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Regulation of double-strand break-induced mammalian homologous recombination by UBL1, a RAD51-interacting protein

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ABSTRACT

Mammalian RAD51 protein plays essential roles in DNA homologous recombination, DNA repair and cell proliferation. RAD51 activities are regulated by its associated proteins. It was previously reported that a ubiquitin-like protein, UBL1, associates with RAD51 in the yeast two-hybrid system. One function of UBL1 is to covalently conjugate with target proteins and thus modify their function. In the present study we found that non-conjugated UBL1 forms a complex with RAD51 and RAD52 proteins in human cells. Overexpression of UBL1 down-regulates DNA double-strand break-induced homologous recombination in CHO cells and reduces cellular resistance to ionizing radiation in HT1080 cells. With or without overexpressed UBL1, most homologous recombination products arise by gene conversion. However, overexpression of UBL1 reduces the fraction of bidirectional gene conversion tracts. Overexpression of a mutant UBL1 that is incapable of being conjugated retains the ability to inhibit homologous recombination. These results suggest a regulatory role for UBL1 in homologous recombination.

INTRODUCTION

To survive DNA double-strand breaks (DSBs), mammalian cells have evolved two major repair mechanisms: non-homologous end-joining (NHEJ) and homologous recombination (HR). Although NHEJ is important for cell survival, it is often imprecise and can be mutagenic (1). In contrast, conservative HR uses a homologous donor as a template to repair the damaged recipient copy of DNA and thus prevent DNA damage-induced mutagenesis. Reduced conservative HR activity may thus result in increased mutagenic repair. Under some conditions, recombination may occur between regions with limited homology, such as *Alu* sequences (2,3), and result in chromosome rearrangements. Therefore, properly regulated HR activity is essential for reducing DNA damage-induced mutagenesis and for maintaining chromosome stability.

A key component of the eukaryotic HR machinery is the RAD51 protein. RAD51 possesses many biochemical activities required for HR, including DNA binding, homologous DNA pairing and strand exchange activities (4–7). Overexpression of hamster RAD51 promotes HR and enhances radiation resistance in the S/G_2 phases (8). Reduced expression of mammalian RAD51 renders cells hypersensitive to ionizing radiation (9). *RAD51* knockout in mice results in early embryonic lethality (10,11), which can be partially rescued by a null *p53* mutation (11).

It is widely accepted that RAD51-mediated HR requires a large protein complex that includes RAD52, RAD54, RAD55 and RAD57 (12–15). While interactions of human and yeast RAD51 with RAD52, RAD54 and RPA proteins facilitate RAD51-mediated recombination activities (16–21), binding of the tumor suppressor protein p53 and phosphorylation of RAD51 by c-abl tyrosine kinase also affect RAD51 activity (22–27). Using RAD51 as 'bait' in a yeast two-hybrid screen, a human ubiquitin homolog was identified and designated UBL1 (ubiquitin-like protein-1) (28). UBL1 shares moderate homology with ubiquitin and several ubiquitin-like and ubiquitin-like domain-containing proteins, including RAD23 and DSK (29–31). UBL1 has since been identified by several other groups (32–35) and has been called PIC1 (PML-interacting clone-1), GMP1 (GAP modifying protein-1), SUMO-1 (small ubiquitin-related modifier-1) and Sentrin, indicating additional functions of UBL1. Compared to ubiquitin, UBL1 has a unique N-terminal domain of 20 amino acids and, following the conserved GlyGly residues at the C-terminus, there are four additional amino acids (28). Removing the six C-terminal amino acids eliminates the capability of UBL1 of being conjugated with other proteins (36–38).

A yeast two-hybrid screen using RAD51 and RAD52 as bait identified a second gene designated UBC9/UBE2I (39,40). Sequence analysis indicates that UBC9 belongs to a family of ubiquitin-conjugating enzymes. We showed that UBL1 and UBC9/UBE2I interact in a yeast two-hybrid system (40), and this was confirmed by others (36). Further studies have led to the conclusion that UBC9 is a UBL1-conjugating enzyme,

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rather than a ubiquitin-conjugating enzyme (38,41–43). It is now well established that UBL1/SUMO-1/PIC-1/Sentrin is conjugated with various proteins to modify target protein functions (44–48) and this process requires UBC9 as an enzyme (38,47,49,50).

Because of its association with RAD51 and RAD52 in the yeast two-hybrid system, it is reasonable to speculate that human UBL1 may regulate recombination and cellular sensitivity in response to DNA-damaging agents. The present study was designed to further characterize the interaction between UBL1 and RAD51 and to clarify the role of UBL1 in HR. We report here that the non-conjugated form of UBL1 co-immunoprecipitates with RAD51 and RAD52 and that overexpression of UBL1 decreases radioresistance and DSB-induced HR in mammalian cells. These data suggest an important role for UBL1 in the regulation of HR.

MATERIALS AND METHODS

Recombinant proteins

Expression and purification of His–RAD51 and His–RAD52 recombinant proteins have been described (13). Vectors pET28c (Novagene, Madison, WI) and pGEX-5x-1 were used to construct recombinant UBL1 protein expression plasmids. The coding region of UBL1 with added *Bam*HI and *Sal*I sites at the N- and C-termini, respectively, was amplified by PCR with the primers 5′-CTA TGG ATC CAT ATC ATG TCT GAC CAG GAG and 5′-TGC AGG TCG ACA TAT CTA AAC TGT TGA ATG ACC. The amplified DNA fragment was cloned into these vectors through their *Bam*HI and *Sal*I sites, generating pET/UBL1 and pGEX/UBL1. Expression of pET/UBL1 in *Escherichia coli* BL21(DE3) cells induced with isopropyl β-D-thiogalactopyanoside (IPTG) produces a UBL1 fusion protein that has a $(His)_6$ tag at the N-terminus (His–UBL1). Vector pGEX/UBL1 expresses glutathione *S*-transferase (GST)-tagged UBL1 (GST–UBL1) in *E.coli* HB101 under induction by IPTG. Similar approaches were used to construct His–UBC9 and GST–UBC9 fusion protein expression vectors. The same procedures for other His-tagged and GST fusion proteins were followed as for His–UBL1 and GST–UBL1 expression and purification, as described previously (13,51). Briefly, His–UBL1 was purified from *E.coli* BL21(DE3) cells using a Ni column according to the recommendations of the manufacturer (Novagene), and GST–UBL1 was purified with glutathione–Sepharose 4B beads (Pharmacia Biotech, Uppsala, Sweden).

Antibodies

Rabbit polyclonal anti-RAD51 antibodies, polyclonal rabbit anti-HA, mouse monoclonal anti-HA antibody (clone 12CA5) and mouse anti-FLAG monoclonal antibody were purchased from CalBiochem (La Jolla, CA), Clonetech Laboratories (Palo Alto, CA), Boehringer Mannheim (Indianapolis, IN) and Eastman Kodak (New Haven, CT), respectively. His–UBL1 and His–UBC9 proteins were used to generate polyclonal antibodies from rabbits. GST–UBL1 and GST–UBC9 were used for affinity purification of anti-UBL1 and anti-UBC9 antibodies from the serum. Rabbit anti-RAD52 antibodies were generated against a synthetic peptide (KSGSWDLQTYSADQR) of the RAD52 protein.

