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13. ABSTRACT (Maximum 200 Words)

We are using the yeast *Saccharomyces cerevisiae* to identify new cancer gene targets that interact with the tumor suppressor Brca1. Expression of Brca1 in diploid WT yeast leads to prolonged G1 arrest and lethality. We identified from a collection of ionizing radiation (IR)-sensitive yeast deletion strains or from a pool of 4746 genetically tagged deletion strains, 34 that rescue Brca1-induced lethality. Two IR resistance genes that rescue Brca1-induced lethality are the transcription factors *CCR4* and *DHH1*. These are checkpoint genes required for cell cycle progression in G1 and S phases following DNA damage. Consistent with a role in radiation resistance, Dhh1p and its highly conserved human ortholog DDX6 were found to physically interact with Brca1 in yeast and human cells. Another transcription factor (*YAF9*) was IR sensitive and rescued Brca1-induced lethality when deleted. This deletion strain and 19 others were subsequently isolated from the deletion strain pool. Most of these deletions (75%) were IR sensitive and hypersensitive to the toxin zymocin which appears to induce DSB damage by inhibiting transcription. Furthermore, most (85%) of these genes are highly conserved suggesting that the human orthologs may interact with Brca1 to maintain genomic stability and suppress the onset of breast cancer.

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INTRODUCTION: Cancer is thought to be a multistep process that encompasses many separate molecular mechanisms. Common cancers such as those of the breast and colon have been associated with defects in genes that regulate DNA repair and/or checkpoint functions thereby implicating a loss of genetic stability as a causative factor in these cancers. . In hereditary breast cancer, changes in DNA repair ability have been identified that result from alterations in the gene products Brca1 and Brca2. These genes have been found to be pleiotropic tumor suppressors that impact on recombinational repair of DSB damage, cell cycle checkpoint arrest following DNA damage, transcriptional regulatory mechanisms, centrosome duplication and transcription coupled repair of DNA damage (9) (12) (19) (18) (1). We are using the yeast *Saccharomyces cerevisiae* as a model organism for the identification of the genetic controls associated with the DNA repair and checkpoint functions of Brca1. We have determined that overexpression of Brca1 in diploid WT yeast leads to a prolonged arrest in G1 and lethality. Furthermore, Brca1 expression also activates the *DIN*::LacZ reporter construct that indicates the presence of DNA damage or replicative stress (17). We therefore used a yeast functional genomics approach to identify from a collection of IR-sensitive yeast gene deletions (2) (16) or from a pool of genetically tagged deletion strains, those that rescue Brca1-induced lethality. We have identified and characterized the function of two IR resistance genes (*CCR4* and *DHH1*) that are transcription factors and rescue Brca1-induced lethality when they are deleted (16). A third IR resistance gene that is a transcription factor (*YAF9*) was found to rescue Brca1–induced lethality when deleted. This highly conserved gene deletion (*YAF9*) was subsequently identified (along with 19 other deletions, see below) from the tagged pool of deletion strains and showed rapid growth on synthetic complete galactose medium lacking uracil (GAL-URA) immediately after transformation of the *GAL::*BRCA1 high copy expression plasmid. The majority of these gene deletions (including *CCR4*, *DHH1* and *YAF9*) show IR sensitivity and/or hypersensitivity to the toxin zymocin which appears to inhibit transcription and results in DSB damage.

BODY: *Task 1.* Identify and characterize in yeast, genes that rescue BRCA1-induced slow growth and lethality. (Months 1-24)

a. Finalize screening of the identified IR^S diploid deletion strains (from Bennett et al., 2001; and unpublished) and identify new deletion mutants from a random pool of deletion mutants for those that rescue slow growth and lethality following BRCA1 expression. Collect~100 deletion mutant clones from pool for sequencing identification (Months 1-12).

We have used a yeast functional genomics approach to identify IR-sensitive (IR^S) gene deletions that partially rescue Brca1-induced lethality in yeast. These factors include known repair proteins including Rad6, Ubc13, Rad51, Rad9 and Sfp1 which are

likely to be involved in mechanisms associated with damage recognition, repair or checkpoints. However, the most efficient rescue of Brca1-induced G1 arrest and lethality was observed in IR^S strains deleted for either *CCR4* or *DHH1*. The protein products of these two genes physically interact together as members of the *CCR4-NOT1* transcriptional complex. Before our discovery of the radiation sensitivity of *CCR4* and *DHH1* mutants (3), little was known about the mechanism of radioresistance determined by these members of the CCR4-NOT1 transcriptional complex. In the first year of this grant we identified the CCR4 network of radiation resistance genes and established that *CCR4* and *DHH1* are members of the RAD9 epistasis group of checkpoint repair genes. This complex appears to mediate resistance to IR or hydroxyurea (HU) through checkpoint arrest functions in G1 and S phases following radiation or replication stress mediated by HU (16) (see Appendix A for publication). Specifically, CCR4 and Dhh1 are required for reentry into the cell cycle following checkpoint arrest at G1/S. Furthermore *CCR4* mutants may have an S phase replication defect which is distinct from that expressed by *RAD52* mutants. Please see (16) for a detailed discussion of these results.

Expression of Brca1 induces a prolonged G1 arrest in WT diploid yeast. Disruption of G1 checkpoint functions by deletion of *CCR4* or *DHH1* allows enhanced survival among cells over expressing Brca1. Enhanced survival is not due to down regulation of Brca1 protein expression in *ccr4*Δ or *dhh1*Δ cells (17). Brca1 induces DNA damage in yeast as measured using a *DIN::*LacZ reporter of *RNR3* transcriptional activation. Deletion of *CCR4* dramatically decreases this DNA damage response and implies that less DNA damage is induced by Brca1 in *ccr4*Δ cells, or alternatively the damage can be repaired more efficiently as the cells continue to progress into later stages of the cell cycle (17). Therefore, we appear to have defined a new G1 and/or S phase specific function for Brca1 in yeast. Similar defects in human cells may be conserved and therefore contribute to the molecular mechanism of breast cancer.

As indicated in Task 1 we have finalized the screening for IR^S mutants and identified a total of 199 yeast genes that are required for ionizing radiation resistance. From among these we have identified the following 15 gene deletions that rescue lethality following Brca1 expression in yeast (Table 1). Eight of these have been implicated in the molecular mechanisms of a variety of cancers including those of the breast (*i.e* RAD54L and RAD51). These mutants have also been screened for chromosome stability by monitoring the spontaneous loss of chromosome III in these diploid deletion strains. Of the strains depicted in Table 1, only strains deleted for *RAD51* or *HFI1* showed enhanced levels of spontaneous chromosome loss suggesting it is not a common feature among these mutants that rescue Brca1-induced lethality.

Table 1. Ionizing radiation sensitive gene deletions that rescue slow growth and lethality following BRCA1 expression in diploid yeast.

IR sensitive Yeast Gene Deletion[1]	Function in Yeast Cells	Corresponding Human Ortholog[2]	Function in Human Cells	GAL-URA (Brca1 expressed)
ccr4	G1/S checkpoint	**hCCR4** (-24)	Transcription interacts with BTG1 tumor suppressor	++++[3]
dhh1	G1/S checkpoint	**DDX6**[4] (-162)	Putative breakpoint oncogene	+++
rad6	DNA repair	UBE2B (-61)	DNA repair	+++
ubc13	DNA repair	UBE2N (-60)	DNA repair	+++
rad18	DNA repair			+
bre1	Interacts with Rad6 Ubiquitination	RNF20 (-28)	unknown	+++
yaf9[5]	uhromatin modification	**AFF9** (-32)	Breakboint oncogene	+++
rad54	Recombination repair	**RAD54L** (-168)	recombination	+++
rad51	Recombination repair	**hRAD51** (-130)	recombination	+
gcn5	transcription	**PCAF** (-84)	associates with p300/CBP and competes with E1A for binding sites in p300/CBP	+++
hfi1	transcription	-	-	++
rad9	checkpoint	**RB binding protein 6** (-05)	binds to underphosphorylated but not phosphorylated pRB	+
mec3	checkpoint	-	-	+
sfp1	checkpoint	JAZF1	Putative transcriptional repressor, G2/M transition	+
hof1	Cytoskeletal binding protein	**GAS7** (-11)	Growth arrest-specific 7	++

[1] Deletions were identified by screening the IR sensitive (IR^S) strain collections described in (2) and (16).

[2] P value as calculated by Blast analysis.

[3] Enhancement of growth as compared to WT after 3 days growth at 30^0C using a dilution replica plating technique: + = 5-fold; ++ = 25-fold; +++ = 125 fold; ++++ = >500-fold; see legend for Fig. 1.

[4] Human orthologs implicated in cancer have been indicated in bold.

[5] Found independently in screen of the diploid deletion strain pool (see Table 2).

In support of the goal of *Task 1a*, we have collected >100 isolates from five independent transformations of the pooled diploid deletion strain collection. The pool of deletion strains were transformed with the high copy *GAL::BRCA1* plasmid that also contains *URA3* as a selectable marker . Following transformation, the deletion cell pool was immediately plated to GAL-URA solid medium to both select for rapidly growing transformants and induce expression of Brca1. Large colony isolates that appeared after 3-4 days incubation at 30°C were purified for single colonies by restreaking to GAL-URA plates. Two separate isolates containing the *GAL::BRCA1* plasmid were then tested

for rescue of Brca1-induced lethality by dilution pronging serial dilutions to GLU-URA and GAL-URA plates (Fig. 1).

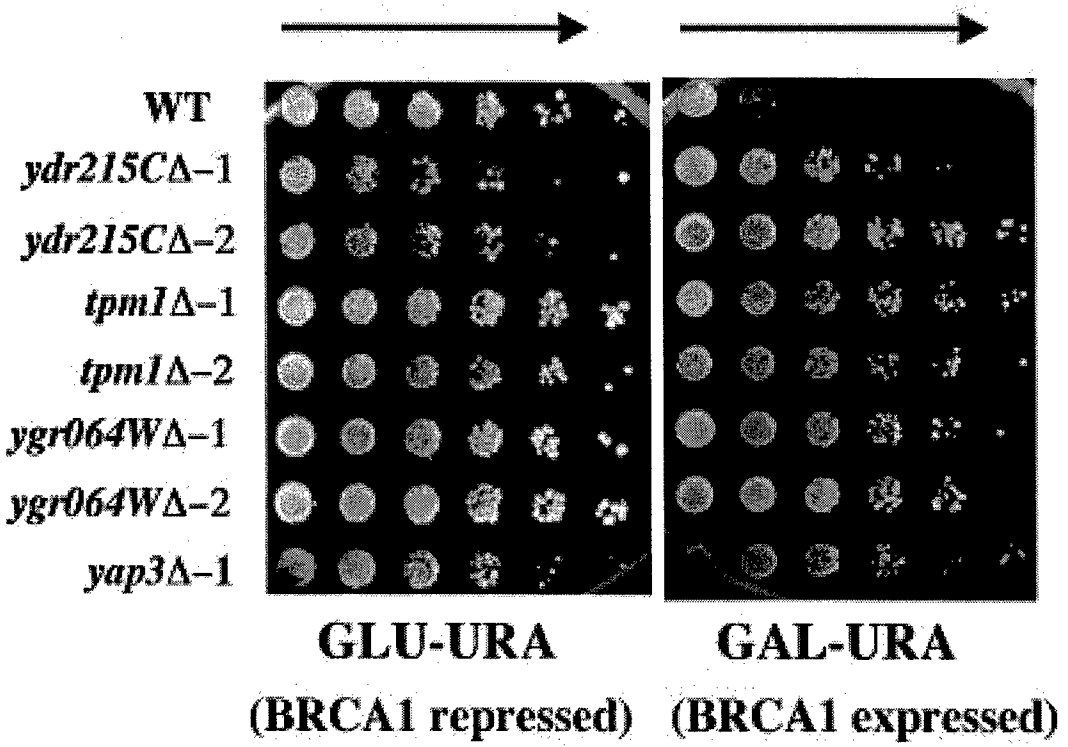

Fig. 1. Typical examples of yeast diploid deletion strains that rescue Brca1-induced lethality upon initial isolation. The depicted strains were isolated from a transformed pool of 4746 isogenic, genetically tagged diploid deletion strains which was transformed with the *GAL::*BRCA1 high copy plasmid and plated to synthetic complete galactose medium lacking uracil (SC GAL-URA) plates. Rapidly growing isolates were subsequently streak purified onto fresh SC GAL-URA plates to obtain single colony isolates. Two separate isolates (1 and 2) of each strain was grown in 200 ul of SC glucose (GLU)-URA liquid medium in 96 well plates for two days at 30°C to repress BRCA1 expression. Serial 5-fold dilutions of each strain was made in sterile water and 2 ul of each dilution was transferred using a 48 pin replica plating device to SC GLU-URA and SG GAL-URA plates. The relative survival of BRCA1 expressing strains was determined by comparing GAL vs. GLU plates lacking uracil. Growth of the WT strain expressing Brca1 (on GAL-URA) is severely inhibited. The deletion strains all show rescue of BRCA1-induced lethality when compared to WT. Deletion strains were identified by sequencing the unique 20 bp upstream tag associated with each deletion that had been amplified using yeast colony PCR. Arrows indicate the direction of decreasing cell concentration.

Deletion strains isolated from the pool that rescue Brca1-induced lethality may have acquired a second site dominant mutation or alternatively the BRCA1 gene contained on the plasmid may have been mutated during the transformation process. To confirm that individual deletions were indeed responsible for the rescue of Brca1-induced lethality, we obtained the putative deletion strain from the diploid deletion library (purchased from Open Biosystems) where each individual deletion strain was isolated in

a single well of a 96 well plate. These "fresh" deletion strains were transformed again with the *GAL::BRCA1* plasmid and examined for the ability to rescue Brca1-induced lethality on GAL-URA plates (Fig. 2). As depicted in Fig. 2, some of the retransformed deletion strains initially isolated from the pool, failed to rescue Brca1-induced lethality when compared to WT. This indicates that some of the original isolates that rescue Brca1-induced lethality were due to mutations that occurred within the plasmid during transformation or alternatively, the strains themselves acquired a second site suppressor of lethality.

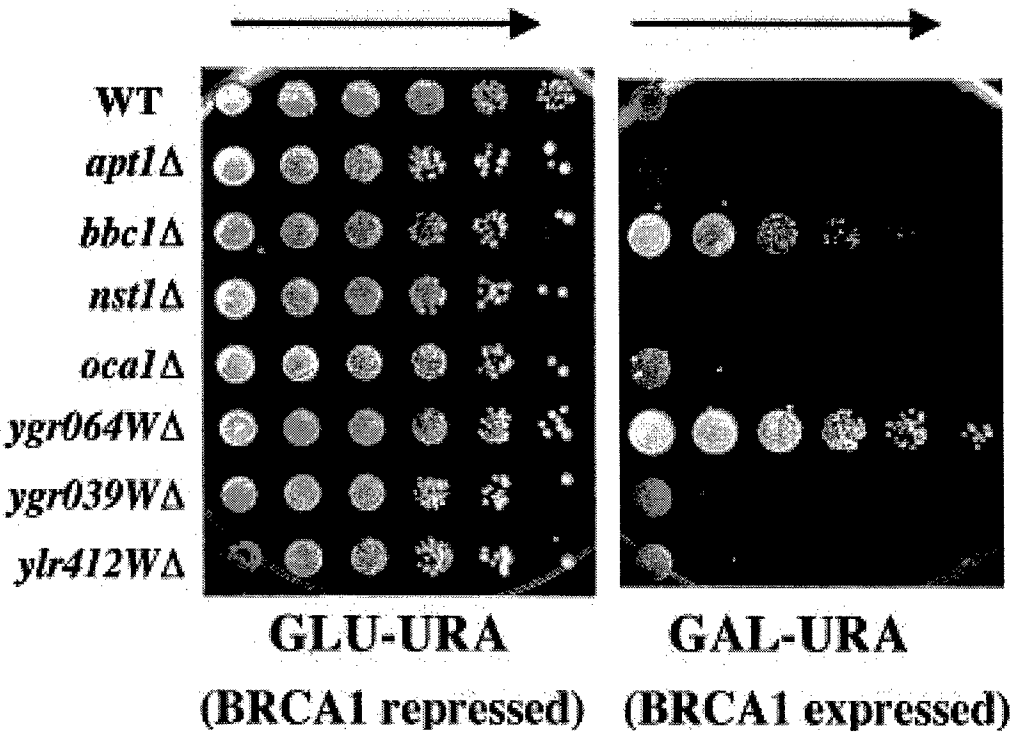

Fig. 2. Rescue of Brca1-induced lethality following retransformation of diploid deletion strains with *GAL::*BRCA1 plasmid. Individual deletion strains that were identified following transformation of the diploid deletion pool were grown on YPD plates from a 5ul aliquot obtained from the diploid deletion library where each individual deletion strain is contained within a separate well in 96 well plates. A single isolated colony of each deletion strain was retransformed with the *GAL::*BRCA1 plasmid. Two isolates of each transformant were dilution plated to SC GLU-URA and SC GAL-URA as described in the legend for Fig. 1. Typical results are depicted above for seven retransformants. Five deletion strains (*apt1*Δ, *nst1*Δ, *oca1*Δ, *ygr039W*Δ and *ylr412W*Δ) failed to rescue Brca1-induced lethality as indicated by a similar degree of growth inhibition when compared to the WT strain containing *GAL::BRCA1*. These have been omitted from further study. Two diploid deletion strains (*bbc1*Δ and *ygr064W*Δ) were confirmed to rescued Brca1-induced lethality following reintoduction of the *GAL::BRCA1* plasmid into deletion strains by transformation. These and other confirmed strains (see Table 2) were subsequently exposed to a variety of DNA damaging agents. Arrows indicate the direction of decreasing cell concentrations.

