

***RAD18* and *RAD54* cooperatively contribute to maintenance of genomic stability in vertebrate cells**

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Translesion DNA synthesis (TLS) and homologous DNA recombination (HR) are two major pathways that account for survival after post-replicative DNA damage. TLS functions by filling gaps on a daughter strand that remain after DNA replication caused by damage on the mother strand, while HR can repair gaps and breaks using the intact sister chromatid as a template. The *RAD18* gene, which is conserved from lower eukaryotes to vertebrates, is essential for TLS in *Saccharomyces cerevisiae*. To investigate the role of *RAD18*, we disrupted *RAD18* by gene targeting in the chicken B-lymphocyte line DT40. *RAD18*^{-/-} cells are sensitive to various DNA-damaging agents including ultraviolet light and the cross-linking agent cisplatin, consistent with its role in TLS. Interestingly, elevated sister chromatid exchange, which reflects HR-mediated post-replicative repair, was observed in *RAD18*^{-/-} cells during the cell cycle. Strikingly, double mutants of *RAD18* and *RAD54*, a gene involved in HR, are synthetic lethal, although the single mutant in either gene can proliferate with nearly normal kinetics. These data suggest that *RAD18* plays an essential role in maintaining chromosomal DNA in cooperation with the *RAD54*-dependent DNA repair pathway.

Keywords: genome instability/homologous DNA recombination/post-replication repair/*RAD18*/translesion DNA synthesis

Introduction

Various types of DNA lesions are generated continuously not only by environmental factors but also by naturally occurring damage during DNA synthesis. Estimates of the number of various types of DNA damage produced daily

per human genome range from a few to several thousand (Lindahl, 1993; Kunkel *et al.*, 1999). The majority of spontaneous and induced DNA damage appears to be repaired efficiently by excision repair pathways. However, some types of DNA damage that are known to arrest a DNA replication fork could cause a daughter strand gap, if excision repair does not eliminate such DNA lesions on mother strands before they encounter replication forks (Wyatt *et al.*, 1999). This daughter strand gap may lead to a double-strand break (DSB) in the sister chromatid (Haber, 1999; Flores-Rozas and Kolodner, 2000). If a DSB is left unrepaired, a DNA damage checkpoint may be activated, leading to programmed cell death (reviewed in van Gent *et al.*, 2001). Thus, DNA replication across an existing lesion can result in more severe damage, including gaps and chromatid breaks. Cells have evolved methods of post-replication DNA repair (PRR) to remove such secondary DNA lesions. It is believed that PRR is carried out mainly by two pathways: translesion DNA synthesis (TLS) and homologous recombination (HR) repair using the intact sister chromatid (reviewed in Friedberg *et al.*, 1995). While the phenotypes of mutants in HR pathway genes in vertebrate cells are relatively well characterized (Sonoda *et al.*, 2001), the role of TLS in maintenance of chromosomal DNA is not clear because of the absence of genetic studies in vertebrate cells. For the same reason, the functional relationship between TLS and HR in vertebrate cells needs to be elucidated.

Genetic studies of *Saccharomyces cerevisiae* revealed that all known components of PRR, including *RAD6* (*UBC2*), *RAD18*, *REV1*, *REV3* and *REV7*, belong to the *RAD6/RAD18* epistasis group ((McDonald *et al.*, 1997; reviewed in Lawrence, 1994; Broomfield *et al.*, 2001). Consistent with a function in TLS, yeast *rad6* and *rad18* mutants are extremely sensitive to killing by DNA-damaging agents, including ultraviolet (UV) light, ionizing radiation (IR), DNA alkylating agents and DNA cross-linking agents such as mitomycin C and cisplatin (Lawrence and Christensen, 1976; Prakash, 1981; Fabre *et al.*, 1989). It is known that Rad6 is a ubiquitin-conjugating enzyme (E2), forming a tight complex with the Rad18 protein. Rad18 has DNA-binding activity, and may recruit Rad6 protein to DNA lesions (Bailly *et al.*, 1994). Rad18 contains a RING-finger motif, which has been shown to be common to E3 ubiquitin ligases. Thus, *RAD18* may be involved in the transfer of a ubiquitin molecule from the Rad6 E2 enzyme onto its target proteins. It is unclear which target molecules are ubiquitylated in the *RAD6/RAD18*-dependent DNA repair pathway. Likewise, the mechanism by which the *RAD6/RAD18* ubiquitin machinery regulates translesion DNA polymerases has still to be elucidated. In mammals, there are two homologs of the *RAD6* gene, *HR6A* and *HR6B*, and a single *RAD18* gene (Koken *et al.*, 1991; Roest *et al.*,