Immunoprecipitation

Full-length RAD51, RAD52, UBL1 and UBC9 cDNAs were cloned into pHA-CMV and pMyc-CMV vectors. Plasmids were transfected into HeLa cells using GenePorter transfection reagent (GeneTherapy Systems) at 7 µl of Geneporter/µg plasmid DNA. Twenty-four hours after transfection, cells were collected, treated with lysis buffer (50 mM HEPES–KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween-20, 10% glycerol, 10 mM β-glycerphosphate, 1 mM NaF, 0.1 mM Na_3VO_5, 0.2 mM PMSF, 20 µg/ml aprotin and 20 µg/ml leupeptin). An aliquot with 200 µg of protein was incubated with 2 µg of mouse monoclonal anti-HA antibody for 1 h on ice, then 20 µl of protein A–agarose beads (Santa Cruz) were added and incubated for an additional 1 h. The protein A beads were blocked with 1 mg/ml BSA for 30 min before use. After incubation with protein A beads, the suspension was washed four times with lysis buffer to retain HA-tagged and their associated proteins. The bead-retained proteins were resuspended in 20 µl of SDS–PAGE sample buffer. Ten microliters of the precipitated proteins were resolved by SDS–PAGE, transferred to a PVD membrane and incubated with anti-RAD51, anti-RAD52 or anti-UBL1 antibodies as specified in the appropriate figure legends. For parallel negative controls, another aliquot of 200 µg protein was immunoprecipitated with 2 µg of non-specific mouse IgG.

In vitro protein binding

Three micrograms of His-tagged recombinant proteins and 6 µg of GST or GST fusion protein were mixed with 50 µl of glutathione–agarose (Sigma Chemical Co., St Louis, MO) in binding buffer (50 mM Tris–HCl, pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$). After incubation at 4°C for 4 h, the slurry was washed three times with wash buffer (50 mM Tris–HCl, pH 7.9, 200 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol) to retain proteins bound to the glutathione–agarose slurry. Protein(s) not directly bound to the slurry but interacting with slurry-bound GST fusion protein was co-precipitated with the GST fusion protein, resolved by SDS–PAGE and detected with RAD51 antibodies (52).

pOPI3/FLAG-UBL1 vector

pOPI3/FLAG-UBL1 expresses FLAG epitope-tagged UBL1 (FLAG–UBL1) in human HT1080 cells. pOPI3/FLAG-UBL1 was constructed as follows. pREPNF was constructed by inserting the following synthesized sequence into pREP10 (Invitrogen, Carlsbad, CA): 5′-TCT GGC AAC ATG CCA CCA AAG AAG AAG CGT AAG GTT GGT GAC TAC AAG GAC GAC GAC GAC AAG CGG ATC CAA CTC GAG. In this sequence: GGC AAC ATG encodes a translation start signal; CCA CCA AAG AAG AAG CGT AAG encodes an SV40 nuclear localization signal (PPKKKRK), GAC TAC AAG GAC GAC GAC GAC AAG encodes a FLAG antigenic epitope (DYKDDDDK); GGA TCC and CTC GAG are *Bam*HI and *Xho*I sites. The coding region of UBL1 was released by *Bam*HI and *Sal*I digestion of pGEX/UBL1 and cloned into pREPNF through the *Bam*HI and *Xho*I sites downstream of the FLAG coding sequence. A pair of primers (5′-ATA AGA TAA GCG GCC GCA GGG ACT TTG AAC ATG GGT GAC TAC AAG GAC GAC and 5′-TGT GGT TTG TCC AAA CTC ATC) were used for PCR amplification of

pREPNF/UBL1. The PCR product was digested with *Not*I and subsequently cloned into pOPI3, which was derived from pOPI3/CAT (Stratagene, La Jolla, CA) by deleting the *CAT* gene. DNA sequencing confirmed the insert orientation and pOPI3/FLAG-UBL1 expressing FLAG-tagged UBL1 (FLAG–UBL1) was identified. Because pOPI3/FLAG-UBL1 has three LacI binding sites between the transcription start site and the UBL1 coding sequence, its expression in mammalian cells is suppressed in the presence of LacI protein.

pOPUR/FLAG-UBL1 vectors

Since recombination in CHO strain 33 cells (described below) is monitored with *neo*, pOPI3 cannot be used in these cells as it includes *neo* as a selection marker. To circumvent this problem, we constructed pOPUR by transferring the mammalian expression cassette from pOPI3 into pPUR (Clontech). This vector is essentially identical to pOPI3 except that it is selected with puromycin rather than neomycin. pOPUR contains a translation start signal (Kozak sequence) at the beginning of the UBL1 cDNA fragments to be expressed. UBL1 cDNA fragments were cloned downstream of the RSV promoter, creating pOPUR/FLAG-UBL1.

A mutant UBL1 lacking the six C-terminal amino acids was subcloned into the pOPUR *Not*I site using a pair of PCR primers (5′-ATA AGC GGC CGC CTA CGT TTG TTC CTG ATA AAC and 5′-ATA AGA TAA GCG GCC GCA GGG ACT TTG AAC ATG GGT GAC TAC AAG GAC GAC) to amplify the coding region of FLAG–UBL1 without the six C-terminal amino acids from pOPUR/FLAG-UBL1, followed by insertion into pOPUR. A construct with the correct gene orientation was identified by DNA sequencing and called pOPUR/FLAG-UBL1-C6.

Homologous recombination (HR) in CHO strain 33 cells

CHO strain 33, described previously (53), contains a chromosomal *neo* direct repeat; one copy of *neo* has an I-*Sce*I recognition sequence. HR is stimulated by DSBs created at the I-*Sce*I site upon transient expression of I-*Sce*I endonuclease. Transfectants of strain 33 with pOPUR/FLAG-UBL1 and a negative control vector (pOPUR) were selected in medium containing 3 μg/ml puromycin. Clones expressing FLAG–UBL1 protein were identified by western blot using anti-FLAG antibody M2 (Eastman Kodak).

To determine the effect of *UBL1* overexpression on DSB-induced HR, 2×10^5 strain 33 cells were seeded in 60 mm diameter dishes, grown for 2 days and transfected with 0.5–3.0 μg of pCMV(I-SceI) (54) using Lipofectamine (Gibco BRL, Gaithersburg, MD). pCMV(I-SceI) expresses NLS-tagged I-*Sce*I endonuclease. Twenty-four hours after transfection, cells from each dish were transferred to two sets of three dishes. One set had 200 cells/100 mm dish to determine the number of viable colony-forming cells and the second set had 200 000 cells/100 mm dish for recombination analysis. Eighteen hours later, G418 (500 μg/ml) was added to the high density dishes and the medium was changed to fresh α-MEM with 10% fetal calf serum and 500 μg G418 per ml every 4 days; G418-resistant colonies formed in ~12 days. The colonies were stained with crystal violet and scored. Since almost all G418-resistant clones arise by HR (53), the frequency of G418-resistant products per viable cell reflects the frequency of HR induced by I-*Sce*I.

Analysis of gene conversion tract lengths and tract directionality

Control and UBL1 overexpressing cells were transfected with 1.0 μg of pCMV(I-SceI) using the standard procedure. Twenty-four hours post-transfection, cell suspensions were inoculated into multiwell dishes at appropriate concentrations so that an average of one G418-resistant clone arose per well. After a 2 week selection with G418, wells with single G418-resistant clones were identified, expanded and genomic DNA was isolated using the Qiagen genomic DNA purification kit. Analyses for gene conversion track and directionality of these recombination products were performed as described previously (53).

