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SIR2 Regulates Recombination between Different rDNA Repeats, but Not Recombination within Individual rRNA Genes in Yeast

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Summary

It is known that mutations in gene *SIR2* increase and those in *FOB1* decrease recombination within rDNA repeats as assayed by marker loss or extrachromosomal rDNA circle formation. *SIR2*-dependent chromatin structures have been thought to inhibit access and/or function of recombination machinery in rDNA. We measured the frequency of *FOB1*-dependent arrest of replication forks, consequent DNA double-strand breaks, and formation of DNA molecules with Holliday junction structures, and found no significant difference between *sir2*Δ and *SIR2* strains. Formal genetic experiments measuring mitotic recombination rates within individual rRNA genes also showed no significant difference between these two strains. Instead, we found a significant decrease in the association of cohesin subunit Mcd1p (Scc1p) to rDNA in *sir2*Δ relative to *SIR2* strains. From these and other experiments, we conclude that *SIR2* prevents unequal sister-chromatid recombination, probably by forming special cohesin structures, without significant effects on recombinational events within individual rRNA genes.

Introduction

In most eukaryotic organisms, the genes for rRNAs (rDNA) are clustered in long tandem repeats on one or a few chromosomes. Although the total number of these chromosomal rDNA repeats appears to be maintained at a level appropriate for each organism, genes with such a repeated structure are in general thought to be unstable because of a high frequency of recombinational events. In fact, extensive alterations in the total number of chromosomal rDNA repeats have been observed in several organisms under a variety of conditions, for example, in certain *bobbed* mutants in *Drosophila* (Hawley and Marcus, 1989). The phenomenon called RNA polymerase switch in yeast also involves rDNA repeat expansion; yeast mutants defective in the essential subunits of RNA polymerase (Pol) I transcription factor UAF were found to transcribe chromosomal rDNA by Pol II, but are able to grow only after an increase in rDNA repeat numbers (Vu et al., 1999; Oakes et al., 1999). The ability to alter the number of rDNA repeats using recombinational mechanisms appears to be beneficial to organisms in such instances. On the other hand, excessively high recombinational events may be harmful to organisms; for example, they may lead to increased production and accumulation of extrachromosomal rDNA circles (ERCs), which have been shown to be toxic and might be a cause of senescence in *Saccharomyces cerevisiae* (Sinclair and Guarente, 1997). DNA double-strand breaks (DSBs) accompanied by recombinational events may also be harmful without efficient repair systems. Thus, it may be expected that organisms have developed systems to regulate recombination within rDNA repeats.

In the yeast *S. cerevisiae*, approximately 150 copies of rDNA are maintained on chromosome XII. Recombinational events within rDNA repeats in growing normal yeast cells appear to be mostly mediated by a *FOB1*-dependent system. This conclusion has been obtained previously by measurements of rDNA repeat expansion and contraction (Kobayashi et al., 1998), ERC formation (Defossez et al., 1999), and recombination frequency using a marker gene integrated within rDNA repeats (Merker and Klein, 2002; Johzuka and Horiuchi, 2002). *FOB1* was originally identified as the gene required for both replication fork blocking activity (RFB activity) at the replication fork barrier (RFB) site within the rDNA repeats (Figure 1A) and *HOT1* activity in a recombination test system outside the rDNA repeats (Kobayashi and Horiuchi, 1996). Because replication fork block takes place at the RFB site (Brewer et al., 1992; Kobayashi et al., 1992) where Fob1p actually binds (Kobayashi, 2003; Mohanty and Basia, 2004), it is likely that *FOB1*-dependent rDNA repeat expansion/contraction involves DSB at the RFB site and repair of the break via gene conversion, as proposed previously [Figure 1B, (i); Kobayashi et al., 1998; Rothstein et al., 2000]. Indeed, the results presented here give direct support to the occurrence of DSB at the RFB site.

It was first shown that the *SIR2* gene plays an important role in decreasing the frequency of recombination in yeast rDNA as assayed by the loss of a marker gene integrated within rDNA repeats (Gottlieb and Esposito, 1989). The frequency of formation of ERCs from rDNA repeats was also shown to increase upon deletion of *SIR2* (Kaeberlein et al., 1999). Sir2p is a protein required for transcriptional silencing at three yeast chromosomal regions, silent mating type loci, telomere regions, and rDNA (for reviews, see Moazed, 2001; Rusche et al., 2003). It is generally believed that Sir2p, perhaps through its NAD^+-dependent histone deacetylase activity (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000), plays an essential role in forming a higher order of repressive

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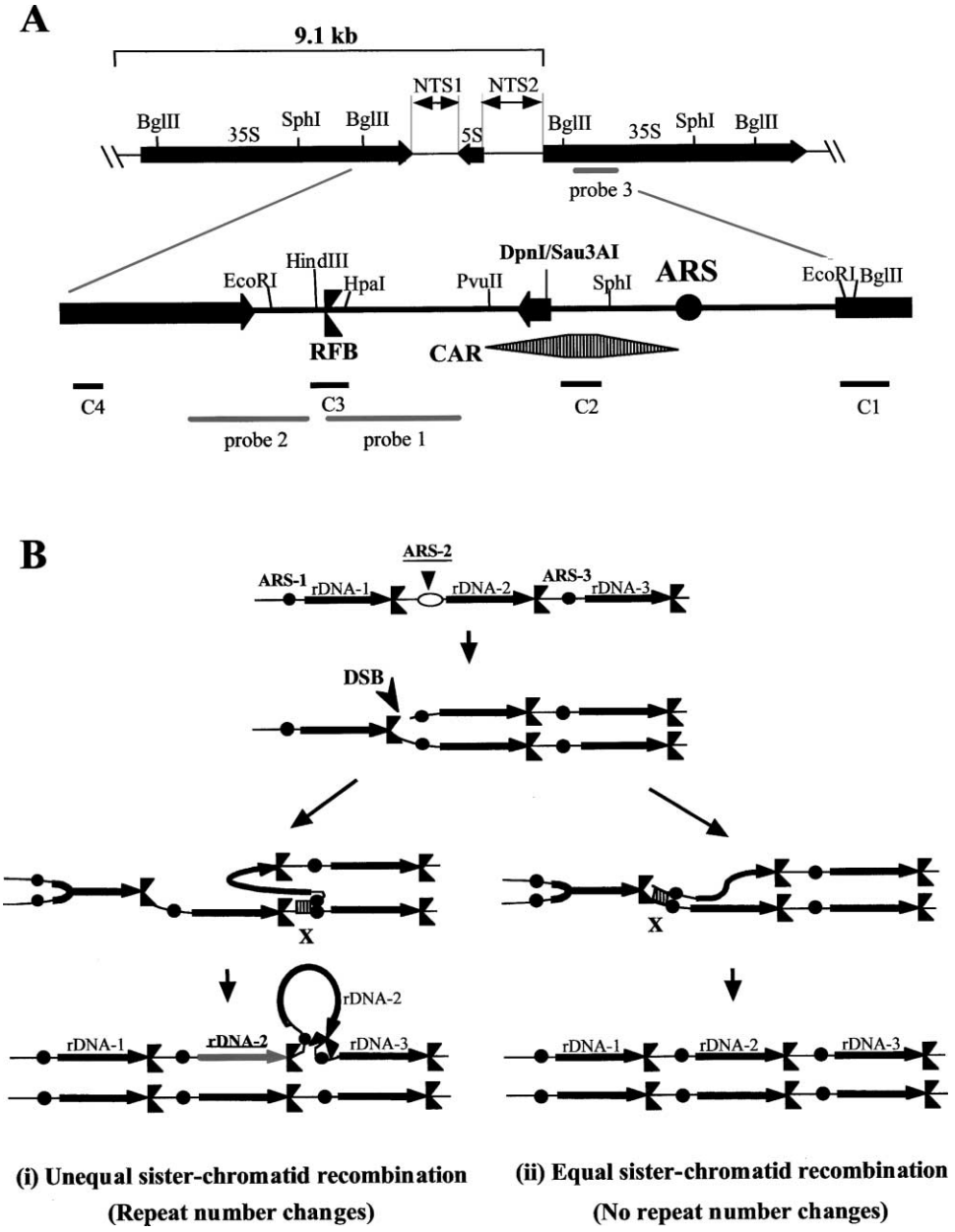

