

Genes required for ionizing radiation resistance in yeast

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The ability of *Saccharomyces cerevisiae* to tolerate ionizing radiation damage requires many DNA-repair and checkpoint genes, most having human orthologs. A genome-wide screen of diploid mutants homozygous with respect to deletions of 3,670 nonessential genes revealed 107 new loci that influence γ -ray sensitivity. Many affect replication, recombination and checkpoint functions. Nearly 90% were sensitive to other agents, and most new genes could be assigned to the following functional groups: chromatin remodeling, chromosome segregation, nuclear pore formation, transcription, Golgi/vacuolar activities, ubiquitin-mediated protein degradation, cytokinesis, mitochondrial activity and cell wall maintenance. Over 50% share homology with human genes, including 17 implicated in cancer, indicating that a large set of newly identified human genes may have related roles in the toleration of radiation damage.

Introduction

Toleration of DNA damage requires lesion recognition and repair as well as many other DNA-associated processes that extend from replication to transcription, cell-cycle progression and chromosome segregation. Knowledge of how cells deal with DNA damage has led to increased understanding of various systems that are required for maintaining genome stability¹. *S. cerevisiae* is an ideal model organism for deducing biological processes in human cells. Many disease genes and most of the genes associated with the repair of ionizing radiation damage in mammalian cells were initially characterized in yeast^{1–3}, and this organism has served as a useful *in vivo* test tube for analyzing human and mammalian gene function¹. Completion of the human and yeast genome sequences has considerably increased opportunities to address human gene functions using yeast. Genome-wide expression analysis in response to DNA-damaging agents⁴ as well as other environmental perturbations^{5,6} is rendering yeast an even more valuable model organism.

Among the many types of damage induced by ionizing radiation, the most biologically relevant lesions are DNA double-strand breaks (DSBs). Approximately 30 genes have been implicated in the repair of radiation-induced DSBs by recombination and/or nonhomologous end-joining repair (NHEJ) pathways in yeast⁷. DSBs in haploid yeast are repaired primarily by homologous recombination between sister chromatids in the S or G2 phase of the cell cycle; in diploid cells, recombinational repair can also occur between homologous chromosomes throughout the cell cycle. The NHEJ pathway, while a relatively minor pathway for repair of radiation-induced DSBs in yeast, is responsible for the repair of most DSBs induced in mammalian cells⁸.

The Yeast Genome Deletion Project⁹ recently created sets of isogenic haploid and diploid strains containing deletions of nonessential genes. A haploid set has already been used to identify new genes affecting rapamycin resistance¹⁰. In a similar manner, we sought to identify new genes that might be associated with toleration of ionizing radiation damage in homozygous diploid mutants; all previous screens for yeast mutants that confer sensitivity to γ -rays were carried out in haploids. As opportunities for recombinational repair are optimal in diploid yeast and mating-type status affects DNA repair capabilities and the effects of a DSB^{11–13}, we anticipated that additional genes affecting sensitivity to ionizing radiation might be detected in a screen of nonessential genes in homozygous diploid mutants. Using this approach, we have increased by nearly three-fold the number of genes known to affect toleration of γ -ray damage. Most loci represent new functional classes of genes having new categories of cross-sensitivities to other DNA damaging agents.

Results

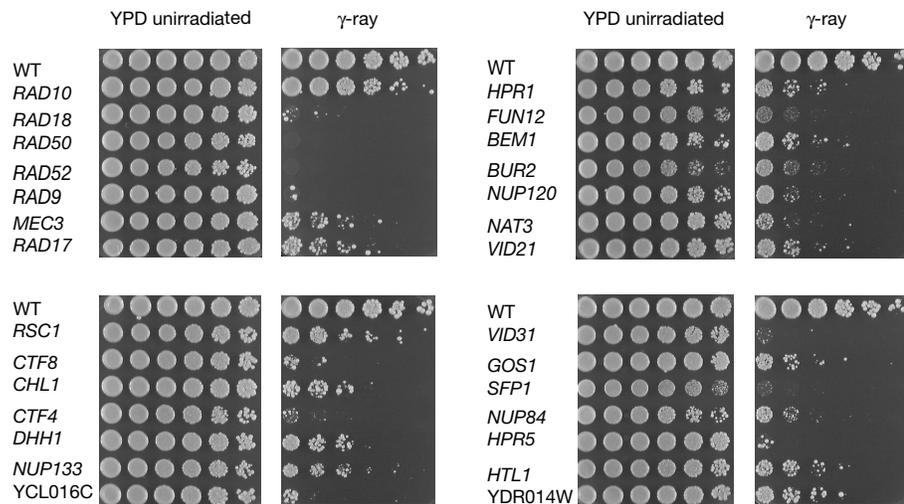
Identification of new genes responsible for γ -radiation resistance

We screened a set of diploid yeast strains homozygous for deletion mutations in nonessential genes or predicted open reading frames (ORFs; Research Genetics, Release I; <ftp://ftp.resgen.com/pub/deletions>) for growth on nutrient-rich yeast extract/peptone/dextrose (YPD) medium after exposure to 80 Krad of γ -irradiation. We confirmed putative γ -ray-sensitive mutants by replica plating serial dilutions of the strains onto YPD medium and exposing them again to 80 Krads (Fig. 1). We then evaluated directly survival responses by determining the reduction in colony-forming ability after irradiation.

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Fig. 1 γ -ray sensitivity of genes required for toleration of ionizing radiation damage. Rad⁺ (WT) and diploid deletions of well-characterized members of the *RAD3*, *RAD6* and *RAD52* epistasis groups of repair genes, checkpoint genes and genes involved in hyper-recombination are compared to newly identified gene deletions that confer sensitivity to γ -irradiation. Cells were grown for 48 h in 200 μ l YPD medium, serially diluted (left to right) by 5-fold dilutions in YPD medium, replica plated to YPD plates and then γ -irradiated (80 Krads) and incubated for 48 h at 30 °C. *RAD10* was classified as 'S' and the other deletions depicted here as 'SSS', where 'S' indicates radiation-induced reductions in colony-forming ability that correspond to one dilution (approximately 5-fold), 'SS' corresponds to two dilutions and 'SSS' to three or more dilutions.



Of the available 3,670 mutants (Methods), 130 showed various levels of γ -ray sensitivity (S, SS or SSS; Web Table A and Fig. 1) and another 8 mutants had a delayed recovery response (S/R). All of the newly identified mutants showed decreased survival on the basis of colony-forming ability (data not shown). For example, the surviving fractions ranged from less than 0.01% to 29% after exposure to 80 Krads, whereas the average for the wild type was 47%. The ability to identify some mutants with modest decreases in survival using replica plating may be enhanced by a combination of increased cell death and slower post-irradiation growth. With the exception of the *ERG3* deletion mutant (70% survival), all of the S/R strains also showed some degree of radiation-induced death. The S/R mutants were included because they were subsequently shown to be markedly sensitive (SS or SSS) to at least one other DNA-damaging agent (Web Table A). None of the mutants seemed respiration deficient (petite), as they grew on plates containing glycerol as the sole carbon source (YPG; data not shown). Some mutants may not have been detected because of errors in the production of haploid deletion mutants¹⁴.