Radiosensitivity assay

HT1080 cells (obtained from ATCC) were grown in α-MEM (Gibco BRL) supplemented with 10% fetal bovine serum (Sigma Chemical Co.). The LacSwitch system from Stratagene was used to establish IPTG-inducible overexpression of UBL1 in HT1080 cells. Plasmid p3′SS (hygromycin selection marker) expressing LacI tagged with a nuclear localization signal (LacI–NLS) was transfected into HT1080 cells by electroporation and independent clones were isolated. These clones were screened for the expression of LacI–NLS by western blot and immunofluorescent staining of cells with polyclonal rabbit anti-LacI antibodies (Stratagene). A clone densely expressing LacI–NLS in the nucleus was selected, and referred to as HT1080(p3′SS). Plasmid pOPI3/FLAG-UBL1 was transfected into HT1080(p3′SS) cells. A derivative over-expressing FLAG–UBL1 upon induction with IPTG was identified by western blot using mouse M5 anti-FLAG mouse monoclonal antibody (Eastman Kodak). This cell line is referred to as HT1080(pOPI3/FLAG-UBL1). A control cell line HT1080(pOPI3) transfected with pOPI3 vector was also generated. These cells were plated for colony formation and treated with 5 mM IPTG for 18 h before γ-ray exposure (dose rate 4 Gy/min). The initial number of cells plated in each γ-ray dose group was determined by a pilot experiment to yield 50–100 surviving colonies/100 mm (diameter) plate for a given dose. The medium was changed 30 h after irradiation (therefore IPTG was present for a total of 48 h). Colonies were visualized after 10–12 days, strained with crystal violet and counted to determine cell survival at each γ-ray dose.

RESULTS

Interactions among UBL1, RAD51 and RAD52

UBL1 associates with RAD51 in a yeast two-hybrid system (28,40). Yeast two-hybrid assays can detect transient protein interactions and indirect protein interactions mediated by an endogenous yeast protein. Therefore, two-hybrid results do not provide direct proof that RAD51 and UBL1 form a stable complex *in vivo* nor that UBL1 directly binds to RAD51. Other possible scenarios to explain the interaction of RAD51 and UBL1 include: (i) RAD51 may be conjugated to UBL1 since UBL1 can be conjugated to other proteins (34,47,50,55–58; see also below); (ii) RAD51 and UBL1 may interact directly via protein–protein binding; (iii) RAD51 may associate with UBL1 indirectly, mediated by other RAD51-associated proteins; (iv) associated proteins may facilitate direct binding

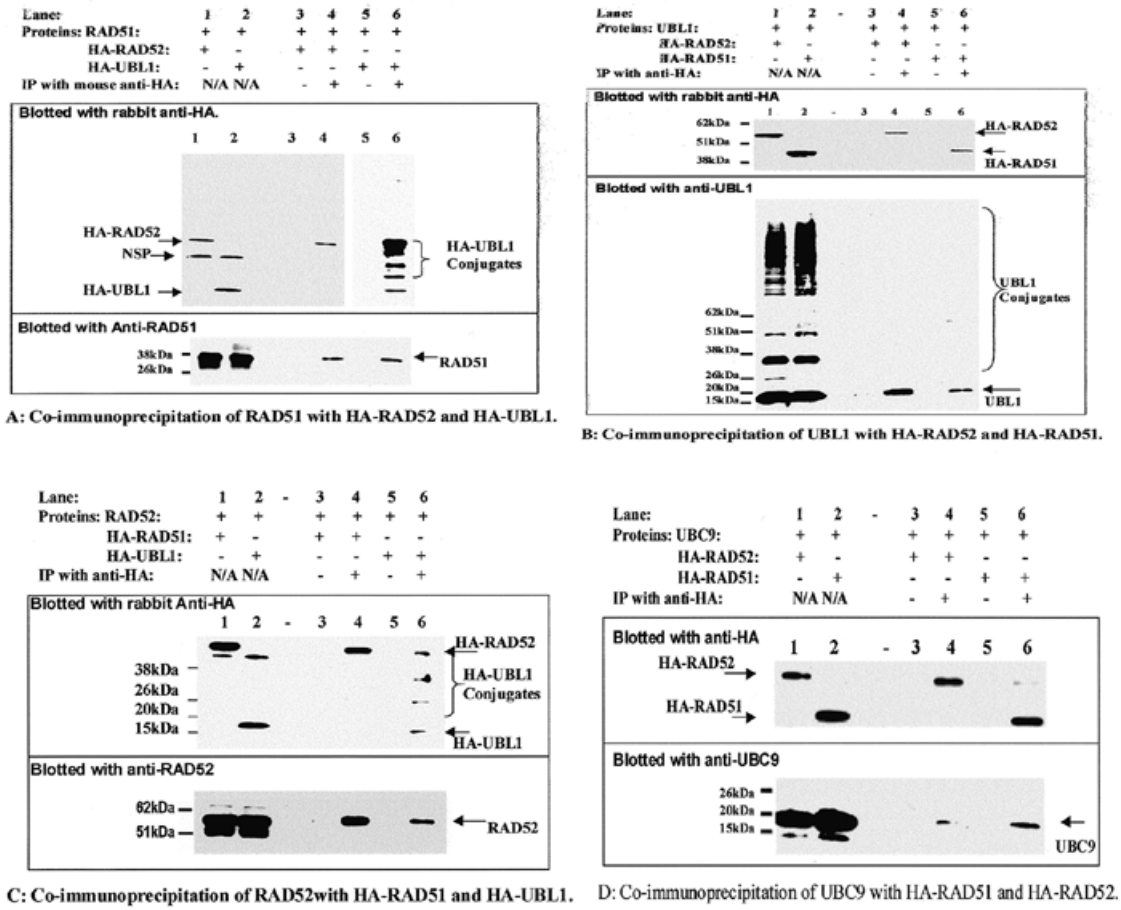

Figure 1. Co-immunoprecipitation of UBL1, RAD51, RAD52 and UBC9 proteins. Proteins to be co-immunoprecipitated (expressed from pMyc-CMV) were transiently co-expressed with HA-tagged proteins in HeLa cells (see individual panels for specific proteins that were expressed). Protein lysates were immunoprecipitated with mouse anti-HA monoclonal antibody. In each panel: lane 1 was loaded with 25 µg of total protein lysate used to prepare samples for lanes 3 and 4; lane 2 was loaded with 25 µg of total protein lysate used to prepare samples for lanes 5 and 6; lanes 3 and 5 have proteins immunoprecipitated with non-specific mouse IgG; lanes 4 and 6 have proteins immunoprecipitated with mouse anti-HA. Proteins were resolved by 4–20% gradient SDS–PAGE in duplicate, transferred to PVD membranes and incubated with specific antibodies. The top panels show detection with rabbit anti-HA antibodies to demonstrate the immunoprecipitation of a HA-tagged protein. The bottom panels show detection with antibodies to specific proteins to be co-immunoprecipitated, to demonstrate co-immunoprecipitation of specific proteins with the HA-tagged proteins detected in the top panels. (**A**) Co-immunoprecipitation of RAD51 with HA–RAD52 and HA–UBL1. (Top) HA–RAD52 and HA–UBL1 immunoprecipitate with anti-HA antibody, but not with non-specific mouse IgG (lanes 3 and 5). The HA–UBL1 conjugates in the whole cell lysate (lane 2) are not visible, but are detectable upon longer exposure, with a similar pattern to lanes 1 and 2 in (B) (data not shown). The common band (~38 kDa) in lanes 1 and 2 in the top panel is an endogenous non-specific protein (NSP) reactive to rabbit anti-HA antibodies. The top panel also demonstrates that at least three UBL1 conjugates are precipitated with mouse anti-HA antibody (lane 6). (Bottom) RAD51 co-precipitates with HA–RAD52 (lane 4) and HA–UBL1 (lane 6). (**B**) Co-immunoprecipitation of non-conjugated UBL1 with RAD51 and RAD52. (Top) HA–RAD52 (lane 4) and HA–RAD51 (lane 6) immunoprecipitate with anti-HA antibody, but not with non-specific mouse IgG (lanes 3 and 5). (Bottom) Non-conjugated UBL1 co-precipitates with HA–RAD52 (lane 4) and HA–RAD51 (lane 6). The patterns in lanes 1 and 2 are different from those in lane 6 of (A), because some of the UBL1 conjugates were not precipatable by anti-HA in (A). (**C**) Co-immunoprecipitation of RAD52 with RAD51 and UBL1 proteins. (Top) HA–RAD51 (lane 4) and HA–UBL1 (lane 6) immunoprecipitate with anti-HA antibody, but not with non-specific mouse IgG (lanes 3 and 5). A few HA–UBL1 conjugates were also detected. The signals from HA–UBL1 conjugates in the whole cell lysate (lane 2) were detectable upon longer exposure (data not shown). (Bottom) RAD52 co-precipitates with HA–RAD51 (lane 4) and HA–UBL1 (lane 6). (**D**) Co-immunoprecipitation of UBC9 protein with HA–RAD52 and HA–RAD51 proteins. (Top) HA–RAD52 (lane 4) and HA–RAD51 (lane 6) immunoprecipitate with mouse anti-HA antibody, but not with non-specific mouse IgG (lanes 3 and 5). (Bottom) UBC9 co-precipitates with RAD52 and RAD51.

