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The Yeast Chromatin Remodeler RSC Complex Facilitates End Joining Repair of DNA Double-Strand Breaks†

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Repair of chromosome double-strand breaks (DSBs) is central to cell survival and genome integrity. Nonhomologous end joining (NHEJ) is the major cellular repair pathway that eliminates chromosome DSBs. Here we report our genetic screen that identified Rsc8 and Rsc30, subunits of the *Saccharomyces cerevisiae* chromatin remodeling complex RSC, as novel NHEJ factors. Deletion of *RSC30* gene or the C-terminal truncation of *RSC8* impairs NHEJ of a chromosome DSB created by HO endonuclease in vivo. *rsc30*Δ maintains a robust level of homologous recombination and the damage-induced cell cycle checkpoints. By chromatin immunoprecipitation, we show recruitment of RSC to a chromosome DSB with kinetics congruent with its involvement in NHEJ. Recruitment of RSC to a DSB depends on Mre11, Rsc30, and yKu70 proteins. Rsc1p and Rsc2p, two other RSC subunits, physically interact with yKu80p and Mre11p. The interaction of Rsc1p with Mre11p appears to be vital for survival from genotoxic stress. These results suggest that chromatin remodeling by RSC is important for NHEJ.

DNA double-strand breaks (DSBs) arise from exposure to ionizing radiation or radiomimetic chemicals and pose a great threat to cell survival and genome integrity. A single unrepaired or improperly repaired break can lead to chromosome loss and rearrangement, and defects in DSB repair have been linked to cancer-prone syndromes (52). Two distinct pathways function to eliminate DSBs in eukaryotes: homologous recombination (HR) and nonhomologous end joining (NHEJ) (49). HR repairs broken chromosomes by recombination between homologous sequences. NHEJ, in contrast, requires little or no homology to join the ends of the broken DNA molecules (21).

Distinct sets of gene products that are largely different from those required for HR seem to play a significant role in NHEJ in yeast and mammals (18). NHEJ in both organisms depends on the heterodimeric DNA binding proteins Ku70 and Ku80 (yKu70p-yKu80p in budding yeast) and the DNA ligase IV and the associated factor, XRCC4 (Dnl4p and Lif1p, respectively, in budding yeast) (36). In *Saccharomyces cerevisiae*, the Rad50p-Mrellp-Xrs2p complex also plays an essential role in promoting intermolecular DNA joining by Lig4p-Lif1p (17, 51). Nej1 modulates the yeast mating type-dependent nuclear localization of Lif1p (32, 48, 67). Pol4p and Fen1p/Rad27p are required for end processing steps of NHEJ in gap synthesis or removal of 5′-flap intermediates, respectively (72, 75).

Chromatin plays an important regulatory role in DNA repair (25). Multiple histone modifications have been implicated in DSB repair and damage signaling (50). Phosphorylation of the carboxy terminus of H2A is induced in a manner that is dependent on DNA damage and on phosphatidylinositol-3-OH kinase-related kinase and is required for efficient end joining repair of DSBs in yeast (8, 16, 22, 53, 58). Acetylation and deacetylation of histone H4 by Esa1p or the Sin3/Rpd3 complex near the DSB site has been linked to NHEJ (10, 29). Mutations in the histone H3 tail and the *HAT1* gene that encodes a histone acetyltransferase result in hypersensitivity to DNA damaging agents due to HR impairment (1). In higher eukaryotes, Gcn5, a histone acetyltransferase, was shown to interact with Ku proteins by yeast two-hybrid analysis (6). Furthermore, Gcn5 is phosphorylated by DNA-dependent protein kinase (DNA-PK), a member of the phosphatidylinositol 3-kinase family composed of a large catalytic subunit ($DNA\text{-}PK_{cs}$) and Ku proteins (6). The ATP-dependent chromatin remodeling complex has also been implicated in DSB repair. Rad54 is the SNF2 family of ATP-dependent chromatin remodeling factor and plays indispensable roles in HR (2, 69). At least two other chromatin remodeling complexes, the Ino80 and the Swr1 complex, have been implicated in repair of DNA damage from agents that create DSBs (23, 30, 34, 38, 43, 46, 57, 68).

RSC is an abundant multisubunit protein complex that functions in the ATP-dependent chromatin remodeling in yeast (42). RSC has been implicated in a variety of cellular activities, including regulation of gene expression in response to stress and cell cycle progression through its ability to mobilize nucleosomes (3, 14, 15, 28). Recently, RSC was also implicated in loading of cohesin onto chromosome arms (5, 28). Interestingly, diploid strains with the homozygous deletion of *RSC1* or *RSC2* show hypersensitivity to γ-irradiation, bleomycin, and methyl methane sulfonate (MMS) treatment (9). Furthermore, the temperature-sensitive mutant allele of Sth1, which has amino acid substitutions within the bromodomain, sensitizes cells to several DNA damaging agents at the permissive temperature (35). Reduced expression of Sth1 also causes cells to become sensitive to DNA damage (35). Taken together, the

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available data suggest that RSC functions in repair of DNA damage, particularly DSBs.

We initiated a genetic screen using the NHEJ assay with a chromosomal DSB to uncover factors required for repair of a DSB in a chromatin context. Through this effort, we have identified *RSC8* and *RSC30*, components of the essential chromatin remodeling complex RSC, as being required for efficient end joining repair. RSC is rapidly recruited to an HO endonuclease-generated DSB and functionally associates with the core NHEJ proteins Mre11 and yKu80. These results implicate a direct role of the RSC complex in NHEJ.

MATERIALS AND METHODS

Media, genetic methods, and strains. Standard procedures were used for media preparation, transformation, sporulation, and tetrad analysis. The cell cycle profiles and the damage-induced checkpoint status were determined by the fluorescence-activated cell sorter analysis using a FACStation (Becton Dickinson Co.) as described previously (39). Strain genotypes are listed in Table S1 of the supplemental material.

HO endonuclease induction. JKM179 cells (*hml*Δ *MAT*α *hmr*Δ *GAL*::*HO*) were grown in preinduction medium (yeast extract-peptone [YEP]–glycerol) at 30°C and then spread onto plates containing either galactose (YEPGAL) or glucose (YEPD) (39). The survival rate was determined by dividing the number of colonies on YEPGAL by the number of colonies on YEPD.

Plasmid end joining assay. The centromeric plasmid pRS314 was linearized by restriction enzymes and was used to transform yeast cells. A parallel transformation was performed using an equal amount of uncut plasmid to normalize the transformation efficiency (40).

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed as described previously with minor modifications (62). Briefly, cultures grown to a density between 1×10^7 and 2×10^7 cells/ml in preinduction medium (YEP-glycerol) were induced for HO endonuclease by adding 2% galactose. To measure dissociation kinetics of Sth1-tandem affinity purification (TAP) and yKu70/80 complex from a DSB, cells were incubated with 2% galactose for 90 min and then 2% glucose was added to the medium to repress HO expression. For immunoprecipitations, the sonicated extracts were incubated with either anti-yKu70/80 antibody–protein G beads (kindly provided by A. Tomkinson), 10 μl of immunoglobulin G (IgG)-agarose beads (for Sth1-TAP), or antihemagglutinin (HA)-protein G beads (for Rsc8-HA) at 4°C for 2 to 4 h. PCR mixtures included 1 pmol (0.3 μl) [α-^{32}P]dATP per 50-μl reaction mixture for quantification of the amplified products. Samples were run on 5% polyacrylamide gels, which were dried and analyzed in the PhosporImager (Amersham Biosciences). Primers used for ChIPs are listed in Table S2 of the supplemental material.