Of the 130 radiation-sensitive mutants, 23 corresponded to previously identified genes associated with some type of DNA repair and/or checkpoint responses, including members of the *RAD52*, *RAD6* and *RAD3* epistasis groups of DNA-repair genes (Table 2 and Web Table A). Although *APN1* has not previously been implicated in the repair of γ -ray damage, it is required for the repair of apurinic sites resulting from damage caused by ionizing radiation¹⁵. We also identified *RAD1* and *RAD10*, which have a role in nucleotide-excision repair of UV-induced lesions. Haploid mutants of these three genes have not been shown previously to have appreciable γ -ray sensitivity¹⁶. This study has thus revealed 107 new genes (excluding *APN1*, *RAD1* and *RAD10*) that affect the γ -irradiation survival response in diploid cells as well as 8 genes that affect the post-irradiation growth response.

Of the 115 newly identified mutants (107 γ -ray-sensitive and 8 S/R mutants), 87 correspond to genes for which a function or genetic role has been suggested or experimentally determined (*Saccharomyces* Genome Database, SGD; <http://genome-www.stanford.edu/Saccharomyces/>). Most of these genes (71 of 87) could be placed into nine functional groups containing at least five members (Web Table A). Three ORFs were placed into functional groups based on predicted homology with previously characterized proteins. The protein products comprising these nine functional groups are associated with: (i) chromatin remodeling, silencing or telomere function (ii) stable chromosome

transmission during mitosis (iii) structural elements of the nuclear pore complex (iv) transcription and mRNA stability (v) Golgi/vacuolar activities (vi) ubiquitin-mediated protein degradation (vii) components of the cytoskeleton/spindle apparatus required for cytokinesis (viii) mitochondrial function or (ix) the cell wall and heat-shock stress responses. Representatives of some groups may have several functions and can also be placed in other functional groups (see the SGD). For example, the transcription factor *Anc1* has a role in chromatin remodeling as well as a cytoskeleton function^{17,18} and the translation initiation factor *Pat1* participates in mRNA stability and is required for chromosome transmission^{19,20}. Before our study, the genes in these functional groups had not been described as affecting responses to ionizing radiation; our approach has thus identified new processes and targets that affect radiation responses.

Ploidy and MAT regulation can affect the γ -ray sensitivity of many mutants

As most loci were not identified in previous screens of haploid mutants, we examined the radiation sensitivity of the haploid parents that gave rise to the 20 most sensitive (SSS) diploids as well as other mutants, such as those affecting checkpoints (Table 1). Unexpectedly, the haploids were generally more resistant than the diploids, even though haploid G1 cells do not carry out recombinational repair processes because they lack a homolog for the genes needing repair. Both haploid parents were highly γ -ray sensitive (SS or SSS) for only 4 of the 20 mutants examined. Moreover, the mutant *MAT α* parents were generally more resistant than the *MAT α* haploids. Similar to our observations, the characteristics of *HPR5* (*SRS2*) and *SGS1* mutants also indicates that mating type and ploidy can greatly affect resistance to γ -ray-induced death²¹. Thus, many of the genes might not have been detectable in previous haploid screens⁷. Mating type and ploidy also affect global expression patterns as determined using microarray analysis²².

Cross-sensitivity to other DNA-damaging agents reveals new categories of γ -ray resistance genes

We examined the 130 radiation-sensitive mutants and the S/R mutants for cross-sensitivity to the following DNA-damaging agents (Table 2 and Web Table A): ultraviolet light (UV), the radio-mimetic chemical bleomycin, the methylating agent methyl methanesulfonate (MMS), the DNA replication inhibitor hydroxyurea (HU) and the topoisomerase I inhibitor camptothecin (CAMP). We grouped the mutants into 22 profiles (A–V) on the

basis of their sensitivities to these agents. Among the previously reported γ -ray-sensitive mutants (Table 1 and Web Table A), all were sensitive to UV and over half (11/19; Table 2, category A) were sensitive to all agents tested. Of the new mutants, however, only half were UV sensitive (categories A–G). Nearly one-quarter of the radiation-sensitive mutants were resistant to bleomycin (categories H–N), even though this anti-neoplastic drug causes DSBs. Radiation-sensitive, bleomycin-resistant mutants have not been reported, which indicates that there may be differences between the two agents in the spectrum of damage and subsequent processing of lesions for repair.

Mutants of the *RAD52* recombinational repair group are sensitive to all DNA-damaging agents examined (Table 2, category A). Included in this category are 21 of the newly identified genes, of which 11 are also associated with the greatest sensitivity (SSS) to γ -rays. The similarity of their response to that of *RAD52* mutants indicates that these genes may also be associated with recombinational repair of DSB damage. Four undesigned ORFs were initially assigned to this category: YCL016C, YDR433W, YLR235C and YLR320W. Because the YDR433W ORF overlaps another gene (*NPL3*) and the YLR235C deletion produces a truncation of *TOP3*, we could not assign them a unique function. Deletion of YCL016C or YLR320W resulted in low survival rates after γ -irradiation (2.9% and 11%, respectively, at 80 Krad), whereas the deletions resulted in less sensitivity to UV (34% and 30% survival, respectively, for the mutants and 42% for the wild type after a 60 J/m² exposure). We also examined the sensitivity of the *MATa* and *MAT α* haploid strains in which YCL016C and YLR320W were deleted. The haploids showed moderate sensitiv-

ity (SS; Web Table A, footnote) to γ -irradiation and slight (S) sensitivity to UV. The haploid strains with YLR320W deleted showed extreme sensitivity (SSS) to MMS and HU, whereas both the wild type and those with YCL016C deleted did not. We therefore assigned the name *MMS22* to YLR320W. A YCL016C mutant recently found to be defective in chromatid cohesion was named *DCC1* (ref. 23).

Newly identified γ -ray resistance genes affect recombination

The deletion of genes in the *RAD52* epistasis group results in extreme γ -ray sensitivity due to reduced recombinational capability^{1,7}. We therefore examined the recombination capability of 19 of the most severely inhibited (SSS) new mutants, including 9 from category A (Table 3; *FUN12* was not included for technical reasons). Also included were *RAI1*, *MRT4* and YPL066W mutants that affect checkpoint responses, the DNA polymerase accessory factor gene *POL32* and YPL055C.