Task 1b. Screen rescued yeast deletion clones from pool (*i.e.* those that do not show inhibited growth following Brca1 expression) for radiation sensitivity. Those that show sensitivity to IR will be analyzed in detail by dose response curve analysis. Those that are not sensitive to IR will be tested for sensitivity to other DNA damaging agents such as UV, bleomycin, doxorubicin, camptothecin, mitomycin C as well as for spontaneous genomic instability resulting in chromosome loss. (Months 6-12)

We have screened the yeast deletion strains identified using the transformed pool protocol to a variety of DNA damaging agents. Deletion strains that were obtained from the library collection (in 96 well plates) were exposed to IR, HU, methyl methane sulfonate (MMS), doxorubicin and the toxin zymocin (Fig. 3).

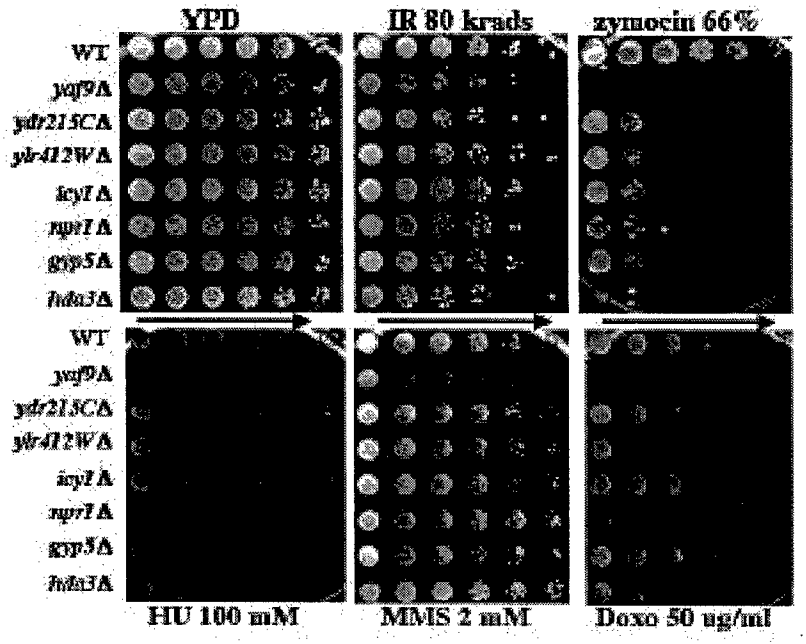

Fig. 3. Response of deletion strains initially isolated from a random pool that "rescue" Brca1-induced lethality to a variety of DNA damaging agents. Deletion strain isolates identified using the method described in Fig. 1 were obtained from the complete deletion library and grown for single colony isolates on YPD plates. Duplicate single colony isolates were grown in 200 ul of liquid YPD for two days at 30°C. Serial 5 fold dilutions of the stationary cell populations were made in fresh YPD liquid and 2ul aliquots were replica plated to YPD plates containing the indicated concentrations of the various DNA damaging agents. Cells were exposed to a single acute dose of IR as previously described (2). Preparation of zymocin containing plates was as previously described (16). With the exception of *ylr412WΔ* and *icy1Δ* (see text below) all deletion strains rescued Brca1-induced lethality following retransformation of the *GAL::BRCA1* plasmid. Arrows indicate the direction of decreasing cell concentrations.

Deletions of *YAF9* or *HDA3* are IR^S and hypersensitive to zymocin induced lethality (Fig. 3). Both of these genes are thought to modulate transcription through the modification of chromatin structure. Interestingly, all strains showed sensitivity to zymocin even though the *ylr412WΔ* strain (and *icy1Δ*, data not shown) failed to significantly rescue Brca1-induced lethality upon retransformation of the *GAL::BRCA1* plasmid and plating to SC GAL-URA (see Fig. 2). However, the growth of *icy1Δ* was inhibited ~5 fold as compared to WT when plated to SC GLU-URA suggesting there may be a small enhancement of survival followingBrca1 expression in this strain. We will therefore determine survival by plating equal cell numbers of logarthmically growing *icy1Δ* cells to SC GLU-URA and SC GAL-URA plates. Furthermore, prior to retransformation the *ylr412WΔ* strain demonstrated enhanced sensitivity to doxorubicin (Fig. 3). We previously examined the ability of the *ylr412WΔ* strain obtained from another library (purchased from Research Genetics) to rescue Brca1-induced lethality. Following retransformation of the *GAL::BRCA1* plasmid, the *ylr412WΔ* strain completely rescued Brca1-induced lethality (*i.e.* equal plating efficiency of colony forming ability on SC GLU-URA *vs.* SC GAL-URA plates). Since the number of *ylr412WΔ* Ura^+ transformants obtained upon retransformation was small (4 colonies) for the experiment depicted in Fig. 2, cross contamination with a WT strain may have occurred during the retransformation process. We will therefore repeat this rescue experiment with a freshly retransformed *ylr412WΔ* where the efficiency of transformation is high (i.e. multiple colonies).

Deletion of *YAF9* also resulted in enhanced sensitivity to the S phase specific inhibitors HU, MMS and doxorubicin when compared to WT indicating a possible replication repair associated defect. Similarly, deletion of *NPR1* resulted in enhanced sensitivity to HU and doxorubicin which again suggests a possible defect in repair specific for S phase. Finally, deletion of *YDR215C* also appears to result in a small (5 fold) enhancement of sensitivity to IR when compared to WT. The cross sensitivity to DNA damaging agents among strains isolated solely by their ability to rescue Brca1-induced lethality strongly suggests that similar to human cells, Brca1 interacts specifically with proteins required to tolerate DNA damage in yeast. Moreover, orthologs of these proteins may similarly interact with Brca1 in human cells.

We have summarized the results for 20 deletion strains confirmed to rescue Brca1-induced lethality that were obtained by transformation screening of the tagged deletion pool (Table 2). Interestingly, among thee mutants, there is a high correlation between confirmed ability to rescue Brca1-induced lethality, enhanced zymocin sensitivity and IR sensitivity. Furthermore, seven of the mutants that rescue Brca1-induced lethality have identified defects in transcription and/or chromatin related functions. This enrichment for mutants in transcription related functions may result from

defects in transcription associated recombination (TAR) in yeast which is analogous to transcription coupled repair (TCR) in human cells. Not surprisingly, TCR is Brca1-dependent in human cells (see discussion below).

Table 2. Yeast strains isolated from a random pool of 4746 isogenic gene deletions that rescue slow growth and lethality following Brca1 expression.

deletedy east gene/ ORF[1]	Function in Yeast Cells	Human Ortholog (p value)[2]	Function in Human Cells	GAL-URA[3]	IR[S]	Zymocin sensitive[4]
bbc1	Actin cytoskeletin organization	**MUC7** (-22)	mucin 7, salivary	+++	S[5]	++++
dan1	anaerobic induced cell wall mannoprotein	**C6orf205** (-13)	unknown	+	S	+++
gyp5 (x3)	GTPase-activating protein for Rab	**RABGAP1** (-51)	RAB GTPase activating protein	++	S	+++
hda3	histone deacetylase	**GCC2** (-08)	localized to the trans-Golgi network	++	S	++++
hcm1	RNA polII transcription factor	**FOXD3** (-20)	forkhead family of transcription factors	++	S	++++
mlp2	myosin like protein nuclear import	**TPR** (-64)	translocated promoter region (to MET oncogene)	++	-	++
npr1	protein kinase	**CHEK1** (-22)	CHK1 checkpoint homolog (S.pombe)	++	S	++++
nup2 x2	sub-unit of the nuclear pore complex	**RANBP2** (-18)	GTP-binding protein at the nuclear membrane	+++	S	++
pom34 x2	nuclear pore protein	-	-	+++	-	++
sub1	transcription co-activator activity	**PC4** (-12)	activated RNA polymerase II transcription cofactor 4	++	S	++++
tpm1	tropomyosin, required for actin cable stability	**TPM2** (-135)	Beta tropomyosin gene	++	S	++++
yaf9	yeast chromatin modifying complex	**GA41** (-32)	homology to MLLT1/ 3 transcription factors	++	S	++++
yap3	bZIP protein; transcription factor	**XBP1** (-05)	over-expression in human breast cancer	++	SS	++++
ymc2	mitochondrial inner membrane transporter	**SLC25A20** (-32)	lipid transporter inner mitochondrial membrane	+	S	++
yal042c-a	deletion overlaps 5' end of *ERV46*	-	-	+	-	++
ydr215c	unknown	-	-	++	S	++
ygr053c	unknown	-	-	+	-	++
ygr064w (x2)	deletion overlaps 5' end of *SPT4*	-	-	+++	S	+++
yjr014w (x2)	RNA binding	**DENR** (-22)	translation initiation factor	+	-	++
ymr172c-a	deletion overlaps the 3' end of *HOT1*	-	-	+	-	++

[1] Deletions were identified by screening the tagged deletion strain pool (see Fig. 1 legend). Some deletion strains such as *gyp5* were independently identified more than once.(x3 or x2) in different transformations.

[2] P value as calculated by Blast analysis.

[3] Enhancement of growth as compared to WT after 3 days growth at 30°C on SC GAL-URA plates (BRCA1 is induced) using a dilution replica plating technique: + = 5-fold; ++ = 25-fold; +++ = 125 fold; ++++ = >500-fold; see legend for Fig. 1.

[4]Enhancement growth inhibition following exposure to zymocin (as compared to WT) after 3-4 days growth at 30°C on YPD + zymocin (66%) plates. A dilution replica plating technique was used as described in the legend to Fig. 3: + = 5-fold inhibition; ++ = 25-fold; +++ = 125 fold; ++++ = >500-fold.

[5]Most deletion strains showed a 5-fold (S) enhanced sensitivity to the killing effects of IR when compared to WT as previously described (2) (16). One strain (*yap3*Δ) showed a 25 fold (SS) increase in sensitivity to IR. IR sensitivity of the *bbc1*Δ, *gyp5*Δ and *dan1*Δ strains were confirmed using survival curve analysis (data not shown).

The yeast mutants confirmed to rescue Brca1-induced lethality (Table 2) are enriched for genes that are required for IR resistance (75%) and have highly conserved human orthologues (85%). These fractions are much larger than would be expected randomly. For example, only 4% of nonessential genes have been found to be required to confer radiation resistance (16) and only 37.5% of the yeast proteome is similar to that of mammals (13). With the exception of *YAF9*, all of the IR resistance genes found in Table 2 are newly identified. This indicates that screens to identify genes that rescue Brca1-induced lethality are able to identify novel IR resistance genes that were previously undetected using conventional screening methods (2) (16). Furthermore, five of the 20 mutants confirmed to rescue Brca1-induced lethality were isolated multiple times. This indicates that the screen (of the unselected pool) is approaching saturation and the total number of gene deletions that rescue Brca1-induced lethality is probably less than 50 genes.

Task 1c. Characterize the IR^S deletion mutants. Determine for IR^S deletion mutants whether IR sensitivity is the result of a defect in recombination or checkpoint functions as previously described. Some Brca1 interacting genes may be members of the post replication repair pathway (*i.e.* similar to *RAD6* and *UBC13*). We will use epistasis analysis in yeast to identify what repair pathway newly identified candidate genes are members of. (Months 8-24).

Characterization of the checkpoint defects and epistasis analysis has been completed for *ccr4*Δ and *dhh1*Δ mutants as described above and in detail in the accompanying appended publication (16). These results will not be discussed further as they are clearly described in (16). Given the correlation between zymocin and IR sensitivity among mutants that rescue Brca1-induced lethality (Table 2), we are examining whether these mutants may be deficient in transcription associated recombination (TAR) repair.

Heterologous expression of Brca1 causes prolonged G1 arrest and lethality in yeast. Brca1 appears to stimulate transcription in yeast following heterologous expression and in some haploid yeast strain backgrounds overexpression in yeast leads to severe growth inhibition (8). Furthermore, mutations in the C terminus that contain the BRCT domains that have been associated with breast cancer disease, abrogate this slow growth phenotype (8). Polymorphysms not associated with breast cancer do not interfere with this slow growth phenotype, suggesting that the biological effects in yeast accurately reflect interactive molecular functions in human cells. We have extended this finding by determining that overexpression of Brca1 in diploid WT yeast leads to a prolonged arrest in G1 and lethality. Furthermore, Brca1 expression leads to activation of the *DIN*::LacZ reporter construct that is specific for DNA damage or replicative stress (17). These results suggest that Brca1 may be interacting in yeast with factors associated with recombinational repair, transcription or cell cycle checkpoints. We have used a yeast functional genomics approach to identify IR-sensitive gene deletions that partially rescue Brca1-induced lethality in yeast. These factors include known repair proteins including Rad6, Ubc13, Rad51, Rad9 and Sfp1 which are likely to be involved in mechanisms associated with damage recognition, repair or checkpoints. However, the most efficient rescue of Brca1-induced G1 arrest and lethality was observed in strains deleted for either *CCR4* or *DHH1*. The protein products of these two genes interact together as members of the *CCR4-NOT1* transcriptional complex. This complex appears to mediate resistance to IR through an arrest checkpoint in G1 (16). Disruption of this G1 checkpoint by deletion of members of the *CCR4-NOT1* complex allows cells over expressing Brca1 to continue to cycle and survive. Furthermore, it implies that in *ccr4*Δ cells, DNA damage induced by Brca1 is reduced or alternatively, the damage can be repaired more efficiently as the cells progress into later stages of the cell cycle (17). Therefore we appear to have defined a new G1 specific function for Brca1 in eukaryotes, defects in which may participate in the molecular mechanism of breast cancer.

We are establishing a link between *CCR4*, zymocin sensitivity and transcription associated recombination which has profound implications for BRCA1 function in yeast and human cells. A role for *CCR4* in regulating G1 to S phase transition in response to radiation was initially suggested by the finding that *CCR4* mutants are required to maintain size homeostasis that result in abnormally small or large cell volumes (10) (20). Many of these genes identified in these genome-wide screens (including *CCR4*) are members of the CCR4-NOT1 complex and are thought to modulate cell size by altering expression of the G1 cyclins required to progress from G1 to S phases of the cell cycle. In addition, *CCR4* mutants were found to be hypersensitive to the toxin zymocin which induces a prolonged G1 arrest in WT haploid cells (11). We have found that, many of the newly identified diploid, IR sensitive yeast deletion mutants including *ccr4* and *dhh1* are sensitive to zymocin (16) and Fig. 4.

The IR resistance mutants that demonstrate the most zymocin sensitivity are those that participate as cofactors for RNA polII mediated transcription such as *CCR4* and *YAF9*. Furthermore, genes required for the repair of IR-induced DSBs including members of the RAD52, RAD6 and RAD9 epistasis groups all show enhanced sensitivity to the lethal effects of zymocin when compared to WT (Fig. 4).

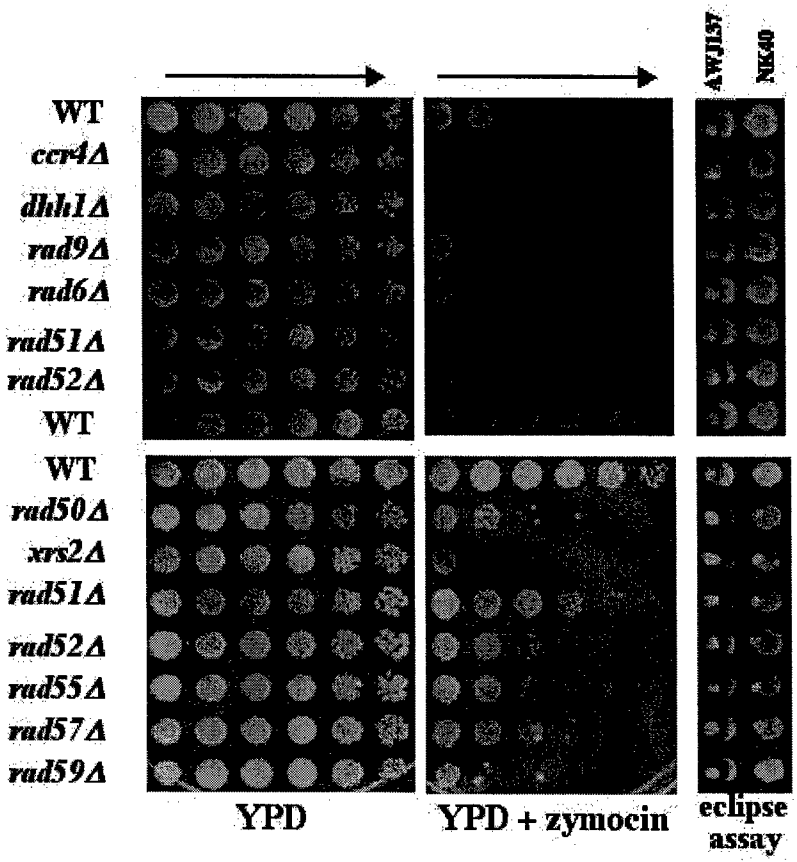

Fig.4. Enhanced zymocin-induced lethality in DNA repair deficient yeast diploid deletion strains. Wild type (WT) and the indicated isogenic *S, cerevisiae* deletion strains were grown in liquid YPD, diluted and replica plated to YPD and YPD plates containing 66% zymocin as described in the legend to Fig. 3. Eclipse assays were performed by diluting stationary phase cells 25-fold in liquid YPD and spotting two replica aliquots (2 ul) onto YPD plates. Using a sterile toothpick zymocin secreting (AWJ137) and zymocin non-secreting (NK40) *Kluyvermyces lactis* cells were positioned to the left side of each deletion strain. Zymocin sensitive repair deficient strains show a distinct zone of inhibition (eclipse) extending from the zymocin secreting strain (AWJ137). No zone of inhibition was seen in the presence of the non secreting strain (NK40). WT strains are zymocin resistant compared to members of the RAD9, RAD6 and RAD52 epistasis repair groups. Strains deleted for the transcription regulators *CCR4*, *DHH1* (above) and *YAF9* (see Fig 3) are hypersensitive to the killing effects of zymocin when compared to WT. Arrows indicate the direction of decreasing cell concentrations.

