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Selenium Toxicity toward Yeast as Assessed by Microarray Analysis and Deletion Mutant Library Screen: A Role for DNA Repair

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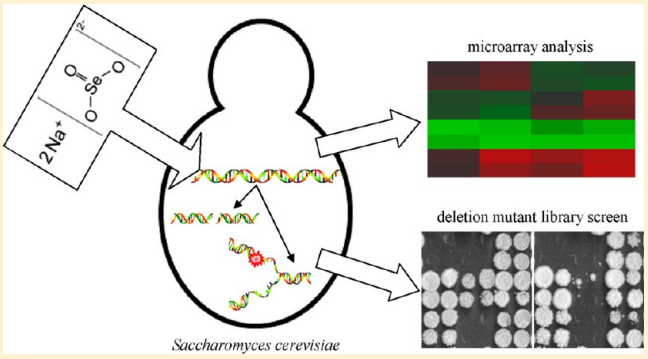
Supporting Information

ABSTRACT: Selenium (Se) is a trace element that is essential for human health as it takes part in many cellular processes. The cellular response to this compound elicits very diverse processes including DNA damage response and repair. Because an inorganic form of Se, sodium selenite (SeL), has often been a part of numerous studies and because this form of Se is used as a dietary supplement by the public, here, we elucidated mechanisms of SeL-induced toxicity in yeast *Saccharomyces cerevisiae* using a combination of systematic genetic and transcriptome analysis. First, we screened the yeast haploid deletion mutant library for growth in the presence of this Se compound. We identified 39 highly SeL sensitive mutants. The corresponding deleted genes encoded mostly proteins involved in DNA damage response and repair, vacuole function, glutathione (GSH) metabolism, transcription, and chromatin metabolism. DNA damage response and repair mutants were examined in more detail: a synergistic interaction between postreplication (PRR) and homologous recombination (HRR) repair pathways was revealed. In addition, the effect of combined defects in HRR and GSH metabolism was analyzed, and again, the synergistic interaction was found. Second, microarray analysis was used to reveal expression profile changes after SeL exposure. The gene process categories "amino acid metabolism" and "generation of precursor metabolites and energy" comprised the greatest number of induced and repressed genes, respectively. We propose that SeL-induced toxicity markedly results from DNA injury, thereby highlighting the importance of DNA damage response and repair pathways in protecting cells against toxic effects of this Se compound. In addition, we suggest that SeL toxicity also originates from damage to cellular proteins, including those acting in DNA damage response and repair.



INTRODUCTION

Selenium (Se) is an essential trace element that is indispensable for human health. This compound is required for numerous cellular processes, and both chemical form and dose determine its bioactivity in living systems. As to dose, an intake of about 55 μg/day optimally fulfills the dietary need for humans. Higher doses of about 200−300 μg/day have been proposed to be required for chemopreventing activities of Se against cancer. Up to doses of 750−800 μg/day, no adverse effects of Se intake have been observed. However, an intake above this level may cause adverse effects that vary from being moderate at doses of 1540−1600 μg/day to the occurrence of selenosis and DNA damage and cell death induction at doses of 3200−5000 μg/day. In contrast, intake of 40 μg/day represents the minimum dietary requirement, and levels below 11 μg/day can lead to deficiency problems in humans.[1−5]

Se exists in different forms, and these are generally classified as inorganic and organic. Sodium selenite (Na_2SeO_3; SeL), an inorganic form of Se, was the first Se compound used in chemopreventing studies. In nature, this Se compound is, however, relatively rare, and its concentrations are quite low. SeL gets converted into hydrogen selenide (H_2Se) and/or elementary Se in the presence of glutathione (GSH) via selenodiglutathione (GSSeSG), generating reactive oxygen species (ROS) as a byproduct.[1,2] Notably, oxidative stress as a consequence of ROS production was proposed to be responsible for the SeL-induced toxic effects,[6−9] which are, at least in part, thought to be caused by direct DNA injury. Indeed, SeL has been demonstrated to cause DNA single-strand (SSBs) and double-strand (DSBs) breaks in murine leukemia cells and other murine mammary carcinoma cell lines.[10,11] SeL exposure also leads to chromosomal damage in Swiss albino mice and in human peripheral lymphocytes.[12,13] In addition to DNA strand breaks, SeL was reported to induce oxidative DNA base damage, 8-oxo-7,8-dihydroguanine, in mouse keratinocytes[14] and in rat liver cells.[15] Expectedly, the induction of DNA damage was accompanied with a loss of cell

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viability in these studies.[10−15] In *Saccharomyces cerevisiae*, SeL has also been shown to generate DSBs,[16−18] which are repaired exclusively by homologous recombination repair (HRR), with virtually no contribution of nonhomologous end-joining (NHEJ).[17] A part of these DSBs, however, seems to be induced indirectly and is rather a consequence of conversion of SSBs and other DNA damage types that are processed via SSBs intermediates into DSBs during process of DNA replication. Thus, a majority of DSBs induced by SeL in yeast has an origin in other DNA damage types, likely in oxidative DNA base damage, as suggested.[16]

Importantly, damage to DNA is not a sole factor causing SeL toxicity; the other factors are also involved. Hence, SeL toxicity is driven by the complex network of different cellular processes. A complexity of this network is still poorly understood, although molecular details of some individual pathways are quite well-characterized. In this context, a combination of systematic genetic and transcriptome analysis achieved by the use of deletion mutant library screen and microarray, respectively, represents a powerful tool to unravel the complexity of SeL toxicity.

Previous microarray analysis revealed that expression of up to 30% of the open reading frames (ORFs) is significantly changed upon SeL exposure in yeast.[19] Iron homeostasis genes were the first to be induced, followed by the genes involved in redox homeostasis and in proteasome activity. In addition, SeL treatment triggered a large environmental stress response,[19] a common response to all stresses that leads to inhibition of the translation apparatus and activation of the energy storage pathways.[20] Several transcription factors have been identified to positively regulate the response of yeast cells to SeL, with the vast majority of them functioning in response to many other environmental factors, however. Unique to SeL treatment appears only Pdr1, a major regulator of pleiotropic drug resistance, whose responsiveness to SeL depends on Rnp4, a proteasome expression regulator. On the basis of this and other observations, it has been proposed that in response to SeL, Rnp4 establishes positive feedback loops with both the oxidative stress response and the pleiotropic drug resistance network.[19]

To reveal a complexity of the cellular processes associated with SeL toxicity, we screened the yeast haploid deletion mutant library for growth in the presence of this compound: we identified 39 mutants as highly SeL sensitive. The corresponding deleted genes encoded mostly proteins involved in DNA damage response and repair, vacuole function, GSH metabolism, transcription, and chromatin metabolism. Along with haploid deletion mutant library screen, we monitored transcriptional changes induced by SeL exposure by microarray analysis. This analysis displayed induction of 281, 788, and 657 and repression of 247, 726, and 657 genes upon 0.1, 1, and 10 mM SeL treatments, respectively (cutoff value 1.0, $p < 0.01$, fold change >2). Throughout all SeL doses, the gene process categories "amino acid metabolism, response to chemical stimulus, and transmembrane transport" comprised the greatest number of induced genes, while "generation of precursor metabolites and energy, cofactor metabolism, and cellular respiration" consisted of the greatest number of repressed genes.