Yeast two-hybrid assay. The NHEJ proteins (yKu70, yKu80, Lif1, Dnl4, Mre11, Rad50, Xrs2, Pol4, and Nej1) fused to the DNA binding domain of Gal4 and the subunits of the RSC complex (Rsc1, Rsc2, Rsc3, Rsc4, Rsc6, Rsc30, Sth1, and Sfh1) fused to the transcription activation domain of Gal4 were expressed in two different haploid yeast strains of opposite mating type (*MAT*α and *MAT***a**). The strains were then mated, and serial dilutions of the resulting diploid strains were plated on yeast synthetic media with or without histidine. Plates were incubated at 30°C for 3 to 4 days, and the activation of the reporter gene (*HIS3*) in the diploid strain indicated an interaction between protein pairs.

To isolate Rsc1 variants that were attenuated for Mre11 binding, the *RSC1* gene was mutagenized by propagating the two-hybrid plasmid that expresses the Gal4 activation domain fusion of Rsc1 (pGADT7-*RSC1*) in the *Escherichia coli mutD5* mutator strain (37). The mutated plasmid collection was then screened in the yeast two-hybrid assay for mutants that were attenuated for Mre11 interaction. After isolation of plasmids from candidate colonies and subsequent sequence analysis, the mutant *rsc1* genes were subcloned into pRS314.

Pull-down assay for detecting protein complexes in extracts. Rsc1-TAP, Rsc2-TAP, Rsc4-TAP, and Swi3-TAP strains (Open Biosystem) that harbor a plasmid expressing glutathione *S*-transferase (GST) (pHQ241) or GST-Mre11 (pSM344) (45) under the control of the *GAL* promoter were induced with 2% galactose for 16 h at 30°C to induce the synthesis of the latter two proteins. Harvested cells were resuspended with lysis buffer (50 mM HEPES, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100) containing protease inhibitors (1 mM benzamidine-HCl, 1 mg/ml bacitracin, 0.1 mM phenylmethylsulfonyl fluoride, 0.4 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin) and then lysed by vortexing with glass beads for 10 min at 4°C. Extracts (0.5 mg) were treated with 20 μg/ml ethidium bromide on ice for half an hour and then incubated with 30 μl of glutathione-Sepharose 4B beads for 4 h at 4°C. After washing with lysis buffer, beads were collected by centrifugation and the bound proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride membrane. The TAP fusion proteins were detected by peroxidase-antiperoxidase-soluble complex (Sigma). GST-Mre11 proteins were detected by immunoblotting with anti-Mre11 antibody (a gift from P. Sung) (64).

Pull-down assay involving ^{35}S-labeled proteins. GST-Mre11 and GST were purified from *E. coli* BL21(pLysS) cells harboring either pGEX4T-1 or pGEX4T-1-Mre11 by affinity chromatography, using glutathione-Sepharose 4B. The plasmids pGAD-T7-*RSC1*, pGAD-T7-*RSC1*-1M, pGAD-T7-RSC1-2M, and pGAD-T7-*RSC2* were used as templates to synthesize ^{35}S-labeled Rsc1, rsc1-1M, rsc1-2M, and Rsc2 by TNT-coupled reticulocyte lysate systems (Promega). GST or GST-Mre11 (1 μg) and in vitro-translated Rsc1, rsc1-1M, rsc1-2M, and Rsc2 were incubated in 20 μl of buffer A (50 mM Tris-HCl [pH 7.5], 100 mM KCl, 1 mM dithiothreitol, 1% Triton X-100), and the beads were collected by centrifugation and then washed extensively with buffer A. Proteins released from the beads were separated by SDS-PAGE and detected by phosporimaging analysis of the dried gels (Amersham Biosciences).

RESULTS

Genetic screen to identify novel NHEJ genes. In *S. cerevisiae*, when HO endonuclease creates a DSB at the mating type (*MAT*) locus, the break can be efficiently repaired by HR with one of the two silent donors of mating type information, *HML* or *HMR* (27). If HR is prevented by deletion of the donor sequences, as in the case with the *hml*Δ *MAT*α *hmr*Δ *GAL*::*HO* strain JKM179, cells repair the DSB by NHEJ only (40). We subjected JKM179 to mTn3::*LEU2* transposon mutagenesis (54) in an effort to identify novel NHEJ genes. A total of 28,000 transformants with random disruptions of the genome were isolated on glucose selective medium and then patched onto master plates. These were then replica plated onto galactose-containing medium to induce the expression of HO. To survive, cells must become HO resistant by end joining reactions that alter the HO recognition sequence to prevent recutting by HO (40, 44). As noted above, HR does not occur in this strain because of the absence of a homologous template. The level of NHEJ repair was such that we were able to clearly detect decreases in the number of papillae (representing independent NHEJ events) that appeared.

We found 22 transposon-tagged mutants that reproducibly showed NHEJ impairment. Tetrad analysis was performed on these NHEJ mutants to demonstrate that the decrease in NHEJ was a result of the transposon insertion. Mutants were then subjected to vectorette PCR and sequencing to locate the transposon insertion (Table 1). This screen identified seven known NHEJ genes, including *POL4*, which encodes a DNA polymerase that is dispensable for the rejoining of fully compatible DNA ends but important for those joining events that require end processing (65, 72). The fact that almost all isolated mutants have transposons inserted at the known NHEJ genes assures the specificity of our screen to NHEJ genes. In addition, one of the mutants, H15, which showed a pronounced defect in NHEJ of a chromosome break, has the transposon inserted in *RSC30*, which encodes a component of the essential chromatin remodeling protein complex RSC (14).

RSC functions in NHEJ. To ascertain the role of *RSC30* in NHEJ, we created an independent *KAN*^{r}-marked deletion of *RSC30* and tested the resulting *rsc30*Δ mutant for NHEJ proficiency using several assays. First, the *rsc30*Δ strain was examined for the ability to repair the HO-induced chromosomal

TABLE 1. Mutants deficient in NHEJ of a chromosome break made by HO endonuclease[a]

Disrupted NHEJ gene	Insertion site of mTn3::*LEU2*[b]	Fold reduction in survival rate in YEP-GAL[c]
Wild type		1
KU80	28	593
NEJ1	262, 376, 462 (two)[d], 628	1,778, 427, 76, 64, 49
RAD50	335, 1244, 1891	4.7, 380, 160
MRE11	342, 488, 1,486	4.7, 291, 107
POL4	322 (two)[d], 376, 959, 1,504	56, 49, 139, 21, 64
LIF1	1,115, 1,214	12.3, 9.4
DNL4	207	94
RSC30	1,278	5.1
RSC8[e]	−239[f]	8.3

[a] The identity of the mutated gene from each mutant was identified by vectorette PCR and sequencing of transposon insertion junctions.
[b] Number of nucleotides from the beginning of the open reading frame (+1).
[c] Fold reduction of survival rate from each mutant was measured by comparison with the wild-type survival rate (3.2×10^{-3}).
[d] We have independently isolated two identical mutants with the transposon insertion at the same place in the genome.
[e] The mutant has a transposon insertion at *yfr038w*. However, the gene responsible for the NHEJ phenotype appears to be *RSC8*. See the text for more details.
[f] Transposon is inserted at the 48th nucleotide from the beginning of *yfr038w* and 239 nucleotides upstream of the *RSC8* coding sequence.

