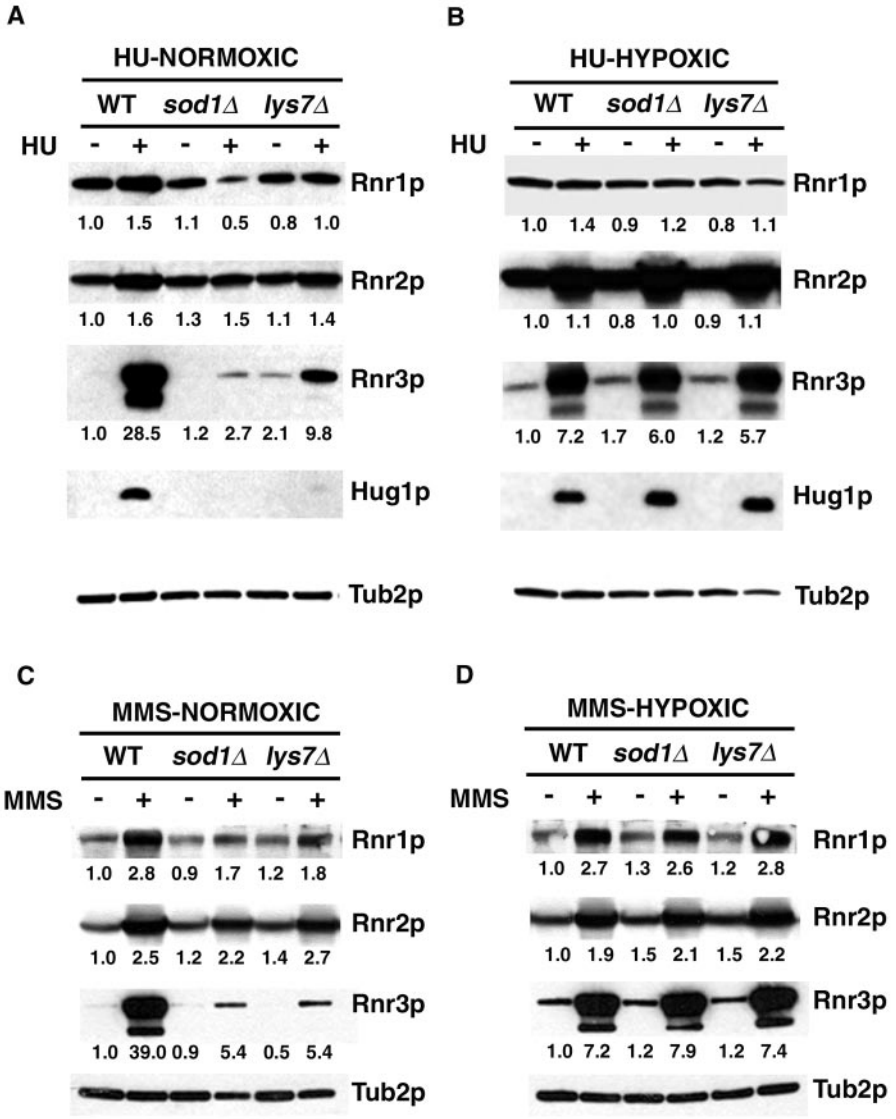


FIG. 4. *sod1*Δ and *lys7*Δ strains show altered levels of *MEC1* checkpoint pathway effector protein Rnr3p and of Hug1p after replication arrest or DNA damage. Wild-type (BY4741), *sod1*Δ, or *lys7*Δ strains were grown for 3 hours in YPD with or without 100 mM HU (A and B) or 0.02% MMS (C and D) under normoxic (A and C) or hypoxic (B and D) conditions. WCE were subjected to Western blotting using Rnr1p, Rnr2p, Rnr3p, Hug1p, or Tub2p polyclonal antibodies. The value below each band indicates the ratio of the signal intensity of each protein band to the signal intensity of the Tub2p band compared to the ratio of these bands in the control lane (untreated WT) for each blot. Signal intensities were compared using ImageQuant TL software (Amersham Biosciences, Piscataway, NJ). Quantitation of proteins from a second set of blots gave similar normalized values. No values are given for Hug1p, since the *HUG1* transcript is not present in the absence of replication arrest or DNA damage (6).

results suggest that the loss of viability of *sod1*Δ strains on HU-containing medium is not due simply to a failure to arrest and that the checkpoint-mediated cell cycle arrest seems to be intact in the *sod1*Δ strains.

