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Identification of Genes Required for Protection from Doxorubicin by a Genome-Wide Screen in *Saccharomyces cerevisiae*

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Abstract

Anthracyclines are chemotherapeutic agents commonly used to treat a broad range of malignancies. Although effective, these drugs present serious complications, most notably cardiotoxicity. To determine the mechanisms that mediate cytoprotection from doxorubicin, we have screened the collection of *Saccharomyces cerevisiae* haploid gene deletion mutants. We have identified 71 deletion strains that display varying degrees of hypersensitivity to doxorubicin at a concentration that does not significantly reduce the viability of wild-type cells. Complementation of the doxorubicin-sensitive phenotype of the deletion strains with the wild-type genes proves that the sensitivity of the strain to doxorubicin is due to the gene deletion. The genes that mediate cytoprotection from doxorubicin belong to multiple pathways including DNA repair, RNA metabolism, chromatin remodeling, amino acid metabolism, and heat shock response. In addition, proteins with mitochondrial, osmosensing, vacuolar, and ribosomal functions are also required for protection from doxorubicin. We tested the sensitivity of the deletion strains to other cytotoxic agents, which resulted in different drug-specific sensitive groups. Most of the identified genes have mammalian homologues that participate in conserved pathways. Our data may prove useful to develop strategies aimed at sensitizing tumor cells to doxorubicin as well as protecting cardiac cells from its cytotoxic effects.

Introduction

The anthracycline antibiotics (1) are among the most effective anticancer drugs available with activity against both hematologic and solid tumors (2), and in some cases, such as in breast cancer, they constitute the primary therapeutic alternative (3). The basis for the antineoplastic effectiveness of anthracyclines (e.g., doxorubicin) is not completely understood, but it is believed that they exert their cytotoxic action by multiple mechanisms including DNA damage through intercalation or direct alkylation of DNA (4). Doxorubicin bound to DNA block s DNA unwinding and helicase activities, leading to inhibition of replication and transcription. It has also been shown that anthracyclines inhibit topoisomerase II, triggering DNA repair and inducing apoptosis (4). Other mechanisms have been proposed to mediate anthracycline activity, including the generation of free radicals, which results in DNA damage and lipid peroxidation (4). However effective, anthracyclines

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can produce serious side effects. These side effects are the result of cumulative exposure to the drug in a dose-dependent manner, which have limited its clinical use. Most notable is cardiotoxicity, which commonly occurs within a year of the completion of anthracycline regimens and may develop into dilative cardiomyopathy and congestive heart failure, which is refractive to therapeutic treatment (5). Extensive studies suggest that the toxicity of doxorubicin involves generation of reactive oxygen species that damage the mitochondria, ultimately leading to apoptosis. In fact, doxorubicin confers susceptibility to free radical formation; in addition, its administration also causes a decrease in intracellular antioxidants normally responsible for preventing free radical damage (4), further enhancing its toxic effects. Therapies aimed at preventing cardiotoxicity by minimizing the generation of reactive oxygen species have been developed and are based on reducing the levels of intracellular iron by use of chelating agents such as dexrazoxane (6). However, they do not completely prevent side effects. In addition to oxidative damage, other potential mechanisms have been suggested to mediate toxicity. These include the activation of signal transduction pathways, which results in altered cardiac gene expression, and the inhibition of various pumps in the cell by the metabolite doxorubicinol, which affects myocardial energy metabolism and ionic currents (7). An additional clinical problem relates to primary and secondary resistance. Among the most relevant are the overexpression of P-glycoproteins and mutations in topoisomerase II (8–10). The ability to overcome this resistance remains a highly sought goal. Anthracyclines are commonly used in combination chemotherapy with cisplatin, cyclophosphamide, platinating agents, Taxol, etc., constituting therapeutic alternatives for the treatment of refractory tumors (11–14). The rationale for combination regimens is based on the distinct antineoplastic mechanisms of action of each individual drug rather than the ability of a certain agent to enhance the antineoplastic properties of any particular drug. To develop strategies that enhance the antineoplastic potential of drugs, we need to understand the underlying mechanisms involved in the sensitivity to these agents.

The budding yeast, *Saccharomyces cerevisiae*, has frequently been used to dissect biological pathways that are conserved in eukaryotes. Studies in yeast have shown that pathways that modulate drug sensitivity (15) are also conserved in humans (16). Recently, the availability of the systematic gene deletion library generated by the *Saccharomyces* Gene Deletion Project (17) has allowed these studies to be carried out at a genome-wide level. This approach has successfully been used to identify genes and pathways required for survival to various cellular stresses such as ionizing radiation and cisplatin (18, 19). In this study, we have characterized the genes and/or pathways that contribute to cytoprotection from doxorubicin. We have screened a gene deletion library for strains that present enhanced sensitivity to doxorubicin. We have identified 71 genes that, when deleted, confer varying degrees of increased sensitivity to doxorubicin.

Materials and Methods

General genetic methods and strains

Yeast extract/peptone/dextrose and synthetic drop-out media were as described (20, 21). Homozygous haploid deletion strain library (parental strain BY4741: MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was obtained from Open Biosystems.

Chemicals

Yeast nitrogen base, yeast extract, peptone, and dextrose were purchased from Fisher Scientific (Fair Lawn, NJ). Doxorubicin-HCl (2 mg/mL), daunorubicin-HCl (5 mg/mL), and cisplatin (1 mg/mL) were obtained from Bedford Laboratories. Camptothecin and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) were obtained from Sigma-Aldrich. Hydroxyurea was obtained from Calbiochem. Stock solutions were prepared as follows: camptothecin (10

mmol/L) and MNNG (3 mmol/L) were prepared in DMSO; hydroxyurea was prepared in sterile water. Drugs were aliquoted and stored at −20°C. Doxorubicin- and daunorubicin-containing plates were made in YPD (1% yeast extract, 2% peptone, 2% dextrose, 2% agar), synthetic complete (SC; 0.67% yeast nitrogen base without amino acid, 0.087% amino acid mixture, 2% dextrose, 2% agar), or the corresponding drop-out media, as indicated. All plates were stored in the dark and used within 24 h.