We analyzed two types of recombination. One assay measured repair of a homothallic switching (HO) endonuclease-induced DSB, whereas the other determined the capacity for targeted recombination at the *HIS3* locus^{24,25}. Expression of HO endonuclease from a plasmid with an inducible *GAL1-10* promoter upstream of the HO gene leads to a site-specific DSB at the *MAT* locus on chromosome III (ref. 24). Repair of the DSB occurs through either recombination between homologous chromosomes or intra-chromosomal gene conversion using the *HML* or *HMR* loci as donors. Both processes require the participation of the *RAD52* group of repair genes.

As expected, overexpression of HO severely inhibited the growth of *rad50 Δ* and *rad51 Δ* diploid mutants (Table 3). Only four SSS mutants, *nup84 Δ* , *dhh1 Δ* , *ctf4 Δ* and *ctf8 Δ* , and the checkpoint mutant *mrt4 Δ* (Fig. 3) had growth inhibition after HO expression (Table 3), indicating that the other mutants are not severely defective in this type of recombinational repair. We also examined the mutants for their ability to target recombination when transformed with a *his3* PCR fragment that generates a functional *HIS3* through recombination with a chromosomal *his3 Δ 1* allele. We classified recombinational capability as wildtype, deficient (similar to that of the *rad50 Δ* or *rad51 Δ* mutants) or enhanced (similar to that of the *hpr1 Δ* mutant). Only the *nup84 Δ* and *chl1 Δ* strains showed significant reductions in targeted recombination (Table 3). Thus, the defects leading to hypersensitivity (SSS) in general do not result from deficiencies in recombinational repair. Among γ -radiation-sensitive mutants, hyper-recombination phenotypes (*hpr1*, *hpr5* and *rad27*)

Table 1 • Comparison of sensitivities to γ -irradiation between highly sensitive (SSS) and other diploids and their haploid deletion parents

Strain	Haploid <i>MATα</i>	Haploid <i>MATa</i>	Diploid <i>MATa/MATα</i>
wildtype	R	R	R
Newly identified γ -sensitive mutants that are SSS as homozygous diploids			
<i>BEM1</i> *	S/R	S	SSS
<i>ANC1</i>	SSS	S	SSS
<i>BUR2</i>	SSS	R	SSS
<i>NUP120</i>	SS	S	SSS
<i>VID31</i>	SS	SS	SSS
<i>NUP133</i>	SS	S	SSS
<i>RSC1</i>	S	S	SSS
<i>GOS1</i>	R	S	SSS
<i>NAT3</i>	SSS	SSS	SSS
<i>CTF4</i>	SS	S	SSS
<i>GRR1</i>	S/R	R	SSS
<i>NUP84</i>	SS	S	SSS
<i>DHH1</i>	SS	S	SSS
YCL016C	SS	SS	SSS
<i>CHL1</i>	SS	S	SSS
<i>VID21</i>	SSS	SS	SSS
<i>FUN12</i>	R	SS	SSS
<i>HTL1</i>	SSS	SS	SSS
YDR014W	SS	S	SSS
<i>CTF8</i>	S	S	SSS
Other mutants			
<i>BCK1</i>	R	R	S
<i>APN1</i>	SS	R	S
<i>SAC6</i>	S	S	SS
<i>MRT4</i>	S	S	SS
<i>RPB9</i>	S	R	SS
<i>RAI1</i>	S	S	S
<i>RAD9</i>	SSS	SSS	SSS
YLR320W	SS	SS	SS
YNR068C	SSS	S	SS
<i>ASF1</i>	S	R	SS

*Bold lettering identifies deletion strains in which sensitivity to γ -irradiation may be under the regulation of mating-type genes.



have been detected^{26,27}. The *hpr1Δ* diploids showed a large increase in targeted recombination (Table 3). The *rsc1Δ*, *bur2Δ*, *yp1055cΔ*, *mrt4Δ*, *ctf8Δ* and *ycl016cΔ* mutants also showed significant increases. The *rsc1Δ* and *bur2Δ* mutants were similar to *hpr1Δ* in that HO expression did not inhibit growth (Table 3), unlike the *ctf8Δ*, *mrt4Δ* and *ycl016cΔ* mutants. These results indicate that unlike the *RAD52* group of genes, *RSC1*, *CTF8*, *BUR2*, *MRT4*, *YCL016C* and *YPL055C* may have a more indirect role in recombinational processes. The *YPL055C* gene was previously found to induce the SOS repair system when overexpressed in *Escherichia coli*²⁸. *MRT4* is a newly identified checkpoint gene (Fig. 2) that also results in cell-cycle perturbation after overexpression²⁹.

Many genes affect spontaneous and DNA-damage checkpoint responses

As cell-cycle checkpoint mutants such as *RAD9* are sensitive to γ -irradiation, we examined all the newly discovered γ -ray-sensitive mutants for checkpoint defects in response to irradiation (Web Table A). After logarithmically growing wildtype cells were exposed to 6 Krad and resuspended in YPD medium, there was an approximate two-fold increase (from 15–20% to approximately 40–45%) in G2/M cells in the population (Web Table A, Fig. 2a and data not shown), whereas checkpoint mutants

showed only a small increase in G2/M cells (<30% of the cells; Fig. 2a and Web Table A). Recombination-deficient mutants (such as the *RAD52* epistasis group) typically showed a dramatic increase (=90%) in the number of cells in G2/M due to the presence of unrepaired damage. Of the 107 new radiation-sensitive mutants, nearly half were altered in cell-cycling and DNA-damage checkpoint responses: 30 showed increased G2/M arrest after irradiation, whereas 21 (including the previously identified mutant *pol32*) showed high spontaneous levels of G2/M cells (Web Table A). The latter phenotype suggests that these cells have elevated levels of unrepaired DNA damage during normal growth. Only *MRT4*, *RAI1*, *BCK1* and *YPL066W* mutants seemed to be checkpoint defective, as they had fewer G2/M-arrested cells after γ -irradiation (<30%; Web Table A and Fig. 2a). These were examined further by micro-manipulating freshly irradiated, unbudded cells onto YPD solid medium and following their progression. Cells were photographed hourly to identify those that passed through G2/M (large-budded stage) forming microcolonies of three or more cells^{13,30}. The *BCK1* mutant did not seem different from the wild type in this assay. Deletions of *RAI1* and *MRT4* resulted in earlier onset of microcolony formation after γ -irradiation as compared with wildtype cells (Fig. 2b). This is a hallmark of checkpoint-defective mutants such as *rad9*

Table 2 • Cross sensitivity of γ -ray sensitive diploid deletion mutants to other agents.

