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Condensin Loaded onto the Replication Fork Barrier Site in the rRNA Gene Repeats during S Phase in a *FOB1*-Dependent Fashion To Prevent Contraction of a Long Repetitive Array in *Saccharomyces cerevisiae*†

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An average of 200 copies of the rRNA gene (rDNA) is clustered in a long tandem array in *Saccharomyces cerevisiae*. *FOB1* is known to be required for expansion/contraction of the repeats by stimulating recombination, thereby contributing to the maintenance of the average copy number. In Δ*fob1* cells, the repeats are still maintained without any fluctuation in the copy number, suggesting that another, unknown system acts to prevent repeat contraction. Here, we show that condensin acts together with *FOB1* in a functionally complemented fashion to maintain the long tandem repeats. Six condensin mutants possessing severely contracted rDNA repeats were isolated in Δ*fob1* cells but not in *FOB1*$^+$ cells. We also found that the condensin complex associated with the nontranscribed spacer region of rDNA with a major peak coincided with the replication fork barrier (RFB) site in a *FOB1*-dependent fashion. Surprisingly, condensin association with the RFB site was established during S phase and was maintained until anaphase. These results indicate that *FOB1* plays a novel role in preventing repeat contraction by regulating condensin association and suggest a link between replication termination and chromosome condensation and segregation.

Repeated sequences and genes are extensively distributed throughout the genomes of many organisms. A common feature of these repeats is instability; the copy number of repeats frequently fluctuates, and they are exposed to the risk of contraction. However, the mechanisms responsible for the fluctuation of repeat copy number and for the maintenance of the repeats remain largely unknown. The eukaryotic rRNA gene (rDNA) family is a typical repeated structure. The genes are generally clustered in long tandem repeats on one or several loci in most eukaryote genomes. In the yeast *Saccharomyces cerevisiae*, 200 copies on average are tandemly arrayed in a central position on the longest chromosome, chromosome XII. A single rDNA unit (9.1 kb) comprises a 35S rRNA coding region which is transcribed by RNA polymerase I, the 5S rRNA coding region transcribed by RNA polymerase III, and two nontranscribed spacers (NTS), i.e., NTS1 and NTS2 (see Fig. 5A). The replication origin (ARS) and the replication fork barrier (RFB) are located within NTS2 and NTS1, respectively (5, 33, 42). The whole length of the tandem array extends ~1.9 Mb.

One system that maintains the average copy number of the rDNA long tandem array has been reported in recent years. High frequencies of recombination events and expansion/contraction of the repeats are observed for *FOB1*$^+$ strains, whereas deletion of *FOB1* (Δ*fob1*) eliminates both events (14, 24, 27). Furthermore, DNA strand break formation around the RFB site was observed to be *FOB1* dependent (7, 29, 47). *FOB1* is therefore thought to be required for the maintenance of average copy number by increasing or decreasing copy number through the regulation of recombination events between the repeats. In Δ*fob1* cells, surprisingly, the rDNA tandem array is still maintained stably without any expansion/contraction. The static rDNA tandem array maintained in Δ*fob1* suggests that a second, unknown system, one that is different from the *FOB1*-stimulated recombinational system, acts to maintain the long tandem repeats. Originally, *FOB1* was identified as the gene required for replication fork blocking in the direction opposite to 35S rRNA transcription at the RFB site (26). Recently, both in vivo and in vitro RFB site binding activity of Fob1p was demonstrated (28, 36). It is believed that the Fob1p/RFB system is required to avoid a head-to-head collision between replication and transcription of RNA polymerase I (5, 26, 33) and to contribute in the maintenance of chromosome integrity.

Condensin in *S. cerevisiae* is a protein complex consisting of five subunits, Smc2p, Smc4p, Brn1p, Ycs4p, and Ycs5p (Ycg1p), and plays an important role in condensation and segregation of chromosomes during mitosis (23, 31). All subunits are conserved across the eukaryotes. The two core subunits, Smc2p and Smc4p, belong to the "structural maintenance of chromosomes" family, whose members are involved in various chromosome dynamics; *SMC1* and *SMC3* encode subunits of cohesin that are required for connection of sister chromatids (35), and *SMC5* and *SMC6* are found to work for

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the segregation of repetitive chromosome regions (48). Condensin is essential for cell growth and has been reported to play a role in mitosis as well as in interphase (2). Cytological observations in *S. cerevisiae* demonstrated that condensin binds throughout the genome during interphase and is concentrated to the nucleolus in anaphase (4, 17, 34). Recently, a novel finding was reported. Although the majority of sister chromatid disjunctions are triggered by cleavage of cohesin during metaphase/anaphase transition (49), sister chromatids on the long arm of chromosome XII that contain the rDNA repeats are still held together despite cohesin destruction. The disjunction of sister chromatids of this locus occurs in mid-anaphase, long after cohesin destruction, and condensin is required for this step (13, 44). However, the mechanisms by which the sister chromatids at the rDNA locus are held together and the role of condensin in chromosome segregation and condensation remain largely unknown.

In this study, we attempted to understand the putative second, unknown system by which the long rDNA arrays are maintained by isolating mutants carrying an unstable, shortened rDNA tandem array in a Δ*fob1* background. We found that condensin mutants showed severe contraction of rDNA tandem array and slow growth in Δ*fob1* cells but not in *FOB1*$^+$ cells. Chromatin immunoprecipitation (ChIP) showed that condensin complex was loaded onto the RFB site in a *FOB1*-dependent fashion during S phase and remained associated until anaphase. These results indicate that there is a novel system to prevent contraction of long rDNA tandem array securely through *FOB1*-dependent condensin complex association to the rDNA.

MATERIALS AND METHODS

Strains and plasmids. Yeast strains and plasmids used in this study are summarized in Table S1 in the supplemental material.

Isolation of the mutants and cloning of the complementing gene. UKJY225 carrying pKJY181 was mutagenized by use of ethyl methanesulfonate (3%) and plated on yeast extract-peptone-dextrose (YPD). Red colonies without any detectable white sector were screened. Candidates were transformed with pKJY053 to test whether the isolated clones still maintained sector formation ability. Subsequently, pKJY181 was removed by selecting for 5-fluoroorotic acid (FOA)-resistant (FOA^R) colonies, and chromosome XII sizes of both FOA-sensitive (FOA^S) and FOA^R cells were analyzed. pKJY181 carried in the mutant was substituted with pKJY182 (*LEU2* marker) to use a *URA3*-marked genomic library (YCp50 based).

CHEF analysis. Chromosomal DNA preparation in gel molds was performed as described in the instruction manual (clamped homogeneous electric field [CHEF]; Bio-Rad). Chromosomes in the gel mold were separated in 0.8% pulse field-certified agarose by using CHEF for 72 h at 14°C with 3 V/cm at a linear pulse of 5 to 15 min. In order to determine the rDNA tandem array length, the chromosomes prepared in the gel molds were digested with BamHI, and the DNA fragments were separated in 1.0% pulse field-certified agarose by using CHEF for 30 h at 14°C with 6 V/cm at a linear pulse of 5 to 25 seconds.