These results strongly suggests that zymocin may be inducing DSB damage at regions of active transcription. Since deletions of *CCR4* and *YAF9* can both rescue lethality induced by expression of Brca1 in yeast, this further suggests that Brca1 may also interfere with transcription and/or transcription associated recombination (TAR) in WT yeast. TAR is a *RAD52*-dependent process and is thought to be responsible for the enhanced levels of gene conversion and meiotic recombination that have been observed at transcriptionally active sites (7) (5). Further support for this model is the finding that the recombination hotspot, *HOT1* corresponds to the transcription initiation site of the 35S ribosomal RNA precursor and an enhancer of RNA polymerase I transcription (14) (15). *HOT1* is a recombination stimulating sequence that retains activity even when moved into other chromosomal locations. The preferential gene conversion of sequences on *HOT1*-containing chromosomes suggests that *HOT1*-promoted gene conversion initiates with a double-strand break in transcriptionally active *HOT1*-adjacent sequences (15).

Since TAR is a *RAD52*-dependent process, exposure to zymocin would be predicted to enhanced lethality in recombination deficient strains when compared to WT. This indeed appears to be the case as all members of the RAD52 epistasis group tested show enhanced sensitivity to zymocin-induced lethality (Fig. 4). Enhanced gene conversion at actively transcribing regions suggests that DSBs are induced during transcription and repaired by TAR. Both zymocin and Brca1 may interfere with TAR and enhance the amount of DSB damage at transcriptionally active sites. This is supported by the observation that WT diploid cells that contain a plasmid encoding Brca1 are hypersensitive to the killing effects of zymocin when compared to WT cells not expressing Brca1 (data not shown). The synergistic lethal effects observed between zymocin and Brca1 indicate that Brca1 may be inhibiting TAR functions in yeast.

Exposure of haploid WT yeast cells containing a low copy selectable plasmid (pGALHOT) with a galactose inducible gene (*GAL::HO*) to zymocin, resulted in a 10 fold increase in plasmid loss when the target gene was expressed (in galactose) as compared to galactose without zymocin (data not shown). This suggests that zymocin may induce plasmid loss by inducing DSBs within actively transcribing genes. Therefore, mutants such as *ccr4*Δ or y*af9*Δ that rescue Brca1-induced lethality may be radiation and zymocin sensitive due to a defect in TAR. This Brca1-dependent effect in yeast may reflect the enhanced transcription coupled repair mediated by Brca1 in human cells following IR (1). Therefore, the human orthologs of highly conserved gene deletions (i.e. *CCR4, DHH1, YAF9* and others) that rescue Brca1-induced lethality in yeast may participate in Brca1-dependent TCR in human cells.

Recently, Brca1 has been found to be a shuttle protein that relocalizes to the cytoplasm following IR (4). Three genes that are associated with the nuclear pore (*MLP2*, *NUP2* and *POM34*) were found to rescue Brca1-induced lethality when deleted in yeast. This effect may result from exclusion of Brca1 protein from the nucleus (or

cytoplasm) due to alterations in mRNA or protein trafficking. Alternatively, the nuclear pore and *MLP2* in particular, may play a critical role in chromosome positioning and dynamics during DNA recombinational repair (6). To investigate these possibilities, we are determining Brca1 protein localization in WT and mutant cells using indirect nuclear immunofluorescence and a panel of anti-Brca1 antibodies both before and after IR.

Task 2. For yeast genes with human orthologs, we will determine if physical protein-protein interactions occur between the newly identified proteins and BRCA1 in yeast and human cells. (Months 13-30).

a. Design and construct a series of plasmids for yeast two hybrid analysis using BRCA1 as the "bait" and candidate yeast (or human orthologs) identified in Task 1 as the "prey". (Months 8-30).

For two hybrid analysis, we have constructed bait and prey vectors containing DHH1, DDX6, the RING domain of Brca1 and the C terminal BRCT domain of Brca1 using the Gateway (Invitrogen) compatible vectors pDEST22 and pDEST32. Construction of the full-length Brca1 vectors has been problematic due to extensive deletion and rearrangements within the Brca1 insert region. We are redesigning our approach to use an "Entry" vector that is Gateway compatible and conventional ligation of a high fidelity PCR amplified Brca1 gene fragment that has unique restriction sites at each end. The PCR amplified Brca1 will be digested with the appropriate enzyme and ligated within the Entry vector. This Brca1 fragment will be subsequently shuttled into the Gateway compatible destination vectors for either yeast or mammalian two hybrid analysis.

Task 2. b. For candidate genes that show physical interaction in yeast, design and construct a series of plasmids for mammalian two hybrid analysis using BRCA1 as the "bait" and candidate human genes as the "prey". (Months 12-36).

This work is just beginning as described above.

Task 2. c. Confirm by co immunoprecipitation techniques the occurrence of physical interaction of BRCA1 protein with candidate human proteins that were previously shown positive for two hybrid interactions in mammalian cells. (Months 16-36).

We are ahead of schedule for this task as we have been able to confirm physical protein-protein interactions occur between Brca1 and Dhh1p in yeast and Brca1 and DDX6 in human cells (Fig. 5). This indicates that DDX6 may have a conserved role in

the toleration of IR damage in human cells similar to the highly conserved yeast orthologue Dhh1p which is 66% identical at the protein level.

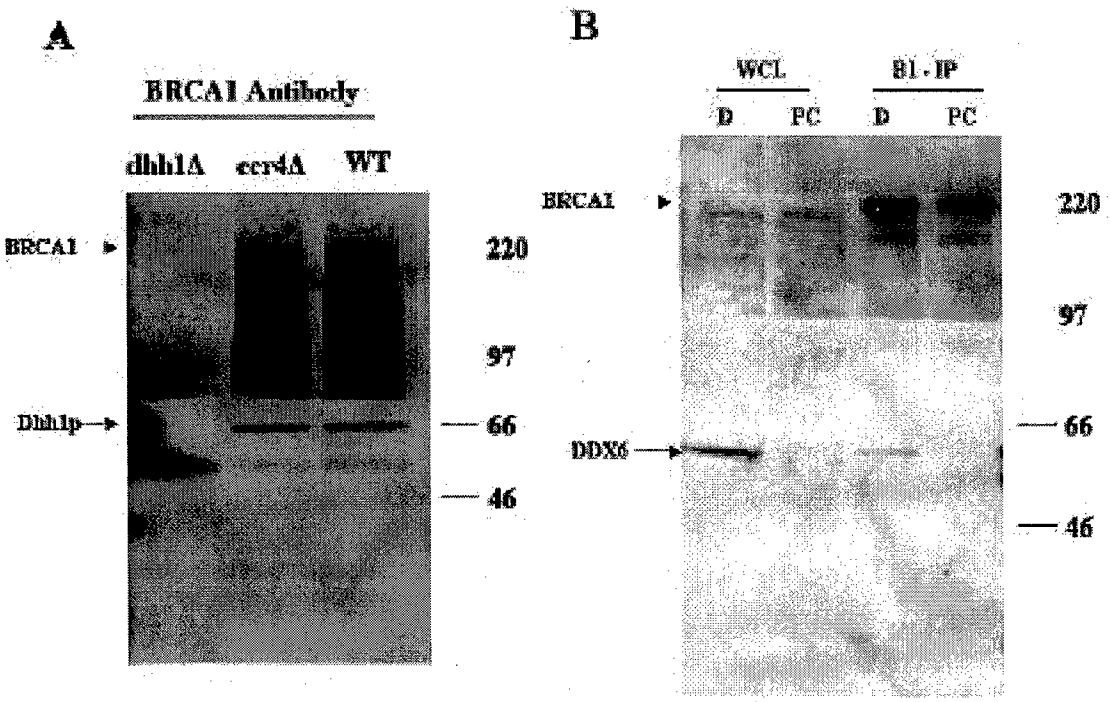

Fig.5 Dhh1p and DDX6 physically interact with BRCA1 in yeast and human cells. (A) BRCA1 was expressed in isogenic diploid yeast strains (*dhh1Δ*, *ccr4Δ* and WT BY4743) containing a high copy, selectable (*URA3*) plasmid and an inducible *GAL::BRCA1* fusion construct. Plasmid bearing cells were grown to logarithmic phase in synthetic complete (SC) liquid medium supplemented with 2% glucose but lacking uracil (GLU-URA) to repress expression of BRCA1. Cells were washed in water then grown for 16 hours in SC plus 2% galactose but lacking uracil (GAL-URA) to induce expression of BRCA1. Pelleted cells were ruptured and protein extracted in a glycerol buffer using glass beads and a bead beater. Crude extracts were then subjected to immunoprecipitation overnight using anti-BRCA1 antibody (Ab-1). Immunoprecipitated protein complexes were run on a gradient SDS-PAGE gel and transferred to nitrocellulose. The membrane was cut in half and subjected to western blotting using anti-BRCA1 antibody (top) and anti Dhh1p antibody (bottom) as probes. (B) Antibodies reactive to BRCA1 (B1; polyclonal) and V5 tagged DDX6 (V5, monoclonal) were used to co-IP whole cell lysates (WCL) of transfected MCF-7 breast cancer cells; (D, pCDNA-DDX6-V5 transfectants; PC, pCDNA parental vector alone).

Task 3. Determine if newly identified BRCA1-interacting genes contribute to genomic stability following IR damage in human cells. (Months 13-36).

a. Using siRNA technology, down regulate the expression of BRCA1 in normal human fibroblast cells and determine if cells are sensitized to the killing effects of IR. (Months 13-18).
b. Using siRNA technology down regulate highly conserved, candidate BRCA1-interacting genes in normal human fibroblasts to demonstrate sensitivity to the killing effects of IR. (Months 16-36).

We have created stable MCF7 (breast cancer) and DU99 (normal) cell lines that overexpress DDX6 as measured using Western blot analysis. Attempts to down regulate the expression of DDX6 in these cells are at an early stage. Preliminary results suggest that overexpression of DDX may modulate resistance to IR (data not shown)

KEY RESEARCH ACCOMPLISHMENTS:

- Discovery of CCR4 damage response network
- Establishment of *CCR4* as a member of the RAD9 checkpoint epistasis group
- Determination that *CCR4* and *DHH1* are required for reentry into the cell cycle following DNA damage induced checkpoint arrest
- Deletion of *CCR4* or *DHH1* results in rescue of Brca1-induced lethality
- Discovery of Zymocin as a DNA damaging agent
- Deletion of genes required for radiation resistance results in Zymocin sensitivity
- Determination that Zymocin is an S phase specific agent in diploid yeast with a mode of action similar to HU
- Linkage between transcription zymocin and Brca1 function in yeast
- Identification and confirmation of 35 gene deletions that rescue Brca1-induced lethality-many are in transcriptional regulators
- Many newly identified gene deletions that rescue Brca1-induced lethality are highly conserved, IR sensitive, zymocin hypersensitive with a role in transcription regulation
- Correlation between defects in transcription, sensitivity to zymocin , IR sensitivity and rescue of Brca1-induced lethality strongly suggests that IR sensitive gene deletions that rescue Brca1 may be defective in transcription associated recombination (TAR)
- Identification of a physical interaction between Dhh1p and Brca1 in yeast
- Identification of a physical interaction between DDX6 (human ortholog of Dhh1p) and Brca1 in human cells

REPORTABLE OUTCOMES: Publication in Eukaryotic Cell (see appendix)

Westmoreland, T. J., Marks, J. R., Olson Jr., J. A., Thompson, E. M., Resnick, M. A. and **Bennett, C. B.** (2004) Cell cycle progression in G1 and S phases is *CCR4* dependent following ionizing radiation or replication stress in *Saccharomyces cerevisiae*. Eukaryot. Cell 3: 430-446.

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Zymocin-induced DNA damage is lethal in the yeast *Saccharomyces cerevisiae.*
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CONCLUSIONS: We have been successful in identifying and confirming 35 genes that appear to interact with Brca1 in yeast to promote cell cycle arrest and lethality. Many of these are highly conserved and appear to be required for IR resistance suggesting that the screen has been successful in identifying putative DNA repair factors. We have also validated the occurrence of a physical interaction with Brca1 for one of the protein products, Dhh1p and its human ortholog DDX6 in yeast and human cells. The success of this screen has motivated us to expand the number of breast cancer pathway genes we will express in yeast in an attempt to define an interactive network of breast cancer genes. For example the kinase STK15 is abnormally expressed in many primary breast cancers. Using the same functional genomic approach we have expressed the human STK15 gene in yeast. Similar to Brca1, there is no ortholog of STK15 in yeast and heterologous expression of STK15 results in lethality in yeast. A number of gene deletions have been found that rescue STK15 lethality. Interestingly, one gene deletion has been found that rescues both STK15 and Brca1-induced lethality. This gene deletion is *SPT4* which is a transcriptional regulator that is highly conserved in humans and the ortholog (SUPT4H1) shares a high degree of identity with Spt4p (p value = 1e –18). Function is also conserved since this gene is required for transcriptional elongation in human cells. These results suggest that our functional genomic approach has successfully identified a new breast cancer gene target (DDX6). Hopefully other cancer gene targets can also be rapidly validated using this approach since mutations in these genes may define previously unknown genetic risk factors for contracting breast cancer.

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APPENDIX: A. Published paper by Westmoreland et al.

B. 2004 Yeast Genetics Meeting Abstract by Westmoreland et al.

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Cell Cycle Progression in G_1 and S Phases Is *CCR4* Dependent following Ionizing Radiation or Replication Stress in *Saccharomyces cerevisiae*†

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To identify new nonessential genes that affect genome integrity, we completed a screening for diploid mutant *Saccharomyces cerevisiae* strains that are sensitive to ionizing radiation (IR) and found 62 new genes that confer resistance. Along with those previously reported (Bennett et al., Nat. Genet. 29:426-434, 2001), these genes bring to 169 the total number of new IR resistance genes identified. Through the use of existing genetic and proteomic databases, many of these genes were found to interact in a damage response network with the transcription factor Ccr4, a core component of the CCR4-NOT and RNA polymerase-associated factor 1 (PAF1)-CDC73 transcription complexes. Deletions of individual members of these two complexes render cells sensitive to the lethal effects of IR as diploids, but not as haploids, indicating that the diploid G_1 cell population is radiosensitive. Consistent with a role in G_1, diploid *ccr4*Δ cells irradiated in G_1 show enhanced lethality compared to cells exposed as a synchronous G_2 population. In addition, a prolonged *RAD9*-dependent G_1 arrest occurred following IR of *ccr4*Δ cells and *CCR4* is a member of the *RAD9* epistasis group, thus confirming a role for *CCR4* in checkpoint control. Moreover, *ccr4*Δ cells that transit S phase in the presence of the replication inhibitor hydroxyurea (HU) undergo prolonged cell cycle arrest at G_2 followed by cellular lysis. This S-phase replication defect is separate from that seen for *rad52* mutants, since *rad52*Δ *ccr4*Δ cells show increased sensitivity to HU compared to *rad52*Δ or *ccr4*Δ mutants alone. These results indicate that cell cycle transition through G_1 and S phases is *CCR4* dependent following radiation or replication stress.

A failure to maintain genome stability following exposure to environmental agents that damage DNA is generally considered to be an early event in cancer progression. This is supported by observations that cancers (such as those of the breast and colon) are associated with defects in genes that normally maintain genomic integrity through DNA repair, recombination, and/or checkpoint functions (20, 38). Also, many physical and chemical agents that damage DNA, including ionizing radiation (IR), are carcinogens that induce a wide array of genome-destabilizing DNA lesions (62, 66). For IR, DNA double-strand breaks (DSBs) are thought to be the most biologically relevant lesion since their persistence appears to be the primary cause of genetic instability as well as lethality (7, 11). The inability to repair DSBs can lead to deletions, gross chromosomal rearrangements, and aneuploidy (18). To avoid the destabilizing effects of IR-induced DSBs, eukaryotes have evolved highly conserved DNA repair and checkpoint pathways that maintain genomic integrity through the accurate repair of DSB damage (41).

The yeast *Saccharomyces cerevisiae* has served as an important model organism for the identification of genetic controls associated with DNA repair and checkpoint functions. Most of the gene products involved in repair of DSBs in humans were first identified in yeast (58). The repair of DSBs in yeast primarily involves the *RAD52* epistasis group of recombinational repair genes (26), while nonhomologous end joining appears to play only a minor role in the repair of IR-induced DSBs (42). Haploid yeast are extremely IR sensitive (IR^S) in G_1, since they lack a homologue for use as a template for the repair of IR-induced DSBs (15). Recombinational repair (using the newly replicated sister chromatid as a template) of DSBs in haploid cells can only occur in the S or G_2 phase of the cell cycle. Conversely, diploid cells are very IR resistant (since recombinational repair can occur throughout the cell cycle using the homologous chromosome); however, mutations of *RAD52* render diploid cells as sensitive to the killing effects of IR as haploid cells in G_1 (59).

Yeast have efficient mechanisms for the detection and signaling of DNA damage that result in the transcriptional activation of damage-inducible genes (*DIN*) as well as the arrest of cells at specific points in the cell cycle (60, 71). Damage-induced cell cycle arrest is regulated by a large number of checkpoint genes that monitor DNA integrity in the G_1/S, S, and G_2/M phases of the cell cycle (24). In the presence of DSBs or replication stress, cells detect the damage and (through transducing pathways) signal an arrest of cell cycle progression. Most checkpoint genes do not participate directly in the repair of DSBs. Instead, their effects are indirect in that they allow additional time for recombinational repair to occur.

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

Following damage-induced cell cycle arrest, another group of checkpoint-associated genes is required for cells to reenter or adapt back into the cell cycle (8, 10, 65). Defects in checkpoint adaptation result in prolonged cell cycle arrest following DNA damage. Prolonged cell cycle arrest can also occur when DNA damage persists due to a defect in a repair gene such as *rad52*, so care must be taken in describing a gene as an adaptation rather than a repair gene. Since loss of function in either checkpoint or adaptation genes can result in sensitivity to IR-induced damage, there appears to be an optimal time window during the cell cycle when repair must be completed and normal cell cycling must be resumed.

