Use of a genome-wide approach to identify new genes that control resistance of *Saccharomyces cerevisiae* to ionizing radiation

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We have used the recently completed set of all homozygous diploid deletion mutants in budding yeast, *S. cerevisiae*, to screen for new mutants conferring sensitivity to ionizing radiation (IR). In each strain a different open reading frame (ORF) has been replaced with a cassette containing unique 20-mer sequences that allow the relative abundance of each strain in a pool to be determined by hybridization to a high-density oligonucleotide array. Putative radiation-sensitive mutants were identified as having a reduced abundance in the pool of 4,627 individual deletion strains following irradiation. Of the top 33 strains most sensitive to IR in this assay, 14 contained genes known to be involved in DNA repair. Eight of the remaining deletion mutants were studied in detail. Only one, that deleted for the ORF YDR014W (which we name *RAD61*), conferred reproducible radiation sensitivity both in the haploid and diploid deletions and had no problem with spore viability when the haploid was backcrossed to wild-type. The rest showed only marginal sensitivity as haploids, and many had problems with spore viability when backcrossed, suggesting the presence of gross aneuploidy or polyploidy in strains initially presumed haploid. Our results emphasize that secondary mutations or deviations from euploidy can be a problem in screening this resource for sensitivity to IR.

Keywords: *Saccharomyces cerevisiae*, radiosensitive mutants, genome-wide, *RAD61*, ionizing radiation

INTRODUCTION

For many years the budding yeast *Saccharomyces cerevisiae* has been an important model system for understanding DNA repair in eukaryotic cells *(1)*. This is primarily a result of the ease of genetic manipulations of this organism and of the remarkable conservation of pathways and genes from yeast to man in the area of DNA repair *(2-4)*.

In mammalian cells the most important lesions leading to cell death by ionizing radiation (IR) are believed to be DNA double strand breaks (DSBs), and mutants defective in the repair of these lesions are highly sensitive to radiation-induced cell killing *(5-7)*. Radiation-induced DSBs are also potentially lethal in yeast based on evidence that a single double strand break can be lethal in repair deficient mutants *(8-10)*. Two major pathways for DNA double strand break repair have been identified in eukaryotic cells. The first, homologous recombination (HR), involves exchange of DNA between broken and non-broken DNA strands. This is the major pathway of repair in *S. cerevisiae* but plays a lesser role in mammalian cells. The second pathway, non-homologous end-joining (NHEJ), which involves direct ligation of the broken ends, is the major pathway for repair of IR-induced DSBs in mammalian cells but plays at most a minor role in repair of IR damage in *S. cerevisiae*. Despite the lesser importance of HR in repairing radiation induced DSBs in mammalian cells *(11, 12)*, it is clearly a pathway of major importance for survival and genomic stability in mammals in the absence of radiation damage. This is apparent from the embryonic lethality in mice of homozygous deletion of many of the individual genes involved in this pathway, as well as the cellular chromosomal instability and predisposition to cancer in individuals with heterozygous mutations in these genes *(13)*.

In yeast, there are several other pathways affecting sensitivity to IR in addition to the HR repair pathway mediated by the *RAD51* epistasis group genes. Dominant among these is the

post-replication repair process mediated by genes in the *RAD6* epistasis family, thought to be mainly involved in repair of potentially lethal or mutagenic DNA damage that depends on replication (the post-replication pathway). Null mutations in the *RAD6* gene itself confer IR hypersensitivity comparable to those in the *RAD51* gene family *(14)*. Mutations in some genes involved in nucleotide excision repair (NER), (the *RAD3* epistasis group), also confer sensitivity to IR *(14)*, though to a much lesser extent than those involved in HR and post-replication repair. Genes that mediate DNA damage checkpoints in the cell cycle (for example *RAD9, RAD17, RAD24, RAD53, MEC3* and *DDC1*) also confer IR sensitivity when mutant, although these genes may also play a direct role in repair and some of them fall into the *RAD6* epistasis group *(11, 12)*.

Increasing evidence points to the important role of HR and DNA damage checkpoints in human cells in controlling genomic stability and suppressing cancer formation *(13, 15)*. Examples of cancer susceptibility syndromes that result from mutations in genes in DNA repair or cell cycle checkpoints include ataxia telangiectasia, Fanconi's anemia, Bloom's syndrome, hereditary non-polyposis colon cancer, Nijmegen breakage syndrome, and xeroderma pigmentosum. Mutations in such genes, termed "caretaker" genes, are proposed to lead to an increased mutation rate of all genes, including those directly responsible for the cancer *(16)*. Consequently, screening yeast mutants for sensitivity to IR has the potential not only of identifying new genes affecting radiation response, but potentially also new genes affecting cancer susceptibility in humans.

Many yeast mutants that confer sensitivity to IR have already been identified and extensively characterized *(14)*. However, in contrast to the situation for UV radiation *(17)*, no systematic effort has been made using classical replica plating techniques to isolate sufficient IR-

sensitive mutants to saturate the genome for this phenotype and thus identify all the genes involved. In addition, the classical methods used are not always effective at identifying mutants with only moderate sensitivity to IR. Furthermore, such methods are inadequate for identifying mutations whose effect may be manifested only in diploid cells. Most classical studies use haploids, since few if any recessive mutations will be identified if diploids are used.

In the current approach, rather than classifying survivors of random mutagenesis as qualitatively sensitive or not sensitive based on replica plating, we obtained a specific number for a deletion mutant involving each yeast gene, reflecting the relative abundance of the mutant compared to every other deletion mutant in a mixed culture at a given time after irradiation. This approach enables one to subsequently test mutants by classical survival assays for IR sensitivity down to any chosen level on this numerical ranking. We started with diploid deletion mutants, as haploid strains do not allow for the effects of recombination repair between homologous chromosomes.

To do this, we took advantage of a new resource, the availability of systematic deletion mutants involving all of the approximately 6,200 known open reading frames (ORFs) of budding yeast *S. cerevisiae*. These have been created and made available by an international group, the *Saccharomyces* Genome Deletion Project Consortium *(18)*. In these mutant strains, a cassette that contains a selectable marker flanked on each side by a different molecular barcode or "tag" has replaced each ORF. The tags for each ORF deletion are unique and consist of a 20 base oligonucleotide sequence. The tags themselves are flanked by sequences common to all the deletion cassettes. This permits PCR amplification of cassette DNA from any of the deletion strains. Following PCR amplification of strains mixed in a pool, these tags can be detected by hybridization to their complementary sequences on a high density oligonucleotide array, thus

enabling the relative abundance of each tag and hence the abundance of each strain in the pool to be determined *(19)*. We have recently shown that this method of screening a pool of 4,627 strains, representing homozygous deletion of each of the non-essential genes in diploid yeast, is a highly reproducible and robust way of detecting known and unknown genes affecting the response of yeast to UV irradiation *(20)*. This hybridization technique using the unique tags with a pool of the deletion mutants has also been used to identify a new gene in yeast involved in non-homologous end joining (NHEJ) *(21)*, and to identify mitochondrial proteins *(22)*. In addition, collections of the deletion mutants have been tested individually for genes affecting sensitivity to IR *(23)* and for genes that participate in the bipolar budding pattern of diploid yeast *(24)*. In the present publication we have used the same pool of 4,627 homozygous deletion strains, representing virtually all non-essential genes, to identify new genes affecting the sensitivity of diploid yeast to IR. In addition we have analyzed several of the new mutants using conventional genetic crosses and survival curve assays to check the results from the hybridization assay. We report several possible new genes involved in IR sensitivity, and have confirmed one of them, which we name *RAD61*, by more detailed assays.

MATERIALS AND METHODS

Yeast strains, genetic methods, and media

Genotypes of the parental yeast strain BY4743 and construction of the homozygous diploid deletion strains have been described previously *(19)*. Information is available in the *Saccharomyces* Genome Deletion Project website (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). All of these deletion strains can be obtained from Research Genetics (Huntsville, AL) or EUROSCARF (Frankfurt, Germany). The homozygous diploid deletion pool was generated as described *(19)*. In the

present study, we used a pool of 4,627 strains containing homozygous deletion mutants of almost all non-essential genes. Rich media (YPD), sporulation media (SPO), and appropriately supplemented minimal media were prepared as described in the literature *(25)*. To score the geneticin-resistance marker in crosses, we used YPD plates supplemented with geneticin (obtained from Sigma) added from filter-sterilized solution shortly before pouring plates, to give a final concentration of 100 μg/ml. Genetic methods, including tetrad dissection, were as described *(27)*.