Screen for doxorubicin-sensitive mutants

The concentration of doxorubicin for the screen was determined experimentally using the parental strain BY4741 and the *rad52* mutant, a hypersensitive strain. A concentration of doxorubicin of 20 μmol/L was determined to be optimal for the screen (Fig. 1). At this concentration, wild-type cells displayed >90% survival versus <0.1% survival for *rad52* mutant. For the screen, the yeast knockout library was grown to saturation (5 days at room temperature) in 96-well plates containing liquid YPD and 200 μg/mL G418 for selective growth and was then replica plated to solid media containing SC and SC plus doxorubicin (20 μmol/L). Cell growth was monitored daily and sensitivity was scored after 3 days at 30°C. The screen was repeated to cover the 4,700 single deletion mutants at least twice.

Confirmation of the doxorubicin-sensitive strains

Strains that showed consistent sensitivity to doxorubicin in each screen were selected. The identity of the strains was determined from their position in the 96-well plate and the strains were then isolated from the original library and grown to saturation at 30°C in liquid YPD medium. Cultures were then diluted to 1×10^7 cells/mL, washed twice, and resuspended in sterile water. Serial dilutions (1×10^{-1} -1×10^{-5}) were spotted onto plates containing SC or SC plus doxorubicin (20 μmol/L) and grown at 30°C for 3 days. Using the wild-type parental strain as a control, the sensitivity was estimated relative to untreated controls and strains were classified as hypersensitive (100–1,000-fold), sensitive (10–100-fold), or slightly sensitive (2–10-fold).

Complementation analysis

For complementation of the *ydj1* strain, the *YDJ1* gene was amplified by PCR using genomic DNA from wild-type strain BY4741 as the template and cloned into vector pYX243 (Ingenious, 2μori, LEU2, and gal promoter). Correct clones were confirmed by sequencing. Complementation of the *rad52* strain was carried out by transformation with a vector (pYEP13, LEU2) containing the *RAD52* coding sequence (gift from Dr. K.J. Myung, NIH, Bethesda, MD). For all other strains, expression plasmids were obtained from yeast open reading frame (ORF) collection (Openbiosystems) and used as suggested by the manufacturer. Expression plasmids and vector controls were transformed into the mutant strains by the lithium acetate method and plated on selective media. To test for complementation of the doxorubicin sensitivity phenotype, transformants were grown to saturation in selective medium and serial dilutions were spotted in duplicate on selective media plates containing galactose (2%) plus or minus doxorubicin (20 μmol/L) and incubated at 30°C. Growth was monitored daily and scored for complementation after 3 days.

Sensitivity to other cytotoxic agents

The concentration of each drug to obtain >90% survival of the wild-type strain BY4741 was determined empirically. These concentrations were used to compare the relative sensitivity of the strains isolated from the screen. The final concentrations of the drugs are daunorubicin, 10 μmol/L; cisplatin, 80 μmol/L; MNNG, 3 μmol/L; camptothecin, 10 μmol/L; and hydroxyurea, 80 mmol/L. Diluted cultures were spotted on plates with or without

drugs as described above. Sensitivity to ionizing radiation was carried by exposing the cells to 50 Gy of γ-rays followed by plating on YPD. Cell sensitivity fold for each treatment is expressed relative to untreated cells (1-fold).

Results

Screen for deletion strains that display increased sensitivity to doxorubicin

To identify genes that protect cells from doxorubicin cytotoxicity, we performed two screens on the set of ~ 4,700 *S. cerevisiae* haploid gene deletion library. The concentration of doxorubicin that was used in the screen was determined empirically using the wild-type strain (BY4741, parental strain for the gene deletion library) and *rad52*, a previously characterized doxorubicin-sensitive strain (15) also derived from the library. A mismatch repair–deficient strain (*msh2*) was used as a doxorubicin-resistant control (15, 16). As shown in Fig. 1, increasing concentrations of drug resulted in reduced viability for every strain, with the *msh2* strain showing higher survival relative to the wild-type at the highest concentration (100 μmol/L) of doxorubicin used. The concentration of 20 μmol/L of doxorubicin was selected for the screen because, at this concentration, wild-type cells retained >80% viability and growth of *rad52* cells was severely affected, displaying <1% survival (Fig. 1*B*).

Taking advantage of the 96-well format of the library, we used a single-step selection method to identify strains that displayed increased sensitivity to doxorubicin. Strains were replica plated onto media containing doxorubicin as well as media lacking the drug to account for the different rates of growth of the different strains. Two independent screenings of the complete collection were carried out, and a total of 67 and 77 genes were recovered from each screen. Identity of the strains was determined from their position in the 96-well array format. All of the mutants from both screens were picked from the original library and retested for semiquantitative determination of their sensitivity to doxorubicin by spotting 10-fold serial dilutions of stationary cultures onto plates with or without 20 μmol/L doxorubicin (Fig. 2). The relative survival of each mutant compared with that of the wild-type strain, normalized to the growth rate of untreated mutant cells, was determined. We found that 71 gene deletions, which constitute ~1.5% of the genome, had varying levels of higher sensitivity to doxorubicin ranging from 2-fold to >1,000-fold over wild-type cells (Fig. 2; Table 1) when retested. Of these, 22 deletion mutants (31% of the identified genes) display hypersensitivity to doxorubicin (>100-fold higher than wild-type), 16 mutants (23%) display intermediate sensitivity (10–100–fold), and 33 (46%) were slightly more sensitive than wild-type cells (2–10-fold). All of the mutants identified in the screen grew well in nonselective media, indicating that the growth defect in doxorubicin-containing plates is a result of the toxicity of the drug (Fig. 2).