DSB, just as we did in the genetic screening. The result showed a degree of NHEJ defect indistinguishable from that observed with the transposon-tagged *rsc30* mutant (Fig. 1A). A significant fraction (13/210) of the repair events were associated with large deletions (>700 bp), characterized by their **a**-like phenotype, which were not found in the *RSC30*$^+$ strain (0/227). The repair events with large deletions have been reported from other NHEJ mutants, including *yku70*Δ, *dnl4*Δ, *mre11*Δ, and *rad50*Δ mutants, and are considered a distinct feature of NHEJ deficiency (12, 44, 71). Sequence analysis of the repair junctions from the *rsc30*Δ survivors also showed that there was an increase (1.6-fold) in the type of repair events associated with ACA deletions along with the 2.4-fold reduction in those with the CA insertions (Table 2). Reduction of the CA insertion event in the *rsc30*Δ mutant was statistically significant by the Student *t* test ($P = 0.05$). More-dramatic reductions of the +CA insertions were reported previously in repair events recovered in the *mre11*Δ or the *rad50*Δ mutants (44). Importantly, exogenous expression of the plasmid-borne *RSC30* gene overcame the NHEJ defect in both the *rsc30*Δ mutant and the H15 strain, which harbors the transposon insertion at *RSC30* (Fig. 1A).

Two distinct types of NHEJ exist (26). Imprecise NHEJ occurs when the substrate ends are not complementary or when the continued presence of an endonuclease (e.g., in our genetic screening method) imposes the need for a sequence alteration at the DNA joint. The other NHEJ route involves the simple religation of complementary overhanging ends without any sequence modifications. To evaluate the role of *RSC30* in the precise NHEJ, HO endonuclease was induced in our *rsc30*Δ strain and the survival rate was determined by plating cells onto the glucose-containing medium, which represses HO expression at various points after addition of galactose. This assay showed that the *rsc30*Δ cells are impaired for precise NHEJ as well (Fig. 1B).

We also determined whether *RSC30* is required for repair of restriction enzyme-generated DSBs using a plasmid-based assay (11). The results showed that joining of linear plasmids with either 5′-BamHI or 3′-protruding ends (SphI) was reduced by about twofold in the *rsc30*Δ strain (Fig. 1C). These results support the role of *RSC30* in NHEJ of DSBs.

Deletion of *RSC30* sensitizes *rad52*Δ to genotoxic stress. The yeast NHEJ mutants display synergistic hypersensitivity to DSB-causing agents when Rad52-dependent HR is also ablated (59). We thus deleted the *RAD52* gene in the *rsc30*Δ strain and analyzed the sensitivity of the double mutant to gamma rays,

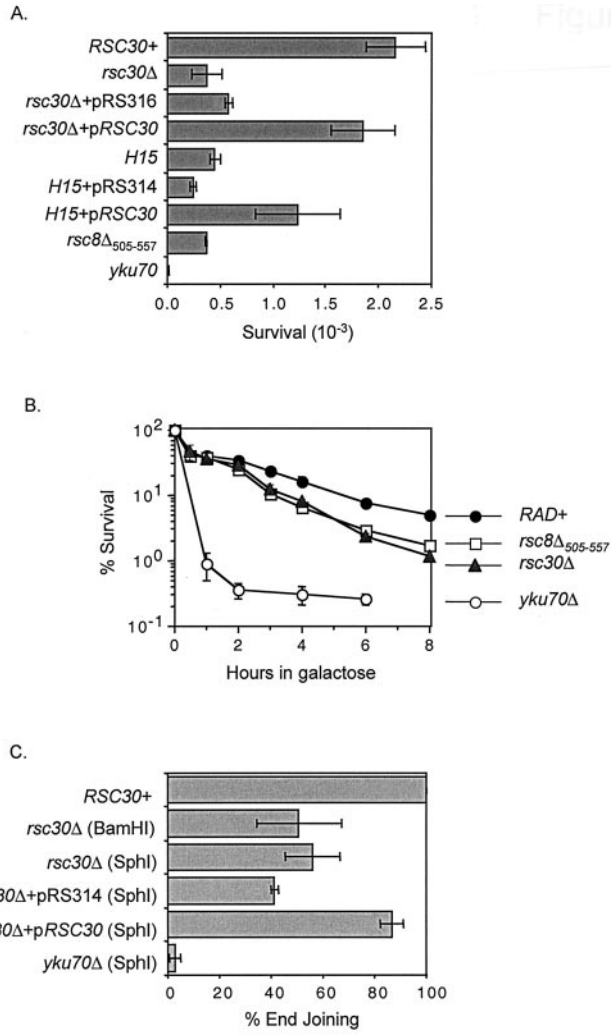

FIG. 1. Involvement of *RSC8* and *RSC30* in NHEJ. (A) NHEJ proficiency of yeast mutants was determined by assessing their survival rate upon induction of an HO break at the *MAT* locus. Lack of a homologous template (*HML* and *HMR* are deleted in these strains) constrained repair of this DSB to occur by NHEJ only. Survival rate was obtained by dividing the number of colonies on YEPGAL by the number of colonies on YEPD. (B) Survival of JKM179 (*RSC30*$^+$), *rsc30*Δ, and the carboxy-terminal-deleted *rsc8* mutant after expression of HO endonuclease for short intervals. Cultures were induced to express HO for the indicated time and plated onto YEPD medium, which shuts off HO expression. In this assay, almost every surviving cell religated and restored the complete *MAT*α locus by precise end joining (40). Each point represents the average of three or more separate experiments. (C) The efficiency of plasmid end joining was determined using a yeast centromeric plasmid linearized by SphI or BamHI. Each point represents the average from at least three independent experiments.

TABLE 2. Sequenced repair events of survivors from an HO break

Derivative	Sequence	No. of repair events	
		RAD30$^+$	*rad30*Δ
Wild type	CGCAACAGTATA[a]		
+CA	CGCAACA**CA**GTATA[b]	19/39	8/40
+ACA	CGCAACA**ACA**GTATA	1/39	1/40
+A	CGC**A**AACAGTATA	1/39	ND[c]
−ACA	CGCA---GTATA[d]	11/39	18/40
−GTA	CGCAACA---TA	1/39	ND
−A	CGC-ACAGTATA	1/39	1/40
−GCA	C---ACAGTATA	1/39	1/40
−AACA	CGC----GTATA	ND	1/40
−GC	C--AACAGTATA	1/39	7/40
−CA	CG--ACAGTATA	2/39	2/40
−C	CG-AACAGTATA	1/39	ND
C→G	CG*G*AACAGTATA[e]	ND	1/40

[a] Sequence starts at the proximal boundary of the Z1 region in *MAT*. The 3′ 4-bp overhang created after HO endonuclease activity is underlined.
[b] Base insertions are indicated by boldface type.
[c] ND, not found.
[d] -, base deletion.
[e] Mutated sequences are indicated by italics.

MMS, hydroxy urea, and phleomycin. Although the *rsc30*Δ *yku70*Δ double mutant strain was no more sensitive to these agents than the isogenic *yku70*Δ strain, the *rsc30*Δ *rad52*Δ double mutant strain was significantly more sensitive to the damage caused by these agents than the isogenic *rad52*Δ strain (Fig. 2A and B). These results are consistent with the *RSC30* being epistatic to *yKu70* and, thus, it likely functions in the NHEJ pathway of DSB repair. *RSC30* appears to be needed primarily for DSB repair, as the *rsc30*Δ mutation did not sensitize cells to UV and mitomycin C, which induce bulky DNA adducts and cross-links, respectively (Fig. 2C and data not shown). Deletion of *RSC30* also does not affect cell cycle checkpoints (see Fig. S1 in the supplemental material), meiosis, or mating type switching (data not shown).