Irradiation

For experiments with the pool, aliquots of the deletion pool representing approximately 10^4 cells of each of the individual strains were grown in YPD medium at 30˚C, and shaken at 300rpm to mid-exponential phase (OD_{600nm} 0.5-1.0). Cells were pelleted (8,000rpm for 3 minutes) and resuspended in ice-cold phosphate buffered saline (PBS) at 10^7 cells/ml immediately before delivering 200 Gy γ-irradiation from a Cs^{137} source (Mark 1 Model 3 from J.L. Shepherd, San Fernando, CA; 33 Gy/min). Mock-irradiated cells were treated identically in parallel. The irradiated or control cells were then pelleted and inoculated into pre-warmed YPD medium at $O.D._{600}$ =0.05 (10^6 cells/ml). Before reaching an $O.D._{600}$ of 1.0, the cultures were diluted into fresh YPD media at a 1:20 dilution to maintain exponential growth. Cultures were harvested and genomic DNA extracted 18 hr after treatment. We performed three identical experiments with γ-irradiation to determine reproducibility. For survival assays using colony formation after Cesium137 exposure, mid-logarithmic phase cultures were diluted in PBS, irradiated with different doses of IR, and various cell dilutions were spread on replicate 100mm YPD plates. Surviving colonies were counted after incubation for 2 to 5 days at 30°C and compared with survival of the parental line, BY4743. For survival curves using X-rays, we used a Machlett

OEG 60 X-ray tube with a beryllium window and a Spellman power supply operated at 30 kilovolts and 15 milliamps to deliver a dose-rate of 1.3 Gy/second of "soft" X-rays. Cells were diluted appropriately from mid-log phase liquid YPD cultures and irradiated on the surface of YPD plates to prevent shielding by liquid.

PCR amplification, microarray hybridization and data acquisition

PCR amplification, microarray hybridization and data acquisition were as described *(19)*. Briefly, following isolation of genomic DNA from the treated and untreated pools, equal amounts of the isolated DNA were used as template in two PCR reactions that amplify the two tags (UPTAG and DOWNTAG) from each strain in the pool using biotinylated PCR primers complementary to common regions in the replacement cassette. For both the treated and untreated pools, we combined the PCR products with oligonucleotides complementary to non-tag regions of the PCR products, heat denatured the mixtures and hybridized them to purpose-built oligonucleotide microarrays (DNA TAG3, Affymetrix, Santa Clara, California) for 16 hours at 42°C. After staining with streptavidin-phycoerythrin (Molecular Probes, Eugene, Oregon), arrays were scanned at an emission wavelength of 560 nm using an Affymetrix GeneChip Scanner (Affymetrix, Santa Clara, California). The hybridization intensities for each of the array elements were determined using the Affymetrix GeneChip software.

Data Analysis

For the analysis of strain prevalence in the pool, each strain was represented by 4 values of signal intensity (sense and antisense array elements for each UPTAG and DOWNTAG sequence). To assess the degree of sensitivity to the IR treatment, a ratio of treated/untreated signal for each of the 4 tags for each strain was calculated. We calculated a mean of these 4 ratios to give a single value for each strain present in the pool. This allowed sensitivity ranking

to be performed for each experiment. Those strains with lower signals in the treated compared to the untreated pool had a low ratio (sensitive strains), while those with similar signals from treated and untreated had ratios close to 1 (unaffected strains). An overall average ratio (and standard error) of treated/control for each strain was determined by pooling the 4 individual tag ratios for 3 separate experiments (12 values for each strain). In some cases the hybridization signals for individual tags were close to the background range. We defined the upper limit of the background range as 2X the mean hybridization signal of the lowest 25% of the 13,798 tag elements on the oligonucleotide arrays not assigned to any ORF tag from the untreated chips, and therefore to which there would be no specific hybridization. This value was 846 and was higher than 98.3% of the signals from the unassigned tags (and higher than 17.6% of the assigned tags). If the average signal from an untreated strain was lower than this number, we considered this strain to be too close to background to evaluate for radiosensitivity. This was the case for 6.4% of the strains.

To determine the total number of strains that were more radiosensitive than wild-type we calculated the mean ratio and standard error of treated/untreated for each strain (using the 12 tag ratios from the 3 experiments) and determined the number whose upper 99.5% confidence level (2.72 x SE given 11 degrees of freedom) did not include 1.0. We note that by chance alone, some 22 strains (0.5% of those that are not radiation sensitive, see below) would have an upper 99.5% confidence interval that does not include 1.0.

Reintroduction of wild-type genes

To determine whether the IR sensitivity of 4 individual strains carrying different deletions was due to the absence of the ORF, we reintroduced the wild-type sequences in each of these strains by means of galactose-inducible and uracil-selectable yeast expression vectors. For *NPL6*, we used the pMYR vector (Stratagene, La Jolla, CA), while for *ARP8*, YDR014W and *SHE1* we used pESC-URA (Stratagene, La Jolla, CA). We PCR-amplified genes or ORFs using genomic DNA from the parental strain, BY4743, using primers that included a hemagglutin (HA) epitope tag on the 3` end of the encoded reading frame as well as suitable restriction sites.

PCR reactions were carried out in a final volume of 50µl containing genomic DNA (200 ng) using Vent DNA polymerase (New England Biolabs, Beverly, MA) according to manufacturer's instructions. The PCR products were cloned into pESC, and the constructs were expanded in *E. coli* (DH5α, Life Technologies, Rockville, MD) and sequenced to ensure that ORF insertion was correct. The yeast deletion strains were transformed using the lithium acetate method *(26)*. The empty vector was also cloned into parallel cultures to provide controls. Transformed cells were selected and grown in synthetic media without uracil with 2% dextrose (SMD). The transformants were subsequently grown in synthetic media minus uracil with 2% galactose (SMG) in place of dextrose for 4 hours to allow for production of the protein prior to IR exposure and clonogenic survival assay. Parallel cultures were treated identically and harvested for western blot analysis as described below.

Western blot analysis

To confirm production of the correct size protein coded by the expression vector, we used western blotting with an anti-HA tag antibody. Briefly, ~$1x10^8$ cells were washed in cold PBS and lysed using glass beads (425-600 microns, acid-washed, Sigma, MO) by vigorous vortexing

for 5 min in Cell Lysis Buffer (140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, 1% Triton X-100 with protease inhibitors (Complete Protease Inhibitor Cocktail, Roche, Germany)). The crude lysate was clarified by centrifugation at 16,000g for 15min at 4°C and 10µg of protein was loaded onto a 4-12% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane and nonspecific sites were blocked by incubation in 5% skim milk in PBS/0.05% Tween 20 (PBS/T). HA-epitope tagged proteins were detected by incubation for 1h in a 1/2000 dilution of the mouse monoclonal antibody, HA.11 (Covance, Cumberland, VA), followed by 1 hr in 1/5000 dilution of goat antimouse IgG-alkaline phosphatase conjugate (Zymed, CA). Specific bands were visualized using the Vistra ECF substrate (APBiotech, Sweden) and scanned on a chemifluoresence detector (Molecular Dynamics Storm, APBiotech, Sweden).

RESULTS

Relationship between Cell Survival and Growth Delay

Hybridization of DNA from the strains comprising the yeast deletion pool gives an indirect method of estimating strain radiosensitivity. This is because the gene-specific hybridization levels provide an estimate of the relative abundance of each strain in the pool. The expectation is that following irradiation, the radiosensitive strains will become depleted in the pool compared to the radioresistant strains. In order to establish the relationship between post-irradiation cell numbers and radiosensitivity, and also to determine suitable doses and times at which to perform the assay, we performed preliminary experiments with the wild-type parental strain (BY4743) and with strains homozygous for *rad50, rad51* and *rad52* deletion mutations. These strains were expected to represent the range of sensitivities expected in the experiment. In parallel with experiments measuring survival by colony formation as a function of radiation

dose, we also measured cellular increase (by $O.D_{600}$) in the same strains following irradiation (Figure 1). As expected from the literature, the *rad51Δ/rad51Δ* and *rad52Δ/rad52Δ* strains showed the greatest radiosensitivity and in addition showed the largest growth delay following irradiation. Based upon the data, we chose a radiation dose of 200 Gy and a time of 18 hours after irradiation to perform the experiment with the deletion pool. These choices were based on wanting minimum cell kill and growth delay in strains of wild-type sensitivity combined with adequate depletion of sensitive strains. We found that this dose and time caused the most sensitive strains to become depleted by a factor of at least 100 relative to wild-type cells. In the wild-type strain BY4743, this dose of 200 Gy IR caused approximately 50% cell killing. Previous experiments using the same system have shown that 50% wild-type cell killing is appropriate to identify deletion strains in a pool that have known sensitivity to cytotoxic agents *(20)*. It can be seen from Figure 1b that larger differences in abundances between strains of wild-type and maximum radiosensitivity could be achieved by sampling the pool at later times than 18 hours after irradiation. However, longer times would be counterproductive for two reasons. First, it is important to minimize post-radiation growth times because individual strains in the pool have different growth rates which causes the slow growing strains to become relatively depleted even in the non-irradiated pool, thereby reducing the ability to see a change in signal in the irradiated pool. Second, the maximum dynamic range of the hybridization signal in our hands was approximately 80 (ratio of highest signal to background, see below) so a ratio of control to treated of greater than this could not be detected.