ChIP assay. The tandem affinity purification-tagged *BRN1* (*BRN1*-TAP) and *SMC4*-TAP strains were used for the ChIP assay. Asynchronous cultures grown at 30°C or cells arrested with α-factor (1.5 μM), hydroxyurea (HU) (180 mM), or nocodazole (10 μg/ml) for 2 h were used for the ChIP assay. To analyze the *SMC4* association in anaphase-arrested cells, the strains possessing the *cdc15-2* mutation (3) were used to arrest cells in anaphase at 37°C for 2 h. ChIP assay was performed in accordance with the method of Claypool et al. (11) with some modifications. Protein concentrations of the whole-cell extracts (WCE) with and without cross-linking were adjusted to 5 mg/ml. Five hundred microliters of WCE was mixed with 35 μl (bed volume) of immunoglobulin G-agarose beads (Sigma), and immunoprecipitation (IP) was performed. Thirty microliters of WCE was stored for use as the Input sample. Input and IP samples were finally suspended in 100 ml Tris-EDTA after reverse cross-linking and phenol extraction. A 30-fold dilution of the Input sample was used for the PCR (17 cycles). The PCR primers in the rDNA and *CUP1* regions were 26-mer oligonucleotides with 50% GC content. The PCR products were separated in 2.5% low-melting-temperature agarose gels (NuSieve GTG agarose; Cambrex) and stained with SYBR green (Cambrex). Signal intensities of the PCR products were measured using a LAS-1000 instrument (Fuji).

FISH observation. Fluorescence in situ hybridization (FISH) was carried out as described in the work of Guacci et al. (20) with some modifications as follows. Zymolyase treatment was performed after the cells had adhered to the poly-lysine-coated slide glass. The 8.5-kb fragment containing most of the rDNA unit was used for probe preparation. The probe was labeled with biotin by use of a biotin-nick translation kit (Roche) and detected with fluorescein isothiocyanate avidin D (Vector).

RESULTS

Isolation of mutants carrying supercontracted rDNA repeats in the Δ*fob1* cells and identification of causal genes. In order to elucidate the second, unknown system involved in rDNA repeat maintenance, we attempted to isolate mutants in which the rDNA copy number is drastically reduced under *FOB1*-defective conditions. However, such cells are expected to exhibit very slow growth or lethality under *fob1*$^-$ conditions because of an insufficient rDNA copy number. To avoid the cessation of cell growth in such mutants, the *FOB1* gene was introduced into the parental strain via a plasmid; this allowed rDNA amplification to occur and thus supplied the rDNA copies. Thus, mutants carrying the shortened rDNA array in *fob1*$^-$ conditions are expected to exhibit *FOB1* plasmid-dependent faster growth or survive. Therefore, a synthetic lethality screen using a colony color-sectoring assay was set up with the Δ*fob1* strain (Fig. 1A). In brief, the UKJY225 strain carrying the pFOB1 plasmid (pKJY181) was mutagenized. Since the pFOB1 plasmid is not essential for growth in the parental cells, segregation of the plasmid can be observed by colony color sectoring in the parental cells, whereas no sector is expected to be observed for the mutants exhibiting pFOB1-dependent growth. The red colonies without any detectable sectors were screened at 30°C. Among ~150,000 colonies, 88 such clones were isolated. pFOB1 was then removed from each mutant by selecting for FOA^R cells, and all 88 FOA^R clones were grown. Subsequently, the sizes of chromosome XII under the *FOB1*$^+$ (FOA^S) and Δ*fob1* (FOA^R) conditions were determined by CHEF electrophoresis. Although most cells carried a long chromosome XII under both conditions, we found six clones (clones #31, #75, #99, #157, #193, and #200) that carried an extremely short chromosome XII in the Δ*fob1* background but carried one of almost normal size in the *FOB1*$^+$ background (Fig. 1B). The parental *FOB1*$^+$ (YMW1) (54) and Δ*fob1* (UKJY225) cells carried a 3.1-Mb-long chromosome XII that was estimated to contain approximately 220 copies of the rDNA (the size of the sum of non-rDNA regions of chromosome XII is approximately 1.1 Mb, and the size of a single rDNA unit is 9.155 kb); however, under the Δ*fob1* condition, the size of the chromosome XII of five mutants (#31, #99, #157, #193, and #200) was reduced to ~1.2 Mb, and that of mutant #75 was reduced to ~1.7 Mb. To confirm the shortening of chromosome XII under Δ*fob1* conditions caused by the contraction of the rDNA tandem array, chromosome DNA samples were digested with BamHI, which does not cut within the rDNA (Fig. 1C), and their fragment sizes were determined by CHEF (Fig. 1D). The copy number of the rDNA repeats of the five mutants was dramatically reduced (7 to 16