UV	Bleo	MMS	HU	Camp	Functional Grouping	n	Gene/ORF Deletions ^a	Cat. ^b	
S ^c	S	S	S	S	Functional Grouping recombination, repair	32	RAD50, XRS2, RAD51, RAD52, RAD55, RAD57, HPR1, HPR5, MUS81, SGS1, REM50, NUP84, NUP120, NUP133, CTF4, CTF8, BUR2, FUN12, VID31, GRR1, BEM1, ASF1, HFI1, CAX4, CLC1, RPB9, RVS161, RVS167, YCL016C, YDR433W, YLR235C, YLR320W	A	
				R	replication, repair	7	RAD6, RAD18, POL32, CDC40, HTL1, GOS1, ADK1	B	
		R	S	S	?	3	CHL1, SPT10, VID21	C	
				R	?	2	MCT1, DIA4	D	
		R	S	S	checkpoint	2	SFP1, YDL151C	E	
				R	?	2	SAC6, DOC1	F	
		R	R	R	chromatin?	5	ARD1, YDL115C, YEL072W, YLR322W, YMR293C	G	
					mRNA localization	2	LOC1, YJL188C	H	
		R	S	S	S	checkpoint	1	RAD17	I
					R	DNA repair	5	RAD1, RAD27, MMS2, UBC13, LIP5	J
	R				transcription	1	CCR4	K	
	R		S	R	kinase	1	PFK2	L	
				S	checkpoint	1	MEC3	M	
	R		S	S	S	S	checkpoint, repair	4	RAD9, RAD10, RSC2, RTF1
R		cytoskeleton, meiosis				3	CNM67, UME6, AKR1	O	
R		repair, transcription				6	APN1, DHH1, ANC1, PAT1, VPH2, REF2	P	
R		R		R	chromatin	6	RSC1, NAT3, BCK1, JEM1, HOF1, YDR014W	Q	
				S	cell wall	2	CWH36, YLR435W	R	
R		R		R	?	23	NUP170, EST1, SCP160, BIK1, MNN11, GRF10, CKB1, BFR1, MRPL31, IMG2, FIL1, TOM37, PDR13, LHS1, SMI1, ZUO1, ADO1, GND1, SHE1, SSE1, YML013C-A, YML014W, YNR068C	S	
					S	nuclear pore, vacuolar	5	NUP188, VMA7, YBL006C, YGR165W, YPL066W	T
					R	checkpoint	3	MRT4, RAI1, RSA1	U
R		R		R	?	14	UBR1, PRE9, RSM7, HSP150, CIS3, IDS2, PFD1, YEL033W, YHR081W, YJL193W, YML010C-B, YML010W-A, YPL055C, YPL071C	V	

^aGenes that have a known role in recombination, repair of DNA damage or DNA damage checkpoint function are in bold. ^bCat. = categories: Damage response genes groupings based on sensitivities to other agents. S or R: Sensitivity or resistance relative to WT.

(ref. 30). The YPL066C deletion mutant differed from the other checkpoint mutants in that it showed a delayed onset of budding (that is, a prolonged time in G1) which resulted in the delayed appearance of microcolonies (Fig. 2*b*). We did not see a prolonged G1 delay in nonirradiated cells (data not shown).

In light of our recent observation with a haploid strain¹³ that mating-type regulation can affect adaptation (defined as re-entry into the cell cycle after induction of DNA damage³¹), we examined the impact of the *rai1Δ*, *mrt4Δ* and *bck1Δ* mutations on the cell-cycle progression of haploid cells after exposure to 6 Krad. Unlike the diploids, the haploid mutants showed prolonged G2 arrest, indicating a possible adaptation defect (Fig. 2*c*). A few cells eventually adapted to the damage and were able to form small microcolonies (Fig. 2*d,e*). This unexpected result suggests that mating-type regulation can influence the affect of these genes on cell-cycle arrest in opposite ways. They are part of the adaptation process in haploids (possibly by attenuating the checkpoint response), whereas in diploids they cause cell-cycle arrest (part of the checkpoint response mechanism). Deletion of *BCK1* in both haploids (Fig. 2*d,e*) and diploids (data not shown) led to a high frequency of lysed cells after radiation-induced G2/M arrest, which was not seen in nonirradiated cells.

Discussion

Yeast has often been the organism of choice for investigating the genetic control of responses to ionizing radiation-induced damage. Our functional genomic screen for radiation sensitivity with homozygous diploid deletion mutants has increased by

more than three-fold the number of genes known to affect resistance to γ -ray damage. As yeast genetic screening is useful for identifying functionally related human genes, the present approach will help both in understanding the totality of the damage response and in characterizing likely mammalian orthologs. This screen has also revealed additional genetic controls of responses to other agents (Web Table A). Many of the genes identified do not have obvious ties with known mechanisms of protection against radiation-induced lethality; however, several gene groups are implicated indirectly through mechanisms of mRNA and protein stability and/or trafficking (Fig. 3). As the γ -ray sensitivity of many mutants seemed to be regulated by *MAT* in a manner similar to that described for *SGS1* (ref. 21), *RAD18* (ref. 12) and *RAD55* (ref. 11), future screens against other agents should take this into account.

Among the 130 radiation-sensitive mutants identified, 23 were previously shown to affect survival after exposure to DNA damage. Most of these include members of the *RAD52*, *RAD6* and *RAD1* epistasis DNA repair groups, with the remaining mutants affecting damage-responsive cell-cycle checkpoints, although they may also have more direct effects on recombination³². All previously identified genes associated with DSB recombinational repair and checkpoint controls in haploid cells were isolated in the present screen for radiation-sensitive diploid cells (Web Table A). *RAD54*, *DDC1* and *RAD24* are among approximately 1,200 nonessential genes not included in this screen (Web Table A). Several of the new genes may influence homologous recombination, although the mechanisms are not clear.

Table 3 • Toleration of an HO-induced DSB and target recombination in highly sensitive and other γ -ray-sensitive mutants

Gene/ORF deletion	Functional defect	γ -ray sensitivity ^a	HO growth inhibition ^b	Targeted recombination at <i>his3ΔT</i> ^c
none	wildtype	R	no	1.0 ± 0.7
<i>RAD50</i>	repair	SSS	yes	0.11 ± 0.003
<i>RAD51</i>	repair	SSS	yes	0.016 ± 0.005
<i>HPR1</i>	recombination	SSS	no	24 ± 10
<i>HPR5</i>	recombination	SSS	yes	2.0 ± 0.6
<i>SFP1</i>	checkpoint	SSS	no	1.8 ± 0.5
<i>NUP84</i>	nuclear pore	SSS	yes	0.081 ± 0.006
<i>NUP120</i>	nuclear pore	SSS	no	6.1 ± 3.8
<i>NUP133</i>	nuclear pore	SSS	no	3.4 ± 1.7
<i>DHH1</i>	helicase	SSS	yes	0.29 ± 0.17
<i>RSC1</i>	chromatin	SSS	no	7.9 ± 4.9
<i>NAT3</i>	chromatin	SSS	no	0.53 ± 0.30
<i>CHL1</i>	chromosome stability	SSS	no	0.18 ± 0.04
<i>CTF4</i>	chromosome stability	SSS	yes	0.85 ± 0.48
<i>CTF8</i>	chromosome stability	SSS	yes	2.8 ± 0.7
<i>GOS1</i>	endocytosis	SSS	no	0.83 ± 0.64
<i>VID21</i>	endocytosis	SSS	no	1.5 ± 1.2
<i>VID31</i>	endocytosis	SSS	no	0.60 ± 0.53
<i>GRR1</i>	ubiquitin degradation	SSS	ND ^d	1.5 ± 0.8
<i>BUR2</i>	transcription	SSS	no	6.4 ± 1.7
YCL016C	chromosome stability (<i>DDC1</i> , see text)	SSS	yes	6.0 ± 0.5
YDR014W	unknown	SSS	no	1.7 ± 1.0
<i>RAI1</i>	mRNA stability	S	no	4.6 ± 4.3
<i>MRT4</i>	mRNA stability	SS	yes	5.5 ± 1.7
<i>BEM1</i>	cytokinesis	SSS	no	1.5 ± 1.3
YPL055C	SOS-inducing	S	no	6.0 ± 4.3
YPL066W	checkpoint	S	no	0.38 ± 0.06
<i>ANC1</i>	transcription factor	SSS	no	1.0 ± 0.3
<i>HTL1</i>	high temperature lethal	SSS	no	3.5 ± 0.4
<i>POL32</i>	polymerase accessory factor	SS	no	0.28 ± 0.11