In accordance with our and other's previous data,[16−18,21,22] we demonstrate that DNA damage repair pathways, HRR and postreplication repair (PRR), considerably contribute to cellular resistance to toxic effects of SeL, where they act synergistically. We further show that DNA lesion channeling between these two pathways that is controlled by the Srs2 helicase does not seem to act after SeL exposure comparably to that after ultraviolet light (UV) or methyl methanesulfonate (MMS) exposure. We also revealed a synergism between the HRR and the GSH metabolism pathway with respect to protecting yeast cells against toxic effects of SeL. Finally, we suggest that SeL also attacks macromolecules other than DNA, namely, cellular proteins acting in DNA damage response and repair.

MATERIALS AND METHODS

Strains and Media. The *S. cerevisiae* strains used in this study were BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), DMBY1 (BY4741 with *rad6::URA3*), DMBY2 (BY4741 with *rad52::LEU2*), DMBY3 (BY4741 with *rad5::kanMX rad6::URA3*), DMBY4 (BY4741 with *rad5::kanMX rad52::LEU2*), DMBY5 (BY4741 with *rad52::LEU2 rad6::URA3*), DMBY6 (BY4741 with *rad5::kanMX rad52::LEU2 rad6::URA3*), DMBY7 (BY4741 with *glr1::URA3*), DMBY8 (BY4741 with *gsh1::kanMX glr1::URA3*), DMBY9 (BY4741 with *gsh1::kanMX rad52::LEU2*), DMBY10 (BY4741 with *glr1::URA3 rad52::LEU2*), DMBY11 (BY4741 with *gsh1::kanMX glr1::URA3 rad52::LEU2*), DMBY12 (BY4741 with *srs2::kanMX rad6::URA3*), DMBY13 (BY4741 with *srs2::kanMX rad52::LEU2*), DMBY14 (BY4741 with *srs2::kanMX rad6::URA3 rad52::LEU2*), the set of viable deletion mutants (approximately 4700) derived from the haploid strain BY4741 (EUROSCARF, http://web.uni-frankfurt.de/fb15/mikro/euroscarf), and *rad5::kanMX*, *srs2::kanMX* and *gsh1::kanMX* single mutants from this set. Furthermore, W303 (*MATα ade2 leu2 his3 trp1 ura3 can1−100*), W303αL (W303 *yku70::LEU2*), JDY1 (W303 *rad52::TRP1*), and JDY2 (W303 *yku70::LEU2 rad52::TRP1*) strains were exploited.

Cells were grown in YPD (1% yeast extract, 2% peptone, and 2% glucose) and YNB (0.67% yeast nitrogen base and 2% glucose). When appropriate, YNB was supplemented with amino acids, uracil, and GSH (a final concentration of 1 mM). G418 sulfate (400 μg/mL) was used to supplement YPD. For plates, 2% agar was added to media.

Growth and Treatment Conditions. For the purpose of DNA damage sensitivity assays, a spot test was used.[18] In this test, serial decimal dilutions of cell suspensions were spotted onto YPD plates containing indicated concentrations of SeL or MMS. In the case of UV, undiluted cell suspensions (2×10^8 cells/mL) were spotted onto YPD plates and irradiated with indicated doses. In some experiments, YNB plates described above were used to determine SeL sensitivity. Plates were cultivated at 30 °C and scored after 5 days. In the case of DSB repair experiments, SeL treatment was carried out in the exponential phase of cell growth. Yeast cells were grown in YPD medium overnight. Overnight culture was used to inoculate fresh YPD. Incubation in YPD continued until the cell suspension reached a density of 2×10^7cells/mL. The yeast culture was then collected by centrifugation, washed with and resuspended in 0.1 M potassium phosphate (pH 7.4) at a density of 2×10^8 cells/mL, and treated with SeL at 30 °C for 3 h with shaking.

Microarray Analysis. BY4741 strain was cultivated overnight at 30 °C. Overnight culture was used to inoculate fresh YPD media supplemented with SeL at different concentrations (0, 0.1, 1, and 10 mM) to a density of 5×10^6 cells/mL. These SeL doses were selected because they had considerably different impact upon cell growth in previous experiments shown in Figure 1 in the Supporting Information. After 12 h of growth, cell cultures were washed in sterile water, and total RNA was isolated by the hot acid phenol method.

One hundred micrograms of total RNA was indirectly labeled with Amino Allyl cDNA Labeling Kit (Ambion, United States). First, total RNA was reverse transcribed incorporating amino allyl-modified dUTP into the synthesized cDNA. The template RNA was then degraded by base hydrolysis, and the reaction was neutralized. The amino allyl-modified cDNA was then purified to remove unincorporated nucleotides and primers. In the second step, the labeled cDNA

Table 1. List of SeL Sensitive Strains as Identified by Individual Rescreening of the SeL Sensitive Strains Revealed by the Haploid Deletion Mutant Library Screen

ORF	gene	molecular/biological function
YDR448W	*ADA2* (*SWI8*)	regulation of RNA polymerase II-transcribed genes
YJR060W	*CBF1* (*CEP1*/*CPF1*)	nucleosome positioning at certain motifs
YMR198W	*CIK1*	karyogamy; mitotic spindle organization
YAL012W	*CYS3* (*CYI1*/*FUN35*/*STR1*)	cystathione γ-lyase catalyzing conversion of homocysteine to cysteine with the intermediary formation of cystathione
YGR252W	*GCN5* (*ADA4*/*SWI9*/*AAS104*)	chromatin modification; positive regulation of transcription elongation by RNA polymerase II
YPL091W	*GLR1* (*LPG17*)	cytosolic and mitochondrial GSH reductase; protects cells from oxidative stress
YJR090C	*GRR1* (*CAT80*/*COT2*/*SSU2*/*SDC1*)	carbon catabolite repression; glucose-dependent divalent cation transport; high affinity glucose transport; morphogenesis; sulfite detoxification
YJL101C	*GSH1*	γ-glutamylcysteine synthetase catalyzing first step of GSH biosynthesis
YJR139C	*HOM6*	homoserine dehydrogenase catalyzing methionine and threonine biosynthesis
YBR098W (YBR100W)	*MMS4* (*SLX2*)	DNA repair; meiotic recombination; replication fork stability
YMR224C	*MRE11* (*RAD58*/*XRS4*/*NGS1*)	DSB repair; meiotic recombination; telomere maintenance; checkpoint signaling
YDR386W	*MUS81* (*SLX3*)	DNA repair; meiotic recombination; replication fork stability
YPR099C		dubious ORF unlike to encode a protein; partially overlaps the verified ORF YPR100W
YDR176W	*NGG1* (*ADA3*/*SWI7*)	chromatin modification; transcriptional regulator involved in glucose repression of Gal4-regulated genes
YBR094W	*PBY1*	putative tubulin tyrosine ligase
YLR032W	*RAD5* (*REV2*/*SNM2*)	replication fork regression during PPR by template switching; stimulation of synthesis of free and PCNA-bound polyubiquitin chains
YGL058W	*RAD6* (*UBC2*/*PSO8*)	ubiquitin-conjugating enzyme (E2); PRR, DSB repair, checkpoint control
YER173W	*RAD24*	DNA damage checkpoint; NER
YNL250W	*RAD50*	DSB repair; meiotic recombination; telomere maintenance; checkpoint signaling
YER095W	*RAD51* (*MUT5*)	DSB repair; meiotic recombination
YML032C	*RAD52*	DSB repair; meiotic recombination
YGL163C	*RAD54* (*XRS1*)	DSB repair; meiotic recombination
YDR076W	*RAD55*	DSB repair; meiotic recombination
YDR004W	*RAD57*	DSB repair; meiotic recombination
YOL143C	*RIB4*	lumazine synthase catalyzing synthesis of immediate precursor to riboflavin
YHL025W	*SNF6*	chromatin remodeling; transcription regulation
YPL129W	*TAF14* (*SWP29*/*TAF30*/*TFG3*/*ANC1*)	RNA polymerase II transcription initiation; chromatin modification
YOL006C	*TOP1* (*MAK1*/*MAK17*)	relaxing supercoiled DNA; replication, transcription, and recombination
YOR295W	*UAF30*	RNA polymerase I specific transcription stimulatory factor
YDL185W	*VMA1* (*CLS8*/*TFP1*)	vacuolar membrane ATPase
YBR127C	*VMA2* (*VAT2*)	vacuolar membrane ATPase
YOR332W	*VMA4*	vacuolar membrane ATPase
YKL080W	*VMA5* (*CSL5*/*VAT3*)	vacuolar membrane ATPase
YEL051W	*VMA8*	vacuolar membrane ATPase
YCL005W-A	*VMA9* (*CWH36*/*LDB10*)	vacuolar membrane ATPase
YOR270C	*VPH1*	vacuolar ATPase; vacuolar pH
YLR240W	*VPS34* (*END12*/*PEP15*/*VPL7*/*VPT29*/*STT8*/*VPS7*)	phosphatidylinositol 3-kinase; protein sorting
YDR369C	*XRS2*	DSB repair; meiotic recombination; telomere maintenance; checkpoint signaling
YCL007C	(*CWH36*)	dubious ORF unlike to encode a protein; overlaps verified ORF YCL005W-A