Role of *RSC8* in NHEJ. We also identified another novel NHEJ mutant (K45) from our screen which harbors a transposon insertion at *YFR038W*. Interestingly, the NHEJ phenotype of this mutant cannot be overcome by the introduction of plasmid-borne *YFR038W* (Fig. 3B). Furthermore, an independent *KAN*^{r}-marked deletion of *YFR038W* did not cause any NHEJ defect in our chromosome break assay (data not shown). Instead, the NHEJ phenotype of K45 was fully complemented by the expression of *RSC8*, whose transcription start site is located only 239 bp away from the inserted transposon (Table 1 and Fig. 3A). Like *RSC30*, *RSC8* encodes a core subunit of the RSC complex (13, 63). We checked a temperature-sensitive allele of *RSC8* (*rsc8*$\Delta_{505\text{-}557}$) that harbors deletions of amino acid residues 505 to 557 at the carboxy terminal (63) and found a degree of NHEJ defect similar to what was seen in the transposon insertion mutant K45 under permissive conditions (Fig. 1A). This mutation also caused an apparent defect in precise NHEJ of a DSB generated by HO endonuclease (Fig. 1B). The results thus indicate that *RSC8* has an NHEJ function. The NHEJ defect seen in K45 likely stems from an altered expression of *RSC8* due to the proximity of the transposon insertion to the *RSC8* promoter region.

RSC is recruited to a DSB. RSC may regulate the transcription of NHEJ genes. However, microarray data cast doubts on this model, because none of the known NHEJ genes changes its transcription pattern more than twofold in the absence of *RSC30* (3). Alternatively, RSC may directly influence the efficiency of the NHEJ reaction. This model predicts that RSC will be physically present at the site of DSB repair. We thus investigated the association of RSC with the DSB at the *MAT* locus in a JKM179 strain by using ChIP. Yeast cells expressing TAP-tagged Sth1 (the catalytic subunit of RSC) or HA-tagged Rsc8 were induced to contain a DSB at *MAT* and were subjected to ChIP at various time points after galactose induction (0, 15, and 30 min and 1, 2, and 3 h) with IgG-agarose, which binds the protein A portion of the TAP tag or the anti-HA antibody that binds to the HA tag, respectively. Association of RSC to the DSB was quantitatively measured by PCR amplification of the immunoprecipitated DNA using five different sets of primers that anneal to the proximal and the distal side of a DSB (Fig. 4A). Recruitment of yeast Ku to the same DSB was monitored as positive control (Fig. 4D). Upon induction of the DSB, which was affirmed by Southern blot hybridization using a radiolabeled probe specific to the *MAT* locus (Fig. 4B), Sth1-TAP, Rsc8-HA, and Ku were all recruited to the HO break but not to a site located at another chromosome (Fig. 4C, D, and E). Association of Sth1-TAP with the break could be detected as early as 15 min after addition of galactose, with maximal accumulation at 3 h of HO expression (Fig. 4C). The signal decreased in the Sth1-TAP, yeast Ku, or Rsc8-HA ChIP upon omission of the formaldehyde cross-linking step (Fig. 4E and data not shown).

The increased cross-linking of Sth1-TAP to the DSB persisted for about 1 h after addition of glucose to the medium, which effectively turned off the *HO* gene expression (40), and then disappeared after 2 h (see Fig. S2A and B in the supplemental material). The cross-linking pattern of the Sth1 protein showed an excellent correlation to that of yeast Ku and to the kinetics of NHEJ product formation as determined by Southern hybridization (Fig. 4C and D; see also Fig. S2C in the supplemental material). These data indicate that RSC associates with the broken chromosome with kinetics congruent with a direct role in the NHEJ reaction.

Mre11, Rsc30, and yeast Ku proteins are required for the RSC recruitment to a DSB. To gain insights into the mechanism of RSC recruitment to a DSB, we analyzed the association of Sth1-TAP with the DSB in the end-joining-defective *mre11*Δ, *rsc30*Δ, or *yku70*Δ cells by the ChIP assay performed at various time points after HO induction. In all three mutants, a DSB was induced rapidly and efficiently (Fig. 5A). We found that the recruitment of Sth1-TAP to the DSB was drastically reduced in the *mre11*Δ strain at every time point after HO induction, indicating that Mre11 plays an indispensable role in this process (Fig. 5C). Similarly, association of Sth1-TAP to the DSB was significantly reduced in the *yku70*Δ strain at 30 min post-DSB induction (Fig. 5D). However, in the *yku70*Δ strain, the Sth1-TAP was gradually accumulated at the DSB, which eventually led to the robust recruitment of Sth1-TAP to the DSB at 2 h of HO expression (Fig. 5D). Deletion of *RSC30* also reduced the enrichment of Sth1-TAP to DNA immediately adjacent to the DSB. Remarkably, however, association of Sth1-TAP to DNA 1.1 kb distal or proximal to the DSB was fully preserved in *rsc30*Δ. The decreased association of Sth1-TAP at a DSB in the *mre11*Δ, the *yku70*Δ, or the *rsc30*Δ strains

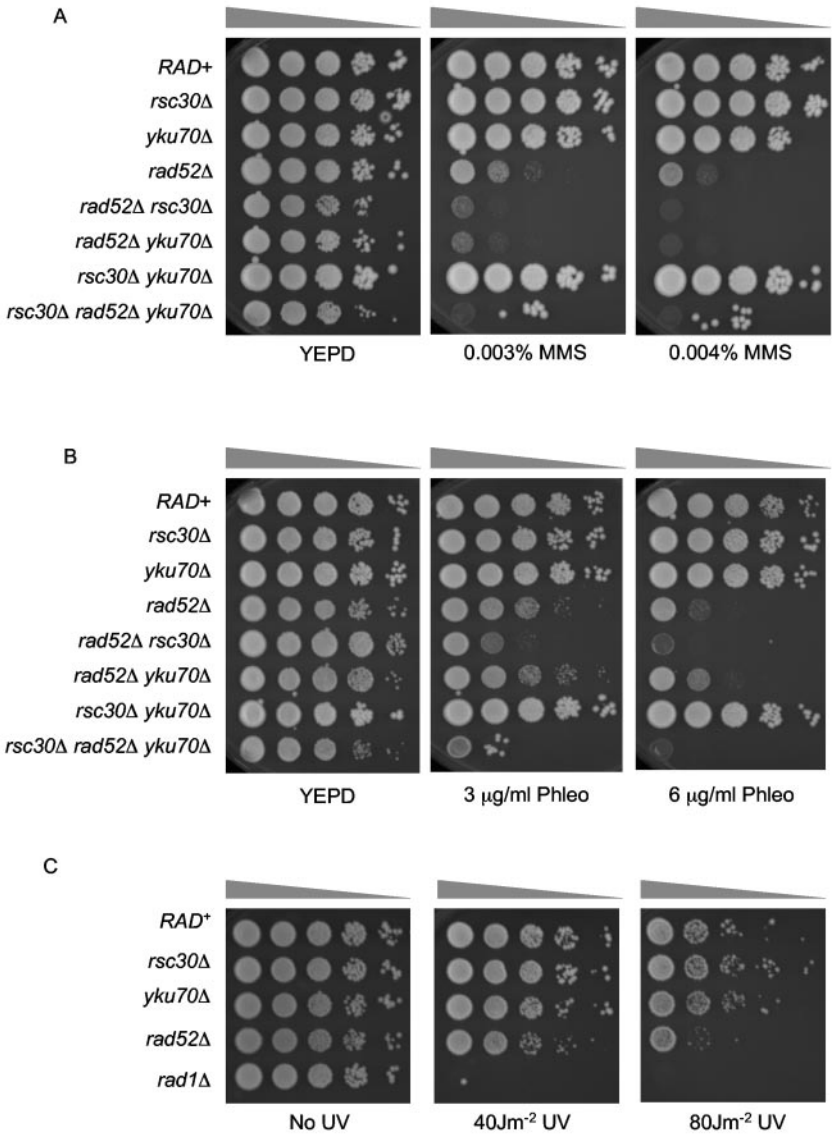

FIG. 2. The *rsc30*Δ mutant exhibits synergistic hypersensitivity to DNA damaging agents with the *rad52*Δ mutation. Serial dilutions of each strain were plated onto YEPD, MMS plates (0.003% and 0.004%), and phleomycin plates (3 μg/ml and 6 μg/ml). To measure UV sensitivity, strains were diluted on YEPD plates, irradiated using a Stratalinker UV source, and incubated for 3 to 4 days.