Figure 1. Structure of rDNA Repeats in *S. cerevisiae* and Model Showing Two Different Ways to Repair the RFB-Dependent DNA Double-Strand Break

(A) Genes for 35S and 5S rRNAs are indicated as well as regions NTS1 and NTS2 that are not transcribed. The locations of the origin of replication (*ARS*), the replication fork barrier (RFB), and the region identified by Laloraya et al. (2000) as the cohesin-associated region (CAR) are shown in the expanded NTS region. The locations of the hybridization probes 1 to 3 and the PCR primer pairs (as PCR products C1 to C4) are also shown. Dpn1/Sau3A1 is the site identified by Fritze et al. (1997) as the site where the access of the structural probe, *dam* methyltransferase, was stimulated by a *sir2*Δ mutation.

(B) In this model, DNA replication starts from one of the ARSs (ARS-2) as indicated by an open bubble. Lines represent chromatids with double-stranded DNA. Recombination intermediates with X-shaped Holliday structures are indicated with an X. (i) An unequal sister-chromatid is used as template for repair of RFB-dependent DSB and the result is a change in rDNA repeat number. (ii) The sister-chromatid at the equivalent position is used as template for DSB repair, which does not lead to any repeat number change.

chromatin structure—heterochromatin structures—which prevents general access of the Pol II machinery and some other macromolecules, thus causing silencing as well as decreasing recombination in the chromosomal rDNA repeats. For example, Fritze and coworkers (1997) suggested that, similar to its role in protecting the silent mating-type regions from HO endonuclease cleavages, *SIR2* may decrease recombination within rDNA repeats by decreasing accessibility of rDNA chromatin to components in the recombination machinery, such as those

responsible for inducing DSB. Until the work described here, critical studies to test the actual role of *SIR2* in regulation of recombinational events in rDNA had not been done.

In this paper, we present experiments that confirm the expected negative role of *SIR2* in rDNA repeat expansion and contraction, and we examine the possible mechanism involved in the increased frequency of repeat expansion and contraction that is observed in mutants defective in *SIR2* functions. Contrary to previous expectations, we did not find any significant increase in the RFB activity, the frequency of DSB or formation of DNA molecules with Holliday junction structures in *sir2* mutants. Instead, we found that *SIR2* plays an important role in the establishment and/or maintenance of sister-chromatid cohesion in rDNA, thus reducing the frequency of unequal sister-chromatid recombination responsible for rDNA repeat expansion and contraction [Figure 1B, (i)]. Finally, we describe the results of formal genetic experiments which support the conclusion that *SIR2* decreases unequal sister-chromatid recombination without significant effects on mitotic recombinational events within individual rRNA genes.

Results

RAD52 Is Required for Expansion of rDNA Repeats

Expecting that rDNA repeat expansion and contraction involve formation of DSBs at the RFB site and their repair as postulated in Figure 1B, we examined effects of mutations in *RAD52* and some other genes in the *RAD52* epistatic group on the efficiency of rDNA repeat expansion. Yeast strain NOY408-1a carries a deletion of an essential subunit of Pol I (*rpa135Δ::LEU2*) and grows on galactose by Pol II transcription of a *GAL7-35S rDNA* fusion gene on a multicopy plasmid (Nogi et al., 1991). This strain carries approximately half (~80) of the normal number (~150) of rDNA repeats, and upon introduction of the missing *RPA135* gene, the number of rDNA repeats increases back to the normal level (~150) (Kobayashi et al., 1998). We followed this increase by measuring the sizes of chromosome XII using contour-clamped homogeneous electric field (CHEF) electrophoresis (Figure 2A, lanes 1–3) and by quantitative Southern hybridization analysis (Figure 2B, lanes 1–3). In contrast to the control *RAD52* strain, introduction of *RPA135* into a *rad52* mutant caused only a slight, if any, increase in the number of rDNA repeats (Figures 2A and 2B, lanes 7–9). The ability to increase rDNA repeat number was restored by introducing the missing *RAD52* gene (on a *CEN* plasmid) into this strain, confirming the importance of *RAD52* for rDNA repeat expansion (Figure 2A, lanes 10 and 11). *MRE11*, together with *RAD50* and *XRS2*, plays a role in resection of broken ends in the process of DSB repair. We found that deletion of *MRE11* decreases the efficiency of repeat expansion, but does not abolish it entirely (Figures 2A and 2B, lanes 4–6). These results support an involvement of DSBs in rDNA repeat expansion, as previously discussed in reference to ERC formation and the X-shaped Holliday junction molecules within rDNA repeats (Park et al., 1999; Gangloff et al., 1996; Zou and Rothstein, 1997).

Deletion of *SIR2* Increases Instability of rDNA in a *FOB1*-Dependent Manner

We examined effects of deletion of *SIR2* on the efficiency of rDNA repeat expansion using the same test system as described above. As shown in Figure 2C, heterogeneity of the size of the chromosome XII and faster increases in its size were obvious in *sir2Δ* samples compared to the wild-type, especially at the earliest time point (44 generations) analyzed (Figure 2C, lanes 15–18 compared with 2–5). Average rDNA copy numbers analyzed by Southern also suggested that the rate of repeat expansion was higher than that of the wild-type from 0 to 44 generations (Figure 2B, lanes 10–12 compared with 1–3). Our interpretation of the observed heterogeneity of rDNA copy numbers in *sir2* mutants is that deletion of *SIR2* stimulates the rate of not only expansion, as shown experimentally, but also contraction of rDNA repeats. In other words, continued expansion/contraction of rDNA repeats that may take place even after repeat numbers reach the normal ~150 copies (in the presence of the intact Pol I machinery) is responsible for increased heterogeneity of rDNA copy numbers in *sir2* mutant cell populations. This interpretation is consistent with the previous observations that *sir2* mutations increase the rate of marker loss by unequal homologous recombination (Gottlieb and Esposito, 1989; see below). As expected from previous work that demonstrated a requirement of *FOB1* for rDNA repeat expansion and contraction (Kobayashi et al., 1998), we observed that deletion of *FOB1* in the *sir2Δ* strain suppressed the "hyperrecombination" phenotype of the *sir2* mutation. Clones of four independent *fob1* transformants obtained from a *sir2* cell population each showed different but more uniform sizes of chromosome XII, and these sizes remained essentially unaltered upon continued subculture in the presence of the *RPA135* gene (compare lanes 6, 7, 8, and 9 with 2, 3, 4, and 5, respectively, in Figure 2D). We conclude that the instability of rDNA seen in *sir2* mutants is due to stimulation of unequal homologous recombination that accompanies the repair of the presumed DSBs induced by *FOB1*-dependent replication fork block.

