Telomere maintenance is required for chromosome stability, and telomeres are typically replicated by the action of telomerase. In both mammalian tumor and yeast cells that lack telomerase, telomeres are maintained by an alternative (ALT) recombination mechanism. In yeast, Sgs1p and its associated type IA topoisomerase, Top3p, may work coordinately in removing Holliday junction intermediates from a crossover-producing recombination pathway. Previous studies have also indicated that Sgs1 helicase acts in a telomere recombination pathway. Here we show that topoisomerase III is involved in telomere-telomere recombination. The recovery of telomere recombination-dependent survivors in a telomerase-minus yeast strain was dependent on Top3p catalytic activity. Moreover, the Rif1p and Rif2p genes are required for the establishment of TOP3/SGS1-dependent telomere-telomere recombination. In human Saos-2 ALT cells, human topoisomerase IIIα (hTOP3α) also contributes to telomere recombination. Strikingly, the telomerase activity is clearly enhanced in surviving si-hTOP3α Saos-2 ALT cells. Altogether, the present results suggest a potential role for hTOP3α in dissociating telomeric structures in telomerase-deficient cells, providing therapeutic implications in human tumors.

Telomeres are dynamic DNA-protein complexes that protect the ends of linear chromosomes, prevent detrimental chromosome rearrangements, and defend against genomic instability and the associated risk of cancer (1–3). Telomeres are composed of telomeric DNA, consisting of tandem repeats of short G-rich sequences that are synthesized by the enzyme telomerase (4, 5). The catalytic core of telomerase is composed of a reverse transcriptase and an RNA subunit. The reverse transcriptase utilizes the RNA component as a template to add the G-rich repeats onto the 3’ ends of the transcript. The structure of type II telomeres in S. cerevisiae resembles that of human telomeres (6). For instance, in mammalian cells, telomerase activity is absent, and telomeres are gradually shortened with successive cell divisions due to incomplete replication, which eventually causes replicative senescence. Once telomeres become sufficiently short, they are thought to lose the ability to protect the ends of the chromosomes from being recognized as broken ends and being subjected to nuclease digestion, homologous recombination, and nonhomologous end-joining repair. Continuous telomere shortening in human fibroblasts leads to chromosome fusions, crisis, and apoptosis (7). Very few human cells can bypass the crisis either through telomerase reactivation (7) or through an alternative (ALT) pathway for telomere lengthening (8–10).

Telomeric DNA in the yeast Saccharomyces cerevisiae consists of ~350 ± 75 bp of TG1/C1-A DNA repeats. Internal to the TG1/C1-A tracts are middle repetitive DNA elements, called X and Y (1, 2). The telomeric TG1/C1-A DNA forms a complex nonnucleosomal heterochromatin (11). The major component in this complex is a double-stranded, sequence-specific DNA-binding protein-Rap1p complex, which includes Rif1p and Rif2p. The copy number of the Rap1p complex regulates telomere length and telomere silencing (12–15).

Even in organisms that normally rely on telomerase, telomerase-independent mechanisms of telomere maintenance exist. Although most cells in S. cerevisiae (16, 17), Kluyveromyces lactis (18), and Schizosaccharomyces pombe (19) lack the genes for telomerase components, multiple tandem copies of the subtelomeric Y′ element and very short terminal tracts of TG1/C1-A DNA (16, 20) (type I survivors). In human cells, telomere sequence are increased heterogeneously from several hundred base pairs to 10 kb or longer (20). The generation of type II survivors depends on the presence of Rap50p, Rap59p, and Sgs1p (21–25), whereas the frequencies of type I and type II formation depend on a number of factors, such as strain background (25), cell type, and various genes involved in nonhomologous end joining (26).

The structure of type II telomeres in Saccharomyces cerevisiae resembles that of 10–15% of human cell lines and tumors that maintain telomeric DNA by the ALT pathway (8–10). These ALT cells display unique nuclear foci termed ALT-associated PML bodies that contain RAD52-dependent homologous recombination. The S. cerevisiae, the majority of cells that survive in the absence of telomerase activity have multiple tandem copies of the subtelomeric Y′ element and very short terminal tracts of TG1/C1-A DNA (16, 20, and type I survivors). In a minor fraction (<10%) of the survivors (type II), the lengths of the telomere sequence are increased heterogeneously from several hundred base pairs to 10 kb or longer (20). The generation of type II survivors depends on the presence of Rap50p, Rap59p, and Sgs1p (21–25), whereas the frequencies of type I and type II formation depend on a number of factors, such as strain background (25), cell type, and various genes involved in nonhomologous end joining (26).
strongly suppresses many phenotypic effects of loss of TOP3 function (31). On the other hand, both Top3 and RecQ family helicases are required to prevent mitotic crossovers by processing and resolving double Holliday junctions generated in response to double-stranded break formation in vivo (32) and in vitro (33). Furthermore, telomere shortening has been detected in the top3Δ strains (34), suggesting that Top3p might be a general mediator in telomere. However, whether Top3p is a general mediator cooperating with the RecQ helicase to resolve recombination intermediates at telomeres is still elusive.