Signal Intensities from Individual Strains: *Hybridization of PCR Products to the Oligonucleotide Array*

We obtained signal intensities for each of the 4,627 deletion strains in the pool, starting with equal amounts of DNA from the cultures with and without irradiation. The intensity values ranged from a minimum level of 386 to a maximum of 29,354 for the most intense strain. Figure 2 shows the distribution of the hybridization signals from the control and IR treated cultures for all 4,627 strains from the pool data averaged from 3 replicate experiments. It is evident from this graph that most of the strains clustered around a line of equal signal intensity between the cultures that received 0 Gy and those that received 200 Gy (the diagonal line in Figure 2). However there are several strains, some of which are identified in Figure 2, that have low signal intensities in the treated compared to untreated pool, and therefore fall considerably below this diagonal line.

Calculation of Sensitivity

After signal intensities for each tag element were obtained for each strain and treatment, a ratio of the hybridization signal in the 200 Gy treated culture to that in the untreated culture was calculated for each tag element for each strain in each experiment. The mean of the treated/untreated ratio for each strain was then calculated individually for the 3 experiments and for all the pooled data. A ratio of 1.0 means that the radiation dose had an effect on that strain equal to that of wild-type cells, whereas a low ratio suggested a post-irradiation growth deficit, and therefore the likelihood of radiation sensitivity of that strain. Ratios above 1 were also obtained but were not constant between the 3 experiments. Table 1 shows a listing of the top 33 strains with the lowest ratio of treated to untreated signal intensity. To display the most sensitive strains in the pool, we used a cutoff of 0.5 in the ratio of signal at 200 Gy to signal at 0 Gy.

Clearly, strains with a higher ranking than 33 are also sensitive to IR but diminishingly so. By our statistical cutoff of the upper 99.5% confidence interval of the mean ratio not including 1.0, we identified 331 strains as radiation sensitive. These, as well as all the hybridization data are shown in the supplementary information (http://cbrl.stanford.edu/mbrown/IR.html). However, as mentioned above, about 0.5% of strains whose true mean ratio is 1.0 may fall below this 99.5% confidence cut-off level by chance alone, and some strains that are truly sensitive will by chance not fall into this group. The latter number cannot easily be estimated, since the probability of any sensitive strain not being identified will vary, depending on its degree of sensitivity. Also shown in Table 1 is the ranking of the mean observed ratio for each strain (from 1 – 4,627) in the 3 individual experiments. The similarity of the rankings in the replicate experiments mirrors our previous data with UV irradiation and demonstrates the level of consistency in the system.

Confirmation of Individual Radiation Sensitivity

Examination of Table 1 shows that the screen identified many genes whose mutation or deletion is already known to confer sensitivity to IR. These include genes involved in post-replication repair, recombinational repair and DNA damage checkpoints. However, there are also a number of genes not previously identified as involved in radiation resistance. In order to determine which of these newly identified genes conferred radiation sensitivity when deleted, we first tested each with a single dose of 300 Gy of Cs^{137} gamma radiation using the colony survival assay. These data are also shown in Table 1. We next obtained multi-dose X-ray survival curves for each of the five newly identified mutant diploids that showed survival below 10% after 300 Gy of gamma rays. These are the deletions of *SHE1*, *NPL6*, *ARP8*, ORF YNR068C and ORF YDR014W (see Table 1). In addition, we obtained X-ray survival curves for three representative

strains from Table 1 that showed survival at 300 Gy gamma rays in the range between 10% and 20%, namely the *thr1Δ,* ydr540CΔ and *rsc1Δ* strains. Figure 3 shows all these survival curves compared to wild-type diploid cells and a known radiation sensitive diploid homozygous for a *rad51::URA3 null* allele in the same genetic background. It can be seen that each of these eight diploid strains exhibits moderate sensitivity with the probable exception of *thr1Δ,* which shows only a borderline increase in killing compared to wild-type. However, none of the eight new mutants shows the extreme sensitivity characteristic of *rad51Δ/rad51Δ* and the other major mutants in recombinational repair (Figure 3 and *(14)*).

To determine whether the sensitivity of the newly identified strains resulted from deletion of the gene (or ORF) or from other genetic alterations, we studied the corresponding haploid strains, performed genetic crosses, and in some cases we also re-introduced the appropriate wild-type genes and expressed their products in the deletion strains.

Haploid strains

X-ray survival curves for presumptive haploid strains containing the same eight deletions that were tested in the diploid assays are shown in Figure 3. Haploid mutants were initially constructed by the Yeast Deletion Project in the same genetic background as the homozygous diploid deletion strains; in most instances they are the actual parents of these diploids. In some cases, we obtained X-ray survival curves for haploids derived by backcrossing these deletion library haploids to wild-type. The data are shown in Figure 4. It can be seen that most of the mutants show either no sensitivity (*MATa* ynr068cΔ) or a moderate sensitivity compared to the wild type strain, a result broadly comparable to that seen in the diploids. (The *MATα* ynr068cΔ strain shows high sensitivity, but this is conferred by a secondary mutation in the *RAD52* gene, see below). However, the *ydr014wΔ* strain (renamed here as *rad61*), which in the diploid

configuration clusters with the other mutants, stands out as being more sensitive than the others as a haploid. Once again, none of the new mutants shows sensitivity comparable to that of the *rad51Δ* haploid strain in Figure 4. With the possible exception of the *rad61Δ* (*ydr014wΔ*) strain, the moderate sensitivity observed makes it unlikely that these mutants would have been identified in conventional mutant assays based on replica plating.

Genetic crosses

We made genetic crosses using the presumptive haploid strains to determine whether the deleted genes were responsible for conferring the observed IR sensitivity, and to further characterize the mutant phenotypes. We crossed each original mutant strain in one or both mating types with very closely related wild-type strains and monitored viability of meiotic spores obtained by tetrad dissection of the heterozygous diploids. We tested viable spore-clones for IR sensitivity and co-segregation of an IR-sensitive phenotype with the deletion allele, as monitored by the geneticin-resistance phenotype conferred by the inserted cassette.

Surprisingly, genetic crosses with the eight mutants for which we had haploid and diploid survival data (see above) revealed only one that showed a clear segregation for IR sensitivity that co-segregated with geneticin resistance. This was the deletion of ORF YDR014W, discussed later (see the section on *RAD61*).

Three of the other mutants gave such poor spore viability when the *MATα* strains were crossed to wild-type that no inferences could be drawn about heritability of X-ray sensitivity. These mutants were the deletion strains for *arp8Δ*, *npl6Δ*, and *she1Δ*. In the case of these three mutants, we also crossed the *MATa* derivatives with wild-type, and found essentially the same very poor (0% to 28%) spore viability. The viability was consistent with triploidy or perhaps other gross genomic aneuploidy in these crosses. Possibly, the starting mutants here had

undergone spontaneous diploidization to form *MATα/MATα* and *MATa/MATa* diploids respectively. We were also unable to obtain live meiotic tetrads from the initial homozygous diploids of these three mutants. Although many IR sensitive mutants are also meiosis defective, triploidy or other major genome changes could alternatively be disrupting meiosis in these strains. Two further mutants, the deletions *thr1Δ* and ydr540CΔ, gave good viability when the *MATα* strains were crossed to wild-type. However, we were unable to confirm reproducible sensitivity of the *thr1Δ* strain with survival assays. We also found no haploid X-ray sensitivity phenotype in plate tests of spore clones from the ydr540CΔ cross. The *MATα rsc1Δ* strain gave about 50% live spores in a cross with wild-type, but again no significant X-ray sensitivity was observed in the progeny. We have not pursued these mutants further.

Another mutant, the deletion ynr068cΔ, demonstrated strong X-ray sensitivity when the original *MATα* haploid strain was tested but no sensitivity when the original *MATa* strain was tested (see Figure 4). A back-cross of the ynr068CΔ *MATα* strain to wild-type confirmed that geneticin-resistance and X-ray sensitivity each segregated 2+: 2-, but were unlinked, demonstrating that a secondary mutation conferring sensitivity was present in this strain. Complementation tests with *MATa* strains containing mutations in known *rad* genes quickly revealed that the *MATα* ynr068cΔ mutant strain contained a separate mutation in the *RAD52* gene. In addition, we found that the *MATa* ynr068cΔ haploid strain failed to mate with normal frequency, and that the ynr068cΔ/Δ diploid strain used in the mutant pool failed to undergo meiosis and was able to mate with *MATa* strains. These observations imply that the diploid strain is behaving phenotypically as a *MATα/MATα* strain rather than as *MATa/MATα,* probably because of an alteration in the mating-type allele contributed by the *MATa* parent. Diploid yeast strains that contain or express only one kind of mating type allele are known to be more IR

sensitive than *MATa/MATα* diploids *(27-29)*. Hence, we interpret the initial identification of the ynr068cΔ/Δ diploid deletion strain as a sensitive mutant as probably resulting from a combination of an unrelated mating type defect in combination with incidental heterozygosity at the *RAD52* locus.

