EUKARYOTIC CELL, Apr. 2004, p. 430–446 Vol. 3, No. 2
1535-9778/04/$08.00+0 DOI: 10.1128/EC.3.2.430–446.2004


# 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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Received 6 November 2003/Accepted 15 February 2004

**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* |

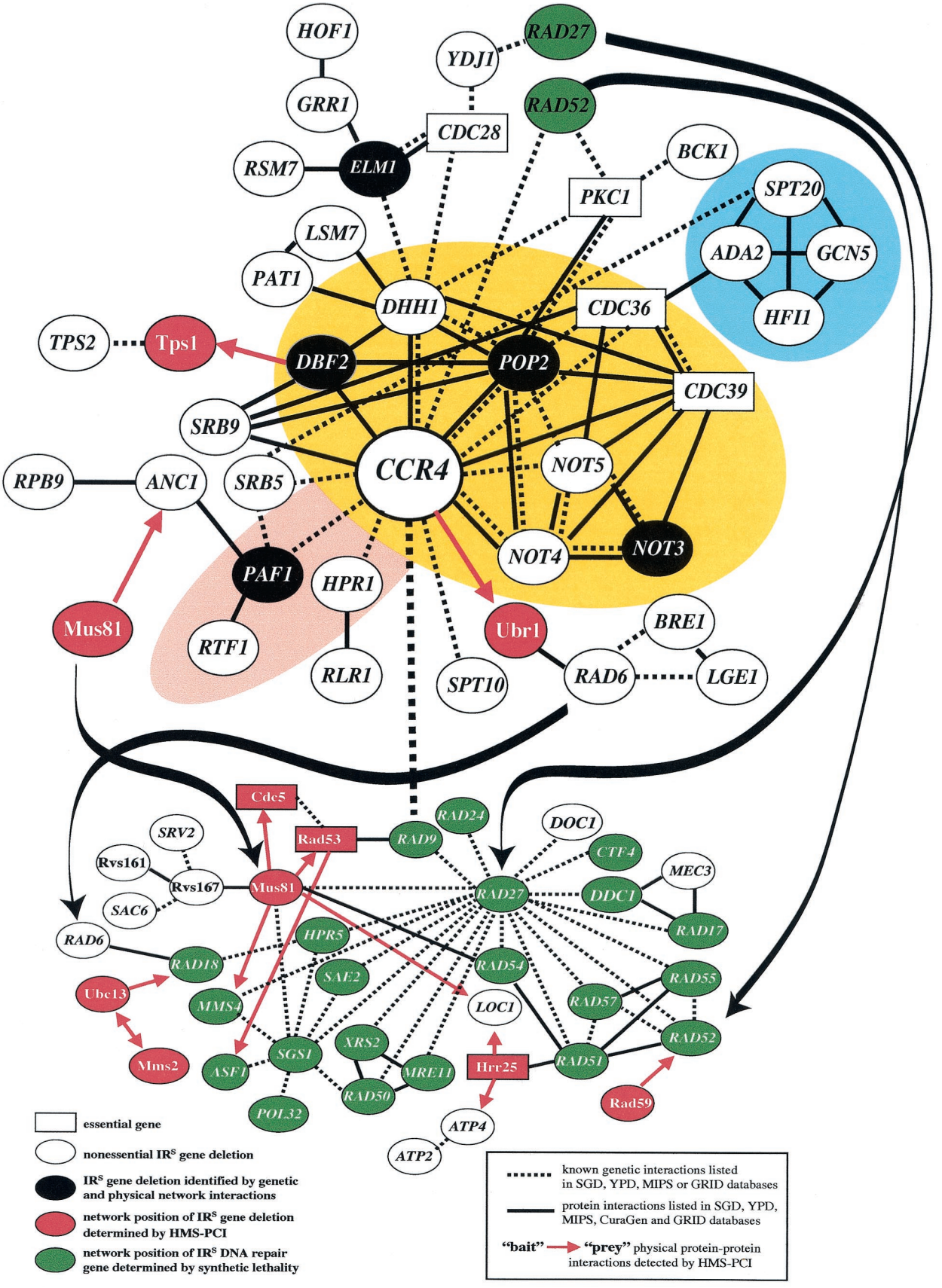
[a] Approximately 1,100 diploid deletion strains were simultaneously screened for sensitivity to seven physical and chemical agents. Relative sensitivity levels were determined by dilution plating, and some strains were more sensitive than others (see Table S1 in the supplemental material). Strains sensitive 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
ADA2
GCN5
HFI1
LSM7
PAT1
DHH1
CDC36
TPS2
Tps1
DBF2
POP2
CDC39
SRB9
CCR4
NOT5
RPB9
ANC1
SRB5
NOT4
NOT3
PAF1
HPR1
Mus81
RTF1
Ubr1
BRE1
RLR1
SPT10
RAD6
LGE1
Cdc5
RAD24
DOC1
SRV2
Rad53
RAD9
CTF4
Rvs161
Rvs167
Mus81
RAD27
DDC1
MEC3
SAC6
RAD6
HPR5
RAD17
RAD18
SAE2
RAD54
RAD55
Ubc13
MMS4
LOC1
RAD57
RAD52
Mms2
SGS1
XRS2
ASF1
MRE11
Hrr25
RAD51
RAD50
Rad59
POL32
ATP4
ATP2
essential gene
nonessential IRS gene deletion
IRS gene deletion identified by genetic and physical network interactions
network position of IRS gene deletion determined by HMS-PCI
network position of IRS 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 ($1.2 \pm 0.7$ versus $1.0 \pm 0.7$). Furthermore, both the diploid *paf1*Δ and the *cdc73*Δ strains produced targeted recombination efficiencies that were similar to that of the WT strain ($0.65 \pm 0.17$ and $2.6 \pm 1.9$, respectively). By comparison, the recombination-deficient *rad51*Δ strain had a significantly reduced targeted-recombination efficiency ($0.016 \pm 0.005$) whereas the hyperrecombination strain *hpr1*Δ had an enhanced recombination efficiency ($24 \pm 10$) 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*