Novel genes identified to be involved in cytoprotection from doxorubicin

The cellular functions of the identified ORFs were obtained from the Saccharomyces Genomics Database.[4] Pathway analysis (supplementary Table S1 and Fig. S1) indicates that a significant number of genes belong to distinct pathways and correlate with the extent of sensitivity displayed by the mutant strains. A search for homologues at the homologene database[5] revealed that 43 of the 71 ORFs identified encode proteins that share sequence and functional similarity with mammalian counter-parts (Table 1). Among the most sensitive strains, the largest group of genes identified is involved in DNA/RNA metabolism (Table 1). Deletion of seven genes involved in homologous recombination exhibited

[4] http://www.yeastgenome.org
[5] http://www.ncbi.nlm.nih.gov/sites/entrez

medium to high levels of sensitivity to doxorubicin. Strains deleted for the genes involved in homologous recombination, *RAD50, RAD51, RAD52, RAD57, MRE11*, and *XRS2*, displayed high sensitivity to doxorubicin (>100-fold) whereas *RAD55* was also sensitive although not as sensitive as the other members of the pathway (~100-fold). Arecent report describes the role of the SUMO ligase SIZ1 in the resistance to doxorubicin (22). We tested if the inactivation of *SIZ1* in the homologous recombination mutants affects their sensitivity to doxorubicin. Only the *rad50* strain was significantly rescued (>1,000-fold relative to the single homologous recombination mutant) by the *siz1* mutation, whereas *rad52, rad55*, and *rad57* were only modestly rescued (5–10-fold) and *mre11* and *xrs2* were not affected (Supplementary Fig. S2). This is consistent with previous observations that the resistance to doxorubicin of *siz1* mutants does not seem to involve an effect on DNA repair (22).

Homologous recombination plays an important role in overcoming stalled replication forks caused by DNA-damaging agents (23). In this pathway, SRS2 acts as an antirecombination factor (23, 24). The inactivation of *SRS2* removes the inhibition to homologous recombination and allows us to determine if the mutants we have identified are required for bypassing doxorubicin-mediated lesions. Inactivation of *SRS2* resulted in a significant rescue of *mre11, rad50*, and *xrs2* mutants (>1,000-fold relative to the single homologous recombination mutant), suggesting that the MRX complex is dispensable for the processing of stalled replication forks. Conversely, *rad52, rad55*, and *rad57* mutations are only modestly (4–10-fold) rescued by the *srs2* mutation, indicating that they play a more important role in lesion bypass (Supplementary Fig. S3).

Several of the genes identified are involved in transcription regulation, with the largest group involved in chromatin remodeling. *SWI3, SWI6*, and *SNF2* are subunits of the SWI/SNF chromatin remodeling complex (25), whereas *HTL1* is a subunit of the RSC chromatin remodeling complex (26) and *ARP8* encodes a nuclear actin-related protein involved in INO80 ATP-dependent chromatin remodeling (27). Additional genes involved in global transcription control that were identified include *HFI1*, which encodes an adaptor protein required for structural integrity of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) complex (28); *UAF30*, a subunit of upstream activation factor (UAF; ref. 29); and nucleosome assembly factor *ASF1* (30). A slightly sensitive phenotype was observed for mutants of *HTZ1*, which encodes the H2 histone variant H2AZ involved in transcriptional regulation by protecting euchromatin from the ectopic spread of silent heterochromatin (31), and for *RTT106* and *RTT109*, which control Ty1 transposition and have roles in genome maintenance. *RTT109* is a histone acetyltransferase required for survival after DNA damage during S phase (32).

In our screen, we isolated several genes with protein chaperone functions that increased the sensitivity to doxorubicin when deleted. The *ydj1* and *zuo1* strains display a hypersensitive phenotype close to 1,000-fold higher than the wild-type strain. Both genes encode proteins that belong to the DnaJ family (33). We also identified the Hsp70 gene *SSZ1*, which displayed high sensitivity (~ 100-fold higher than wild-type) when deleted. Deletion of *NEW1* also results in hypersensitivity to doxorubicin. NEW1 is involved in protein aggregation, although it is not a chaperone. It contains a Gln/Asn–rich domain that may function as a prion-like element (34).

Another major group of genes identified comprises pathways involved in amino acid biosynthesis. Interruption of the synthesis of four amino acids results in increased toxicity of doxorubicin. These include *HOM6, THR1*, and *THR4*, which are required for the synthesis of threonine and methionine; *SER1* and *SER2*, which are involved in serine biosynthesis; and *TRP1*, which is necessary for the synthesis of tryptophan. The sensitivity displayed by these strains is not the result of the absence of the amino acids because doxorubicin is still

toxic in SC or YPD plates that contain all necessary amino acids. However, it is most likely that the sensitivity is produced by the accumulation of toxic intermediates that enhance the effects of doxorubicin. Mitochondrial integrity is also necessary for cytoprotection from doxorubicin. Mutants in genes *ILM1, MSH1*, and *PTC1*, which are required for mitochondrial genomic stability, display moderate sensitivity (35–37). Other mitochondrial genes identified in the screen are *MRPL37* and *MRPL6*, which encode mitochondrial ribosomal proteins; *ADK1*, an adenylate kinase; *FLX1*, a FAD transporter; and *AFG3*, a component of the m-AAA protease. We have tested the ability of the doxorubicin-sensitive mutants to grow in media containing a nonfermentable carbon source (i.e., glycerol). Among the mitochondrial genes, we find that only the *cox6* and *msh1* strains are petites failing to grow on YPG; *mrpl6, mrpl37*, and *afg3* display slow growth (Supplementary Table S2); and *ilm1* and *flx1* grow normally.

A highly sensitive group of genes identified is involved in vacuolar functions and protein sorting into the endosomes. *TFP3, VPH2*, and *VMA21* were at least 100-fold more sensitive, whereas *VPS28* and *SNF8* present more moderate sensitivity.

The toxicity of doxorubicin has been ascribed, in part, to its ability to generate reactive oxygen species. We identified mutants of genes required for oxidative stress as sensitive to doxorubicin. *SOD1* encodes the superoxide dismutase and *CCS1* encodes the copper chaperone of SOD1. Direct damage to membranes and associated structures by doxorubicin has previously been described (4). We identified several strains involved in cell wall integrity and maintenance, such as *GAS1* (glucanosyltransferase), which is required for cell wall assembly; *TCO89*, a subunit of TORC1, which cooperates with SSD1 for maintenance of the cell wall integrity; and *SAC7* and *BEM4*, which are involved in actin cytoskeleton organization and biosynthesis. Defects in ribosomal genes *RPL37A, RPL13B, RPS9B, RPP1A*, and *DBP3*, which encode ribosomal subunits, show a modest increase in the sensitivity to doxorubicin.

