THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 21, pp. 15064–15079, May 23, 2014

Rad52/Rad59-dependent Recombination as a Means to Rectify Faulty Okazaki Fragment Processing*

Received for publication, January 7, 2014, and in revised form, April 3, 2014 Published, JBC Papers in Press, April 7, 2014, DOI 10.1074/jbc.M114.548388

Miju Lee[‡], Chul-Hwan Lee[‡], Annie Albert Demin[‡], Palinda Ruvan Munashingha[‡], Tamir Amangyeld[‡], Buki Kwon[‡], Tim Formosa[§], and Yeon-Soo Seo[‡1]

From the [‡]Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea and the [§]Department of Biochemistry, University of Utah School of Medicine, Salt Lake City, Utah 84112

Background: How faulty Okazaki fragments are repaired remains unclear.
Results: The DNA annealing activity of Rad52 and sister chromatid cohesion are important in the repair of faulty Okazaki fragments.
Conclusion: Rad52/Rad59-mediated sister chromatid recombination is a major means of repairing faulty Okazaki fragments.
Significance: The faithful repair of faulty Okazaki fragments is critical for genome stability.

The correct removal of 5′-flap structures by Rad27 and Dna2 during Okazaki fragment maturation is crucial for the stable maintenance of genetic materials and cell viability. In this study, we identified *RAD52*, a key recombination protein, as a multicopy suppressor of *dna2-K1080E*, a lethal helicase-negative mutant allele of *DNA2* in yeasts. In contrast, the overexpression of Rad51, which works conjointly with Rad52 in canonical homologous recombination, failed to suppress the growth defect of the *dna2-K1080E* mutation, indicating that Rad52 plays a unique and distinct role in Okazaki fragment metabolism. We found that the recombination-defective Rad52-QDDD/AAAA mutant did not rescue *dna2-K1080E*, suggesting that Rad52-mediated recombination is important for suppression. The Rad52-mediated enzymatic stimulation of Dna2 or Rad27 is not a direct cause of suppression observed *in vivo*, as both Rad52 and Rad52-QDDD/AAAA proteins stimulated the endonuclease activities of both Dna2 and Rad27 to a similar extent. The recombination mediator activity of Rad52 was dispensable for the suppression, whereas both the DNA annealing activity and its ability to interact with Rad59 were essential. In addition, we found that several cohesion establishment factors, including Rsc2 and Elg1, were required for the Rad52-dependent suppression of *dna2-K1080E*. Our findings suggest a novel Rad52/Rad59-dependent, but Rad51-independent recombination pathway that could ultimately lead to the removal of faulty flaps in conjunction with cohesion establishment factors.

DNA replication, repair, and recombination are intricately networked to coordinate their functions for the stable maintenance of a vast amount of genetic materials. Most factors involved in these processes display a large number of physical and genetic interactions in a complicatedly interwoven manner (1–7). Lagging strand DNA synthesis is one such notable example that depends heavily on the collaborative actions of a number of proteins involved in all three processes because it is associated with a great risk of formation of a variety of aberrant DNA structures (8–11). Unlike leading strand DNA synthesis, lagging strand DNA synthesis proceeds discontinuously via a series of linked events that include the generation of Okazaki fragments, the removal of primer RNAs, and ligation as the final step (8, 12–14). In eukaryotes, the synthesis of an Okazaki fragment is initiated by DNA polymerase (Pol)[2] α-primase, which synthesizes ~10 nt of RNA followed by the addition of short stretches of DNA. The second DNA polymerase, Pol δ, elongates the nascent primer RNA-DNA to form a new Okazaki fragment with the aid of proliferating cell nuclear antigen (PCNA) and replication factor C (15). As Pol δ, a lagging strand DNA polymerase, encounters the previously synthesized downstream Okazaki fragment, it displaces the 5′-end region of the downstream fragment, creating a flap. The flaps are then removed by the combined action of Dna2 and Rad27, the two critical 5′-flap endonucleases, to create nicks that are sealed by Cdc9, a DNA ligase, to complete Okazaki fragment maturation (16–20). Considering that an extraordinary number of Okazaki fragments (for example, 2×10^7 in humans) are produced and processed per cell cycle, it would be highly deleterious to the cell if faulty processing of Okazaki fragments was not efficiently repaired.

Among the enzymes involved in the maturation of Okazaki fragments, Dna2 appears to link diverse DNA metabolisms by playing multiple roles in Okazaki fragment processing (11, 17, 21, 22), long-range resection of double strand break (DSB) repair (23–26), and S-phase checkpoint activation (27, 28). It has been shown that yeast Dna2 consists of three domains, each of which encodes a distinct biochemical activity: helicase, endonuclease, and secondary structure-specific DNA binding activity (28–32). The endonuclease activity of Dna2 is essential (33, 34), whereas other activities such as DNA helicase and struc-

* This work was supported by National Research Foundation of Korea Grant 2012R1A2A2A01047260 from the Ministry of Education, Science, and Technology.
[1] To whom correspondence should be addressed. Tel.: 82-42-350-2637; Fax: 82-42-350-2610; E-mail: yeonsooseo@kaist.ac.kr.

[2] The abbreviations used are: Pol, polymerase; PCNA, proliferating cell nuclear antigen; DSB, double strand break; aa, amino acid; ts, temperature-sensitive; HR, homologous recombination; SSA, single strand annealing; MRX, Mre11-Rad50-Xrs2; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; BIR, break-induced replication; SCR, sister chromatid recombination; RPA, replication protein A.

ture-specific DNA binding activities are dispensable for cell survival under some growth conditions (31, 35). For example, the Dna2 helicase activity, although normally essential, becomes dispensable when cells are grown in the presence of poor carbon sources such as lactate or glycerol (35). Cells with the *dna2Δ405N* mutant allele lacking the N-terminal 405-amino acid (aa) display temperature-sensitive (ts) growth defects (31). The endonuclease activity of Dna2 is critical for both Okazaki fragment processing and DSB resection (23, 24), whereas the N-terminal 405-aa domain of Dna2 is required for the checkpoint function and also for DSB resection to some extent (27, 36). This N-terminal domain of Dna2 becomes critical in the processing of secondary structured flaps in Okazaki fragment (28). In contrast to the multiple functions of the endonuclease and N-terminal DNA binding activities of Dna2, the helicase activity of Dna2 seems to be involved exclusively in DNA replication, most likely in the processing of secondary structure or higher order structure flaps present in a subset of Okazaki fragments (23, 27, 32, 36, 37). The failure to deal with this problem could result in DNA damages, checkpoint activation, and cell death. Therefore, the coordinated action of lagging strand replication and DNA repair would be important for the maintenance of genome integrity.

Rad52 is a key recombination protein that possesses two important functions in homologous recombination (HR). First, it facilitates the formation of the Rad51 nucleoprotein filament by replacing RPA with Rad51 (38, 39), which is termed the "recombination mediator activity" of Rad52. The C-terminal 409–420-amino acid region of Rad52, which is responsible for its interaction with Rad51, is identified as crucial for the recombination mediator function of Rad52 (40, 41). Second, Rad52 has single-stranded (ss) DNA annealing activity in its N-terminal region (42–44), which is required for the second-end capture during the canonical HR event (45) or other alternative recombination events such as synthesis-dependent strand annealing (46, 47) and single strand annealing (SSA) (43, 48). Located in the central part of Rad52, the RPA-interacting domain is responsible for the displacement of RPA from ssDNA (49, 50). The RPA displacement function of Rad52 is important for both HR mediator (50) and ssDNA annealing activities (51) because the RPA-coated ssDNA is inhibitory to Rad51-ssDNA filament formation or DNA annealing between two complementary ssDNA.

It has been suggested that HR works as a backup pathway for faulty Okazaki fragment processing. Previous genetic data show that *rad27Δ*, which is hyper-recombinogenic by itself, exhibits synthetic lethal phenotypes in combination with deletions of the genes involved in HR, implying important roles for HR in the repair of faulty Okazaki fragments (52, 53). In addition, the genetic data showing that *dna2-1 rad52Δ* and *dna2-2 rad52Δ* double mutants are synthetic lethal (9), whereas *dna2-22* and *dna2Δ405N* single mutants show increased rates of recombination, are also consistent with the above possibility (54, 58). In this article, we have identified the *RAD52* gene as a suppressor of the *dna2-K1080E* mutant in *Saccharomyces cerevisiae* that lacks ATPase/helicase activity (30, 55). The suppression was dependent on both the strand annealing activity and the ability of Rad52 to interact with Rad59 but not its recombination mediator activity. Thus, a Rad52/Rad59-dependent, but Rad51-independent, recombination pathway appears crucial for Okazaki fragment metabolism. This pathway also requires several factors for cohesion establishment. We will discuss the interplay among these factors for faithful lagging strand DNA synthesis.

MATERIALS AND METHODS

Strains—The genotypes of the *S. cerevisiae* strains used in this study are summarized in Table 1. All strains are haploid and were derived from YPH499. YJA2 has the *dna2Δ405N* allele that lacks the N-terminal 405-aa domain, whereas YJA1B lacks a chromosomal version of *DNA2* but harbors pRS316-*DNA2*, a low copy plasmid with *URA3* as a selection marker expressing wild type Dna2. YJA1BJK65, a derivative of YJA1B, possesses pRS314-*dna2-K1080E*, a low copy plasmid with *TRP1* as a selection marker containing helicase-negative *dna2-K1080E*. YMJ1 is a derivative of YJA1BJK65 that harbors pRS325-*ADH1-RAD52*, a multicopy plasmid with *LEU2* as a selection marker expressing Rad52. The *RAD50*, *RAD51*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *ELG1*, *RSC2*, and *RSC7* genes were deleted separately from YMJ1 by replacing the genes with *KanMX4* as described previously (56) and termed YMJ2 to YMJ10, respectively. The *dna2-1*, *-4*, *-5*, *-7*, *-8*, *-9*, *-12*, *-13*, *-15*, and *-16* mutant alleles were isolated previously (35).

Chemicals, Nucleotides, and Enzymes—Methyl methanesulfonate was obtained from Sigma-Aldrich. Restriction endonucleases and DNA polymerases for PCR were purchased from either Enzynomics (Daejeon, Korea) or New England Biolabs (Beverly, MA). The pRS plasmids were purchased from New England Biolabs. The pET28 vectors used for protein expression in *Escherichia coli* were from Novagen (Darmstadt, Germany). Isopropyl β-D-1-thiogalactopyranoside and X-Gal were from ElpisBiotech (Daejeon, Korea). Imidazole was from Acros Organics (Geel, Belgium). A uracil analog, 5-fluorooritic acid, was obtained from Duchefa Biochemie (Haarlem, Netherlands). Antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX), Sigma-Aldrich, and Abcam (Cambridge, UK) or kindly provided (anti-H3 polyclonal antibodies) by Dr. Daeyoup Lee (Korea Advanced Institute of Science and Technology). The antibodies used are shown in Figs. 1, 3, and 4. Rad27, the yeast Fen1, was purified as described previously (17). Oligonucleotides and α-factor used in this study were synthesized commercially by Genotech (Daejeon, Korea) and Peptron (Deajeon, Korea), respectively.

Alkaline Protein Extraction from S. cerevisiae—Expression levels of proteins in yeast were determined by alkaline protein extraction (57). Briefly, cells (5×10^7, total) were harvested and resuspended in lysis buffer (150 μl) (1.85 M NaOH and 7.5% β-mercaptoethanol) followed by incubation on ice for 10 min. Trichloroacetic acid (50% v/v, 150 μl) was added, and the mixture was incubated on ice for an additional 10 min. Samples were then centrifuged at 4 °C for 10 min at 14,000 rpm. Supernatants were discarded, and 1× SDS sample buffer (50 mM Tris-HCl, pH 7.5, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromphenol blue) was added to the precipitated materials. The samples were then neutralized with 1–2 μl of 2 M Tris-HCl, pH 8.0, boiled for 10 min, and subjected to electro-

phoresis in a 10% (Tris-glycine) SDS-PAGE in Tris-glycine buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS, pH 8.5). Histone H3 used as a loading control was analyzed by a 10% (Tris-Tricine) SDS-PAGE using Tris-Tricine buffer (cathode buffer: 100 mM Tris, 100 mM Tricine, 0.1% SDS, pH 8.3; anode buffer: 200 mM Tris, pH 8.8). The levels of protein expression were examined by Western blot using antibodies as described for each experiment.

Purification of Recombinant Rad52 and Rad52-QDDD/AAAA—The *RAD52* ORF was inserted into the pET28 vector, which expresses the inserted ORF as an N-terminal His_6-tagged protein. The resulting pET28-*RAD52* plasmid was transformed into *E. coli* BL21 (CodonPlus-RIL), and cells (0.5 liter) were grown at 25 °C until the A_{600} value reached 0.5. At this point, cells were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (final concentration) for 4 h. Cells (1 liter) were harvested by centrifugation, and the cell pellet was resuspended in 30 ml of buffer T_{200} (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 1.25 μg/ml leupeptin, and 0.625 μg/ml pepstatin A). The subscript number in T_{200} indicates the concentration of NaCl in mM. Then, cells were sonicated and centrifuged at 45,000 rpm for 30 min. The supernatant was loaded onto a 4-ml SP Sepharose column (GE Healthcare) that was pre-equilibrated with T_{200}. The proteins were then eluted by a linear gradient (100 ml) of T_{200} to T_{800}. Fractions that possessed protein peaks of Rad52 were collected and adjusted to 10 mM imidazole (final concentration) and 500 mM NaCl (final concentration) followed by loading onto a 1-ml Ni^{2+}-nitrilotriacetic acid-agarose column (Qiagen, Valencia, CA) pre-equilibrated with T_{500} plus 10 mM imidazole. The column was then washed successively with 10-column volumes of T_{500} plus 10 mM imidazole and T_{500} plus 50 mM imidazole. The protein was eluted with T_{500} plus 500 mM imidazole. The eluate was subjected to glycerol gradient sedimentation (5 ml, 15–35% glycerol in buffer T_{500}) at 45,000 rpm for 24 h in a SW55 Ti rotor. Fractions (250 μl each) were collected from the bottom of the tube. Peak fractions were stored at −80 °C. The vector to express Rad52-QDDD/AAAA was constructed similarly, and the proteins were purified using the same procedure as described above.