The availability of haploid and diploid yeast with a complete set of deletion mutations in nonessential genes has enabled a number of successful genome-wide screenings to be performed (5, 6, 8, 12–14, 16, 39, 56, 73). To identify new recombination or checkpoint genes that are required for the maintenance of genetic integrity following induction of DSBs, Bennett et al. previously examined 3,670 nonessential genes for the consequences of diploid homozygous mutations for growth and/or lethality following a single acute dose of IR (8). A total of 107 new genes that were required for radiation resistance were initially found. Many of these appear to affect replication, recombination, and checkpoint functions, and >50% share homology with human genes (including 17 implicated in cancer).

In this study, we report the completion of the genome-wide screening of nonessential genes and identify a total of 169 new genes that are required for radiation toleration. Many (35) of the new IR resistance genes interact genetically and/or physically in a network with the transcription factor Ccr4, which is a core component of the CCR4-NOT (CNOT) and RNA polymerase-associated factor 1-CDC73 (PAF) transcriptional complexes. We show that deletions of genes within the Ccr4 transcription complex render cells sensitive to the lethal effects of IR as diploids but not as haploids. Deletion of two core members (*CCR4* and *DHH1*) of the CNOT complex does not directly affect recombination; instead, these mutants show reduced viability in G_1 following IR due to a defect in G_1 checkpoint transition. Moreover, *ccr4* and *rad9* mutants were found to be within the same checkpoint epistasis group and *ccr4*Δ cells demonstrate a prolonged IR-induced G_1 arrest that is *RAD9* dependent. Since *ccr4*Δ, *pop2*Δ, and *dhh1*Δ cells are also sensitive to the S-phase-specific agent hydroxyurea (HU), these results suggest that (following checkpoint arrest in G_1) CNOT functions to promote cell cycle transition from G_1 into S phase with effects that also extend into S phase. Furthermore, *ccr4*Δ cells that transit S phase in the presence of HU show prolonged arrest as large budded cells followed by cellular lysis, suggesting a replication defect. The synthetic slow growth and hypersensitivity to HU exhibited by *rad52*Δ *ccr4*Δ cells further suggests an S-phase replication defect in *ccr4*Δ cells that is *RAD52* independent.

MATERIALS AND METHODS

Yeast strains and gamma-ray screening. Deletions of individual nonessential genes (or open reading frames [ORFs]) were performed with *MAT***a** (BY4741) and *MAT*α (BY4742) haploid *S. cerevisiae* strains as part of the Saccharomyces Gene Deletion Project. The diploid deletion strains (1,076 mutants) were purchased in 96-well microtiter dishes from Research Genetics (release II). Strains were screened for radiation and chemical sensitivity as previously described (8). Sensitivity to doxorubicin (dissolved in dimethyl sulfoxide; 20 mg/ml was then added to warm yeast extract-peptone-dextrose [YPD] agar) was determined at a final concentration of 50 μg/ml. YPD plates were immediately irradiated with 80 krads of gamma irradiation from a ^{137}Cs source (J.L. Sheppard & Assoc., San Fernando, Calif.) at a dose rate of 2.4 krad/min or 60 J of UV light/m^2 (dose rate of 1 J/m^2/s). These plates (along with unirradiated control plates) were examined after 24 and 48 h of growth at 30°C. Putative gamma-ray-sensitive mutants were confirmed by (i) plating serial dilutions of the strains grown for 48 h at 30°C to YPD and again exposing them to 80 krads and (ii) using survival curve analysis as previously described (8). Briefly, following 3 to 5 days of growth at 30°C, relative survival levels were determined as the ratio of viable CFU levels on gamma-irradiated versus unirradiated plates. Haploid deletion strains used to derive the IR^S diploids were also obtained from Research Genetics and individually examined for sensitivity to IR by survival curve analysis of logarithmically growing cultures.

Diploid double-deletion strains were constructed as follows. A haploid *MAT*α *rad9*Δ::*URA3* deletion strain was constructed by transforming plasmid pRR330 (cut with SalI and EcoRI) into BY4742. Putative deletions were identified by enhanced sensitivity of Ura$^+$ transformants to UV and gamma irradiation. Successful deletion of *RAD9* was confirmed by PCR using genomic template DNA obtained from an isolated Ura$^+$ colony and the appropriate *RAD9* flaking primer and an internal *URA3* primer (sequences available upon request). The *rad9*Δ strain was mated to either a *MAT***a** *dhh1*Δ::G418^R or a *MAT***a** *ccr4*Δ::G418^R strain constructed in the isogenic BY4741 background (Research Genetics). The heterozygote diploids were selected on synthetic complete (SC) glucose-uracil plates containing G418 (200 μg/ml). Through the use of standard genetic techniques, the heterozygotes were sporulated and 4 spore asci were dissected to obtain haploid *rad9*Δ *dhh1*Δ and *rad9*Δ *ccr4*Δ segregants of each mating type. *MAT***a** and *MAT*α haploid double-deletion strains were mated, and diploids were visually identified by zygote formation during mating. Diploidy of the double-deletion strains was confirmed using appropriate mating type tester strains. The *rad52*Δ *ccr4*Δ diploid strain was constructed in a similar manner by crossing a *MAT*α *rad52*Δ::*LEU2* disruption in BY4742 (obtained from K. Lewis) to the *MAT***a** *ccr4*Δ::G418^R strain described above and selecting the heterozygous diploid on SC glucose medium lacking leucine (SC GLU−LEU) and containing G418. Sporulation, selection of haploid double-mutant segregants, and construction of the diploid double mutant were similar to the procedures described above. The *rad6*Δ *ccr4*Δ diploid strain was also prepared in a manner similar to that used for the *rad52*Δ *ccr4*Δ diploid strain. Initially, we created a haploid *MAT*α *rad6*::*LEU2* deletion by transforming BY4742 with the deletion plasmid pDG315 (obtained from W. Xiao) cut with BamHI and HindIII. Successful deletion of *RAD6* was confirmed by PCR using genomic template DNA obtained from an isolated Leu$^+$ colony, the appropriate *RAD6* flaking primer, and an internal *LEU2* primer (sequences available upon request). The resulting *rad6*Δ::*LEU2 MAT*α strain was also shown to be sensitive to radiation. This *rad6* strain was mated with the *MAT***a** *ccr4*Δ::G418^R strain described above, and heterozygote diploids were selected on SC GLU−LEU containing G418. Sporulation, selection of haploid double-mutant segregants, and construction of the diploid double mutant were similar to the procedures described above. The *ccr4*Δ *his3Δ1* diploid strain was obtained by mating haploid *ccr4*Δ::G418^R Ura$^+$ his$^-$ or *ccr4*Δ::G418^R Leu$^+$ his$^-$ segregants from the sporulations of diploid *rad9*/*RAD9 ccr4*/*CCR4* or *rad52*/*RAD52 ccr4*/*CCR4* heterozygotes.

Targeted recombination at *his3Δ1*. Cells were grown to logarithmic phase in YPD liquid culture and then transformed (as described previously) with 200 ng of pRS315 and 1 μg of a partial *HIS3* PCR fragment that spanned the *his3Δ1* deletion (8). 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 only occur by targeted integration of the amplified PCR fragment into the genomic *his3Δ1* sequences following transformation. Targeted integration efficiencies were determined by calculating the ratio of the colony-forming abilities of wild-type (WT) and deletion strains on SC medium lacking histidine. Ratios were then corrected for the relative transformation efficiency of circular plasmid DNA (pRS315; *LEU2*-selectable marker on SC GLU-LEU).

Zymocin production and killer eclipse assay. WT and deletion strains were exposed (using a dilution plating technique described above) to zymocin on plates. Briefly, cells were grown for 2 days in liquid YPD (filter sterilized) in 96-well plates and serial fivefold dilutions were made in water. Cells (~2 μl of each dilution) were then transferred to YPD and YPD-zymocin plates using a 48-pin replica-plating device. YPD plates containing zymocin were made by growing *Kluyveromyces lactis* strain AWJ137 on filter-sterilized liquid YPD for 2 days at room temperature. Briefly, 2 parts of a sterile YPD filtrate of conditioned medium from the 48-h culture of the *K. lactis* strain were mixed with 1 part of 3×

agar made in fresh 1× YPD. Plates were immediately poured and allowed to solidify at room temperature. The killer eclipse assay using the *K. lactis* strains AWJ137 (zymocin producing) and NK40 (zymocin nonproducing) was performed on YPD plates as previously described (40).

Irradiation of synchronized cells. Logarithmically growing cells (~10^7 cells/ml) were exposed to benomyl (a 10 mg/ml solution of benomyl dissolved in dimethyl sulfoxide was added to cells in 5 ml of YPD to give a final concentration of 40 μg/ml) for a total of 4 h with vigorous shaking at 30°C. Exposure to benomyl by this method resulted in the arrest of ~90% of logarithmically growing cells in G_2, with no decrease in survival. Arrested cells were pelleted by low-speed centrifugation and irradiated (80 krads) following suspension of cells in water containing benomyl (40 μg/ml) as described above. Unirradiated and irradiated benomyl-arrested cells were diluted in water and plated to YPD as described above. Cells arrested by benomyl were released from the block by resuspending pelleted cells in liquid YPD (no benomyl) and grown at 30°C with vigorous shaking for 45 min. This release was asynchronous such that ~50% of cells entered into G_1 before the onset of S phase (i.e., in previous experiments newly budded cells were observed at 1 h following resuspension of benomyl arrested cells in fresh YPD). Following release from the block (after 45 min of YPD growth), cells were pelleted, irradiated (80 krads) in water, and plated to YPD as described above.

Checkpoint analysis. Position in the cell cycle can be morphologically distinguished in yeast (unbudded cells are in G_1; the beginning of S phase is marked by bud emergence; G_2 cells are large budded). To examine the checkpoint transition from single (G_1) cells into budded cells and microcolonies, logarithmically growing cells were plated to YPD, YPD-HU (200 mM), or YPD followed by exposure to 8 krads of IR. The time of transition from G_1 to S phase was determined by marking the positions of cell fields (60 to 150 cells) from each strain and repeatedly photographing the same cells at hourly intervals with a Singer MSM dissecting microscope as previously described (8). Alternatively, single G_1 cells were plated and repositioned into a grid pattern within one field of view. Cells were monitored hourly to determine the precise transition times for G_1 to S phase (single cells to small budded cells) and G_2 to M phase (large budded cells to microcolonies of 3 or more cells).

RESULTS

Genome-wide screening reveals 169 new genes that are required for toleration of gamma-ray damage in diploid yeast. We completed the genome-wide screening of the yeast diploid deletion strain collection for sensitivity to a single acute dose (80 krads) of IR (reference 8 and this study). As previously described (8), sensitivity in this screening system may be determined by decreased survival and/or slower postirradiation growth rate compared to that of the WT strain. The survival response is ascertainable only with additional tests (see below). The first study examined 3,670 genes. This study completes the genome-wide screening of nonessential genes. The remaining gene deletion mutants (1,076) were screened for sensitivity to IR and a number of other DNA-damaging agents, including UV light, methyl methanesulfonate (MMS), HU, bleomycin, camptothecin, and doxorubicin (Table 1; see Table S1 in the supplemental material).

Among the members of this collection, we have identified a further 65 diploid deletion strains that are IR^S. These were confirmed to be IR^S by replica plating serially diluted stationary-phase cells to YPD plates and exposing these to 80 krads as previously described (8). Of these, 57 have not been previously associated with sensitivity to DNA damage (Table 1; see Table S1 in the supplemental material). Therefore, 164 new genes (57 in this study plus 107 previously described) that are required for the toleration of IR damage have been identified. In addition, we identified in the combined screenings all 31 of the expected, well-characterized recombination, checkpoint, and postreplication repair genes (such as *RAD52*, *RAD9*, and *RAD6*) that are required for radiation resistance. Five gene deletion strains (*pop2*, *dbf2*, *not3*, *paf1*, and *elm1*) were not detected as radiation sensitive in the initial screening. On the basis of their described physical or genetic network interactions with identified IR^S gene deletions, however, these mutants were predicted to be IR^S. Subsequent retesting confirmed them to be IR^S. In this genome-wide screening, therefore, 4.2% (200/4,746) of the nonessential yeast genes were found to contribute to the toleration of IR damage.

Of the 169 new genes, 131 correspond to genes for which a function or genetic role has been suggested on the basis of experimental evidence (see *Saccharomyces* Genome Database [SGD]; http://www.yeastgenome.org/). Most (90%) of these deletion mutants show cross-sensitivity to one or more of the damaging agents described above (Table 1) (8). On the basis of the cross-sensitivities to other DNA-damaging agents, we can group these new IR resistance genes into 24 functional groupings, of which 6 contain previously identified DNA damage or checkpoint repair genes (Table 1). As in our previous study, many genes can also be grouped on the basis of shared functions such as transcription or protein synthesis (Table 1; see Table S1 in the supplemental material). When screened for sensitivity to other DNA-damaging agents, some of the new IR^S deletions show cross-sensitivity profiles similar to those of known recombinational repair or checkpoint genes (Table 1). With the exception of the *RAD59* deletion mutant (see Table S1 in the supplemental material), strains lacking members of the *RAD52* group of recombinational repair genes were cross-sensitive to each of the DNA-damaging agents tested.

Genetic and physical relationships among newly identified IR resistance genes identify a novel damage response network. Using literature annotated in the SGD, the Yeast Proteome Database (https://www.incyte.com/proteome/YPD/), the Munich Information Center for Protein Sequences (http://mips.gsf.de/), and the General Repository for Interaction Datasets (http://biodata.mshri.on.ca:80/grid/servlet/Index) plus recently published data describing large interactive genetic and proteomic networks, we have identified genetic and/or physical interactions among the genes and gene products required for the toleration of IR damage. The criteria for these interactions include epistasis analysis, synthetic lethal interactions, two-hybrid analysis, and mass spectrometry of immunoprecipitated protein complexes. This has allowed us to create networks that overlay our functional genomic screening with genomic and proteomic interaction maps. Using this approach, we have identified a new damage response network (as described for Fig. 1) that directly links three separate well-characterized transcriptional complexes through their interactions with *CCR4*. These transcription complexes include the CNOT complex (seven genes: *CCR4*, *DHH1*, *POP2*, *NOT3*, *NOT4*, *NOT5*, and *DBF2*), the PAF complex (four genes: *CCR4*, *PAF1*, *HPR1*, and *RTF1*) and the SRB transcription complex (*SRB5* and *ANC1*). Furthermore, *RLR1* (*THO2*) interacts with *HPR1* in the THO complex that is required for transcriptional elongation. Individual deletions of these genes render cells IR^S as diploids.

Members of the SAGA complex (*SPT20*, *ADA2*, *GCN5*, and *HFI1*) which are required for transcriptional activation of a subset of RNA polymerase (Pol) II-dependent genes are linked to the CNOT complex through interactions with the essential gene *CDC36* (*NOT2*). The *CCR4* gene product ap-

TABLE 1. Cross-sensitivity of gamma-ray-sensitive diploid deletion strains to other DNA-damaging agents[a,b]

UV	Bleo	MMS	HU	Camp	Doxo	Functional grouping(s)	Gamma-ray-sensitive gene-ORF deletion(s) (n = 65)
S	S	S	S	S	R	Bud site selection	*BUD32*
S	S	S	S	R	S	Recombination, transcription	***RLR1***, *NOT4*, *NOT5*, *SRB5*, *MDM20*
S	S	S	S	R	R	Cytoskeleton, sporulation	*SRV2*
S	S	S	R	R	S	Transcription, DNA recombination	*YAF9*, *SLX8*
S	S	S	R	R	R	Transcription	*BDF1*
S	S	R	S	R	S	α-1,6-Mannosyltransferase	*OCH1*
S	S	R	R	R	S	Trehelose synthesis	*TPS1*
S	S	R	R	R	R	Checkpoint	***DDC1***, *EAP1*
S	R	S	R	S	S	?	*YJL184W*[d]
S	R	R	R	R	S	mRNA processing	*LSM7*
S	R	R	R	R	R	Checkpoint	***RAD24***
R	S	S	S	S	S	60S ribosomal protein subunit	*RPL31A*
R	S	S	S	R	S	Vacuolar organization-biogenesis	*FAB1*, *YGL218W*
R	S	S	R	S	S	?	*YBR100W*
R	S	S	R	S	R	Recombination	***RAD59***
R	S	S	R	R	S	DNA repair, mitochondrial	***SAE2***, *MDM10*
R	S	R	S	R	S	? (Diverse functions)	*ATP4*, *PLC1*, *VPS33*, *MAP1*, *YDJ1*[d]
R	S	R	R	R	S	Transcription, protein synthesis	*GLO3*, *ADA2*, *GCN5*, *RPL34B*, *TIF4631*, *YDL041W*, *YDR532C*, *YBR077C*
R	S	R	R	R	R	Checkpoint, DNA repair	***RDH54***, ***PSO2***, *SCO1*, *YML036W*
R	R	S	S	R	S	Elongated bud morphology[c]	*YJL075C*
R	R	S	R	S	S	DNA repair	***MMS4***
R	R	S	R	R	R	Chromatin	*NAT1*, *YLR358C*
R	R	R	S	R	S	Transcription	*SPT20*, *TUP1*
R	R	R	S	R	R	Actin cytoskeleton	*ARP8*, *ARP5*
R	R	R	R	R	S	Chromatin, mitochondrial, transcription	*ASM4*, *HMO1*, *MBP1*, *ATP2*, *MRP10*, *DEG1*, *ADE12*, *BMH1*, *MDJ1*, *YGR272C*
R	R	R	R	R	R	? (Diverse functions)	*DOT1*, *RIM1*, *BRE1*, *TPS2*, *YDR417C*, *YNL080C*