Our results showed that *sod1*Δ strains are inviable in the presence of HU and maintained G_1/S-phase arrest even upon prolonged incubation (Fig. 2B). Hence, we wanted to determine whether *sod1*Δ strains were viable after release from transient HU treatment. When treated with HU (100 mM) under normoxic conditions, the viability of *sod1*Δ strains was 84.6%, 40.0%, and 13.5% that of wild-type strains following 3, 6 and 9 hours of treatment, respectively (Fig. 2D). The inviable *sod1*Δ cells did not proceed beyond the large-budded stage (data not shown), similar to the cell morphology shown in Fig. 2B. The increased loss of viability of *sod1*Δ strains in response to increasing times of HU treatment may be due to an inability of the cells to reenter the cell cycle as shown in Fig. 2C. This effect was oxygen dependent, because the viabilities of the *sod1*Δ strains were near those of wild-type strains under hypoxic conditions (data not shown). The viability of *lys7*Δ strains was similar to that of the wild type under both normoxic (Fig. 2D) and hypoxic (data not shown) conditions. In comparison, the *mec1*Δ *sml1*Δ strain failed to produce colonies under both normoxic (Fig. 2D) and hypoxic (data not shown) conditions when released from 3, 6, or 9 hours of HU treatment. From these results we conclude that the growth

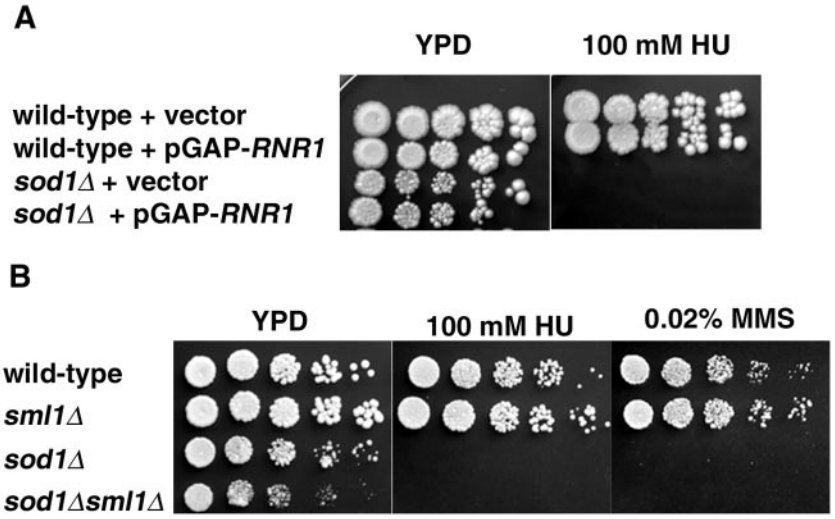


FIG. 5. Overexpression of *RNR1* and deletion of *SML1* do not suppress *sod1*Δ phenotypes. (A) Three microliters of fivefold serial dilutions of wild-type (EG103) or *sod1*Δ (KS101) strains transformed with either vector alone (pBAD54) or *RNR1* expressed from a constitutive promoter (pBAD790) was spotted on YPD and YPD containing 100 mM HU. At least two independent transformants were tested for each strain, and expression of *RNR1* was verified by Western blot analysis (data not shown). (B) Three microliters of fivefold serial dilutions of wild-type (YMB3233), *sml1*Δ (YMB3234), *sod1*Δ (YMB3235), and *sod1*Δ *sml1*Δ strains (YMB3236) was spotted onto YPD, YPD containing 100 mM HU, or 0.02% MMS and then incubated under normoxic conditions at 30°C for 2 to 3 days. At least two independent strains were tested for each genotype.

phenotypes of *sod1*Δ and *lys7*Δ strains treated with HU are not similar to those observed for checkpoint mutants such as *mec1*Δ *sml1*Δ and that the cell cycle arrest aspect of the DNA damage checkpoint seems intact in the absence of *SOD1* and *LYS7*.