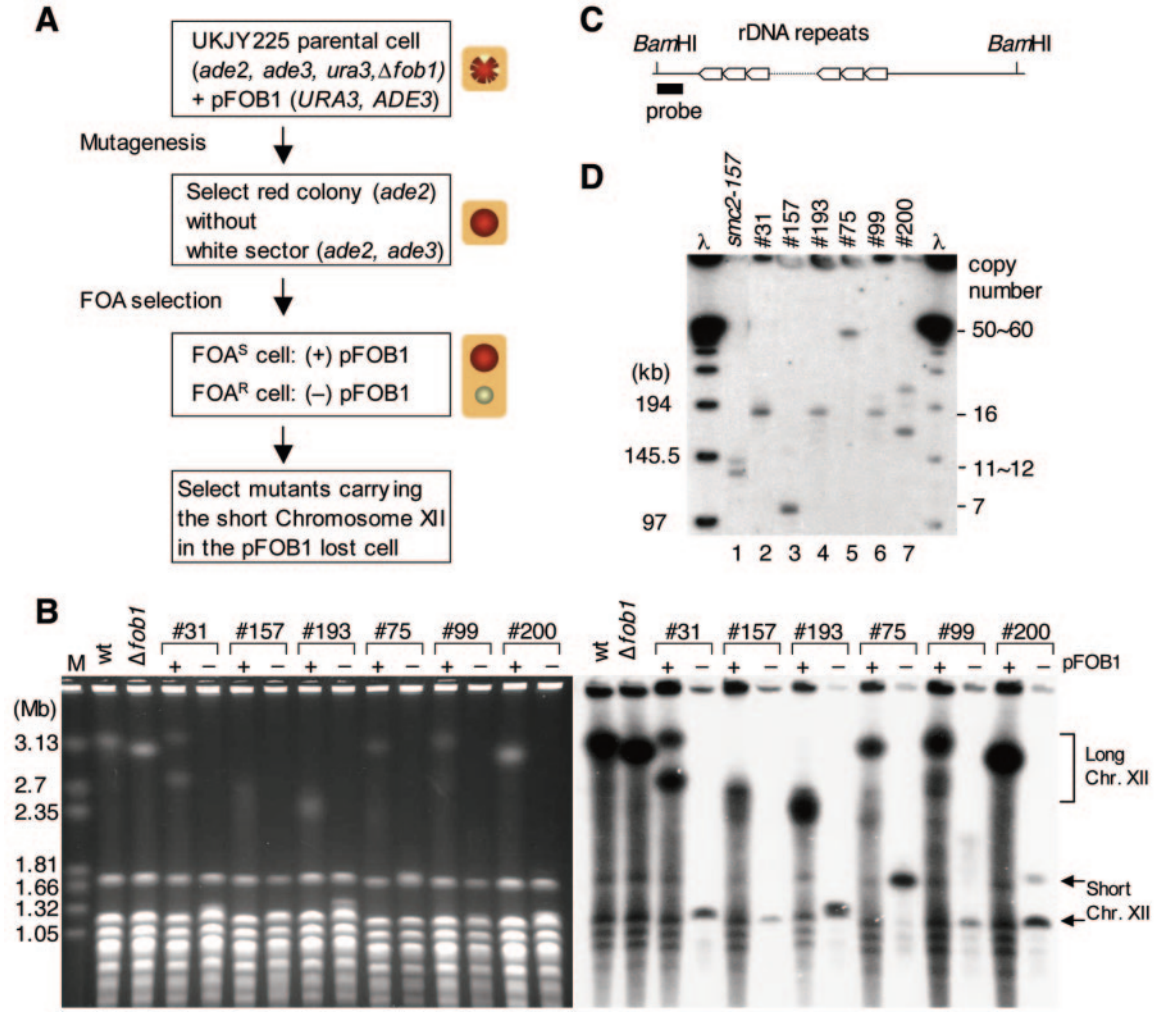


FIG. 1. (A) Schematic representation of mutant screening. The genotype of the parental cells (UKJY225) carrying the pFOB1 plasmid is *ade2*; this genotype produces red-colored cells. If the plasmid is lost, the genotype of the cells changes to *ade2 ade3*, resulting in the production of a white sector. Since the pFOB1 plasmid (pKJY181) is not essential for the growth of the parental cells, plasmid segregation is observed as red-colored colonies with white sectors, as shown on the right. Based on this genetic rationale, the cells that exhibited pFOB1-dependent fast growth and produced red colonies without any detectable white sector were screened. *URA3*$^+$ cells are sensitive to FOA, but *ura3*$^-$ cells are resistant to it. Therefore, the cells carrying pFOB1 (*URA3* marker) cannot be grown on a medium containing FOA (FOA^S), but the cells that have lost the plasmid can be grown on this medium (FOA^R). (B) Mutants carrying the supercontracted rDNA tandem repeats in Δ*fob1* cells. Chromosomes of the six mutants with (+) and without (−) pFOB1 (pKJY181) were separated by CHEF electrophoresis and visualized by staining with ethidium bromide (EtBr) (left panel). The size of each marker band is indicated on the left. Chromosome XII was detected by Southern hybridization with an rDNA probe (right panel). The background cross-hybridization of the rDNA probe to other chromosomes without rDNA is not uniform. The reason is unclear, but the cross-hybridization pattern was not reproducible. The *Hansenula wingei* genome (Bio-Rad) was used as a chromosome size marker (M). YMW1, wild type (wt); UKJY225, Δ*fob1*. (C) The rDNA repeats (boxes) and their flanking regions are shown. The black bar indicates the probe position, which is located outside the rDNA array. Digestion with BamHI, which does not have any sites within the rDNA, produces a fragment containing the rDNA tandem array; the size of this fragment can be calculated as follows: (number of rDNA repeats × 9.15 kb) + 38.7 kb; 38.7 kb is the size of the non-rDNA region. For instance, if seven rDNA repeats are present, the BamHI digestion will yield a 103-kb fragment based on the above calculation. (D) CHEF analysis of the length of the rDNA array in the mutants without the pFOB1 plasmid (Δ*fob1*) is shown. The BamHI fragment of each mutant was separated and detected by Southern hybridization. A lambda ladder (λ) was used as the size standard and was detected simultaneously using a lambda probe. The lambda band sizes are indicated on the left, and the estimated copy numbers of the BamHI fragment are indicated on the right. Strains: N2KJY173 (lane 1), #31 (lane 2), #157 (lane 3), #193 (lane 4), #75 (lane 5), #99 (lane 6), and #200 (lane 7).

copies; lanes 2, 3, 4, 6, and 7) and that of mutant #75 (lane 5) was reduced to approximately 60 copies (based on the results shown in Fig. 1B). Therefore, the shortening of chromosome XII was due to the supercontraction of the rDNA tandem array. The growth rate of the six mutants carrying p*FOB1* (doubling time = 90 to 100 min) was approximately equal to or slightly lower than that of the parental strain (90 min), whereas the mutants in the Δ*fob1* background showed a severe slow-growth phenotype (doubling time = 170 to 260 min).

In order to identify the genes responsible for the shortening of the rDNA array in the six mutants, we screened the genomic library and identified the gene that was capable of comple-

menting the lack of sector formation in mutants #193 and #31. In the case of mutant #193, the *BRN1* gene—a gene encoding a subunit of the condensin complex—was identified as the gene complementing sector formation at the wild-type level. We then examined whether the *BRN1* gene was able to complement the supercontraction of the rDNA array in mutant #193 after the removal of pFOB1. As shown in Fig. 2A, the loss of pFOB1 in mutant #193 led to the shortening of its chromosome XII (compare lanes 2 and 3). However, in the presence of the *BRN1* gene, the length of chromosome XII remained unchanged even after pFOB1 loss (compare lanes 4 and 5), confirming that *BRN1* complemented the supercontraction of the rDNA array in mutant #193. Further, the subsequent removal of the *BRN1* plasmid led to the shortening of chromosome XII (lane 6), indicating that the *BRN1* gene was required to prevent repeat contraction in Δ*fob1* cells. In the case of mutant #31, *SMC4*, whose product is another subunit of the condensin complex, was identified.

Since all six mutants exhibited similar phenotypes, we asked whether condensin could complement the defect in the other four mutants as well. Five condensin genes, *SMC2*, *SMC4*, *BRN1*, *YCS4*, and *YCS5*, were cloned on a centromere plasmid (*LEU2* marker) and introduced into the remaining four mutants carrying pFOB1; sector formation was then examined. The results of this complementation test revealed that two condensin genes were able to complement the defects in all four mutants; *SMC2* and *SMC4* complemented in mutants #157 and #99 and mutants #75 and #200, respectively (data not shown). Therefore, all six mutants were identified as condensin mutants, suggesting that condensin plays an extremely important role in preventing the contraction of the long rDNA tandem repeats, at least under Δ*fob1* conditions. Each mutation was identified as a single base substitution within the coding region, leading to a single amino acid substitution (summarized in Fig. 2B). We refer to these mutations as indicated in Fig. 2B; for instance, we refer to mutation in the mutant #193 as *brn1-193*. Four mutations in the *SMC* genes (*smc2-99*, *smc2-157*, *smc4-31*, and *smc4-75*) are located near the ATP binding Walker B motif. The *brn1-193* mutation is located within the N-terminal domain conserved among the kleisin superfamily.

Construction and characterization of the *smc2-157* mutant. To confirm and further analyze the effect of condensin mutation on rDNA array size, we introduced the most severe mutation (*smc2-157*) of mutant #157 into the wild-type strain. Mutant #157 under Δ*fob1* conditions carries the least number of rDNA repeats (seven copies; Fig. 1D, lane 3). The *smc2-157* mutation was fused with a *kanMX* marker and introduced into the diploid strain (N2KJY038). The resulting *smc2-157* heterozygous diploid strain (*smc2-157*/*SMC2* Δ*fob1*/Δ*fob1*; N2KJY091) carried long rDNA repeats. In addition, the diploid strain carrying pFOB1 (N2KJY073) produced a wild-type level of sectored colonies, indicating that the *smc2-157* mutation was recessive. A haploid Δ*fob1 smc2-157* strain carrying pFOB1 (N2KJY075), which is created by dissection of N2KJY073, produced sectorless colonies similar to those seen for the original mutant. The rDNA array size of the *smc2-157* mutant was then analyzed by monitoring the chromosome XII size in the *FOB1*$^+$ and Δ*fob1* cells. While chromosome XII in the *FOB1*$^+$ condition (N2KJY075) was observed as a broad band with a