Preparation of DNA Substrates—The preparation of DNA substrates and their labeling at the 5′-end were as described (16). The structure and position of radioisotopic labels (indicated by *asterisks* in *D* and *E*) in substrates are shown in Fig. 5. To construct 5′-flap-structured substrates, the two oligonucleotides (referred to as upstream and downstream) were annealed to the same template oligonucleotide. The sequences of oligonucleotides used were as follows: the template oligonucleotide, 5′-GAA AAC ATT ATT AAT GGC GTC GAG CTA GGC ACA AGG CGA ACT GCT AAC GG-3′ (50 mer); the upstream oligonucleotide for Dna2 substrate, 5′-CCG TTA GCA GTT CGC CTT GTG CCT A-3′ (25 mer); the upstream oligonucleotide for Rad27 substrate, 5′-CCG TTA GCA GTT CGC CTT GTG CCT AG-3′ (26 mer); the downstream oligonucleotide for Dna2 substrate, 5′-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT CGG ACG CTC GAC GCC ATT AAT AAT GTT TTC-3′ (60 mer); and the downstream oligonucleotide for Rad27 substrate, 5′-CGA ACA ATT CAG CGG CTT TAA CCG GAC GCT CGA CGC CAT TAA TAA TGT TTT C-3′ (52 mer).

Nuclease Assays—Standard nuclease assays were performed in reaction mixtures (20 μl) containing 50 mM Tris-HCl, pH 7.8, 2 mM $MgCl_2$, 2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, and DNA substrate (15 fmol). Reactions were incubated at 37 °C for 15 min followed by the addition of 4 μl of 6× stop solution (60 mM EDTA, pH 8.0, 40% sucrose, 0.6% SDS, 0.25% bromphenol blue, and 0.25% xylene cyanol). The products were subjected to electrophoresis for 30 min at 150 V in 0.5× TBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA). The gels were dried on a DEAE-cellulose paper and autoradiographed. Labeled DNA products were quantified with the use of a phosphorimaging device (Bas-1500, Fujifilm).

Measurement of Viability, Growth, and Doubling Time—Cells were grown to saturation at 25 °C for 2 days, and the cell density was measured by the use of a hemocytometer. The percentage of viable cells in the culture was determined as follows. Cells were diluted appropriately so that 400 cells were spread onto YPD plates followed by incubation at 25 °C for 3–4 days. The number of colonies formed on YPD plates was determined. Although the Rad52-mediated suppression of *dna2-K1080E* allowed cells to survive, the rescued cells were not as healthy as the wild type and were significantly larger. Therefore, the spectrophotometric determination of doubling time could not be accurate. Thus, we decided to determine the doubling time of live cells as follows. The culture of saturated cells obtained above was diluted (2×10^6 cells/ml) to a fresh medium followed by incubation at 25 °C. Samples were taken at 4-h intervals, properly diluted, and spread onto YPD plates followed by incubation at 25 °C for 3–4 days. Cell densities were determined based on the number of colonies formed. Doubling time was calculated using the software provided by Doubling Time. Each experiment was repeated three to four times, and the results are presented with *error bars* (Fig. 4*B*).

RESULTS

Rad52 Acts as a Multicopy Suppressor of dna2-K1080E—As a means of discovering novel factors involved in Okazaki fragment processing, we have continued to identify genetic suppressors that can rescue the growth defects of *dna2* mutations. We isolated a DNA fragment containing the open reading frame of *RAD52* that suppressed the lethal phenotype of *dna2-K1080E*, a helicase-defective allele of *DNA2* (32, 55) using the same screening method as described previously (58–60). Subsequent subcloning analyses using the multicopy pRS325-*ADH1* vector (containing the 2μ origin and constitutive *ADH1* promoter) confirmed that overexpression of Rad52 alone was sufficient and necessary to suppress *dna2-K1080E* mutation (Fig. 1*A*). Interestingly, overexpression of Rad51, which is loaded onto ssDNA by Rad52 to form a nucleofilament for canonical HR, failed to suppress *dna2-K1080E* (Fig. 1*A*). We examined the expression levels of Rad51 and Rad52 by Western blotting using monoclonal and polyclonal antibodies, respectively, against native forms of each protein, and we detected both Rad51 and Rad52 proteins when they were expressed exogenously (Fig. 1*B*).

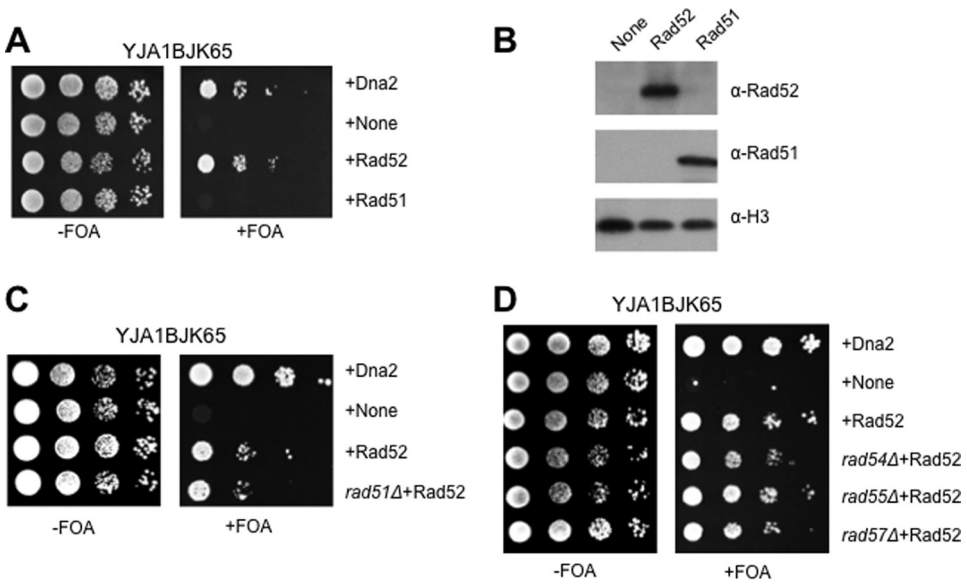

FIGURE 1. **Rad52 suppresses the lethality of the helicase-deficient *dna2-K1080E* mutation in a Rad51-independent manner.** *A*, YJA18JK65 (*dna2*::*HIS3*, pRS316-*DNA2*, and pRS314-*dna2-K1080E*) was transformed with pRS325-*ADH1* plasmids expressing Dna2 (positive control), none (negative control), Rad52, or Rad51. Transformants were inoculated in SD-HWL liquid medium (2 ml) and grown for 2–3 days until saturated. Each saturated culture was spotted in 10-fold serial dilutions on SD-HWL plates without (−*FOA*) or with (+*FOA*) 5-fluoorooritic acid (0.1%) followed by incubation for 5–6 days at 25 °C. *B*, expressions of Rad51 and Rad52 in each strain used in *A* were examined by Western blot using anti-Rad51 (α-Rad51; sc-133089, Santa Cruz Biotechnology) or anti-Rad52 antibodies (α-Rad52; sc-8939, Santa Cruz Biotechnology). Whole cell extracts were prepared by TCA precipitation as described under "Materials and Methods." Histone H3 was used as the loading control. α-*H3*, antibodies against histone H3. *C* and *D*, the Rad52-dependent suppression of *dna2-K1080E* was examined in mutant cells lacking a gene belonging to the *RAD52*-epistatic group; Rad52 overexpression in *rad51*-null cells (*C*, indicated as *rad51*Δ+Rad52); Rad52 overexpression in *rad54*-, *rad55*-, and *rad57*-null cells (*D*, indicated as *rad54*Δ+Rad52, *rad55*Δ+Rad52, and *rad57*Δ+Rad52, respectively). Cells were grown and spotted as described for *A*.

A marked difference in the abilities of Rad51 and Rad52 to suppress *dna2-K1080E* prompted us to further verify that the suppression is independent of *RAD51*. To this end, we overexpressed Rad52 in the *rad51*-null strain (termed YMJ3; see Table 1 for genotype) and found that the suppression is independent of *RAD51* (Fig. 1*C*). This result suggests that the suppression observed is primarily due to a function of Rad52. We also investigated whether *RAD54*, *RAD55*, and *RAD57*, which work cooperatively with Rad51 in Rad51-dependent HR, are also involved in the suppression. Rad54 interacts with Rad51 nucleoprotein filaments and promotes the unwinding of duplex DNA to facilitate pairing between the recipient and the incoming ssDNA (3, 47). Rad55 forms a heterodimer complex with Rad57, and this complex stabilizes Rad51 nucleoprotein filaments (3, 47). Considering that they all are related to a Rad51 function, it is predicted that overexpression of Rad52 is able to suppress *dna2-K1080E* in the absence of Rad54, Rad55, or Rad57. To address this issue, we examined the influence of Rad52 overexpression in the *rad54*-, *rad55*-, and *rad57*-null strains (YMJ4, YMJ5, and YMJ6, respectively). Consistent with this prediction, we found that the *Rad52*-mediated suppression was not affected by the deletion of *RAD54*, *RAD55*, and *RAD57* (Fig. 1*D*). These observations indicate that the suppression is independent of the formation of Rad51 nucleoprotein filament and its invasion to homologous sequence.

Rad52 Fails to Suppress the N-terminally Deleted dna2 but Is Able to Suppress Mutant dna2 Alleles in Catalytic Domains—To confirm that the suppression is not restricted to the helicase-negative mutant *dna2* allele, we examined whether all ts mutant alleles of *dna2* isolated previously (35) could be suppressed by overexpression of Rad52 but not Rad51. These ts *dna2* mutants contained one or two aa substitutions, largely in the nuclease or helicase domain, and some of them (*dna2-1*, *dna2-8*, and *dna2-16*) displayed methyl methanesulfonate sensitivity (Fig. 2*A*). In this test, we also included the *dna2*Δ*405N* allele, which is devoid of the N-terminal 405-aa domain that confers a ts phenotype. Neither Rad51 nor Rad52 suppressed the ts growth defect of *dna2*Δ*405N* upon overexpression (Fig. 2*B*). It was shown that the N-terminal 405-aa deletion is not a loss-of-function mutation in enzymatic activity; the Dna2Δ405N mutant enzyme is catalytically as active or more active than wild type Dna2 under some circumstances (28, 31). This observation suggests that the types of DNA damages caused by *dna2*Δ*405N* are not critically dependent on the elevated levels of Rad51 or Rad52 for repair. In contrast, most ts *dna2* mutations mapped in the catalytic domains were suppressed specifically by overexpression of Rad52 but not by Rad51, although the levels of suppression varied (Fig. 2*C*). Among them, the *dna2-7* and *dna2-16* mutants were most efficiently rescued by Rad52 overexpression (Fig. 2*C*). These findings indicate that the Rad52-dependent, but Rad51-independent, pathway contributes to the repair of DNA damages that could occur in catalytically defective *dna2* cells.

Overexpression of Rad52 Suppresses the Defects Associated Specifically with Processing of Okazaki Fragments—Dna2 plays multiple roles during DNA metabolisms by participating in the processing of Okazaki fragments, long-range resection of DSB repair, and S-phase checkpoint activation. Among these, Okazaki fragment processing is an essential process, depending on the coordinated interplay between the N-terminal DNA-binding domain and the endonuclease and helicase activities of Dna2. Because the helicase activity of Dna2 was shown to be