Here, we examined whether a TOP3 deficiency affected telomere maintenance in telomerase-deficient human and yeast cells. We show that TOP3 is required for type II recombination in yeast. The recovery of type II survivors is dependent on Top3p supercoiling activity. In addition, the Rif genes are involved in the establishment of TOP3/SGS1-dependent telomere-telomere recombination. In human si-hTOP3 Saos-2 ALT cells, the heterogeneous telomeric pattern of ALT is switched to uniform telomere lengths. Strikingly, the telomerase activity is enhanced in surviving si-hTOP3 Saos-2 ALT cancer cells. These observations indicate that, in human Saos-2 cells, hTOP3 is required to prevent mitotic crossovers by processing and resolving dou-

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Yeast Culture Condition, DNA Preparation, Enzyme Digestion, Gel Electrophoresis, and Southern Blot Analysis—Each spore colony was inoculated into YEPD and grown at 30 °C. Several independent isolates for each genotype were analyzed. Genomic DNA preparation and Southern blot analysis were performed as previously described (35). To examine the DNA from individual colonies, each colony was expanded in 2 ml of liquid medium to obtain DNA for Southern blot analysis. The DNA was digested with XhoI in order to observe the type I pattern or with a mixture of HaeIII, Hinfl, HindIII, andMspI four-base cutters to observe the type II pattern. A 270-bp C1–3A fragment was randomly labeled with the random prime labeling system (Invitrogen) and used in Southern hybridization. Data shown under “Results” are representative of two or more experiments from independent spores.

**Mammalian Cell Culture, Transfection, and Western Blotting—**Cells were transfected with the CaPO4 method (Saos-2) or Effectene transfection reagent (Qiagen) (HeLa) according to the manufacturer’s recommendations. Cells were cultured in Dulbecco’s modified Eagle’s medium (HeLa) and RPMI (Saos-2) containing fetal calf serum, penicillin, streptomycin, glutamine, nonessential amino acids (Hyclone), and Geneticin G418 (400 μg/ml; Invitrogen). Untransfected HeLa and Saos-2 cells were cultured in the same media without Geneticin. Nuclear extracts for protein analysis were prepared by nuclear extraction reagents (Pierce). Nuclear extracts were loaded on an 8% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred onto an ECL membrane. The membrane was then blocked with 5% low fat milk in phosphate-buffered saline (PBS) for 1 h at room temperature. Primary antibodies used to detect hTOP3α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), hTERT (Santa Cruz Biotechnology), and proliferating cell nuclear antigen (Santa Cruz Biotechnology) as loading control were used at a dilution of 1:100 in the blocking buffer and incubated for 1 h at room temperature. After three washes with PBS containing 1% Tween, secondary antibodies were applied for 1 h at room temperature at a dilution of 1:5000. A conventional chemiluminescence system was used to visualize proteins following the manufacturer’s instructions (PerkinElmer Life Sciences). Data shown here are representative of three or more independent experiments. Imaging and quantification of the data were performed by an Alphalnnotech photodocumentation system.

**Human Telomere Repeat Amplification Protocol and Telomere Restriction Fragment (TRF) Analysis—**Telomerase activity was measured using the TRAPeze telomerase detection kit (Chemicon) with 1 μg of protein/assay system. All telomerase assays were performed in duplicate and repeated at least twice. Genomic DNA was isolated from cells by a genomic DNA purification kit (Promega), and 10 μg of DNA was digested by MspI and HindIII. The digested DNA was resolved on a 0.5% agarose gel and transferred onto a Hybond N+ nylon membrane and hybridized overnight to a 32P-labeled 800-bp telomere-specific probe.

