Rad18 Is Required for DNA Repair and Checkpoint Responses in Fission Yeast

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To survive damage to the genome, cells must respond by activating both DNA repair and checkpoint responses. Using genetic screens in the fission yeast *Schizosaccharomyces pombe*, we recently isolated new genes required for DNA damage checkpoint control. We show here that one of these strains defines a new allele of the previously described *rad18* gene, *rad18-74*. *rad18* is an essential gene, even in the absence of extrinsic DNA damage. It encodes a conserved protein related to the structural maintenance of chromosomes proteins. Point mutations in *rad18* lead to defective DNA repair pathways responding to both UV-induced lesions and, as we show here, double-stranded breaks. Furthermore, rad18p is required to maintain cell cycle arrest in the presence of DNA damage, and failure of this leads to highly aberrant mitoses. A gene encoding a BRCT-containing protein, *brc1*, was isolated as an allele-specific high-copy suppressor of *rad18-74*. *brc1* is required for mitotic fidelity and for cellular viability in strains with *rad18* mutations but is not essential for DNA damage responses. Mutations in *rad18* and *brc1* are synthetically lethal with a topoisomerase II mutant (*top2-191*), indicating that these proteins play a role in chromatin organization. These studies show a role for chromatin organization in the maintenance or activation of responses to DNA damage.

INTRODUCTION

Strict control of the fidelity of cell cycle events is important for cells to prevent heritable damage and changes in ploidy. The importance of this control is manifest in the occurrence of cell cycle checkpoints. Checkpoint pathways maintain the interdependency of cell cycle transitions and pause cell cycle progression when cellular defects are detected. Checkpoints have been described that monitor DNA damage, completion of DNA replication, spindle integrity, and cell mass, delaying cell cycle progression until the requirements for continuation of the cell cycle are met (Nurse, 1975, 1994; Hartwell and Weinert, 1989; Murray, 1995).

Entry into mitosis is controlled by the activity of the cdk/cyclin complex $p34^{cdc2}$/cyclinB (Nurse, 1990). This complex accumulates during G2 and is maintained in an inactive state through the phosphorylation of tyrosine 15 (Y15) of $p34^{cdc2}$ by wee1p and related kinases. $p34^{cdc2}$ becomes active when Y15 is dephosphorylated by the cdc25p family of phosphatases, and the cell then proceeds into mitosis. The activation of $p34^{cdc2}$/cyclinB is the rate-limiting step for entry into mitosis. Analysis of the G2 DNA damage checkpoint in fission yeast has identified many genes that are essential for the DNA damage checkpoint (Al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994; Saka *et al.*, 1997; Willson *et al.*, 1997). This checkpoint arrests the cell cycle through maintenance of the inhibitory Y15 phosphorylation of $p34^{cdc2}$, which is achieved through chk1p-dependent signaling via wee1p and cdc25p (Furnari *et al.*, 1997; O'Connell *et al.*, 1997; Peng *et al.*, 1997; Rhind *et al.*, 1997; Sanchez *et al.*, 1997). Chk1p becomes phosphorylated after DNA damage, dependent on the checkpoint rad genes, which function upstream of *chk1* (Walworth and Bernards, 1996). Similar signaling pathways appear to be activated by blocks to DNA replication where another kinase, cds1p, may also be involved in transducing signals to the cell cycle machinery (Boddy *et al.*, 1998; Lindsay *et al.*, 1998; Zeng *et al.*, 1998).

Maintenance of genomic integrity requires the DNA to be held in highly structured proteinaceous complexes called chromatin. Chromatin undergoes structural alterations coincident with DNA replication and chromosome segregation, and many of the proteins involved in maintaining or

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altering chromatin structure are essential for stability and integrity of the genome (for review, see Heck, 1997). Throughout S and G2 phases, sister chromatids associate tightly along their lengths. Perturbation of sister chromatin cohesion results in chromosome instability and chromosome breaks and may cause an inability to repair double-stranded breaks (Birkenbihl and Subramani, 1992; Strunnikov *et al.*, 1993; Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Losada *et al.*, 1998). In *Xenopus,* 9S and 14S protein complexes, cohesins, are required to generate and maintain this cohesion (Losada *et al.*, 1998). Cohesins contain the structural maintenance of chromosomes (SMC) proteins XSMC1p and XSMC3p, homologues of which are required for sister chromatid cohesion in *Saccharomyces cerevisiae* (*SMC1* and *SMC3*) (Michaelis *et al.*, 1997). The 14S cohesin particle also contains XRAD21p, which has homologues in both budding yeast (Scc1p/ Mcd1p) and fission yeast (rad21p) (Birkenbihl and Subramani, 1995; Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Losada *et al.*, 1998). In budding yeast Scc1p/Mcd1p is required to establish cohesion in S-phase (Uhlmann and Nasmyth, 1998), dissociates from chromatin as chromatids segregate, and is then degraded (Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Ciosk *et al.*, 1998). Mutations in fission yeast *rad21* also lead to phenotypes that are consistent with defective sister chromatid cohesion: chromosome instability, failure of double-stranded break repair, and defects in chromosome segregation (Birkenbihl and Subramani, 1995; Tatebayashi *et al.*, 1998). Another key element required for stable inheritance of chromosomes is budding yeast scc2p which, although not a stoichimetric subunit of the cohesin, is required for the association of the cohesin with chromosomes, suggesting that it loads the cohesin complex onto the chromosomes (Toth *et al.*, 1999). Likewise, mutations in the fission yeast homologue *mis4* lead to defects in sister chromatid cohesion and chromosome stability, and mis4p also appears to function mainly during S-phase (Furuya *et al.*, 1998). Strains containing the temperature-sensitive *mis4-242* allele show sensitivity to UV-C and the DNA replication inhibitor hydroxyurea at permissive temperature, although G2 checkpoints are intact (Furuya *et al.*, 1998).

Once paired, sister chromatids become intertwined, and their subsequent decatenation requires the activity of topoisomerase II. Failure to resolve the sister chromatids before segregation results in chromosome breakage and subsequent instability of the genome and loss of genetic material. In the extreme this leads to increased rates of molecular evolution or to cell death (Holm *et al.*, 1985, 1989; Uemura and Yanagida, 1986; Uemura *et al.*, 1987; Downes *et al.*, 1991).

The onset of mitosis is marked by chromatin condensation, an alteration in chromatin organization that compacts chromosomes many-fold. Two protein complexes with chromatin condensation activity, the condensins, have been characterized in *Xenopus* (Kimura and Hirano, 1997). These contain the SMC proteins XCAP-Ep (*SMC2* homologue) and XCAP-Cp (*SMC4* homologue) (Hirano and Mitchison, 1994; Hirano *et al.*, 1997), homologues of which are found across several species. Loss of function of the budding yeast XCAP-E homologue (*SMC2*) or the fission yeast SMC2 and SMC4 homologues (*cut14* and *cut3*) causes an inability to condense chromatin and to complete chromosome segregation (Saka *et al.*, 1994b; Strunnikov *et al.*, 1995). Topoisomerase II is also required for chromatin condensation through-

out G2 and into mitosis, in addition to its role in sister chromatid resolution (Uemura *et al.*, 1987; Holm *et al.*, 1989).

The SMC1–4 proteins are therefore central to chromatin organization. A third class of proteins that are structurally related to SMC1–4 are defined by rad18p of fission yeast and Rhc18p in budding yeast (Lehmann *et al.*, 1995). *Rad18* is an essential gene. Its role is inferred from the phenotype of the point mutant *rad18-X*, which shows sensitivity to ionizing and UV-C irradiation, an inability to repair UV-C-induced lesions, and chromosomal instability accompanied by accumulation of mitotic defects (Murray *et al.*, 1994; Lehmann *et al.*, 1995). Genetic studies place *rad18* in the same epistasis group as *rad2* and the fission yeast RAD51 homologue *rhp51* (Lehmann *et al.*, 1995). Mutations in either of these genes also results in chromosomal instability (Murray *et al.*, 1994; Lehmann *et al.*, 1995). However, rad2p is only required for repair of UV-C-induced lesions, and acts independently from classical excision repair, whereas rhp51p, by analogy to functional studies of other *RAD51* proteins, is required for repair of double-stranded breaks. Both pathways may be linked by a requirement for recombination in these processes (Lehmann *et al.*, 1995). With its structural similarity to the SMC proteins, rad18p may play a role in forming chromosomal structures, including those required for different DNA repair mechanisms.

Of the DNA damage checkpoint genes described for fission yeast, only *cut5* is essential in the absence of irradiation. It is possible that genes involved in other aspects of genomic integrity may have a checkpoint role as part of their repertoire of function. Proteins required for detection of DNA damage and activation of checkpoint and repair pathways have not been described. It is likely that such proteins would be chromatin associated and may define essential genes. Thus, to identify genes that are essential for viability as well as checkpoint function, specific alleles would need to be isolated, such as the T45M mutation in *cut5*, present in all *cut5* alleles isolated thus far (Saka *et al.*, 1997; Verkade and O'Connell, 1998). Further screening to approach saturation of the genome would be needed to find specific mutations in essential genes that lead to checkpoint deficiencies but do not alter the other, essential, functions.

We performed a genetic screen in fission yeast to identify new genes involved in the DNA damage checkpoint (Verkade and O'Connell, 1998). We show here that one strain isolated in this screen, previously termed NA74, defines a novel allele of *rad18* and is hence renamed *rad18-74*. Unlike the previously characterized allele, *rad18-X*, *rad18-74* has defects in the DNA damage checkpoint, but both alleles are defective in DNA repair. Cells deleted for *rad18* were also unable to arrest in G2 in the presence of DNA-damaging agents. Expression of an allele mutated in the putative *rad18* ATP-binding domain failed to rescue the DNA damage sensitivity of *rad18-X* and *rad18-74*, and its overexpression was dominant negative and lethal. This indicates an essential role for ATP binding or hydrolysis for rad18p function. Rad18 mutants still show phosphorylation of chk1p in response to DNA damage, an event that is proposed to be indicative of checkpoint initiation. Therefore, rad18p is required to maintain a checkpoint-mediated G2 arrest. We also isolated an allele-specific high-copy suppressor of *rad18-74*. This gene, *brc1*, encodes a BRCT domain–containing protein (Koonin *et al.*, 1996; Callebaut and Mornon, 1997), deletion of

which leads to genomic instability and mitotic defects but not defects in DNA repair or checkpoint function. Genetic interactions among *rad18*, *brc1*, and other genes involved in chromatin organization suggest a role for rad18p in the regulation of chromatin structure that is essential for genomic integrity and responses to DNA damage.

MATERIALS AND METHODS

Fission Yeast Methods

All strains are derivatives of *972h⁻* and *975h⁺*. Standard procedures and media were used for propagation and genetic manipulation (Moreno *et al.*, 1991). Methods for transformation and microscopy have been described previously (O'Connell *et al.*, 1994). The procedure used to screen for new genes involved in the DNA damage checkpoint has been described previously (Verkade and O'Connell, 1998). To test the temperature sensitivity of strains for the genetic analyses, the strains were streaked onto YES and placed at 25, 30, or 36°C for 3–4 d. Strains were grown at 25°C in defined minimal media to exponential phase and shifted to 30 or 36°C. Samples were fixed with ethanol or 0.25% gluteraldehyde plus 3.7% formaldehyde (O'Connell *et al.*, 1994) and were stained with DAPI for microscopy (Moreno *et al.*, 1991). Immunolocalization of rad18p tagged at the N terminus with myc and expressed from the medium-strength *nmt1* promoter was performed using 9E10 and Cy3-coupled anti-mouse immunoglobulin G (Krien *et al.*, 1998).