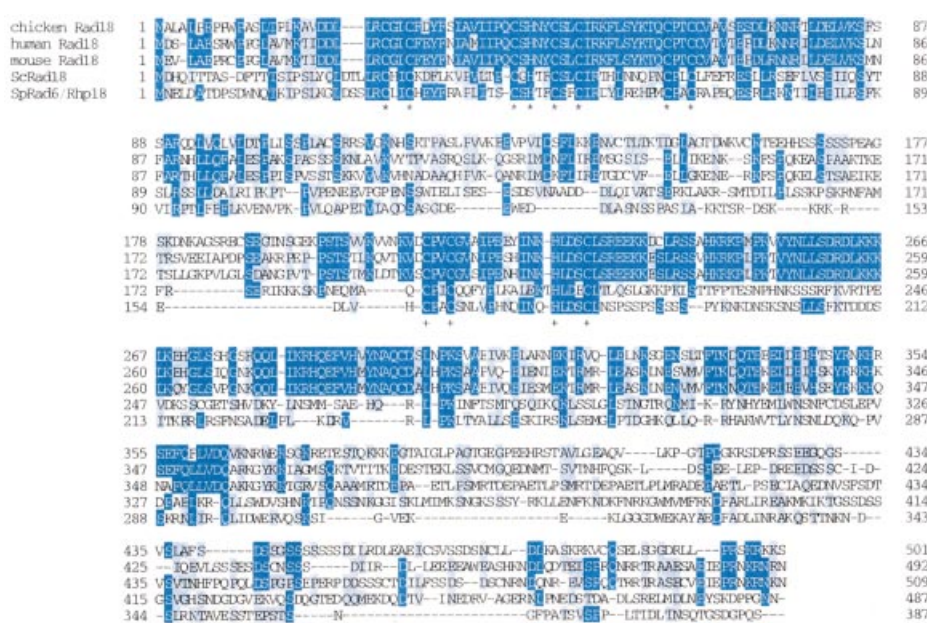


Fig. 1. Comparison of amino acid sequences of the Rad18 proteins. Amino acid sequence comparison between chicken, human, mouse, budding yeast (Sc) and fission yeast (Sp) Rad18 proteins. RING-finger motifs are marked with asterisks (*). The B-box is marked with +. Highlighted letters represent identical amino acids among more than three species. Shaded letters represent similar (P, A, G, S and T; E, D, N and Q; V, I, L and M; F, W and Y; R, K and H) amino acids conserved among more than three species. Numbers denote amino acid positions.

1996; Baarends *et al.*, 1999; van der Laan *et al.*, 2000). Human and mouse Rad18 proteins can interact with both HR6A and HR6B proteins (Tateishi *et al.*, 2000; Xin *et al.*, 2000), implying that mammalian *RAD6* and *RAD18* could work cooperatively, as in yeast. Mice deficient in the *HR6B* gene showed defective spermatogenesis but no obvious defects in DNA repair, probably because of the redundant function of *HR6B* and *HR6A* (Roest *et al.*, 1996). However, the function of *RAD18* in vertebrates remains to be elucidated due to the lack of genetic studies.

In order to investigate the role of *RAD18* in vertebrate cells, we generated *RAD18*-deficient cells from the chicken B-lymphocyte line DT40 (Buerstedde and Takeda, 1991). Here we show that deletion of *RAD18* leads to UV sensitization, consistent with a role in TLS. We also found that *RAD18*^{-/-} *RAD54*^{-/-} double mutant cells are unable to proliferate, suggesting that these two genes cooperatively play an essential role for cell survival. The functional relationship between *RAD18* and the *RAD54*-dependent HR pathway is discussed.

Results

Isolation of the chicken *RAD18* gene and generation of *RAD18*-deficient cells

We isolated the chicken *RAD18* cDNA, which encodes a protein of 501 amino acids with a predicted mol. wt of 55 kDa (Figure 1). The chicken Rad18 protein shows ~50% identity to the human and mouse Rad18 proteins, and ~25% identity to the yeast Rad18 proteins (*S.cerevisiae* Rad18 and *Schizosaccharomyces pombe* Rhp18/Rad6). Sequence comparison between the homologs shows that Rad18 protein is highly divergent. Even mouse and human homologs show only ~65% identity overall. However, sequence conservation was significant

within the RING-finger domain located at the N-terminus, with ~70% identity to human and mouse Rad18. All the cysteine and histidine residues that constitute the RING-finger motifs (C-C-C-H-C-C-C) were perfectly conserved at precise intervals from yeast to human (Figure 1, asterisks), suggesting an important role of the RING-finger for *RAD18* function.

Genomic DNA fragments around the *RAD18* coding region (Figure 2A) were amplified by PCR from DT40 cells. Gene targeting constructs were generated from the amplified genomic DNA. Gene targeting events are expected to insert a premature stop codon immediately after amino acid 162 and to delete the genomic sequences that encode amino acids 163–182 of the chicken Rad18 protein. Gene targeting of the *RAD18* locus was confirmed by Southern blot analysis of *Bam*HI-digested genomic DNA for the appearance of a 2.9 kb band as well as the disappearance of a >20 kb band, which corresponds to the intact allele (Figure 2B). Because all five *RAD18*^{-/-} DT40 clones obtained showed essentially the same phenotype, we only show data from two representative clones hereafter. The proliferative properties of *RAD18*^{-/-} cells were monitored using growth curves (Figure 2C), showing that *RAD18*^{-/-} cells are able to proliferate with normal kinetics. We conclude that *RAD18* is not essential for cell viability.

***RAD18*^{-/-} cells are highly sensitive to UV and cisplatin**

To analyze the DNA repair capacity of *RAD18*^{-/-} cells, we examined the viability of wild-type and *RAD18*^{-/-} cells after genotoxic treatment by colony survival assays. *RAD18*^{-/-} cells were hypersensitive to cisplatin, moderately sensitive to UV and mildly sensitive to IR, when compared with wild-type DT40 cells (Figure 3). Elevated

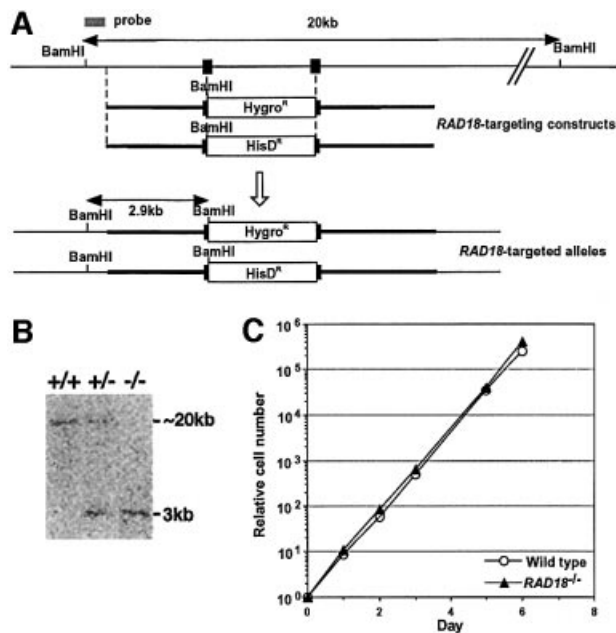


Fig. 2. Gene targeting of the *RAD18* locus. (A) Schematic representation of part of the *RAD18* locus, the gene disruption constructs and the configuration of the targeted alleles. Solid boxes indicate the positions of the exons. Only disrupted exons are indicated. (B) Southern blot analysis of *Bam*HI-digested genomic DNA from cells with the indicated genotypes of the *RAD18* gene, using the probe shown in (A). The positions and sizes of hybridizing fragments of the wild-type and targeted loci are indicated. (C) Growth curves of cells of the indicated genotypes. Similar results were obtained in at least three independent experiments.