Deletion of *SIR2* Does Not Affect the Efficiency of Arrest at the RFB Site, *FOB1*-Dependent DSB or Formation of X-Shaped Recombination Intermediates

Because the hyperrecombination phenotype seen for *sir2* mutants requires the presence of *FOB1*, we considered three possible steps to be affected by the loss of *SIR2* activity: (1) the RFB activity, (2) DSBs caused by replication fork blocks, and (3) actual recombinational events including single-strand DNA invasion, formation of Holliday junction structures and their resolution, as indicated in the model shown in Figure 1B, (i).

We first examined effects of a *sir2* mutation on RFB activity by carrying out analysis of DNA restriction fragments containing a replication fork using two-dimensional (2D) gel electrophoresis (Brewer and Fangman, 1988). RFB activity can be recognized by an accumulation of a particular Y-shaped molecule (arrowhead in Figure 3A) as detected by a suitable probe. As shown in Figure 3A (and 3D, a), the number of replication fork

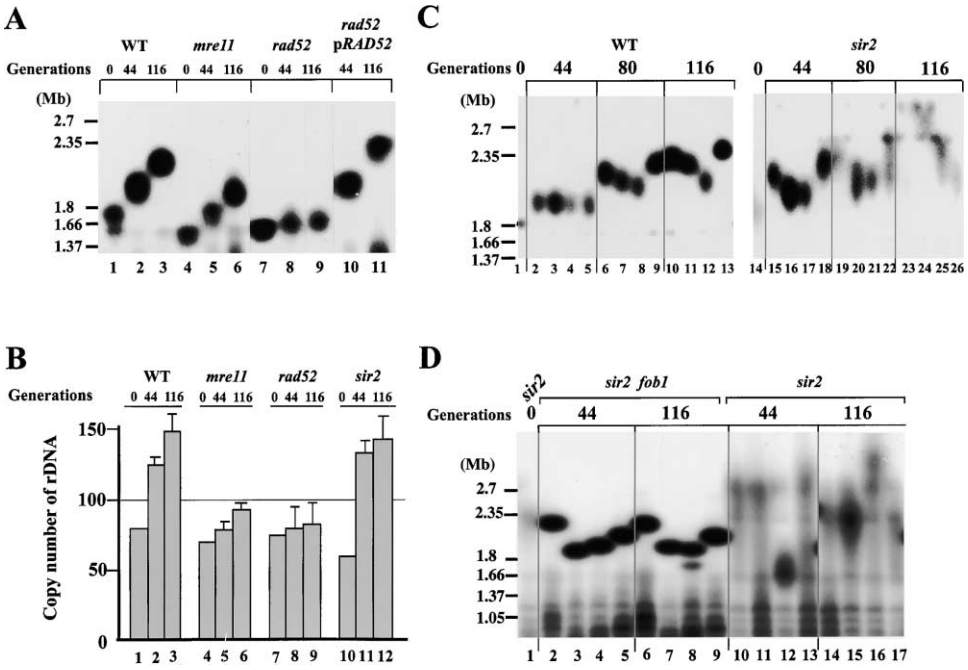

Figure 2. Expansion of rDNA in Various Mutants

(A) Analysis of the size of chromosome XII by CHEF electrophoresis. The *RPA135* gene carried by pNOY117 was introduced by transformation into strains NOY408-1a (*rpa135*, lane 1–3), TAK601 (*rpa135 mre11*, lane 4–6), and TAK603 (*rpa135 rad52*, lane 7–9). DNA samples were prepared before (time 0; lanes 1, 4, 7), and 44 (lanes 2, 5, 8) and 116 generations (lanes 3, 6, 9) after the introduction of *RPA135*. A strain derived from TAK603 carrying the *RAD52* gene on a plasmid (pTAK606) was also analyzed at 44 and 116 generations (lanes 10 and 11, respectively). The positions of chromosome size markers (*Hansenula wingei* chromosomes) are indicated. The probe used for hybridization is probe 1 (Figure 1A).

(B) Copy numbers of rDNA determined by Southern hybridization. Averages of four independent experiments and standard deviations are shown. DNA samples analyzed correspond to lanes 1 to 9 of (A) and lanes shown for *sir2* are from the results of experiments similar to those shown in (C).

(C) Increased heterogeneity in rDNA repeat numbers observed in repeat expansion experiments. The *RPA135* gene was introduced by transformation into strains NOY408-1a (*rpa135*, lane 1–13), TAK604 (*rpa135 sir2*Δ, lane 14–26), and the size of chromosome XII from four independent transformants was analyzed as in (A).

(D) A *fob1* mutation suppresses the instability of rDNA repeat numbers observed in a *sir2*Δ strain. The *FOB1* gene of TAK604 (*rpa135 sir2*Δ), which had grown more than 116 generations after the introduction of the *RPA135* gene, was disrupted by transformation and the size of chromosome XII from four independent transformants was analyzed by CHEF electrophoresis before (lane 1) and at 44 (lanes 2–5) and 116 (lanes 6–9) generations after the disruption of *FOB1*. Lanes 10–17 show control clones without disruption of *FOB1*. The cell population used in lane 1 was plated repeatedly and independent colonies were analyzed by CHEF analysis, four at 44 generations and another four at 116 generations, as indicated.

molecules paused at the RFB site was approximately the same in both the *sir2* mutant and the control ("WT") strains (see also the 9.4 kb band in Figure 3B, lanes 1 and 3, discussed below). No accumulation was observed in the *fob1*Δ mutant as shown previously (Kobayashi and Horiuchi, 1996). We conclude that the loss of *SIR2* function does not significantly affect the Fob1-dependent RFB activity.

We have obtained direct evidence for previously proposed Fob1-dependent DSBs. DNA was isolated from early log phase cells in agarose gel blocks to minimize damage to the DNA, digested with BglII in the block and digests were subjected to 1D gel electrophoresis, followed by Southern analysis using probe 1. In addition to the 4.6 kb BglII fragment present in all the samples, two fragments were present in the *FOB1* control (WT; Figure 3B, lane 1), but not in the *fob1*Δ strain (lane 2). The size of the smaller fragment is approximately 2.3 kb, which is the distance between the BglII site and the RFB site where the DSB is expected to take place. In addition, this fragment hybridizes to probe 1, but not probe 2 (data not shown; for probe 2, see Figure 1A), supporting our expectation of the presence of a DSB at the RFB site. The larger fragment at the position of ~9.4 kb corresponds to the Y-shaped intermediate molecule accumulated at the RFB site that was observed in 2D-gel analysis. The fragment moved considerably more slowly than the actual mass (4.6 kb plus 2.3 kb) of the Y-shaped intermediate molecule, as we expect for this shape of molecules (cf. behavior of Y-shaped molecules in the 2D gel electrophoresis shown as an inset in Figure 3A). Importantly, intensities of the 2.3 kb and 9.4 kb bands relative to the main 4.6 kb BglII band were not significantly different (less than 10%) between the *sir2* mutant and the control *SIR2* strain (Figure 3B, lanes 1 and 3; Figure 3D, b). We conclude that deletion of *SIR2* does not significantly increase the frequency of *FOB1*-dependent DSB events.