Rad53p is expressed and phosphorylated in *sod1*Δ and *lys7*Δ strains treated with HU under normoxic conditions. An indicator of activation of the *MEC1*-dependent DNA damage checkpoint pathway is the phosphorylation of Rad53p, which mediates cell cycle arrest and transcriptional induction of downstream effectors in response to DNA damage or replication arrest (46, 52). Our results for the cell cycle arrest phenotype of the *sod1*Δ and *lys7*Δ strains in response to HU treatment led us to investigate the expression of Rad53p in these strains. We analyzed WCE of wild-type, *sod1*Δ, and *lys7*Δ strains grown under normoxic or hypoxic conditions in the presence or absence of HU. Extracts were analyzed by Western blotting using an antibody to the C terminus of Rad53p, and extracts from a *rad53*Δ *sml1-1* strain served as a negative control. Our results showed that like the wild-type strain, *sod1*Δ and *lys7*Δ strains treated with HU under normoxic (Fig. 3A) or hypoxic (Fig. 3B) conditions express both unphosphorylated and phosphorylated forms of Rad53p, as indicated by multiple bands. We did observe, however, that the mobility shift of Rad53p that occurs upon HU treatment of *sod1*Δ strains is not identical to that of wild-type and *lys7*Δ strains.

Induction of *MEC1* pathway effector protein Rnr3p and of Hug1p in the presence of HU under normoxic conditions is dependent on *SOD1* and *LYS7*. Another indicator of signaling through the *MEC1* pathway is transcriptional induction of effectors such as *RNR1*, *RNR2*, *RNR3*, and *RNR4* (32) and of *HUG1* (6) in response to replication arrest or DNA damage. The DNA damage and HU sensitivity phenotypes of *sod1*Δ and *lys7*Δ strains prompted us to investigate, by Western blot analysis, whether expression of these effectors was altered in the *sod1*Δ and *lys7*Δ strains. Following HU exposure under normoxic conditions (Fig. 4A), Rnr3p and Hug1p, and to a lesser extent, Rnr1p and Rnr2p, were induced in wild-type cells. In *sod1*Δ strains, however, we observed a striking lack of induction of Rnr3p or Hug1p following HU treatment under normoxic conditions compared to wild-type strains (for Rnr3p, 2.7-fold versus 28.5-fold). In addition, these proteins were less induced in *lys7*Δ strains compared to the wild-type strains (for Rnr3p, 9.8-fold versus 28.5-fold). Rnr2p was induced to approximately the same levels in *sod1*Δ, *lys7*Δ, and wild-type strains while Rnr1p was slightly less induced in *sod1*Δ strains than in *lys7*Δ and wild-type strains. The reliance on *SOD1* and, to a lesser extent, *LYS7* for Rnr3p and Hug1p induction is entirely dependent on the presence of oxygen, as Rnr3p and Hug1p were each expressed similarly in wild-type, *sod1*Δ, and *lys7*Δ strains under hypoxic conditions (Fig. 4B). Exposure of *sod1*Δ and *lys7*Δ strains to MMS (0.02%) under normoxic conditions also resulted in a sharp decrease in Rnr3p induction compared to that in wild-type strains (5.4-fold versus 39.0-fold) (Fig. 4C). No changes in induction of Rnr3p compared to wild-type strains were found following MMS exposure under hypoxic conditions (Fig. 4D), however, and Hug1p was not detected under either condition (data not shown). We conclude that in *sod1*Δ and *lys7*Δ strains exposed to replication arrest under normoxic but not hypoxic conditions, induction of the *MEC1* pathway effector Rnr3p and of Hug1p is significantly defective, suggesting that this aspect of the *MEC1*-dependent DNA damage checkpoint is not fully functional under normoxic conditions in the absence of *SOD1* and *LYS7*.

Overexpression of *RNR1* or deletion of *SML1* does not suppress the HU sensitivity of *sod1*Δ strains. We reasoned that the growth inhibition phenotypes of *sod1*Δ strains in response to replication arrest and DNA damage may be due to lower