**Immunofluorescence Staining—**Cells were grown on glass coverslips, growth-arrested by withdrawal of methionine for 4 days, rinsed with PBS, and fixed in PBS-buffered 3% paraformaldehyde and 2% sucrose at room temperature for 10 min, followed by permeabilization in Triton X buffer (0.5% Triton X-100, 20 mM Hepes-KOH, pH 7.9, 50 mM NaCl, 3 mM MgCl2, 300 mM sucrose) at room temperature for 10 min. For dual immunostaining, we blocked cells with 0.5% bovine serum albumin in PBS and incubated at 4 °C for overnight with either mouse anti-Nbs1 (1:500; GeneTex) or rabbit anti-PML (1:500; Santa Cruz Biotechnology) Abs. The primary antibodies were detected using Rhodamine Red-conjugated goat anti-rabbit and fluorescein isothiocyanate-conjugated goat anti-mouse antibodies (1:500). DNA was stained with Hoechst at room
temperature for 1 h. Immunofluorescence was analyzed with a Zeiss Axiosplan fluorescence microscope.

RESULTS

TOP3 Contributes to Telomere Homeostasis in Wild-type and Telomerase-minus (tlc1Δ) Strains—To investigate whether TOP3 contributes to telomere replication, telomere lengths in tlc1, top3, and tlc1 top3 strains were assessed by Southern blot analysis using telomeric DNA probes. Consistent with a previous report (34), when the telomere lengths of TOP3 and top3 strains were compared, a small (~150 bp) but reproducible TOP3-dependent telomere shortening was observed in top3 mutants. This telomere length defect was stably maintained, and the top3 strain showed no sign of progressive telomere shortening or cellular senescence (Fig. 1A) (data not shown). However, the tlc1 top3 cells displayed slow growth and a cellular senescence phenotype (data not shown). Thus, TOP3 partially contributes to the telomere maintenance. In previous studies, we demonstrated that when a tlc1 strain is propagated in liquid, only long and heterogeneous telomeres, representing a pattern of type II survivors, are eventually developed, due to the selective growth advantage over type I survivors (20). However, examination of the telomeres of the tlc1 top3 strain in liquid did not reveal the type II pattern (Fig. 1B), an observation consistent with the notion that Top3p is important for generating type II survivors. To further examine this hypothesis, we took advantage of the solid plate analysis. On the solid plate until the survivors appeared. DNA was prepared from individual survivors, and the telomere pattern was determined by Southern blot analysis. Significantly, all of the tlc1 top3 survivors (88 of 88) displayed the type I pattern, whereas both type I and type II survivors were recovered from a tlc1 strain (Fig. 1C). These data suggested that type II recombination is blocked in tlc1 top3 cells. Together, these results demonstrate that TOP3 affects telomere length maintenance in both telomerase-plus and telomerase-minus cells.

It was shown previously that Rad51p is essential for type I recombination (21, 23). To test whether tlc1, rad51, and top3 mutants could abolish telomerase, type I and type II recombination pathways, strains generated from freshly dissected spores with different mutation backgrounds were examined by serial single colony restreaking on solid plates (Fig. 1D). We found that in sharp contrast to tlc1, tlc1 rad51, and tlc1 top3 strains, which showed a cellular senescence phenotype on streaking plates, the tlc1 rad51 top3 spores could be characterized by microcolony formation from the tetrad dissections and completely lost their viabilities at the first restreak. These results suggest that the triple mutant completely knocks out all three pathways for telomere maintenance, which causes accelerated cellular senescence.

Sgs1p-Top3p Interaction Is Critical for Type II Recombination—Previous work has shown that the loss of Top3p function confers a slow growth phenotype, which is partially alleviated in an sgs1 strain (31). Strains that are deficient in sgs1 also exhibit altered regulation of telomere-telomere recombination in telomerase-minus cells (21, 25). We therefore tested whether the loss of SGS1 could similarly influence the telomere phenotype in a tlc1 top3 double mutant strain. Freshly generated tlc1 top3 sgs1 spore colonies were subjected to liquid culture analysis. Fig. 2A shows that an additional sgs1 mutation did not rescue the type I pattern displayed by the tlc1 top3 double mutant strain. Examining the 56 tlc1 top3 sgs1 survivors that were generated by the serial single colony isolation, we only observed TRF profiles of type I survivors (Fig. 1C). These results demonstrate that type II recombination does not initiate in a tlc1 strain deficient in both Top3p and Sgs1p. The results in Fig. 1 and Fig. 2A indicated that both Top3p and Sgs1p are required for type II recombination. In the absence of telomerase, top3 and top3 sgs1 mutants may be defective in resolving the same structural hindrance as in sgs1 cells. Top3p along with Sgs1p may contribute to resolve the Holliday junctions formed during telomere-telomere recombination. To examine whether the interaction between Sgs1p and Top3p is critical for their activities on type II recombination, telomere patterns of freshly dissected tlc1 sgs1 spores containing an empty plasmid, a CEN plasmid expressing the wild-type SGS1, or a mutated sgs1 with the abolished Sgs1p-Top3p interaction domain (36) were analyzed. Compared with the wild-type SGS1-complemented strain displaying the type II pattern after several dilutions, the sgs1 mutant-complemented strain maintained the type I pat-
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Yeast TOP3 and Human TOP3a Rescue Type I Phenotype Recovered from Telomerase-defective top3Δ Strains—To further confirm the role that TOP3 may play in the generation of type II survivors, we first tested the effect of reintroducing TOP3 on a low copy plasmid in a tlc1 top3 strain. Telomere patterns of freshly dissected tlc1 top3 spores containing an empty plasmid or a yeast TOP3 plasmid were analyzed. The reintroduction of TOP3 rescued the slow growth of the tlc1 top3 cells (data not shown). As shown in Fig. 3A, unlike tlc1 top3 strains that produced only the type I pattern, this complement recovered the type II pattern as observed in the tlc1 cells.