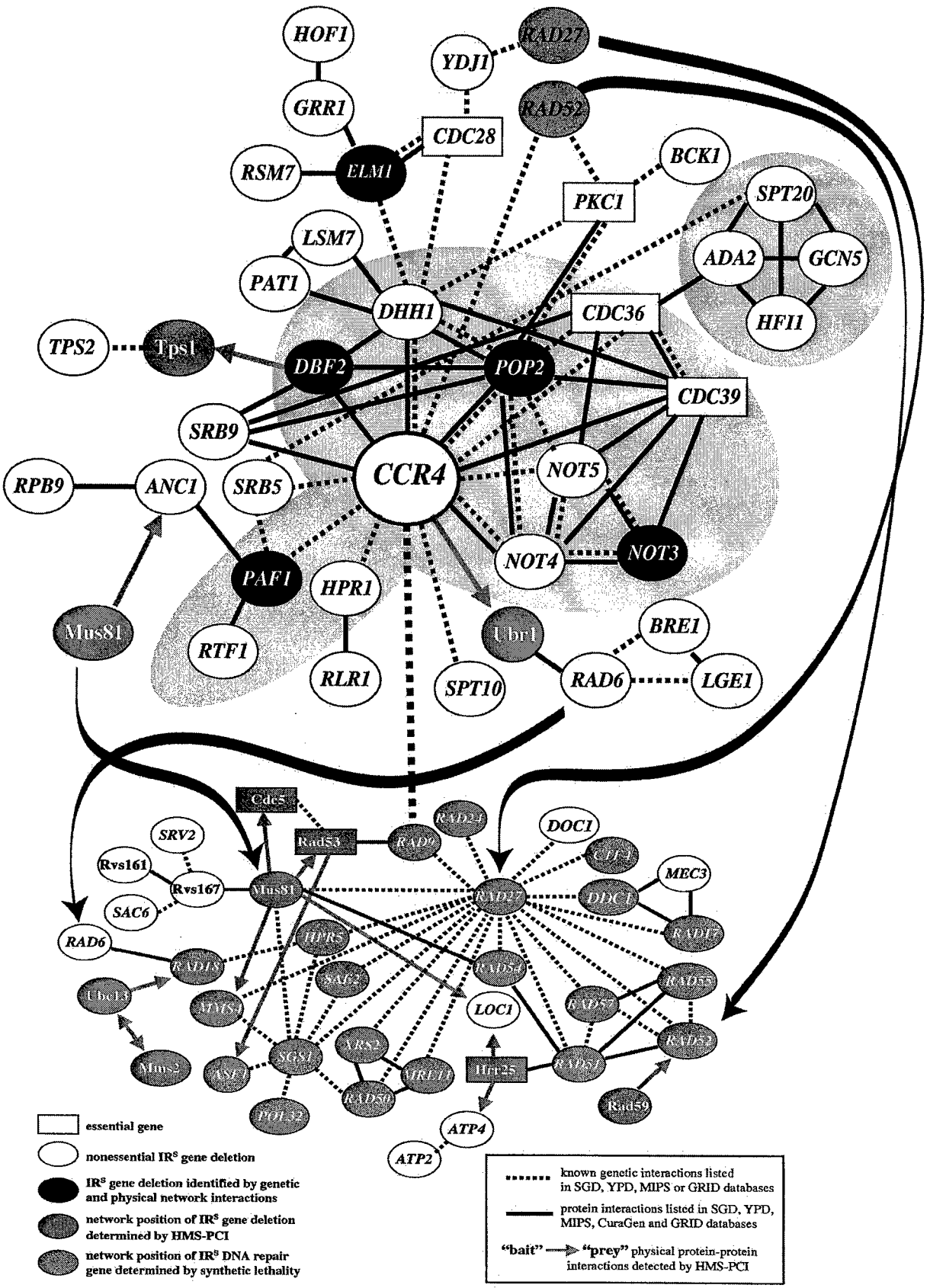
[a] Approximately 1,100 diploid deletion strains were simultaneously screened for sensitivity to seven physical and chemical agents. Relative sensitivity levels were determined by dilution plating, and some strains were more sensitive than others (see Table S1 in the supplemental material). Strains sensitive to IR are listed (cross-sensitivity to other agents is indicated). Several of the genes have multiple functions. Eight genes (indicated with boldface characters) have been previously characterized as participating in DNA repair or checkpoint functions.

[b] UV, 60 J/m^2 (sensitivity defined as described above); Bleo, 4 ug of bleomycin/ml (sensitivity defined as described above); MMS, 2 mM methyl methane sulfonate (sensitivity defined as described above); HU, 100 mM hydroxyurea (sensitivity defined as described above); Camp, 10 ug of camptothecin/ml in 25 mM HEPES buffer, pH 7.2 (sensitivity defined as described above); Doxo, 50 ug of doxorubicin (sensitivity defined as described above).

[c] *APQ13* enhanced apical growth detected by quantitative analyses (Yoshikazu Ohya, personal communication).

[d] Poor survival upon refrigeration at 4°C on YPD plates; unable to be grown from frozen stocks at Research Genetics.

pears to play a central role in the toleration of IR damage, since it interacts with at least 13 other nonessential genes (including *RAD9*) whose absence confers IR^S (this study; see below and Fig. 1). Another 23 radiation resistance genes are indirectly linked to *CCR4* (through pathways that connect to the 13 genes that directly interact with *CCR4*) (Fig. 1). Because *CCR4* has the largest number of genetic and/or physical associations among the members of our combined collection of


HOF1
YDJ1
RAD27
GRR1
RAD52
CDC28
RSM7
ELM1
BCK1
PKC1
SPT20
LSM7
ADA2
GCN5
PAT1
DHH1
CDC36
HFI1
TPS2
Tps1
DBF2
POP2
CDC39
SRB9
CCR4
NOT5
RPB9
ANC1
SRB5
NOT4
NOT3
PAF1
HPR1
Mus81
RTF1
Ubr1
BRE1
RLR1
SPT10
RAD6
LGE1
Cdc5
SRV2
Rad53
RAD24
DOC1
Rvs161
Rvs167
Mus81
MEC3
SAC6
RAD6
RAD18
RAD54
RAD55
LOC1
RAD57
RAD52
Ubc13
Mms2
SGS1
ATR1
RAD50
Hrr25
RAD59
ATP4
ATP2
essential gene
nonessential IR^S gene deletion
IR^S gene deletion identified by genetic and physical network interactions
network position of IR^S gene deletion determined by HMS-PCI
network position of IR^S DNA repair gene determined by synthetic lethality
known genetic interactions listed in SGD, YPD, MIPS or GRID databases
protein interactions listed in SGD, YPD, MIPS, CuraGen and GRID databases
"bait" → "prey" physical protein-protein interactions detected by HMS-PCI

newly identified damage toleration genes, we have collectively named these genes the CCR4 damage response network.

The CCR4 damage response network has a number of genetic and/or physical interactions with characterized repair genes (including *RAD9*, *RAD52*, *RAD6*, *RAD27*, and *MUS81*) (Fig. 1). Furthermore, members of the PAF complex (*HPR1*) as well as *RLR1* play a role in transcription elongation and confer a hyperrecombination phenotype when deleted. The repair genes *RAD9*, *RAD52*, *RAD6*, *RAD27*, and *MUS81* participate in another interactive damage response network that includes a large number of the IR resistance genes detected in our screening and elsewhere (Fig. 1). In total, 68 genes form an overlapping interactive network that includes previously characterized repair genes and those from our combined collection of newly identified radiation resistance genes (Fig. 1).

We have also found from our combined studies IR resistance genes that belong to smaller groups within which the genes and/or protein products interact genetically or physically. Six interacting genes within the nuclear pore complex (*NUP84*, *NUP120*, *NUP133*, *NUP170*, *NUP188*, and *ASM4*) are sensitive to IR following deletion (12) (see Table S1 in the supplemental material). Another group of six IR toleration genes (*PDR13*, *ZUO1*, *SRO9*, *TIF4631*, *SCP160*, and *BFR1*) can be grouped through genetic and/or physical interactions but share no apparent common function. A group of interacting IR resistance genes (*RVS161*, *RVS167*, *SAC6*, and *SRV2*) have been implicated in actin-related, cytoskeletal functions. Recently these actin-related genes have been found to interact with the repair protein Mus81 through Rvs167 (Fig. 1). Three groups (a group consisting of *NAT1*, *NAT3*, and *ARD1*, a group consisting of *BUD32*, *DIA4*, and *YML036W*, and a group consisting of *RAD6*, *YPL055C* [*LGE1*], and *BRE1*) containing three IR resistance genes each were also found to interact physically or genetically. Finally, three pairs of IR resistance genes (pair *CIS3* and *BUR2*, pair *BEM1* and *AKR1*, and pair *RAD1* and *RAD10*) were also found to interact genetically and/or physically. Thus, 47% (94/200) of the IR^S gene deletions show genetic or physical interactions as part of a large damage response network or within smaller interactive groups.

Members of the CCR4 damage response network have overlapping functions in cell size homeostasis and zymocin resistance. Recently, genome-wide screenings have identified gene deletions that are required to maintain cell size homeostasis (39, 73). Surprisingly, a large number (80/200 = 40%) of the gene deletions that have been identified as IR^S from our combined studies have also been characterized as having abnormally small or large cell volumes compared to the results seen with WT cells (Table 2). Many of these genes are members of the CNOT or PAF complexes and are thought to modulate cell size by altering expression of G_1 cyclins required to progress from G_1 to S phase of the cell cycle. Of the genes that interact with *CCR4* (see Fig. 1, upper panel), deletion of 16 (*POP2*, *DBF2*, *NOT4*, *PAF1*, *HPR1*, *SRB5*, *RLR1*, *ANC1*, *RPB9*, *SPT10*, *HFI1*, *PAT1*, *TPS1*, *HOF1*, *YDJ1*, and *BCK1*) (in addition to *CCR4*) has been shown to cause altered cell size homeostasis. With the exception of *BCK1* and *TPS1*, all of these gene deletions result in cells that are larger than WT cells. Since the *cln3* mutant strain also produces large cells, many of these gene deletions appear to affect the G_1- to S-phase transition by delaying *CDC28*-dependent Start function.

Many of the IR^S strains that interact with Ccr4 and are defective for RNA Pol II transcription (strains *CCR4*, *POP2*, *NOT3*, *NOT4*, *NOT5*, *RTF1*, *SRB5*, *SPT20*, *ADA2*, *GCN5*, and *DHH1*) are also hypersensitive to the killer toxin zymocin that is produced by the yeast *K. lactis*. This toxin causes a prolonged G_1 arrest and lethality in haploid *S. cerevisiae*. The overlap between IR^S deletion strains that are also sensitive to zymocin and show defects in cell size control suggests that these mutant phenotypes all share a common underlying molecular defect. Furthermore, the overlap of mutants sensitive to both zymocin and IR suggests that the presence of zymocin might induce DNA DSB damage. Since abnormal cell cycle regulation at the G_1/S phase boundary has been observed for zymocin-hypersensitive cells and for cells with altered cell size homeostasis, this suggests that IR^S mutants that share these phenotypes might also have abnormal regulation at the G_1/S boundary in response to IR. Interestingly, deletions of four genes (*MEC3*, *SFP1*, *BCK1*, and *MRT4*) that have been previously associated with defective damage checkpoint arrest produced cells that were abnormally small compared to WT cells (Table 2). Therefore, IR^S deletions that overlap with those that fail to inhibit G_1- to S-phase transition in response to growth signals may

FIG. 1. Interaction of the CCR4 damage response network with known repair and checkpoint genes. Nonessential, IR^S gene deletions are depicted by an oval enclosing the gene name. Genes or their protein products that interact either genetically (dotted lines) or physically (solid black lines without arrows or red lines with arrows) in the network are shown. Critical essential genes that have described roles in recombination and checkpoint functions or link nonessential genes within the *CCR4* network have been indicated with a rectangular box enclosing the gene name. Ccr4 is a core member of two separate transcription complexes. IR^S members of the CNOT complex are highlighted in yellow, while members of the PAF complex are highlighted in pink. CCR4 appears to be the core of this network, since it shows the largest number (13) of genetic and physical interactions with other radiation resistance genes. Members of the SAGA transcriptional complex that confer radiation resistance have been highlighted in blue. On the basis of their network interactions, gene deletions not initially identified in the primary screening as IR^S (indicated with an oval containing white characters on a black background) were subsequently identified (using dilution pronging or survival curve analysis) as radiation sensitive (see Fig. 2). Genetic and physical interactions were determined using the following databases: SGD, the Yeast Proteome Database, the Munich Information Center for Protein Sequences, the Pathcalling yeast interaction database at CuraGen Corporation, and the General Repository for Interaction Datasets. Network positions of the genes indicated with white characters on a red background have been determined by high-throughput mass-spectrometric protein complex identification (HMS-PCI). Red arrows indicate the direction (from the bait protein to the prey) of the interaction (33). Network positions of the genes indicated with white characters on a green background have been determined from a systematic examination of synthetic lethal interactions (67). Some proteins (Ydj1 and Atp2) identified by HMS-PCI but omitted due to high frequencies of interaction have been included in this figure on the basis of supporting data indicating genetic interactions with other known IR resistance genes. The CCR4 network (upper grouping) interacts with other established repair networks or pathways (lower grouping of genes) through at least four intermediary IR resistance genes (*MUS81*, *RAD6*, *RAD27*, and *RAD52* [indicated with black lines with arrows]) that serve as a linkage between the two networks. The genetic linkage of *CCR4* with *RAD9* was determined by epistasis analysis in this study.

TABLE 2. IR-sensitive gene deletions that show overlapping defects in G1-regulated responses (including maintenance of cell size homeostasis and sensitivity to zymocin)

Cell size homeostasis defect[a]	Sensitivity to zymocin[b]	IR-sensitive gene deletion[c]
Large	Yes (n = 39)	*ADK1, AKR1, ANC1, APN1, ASF1, BEM1, BFR1, BUR2,* ***CCR4****, CDC40, CLC1, DEG1, DHH1, EAP1, EST1, HFI1, HPR1, HTL1, MMS22,* ***NOT3****,* ***NOT4****,* ***NOT5****, PAT1,* ***POP2****, RAD50, REF2, RPB9, RSC1, RSC2,* ***RTF1****, RVS161, SCP160,* ***SRB5****, VID21, VID31, YBL006C, YCL016C, YLR322W, YPL055C*
Large	No (n = 2)	*FUN12, YML014W*[d]
Large	ND[e] (n = 18)	*ARP5, ARP8, BDF1, BRE1, BUD32, CDC73, DBF2, FAB1, HOF1, PAF1, PLC1, RLR1, SLX8, SPT10, SRV2, YDJ1, YDR532C, YLR358C*
Small	Yes (n = 10)	*BCK1, DIA4, MEC3, MRPL31, RSA1, SFP1, TOM37, ZUO1, YGR165W, YJL188C*
Small	No (n = 2)	*LOC1, MRT4*
Small	ND (n = 10)	*ATP4, GLO3, MAP1, MDM10, RIM1, RPL34B, SCO1, TPS1, YDR417C, YGL218W*

[a] Yeast deletion strains that fail to maintain cell size homeostasis (i.e., cells are either larger or smaller than wild-type cells) as described by Jorgensen et al. (39) and Zhang et al. (73).

[b] Enhanced sensitivity of diploid deletion strains to zymocin was determined using the zymocin eclipse assay or dilution plating to zymocin-containing plates as previously described by Kitamoto et al. (40). Sensitivity of the *dhh1* Δ to zymocin was previously determined by Westmoreland et al. (72). Haploid deletion strains previously described by Kitamoto et al. (40) as hypersensitive to zymocin are highlighted in boldface characters.

[c] IR-sensitive diploid deletion strains identified in this study and by Bennett et al. (8). The *cdc73*Δ strain was IR sensitive only at a high dose (120 krads).

[d] The *yml014w*Δ strain showed enhanced resistance to zymocin (i.e., enhanced growth rate and survival) compared to the WT.

[e] ND, not yet determined.

represent a subset of genes required for DNA damage-dependent checkpoint functions. Taken together, these results suggest that many of the newly identified IR^S gene deletions might exhibit defects in cell cycle transition at the G_1/S boundary. Since individual deletions of at least seven members of the CNOT complex render cells IR^S (and since *CCR4* appears to be the hub of an interactive damage response network), we investigated in detail the mechanistic role the CNOT transcriptional complex plays in radiation resistance in diploid yeast cells.

Two *CCR4*-dependent transcription complexes are required for toleration of radiation in diploid cells. In our previous IR screening, we determined that two core members (*CCR4* and *DHH1*) of the CNOT complex were required for radiation resistance in diploid yeast (8). However, several members of the CNOT complex were not present in our first screening (which included only 3,670 of the 4,746 nonessential genes). Screening the remaining genes identified another two members (*NOT4* and *NOT5*) of the CNOT complex that were IR^S. In our previous screening, we also found enhanced IR sensitivity for the isogenic diploid *hpr1*Δ and *rtf1*Δ strains. Both these genes are members of the PAF transcription complex, which is distinct from the CNOT complex even though both complexes contain Ccr4 (17, 19, 54). Deletion of *HPR1* has been shown to cause hyperrecombination but does not result in radiation sensitivity in haploid cells (2, 3). These results suggest that the two *CCR4*-dependent transcriptional complexes (CNOT and PAF) are required for IR resistance in diploid yeast.

To confirm that the mutations in the CNOT complex were IR^S due to single recessive gene deletions and not due to errors in strain construction, we transformed the diploid *ccr4*Δ and *dhh1*Δ strains with plasmids containing full-length copies of *CCR4* and *DHH1*. These strains showed WT survival when exposed to a single dose of IR (80 krads; Fig. 2A). In addition, we found that a reconstructed diploid *dhh1*Δ strain (haploid strains BY4741 and BY4742 [each individually lacking *DHH1*] were mated) was also IR^S (data not shown).