is not due to the reduced stability of Sth1-TAP, as the level of Sth1-TAP remains unchanged in these cells (data not shown). Taken together, we concluded that Mre11, yKu70, and Rsc30 all play important roles in the recruitment of RSC to the DSB. This result also provides evidence that efficient NHEJ is contingent upon the recruitment of RSC to the break site.

RSC interacts with the Mre11 and yKu proteins. Physical interactions among the NHEJ factors have been noted (17, 65). We used the yeast two-hybrid system in all permutations to examine possible physical interactions of the RSC subunits (Rsc1, Rsc2, Rsc3, Rsc4, Rsc6, Rsc8, Rsc30, Sfh1, and Sth1) with the known NHEJ factors (yKu70 and yKu80, Mre11, Rad50, Xrs2, Lif1, Lig4, Nej1, and Pol4). In this analysis, interactions of Rsc1 and Rsc2 with the two core NHEJ proteins, yKu80 and Mre11, were detected (Fig. 6A). A weaker interaction between Rsc1 and Lig4 was also seen (data not shown). However, the strength of such an interaction casts doubt on its physiological relevance in vivo. In contrast, no significant interactions with other subunits of RSC and NHEJ proteins were observed (Fig. 6A and data not shown).

To ascertain the interaction of Rsc1 and Rsc2 with Mre11 biochemically, we coexpressed GST-Mre11 and Rsc1-TAP or Rsc2-TAP in yeast cells and determined whether either of the latter proteins is tethered to GST-Mre11 in a pull-down assay. The results showed that both Rsc1-TAP and Rsc2-TAP physically associate with GST-Mre11, but not with GST (Fig. 6B). These protein complexes are resistant to ethidium bromide treatment, which disrupts DNA-protein interactions, providing evidence for a direct binding of Rsc1-TAP or Rsc2-TAP to GST-Mre11 (Fig. 6B). Additionally, ^{35}S-labeled Rsc1 or Rsc2 that was synthesized by coupled in vitro transcription and translation also formed a complex with GST-Mre11 (Fig. 6C, lanes 2 and 8) but not with GST (Fig. 6C, lanes 1 and 7). The noted interactions of Rsc1 and Rsc2 with Mre11 are specific,

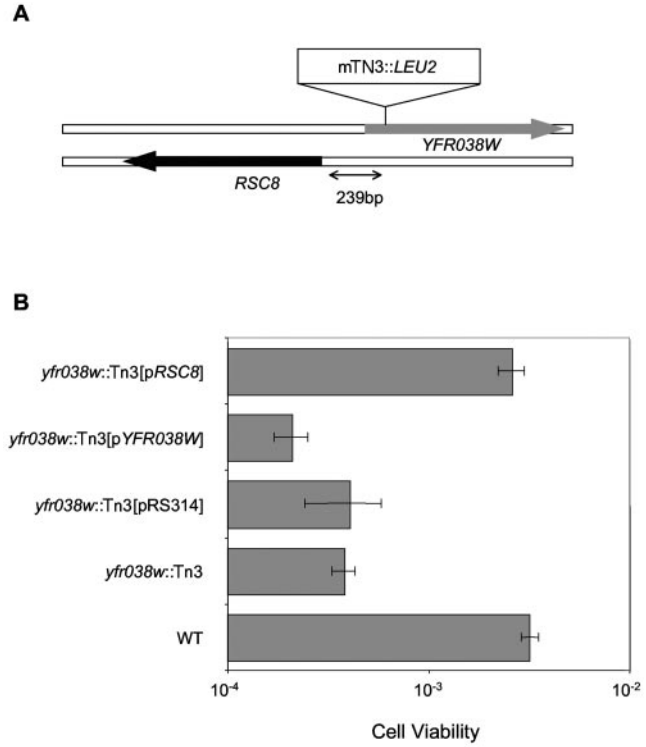

FIG. 3. Complementation of the NHEJ phenotype of the *yfr038w*::mTn3::*LEU2* mutant by *RSC8* gene expression. (A) Schematic diagram of transposon insertion in the *yfr038w*::mTn3::*LEU2* mutant and the location of *RSC8*. (B) *yfr038w*::mTn3::*LEU2* mutants transformed with either a yeast centromeric plasmid containing *YFR038W* or pIT399 (2μm; *RSC8*; a gift from M. Carlson) were induced for an HO-created DSB at *MAT* by plating onto YEPGAL. The NHEJ phenotype of this mutant can be rescued by exogenous expression of *RSC8*, but not by that of *YFR038W*. We attribute the NHEJ phenotype of this mutant to the alteration of *RSC8* gene expression because of the nearby transposon insertion.

because neither TAP-tagged Rsc4 (another bromodomain-containing RSC subunit) nor Swi3-TAP (a core component of Swi/Snf chromatin remodeler) associated with GST-Mre11 (Fig. 6B, compare lanes 2 and 4 with 6 and 8) (13, 31). These results thus revealed direct and specific interactions of Rsc1 and Rsc2 with Mre11.

Interactions between Rsc1 and Mre11 are indispensable for DSB repair. Deletion of either the *RSC1* or *RSC2* gene causes hypersensitivity to genotoxic agents, including bleomycin, MMS, and γ-irradiation (9). To ascertain whether the interaction of Rsc1 with Mre11 is important for the cellular resistance to DNA damaging agents, we used the yeast two-hybrid system to identify two *rsc1* mutations that ablate the interaction with Mre11 but do not affect binding to yKu80 (Fig. 6A) (37). Attenuated interaction of the rsc1 mutant proteins with Mre11 was confirmed by the pull-down assay using GST-Mre11 (Fig. 6C, compare lanes 4 and 6 with lane 2). Western blotting of whole-cell lysates revealed that these mutant proteins were expressed normally (Fig. 7B). The mutant rsc1 proteins also retained the ability to associate with Sth1 and appeared to be incorporated into the RSC complex (Fig. 7B). Interestingly, both mutations are present within the two bromodomains of Rsc1, raising the possibility that the bromodomains are critical for Mre11 binding (Fig. 7A). Consistent with this idea, truncation of one or both of the bromodomains weakens the interaction of Rsc1 with Mre11 (Fig. 6A and data not shown). Expression of these interaction-deficient alleles of *RSC1* either in an *rsc1*Δ or *rsc1*Δ *rsc2*Δ strain does not increase the cellular resistance to MMS or phleomycin (Fig. 7C and data not shown). This suggests that the interaction between Rsc1 and Mre11 is important for survival from DNA damage.