Reintroduction of the wild-type gene (or ORF).

For four of the diploid strains studied, we reintroduced the wild-type sequences and expressed their products in the deletion strains using a galactose-inducible expression vector. In these cases, we confirmed the expression of the relevant protein by Western blotting by using an antihemagglutin antibody before testing for IR sensitivity. As a negative control we introduced the expression vector made without the coding insert. Although its biological function is unknown, the *SHE1* gene is lethal when overexpressed *(30)*. Our *SHE1* expression vector also conferred lethality when expressed in cells grown in SMG. However, when a mixed medium containing 90% SMG and 10% SMD was used, we were able to induce She1 protein to a sub-lethal level, thus allowing us to perform IR clonogenic survival assays.

Figure 5 shows the data for survival after 300 Gy of four of the newly identified IR sensitive diploid strains with introduction of the vector or with the vector containing the relevant gene or ORF. We also performed the same procedure with the known IR-sensitive *rad18Δ/rad18Δ* strain. The data show that for strains deleted for YDR014W, *ARP8* and *RAD18,* the introduced sequence improved survival to a level consistent with that of the wild-type strain BY4743 (~40% at this dose). However, correction of the mutant phenotype is only partial in the case of *NPL6* and *SHE1*.

The RAD61 gene

In contrast to the other tested mutants, the *MATa* strain deleted for the ORF YDR014 gave good spore viability in a cross with wild type, and an IR sensitive phenotype could be observed in all spore clones that were geneticin-resistant. 24 dissected tetrads combined from 4 crosses gave 89 lives spore clones (93% viability). The 18 tetrads that gave four live spores were monitored for geneticin-resistance and IR-sensitivity as judged by replica-plating assays. Each tetrad gave two geneticin-resistant IR sensitive spore-clones, and two geneticin-sensitive spore-clones that were wild-type for IR response.

We took a *MATa* geneticin-resistant spore clone (g1185-2b) from a cross of the *MATa* ydr014wΔ strain with wild-type, and mated it with the original *MATα* ydr014wΔ deletion mutant to make diploid g1197. We also mated the original *MATα* and *MATa* ydr014wΔ strains with each other. IR survival curves for both the resultant diploids are shown in Figure 6. It can be seen that both strains show similar sensitivity, which is significantly greater than that of the wild-type diploids. This demonstrates genetically that the IR-sensitivity phenotype in *MATα* and *MATa* ydr014wΔ strains is conferred by the same mutant gene in each case. The X-ray sensitive phenotype conferred by the deletion is recessive, since a heterozygous YDR014W/ydr014wΔ diploid strain shows a survival curve equivalent to that of wild-type (Figure 6).

Figure 6 also shows survival curves for two ydr014wΔ haploid strains, the original two haploid parents of the displayed diploid g1197. Again, it can be seen that there is good agreement in sensitivity between these two strains, and that both are substantially more sensitive than wild-type haploids. Consistent with its ranking of 950 (out of 4,627 strains) in our hybridization screen for UV sensitivity, we found no UV sensitivity in a survival assay of a diploid *rad61Δ/rad61Δ* strain compared to wild-type (data not shown). Our hybridization screen

for sensitivity to hydrogen peroxide also revealed no sensitivity of the *rad61Δ/rad61Δ* strain (rank 4,051).

The ORF YDR014W does not correspond to the physical position of any known *RAD* gene and no phenotype has previously been reported for its deletion mutant. We have confirmed from tetrad data that the designated ydr014w deletion shows the expected close genetic linkage to the *TRP1* locus (YDR007W) near the centromere of chromosome IV. The genome sequence shows a short physical distance (~ 13 kb of DNA) between the two loci (http://genome-www.stanford.edu/Saccharomyces/). Given our observation of significant IR sensitivity in the ydr014wΔ mutant, and its lack of UV sensitivity, we propose the gene name *RAD61* for ORF YDR014W. This is consistent with accepted nomenclature in which *RAD* genes are numbered from *RAD50* upwards if their mutant alleles primarily affect sensitivity to IR rather than UV radiation *(31)*. The previously highest numbered *RAD* gene is *RAD60*, proposed as an alternate name within *RAD* nomenclature for the gene also known as *XRS2 (14)*.

DISCUSSION

We have used methodology developed earlier by us *(20)* to screen a pool of 4,627 diploid yeast mutants for sensitivity to ionizing radiation, based on relative strengths of DNA hybridization signals on a high density oligonucleotide array. It should be noted that relative cellular abundance at a fixed time after irradiation (18hr in our case) is a different endpoint from ultimate survival as determined by colony forming ability. Thus, mutations that extend division delay after radiation without leading to increased killing would be detected via the microarray screen but would not affect clonogenic survival. The ranking of any strain by relative abundance in the irradiated pool is also dependent on the abundance of that strain in the unirradiated pool, and in an extreme case when low abundance in the unirradiated pool gives a hybridization signal

close to background levels, no conclusion can be drawn as to the sensitivity of that strain. Despite this, the hybridization screen using the molecular "barcodes" is an effective and rapid way of identifying mutants sensitive to killing by IR, as we showed earlier for mutants sensitive to UV *(20)*. As can be seen in Table 1, the degree of reproducibility in ordering the mutants in repeat experiments is extremely good. In contrast, unless several thousand clonogenic survival assays are performed, classical methods address only those mutants showing up as sensitive on a qualitative assay, even when genome-wide mutant collections are used *(23)*.

In our study, 8 of the 33 strains identified as most sensitive by this screen contained deletions of genes already designated as *RAD* loci and specifically known to control X-ray sensitivity (reviewed in (*14*)). At least six more of these thirty-three mutants are also deleted for genes known to be involved in DNA repair, recombination, or DNA damage checkpoints. These genes are *DDC1, MEC3, MUS81, SAE2, MMS4,* and *RDH54*. Another ORF, YBR099C, overlaps the *MMS4* gene on the opposite strand so it can be assumed that ybr099CΔ is co-deleted for part of the DNA repair gene *MMS4*, and strikingly, the two deletion strains are identified next to each other in the sequence in Table 1.

Despite this high success rate for identifying genes known to be involved in X-ray sensitivity, there are several genes whose deletion is known to confer radiosensitivity that are not in Table 1. However, most of these were ranked as significantly radiosensitive including (with rank order): *RAD18* (42), *HPR5* (45), *RAD51* (48), *REV7* (109), *XRS2* (163), *RAD50* (218), and *REV1* (280). All of the most highly radiosensitive of these in terms of survival curves (strains with deletion of *RAD18, RAD51, XRS2,* and *RAD50*) had relatively low hybridization signals in the control samples and signal levels in the treated samples in the background range. Thus, they would be expected to be more radiosensitive than the ratios suggest. Only 3 strains expected to

be highly radiosensitive – those with deletion of *MRE11, RAD6* and *RAD52* - were not identified in our screen, and all had hybridization signals in the control samples at background levels so could not be interrogated. Thus, given the limitation of low signal strength for some 6-7% of the strains in the pool, the screen identified all of the previously known radiosensitive strains, and therefore has a zero, or very low, false negative rate. Surprisingly, a number of excision repair genes whose mutants by themselves are not expected to confer sensitivity in survival assays are also identified as IR sensitive in the hybridization assay. These include (with rank order) *RAD10* (50), *RAD1* (85), *RAD14* (170) and *RAD4* (186). *RAD10* and *RAD1* are known to play a role in recombination in addition to excision, and excision mutants in general may confer some X-ray sensitivity when other pathways are blocked (*14*). Possibly, these mutants may be slower to complete repair than wild-type, leading to lower abundance in the pool assay 18 hours after radiation, but an essentially wild-type survival when assessed by final colony formation.

To identify new genes involved in radiosensitivity (and to address the question of a possible false positive rate), we examined eight of the 19 strains in the top thirty-three that had not previously been reported to be involved in DNA repair or cell cycle checkpoints, and determined if they demonstrated X-ray sensitivity in standard survival assays, and if so, whether this sensitivity was conferred by the deletion mutation defined by the *KANMX4* insert. In one case, we were able to determine that the MATα ynr068cΔ strain contained a secondary mutation in the *RAD52* gene. Although we observed that sensitivity to IR was greater than that of wild-type for at least six of the other strains, none of these newly identified strains showed the extreme IR sensitivity typical of the major mutants in recombinational repair (e.g., *rad50null*, *rad52null*) or of *rad6null* mutants. More importantly, we encountered significant difficulties when we attempted to test whether the IR-sensitive phenotypes of the strains we identified were

conferred by the deletion mutations they contained. Surprisingly, we found that both the *MATa* and the *MATα* presumptive haploid strains for three mutants (*she1Δ, arp8Δ* and *npl6Δ*) gave such poor spore germination in back-crosses to wild-type strains that we were unable to determine if an X-ray sensitive phenotype co-segregated with the geneticin resistance phenotype conferred by the deletion alleles.