sensitivity to these DNA-damaging agents is reminiscent of the phenotype of yeast mutants of the *RAD6/RAD18* epistasis group genes (McKee and Lawrence, 1980; Keszenman *et al.*, 1992). To investigate the cause of cell death following exposure to DNA-damaging agents, we measured cytologically detectable chromosomal breaks, which are believed to reflect DSBs, as previously described (Sonoda *et al.*, 1998). After the treatment with each DNA-damaging agent, *RAD18*^{-/-} cells showed ~2- to 4-fold more chromosome aberrations compared with wild-type cells (data not shown). We assume that the elevated chromosome aberrations account for the sensitivity of *RAD18*^{-/-} cells to various types of DNA-damaging agents.

***RAD18*^{-/-} cells are defective in post-replication DNA repair of UV damage**

To address whether or not *RAD18* is involved in PRR as in yeasts, we examined the cell cycle-specific sensitivity of *RAD18*^{-/-} cells to UV damage. Cells were synchronized at G₁ phase by an elutriation method (Takata *et al.*, 1998), and released into fresh media. At various times after the release, the UV sensitivity of wild-type and *RAD18*^{-/-} cells was measured by colony survival assay. Consistent with the idea that *RAD18* is involved in PRR, the sensitivity of *RAD18*^{-/-} cells to UV damage was most striking after the onset of S phase (Figure 4A).

To assess directly the ability of *RAD18*^{-/-} cells to carry out PRR, we measured the size of newly replicated DNA following UV irradiation (Prakash, 1981). In the absence of UV irradiation (Figure 4B, top panels), the size of

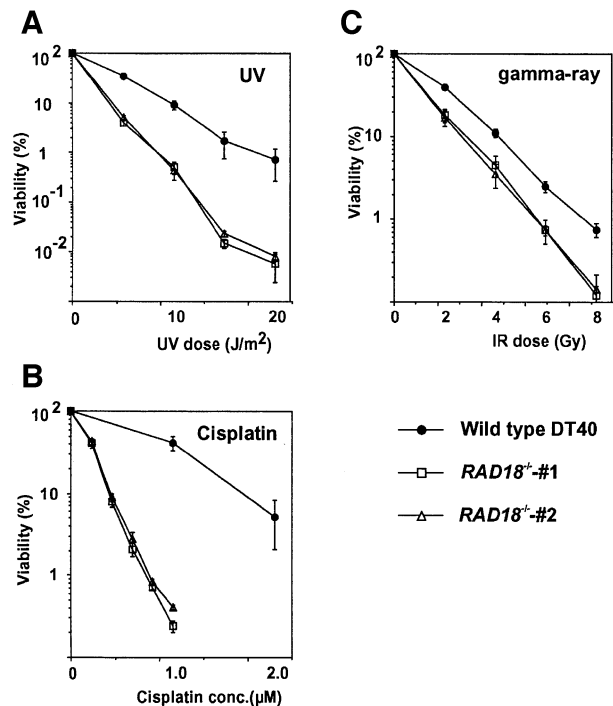


Fig. 3. Sensitivity of clones of the wild-type and *RAD18*^{-/-} cells to DNA-damaging agents. The fractions of surviving colonies after the indicated treatment of cells compared with untreated controls of the same genotype are shown on the y-axis on a logarithmic scale. (A) UV; (B) cisplatin (CDDP; continuous exposure); (C) γ rays. The dose of ¹³⁷Cs γ and UV, and concentrations of cisplatin are displayed on the x-axis on a linear scale in each graph. The data shown are means \pm SE of at least three separate experiments.

labeled DNA in *RAD18*^{-/-} cells was increased to the same extent as that of wild-type cells at 30 min after pulse labeling (open triangles) when compared with 0 min (open circles), suggesting that both types of cells underwent constant DNA synthesis during the chase period. After UV irradiation, wild-type cells showed a constant increase in DNA size from 30 min (open triangles) to 90 min (closed squares) after pulse labeling (bottom left panel). In contrast, UV-irradiated *RAD18*^{-/-} cells showed no significant increase in DNA size especially from 30 to 90 min after pulse labeling (bottom right panel), suggesting that *RAD18*^{-/-} cells were not able to continue DNA synthesis after UV irradiation. This result is reminiscent of that seen in XP-V cells, and indicates that depletion of *RAD18* results in an accumulation of DNA gaps. Taken together, we conclude that *RAD18* is involved in PRR in vertebrate cells as well as in yeast.

Sister chromatid exchange is enhanced in *RAD18*^{-/-} cells

Since TLS and HR constitute post-replication repair pathways in yeast (Broomfield *et al.*, 2001), we wished to know which pathway is mainly affected in *RAD18*^{-/-}. To evaluate HR-mediated repair events, we determined the level of microscopically visible sister chromatid exchange (SCE) events (Sonoda *et al.*, 1999; Dronkert *et al.*, 2000). We first estimated the contribution of bromodeoxyuridine (BrdU), which is used for SCE staining (see Materials and methods), to the induction of SCE. We measured SCE