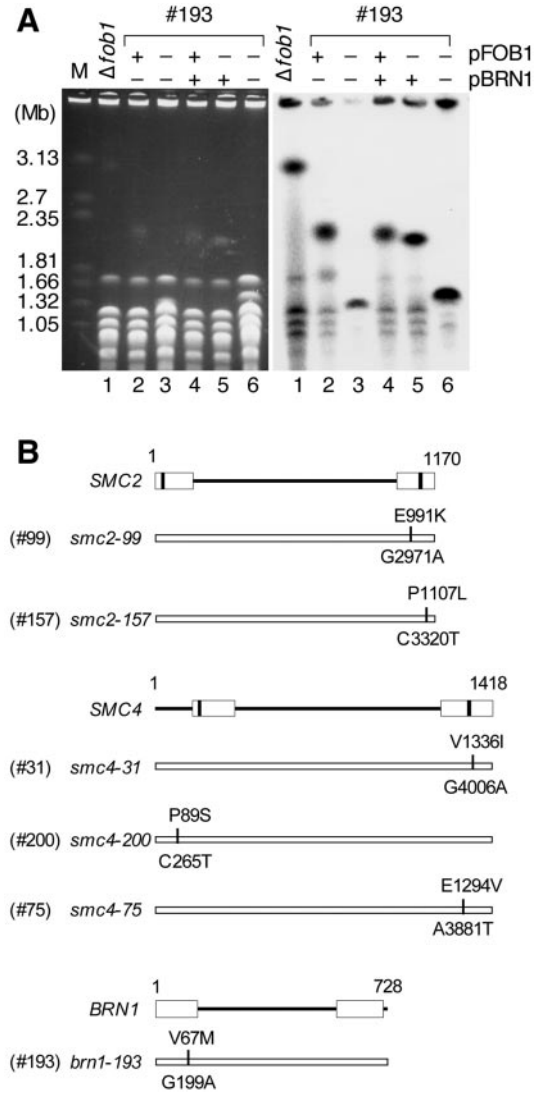
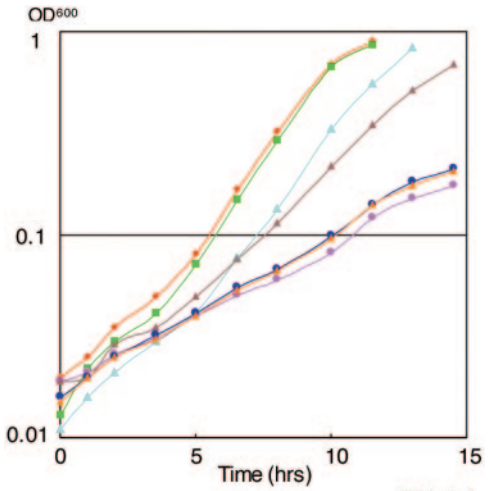


FIG. 2. (A) Complementation of supercontraction in mutant #193 by the *BRN1* gene. CHEF analysis of the chromosome XII length in mutant #193 is shown. All the chromosomes are visualized by staining with EtBr (left panel), and chromosome XII is detected by Southern hybridization with an rDNA probe (right panel). The fragment of the *FOB1* gene in pKJY182 was prepared by PCR amplification (27), and its ability to stimulate *HOT1* recombination was partial in case the fragment was cloned on a low-copy-number plasmid (T. Kobayashi, personal communication). The slightly shorter chromosome XII length in mutant #193 carrying pKJY182 (lanes 2 and 4) is probably due to the partially reduced *FOB1* function. M (*H. wingei*); UKJY225 (Δ*fob1*; lane 1); mutant #193 (lanes 2 to 6); pFOB1 (pKJY182); pBRN1 (pKJY190). (B) Schematic representation of each position of mutation identified from the six mutants. The vertical line in each open reading frame indicates the position of the mutation. The base substitution of each mutation is indicated below the line along with its position relative to the first ATG. The resulting amino acid change is shown above the line along with its position relative to the first Met. For Smc2p and Smc4p, the globular domains located at the N terminus and C terminus are represented as rectangles, and the ATP binding Walker A and B motifs are shown as vertical bars in the N-terminal and C-terminal globular domains, respectively. For Brn1p, the N-terminal and C-terminal conserved domains among the kleisin superfamily are represented as rectangles.



		Td (min)
YMW1	wild type	90
UKJY225	Δ*fob1*	90
N2KJY075	*smc2* Δ*fob1* + pFOB1	95
N2KJY173	*smc2* Δ*fob1*	260
N2KJY173*1	*smc2* Δ*fob1* + pRDN-wt	240
N2KJY173*2	*smc2* Δ*fob1* + pFOB1	140
N2KJY173*3	*smc2* Δ*fob1* + vector	250

FIG. 3. Growth rates of the *smc2-157* mutants. An aliquot of a full growth culture of each strain was transferred into fresh YPD medium, and the growth was monitored to determine the cell density (optical density at 600 nm [OD_{600}]) at 30°C for several hours. The cell densities at each time point were plotted. The doubling time (Td) for each strain is shown below the growth curve. The bottom three strains, N2KJY173*1 to N2KJY173*3, were created by introducing the pRDN-wt, pFOB1-*TRP1* (pKJY053), and YCplac22 plasmids, respectively, and each fresh transformant was used for the assay.

major intense band of 2.35 Mb, corresponding to ~130 rDNA copies, it was dramatically shrunk to ~1.2 Mb in the Δ*fob1* condition (N2KJY173) (see Fig. S1 in the supplemental material), as observed in the original mutant #157. The BamHI digestion to analyze the rDNA array length in the Δ*fob1 smc2-157* double mutant showed two discrete bands of approximately ~140 kb, corresponding to 11 and 12 copies of rDNA (Fig. 1D, lane 1). Therefore, it was confirmed that the *smc2-157* mutation itself caused destabilization of the rDNA array even under the *FOB1*$^+$ condition and supercontraction of the rDNA array under the Δ*fob1* condition.

The destabilized rDNA repeat observed in the mutant led us to analyze the recombination event within the repeats. In the *FOB1*$^+$ *smc2-157* strain, the recombination event measured by *URA3* marker loss frequency increased to 9.5-fold that in the *FOB1*$^+$ *SMC2*$^+$ strain ($82.5 \pm 6.3 \times 10^{-4}$ versus $8.8 \pm 1.3 \times 10^{-4}$). We also analyzed the production extrachromosomal rDNA circle (ERC) molecules. However, the accumulation of ERC molecules in the *FOB1*$^+$ *smc2-157* strain (N2KJY075) was only twofold that in the wild type (YMW1); in the Δ*fob1 smc2-157* double mutant (N2KJY173), ERC molecules were barely detected (unpublished results). Thus, the total copy number (chromosomal repeats and ERC molecules) of the rDNA in the Δ*fob1 smc2-157* double mutant is extremely low.

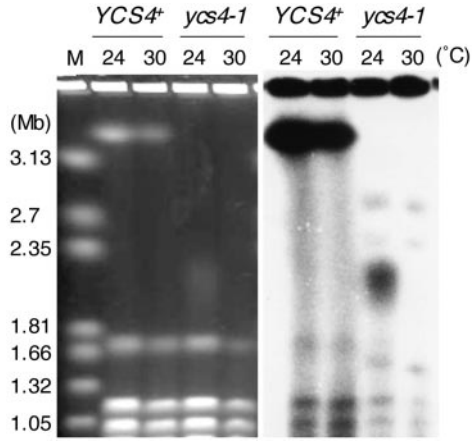


FIG. 4. Length of the rDNA array in the *ycs4-1* mutant. CHEF analysis of chromosome XII length in *ycs4-1* and *YCS4*$^+$ cells is shown. The left panel shows the CHEF gel stained by EtBr, and the right panel shows the same gel observed by Southern hybridization with an rDNA probe. The *ycs4-1* strain (NBY521) and its parental *YCS4*$^+$ strain (NBY522) grown in YPD medium under permissive (24°C) and semipermissive (30°C) conditions were used. M (*H. wingei* genome).