of RAD51 and UBL1. To distinguish between these possibilities we used immunoprecipitation assays in HeLa cells.

When RAD51 is transiently co-expressed with HA–RAD52 or HA–UBL1, immunoprecipitation of both HA–RAD52 and HA–UBL1 co-precipitate RAD51 (Fig. 1A). The co-precipitation of RAD51 with HA–UBL1 indicates an interaction between RAD51 and UBL1. The co-precipitation of RAD51 with HA–RAD52 confirms the RAD51–RAD52 interaction reported earlier (13). Immunoprecipitation of HA–UBL1 also precipitates some, if not all, proteins that are covalently conjugated to UBL1 (Fig. 1A, top). Therefore, it is possible that the co-precipitation of RAD51 with anti-HA–UBL1 is due to an interaction of RAD51 with a UBL1-conjugated protein, rather than an interaction between RAD51 and UBL1. To further characterize this interaction, HA–RAD52 and HA–RAD51 were co-expressed with UBL1 and immunoprecipitated. As shown in

Figure 1B, various forms of UBL1-conjugated proteins are detected using anti-UBL1 antibodies in the total protein lysate before immunoprecipitation (Fig. 1B, bottom, lanes 1 and 2). However, after immunoprecipitation of HA–RAD51 and HA–RAD52, only the non-conjugated form of UBL1 was detected, indicating that non-conjugated UBL1 is sufficient to form a complex with RAD51 and RAD52. RAD52 also forms a stable complex with UBL1 (Fig. 1B).

To further confirm the RAD52–UBL1 interaction, RAD52 was co-expressed with HA–RAD51 and HA–UBL1 (Fig. 1C, lanes 1 and 2). Precipitations of HA–RAD51 and HA–UBL1 co-precipitate RAD52 (Fig. 1C, top, lanes 3–6), confirming an interaction between RAD52 and RAD51 or UBL1. It has been reported that UBC9 and UBL1 directly bind to each other (42) and we also found that UBC9 co-precipitates with UBL1 (data not shown). Since UBC9 also interacts with RAD51 and RAD52 in a yeast two-hybrid system (40), we tested whether UBC9 can be co-precipitated with RAD51 and RAD52. As shown in Figure 1D, UBC9 indeed co-immunoprecipitates with RAD51 and RAD52.

In vitro binding of UBL1 with RAD51 in the presence of RAD52 and UBC9

The data in Figure 1 indicate that RAD51 interacts with UBL1 in HeLa cells and that RAD52 and UBC9 may also be present in this complex. Since endogenous proteins were present during immunoprecipitation and previous yeast two-hybrid analysis, such assays cannot distinguish whether particular interactions are direct or occur within a large protein complex. To resolve this matter, we mixed purified GST–UBL1 with His–RAD51 *in vitro*. When only GST–UBL1 and His–RAD51 were mixed, precipitation of GST–UBL1 with glutathione beads did not co-precipitate His–RAD51, although RAD51 alone binds to RAD52 and UBL1 alone binds to UBC9 (data not shown). These results suggest that RAD51 and UBL1 do not form a stable complex by themselves, at least in recombinant protein form. Since RAD52 and UBC9 interact with RAD51 *in vivo* (40), we thought that the interaction of UBL1 with RAD51 may require these interacting proteins. To test this we mixed His–RAD52, His–RAD51 and His–UBC9 with GST–UBL1. As shown in Figure 2, precipitation of GST protein with glutathione–agarose does not precipitate RAD51 (lane 1), thus non-specific binding of His–RAD51 to the agarose slurry was not detected. However, when GST–UBL1 (lane 2) was used in place of GST, His–RAD51 was precipitated with glutathione beads (Fig. 2, lane 2), suggesting that RAD52 and/or UBC9 facilitate the interaction of RAD51 with UBL1. There is no ATP in the binding solution and thus no possibility for covalent UBL1 conjugation. Again, these data suggest a non-covalent protein interaction between RAD51 and UBL1 in the presence of RAD52 and UBC9, in agreement with the data in Figure 1B.

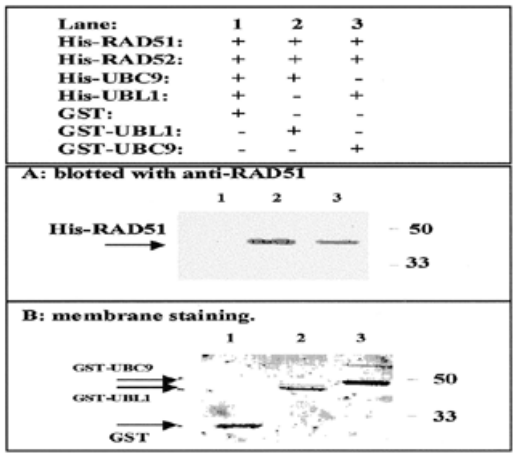

Figure 2. RAD51 and UBL1 form a stable complex in the presence of RAD52 and UBC9 *in vitro*. Three micrograms of His-tagged recombinant proteins were mixed with one of the GST proteins (GST, GST–UBL1 or GST–UBC9) and 50 µl of glutathione–agarose in binding buffer. After incubation at 4°C for 4 h, the slurry was washed and proteins retained by the glutathione–agarose were analyzed. Because the majority of the His protein did not bind to the GST protein, co-precipitated His–RAD51 was not visible by Coomassie blue staining. Western blotting was used to visualize co-precipitated His–RAD51 (**A**). The membrane was stained with Coomassie blue after western blotting (**B**), showing that equal amounts of GST, GST–UBL1 and GST–UBC9 proteins are bound to the agarose in each of the binding reactions.

Overexpression of UBL1 down-regulates DSB-induced homologous recombination (HR)

Under the immunoprecipitation conditions, we have shown that UBL1 forms a stable complex with RAD51 protein. We next investigated whether overexpression of UBL1 would affect DSB-induced HR in CHO strain 33 (53). Strain 33 carries two chromosomal copies of *neo* flanking an SV40 promoter-driven *gpt* gene. One copy (recipient) is inactivated by insertion of 29 bp containing an I-*Sce*I site and is regulated by the MMTV promoter, designated MMTV*neo*(I-SceI). The second (donor) copy, designated *neo12*, is inactive because it lacks a promoter, but otherwise has wild-type coding capacity. *neo12* has 12 phenotypically silent RFLP mutations at ~100 bp intervals to allow analysis of conversion tract lengths and directionality. Constitutive expression from the MMTV promoter is sufficient to confer G418 resistance to cells that have undergone recombination to yield a functional MMTV*neo* gene (53,59). Transient expression of I-*Sce*I leads to cleavage of MMTV*neo*(I-SceI) and this increases HR by at least 6000-fold, nearly all of which is gene conversion (53).