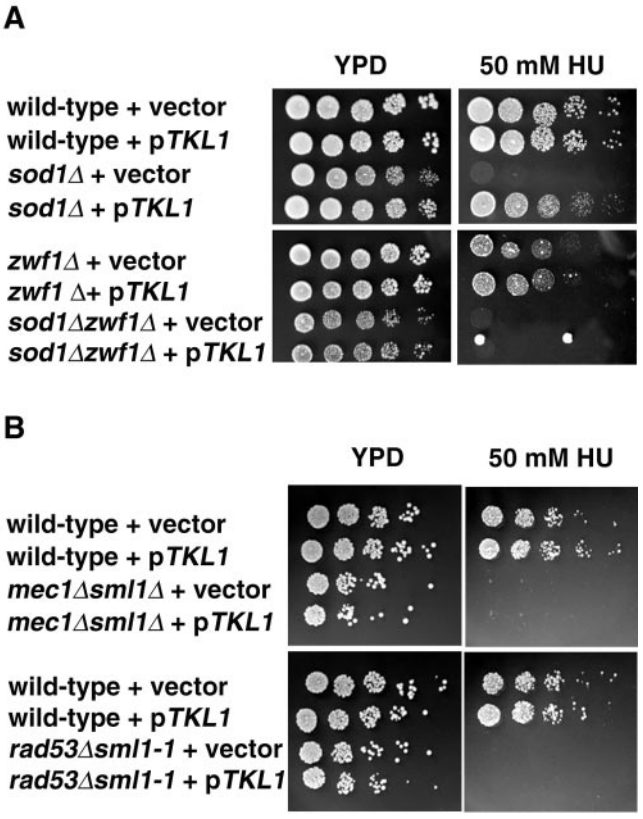


FIG. 6. Overexpression of *TKL1* in *sod1*Δ strains, but not in *mec1* or *rad53* strains, restores HU resistance. (A) Three microliters of fivefold serial dilutions of wild-type (1783), *sod1*Δ (KS105), *zwf1*Δ (KS113), or *sod1*Δ *zwf1*Δ (KS117) strains transformed with vector alone or pKS10 (p*TKL1*/2μm/*LEU2*) was spotted onto YPD with or without 50 mM HU and incubated under normoxic conditions at 30°C for 2 days. At least two independent transformants were tested for each strain. Expression of pKS10 (p*TKL1*/2μm/*LEU2*) in *lys7*Δ strains also complemented the HU sensitivity of these strains (data not shown). (B) Three microliters of fivefold serial dilutions of wild-type (W1588-4A), *mec1*Δ *sml1*Δ (U953-61D), or *rad53*Δ *sml1-1* (U960-5C) strains transformed with vector alone or pKS10 (p*TKL1*/2μm/*LEU2*) was spotted onto YPD with or without 50 mM HU and incubated under normoxic conditions at 30°C for 2 days.

levels of dinucleoside triphosphates (dNTPs) being available for DNA synthesis and repair, which occurs when levels and/or activity of RNR is altered (9, 33). The lack of induction of Rnr3p upon HU and MMS treatment prompted us to test whether increasing the abundance of Rnr3p would suppress these phenotypes in the *sod1*Δ and *lys7*Δ strains. However, we found that overexpression of *RNR3* rendered wild-type cells sensitive to HU and resulted in extremely slow growth of the *sod1*Δ strains even in the absence of HU (data not shown). The slight reduction that we see in the induction of Rnr1p in the *sod1*Δ and *lys7*Δ strains under these conditions might be sufficient to affect the replication arrest and DNA damage sensitivity phenotypes of these strains, since a single copy of *RNR1* can rescue the lethality of *mec1* and *rad53* mutants (17). Since Rnr1p is the rate-limiting subunit for RNR activity (9), we reasoned that increasing *RNR1* levels might be sufficient to overcome the HU sensitivity of the *sod1*Δ strains. However, neither *sod1*Δ (Fig. 5A) nor *lys7*Δ strains (data not shown) transformed with *RNR1* under the control of a constitutive glyceraldehyde-3-phosphate promoter (GAP-*RNR1*) were able to grow on HU-containing medium, even though Rnr1p was constitutively expressed in these strains as analyzed by Western blotting (data not shown). An alternate method to increase RNR activity is the removal of Sml1p, which binds to Rnr1p and renders it unavailable for incorporation into active RNR (8, 61). We constructed a strain lacking both *SML1* and *SOD1*. While wild-type and *sml1*Δ strains grew on HU- and MMS-containing media, *sod1*Δ and *sod1*Δ *sml1*Δ strains did not (Fig. 5B). Combined, these data lead us to suggest that increasing the amount of Rnr1p, either by overexpression of *RNR1* or removal of *SML1*, is not sufficient to suppress the replication arrest and DNA damage sensitivity of *sod1*Δ strains.