***FOB1* can suppress the slow-growth phenotype of the Δ*fob1* condensin double mutant, but a high-copy-number rDNA plasmid cannot.** As described above, the growth rate of the six condensin mutants was almost at the wild-type level in the *FOB1*$^+$ cells; however, the growth rate in the Δ*fob1* cells showed a severe decrease. A plausible reason may be that the copy number of rDNA was insufficient to ensure maximal growth. In order to verify this, the full length of the rDNA unit cloned on a high-copy-number plasmid, pRDN-wt (9), was introduced into the Δ*fob1 smc2-157* double mutant to supply sufficient copy numbers, and the growth rate was examined. Unexpectedly, as shown in Fig. 3, pRDN-wt hardly improves the slow growth of the double mutant (N2KJY173*1), indicating that the dramatic reduction in rDNA copy number is not the primary cause of slow growth. On the other hand, growth rate improvement was observed by the introduction of the *FOB1* gene (N2KJY173*2). The rDNA copy number of N2KJY173*2 cells gradually increased and possessed approximately 30 to 40 copies in this assay (data not shown). Despite the fact that N2KJY173*2 had a copy number lower than that of N2KJY173*1, which possesses the rDNA on the 2μm plasmid (an average of 60 copies per haploid cell) (10), growth rate recovery was observed only in the N2KJY173*2 transformant. These results suggest that *FOB1* suppresses the slow growth defect of condensin mutation by a reason other than insufficient rDNA copy number. The slightly slower growth of N2KJY173*2 in comparison to that of N2KJY075, which has harbored pFOB1 for multiple generations, is probably due to the lower rDNA copy number (30 to 40 versus 150).

Mutation of another condensin subunit, *ycs4-1*, results in the contraction of the rDNA array in *FOB1*$^+$ cells and is lethal in Δ*fob1* cells. In order to examine the effect of mutation in the other condensin subunit, the rDNA array size was analyzed by monitoring the length of chromosome XII in the previously isolated *ycs4-1* temperature-sensitive mutant (NBY521) (4) under both permissive (24°C) and semipermissive (30°C) conditions (Fig. 4). In the parental *YCS4*$^+$ strain (NBY522), the

length of chromosome XII was normal, whereas in the *ycs4-1* mutant, its length decreased to ~2 Mb under the permissive condition and it was either too dispersed to detect or stacked in a well under the semipermissive condition. Thus, the mutation of another condensin subunit also resulted in shortened rDNA repeats, even in *FOB1*$^+$ cells. We then asked whether the supercontraction of the array occurs in a Δ*fob1 ycs4-1* double mutant. We first attempted to delete *FOB1* in the *ycs4-1* strain (NBY521); however, we failed to disrupt the *FOB1* gene. Subsequently, the diploid strain was dissected; this strain was constructed by crossing the Δ*fob1* strain (N2KJY 002) with the *ycs4-1* strain (NBY521). Surprisingly, none of the Δ*fob1 ycs4-1* double mutant progeny grew. We concluded that *ycs4-1* is synthetically lethal with Δ*fob1*, and the six mutants isolated in this study have properties similar to but less severe than those of *ycs4-1*. While a 9.5-fold increase in the frequency of marker loss was observed in the *smc2-157* mutant, the 63-fold increase observed in the *ycs4-1* mutants in comparison with the parental *YCS4*$^+$ strain (4) is consistent with the above conclusion.

***FOB1*-dependent condensin complex association with the RFB site.** It was reported that the condensin complex plays an important role in sister chromatid segregation of the rDNA region (4, 17, 34). By using the ChIP assay, it was demonstrated that condensin physically binds to the rDNA in vivo (17, 34, 51). The results of the suppression in condensin mutants by *FOB1* raise the possibility that *FOB1* may affect the association of condensin with rDNA. We examined this possibility to analyze the profile of condensin complex association across the rDNA by a ChIP assay using TAP-tagged (41) *BRN1* or *SMC4*, in which the endogenous copy of each gene was modified at its C terminus by TAP (*BRN1*-TAP and *SMC4*-TAP). Both the strains exhibited normal growth rates and did not exhibit temperature sensitivity, suggesting that the TAP-tagged condensin genes are fully functional. As shown in Fig. 5B, both *BRN1*-TAP and *SMC4*-TAP showed similar association patterns in asynchronous cultures of *FOB1*$^+$ cells. The major peak in the association profiles of both subunits coincided with the RFB site, and the base of the peak was widely extended within the NTS region. In contrast, both subunits did not associate strongly with the 35S rRNA region; the binding was even throughout the 35S rRNA coding region and did not show any characteristic peaks. The major peak of association with the RFB site was consistent with results from a previous report (51), but no other significant peaks were observed within the 35S rRNA coding region. The virtually identical profiles of *BRN1*-TAP and *SMC4*-TAP association across the rDNA strongly suggest that both components associate as a condensin complex. In the Δ*fob1* cells, the peak of association at the RFB site was almost lost, but a small increase in the association profile within the NTS region was observed (Fig. 5B). Therefore, we concluded that there were three modes of condensin complex association within the rDNA. The first mode was identified based on the peak of *FOB1*-dependent association with the RFB site, the second by the small increase in association observed within the NTS region in the Δ*fob1* cells, and the third by a low basal level of association observed in the 35S rRNA coding region without any characteristic peaks in both *FOB1*$^+$ and Δ*fob1* cells. The preferential association of condensin with the RFB site in a *FOB1*-dependent fashion was also confirmed by monitoring the relative severalfold increases in the intensities of the PCR fragments around the RFB site from that of a fragment in the 35S rRNA coding region (see Fig. S2 in the supplemental material).

We also observed the *BRN1*-TAP association in an asynchronous culture of the *smc2-157* mutant. As shown in Fig. 5D, while a peak at the RFB site was still observed in the *smc2-157* mutant in *FOB1*$^+$ cells, the association with the RFB site as well as the base of the peak extended within the NTS region was severely decreased; weak associations in the 35S rRNA coding region were also decreased. These results indicate that the *smc2-157* mutation decreases the rDNA binding ability of the condensin complex.

Cytological observations demonstrated that condensin was dispersed within the nucleus during interphase and was concentrated in the nucleolus during mitosis (4, 17, 34). In order to determine whether the *FOB1*-dependent condensin association peak at the RFB site was cell cycle regulated, the *SMC4*-TAP strain was arrested in G_1 phase (α-factor) or in metaphase (nocodazole), and a ChIP assay was carried out. As shown in Fig. 5E (the topmost graph and the third graph from the top), the *FOB1*-dependent condensin association with the RFB site is not observed in G_1-phase cells but is observed in metaphase cells. Thus, condensin association with the RFB site fluctuates during the cell cycle. The small peak at the RFB site observed in G_1-phase-arrested cells may be the result of leaky arrest by the α-factor, which was observed in the fluorescence-activated cell sorter (FACS) profiles.

Despite the low population of metaphase cells in asynchronous cultures, the peak value of condensin association of asynchronous cells is relatively high compared to that for metaphase-arrested cells. This raises the possibility that condensin association occurs in a cell cycle phase earlier than metaphase. We examined the *SMC4*-TAP association in S phase by arresting the cells for 2 h using HU, which arrested the cell cycle in early S phase. As shown in Fig. 5E (second graph from the top), the *FOB1*-dependent condensin association with the RFB site is observed in S-phase-arrested cells, and the peak value is almost the same as that observed in metaphase-arrested cells. A *FOB1*-dependent condensin association peak at the RFB site was also observed in the cells synchronized in S phase (the cells incubated for 30 minutes after being released from α-factor arrest) (data not shown). These results indicated that condensin was loaded onto the RFB site during the early S phase.