We then asked whether deletion of *SIR2* stimulates recombinational events that follow DSBs, namely, strand invasion and formation of Holliday junction structures. Recombination intermediates with Holliday struc-

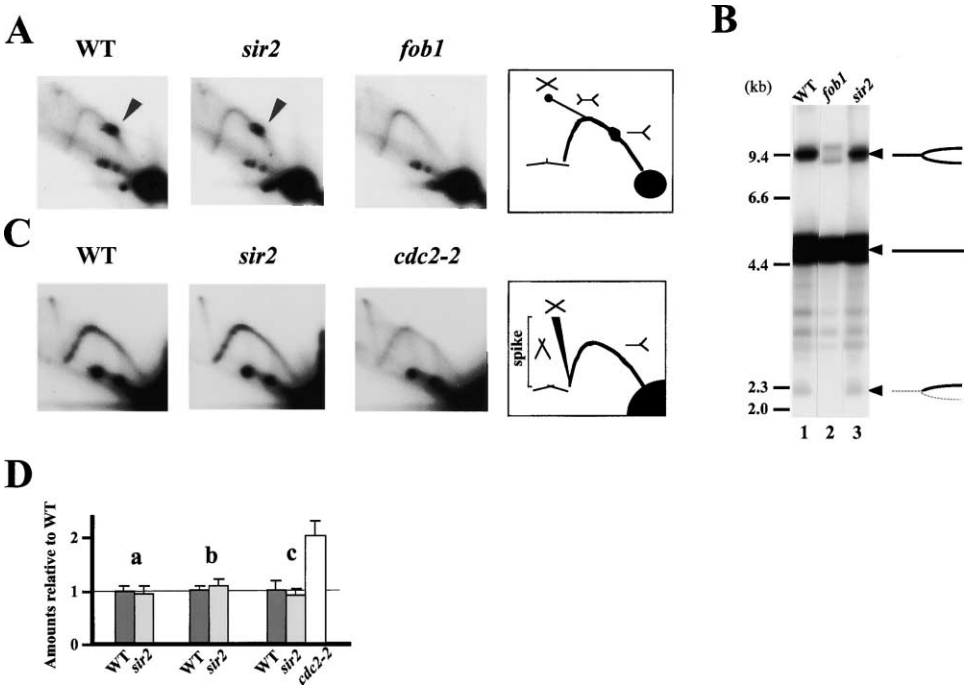

Figure 3. Deletion of *SIR2* Does Not Affect RFB Activity, Frequency of Double-Strand Breaks or Formation of Recombination Intermediates

(A) RFB activity analyzed by 2D-gel analysis in a *sir2*Δ strain. DNA was prepared from NOY408-1a carrying pNOY117 (WT), TAK604 carrying pNOY117 *(sir2*), and TAK600 carrying pNOY117 (*fob1*), digested with BglII and SphI and subjected to 2D-gel analysis followed by Southern hybridization using probe 1 (Figure 1A). Migration patterns of pertinent DNA molecules are explained in the right image. Spots indicated by arrowheads show accumulation of Y-shaped DNA molecules paused at the RFB site.

(B) Detection and quantitation of DNA molecules produced by *FOB1*-dependent activities. DNA preparations used in (A) were digested with BglII and subjected to 1D gel electrophoresis followed by Southern hybridization using probe 1 (Figure 1A). Structures of DNA molecules deduced are shown for three bands. The two bands with sizes close to 9.4 kb recognized in lane 2 are background bands different from the 9.4 kb band seen in lanes 1 and 3.

(C) Detection of the spike signal that represents molecules with Holliday structures. Migration patterns are explained in the right image. DNA prepared from the strains used in (A) for the wild-type (WT) and the *sir2* mutant (*sir2*) and from strain 2774-21 (*cdc2-2*) was analyzed as in (A), but using probe 3 (Figure 1A).

(D) RFB activity, the formation of double-strand breaks and recombination intermediates in *sir2*Δ strain normalized to WT. (a) From (A), WT and *sir2*Δ, the amount of DNA accumulated at the RFB site indicated by arrowheads was normalized to that of Y-shaped molecules and then normalized to WT. (b) From (B), WT and *sir2*Δ, the amount of 2.3 kb fragment was normalized to that of the 9.4 kb Y-shaped fragment and then normalized to WT. (c) From (C), WT, *sir2*Δ and *cdc2-2*, the amount of DNA in the spike was normalized to that of Y-shaped molecules and then normalized to WT.

tures can be identified by 2D gel electrophoresis as X-shaped molecules that give a "spike" signal (Zou and Rothstein, 1997). Thus, we compared the frequency of occurrence of the X-shaped molecules between the *sir2*Δ strain and the wild-type strain. The probe used here (probe 3, Figure 1A) anneals with the beginning of the 35S rRNA coding region. Since this region should contain only rightward replication forks, the spike signal must correspond to Holliday structures. As a positive control, we examined a DNA sample obtained from a temperature sensitive (ts) *cdc2* mutant grown at a semi-permissive temperature. The *CDC2* gene encodes the DNA polymerase III catalytic subunit, and its ts mutants were previously shown to accumulate a higher level of the X-shaped DNA molecules than the wild-type strain (Zou and Rothstein, 1997). As shown in Figure 3C and 3D(c), no significant difference was observed between the wild-type and *sir2*Δ strains in the amounts of the X-shaped molecules (when normalized to Y-shaped molecules), which were smaller than the amount found for the *cdc2-2* mutant strain. We conclude that deletion of the *SIR2* gene does not cause a significant increase in overall recombinational events as judged from the amounts of the X-shaped Holliday junction recombination intermediates.

Deletion of *SIR2* Decreases the Association of Cohesin Subunit Mcd1p (Scc1p) with rDNA

In order to explain the above-described observations, we considered the possibility that unequal sister-chromatid recombination shown in Figure 1B, (i), may represent only a small fraction of the total recombinational events (see Zou and Rothstein, 1997), and that its large increase in *sir2* mutants may not be detected by measurements of overall frequency of RFB, DSBs, or formation of Holliday junction molecules. More specifically, we considered the possibility that *SIR2* is important for proper execution of DSB repair by equal sister-chromatid recombination [Figure 1B, (ii)], thereby preventing chromosomal rearrangements that would accompany repair using unequal sister-chromatid recombination [Figure 1B, (i)]. One particular mechanism that could