^aData taken from Web Table A. ^bCells containing plasmid pJH132 (containing *GAL:HO*) were grown in liquid SC + glu + ura overnight, diluted 10-fold and replica plated to SC + gal + ura or SC + glu + ura to induce HO. Cells were scored 2 d later for relative growth on gal vs. glu. ^cA functional *HIS3* could occur only by targeted integration of the PCR fragment into the genomic *his3* sequences. Ratios have been normalized to the transformation efficiencies of the covalently closed circular plasmid pRS315. Ratios are the means of three to nine replica transformations. Errors are ± 1 s.e.m.. Ratios in bold indicate results significantly different from wildtype. ^dND, not determined (as cells grow very poorly on galactose-containing medium).



Among the newly identified genes, nine new functional groups were identified whose mutants could influence radiation sensitivity. These correspond to over 80% (71/87) of the genes identified for which a function has been assigned. The functions of the genes in these groups are based on experimental results summarized in the Yeast Protein Database (YPD; www.proteome.com), which provides a literature summary for each mutant. In some cases, the genes are associated with more than one functional group; thus, the functional significance regarding radiation resistance may be ambiguous.

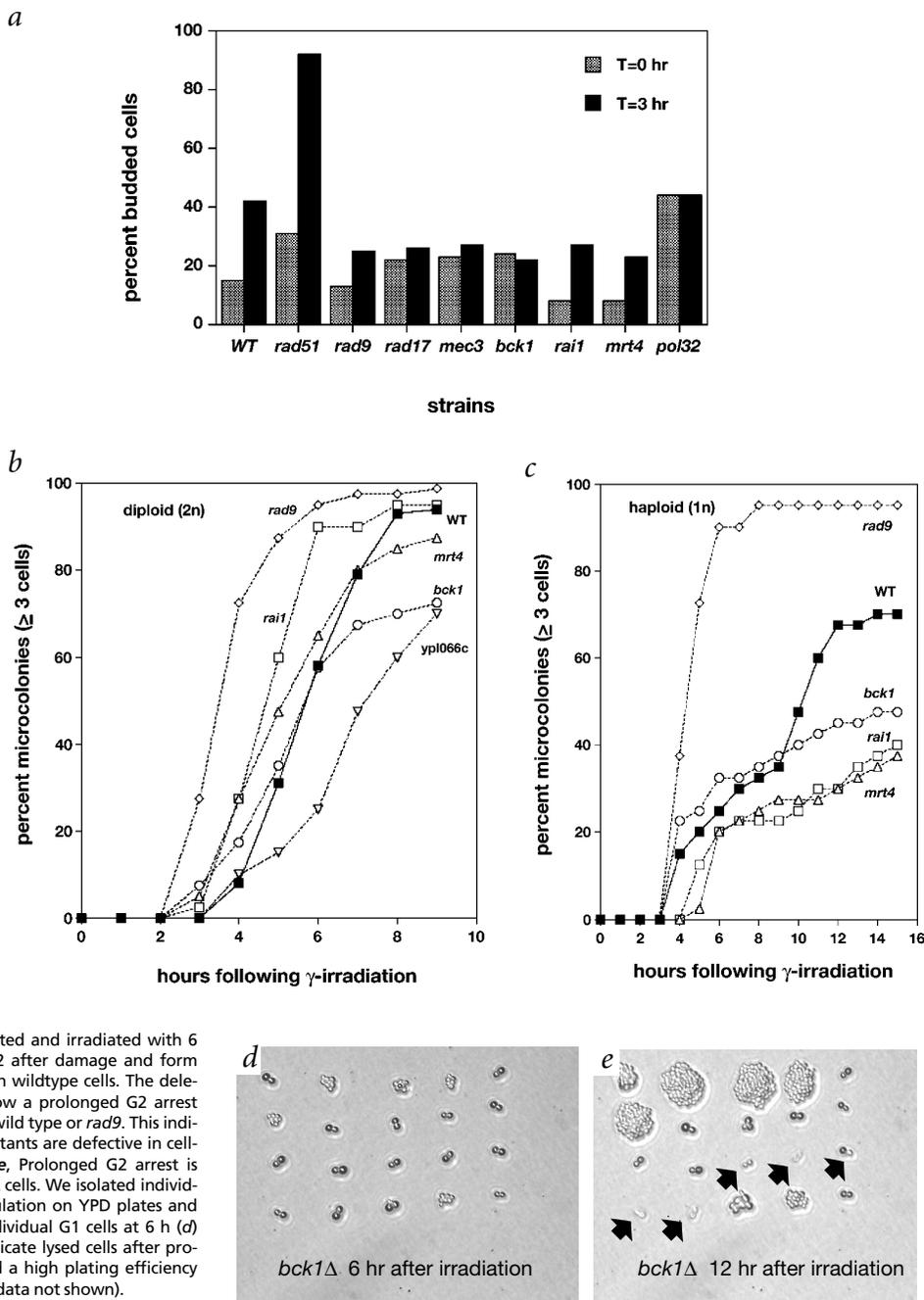
The involvement of chromatin organization and segregation proteins could be anticipated based on processes associated with repair and cell-cycle response. The reasons for the sensitivity among the mitochondrial activity, cytokinesis and cell-wall maintenance groups are less clear. Orderly mitochondrial func-

tion or modulation of radical production may be important after irradiation. The latter two groups may also correspond to sensitive structural and organelle targets.

A common feature among the four remaining functional groups (corresponding to roughly 30 genes, as some have overlapping functions) is their ability to affect protein metabolism through transcription, trafficking (nuclear pore formation), translation and degradation. An additional 5–10 genes relate to protein metabolism and stabilization, suggesting that nearly half of the newly identified genes involve protein production and stability.