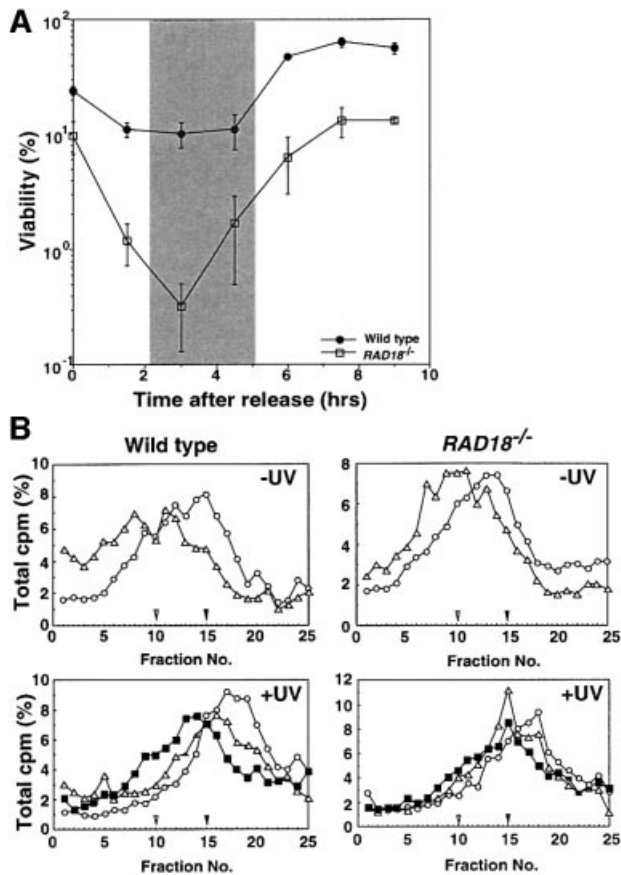


Fig. 4. Defective PRR following UV irradiation in the absence of *RAD18*. (A) Cell cycle-specific sensitivity of *RAD18*^{-/-} cells. Wild-type and *RAD18*^{-/-} cells were synchronized at G₁ phase by elutriation. Cell cycle phase was determined by the incorporation of BrdU followed by fluorescence-activated cell sorting analysis (data not shown) and is shown as a shaded box. At the time points indicated after release, cells were irradiated with UV at 2.5 J/m² and cell survival was measured. *RAD18*^{-/-} cells are most sensitive to UV irradiation after the onset of DNA replication. (B) Impaired post-replication repair of *RAD18*-deficient DT40 cells. Top panels: wild-type cells or *RAD18*-deficient cells were pulse labeled with [³H]thymidine (0.93 MBq/ml) for 15 min (open circles). In a pulse-chase experiment, the pulse-labeled cells were incubated further for 30 min (open triangles) in fresh medium containing 10 μM unlabeled thymidine and uridine. Samples were sedimented on 5–20% alkaline sucrose gradients from right to left. Bottom panels: wild-type and *RAD18*-deficient DT40 cells were irradiated with UV light (8 J/m²), incubated for 10 min, and then pulse-labeled with [³H]thymidine (0.93 MBq/ml) for 15 min (open circles). In a pulse-chase experiment, the pulse-labeled cells were incubated further for 30 min (open triangles) or 90 min (closed squares) in the chase medium. Left: wild-type cells. Right: *RAD18*-deficient cells. Closed and open arrowheads indicate the positions of bacteriophage λ DNA (42 kb) and T4GT7 DNA (165.6 kb), respectively.

levels with various concentrations of BrdU and assumed that the extrapolation of SCE events to the y-axis represents the spontaneous SCE level without BrdU (Figure 5A). This estimation indicates that the few SCEs observed in normally cycling wild-type DT40 cells are not solely induced by BrdU, but occur mostly spontaneously, as observed in human cells (Galloway and Evans, 1975; Crossen *et al.*, 1977). Remarkably, *RAD18*^{-/-} cells showed an ~3-fold higher spontaneous SCE level than did wild-type cells (Figure 5B and C), indicating that a defect in *RAD18* leads to enhancement of HR-mediated repair events even in the absence of genotoxic treatments.

We next measured the level of SCE following exposure of cells to 4-nitroquinoline 1-oxide (4NQO), which damages base residues in a manner similar to UV irradiation. The level of induced SCE was 6.3 ± 2.2 SCEs/cell for wild-type cells and 17 ± 5.3 SCEs/cell for *RAD18*^{-/-} cells (Figure 5B and C). Thus, a defect in *RAD18* indeed elevated HR-mediated repair events.

We interpreted the increased level of SCE in *RAD18*^{-/-} cells as preferential usage of the HR pathway due to the lack of the TLS pathway. However, it is also possible that the HR pathway is hyperactivated in *RAD18*^{-/-} cells. To address this question, we evaluated the capability of the HR pathway by examining the frequency of targeted integration at the ovalbumin locus (Buerstedde and Takeda, 1991). The frequency of targeted integration was reduced in *RAD18*^{-/-} cells compared with wild-type cells (Table I), indicating that hyperactivation of the HR pathway is not likely. Thus, we conclude that the elevated SCE in *RAD18*^{-/-} cells may be caused by more frequent usage of HR-mediated repair in the mutant cells than in wild-type cells.

Double mutants of *Rad18* and *Rad54* are unable to proliferate

To investigate further the functional relationship between the *RAD18*-dependent PRR pathway and HR, we generated cells deficient in both *RAD18* and *RAD54*. We previously showed that *RAD54*-deficient DT40 cells exhibit hypersensitivity to γ rays and marked reduction in gene targeting efficiencies, but are able to proliferate with nearly normal kinetics (Bezzubova *et al.*, 1997; Takata *et al.*, 1998). We have failed to generate cells deficient in both *RAD18* and *RAD54*. Thus, we generated conditional *RAD18*^{-/-}*RAD54*^{-/-} double mutant cells using the tamoxifen-inducible Cre-loxP system (Zhang *et al.*, 1998; Fujimori *et al.*, 2001) as summarized in Figure 6A. Upon the addition of tamoxifen to the culture media, the *RAD54* transgene (designated as *loxP-RAD54* hereafter) is eliminated by Cre-recombinase, generating mutant cells that completely lost functional *RAD18* and *RAD54* genes. As we reported previously, the Cre-mediated recombination worked efficiently in DT40 cells; *loxP-RAD54* was eliminated in virtually all the cells within 3 days after the addition of tamoxifen, as verified by western blot analysis of Rad54 as well as the loss of transgene marker [green fluorescent protein (GFP) fluorescence; data not shown]. Since the tamoxifen-inducible Cre-loxP system is known to have genotoxic effects to some extent (Loonstra *et al.*, 2001; Silver and Livingston, 2001), we generated *RAD18*^{-/-} and *RAD54*^{-/-} clones that carry the Cre-recombinase (hereafter called *RAD18*^{-/-} w/*CRE* and *RAD54*^{-/-} w/*CRE* cells) to normalize the toxic effect of the Cre-loxP system in the experiments.