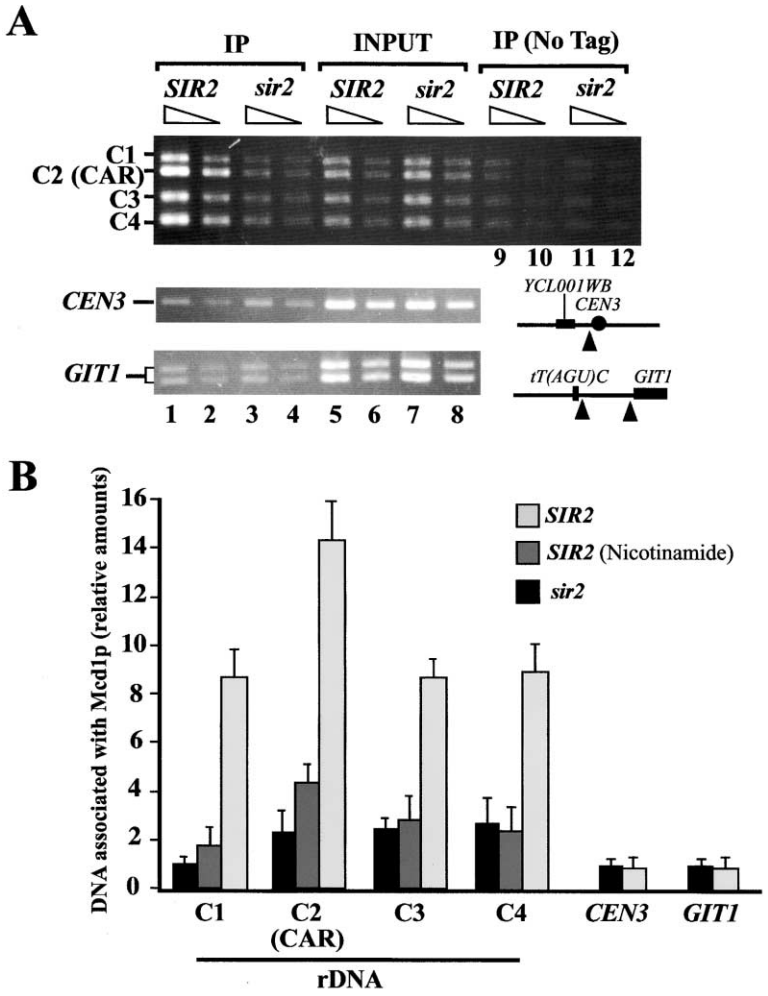

Figure 4. Functional Interaction of Sir2p with Cohesin

(A) Association of cohesin with rDNA analyzed by ChIP assays. TAK606 (*sir2*Δ) and TAK607 (*SIR2*), both of which carry FLAG-tagged *MCD1*, as well as control strains without tag ("No Tag") were treated with formaldehyde and DNA associated with Mcd1p was immunoprecipitated with an anti-FLAG monoclonal antibody. Four regions within rDNA (C1 to C4 shown in Figure 1A) as well as control regions, one at *CEN3* and two at the *HMR-GIT1* region (indicated with triangles; *tT (AGC)C* is the tRNA gene at the right boundary of *HMR*, see Donze et al., 1999) were analyzed by PCR. The products obtained for two sample concentrations (2-fold dilution) were separated on 2.6% agarose gels and stained with ethidium bromide.

(B) PCR products shown in (A) and other independent experiments were quantified. The values obtained for immunoprecipitated (IP) DNA were first corrected for small background values obtained for controls without tag, and then normalized to the values for corresponding input DNAs obtained without IP. In addition, ChIP assays were done using the *SIR2* strain with and without nicotinamide, an inhibitor of the Sir2p histone deacetylase activity. Average values from three independent experiments are shown in arbitrary units. Error bars represent standard deviations.

prevent unequal sister-chromatid recombination is an establishment of sister-chromatid cohesion, as previously suggested by Laloraya et al. (2000) (for reviews on cohesin, see Nasmyth, 2002; Meluh and Strunnikov, 2002). Therefore, we considered the possibility that *SIR2* plays a role in establishment and/or maintenance of cohesion in rDNA, and examined effects of the *SIR2* deletion on the association of cohesin subunit Mcd1p (Scc1p) with rDNA using chromatin immunoprecipitation (ChIP) assay. We used *sir2*Δ cells and control *SIR2* cells both carrying FLAG-epitope-tagged Mcd1p. Cells were treated with formaldehyde and rDNA fragments (sheared to the sizes of ~500 bp to 1 kb) were coimmunoprecipitated with FLAG-tagged Mcd1p using anti-FLAG-antibodies followed by PCR analysis to measure their relative amounts. Four PCR primer pairs were used (see Figure 1A, C1–C4). In agreement with the previous report (Laloraya et al., 2000), primer set C2 covering the CAR region near the 5S gene gave a signal significantly stronger than the signals obtained for the other three primer pairs (and 11 other primer pairs not shown in the figures) in control *SIR2* cells (Figure 4A and data not shown; see below). Importantly, signals observed for *sir2*Δ mutant cells were in general significantly weaker than those for the control cells in all the regions analyzed (Figures 4A and 4B, and other data not shown). The association of Mcd1p with the CAR region in rDNA was ~6-fold lower in *sir2* strains than in WT (Figure 4B). As controls, we examined the effects of the *sir2* deletion on the association of Mcd1p with the known cohesin-association regions at *CEN3* (Blat and Kleckner, 1999) and at or near the right *HMR* boundary element ("*GIT1*"; Laloraya et al., 2000). No significant difference was observed between the *sir2*Δ and *SIR2* strains at these regions (Figures 4A and 4B). In addition to the above four regions, eleven other regions in rDNA were analyzed by PCR for their association with cohesin in the *sir2*Δ relative to WT strains. In these regions analyzed, the association with cohesion was decreased in *sir2*Δ relative to WT; the decrease ranged from 2.6- to 8.8-fold (data not shown). Thus, altogether 3.1 kb within the 9.1 kb rDNA was analyzed by PCR with gaps between the regions averaging ~400 bp. Since the size of the sheared DNA is 500 bp to 1 kb, the data strongly suggest that no "new" cohesion was created in the *sir2*Δ strain which would compensate for the cohesion at the CAR that was weakened by the *sir2*Δ mutation. We conclude that the *sir2* mutation causes an alteration of rDNA chromatin, leading to a significantly decreased association of cohesin with rDNA.

Table 1. Cohesin Mutation *smc1-2* Increases Unequal Recombination in rDNA Repeats

	Genotype		23°			31°			
	SMC1	FOB1			smc1/SMC1			smc1/SMC1	31°/23°
Frequency of Ura$^-$ ($\times 10^{-3}$)	W	W	0.75	(0.2)	---	0.74	(0.3)	---	---
	ts	W	1.6	(0.7)	2.1	6.5	(1.9)	8.8	4.2
	W	Δ	0.23	(0.03)	---	0.24	(0.02)	---	---
	ts	Δ	0.42	(0.12)	1.8	0.43	(0.21)	1.8	1.0
Viability (Relative to WT at 23°)	W	W	1.0	(0.1)	---	0.9	(0.1)	---	---
	ts	W	1.1	(0.2)	1.1	0.13	(0.04)	0.14	0.13
	W	Δ	0.9	(0.1)	---	0.8	(0.2)	---	---
	ts	Δ	1.1	(0.2)	1.2	0.32	(0.05)	0.40	0.33

Four strains were used: MAY589 (*SMC1 FOB1*), 1bAS172 (*smc1-ts FOB1*), TAK609 (*SMC1 fob1*Δ) and TAK610 (*smc1-ts fob1*Δ). The frequency of marker (*URA3* in NTS2 at the EcoRV site, 8757) loss was determined by growing cells to stationary phase in SC-Ura medium at 23°C or 31°C, followed by measuring viable cells on SC with and without 5-FOA at 23°C. For measurements of viability, cells growing exponentially in SC at 23°C or 31°C were plated at 23°C and their viabilities were compared first by calculating the number of viable colonies relative to the number of cells determined by microscopy and then by normalizing the values to the value obtained for the control culture (*SMC1 FOB1*) at 23°C. In both marker loss and viability measurements, averages of values from three independent experiments are shown together with standard deviations (in parentheses). "W" stands for wild type, and "*smc1/SMC1*" columns are ratios of the numbers in the preceding columns.

Because nicotinamide, a product of NAD$^+$-dependent Sir2p deacetylase reaction, is known to inhibit Sir2p deacetylase strongly (Bitterman et al., 2002), we examined its effects on the association of Mcd1p with rDNA. As shown in Figure 4B, we observed that nicotinamide decreases association of Mcd1p with rDNA as analyzed by ChIP. These data suggest that the deacetylase activity of Sir2p is required for the stimulation of association of cohesin with rDNA.