To determine whether the IR sensitivity of mutants within these two CCR4-dependent complexes was due to altered survival responses and not to slow growth recovery, we compared cell survival of the deletion strains described above to that of the recombination-deficient *rad51*Δ and WT strains following exposure to various doses of IR (Fig. 2B). On the basis of reported protein interactions of Ccr4 with Dbf2 (45) and Pop2 (32) and Not4 and Not5 with Not3 (44) (as well as the genetic interaction of *DHH1* with *ELM1*) (53), we also used dilution pronging and survival curve analysis to examine *dbf2*Δ, *pop2*Δ, *not3*Δ, and *elm1*Δ cells for IR sensitivity. We similarly used survival curve analysis to examine strains lacking two core members of the PAF complex (*PAF1* and *CDC73*) for sensitivity to IR. These six deletion strains were not initially identified as IR^S, possibly due to insensitivity of the spot-testing screening technique.

As predicted, all CNOT complex mutations (including *dbf2*Δ, *pop2*Δ, and *not3*Δ) demonstrated increased IR sensitivity (Fig. 2B and data not shown). However, the dose-dependent decreases in survival were intermediate between those seen for WT and *rad51*Δ diploid strains, suggesting they do not play a direct role in recombination. Similar results were found for the *srb5*Δ, *hpr1*Δ, and *paf1*Δ strains (Fig. 2C), but enhanced IR sensitivity was observed only at a high radiation dose (120 krads; Fig. 2C) for the *cdc73*Δ strain. The survival of these deletion strains was greater than that for the recombination-deficient *rad51*Δ or *rad52*Δ strains following IR (Fig. 3A). Therefore, the CNOT and PAF complexes do not appear to play a direct role in recombinational repair (although a minor or indirect role cannot be ruled out without epistasis analysis). The capability of these strains to undergo *RAD52*-dependent PCR-mediated gene targeting (see below) further suggests they do not directly affect recombination.

Deletion mutants within the *CCR4* dependent transcriptional complexes are radiation resistant in G_2. In yeast, unrepaired DSBs are the primary source of lethality caused by IR. Haploid strains are extremely sensitive in the G_1 phase of the cell cycle, since DSBs are not able to utilize recombinational

repair due to the lack of an available homolog. WT haploid cells that have replicated their DNA prior to segregation (i.e., G_2 cells) are IR resistant due to their ability to repair DSBs by recombination using the undamaged sister chromatid as a template. Diploid cells are IR resistant in G_1 because of the presence of homologous chromosomes. Among some of the previously identified IR^S diploid deletion strains, the isogenic haploid derivatives were more resistant to radiation (8). We therefore examined the IR sensitivity of logarithmically growing *MAT***a** haploid strains lacking individual members (*ccr4*Δ, *dhh1*Δ, *not4*Δ, *hpr1*Δ, and *paf1*Δ) of the two *CCR4*-dependent transcription complexes. These haploid strains did not show enhanced IR sensitivity compared to the WT strain (Fig. 2D), thus indicating that these mutants lacking members of the two *CCR4*-dependent transcription complexes are recombination proficient for DSBs induced in G_2. Furthermore, this suggests that a mechanism other than reduced recombination between sister chromatids in G_2 is responsible for the IR sensitivity of diploid mutants.

The resistance of G_2 haploid cells led us to consider whether IR sensitivity of the *CCR4* diploid mutants is primarily due to killing of the G_1 population. We therefore compared the radiation sensitivities of logarithmically growing versus stationary cultures of WT and mutant *ccr4*Δ cells. A threefold increase in the survival fractions was obtained for irradiated logarithmically growing *ccr4*Δ diploid cells (~80% budded) compared to the results seen with stationary cells (<10% budded) which were exposed to 80 krads of IR. The survival fractions for *ccr4*Δ cells relative to those of WT cells were 0.82 versus 0.27 (means of four experiments [80 krads]) for the logarithmically growing and stationary cells, respectively.

To confirm that the G_1 cell population of the *ccr4*Δ strain was radiosensitive compared to the G_2 cell population, we used the tubulin inhibitor benomyl to synchronize WT and *ccr4*Δ cells in G_2 (92 and 88% [means of five experiments] large budded cells for WT and *ccr4*Δ strains, respectively). These synchronized cells were exposed to IR (80 krads), and viability was determined by plating unirradiated and gamma-irradiated synchronized cells to YPD. We similarly determined the relative survival levels of unirradiated and IR (80 krads)-exposed *ccr4*Δ and WT cells following release from the benomyl cell cycle block (when irradiated, 59 and 45% of the cells were unbudded [i.e., in G_1] for the WT and *ccr4*Δ strains, respectively). The resulting survival fractions for *ccr4*Δ cells relative to those for the WT cells were 0.81 for the synchronized G_2 cells and 0.57 for the released asynchronous population of G_1 and G_2 cells. If the G_2 cells within the asynchronous *ccr4*Δ cell population are assumed to have the same survival rate as the WT benomyl-treated cells, then the relative survival of the *ccr4*Δ G_1 cell fraction was 0.28. This relative decrease in the level of survival (compared to that of the WT cells) for the *ccr4*Δ G_1 cells released from the benomyl block was very similar to that obtained for cells that were irradiated as stationary G_1 cultures (0.27). These results are consistent with the IR sensitivity of the diploid *ccr4*Δ strain being primarily associated with the G_1 population of cells.

Strains lacking *CCR4* are recombination proficient. We previously reported that targeted chromosomal recombination, which is a *RAD52*-dependent process, was not significantly decreased in the diploid *dhh1*Δ strain but was enhanced in the *hpr1*Δ strain (8). To confirm that diploid *CCR4* complex mutants were radiation sensitive due to a defect in a process other than *RAD52*-dependent recombination, we similarly assayed the *ccr4*Δ, *paf1*Δ, and *cdc73*Δ strains for targeted PCR fragment-mediated recombination at the chromosomal *his3*Δ*-1* locus. For the *ccr4*Δ strain, the efficiency of targeted recombination at the *his3*Δ*-1* locus was comparable to that observed in the WT strain (1.2 ± 0.7 versus 1.0 ± 0.7). Furthermore, both the diploid *paf1*Δ and the *cdc73*Δ strains produced targeted recombination efficiencies that were similar to that of the WT strain (0.65 ± 0.17 and 2.6 ± 1.9, respectively). By comparison, the recombination-deficient *rad51*Δ strain had a significantly reduced targeted-recombination efficiency (0.016 ± 0.005) whereas the hyperrecombination strain *hpr1*Δ had an enhanced recombination efficiency (24 ± 10) compared to the WT strain (8). These results suggest that the *ccr4*Δ, *paf1*Δ, and *cdc73*Δ strains are recombination proficient for PCR fragment-mediated targeted recombination at *his3*Δ*-1*.

Following exposure of recombination-deficient diploid *rad52* cells to IR (20 krads), chromosome integrity is lost as measured by pulse-field gel analysis and only partially restored after prolonged liquid holding (resuspension of cells in water) of the damaged cells for 24 to 48 h (52). However, lost chromosome integrity was completely restored in diploid *ccr4*Δ cells at 4 to 6 h following irradiation at a much higher dose (40 krads) when chromosome integrity was examined by pulsed-field gel analysis (data not shown). These results further indicate that *ccr4*Δ cells are recombination proficient and are able to repair IR-induced DSB damage.

CCR4 is a member of the RAD9 checkpoint repair epistasis group. Epistasis analysis can be used to determine whether two IR resistance genes are members of the same genetic pathway. IR resistance genes are within the same epistasis repair group if the IR sensitivity of a strain containing both mutations is no greater than the sensitivity of the more sensitive of the two single gene mutation strains (26). Since a *CCR4* mutation was reported to suppress the IR sensitivity of an allele of *rad52* (*rad52-20*) (61), we determined whether *CCR4* was a member of the *RAD52* radiation repair epistasis group. Although *RAD52* is responsible for the majority of DSB repair in yeast, we also examined whether *CCR4* was a member of two other epistasis groups (*RAD6* and *RAD9*) which are responsible for most of the remaining IR repair.

IR-induced killing of the diploid *rad52*Δ *ccr4*Δ and the *rad6*Δ *ccr4*Δ strains was enhanced by an order of magnitude compared to that of the *rad52*Δ or *rad6*Δ strain alone (Fig. 3A). However, sensitivity to IR was not enhanced for the *rad9*Δ *ccr4*Δ or the *rad9*Δ *dhh1*Δ strain compared to that of the *rad9*Δ strain alone (Fig. 3A), suggesting that the *CCR4* and *DHH1* genes function in the same pathway as *RAD9*. Both the diploid *ccr4*Δ and *dhh1*Δ survival curves (Fig. 2B) were similar to that expressed by the diploid *rad9*Δ strain (Fig. 3A). This epistasis analysis therefore indicates that *CCR4* is a member of the *RAD9* checkpoint pathway for IR-induced cell killing.

Strains lacking *CCR4* have an S-phase cell cycle defect following replication stress. Since the CNOT complex plays a role in cell cycle responses to stress, we examined its impact when a *ccr4*Δ was combined with a *rad52*Δ and the double mutant exposed to the replication inhibitor HU. We observed a synergistic decrease in growth rate when *CCR4* and *RAD52*

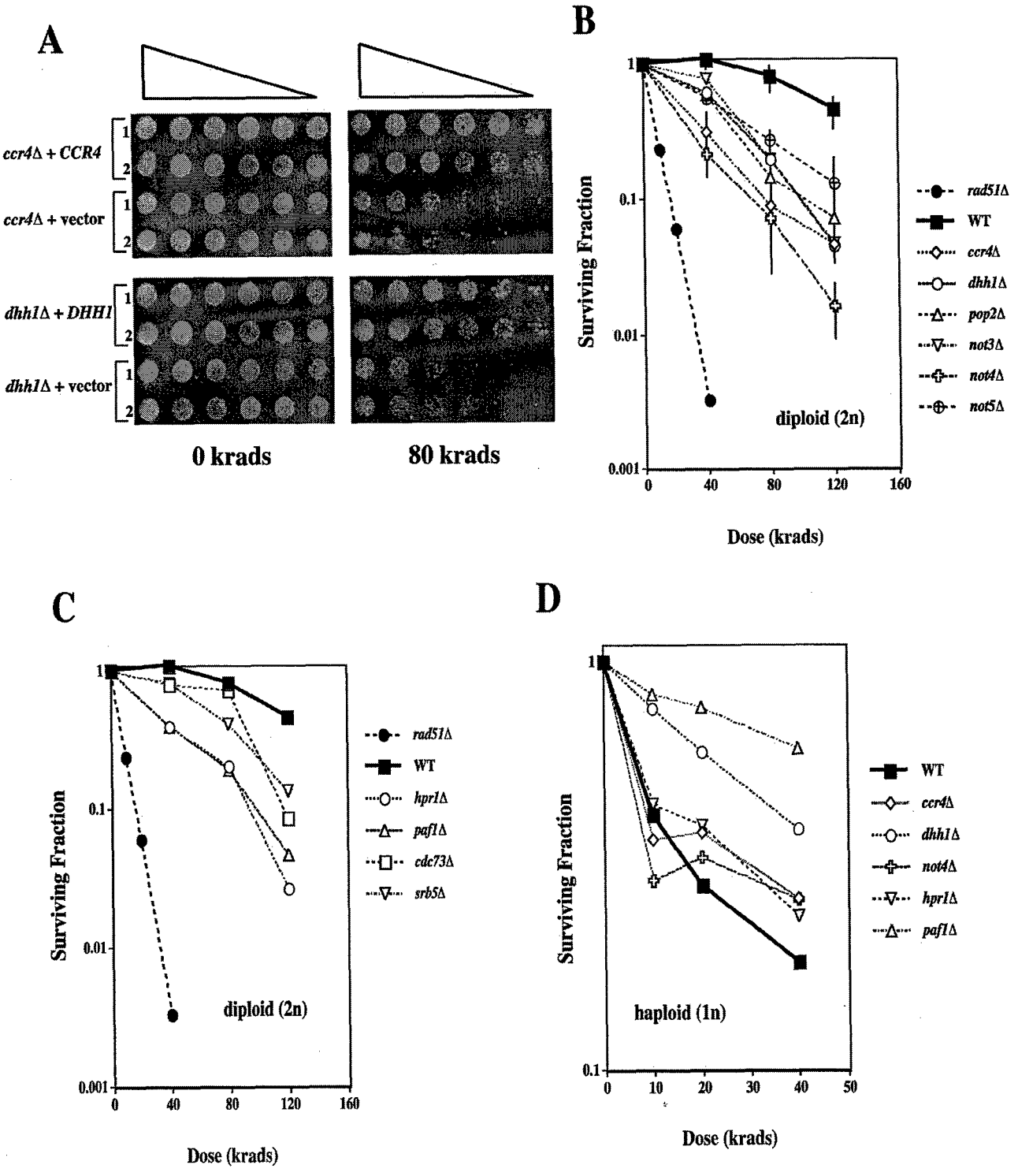


FIG. 2. Reduced survival of gamma-irradiated diploid stationary cells lacking various members of two CCR4-dependent transcriptional complexes. (A) *ccr4*Δ and *dhh1*Δ diploid strains containing the plasmid YEP13-CCR4 or pRS425-DHH1 (49) that express full-length copies of *CCR4* and *DHH1* or vector alone were grown for 2 days in SC GLU−LEU and serially diluted fivefold. Cells were replica pronged to SC GLU−LEU plates, immediately irradiated with 80 krads of gamma rays, and grown for 3 days at 30°C. Downward-sloping triangles indicate decreasing cell concentration gradients. Complementation of the *ccr4*Δ and *dhh1*Δ strains for radiation resistance was similar to that observed for the WT (data not shown). (B) Diploid strains were grown to stationary phase (4 days at 30°C) in liquid YPD such that >95% of cells were in G_1 (i.e., unbudded) at the time of irradiation. A dose-dependent decrease in survival of colony-forming ability was seen for diploid strains lacking members of the CNOT complex. Typical error measurements (± 1 standard error) have been shown for WT, *ccr4*Δ, *not4*Δ, and *not5*Δ strains. (C) Diploid cells lacking members of the PAF complex were grown and irradiated as described above. (D) Haploid strains were grown

deletions were combined in the same strain (Fig. 3B); this decrease was not due to loss of mitochondrial function. The generation time of the double mutant was 4.9 h compared to 2.6 and 1.9 h for the *rad52*Δ and *ccr4*Δ mutants, respectively (Fig. 3B), and 1.7 h for the WT strain. There was no decreased growth rate for the *rad6*Δ *ccr4*Δ or *rad9*Δ *ccr4*Δ strain (data not shown).

Diploid strains lacking *CCR4* and other members (including *DHH1* or *POP2*) of the CNOT complex are sensitive to HU and MMS compared to WT strains (Fig. 3C). Similar to the diploid strains, haploid strains lacking *CCR4* and *DHH1* also demonstrated enhanced sensitivity to HU and MMS compared to WT strains (data not shown). Diploid *rad52*Δ strains are also sensitive to HU (Fig. 3C) because of the inability to repair DSBs produced during HU-induced replication arrest (51). We therefore compared the HU sensitivity of the *rad52*Δ *ccr4*Δ double mutant to that of the single *rad52*Δ and *ccr4*Δ mutants to determine whether these genes could be placed in the same epistasis group for HU-induced lethality. The *rad52*Δ *ccr4*Δ strain was hypersensitive to the killing effects of HU (Fig. 3C). Similar results were observed with MMS, an alkylating agent that is also S phase specific for the induction of DNA damage (Fig. 3C), as well as with doxorubicin, which is a potent topoisomerase inhibitor (data not shown). After extended incubation times at lower doses of HU (25 mM) and MMS (0.5 mM), the enhanced lethality of the slow-growing *rad52*Δ *ccr4*Δ strain compared to that of the *ccr4*Δ and *rad52*Δ stains is apparent (Fig. 3C). These results indicate that for HU survival, *CCR4* is in an epistasis group separate from that defined by the *RAD52* repair group. Moreover, *CCR4* may be required for cell cycle progression through S phase in the presence of chemical agents that induce replication stress.

When HU blocks DNA replication, WT cells arrest as budded cells until S phase is completed; this is referred to as the S/M checkpoint. To examine whether cell cycle progression of *ccr4*Δ cells is inhibited by the presence of HU, single G_1 cells from logarithmically growing cultures of WT and *ccr4*Δ strains were examined hourly for cell cycle progression on YPD plates containing 200 mM HU (Fig. 3D). After 6 h on HU, most of the WT and *ccr4*Δ cells initially plated in G_1 progressed into S phase and arrested as budded cells. In the absence of HU, the majority (85%) of G_1 cells from these two strains progressed to form microcolonies at 6 h (data not shown). Although all of the WT cells completed S phase and progressed to form viable microcolonies after 24 h of exposure to HU, most (53%) of the *ccr4*Δ cells remained as large budded cells (Fig. 3D) and most (60%) were lysed (data not shown). Similarly, nearly 65% of the G_1 *rad52*Δ *ccr4*Δ cells were large budded cells after 6 h; almost all (88%) were lysed after 24 h, however, and none progressed further than the three-cell microcolony stage (data not shown). This severe growth arrest which was followed by cellular lysis was not observed with *rad52*Δ strains and may account for the enhanced hypersensitivity of the *rad52*Δ *ccr4*Δ strain when it is plated to HU (Fig. 3C) compared to the results seen with *ccr4*Δ or *rad52*Δ strains alone. Taken together, these results suggest that HU inhibited cell cycle progression in S phase in *ccr4*Δ cells. Since this effect is enhanced in the absence of *RAD52*, *ccr4*Δ strains appear to have a defect in replication or adaptation to the S/M checkpoint which is independent of recombination.