We analyzed the proliferative properties of *RAD18*^{-/-} w/*CRE* and *RAD18*^{-/-} *RAD54*^{-/-} (w/*loxP-RAD54*, *CRE*) cells in the absence and presence of tamoxifen by measuring their growth rate (Figure 6B) as well as by colony formation assay (data not shown). Although continuous exposure of *RAD18*^{-/-} cells to tamoxifen reduced their proliferation rates to some extent (Figure 6B), *RAD18*^{-/-} cells could proliferate exponentially. Expression of the Cre-recombinase did not affect the viability of *RAD18*^{-/-} and *RAD54*^{-/-} cells irrespective of

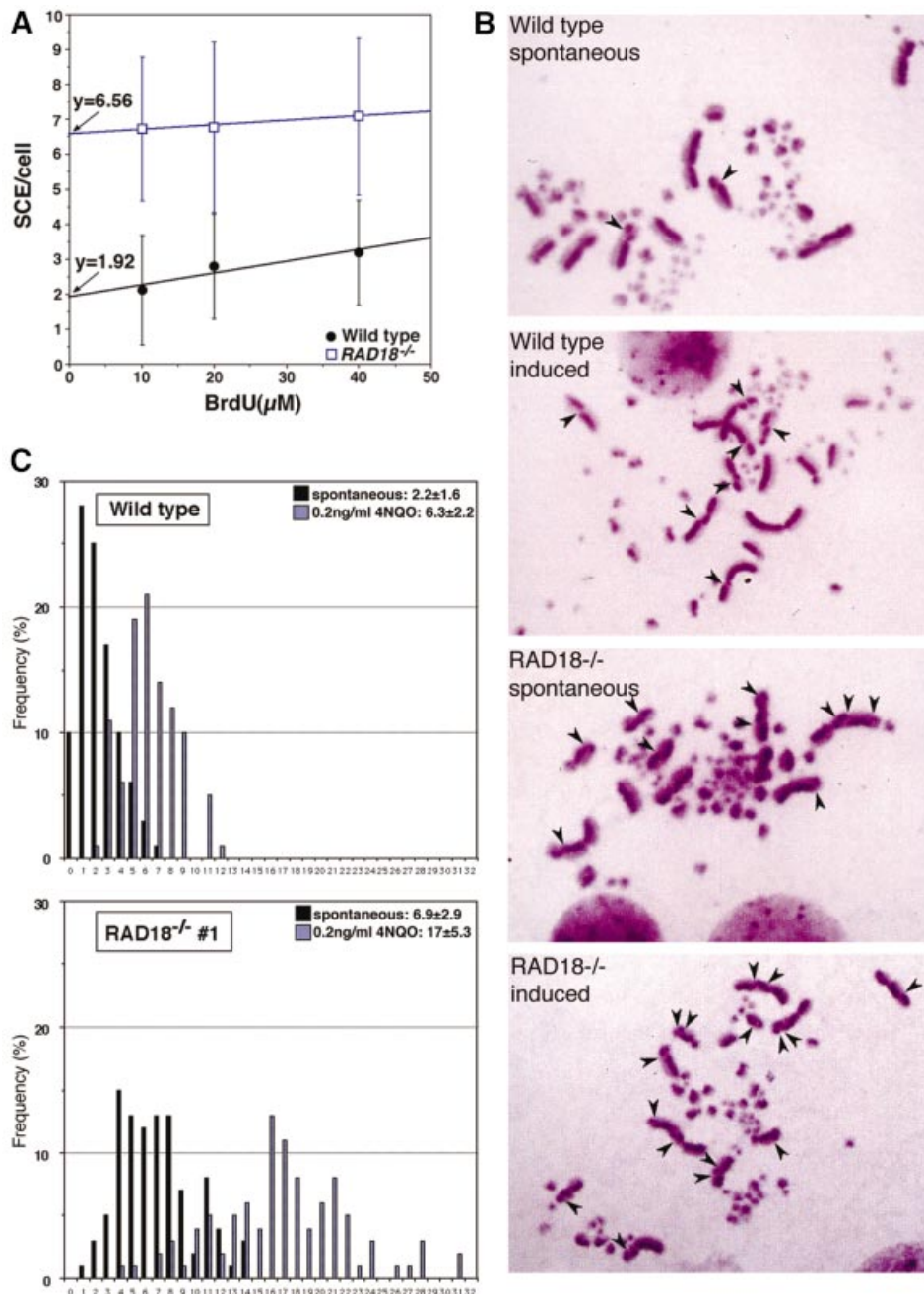


Fig. 5. Sister chromatid exchange is elevated in *RAD18*^{-/-} cells. (A) Wild-type DT40 and *RAD18*^{-/-} cells were cultured with various concentrations of BrdU for two rounds of the cell cycle (16 h), and SCE levels were measured. The value of extrapolation to the y-axis is shown. (B) SCEs of wild-type DT40 and *RAD18*^{-/-} cells. Sister chromatid stain of wild-type and *RAD18*^{-/-} cells treated or not with 4NQO is shown as indicated. Arrowheads indicate the sites of SCE. (C) Level of SCE per cell in wild-type DT40 (top panel) and *RAD18*^{-/-} (bottom panel). Mean ± SE is shown at the top of each panel. Solid box, distribution of SCEs/cell without 4NQO treatment; shaded box, distribution of SCEs/cell with 0.2 ng/ml 4NQO treatment.