was coupled with the monoreactive succinimide ester derivative of an AlexaFluor 647 (Invitrogen, United States). The fluorescently labeled cDNA was purified with a NucAway Spin Column (Ambion), to remove any unreacted dye. The 2 μg of labeled and purified cDNA was dried and resuspended again in 5 μL of nuclease-free water and used for hybridization in a one color experiment.

The hybridization was carried out using MAUI Hybridization system (BioMicro, United States) and required adhering a NimbleChip X1 mixer to the microarray slide *S. cerevisiae* 385K Array TI4932 60mer (Roche Nimblegen Inc., United States). The hybridization master mix solution was prepared using components from a NimbleGen Hybridization Kit according to the NimbleGen protocol. Hybridization solution and samples were mixed, denatured, and loaded into the fill port of a mixer. Then, the samples were hybridized for 20 h at 42 °C in the mix mode B.

Microarrays were scanned with InnoScan 700 (Innopsys, France) at resolution 3 μm. Image analysis was performed in NimbleScan 2.5 software (Roche NimbleGen Inc.) according to the appropriate .ndf file. The data analysis was performed using GeneSpring GX 11 (Agilent Technologies Inc., United States). For each chip, raw intensity data were normalized by interarray normalization to quantile, and baseline was set to the median of all samples. The low 20% and top 1% of genes were filtered out as well as features with SD < 0.2. Evaluation of expression changes at each SeL concentration was statistically done by unpaired *T* test (SeL treatment vs control). The filtered and normalized values are available as Table 2 in the Supporting Information. Expression profile throughout all SeL concentrations was statistically tested using one-way ANOVA and posthoc Tukey's HSD test. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-3449. The reader should be aware of the fact that experiments deposited at this web address are numbered differently (2, 3, and 5) as compared with those in this paper (1, 2, and 3, respectively). The significant data sets (Benjamini-Hochberg corrected

$p < 0.01$) were further interpreted using Gene Ontology (GO) Slim Mapper at http://yeast-genome.org. Microarray was carried out in triplicate.

Deletion Mutant Library Screen. The collection of deletion mutants was screened for cellular response to SeL exposure as follows. Mutants were transferred from 96-well master plates onto YPD plates containing G418 sulfate. After 3 days, cells from grown colonies were inoculated into the corresponding wells of a 96-well microtiter plates containing 200 μL of YPD and G418 sulfate. After overnight cultivation with shaking at 30 °C, cells were diluted 10 and 50 times in sterile water and replica pinned onto YPD plates containing 0, 0.05, 0.1, and 0.5 mM SeL using a 96 floating pin replicator. Each of the mutant strains on a 96-well plate was replicated in four copies forming a square. YPD plates were cultivated at 30 °C and scored after 3 and 5 days. The cellular response to SeL was assessed visually from the growth on the SeL-containing medium as compared to growth on the YPD control plate. The sensitivity score presented in Table 1 in the Supporting Information was calculated by multiplying three parameters: sensitivity, growth, and dilution. The sensitivity was rated from 3 to 5, where score 3 was given to the strain that was killed only by the highest SeL dose used (0.5 mM) and score 5 was given to the strain killed even by the lowest SeL dose (0.05 mM). Growth was scored from 1 (very poor growth) to 3 (normal growth). If strain was not able to growth at both 10 and 50 times dilutions, it was given a dilution score 2. A dilution score of 1 was given to strain being able to grow at 10 times diluted but not at 50 times diluted concentration.

A set of SeL sensitive mutants revealed by deletion mutant library screen was individually rescreened for SeL sensitivity in the spot test. Experimental conditions of spot test enabled us to eliminate many false positives identified by primary deletion mutant library screen. Spot tests were carried out as previously described.[18] To eliminate a problem with different cell culture densities occurring in the deletion mutant library screen, all overnight cultures in the spot test were adjusted to achieve a final concentration of 1×10^7 cells/mL. Subsequently, 10-fold serial dilutions of cell suspensions were spotted onto YPD plates containing 0, 0.05, 0.1, and 0.5 mM SeL and cultivated at 30 °C for 5 days. A strain was considered as sensitive if it was unable to grow on a plate containing 0.05 mM SeL when spotted from a culture of density of 1×10^6 cells/mL.

DSB repair. DSB repair experiments were monitored using pulsed-field gel electrophoresis (PFGE). These experiments were performed as previously described,[17] except that postincubation (0, 2, 4, and 24 h, where 0 h means 3 h treatment with no postincubation time) was carried out in YPD medium supplemented with cycloheximide (EMD Biosciences, La Jolla, Canada) at a final concentration of 20 μg/mL to inhibit de novo protein synthesis. After postincubation, the cells were collected by centrifugation, washed twice with and resuspended in 50 mM EDTA (pH 7.5) at a density of 6.25×10^8 cells/mL, and processed as reported.[17]

RESULTS

Identification of Yeast Mutants Sensitive to SeL. To identify factors and processes that play a role in the cellular response to SeL, we screened the yeast haploid deletion mutant library for growth in the presence of this compound. Three different concentrations of SeL (0.05, 0.1, and 0.5 mM) and two different dilutions of cell cultures to be plated (10 and 50 times) were used (for further details, see the Materials and Methods). The primary screen identified 173 potentially SeL sensitive mutants (Table 1 in the Supporting Information). These mutants were individually rescreened using the spot test, and 39 of them appeared to be truly and highly sensitive to SeL (only these mutants are referred to hereafter as SeL sensitive mutants) (Table 1). The corresponding deleted genes encoded mostly proteins involved in DNA damage response and repair, vacuole function, GSH metabolism, transcription, and chromatin metabolism.

Mutants defective in DNA damage response and repair constituted the largest group of strains sensitive to SeL, suggesting that damage to DNA considerably contributes to the toxic effects of SeL. In this group, HRR mutants were the most prevalent, implying that SeL induces DNA lesions that are primarily repaired by HRR. Those lesions could likely be typical two-ended DSBs. However, such DNA lesions could also be those that cause collapse of replication forks (e.g., SSBs), resulting in one-ended DSBs that are also repaired by HRR.[23] Stalled replication forks may occur in SeL-treated cells as well. If this is the case, both the repair of these forks after their collapse into one-ended DSBs and their restart would require HRR.[23] We assume that all above-mentioned DNA lesions/structures are generated in SeL-exposed yeast cells. Our opinion is in line with the demonstration that SeL induces DSBs in stationary SeL-treated yeast cells,[16] providing evidence for DNA replication-independent DSBs, and that both replication fork stability (*mus81* and *mms4*) and replication fork regression (*rad5*) mutants are present in a group of the SeL sensitive strains (Table 1).