UV Irradiation

Cells grown to exponential phase at 30°C were plated onto YES, allowed to dry, and irradiated with a range of doses of UV-C using a Stratalinker (Stratagene, La Jolla, CA). Colonies were counted after 3 d at 30°C. To test the ability of *cdc25-22* to rescue the radiation sensitivity, strains in a *cdc25-22* background were grown at 25°C, plated onto YES, and irradiated as above. These plates were placed directly at 25°C or were incubated at 36°C for 4 h and then placed at 25°C. Colonies were counted after 4–5 d incubation at 25°C. Checkpoint analysis was performed on cells synchronized with a *cdc25-22* block and release protocol. Strains in a *cdc25-22* background were grown at 25°C, shifted to 36°C for 3 h, irradiated with 50 J/m² UV-C as described (O'Connell *et al.*, 1997), and resuspended in prewarmed media at 25°C. Cell cycle progression was monitored by DAPI straining of ethanol-fixed samples, counting the number of cells passing mitosis, assessed as cells in mitosis, with two nuclei or undergoing septation. The proportion of cells failing to arrest was estimated from the area under the curve spanning the time until control cells entered mitosis.

Ionizing Radiation

Exponentially growing cells at 30°C were irradiated with a Varian (Palo Alto, CA) linear accelerator. The focus was set 100 cm below the flasks, and they were irradiated with a 6-MeV electron beam at a dose rate of ~22 Gy/min. Samples were taken and plated onto YES, and colonies were counted after 3 d at 30°C. Synchronous cultures were obtained using lactose synchronization of exponentially growing cells (Mitchison and Carter, 1975). Ionizing radiation of synchronous cultures was delivered using a Gammacell (Gamma Elektric, Stenløse, Denmark) 1000 ¹³⁷Cs source at 12 Gy/min. Cell cycle progression was monitored by DAPI staining of ethanol-fixed samples, counting the number of cells passing mitosis.

Analysis of Double-stranded Break Repair

Double-stranded breaks were induced in exponentially growing cells by treating the cultures with 450 Gy using the Varian linear accelerator as described above. The culture was placed back at 30°C to recover. Fifteen-milliliter samples were harvested by centrifuga-

tion, washed twice in CSE (20 mM citrate/phosphate, pH 5.6, 40 mM EDTA, 1.2 M sorbitol), and incubated for 1 h at 37°C in 1 ml CSE and 1.5 mg Zymolyase 20-T (ICN, Costa Mesa, CA). The cells were washed once in CSE and resuspended to ~6 × 10⁸ cells/ml in TSE (10 mM Tris, pH 7.5, 45 mM EDTA, 0.9 M sorbitol) at 37°C. An equal volume of 1% low gelling temperature agarose in TSE (at 55°C) was added, and agarose plugs of the samples were made and set on ice for 15 min. The plugs were transferred to a solution of 1% sarcosyl, 0.5 M EDTA, pH 9.5, 0.5 mg/ml proteinase K and incubated at 55°C for 48 h, with readdition of proteinase K after 24 h. The plugs were stored at 4°C and washed in Tris-EDTA before loading. Sample plugs were run on a 0.6% gel of chromosomal grade agarose (Bio-Rad, Hercules, Ca) in 0.5× Tris borate-EDTA for 220 h at 40 V with a pulse time of 75 min in a Gene Navigator PFGE apparatus (Pharmacia Biotech, Piscataway, NJ).
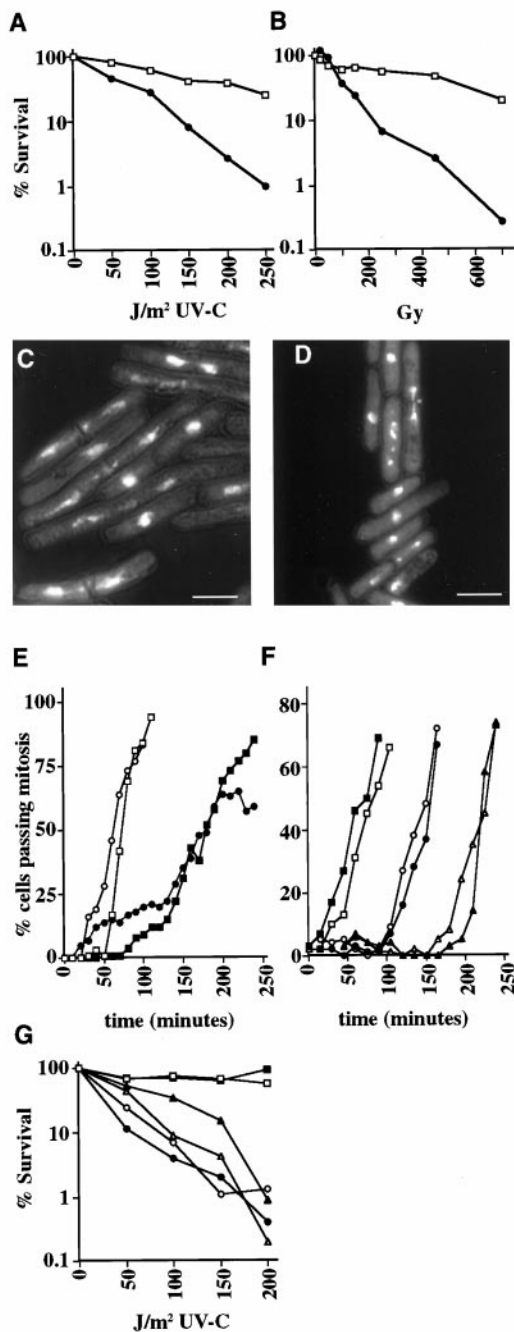
Chromosome Loss Assays

Mutant alleles were crossed into an *ade6-704* background and then crossed to a strain containing the artificial minichromosome (Ch10), which contains the *ade6-704* tRNA suppressor *sup3-5* (Niwa *et al.*, 1989). The resulting progeny were grown at 30°C under selective conditions (minimal medium lacking adenine), and transferred to nonselective conditions (YES) for 29 generations, and samples were plated onto minimal media containing 10 mg/l adenine. A sample was taken at the beginning of the time course and used as a background correction. After 3 d at 30°C the number of red colonies was scored as a percentage of the total number of colonies, and this was used to determine the rate of chromosome loss, calculated as the percentage of colonies auxotrophic to adenine divided by the number of generations.

Cloning of NA74 and Recombinant Techniques

Standard procedures were used for the construction of recombinant plasmids (Sambrook *et al.*, 1989). DNA sequencing was performed on double stranded templates and processed on an ABI377 sequencer (Applied Biosystems, Foster City, CA). The NA74 strain (in a *ura4-D18* background) was transformed with a genomic library pURSP1 (Barbet *et al.*, 1992). The transformants were replica plated on YES and 0.01% methyl methanesulfonate (MMS) to select for complementation. DNA was extracted from the complementing transformants and was used to electroporate *Escherichia coli*. The plasmids were retested for complementing activity by transforming the NA74 strain and assessing complementation. Complementation was assessed by sensitivity to a range of doses of UV-C irradiation (as described above). Three deletion versions of a suppressing clone were constructed by removing either the internal 1.4-kb *Hin*dIII fragment or the 3.8-kb *Hin*dIII fragment extending to the end of the clone and religating or the 2.7-kb *Pme*I–*Sma*I fragment, end filling with Klenow and religating. An ATP-binding domain mutation (G129A,K130R) was constructed by in vitro mutagenesis (Kunkel *et al.*, 1987) of a rad18 cDNA with the following oligonucleotide: 5'-GGTCATAATGGTAGCG<u>C</u>T<u>A</u>GAAGTGCTATACTTACAGGA-3'. Base substitutions are underlined and create an *Eco*47III site. Mutant (referred to as *rad18-DN*) and wild-type *rad18* were then cloned onto the full-strength *nmt1* promoter (pREP1), and complementation was assessed in the presence of thiamine.

Spore Germination of rad18::ura4

Construction of the rad18 deletion strain has been described previously (Lehmann *et al.*, 1995). Sporulating colonies of a wild-type strain with the genotype *ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M210/ade6-M216 his3-D1/his3-D1 h+/h⁻* and the rad18 deleted strain of the genotype *rad18⁺/rad18::ura4 ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-704/ade6-704 h+/h90* were grown in YE to exponential phase and then starved in minimal media lacking ammonium at 30°C to induce sporulation. This cell mix was digested overnight in

0.05% β-glucuronidase and washed in water three times; spores were harvested by layering this mix onto 25% glycerol in minimal media lacking ammonium, collected by centrifugation at 1500 × g for 15 min, and washed with water. Spores (6 × 10⁶) were inoculated into minimal media lacking uracil for the *rad18::ura4* spore culture and including uracil for the wild-type spore culture. These were allowed to germinate at 30°C until polar growth was observed as a marker of germination (10–12 h) and then incubated with 0.01% MMS or 0.31 μg/ml 4-nitroquinoline-*N*-oxide (4-NQ) or with no treatment for a further 6 h. Cells were fixed in ethanol and DAPI stained for counting mitoses or were fixed in glutaraldehyde and formaldehyde as described above for photography.

Construction of the brc1::ura4 Strain

For construction of a knockout plasmid, the 7.5-kb fragment was subcloned into pBluescript KS+ from which the *Hin*dIII site had been ablated by digestion, end filling, and ligation. From this, the 1.4-kb *Hin*dIII fragment, which spans from amino acid 48 to amino acid 490 of the *brc1* coding open reading frame was excised and replaced with a 1.8-kb *ura4* fragment. A diploid strain was constructed with the genotype: *ura4-D18/ura4-D18 leu1–32/leu1–32 ade6-M210/ade6-M216 h+/h−*. The *brc1* deletion plasmid was used to transform this strain to uracil prototrophy. Transformants were propagated in continuous log-phase for 4 d to allow integration and replated back under selective conditions. Colonies were screened by Southern blotting for the replacement event. Several appropriate strains were derived, and one was chosen for further analysis. This strain was induced to sporulate by plating onto MEA medium for 2 d. Tetrads from 20 azygotic asci were dissected, and in all cases all four spores were viable and gave a 2:2 segregation of the ura+ phenotype. *brc1::ura4* strains were back-crossed to wild type to exclude the possibility of suppressor mutations.