Recently, it was reported that condensin was required for the proper separation of sister chromatids in the rDNA region during anaphase (13, 44). We investigated whether the *FOB1*-dependent condensin association with the RFB site was maintained until the anaphase, in which cells were arrested by the *cdc15-2* mutation. This mutation arrests the cell cycle in the anaphase at 37°C, and nucleolar localization of Ycs4p was observed in the cells arrested in anaphase (13). As shown in Fig. 5E (bottom graph), the *FOB1*-dependent condensin association peak at the RFB site was also observed in the anaphase-arrested cells. Therefore, we concluded that the *FOB1*-dependent condensin association with the RFB site was established during the early S phase and maintained until anaphase.

Slow growth of the Δ*fob1 smc2-157* double mutant is due to a defect in rDNA segregation. What causes the slow growth

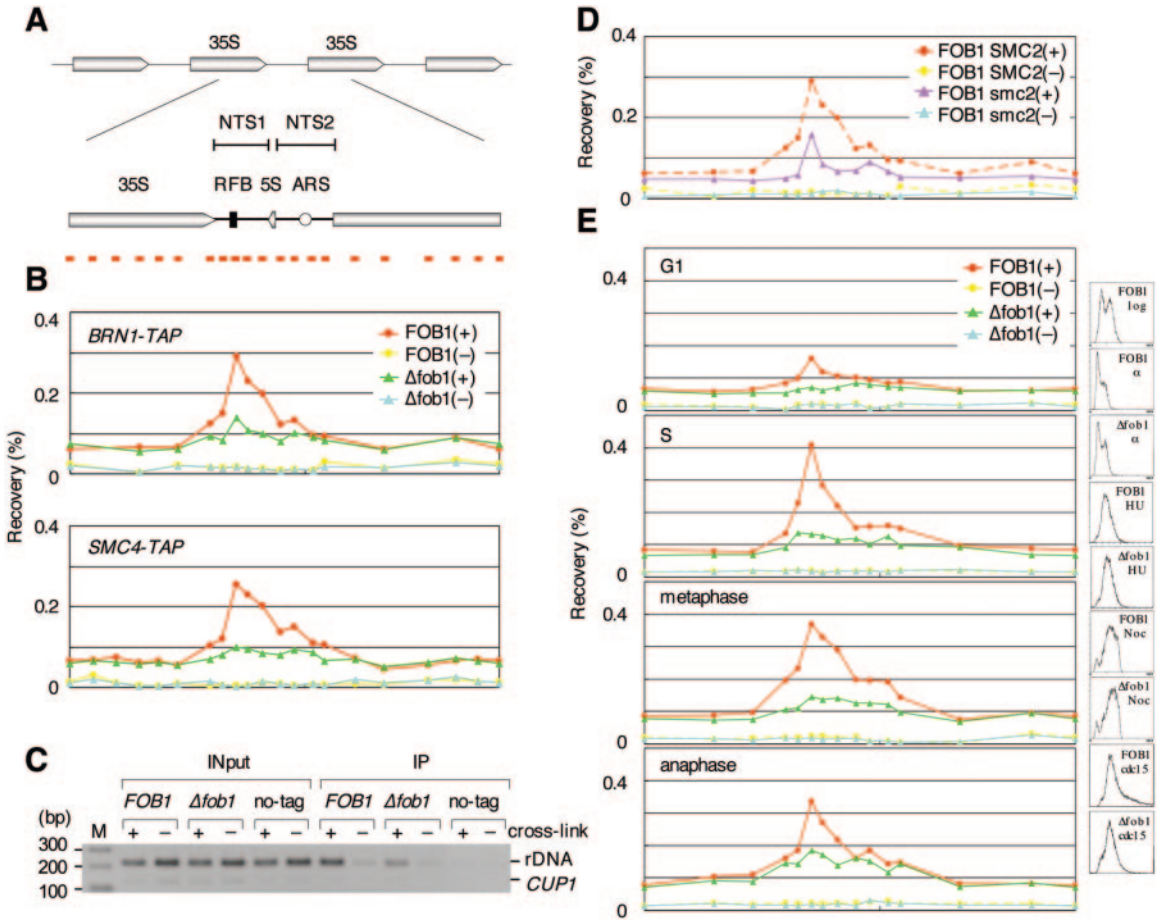


FIG. 5. *FOB1*-dependent condensin association with the RFB site in the rDNA. (A) The structure of the rDNA tandem array is shown above, and an enlarged single (9.1-kb) copy is shown below. The positions of the PCR fragments used for the ChIP assay are indicated as short red bars. The black rectangle within NTS1 shows the RFB site, and the circle within NTS2 shows the ARS. (B) Condensin association levels across a single rDNA unit in asynchronous cultures of *FOB1*$^+$ and Δ*fob1* are shown. The upper graph shows Brn1p association in *FOB1*$^+$ (red circles) or Δ*fob1* (green triangles) cells with cross-linking (+). Brn1p associations in *FOB1*$^+$ (yellow circles) and Δ*fob1* (blue triangles) cells without cross-linking (−) are also shown as controls. Similarly, the Smc4p associations analyzed using more-detailed primer sets are shown in the bottom graph. Percent recovery refers to the ratio of the signal intensities of the PCR products of IP materials divided by those of the Input materials at each position. Strains: N2KJY104 (*BRN1*-TAP *FOB1*$^+$); N2KJY131 (*BRN1*-TAP Δ*fob1*); N2KJY105 (*SMC4*-TAP *FOB1*$^+$); N2KJY133 (*SMC4*-TAP Δ*fob1*). (C) Examples of results from the ChIP assay for analysis of the Brn1p associations (at the RFB site) are shown. Both rDNA and *CUP1* primers were used in the PCRs, and specific enrichment of the rDNA was observed only in the IP samples with cross-linking (+) and not in samples without cross-linking (−). As a control experiment, a strain with no tag was also used for the ChIP assay (no-tag). M, 100-bp ladder. (D) The graph shows the Brn1p association profile in the *smc2-157* mutant (purple triangles) in asynchronous cells. Brn1p associations in the *SMC2*$^+$ strain (red circles with dotted line) are also shown. *FOB1*$^+$ *SMC2*$^+$ (yellow circles) and *FOB1*$^+$ *smc2-157* (blue triangles) cells without cross-linking (−) were included as a control. Strains: N2KJY159 (*BRN1*-TAP *FOB1*$^+$ *smc2-157*); N2KJY104 (*BRN1*-TAP *FOB1*$^+$). (E) The Smc4p association profiles across the rDNA in cells arrested at various stages of the cell cycle are shown. G_1-phase arrest, S-phase arrest, and metaphase arrest were carried out using N2KJY105 and N2KJY133 strains. Anaphase arrest was carried out using N2KJY237 (*SMC4*-TAP *cdc15-2*) and N2KJY241 (*SMC4*-TAP Δ*fob1 cdc15-2*) strains that possessed the *cdc15-2* mutation. Cell cycle arrest was monitored by FACS, as indicated on the right. *FOB1*$^+$ cell cross-link (+) (red circles); *FOB1*$^+$ cell cross-link (−) (yellow circles); Δ*fob1* cell cross-link (+) (green triangles); Δ*fob1* cell cross-link (−) (blue triangles).