In addition, several ORFs that encode proteins of unknown function have been identified, including some with high sensitivity to doxorubicin such as *YOR199W* (>100-fold), *YHR151C*, and *YKL098W* (~100-fold), which need to be further characterized.

Reversion of the hypersensitive phenotype by complementation of the deletion strains with the wild-type gene

Multiple pathways were identified in the screen that, when compromised, lead to hypersensitivity to doxorubicin. To confirm that the sensitivity was due to the specific gene deletion and not a consequence of secondary mutations during the screening process, we proceeded to revert the phenotype by complementation of the strains with their specific genes. We selected strains with mutations in the pathways that were most commonly represented and also presented the highest sensitivity to the drug. These include homologous recombination/DNA repair (*RAD52*), transcription regulation (*HFI1*), amino acid biosynthesis (*HOM6*), oxidative stress (*SOD1*), and chromatin remodeling (*SWI3*). As shown in Fig. 3, all the strains tested could be efficiently complemented by plasmids containing wild-type copies of the genes. The plasmid used to complement the *rad52* strain contains a genomic fragment carrying the RAD52 gene under its native promoter and efficiently complemented the growth defect of *rad52* strain in doxorubicin-containing plates, whereas the empty vector could not. The HFI1, HOM6, SWI3, and YDJ1 plasmids are galactose-inducible overexpressors and could complement the doxorubicin sensitivity of their respective strains. In addition, the HFI1 plasmid also restored the growth defect of the *hfi1* strain in nonselective medium (Fig. 3). The results from these experiments confirm that the growth defect in doxorubicin-containing plates was due to the specific gene defect of the strains.

Analysis of the spectrum of sensitivity of the doxorubicin-sensitive deletion strains to other cytotoxic treatments

To determine if the doxorubicin-sensitive strains isolated in the screen displayed a general sensitive phenotype, we tested their response to different cytotoxic agents, some of which are commonly used chemotherapeutic drugs. We selected a broad range of stress conditions including heat sensitivity, ability to grow in hydroxyurea, and sensitivity to ionizing radiation and DNA-damaging agents such as cross-linkers (cisplatin), alkylating agents (MNNG), topoisomerase I inhibitors (camptothecin), and another commonly used anthracycline (daunorubicin). We tested all 71 ORFs identified. Serial dilutions of stationary cell cultures were spotted on SDM plates containing different drugs at concentrations in which the wild-type cells retain ~90% survival. The group of strains that displays hypersensitivity to doxorubicin is presented in Table 2. Interestingly, although this group of genes displays >100-fold sensitivity to doxorubicin, some of them, including *RAD57, RAD51, RAD55, VPH2, ASC1*, and *MAC1*, are only modestly sensitive to daunorubicin. Conversely, *ASF1*, which is moderately sensitive to doxorubicin (~100-fold), is hypersensitive to daunorubicin (~10,000-fold). Although highly analogous (doxorubicin differs from daunorubicin by a single hydroxyl group addition in the methyl group, at carbon 14 of doxorubicin; ref. 4), they display different therapeutic spectra. Strains defective in genes involved in homologous recombination, such as *RAD50, RAD51, RAD52, RAD55, RAD57, XRS2*, and *MRE11*, are also highly sensitive to other DNA-damaging agents like ionizing radiation, MNNG, cisplatin, and camptothecin and cannot grow in the presence of hydroxyurea. They, however, are not sensitive to heat. Another group of genes is sensitive only to anthracyclines. These include *NEW1, ASC1*, and *MAC1*. Most of the genes have moderate sensitivity to other agents including those involved in amino acid biosynthesis (*HOM6* and *TRP1*) and the heat shock response (*YDJ1, SSZ1*, and *ZUO1*). Only two strains were heat sensitive (*YDJ1* and *VPH2*). Interestingly, *ydj1* and *zuo1* also display hypersensitivity to cisplatin. Among the group of strains that are less sensitive to doxorubicin, only *asf1* and *rtt109* showed significant sensitivity to other DNA-damaging agents (supplementary Table S2). The results presented here indicate that whereas some genes (recombination/DNA repair) are sensitive to most DNA-damaging agents, other genes confer specific protection from anthracyclines and, furthermore, they display differential sensitivity between doxorubicin and daunorubicin.

Analysis of gene networks

The 71 hits were analyzed using Osprey 1.2.0 (38) for all physical and genetic interactions between the hits using the Yeast BioGRID version 2.0.29 release of 1 June 2007. This analysis revealed that 24 genes were not interrelated, 5 of which are ORFs with no known functions; 4 genes (*TFP2, VMA21, VPH2*, and *SER2*) had one interaction each (TFP2 and VMA21 physically interacted and VPH2 and SER2 interacted genetically); and *VPS28, VPS36*, and *SNF8* physically interacted with each other. The remaining 40 genes were heavily networked and contained several genes involved in DNAre pair (*RAD52, RAD51, RAD55, RAD57*, subunits of the MRX complex), chromatin remodeling (*HTL1, HTZ1, HFI1, ASF1, SNF2, SWI3*, and *SWI6*), amino acid metabolism, protein synthesis, protein folding, and signal transduction (Supplementary Fig. S1). The identification of such a network of 40 genes strongly suggests the potential molecular pathways that confer doxorubicin resistance in cells.