Phosphorylation of chk1p

All strains were in the background of chk1::HA (Walworth and Bernards, 1996). Cultures were irradiated with UV-C or ionizing radiation as described above, and samples were harvested and frozen. The frozen cells were disrupted using a bead beater with glass beads and extracted into lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 50 mM NaF, 2 mM EDTA, 1 mM DTT, 80 mM β-glycerophosphate, 0.1% NP40, 0.1 mM Na-orthovanadate, 1 mM PMSF, plus protease inhibitors). The extract was cleared by centrifugation at 13,000 × g for 15 min, and the supernatant was boiled in SDS sample buffer. Samples were analyzed by SDS-PAGE and Western blotting using the 12CA5 antibody. The band intensity was quantified on a Molecular Dynamics (Sunnyvale, CA) densitometer using ImageQuant software.

Figure 1. NA74 is defective in the response to DNA damage. (A) UV-C survival curve for wild type (□) and NA74 (●). (B) Ionizing radiation survival curve for wild type (□) and NA74 (●). (C) DAPI-stained cells for NA74 that have been synchronized by *cdc25-22* block and release and irradiated with 50 J/m² UV-C. Bar, 10 μm. (D) DAPI-stained cells for asynchronous NA74 irradiated with 450 Gy ionizing radiation. Bar, 10 μm. (E) Wild-type cells (■ and □) and NA74 (● and ○) were synchronized by *cdc25-22* block and release and then irradiated with 50 J/m² UV-C (closed symbols) or mock irradiated (open symbols). Cultures were incubated at 25°C, and ethanol-fixed samples were taken for DAPI staining. The percentages of cells that had passed mitosis were assessed from each sample

Figure 1 (cont). by fluorescence microscopy. (F) Wild-type cells (closed symbols) and NA74 (open symbols) were synchronized by lactose gradient centrifugation and then irradiated with 100 Gy (● and ○) or 500 Gy (▲ and △) or mock irradiated (■ and □). Cultures were incubated at 30°C, and ethanol-fixed samples were taken for DAPI staining. The percentages of cells that had passed mitosis were assessed from each sample by fluorescence microscopy. (G) *cdc25-22* (■ and □), *cdc25-22* NA74 (● and ○), and *cdc25-22 rad3-136* (▲ and △) were grown at 25°C, plated on YES medium, and irradiated with the indicated dose of UV-C. Plates were then placed at 25°C (open symbols) or at 36°C for 4 h and then shifted to 25°C (closed symbols). Surviving colonies, expressed as a percentage of unirradiated controls, were counted after 4 d.
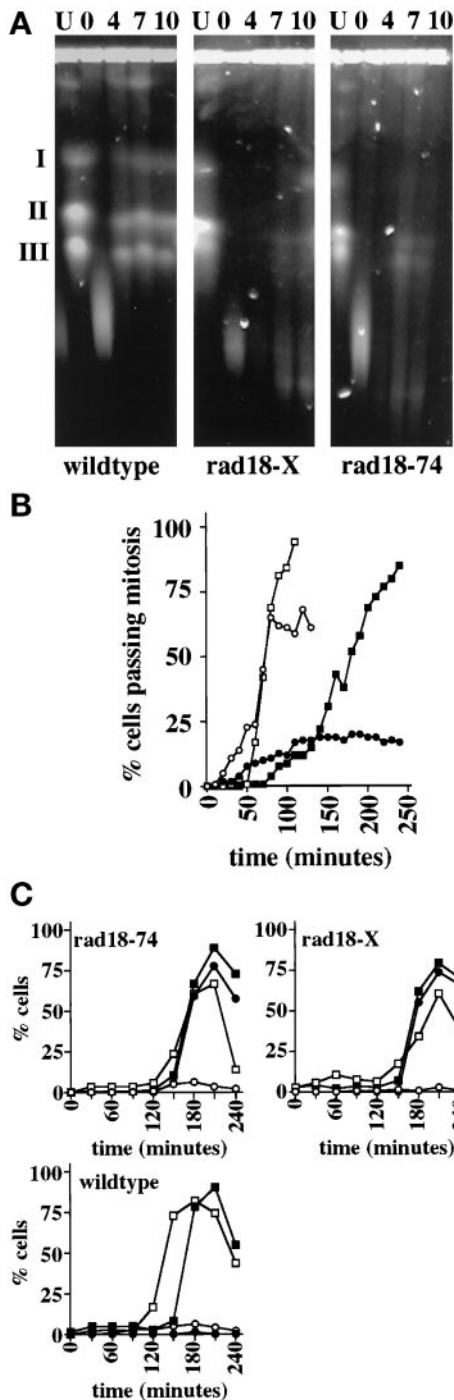
Figure 2. Rad18 mutants are defective in DNA repair. (A) The indicated strains were grown to midlogarithmic phase and then irradiated with 450 Gy of ionizing radiation. Samples were taken at the indicated times (hours) and processed for pulse field gel electrophoresis. Unirradiated controls (U) were included and used as standards for the positions of the three chromosomes. (B) Wild-type cells (■ and □) and *rad18-X* (● and ○) were synchronized by *cdc25-22* block and release and then irradiated with 50 J/m² UV-C (closed symbols) or mock irradiated (open symbols). Cultures were incubated at 25°C, and ethanol-fixed samples were taken at the

RESULTS

The NA74 Mutation Causes an Altered Response to DNA Damage in G2

The NA74 mutation defines a gene that we recently identified in a screen for novel G2 checkpoint genes in fission yeast (Verkade and O'Connell, 1998). This strain is MMS sensitive and enters lethal mitoses in the presence of this DNA-damaging agent. To characterize further this mutation we performed a phenotypic analysis of its effect on the response to DNA damage. Survival curves with both UV-C and ionizing radiation showed NA74 to be hypersensitive to both these DNA damaging agents (Figure 1, A and B). DAPI staining of irradiated cells confirmed that they were progressing aberrantly through mitosis. In many cells, the DNA was stretched along the division plane, and cytokinesis had progressed in the absence of completed chromosome segregation (Figure 1, C and D).

Because defective mitoses after DNA damage is consistent with a failure of the G2 DNA damage checkpoint, we assessed cell cycle progression kinetics in synchronous cultures after irradiation. In wild-type controls, a normal checkpoint-induced delay was seen at doses of irradiation that had minimal effects on cell survival. The response of NA74 cells was, however, more complex. In UV-C-irradiated cells, ~30% of the cells in the culture failed to arrest, entering mitosis with kinetics similar to unirradiated controls. The remainder progressed through mitosis with kinetics similar to wild-type cells (Figure 1E). Consequently, both cultures of NA74 cells showed a doubling of cell number over the time course of the experiment. However, unlike wild-type cells, the mitoses in the irradiated NA74 cells were highly aberrant, with failed chromosome segregation (Figure 1C), suggesting they had not fully resolved lesions caused by UV-C irradiation before reinitiating mitosis. This response is not due to a lack of synchrony (as assessed by unirradiated controls) or an incomplete arrest by *cdc25-22* (see Figure 2C). These data are therefore consistent with a checkpoint defect in which the delay is not maintained for sufficient time to ensure mitotic fidelity. In the γ-irradiated cells, the NA74 strain showed a dose-dependent delay to mitosis identical to the wild-type strain (Figure 1F). This was despite the fact that the NA74 cells irradiated with 500 Gy showed a considerable reduction in viability (2%) compared with wild-type controls (48%), and failed to faithfully complete mitosis (Figure 1D). Because the physiological role of this checkpoint is to prevent mitosis in the presence of DNA damage, these data are also consistent with a defective cell cycle response to DNA damage.

Figure 2 (cont). indicated times for DAPI staining. The percentages of cells that had passed mitosis were assessed from each sample by fluorescence microscopy. (C) The indicated strains in the background of *cdc25-22* were arrested in late G2 by incubation at 36°C for 3 h. Cells were then irradiated with 100 J/m² UV-C (closed symbols) or mock irradiated (open symbols; time = 0) and were maintained at 36°C for 2 h after irradiation and then shifted to 25°C. Samples were assayed by DAPI staining, and normal mitoses (■ and □) and aberrant mitoses (● and ○) as a percentage of total cells were counted from three samples of 100–150 cells per time point. Data represent the mean of these samples; SE ≤ 5%.

If a defective G2 DNA damage checkpoint were the sole cause of the radiation sensitivity of NA74, then an imposed cell cycle delay after irradiation should at least partially rescue the radiation-induced cell death, as has been described for other checkpoint mutants (Al-Khodairy and Carr, 1992). However, no such rescue was observed when NA74 was delayed in G2 after UV-C irradiation by imposition of a *cdc25-22* arrest (Figure 1G), whereas rescue was clearly evident for the checkpoint-defective *rad3-136* control. Hence, checkpoint defects alone do not account for the radiation sensitivity of NA74, and this may be consistent with an additional defect in DNA repair.
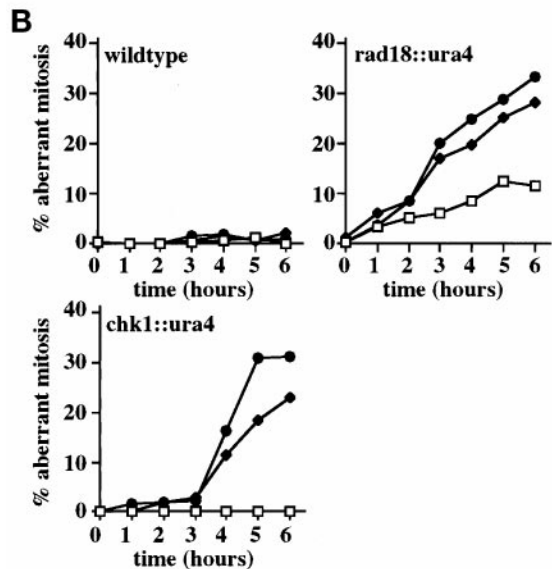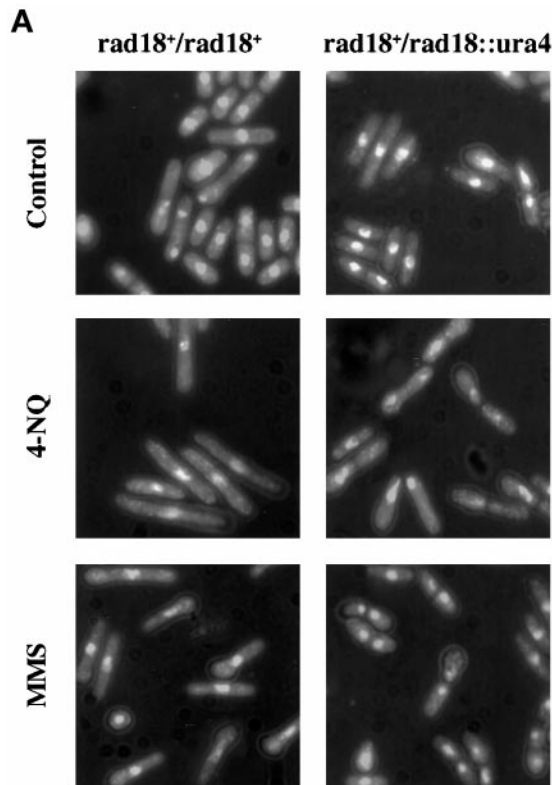
NA74 Is an Allele of rad18