Stability of rDNA Repeats in a Cohesion Defective Strain

If the function of Sir2p in preventing unequal sister-chromatid recombination is achieved through its activity to increase the association of cohesin with rDNA, as the above experiments suggest, then some mutational defects in cohesins might also increase unequal sister-chromatid recombination [Figure 1B, (i)] relative to equal sister-chromatid recombination [Figure 1B, (ii)]. This possibility was examined by measuring loss of a marker integrated in rDNA repeats. Since the genes for cohesin are essential for cell growth, we used the *smc1-2* ts strain (Strunnikov et al., 1993). Smc1p is a component of the cohesin complex. We integrated a marker, *URA3*, into rDNA repeats and compared the rate of its loss with that in the control *SMC1* strain at both permissive (23°C) and semipermissive (31°C) temperatures. In addition, we carried out the same experiments using *fob1* derivatives of these two strains. As shown in Table 1, the *smc1-2* mutation caused increases in marker loss, especially at the semipermissive temperature relative to the *SMC1* strain used as a reference. The ratio of the rate of marker loss at 31°C to that at 23°C was approximately 4-fold higher in the *smc1-2* mutant than in the control *SMC1* strain. In addition, in the *fob1* background, there was no significant difference between the *smc1* mutant and the wild-type in this ratio, confirming that the unequal sister-chromatid recombination responsible for the elevated loss of the marker in the cohesin mutant is *FOB1*-dependent.

SMC1 is an essential gene, and the semipermissive temperatures used in the above experiments led to a substantial loss of viability of the *smc1-2* mutant cells (Table 1). We found that viability of *smc1-2* cells at 31°C increased 2.5-fold (0.32/0.13) by introduction of the *fob1* deletion. It appears that DSBs in S phase caused by the *FOB1*-dependent replication fork block occur at a significant frequency within rDNA repeats, but are efficiently repaired in the presence of intact chromatid cohesion.

We also examined whether accumulation of ERCs in the above cohesin mutant increases as in the case of *sir2* mutants. For cells growing at 23°C, the amount of ERCs relative to genomic rDNA was approximately the same for the wild-type and *smc1-2* strains. After shifting the cultures from 23°C to 31°C for 3 hr, we found that the amount of ERCs (relative to genomic DNA) increased approximately 53% in the mutant, but only a slight (~6%) increase was observed for the WT strain (data not shown). These results also support the conclusion that the increase in unequal sister-chromatid recombination caused by *sir2* mutations is mediated by their effects on cohesin-rDNA association.

Recombinational Events within a Single rRNA Coding Region Are Not Affected by *sir2* Mutation

The results described in the previous sections led to the conclusion that special rDNA chromatin structures formed by the activity of Sir2p decrease unequal sister-chromatid recombination without preventing access of recombinational machinery to rDNA. We carried out formal genetic experiments to test this conclusion. We constructed an integration plasmid, pNOY705 (Figure 5A), cleaved with PflmI and integrated into rDNA repeats. His$^+$ (and Ura$^-$) transformants obtained in this way carry two incomplete fragments of *URA3*, one missing the 5′-end and the other missing 3′-end, within a single 35S rRNA gene in rDNA repeats. Recombination between these two *ura3* fragments to form *URA3* may take place using the 248 bp region shared by both fragments, presumably during S or G2 phase between sister-chromatids, or alternatively, by an intrachromosomal recombination. In any event, if *SIR2*-dependent rDNA structures prevent access of recombination machinery to rDNA,

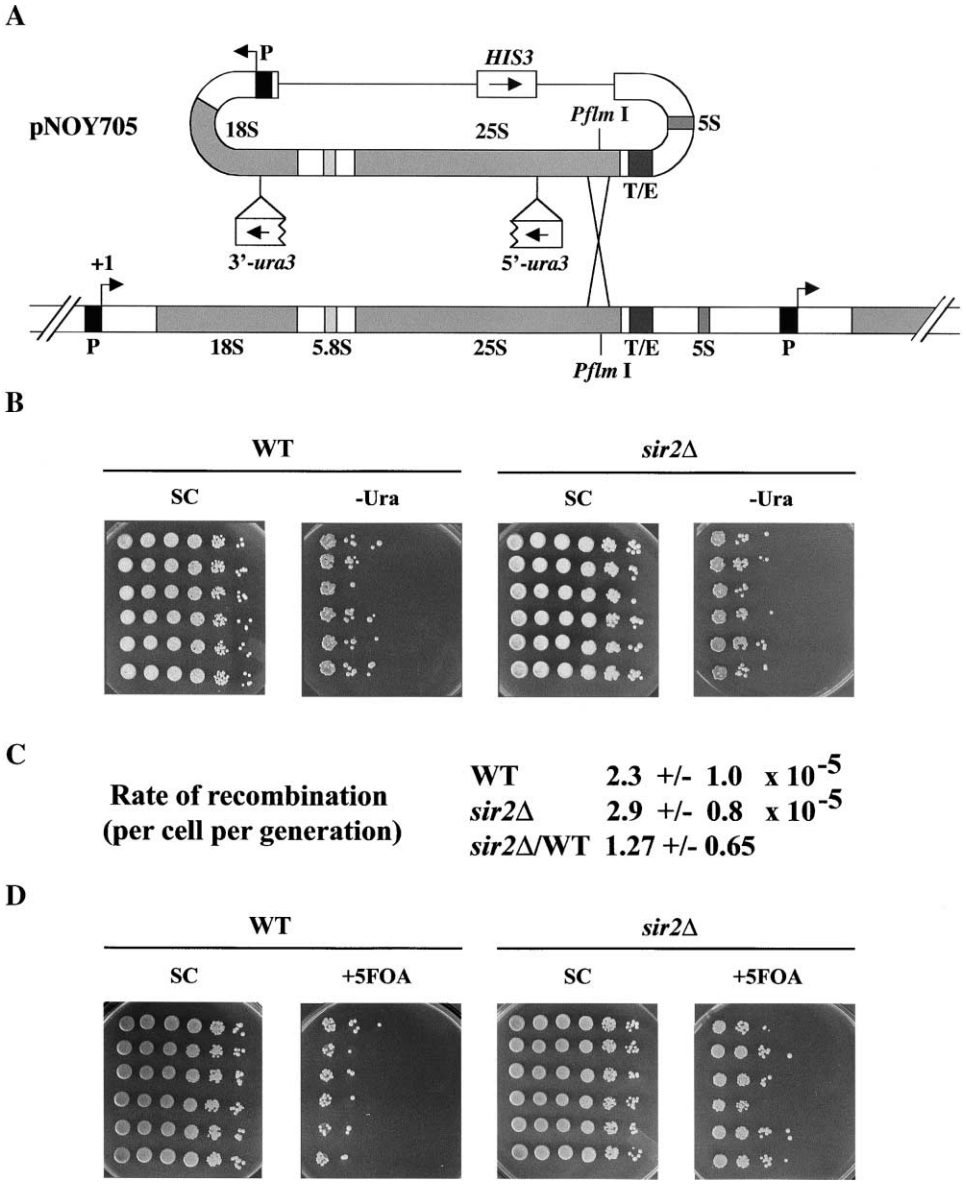

Figure 5. Mutation of *SIR2* Increases Unequal Sister-Chromatid Recombination in rDNA but Does Not Affect Recombination within a Single 35S rRNA Gene

(A) Structure of plasmid pNOY705 and its use for integration into rDNA. The Pol I promoter (P), the region containing the Pol I terminator, the E element, and RFB (T/E) are indicated.