rate of the Δ*fob1 smc2-157* double mutant? Microscopic observations showed that dumbbell-shaped cells of the Δ*fob1 smc2-157* double mutant were accumulated at a high level in an asynchronous culture. FACS analysis confirmed that a single peak corresponding to 2C cells was observed in the double mutant strain (data not shown); this indicates that the slow growth of the double mutant was caused mainly by slow progression during M phase. By FISH, we analyzed the segregation of the nucleolus and nucleus in these dumbbell-shaped cells, which had accumulated in the asynchronous culture of the Δ*fob1 smc2-157* double mutant. As shown in Fig. 6A, the rDNA signals decreased in size and intensity in the Δ*fob1 smc2-157* double mutant; this is consistent with the drastic reduction of the number of rDNA repeats (11 or 12 copies). In addition, despite our use of asynchronous cells, we observed a higher population of mitotic cells in which nuclei were completely separated but the nucleoli were still in the process of separation (Fig. 6A). In contrast, most of the cells were in interphase in the cases of the wild-type, Δ*fob1*, and *smc2-157* strains. Accumulation of the cells in the process of rDNA

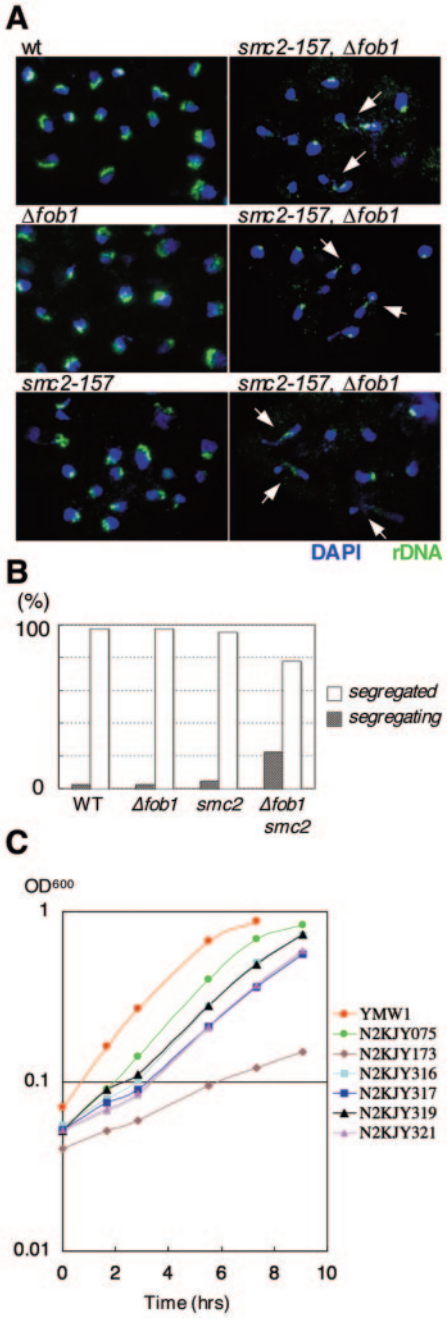


FIG. 6. Slow growth of the Δ*fob1 smc2-157* double mutant is due to a defect in rDNA segregation. (A) rDNA segregation of asynchronously growing cultures of wild-type (wt; YMW1), Δ*fob1* (UKJY225), *smc2-157* (N2KJY075), and *smc2-157* Δ*fob1* (N2KJY173) strains was observed by FISH. Arrows show the segregating rDNA observed in the Δ*fob1 smc2-157* double mutant. rDNA (green); DAPI (4′,6′-diamidino-2-phenylindole) (blue). (B) Graph showing the percentages of cells that have already segregated or are in the process of segregating rDNA in the cultures. (C) Growth curve of the Δ*fob1 smc2-157* double mutants lacking chromosomal rDNA array are shown. An aliquot of overnight culture was transferred into fresh YPD medium, and the growth was monitored to determine the cell density (optical density at 600 nm [OD_{600}]) at 30°C. The cell densities at each time point were plotted. Strains: YMW1 (wild type); N2KJY075 (*FOB1*$^+$ *smc2-157*); N2KJY173 (Δ*fob1 smc2-157*); N2KJY316 (*rdn*ΔΔ *FOB1*$^+$ *SMC2*$^+$); N2KJY317 (*rdn*ΔΔ *FOB1*$^+$ *smc2-157*); N2KJY319 (*rdn*ΔΔ Δ*fob1 SMC2*$^+$); N2KJY321 (*rdn*ΔΔ Δ*fob1 smc2-157*).

segregation suggests that the slow growth of the Δ*fob1* condensin double mutant was caused primarily by the delay of rDNA segregation.

If this is true, then the mitotic delay in the double mutant is due to the presence of an rDNA repeat; thus, the complete removal of the rDNA array from chromosome XII must restore the growth rate of the Δ*fob1* condensin double mutant. We examined this possibility by constructing a Δ*fob1 smc2-157* double mutant lacking a chromosomal rDNA array by crossing the double mutant with the *rdn*ΔΔ strain (50). The strains lacking chromosomal rDNA array constructed in this study carry a helper plasmid that can supply 35S rRNA by transcription of a galactose-inducible RNA polymerase II promoter. In addition, those strains also carry multiple copies of ERC molecules in which the 35S rRNA is transcribed by the native RNA polymerase I promoter, thus allowing cell growth even in a glucose medium (50). As shown in Fig. 6C, the growth rate of the Δ*fob1 smc2-157* double mutant lacking chromosomal rDNA array was almost same as those of the *smc2-157* single mutant (N2KJY317) and the *SMC2*$^+$ strains (N2KJY319 and N2KJY316). Thus, we confirmed that the slow growth observed in the Δ*fob1 smc2-157* double mutant was due to the defect in rDNA segregation. In addition, although the pFOB1 segregation frequency in the Δ*fob1 smc2-157* double mutant was particularly low, deletion of the chromosomal rDNA array restored the segregation frequency to the wild-type level (see Fig. S3 in the supplemental material). We therefore concluded that *FOB1* plays an important role in rDNA segregation, especially in the condensin mutant.

DISCUSSION

A long rDNA tandem array is important for cell growth, but it is vulnerable to the risk of copy number decrease. Here, we found a condensin complex-based system that prevents the contraction of long rDNA array in *S. cerevisiae*. The condensin mutants isolated in this study exhibited a severely slowed growth and a supercontraction of the rDNA tandem array under Δ*fob1* conditions, whereas both phenotypes were nearly suppressed under *FOB1*$^+$ conditions. Surprisingly, a sufficient number of rDNA copies supplied by a plasmid could not suppress the slow-growth phenotype of the Δ*fob1* condensin double mutant, whereas the *FOB1* gene could suppress the slow-growth phenotype. Because the tandem array was stably maintained in Δ*fob1* cells, we initially assumed the existence of a system that maintains the long tandem array independent from *FOB1* function. However, our results indicated that the condensin complex functions in concert with *FOB1* to prevent contraction of the long tandem array. The *FOB1*-dependent condensin complex association peak at the RFB site is the basis of the genetic relationship between *FOB1* and condensin.

Condensin complex loading onto the RFB site occurs during the DNA replication stage. Condensin is required for chromosome condensation and segregation during the mitotic stage. Hence, it is believed that condensin is loaded onto chromatin during mitosis. However, our results clearly indicated that condensin complex loading onto the RFB site occurred in the early S phase at the rDNA locus. This suggests that condensin also plays some role during the S phase. In the fission yeast *Schizosaccharomyces pombe*, it was reported that condensin played a

dual role during both mitosis and interphase (2). It was demonstrated that the condensin mutant exhibited a higher sensitivity to hydroxyurea and failed to activate the replication checkpoint. Thus, our results are consistent with the results demonstrated for *S. pombe* and suggest that condensin also plays an important role in the S phase of *S. cerevisiae*.

Cytological observations showed that the condensin complex is enriched at nucleolus during anaphase (4, 17, 34). Observation of condensin association by the ChIP method used in this study showed that there was almost no difference in the amounts of condensin bound to the rDNA from S phase until anaphase. Probably, high condensation of rDNA might produce an apparent increase in signal intensity in cytological observations, despite the fact that the total amount of condensin bound to rDNA could remain the same. Alternatively, condensin distribution during interphase is uniform on multiple chromosomal loci, but condensin remains bound only to the rDNA locus in anaphase. This might also produce an intense signal only in the nucleolus.