We aimed to clone the NA74 gene by complementation of its MMS sensitivity and isolated seven complementing plasmids, which fell into two classes. One set, defined by three plasmids, included the *rad18* gene. NA74 was subsequently shown to be an allele of *rad18* by linkage analysis and is hitherto referred to as *rad18-74*. Sequencing of *rad18-74* showed a single base substitution (G→A) leading to an amino acid substitution (A151T), which is located 21 residues C-terminal to the putative ATP-binding domain. The remaining four plasmids were shown by restriction and hybridization analyses to possess overlapping inserts and represented a high-copy suppressor that completely rescued the radiation sensitivity of *rad18-74*. This suppressor, *brc1*, is discussed below.
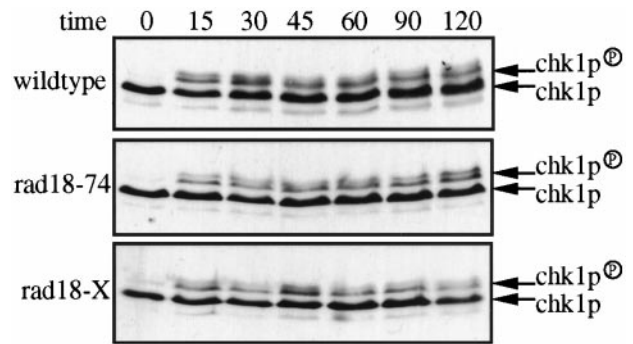
Rad18p has previously been shown to be required for repair of UV-C-induced lesions (Lehmann *et al.*, 1995). Because both *rad18-74* and the previously isolated allele, *rad18-X*, are also sensitive to MMS and ionizing radiation, we assayed their ability to repair double-stranded DNA breaks using pulse field gel electrophoresis (Figure 2A). Wild-type cells had efficiently rejoined chromosomal fragments by 4 h after irradiation with 450 Gy, whereas little repair was evident in either *rad18* strain through 10 h after irradiation. Thus, rad18p is required for repair of double-stranded DNA breaks, and yet rad18-74 enters mitosis after this dose of ionizing radiation without completing repair. These data indicate that although γ-irradiated *rad18-74* cells showed checkpoint kinetics similar to wild-type cells, they cycle through G2 into mitosis in the absence of successfully completing DNA repair, which explains the appearance of mitotic defects in these cells. Conversely, *rad18-X* is largely checkpoint proficient in response to ionizing radiation (Al-Khodairy and Carr, 1992). Because neither *rad18* strain is capable of repairing DNA damage induced by ionizing radiation, the difference in cell cycle responses is dramatic. We assayed the UV-C checkpoint of *rad18-X* in a *cdc25-22* block (36°C) and release (25°C) synchronization and found that, unlike *rad18-74*, only a small proportion of *rad18-X* cells entered mitosis, and there was subsequently little increase in cell number (Figure 2B). When strains defective in nucleotide excision repair (*rad13-A*, *rad15-P*, or *rad16-U* [Murray *et al.*, 1992; Carr *et al.*, 1993, 1994]) are irradiated with UV-C, the cells elongate and fail to enter mitosis, demonstrating the effect of a wild-type checkpoint responding to unrepairable DNA damage (Al-Khodairy and Carr, 1992). Like *rad18-74*, the UV-C sensitivity of *rad18-X* was not rescued by a *cdc25-22* G2 arrest imposed for 2 h after irradiation of synchronized G2 cells, which is consistent with the DNA repair

defects of *rad18-X* (Lehmann *et al.*, 1995) (Figure 2C and our unpublished results). Both *rad18* strains were prevented from entering mitosis until the arrest was relieved. These data indicate that both *rad18* mutants require p34^{cdc2} activation for entry into these aberrant mitoses, and the nuclear abnormalities are a result of passage through mitosis rather than an interphase event. However, the following mitoses in *rad18-X* using this protocol were as highly aberrant as those seen with irradiated *rad18-74* cells with or without this postirradiation arrest (Figures 1E and 2C). This may be due to the reported enhancement of *rad18-X* defects at 36°C (Lehmann *et al.*, 1995) and suggests that a failure to maintain a checkpoint arrest is a general feature of *rad18* alleles. Neither *rad18* allele was defective in its DNA replication checkpoint as assessed by hydroxyurea-induced cell cycle arrest or imposition of temperature-sensitive S-phase mutants. However, like the checkpoint-proficient *mis4-242* strain, *rad18-74* and *rad18-X* are also hydroxyurea sensitive (our unpublished results).

Because *rad18* is an essential gene, the *rad18-74* and *rad18-X* alleles are not completely null for rad18p function. To determine the checkpoint status of *rad18Δ* cells, we assessed the ability of wild-type spores and spores deleted for *rad18* (*rad18::ura4*) to maintain a checkpoint arrest after DNA damage induced by MMS or the UV-mimetic 4-NQ. After germination, these radiomimetic drugs were added to cultures, and we followed mitotic figures for a further 6 h. This continuous exposure to the drugs circumvents the asynchrony of spore germination that precludes spore germination experiments using UV-C or γ-irradiation. In the presence of these drugs, wild-type spores maintained a G2 arrest, as assessed by fluorescence-activated cell-sorting analysis for the duration of the experiment (Figure 3 and our unpublished results). Conversely, *rad18::ura4* spores germinated and entered defective mitoses in the presence of both drugs. After 6 h, these aberrant mitoses represented ~30% of the germinated spores, and no normal mitoses were observed (Figure 3). After 3 h, ~20% of these aberrant mitoses were characterized by the stretching of DNA along the division plane, with the remainder resembling a classical "cut" phenotype. By 6 h, cytokinesis through nuclear material had occurred in all mitotic cells. In the absence of MMS or 4-NQ, ~10% of *rad18::ura4* spores displayed a cut or "stretched" phenotype, accounting for 75% of mitotic figures. These data indicate that rad18p is required for successful passage into mitosis even in the absence of DNA-damaging agents. Germination of *rad18::ura4* spores in 11 mM hydroxyurea caused prolonged cell cycle arrest (our unpublished results), indicating that the replication checkpoint is intact, and these cells are capable of undergoing interphase arrest. We compared the kinetics of aberrant mitoses in these cultures with that of a *chk1::ura4* strain, which completely lacks G2 DNA damage control (Walworth *et al.*, 1993). These cells accumulated similar levels of aberrant mitoses caused by checkpoint failure in the presence of MMS or 4-NQ compared with *rad18::ura4*, although all these were of a cut phenotype. The appearance of these aberrant mitoses was delayed compared with those in the *rad18::ura4* strain. The earlier appearance in *rad18::ura4* may be due to the inherent requirement for rad18p for mitosis and/or may be partially affected by different germination kinetics in the two strains.

Figure 4. Rad18p is not required for phosphorylation of chk1p after DNA damage. The indicated strains, in the background of hemagglutinin-tagged chk1p, were irradiated at 100 J/m², and samples were taken at the indicated times (minutes). Western blot analysis using the 12CA5 antibody was used to detect the forms of hemagglutinin-tagged chk1p. Time 0 = unirradiated. Essentially identical results were obtained with cultures irradiated with 250 Gy ionizing radiation.

Nevertheless, these data indicate that rad18p is essential to prevent entry into mitosis in the presence of DNA damage.

Rad18-X and rad18-74 Show Normal DNA Damage-induced chk1p Phosphorylation

The chk1p protein kinase is the final element in the signaling cascade activated by the G2 DNA damage checkpoint. DNA damage induces a phosphorylation-dependent mobility shift on chk1p, in a *rad* checkpoint gene-dependent manner (Walworth and Bernards, 1996). This phosphorylation promotes binding to 14-3-3 proteins and is used a surrogate marker of checkpoint activation (Chen *et al.*, 1999). We used this marker to investigate whether the defective DNA damage checkpoint responses of *rad18* mutant strains are due to failed checkpoint activation or checkpoint maintenance. We assayed the ability of *rad18-X* and *rad18-74* cultures to promote chk1p phosphorylation in response to ionizing or UV-C irradiation. Both strains showed wild-type chk1p phosphorylation kinetics in response to the DNA damage, with two phosphorylated forms appearing after 15 min, and being maintained, albeit at slightly lower levels in the *rad18* mutants, for the duration of the time course (Figure 4). Phosphorylated chk1p continued to be maintained after the wild-type cells had recovered from the checkpoint arrest and entered mitosis, suggesting that chk1p phosphorylation is not a marker of checkpoint release. These data are consistent with the model that rad18p is required for checkpoint maintenance rather than initiation. It was not possible to assay chk1p phosphorylation in a *rad18::ura4* background,

Figure 3. Rad18p is essential for cell cycle arrest after DNA damage. (A) Spores from rad18⁺/rad18::ura4 were germinated until the appearance of polar growth (10 h) at 30°C in medium lacking uracil to select only rad18::ura4 cells, with *rad18⁺* spores as a control. MMS or 4-NQ was then added, and cultures continued incubation for a further 3 h. Samples were taken, fixed, and stained with DAPI. Note the presence of cells with septa bisecting *rad18::ura4* nuclei in the presence of both 4-NQ and MMS. Bar, 10 μm. (B) Kinetics of aberrant mitoses in spore germination experiments. Spores were germinated as above for wild-type (ura4⁺/ura4-D18), rad18::ura4

Figure 3 (cont.) (rad18⁺/rad18::ura4), and chk1::ura4 (chk1⁺/chk1::ura4) strains and either left untreated (□), or MMS (♦) or 4-NQ (●) was added at time 0. Samples were then taken hourly, fixed, and stained with DAPI, and the mean percent of cells with aberrant mitoses (either cut or DNA stretched along the division plane; see text) was recorded from three samples of 100–150 cells. SE ≤ 5%.
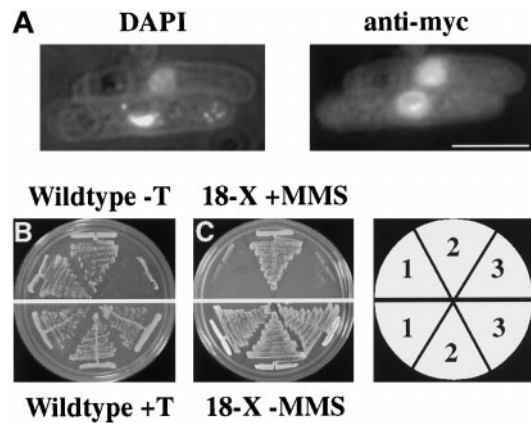
Figure 5. Rad18p is a nuclear protein with an essential ATP-binding domain. (A) Rad18p localizes to the chromatin compartment of the nucleus. Cells expressing myc-tagged rad18p were stained with 9E10 anti-myc antibody, visualized with Cy3, and counterstained with DAPI. (B) *rad18-DN* is dominant negative when overexpressed. Wildtype colonies were grown in the presence (+T) or absence (−T) of thiamine for 3 d at 30°C. Plasmids: (1) pREP1 empty vector; (2) pREP1 wild-type *rad18*; (3) pREP1 *rad18-DN*. (C) *rad18-DN* cannot complement *rad18-X*. *rad18-X* cells were grown in the presence (+MMS) or absence (−MMS) of 0.0075% MMS on thiamine-containing media (which represses lethal expression) for 3 d at 30°C. Plasmids: (1) pREP1 empty vector; (2) pREP1 wild-type *rad18*; (3) pREP1 *rad18-DN*. *rad18-DN* cannot complement *rad18-74* (our unpublished results).

because the presence of an equal number of ungerminated (*rad18⁺*) spores makes the data impossible to interpret. These spores are in G1, a time when cells are not competent for chk1p phosphorylation (Martinho *et al.*, 1998), and so any observed absence of phoshorylation could be attributed to cell cycle status rather than a defect in signaling to chk1p.