Poor meiotic spore viability in yeast can be caused by triploidy or other gross chromosomal abnormalities. If this is the case here, it implies that the presumed haploid cultures of these mutants in the deletion collection could have been *MATa/MATa* or *MATα/MATα* diploids, and the corresponding diploids in the pool could have been triploid or tetraploid or perhaps grossly aneuploid. Polyploid yeast strains are usually more sensitive to X-rays than the corresponding diploids *(27, 28)*, hence if tetraploids or cultures with other gross genomic changes are present at low frequency in the pool of diploids, selective identification of these strains as sensitive in the IR assay might be expected. However, this is unlikely to be the whole explanation in the case of *npl6* and *she1*, since re-introducing the gene itself into the mutant strains partially restores IR resistance. Possibly, a combination of altered ploidy plus a mutation that confers moderate IR sensitivity is responsible for the phenotype of these strains in the mutant pool.

We note that Bennett and colleagues *(23)* tested IR sensitivity in 3,670 deletion strains that form a subset of those in the pool used here. They reported IR sensitivity qualitatively in the diploid deletion mutants of 107 genes or ORFs that were not previously known to be involved in this phenotype. Although they concluded that these genes were involved in IR sensitivity, they did not test whether the sensitivity was conferred by the putative deletions that the strains contained. They tested the haploid parents for a subset of 29 of the new diploids they reported as

sensitive, and found that in more than one third of these, one or both parents were either not sensitive at all (8 mutants) or showed marginal sensitivity much less than the other parent (3 mutants). In these cases we consider it likely that the deletion is not primarily responsible for the IR sensitivity, and this is consistent with our own conclusions discussed above, that other factors could be responsible for deletion mutants initially testing as IR sensitive. As in our study, few if any of the new strains identified by Bennett and colleagues show the extreme IR sensitivity typical of many known mutants in recombinational repair.

In the present study we highlight only the most sensitive 33 strains (Table 1) found in our hybridization assay since these are probably of most biological interest. In fact there is no cutoff between strains that are radiosensitive and those that are of normal sensitivity. As shown in Fig 1 of the supplementary information (http://cbrl.stanford.edu/mbrown/IR.html) there is a continuum of sensitivities from which we identify 331 strains as more sensitive than wild-type at the 99.5% confidence level. Missing from this list will be strains that we cannot assign because of the low level of hybridization signal in the untreated control. Using our cutoff for not being able to evaluate strains with a mean hybridization signal of less than 2x background levels we could not assign sensitivities to 295 strains (6.4% of the total). These strains can be assessed either by individual testing (as was done for all the strains studied by Bennett and colleagues *(23))*, or, as we plan to do in the future, by using pools appropriately adjusted to give adequate abundance for the under-represented strains.

Of the mutants we have studied here, three were among those reported as "highly sensitive" in a spot test of diploids by Bennett and colleagues *(23)*. These were ORF ynr068cΔ, *rsc1Δ* and ORF ydr014wΔ. Our data demonstrate that ynr068cΔ does not confer sensitivity when separated from a *rad52* mutation also present in the original strain. For *rsc1Δ*, we find

only modest sensitivity in full survival curves for the designated haploid and diploid strains, and have been unable to successfully outcross this mutant, leaving open the possibility that the sensitivity arises from other genetic changes.

In contrast, we have demonstrated by genetic crosses and survival assays that ORF ydr014WΔ deletion does consistently confer sensitivity, although we consider this moderate rather than high. We have proposed the gene name *RAD61* for this locus, following the nomenclature of Game and Cox *(31)*, and are currently investigating the mutant phenotype in more detail. The predicted amino acid sequence of YDR014W suggests that it encodes a nuclear localized protein of 647 amino acids and molecular weight 74.7 kD, with a coiled-coil domain potentially mediating protein-protein interactions *(32, 33)*. However no interactions have been reported for this protein in high throughput two-hybrid studies *(34, 35)*. Weak similarity to several other proteins such as those encoded by *SLK19*, *MAD1*, *NUM1* and *SMC3* in *S. cerevisiae* as well as *SCP1* in *Rattus norvegicus* is seen primarily through the shared coiled-coil domains. It is intriguing that of the approximately 300 predicted coiled-coil domain-containing proteins in budding yeast, YDR014W encodes a protein most similar to those that have been shown to be involved in meiotic and/or mitotic chromosome fate *(36)*.

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REFERENCES

1. M. A. Resnick and B. S. Cox, Yeast as an honorary mammal. *Mutat Res* **451,** 1-11 (2000).

2. E. M. Taylor and A. R. Lehmann, Conservation of eukaryotic DNA repair mechanisms. *Int J Radiat Biol* **74,** 277-286. (1998).

3. S. E. Critchlow and S. P. Jackson, DNA end-joining: from yeast to man. *Trends Biochem Sci* **23,** 394-398. (1998).

4. J. A. Eisen and P. C. Hanawalt, A phylogenomic study of DNA repair genes, proteins, and processes. *Mutat Res* **435,** 171-213. (1999).

5. L. M. Kemp, S. G. Sedgwick and P. A. Jeggo, X-ray sensitive mutants of Chinese hamster ovary cells defective in double-strand break rejoining. *Mutat Res* **132,** 189-196 (1984).

6. G. Iliakis, The role of DNA double strand breaks in ionizing radiation-induced killing of eukaryotic cells. *Bioessays* **13,** 641-648 (1991).

7. C. U. Kirchgessner and J. M. Brown Radiosensitivity, DNA double-strand break repair and VDJ recombination. *In:* J.-M. H. Vos (ed.), DNA Repair Mechanisms: Impact on Human Disease and Cancer, pp. 349-373: R.G.Landes Company, 1994.

8. K. S. Ho, Induction of DNA double-strand breaks by X-rays in a radiosensitive strain of the yeast Saccharomyces cerevisiae. *Mutat Res* **30,** 327-334. (1975).

9. M. A. Resnick and P. Martin, The repair of double-strand breaks in the nuclear DNA of Saccharomyces cerevisiae and its genetic control. *Mol Gen Genet* **143,** 119-129. (1976).

10. D. Frankenberg, M. Frankenberg-Schwager, D. Blocher and R. Harbich, Evidence for DNA double-strand breaks as the critical lesions in yeast cells irradiated with sparsely or

densely ionizing radiation under oxic or anoxic conditions. *Radiat Res* **88,** 524-532. (1981).

11. T. A. Weinert and L. H. Hartwell, The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. *Science* **241,** 317-322 (1988).

12. J. A. Simon, P. Szankasi, D. K. Nguyen, C. Ludlow, H. M. Dunstan, C. J. Roberts, E. L. Jensen, L. H. Hartwell and S. H. Friend, Differential toxicities of anticancer agents among DNA repair and checkpoint mutants of Saccharomyces cerevisiae. *Cancer Res* **60,** 328-333 (2000).

13. K. K. Khanna and S. P. Jackson, DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet* **27,** 247-254. (2001).

14. J. C. Game, The Saccharomyces repair genes at the end of the century. *Mutat Res* **451,** 277-293 (2000).

15. D. C. van Gent, J. H. Hoeijmakers and R. Kanaar, Chromosomal stability and the DNA double-stranded break connection. *Nat Rev Genet* **2,** 196-206 (2001).

16. B. Vogelstein and K. W. Kinzler The genetic basis of human cancer. New York: McGraw-Hill, 1998.

17. B. S. Cox and J. M. Parry, The isolation, genetics and survival characteristics of ultraviolet light-sensitive mutants in yeast. *Mutat Res* **6,** 37-55 (1968).

18. G. Giaever A. M. Chu L. Ni C. Connelly L. Riles S. Veronneau S. Dow A. Lucau-Danila K. Anderson B. Andre, et al., Functional profiling of the Saccharomyces cerevisiae genome. *Nature* **418,** 387-391 (2002).

19. E. A. Winzeler D. D. Shoemaker A. Astromoff H. Liang K. Anderson B. Andre R. Bangham R. Benito J. D. Boeke H. Bussey, et al., Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. *Science* **285,** 901-906 (1999).

20. G. W. Birrell, G. Giaever, A. M. Chu, R. W. Davis and J. M. Brown, A genome-wide screen in Saccharomyces cerevisiae for genes affecting UV radiation sensitivity. *Proc Natl Acad Sci U S A* **98,** 12608-12613 (2001).

21. S. L. Ooi, D. D. Shoemaker and J. D. Boeke, A DNA microarray-based genetic screen for nonhomologous end-joining mutants in Saccharomyces cerevisiae. *Science* **294,** 2552-2556 (2001).