TABLE 1
Yeast strains used in this study

Strain	Genotype	Reference
YPH499	*MATa ade2–101 ura3–52 lys2–801 trp1–63 his3–200 leu2-Δ1 GAL+*	Ref. 103
YJA2	*MATa ade2–101 ura3–52 lys2–801 trp1–63 his3–200 leu2-Δ1 GAL + dna2Δ405N*	Ref. 31
YJA1B	*MATa ade2–101 ura3–52 lys2–801 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL + dna2::HIS3* [pRS316-*DNA2*]	Ref. 58
YJA1BJK65	*MATa ade2-101 ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL+ dna2::HIS3* [pRS316-*DNA2*] [pRS314-*dna2K1080E*]	Ref. 58
YMJ1	*MATa ade2-101 ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL+ dna2::HIS3* [pRS316-*DNA2*] [pRS314-*dna2K1080E*] [pRS325-*ADH1-RAD52*]	This study
YMJ2	*MATa ade2-101 ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL+ dna2::HIS3 rad50::KanMX4* [pRS316-*DNA2*] [pRS314-*dna2K1080E*] [pRS325-*ADH1-RAD52*]	This study
YMJ3	*MATa ade2–101 ura3–52 lys2–801 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL+ dna2::HIS3 rad51::KanMX4* [pRS316-*DNA2*] [pRS314-*dna2K1080E*] [pRS325-*ADH1-RAD52*]	This study
YMJ4	*MATa ade2-101 ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL+ dna2::HIS3 rad54::KanMX4* [pRS316-*DNA2*] [pRS314-*dna2K1080E*] [pRS325-*ADH1-RAD52*]	This study
YMJ5	*MATa ade2-101 ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL+ dna2::HIS3 rad55::KanMX4* [pRS316-*DNA2*] [pRS314-*dna2K1080E*] [pRS325-*ADH1-RAD52*]	This study
YMJ6	*MATa ade2-101 ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL+ dna2::HIS3 rad57::KanMX4* [pRS316-*DNA2*] [pRS314-*dna2K1081E*] [pRS325-*ADH1-RAD52*]	This study
YMJ7	*MATa ade2-101 ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL+ dna2::HIS3 rad59::KanMX4* [pRS316-*DNA2*] [pRS314-*dna2K1082E*] [pRS325-*ADH1-RAD52*]	This study
YMJ8	*MATa ade2-101 ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL+ dna2::HIS3 elg1::KanMX4* [pRS316-*DNA2*] [pRS314-*dna2K1082E*] [pRS325-*ADH1-RAD52*]	This study
YMJ9	*MATa ade2-101 ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL+ dna2::HIS3 rsc2::KanMX4* [pRS316-*DNA2*] [pRS314-*dna2K1083E*] [pRS325-*ADH1-RAD52*]	This study
YMJ10	*MATa ade2-101 ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL+ dna2::HIS3 rsc7::KanMX4* [pRS316-*DNA2*] [pRS314-*dna2K1083E*] [pRS325-*ADH1-RAD52*]	This study

dispensable for DSB resection or checkpoint activation as described above, it is assumed that Dna2 helicase activity participates exclusively in DNA replication by resolving the structured flaps present in a subset of Okazaki fragments. Nevertheless, it is still possible that the helicase function of Dna2 could contribute to DSB resection under specific circumstances, for example, when DSB occurs in structure-forming sequences. However, we wanted to confirm that the lethality of *dna2-K1080E* is not caused by the failure of DSB resection in the absence of the helicase activity of Dna2. To this end, we constructed a *dna2-K1080E rad50*Δ strain (YMJ2). In the absence of a functional copy of *RAD50*, Dna2 could not be recruited to the sites of DSB for the subsequent long-range resection, because the Mre11-Rad50-Xrs2 (MRX) complex bound to DSB provides a structural platform to recruit the Sgs1-Dna2 resection machinery (61). As shown in Fig. 3*A*, the overexpression of Rad52 allowed the *dna2-K1080E rad50*Δ double mutant cells to grow, suggesting that the suppression of *dna2-K1080E* by Rad52 is independent of the MRX complex required for the Dna2-mediated long-range resection. It should be noted that the control *rad50*Δ (*rad50*Δ *dna2*Δ + pRS316-*DNA2* + pRS314-*dna2-K1080E*) cells displayed a slight growth defect compared with *RAD50* control cells. Hence, we concluded that the Rad52-dependent suppression of *dna2-K1080E* occurs in the absence of Dna2-mediated DSB resection.

Then we examined whether *RAD27*, encoding the alternative nuclease yeast FEN1 for 5′-flap cleavage during Okazaki fragment processing, could rescue the lethality of *dna2-K1080E* upon overexpression. If the lethality of *dna2-K1080E* was attributed to the reduced efficiency of Okazaki fragment processing, overexpression of Rad27 could suppress the lethal phenotype of *dna2-K1080E*. In support of this assumption, overexpression of Rad27 (and Rad27-FLAG, a C-terminally FLAG epitope-tagged Rad27) efficiently suppressed the lethality of *dna2-K1080E* (Fig. 3*B*). In contrast, overexpression of Rad27-D179A (Rad27DA, a catalytically dead enzyme with an Asp to Ala substitution at amino acid 179) (62–64) could not suppress *dna2-K1080E* (Fig. 3*B*), although the expression level of this mutant protein was comparable with that of wild type Rad27 (Fig. 3*C*). Our finding that the catalytically active Rad27 only was able to suppress *dna2-K1080E* underscores the importance of endonucleolytic cleavage of the 5′-flap. Therefore, we concluded that the helicase function of Dna2 contributes critically to Okazaki fragment processing and that the lethality of *dna2-K1080E* is most likely caused by defective Okazaki fragment processing.

Overexpression of Rad52 Allows dna2-K1080E Cells to Grow but Does Not Fully Restore Their Defects—To investigate the influence of Rad52 overexpression on cell growth, we examined the viability of wild type and *dna2-K1080E* cells overexpressing Rad52 that grew mitotically (indicated as WT+Rad52 and *dna2-KE*+Rad52, respectively) as described under "Materials and Methods." Overexpression of Rad52 allowed approximately half (~57%) of the *dna2-K1080E* mutant cells to form visible colonies (Fig. 4*A*). Subsequently, we measured the doubling time of both WT+Rad52 and *dna2-KE*+Rad52 cells and found that the *dna2-KE*+Rad52 cells grew significantly more slowly compared with the WT+Rad52 cells. The doubling times of the wild type and mutant cells were ~3.0 and 4.2 h, respectively, in the presence of Rad52 overexpression (Fig. 4*B*). Considering that the repair of HO-induced DSB takes 2–4 h to complete (65), this result raises the possibility that the structure of DNA damages or the repair pathway could differ from HO-induced DSB and thus does not rely on long-range or extensive DSB resection for repair.

The slow growth and reduced viability observed with *dna2-KE*+Rad52 cells indicate that they are still defective in overall DNA replication and repair process. Thus, it is highly likely that the genomic DNA in the mutant cells may not be intact and contains damages. To address this issue, we decided to investigate the extent of serine 129 phosphorylation of H2A (H2A-Ser^{129}), a landmark event indicative of DSB occurrence *in vivo*

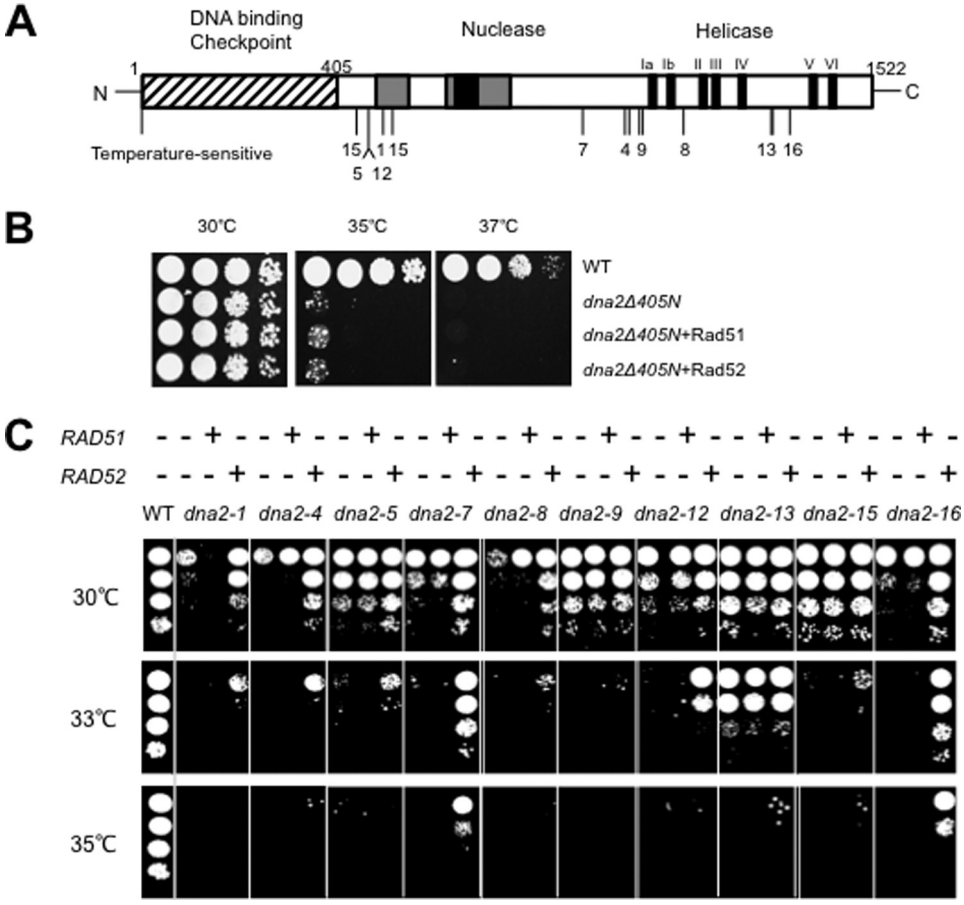

FIGURE 2. **Overexpression of Rad52 suppresses temperature sensitivity of *dna2* mutant alleles.** *A*, a schematic representation of Dna2. Locations of three domains and various ts *dna2* alleles isolated previously by others (35) are as indicated. The mutant alleles include *dna2-1* (P504S), *-4* (D1015N P1031L), *-5* (H471Y), *-7* (G913D), *-8* (H1129Y), *-9* (A1036V P1031S), *-12* (H471Y), *-13* (P1311L, T1312I), *-15* (G446A, R521K), and *-16* (G1350E). Amino acid substitutions are indicated in *parentheses*. The N-terminal 405-aa domain is indicated by a *hatched box*. The conserved parts of the Dna2 nuclease domain are indicated by *gray boxes*. The *black box* in the nuclease domain denotes the RecB homology region. The seven motifs (denoted *Ia* to *VI*) common to helicase superfamily I are indicated by *black strips*. *B*, overexpression of Rad51 and Rad52 was examined in *dna2Δ405N* cells. The pRS325-*ADH1*, pRS325-*ADH1-RAD51*, and pRS325-*ADH1-RAD52* plasmids were introduced in *dna2Δ405N* cells (indicated as *dna2Δ405N dna2Δ405N*+RAD51, and *dna2Δ405N*+RAD52, respectively). Transformants were grown in liquid SD-L medium and spotted in 10-fold serial dilutions in YPD plates followed by incubation at 30, 35, and 37 °C for 3 days. *C*, pRS325-*ADH1-RAD51* or pRS325-*ADH1-RAD52* plasmid was introduced into the ts *dna2* mutants shown in *A*. Transformants were grown in liquid SD-L medium and spotted in 10-fold serial dilutions in YPD plates and incubated at 30, 33, and 35 °C for 3 days.

(66). When we examined asynchronous logarithmically growing cells ($0.4 < A_{600} < 0.6$), no significant difference was observed on the levels of H2A-Ser^{129} phosphorylation between WT+Rad52 and *dna2-KE*+Rad52 (Fig. 4*C*, compare *lanes 1* and *7*). We then treated cells with α-factor, which arrests cells at the G_1/S boundary, and found that the extent of H2A-Ser^{129} phosphorylation markedly (>70%) decreased in WT+Rad52, whereas no significant change was observed in *dna2-KE*+Rad52 (Fig. 4*C*, compare *lanes 2–5* with *8–11*). This result suggests that cell cycle progression is retarded in *dna2-KE*+Rad52 cells because of persistent DNA damages that can be converted into DSBs.

We also analyzed under a microscope the morphology of both wild type and *dna2-K1080E* cells overexpressing Rad52 as described previously (67, 68). Microscopic analyses revealed significant differences in size and morphology between WT+Rad52 and *dna2-KE*+Rad52 cells; *dna2-KE*+Rad52 cells were considerably larger than wild type (data not shown), and the population of dumbbell-shaped (G_2/M phase) cells increased markedly (2.3 times) compared with WT+Rad52 (Fig. 4*D*). In addition, we found that the duplicated nuclei of dumbbell-shaped cells were not properly segregated to daughter cells, accumulating in the bud neck in *dna2-KE*+Rad52, although nuclei were normally segregated to daughter cells in WT+Rad52. > 50% of the cells in *dna2-KE*+Rad52 had an unsegregated single nucleus in the bud neck, whereas ~10% of the WT+Rad52 cells displayed such a phenotype (Fig. 4*E*). This result is in keeping with our prediction that the *dna2-K1080E* cells still possess some problems in their DNA, despite the fact that overexpression of Rad52 renders *dna2-K1080E* cells viable.

Direct Stimulation of Endonuclease Activity of Dna2 and Rad27 by Rad52 Is Not the Underlying Mechanism for the Suppression of dna2-K1080E—The simplest explanation for the suppression mechanism could be the direct stimulation of nuclease activity of either Dna2 or Rad27 or both by Rad52, as Rad27 and nuclease-proficient Dna2K1080E/Dna2Δ405N enzymes, upon overexpression, were able to suppress the lethal phenotype of *dna2-K1080E* (21, 31, 33, 60). Alternatively, the Rad52-mediated suppression of *dna2* mutations could be explained by efficient recombination-mediated repair of dam-