Screen for suppressors of *sod1*Δ and *lys7*Δ replication arrest and DNA damage sensitivity identifies *TKL1*. We established that addition of an antioxidant to the medium suppressed the oxygen-dependent HU sensitivity of the *sod1*Δ and *lys7*Δ strains (Fig. 1D). This prompted us to screen for cellular factors that might mimic this antioxidant effect by suppressing the HU sensitivity phenotype of *sod1*Δ and *lys7*Δ strains when expressed at high copy number. We transformed *sod1*Δ with a 2μm-*URA3* library and screened the primary transformants for ability to grow on YPD containing HU. Of 2000 colonies screened, we identified two Ura+/HU-resistant transformants that each carried a plasmid containing *TKL1*. Interestingly, Tkl1p is known to suppress the methionine auxotrophy and oxygen sensitivity, but not the lysine auxotrophy, of *sod1*Δ strains through its ability to increase intracellular levels of NADPH (49).

We verified that *TKL1* was responsible for the suppression of the HU sensitivity since *sod1*Δ transformed with a 2μm *TKL1* plasmid grew on HU-containing medium (Fig. 6A). *TKL1* similarly suppressed the MMS sensitivity of *sod1*Δ strains and the HU and MMS sensitivities of *lys7*Δ strains (data not shown). One explanation for the suppression of *sod1*Δ phenotypes by *TKL1* may be its ability to increase NADPH levels in the cell through its role in the nonoxidative branch of the pentose phosphate pathway (54). If suppression of *sod1*Δ phenotypes by *TKL1* is due to increased NADPH, then the suppression should be dependent on Zwf1p, which catalyzes the rate-limiting step of the pentose phosphate pathway (49). Therefore, we tested the phenotypes of *zwf1*Δ and *sod1*Δ *zwf1*Δ strains carrying plasmids bearing *TKL1*. While *zwf1*Δ strains were slightly more sensitive to HU than wild-type strains, *TKL1* in these strains did not affect their growth on medium containing HU (Fig. 6A). The *sod1*Δ *zwf1*Δ strains were as sensitive to HU as the *sod1*Δ strains, but *TKL1* did not confer HU resistance to the double mutant, verifying the strains' dependence on *ZWF1* for suppression of HU sensitivity (Fig. 6A). The ability of *TKL1* to restore HU resistance to *sod1*Δ strains was not a general effect on a compromised *MEC1* pathway, since *TKL1* did not suppress the HU sensitivity of *mec1*Δ *sml1*Δ or *rad53*Δ *sml1-1* strains (Fig. 6B).

We next asked whether suppression of *sod1*Δ phenotypes by *TKL1* restored expression of *MEC1* pathway effectors. WCE made from wild-type, *sod1*Δ and *lys7*Δ strains transformed with vector alone or *TKL1* and treated with HU were subjected to Western blotting and probed with polyclonal antibodies to Rnr1p, Rnr2p, Rnr3p, and Hug1p. In HU-treated *sod1*Δ and *lys7*Δ strains carrying *TKL1*, Rnr3p and Hug1p expression was