***CCR4* and *DHH1* are required for cell cycle progression in G_1 and G_2 following gamma irradiation.** Since *CCR4* and the *RAD9* checkpoint gene reside within the same epistasis group, we examined cell cycle progression of irradiated *ccr4*Δ and *dhh1*Δ cells. In budding yeast, cell cycle arrest following IR damage occurs at G_1 as well as G_2 stages of the cell cycle and both checkpoints are under the control of the *RAD9* gene product (63, 71). However, *RAD9* has also been implicated in S-phase checkpoint arrest and is required for the damage-induced transcription of a number of repair genes normally expressed in S phase. Since diploid deletions of *CCR4* are IR^S in G_1 and also show reduced G_1 arrest following nitrogen starvation (72), we examined whether the transition from G_1 to S phase (i.e., at the G_1 checkpoint) was abnormal following IR in diploid strains lacking *CCR4* or *DHH1* as well as *RAD9*. A rapid and comparable G_1 to S transition was observed for all unirradiated strains (Fig. 4A). As previously reported (31), irradiated *rad9*Δ cells had a more rapid (1.1 ± 0.5 h earlier on average) G_1- to S-phase transition compared to WT cells (Fig. 4A). However, the *dhh1*Δ and *ccr4*Δ strains showed prolonged G_1 arrest following IR. The transition times from G_1 to S phase in these strains were longer (1.4 ± 0.6 and 2.5 ± 1.0 h for *dhh1*Δ and *ccr4*Δ, respectively) than that observed in WT (Fig. 4A).

Similarly, in the *dhh1*Δ and *ccr4*Δ strains, we found a prolonged delay in cell cycle progression among the irradiated budded (S plus G_2) cell populations following IR compared to the results seen with WT cell populations (Fig. 4B). For all strains examined, the transition of unirradiated budded cells was more rapid than that observed for irradiated cells. Therefore, *dhh1*Δ and *ccr4*Δ cells exhibit prolonged cell cycle delay at two morphological checkpoint "landmarks" following IR exposure.

Prolonged damage-induced cell cycle arrest in *ccr4*Δ and *dhh1*Δ strains is *RAD9* dependent. Prolonged cell cycle delays at G_1 and S phase may result from the persistence of unrepaired DSB damage due to a repair defect; alternatively, cells may be defective in reentering the cell cycle following DNA repair. This latter process has been termed checkpoint adaptation and has been shown to be under genetic control (65). Defects in genes controlling checkpoint adaptation result in prolonged arrest and reduced survival following DNA damage. Therefore, *CCR4* and *DHH1* could also be required for adaptation to *RAD9*-dependent checkpoints at G_1/S or in S phase following DNA damage. Among the components of the DNA damage checkpoint pathway, *RAD9* has been proposed to per-

overnight in liquid YPD, diluted 1 to 4 in fresh YPD, and allowed to grow into logarithmic phase with vigorous shaking for 4 h at 30°C. No decrease in survival relative to that of the WT was seen for haploid strains lacking members of either CCR4-dependent transcription complex. The two-component nature of these curves results from the extreme IR sensitivity of haploid unbudded G_1-phase cells (see text), while the budded (S and G_2 phase) population is radioresistant. The lack of an IR^S component in the *dhh1*Δ and *paf1*Δ cells was due to a high percentage of radioresistant budded cells in the population at the time of irradiation. All data points represent the averages of three to eight replica experiments.

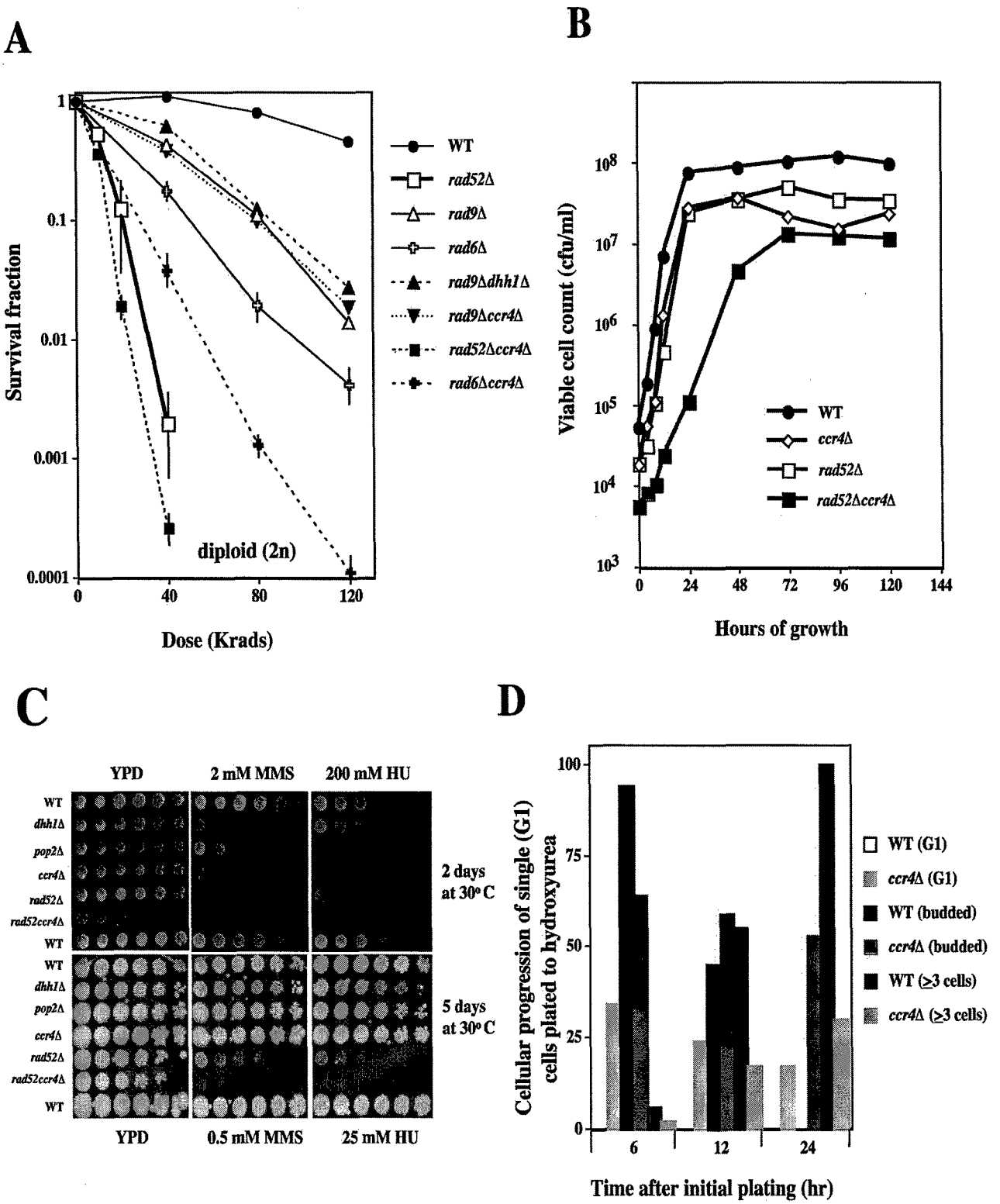


FIG. 3. *CCR4* is a member of the *RAD9* checkpoint pathway and exhibits defects in cell cycle progression in S phase. (A) *CCR4* and *RAD9* show the same epistatic relationship with respect to radiation-induced lethality. Diploid cells were grown to stationary phase and gamma irradiated at various doses as described above. Survival of colony-forming ability was determined on YPD following irradiation. No increase in lethality was seen for the *rad9Δ dhh1Δ* or *rad9Δ ccr4Δ* strains compared to the results seen with the *rad9Δ* strain. However, the *rad52Δ ccr4Δ* and *rad6Δ ccr4Δ* strains showed dose-dependent decreases in survival that were greater than that observed for the *rad52Δ* and *rad6Δ* strains, respectively. The data points represent the averages of three to eight replica experiments. Error bars are ± 1 standard error. (B) The growth rate of the diploid *rad52Δ ccr4Δ* strain is decreased compared to the results seen with the isogenic *rad52Δ* or *ccr4Δ* strains. Strains growing logarithmically in YPD were diluted

form a damage sensor function early in the pathway (48). In *rad9*Δ cells, unrepaired DSBs have no effect on the rapid onset of cell cycle progression. Therefore, if *CCR4* and *DHH1* were strictly repair genes their absence would not affect the rapid progression of a *rad9*Δ strain following damage. To identify whether *CCR4* and *DHH1* behave like repair- or damage-specific checkpoint adaptation genes, we determined transition times for G_1- to S-phase cell cycle progression for *rad9*Δ *ccr4*Δ and *rad9*Δ *dhh1*Δ diploid strains following IR (Fig. 4C). The double-deletion strains did not show the prolonged G_1 arrest that was characteristic of the *ccr4*Δ and *dhh1*Δ strains or the rapid G_1 to S transition characteristic of *rad9*Δ single-mutant strains following IR (Fig. 4A and C). Instead, the double-mutant cells transit through the G_1 checkpoint earlier (1.3 ± 0.3 and 2.3 ± 0.1 h earlier for the respective double-mutant strains) than the *dhh1*Δ or *ccr4*Δ cells. Compared to the *rad9*Δ strain, the double mutants showed a delayed progression in ~60% of the G_1 cell population (Fig. 4C). This suggests that *CCR4* and *DHH1* are required in part for G_1 checkpoint adaptation in a pathway that requires the damage-sensing function of *RAD9*. However, ~40% of the G_1 cells in the double mutants progressed as rapidly as the cells of the *rad9*Δ strain (Fig. 4C), suggesting the presence of a possible second adaptation pathway similar to that seen for cells arrested at the G_2 damage checkpoint at which G_2 arrest requires two parallel pathways (29). Alternatively, there may also be a minor *RAD52*-independent repair pathway in which *CCR4* contributes to the repair of DSBs.

A more rapid transition of irradiated G_2 (budded cells) into microcolonies was also observed among the *rad9*Δ *ccr4*Δ and *rad9*Δ *dhh1*Δ diploid strains compared to the results seen with the *ccr4*Δ or *dhh1*Δ strains (Fig. 4B). Therefore, deletion of *RAD9* can greatly suppress the prolonged IR-induced cell cycle delays observed at both G_1/S and G_2/M for the *ccr4*Δ strain.

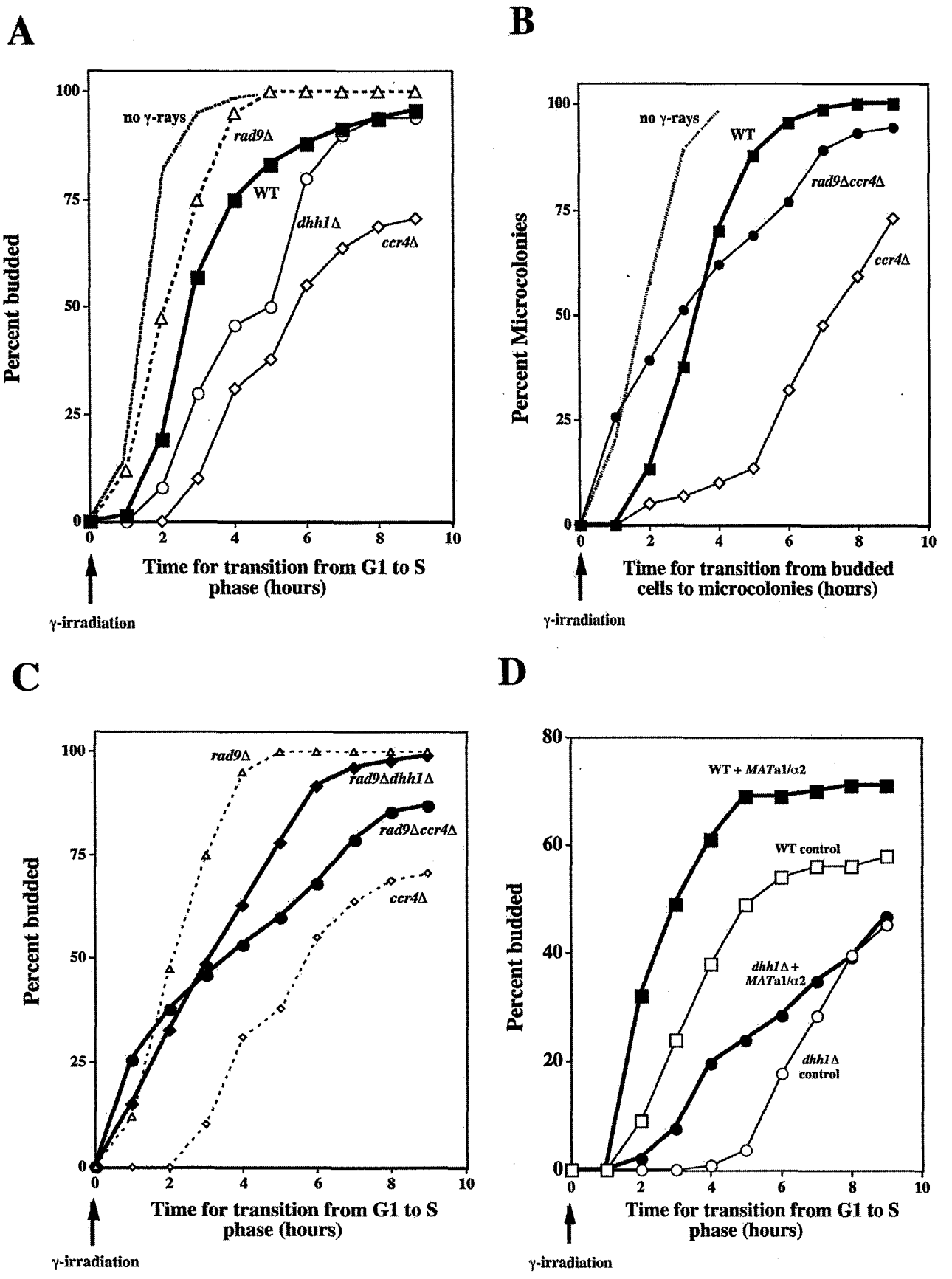
Checkpoint adaptation at G_1/S is not dependent on *MAT*. Slow adaptation to the DSB-induced checkpoint at G_2/M is dependent in part on expression of the mating-type (*MAT*) transcriptional regulators *MAT***a**1 and *MAT*α2 that confer a diploid phenotype (10). We, therefore, examined WT and *dhh1*Δ haploid cells that were transformed with a plasmid (pCB115) that expresses both *MAT***a**1 and *MAT*α2 to determine whether the prolonged G_1 arrest in diploid *dhh1*Δ cells is *MAT* dependent (Fig. 4D). Similar to the results seen with the diploid strains, a prolonged damage-induced G_1 arrest was found in the *dhh1*Δ haploid strain compared to that seen with the WT. The length of the G_1 delay was increased for both the WT and the *dhh1*Δ haploid strains compared to the results seen with their isogenic diploid counterparts (Fig. 4A). For example, the time required for 50% of the diploid and haploid WT cells to exit G_1 was 3 and 5 h, respectively. The corresponding G_1 delays for the *dhh1*Δ diploid and haploid strains were 5 and >9 h, respectively. Following coexpression of the *MAT***a**1 and *MAT*α2 transcriptional regulators which are normally jointly expressed in diploids, but not in haploids, the time required for cell cycle progression from G_1 to S decreased for both the WT and *dhh1*Δ haploid stains. For these strains the time of G_1 to S transition occurred earlier when the *MAT* transcriptional regulators were present (1.9 + 0.8 and 1.8 + 0.7 for the WT and *dhh1*Δ strain, respectively). Thus, there is a "diploid" effect for adaptation to damage in both the WT and the *dhh1* mutants that results in a decreased time for G_1- to S-phase transition. This comparison suggests that the *DHH1*-controlled adaptation in diploids is not dependent on *MAT* expression.

MAT heterozygosity has also been shown to suppress the IR sensitivity of the *rad52-20* allele (61) and *rad55* deletion mutants (46). Since the radiosensitivity of *ccr4*Δ and *dhh1*Δ has been observed with diploids but not with haploids, the diploid IR sensitivity could similarly be *MAT*-dependent. We therefore examined the relative survival rates of haploid *ccr4*Δ, *dhh1*Δ, and WT (*MAT***a**) strains expressing *MAT***a**1 and *MAT*α2 transcriptional regulators (transformed with pCB115) versus the results seen with identical control strains (transformed with vector alone) following a single acute IR dose (80 krads). No difference in survival rates was seen between any of the haploid control strains or those expressing *MAT***a**1 and *MAT*α2 (data not shown). Therefore, *MAT* expression is not responsible for the IR sensitivity of diploid *ccr4*Δ or *dhh1*Δ strains.

DISCUSSION

The recent availability of the complete isogenic haploid and diploid yeast deletion strain collections has facilitated the rapid genome-wide identification of new genetic determinants required for yeast to survive exposure to a variety of physical or chemical environmental agents. An inherent feature of these functional genomic screenings is that they often lead to the listing of a large number of new yeast genes required to tolerate a specific inhibitory agent. Often these lists contain a bewildering array of seemingly unrelated genes that define many

into fresh YPD with vigorous shaking at 30°C, and the viable cell counts were determined according to the colony-forming ability of samples selected at the indicated times. All data points represent the averages of three determinations. (C) Strains lacking members of the CNOT complex are sensitive to the replication inhibitors MMS and HU. Strains were grown in liquid YPD for 2 days and serially diluted as described for Fig. 2A. Diluted cells were replica pronged to YPD plates containing either the alkylating agent MMS or the ribonucleotide reductase inhibitor HU at the indicated concentrations and allowed to grow for 2 days (high doses of MMS and HU) or 5 days (low doses of MMS and HU) at 30°C. No growth of the *rad52*Δ *ccr4*Δ strain was observed on either the high- or low-dose plates after extended incubation (5 days), indicating a hypersensitivity of the double mutant to these S-phase inhibitors. Similar inhibition results were observed for plates containing zymocin (66%) or doxorubicin (50 μg/ml; data not shown). (D) Prolonged S/M arrest of *ccr4*Δ cells in the presence of HU. WT and *ccr4*Δ diploid cells were grown to logarithmic phase and plated to YPD or YPD plus 200 mM HU. Individual G_1 cells were micromanipulated into a grid pattern, and the time required for the transition from single (G_1) cells to budded (S and G_2) cells and into microcolonies (≥3 cells) was determined. More than 50% of the *ccr4*Δ cells remained budded (in G_2) at 24 h after plating to the S-phase-specific inhibitor HU. At 6 h, all of the WT cells initially plated, as single cells had progressed to budded cells or formed microcolonies; by 24 h, all (100%) of the WT cells had progressed to form viable microcolonies on HU. For both strains, 85% of G_1 cells (in the absence of HU) progressed to microcolonies following 6 h on YPD plates. Cell progression (vertical bars) was calculated as average percentages from four replicate experiments.