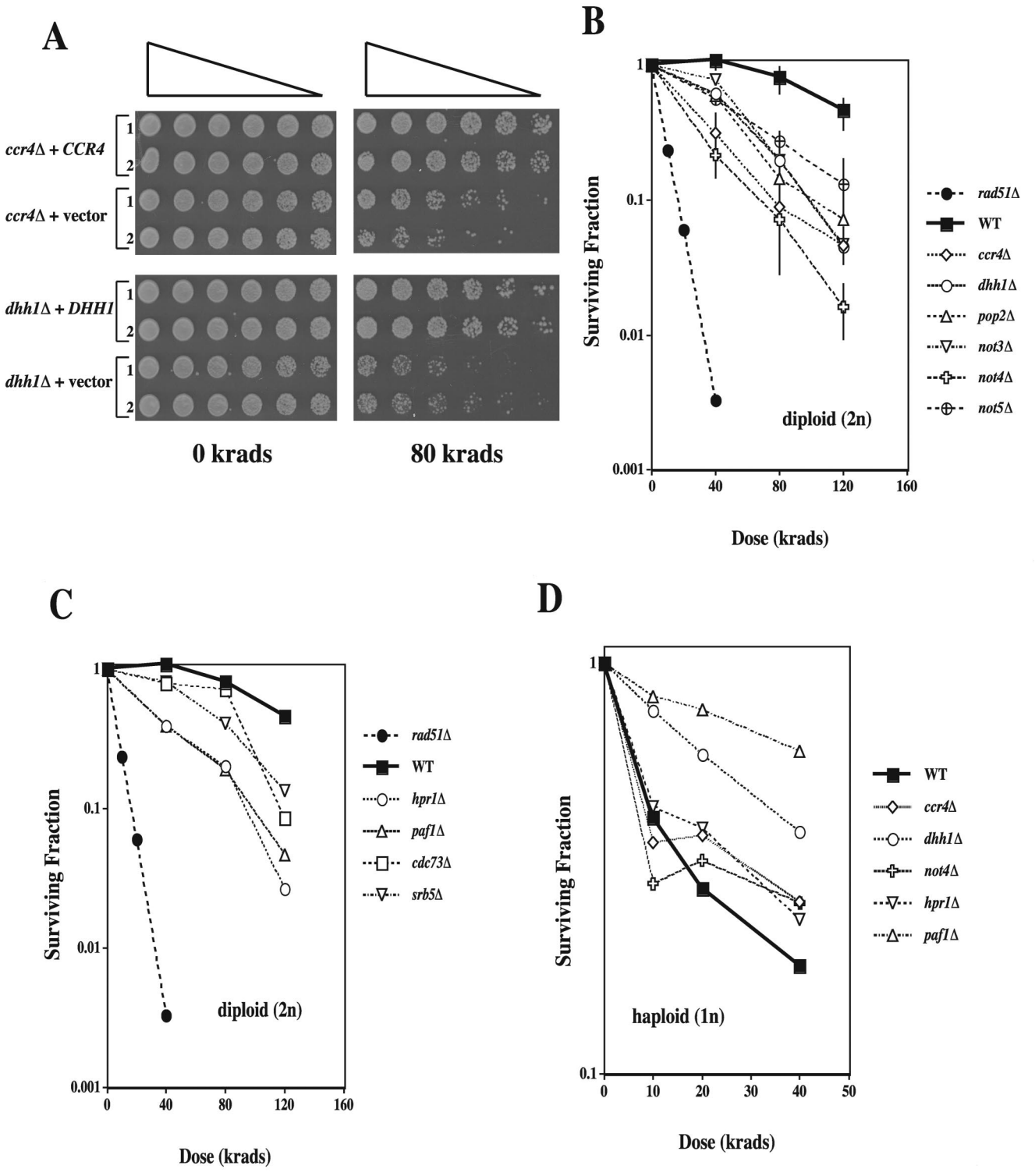



FIG. 2. Reduced survival of gamma-irradiated diploid stationary cells lacking various members of two CCR4-dependent transcriptional complexes. (A) *ccr4*Δ and *dhh1*Δ diploid strains containing the plasmid YEP13-CCR4 or pRS425-DHH1 (49) that express full-length copies of *CCR4* and *DHH1* or vector alone 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 ($1.1 \pm 0.5$ 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 ($1.4 \pm 0.6$ and $2.5 \pm 1.0$ 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.