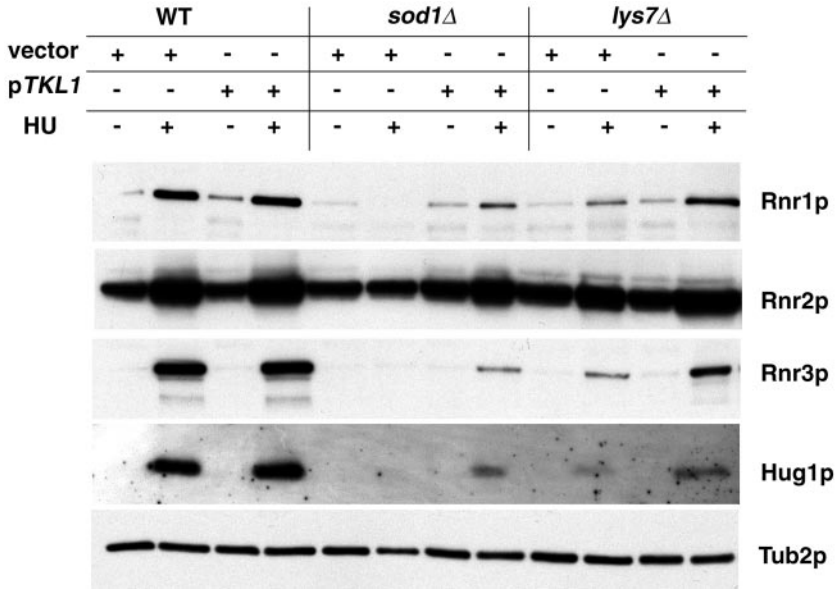


FIG. 7. Overexpression of *TKL1* in *sod1*Δ and *lys7*Δ strains partially restores expression of the *MEC1* pathway effector protein Rnr3p and of Hug1p. Wild-type (WT; BY4741), *sod1*Δ, or *lys7*Δ strains transformed with vector alone or with p*TKL1* (pKS10) were grown under hypoxic conditions overnight in minimal medium lacking leucine. Cultures were diluted, grown to logarithmic phase, and then grown for 3 hours in YPD with or without 100 mM HU under normoxic conditions. WCE were subjected to Western blotting. Blots were probed with polyclonal antibodies to Rnr1p, Rnr2p, Rnr3p, Hug1p, and Tub2p.

increased compared to that in strains carrying vector alone, although not to wild-type levels (Fig. 7). Taken together with results shown in Fig. 6, *TKL1* partially restored the expression of effectors of the *MEC1*-dependent signaling pathway in *sod1*Δ and *lys7*Δ strains and made these strains resistant to replication arrest and DNA damaging agents, suggesting that alteration of NADPH levels in the cell may counteract the elevated levels of superoxide anions present in strains lacking *SOD1* and *LYS7*.

DISCUSSION

In this study, we show that loss of the primary cytosolic superoxide anion scavenger, Sod1p, or its copper chaperone Lys7p, leads to a defect in signaling of the replication arrest and DNA damage mediated by the *MEC1* pathway and that this defect correlates with an inability to cope with genotoxic stress. We have determined that under normoxic, but not hypoxic, conditions, the absence of *S. cerevisiae SOD1*, and to a lesser extent the absence of *LYS7*, leads to (i) sensitivity to replication stress and DNA damaging agents and (ii) defective induction of an effector of the *MEC1* pathway, Rnr3p, and of Hug1p, following HU treatment. These results suggest that HU and MMS sensitivities of *sod1*Δ and *lys7*Δ strains coincide with an impaired response of the *MEC1*-dependent checkpoint pathway. A high-copy-number suppressor screen for genes that suppress the oxygen-dependent HU sensitivity of *sod1*Δ strains identified *TKL1*, which was capable of restoring HU and MMS resistance and partial induction of Rnr3p and Hug1p in *sod1*Δ and *lys7*Δ strains. Based on these results we propose that these *sod1*Δ phenotypes may arise in part due to defective induction through the *MEC1* pathway and low NADPH levels resulting from excess superoxide anions and that *TKL1* complements these phenotypes by increasing NADPH and restoring a normal redox balance. Our results establish that the *MEC1* pathway is sensitive to an altered cellular redox state due to an overabundance of superoxide anions and suggests a functional relationship between *SOD1* and *LYS7* and the *MEC1*-mediated checkpoint response to replication arrest and DNA damage in *S. cerevisiae*.

Analysis of the cell cycle arrest phenotype in response to HU treatment shows that the *sod1*Δ strains arrest in G_1/S phase unlike checkpoint mutants that do not arrest. In addition, the viability of *sod1*Δ released from a HU treatment decreases as the time of treatment is increased, and the terminal phenotype of HU-treated *sod1*Δ strains is distinct from that of *mec1*Δ *sml1*Δ strains. From these data we infer that the checkpoint mechanism that leads to cell cycle arrest in response to HU seems to be intact in the *sod1*Δ strains. Furthermore, we have also observed that the cell cycle arrest response is not HU-specific, since *sod1*Δ strains treated with MMS (0.02%) for up to 5 hours show S-phase morphology as evidenced by flow cytometry (data not shown) unlike *mec1*Δ *sml1*Δ strains, which progress through the cell cycle (39). The cell cycle arrest in the *sod1*Δ strains is most likely mediated by the Rad53p branch of the *MEC1* pathway as Rad53p is expressed and phosphorylated in these strains. Additional support for this conclusion is based on the observation that the arrest phenotype of *sod1*Δ is not due to the *CHK1*-dependent, *RAD53*-independent branch of the *MEC1* pathway, since *sod1*Δ *pds1*Δ strains also arrest in response to HU treatment (data not shown).

Our data showed that the mobility shift of Rad53p that occurs upon HU treatment of *sod1*Δ strains is not identical to that observed for wild-type and *lys7*Δ strains. To our knowledge, previous reports have not established what levels of phosphorylated Rad53p are necessary for cell cycle arrest and

induction of downstream effectors of the *MEC1* pathway. The levels of Rad53p in the *sod1*Δ strains seem to be sufficient for cell cycle arrest in response to HU but may affect the optimal induction of Rnr3p and Hug1p.