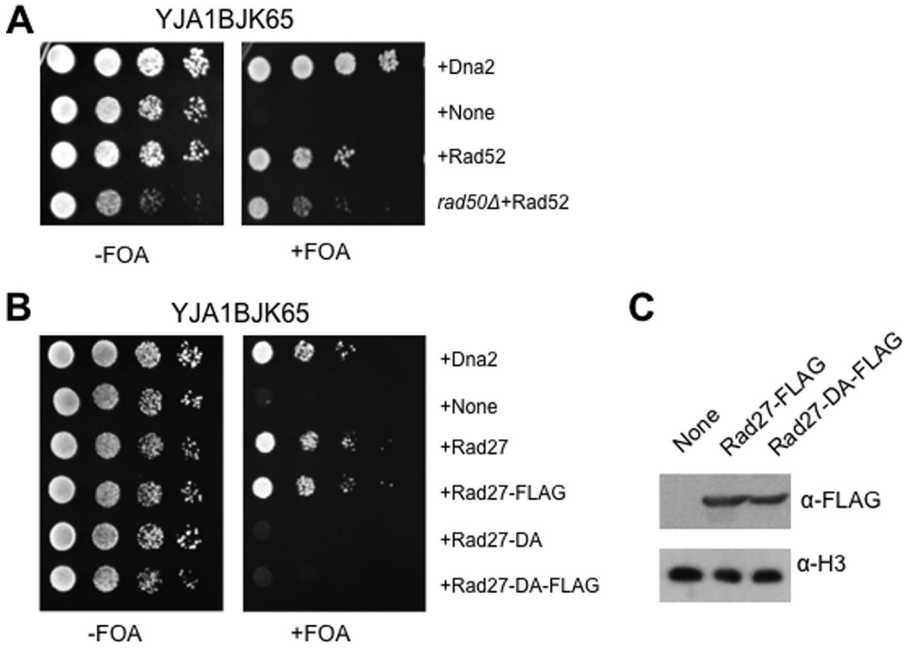

FIGURE 3. **Defects of *dna2-K1080E* can be corrected by active Rad27.** *A*, the Rad52-dependent suppression of *dna2-K1080E* was examined in *rad50*-null cells. Overexpression of Rad52 in *rad50*-null cells is indicated as *rad50*Δ+Rad52. Cells were grown and spotted as described in the legend for Fig. 1*A*. *FOA*, 5-fluorooritic acid. *B*, the YJA1BJK65 cells were transformed with pRS325-*ADH1* plasmids expressing Rad27, Rad27-FLAG (Rad27 with a FLAG tag fused to its C terminus), Rad27-DA (Rad27-D179A; catalytically defective), and Rad27-DA-FLAG. Transformants were grown and spotted as described above in the legend for Fig. 1*A*. *C*, expression levels of Rad27-FLAG and Rad27-DA-FLAG were examined by Western blot using anti-FLAG antibody (α-FLAG; F-3165, Sigma-Aldrich). Histone H3 was used as the loading control. *α-H3*, antibodies against histone H3. The experiment was done as described in the legend for Fig. 1*B*.

aged DNA lesions generated by *dna2* mutations. To distinguish between these two possibilities, we constructed another pRS325-*ADH1* vector that expresses the Rad52-QDDD-308-311-AAAA (Rad52-QDDD/AAAA) mutant protein. It was shown that Rad52-QDDD/AAAA did not support HR because of its reduced recombination mediator activity and inability to interact with RPA, although it retained normal DNA binding and annealing activities in the absence of RPA (50). Interestingly, we found that overexpression of Rad52-QDDD/AAAA failed to suppress the lethality of *dna2-K1080E* (Fig. 5*A*). This was not due to the failure of protein expression *in vivo*, as the expression level of Rad52-QDDD/AAAA was comparable to that of wild type Rad52 (Fig. 5*B*).

These data suggest that the suppression observed requires a recombination function of the Rad52 protein. However, we cannot exclude the possibility that the failure suppression was due to the failure of the mutant Rad52 to stimulate the nuclease activities of Dna2 or Rad27. To rule out this possibility, we purified both Rad52 and Rad52-QDDD/AAAA from *E. coli* to near homogeneity (Fig. 5*C*) as described under "Materials and Methods." Neither the wild type nor the mutant Rad52 proteins showed any detectable nuclease activity (Fig. 5*D*, *lanes 3* and *4*). Using these preparations, we first carried out nuclease assays with Dna2 and found that both Rad52 and Rad52-QDDD/AAAA significantly stimulated the Dna2-catalyzed cleavage of the 5′-flap substrate to a comparable extent (>4-fold with 25 fmol of Rad52 proteins) (Fig. 5*D*, *lanes 6–8* and *9–11*, respectively). Next, we examined the influence of Rad52 and Rad52-QDDD/AAAA on the endonuclease activity of Rad27. Rad27 was also stimulated markedly (~11-fold) by Rad52 (Fig. 5*E*, *lanes 5–8*) almost to the same extent as by Rad52-QDDD/AAAA (Fig. 5*E*, compare *lanes 6–8* with *9–11*). These results suggest that stimulation *in vitro* of the endonuclease activity of either Dna2 or Rad27 is dispensable for the suppression *in vivo* of the lethal phenotype of *dna2-K1080E*. Another possible explanation for the failure of the mutant Rad52 to suppress *dna2-K1080E in vivo* is an impairment of a cryptic function of Rad52 in the mutant Rad52 used that, together with its Dna2/Rad27 stimulation activity, is needed for the suppression. We also investigated the possibility, although highly remote, that the Rad52-QDDD/AAAA mutant did not stimulate the endonuclease activity of the Dna2-K1080E enzyme, unlike wild-type Rad52. We found that both wild type and mutant Rad52-QDDD/AAAA protein stimulated the Dna2-K1080E-catalyzed cleavage reaction to a comparable extent (data not shown).

We were also interested in the effect of Rad51 on the endonuclease activity of Dna2 or Rad27 because of its proximity to Rad52 in the Rad51-dependent recombination pathway. We repeated the nuclease assays using purified Rad51 protein and found that the endonuclease activities of Dna2 and Rad27 were stimulated but less efficiently by Rad51 than by Rad52 (2.7- and 4.6-fold, respectively, with 100 fmol of Rad51; data not shown). This is in keeping with our finding above that the stimulation of the endonuclease activities of Dna2 or Rad27 is not responsible for the suppression of *dna2-K1080E.*

The Rad52-dependent Suppression of dna2-K1080E Requires the DNA Annealing Activity of Rad52—The suppression of *dna2-K1080E* in a manner dependent on Rad52, but not Rad51, suggests that the recombination mediator activity of Rad52, which functions to nucleate the Rad51 recombinase onto RPA-coated ssDNA, is dispensable for the suppression observed. If this is the case, it is predicted that the Rad52 mutant protein

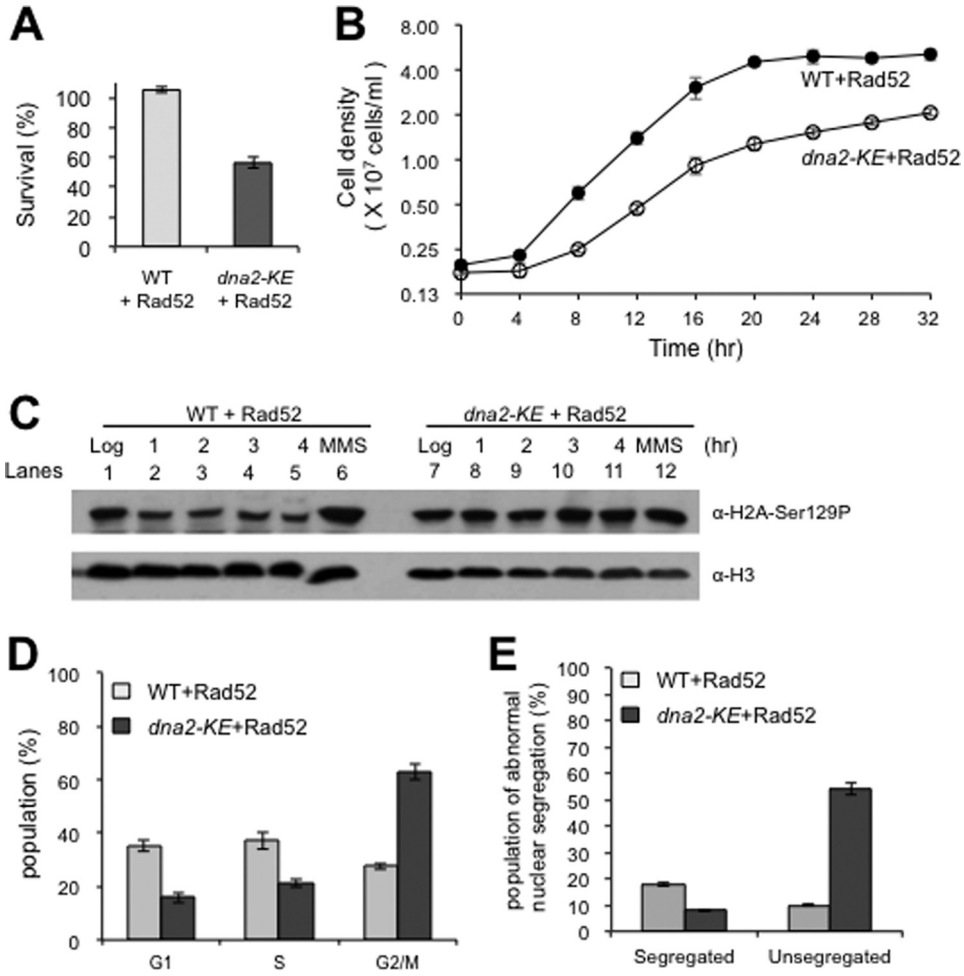

FIGURE 4. **Overexpression of Rad52 rescues the lethality *of dna2-K1080E*, but it still displays growth defects.** *A* and *B*, the survival (*A*) and growth (*B*) rates of wild type (WT+Rad52) and *dna2-K1080E* (*dna2-KE*+Rad52) cells overexpressing Rad52 were measured as described under "Materials and Methods." The experiment was repeated three times, and the results are presented with *error bars*. *C*, the levels of H2A-Ser^{129} phosphorylation were examined. The logarithmically growing cells were treated with α-factor (3 μg/ml) followed by incubation at 25 °C for 4 h. The cells were withdrawn at the indicated time point, and the total cellular proteins were prepared as described under "Materials and Methods" followed by immunoblotting with α-H2A-Ser129P antibodies raised against phosphorylated serine 129 residue of H2A (ab17353, Abcam). Cells grown for 1 h in the presence of 0.033% methyl methanesulfonate were used as a positive control for the detection of phosphorylated H2A-Ser^{129}. Histone H3 was used as loading control. *α-H3*, antibodies against histone H3. *D*, the relative distribution of the G_1-, S-, and G_2/M-phase cells in logarithmically growing cells was determined using microscopic analyses. Cells were categorized into three groups by their budding states: G_1, unbudded cells; S, budding cells; G_2/M, dumbbell-shaped cells. The experiment was repeated three times, and the results are presented with *error bars*. *E*, the morphology of nuclei was examined microscopically using cells stained with 4′,6-diamidino-2-phenylindole. The population of G_2/M-phase cells with normal (two nuclei, segregated) and defective nuclear segregation (one nucleus, unsegregated) was determined. The experiment was repeated three times, and the results are presented with *error bars*.

devoid of the recombination mediator activity could still suppress *dna2-K1080E* as efficiently as wild type Rad52. To prove this point, we constructed expression vectors that expressed Rad52Δ327 and Rad52Δ198N, the two truncated versions of Rad52, as illustrated in Fig. 6*A*. Rad52Δ327 lacks the C-terminal one-third region of Rad52 required for the interaction with Rad51 (40, 41), but it is able to support Rad51-independent recombination events such as break-induced replication (BIR) and SSA (69). In support of our prediction, we found that overexpression of Rad52Δ327 could suppress *dna2-K1080E* as efficiently as wild type (Fig. 6*B*), confirming again that suppression does not rely on the function of Rad51. In strong contrast, overexpression of Rad52Δ198N, which lacked the N-terminal 198-aa region, failed to suppress the lethality of *dna2-K1080E* (Fig. 6*B*). As the N-terminal part of Rad52 is required to facilitate the annealing of two complementary ssDNA (70), this result indicates that the DNA annealing activity of Rad52 is essential for the efficient repair of DNA damages caused by *dna2-K1080E* mutation. We were not able to show the expression of Rad52Δ327 because the available polyclonal antibodies were raised against a peptide mapped near the C terminus of yeast Rad52. We also attempted to detect the Rad52Δ198N using the same antibody but in vain; this may have been due to the low expression level or rapid degradation of this mutant protein. Nevertheless, the results obtained with overexpression of truncated Rad52 fragments support the idea that the C-terminal region (aa 328–504) of Rad52 is utterly dispensable for the suppression of *dna2-K1080E*.

As shown above, the DNA annealing activity, but not the recombination mediator activity, of Rad52 was essential for the suppression of *dna2-K1080E* mutation. The result obtained using the Rad52-QDDD/AAAA mutant protein needs further clarification, as Rad52-QDDD/AAAA is impaired in both its recombination mediator activity and its interaction with RPA

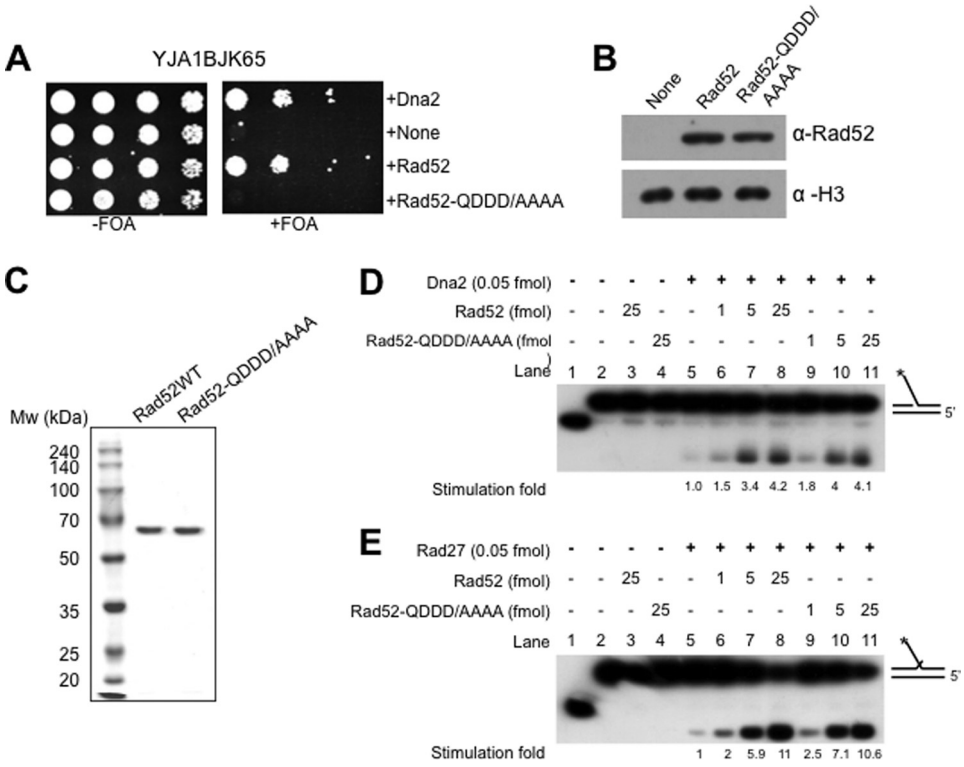

FIGURE 5. **Rad52 stimulates endonuclease activities of Dna2 and Rad27.** *A*, the pRS325-*ADH1* plasmids expressing Dna2, Rad52, and Rad52-QDDD/AAAA were introduced into YJA1BJK65. *None*, empty vector (negative control). Transformants were grown and spotted as described in the legend for Fig. 1*A*. *B*, expression levels of wild type (Rad52) and mutant (Rad52-QDDD/AAAA) proteins were examined as described in the legend for Fig. 1*B*. *C*, SDS-PAGE analysis of purified recombinant Rad52 and Rad52-QDDD/AAAA proteins. Both proteins were purified as described under "Materials and Methods." *D* and *E*, purified Rad52 proteins stimulate endonuclease activities of Dna2 and Rad27 *in vitro*. The substrates used in this experiment are indicated at the *right* of each panel. The *asterisk* represents the position of ^{32}P radiolabel. The reaction mixtures were assembled with components as indicated *above* the gel and incubated at 37 °C for 30 min. The additions and omissions are indicated + and − *signs*, respectively. Cleavage products were analyzed on a 10% denaturing polyacrylamide gel and quantified as described under "Materials and Methods." The stimulation fold obtained (with respect to Dna2 or Rad27 alone) by the addition of Rad52 proteins is indicated *below* the gel.