the absence or presence of tamoxifen (data not shown). In marked contrast to these populations, the *RAD18*^{-/-} *RAD54*^{-/-} (*w/loxP-RAD54, CRE*) cells exhibited a dramatic reduction in growth rate in the presence of tamoxifen and eventually ceased proliferation (Figure 6B), suggesting that *RAD18*^{-/-} *RAD54*^{-/-} cells are lethal. However, since tamoxifen is known to have genotoxic effects and indeed *RAD18*^{-/-} cells show a slight reduction of growth rate in the presence of tamoxifen, it is possible that *RAD18*^{-/-} *RAD54*^{-/-} cells are viable but extremely sensitive to tamoxifen. To exclude this

Table I. Targeted integration frequencies in *RAD18*^{-/-} cells

| Genotype analyzed | Targeted integration frequency at the ovalbumin locus |
|-----------------------------------|---|
| Wild type | 44/50 (88%) |
| <i>RAD18</i> ^{-/-} no. 1 | 22/54 (41%) |
| <i>RAD18</i> ^{-/-} no. 2 | 17/57 (30%) |

Wild-type and *RAD18*^{-/-} cells were transfected with the targeting construct of the ovalbumin locus. Two independent clones of *RAD18*^{-/-} cells were examined.

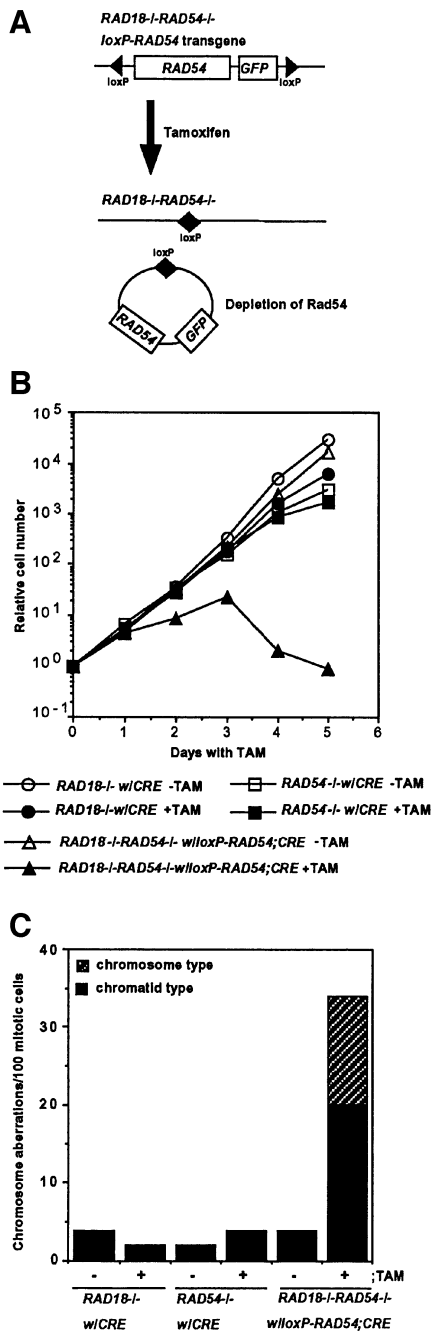


Fig. 6. *RAD18*^{-/-} *RAD54*^{-/-} double mutant cells are not viable. (A) Schematic representation of conditional *RAD18*^{-/-} *RAD54*^{-/-} double mutant cells. We generated *RAD18*^{-/-}*RAD54*^{-/-} cells that carry the chimeric Cre-recombinase and a transgene containing both the chicken *RAD54* and *GFP* genes flanked by *loxP* sequences on both sides (*loxP-RAD54*) (upper panel). Upon addition of tamoxifen to the culture media, both *GdRAD54* and *GFP* transgenes are expected to be eliminated (lower panel). The efficiency of *loxP-RAD54* transgene deletion was verified by flow cytometric analysis of GFP fluorescence. (B) Growth curves of the indicated cell cultures in the absence and presence of tamoxifen (TAM). The data shown are the averaged results from two separate clones of each genotype. (C) Chromosomal breaks are accumulated in dying *RAD18*^{-/-}*RAD54*^{-/-} double mutant cells. Cells were exposed to tamoxifen for 3 days. More than 50 mitotic cells were analyzed in each case.

possibility, we carried out the following experiments: *RAD18*^{-/-} *RAD54*^{-/-} (*w/loxP-RAD54, CRE*) cells were treated with tamoxifen for only 24 h, when the amount of

Rad54 protein is reduced by <50% (data not shown), and transferred to tamoxifen-free media. About 20% of these cells were able to form colonies, all of which were found to retain the *loxP-RAD54* transgene as verified by the presence of the transgene marker, GFP fluorescence (data not shown). From this result, together with the fact that no *RAD18*^{-/-} *RAD54*^{-/-} clone was obtained by directly targeting the *RAD54* gene of *RAD18*^{-/-}*RAD54*^{+/-} cells, we conclude that cells deficient in both *RAD18* and *RAD54* are unable to proliferate.

To investigate the cause of cell death, we analyzed chromosomal breaks in dying *RAD18*^{-/-} *RAD54*^{-/-} cells. *RAD18*^{-/-} (*w/CRE*) and *RAD54*^{-/-} (*w/CRE*) cells showed few chromosomal aberrations (Figure 6C). These values were not dependent on tamoxifen. Remarkably, in contrast to these control populations, the *RAD18*^{-/-} *RAD54*^{-/-} cells had ~0.35 aberrations per cell at day 3 after adding tamoxifen (Figure 6C). We previously showed that the level of spontaneous chromosomal breaks of various HR-deficient DT40 clones is closely correlated with the rate of cell death during the cell cycle (reviewed in Morrison and Takeda, 2000; Sonoda *et al.*, 2001). These observations indicate that the increased chromosomal breaks may account for the massive cell death of *RAD18*^{-/-} *RAD54*^{-/-} cells.