While the cell cycle arrest response of *sod1*Δ strains in the presence of HU under normoxic conditions is seemingly wild type, *sod1*Δ strains show increased sensitivity on HU-containing medium, reduced viability following transient HU exposure, and defective induction of Rnr3p and Hug1p. These phenotypes may be due to altered activity of the *MEC1* pathway enzymes or other cellular targets in response to excess superoxide anions. Alternatively, *sod1*Δ strains may fail to reenter the cell cycle and thus fail to survive after a prolonged arrest, thereby resulting in increased HU sensitivity. We do not favor the latter possibility, since *sod1*Δ strains treated with MMS (0.02% for 3 hours), which causes a slowed progression in S phase, showed only a slight (<10%) reduction in viability when plated on medium lacking MMS (data not shown). The phenotypes of *sod1*Δ strains may also stem from a decrease in dNTP levels due to a compromised signaling through the *MEC1* pathway. Our attempts to increase dNTP levels by overexpression of *RNR1* or removal of the negative regulator of *RNR1* activity, *SML1*, did not complement the mutant phenotypes of *sod1*Δ strains. Since mere alteration of Rnr1p levels available for incorporation into active RNR does not seem to suppress the HU-sensitive *sod1*Δ strains, we hypothesize that *sod1*Δ strains may have altered RNR activity, no matter the level of Rnr1p. Several factors regulate the activity of RNRs, including the cellular redox state and the availability of NADPH (29, 42). Class I RNR enzymes from eukaryotes and *Escherichia coli* are extremely sensitive to ROS (20, 22). Oxidative stress also leads to decreased levels of reduced forms of thioredoxins and glutathione, cofactors for RNR that utilize NADPH (7). In *E. coli*, superoxide anions can also directly inactivate the small subunit of RNR (R2) by irreversible oxidation of a stable tyrosyl radical essential for its activity (20). In addition, the large subunit (R1) contains three redox-active cysteine residues that participate in ribonucleotide reduction; these redox-sensitive moieties exist in eukaryotic RNR as well (20). The *ZWF1*-dependent suppression of *sod1*Δ phenotypes by *TKL1* is consistent with our hypothesis that RNR activity is affected in *sod1*Δ strains, since NAPDH is required for RNR activity. We hypothesize that in wild-type cells (Fig. 8A) Sod1p scavenges superoxide anions while a reducing environment is maintained in the cytosol by a certain level of NADPH (31, 37, 47). In the absence of *SOD1* (Fig. 8B), however, the increased levels of superoxide anions lead either directly or indirectly (through conversion to hydroxyl radicals) to oxidation of redox-sensitive cellular targets, such as thioredoxins and glutaredoxins and perhaps *MEC1* pathway components, thereby requiring more NADPH to maintain these targets in a reduced and active state. The decreased general availability of NADPH in *sod1*Δ strains would lead to decreased RNR activity, resulting in lowered dNTP levels and replication arrest and DNA damage sensitivity. We propose that *TKL1* (Fig. 8C) suppresses the *sod1*Δ phenotypes via an alteration of the cellular redox environment, perhaps through a concomitant increase of NADPH levels that restores signaling through the *MEC1* pathway. This model is further supported by our data that the antioxidant NAC suppresses the HU sensitivity phenotype of *sod1*Δ and *lys7*Δ strains, presumably by removing hydroxyl radicals which are created from superoxide anions (2) and that lead to the more oxidized state of the cell.