(50). We were interested in the influence of Rad52 on RPA-governed events such as DNA annealing. It was shown that RPA interferes with spontaneous annealing between two complementary ssDNA, which can be overcome by the addition of wild type Rad52 (44, 51). To this end, we decided to construct an expression vector that expresses the Rad52-R70A mutant protein, which has a specific defect, *i.e.* impairment in annealing RPA-coated ssDNA, but is normal in recombination mediator and DNA binding activities (51). When the Rad52-R70A mutant protein was overexpressed in *dna2-K1080E*, it suppressed the lethality of *dna2-K1080E* poorly, as shown in Fig. 6*C*, unlike wild type Rad52. This was not due to the failure of Rad52-R70A expression, because its expression level was higher than the wild type (Fig. 6*D*). This result indicates that the repair of DNA damages formed in *dna2-K1080E* mutant cells is critically dependent on a step that requires the annealing of RPA-coated complementary ssDNA.

Rad52 Works in Conjunction with Rad59 to Rescue dna2-K1080E Lethality—One additional important factor involved in Rad51-independent HR is Rad59. Rad59 shares significant homology with the N-terminal part of Rad52, and it possesses DNA annealing activity (71, 72). Rad59 was first isolated in an effort to identify genes that play roles in Rad51-independent spontaneous mitotic recombination between inverted repeats (71). Strong support for the importance of Rad59 in Rad51-independent recombination is provided by several genetic and biochemical observations. First, the *rad51*Δ *rad59*Δ double mutant displays severely reduced recombination rates that are comparable with those observed with *rad52*Δ (71, 73). Second, overexpression of Rad52 is able to restore the DNA repair or recombination defects caused by the *rad59* mutations, suggesting that Rad59 has overlapping roles with Rad52 (71, 74). Third, it has been shown that Rad59 physically interacts with Rad52 (72). These results together strongly support the possibility that the suppression of *dna2-K1080E* by *RAD52* requires a functional copy of *RAD59.* To verify this likelihood, we examined the influence of Rad52 overexpression in the *rad59*-null strain, YMJ7. As shown in Fig. 7*A*, the overexpression of Rad52 failed to suppress the lethal phenotype of *dna2-K1080E* in the absence of Rad59.

We attempted to further confirm this observation using the *rad52-L89F* mutant, which displays phenotypes similar to *rad59*Δ, most likely due to its inability to bind Rad59 (73). The intrachromosomal recombination rate between two inverted repeats is similarly diminished in *rad51*Δ *rad52-L89F* and *rad51*Δ *rad59*Δ or *rad52*Δ (73). To test whether Rad59 is involved in the Rad52-dependent suppression of *dna2-K1080E*, we constructed a plasmid overexpressing the *rad52-L89F* allele. The result was that the overexpression of Rad52-L89F poorly suppressed *dna2-K1080E* lethality, as shown in Fig. 7*B*, although the expression level of Rad52-L89F was higher (~2-fold) than that of wild type Rad52 (Fig. 7*C*). This result indicates

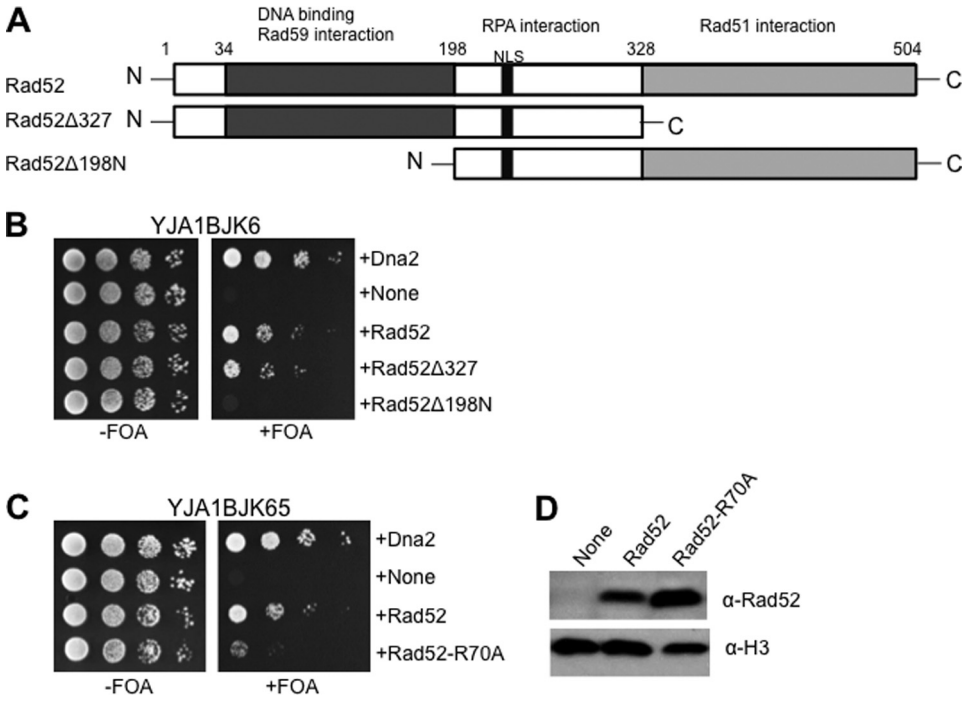

FIGURE 6. **The Rad52-dependent suppression of *dna2-K1080E* depends on the DNA annealing activity of Rad52.** *A*, schematic representation of Rad52. Domains required for DNA binding and interactions with Rad59, RPA, and Rad51 are as shown. The two truncated fragments, Rad52Δ327 and Rad52Δ198N, are as indicated. *NLS*, nuclear localization signal. *B*, the YJA1BJK56 strain was transformed with each of the five pRS325-*ADH1* plasmids expressing Dna2, empty vector (*None*), Rad52, Rad52Δ327, and Rad52Δ198N. The resulting transformants were grown and spotted as described in the legend for Fig. 1*A*. *C*, the pRS325-*ADH1* plasmids expressing the proteins indicated at the *right* of the figure were transformed into YJA1BJK65. Transformants were grown and spotted as described in the legend for Fig. 1*A*. *D*, expression levels of Rad52 and Rad52-R70A proteins were examined as described in legend for Fig. 1*B*. Histone H3 was used as the loading control. *α-H3*, antibodies against histone H3.

that the physical interaction of Rad52 with Rad59 and/or their cooperative function may be important for the suppression of the *dna2-K1080E* defect.

Suppression of dna2-K1080E by Rad52 Depends on Several Factors Required for Sister Chromatid Cohesion—Previous studies on HR revealed several recombination pathways that do not require Rad51; they include SSA and BIR, although a majority of BIR is dependent on Rad51 (43, 46, 75–77). In addition, a subset of sister chromatid recombination (SCR) events, which is facilitated by the RSC chromatin-remodeling complex, also can occur independently of Rad51 (78, 79). This Rad51-independent SCR pathway has been shown to require Rsc2 and Rsc7, which are the two critical components of the RSC chromatin-remodeling complex (79). For this reason, we were interested in the involvement of the RSC complex in the suppression of *dna2-K1080E*. When Rad52 was overexpressed in *rsc2*Δ and *rsc7*Δ mutant cells (YMJ9 and YMJ10, respectively), the Rad52-dependent suppression of *dna2-K1080E* was totally abrogated in *rsc2*Δ cells but only partially in *rsc7*Δ cells (Fig. 8*A*). The levels of Rad52 expression in *rsc2*Δ and *rsc7*Δ cells were comparable with that of the wild type (Fig. 8*B*), indicating that the abrogation of suppression observed in *rsc2*Δ was not due to the lack of Rad52 protein expression (Fig. 8*B*). Therefore, we concluded that Rsc2 plays a crucial role in the Rad52-dependent suppression of *dna2-K1080E*.

Because Rsc2, a critical component for sister chromatid cohesion establishment (80, 81), is essential in the Rad52-dependent suppression of *dna2-K1080E*, it is possible that other cohesion establishment factors could also contribute to the suppression of *dna2-K1080E*. For example, Elg1 is a well known factor that is required for the establishment of sister chromatid cohesion (82, 83); deletion of *ELG1* results in a higher frequency of precocious sister chromatid separation (82). In addition, Elg1 genetically interacts with Dna2, and a synthetic lethal interaction between *elg1*Δ and *dna2-2* is reported (9). Consistent with these observations, we isolated Elg1 as a *dna2-K1080E* suppressor as shown in Fig. 8*C*. In addition, the overexpression of Elg1 resulted in the efficient suppression of the ts growth defect of *dna2*Δ*405N* (Fig. 8*D*). We also examined the influence of Rad52 overexpression on the growth of the *elg1*-null strain (YMJ8) and discovered that the Rad52-dependent suppression of *dna2-K1080E* requires a functional copy of *ELG1* because Rad52 overexpression failed to suppress the lethality of *dna2-K1080E* in the absence of Elg1 (Fig. 8*E*). Interestingly, the suppression of *dna2-K1080E* by Elg1 overexpression was not observed in the absence of Rad52 (Fig. 8*F*), indicating the functional interaction between the two proteins in repair of damages during lagging strand DNA synthesis. We discuss how they work together below.

DISCUSSION

In this study, we found that Rad52 upon overexpression rescued the lethality of *dna2-K1080E*, a DNA helicase-negative *dna2* allele, and showed that Rad52-mediated recombination could play an important role in rectifying faulty Okazaki fragment processing. The suppression observed was not attributable to the ability of Rad52 to stimulate the endonuclease activities of Dna2 or Rad27 but to the *bona fide* recombination

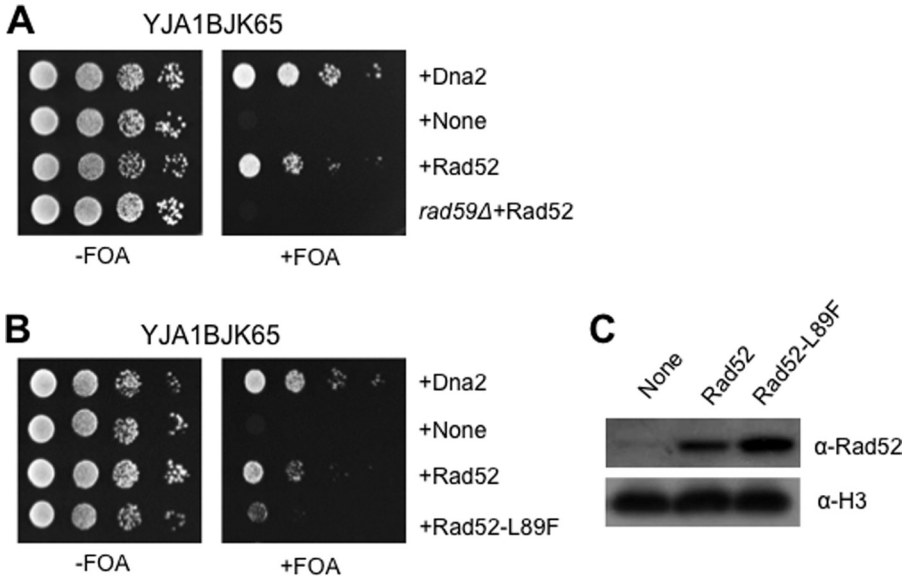

FIGURE 7. **Rad59 is essential for Rad52-mediated suppression of *dna2-K1080E*.** *A*, the Rad52-dependent suppression of *dna2-K1080E* was examined in *rad59*-null cells. Overexpression of Rad52 in *rad59*-null cells is indicated as *rad59*Δ+Rad52. Transformants were grown and spotted as described above in legend for Fig. 1*C*. *FOA*, 5-fluoroorotic acid. *B*, the pRS325-*ADH1* plasmids expressing Dna2, Rad52, and Rad52-L89F were introduced into YJA1BJK65, and the resulting transformants were grown and spotted as described in the legend for Fig. 1*A*. *C*, expression levels of Rad52 and Rad52-L89F proteins were examined as described in legend for Fig. 1*B*. Histone H3 was used as loading control. *α-H3*, antibodies against histone H3.