A
Percent budded
100
75
50
25
0
no γ-rays
rad9Δ
WT
dhh1Δ
ccr4Δ
0
2
4
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8
10
Time for transition from G1 to S phase (hours)
γ-irradiation
B
Percent Microcolonies
100
75
50
25
0
no γ-rays
WT
rad9Δccr4Δ
ccr4Δ
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8
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Time for transition from budded cells to microcolonies (hours)
γ-irradiation
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rad9Δ
rad9Δdhh1Δ
rad9Δccr4Δ
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10
Time for transition from G1 to S phase (hours)
γ-irradiation
D
Percent budded
80
60
40
20
0
WT + MATa1/α2
WT control
dhh1Δ + MATa1/α2
dhh1Δ control
0
2
4
6
8
10
Time for transition from G1 to S phase (hours)
γ-irradiation

distinct functional groupings. We therefore used a reductionist approach to identify from our gene list the largest group of IR resistance genes that share a common underlying mechanistic function. Using recent advances in genome-wide determinations of proteomic and genomic interactions (30, 33, 37, 67, 69), we could place many of our newly identified IR resistance genes in a large interactive network (Fig. 1).

This approach has successfully shown that the CCR4 radiation response network is required for survival following IR damage. At least 13 interactions between *CCR4* and other radiation resistance genes are present in this network. The CCR4 network also interconnects to another established repair network (67) through at least five well-characterized recombination and repair genes (including *RAD9*), as described in this study (Fig. 1). Separate experimental screenings have shown that many of the IR^S gene deletions within the CCR4 damage response network are also required for maintaining cell size homeostasis and/or zymocin resistance (39, 40, 73). Furthermore, the CNOT mutants *ccr4*Δ and *dhh1*Δ (72), as well as *pop2*Δ, *not4*Δ, and *not5*Δ (data not shown), all demonstrated reduced viability following 4 to 5 days of nitrogen starvation. Since these are all G_1/S-regulated responses, we propose that the radiation sensitivity of mutants in the CCR4 network also results from defects in cell cycle progression at G_1/S. By examining two central members of the CCR4 damage response network, *CCR4* and *DHH1*, we found that they are indeed required for cell cycle progression following *RAD9*-dependent checkpoint arrest, which is consistent with the apparent G_1 sensitivity of the diploid mutants. Furthermore, *ccr4*Δ strains are also sensitive to the S-phase-specific replication inhibitor HU and show a prolonged arrest at the S/M checkpoint following exposure to HU. This indicates that *ccr4*Δ strains have cell cycle progression defects in both G_1 and S phase following DNA damage.

***CCR4*-mediates IR resistance in the G_1 phase of the cell cycle.** Ccr4 is a highly conserved protein that has multiple roles in the control of mRNA metabolism (including transcription initiation, mRNA elongation, and degradation) (21, 22, 68). It is a core component of two distinct transcriptional complexes that affect diverse processes in yeast. One complex (CNOT) is a global regulator of gene expression that can have both positive and negative effects on RNA Pol II-mediated transcription and is required for the G_1 arrest following nitrogen starvation (72) as well as hypersensitivity to zymocin (40). In *ccr4*Δ diploid strains there is reduced sensitivity to IR when they are irradiated as benomyl-arrested cultures containing a high percentage of G_2 cells compared to IR^S stationary G_1 cultures, further supporting the importance of *CCR4* in dealing with damage in the diploid G_1 phase. IR^S members of the CCR4 network were previously undetected, because all prior radiation screenings utilized haploids in which WT G_1 cells are IR^S due to the lack of recombinational repair. Therefore, screening of the diploid strain collection has facilitated the detection of a new set of IR^S mutants enriched for checkpoint or repair defects specific to the G_1 and S phases of the cell cycle.

Similar to the results of this study, IR-induced loss in survival has been observed in diploid but not haploid strains lacking the DNA helicases *SGS1* or *HPR5* (*SRS2*) (28). In a separate screening using the same diploid deletion collection, moreover, six deletion strains with identical phenotypes (i.e., IR sensitivity in diploid but not haploid strains) were identified (27). These include five IR^S deletion strains (*SHE1*, *ARP8*, *RSC1*, *YDR014W*, and *YNR068C*) identified in this study or previously (8). For three of these mutants (*ydr014W*Δ, *she1*Δ, and *arp8*Δ) plasmid expression of the deleted gene restored radiation resistance, indicating that the genomic mutation was indeed responsible for radiation sensitivity (27). Furthermore, deletion of *YDR014W* was found in both screenings to result in IR^S as a diploid. This gene was renamed *RAD61*, because both diploids and haploids showed enhanced IR sensitivity compared to the WT (27). These results suggest that diploid screenings might be useful for the discovery of new mutants that function specifically in the G_1 or early S phases of the cell cycle.

Rapid reentry into the cell cycle following *RAD9*-dependent checkpoint arrest requires *CCR4* and *DHH1*. We have shown a prolonged delay in cell cycle transition from G_1 to S as well as delay at the G_2/M phase of the cell cycle for *ccr4*Δ and *dhh1*Δ strains following IR or HU. Furthermore, both the *rad9* and *ccr4* deletion mutants are within the same epistasis group,

FIG. 4. Deletion of *CCR4* or *DHH1* results in a prolonged, *RAD9*-dependent G_1- to S-phase cell cycle transition following IR damage. (A) The time required for cell cycle progression of individual G_1 cells into budded (S-phase) cells was determined for the diploid WT, *dhh1*Δ and *ccr4*Δ mutants, and the G_1 checkpoint mutant *rad9*Δ strains following exposure to 8 krads of IR. Cells were grown to logarithmic phase, plated to YPD, and gamma irradiated. The *rad9*Δ cells showed a more rapid transition from single (G_1-phase) to budded (S-phase) cells than WT cells. Compared to the results seen with the *rad9*Δ strain, the average increase in time required for the WT cells to transit from G_1 to S was 1.1 ± 0.5 h following IR. Compared to the results seen with the WT strain, both the *dhh1*Δ and *ccr4*Δ strains required more time (1.3 ± 0.6 and 2.5 ± 1.0 h, respectively) to transit from G_1 to S following IR damage. No difference was seen in the onset of cell cycle progression for unirradiated cells (no gamma rays; averaged pooled data for all strains examined). (B) The time required for cell cycle progression of diploid budded cells (S and G_2) into microcolonies following 8 krads of IR. The *dhh1*Δ and *rad9*Δ *dhh1*Δ cells showed delays similar to those seen with the *ccr4*Δ and *rad9*Δ *ccr4*Δ cells; the results obtained with those cells have been omitted for clarity. No difference was seen in the onset of cell cycle progression for unirradiated cells (no gamma rays; averaged pooled data for all strains examined). (C) The time required for cell cycle progression of individual G_1 cells into budded (S-phase) cells was determined for the diploid mutant *rad9*Δ *dhh1*Δ and *rad9*Δ *ccr4*Δ strains following exposure to 8 krads of IR. Data for the *rad9*Δ and *ccr4*Δ strains have been included for comparison. Cell cycle progression results for individual G_1 cells of the diploid deletion mutant *dhh1* and *ccr4* strains were compared to the results seen with the WT and G_1 checkpoint deletion mutant *rad9* strains following exposure to 8 krads of IR. The time required to transit from G_1 (unbudded) to S (budded) phase for the *rad9*Δ *dhh1*Δ and *rad9*Δ *ccr4*Δ strains was significantly shorter than that seen with the *dhh1*Δ or *ccr4*Δ single-mutant strains. The progress of the unirradiated cells was identical to that shown in panel A. (D) Effects of *MAT* expression on G_1- to S-phase cell cycle transition following IR damage in haploid cells. WT and *dhh1*Δ haploid *MAT***a** cells were transformed with either pRS315 (control) or pCB115 (*MAT***a**1α2). The plasmid pCB115 expresses the *MAT***a**1 and *MAT*α2 genes which confer a nonmating, pseudodiploid phenotype on haploid cells containing the plasmid. The time required for G_1- to S-phase transition was determined following IR as described above, with the exception that plasmid-containing cells were grown in SC GLU−LEU to maintain the plasmid and subsequently plated to SC GLU-LEU following irradiation.

as determined for IR-induced cell lethality. Moreover, the prolonged cell cycle arrest of these mutants is *RAD9* dependent, further indicating these genes have checkpoint-associated functions within the *RAD9* pathway. IR sensitivity resulting from prolonged radiation or DSB-induced arrest at the G_2/M checkpoint has been described for numerous mutated genes (including *CDC5*, *YKU70*, *RDH54*, *BCK1*, *MRT4*, *RAI1*, *SIR2*, *SIR3*, and *SIR4*) (9). These genes have been described as checkpoint adaptation genes, since they are required for reentry into the cell cycle following checkpoint arrest. Both *CCR4* mutations (this study) and *MAT* heterozygosity (10) delay reentry into the cell cycle following G_2/M checkpoint arrest as well as suppressing the radiation sensitivity of the recombination-proficient *rad52-20* allele (61). Therefore, IR sensitivity of this unusual *rad52-20* allele may be associated with a defect in checkpoint arrest (at G_2/M) which can be independently suppressed by the prolonged, damage-induced G_2 arrest mediated by *ccr4* or *MAT*.

Transcriptional regulation of the G_1/S damage checkpoint. Damage-induced checkpoint functions in yeast at G_1/S are poorly understood. WT yeast undergo a significant damage-induced G_1 arrest, and several G_1/S checkpoint genes (*RAD9*, *RAD17*, *RAD24*, and *MEC1*) have been described and characterized (although the precise molecular mechanism of their action is unknown) (31, 55, 63). However, Rad9 is hyperphosphorylated following damage which is dependent on *MEC1*, *RAD17*, *RAD24*, *MEC3*, and *DDC1*, suggesting involvement of the products of these genes in checkpoint signal transduction (70). *RAD9* is also required for the damage-mediated transcriptional activation of a number of repair or checkpoint genes (including *RAD6*, *RAD18*, *RAD51*, *RAD54*, *RAD53*, *DUN1*, and others) (1). Since *CCR4* and *RAD9* share the same epistasis group, it is possible that transcription functions of *CCR4* are also required for this "SOS-like" transcriptional regulation. Since the CNOT genes are also involved in nitrogen starvation-induced G_1 arrest (72), cell-size regulation (homeostasis), and adaptation to the IR-induced G_1 checkpoint, however, it is likely that *CCR4* can regulate G_1/S cell cycle functions through Cdc28 activation or inhibition. Preexisting functional data for genes that interact within the CCR4 network suggest a molecular model in which transcriptional and posttranscriptional regulation of G_1- or S-phase-specific cyclins could account for the cell cycle responses of these deletions to a variety of environmental perturbations. Environmental agents (including IR) (see below) that cause cellular stress are proposed to activate the protein kinase C–mitogen-activated protein (PKC-MAP) signaling pathway that, in turn, activates Ccr4-dependent transcription complexes. This would transiently down regulate a critical subset of cyclin genes involved in G_1- and S-phase transition.

The *PKC1* stress response pathway is involved in recombination and checkpoint processes. Ccr4p is a component of the PAF1-CDC73 transcriptional complex which is a downstream effector of the PKC-MAP kinase pathway (17). Genes in this pathway play an essential role in the maintenance of cell wall integrity in response to external environmental signals (including heat shock, hypoosmotic shock, and treatment with α-factor) and possibly to the *K. lactis* killer toxin zymocin (40). IR appears to be similarly sensed by the PKC-MAP kinase pathway, since the IR^S MAP kinase mutant *bck1*Δ expressed a prolonged G_2 arrest followed by cellular lysis in response to IR (9). Since lethality in irradiated *ccr4*Δ or *dhh1*Δ strains could not be rescued by plating to YPD plates containing sorbitol (1 M; data not shown), IR-induced lethality in these strains is not mediated solely by the loss of cell wall integrity.

The PKC-MAP kinase stress pathway genes similarly play overlapping roles in the signaling of checkpoint arrest and DNA repair, since the MAP kinase mutant *bck1* expresses a prolonged IR-induced G_2 arrest (9) and *PKC1* mutations result in hyperrecombination (35, 47). Moreover, IR^S mutations in members (including *PAF1*) of the PAF1-CDC73 complex have been reported to enhance intrachromosomal recombination (17); in the case of *hpr1*Δ mutants, this is *RAD52* dependent (3). While the interactive roles that genes within the PKC-MAP kinase pathway play in the stress and DNA damage responses are complex and remain to be fully elucidated, similar overlapping roles in stress response and DNA damage-induced checkpoints has been described for mammalian genes such as p38 (57).

***CCR4* has a role in the repair of S-phase damage.** The *rad52*Δ *ccr4*Δ strain showed a severe synthetic growth defect compared to either the *rad52*Δ or *ccr4*Δ strains alone. A slower growth rate for *rad52*Δ mutants has been attributed to a requirement for recombination functions to repair replication-induced DSBs, especially in mutants partially defective in replication (51). Thus, the greatly decreased growth rate of the *rad52*Δ *ccr4*Δ strain may be due to the influence of *CCR4*-mediated checkpoint adaptation on another replication and/or repair system that acts on spontaneous or replication-associated DSBs occurring in S phase. The fact that *rad52*Δ *ccr4*Δ strains are more sensitive to MMS or HU than either *rad52*Δ or *ccr4*Δ strains supports the idea that *CCR4* regulates a pathway other than recombination that is required for the repair of S-phase damage. An explanation for such an *RAD52*-independent repair system could be that *CCR4* participates in the transcriptional induction of genes required for DNA replication in the presence of S-phase damage. We have shown that *ccr4*Δ strains are defective in spontaneous and MMS-induced activation of the ribonucleotide reductase (*RNR3*) promoter (72). It is possible that similar to *hrr25* mutant strains (34), *ccr4*Δ strains are HU sensitive due to a defect in the transcriptional induction of *RNR* genes in response to ribonucleotide depletion following HU treatment. Furthermore, *ccr4* mutants were also shown to reduce transcription of the damage-inducible repair gene *RAD51* (61) which is expressed specifically in G_1 at Start (50) as well as in response to DNA damage. Not surprisingly, *RAD9* is also required for the DNA damage-induced transcriptional activation of *RAD51* and *RNR3* (1), further indicating that *RAD9* and *CCR4* share the same damage response pathway.

Other radiation resistance genes. Many of the newly described IR resistance genes do not appear to be associated with the CCR4 damage response network. This may be due in part to an incomplete functional understanding of the complex interrelationships within the yeast genome as a whole. For example, the radiation resistance gene *SCP160* is associated with five other IR resistance genes (see Results) that share no other apparent common function. Recently, Scp160p has been shown to be part of a mRNP complex that binds to a number of specific mRNAs (including that of *DHH1*) (43). Since the

SCP160 deletion can affect abundance and distribution of these mRNAs, the IR sensitivity caused by *scp160Δ* may be indirect (affecting the expression or cellular distribution of Dhh1p). Similar to the results seen with *SCP160*, many of the new IR resistance genes may not have direct checkpoint or repair functions (see reference 8 for a review). Instead, their roles may be indirect, affecting metabolism, trafficking, and/or the abundance of critical DNA repair proteins with a variety of basic cellular functions. These include chromatin organization (*ARP5* and *ARP8*), nuclear pore function (*ASM4*), Golgi and vacuolar function (*FAB1* and *VPS33*), transcription (*YAF9*, *BDF1*, and others), cytoskeletal organization (*SRV2*), mitochondrial function (*MDM10* and *MDM20*), and protein synthesis (*NAT1*, *EAP1*, *TIF4631*, and others).

Many newly identified IR^S gene deletions (including those of members of the *CCR4* damage response network) are highly conserved in eukaryotes and have been implicated in cancer (see Table S1 in the supplemental material). For example, the proteins Dhh1p (4, 36), Pop2p (23), Yaf9p (64), and Bdf1p (25) are all transcription factors with human orthologs that are putative tumor suppressor genes or translocation-activated fusion oncoproteins implicated in cancers of myeloid or epithelial origin. By virtue of their evolutionarily conserved nature and association with cancer onset and/or progression, these proteins may play a critical and therefore more direct role in the maintenance of genomic integrity following DNA damage in human or yeast cells.

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Zymocin-induced DNA damage is lethal in the yeast *Saccharomyces cerevisiae.*

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Secretion of the heterotrimeric toxin zymocin by *Kluyveromyces lactis* inhibits the growth of other yeast species including *Saccharomyces cerevisiae*. Although this protein toxin affects transcription in susceptible yeast, little is known about its lethal mode of action. Here we show that diploid yeast strains defective in the repair of ionizing radiation (IR) induced double-strand breaks (DSBs) including members of the *RAD52*, *RAD6* and *RAD9* epistasis groups have enhanced sensitivity to the killing effects of zymocin. Furthermore, IR sensitive mutant strains that are defective in transcriptional regulation and/or replication such as *ccr4*Δ or *mms22*Δ strains show extreme hypersensitivity to the killing effects of zymocin. Following exposure to zymocin, most unbudded *ccr4*Δ and *mms22*Δ cells rapidly transit G1 and S phases to permanently arrest in G2 as budded cells which undergo lysis. This S/M checkpoint arrest is similar to that induced by the replication inhibitor hydroxyurea which is consistent with zymocin acting as an S phase specific replication inhibitor. Similar to IR-induced DSB damage, recombination proficient diploid cells were much more resistant to the killing effects of zymocin than haploid cells. Moreover, we show that zymocin exposure induced transcription of a *DIN::*LacZ reporter construct (an indirect assay of DNA damage) in repair proficient yeast. Therefore zymocin-induced lethality appears to result from persistent DSB damage in the yeast *S. cerevisiae*.