DISCUSSION

In vivo, DNA is packaged into chromatin, and thus the chromatin remodeling mechanisms that enhance access to the DNA should have important consequences for DNA metabolic processes, including DNA repair (24, 50, 74). Here, we report several lines of evidence supporting that RSC, an abundant ATP-dependent chromatin remodeling complex in yeast, functions in the NHEJ pathway of DSB repair. First, we identified two subunits of the RSC complex, *RSC8* and *RSC30*, as novel NHEJ genes from our genetic screen using an NHEJ assay with a chromosome break. Second, Rsc8 and Rsc30 are needed for the precise and the imprecise NHEJ of a chromosome break in vivo and the restriction enzyme-created DSB on a plasmid. Third, deletion of the *RSC30* gene causes elevated sensitivity to DSB-causing agents in the absence of the core HR gene, *RAD52*. Fourth, RSC is specifically and rapidly recruited to the HO break site, and the efficient association of RSC to a DSB depends on Mre11, yKu70, and Rsc30 proteins. Finally, two other subunits of RSC, Rsc1 and Rsc2, specifically interact with two core NHEJ subunits, Mre11 and yKu80, and the attenuation of interactions between Rsc1 and Mre11 compromised survival from genotoxic agents. The available data thus strongly support the role of RSC in DSB repair, particularly the NHEJ process.

How is RSC involved in NHEJ? Given the ability of RSC to mobilize nucleosomes at the expense of ATP hydrolysis (55), it is tempting to propose that RSC facilitates NHEJ by altering the local chromatin structure at or near DSBs and allowing better access to other NHEJ proteins. Rapid association of RSC with an HO-induced DSB and the physical and functional interactions of RSC with yKu80 and Mre11 support this view. Alternatively, a distinct chromatin structure induced by RSC at or near DSBs may promote efficient tethering of the broken chromosome ends and facilitate their end joining directly (7). It is worth noting in this regard that RSC loads cohesin onto chromosome arms (5, 28). Since human cohesin is recruited to the site of DNA damage via an association with the Mre11/Rad50/Nbs1 complex (33), and in yeast mutations that disrupt sister chromatid cohesion impair DNA repair during the G_2 phase of the cell cycle (60) and NHEJ of a linearized plasmid (56), we reason that the role of RSC in NHEJ may stem from loading cohesin to the region at or near DSBs. The elevated cohesin loading at or near DSBs could hold broken ends together to promote NHEJ of DSBs that occurs in the context of chromatin (61, 66). Lastly, while our data strongly suggest that RSC participates directly in the NHEJ reaction, we cannot yet completely rule out the possibility that it also affects NHEJ efficiency by regulating expression of an as-yet-unidentified NHEJ gene.

One interesting aspect of our results is the demonstration of

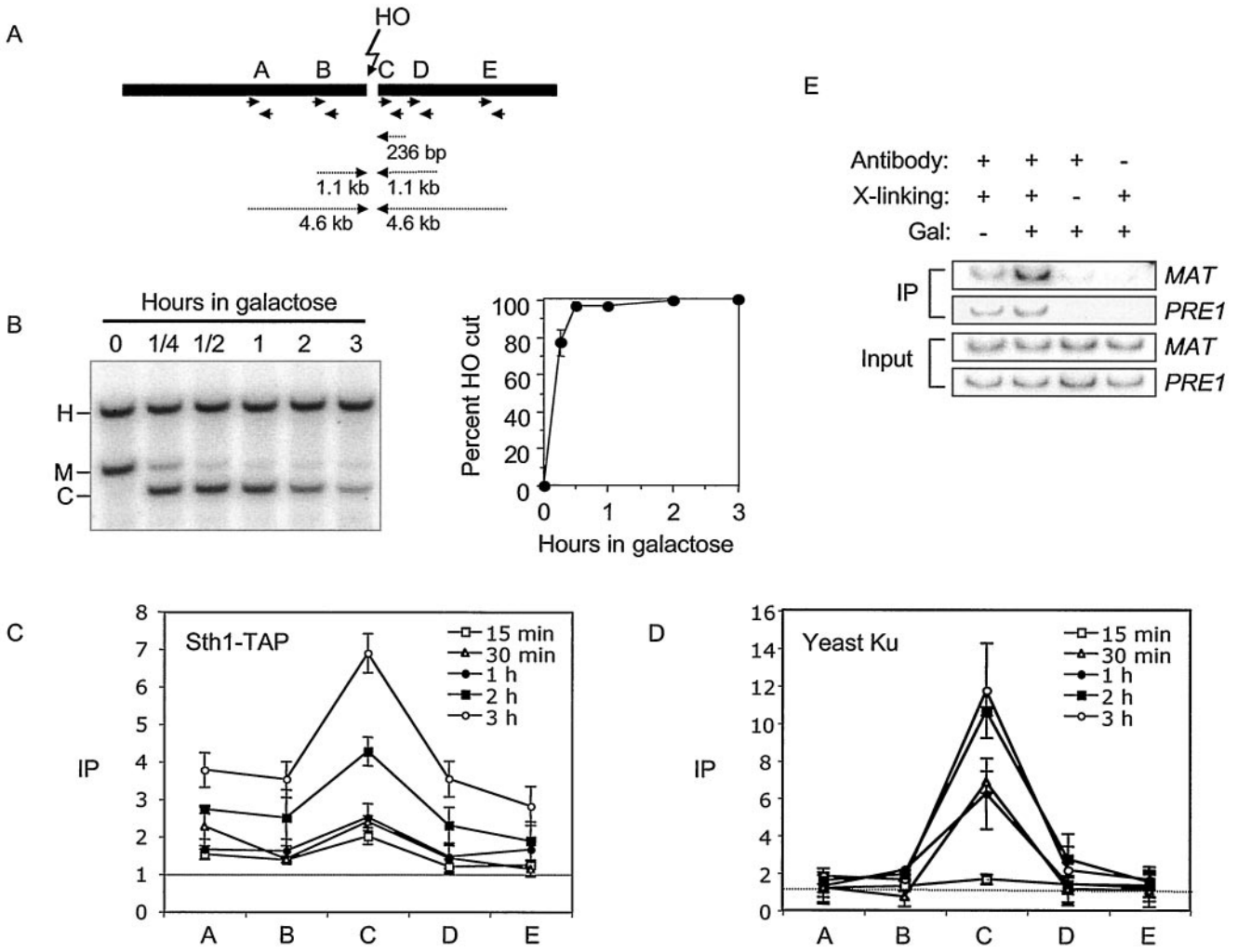

FIG. 4. Recruitment of Sth1 and Rsc8 to an HO break. (A) Locations of the five sets of primers (A to E) used in the ChIP assays in relation to the HO break. (B) Kinetics of DSB induction at the *MAT* locus. Strain SLY413 containing Sth1-TAP was grown in galactose for the indicated times to induce HO, and DSB formation was monitored by Southern blot hybridization (39). H, a signal from the *HIS3* locus that serves as a control; M, a signal from the *MAT* locus that represents uncut fragment; C, a signal resulting from the HO cleavage. Percent HO cleavage was determined by the amount of uncut fragment signals (C) relative to the control *HIS3* band (H), normalized to the value obtained from uninduced (0 h) sample and was plotted as a function of time for HO expression. (C) SLY413 cells grown in galactose were subjected to ChIP with rabbit IgG-agarose. DNA was extracted from the immunoprecipitates and PCR amplified with five sets of primers specific for the *MAT* locus on chromosome III, or with primers specific for the *PRE1* gene situated on chromosome V as control. PCR signals from each primer set at different times of HO expression (15 and 30 min and 1, 2, and 3 h) were quantified and plotted as a graph. IP represents the ratio of the Sth1-TAP PCR signal before and after HO induction, normalized by the PCR signal of the *PRE1* control. Each point is the average of two separate experiments. (D) SLY413 cells grown in galactose were subjected to ChIP with anti-yKu antibody–protein G beads as described for panel C. (E) SLY467 cells expressing the HA-tagged Rsc8(pJMH2) were incubated in galactose-containing medium for 3 hours and subjected to ChIP with anti-HA–protein G beads as described for panel C. DNA was extracted from the immunoprecipitates and PCR amplified with primers specific for a region immediately adjacent to the HO break (primer set C) or with primers specific for the *PRE1* gene situated on a different chromosome (chromosome V) as control. Note that the PCR signal is greatly diminished upon omission of the formaldehyde cross-linking step. The input DNA samples (i.e., samples before immunoprecipitation) were also PCR amplified.