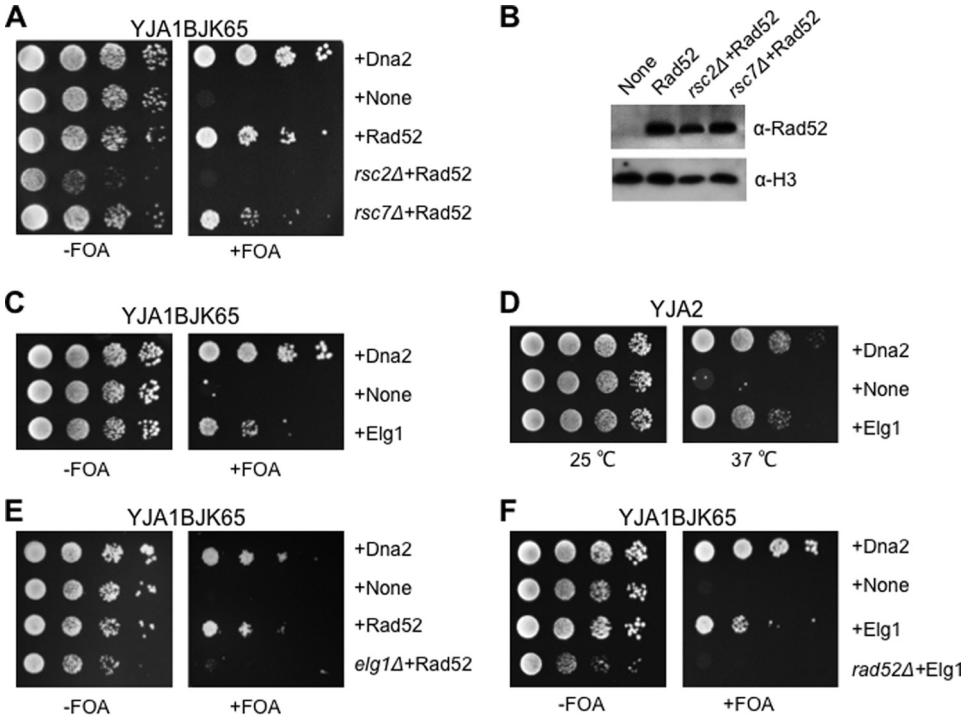

FIGURE 8. **Rsc2 and Elg1 are essential for the Rad52-dependent suppression of *dna2-K1080E*.** *A*, the Rad52-dependent suppression of *dna2-K1080E* was examined in *rsc2*- and *rsc7*-null cells. Overexpression of Rad52 in *rsc2*- or *rsc7*-null cells is indicated as *rsc2*Δ+Rad52 and *rsc7*Δ+Rad52, respectively. *FOA*, 5-fluoroorotic acid. *B*, expression levels of Rad52 proteins were examined in the absence of Rsc2 or Rsc7 as described in the legend for Fig. 1*B*. *C*, overexpression of Elg1 suppresses the lethality of *dna2-K1080E*. The pRS325-*ADH1* plasmids expressing Dna2 and Elg1 were introduced into YJA1BJK65 (*dna2*::*HIS3*, pRS314-*dna2-K1080E*, and pRS316-*DNA2*), and the resulting transformants were grown and spotted as described in the legend for Fig. 1*A*. *D*, the pRS325-*ADH1* plasmids expressing Dna2 and Elg1 were introduced into YJA2 (*dna2*Δ*405N*). Cells were grown until saturated and spotted on an SD-L plate followed by incubation at 25 and 37 °C for 3 days. *E*, the Rad52-dependent suppression of *dna2-K1080E* was examined in *elg1*-null cells as described in the legend for Fig. 1*C*. Overexpression of in *elg1*-null cells is indicated as *elg1*Δ+Rad52. *F*, the Elg1-dependent suppression of *dna2-K1080E* was examined in *rad52*-null cells. Overexpression of Elg1 in *rad52*-null cells is indicated as *rad52*Δ+Elg1.

function of Rad52. This finding suggests that stimulation of the nuclease activity of Rad27 (Mgs1 and Mus81), Dna2 (RPA), or both (Vts1, Mph1, and Rad27) might not be the only mechanism by which suppression occurred (17, 58–60, 64, 84). Previously, it was shown that the overexpression of nuclease-attenuated (34) or helicase-negative mutant Dna2 enzymes (60) or of wild type Rad27 (21) allows several *dna2* mutant cells to grow, indicating that the enhanced nuclease activity of Dna2 or Fen1

is the most plausible means to suppress the *dna2* mutations. One likely scenario accounting for our finding that robust stimulation *in vitro* of the Dna2 and Rad27 activities by Rad52 did not result in suppression *in vivo* is that the interactions between Rad52 and Dna2/Rad27 may be restricted *in vivo*. Alternatively, these levels of stimulation may not be enough to rescue *dna2-K1080E*. We, however, prefer the first possibility for the following reason: in many cases, the 5–10-fold stimulation of either Dna2 or Rad27 is sufficient to suppress growth defects of *dna2* alleles (58, 60, 84). Therefore, we believe that the underlying mechanism for the Rad52-mediated suppression of *dna2-K1080E* is due to the increased levels of recombination events.

Because the overexpression of catalytically active Rad27 suppressed the *dna2-K1080E* lethal phenotype (Fig. 3*B*), the lethality of *dna2-K1080E* could be attributed to the failure of 5′-flap cleavage, most likely leading to the generation of long flaps, which have a greater potential to form structured ones. This is consistent with the results obtained from our previous *in vitro* studies that Dna2-K1080E is not able to cleave 5′-flaps containing hairpin structures (32). This is also in keeping with the observation that overexpression of RPA suppresses *dna2-K1080E* (32); RPA is known to have the ability to destabilize short duplexes such as hairpins in 5′-flaps. Previously, it has been shown that Rad27 disengages nonproductively bound Dna2 molecules, facilitating Dna2 recycling (85). Theoretically, the Rad27-mediated recycling of Dna2 could lead to a net increase in the effective concentrations of Dna2 in cells, which in turn could lead to the suppression of *dna2-K1080E*. Our finding that nuclease-dead Rad27-DA, however, was not able to suppress *dna2-K1080E* (Fig. 3*B*) rules out the possibility that the suppression observed by Rad27 is due to the ability of Rad27 to dissociate Dna2 from DNA. It was shown that Dna2Δ405N, mutant Dna2 lacking the N-terminal 405-aa domain, was not able to recognize and bind to secondary structured flaps and that the inability of Dna2Δ405N to target to a secondary structured flap rendered this mutant enzyme severely defective in resolving secondary structure (28). Although Dna2-K1080E and Dna2Δ405N are similar with respect to their inability to process secondary structured flaps, *dna2-K1080E* cells displayed much more severe growth defects than those of *dna2Δ405N*; the former are lethal, whereas the latter grow in a temperature-sensitive manner. One conceivable explanation for this is as follows. In cells producing Dna2Δ405N, the unprocessed secondary structured flaps could be processed with the aid of other factors, such as Mph1 helicase, which may constitute redundant pathways in parallel with Dna2. In contrast, Dna2-K1080E is supposed to remain firmly bound to the structured 5′-flap, forming a nonproductive DNA-protein complex that interferes with the access of other enzymes required to process the flap. Analogous to and in support of this possibility, it has been shown that *rad27-p*, a mutant allele defective in PCNA binding, ameliorates the growth defect of nuclease-dead *rad27-n* when both mutations are combined (63). It was interpreted that the Rad27-n,p allows Okazaki fragments to be processed by other alternative nucleases such as Dna2 or even by the 3′ to 5′ exonuclease activity of Pol δ (63). Recently, it was shown that Dna2 is involved in the activation of the Mec1 kinase, a sensor protein for the intra-S-phase checkpoint activation (27, 28). Therefore, it is possible that Dna2-K1080E in a complex with the 5′-structured flap could cause cell death due to the hyperactivation of the intra-S-phase checkpoint. This possibility remains to be tested.

Our results shown above support the idea that the helicase activity of Dna2 is important in the replication of DNA sequences or chromosomal regions that can be converted into secondary or higher ordered structures when replication forks pass. They may include ribosomal and telomeric repeats and other repeats containing palindromes or simple trinucleotide repeats. Results from several studies have revealed that Dna2 is implicated in the replication or the maintenance of telomeric repeat sequences or rDNA arrays (37, 86–90). For example, the *dna2-2* mutant displayed elevated rates of DSB-associated recombination and increased levels of replication pause and fork convergence at the rDNA region (86, 91). Moreover, Dna2 is maximally associated with rDNA during S phase (86), supporting the possibility that Dna2 is importantly involved in rDNA replication. Therefore, the helicase activity of Dna2 becomes more important particularly when replication forks proceed through such structure-forming DNA regions. It would be interesting to examine which parts of chromosomes become unstable in the absence of the helicase activity of Dna2.

There have been a number of experimental data suggesting that Rad51-independent HR could play a crucial role in the repair of DNA damages associated with lagging strand DNA replication. It was shown that the *pol12-100*, *cdc9-1*, *cdc2-1* mutants, which have defects in lagging strand DNA replication, accumulated significant amounts of recombination intermediates during S phase in rDNA, whereas the recombination intermediates were not observed in *pol2-1* and *dpb2-1* mutants, which are defective in leading strand DNA replication (92). These recombination intermediates were formed in a manner dependent on Rad52 but not on Rad51 and its other paralogs (92). In addition, it was reported that Rad52, but not Rad51, is associated with stalled replication forks in rDNA (93). The genetic data that *rad59*Δ or *rad59-K166A* is synthetic lethal or sick with *rad27*Δ or *cdc9-1* (52, 94, 95) also support the importance of Rad59-dependent recombination in the repair of lagging strand associated damages. Our findings strongly suggest that the Rad52/Rad59-dependent pathway is a highly preferred choice, whereas the Rad51-dependent pathway is negatively regulated during DNA replication. The genetic observation that *rad59-Y92A* cells display increases in Rad51-dependent HR events (94) raises the possibility that the two pathways are tightly regulated. For example, Rad59 could play a role in the prevention of unscheduled Rad51-dependent recombination. In addition, it has been shown that during S phase, sumoylated PCNA recruits Srs2, which functions to dismantle Rad51 from nucleofilament, leading to suppression of unwanted HR (96, 97). Considering that the Rad51-dependnet HR requires many more accessory factors and a longer time to complete, the Rad51-independent HR-mediated repair of DNA damages occurring during DNA replication, particularly in lagging strand, could be a preferred choice.

Previous studies have revealed that SSA and BIR could occur in the absence of Rad51 function, although a majority of BIR occurs more efficiently in the presence of Rad51 (43, 46, 75–77).

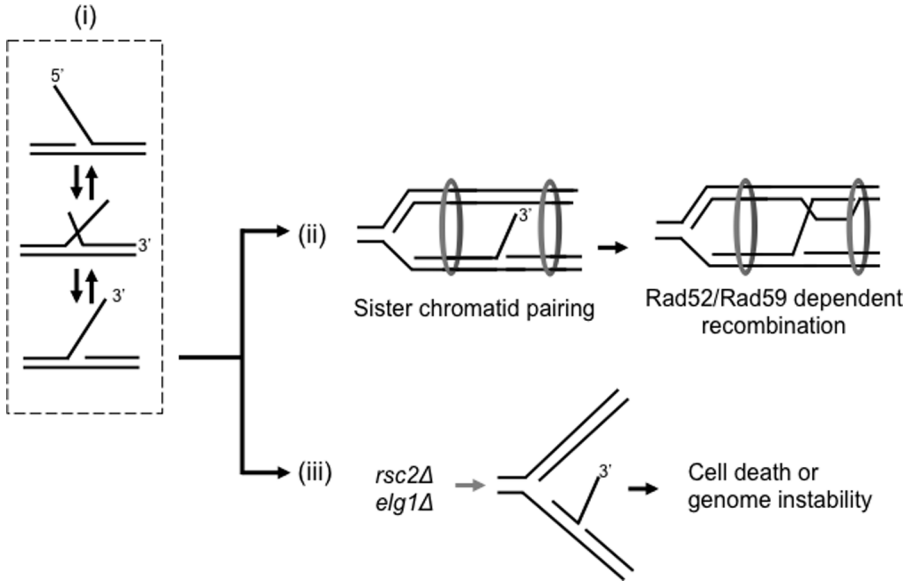

FIGURE 9. **A model for Rad52/59-mediated repair of faulty Okazaki fragment processing.** *i*, unprocessed 5′-flaps can be converted to 3′-flaps via a process called "flap equilibration" (see "Discussion" for details). *ii*, the 3′-flaps can invade the homologous DNA sequences located on a sister chromatid with the aid of Rad52 and Rad59 proteins. The sister chromatid pairing appears critical for this invasion step. *iii*, in the absence of cohesion establishment factors, sister chromatids are not able to pair properly, causing the failure of error-free recombination-mediated repair of erroneous flaps, which in turn leads to cell death or genome instability.

We examined whether SSA and BIR could contribute to the Rad52-dependent suppression of *dna2-K1080E* and found that the deletion of Rad10, Saw1, and Tid1, important factors for SSA or BIR (76, 98, 99), did not affect the suppression (data not shown). It has also been shown that a subset of SCR events, which is facilitated by the RSC complex, could occur independently of Rad51 (78, 79). We found that the suppression was completely abolished in *rsc2*Δ and *elg1*Δ cells, raising the possibility that the same mechanism for SCR is responsible, at least in part, for the repair of faulty flaps. We also investigated whether Ctf4 and Ctf18, two other sister chromatid cohesion establishment factors, had a role in the suppression of *dna2-K1080E* by Rad52 overexpression. It has been found that *ctf4* shows synthetic lethality or synthetic growth defects when combined with *dna2* ts mutant alleles (35), and *CTF18* has been isolated as a multicopy suppressor of *dna2*Δ*405N* (data not shown). However, we were not able to determine the requirement of Ctf4 and Ctf18 for the Rad52-mediated suppression, because overexpression of Rad52 rendered *ctf4*Δ and *ctf18*Δ cells inviable (data not shown).