In the second largest group were mutants in genes encoding five subunits (A, B, C, D, and E) of the eight-subunit V1 peripheral membrane domain of the vacuolar H^+-ATPase (V-ATPase) as well as two subunits (E and one of the two isoforms of A) of the V-ATPase V0 domain (Table 1). In accordance with our findings are previous data showing that detoxification of SeL in yeast is also mediated via its reduction to elementary Se that is subsequently sequestrated in vacuoles.[24−26] Importantly, cytosolic enzymatic reduction of SeL to elementary Se was suggested to be related to activity of the V-ATPase. As GSH is one of the most active SeL-reducing components in a cell, mutants affected in genes of GSH metabolism expectedly constituted another group of strains sensitive to SeL (Table 1).

SeL Sensitivity in Mutants with Combined DNA Repair Defects. Various surveillance and DNA damage repair and tolerance mechanisms that ensure the processing or stabilization of stalled replication forks have evolved in yeast cells. Replication can be re-established by HRR and/or by shuttling the interfering lesions into the pathways of PRR that can lead to lesion bypass. This process is primarily controlled by Srs2, a helicase that is recruited to replication forks by SUMOylated proliferating cell nuclear antigen (PCNA), where it removes Rad51 filaments, thus preventing HRR and favoring PRR.[27] In line with this, HRR contributes to hyperrecombination phenotype of the cells defective in PRR pathway.[28] In addition, it is required for the suppressive effect of *srs2* on *rad6* and *rad18* repair defects.[29] The main HRR factor in yeast is Rad52 because virtually no HRR events occur in *rad52* cells.[30]

A central role to PPR in yeast plays the Rad6−Rad18 complex that regulates several distinct pathways of PRR and translesion DNA synthesis (TLS) via modification of PCNA. While PRR controlled by Rad6−Rad18 is an error-prone process, the PPR branch that involves template switching by fork regression, and is dependent on the Mms2−Ubc13−Rad5 complex, is error-free.[31]

As suggested, re-establishment of stalled DNA replication may account for a requirement for Rad52 for survival of yeast cells after SeL exposure.[17] As our deletion mutant library screen data presented here, as well as previous data by others,[21] showed an involvement of Rad5 and Rad6 in cellular response to SeL, we wished to address the question whether DNA damage response and repair pathways engaging Rad52, Rad6,

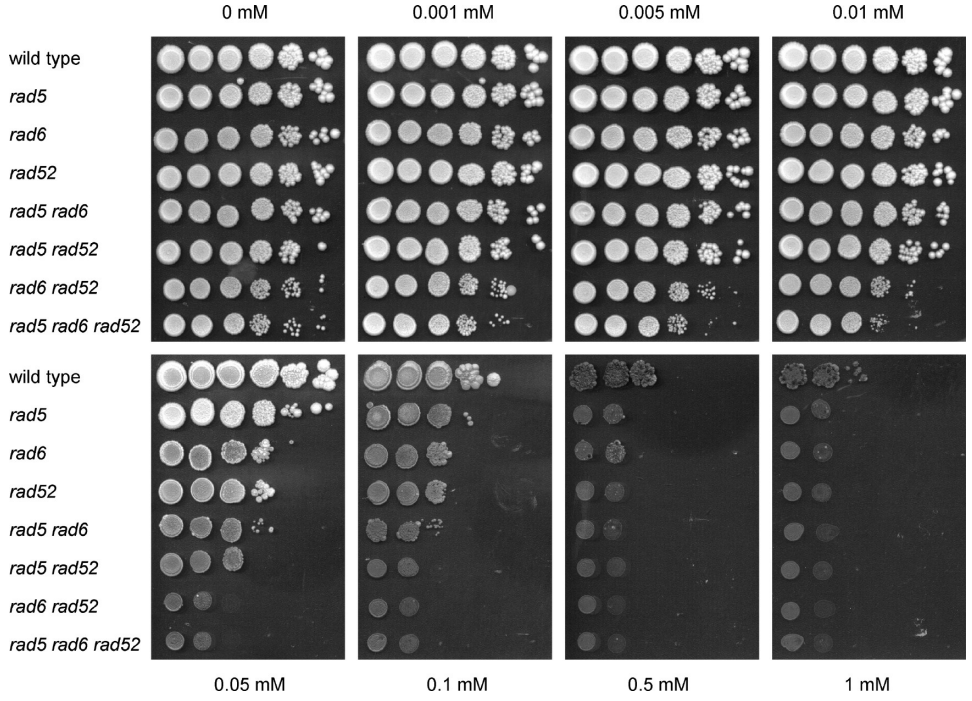

Figure 1. Analysis of the effects of combining *RAD5*, *RAD6* and *RAD52* gene inactivations on SeL sensitivity. Serial decimal dilutions of exponential cultures were spotted onto YPD plates containing indicated concentrations of SeL. Plates were cultivated at 30 °C and scored after 5 days.

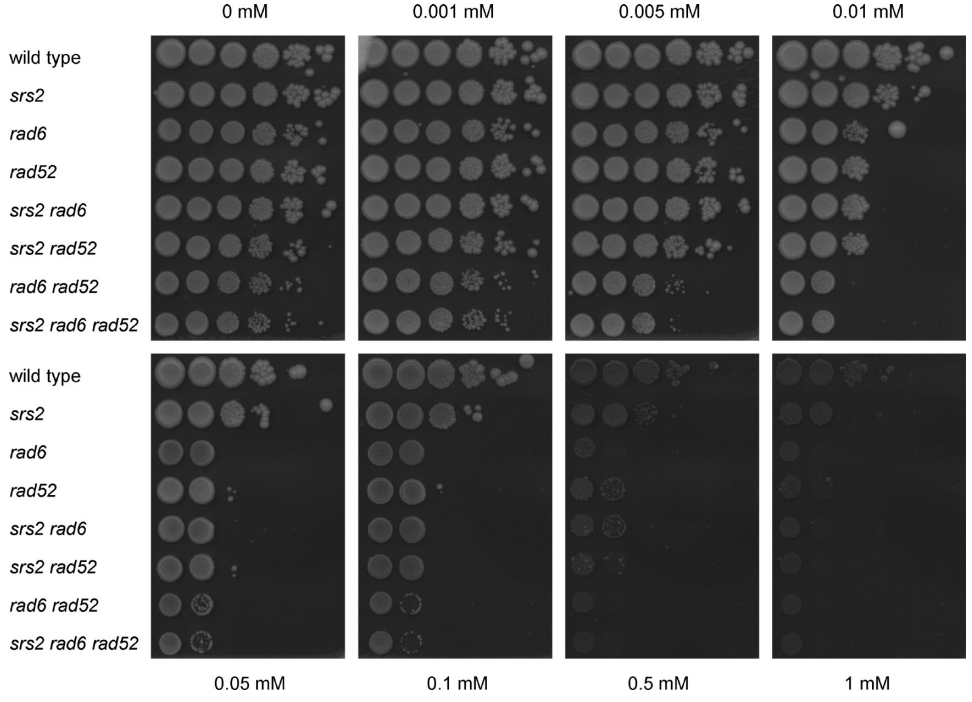

Figure 2. Analysis of the effects of combining *SRS2*, *RAD6* and *RAD52* gene inactivations on SeL sensitivity. Serial decimal dilutions of exponential cultures were spotted onto YPD plates containing indicated concentrations of SeL. Plates were cultivated at 30 °C and scored after 5 days.

and Rad5 act epistatically or synergistically with respect to SeL sensitivity. Consequently, we generated the corresponding double and triple mutants and tested their sensitivity toward SeL along with single mutants and parental wild-type strain. As evident (Figure 1), the *rad52* and *rad6* single mutants were sensitive to SeL, in line with our and other's previous data.[17,21] The *rad5* single mutant also displayed SeL sensitivity, although slightly less pronounced as compared to *rad52* or *rad6* cells. Interestingly, PRR inactivation in *rad52* cells further increased their sensitivity toward SeL. Notably, *RAD5* deletion had virtually no extra effect on response of the *rad6 rad52* double mutant cells to this compound. Hence, we propose that PRR and HRR repair pathways act synergistically in the removal of the toxic DNA lesions induced by SeL. With respect to function of PRR in the process, recovery of stalled replication after SeL exposure via TLS seems to be more prevalent event as compared to that via template switching by fork regression.