Discussion

Use of anthracyclines as antineoplastic agents has spanned more than 30 years. Although they remain highly effective, their associated side effects, most notably cardiotoxicity, have limited their clinical use. Liposomal preparations that are mostly directed to the tumor have

reduced this problem. In addition, the development of resistance by tumors renders the use of anthracyclines ineffective. To better understand the mechanisms that participate in the cyto-protection from anthracyclines, we have done a screen for mutations that sensitize cells to doxorubicin. We have identified 71 genes, which, when deleted, result in varying degrees of sensitivity, some as high as >1,000-fold more sensitive compared with wild-type cells. We expect that some of these genes may have potential clinical applications. Among the relevant genes are those that are required for DNA repair/recombination because they seem to sensitize cells to a broad spectrum of DNA-damaging agents. This result is consistent with the mechanism of action of doxorubicin, which results in the generation of double-strand breaks (39, 40) that require homologous recombination for repair (15). In addition, homologous recombination is required to bypass lesions that block DNA replication forks. In fact, most of the recombination mutants remain sensitive to doxorubicin even in the antirecombination defective *srs2* background. The exceptions are the *mre11, rad50*, and *xrs2* strains, which constitute the MRX complex, an exonuclease required to process DNA end s in double-strand breaks. This is consistent with a role of homologous recombination in the bypass of replication blocks that do not require DNA end processing, making the MRX complex dispensable in this process. Because SRS2 is recruited to replication forks by SUMO-modified proliferating cell nuclear antigen (24), we tested the effect of deleting the *SIZ1* gene, which encodes the SUMO ligase, on the homologous recombination mutants. *SIZ1* has recently been involved in the resistance to doxorubicin by a mechanism independent of activation of DNA repair (22). With the exception of *rad50*, only modest rescue was observed in the *siz1* homologous recombination double mutants, consistent with previous reports (22). The slight increase in survival may be due to reduced accumulation of doxorubicin by *SIZ1* inactivation (22) because exposure of the double mutants to higher concentrations of the drug (50 μmol/L) resulted in no rescue at all (data not shown).

Of particular interest are the genes involved in the heat shock response. There is already literature indicating that the heat shock response prevents cytotoxicity of doxorubicin; however, these have mostly focused on Hsp70 and Hsp27 (41, 42). We have identified *YDJ1*, a homologue of the DNAJA2 Hsp40, as a crucial factor for survival under doxorubicin stress. There are currently agents such as geldanamycin (43) that can activate the heat shock response. Interestingly, SSZ1 is also known as PDR13 because it has been previously shown, together with ZUO1, to activate pleiotropic drug resistance (PDR), and that this activity is independent of their chaperone function at the ribosome (44). *YDJ1* has not been shown to mediate PDR. These studies are currently being followed up in the mammalian system.

Several mutants display growth defect on a nonfermentable carbon source. However, only two mutants are petites, *msh1* and *cox6*, which completely fail to grow on YPG. There seems to be no correlation between the inability to use nonfermentable sugars and sensitivity to doxorubicin because some of the most sensitive strains grow normally on plates containing glycerol (e.g., *rad50, rad51, ydj1, hom6*, etc.).

Also of interest are genes that are required for global transcription control, such as those involved in chromatin remodeling, histone deacetylation, and chromatin silencing. Intriguing results are those that point to a potential role of some amino acid metabolites in the sensitivity to doxorubicin. Inactivation of the *HOM6* gene, which encodes the homoserine dehydrogenase, results in accumulation of L-aspartate-semialdehyde, a precursor of homoserine. L-Aspartate-semialdehyde has been shown to be toxic in certain genetic backgrounds (45). Interestingly, inhibition of the subsequent steps of the reaction also results in sensitivity to doxorubicin. Strains defective in *THR1*, which encodes the homoserine kinase, and *THR4*, which encodes the threonine synthase, also display sensitivity to doxorubicin, although not as high as *hom6* strains. It is possible that inhibition

of these steps downstream to *HOM6* may result in some accumulation of L-aspartate-semialdehyde as well. The mechanism of the toxicity of L-aspartate-semialdehyde is still not understood. Inactivation of *TRP1* in tryptophan biosynthesis blocks the third step of the pathway resulting in accumulation of *N*-(5′-phosphoribosyl)-anthranilate. Although some anthranilates have been reported to be carcinogenic (46), no report has described the toxicity of *N*-(5′-phosphoribosyl)-anthranilate. Inhibition of the *SER1* gene, which encodes the 3-phosphoserine aminotransferase, results in accumulation of 3-phospho-hydroxypyruvate. Strains defective in *SER2*, which encodes the phosphoserine phosphatase required for the third step of the serine biosynthesis pathway, result in the accumulation of 3-phospho-serine. The mechanism involved in the sensitivity of the *ser1* and *ser2* strains needs to be further investigated. If these metabolites can be produced synthetically, they should be tested in mammalian cultures.