One question that arises is how the Rad52-dependent recombination is initiated to repair the problematic flaps that have not been appropriately processed due to a defective processing enzyme such as helicase-deficient Dna2. For the HR-mediated repair of general DSB or collapsed replication forks, the initial step is the generation of 3′-ssDNA overhangs by the combined action of the MRX complex and DSB resection machineries (23, 47, 100). Contrary to this, we found that the overexpression of Rad52 in a *rad50*-null (Fig. 3*A*) or *rpd3-null* (data not shown) strain defective in DSB resection (61, 101) resulted in efficient suppression of *dna2-K1080E*. These findings suggest that the 3′-ssDNA overhangs may not be generated by the canonical DSB resection. One likely alternative mechanism for the generation of 3′-ssDNA would be flap equilibration, the spontaneous process of competitive annealing between the two 5′- and 3′-strands. The 5′-flaps, presumably with hairpin or other aberrant structures and thus longer than the average ones, are expected to accumulate in *dna2-K1080E*, and they can be converted to 3′-flaps through energetically allowed flap equilibration (Fig. 9, *step i*). One role of Rad52 in this spontaneous process is to facilitate the interconversion between the 5′- and 3′-flaps by participating in the following process. The RPA-coated long 5′-flaps could recruit Rad52, because RPA-coated ssDNA recruits Rad52 through specific protein-protein interactions (50). Then, the 5′-flap-bound Rad52 proteins could rapidly generate 3′-flaps by facilitating the annealing of the 5′-flap to the template using the strand exchange activity of Rad52 (102). As a result of flap equilibration, the newly generated 3′-flap could form a complex with Rad52 molecules and invade a homologous region in the sister chromatid, readily available because of the close proximity by sister chromatid cohesion (Fig. 9, *step ii*). This proximity may allow the 3′-ssDNA to find its homologous sequence without the aid of Rad51. Subsequently, the strand exchange activity of Rad52 could be used to promote strand invasion between the invading 3′-ssDNA and donor sister chromatid to establish D-loop structure. This later step may not be efficient without the proper paring of the two sister chromatids (Fig. 9, *step iii*). An alternative fate of the 3′-flaps formed via flap equilibration is that they could be newly processed by a 3′-endonuclease such as Mus81 or its related complexes. This is in keeping with the observation that the overexpression of Mus81-Mms4 suppresses the lethality of *dna2-K1080E* (59).

Acknowledgment—We are grateful to the members of our laboratory for critical reading of the manuscript.

REFERENCES

1. Haber, J. E. (1999) Sir-Ku-itous routes to make ends meet. *Cell* **97,** 829–832
2. Kowalczykowski, S. C. (2000) Initiation of genetic recombination and recombination-dependent replication. *Trends Biochem. Sci.* **25,** 156–165
3. Krogh, B. O., and Symington, L. S. (2004) Recombination proteins in yeast. *Annu. Rev. Genet.* **38,** 233–271
4. Alvaro, D., Lisby, M., and Rothstein, R. (2007) Genome-wide analysis of Rad52 foci reveals diverse mechanisms impacting recombination. *PLoS Genet.* **3,** e228
5. Hanawalt, P. C. (2007) Paradigms for the three Rs: DNA replication, recombination, and repair. *Mol. Cell* **28,** 702–707
6. Li, X., and Heyer, W. D. (2008) Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res.* **18,** 99–113
7. Maher, R. L., Branagan, A. M., and Morrical, S. W. (2011) Coordination of DNA replication and recombination activities in the maintenance of genome stability. *J. Cell. Biochem.* **112,** 2672–2682
8. Hübscher, U., and Seo, Y. S. (2001) Replication of the lagging strand: a concert of at least 23 polypeptides. *Mol. Cells* **12,** 149–157
9. Budd, M. E., Tong, A., Peng, X., Polaczek, A., Boone, A., and Campbell, J. L. (2005) A network of multi-tasking proteins at the DNA replication fork preserves genome stability. *PLoS Genet.* **1,** 634–650
10. Loeillet, S., Palancade, B., Cartron, M., Thierry, A., Richard, G. F., Dujon, B., Doye, V., and Nicolas, A. (2005) Genetic network interactions among replication, repair, and nuclear pore deficiencies in yeast. *DNA Repair* **4,** 459–468
11. Kang, Y. H., Lee, C. H., and Seo, Y. S. (2010) Dna2 on the road to Okazaki fragment processing and genome stability in eukaryotes. *Crit. Rev. Biochem. Mol. Biol.* **45,** 71–96
12. Garg, P., and Burgers, P. M. (2005) DNA polymerases that propagate the eukaryotic DNA replication fork. *Crit. Rev. Biochem. Mol. Biol.* **40,** 115–128
13. Zheng, L., and Shen, B. (2011) Okazaki fragment maturation: nucleases take centre stage. *J. Mol. Cell. Biol.* **3,** 23–30
14. Balakrishnan, L., and Bambara, R. A. (2013) Flap endonuclease 1. *Annu. Rev. Biochem.* **82,** 119–138
15. Burgers, P. M. (2009) Polymerase dynamics at the eukaryotic DNA replication fork. *J. Biol. Chem.* **284,** 4041–4045
16. Bae, S. H., and Seo, Y. S. (2000) Characterization of the enzymatic properties of the yeast Dna2 helicase/endonuclease suggests a new model for Okazaki fragment processing. *J. Biol. Chem.* **275,** 38022–38031
17. Bae, S. H., Bae, K. H., Kim, J. A., and Seo, Y. S. (2001) RPA governs endonuclease switching during processing of Okazaki fragments in eukaryotes. *Nature* **412,** 456–461
18. MacNeill, S. A. (2001) DNA replication: partners in the Okazaki two-step. *Curr. Biol.* **11,** R842–844
19. Ayyagari, R., Gomes, X. V., Gordenin, D. A., and Burgers, P. M. (2003) Okazaki fragment maturation in yeast. I. Distribution of functions between *FEN1* and *DNA2*. *J. Biol. Chem.* **278,** 1618–1625
20. Jin, Y. H., Ayyagari, R., Resnick, M. A., Gordenin, D. A., and Burgers, P. M. (2003) Okazaki fragment maturation in yeast. II. Cooperation between the polymerase and 3′-5′ exonuclease activities of Pol δ in the creation of a ligatable nick, *J. Biol. Chem.* **278,** 1626–1633
21. Budd, M. E., and Campbell, J. L. (1997) A yeast replicative helicase, Dna2 helicase, interacts with yeast FEN-1 nuclease in carrying out its essential function. *Mol. Cell. Biol.* **17,** 2136–2142
22. Kang, H. Y., Choi, E., Bae, S. H., Lee, K. H., Gim, B. S., Kim, H. D., Park, C., MacNeill, S. A., and Seo, Y. S. (2000) Genetic analyses of *Schizosaccharomyces pombe dna2*(+) reveal that Dna2 plays an essential role in Okazaki fragment metabolism. *Genetics* **155,** 1055–1067
23. Zhu, Z., Chung, W. H., Shim, E. Y., Lee, S. E., and Ira, G. (2008) Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double strand break ends. *Cell* **134,** 981–994
24. Cejka, P., Cannavo, E., Polaczek, P., Masuda-Sasa, T., Pokharel, S., Campbell, J. L., and Kowalczykowski, S. C. (2010) DNA end resection by Dna2-Sgs1-RPA and its stimulation by Top3-Rmi1 and Mre11-Rad50-Xrs2. *Nature* **467,** 112–116
25. Nimonkar, A. V., Genschel, J., Kinoshita, E., Polaczek, P., Campbell, J. L., Wyman, C., Modrich, P., and Kowalczykowski, S. C. (2011) BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN constitute two DNA end resection machineries for human DNA break repair. *Genes Dev.* **25,** 350–362
26. Symington, L. S., and Gautier, J. (2011) Double-strand break end resection and repair pathway choice. *Annu. Rev. Genet.* **45,** 247–271
27. Kumar, S., and Burgers, P. M. (2013) Lagging strand maturation factor Dna2 is a component of the replication checkpoint initiation machinery. *Genes Dev.* **27,** 313–321
28. Lee, C. H., Lee, M., Kang, H. J., Kim, D. H., Kang, Y. H., Bae, S. H., and Seo, Y. S. (2013) The N-terminal 45-kDa domain of Dna2 endonuclease/helicase targets the enzyme to secondary structure DNA. *J. Biol. Chem.* **288,** 9468–9481
29. Budd, M. E., and Campbell, J. L. (1995) A yeast gene required for DNA replication encodes a protein with homology to DNA helicases. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7642–7646
30. Bae, S. H., Choi, E., Lee, K. H., Park, J. S., Lee, S. H., and Seo, Y. S. (1998) Dna2 of *Saccharomyces cerevisiae* possesses a single-stranded DNA-specific endonuclease activity that is able to act on double-stranded DNA in the presence of ATP. *J. Biol. Chem.* **273,** 26880–26890
31. Bae, S. H., Kim, J. A., Choi, E., Lee, K. H., Kang, H. Y., Kim, H. D., Kim, J. H., Bae, K. H., Cho, Y., Park, C., and Seo, Y. S. (2001) Tripartite structure of *Saccharomyces cerevisiae* Dna2 helicase/endonuclease. *Nucleic Acids Res.* **29,** 3069–3079
32. Bae, S. H., Kim, D. W., Kim, J., Kim, J. H., Kim, D. H., Kim, H. D., Kang, H. Y., and Seo, Y. S. (2002) Coupling of DNA helicase and endonuclease activities of yeast Dna2 facilitates Okazaki fragment processing. *J. Biol. Chem.* **277,** 26632–26641
33. Budd, M. E., Choe, Wc., and Campbell, J. L. (2000) The nuclease activity of the yeast Dna2 protein, which is related to the RecB-like nucleases, is essential *in vivo*. *J. Biol. Chem.* **275,** 16518–16529
34. Lee, K. H., Kim, D. W., Bae, S. H., Kim, J. A., Ryu, G. H., Kwon, Y. N., Kim, K. A., Koo, H. S., and Seo, Y. S. (2000) The endonuclease activity of the yeast Dna2 enzyme is essential *in vivo*. *Nucleic Acids Res.* **28,** 2873–2881
35. Formosa, T., and Nittis, T. (1999) Dna2 mutants reveal interactions with Dna polymerase α and Ctf4, a Pol α accessory factor, and show that full Dna2 helicase activity is not essential for growth. *Genetics* **151,** 1459–1470
36. Chen, X., Niu, H., Chung, W. H., Zhu, Z., Papusha, A., Shim, E. Y., Lee, S. E., Sung, P., and Ira, G. (2011) Cell cycle regulation of DNA double-strand break end resection by Cdk1-dependent Dna2 phosphorylation. *Nat. Struct. Mol. Biol.* **18,** 1015–1019
37. Masuda-Sasa, T., Polaczek, P., Peng, X. P., Chen, L., and Campbell, J. L. (2008) Processing of G4 DNA by Dna2 helicase/nuclease and replication protein A (RPA) provides insights into the mechanism of Dna2/RPA substrate recognition. *J. Biol. Chem.* **283,** 24359–24373
38. Sung, P. (1997) Function of yeast Rad52 protein as a mediator between replication protein A and the Rad51 recombinase. *J. Biol. Chem.* **272,** 28194–28197
39. Song, B., and Sung, P. (2000) Functional interactions among yeast Rad51 recombinase, Rad52 mediator, and replication protein A in DNA strand exchange. *J. Biol. Chem.* **275,** 15895–15904
40. Milne, G. T., and Weaver, D. T. (1993) Dominant negative alleles of *RAD52* reveal a DNA repair/recombination complex including Rad51 and Rad52. *Genes Dev.* **7,** 1755–1765
41. Krejci, L., Song, B., Bussen, W., Rothstein, R., Mortensen, U. H., and Sung, P. (2002) Interaction with Rad51 is indispensable for recombination mediator function of Rad52. *J. Biol. Chem.* **277,** 40132–40141
42. Mortensen, U. H., Bendixen, C., Sunjevaric, I., and Rothstein, R. (1996) DNA strand annealing is promoted by the yeast Rad52 protein. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 10729–10734
43. Shinohara, A., Shinohara, M., Ohta, T., Matsuda, S., and Ogawa, T. (1998) Rad52 forms ring structures and cooperates with RPA in single-strand DNA annealing. *Genes Cells* **3,** 145–156
44. Sugiyama, T., New, J. H., and Kowalczykowski, S. C. (1998) DNA annealing by Rad52 protein is stimulated by specific interaction with the complex of replication protein A and single-stranded DNA. *Proc. Natl. Acad.*