22. L. M. Steinmetz C. Scharfe A. M. Deutschbauer D. Mokranjac Z. S. Herman T. Jones A. M. Chu G. Giaever H. Prokisch P. J. Oefner, et al., Systematic screen for human disease genes in yeast. *Nat Genet* **31,** 400-404 (2002).

23. C. B. Bennett, L. K. Lewis, G. Karthikeyan, K. S. Lobachev, Y. H. Jin, J. F. Sterling, J. R. Snipe and M. A. Resnick, Genes required for ionizing radiation resistance in yeast. *Nat Genet* **29,** 426-434 (2001).

24. L. Ni and M. Snyder, A genomic study of the bipolar bud site selection pattern in Saccharomyces cerevisiae. *Mol Biol Cell* **12,** 2147-2170 (2001).

25. F. Sherman, G. R. Fink and J. B. Hicks Methods in yeast genetics. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press, 1982.

26. R. D. Gietz and R. H. Schiestl, Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. *Yeast* **7,** 253-263. (1991).

27. R. K. Mortimer, Radiobiological and genetic studies on a polyploid series (haploid to hexaploid) of Saccharomyces cerevisiae. *Radiation Res* **9,** 312-316 (1958).

28. W. Laskowski, Inaktivierungsversuche mit homozygoten hefestammen verschiedenen ploidiegrades. *Z. Naturforsch* **15b,** 495-506 (1960).

29. J. C. Game Radiation-sensitive mutants and repair in yeast. *In:* J. F. T. Spencer, D. Spencer, and A. R. W. Smith (eds.), Yeast Genetics, Fundamental and Applied Aspects, pp. 109-137. New York: Springer-Verlag, 1983.

30. C. Espinet, M. A. de la Torre, M. Aldea and E. Herrero, An efficient method to isolate yeast genes causing overexpression-mediated growth arrest. *Yeast* **11,** 25-32 (1995).

31. J. C. Game and B. S. Cox, Allelism tests of mutants affecting the sensitivity to radiation in yeast and a proposed nomenclature. *Mutat Res* **20,** 35-44 (1971).

32. M. Dardalhon, N. Magana-Schwencke and D. Averbeck, Disruption and functional analysis of six ORFs on chromosome IV: YDR013w, YDR014w, YDR015c, YDR018c, YDR020c, YDR021w (FAL1). *Yeast* **16,** 267-276 (2000).

33. A. Drawid and M. Gerstein, A Bayesian system integrating expression data with sequence patterns for localizing proteins: comprehensive application to the yeast genome. *J Mol Biol* **301,** 1059-1075 (2000).

34. T. Ito, T. Chiba, R. Ozawa, M. Yoshida, M. Hattori and Y. Sakaki, A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc Natl Acad Sci U S A* **98,** 4569-4574 (2001).

35. P. Uetz L. Giot G. Cagney T. A. Mansfield R. S. Judson J. R. Knight D. Lockshon V. Narayan M. Srinivasan P. Pochart, et al., A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. *Nature* **403,** 623-627 (2000).

36. J. R. Newman, E. Wolf and P. S. Kim, A computationally directed screen identifying interacting coiled coils from Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **97,** 13203-13208 (2000).

FIGURE CAPTIONS

Figure 1: Radiation survival curves (a) and post irradiation growth curves (b) of wild-type yeast and yeast with homozygous deletions of *RAD50, RAD51,* or *RAD52.* These data form the basis for the choice of 18 hr post irradiation as a time that would give approximately 100-fold difference between the abundance in the pool of the most sensitive strains and the strains with wild type sensitivity.

Figure 2: Scatter plot showing hybridization intensities of the 4,627 deletion strains in the untreated pool (0 Gy) versus the treated pool (200 Gy), using pooled results of three experiments. Data for most strains fall close to the line of equivalence, whereas IR-sensitive strains fall near the X axis. Seven known IR-sensitive strains are shown with arrows.

Figure 3: Representative X-ray survival curves for wild-type strain BY4743 and eight homozygous diploid deletion mutants in strain BY4743 identified as radiosensitive by the hybridization assay. Shown also on this figure is the X-ray survival curve of a homozygous *rad51Δ/rad51Δ* diploid strain (g1227) demonstrating that none of the deletion mutants are as sensitive as this strain.

Figure 4: X-ray survival curves of the haploid stains of the eight diploid deletion mutants shown in Figure 3. Shown also on this figure is the X-ray survival curve of the *rad51Δ* haploid strain demonstrating that none of the deletion mutants are as sensitive as this strain. Note that *MAT*α (BY4742) and *MATa* (BY4741) haploid strains are both shown for the ynr068C deletion, because these differed sharply. The *MAT*α ynr068CΔ strain contained a secondary mutation in the *RAD52* gene (see text).

Figure 5: Reintroduction of deleted ORF abrogates IR sensitivity in some, but not all, of the homozygous deletion strains tested. We transformed four of the newly discovered IR-sensitive

strains along with *rad18Δ*/rad18*Δ* as a positive control with vector alone (black bars) or vector containing the deleted ORF with a galactose-inducible promoter (white bars) and tested survival to a single dose of 300 Gy. The pooled results of three experiments are shown with the standard error. Below the bars are shown immunoblots for the introduced protein for yeast transformed with the empty vector or with the vector containing the ORF. The primers used to PCR-amplify the ORFs using DNA from the parental strain are as follows:

NPL6 HindIII forward 5`-AAGCTTATGTCAGATTCAGAGGGAG-3`, *NPL6 Xho1* reverse 5`-ACTCGAGAGCGTAGTCTGGGACGTCGTATGGGTACTAAATATATTCGTTGCA-3`. *ARP8 XhoI* forward 5`-GTGAGACTCGAGATGTCGCAAGAAGAAGCAGAATC-3`, *ARP8 NheI* reverse 5`-CTCTCAGCTAGCCTACTAAGCGTAGTCTGGGACGTCGTATGGGTAGTACGTGAAAATACATTTAT-3`; YDR014w *XhoI* forward 5`-GTGAGACTCGAGATGAGAGCATATGGAAAGAGGG-3`, YDR014w *NheI* reverse 5`-CTCTCAGCTAGCCTACTAAGCGTAGTCTGGGACGTCGTATGGGTATATTGTAATTCTTTCACTCGTGT-3`; *SHE1 BamHI* forward 5`-CTCTCGGGATCCGATGAATGATAAACTCCAAGAAGAGC-3`, *SHE1 HindIII* reverse 5`-GTGAGCAAGCTTCTACTAAGCGTAGTCTGGGACGTCGTATGGGTACCGCCAAATAGGTCTATCACTCT

Figure 6: Radiation sensitivity of the *rad61* deletion mutant: X-ray survival curves for haploid and diploid wild-type, two independently constructed *rad61Δ* haploid and diploid strains, a heterozygous *rad61* strain and haploid and homozygous diploid *rad51* deletion strains for comparison.

TABLE 1

Ranking of the most radiosensitive deletion strains identified in three separate hybridization assays, the genes deleted in the strains, the known function of the genes and the survival of the individual strains to a single dose of 300Gy of ^{137}Cs γ-irradiation.

IR Rank	Gene	Description/Function	Exp 1	Exp 2	Exp 3	Ave[a] Ratio	SF 300 Gy
1	*RAD5*	DNA helicase in postreplication repair	1	2	1	0.101	-
2	*YNR068C*	Similarity to Bul1p ubiquitin ligase (but see text)	3	1	2	0.111	0.005
3	*RAD9*	DNA damage checkpoint	2	3	3	0.120	-
4	*DDC1*	DNA damage checkpoint	6	5	5	0.162	-
5	*MEC3*	G2-specific checkpoint with Rad17p and Ddc1p	4	4	7	0.165	-
6	*RAD59*	*RAD52* epistasis group in DNA repair	7	6	4	0.170	-
7	*MUS81*	DNA repair; interacts with Rad54p	5	7	5	0.171	0.03
8	*SAE2*	meiotic recombination protein	10	8	8	0.225	0.02
9	*RAD57*	*RAD52* epistasis group; dimer with Rad55p	11	12	9	0.256	-
10	*YKL076C*	Unknown function	8	15	14	0.275	0.18
11	*YBR099C*	Hypothetical ORF on opposite strand to MMS4	12	11	15	0.289	0.11
12	*MMS4*	Repairs alkylating agent damage	14	14	11	0.290	0.07
13	*SHE1*	Unknown function	9	17	18	0.296	0.04
14	*RAD24*	Involved in G2 checkpoint after DNA damage	15	10	16	0.302	-
15	*YDR540C*	Unknown function	13	13	19	0.316	0.14
16	*THR1*	Homoserine kinase; in threonine synthesis	16	23	17	0.343	0.18
17	*RPL20A*	60s large subunit ribosomal protein	21	22	12	0.344	0.10
18	*YDR014W*	Unknown function, renamed *RAD61*	20	9	30	0.363	0.03
19	*MMS2*	Postreplication repair; complex with Ubc13p-Rad5p	17	20	33	0.386	-
20	*NPL6*	Nuclear protein localization factor	19	24	28	0.401	0.003
21	*YML117W*	Unknown function	18	32	25	0.409	0.21
22	*RAD55*	*RAD52* epistasis group	22	28	23	0.413	-
23	*UBP8*	Putative ubiquitin-specific protease	28	26	22	0.420	0.31
24	*RAD54*	DNA repair; interacts with Rad51p and Mus81p	29	27	21	0.423	-
25	*RSC1*	Chromatin remodeling	31	18	37	0.429	0.14
26	*ARP8*	Actin-related protein	24	19	48	0.432	0.05
27	*GPX2*	Glutathione peroxidase	23	34	31	0.443	0.17
28	*UBR1*	Ubiquitin-protein ligase	43	29	20	0.446	0.29
29	*RDH54*	Mitotic diploid-specific recombination and repair	36	16	54	0.459	-
30	*RAD17*	DNA damage checkpoint required for G2 arrest	32	35	32	0.461	-
31	*YLR426W*	Alcohol dehydrogenase family	39	21	39	0.466	0.19
32	*YPL208W*	Unknown function	27	33	46	0.472	0.29
33	*IDP1*	NADP-specific isocitrate dehydrogenase	26	46	40	0.489	-
	BY4743	Wild type	-	-	-	-	0.40