It is our hope that some of the genes or pathways identified in our study may provide clues to address the issues of cardiotoxicity as well as drug resistance. It seems possible that by selecting a combination of genes to target, we can enhance the therapeutic efficacy of some of the drugs tested here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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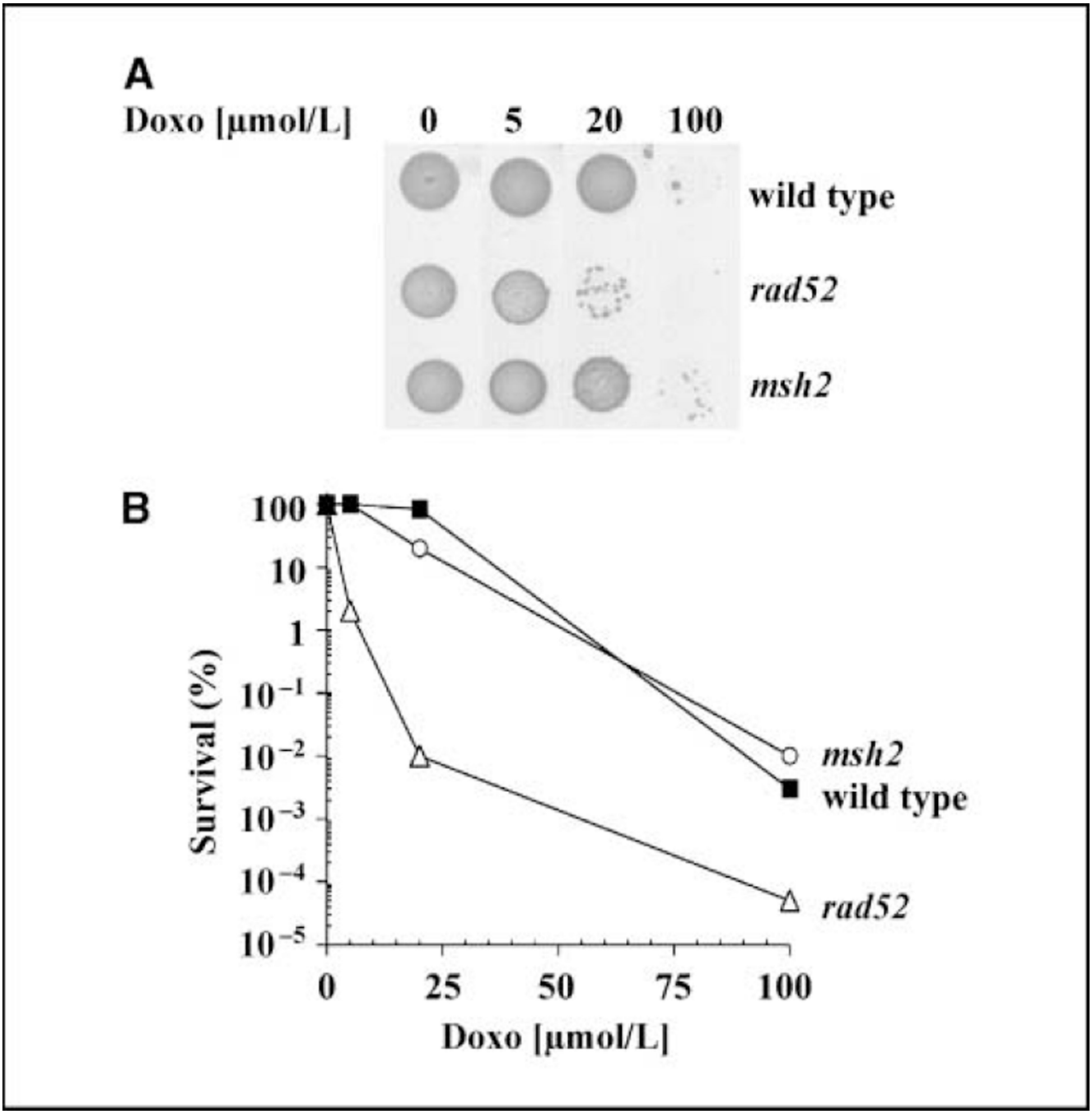

Figure 1.

Determination of the concentration of doxorubicin for the screen. *A*, wild-type parental strain (BY4741) and *rad52* and *msh2* mutants were tested for growth on media containing different concentrations of doxorubicin (*Doxo*) as indicated in Materials and Methods. Serial dilutions (1:10–1:10^5) of saturated stationary cultures (cell density, ~2 × 10^7) were spotted in the plates containing 0, 5, 20, and 100 μmol/L doxorubicin, respectively. Growth was scored after 3 d of incubation at 30°C. The 1:10 dilution of the different concentration plates is shown. *B*, quantification of the survival of the tested strains. Survival was determined by counting the number of colonies in the respective dilutions and calculated based on the growth in plates lacking doxorubicin.

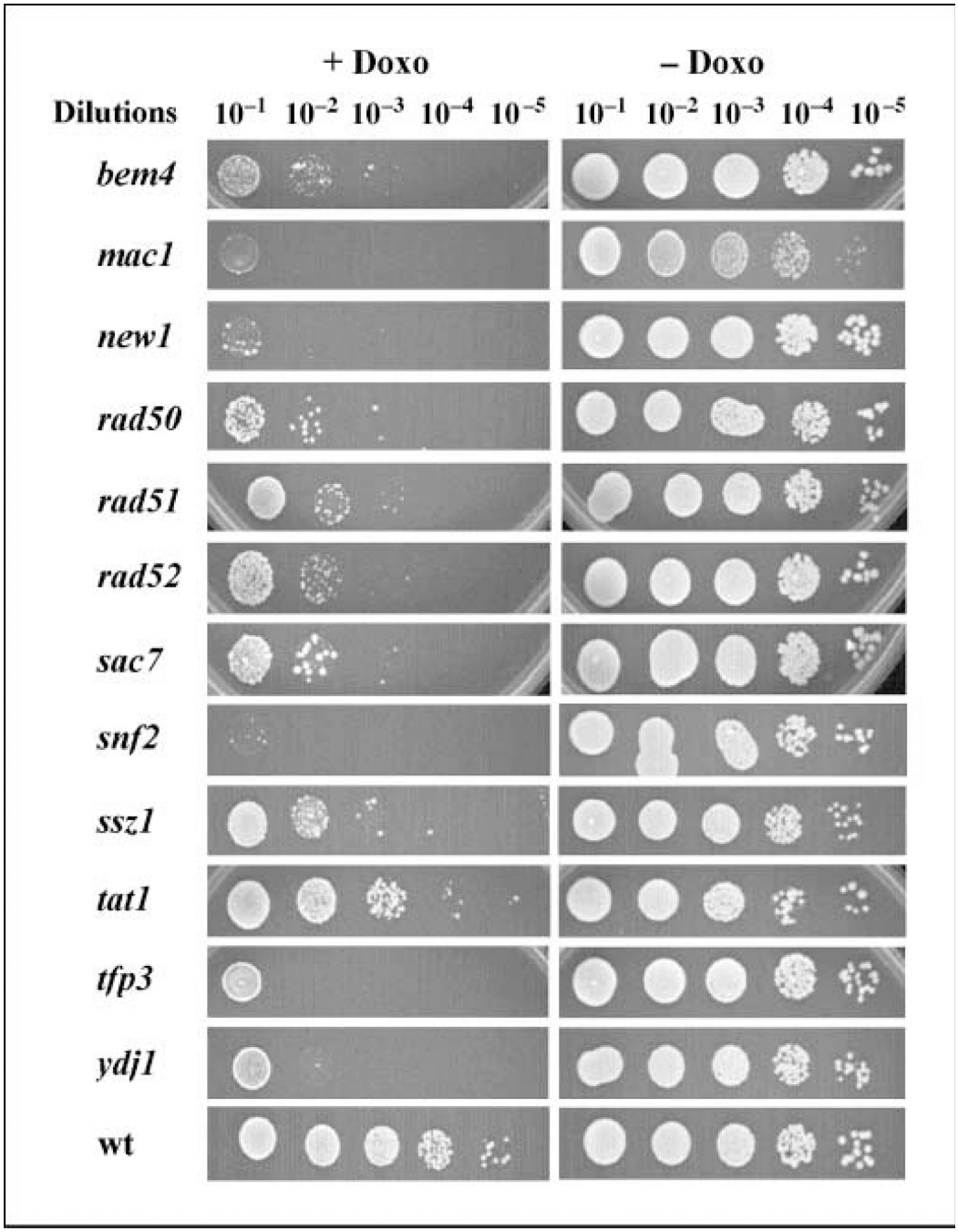

Figure 2.