We transfected strain 33 cells with pOPUR (control) or pOPUR/FLAG-UBL1 and three independent derivatives of each were isolated. FLAG–UBL1 expression was confirmed by western blot (Fig. 3A). Using a green fluorescent protein expression vector, we confirmed that these derivatives had similar (~30%) transfection efficiencies using lipofection (data not shown). Various amounts of pCMV(I-SceI) plasmid were transfected into these clones and HR frequencies were measured. As shown in Figure 3B, expression of FLAG–UBL1 reduces DSB-induced HR by 2- to 3-fold. The reductions were statistically significant at all input DNA concentrations tested ($P < 0.01$, *t*-test).

Removing the six C-terminal amino acids of UBL1 eliminates its ability to conjugate with other proteins (36–38), and this ability is crucial to the regulatory functions of UBL1 in some cellular processes (45,48,55,57,60). However, removal of these six C-terminal amino acids did not prevent the UBL1–RAD51 interaction in a yeast two-hybrid assay (data not shown). In addition, *in vitro* binding assays demonstrate that UBL1 binds

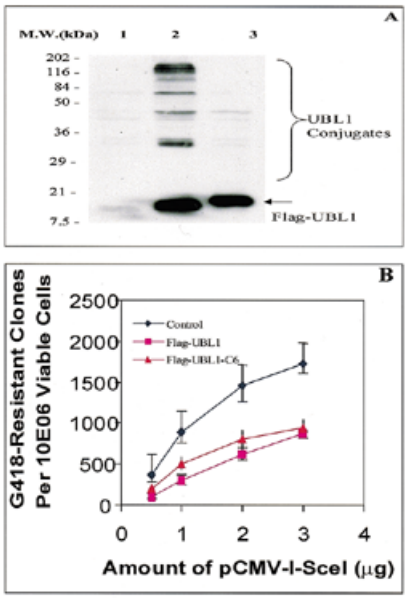

Figure 3. Effect of FLAG–UBL1 overexpression on homologous recombination (HR). (**A**) anti-FLAG M2 immunoblot of total cell lysate from derivatives of strain 33. Lane 1, cells transfected with pOPUR vector; lane 2, cells transfected with pOPUR/FLAG-UBL1; lane 3, cells transfected with pOPUR/FLAG-UBL1-C6, which express a C-terminus truncated UBL1 protein lacking conjugation activity. (**B**) I-*Sce*I-induced HR frequencies with increasing amounts of pCMV(I-SceI) vector in strain 33 derivatives as described in (A). Data points represent the average of nine experiments from three independent clones (± SEM).

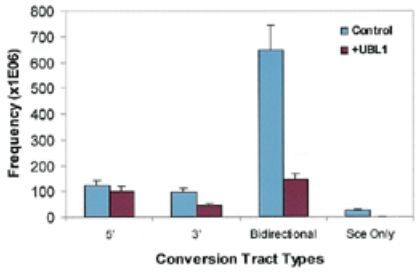

Figure 4. Absolute frequencies of specific gene conversion tract types. Data were calculated from tract spectra generated from 65 and 46 independent recombination products of control and UBL1-overexpressing cells, respectively, and from recombination frequencies determined with 1 μg of pCMV(I-SceI) (Fig. 3; see text for details). All products converted the I-*Sce*I site. 5′ indicates conversion only of markers 5′ of the DSB, 3′ indicates conversion only of the markers 3′ of the DSB, Bidirectional indicates conversion of markers on both sides of the DSB and Sce Only indicates no silent marker converted. Error bars indicate standard errors.

to RAD51 in the absence of ATP and without activation of UBL1 conjugation (Fig. 2). To test whether UBL1 conjugation is required for the down-regulation of HR by UBL1, we transfected pOPUR/FLAG-UBL1-C6 into strain 33 cells. As expected, the mutant protein did not conjugate with other proteins (Fig. 3A, lane 3). However, it retained the ability to significantly reduce the frequency of HR (Fig. 3B) ($P < 0.01$, *t*-test). The slight differences between wild-type and mutant UBL1 in down-regulating HR are not statistically significant. These data indicate that conjugation is not required for negative regulation of HR by UBL1.

Overexpressed UBL1 reduces bidirectional gene conversion tracts

The recombination substrate in strain 33 allows determination of the ratio of gene conversions to pop-outs (deletion of one copy of *neo* and DNA between the *neo* direct repeats by crossing over, single-strand annealing or unequal sister chromatid exchange), conversion tract lengths and directionality and conversion frequencies for individual markers as a function of distance from the initiating DSB. To investigate whether UBL1 expression influences recombinant product spectra, we analyzed the structures of 116 independent G418-resistant products, including 66 from strain 33/pOPUR (control) and 50 from strain 33/pOPUR/FLAG-UBL1. Each set had a single G418-resistant product that did not arise by HR, as recombination substrates in these had the parental structures. These rare products may reflect activation of multidrug resistance and were not studied further. The rest of the G418-resistant products (65 from the control and 49 from the UBL1 group) arose by HR. It was shown previously that nearly all DSB-induced HR in strain 33 involve gene conversion without associated cross-overs (53). All 65 HR products in the control group arose by gene conversion, as did 46 of 49 in the UBL1 overexpression group. This slight difference is not statistically significant ($P > 0.08$, Fisher's exact test).

Tract spectra and values for percent conversion of markers as a function of distance from the initiating DSB for control and UBL1-overexpressing cells were not significantly different (data not shown) and were similar to those obtained previously (53). Control and UBL1-overexpressing cells also had similar average tract lengths (~200 bp). However, 47 of 65 (72%) recombination products from control cells had bidirectional tracts and only 23 of 46 (50%) recombination products from UBL1-expressing cells were bidirectional. Thus, UBL1 expression led to significantly fewer bidirectional tracts than the control ($P = 0.028$, Fisher's exact test). Although the frequencies of all four tract types were reduced upon overexpression of UBL1, the decrease in HR was largely due to a 4-fold decrease in bidirectional tracts (Fig. 4).

Overexpression of UBL1 reduces cellular radioresistance

Because UBL1 interacts with RAD51 and influences HR, we thought that overexpression of UBL1 might affect radiosensitivity. Although inhibition of RAD51 confers hypersensitivity to radiation (9) and stable overexpression of hamster RAD51 confers slightly higher radioresistance in S/G_2 phase cells (8), overexpression of RAD51 has little effect on cellular sensitivity to radiation damage in heterogeneous cell populations (8). We anticipated that UBL1 overexpression would have at most modest effects on radiosensitivity and thus used an inducible overexpression system to eliminate potential interstrain variation.