Discussion

Vertebrate *RAD18* is involved in post-replication DNA repair

Our data show that *RAD18* is involved in the repair of diverse types of DNA lesions, including UV-, IR- and cisplatin-induced DNA damage. In addition to the hypersensitivity to UV and cisplatin, *RAD18*^{-/-} cells exhibited higher levels of UV-induced chromosomal breaks, when compared with wild-type cells (data not shown). Presumably, DNA gaps caused by UV damage and subsequent DNA replication were left unrepaired more frequently in *RAD18*^{-/-} cells than in wild-type cells. Furthermore, after UV irradiation, the size of newly replicated DNA strands in *RAD18*^{-/-} cells was significantly smaller than that of wild-type DT40 cells (Figure 4B). Similar results were obtained in murine embryonic cells deficient in *RAD18* (S.Tateishi and M.Yamaizumi, submitted) as well as in human cells overexpressing a dominant-negative form of the human *RAD18* gene (Tateishi *et al.*, 2000). These observations demonstrate that *RAD18* is indeed involved in PRR in vertebrate cells as well as in yeast.

It is unclear how *RAD18* controls PRR. Two major pathways for PRR are TLS and HR. We favor the possibility that *RAD18* is involved in PRR mainly by regulating the TLS pathway, for the following reasons. First, *RAD18*^{-/-} cells showed elevated levels of SCE in the presence and the absence of genotoxic reagents, which indicates that the activity of HR is not abolished in *RAD18*^{-/-} cells. Secondly, the double mutant *RAD18*^{-/-} *RAD54*^{-/-} showed synthetic lethality. If *RAD18* mainly controls the HR pathway, depletion of the HR pathway gene *RAD54* from *RAD18*^{-/-} cells might not cause such a dramatic effect.

The possibility that *RAD18* also controls HR and/or other PRR pathways in addition to TLS remains to be

verified in a future study. Indeed, we observed the decreased frequency of targeted integration (Table I), which indicates that a certain type of HR is affected in *RAD18*^{-/-} cells, although the majority of HR is not halted in *RAD18*^{-/-}, as exemplified by the elevated SCE level. Involvement of *RAD18* in a copy choice DNA synthesis mechanism (Broomfield *et al.*, 2001) is also plausible. Recently, it has been proposed that error-free PRR genes, *RAD5* and *MMS2*, are involved in a copy choice DNA synthesis mechanism (Torres-Ramos *et al.*, 2002). Because *RAD5* and *MMS2* genes are epistatic to *RAD6* and *RAD18*, *RAD18* could control PRR through a copy choice mechanism by controlling *RAD5* and/or *MMS2*. Future study, including the generation of mutants in TLS genes (i.e. TLS polymerases, *RAD5* and *MMS2*, etc.) and double mutants between *RAD18* and those genes, would establish whether and how *RAD18* controls TLS and other PRR mechanisms in vertebrate cells.

***RAD18* plays an important role in maintaining chromosomal integrity during the cell cycle, by cooperating with *RAD54*-dependent HR pathways**

Synthetic lethality of *RAD18*^{-/-} and *RAD54*^{-/-} mutations is surprising, given the fact that either single mutant causes only a slight phenotypes in terms of cell proliferation. *RAD18*^{-/-} cells can grow at a normal rate compared with wild type (Figure 2C). Among the genes involved in HR, the *RAD54* gene, when mutated, causes a relatively mild phenotype in both murine embryonic stem cells and DT40 cells, i.e. normal mouse embryogenesis, nearly normal rates of cell proliferation and only moderately elevated IR sensitivity (Bezzubova *et al.*, 1997; Essers *et al.*, 1997). Synthetic lethality of *RAD18*^{-/-} and *RAD54*^{-/-} mutations demonstrates that a defect in *RAD18* is fully substituted by the *RAD54*-dependent HR pathway, while a defect in *RAD54* can be substituted by the *RAD18* in maintenance of chromosomal DNA during the cell cycle. Thus, the roles of a *RAD18*-dependent PRR pathway and *RAD54*-dependent HR pathways may be significantly redundant in repairing replication-associated DNA damage. Presumably, a large number of gaps are generated during DNA replication and, in the absence of *RAD18*, even a minor defect in HR, i.e. a defect in *RAD54*, may be lethal to the cells.

Cisplatin sensitivity of *RAD18*^{-/-} cells

RAD18^{-/-} cells showed an extreme sensitivity to cisplatin (Figure 3B). The cross-linking agent cisplatin, which is widely used in chemotherapy of cancer, forms covalent adducts with chromosomal DNA. Cross-linking agents form a variety of DNA adducts: intrastrand cross-links, interstrand cross-links and protein–DNA cross-links (reviewed in Zamble and Lippard, 1995). A number of repair pathways could be involved in repairing DNA damage caused by cisplatin, depending on the type of damage (reviewed in Dronkert and Kanaar, 2001). While yeast mutants deficient in the nucleotide excision repair (NER) or HR pathway show mildly increased sensitivity to cisplatin, *rad6* and *rad18* mutants exhibit dramatically increased sensitivity (Lawrence and Christensen, 1976). Similarly, loss of *RAD18* in DT40 cells dramatically increased sensitivity to cisplatin. *RAD18*^{-/-} cells showed the same pattern of chromosomal breaks following UV

radiation (data not shown). These observations suggest that *RAD18* may be involved in repairing cisplatin-induced DNA damage mainly in the same manner as it is involved in repairing UV damage. Further investigation based on these findings would provide insight into the molecular mechanisms of the chemotherapeutic function of cisplatin.

Materials and methods

Isolation of the chicken *RAD18* gene

To isolate the chicken *RAD18* gene, we designed oligonucleotide PCR primers based on conserved sequences between the human and mouse *RAD18* homologs (Tateishi *et al.*, 2000; van der Laan *et al.*, 2000; Xin *et al.*, 2000). By using these PCR primers, chicken cDNA fragments were isolated and proved to encode a protein that is highly homologous to human and mouse *RAD18*. RACE PCR was carried out to isolate the 3' and 5' termini of the chicken *RAD18* gene.