Given the large number of genes that affect protein metabolism, we suggest that many involve proteins required for known DNA metabolic processes including repair, replication, recombination and checkpoint controls (Fig. 3). Amounts of some repair proteins must be maintained, induced or decreased, and they

Fig. 2 Characterization of radiation-sensitive mutants. **a**, Initial identification of new genes involved in checkpoint functions after DNA damage. Percentages of cells that were large budded (in the G₂/M phase of the cell cycle) were determined for nonirradiated and γ -irradiated diploid cells in liquid (YPD) cultures. Cells were diluted and examined 3 h after irradiation (6 Krads). Shown are the results for Rad⁺ (wildtype), a recombination repair-deficient deletion mutant (*rad51*), and for deletion mutants known to be required for G₂ arrest after damage (*rad9*, *rad17* and *mec3*). We identified four new putative checkpoint deletion mutants, *bck1*, *rai1* and *mrt4* (ypl066C not shown), by virtue of their similarity to known checkpoint mutants. Another deletion mutant (*pol32*) is typical of those that show a high constitutive level of G₂ arrest with no enhanced arrest after damage. **b**, Early onset of microcolony formation in the putative diploid checkpoint mutants *mrt4* and *rai1* after γ -irradiation. Cell-cycle progression of individual G₁ cells of the putative diploid checkpoint deletion mutants *bck1*, *rai1*, *mrt4* and ypl066C were compared to Rad⁺ and the *rad9* checkpoint mutant after γ -irradiation. Cells were plated and irradiated with 6 Krads of γ -irradiation. The *rad9* cells do not arrest at G₂ after damage and form three-cell microcolonies earlier than wildtype cells. The *rai1*, *mrt4* and, to a lesser extent, *bck1* mutants all show a reduced period of time in G₂ arrest after damage when compared with the wild type. **c**, Delayed onset of microcolony formation in the haploid MAT α checkpoint deletion mutants *bck1*, *mrt4* and *rai1* after γ -irradiation. We compared the cell-cycle progression of individual G₁ cells of the putative checkpoint deletion mutants *bck1*, *rai1* and *mrt4* with Rad⁺ and the *rad9* checkpoint deletion mutant after γ -irradiation. Cells were plated and irradiated with 6 Krads. The *rad9* cells do not arrest at G₂ after damage and form three-cell microcolonies much earlier than wildtype cells. The deletion mutants *bck1*, *rai1* and *mrt4* all show a prolonged G₂ arrest after exposure when compared with the wild type or *rad9*. This indicates that, as haploids, these deletion mutants are defective in cell-cycle adaptation after DNA damage. **d,e**, Prolonged G₂ arrest is followed by cell lysis in γ -irradiated *bck1* Δ cells. We isolated individual haploid (1n) G₁ cells by micromanipulation on YPD plates and irradiated (6 Krads) them. Shown are individual G₁ cells at 6 h (**d**) and 12 h (**e**) after irradiation. Arrows indicate lysed cells after prolonged G₂ arrest. Nonirradiated cells had a high plating efficiency (>95%) and did not show enhanced lysis (data not shown).



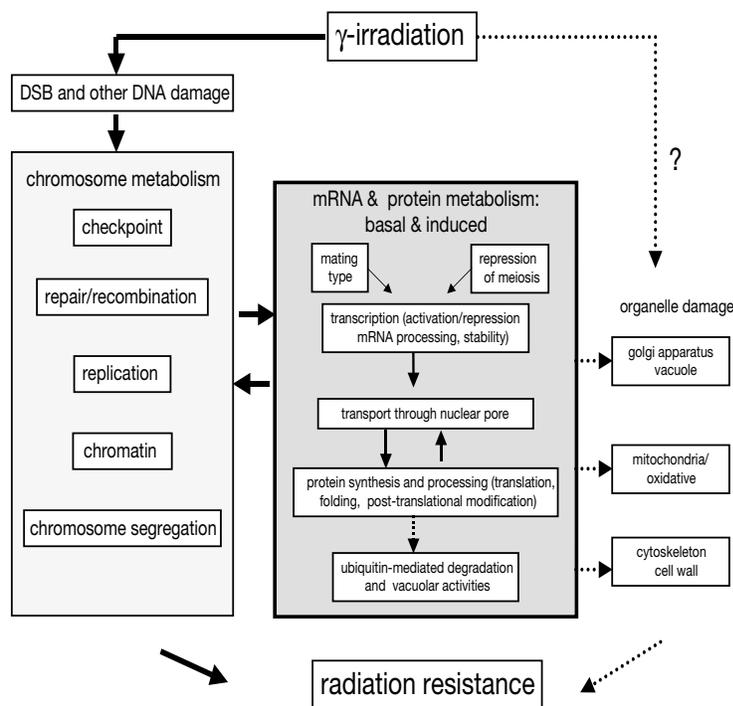


Fig. 3 Possible functional interactions of genes affecting resistance to radiation. In addition to chromosomal and repair functions, many groups of genes were identified in the present screen that are associated with some aspect of protein production and metabolism, including degradation (heavily shaded box). In addition, several genes are important in the functions of various organelles. Although defined relationships and roles in resistance are not established, this scheme shows how they might interrelate. Protein synthesis, trafficking and degradation would be important to many processes, including DNA damage-inducible responses and post-irradiation cellular responses as well as degradation of proteins induced in response to DNA damage. The organelles could be important to this processing. Radiation could also directly damage key organelles, such as Golgi apparatus, vacuoles and mitochondria. Mitochondria mutants could have either altered energy production or increased free radical production that might affect nuclear functions (G.K., L.K.L. and M.A.R., unpublished data).

and possibly kinetochore function⁴¹, suggesting a possible mechanism for the radiation sensitivity of other chromosome segregation mutants such as *CHL1* (Web Table A).

We have identified several mutants with cell-cycle checkpoint defects. Unlike wildtype diploid cells, *rai1Δ*, *mrt4Δ*, *bck1Δ* and *ypl066wΔ* mutants did not accumulate in G2/M after irradiation. Little is known about the molecular

need to be transported into the nucleus. For example, the recombinational repair protein, Rad51, has been localized to the cytoplasm; upon irradiation of cells, it is rapidly transported to the nucleus³³. After repair, the amounts may need to decline to allow cell progression, possibly by targeted degradation. Rad6 and the damage-inducible Ubc13 protein are both required for ubiquitin degradation functions as well as error-free and error-prone post-replication repair (PRR) pathways in yeast³⁴. In addition, the deletion of genes involved in vacuolar import and degradation (*VID21*, *VID31*) result in enhanced radiation-induced cell death (Web Table A). The prolonged persistence of transiently expressed, damage-inducible repair proteins such as Ubc13 may be deleterious to cell survival and could require rapid degradation after damage repair.