Next, the issue of whether hTOP3 could complement the type I telomere-related phenotypes in yeast was examined. Human cells encode two topoisomerase III isoenzymes, hTOP3α and hTOP3β, and it is not clear which one is the functional equivalent of yeast Top3α. For unknown reasons, we could not obtain any viable spore over 100 tetrads from the tlc1 top3 plus an hTOP3β plasmid background (data not shown). In contrast, the freshly dissected tlc1 top3 spores containing an hTOP3α plasmid could produce a type II pattern (Fig. 3A). Moreover, the hTOP3α-expressing tlc1 top3 strains exhibit growth rates and cell cycle distributions similar to those in tlc1 strains (data not shown). Thus, hTOP3α can functionally complement both the slow growth and the defect in type II pathway phenotypes of tlc1 top3 cells.

To investigate whether the supercoiling activity of TOP3 is required for type II recombination, the telomere pattern was tested using a yeast top3 complementary plasmid containing a Tyr → Phe mutation at the active site of topoisomerase. This mutation was previously shown to abolish the topoisomerase activity (36). In the liquid assay, we observed that the type I pattern was maintained throughout serial dilutions (Fig. 3B). Taken together, these data suggest that the Top3p catalytic activity is essential for the type II recombination and that the hTOP3α may be functionally equivalent to the yTop3p for type II recombination.

Mutations in RIF1 and RIF2 Restore Telomere-Telomere Recombination in top3 Mutant Cells—Purified recombinant Drosophila topoisomerase III displayed relaxation activity only with a hypernegatively supercoiled substrate, suggesting that Top3p is specifically performed at hypernegatively supercoiled DNA in vivo (37). Hypernegatively supercoiled DNA might be generated during recombination through telomeres. Rap1 protein, together with Rfi1p and Rfi2p, is involved in forming the higher order telomere structure that regulates telomere length and telomere silencing (12–15). Therefore, we next tested whether TOP3-dependent type I is abolished in rif mutants. Strikingly, rif mutations completely restored telomere-telomere recombination in tlc1 top3 mutant cells, such that tlc1 top3 rif1, tlc1 top3 rif2, and tlc1 top3 rif1 rif2 mutants generated the type II pattern (Fig. 4A). The same effect was observed with tlc1 sgs1 rif mutant combinations (Fig. 4B). Altogether, our data suggest that a role for Top3p-Sgs1 proteins in telomere-telomere recombination might be to overcome the inhibitory effect provided by the Rap1-Rif complex.

Reduction of TOP3α Expression Influences Telomere Maintenance in Saos-2 ALT Cells—ALT cells have very long and heterogeneous telomeres that are reminiscent of yeast type II telomeres (38). Although the proteins that mediate ALT are not well known, evidence suggests that ALT is mediated by gene conversion (9), the same model proposed for the generation of type II telomeres in yeast. These results, combined with our findings, raise the possibility that human TOP3 might be an ALT factor. To address this, hTOP3α and hTOP3β knockdown were generated by the plasmid-mediated RNA interference technology (siRNA) in telomerase-positive HeLa and telomerase-negative Saos-2 ALT cells to create stable si-hTOP3α (Fig. 5A) and si-hTOP3β (data not shown) cell lines. To examine their roles in telomere formation, their telomere lengths following population doublings (PDs) were analyzed. The cell lines were maintained under selection in the media for 90 PDs (PD 0 is defined as the point at which the pooled selected
population was subcloned by limited dilution), and their telomere lengths were assessed by Southern blot analysis using a telomeric probe. An indication of the ALT telomere is the pattern of heterogeneous sizes (8, 38). Whereas cells with repression of TOP3β/H9252 or insufficient repression of TOP3α/H9251 did not result in any alteration of the telomere pattern (data not shown), the heterogeneous sizes of telomeres gradually became less clear in si-hTOP3α/H9251 Saos-2 cells (Fig. 5B). Instead, equally sized bands, similar to those of telomerase-positive HeLa cells, progressively developed in si-hTOP3α Saos-2 cells. Interestingly, there was no observable growth difference in these cells (Fig. 5C). These results suggest that the surviving si-hTOP3α Saos-2 ALT cells might have switched to the telomerase-positive cells.