We constructed a FLAG-tagged UBL1 expression vector (pOPI3/FLAG-UBL1) in which UBL1–FLAG is regulated by an IPTG-inducible promoter. When this vector was transfected into a LacI-expressing derivative of HT1080 cells [HT1080(p3′SS)], FLAG–UBL1 fusion protein expression was repressed; when these cells were treated with IPTG, FLAG–UBL1 expression was induced. Total protein extracts

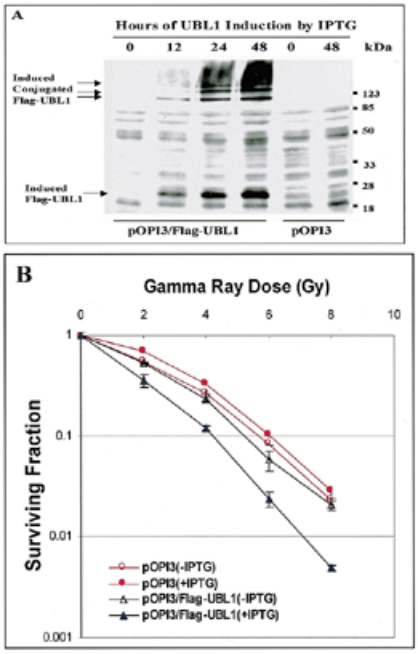

Figure 5. Overexpression of FLAG–UBL1 reduces HT1080 radioresistance. (**A**) Expression of FLAG–UBL1 and conjugation of FLAG–UBL1 with other proteins in HT1080 cells. Western analysis of total cell extracts from HT1080 (pOPI3) and HT1080(pOPI3/FLAG-UBL1) was performed using mouse monoclonal anti-FLAG M5 antibody. IPTG induction times are given at the top; molecular weights (kDa) are shown on the right. (**B**) Radiation survival of HT1080(pOPI3) and HT1080(pOPI3/FLAG-UBL1) with or without treatment with 5 mM IPTG. Error bars indicate standard errors from three to five independent experiments.

were analyzed by western blotting using mouse monoclonal anti-FLAG antibody M5. The M5 antibody was generated against an eight amino acid peptide resulting in some background reactivity to cellular proteins in HT1080 cells (Fig. 5A). After IPTG treatment, cells transfected with pOPI3/FLAG-UBL1 displayed several additional bands. Moreover, the intensities of these additional bands corresponded to the length of IPTG treatment in SDS–PAGE, indicating that they are derived from FLAG–UBL1. Because antibody against the FLAG tag was used, we detected high molecular weight IPTG-inducible bands representing FLAG–UBL1-conjugated proteins and an ~20 kDa IPTG-inducible band representing non-conjugated FLAG–UBL1 protein. These data suggest that FLAG–UBL1 is conjugated to several proteins in HT1080 cells. No IPTG-dependent bands were evident when extracts were prepared from control cells (HT1080/pOPI3) (Fig. 5A).

We used the HT1080 (pOPI3/FLAG-UBL1) cell line and control HT1080/pOPI3 cells to test the effect of UBL1 overexpression on cellular radiosensitivity. IPTG treatment did not change the radiation sensitivity of the control cells (closed and open circles in Fig. 5B). Without IPTG treatment, HT1080/FLAG–UBL1 cells displayed similar radiation sensitivity to the control (open triangles, Fig. 5B). However, HT1080/FLAG–UBL1 cells treated with IPTG were more sensitive to radiation for a given dose (2–8 Gy) (closed triangles, Fig. 5B) than in the absence of IPTG ($P < 0.01$, *t*-test).

DISCUSSION

UBL1/PIC-1/Sentrin/SUMO-1 is likely to be involved in many cellular processes, including apoptosis, regulation of mitosis, protein translocation, cell proliferation and transcriptional regulation (32–35,48,61,62). The present study suggests a role for UBL1 in DSB-initiated HR. UBL1 belongs to a family of ubiquitin-like proteins with roles in protein complex assembly, such as DNA repair complexes, cytoskeleton structure and microtubule organization (30,31,63,64). It is likely that UBL1-mediated and other ubiquitination-like pathways constitute important cellular mechanisms that regulate the formation and disassembly of various protein complexes. Although it is known that UBL1 functions in some cellular processes by covalently conjugating with some target proteins, the present study suggests novel UBL1 functions, mediated by interactions between the non-conjugated form of UBL1 and other target proteins, that modulate distinct cellular processes.

An interaction between UBL1 and RAD51 was identified previously in yeast two-hybrid screens (28,40). However, the two-hybrid assay cannot address whether the proteins form a stable complex, since transient protein interactions would also activate the reporter gene in a yeast two-hybrid assay. In the present study, co-immunoprecipitation was used to confirm this interaction and to show that the interaction occurs independently of UBL1 conjugation activity. Co-immunoprecipitation also identified interactions between UBL1 and RAD52 and between UBC9 and both RAD51 and RAD52.

RAD51 is a member of the RecA family of strand exchange proteins (65,66). *In vitro* studies with yeast and human RAD51 indicate that, as with RecA, RAD51 promotes homologous DNA pairing and strand exchange (4–6,67–69). Eukaryotic RAD52 binds to single-stranded DNA and to DNA ends at DSBs (70–73) and is thought to mediate RAD51 function (16–19). By extension from yeast, it is likely that RAD51 and RAD52 play key roles in HR in mammalian cells. We found that overexpression of UBL1 decreases DSB-induced HR in mammalian cells, suggesting that UBL1 acts as a negative regulator of this process. Our results indicate that negative regulation of DSB-induced HR by UBL1 is independent of UBL1 conjugation ability. However, the precise role of endogenous level of UBL1 in regulating recombination remains unclear.

Although eukaryotic *RAD52* null mutants are defective in non-crossover and crossover recombination and single-strand annealing, eukaryotic *RAD51* mutants are mainly defective in gene conversion (74–76). Because most HR in strain 33 involves gene conversion, it is likely that these events are RAD51 dependent. The reduction in HR in strain 33 overexpressing UBL1 may indicate a repression of RAD51-dependent recombination activity. It has been shown that RAD51-associated p53 also down-regulates HR (22,24).

Since RAD51 stimulates homologous pairing and strand exchange, UBL1-dependent down-regulation of RAD51 activity might be expected to reduce gene conversion tract lengths. However, we found that overexpression of UBL1 does not alter conversion tract lengths. Average tract lengths and percent conversion of individual markers determined in the present study are similar to results obtained previously (53). These results suggest that UBL1 does not influence the extent of strand exchange once strand invasion has occurred. However, overexpression of UBL1 reduces the relative

frequency of bidirectional conversion tracts. Tract directionality is a reflection of several factors, including strand invasion on either side of a DSB, branch migration and mismatch repair activity, but the relative contributions of these factors remain unclear. For example, current recombination models suggest that uni- and bidirectional tracts can arise from either single- or two-ended invasion (77). If end invasion is a significant factor controlling tract directionality, it is possible that UBL1 regulates this aspect of RAD51 function.

In yeast, HR is the dominant mode of DSB repair, and *rad51* mutants are markedly radiosensitive and defective in mitotic and meiotic recombination. However, it is not clear how significant HR is in mammalian DSB repair. In mammalian cells, mutations in genes involved in NHEJ, including *XRCC4* (accessory to ligase IV), *XRCC5* (Ku80), *XRCC6* (Ku70) and *XRCC7* (catalytic subunit of DNA protein kinase), strongly decrease radioresistance (78–81). In contrast, mutations in mammalian genes involved in HR generally have smaller effects on radioresistance. For example, mutation of RAD52 in mouse reduces HR but does not decrease radioresistance (82,83). Parallel experiments in RAD51 mutants are not possible due to early embryonic lethality and apparent cellular inviability (10,11). These studies favor the idea that HR is not the primary DSB repair mechanism in mammalian cells. However, it is possible that mammalian HR mutants are not completely defective in recombination and recent studies have suggested that HR plays a significant role in the repair of DSBs in mammalian chromosomal DNA (53,84,85).

XRCC2 and *XRCC3* are *RAD51* paralogs and mutations in these genes produce several-fold decreases in radioresistance (86–92), similar in magnitude to the decrease observed in cells overexpressing UBL1 (Fig. 5B). The UBL1-dependent decrease in radioresistance likely reflects effects on RAD51-dependent HR, consistent with the observed UBL1-dependent decrease in I-*Sce*I-induced HR.