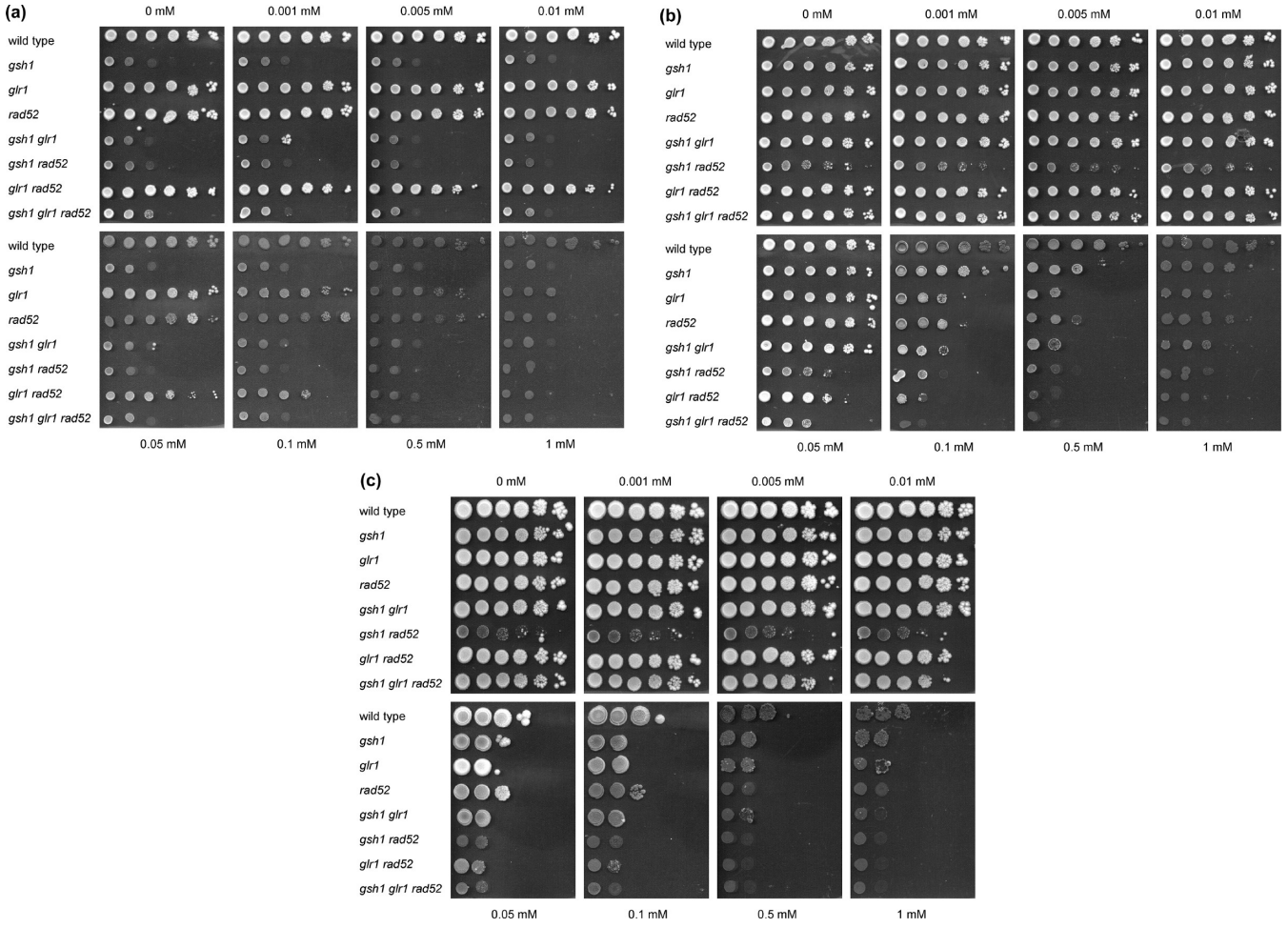

Figure 3. Analysis of the effects of combining *GSH1*, *GLR1* and *RAD52* gene inactivations on SeL sensitivity. Serial decimal dilutions of exponential cultures were spotted onto plates containing indicated concentrations of SeL. (A) YNB supplemented with histidine, leucine, methionine, and uracil, (B) as A plus GSH, and (C) YPD. Plates were cultivated at 30 °C and scored after 5 days.

To get deeper insights into the mechanism of stalled replication fork recovery after SeL exposure, we inactivated *SRS2* in *rad6, rad52,* and *rad6 rad52* mutant cells and examined their sensitivity to this compound. As shown (Figure 2, Table 1, and Table 1 in the Supporting Information), the *srs2* single mutant displayed mild SeL sensitivity. Astonishingly, *SRS2* inactivation did not lead to any further increase in SeL sensitivity in *rad6*, *rad52*, and *rad6 rad52* backgrounds and only negligibly suppressed the repair defect of *rad6* cells in repairing the toxic DNA lesions induced by SeL, in sharp contrast to those induced by UV and MMS (Figures 2 and 3 in the Supporting Information). UV and MMS treatments were used as controls, because their toxicity toward mutants being tested here for possibility of genetic interaction after SeL exposure has already been well-documented.[32−34] Notably, our survival data after UV and MMS exposures were in good agreement with those already published,[32−34] showing correct functioning of our epistatic/synergistic analysis in case of UV and MMS exposures, thereby validating data obtained after SeL exposure.

It seems that channeling of DNA lesions into HRR after SeL treatment in *rad6* cells does not have that strong association with toxicity as in case of UV- and MMS-induced DNA damage. Hence, a proportion of DNA lesions that is channeled into HRR in *rad6* cells after SeL exposure is likely significantly lower than that after UV or MMS exposure. Therefore, it appears that in *rad6* cells, a third mechanism (probably controlled by Rad5) has a considerably higher contribution to dealing with the toxic DNA lesions induced by SeL as compared with those induced by UV or MMS.