[a] Ratio of the hybridization signals for each strain in the irradiated/unirradiated samples averaged over 3 experiments.

Fig 1

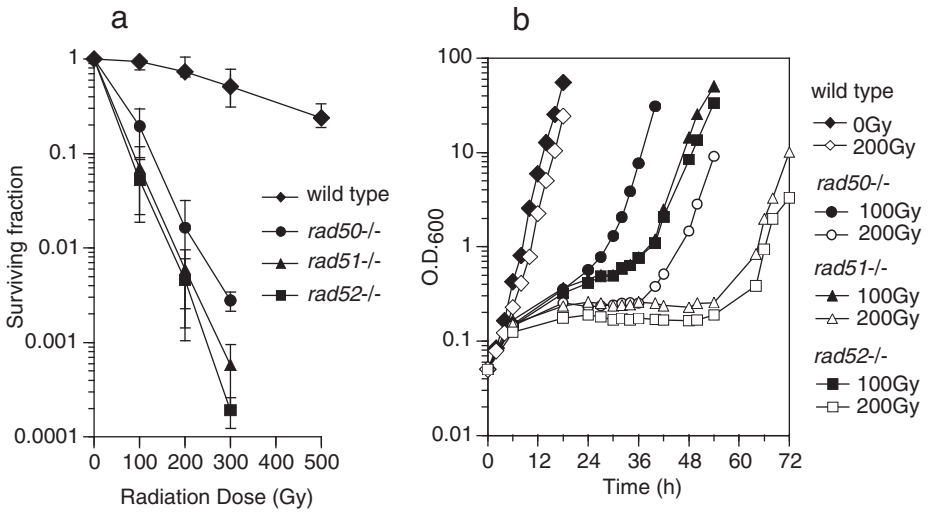

a
Surviving fraction
1
0.1
0.01
0.001
0.0001
0 100 200 300 400 500
Radiation Dose (Gy)
wild type
rad50-/-
rad51-/-
rad52-/-
b
O.D.600
100
10
1
0.1
0.01
0 12 24 36 48 60 72
Time (h)
wild type
0Gy
200Gy
rad50-/-
100Gy
200Gy
rad51-/-
100Gy
200Gy
rad52-/-
100Gy
200Gy

Fig 2

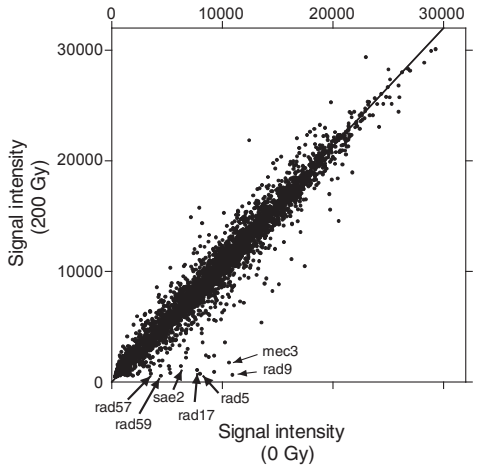

0
10000
20000
30000
30000
20000
10000
0
Signal intensity
(200 Gy)
mec3
rad9
rad57
rad59
sae2
rad17
rad5
Signal intensity
(0 Gy)

Fig 3

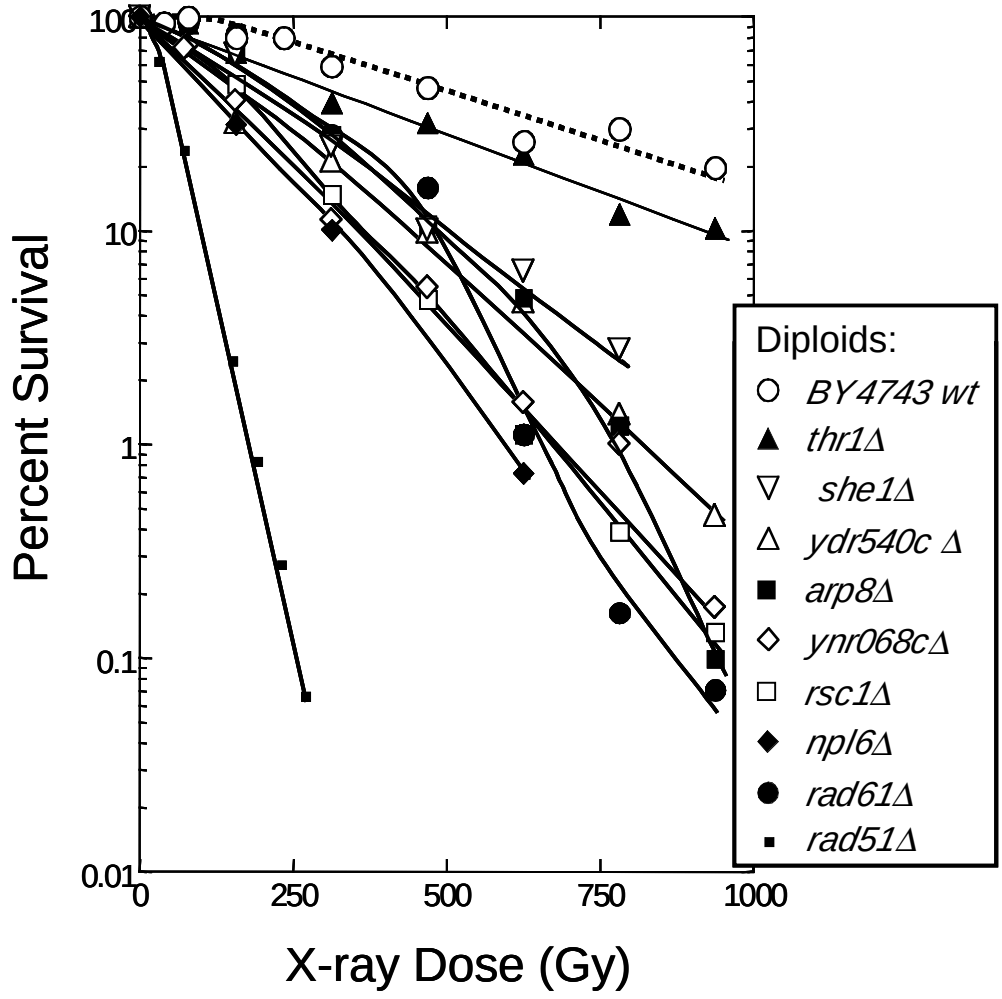

Percent Survival
100
10
1
0.1
0.01
0
250
500
750
1000
X-ray Dose (Gy)
Diploids:
BY4743 wt
thr1Δ
she1Δ
ydr540c Δ
arp8Δ
ynr068cΔ
rsc1Δ
npl6Δ
rad61Δ
rad51Δ