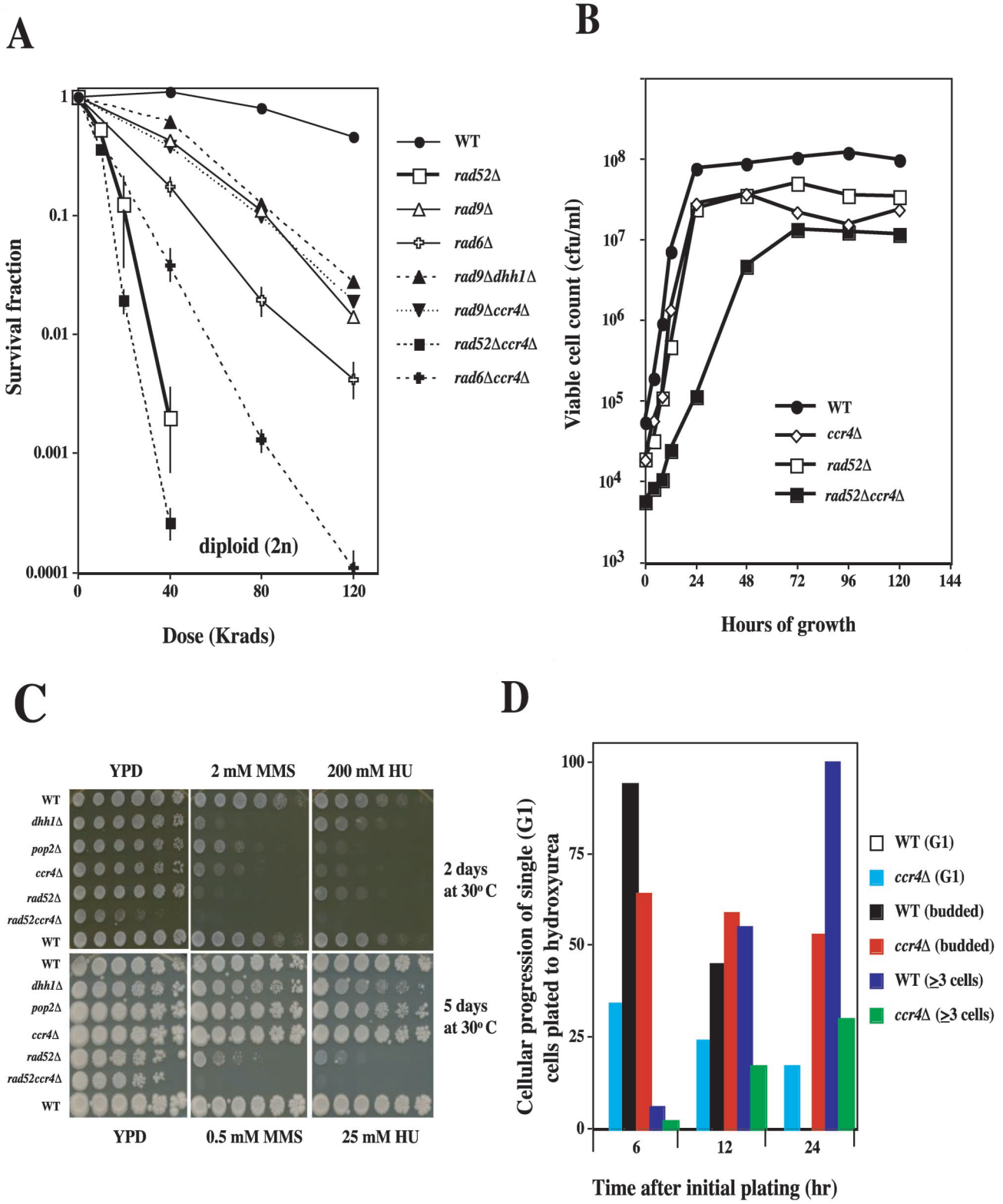



FIG. 3. *CCR4* is a member of the *RAD9* checkpoint pathway and exhibits defects in cell cycle progression in S phase. (A) *CCR4* and *RAD9* show the same epistatic relationship with respect to radiation-induced lethality. Diploid cells were grown to stationary phase and gamma irradiated at various doses as described above. Survival of colony-forming ability was determined on YPD following irradiation. No increase in lethality was seen for the *rad9*Δ *dhh1*Δ or *rad9*Δ *ccr4*Δ strains compared to the results seen with the *rad9*Δ strain. However, the *rad52*Δ *ccr4*Δ and *rad6*Δ *ccr4*Δ strains showed dose-dependent decreases in survival that were greater than that observed for the *rad52*Δ and *rad6*Δ 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 *rad52*Δ *ccr4*Δ strain is decreased compared to the results seen with the isogenic *rad52*Δ or *ccr4*Δ 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.