SeL Sensitivity in Mutants with Combined Defects in DNA Repair and GSH Biosynthesis. To find out the effect of combining defects affecting GSH pool with that in HRR on SeL sensitivity, the following set of deletion mutants was prepared and used to examine its sensitivity toward SeL: *gsh1*, *glr1*, *rad52*, *gsh1 glr1*, *gsh1 rad52*, *glr1 rad52*, and *gsh1 glr1 rad52*. Both *GSH1* (encodes γ-glutamylcysteine synthetase) and *GLR1* (encodes GSH reductase) are involved in GSH biosynthesis in a cell. While the former catalyzes the formation of GSH from its constituent amino acids, the latter increases GSH pool via its regeneration from glutathione disulfide (GSSG).[35]

To strictly control GSH pools, our original experiment was carried out under conditions where virtually no extracellular GSH levels were present, that is, on YNB plates supplemented only with all essential amino acids and nitrogen base. In line with previous data,[36] such conditions lead to substantial growth defect in strains that were unable to synthesize GSH as a consequence of *GSH1* inactivation (Figure 3A), indicating that biosynthesis of GSH from its constituent amino acids is essential for providing yeast cells with conditions allowing normal physiological growth. Interestingly, *GLR1* inactivation

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Table 2. List of 10 Most Highly SeL-Induced ORFs/Genes

SeL					
0.1 mM		1 mM		10 mM	
ORF/gene	fold of induction[a]	ORF/gene	fold of induction	ORF/gene	fold of induction
YBR244W/*GPX2* (*AMI1*)	93	YML058W-A/*HUG1*	222	YJR005C-A	95
YJR005C-A	58	YDR345C/*HXT3*	146	YLR412C-A	74
YFL056C/*AAD6*	43	YJR005C-A	46	YML058W-A/*HUG1*	72
YDL243C/*AAD4*	33	YHR092C/*HXT4* (*LGT1*/*RAG1*)	31	YOR072W−B	68
YFL057C/*AAD16*	29	YGL157W/*ARI1*	23	YOR385W	65
YLR108C	25	YHR094C/*HXT1* (*HOR4*)	23	YFL067W	56
YPL250C/*ICY2*	19	YKL071W	23	YFL057C/*AAD16*	47
YGR142W/*BTN2*	17	YLR412C-A	22	YDL243C/*AAD4*	44
YGR211W/*ZPR1*	16	YAL012W/*CYS3* (*CYI1*/*FUN35*/*STR1*)	22	YGR152C/*RSR1* (*BUD1*)	44
YOL165C/*AAD15*	16	YFL057C/*AAD16*	21	YBR244W/*GPX2* (*AMI1*)	37

[a] $p < 0.01$.

Table 3. List of 10 Most Highly SeL-Repressed ORFs/Genes

SeL					
0.1 mM		1 mM		10 mM	
ORF/gene	fold of repression[a]	ORF/gene	fold of repression	ORF/gene	fold of repression
YJR095W/*SFC1* (*ACR1*)	48	YLR377C/*FBP1* (*ACN8*)	141	YLR377C/*FBP1* (*ACN8*)	23
YLR377C/*FBP1* (*ACN8*)	20	YKL217W/*JEN1*	83	YKL217W/*JEN1*	18
YOR375C/*GDH1* (*URE1*/*DHE4*)	12	YNL117W/*MLS1*	67	YEL024W/*RIP1*[b]	18
YKL182W/*FAS1*	12	YJR095W/*SFC1* (*ACR1*)	66	YNL052W/*COX5A*	15
YLR304C/*ACO1* (*GLU1*)	9	YDR536W/*STL1*	60	YBL015W/*ACH1*	15
YDL174C/*DLD1*	8	YMR206W	42	YNL117W/*MLS1*	15
YNL333W/*SNZ2*	7	YDR119W-A/*COX26*	38	YLL041C/*SDH2* (*ACN17*)	14
YKR013W/*PRY2* (*YFW12*)	6	YNL194C	36	YOR228C	13
YFL059W/*SNZ3*	6	YKR097W/*PCK1* (*JPM2*/*PPC1*)	34	YBR067C/*TIP1*	13
YIL136W/*OM45*	6	YGR236C/*SPG1*	30	YDL174C/*DLD1*	13

[a] $p < 0.01$. [b] *RIP1* has been used in the literature to refer to two ORFs: YEL024, which encodes a Rieske iron−sulfur protein, and YDR192C, which encodes a nuclear pore complex subunit.

did not cause any growth defect in the absence of extracellular GSH but sensitized yeast cells to SeL treatment (Figure 3A), suggesting that inability of cells to reduce GSSG to GSH results in sensitivity to SeL.

In extended experimental setup, above-mentioned plates were supplemented with GSH. In addition, YPD plates were used in this setup. As obvious, extracellularly added GSH solved the growth problem caused by *GSH1* inactivation, although not completely in the *rad52 gsh1* double mutant cells (Figure 3B,C), indicating that extracellular GSH can efficiently be uptaken by cells to ensure their normal physiological growth. However, extracellularly added GSH could not fully protect yeast cells against the toxic effects of SeL. We expect that extracellularly added GSH gets at least in part oxidized upon its reaction with SeL before it enters the cells. Therefore, it appears that extracellular GSH, represented by a mixture of its reduced and oxidized form, can rescue growth problem of cells that are unable of GSH synthesis de novo, but it cannot fully provide cells with protective effects against SeL toxicity.

We observed that the resultant SeL sensitivity after combining defect in GSH biosynthesis with that in HRR is increased as compared to either single defect, indicating that GSH biosynthesis and HRR protect yeast cells from the toxic effects of SeL in a synergistic way. On the basis of the fact that GSH is ultimately required for conversion of SeL into H_2Se, a highly toxic Se form that can produce ROS upon oxidation,[6−9] epistatic interaction between these two pathways would rather be expected. Because ROS generate a broad range of DNA damage including strand breaks and replication stalling structures,[37] GSH-catalyzed conversion of SeL into H_2Se is expected to lead to an increased production of substrates for HRR. A synergistic interaction between the two pathways that we observed indicates that GSH also has a protective role in SeL-exposed yeast cells, and we propose that this role obviously overcomes that with deleterious effects.

Transcriptional Profile Analysis of SeL Treated Yeast Cells. To get deeper insights into cellular processes that are associated with toxicity of SeL, we analyzed transcriptional profiles of cells that have been treated with this compound. For this purpose, we used three doses of SeL, each having a substantially different impact on cell growth: 0.1 (virtually no effect), 1 (moderate effect), and 10 mM (nearly complete inhibition) (Figure 1 in the Supporting Information). Among all ORFs that exhibited intensities over the cutoff value 1.0 and had p values of <0.01, 281, 788, and 657, genes were induced >2-fold upon 0.1, 1, and 10 mM SeL treatments, respectively. At the same parameters, 247, 726, and 657 genes, respectively, were repressed (Table 2 in the Supporting Information). The 10 most highly SeL-induced and -repressed genes are listed in Tables 2 and 3. As obvious, YJR005C-A (unknown function) and YFL057C (encodes the Aad16 protein with putative aryl-alcohol dehydrogenase activity)[38] belong to the 10 most highly induced ORFs/genes at all SeL doses used. On the other hand, YLR377C (encodes Fbp1/Acn8, a fructose-1,6-bisphosphatase

functioning as key regulatory enzyme in the gluconeogenesis pathway)[39] is part of the 10 most highly repressed ORFs/genes. Throughout all SeL doses, the gene process category "amino acid metabolism" comprised the greatest number of induced genes, while "generation of precursor metabolites and energy" consisted of the greatest number of repressed genes (Table 3 in the Supporting Information).