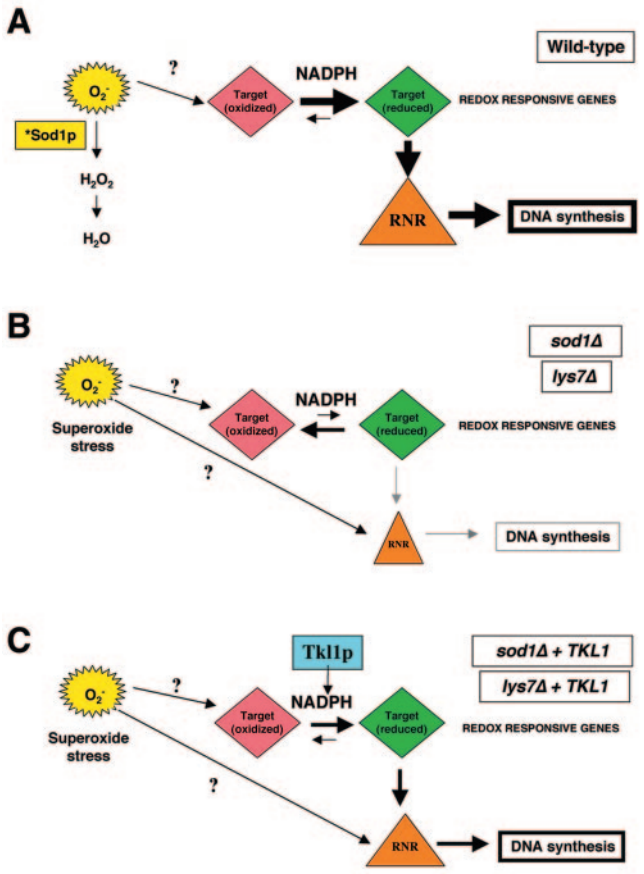


FIG. 8. Model for relationship between superoxide anions, NADPH levels, and RNR activity. (A) In wild-type strains, superoxide anions are scavenged by active Sod1p (*Sod1p), and sufficient levels of NADPH are provided through the pentose phosphate shunt to maintain redox-sensitive proteins in a reduced state. Redox-sensitive cellular targets, such as thioredoxins and glutaredoxins, transfer reductive capacity to RNR, and sufficient levels of active RNR are therefore available for DNA synthesis and repair. (B) In *sod1*Δ and *lys7*Δ strains, increases in superoxide anions and lowered NADPH levels shift the equilibrium of redox-sensitive cellular targets to more oxidized forms, resulting in lower RNR activity, decreased DNA synthesis and repair, and sensitivity to replication arrest and DNA damaging agents. Activity of RNR and induction of the *MEC1* pathway effector Rnr3p and of Hug1p may also be decreased by the redox sensitivity of *MEC1* pathway components. (C) Overexpression of *TKL1* in *sod1*Δ or *lys7*Δ strains increases the available NADPH in a *ZWF1*-dependent manner and shifts the equilibrium of redox-sensitive cellular targets towards their reduced forms, thereby increasing RNR activity and suppressing the replication arrest and DNA damage phenotypes of *sod1*Δ and *lys7*Δ strains. In addition, induction of Rnr3p and Hug1p is partially restored in the *sod1*Δ and *lys7*Δ strains.

Our observation that the *MEC1*-dependent response to replication arrest and DNA damage requires *SOD1* but shows less dependence on the presence of *LYS7* suggests that the sensitivity of *sod1*Δ mutants does not rely entirely on the Lys7p-dependent activity of Sod1p. Induction of Rnr3p and Hug1p, and to a lesser extent Rnr1p, upon HU exposure and viability after release from replication arrest also seem to differ between the *sod1*Δ and *lys7*Δ strains. Taken together, these data

support either a nonenzymatic role for Sod1p or the presence of a low level of active Sod1p in the absence of Lys7p. A low level of active Sod1p might be present in *sod1*Δ strains due to unassisted copper loading or copper loading dependent on a cellular protein other than Lys7p. In addition, a role for Sod1p that is independent of its Lys7-dependent SOD activity in response to replication arrest and DNA damage may exist. While both *SOD1* and *LYS7* have been reported to exhibit genetic interactions with some genes involved in human disease and DNA synthesis and repair, including *MRE11*, *RAD27*, *RAD50*, and *RAD52*, *SOD1* alone showed genetic interactions with *POL32*, *RTT107*/*ESC4*, and *SGS1* (56, 57), further supporting the stronger phenotypes we have observed for the *sod1*Δ strains.

We have identified an important relationship between alteration of cellular redox state due to loss of superoxide anion scavengers and the *MEC1*-dependent checkpoint response in *S. cerevisiae*. *TKL1*, which may alter the cellular redox state by increasing intracellular NADPH levels, suppresses HU sensitivity caused by the absence of *SOD1* and restores expression of the *MEC1* pathway effector, Rnr3p, and of Hug1p, in *sod1*Δ strains. Future experiments addressing the involvement of *S. cerevisiae* Sod1p and Lys7p with the *MEC1* pathway may shed light on the role of a compromised redox state due to excess superoxide anions in signaling through checkpoint pathways responding to replication arrest and DNA damage in other systems.

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