ALT cells display unique nuclear foci termed ALT-associated PML bodies (APBs) (27). The presence of APB foci is another signature of...
To determine whether the APB status was altered in si-hTOP3a/Saos-2 ALT cells, APBs were detected by immunostaining. To facilitate the observation of APBs, the cells were subjected to growth arrest by withdrawal of methionine from the medium, which increased the proportion of cells containing APBs to 50–60%, in contrast to 5% in exponentially dividing populations (39). We observed that the majority of the APB foci diminished in si-hTOP3a/Saos-2 cells (Fig. 6, A and B). Collectively, the ALT pattern in Saos-2 cells is inactivated in cells with reduced hTOP3a, indicating that TOP3a may play a critical role at telomeres in telomerase-deficient Saos-2 cells.

To further understand the molecular basis of this phenotype, we analyzed the telomerase activity in si-hTOP3a/Saos-2 cells. Although no telomerase activity was detected in empty vector-transfected and selected Saos-2 cell lines, strong telomerase activity was observed in si-hTOP3a/Saos-2 cells (Fig. 7A). Moreover, the expression of telomerase was detected in si-hTOP3a/Saos-2 cells (Fig. 7B). It was notable that a milder repression of hTOP3a was observed in si-hTOP3a Saos-2 clone 16 (Fig. 5A, lane 6). Consistently, clone 16 also displayed weaker telomerase activity (Fig. 7A, lane 6) and a delayed development of telomerase-positive cell-like telomere patterns (Fig. 5B) compared with other si-hTOP3a Saos-2 clones. Altogether, these data demonstrate that telomerase is activated in si-hTOP3a-selected stable Saos-2 cells.

**DISCUSSION**

**TOP3 Mediates Telomerase-independent Telomere Maintenance**—
The above observations indicate that TOP3 is involved in telomere maintenance in yeast as well as human cells. Top3p represses telomere shortening in telomerase-proficient cells. Additionally, Top3p appears to directly participate in telomere-telomere recombination in telomerase-minus cells. Telomere maintenance in the absence of telomerase has been proposed to employ a break-induced replication mechanism (40, 41). Our results indicate that there exist two similar genetic pat-
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ways for telomerase-independent telomere maintenance and break-induced replication, namely the RAD50/SGS1/TOP3-dependent type II pathway and the RAD51-dependent type I pathway.

The Role of TOP3α in Saos-2 ALT Cells—BLM and WRN, two of the RecQ families in humans, have the ability in vitro to promote ATP-dependent branch migration of Holliday junctions (42, 43). That the repression of the ALT pattern in si-hTOP3α caused telomerase activation in Saos-2 ALT cells implies that recombination intermediates may be a possible substrate for TOP3α. The TOP3 and RecQ helicase may act together to resolve Holliday junctions generated during telomere-telomere recombination (Fig. 7C). Unresolved structures at the telomeres may lead to chromosomal breakage and/or end fusions. Thus, even in telomerase-positive cells, TOP3α defects may lead to telomere loss. Alternatively, the Top3-Sgs1 complex breakage and/or end fusions. Thus, even in telomerase-positive cells, TOP3α pathway. The surviving Saos-2 si-hTOP3α repressed, cells try to survive through the activation of the telomerase enzyme. Activation of the ALT pathway was previously observed in individuals with cancer. Given that telomere maintenance is a required step for the importance of understanding the molecular mechanism of ALT pathway and the role of the ALT pathway in telomere biology, since the alternative ALT pathway might cause therapeutic failures and/or acquired resistances in telomerase inhibition-based anticancer therapy. Therefore, identification of molecular targeting of pathways responsible for ALT is clearly an important issue with respect to the therapeutic efficacy of telomerase inhibitors in clinics and would have a tremendous impact in the clinical management of individuals with cancer. Given that telomere maintenance is a required step for all cancer cells, the combination of a telomerase inhibitor and an innovated topoisomerase III inhibitor might represent a valuable anticancer therapeutic strategy to simultaneously block both telomerase and ALT pathways in cancer cells.

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