Relationship between *FOB1* and the condensin complex. The simple explanation for the relationship between *FOB1* and the condensin complex is that condensin is recruited to the RFB site by direct interaction with Fob1p. However, if that is the case, it is difficult to explain why the small amount of condensin association is observed within the NTS region in Δ*fob1* cells. In addition, the specific interaction between Fob1p and the condensin complex is not observed by use of the immunoprecipitation assay (unpublished data). Alternatively, the *FOB1*-dependent condensin loading onto the RFB site during the early S phase suggests that there is an intimate relationship between condensin loading and replication fork termination. In fact, most of ARS in the rDNA repeats fire in the early S phase (40), and replicating bubbles of an average size of 7 ± 4 kb were observed in the cells arrested for 2 h by HU treatment (43). At the RFB site, the progression of the replication fork in the direction opposite to that of 35S rRNA transcription is stopped in a *FOB1*-dependent manner during the early S phase. Subsequently, the fork moving in the same direction as that of 35S rRNA transcription approaches, and the two forks converge at the RFB site. Thus, the site of convergence of the replication fork is restricted to the RFB site in a *FOB1*-dependent manner. Completion of replication by the convergence of the two forks produces multiple intertwinings between sister chromatids (15, 45). Therefore, in the rDNA locus, the localization of the intertwinings must be restricted to the RFB site by *FOB1*. It is likely that condensin recognizes a certain DNA structure that is created at the RFB site, such as an entangled DNA structure formed by two converging replication forks or a Holliday-like structure formed by replication fork regression. In fact, biochemical analyses demonstrated that the human 13S condensin complex preferentially bound to cruciform DNA and DNA molecules with higher-order structures (25). Further study is necessary to elucidate the mechanism by which condensin is loaded onto the RFB site during S phase.

Possible role of condensin in preventing rDNA array contraction in concert with *FOB1*. Multiple intertwinings between sister chromatids produced by converging replication forks must be untangled completely before their separation during mitosis. Condensin is loaded onto the RFB site in the S phase when the intertwinings occur, and it remains associated with the RFB site until the M phase, when the catenanes must be untangled. It can be stated that condensin, along with topoisomerase II (Top2p), plays a role in managing the untangling of the catenanes. In fact, it was reported that condensin was required to localize Top2p onto the chromatin (4). In addition, it was recently reported that although the disjunction of the majority of chromosomal regions occurred through cohesin destruction during the metaphase/anaphase transition, the rDNA locus on sister chromatids remained connected even after the destruction of cohesin, and both condensin and Top2p were required for proper disjunction of this region during mid-anaphase (13, 44). Efficient untangling by condensin and Top2p may be required for the proper segregation of the rDNA locus. Although it is still unclear why cohesin-independent connection remains until anaphase, it can be interpreted that catenation connects the sister chromatids of the rDNA region even after cohesin destruction.

In the Δ*fob1* condensin double mutant, there is some defect in rDNA segregation. As a result, segregation of sister chromatids is expected to occur without complete untangling of the catenanes accumulated in the rDNA region. This may lead to fatal damage and result in the supercontraction of the tandem array. Although the *rad52* Δ*fob1 smc2-157* triple mutant was able to survive, the segregation frequency of the pFOB1 plasmid in the triple mutant was lower than that in *RDA52*$^+$ cells (unpublished results). It appears that the supercontraction may require the double-strand break repair pathway; however, the single-strand annealing pathway is probably responsible for the rescue of the *rad52* mutant. However, when supercontraction occurs and it results in carrying short tandem array in the Δ*fob1* condensin double mutant, the amounts of mutant condensin available for single rDNA copy are expected to increase. This would make the disjunction of short tandem array possible, although difficulties in disjunction still remain. This explanation is supported by the accumulation of dumbbell-shaped cells with still segregating rDNA regions in the asynchronous culture of the Δ*fob1 smc2-157* strain.

Why was the supercontraction observed in the condensin mutant under the Δ*fob1* condition but not under the *FOB1*$^+$ condition? In Δ*fob1* cells, as the replication fork proceeds in the direction opposite to that of RNA polymerase I transcription, it moves through the RFB site and reaches the 35S rRNA coding region (46); therefore, converging replication forks are not restricted to the RFB site but are distributed randomly throughout the rDNA. This results in the random distribution of intertwinings throughout the rDNA. Approximately 72% (6.6 kb of a single 9.1-kb copy) of these positions are expected to be located within the 35S rRNA coding region. Further, more than half of the copies of rDNA repeats are actively transcribed (18), and active replication origins are clustered (40) and are localized downstream of the transcriptionally active copies (40, 38). These findings imply that most replication fork convergence sites must be localized in the active 35S rRNA coding region. Within the active copies, strong and ceaseless RNA polymerase I transcription that can dissociate even histone-DNA associations (12) continues throughout the cell cycle (16). Such strong transcription may have an inhibitory effect on condensin association with the rDNA. Furthermore, the condensin mutant isolated in this study exhibited a

reduced ability to associate with the rDNA (Fig. 5D). Thus, in the Δ*fob1* condensin double mutant, strong RNA polymerase I transcription may dramatically inhibit the association of the mutant condensin complex with the rDNA. It was reported that the distribution of a similar SMC family complex, cohesin, is also affected by RNA polymerase II transcription (30, 32).

In the case of *FOB1*$^+$ cells, *FOB1* restricts the position of replication fork convergence to the RFB site; therefore, the inhibitory effect of RNA polymerase I transcription on the reduced associative ability of the mutant condensin complex can be suppressed by keeping the condensin interaction sites out of the RNA polymerase I transcription zone. This would enable a level of untangling sufficient to allow the maintenance of a long tandem array even in the case of the condensin mutants. The importance of the *FOB1*-dependent condensin association system observed only in the condensin mutants is that it appears to ensure prevention of the contraction of the long repeats. In fact, in the case of wild-type condensin, the long tandem array is stably maintained even under the Δ*fob1* condition. The *FOB1*-independent condensin association observed within the NTS region under the Δ*fob1* condition may be sufficient to manage 200 copies, if the condensin is wild type, because the degree of condensin association with the rDNA in Δ*fob1 SMC2*$^+$ cells is higher than that in *FOB1*$^+$ *smc2-157* cells, in which ~150 copies are maintained by the mutant condensin.

Dual roles are conserved for maintenance of long rDNA tandem array. Previous studies have demonstrated that cells possess an average copy number maintenance system, in which the *FOB1* stimulates recombination, thus allowing expansion/contraction of the repeats (27). Here, we demonstrate that another task performed by *FOB1* is prevention of the contraction of the tandem array via the regulation of condensin association with the RFB site. In performing both of these roles, the cells seem to acquire a higher potential for stably maintaining long repeat structures; this is because replication fork blocking activity and condensin are conserved in various organisms. From this point of view, it is very interesting that condensin in human cells is concentrated in the nucleolus during the interphase (8). Further, in bacteria, both SMC-like proteins (e.g., that encoded by *mukB* in *Escherichia coli*) and replication fork blocking systems (*Ter* site and Tus protein) are found, and chromosome segregation defects were observed in mutants of SMC-related genes (6, 19, 21, 22, 37, 39, 53). Therefore, the relationship between replication forks converging at specific sites and condensin association may be important not only for repeat but also for nonrepeat regions. In fact, it was reported very recently that condensin localized in the zone of converging DNA replication (52).

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