Transcriptional changes of the genes associated with HRR[40] and PRR[41] processes in SeL-treated cells are summarized in Table 4. Apart from some exceptions such as *RAD51*, *RAD52*, *RDH54*, *RFA1*−*3*, *RAD6*, and *POL30* (these factors, however, do not constitute the whole DNA repair pathway), virtually no expression profile changes have been found for these categories of DNA damage response and repair genes after SeL treatment even though strains defective in HRR and PRR are highly sensitive to this compound, as mentioned above. Hence, it looks like steady state levels of the HRR and PRR proteins are sufficient to provide yeast cells with protection against toxic effects of SeL. In agreement with our data, others have also shown that DNA repair genes, including those being subject of this study, hardly respond to DNA damaging agents at transcriptional level in yeast.[42] Importantly, those DNA repair genes that change transcriptional profile upon DNA damaging treatment usually do so regardless of whether their induction provides cells with an advantage of higher survival. In other words, the nature of the DNA lesions generated by DNA damaging agents is inadequately related to gene expression profiling.[43] A lack of specific relationship between transcriptional change after DNA damage and survival to that damage has led to conclusion that yeast cells rather use posttranslational modifications such phosphorylation, ubiquitination and SUMOylation, and a sophisticated signal transduction cascade to regulate gene expression in response to DNA damage.

Table 4. Expression Change of the HRR and PRR Factors after SeL Exposure

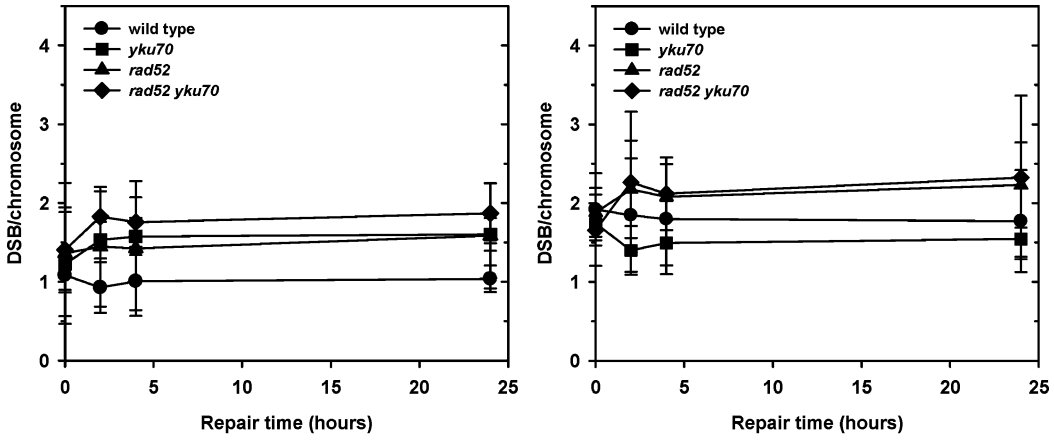
	fold change		
gene/ORF/pathway	0.1 mM	1 mM	10 mM
DNA2/YHR164C/HRR	−1.12	+1.04	−1.27
EXO1/YOR033C/HRR	+1.89	+1.87	+2.11
MMS4/YBR098W/HRR	+1.33	+1.09	+1.06
MPH1/YIR002C/HRR	+1.40	+1.01	+1.00
MRE11/YMR224C/HRR	−1.68	−1.49	−1.66
MUS81/YDR386W/HRR	+1.09	+2.01	+1.03
RAD50/YNL250W/HRR	−1.01	−1.44	−1.27
RAD51/YER095W/HRR	+3.44	+1.62	+1.14
RAD52/YML032C/HRR	+3.08	+1.69	+2.83
RAD54/YGL163C/HRR	+2.34	+1.57	+1.75
RAD55/YDR076W/HRR	−1.13	−1.37	−1.47
RAD57/YDR004W/HRR	+1.11	+1.04	+1.12
RDH54/YBR073W/HRR	+2.30	+4.58	+3.47
RFA1/YAR007C/HRR	+3.51	+2.37	+3.30
RFA2/YNL312W/HRR	+4.17	+3.43	+3.70
RFA3/YJL173C/HRR	+1.32	+1.86	+1.35
RMI1/YPL024W/HRR	−1.88	−1.38	−1.42
SAE2/YGL175C/HRR	−1.12	−1.27	+1.09
SGS1/YMR190C/HRR	−1.32	+1.13	+1.01
SLX1/YBR228W/HRR	+1.02	−1.27	−1.38
SLX4/YLR135W/HRR	+1.75	+1.44	+2.22
TOP3/YLR234W/HRR	+1.23	+1.02	+2.01
XRS2/YDR369C/HRR	−1.12	−1.29	−1.18
YEN1/YER041W/HRR	−1.08	+1.04	+1.92
SRS2/YJL092W/HRR/PRR	−1.00	+1.22	+1.39
MMS2/YGL087C/PRR	+1.37	−1.19	−2.70
POL3/YDL102W/PRR	−1.31	+1.05	−1.40
POL30/YBR088C/PRR	+3.30	+10.38	+6.85
RAD18/YCR066W/PRR	−1.11	−1.06	−1.31
RAD5/YLR032W/PRR	+1.51	+1.38	+1.40
RAD6/YGL058W/PRR	+2.97	+2.21	+1.54
REV1/YOR346W/PRR	−1.13	+1.31	+1.49
REV3/YPL167C/PRR	−1.53	−1.42	−1.92
REV7/YIL139C/PRR	−1.35	+1.95	+2.50
UBC13/YDR092W/PRR	+2.22	+1.55	+1.32

DSB Repair in SeL-Treated Yeast Cells. Previously, we have reported that DSBs were efficiently repaired in the wild-type and NHEJ defective (*yku70* mutant) cells under growing conditions (growth in complete medium). In sharp contrast, no DSB repair was observed in cells debilitated in HRR (*rad52* single or *rad52 yku70* double mutant). Because DSB repair data corresponded to that from survival, we proposed that the inability to repair DSBs determines cellular sensitivity to SeL in yeast.[17] As aforementioned, the HRR proteins that are necessary for the repair of SeL-induced DSBs seem to be at sufficient levels within the cell because transcription of the related genes does not significantly change after SeL exposure. Consequently, we carried out DSB repair experiments in complete medium supplemented with cycloheximide, a drug inhibiting protein synthesis, to find out whether there may be a requirement for de novo synthesis of the HRR proteins for efficient DSB repair after SeL exposure. As obvious, HRR-proficient cells failed to repair the SeL-induced DSBs in the presence of cycloheximide (Figure 4), indicating that de novo protein synthesis is indeed required for efficient DSB repair by HRR in SeL-treated cells. In addition, it implies that cellular proteins, including those acting in HRR, are also targets of SeL that can damage them likely via the process of oxidation.

DISCUSSION

In this study, we identify genes whose products are associated with SeL toxicity in yeast. If impaired, mainly three cellular processes sensitize yeast cells to SeL: DNA damage response and repair, vacuole function, and GSH metabolism. On the other hand, there are factors that can increase resistance to toxic effects of SeL, when mutated. Almost solely these factors are involved in mitochondrial biology (data not shown), indicating that functional mitochondria potentiate the toxic effects of SeL. In line with this opinion are findings by Lewinska and Bartosz[44] showing that nonfermentable carbon sources glycerol and ethanol increase the toxic effects of SeL via an enhancement of mitochondrial ROS production.