(B) Six independent His^+ transformants carrying pNOY705 integrated at the PflM1 site were obtained from the WT (NOY388) and *sir2*Δ (NOY1045) strains. They were grown in SC-His medium to saturation and aliquots of 10-fold serial dilutions were spotted on SC and SC-Ura plates to observe the frequency of Ura^+ recombinants in these independent clones.

(C) Four independent transformants from each of the WT and *sir2*Δ strains similar to those shown in (B) were analyzed to determine the rate of recombination as described in Experimental Procedures. Averages and standard deviations are shown.

(D) Six independent Ura^+ recombinants were obtained from each of the −Ura plates of the WT and *sir2*Δ strains shown in B. They were grown in SC-Ura medium to saturation and aliquots of 10-fold serial dilutions were spot tested on SC and SC containing 5FOA to measure the frequency of marker (*URA3*) loss.

as suggested previously, one would expect a large increase in the rate of Ura^+ recombinant formation in *sir2* mutants. Six independent His^+ transformants carrying integrated pNOY705 in rDNA were obtained from *sir2*Δ and control *SIR2* strains. They were grown in complete medium to saturation and the frequency of Ura^+ recombinants in these independent cultures was determined by spot test. As shown in Figure 5B, no significant difference was observed between the *sir2* and *SIR2* strains. We then measured the rate of recombination more quantitatively using the method of median according to Lea and Coulson (1949). Four independent experiments were carried out, each using independent transformant clones of the *SIR2* and *sir2*Δ strains. The rate (recombinant formation per cell per generation) in the *sir2* strain was found to be 1.27 (± 0.65) times that found for the

SIR2 strain (Figure 5C). Clearly, there was no significant difference in recombination frequency between the two strains.

We determined the rate of *URA3* marker loss from rDNA using the method similar to that described in Table 1. As originally found by Gottlieb and Esposito (1989), we found the rate in *sir2*Δ is 10- to 20-fold higher than that in *SIR2* (data not shown). To confirm this conclusion further, we analyzed Ura$^+$ recombinant clones obtained in the experiments described above (shown in Figures 5A and 5B). Six independent Ura$^+$ recombinants were obtained from each of the −Ura plates of *sir2*Δ and *SIR2* strains shown in Figure 5B. They were then grown in SC-Ura medium to saturation and the frequency of Ura$^-$ cells, which reflect approximately the rate of loss of *URA3* per cell per generation, was determined by spot testing on plates with and without 5FOA. The results are shown in Figure 5D. It is clear that, in contrast to the formation of *URA3* by recombination, the rate of loss of the *URA3* genes (*URA3* genes formed in this recombination experiment) from rDNA by unequal sister-chromatid recombination was significantly (roughly 10- to 100-fold) increased by the *sir2*Δ mutation.

Discussion

SIR2 Functions in Decreasing the Frequency of Unequal Sister-Chromatid Recombination without Decreasing the Overall Frequency of Recombinational Events in rDNA

Mutations in *SIR2* have been known to abolish transcriptional silencing at silent mating type loci, telomere regions, and rDNA (for reviews, see Moazed, 2001; Rusche et al., 2003), and to increase the frequency of recombination as assayed by marker loss or ERC formation (Gottlieb and Esposito, 1989; Kaeberlein et al., 1999). It has been generally believed that Sir2p plays an important role in forming repressive heterochromatin structures, presumably through its NAD$^+$-dependent histone deacetylase activity, and that such structures prevent access of recombinational machinery as well as transcription machinery. However, we have not observed any increase in the frequency of *FOB1*-dependent replication fork blocks, consequent DSBs, or formation of DNA molecules with Holliday junction structures in rDNA repeats in *sir2*Δ mutants relative to the control strain. Therefore, our data suggest that proteins involved in these events, such as Rad52p, have equal access to rDNA with or without rDNA chromatin structures that involve Sir2p and are apparently repressive to transcription of Pol II reporter genes. We suggest that Sir2p interacts with cohesin, directly or indirectly, and decreases the frequency of unequal sister-chromatid recombination. Zou and Rothstein (1997) compared the frequency of formation of Holliday junction structures with the frequency of marker loss in rDNA repeats and concluded that most (~85%) of the recombinational events involving Holliday junction structures do not lead to deletion of rDNA repeats, that is, recombinational repair is largely based on equal sister-chromatid recombination [Figure 1B, (ii)]. Although *sir2* mutations decrease the association of cohesin with rDNA as judged by the results of ChIP experiments, *sir2* mutations do not generally decrease cell viability, and hence, frequent DSBs (Zou and Rothstein, 1997; see below) observed in rDNA during S phase are apparently repaired as efficiently in *sir2* mutants as in the control *SIR2* strains. We found that the *smc1-2* ts mutant defective in the Smc1p cohesin subunit shows an increase in unequal sister-chromatid recombination among viable cells and an increase in ERC formation at semipermissive temperatures. These observations combined with our ChIP analysis suggest that *sir2* mutations increase unequal sister-chromatid recombination by altering cohesin structures that hold the replicated sister-chromatids together, leading to an increase in the rates of marker loss as well as rDNA repeat expansion and contraction without a significant decrease in the overall efficiency of DSB repair in rDNA.

It was previously reported that *sir2* mutations cause a decrease in accessibility of rDNA to structural probes, e.g., *dam* methyl transferase in vivo (Fritze et al., 1997), and such observations led to a suggestion of a general inaccessibility of rDNA to transcription and recombination machineries as the basis of silencing of Pol II reporter genes and of the decrease in recombination frequency measured by marker loss. This suggestion does not appear to be correct in view of the present work and of the recent work on silencing of Pol II reporter genes in rDNA which showed that the rDNA chromatin structures responsible for silencing of Pol II genes do not prevent access of Pol I machinery to rDNA (Buck et al., 2002; Cioci et al., 2003). Nevertheless, it is interesting to note that the DpnI/SauIIIAI site which displayed an increased accessibility to *dam* methyl transferase in *sir2* mutants (Fritze et al., 1997) is in the CAR region (see Figure 1A) and this altered accessibility may reflect an alteration in the cohesin-rDNA association in the *sir2*Δ mutant as found in the present study.

Pasero et al. (2002) recently reported that in a *sir2* mutant, the frequency of active replication origins (ARSs) increased 2-fold, and that the number of replication forks arrested at the RFB site detected by 2D gel electrophoresis was also increased by 43% compared to the wild-type strain. Thus, there is an apparent discrepancy regarding the effects of *sir2* mutations on the RFB activity between their results and our results. According to Pasero et al. (2002), the 2-fold increase in the number of active ARSs is expected to increase the rate of formation of arrested replication forks 2-fold. However, since replication initiated at the active ARSs is bidirectional, the rate of disappearance of the arrested replication forks by rightward replication forks should also increase 2-fold. Therefore, the steady state amount of replication forks arrested at the RFB site in *sir2*Δ mutants may be expected to be similar to that in control *SIR2* strains, and hence the frequency of DSBs at the RFB site may also be similar between *sir2* mutants and control strains. Our results are consistent with these expectations.