In summary, we have shown that UBL1 binds to RAD51 and that UBL1 decreases radioresistance and down-regulates DSB-induced HR. These provide bases for further investigation of the influence of UBL1 on RAD51-dependent cellular processes.

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REFERENCES

1. Thacker,J. (1999) *Biochimie*, **81**, 77–85.
2. Petrij-Bosch,A., Peelen,T., van Vliet,M., van Eijk,R., Olmer,R., Drusedau,M., Hogervorst,F.B., Hageman,S., Arts,P.J., Ligtenberg,M.J., Meijers-Heijboer,H., Klijn,J.G., Vasen,H.F., Cornelisse,C.J., van't Veer,L.J., Bakker,E., van Ommen,G.J. and Devilee,P. (1997) *Nature Genet.*, **17**, 341–345.
3. Centra,M., Memeo,E., d'Apolito,M., Savino,M., Ianzano,L., Notarangelo,A., Liu,J., Doggett,N.A., Zelante,L. and Savoia,A. (1998) *Genomics*, **51**, 463–467.
4. Sung,P. (1994) *Science*, **265**, 1241–1243.
5. Baumann,P., Benson,F.E. and West,S.C. (1996) *Cell*, **87**, 757–766.
6. Sung,P. and Stratton,S.A. (1996) *J. Biol. Chem.*, **271**, 27983–27986.
7. Namsaraev,E. and Berg,P. (1997) *Mol. Cell. Biol.*, **17**, 5359–5368.
8. Vispe,S., Cazaux,C., Lesca,C. and Defais,M. (1998) *Nucleic Acids Res.*, **26**, 2859–2864.
9. Taki,T., Ohnishi,T., Yamamoto,A., Hiraga,S., Arita,N., Izumoto,S., Hayakawa,T. and Morita,T. (1996) *Biochem. Biophys. Res. Commun.*, **223**, 434–438.
10. Tsuzuki,T., Fujii,Y., Sakumi,K., Tominaga,Y., Nakao,K., Sekiguchi,M., Matsushiro,A., Yoshimura,Y. and Morita,T. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 6236–6240.
11. Lim,D.S. and Hasty,P. (1996) *Mol. Cell. Biol.*, **16**, 7133–7143.
12. Clever,B., Interthal,H., Schmuckli-Maurer,J., King,J., Sigrist,M. and Heyer,W.D. (1997) *EMBO J.*, **16**, 2535–2544.
13. Shen,Z., Cloud,K.G., Chen,D.J. and Park,M.S. (1996) *J. Biol. Chem.*, **271**, 148–152.
14. Hays,S.L., Firmenich,A.A. and Berg,P. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 6925–6929.
15. Johnson,R.D. and Symington,L.S. (1995) *Mol. Cell. Biol.*, **15**, 4843–4850.
16. New,J.H., Sugiyama,T., Zaitseva,E. and Kowalczykowski,S.C. (1998) *Nature*, **391**, 407–410.
17. Shinohara,A. and Ogawa,T. (1998) *Nature*, **391**, 404–407.
18. Benson,F.E., Baumann,P. and West,S.C. (1998) *Nature*, **391**, 401–404.
19. Sung,P. (1997) *J. Biol. Chem.*, **272**, 28194–28197.
20. Petukhova,G., Stratton,S. and Sung,P. (1998) *Nature*, **393**, 91–94.
21. Golub,E.I., Kovalenko,O.V., Gupta,R.C., Ward,D.C. and Radding,C.M. (1997) *Nucleic Acids Res.*, **25**, 4106–4110.
22. Xia,S.J., Shammas,M.A. and Reis,R.J. (1997) *Mol. Cell. Biol.*, **17**, 7151–7158.
23. Buchhop,S., Gibson,M.K., Wang,X.W., Wagner,P., Sturzbecher,H.W. and Harris,C.C. (1997) *Nucleic Acids Res.*, **25**, 3868–3874.
24. Mekeel,K.L., Tang,W., Kachnic,L.A., Luo,C.M., DeFrank,J.S. and Powell,S.N. (1997) *Oncogene*, **14**, 1847–1857.
25. Yuan,Z.M., Huang,Y., Ishiko,T., Nakada,S., Utsugisawa,T., Kharbanda,S., Wang,R., Sung,P., Shinohara,A., Weichselbaum,R. and Kufe,D. (1998) *J. Biol. Chem.*, **273**, 3799–3802.
26. Sturzbecher,H.W., Donzelmann,B., Henning,W., Knippschild,U. and Buchhop,S. (1996) *EMBO J.*, **15**, 1992–2002.
27. Chen,G., Yuan,S.S., Liu,W., Xu,Y., Trujillo,K., Song,B., Cong,F., Goff,S.P., Wu,Y., Arlinghaus,R., Baltimore,D., Gasser,P.J., Park,M.S., Sung,P. and Lee,E.Y. (1999) *J. Biol. Chem.*, **274**, 12748–12752.
28. Shen,Z., Pardington-Purtymun,P.E., Comeaux,J.C., Moyzis,R.K. and Chen,D.J. (1996) *Genomics*, **36**, 271–279.
29. van der Spek,P.J., Visser,C.E., Hanaoka,F., Smit,B., Hagemeijer,A., Bootsma,D. and Hoeijmakers,J.H. (1996) *Genomics*, **31**, 20–27.
30. Watkins,J.F., Sung,P., Prakash,L. and Prakash,S. (1993) *Mol. Cell. Biol.*, **13**, 7757–7765.
31. Biggins,S., Ivanovska,I. and Rose,M.D. (1996) *J. Cell Biol.*, **133**, 1331–1346.
32. Boddy,M.N., Howe,K., Etkin,L.D., Solomon,E. and Freemont,P.S. (1996) *Oncogene*, **13**, 971–982.
33. Okura,T., Gong,L., Kamitani,T., Wada,T., Okura,I., Wei,C.F., Chang,H.M. and Yeh,E.T. (1996) *J. Immunol.*, **157**, 4277–4281.
34. Mahajan,R., Delphin,C., Guan,T., Gerace,L. and Melchior,F. (1997) *Cell*, **88**, 97–107.
35. Matunis,M.J., Coutavas,E. and Blobel,G. (1996) *J. Cell Biol.*, **135**, 1457–1470.
36. Gong,L., Kamitani,T., Fujise,K., Caskey,L.S. and Yeh,E.T. (1997) *J. Biol. Chem.*, **272**, 28198–28201.
37. Johnson,E.S., Schwienhorst,I., Dohmen,R.J. and Blobel,G. (1997) *EMBO J.*, **16**, 5509–5519.
38. Johnson,E.S. and Blobel,G. (1997) *J. Biol. Chem.*, **272**, 26799–26802.
39. Kovalenko,O.V., Plug,A.W., Haaf,T., Gonda,D.K., Ashley,T., Ward,D.C., Radding,C.M. and Golub,E.I. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 2958–2963.
40. Shen,Z., Pardington-Purtymun,P.E., Comeaux,J.C., Moyzis,R.K. and Chen,D.J. (1996) *Genomics*, **37**, 183–186.
41. Desterro,J.M., Thomson,J. and Hay,R.T. (1997) *FEBS Lett.*, **417**, 297–300.