With regard to DNA damage response and repair, HRR and PRR are the two principal pathways dealing with SeL-induced DNA damage. Involvement of HRR suggests that DSBs account for a remarkable part of DNA damage induced by SeL. As already shown,[16] this opinion holds true even though DSBs do not largely arise as primary DNA lesions upon SeL treatment. Rather, SeL-induced DSBs are the result of DNA replication fork collapse. Hence, it is not surprising that the mutants defective in NHEJ, a second main DSB repair pathway,[45] are not sensitive to this compound even in the absence of HRR.[17] In addition to HRR, collapsed DNA replication can be re-established by PRR. Indeed, we observed an involvement of this pathway in the removal of toxic DNA lesions upon SeL exposure but have no idea what type of SeL-



Strain	% remaining DSB			
	Repair time (hours)			
	0	2	4	24
wild type	100	85.4	92.9	95.7
yku70	100	>100	>100	>100
rad52	100	>100	>100	>100
yku70 rad52	100	>100	>100	>100

Strain	% remaining DSB			
	Repair time (hours)			
	0	2	4	24
wild type	100	96.2	93.6	92.3
yku70	100	80.3	85.8	88.7
rad52	100	>100	>100	>100
yku70 rad52	100	>100	>100	>100

Figure 4. DSB repair in the wild-type and the isogenic *yku70*, *rad52*, and *yku70 rad52* mutant cells treated with 5 (left) and 10 (right) mM SeL. DSB levels were calculated according to previously published formula.[17] Error bars (standard deviations from at least three independent experiments) are indicated. Tables summarize the data plotted in the graphs.

induced DNA damage requires bypass by TLS and which polymerase is thus being involved. Addressing these questions is an important future area of study, even though according to previous work,[21,22] the corresponding TLS polymerase is likely to be DNA polymerase ζ (Rev3-Rev7).

In contrast to the *gsh1* mutant, *gsh2* cells were not identified as SeL sensitive after retesting the potentially SeL sensitive mutants from the primary screen (compare Table 1 in the Supporting Information and Table 1). The *gsh2* mutant lacks a GSH synthetase that catalyzes the ligation of γ-glutamylcysteine with glycine,[46] the second reaction in the consecutive two-step GSH biosynthesis.[35] Like *gsh1*, the *gsh2* mutant is expected to be completely devoid of intracellular GSH. We propose that in cells lacking Gsh2, γ-glutamylcysteine accumulated due to a regular action of γ-glutamylcysteine synthetase encoded by *GSH1*, represents the major thiol that acts as an antioxidant, and hence protects yeast cells from toxic effects of SeL. The level of protection provided by γ-glutamylcysteine in *gsh2* cells is comparable to that provided by GSH in the wild-type cells.

Another mutant that displayed SeL sensitivity in deletion mutant library screen, but not in individual spot test, is the *grx1* mutant (compare Table 1 in the Supporting Information and Table 1). The *GRX1* gene encodes one of the two components of the yeast glutaredoxin system containing two cysteine residues at their active sites. Its product confers resistance toward oxidative stress caused by superoxide anions ($O_2^{\bullet-}$), in contrast to the second component of this system, Grx2, which protects yeast cells primarily from effects of hydroxyl radicals ($^{\bullet}OH$).[47] Notably, the *grx1 grx2* double mutant was previously shown to have SeL sensitive phenotype, suggesting an overlap between Grx1 and Grx2 in protection against toxic effects of this Se compound in yeast.[47] Nevertheless, as we did not find *grx2* cells SeL sensitive at least in the deletion mutant library screen (Table 1 in the Supporting Information), as observed for *grx1* cells, we are prompt to suggest that SeL executes oxidative stress mostly through $O_2^{\bullet-}$ generation, although some contribution of $^{\bullet}OH$ cannot still be ruled out, as already proposed by others.[8] In agreement, in vitro transformation of SeL into H_2Se followed by oxidation of H_2Se by oxygen has been shown to generate $O_2^{\bullet-}$.[6,48]

The toxic effects of Se compounds may become strongly beneficial if they are selectively targeted.[17] A highly important candidate for selective targeting appears to be DNA damage response and repair deficiency in cancer cells. On the basis of our yeast haploid deletion mutant library screen, we propose that HRR and PRR deficient tumors could represent specific targets for Se compounds. An existence of such tumors has already been reported. In the case of PRR deficiency, a significant reduction of *REV3* gene expression has been found in colon carcinomas.[49] HRR deficiency is much more prevalent than PRR deficiency due to causative mutations in more key genes such as *BRCA1*, *BRCA2*, *RAD51B*, *RAD51C*, *RAD51D*, *RAD54B*, etc.[50] Furthermore, HRR deficiency may also result from other than genetic changes, including hypoxia, an insufficient oxygen supply to tumor cells. Hypoxic cells have specifically down-regulated two key HRR genes, *BRCA1* and *RAD51*, and accordingly, they display reduced capacity to carry out HRR.[51] HRR can also be specifically down-regulated via RAD51 factor in cell carcinomas by some phytochemicals such as berberine.[52] We propose that, whatever is the basis, HRR deficiency/impairment could be targeted by Se, which could hence be a useful tool in clinical strategies aimed at selective combating of cancer cells.

Because of its presence in the active sites of proteins in the form of SeC, Se is an essential trace element in higher organisms. In humans, 25 SeC-containing proteins called selenoproteins have been identified. Importantly, there are also organisms that have lost a pathway of incorporation of SeC into cellular proteins during evolution. These include the yeast *S. cerevisiae*. In this context, *S. cerevisiae* would appear to be a suitable tool for addressing fundamental questions as to Se toxicity, because no incorporation of Se into proteins permits separation of effects of Se metabolism from Se function within the active sites of selenoproteins. A caveat against using this

model system could lie in the interpretation of Se toxicity data obtained with yeast to humans or other selenoprotein-containing organisms. However, the only Se compound that is metabolized via SeC is SeM. Therefore, only SeM toxicity data could potentially be influenced by disrupting the arm of Se metabolism that involves SeC, because one can expect that in the absence of SeC incorporation pathway, more SeC is converted into H_2Se, a highly toxic Se metabolite. Hence, SeM or SeC toxicity could be overestimated in yeast. In contrast, this should not be the case of SeL, a compound relevant to present study.

We found that amino acid biosynthesis belongs to processes that are highly up-regulated upon SeL treatment (Table 3 in the Supporting Information). This indicates that de novo protein synthesis is rapidly triggered upon this treatment. The requirement for de novo protein synthesis may reflect the fact that SeL can considerably damage cellular proteins. In line with this, Izquierdo and co-workers[53] showed that protein carbonylation, an indicator of oxidative stress on the protein level,[54] occurs in SeL-exposed yeast. Consequently, we used cycloheximide, a drug inhibiting protein synthesis, to follow the repair of SeL-induced DSBs under conditions where de novo protein synthesis could not occur. We did not observe any repair of SeL-induced DSBs even in HRR-proficient background, indicating that damaged cellular proteins need to be replaced to ensure efficient repair of SeL-induced DSBs in yeast cells. Hence, we imply that HRR factors are also damaged by SeL exposure and that their turnover is required for efficient repair of SeL-induced DSBs.

■ ASSOCIATED CONTENT

Supporting Information

SeL sensitive mutant strains revealed by the haploid deletion mutant library screen (Table 1); gene expression profile after 0.1, 1, and 10 mM SeL exposures (Table 2); process classification of SeL-affected genes (Table 3); effect of SeL on yeast growth (Figure 1); effects of combining *RAD5*, *RAD6*, and *RAD52* gene inactivations on UV and MMS sensitivity (Figure 2); and effects of combining *SRS2*, *RAD6*, and *RAD52* gene inactivations on UV and MMS sensitivity (Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

Se, selenium; SeL, sodium selenite; GSH, glutathione; PRR, postreplication repair; HRR, homologous recombination repair; H_2Se, hydrogen selenide; GSSeSG, seleno-diglutathione; ROS, reactive oxygen species; SSBs, single-strand breaks; DSBs, double-strand breaks; ORFs, open reading frames; UV, ultraviolet light; MMS, methyl methanesulfonate; PCNA, proliferating cell nuclear antigen; TLS, translesion DNA synthesis; GSSG, glutathione disulfide; NHEJ, nonhomologous end-joining; O_2^-, superoxide anion; $^{\bullet}OH$, hydroxyl radical

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