Although a role in radiation resistance for genes involved in endocytosis or Golgi apparatus function has not previously been reported, treatment of mammalian cells with 3-methyladenine, an inhibitor of H⁺ ATPase activity and vacuolar acidification, can sensitize cells to radiation-induced death³⁵. A similar effect may be responsible for the radiation sensitivity of *VPH2* or *VMA7* mutants (Web Table A), as both genes are required for H⁺ ATPase function and vacuolar acidification in yeast (see the YPD).

Proteins associated with critical structures may represent additional targets for radiation damage. Ionizing radiation is capable of producing both direct and indirect damage to protein and membrane structures of cells and viruses^{36–38}. Moreover, drugs such as benomyl do not directly damage DNA but exert a lethal effect through the disruption of tubulin and cytoskeletal functions, resulting in chromosome malsegregation³⁹. The sensitivity of a number of mutants with deletions of genes affecting cytoskeletal functions indicates that radiation-induced lethality may result from damage to the sensitized protein components of these large structures. Tethering of chromosomes to the nuclear envelope by Nup and/or Mlp proteins⁴⁰ may be important to radiation resistance. The tethering could involve telomere structures associated with silencing and telomere length maintenance, such as the DSB repair protein Ku70 and Rap1. Notably, Nup170 (Web Table A) has recently been shown to be required for chromosome segregation

functions of *RAI1* and *MRT4*, except that both seem to affect the stability of mRNA (functions summarized in the YPD). Bck1 shares homology with the Chk1 checkpoint proteins of *Schizosaccharomyces pombe* (BLASTP analysis: $P=10^{-26}$) and humans (10^{-29}) as well as the essential checkpoint kinase Rad53 of *S. cerevisiae* (10^{-31}) required for G1, S and G2 checkpoints⁴².

Radiation-induced death of diploid *RAD55*-null mutant strains is suppressed by mating-type heterozygosity¹¹. In a similar manner, prolonged radiation-induced G2 arrest is suppressed by *MAT* heterozygosity in *bck1Δ*, *rai1Δ* and *mrt4Δ* diploids (Fig. 2*b,c*). The diploid *rai1Δ* and *mrt4Δ* mutants are like *rad9*, having little or no G2 checkpoint arrest after DNA damage. In an isogenic haploid *MATα* background, both *rai1Δ* and *mrt4Δ* as well as the *bck1Δ* cells showed prolonged arrest at G2/M after irradiation. These genes are therefore required in haploids for adaptation after DNA damage-induced arrest at G2/M. Recently, we found that *SIR4* functions, one of which is *MAT* repression, are also required for rapid adaptation to the G2 checkpoint¹³. This suggests that differential expression of *MAT*-regulated genes²² may affect checkpoint adaptation to DNA damage through a pathway(s) involving *RAI1*, *MRT4* and/or *BCK1*. These genes may exert their effect indirectly through the regulation of other adaptation genes such as *YKU70* (ref. 43), *CDC5* and *CKB2* (ref. 31).

We observed large amounts of G2/M cells in untreated (log phase) populations of 21 of the radiation-sensitive mutants (Fig. 2*a* and Web Table A), suggesting that deletion of these genes results in the accumulation of unrepaired spontaneous DNA lesions. *POL32*, *CTF4*, *EST1*, *ASF1* and *CDC40* have all been implicated in DNA replication, indicating that some lesions may arise during replication⁴⁴. Synthetic lethal interactions have been described for *ctf4Δ* and *nup84Δ* in combination with *rad52Δ* and *spo7Δ* (see the YPD for references), indicating that DSBs arising during mitotic or premitotic DNA synthesis may be repaired through recombination. *ANCI* may also be implicated in replication, as overexpression of this gene can suppress the lethality associated with a deletion of *MEC1*, which is required for the progression of DNA synthesis⁴⁵.



The other genes whose mutants had constitutively high proportions of G2/M cells have not been implicated in DNA replication. *Doc1* is part of the anaphase-promoting complex. *HF11*, *CWH8* and *CNM67* are involved in transcription and actin-related cytoskeleton functions, and *YCL016C* is involved in chromosome cohesion (functions summarized in the YPD). Little is known about the involvement of *VID21*, *VID31*, *LHS1*, *LIP5*, *GND1*, *YJL193W* and *YLR235C* in DNA metabolism or cytoskeletal functions. Deletion of *YLR235C* results in an overlapping 3' truncation of *TOP3* that may have altered function. *Lip5* is required for synthesis of lipoic acid, an antioxidant and free-radical scavenger (functions summarized in the YPD). Increased amounts of radicals resulting from γ -irradiation may lead to additional nuclear damage, affecting cellular replication.

The identification of genes controlling DNA-damage responses in yeast has often led to the discovery of functionally related genes in mammalian cells, including many DNA repair genes. Sequence comparisons based on the BLASTP algorithm⁴⁶ show that approximately 60% of the 138 genes implicated in γ -ray responses encode proteins that share significant homology with human proteins, based on *P* values equal to 10^{-4} (Web Table A, footnote 'b'). We found homologous sequences for proteins in each of the above nine functional categories, including seven of the eight proteins conferring an S/R phenotype. Given the similarity of yeast and human cells at the genetic and mechanistic level, the identification of these genes will expand opportunities to understand tolerance to radiation damage in human cells.

Recently, a genome-wide microarray analysis identified 36 human genes that have an altered transcriptional response to ionizing radiation⁴⁷. Seven of these have significant homology to one or more of the yeast genes identified in the present study: *CENPE*, *E2-EPF*, *P55cdc* (*CDC20*), *NOF1*, *C-TAK1* (*MAP3K7*), *PLK* and *WEE1* (Web Table A). Notably, *CENPE* which encodes a putative kinetochore motor protein with extensive coiled-coiled domains was found to share homology at a *P* value less than 5×10^{-4} with six of the genes in the present study that also coded for the following coiled-coil proteins: *Rad50*, *Rud3*, *Bfr1*, *Bik1*, *Vid31* and *Cnm67*. As coiled-coil domains such as those in *Rad50* are thought to mediate protein-protein interactions, it will be interesting to determine the role of these genes in resistance to ionizing radiation.