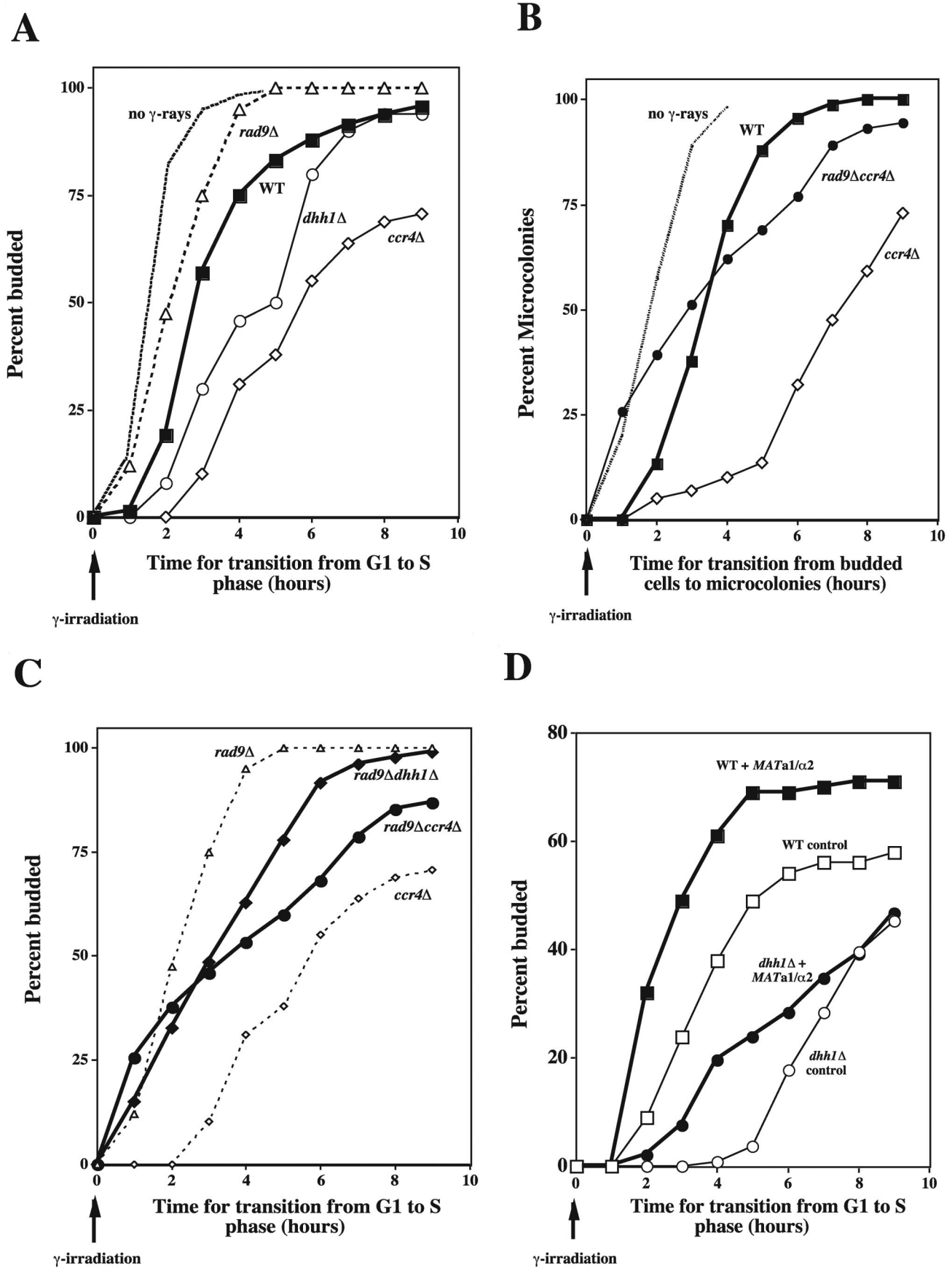
**Checkpoint adaptation at $G_1$/S is not dependent on *MAT*.** Slow adaptation to the DSB-induced checkpoint at $G_2$/M is dependent in part on expression of the mating-type (*MAT*) transcriptional regulators *MAT***a**1 and *MAT*α2 that confer a diploid phenotype (10). We, therefore, examined WT and *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
6
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
0
2
4
6
8
10
Time for transition from budded cells to microcolonies (hours)
γ-irradiation
C
Percent budded
100
75
50
25
0
rad9Δ
rad9Δdhh1Δ
rad9Δccr4Δ
ccr4Δ
0
2
4
6
8
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 *scp160*Δ 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.

## ACKNOWLEDGMENTS

We thank M. Collart, T. Weinert, K. Lewis, W. Xiao, and R. Schaffrath for the generous gift of yeast plasmids and strains. The technical assistance of A. Kelkar, D. Bailey, J. Westmoreland, J. Sterling, and A. Falae in some of the experiments is greatly appreciated. We also thank M. Kupiec, Y. Jin, and K. Lewis for critical comments during the preparation of the manuscript.

This work was supported in part by a grant from the DOD Breast Cancer Research Program (DAMD17-03-1-0232) to C.B.B.

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