a link between RSC and the Mre11 complex. RSC and the Mre11 complexes are needed for NHEJ of DSBs, and the repair events detected in the absence of *RSC30* are strikingly similar to those in the absence of the Mre11 complex. Secondly, both RSC and the Mre11 complex are recruited to the HO-induced chromosomal DSB, with RSC recruitment dependent on the Mre11 protein (41). Third, Rsc1 and Rsc2, two subunits of RSC, physically interact with Mre11. This interaction appears important for cellular resistance to genotoxic stress, including MMS and phleomycin. We also noted that the deletion of *RSC2* led to telomere shortening without affecting the silencing of genes placed near telomeres, a phenomenon known as telomere position effect (4; S. E. Lee and E. Y. Shim, unpublished data). The Mre11 complex also plays a role in telomere length maintenance, but it is dispensable for the telomere position effect (47). Based on these observations, we propose that RSC regulates the accessibility and/or the enzymatic activity of the Mre11 complex to the damaged DNA, which is the key step to the DNA damage response and repair by mobilizing nucleosomes at or near DSBs. This model fits to

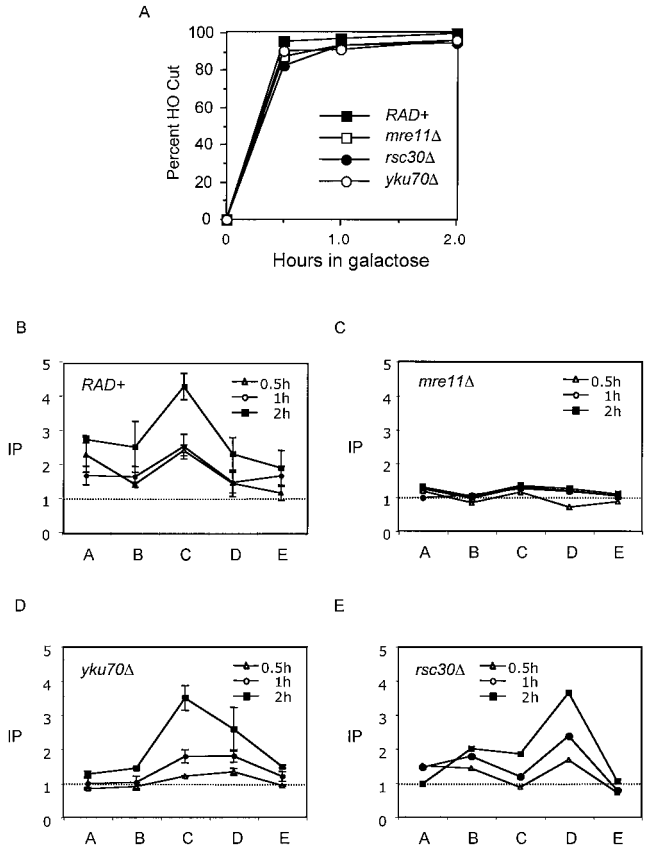

FIG. 5. Effects of *mre11*Δ, *rsc30*Δ, or *yku70*Δ on the recruitment of Sth1-TAP to a DSB were determined using the SLY460 (*rsc30*Δ), SLY480 (*mre11*Δ), and SLY481 (*yku70*Δ) strains expressing the Sth1-TAP fusion proteins by ChIP assay as described in the legend for Fig. 4. (A) Efficiency of DSB formation at the *MAT* locus from the yeast mutant strains. Cleavage efficiency was calculated as described in the legend for Fig. 4B. (B to E) Shown is the quantification of PCR signals obtained from SLY413 (*RSC30*$^+$) (B), SLY480 (*mre11*Δ) (C), SLY481 (*yku70*Δ) (D), and SLY460 (*rsc30*Δ) (E). See the legend to Fig. 4 for the definition of IP.

the pleiotropic nature of a few *rsc* mutations that display deficiency in a wide variety of DNA metabolisms, including sister chromatid cohesion, DSB repair, cell cycle progression, and chromosome segregation (3, 28, 35, 73).

In addition to its NHEJ role, we believe that RSC functions in HR as well. In fact, the hypersensitivity of cells with *RSC1* or *RSC2* deleted to genotoxic agents cannot be explained solely by an NHEJ defect (Fig. 7C) (9). Mre11p, which we have shown to associate with RSC, is required for both the HR and NHEJ pathways of DSB repair (36). We postulate that RSC-mediated alterations in chromatin architecture at DSBs influence both NHEJ and HR. Alternatively, the previously identified RSC isoforms comprised of different RSC subunits are needed for subsequent stages of the DNA repair process (3, 15).

Finally, we noted that the mutation in the *RSC30* or the *RSC8* gene caused less profound deficiency in the NHEJ of a chromosome DSB or a DSB on a plasmid than other NHEJ mutants, such as *yku70*Δ. The mild NHEJ defect of *rsc* mutations may account for the relatively moderate requirement of chromatin remodeling in DSB repair, or for the redundancy between different chromatin remodelers in DSB repair. The recruitment of at least three distinct chromatin remodelers to the DSB reinforces the latter scenario (20, 46, 68). One should also bear in mind that the *rsc* mutants examined in this work must retain some biological activity, as null mutants of RSC would be inviable (14).