Among the 69 new yeast genes with at least some homology to human genes, 17 share significant homology with known oncogenes or other cancer-related genes (Web Table A). Several of these, including *SAC6* (a putative ortholog of the human oncogene *LCP1*, encoding plastin), affect actin and cytoskeleton function. *Dhh1*, which has similarity with the protein of the human oncogene *DDX6*, is notable because of its complex relationship with other genes identified in this screen. *DHH1* encodes an RNA helicase and is required for *Rap1* localization to telomeres⁴⁸. It is a multicopy suppressor of *pop2* and *ccr4* mutants and interacts with these proteins in a transcriptional complex⁴⁸. We also detected the *ccr4Δ* mutant in this screen as being radiation sensitive (Web Table A), suggesting that the complex is required for radiation resistance. *Dhh1* is highly homologous to the human DEAD/H-box RNA helicases *DDX6* and *DDX10*. The *DDX10* oncogene is translocation activated in treatment-related malignancies as a result of fusion to *NUP98* (a human nuclear-pore protein⁴⁹) which, similar to *NUP* genes described in Web Table A, may also function in radiation resistance. Another DEAD/H-box RNA helicase protein, *Chl1*, found in this screen is homologous to *DDX11* and has significant homology to the human breakpoint oncogene *TNFRSF6B* (Web Table A).

Through an analysis of most of the nonessential genes (3,670) in yeast, the number of genes known to affect radiation resistance has been increased considerably, bringing the total to approximately

130, or 3.5%, of the genes examined. This will probably increase to approximately 170 when the remaining nonessential genes (approximately 1,200) are tested. The diversity of metabolic pathways affecting radiation sensitivity is consistent with the view that many factors other than those directly affecting DNA can affect resistance to radiation. It will be useful to determine the genetic pathways involved and to assess whether multiple mutants can have increased radiation sensitivity. The approach taken here is relevant to primary screening for sensitivities to other agents. Not only were many new genes identified that affected radiation sensitivity, all but 14 of them affected sensitivity to other agents. Thus, direct screens against other agents will probably reveal large numbers of additional genes. The identification of nearly three times the number of genes previously reported to be radiation sensitive could be due to the use of complete deletion mutants. As previous screens examined mutants generated by agents causing random mutational changes, many of them might have retained partial function.

The observation that yeast genes identified in this study (Web Table A) had homology with nearly 20% (7/36) of human genes having altered post-irradiation expression⁴⁷ suggests that a combination of expression and functional genetic approaches can greatly enhance opportunities to identify genes relevant to damage responses. Whereas global analyses of genome expression patterns using microarrays have increased knowledge about the circuitry of biological responses, the functional roles of the various genes is generally poorly understood. In this regard, it is notable that among the approximately 50 new mutants that were sensitive to MMS as well as γ -rays, only three (*DHH1*, *UBC13* and *ERG3*) correspond to genes that show large changes in expression after MMS exposure of haploid yeast⁵⁰.

The characterization of possible mammalian cell functions associated with the genetic groups identified in this yeast-based genetic screen may offer new insights into the mechanisms by which radiation or anti-cancer drugs produce their effects. The identification of many new genes and homologies with human genes will be useful both in identifying new chemotherapeutic targets and in developing screening tools for compounds that might enhance existing chemical and radiation cancer therapies.

Methods

Yeast strains and γ -ray screening. Deletions of individual nonessential genes (or ORFs) were made in *MATa* (BY4741) and *MAT α* (BY4742) haploid *S. cerevisiae* strains as part of The Saccharomyces Gene Deletion Project⁹. We obtained the diploid deletion strains (3,670 mutants) in 96-well microtiter dishes (Research Genetics, Release I). Initially, the diploid deletion strains were thawed and transferred to YPD and YPG plates (containing 2% glycerol, as a nonfermentable carbon source, instead of dextrose) using a multiprong replica-plating device. Slow growth on YPG is indicative of a defect in mitochondrial respiratory function. We immediately irradiated the YPD plates (80 Krads; ¹³⁷Cs source at a dose rate of 2.5 Krad/min) and examined them along with the unirradiated control plates after 24 h and 48 h of growth at 30 °C. Putative γ -ray-sensitive mutants were confirmed by: (i) replica-plating serial dilutions of the strains grown overnight at 30 °C onto YPD medium and exposing them again to 80 Krads and (ii) generating single-point survival curves where cells diluted in water (10^{-4}) were irradiated (80 Krads) in microtiter dishes. We plated aliquots onto YPD medium before and after irradiation from the same dilution. After three days of growth at 30 °C, we determined relative survival as the ratio of colony-forming units on γ -irradiated versus nonirradiated plates. We also obtained haploid deletion strains of γ -ray-sensitive diploids (Research Genetics) and examined them individually for sensitivity to γ -irradiation by dilution replica plating as described above.

HO-induced cell lethality. We transformed deletion strains with plasmid pJH132, a low-copy (CEN), selectable (*URA3*) plasmid containing the *HO* endonuclease fused to the galactose promoter (*GAL:HO*). We grew transformed cells in liquid synthetic-complete (SC) glucose-uracil medium (SC + glu – ura) overnight to repress *HO* and maintain selection for



the plasmid. We diluted cells 10-fold and then replica plated them to SC galactose-uracil (SC + gal – ura) to induce or SC + glu – ura plates to repress HO expression. Cells were scored two days later for relative growth on galactose versus glucose. The growth of recombination-deficient strains is greatly inhibited on galactose-containing plates.

Targeted recombination at his3Δ1. Cells were grown to logarithmic phase in YPD liquid culture and then transformed as previously described¹³ with 200 ng of pRS315 and 1 μg of a partial *HIS3* PCR fragment that spanned the *his3Δ1* deletion. Primers used for producing the partial *his3* PCR fragment were: 5' *HIS3.2*: (5'–CCCTAGCGATAGAGCACTCGATCTTC–3') and 3' *HIS3.2*: (5'–CCTCGTTTCAGAATGACACGTATAGA–3'). PCR amplification of *HIS3* produced a 729-bp fragment with an overlap of 225 bp 5' and 317 bp 3' of the *his3Δ1* deletion. A functional *HIS3* gene could occur only by targeted integration of the amplified PCR fragment into the genomic *his3Δ1* sequences after transformation. We determined targeted integration efficiencies by calculating the ratio of the colony-forming ability of wildtype and deletion strains on SC medium lacking histidine. We then corrected ratios for the relative transformation efficiency of circular plasmid DNA (pRS315, containing a *LEU2* selectable marker, on SC-leucine).

Checkpoint analysis. Position in the cell cycle can be morphologically distinguished in yeast (unbudded cells are in G1; the beginning of S phase is marked by bud emergence and cells containing large buds are in G2/M)³⁰. To examine the checkpoint responses, we exposed logarithmically growing cells to 6 Krads of ionizing radiation and examined them microscopically 3 h later. Random microscopic fields were scored for the percentages of cells in G1, S or G2.

Mutants that were checkpoint deficient in liquid culture (<15% of cells were large budded, unirradiated; <30% of cells were large budded 3 h after irradiation) were examined by irradiating (6 Krads) a logarithmically growing cell population on YPD plates. We micromanipulated single unbudded (G1) cells into a grid pattern within one microscopic field of view and determined the cell-cycle progression of individual cells by photographing at hourly intervals as previously described¹³.

Note: Supplementary information is available on the Nature Genetics web site (http://genetics.nature.com/supplementary_info/).

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