Rad18p Is a Nuclear Protein with an Essential ATP-binding Domain

The structural similarity between rad18p and other SMC proteins suggests a chromatin organization role for rad18p. Immunostaining of cells expressing a myc-tagged version of rad18p showed a localization pattern identical to DAPI staining, indicating that, like other SMC proteins, rad18p is found in the chromatin compartment of the nucleus (Figure 5A) (Strunnikov *et al.*, 1993, 1995; Hirano and Mitchison, 1994; Saka *et al.*, 1994b; Saitoh *et al.*, 1995). Because ATPase activity has been demonstrated for SMC family members, we mutated the ATP-binding domain of *rad18*, to construct the allele *rad18-DN*. Overexpression of *rad18-DN* in wild-type cells was lethal, indicating that this allele has a dominant negative effect on essential *rad18* function with cells resembling *rad18Δ* with an accumulation of mitotic defects (Figure 5B and our unpublished results) (Lehmann *et al.*, 1995). Lower-level expression of wild-type *rad18* rescued the MMS sensitivity of *rad18-X* and *rad18-74*, but expression of *rad18-DN* could not complement either *rad18-X* or *rad18-74* (Figure 5C). Together, these data indicate that the ATP-binding domain of *rad18* is essential for function, indicating an essential role for ATP hydrolysis, as has been described
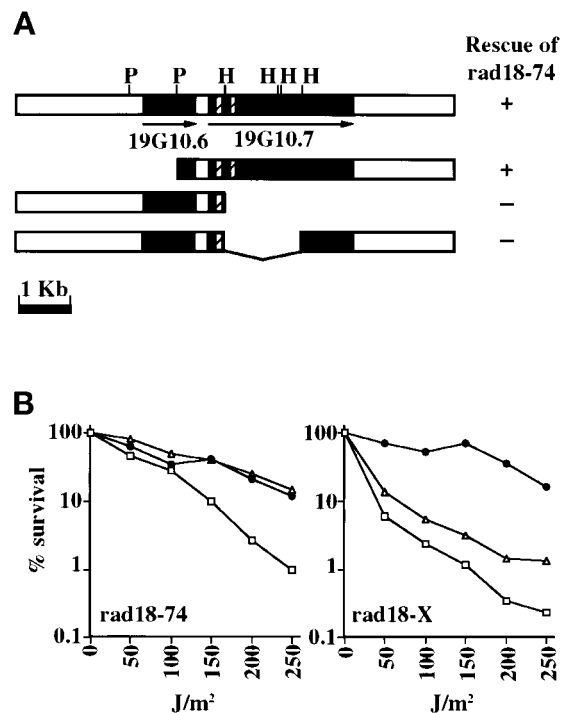
Figure 6. Isolation of an allele-specific high-copy suppressor of *rad18-74*. (A) Partial restriction map of a *rad18-74*-suppressing plasmid. All isolates contained the open reading frames 19G10.6 and 19G10.7 (indicated as solid bars; introns in 19G10.7 [*brc1*] are indicated as hatched bars). P, *Pme*I; H, *Hin*dIII. Rescue of *rad18-74* was assessed by UV-C irradiation survival curves. (B) *rad18-74* and *rad18-X* were transformed with vector alone (□), C19G10.7 (*brc1*) (△), or *rad18* (●). Transformants were grown in selective media, and UV-C survival curves were determined as a percent survival compared with unirradiated controls.

for activity of the SMC2/SMC4 heterodimer (Kimura and Hirano, 1997).

An Allele-specific High-Copy Suppressor of rad18 Encodes a BRCT Protein

In complementation of *rad18-74* with a genomic DNA library, four plasmids carrying a high-copy suppressor of *rad18-74* were isolated in addition to three *rad18*-containing plasmids. Sequence analysis of these high-copy suppressor plasmids showed they corresponded to a locus on chromosome I covered by cosmid C19G10 (GenBank accession number Z69909). Two open reading frames were present on the suppressing plasmids, and deletion mapping of one of these plasmids showed the suppressing activity resided in, and was dependent on, an open reading frame designated C19G10.7 (Figure 6A). C19G10.7 encodes a protein with six BRCT domains, and hence we have termed this gene *brc1*. The suppression of *rad18-74* by *brc1* was allele specific, as it had no suppressive activity on the radiation sensitivity of *rad18-X* (Figure 6B). The full suppression of radiation sensitivity of *rad18-74* was also reflected by the suppression of the mitotic defects seen with checkpoint failure. These effects are unlikely to be due to perturbation of cell cycle distribution,

because even high-level overexpression of *brc1* from the *nmt1* promoter has no effect on cell cycle progression in wild-type or *rad18* mutant cells (our unpublished results). Two other fission yeast BRCT proteins, encoded by *cut5* and *rhp9*, had no effect on the radiation sensitivity of *rad18-74* when expressed from a multicopy plasmid. Therefore, BRCT domains alone are insufficient to suppress *rad18-74*.

brc1 Is Required for Mitotic Fidelity

To determine function for brc1p, we deleted *brc1* and replaced it with the *ura4* marker by homologous recombination in diploid cells (Figure 7A). Dissection of tetrads showed a 2:2 segregation of $ura4^+$:$ura4^-$, indicating *brc1* is not an essential gene. Given the allele-specific interaction with *rad18*, we tested whether *brc1::ura4* cells are radiation sensitive. Radiation survival curves showed *brc1::ura4* cells were not sensitive to UV-C irradiation, and were only mildly sensitive to ionizing radiation (Figure 7, B and C), nor did we observe defects in DNA damage or replication checkpoint kinetics (our unpublished results). However, other phenotypes of *brc1::ura4* cells indicate that brc1p plays a role in the fidelity of mitotic chromosome segregation. First, assays of chromosome stability showed that *brc1::ura4* cells lost an artificial chromosome with a frequency 200-fold greater than a wild-type cell (Figure 7D). Chromosome instability was also seen in *rad18-74* (16-fold) and has been described for *rad18-X* (74-fold) (Lehmann *et al.*, 1995). This has also been described for the other *rad18* epistasis group members *rad2* and *rhp51* (Murray *et al.*, 1994; Muris *et al.*, 1996). Second, *brc1::ura4* cells showed a consistent although variable delay to entry into mitosis, dividing at 19 ± 3.8 μm, compared with 13.8 ± 0.4 μm for wild-type controls (Figure 7E). Fluorescence-activated cell-sorting analysis combined with mitotic indices showed that this delay was confined to the G2 period of the cell cycle. Furthermore, ~6% of cells showed a variety of chromosome segregation defects resulting in classical cut phenotypes, nuclear fragmentation, hypercondensation, mis-segregation, and stretching of DNA along the division axis (Figure 7, F–J). These phenotypes resemble those observed in *rad18::ura4* spores (Figure 3A). Together, these data are consistent with defective organization of chromatin in the absence of brc1p function. Finally, no nonparental ditypes were recovered in crosses between *brc1::ura4* and either *rad18-74* or *rad18-X*. In every case, the presumptive double mutants divided two or three times, arresting as microcolonies of four to eight elongated and/or lysed cells, as has been described in strains deleted for *rad18* (Lehmann *et al.*, 1995). These data indicated that mutations in both *brc1* and *rad18* result in synthetic lethality, and hence *brc1* is essential when *rad18* function is impaired.

Interactions between Genes Involved in Higher-Order Chromosome Structure

The defects in chromosome segregation and the G2 delay seen in *brc1* and *rad18* mutants were reminiscent of phenotypes described of strains defective in chromatin organization. The temperature-sensitive allele of type II topoisomerase, *top2-191*, displays similar phenotypes when shifted to semipermissive temperature (Krien *et al.*, 1998). Top2p appears to play many roles in chromatin organization, including chromatin condensation. Fin1p, the fission yeast homologue of the *Aspergillus* NIMA kinase, is implicated in the control of chromatin condensation, because activation of these kinases alone can prematurely induce chromatin condensation independently of cell cycle progression (O'Connell *et al.*, 1994). When *fin1* is deleted, the cells again show similar phenotypes of G2 delay and mitotic defects. In fact, double mutants between *fin1Δ* and *top2-191* show synthetic lethality because of a greatly increased frequency of mitotic abnormalities (Krien *et al.*, 1998). This suggests that they cooperate in chromatin condensation. To determine whether rad18p plays a role in chromatin condensation, we examined any genetic interactions between these and other genes involved in chromatin organization. The data are presented in Table 1.

Both *rad18-74* and *rad18-X* were found to be synthetically lethal with *top2-191*, failing to form colonies at the semipermissive temperature of 30°C (Figure 8A). Under these conditions *top2-191* cells delay in G2 and show mitotic defects in ~10% of cells but are able to form colonies (Krien *et al.*, 1998). DAPI staining of double mutant strains grown at the permissive temperature of 25°C and then shifted to 30°C for 4 h showed a dramatic increase in the frequency of aberrant mitoses. Most of these were characterized by failure to completely segregate chromosomes before cytokinesis (Figure 8, C–I, and Table 1). Inactivation of topoisomerase II by drugs such as mitoxantrone leads to G2 arrest and double-stranded DNA breaks, and hence these drugs are highly toxic to strains defective in double-stranded break repair in budding yeast (Hartwell *et al.*, 1997). As the *rad18-X;top2-191* and *rad18-74;top2-191* double mutants proceed into aberrant mitosis, rad18p is required to maintain the G2 delay caused by the reduction in top2p activity at the semipermissive temperature. An inability to repair double-stranded breaks caused by top2p inactivation would not account for these mitotic phenotypes, and therefore the repair defect of *rad18-X* and *rad18-74* cannot be the sole cause of the synthetic lethality. Alternatively, the synthetic phenotypes could suggest that top2p and rad18p cooperate in chromatin organization. We attempted to suppress both *rad18* mutants by overexpressing *top2* from the *nmt1* promotor but observed no effect on radiation sensitivity or mitotic fidelity. Overexpression of top2 had no effect on wild-type cells (our unpublished results). *brc1::ura4* was also synthetically lethal with *top2-191* (Figure 8B), with increased defects in chromosome segregation (Figure 8, C–J, and Table 1).