Fig 4

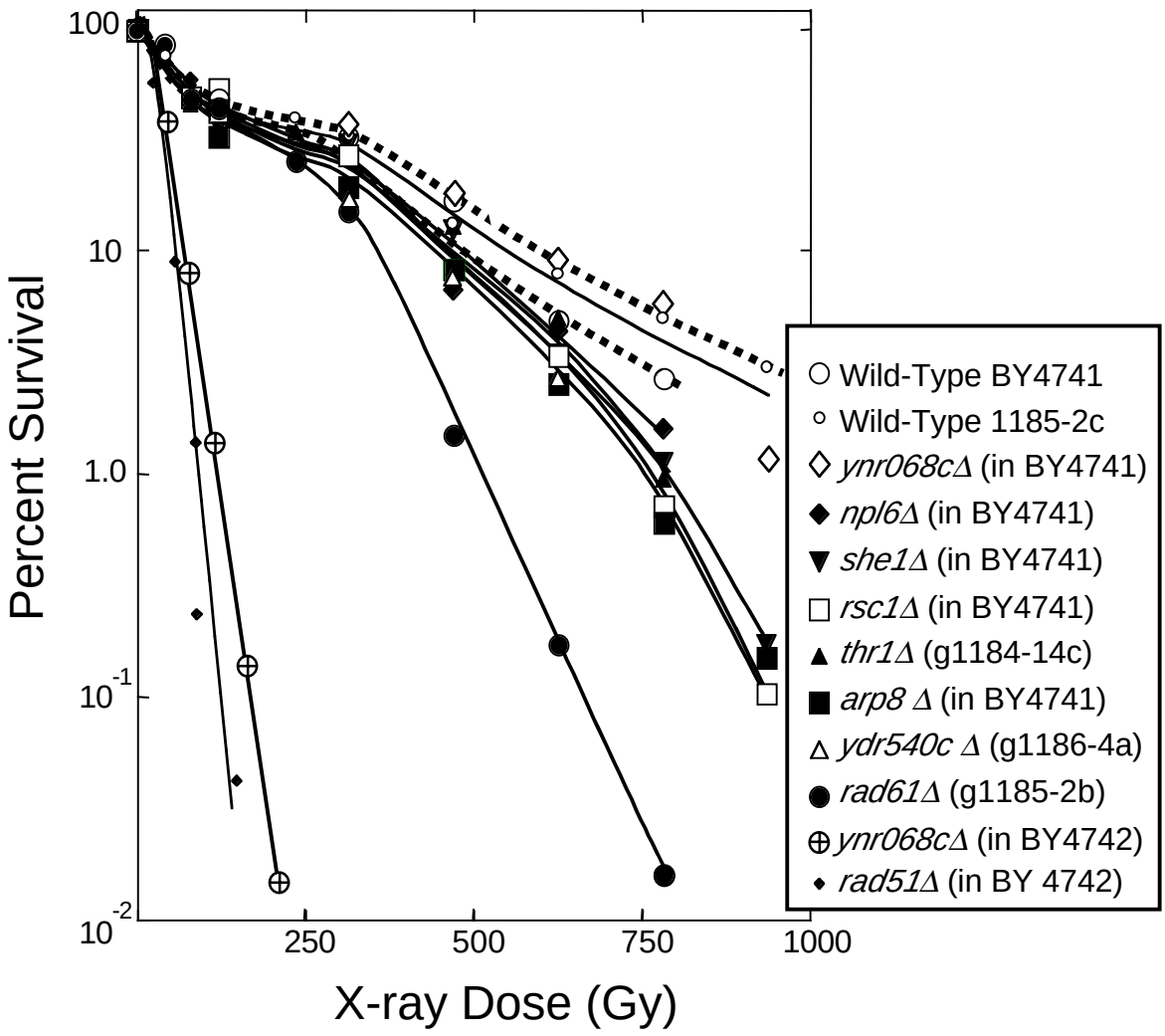

100
10
1.0
10^{-1}
10^{-2}
Percent Survival
250
500
750
1000
X-ray Dose (Gy)
Wild-Type BY4741
Wild-Type 1185-2c
ynr068cΔ (in BY4741)
npl6Δ (in BY4741)
she1Δ (in BY4741)
rsc1Δ (in BY4741)
thr1Δ (g1184-14c)
arp8 Δ (in BY4741)
ydr540c Δ (g1186-4a)
rad61Δ (g1185-2b)
ynr068cΔ (in BY4742)
rad51Δ (in BY 4742)

Fig 5

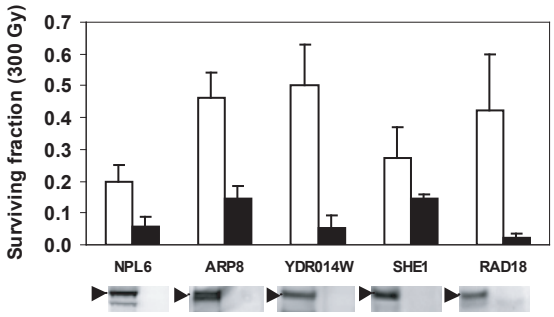
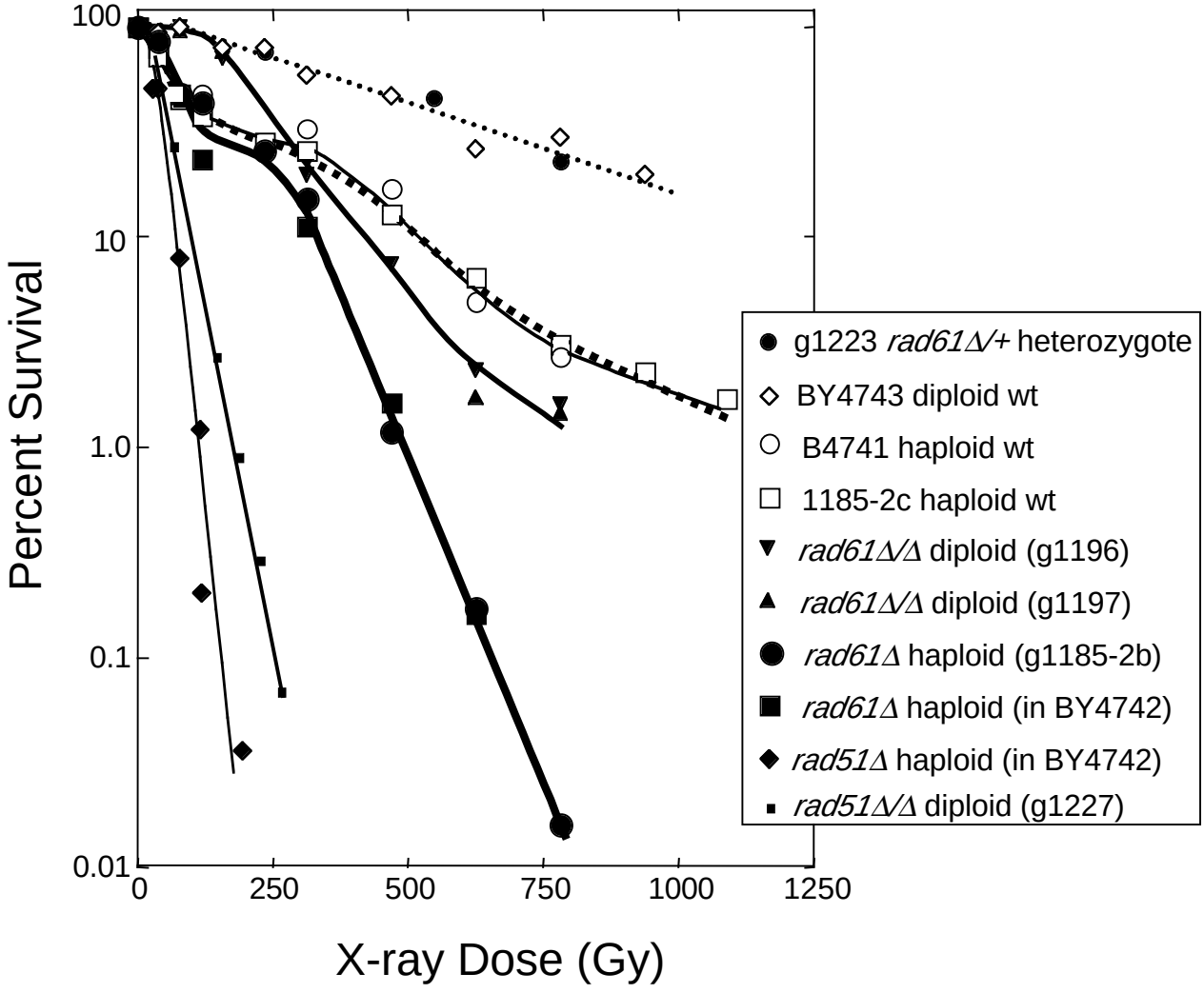

Surviving fraction (300 Gy)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
NPL6
ARP8
YDR014W
SHE1
RAD18

Fig 6


100
10
1.0
0.1
0.01
Percent Survival
0
250
500
750
1000
1250
X-ray Dose (Gy)
g1223 rad61Δ/+ heterozygote
BY4743 diploid wt
B4741 haploid wt
1185-2c haploid wt
rad61Δ/Δ diploid (g1196)
rad61Δ/Δ diploid (g1197)
rad61Δ haploid (g1185-2b)
rad61Δ haploid (in BY4742)
rad51Δ haploid (in BY4742)
rad51Δ/Δ diploid (g1227)