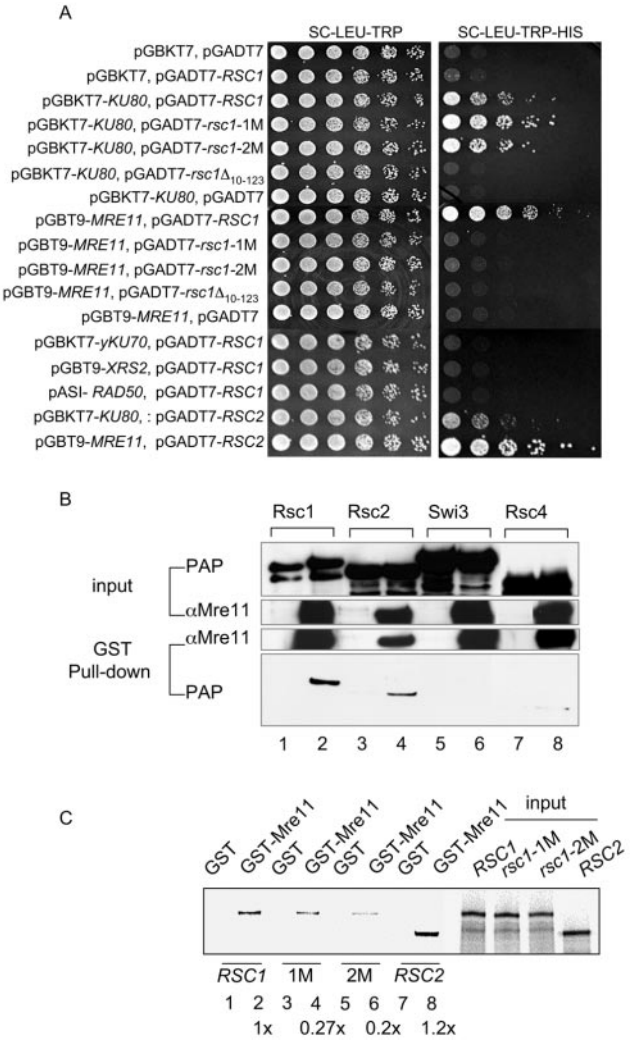

FIG. 6. Interaction of Rsc1 and Rsc2 with yKu80 and Mre11. (A) A series of yeast two-hybrid assays were carried out to examine interactions of Rsc1, the rsc1-1M and rsc1-2M mutants, and Rsc2 with several NHEJ factors. Interactions were discerned based on the ability to express the *HIS3* reporter gene by spotting cells on synthetic medium in the presence or absence of histidine. (B) Lysates from yeast cells coexpressing GST-Mre11 or GST and Rsc1-TAP, Rsc2-TAP, Rsc4-TAP, or Swi3-TAP were incubated with glutathione-Sepharose 4B, and the TAP-tagged proteins associated with GST-Mre11 were detected by immunoblotting with peroxidase-antiperoxidase (PAP). GST-Mre11 was detected by anti-Mre11 antibody (α-Mre11). The 1/10 volume of the yeast extracts used for each pull-down experiment is shown as input. (C) ^{35}S-labeled Rsc1, rsc1-1M and rsc1-2M mutant proteins, and Rsc2, obtained by coupled in vitro transcription-translation, were incubated with GST-Mre11 or GST. Proteins associated with GST-Mre11 or GST were isolated on glutathione-Sepharose beads, resolved by SDS-PAGE, and visualized by autoradiography. The numbers below lanes 2, 4, 6, and 8 report the relative affinities of the ^{35}S-labeled proteins for GST-Mre11, which were calculated by comparing the signal intensities of the Rsc1 or the rsc1 mutant derivatives pulled down from the identical amount of GST-Mre11, normalized by the amount of Rsc1 or rsc1 mutants before pull down.

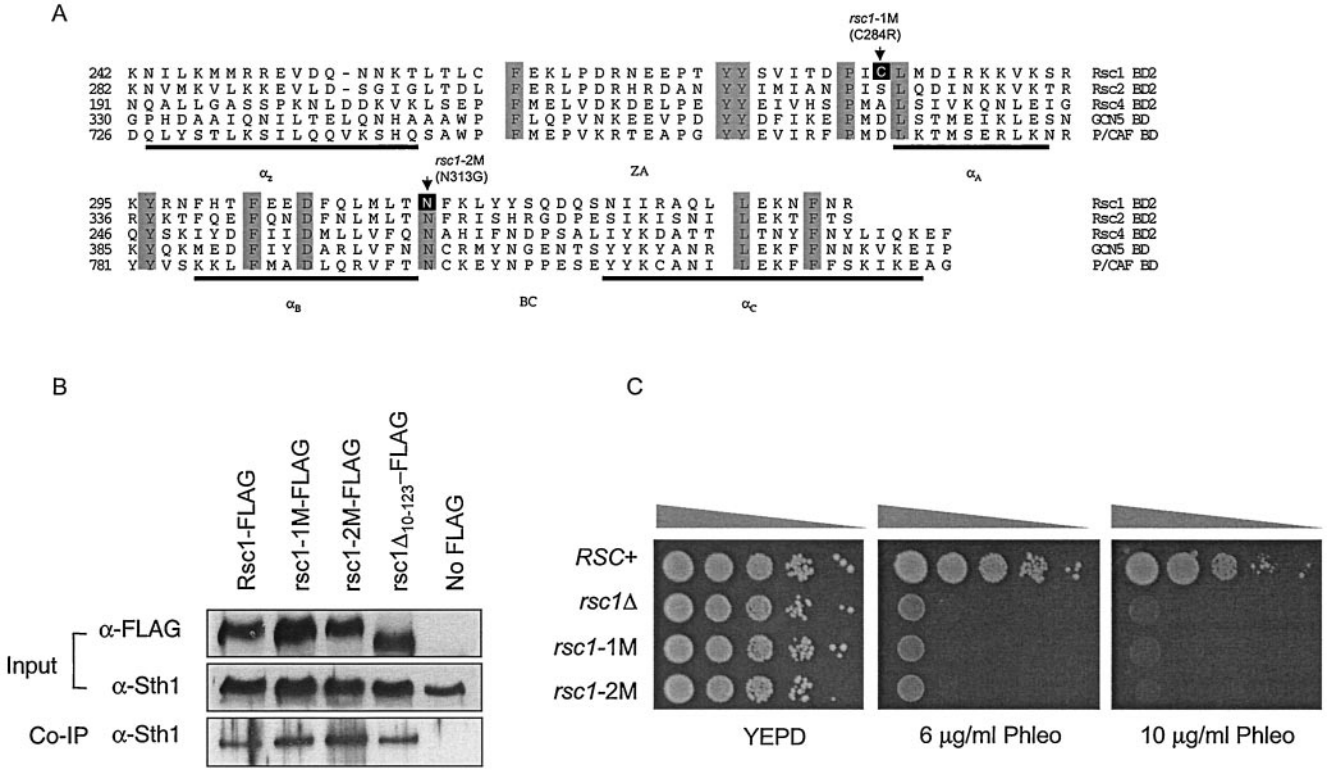

FIG. 7. Attenuated interactions between Rsc1 and Mre11 sensitized cells to genotoxic stresses. (A) Alignment of Rsc1 bromodomains with the bromodomains of yeast Gcn5, P/CAF, Rsc2, and Rsc4. Regions of identity among the five bromodomains are highlighted in gray. Four alpha-helix bundles and two long loop structures predicted by nuclear magnetic resonance studies are shown (19). Locations of the mutations in Rsc1 that reduced the interaction with Mre11 without affecting binding to yKu80 are marked by arrows. (B) Yeast extracts (200 μg) with a centromeric plasmid expressing FLAG-tagged RSC1, rsc1-1M, or rsc1-2M were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were separated on SDS-PAGE, transferred to polyvinylidene difluoride membrane, and then probed with anti-Sth1 antiserum (a gift from B. Laurent). The 1/10 volume of extract used for immunoblotting with anti-Sth1 antibody is shown as input. The level of Rsc1 or rsc1 mutant protein expression was analyzed by Western blot analysis of whole-cell extracts using anti-FLAG antiserum (Sigma). (C) Yeast strains with *RSC1* deleted but harboring a plasmid that expresses either wild-type RSC1, rsc1-1M, or rsc1-2M were generated by standard yeast transformation procedures. Serial dilutions of mid-log-phase cultures were plated onto YEPD medium with or without phleomycin. Plates were incubated at 30°C for 3 to 4 days before they were photographed.

In summary, we identified a previously undisclosed role of the RSC complex in DNA repair, in particular, NHEJ of chromosome DSBs. Human cells contain two chromatin-remodeling complexes, one of which appears to be a homolog of RSC (70). It will be of interest to test whether the human equivalent of *S. cerevisiae* RSC also functions in the repair of chromosome breaks.

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