Double mutants constructed between *rad18-X*, *rad18-74* and *fin1Δ* also showed higher levels of mitotic abnormalities (7- to 10-fold), suggesting cooperation of function. To further characterize this, we investigated functional links with the condensin SMC proteins encoded by *cut3* and *cut14*. No synthetic phenotypes were seen with double mutants constructed with the only conditional alleles, *cut3-477* and *cut14-208*. This may be specific for these alleles, which do not show intermediate phenotypes at temperatures ranging from 25 to 34°C (our unpublished results), and thus we cannot rule out functional links at this time. The functional interactions with *top2* and *fin1* suggest a possible role for rad18p in chromatin condensation. Double mutants of *rad18* alleles with the *rad21-45* strain, which by analogy to homologous proteins in other systems is likely to be defective in sister chromatid cohesion (Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Losada *et al.*, 1998), showed no synthetic interactions.
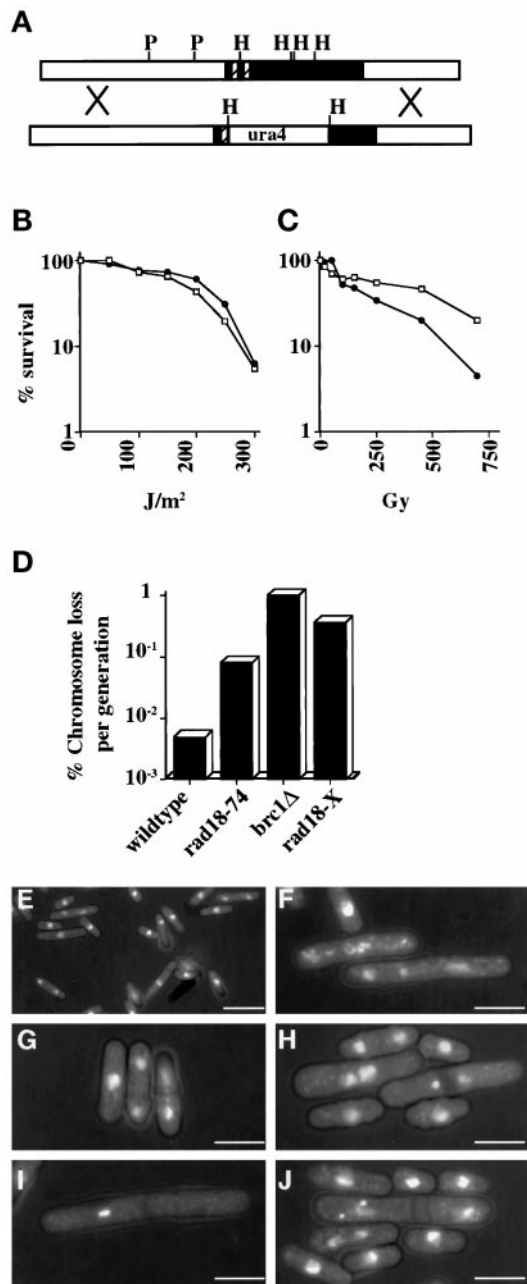
Figure 7. Mitotic defects and chromosome instability in strain deleted for *brc1*. (A) Strategy for creating a null allele of *brc1*. See MATERIALS AND METHODS for details. (B) UV-C survival curve for wild-type (□) and *brc1::ura4* (●). (C) Ionizing radiation survival curve for wild-type (□) and *brc1::ura4* (●). (D) Rates of loss of artificial chromosome 10 per generation. Data are expressed as a percentage of total colonies and are corrected for loss at generation zero. Values are wild-type, 0.005; *rad18-74*, 0.080; *brc1::ura4*, 0.997; *rad18-X*, 0.368. (E) DAPI and phase-contrast image of exponentially growing *brc1::ura4* cells. Note variability in cell length and mis-segregated chromosomes (top left). Bar, 20 μm. (F–J) Examples of mitotic abnormalities of *brc1::ura4*. Bars, 10 μm. (F) Nuclear fragmentation and mis-segregation. (G) Stretching of chromosomes along division axis and bisection by septum. (H) Bisection of nucleus by septum and hypercondensation. (I) Hypercondensation and mis-segregation. (J) Fragmentation (above) and incomplete segregation of chromosomes (below left).

Similarly, no genetic interactions were observed with *mis4-242*, which is also defective in sister chromatid cohesion (Furuya *et al.*, 1998). Furthermore, double mutants between *rad18* alleles and either *rad21-45* or *mis4-242* showed enhanced radiation sensitivity compared with either parent, which is further indication of separable function (our unpublished results). Therefore, the genetic interactions we observed are consistent with a role for rad18p in chromatin organization, which is independent of sister chromatid cohesion.

DISCUSSION

Cells respond to DNA damage in G2 by delaying mitotic onset through maintenance of p34^{cdc2} Y15 phosphorylation (O'Connell *et al.*, 1997; Rhind *et al.*, 1997). Studies of the molecular events controlling this checkpoint response are beginning to unravel biochemical interactions between different checkpoint proteins and between checkpoint proteins and core cell cycle regulators (Walworth and Bernards, 1996; Furnari *et al.*, 1997; O'Connell *et al.*, 1997; Peng *et al.*, 1997; Rhind *et al.*, 1997; Saka *et al.*, 1997; Sanchez *et al.*, 1997; Kostrub *et al.*, 1998; Kumagai *et al.*, 1998; Chen *et al.*, 1999; Furnari *et al.*, 1999; Kumagai and Dunphy, 1999; Lopez-Girona *et al.*, 1999). Classically, DNA damage checkpoint genes have been defined by mutations that abolish the checkpoint response but have no phenotypic consequence in the absence of DNA damage. However, in some cases, a role in checkpoint control is only part of the repertoire of function of an essential gene, as is the case for cut5p (Saka and Yanagida, 1993; Saka *et al.*, 1994a; Verkade and O'Connell, 1998). Extensive genetic screens will be needed to identify specific checkpoint-defective alleles of essential genes to fully define the molecules involved in the response to DNA damage.

rad18 is an example of an essential gene that is required for checkpoint control. Although the precise essential function of rad18p is not known, its structural similarity to the SMC family of proteins, its localization to chromatin, and the phenotypes of *rad18* mutants support a role in chromatin organization. Deletion of *rad18* is lethal and is associated with mitotic failure. Analysis of a point mutation in *rad18*, *rad18-X*, has previously shown rad18p to be required for repair of UV-induced lesions (Lehmann *et al.*, 1995), and we showed here that rad18p is also essential for the repair of double-stranded DNA breaks. Furthermore, we have also shown that rad18p is required to maintain a checkpoint arrest after DNA damage, and that this failed checkpoint response leads to highly aberrant mitoses and subsequent cell death. We have described here the isolation and analysis of a checkpoint-defective allele of *rad18*, *rad18-74*. In response to either UV-C or ionizing radiation, strains carrying the *rad18-74* allele failed to maintain a checkpoint arrest. Moreover, *rad18-X* cells also showed defective checkpoint responses when held at 36°C after irradiation. Importantly, cells deleted for *rad18* had checkpoint deficiencies comparable with those of a *chk1* deleted strain.

The response to DNA damage could be considered a multistage process involving damage detection, initiation of cell cycle arrest and DNA repair pathways, maintenance of arrest during repair, and subsequent recovery and cell cycle reentry. To assess where the checkpoint defect lies in *rad18*

Table 1. Genetic interactions among *rad18*, *brc1*, *top2*, *fin1*, *rad21*, and *mis4*

	rad18-74	rad18-X	brc1Δ	fin1Δ	top2-191	rad21-45	mis4-242
Wild type	2	6	6	4	11	7	11
rad18-74	–	Allelic	Synthetic lethal	27	52	12	6
rad18-X		–	Synthetic lethal	41	52	10	12
brc1Δ			–	8	40	6	16

Numbers represent percent aberrant mitoses in double mutants. All strains not containing *top2-191* or *mis4-242* were grown to midlogarithmic phase at 30°C before fixation, DAPI staining, and observation by fluorescence microscopy. *top2-191*- and *mis4-242*-containing strains were grown to midlogarithmic phase at 25°C and then shifted to 30°C for 4 h before fixation, DAPI staining, and observation by fluorescence microscopy. Data represents the mean from five samples of 100 cells.

mutants, we assayed phosphorylation of the chk1p protein kinase as a marker of checkpoint activation (Walworth and Bernards, 1996). In time course experiments, both *rad18-74* and *rad18-X* showed wild-type kinetics of chk1p phosphorylation. This observation, together with the kinetics of mitotic entry in irradiated synchronous cultures, is consistent with a model whereby rad18p is essential in the maintenance of a checkpoint arrest, rather than its initiation. This is in contrast to the rad3p protein, which is required to initiate checkpoint arrest but not to maintain it (Martinho *et al.*, 1998). These analyses show that the DNA damage checkpoint is genetically separable into initiation and maintenance events, both of which are required for survival of DNA damage. Because chk1p phosphorylation persists in wild-type cells after they reenter the cell cycle, and *rad18* mutant cells also maintain chk1p phosphorylation despite defective checkpoint maintenance, chk1p itself may also be specific for checkpoint initiation.

In addition to its role in checkpoint maintenance, rad18p is also required for DNA repair. These defects may not be unrelated and present a model explaining the response of *rad18* mutants to DNA damage. With its similarity to the SMC family of DNA-binding and chromatin organization proteins, rad18p may be involved in establishing or maintaining chromosomal structures that are necessary for DNA repair. The presence of these structures on the chromosomes then acts as the origin of the signal to maintain checkpoint arrest. In this scenario, defective rad18p function would, as we observed, result in a failure to complete repair and to maintain arrest. These structures are presumably essential for normal chromatin organization given the essential nature of *rad18* and the mitotic defects that occur in its absence.

This phenomenon may be related to the checkpoint that monitors completion of S-phase and in particular the role of DNA polymerase α (*pol1/swi7*) in this response (D'Urso *et al.*, 1995). When pol1p is present, although not functional, S-phase progression is stalled. This results in checkpoint activation and prevents entry into mitosis. When pol1p is deleted, cells fail to recognize incomplete DNA synthesis and enter catastrophic mitoses. Thus, the presence of pol1p on the stalled replication forks appears to signal incomplete S-phase and activates the checkpoint. Similarly, checkpoint pathways may monitor the presence of proteins involved in chromatin organization, and these influence the timing of entry into mitosis and serve to alert the cell where it is positioned in the cell cycle.

The radiation sensitivity and checkpoint defects of *rad18-74* were completely suppressed by the expression of brc1p from a multicopy plasmid. This suppression was allele specific for *rad18-74* and was not a general feature of increased expression of BRCT proteins. BRCT domains show homology to the C-terminus of the breast and ovarian cancer susceptibility gene BRCA1 (itself a G2 checkpoint protein; Xu *et al.*, 1999) and are found in a variety of proteins involved in DNA repair, DNA replication, and checkpoint control (Callebaut and Mornon, 1997). In several instances they have been shown to act as protein interaction domains (Saka *et al.*, 1997; Grawunder *et al.*, 1998; Taylor *et al.*, 1998). One possible interpretation of the suppression of *rad18-74* is that rad18p and brc1p physically interact and that this interaction is defective in the *rad18-74* mutant. We attempted to coimmunoprecipitate epitope-tagged derivatives of rad18p and brc1p expressed in wild-type and irradiated cells but saw no evidence of physical association despite efficient immunoprecipitation of each protein. Furthermore, we saw no evidence for interaction by yeast two-hybrid analysis or between in vitro–transcribed and translated proteins (our unpublished results). Therefore it is likely that *brc1* acts downstream of *rad18* as a bypass suppressor of *rad18-74*, and that this suppression represents only a subset of rad18p functions. Deletion of *brc1* results in mitotic defects and chromosome instability, which may be phenotypes of the same primary defect. These phenotypes are also seen in *rad18* mutants. Furthermore, deletion of *brc1* is synthetically lethal with *rad18-X* and *rad18-74*, indicating that brc1p is required for viability when *rad18* function is compromised. These cells are not, however, hypersensitive to UV-C or ionizing radiation, and so brc1p is not essential for DNA repair or checkpoint responses, as seen for rad18p. However, in the presence of *rad18-X* or *rad18-74*, *brc1* is essential, suggesting that *brc1* plays a downstream role, which is able to compensate for subfunctional *rad18*.