Sci. U.S.A. **95,** 6049–6054
45. Nimonkar, A. V., Sica, R. A., and Kowalczykowski, S. C. (2009) Rad52 promotes second-end DNA capture in double-stranded break repair to form complement-stabilized joint molecules. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 3077–3082
46. Pâques, F., and Haber, J. E. (1999) Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **63,** 349–404
47. Symington, L. S. (2002) Role of *RAD52* epistasis group genes in homologous recombination and double-strand break repair. *Microbiol. Mol. Biol. Rev.* **66,** 630–670
48. Van Dyck, E., Stasiak, A. Z., Stasiak, A., and West, S. C. (2001) Visualization of recombination intermediates produced by RAD52-mediated single-strand annealing. *EMBO Rep.* **2,** 905–909
49. Sugiyama, T., and Kowalczykowski, S. C. (2002) Rad52 protein associates with replication protein A (RPA)-single-stranded DNA to accelerate Rad51-mediated displacement of RPA and presynaptic complex formation. *J. Biol. Chem.* **277,** 31663–31672
50. Plate, I., Hallwyl, S. C., Shi, I., Krejci, L., Müller, C., Albertsen, L., Sung, P., and Mortensen, U. H. (2008) Interaction with RPA is necessary for Rad52 repair center formation and for its mediator activity. *J. Biol. Chem.* **283,** 29077–29085
51. Shi, I., Hallwyl, S. C., Seong, C., Mortensen, U., Rothstein, R., and Sung, P. (2009) Role of the Rad52 amino-terminal DNA binding activity in DNA strand capture in homologous recombination. *J. Biol. Chem.* **284,** 33275–33284
52. Symington, L. S. (1998) Homologous recombination is required for the viability of *rad27* mutants. *Nucleic Acids Res.* **26,** 5589–5595
53. Debrauwère, H., Loeillet, S., Lin, W., Lopes, J., and Nicolas, A. (2001) Links between replication and recombination in *Saccharomyces cerevisiae*: a hypersensitive requirement for homologous recombination in the absence of Rad27 activity. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 8263–8269
54. Fiorentino, D. F., and Crabtree, G. R. (1997) Characterization of *Saccharomyces cerevisiae dna2* mutants suggests a role for the helicase late in S phase. *Mol. Biol. Cell* **8,** 2519–2537
55. Budd, M. E., Choe, Wc., and Campbell, J. L. (1995) *DNA2* encodes a DNA helicase essential for replication of eukaryotic chromosomes. *J. Biol. Chem.* **270,** 26766–26769
56. Wach, A., Brachat, A., Pöhlmann, R., and Philippsen, P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10,** 1793–1808
57. Riezman, H., Hase, T., van Loon, A. P., Grivell, L. A., Suda, K., and Schatz, G. (1983) Import of proteins into mitochondria: a 70 kilodalton outer membrane protein with a large carboxy-terminal deletion is still transported to the outer membrane. *EMBO J.* **2,** 2161–2168
58. Kang, Y. H., Kang, M. J., Kim, J. H., Lee, C. H., Cho, I. T., Hurwitz, J., and Seo, Y. S. (2009) The *MPH1* gene of *Saccharomyces cerevisiae* functions in Okazaki fragment processing. *J. Biol. Chem.* **284,** 10376–10386
59. Kang, M. J., Lee, C. H., Kang, Y. H., Cho, I. T., Nguyen, T. A., and Seo, Y. S. (2010) Genetic and functional interactions between Mus81-Mms4 and Rad27. *Nucleic Acids Res.* **38,** 7611–7625
60. Lee, C. H., Shin, Y. K., Phung, T. T., Bae, J. S., Kang, Y. H., Nguyen, T. A., Kim, J. H., Kim, D. H., Kang, M. J., Bae, S. H., and Seo, Y. S. (2010) Involvement of Vts1, a structure-specific RNA-binding protein, in Okazaki fragment processing in yeast. *Nucleic Acids Res.* **38,** 1583–1595
61. Shim, E. Y., Chung, W. H., Nicolette, M. L., Zhang, Y., Davis, M., Zhu, Z., Paull, T. T., Ira, G., and Lee, S. E. (2010) *Saccharomyces cerevisiae* Mre11/Rad50/Xrs2 and Ku proteins regulate association of Exo1 and Dna2 with DNA breaks. *EMBO J.* **29,** 3370–3380
62. Shen, B., Nolan, J. P., Sklar, L. A., and Park, M. S. (1997) Functional analysis of point mutations in human flap endonuclease-1 active site. *Nucleic Acids Res.* **25,** 3332–3338
63. Gary, R., Park, M. S., Nolan, J. P., Cornelius, H. L., Kozyreva, O. G., Tran, H. T., Lobachev, K. S., Resnick, M. A., and Gordenin, D. A. (1999) A novel role in DNA metabolism for the binding of Fen1/Rad27 to PCNA and implications for genetic risk. *Mol. Cell. Biol.* **19,** 5373–5382
64. Munashingha, P. R., Lee, C. H., Kang, Y. H., Shin, Y. K., Nguyen, T. A., and Seo, Y. S. (2012) The trans-autostimulatory activity of Rad27 suppresses *dna2* defects in Okazaki fragment processing. *J. Biol. Chem.* **287,** 8675–8687
65. Chung, W. H., Zhu, Z., Papusha, A., Malkova, A., and Ira, G. (2010) Defective resection at DNA double-strand breaks leads to *de novo* telomere formation and enhances gene targeting. *PLoS Genet.* **6,** e1000948
66. van Attikum, H., and Gasser, S. M. (2005) The histone code at DNA breaks: a guide to repair? *Nat. Rev. Mol. Cell Biol.* **6,** 757–765
67. Hollenhorst, P. C., Bose, M. E., Mielke, M. R., Müller, U., and Fox, C. A. (2000) Forkhead genes in transcriptional silencing, cell morphology and the cell cycle: overlapping and distinct functions for FKH1 and FKH2 in *Saccharomyces cerevisiae*. *Genetics* **154,** 1533–1548
68. IJpma, A. S., and Greider, C. W. (2003) Short telomeres induce a DNA damage response in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **14,** 987–1001
69. Tsukamoto, M., Yamashita, K., Miyazaki, T., Shinohara, M., and Shinohara, A. (2003) The N-terminal DNA-binding domain of Rad52 promotes *RAD51*-independent recombination in *Saccharomyces cerevisiae*. *Genetics* **165,** 1703–1715
70. Singleton, M. R., Wentzell, L. M., Liu, Y., West, S. C., and Wigley, D. B. (2002) Structure of the single-strand annealing domain of human *RAD52* protein. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 13492–13497
71. Bai, Y., and Symington, L. S. (1996) A Rad52 homolog is required for *RAD51*-independent mitotic recombination in *Saccharomyces cerevisiae*. *Genes Dev.* **10,** 2025–2037
72. Davis, A. P., and Symington, L. S. (2001) The yeast recombinational repair protein Rad59 interacts with Rad52 and stimulates single-strand annealing. *Genetics* **159,** 515–525
73. Cortés-Ledesma, F., Malagón, F., and Aguilera, A. (2004) A novel yeast mutation, rad52-L89F, causes a specific defect in Rad51-independent recombination that correlates with a reduced ability of Rad52-L89F to interact with Rad59. *Genetics* **168,** 553–557
74. Feng, Q., Düring, L., de Mayolo, A. A., Lettier, G., Lisby, M., Erdeniz, N., Mortensen, U. H., and Rothstein, R. (2007) Rad52 and Rad59 exhibit both overlapping and distinct functions. *DNA Repair* **6,** 27–37
75. Malkova, A., Ivanov, E. L., and Haber, J. E. (1996) Double-strand break repair in the absence of *RAD51* in yeast: a possible role for break-induced DNA replication. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 7131–7136
76. Ira, G., and Haber, J. E. (2002) Characterization of *RAD51*-independent break-induced replication that acts preferentially with short homologous sequences. *Mol. Cell. Biol.* **22,** 6384–6392
77. Pohl, T. J., and Nickoloff, J. A. (2008) Rad51-independent interchromosomal double-strand break repair by gene conversion requires Rad52 but not Rad55, Rad57, or Dmc1. *Mol. Cell. Biol.* **28,** 897–906
78. Liefshitz, B., and Kupiec, M. (2011) Roles of RSC, Rad59, and cohesin in double-strand break repair. *Mol. Cell. Biol.* **31,** 3921–3923
79. Oum, J. H., Seong, C., Kwon, Y., Ji, J. H., Sid, A., Ramakrishnan, S., Ira, G., Malkova, A., Sung, P., Lee, S. E., and Shim, E. Y. (2011) RSC facilitates Rad59-dependent homologous recombination between sister chromatids by promoting cohesin loading at DNA double-strand breaks. *Mol. Cell. Biol.* **31,** 3924–3937
80. Huang, J., Hsu, J. M., and Laurent, B. C. (2004) The RSC nucleosome-remodeling complex is required for Cohesin's association with chromosome arms. *Mol. Cell* **13,** 739–750
81. Huang, J., and Laurent, B. C. (2004) A Role for the RSC chromatin remodeler in regulating cohesion of sister chromatid arms. *Cell Cycle* **3,** 973–975
82. Maradeo, M. E., and Skibbens, R. V. (2009) The Elg1-RFC clamp-loading complex performs a role in sister chromatid cohesion. *PLoS One* **4,** e4707
83. Parnas, O., Zipin-Roitman, A., Mazor, Y., Liefshitz, B., Ben-Aroya, S., and Kupiec, M. (2009) The *ELG1* clamp loader plays a role in sister chromatid cohesion. *PLoS One* **4,** e5497
84. Kim, J. H., Kang, Y. H., Kang, H. J., Kim, D. H., Ryu, G. H., Kang, M. J., and Seo, Y. S. (2005) *In vivo* and *in vitro* studies of Mgs1 suggest a link between genome instability and Okazaki fragment processing. *Nucleic Acids Res.* **33,** 6137–6150
85. Stewart, J. A., Campbell, J. L., and Bambara, R. A. (2009) Significance of the dissociation of Dna2 by flap endonuclease 1 to Okazaki fragment processing in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **284,** 8283–8291

86. Hoopes, L. L., Budd, M., Choe, W., Weitao, T., and Campbell, J. L. (2002) Mutations in DNA replication genes reduce yeast life span. *Mol. Cell. Biol.* **22,** 4136–4146
87. Lin, W., Sampathi, S., Dai, H., Liu, C., Zhou, M., Hu, J., Huang, Q., Campbell, J., Shin-Ya, K., Zheng, L., Chai, W., and Shen, B. (2013) Mammalian DNA2 helicase/nuclease cleaves G-quadruplex DNA and is required for telomere integrity. *EMBO J.* **32,** 1425–1439
88. Chai, W., Zheng, L., and Shen, B. (2013) DNA2, a new player in telomere maintenance and tumor suppression. *Cell Cycle* **12,** 1985–1986
89. Tomita, K., Kibe, T., Kang, H. Y., Seo, Y. S., Uritani, M., Ushimaru, T., and Ueno, M. (2004) Fission yeast Dna2 is required for generation of the telomeric single-strand overhang. *Mol. Cell. Biol.* **24,** 9557–9567
90. Choe, W., Budd, M., Imamura, O., Hoopes, L., and Campbell, J. L. (2002) Dynamic localization of an Okazaki fragment processing protein suggests a novel role in telomere replication. *Mol. Cell. Biol.* **22,** 4202–4217
91. Weitao, T., Budd, M., Hoopes, L. L., and Campbell, J. L. (2003) Dna2 helicase/nuclease causes replicative fork stalling and double-strand breaks in the ribosomal DNA of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **278,** 22513–22522
92. Zou, H., and Rothstein, R. (1997) Holliday junctions accumulate in replication mutants via a RecA homolog-independent mechanism. *Cell* **90,** 87–96
93. Irmisch, A., Ampatzidou, E., Mizuno, K., O'Connell, M. J., and Murray, J. M. (2009) Smc5/6 maintains stalled replication forks in a recombination-competent conformation. *EMBO J.* **28,** 144–155
94. Liddell, L. C., Manthey, G. M., Owens, S. N., Fu, B. X., and Bailis, A. M. (2013) Alleles of the homologous recombination gene, *RAD59*, identify multiple responses to disrupted DNA replication in *Saccharomyces cerevisiae*. *BMC Microbiol.* **13,** 229
95. Nguyen, H. D., Becker, J., Thu, Y. M., Costanzo, M., Koch, E. N., Smith, S., Myung, K., Myers, C. L., Boone, C., and Bielinsky, A. K. (2013) Unligated Okazaki fragments induce PCNA ubiquitination and a requirement for Rad59-dependent replication fork progression. *PLoS One.* **8,** e66379
96. Krejci, L., Van Komen, S., Li, Y., Villemain, J., Reddy, M. S., Klein, H., Ellenberger, T., and Sung, P. (2003) DNA helicase Srs2 disrupts the Rad51 presynaptic filament. *Nature* **423,** 305–309
97. Veaute, X., Jeusset, J., Soustelle, C., Kowalczykowski, S. C., Le Cam, E., and Fabre, F. (2003) The Srs2 helicase prevents recombination by disrupting Rad51 nucleoprotein filaments. *Nature* **423,** 309–312
98. Li, F., Dong, J., Eichmiller, R., Holland, C., Minca, E., Prakash, R., Sung, P., Yong Shim, E., Surtees, J. A., and Eun Lee, S. (2013) Role of Saw1 in Rad1/Rad10 complex assembly at recombination intermediates in budding yeast. *EMBO J.* **32,** 461–472
99. Li, F., Dong, J., Pan, X., Oum, J. H., Boeke, J. D., and Lee, S. E. (2008) Microarray-based genetic screen defines *SAW1*, a gene required for Rad1/Rad10-dependent processing of recombination intermediates. *Mol. Cell* **30,** 325–335
100. Mimitou, E. P., and Symington, L. S. (2009) DNA end resection: many nucleases make light work. *DNA Repair* **8,** 983–995
101. Robert, T., Vanoli, F., Chiolo, I., Shubassi, G., Bernstein, K. A., Rothstein, R., Botrugno, O. A., Parazzoli, D., Oldani, A., Minucci, S., and Foiani, M. (2011) HDACs link the DNA damage response, processing of double-strand breaks and autophagy. *Nature* **471,** 74–79
102. Bi, B., Rybalchenko, N., Golub, E. I., and Radding, C. M. (2004) Human and yeast Rad52 proteins promote DNA strand exchange. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 9568–9572
103. Sikorski, R. S., and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122,** 19–27