Confirmation of the sensitivity of individual deletion mutant strains to doxorubicin. Candidate doxorubicin-sensitive strains identified in the screen were retested by plating serial dilutions of overnight cultures from the original library onto plates containing 20 μmol/L doxorubicin. To account for growth rate differences between the strains, growth in a control plate lacking doxorubicin was also tested. Sensitivity was determined by growth in doxorubicin-containing plates relative to the growth in plates with no drug. The wild-type control shows no significant growth defect (>83% survival) in doxorubicin at the concentration used, consistent with the data in Fig. 1. The sensitive strains displayed varying degrees of sensitivity in doxorubicin plates and were classified as slightly sensitive strains

(2–10-fold sensitivity; e.g., *tat1*), sensitive strains (10–100-fold sensitivity; e.g., *ssz1*), or hypersensitive strains (>100-fold sensitivity; e.g., *snf2, ydj1*, etc). The strains were retested twice, displaying consistent phenotype. Selected strains are shown.

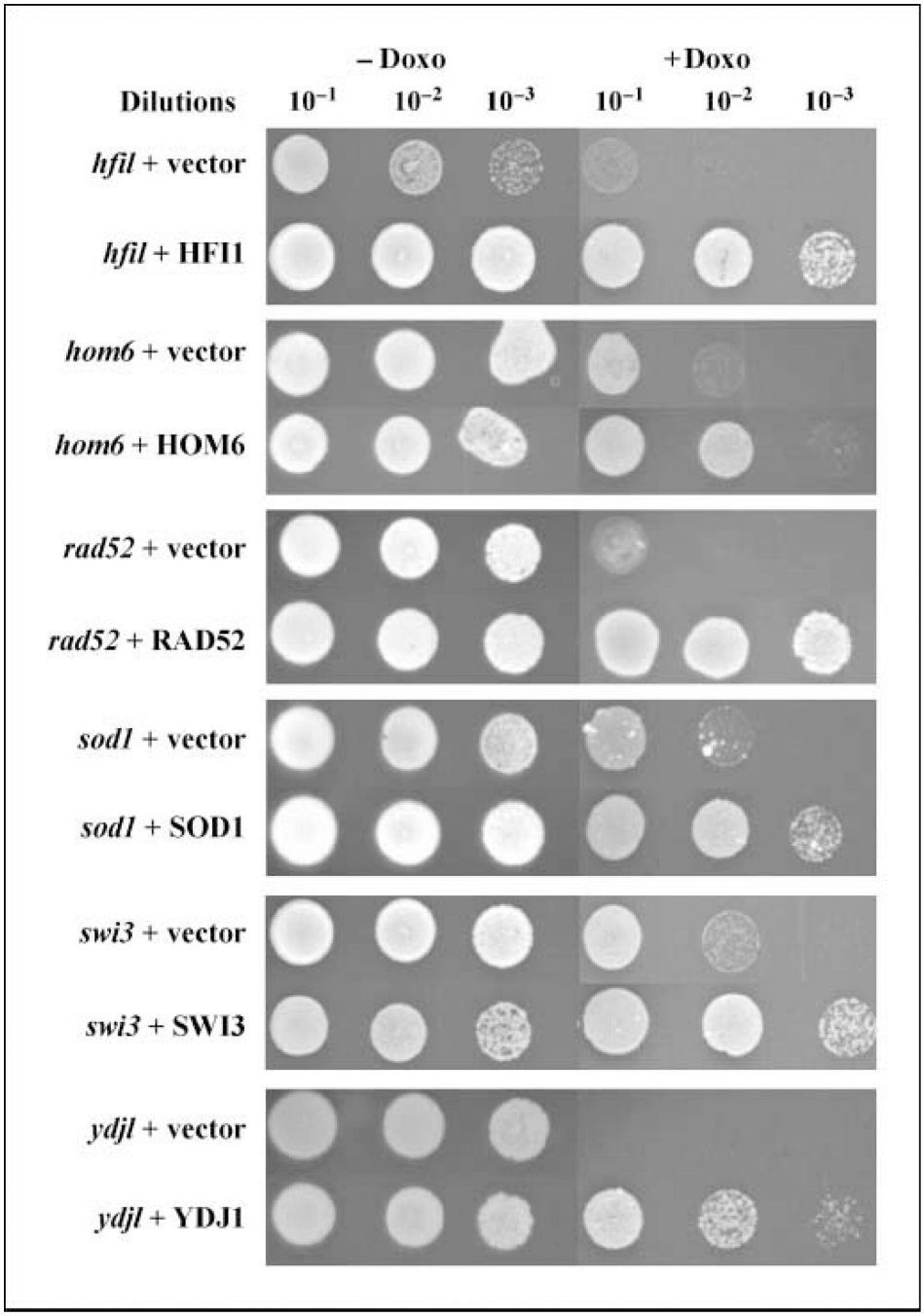

Figure 3.

Complementation of the doxorubicin sensitivity of selected deletion strains. Strains were transformed with expression plasmids containing their specific deleted genes or the empty vector and tested for complementation. Serial dilutions of stationary cultures were spotted in selective media and selective media plus doxorubicin. Growth was determined after 3 d of incubation at 30°C.