With the presence of six BRCT domains, it is likely that brc1p facilitates protein–protein interactions, perhaps interacting with another BRCT domain protein. By analogy to the cohesin and condensin complexes, rad18p may be part of one or more larger complexes (Jessberger *et al.*, 1998). brc1p may function as an adaptor molecule between other proteins, as part of a complex involved in a rad18p-dependent process required for mitosis. We attempted to localize brc1p by indirect immunofluorescence but found no specific localization in cells despite detecting brc1p on Western blots. It may be possible that the epitopes are obscured in vivo. We also attempted to localize GFP-brc1p fusions but found no localization of fluorescence in cells expressing the fusion protein from the *nmt1* promoter. Multiprotein complexes
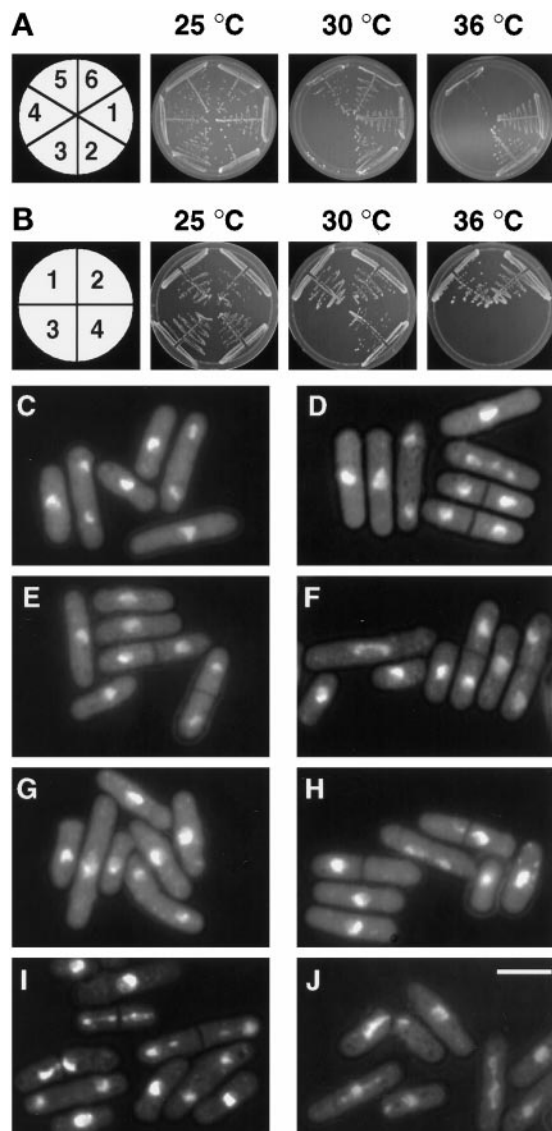
Figure 8. Interactions among *top2, rad18* and *brc1*. (A) *top2-191* is synthetically lethal with *rad18-74* and *rad18-X*. Strains were plated on YES and incubated at the indicated temperatures for either 4 d (25°C) or 3 d (30 and 36°C). Strains: (1) wild type; (2) *rad18-74*; (3) *rad18-74 top2-191*; (4) *rad18-X top2-191*; (5) *rad18-X; 6, top2-191*. (B) *top2-191* is synthetically lethal with *brc1::ura4*. Strains were plated on YES and treated as above. Strains: (1) wild type; (2) *brc1::ura4*; (3) *brc1::ura4 top2-191*; (4) *top2-191*. (C–J) DAPI and phase-contrast images of cells grown to midlogarithmic phase at 25°C and then shifted to 30°C for 4 h. Strains: (C) wild type; (D) *rad18-X;* (E) *rad18-74;* (F) *top2-191;* (G) *brc1::ura4;* (H) *top2-191 rad18-X;* (I) *top2-191 rad18-74;* (J) *top2-191 brc1::ura4.* Bar, 10 μm.

may impede the fluorescence of GFP, or the protein may be diffusely localized throughout the cell.

We investigated functional links between *brc1* and genes implicated in chromatin organization by constructing double mutants and found several combinations that lead to enhancement of phenotype, which is indicative of coopera-

tion between these proteins in mitotic fidelity. Mutations in *rad18, brc1* and *fin1* are synthetically lethal with temperature-sensitive topoisomerase II at semipermissive temperature. This is consistent with the models proposing multiple roles for topoisomerase II in chromatin organization (Heck, 1997). Although rad18p may play a role in top2p-dependent decatenation of chromatin, defects in this could not explain all the *rad18* phenotypes. *Rad18-74* and *rad18-X* mutants, unlike *top2-191*, show only minor mitotic defects in the absence of irradiation. Furthermore, these mutants are defective in maintaining a checkpoint arrest when irradiated late in G2 (*cdc25-22*-arrested cells), by which time the bulk of decatenation is likely to be completed. Thus, the defective mitoses are presumably a result of failed repair and checkpoint maintenance rather than a defect in topoisomerase II activity. Indeed, overexpression of *top2* could not rescue the radiation sensitivity or the mitotic abnormalities of *rad18-X* or *rad18-74. Brc1*-deleted cells delay entry into mitosis. A combination of G2 delay and mitotic defects is also seen in strains carrying mutations in *rad18* (Lehmann *et al.*, 1995) or *fin1* (Krien *et al.*, 1998) and also in temperature-sensitive *top2* mutants at semipermissive temperature (Krien *et al.*, 1998). Such a delay is consistent with a model in which defects in chromatin organization are monitored before mitosis and lead to activation of a checkpoint that influences the timing of mitotic onset.

It is becoming increasingly evident that regulation of higher-order chromatin structure is vital to maintenance of genomic integrity. SMC1–4 proteins are central to regulating chromosome dynamics, functioning in key components in both chromatin condensation and sister chromatid cohesion. The phenotypes and genetic interactions of the *rad18-74*, *rad18-X*, and *rad18::ura4* mutants provide a link among chromatin organization, DNA repair, and the control of mitotic entry. The conserved structure, localization, and requirement for chromosome stability suggest that rad18p carries out related functions to SMC1–4. Genetic interactions of *rad18* with *top2* and *fin1* suggest a role in chromatin condensation, but extensive analyses of rad18p are needed to determine its precise function. Defects in both DNA repair and checkpoint maintenance in the absence of rad18p function are consistent with a role for rad18p in forming essential structures that are required for DNA repair and are recognized by proteins that signal checkpoint maintenance. In recent years, extensive progress has been made in understanding checkpoint initiation, and further analysis of rad18p may uncover how this arrest is maintained to ensure completion of DNA repair before progression into mitosis.

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REFERENCES

Al-Khodairy, F., and Carr, A.M. (1992). DNA repair mutants defining G2 checkpoint pathways in *Schizosaccharomyces pombe*. EMBO J. *11*, 1343–1350.

Al-Khodairy, F., Fotou, E., Sheldrick, K.S., Griffiths, D.J.F., Lehman, A.R., and Carr, A.M. (1994). Identification and characterization of new elements involved in checkpoint and feedback controls in fission yeast. Mol. Biol. Cell *5*, 147–160.

Barbet, N., Muriel, W.J., and Carr, A.M. (1992). Versatile shuttle vectors and genomic libraries for use with *Schizosaccharomyces pombe*. Gene *114*, 59–66.

Birkenbihl, R.P., and Subramani, S. (1992). Cloning and characterization of rad21 an essential gene of *Schizosaccharomyces pombe* involved in DNA double-strand-break repair. Nucleic Acids Res. *20*, 6605–6611.

Birkenbihl, R.P., and Subramani, S. (1995). The rad21 gene product of *Schizosaccharomyces pombe* is a nuclear, cell cycle-regulated phosphoprotein. J. Biol. Chem. *270*, 7703–7711.

Boddy, M.N., Furnari, B., Mondesert, O., and Russell, P. (1998). Replication checkpoint enforced by kinases Cds1 and Chk1. Science *280*, 909–912.

Callebaut, I., and Mornon, J.P. (1997). From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair. FEBS Lett. *400*, 25–30.

Carr, A.M., Schmidt, H., Kirchhoff, S., Muriel, W.J., Sheldrick, K.S., Griffiths, D.J., Basmacioglu, C.N., Subramani, S., Clegg, M., and Nasim, A. (1994). The rad16 gene of *Schizosaccharomyces pombe*: a homolog of the RAD1 gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. *14*, 2029–2040.

Carr, A.M., Sheldrick, K.S., Murray, J.M., al-Harithy, R., Watts, F.Z., and Lehmann, A.R. (1993). Evolutionary conservation of excision repair in *Schizosaccharomyces pombe*: evidence for a family of sequences related to the *Saccharomyces cerevisiae* RAD2 gene. Nucleic Acids Res. *21*, 1345–1349.

Chen, L., Liu, T.H., and Walworth, N.C. (1999). Association of Chk1 with 14-3-3 proteins is stimulated by DNA damage. Genes & Dev. *13*, 675–685.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M., and Nasmyth, K. (1998). An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. Cell *93*, 1067–1076.

D'Urso, G., Grallert, B., and Nurse, P. (1995). DNA polymerase alpha, a component of the replication initiation complex, is essential for the checkpoint coupling S phase to mitosis in fission yeast. J. Cell Sci. *108*, 3109–3018.

Downes, C.S., Mullinger, A.M., and Johnson, R.T. (1991). Inhibitors of DNA topoisomerase II prevent chromatid separation in mammalian cells but do not prevent exit from mitosis. Proc. Natl. Acad. Sci. USA *88*, 8895–8899.

Enoch, T., Carr, A., and Nurse, P. (1992). Fission yeast genes involved in coupling mitosis to completion of DNA replication. Genes & Dev. *6*, 2035–2046.

Furnari, B., Blasina, A., Boddy, M.N., McGowan, C.H., and Russell, P. (1999). Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1. Mol. Biol. Cell *10*, 833–845.

Furnari, B., Rhind, N., and Russell, P. (1997). Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase. Science *277*, 1495–1497.

Furuya, K., Takahashi, K., and Yanagida, M. (1998). Faithful anaphase is ensured by Mis4, a sister chromatid cohesion molecule required in S phase and not destroyed in G1 phase. Genes & Dev. *12*, 3408–3018.

Grawunder, U., Zimmer, D., and Leiber, M.R. (1998). DNA ligase IV binds to XRCC4 via a motif located between rather than within its BRCT domains. Curr. Biol. *8*, 873–876.