Cell culture, DNA transfection and irradiation

Cells were cultured in RPMI 1640 supplemented with 10⁻⁵ M β-mercaptoethanol, 10% fetal calf serum and 1% chicken serum (Sigma, St Louis, MO) at 39.5°C. Methods for DNA transfection and genotoxic treatments have been described previously (Takata *et al.*, 1998).

Plasmid construction

Two *RAD18* disruption constructs, *RAD18-hisD* and *RAD18-hygro*, were generated from genomic PCR products combined with *hisD* and *hygro* selection marker cassettes. Genomic DNA sequences were amplified using the primers 5'-CCCATAACTATTGTTCCCTTTGCATACGG-3' and 5'-TTTGGAAACTTTCCAATCTGTTCTGCCAGACC-3' (for the left arm of the KO construct); and 5'-GCAGGATCAAGAGAGTGTTCTGAAGGC-3' and 5'-GGGATTAGAGAATCACACTGAGCAT-TATACACGTGC-3' (for the right arm of the KO construct). Amplified PCR products were cloned into pCRII-TOPO vector (Invitrogen). The 2.9 kb *NheI* (in genomic DNA)–*BamHI* (in pCRII) fragment from the left arm and the 3.1 kb *EcoRV* (in pCRII)–*ApaI* (in genomic DNA) fragment were cloned into pBluescript (Stratagene). The *BamHI* site was used to clone marker gene cassettes. The 0.3 kb *BamHI*–*NheI* fragment from the left arm was used to make the probe for Southern blot analysis. *RAD54-puro* and *RAD54-bsr* disruption constructs were described previously (Takata *et al.*, 1998; Fukushima *et al.*, 2001). We constructed an expression vector pCR3-*loxP*-*RAD54*/IRES-EGFP-*loxP* (named *loxP-RAD54*), in which *RAD54* and GFP (*EGFP*) genes are flanked by the *loxP* sequences, by inserting the *RAD54 BamHI* fragment into the *BamHI* site of pCR3-*loxP*-MCS-*loxP* (Fujimori *et al.*, 2001).

Generation of *RAD18*^{-/-} and *RAD18*^{-/-} *RAD54*^{-/-} (w/*loxP*-*RAD54*, CRE) cells

The wild-type DT40 cells were transfected sequentially with *RAD18-hygro* and *RAD18-hisD* targeting constructs to obtain *RAD18*^{-/-} cells. *RAD18*^{-/-} cells were transfected with the *RAD54-puro* targeting construct. The resulting *RAD18*^{-/-} *RAD54*^{+/-} clone was transfected with *loxP-RAD54*, which can be selected by a neo selection marker. *RAD18*^{-/-} *RAD54*^{+/-} (w/*loxP*-*RAD54*) cells subsequently were transfected with the *RAD54-bsr* targeting construct to produce *RAD18*^{-/-} *RAD54*^{-/-} (w/*loxP*-*RAD54*) cells. Then, the plasmid containing CreER chimeric recombinase (pANMerCreMer) (Zhang *et al.*, 1998) was co-transfected with an Eco-gpt marker plasmid into *RAD18*^{-/-} *RAD54*^{-/-} (w/*loxP*-*RAD54*, CRE) cells. We also transfected the plasmid containing the CreER chimeric recombinase into *RAD18*^{-/-} and *RAD54*^{-/-} cells, and obtained *RAD18*^{-/-} w/*CRE* and *RAD54*^{-/-} w/*CRE* cells.

Chromosome aberration analysis

Karyotype analysis was performed as described previously (Sonoda *et al.*, 1998). For the morphological analysis of chromosome aberrations, cells were treated with colcemid for 3 h to enrich mitotic cells.

Measurement of SCE levels

Measurement of SCE levels was performed as described previously (Sonoda *et al.*, 1999). Briefly, cells were cultured in the presence of 10 μM BrdU for 18 h and pulsed with 0.1 μg/ml colcemid for the last 2 h. 4NQO (0.2 ng/ml) was added 8 h before harvest. Harvested cells were treated

with 75 mM KCl for 15 min, and subsequently fixed with methanol:acetic acid (3:1) for at least 30 min. Cells were fixed onto wet (50% ethanol) glass slides and dried on a 40–42°C plate. Dried slides were incubated with 10 µg of Hoechst 33258/ml in phosphate buffer pH 6.8 for 20 min, followed by rinsing with MacIlvaine solution (164 mM Na₂HPO₄, 16 mM citric acid pH 7.0). Slides were irradiated with black light ($\lambda = 352$ nm) for 60 min and incubated in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) solution at 62°C for 1 h before staining with 3% Giemsa solution pH 6.8 and subsequent microscopic observation.

Measurement of the size of newly synthesized DNA strands following UV irradiation

Post-replication repair was analyzed by the sedimentation velocity method. In brief, actively growing cells (10⁵ cells/dish) were irradiated with UV light at 8 J/m², incubated for 10 min in 2 ml of pre-warmed medium, and then pulse-labeled with 0.93 MBq/ml [methyl-³H]thymidine for 15 min. In pulse-chase experiments, the pulse-labeled cells were incubated further for 30 or 90 min in fresh medium containing 10 µM unlabeled thymidine and uridine. As a control, cells were treated in the same way without UV irradiation. The cells were lysed in the dishes by addition of 0.1 ml of lysis solution containing 0.2 M NaOH and 20 mM EDTA, and the lysates were irradiated with 20 Gy of X-rays on ice. Aliquots of the cell suspension were layered on the top of 3.8 ml of 5–20% (w/v) alkaline sucrose density gradients containing 0.2 M NaOH, 0.5 M NaCl, 10 mM EDTA and 0.5% sodium *n*-dodecanoyl-salcosinate, and centrifuged at 25 000 r.p.m. for 6 h at 4°C in a P56ST rotor (Hitachi). After centrifugation, drop fractions were collected directly onto GF/C glass filters (Whatman), and the acid-insoluble radioactivities were counted using a liquid scintillation counter.

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