Table 1

Genes identified in the doxorubicin sensitivity screen

Sensitivity*	Gene/ORF	Function†	Mammalian orthologues‡
SSS	*RAD50, RAD51, RAD52*	Recombination, DNA repair	RAD50, RAD51, RAD52
	RAD57, MRE11, XRS2	Recombination, DNA repair	RAD51L1, MRE11, NBS1
	SNF2, SWI3, HFI1	Chromatin remodeling, transcription	SNF/SWI complex, TADA1L
	TFP3, VPH2, VMA21	Vacuolar function	ATP6V0C, —, —
	HOM6, TRP1	Amino acid biosynthesis	—
	YDJ1, ZUO1, NEW1	Protein folding, prion formation	DNAJA2, ZRF1, —
	SAC7	Cytoskeleton	—
	MRPL6	Mitochondrial ribosome	—
	GND1	6-Phosphogluconate dehydrogenase	PGD
	ASC1	Translation regulation	RACK-1
	MAC1	Copper-sensing transcription factor	—
SS	*THR1, THR4, SER2*	Amino acid biosynthesis	—
	MRPL37, AFG3	Mitochondrial function	JMJD2C, AFG3L2
	RAD55	Recombination, DNA repair	RAD51 paralogue
	PBP1	mRNA polyadenylation	—
	SWI6, ARP8	Chromatin remodeling, transcription	SWI complex, ACTR8
	ERG3	Ergosterol biosynthesis	SC5DL
	BEM4	Cell polarity	—
	RPL37A	Ribosomal protein	RPL37A
	VPS36	Protein sorting to endosome	VPS36
	SSZ1	Hsp70 involved in drug resistance	Hsp70B
	SOD1	Superoxide dismutase	SOD1
	YOR199W	Uncharacterized ORF	—
S	*RTT106, RTT109*	Ty1 transposition control	—
	BUD22	Bud site selection	—
	GAS1	Cell wall assembly	—
	UAF30	RNA polymerase I transcription factor	SMARCD1
	HTZ1	Histone H2 variant	H2AFZ
	TCO89	Subunit of TORC1	—
	BEM1	Cell polarity	SH3PXD2B
	COX6	Subunit of cytochrome *c* oxidase	COX5A
	MSH1, ILM1	Mitochondrial maintance	—
	SER1	Amino acid biosynthesis	PSAT1
	TOP3	Topoisomerase	TOP3
	NBP2	Hyperosmotic response	SH3RF1
	ADK1	Adenylate kinase	AK2
	VPS28, SNF8	Vacuolar function, endosome sorting	VPS28, SNF8
	PHO4	Phosphate availability transcription factor	USF2
	DBP3	Ribosome biogenesis	DDX17

Sensitivity*	Gene/ORF	Function†	Mammalian orthologues‡
	RPL13B, RPS9B, RPP1A	Ribosomal subunit	RPL13, RPS9, RPLP1
	ASF1	Chromatin assembly	ASF1
	HTL1	Chromatin remodeling	—
	TAT1	Amino acid transporter	—
	FLX1	FAD transport from mitochondria	SLC25A32
	PTC1	Type 2C protein phosphatase	PPM1A
	CCS1	Oxidative stress, SOD1 chaperone	CCS
	YPL205C, YHR151C,	Uncharacterized ORF	—
	YKL098W, YGL218W	Uncharacterized ORF	—
	YNL198C	Uncharacterized ORF	—

*Survival was determined by plating serial dilutions (10-fold) of stationary-phase cultures grown on plates with or without doxorubicin. SSS, survival was decreased by three or more serial dilutions (100–1,000-fold sensitivity); SS, survival was decreased by two to three serial dilutions (10–100-fold sensitivity); S, survival was decreased by one to two serial dilutions (2–10-fold sensitivity) following doxorubicin treatment.

†Cellular role as indicted in Saccharomyces Genome Database (SGD) and Munich Information Center for protein Sequences (Mips, http://mips.gsf.de).

‡Potential human orthologue genes or functional counterparts found in SGD, National Center for Biotechnology Information Entrez Gene, and GenBank database.

Table 2

Comparison of the cross-sensitivity of doxorubicin hypersensitive strains to other cytotoxic agents

Gene name	Growth			Sensitivity (fold)			
	Irradiation (50 Gy)	Heat (37°C)	Hydroxyurea (80 mmol/L)	Cisplatin (80 μmol/L)	MNNG00 (3 μmol/L)	Camptothecin (10 μmol/L)	Daunorubicin (10 μmol/L)
RAD57	−		S	10	10,000	10,000	10
RAD50	−		S	10	100,000	10,000	1,000
RAD51	−			1,000	50,000	100,000	10
RAD52	−		S	10,000	100,000	100,000	1,000
XRS2	−		S	10,000	100,000	100,000	1,000
RAD55	+		S	<5	10,000	10,000	10
MRE11	−		S	10,000	100,000	100,000	1,000
ZUO1	+			1,000	5	5	1,000
YDJ1	+	S		10,000		10	10,000
NEW1	++						1,000
SSZ1*	−			1,000	10	10	1,000
TFP3	++		S	10			100
VPH2	N.D.	S	S	100			10
VMA21	++			10	100		10
SWI3	+		S				
SNF2	+		S	10	10	10	1,000
HFI1	+		S	10	10	10	100
HOM6	++			<10		10	100
TRP1	++			10			100
MRPL6	++					10	1,000
ASC1	+						10
MAC1	++		S				10
GND1	++			100		10	1,000
ASF1†	+			10,000	10,000	10,000	10,000

NOTE: Growth after irradiation was determined relative to wild-type cells. Strains were classified as +++, not sensitive (≥50% survival); ++, slightly sensitive (10–50% survival); +, sensitive (1–10%); and −, hypersensitive (<1%). Survival of wild-type cells was 50% after irradiation. Heat-sensitive strains did not grow when patched onto YPD plates and incubated at 37jC for 3 d (indicated by an S). Strains

sensitive to hydroxyurea displayed <10% growth relative to untreated cells after incubation for 2 d (indicated by an S). Sensitivity to cisplatin, MNNG, camptothecin, and daunorubicin was calculated by determining cell survival and is expressed as fold reduction in viability. Survival of the wild-type strain was >90% at the concentrations of drugs used. Abbreviation: N.D., not determined.

*SSZ1 is a doxorubicin-sensitive strain (10–100-fold).

†ASF1 is a slightly sensitive strain (2–10-fold).