Guacci, V., Koshland, D., and Strunnikov, A. (1997). A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD in *S. cerevisiae*. Cell *91*, 47–57.

Hartwell, L., and Weinert, T. (1989). Checkpoints: controls that ensure the order of cell cycle events. Science *246*, 629–634.

Hartwell, L.H., Szankasi, P., Roberts, C.J., Murray, A.W., and Friend, S.H. (1997). Integrating genetic approaches into the discovery of anticancer drugs. Science *278*, 1064–1068.

Heck, M.M.S. (1997). Condensins, cohesins and chromosome architecture: how to make and brake a mitotic chromosome. Cell *91*, 5–8.

Hirano, T., Kobayashi, R., and Hirano, M. (1997). Condensins, chromosome condensation protein complexes containing XCAP-C, XCAP-E and a *Xenopus* homolog of the *Drosophila* Barren protein. Cell *89*, 511–521.

Hirano, T., and Mitchison, T.J. (1994). A heterodimeric coiled-coil protein required for mitotic chromosome condensation in vitro. Cell *79*, 449–458.

Holm, C., Goto, T., Wang, J., and Bostein, D. (1985). DNA topoisomerase II is required at the time of mitosis in yeast. Cell *41*, 553–563.

Holm, C., Stearns, T., and Botstein, D. (1989). DNA topoisomerase II must act at mitosis to prevent nondisjunction and chromosome breakage. Mol. Cell. Biol. *9*, 159–168.

Jessberger, R., Frei, C., and Gasser, S.M. (1998). Chromosome dynamics: the SMC protein family. Curr. Opin. Genet. Dev. *8*, 254–259.

Kimura, K., and Hirano, T. (1997). ATP-dependent positive supercoiling of DNA by 13S condensin: a biochemical implication for chromosome condensation. Cell *90*, 625–634.

Koonin, E., Altschul, S., and Bork, P. (1996). Functional motifs. Nat. Genet. *13*, 266–267.

Kostrub, C.F., Knudsen, K., Subramani, S., and Enoch, T. (1998). Hus1p, a conserved fission yeast checkpoint protein, interacts with Rad1p and is phosphorylated in response to DNA damage. EMBO J. *17*, 2055–2066.

Krien, M.J.E., Bugg, S.J., Palatsides, M., Ausoline, G., Morimyo, M., and O'Connell, M.J. (1998). A NIMA homologue promotes chromatin condensation in fission yeast. J. Cell Sci. *111*, 967–976.

Kumagai, A., and Dunphy, W.G. (1999). Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer cdc25. Genes & Dev. *13*, 1067–1072.

Kumagai, A., Yakowec, P.S., and Dunphy, W.G. (1998). 14-3-3 proteins act as negative regulators of the mitotic inducer Cdc25 in *Xenopus* egg extracts. Mol. Biol. Cell *9*, 345–354.

Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. *154*, 367–382.

Lehmann, A.R., Walicka, M., Grittiths, D.J.F., Murray, J.M., Watts, F.Z., McCready, S., and Carr, A.M. (1995). The rad18 gene of *Schizosaccharomyces pombe* defines a new subgroup of the SMC superfamily involved in DNA repair. Mol. Cell. Biol. *15*, 7067–7080.

Lindsay, H.D., Griffiths, D.J.F., Edwards, R.J., Christensen, P.U., Murray, J.M., Osman, F., Walworth, N., and Carr, A.M. (1998). S-phase-specific activation of Cds1 kinase defines a subpathway of the checkpoint response in *Schizosaccharomyces pombe*. Genes & Dev. *12*, 382–395.

Lopez-Girona, A., Furnari, B., Mondesert, O., and Russell, P. (1999). Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. Nature *397*, 172–175.

Losada, A., Hirano, M., and Hirano, T. (1998). Identification of *Xenopus* SMC protein complexes required for sister chromatid cohesion. Genes & Dev. *12*, 1986–1997.

Martinho, R.G., Lindsay, H., Flaggs, G., DeMaggio, A.J., Hoekstra, M.F., Carr, A.M., and Bently, N.J. (1998). Analysis of rad3 and chk1 protein kinases defines different checkpoint responses. EMBO J. *17*, 7239–7249.

Michaelis, C., Ciosk, R., and Nasmyth, K. (1997). Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell *91*, 35–45.

Mitchison, J.M., and Carter, B.L. (1975). Cell cycle analysis. Methods Cell Biol. *11*, 201–219.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. *194*, 795–823.

Muris, D.F.R., Vreeken, K., Carr, A.M., Murray, J.M., Smit, C., Lohman, P.H.M., and Pastink, A. (1996). Isolation of the *Schizosaccharomyces pombe* RAD54 homologue, rhp54, a gene involved in the repair of radiation damage and replication fidelity. J. Cell Sci. *109*, 73–81.

Murray, A. (1995). The genetics of cell cycle checkpoints. Curr. Opin. Genet. Dev. *5*, 5–11.

Murray, J.M., Doe, C.L., Schenk, P., Carr, A.M., Lehmann, A.R., and Watts, F.Z. (1992). Cloning and characterization of the *S. pombe* rad15 gene, a homologue to the *S. cerevisiae* RAD3 and human ERCC2 genes. Nucleic Acids Res. *20*, 2673–2678.

Murray, J.M., Tavassoli, M., Al-Harithy, R., Sheldrick, K.S., Lehmann, A.R., Carr, A.M., and Watts, F.Z. (1994). Structural and functional conservation of the human homolog of the *Schizosaccharomyces pombe* rad2 gene, which is required for chromosome segregation and recovery from DNA damage. Mol. Cell. Biol. *14*, 4878–4888.

Niwa, O., Matsumoto, T., Chikashige, Y., and Yanagida, M. (1989). Characterization of *Schizosaccharomyces pombe* minichromosome deletion derivatives and a functional allocation of their centromere. EMBO J. *8*, 3045–3052.

Nurse, P. (1975). Genetic control of cell size at cell division in yeast. Nature *256*, 547–55l.

Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. Nature *344*, 503–508.

Nurse, P. (1994). Ordering S phase and M phase in the cell cycle. Cell *79*, 547–550.

O'Connell, M.J., Norbury, C., and Nurse, P. (1994). Premature chromatin condensation upon accumulation of NIMA. EMBO J. *13*, 4926–4937.

O'Connell, M.J., Raleigh, J.M., Verkade, H.D., and Nurse, P. (1997). Chk1 is a wee1 kinase in the G2 DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation. EMBO J. *16*, 545–554.

Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S., and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science *277*, 1501–1505.

Rhind, N., Furnari, B., and Russell, P. (1997). Cdc2 tyrosine phosphorylation is required for the DNA damage checkpoint in fission yeast. Genes & Dev. *11*, 504–511.

Saitoh, N., Goldberg, I., and Earnshaw, W.C. (1995). The SMC proteins and the coming of age of the chromosome scaffold hypothesis. Bioessays *17*, 759–766.

Saka, Y., Esashi, F., Matsusaka, T., Mochida, S., and Yanagida, M. (1997). Damage and replication checkpoint control in fission yeast is ensured by interactions of Crb2, a protein with BRCT motif, with Cut5 and Chk1. Genes & Dev. *11*, 3387–3400.

Saka, Y., Fantes, P., Sutani, T., McInerny, C., Creanor, J., and Yanagida, M. (1994a). Fission yeast cut5 links nuclear chromatin and M phase regulation in the replication checkpoint control. EMBO J. *13*, 5319–5329.

Saka, Y., Sutani, T., Yamashita, Y., Saitoh, S., Takeuchi, M., Nakaseko, Y., and Yanagida, M. (1994b). Fission yeast cut3 and cut14, members of a ubiquitous protein family, are required for chromosome condensation and segregation in mitosis. EMBO J. *13*, 4938–4952.

Saka, Y., and Yanagida, M. (1993). Fission yeast *cut5*$^+$, required for S phase onset and M phase restraint, is identical to the radiation-damage repair gene rad4$^+$. Cell *74*, 383–393.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). Molecular Cloning: a Laboratory Manual, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanchez, Y., Wong, C., Thoma, R.S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S.J. (1997). Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science *277*, 1497–1501.

Strunnikov, A.V., Hogan, E., and Koshland, D. (1995). SMC2, a *Saccharomyces cerevisiae* gene essential for chromosome segregation and condensation, defines a subgroup within the SMC family. Genes & Dev. *9*, 587–599.

Strunnikov, A.V., Larionov, V.L., and Koshland, D. (1993). SMC1: an essential yeast gene encoding a putative head-rod-tail protein is required for nuclear division and defines a new ubiquitous protein family. J. Cell Biol. *123*, 1635–1648.

Tatebayashi, K., Kato, J., and Ikeda, H. (1998). Isolation of a *Schizosaccharomyces pombe* rad21ts mutant that is aberrant in chromosome segregation, microtubule function, DNA repair and sensitive to hydroxyurea: possible involvement of Rad21 in ubiquitin-mediated proteolysis. Genetics *148*, 49–57.

Taylor, R.M., Wickstead, B., Cronin, S., and Caldecott, K.W. (1998). Role of a BRCT domain in the interaction of DNA ligase III-alpha with the DNA repair protein XRCC1. Curr. Biol. *8*, 877–880.

Toth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleiffer, A., and Nasmyth, K. (1999). Yeast Cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. Genes & Dev. *13*, 320–333.

Uemura, T., Ohkura, H., Adachi, Y., Morino, K., Shiozaki, K., and Yanagida, M. (1987). DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in *S. pombe*. Cell *50*, 917–925.

Uemura, T., and Yanagida, M. (1986). Mitotic spindle pulls but fails to separate chromosomes in type II DNA topoisomerase mutants: uncoordinated mitosis. EMBO J. *5*, 1003–1010.

Uhlmann, F., and Nasmyth, K. (1998). Cohesion between sister chromatids must be established during DNA replication. Curr. Biol. *8*, 1095–1101.

Verkade, H.M., and O'Connell, M.J. (1998). Cut5 is a component of the UV responsive DNA damage checkpoint in fission yeast. Mol. Gen. Genet. *260*, 426–433.

Walworth, N., Davey, S., and Beach, D. (1993). Fission yeast *chk1* protein kinase links the rad checkpoint pathway to. cdc2. Nature *363*, 368–371.

Walworth, N.C., and Bernards, R. (1996). rad-dependent response of the *chk1*-encoded protein kinase at the DNA damage checkpoint. Science *271*, 353–356.

Willson, J., Wilson, S., Warr, N., and Watts, F.Z. (1997). Isolation and characterization of the *Schizosaccharomyces pombe* rhp9 gene: a gene required for the DNA damage checkpoint but no the replication checkpoint. Nucleic Acids Res. *25*, 2138–2145.

Xu, X., Weaver, Z., Linke, S.P., Li, C., Gotay, J., Wang, X.W., Harris, C.C., Ried, T., and Deng, C.X. (1999). Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells. Mol. Cell *3*, 389–395.

Zeng, Y., Forbes, K.C., Wu, Z., Moreno, S., Piwnica-Worms, H., and Enoch, T. (1998). Replication checkpoint requires phosphorylation of the phosphatase cdc25 by cds1 or chk1. Nature *395*, 507–510.