42. Liu,Q., Jin,C., Liao,X., Shen,Z., Chen,D.J. and Chen,Y. (1999) *J. Biol. Chem.*, **274**, 16979–16987.
43. Giraud,M.F., Desterro,J.M. and Naismith,J.H. (1998) *Acta Crystallogr. D Biol. Crystallogr.*, **54**, 891–898.
44. Lee,G.W., Melchior,F., Matunis,M.J., Mahajan,R., Tian,Q. and Anderson,P. (1998) *J. Biol. Chem.*, **273**, 6503–6507.
45. Duprez,E., Saurin,A.J., Desterro,J.M., Lallemand-Breitenbach,V., Howe,K., Boddy,M.N., Solomon,E., de The,H., Hay,R.T. and Freemont,P.S. (1999) *J. Cell Sci.*, **112**, 381–393.
46. Okuma,T., Honda,R., Ichikawa,G., Tsumagari,N. and Yasuda,H. (1999) *Biochem. Biophys. Res. Commun.*, **254**, 693–698.
47. Saitoh,H., Sparrow,D.B., Shiomi,T., Pu,R.T., Nishimoto,T., Mohun,T.J. and Dasso,M. (1998) *Curr. Biol.*, **8**, 121–124.
48. Desterro,J.M., Rodriguez,M.S. and Hay,R.T. (1998) *Mol. Cell*, **2**, 233–239.
49. Schwarz,S.E., Matuschewski,K., Liakopoulos,D., Scheffner,M. and Jentsch,S. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 560–564.
50. Lee,G.W., Melchior,F., Matunis,M.J., Mahajan,R., Tian,Q. and Anderson,P. (1998) *J. Biol. Chem.*, **273**, 6503–6507.
51. Shen,Z., Peterson,S.R., Comeaux,J.C., Zastrow,D., Moyzis,R.K., Bradbury,E.M. and Chen,D.J. (1996) *Mutat. Res.*, **364**, 81–89.
52. Chen,F., Nastasi,A., Shen,Z., Brenneman,M., Crissman,H. and Chen,D.J. (1997) *Mutat. Res.*, **384**, 205–211.
53. Taghian,D.G. and Nickoloff,J.A. (1997) *Mol. Cell. Biol.*, **17**, 6386–6393.
54. Choulika,A., Perrin,A., Dujon,B. and Nicolas,J.F. (1995) *Mol. Cell. Biol.*, **15**, 1968–1973.
55. Saitoh,H., Pu,R.T. and Dasso,M. (1997) *Trends Biochem. Sci.*, **22**, 374–376.
56. Muller,S., Matunis,M.J. and Dejean,A. (1998) *EMBO J.*, **17**, 61–70.
57. Matunis,M.J., Wu,J. and Blobel,G. (1998) *J. Cell Biol.*, **140**, 499–509.
58. Mahajan,R., Gerace,L. and Melchior,F. (1998) *J. Cell Biol.*, **140**, 259–270.
59. Nickoloff,J.A. (1992) *Mol. Cell. Biol.*, **12**, 5311–5318.
60. Sternsdorf,T., Jensen,K. and Will,H. (1997) *J. Cell Biol.*, **139**, 1621–1634.
61. Kamitani,T., Nguyen,H.P., Kito,K., Fukuda-Kamitani,T. and Yeh,E.T. (1998) *J. Biol. Chem.*, **273**, 3117–3120.
62. Sternsdorf,T., Guldner,H.H., Szostecki,C., Grotzinger,T. and Will,H. (1995) *Scand. J. Immunol.*, **42**, 257–268.
63. Loeb,K.R. and Haas,A.L. (1994) *Mol. Cell. Biol.*, **14**, 8408–8419.
64. Guzder,S.N., Bailly,V., Sung,P., Prakash,L. and Prakash,S. (1995) *J. Biol. Chem.*, **270**, 8385–8388.
65. Thacker,J. (1999) *Trends Genet.*, **15**, 166–168.
66. Thompson,L.H. and Schild,D. (1999) *Biochimie*, **81**, 87–105.
67. Gupta,R.C., Bazemore,L.R., Golub,E.I. and Radding,C.M. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 463–468.
68. Sugiyama,T., Zaitseva,E.M. and Kowalczykowski,S.C. (1997) *J. Biol. Chem.*, **272**, 7940–7945.
69. Sung,P. and Robberson,D.L. (1995) *Cell*, **82**, 453–461.
70. Van Dyck,E., Hajibagheri,N.M., Stasiak,A. and West,S.C. (1998) *J. Mol. Biol.*, **284**, 1027–1038.
71. Van Dyck,E., Stasiak,A.Z., Stasiak,A. and West,S.C. (1999) *Nature*, **398**, 728–731.
72. Mortensen,U.H., Bendixen,C., Sunjevaric,I. and Rothstein,R. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 10729–10734.
73. Sugiyama,T., New,J.H. and Kowalczykowski,S.C. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 6049–6054.
74. Rattray,A.J. and Symington,L.S. (1995) *Genetics*, **139**, 45–56.
75. Rattray,A.J. and Symington,L.S. (1994) *Genetics*, **138**, 587–595.
76. Ivanov,E.L., Sugawara,N., Fishman-Lobell,J. and Haber,J.E. (1996) *Genetics*, **142**, 693–704.
77. Cho,J.W., Khalsa,G.J. and Nickoloff,J.A. (1998) *Curr. Genet.*, **34**, 269–279.
78. Collins,A.R. (1993) *Mutat. Res.*, **293**, 99–118.
79. Gu,Y., Jin,S., Gao,Y., Weaver,D.T. and Alt,F.W. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 8076–8081.
80. Ouyang,H., Nussenzweig,A., Kurimasa,A., Soares,V.C., Li,X., Cordon-Cardo,C., Li,W., Cheong,N., Nussenzweig,M., Iliakis,G., Chen,D.J. and Li,G.C. (1997) *J. Exp. Med.*, **186**, 921–929.
81. Jeggo,P.A. (1998) *Adv. Genet.*, **38**, 185–218.
82. Rijkers,T., Van Den Ouweland,J., Morolli,B., Rolink,A.G., Baarends,W.M., Van Sloun,P.P., Lohman,P.H. and Pastink,A. (1998) *Mol. Cell. Biol.*, **18**, 6423–6429.
83. Yamaguchi-Iwai,Y., Sonoda,E., Buerstedde,J.M., Bezzubova,O., Morrison,C., Takata,M., Shinohara,A. and Takeda,S. (1998) *Mol. Cell. Biol.*, **18**, 6430–6435.
84. Liang,F., Han,M., Romanienko,P.J. and Jasin,M. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 5172–5177.
85. Johnson,R.D., Liu,N. and Jasin,M. (1999) *Nature*, **401**, 397–399.
86. Jones,N.J., Cox,R. and Thacker,J. (1987) *Mutat. Res.*, **183**, 279–286.
87. Fuller,L.F. and Painter,R.B. (1988) *Mutat. Res.*, **193**, 109–121.
88. Tucker,J.D., Jones,N.J., Allen,N.A., Minkler,J.L., Thompson,L.H. and Carrano,A.V. (1991) *Mutat. Res.*, **254**, 143–152.
89. Caldecott,K. and Jeggo,P. (1991) *Mutat. Res.*, **255**, 111–121.
90. Okayasu,R., Pantelias,G.E. and Iliakis,G. (1993) *Mutat. Res.*, **294**, 199–206.
91. Tebbs,R.S., Zhao,Y., Tucker,J.D., Scheerer,J.B., Siciliano,M.J., Hwang,M., Liu,N., Legerski,R.J. and Thompson,L.H. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 6354–6358.
92. Liu,N., Lamerdin,J.E., Tebbs,R.S., Schild,D., Tucker,J.D., Shen,M.R., Brookman,K.W., Siciliano,M.J., Walter,C.A., Fan,W., Narayana,L.S., Zhou,Z.Q., Adamson,A.W., Sorensen,K.J., Chen,D.J., Jones,N.J. and Thompson,L.H. (1998) *Mol. Cell*, **1**, 783–793.