A Unique Role of Sister-Chromatid Cohesion in rDNA Repeats

Sister-chromatid cohesion is essential in S and G2 phases to hold the copies of each chromosome together and ensure their proper segregation during mitosis. Al-

though cohesin binding sites exist along the entire length of the chromosome, preferential association of cohesins with the centromere regions has been observed for *S. cerevisiae* as for other eukaryotic organisms (Blat and Kleckner, 1999; Tanaka et al., 1999; Nasmyth, 2002). Recent studies using *Schizosaccharomyces pombe* have demonstrated that the mode of cohesin binding to centromeres is different from that to chromosome arms and specifically requires heterochromatin protein Swi6, a homolog of mammalian heterochromatin protein HP1, which in turn requires histone methyl transferase Clr4 for binding to centromeres (Nonaka et al., 2002; Bernard et al., 2001). It has been suggested that cohesion at the centromere plays a crucial role in ensuring the proper segregation of chromosomes, and this is reflected in the unique requirement of heterochromatin for cohesion at centromeres.

Our discovery of a Sir2p requirement for efficient association of cohesin subunit Mcd1p/Scc1p to rDNA might also reflect some unique function(s) of cohesin in rDNA. A requirement for sister-chromatid cohesion in postreplicative DSB repair was previously demonstrated by irradiating yeast cells with X-rays and measuring the amount of intact chromosome XVI after its separation using pulse field gel electrophoresis (Sjogren and Nasmyth, 2001). DSBs in normal growing yeast cells appear to take place frequently at rDNA during the S phase. By measuring the amount of Holliday structure molecules, which are almost certainly derived from DSBs, Zou and Rothstein (1997) calculated that the average number of such molecules is 3.6 in rDNA per genome. In the present study, we found that the reduced viability of *smc1-2* mutant at 31°C is reproducibly increased by deletion of *FOB1* (0.32 relative to 0.13, i.e., a 2.5-fold increase in the experiment shown in Table 1) and this increase estimated at the semipermissive temperature represents a significant fraction (0.32–0.13 or ~19%) of the total cell population. Thus, although cohesin subunit Smc1p is essential for viability mostly because of its essential function for proper chromosome segregation, as previously recognized, its function in repair of "spontaneous" DSBs in rDNA is also vital and defects in this function contribute to cell lethality in *smc1* mutant cells. Perhaps, a single strong cohesion binding site for every rDNA repeat, as discovered by Laloraya et al. (2000), may have evolved to ensure efficient repair of the DSBs which accompany the *FOB1*-dependent RFB activity and also to decrease other harmful effects of unequal sister-chromatid recombination, such as ERC formation (Sinclair and Guarente, 1997). The unique requirement for Sir2p in the association of cohesin with rDNA may reflect the importance of this aspect of cohesin function in rDNA repeats.

The NAD^+-dependent protein deacetylase catalytic activity of Sir2p is required for silencing of Pol II reporter genes in rDNA (Imai et al., 2000; Bitterman et al., 2002). We have now observed that the association of cohesin with rDNA decreases not only as a result of a *sir2* deletion mutation, but also by the presence of nicotinamide, a specific inhibitor of the deacetylase activity of Sir2p. Therefore, formation and/or maintenance of the cohesion structure on rDNA appears to require the deacetylase activity of Sir2p and might be subject to metabolic regulation, as suggested for the role of Sir2p in senescence (Rusche et al., 2003; Koubova and Guarente, 2003). Related to the role of Sir2p in cohesion on rDNA, it is interesting to note that Eco1p, which is essential for establishment of sister-chromatid cohesion in S phase, but not for its further maintenance during G2 or M phase, shows protein acetyl transferase activity and cohesin subunits, including Mcd1p/Scc1p, but not histones, are acetylated by Eco1p and acetyl CoA in vitro (Ivanov et al., 2002). The question of whether the requirement for NAD^+-dependent deacetylase activity in the efficient association of cohesin with rDNA is connected with such acetylation reactions involving cohesin subunits or with deacetylation of histones is a subject for future studies.

In conclusion, the results of our experiments strongly suggest that rDNA chromatin structures involving Sir2p do not cause a general decrease in accessibility of recombinational machinery to rDNA and the overall frequency of mitotic sister-chromatid recombination/DNA repair in *sir2* mutants is not significantly different from that seen in control *SIR2* strains. Rather, Sir2p plays a role in forming a special cohesion structure to decrease the frequency of unequal mitotic sister-chromatid recombination. The emergence of the Pol I transcription machinery during evolution of eukaryotes was accompanied by separation of its location, the nucleolus, and by tandemly repeated rRNA gene structures, perhaps for the sake of efficiency and regulation of rRNA transcription (see discussion in Nomura, 2001). The presence of RFB elements at the ends of pre-rRNA coding regions is conserved in eukaryotic cells from yeast (Brewer and Fangman, 1988; Linskens and Huberman, 1988) to human (Little et al., 1993; Gerber et al., 1997). Thus, recombination systems similar to the yeast *FOB1*-dependent system may be used for rDNA repeat expansion/contraction in all eukaryotes. As discussed above, the evolution of a special cohesion structure in the yeast rDNA may have been advantageous to regulate unequal sister-chromatid recombination. The question of whether special cohesion structures in rDNA are also present in other eukaryotes must await future studies.

Experimental Procedures

Media, Strains, and Plasmids

SD is a synthetic glucose medium (Kaiser et al., 1994). SGal is the same as SD, except that 2% glucose is replaced by 2% galactose. Both SD and SGal were supplemented appropriately with amino acids and bases to satisfy nutritional requirements and also to retain unstable plasmids (Kaiser et al., 1994), and are called SD complete (SC) and SGal complete, respectively. SGal media were used to grow *rpa135*Δ strains such as NOY408-1a.

Yeast strains and plasmids are listed in Table 2. Disruption of *FOB1* was described previously (Kobayashi and Horiuchi, 1996). Disruption of *RAD52*, *MRE11*, and *SIR2* were performed by the method of gene replacement (Kaiser et al., 1994). Two DNA sequences of approximately 500 bp that flank an ORF to be replaced were amplified by PCR using DNA prepared from NOY408-1b. Each of the primers used for PCR had appropriate restriction enzyme recognition sites at the 5′-ends. The two PCR products and the 1.4 kb *HIS3* fragment were first cloned into pUC18. The *HIS3* gene with flanking sequences was then excised from the vector, and used for gene replacement by transformation and selecting for His^+ colonies. Replacement of *SIR2* with *LEU2* was done in a similar way. PCR was used to confirm the positions and the size of the insert expected from the correct replacement. TAK606 was con-

Table 2. Yeast Strains and Plasmids Used

Designation	Genotypes and Comments
Strain	
W303-1a (NOY388)	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-100*
NOY408-1a	*MAT*α *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 rpa135*Δ*::LEU2* pNOY102 (Nogi et al., 1991)
NOY408-1b	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100* (Nogi et al., 1991)
NOY1045	Same as NOY388 except for *sir2*Δ*::LEU2*
TAK600	Same as NOY408-1a except for *fob1*Δ*::HIS3*
TAK601	Same as NOY408-1a except for *mre11*Δ*::HIS3*
TAK603	Same as NOY408-1a except for *rad52*Δ*::HIS3*
TAK604	Same as NOY408-1a except for *sir2*Δ*::HIS3*
TAK606	Same as NOY408-1b except for *sir2*Δ*::HIS3* and *MCD1-FLAG* replacing *MCD1*
TAK607	Same as TAK606, but carries pTAK607 making the strain *SIR2*
MAY589	*MAT***a** *ade2-101 ura3-52 his3-*Δ*200 leu2-3,112* (Strunnikov et al., 1993)
1bAS172	*MAT*α *leu2-3,112 his3 smc1-*Δ*2::HIS3 ura3::smc1-2* (Strunnikov et al., 1993)
TAK609	Same as MAY589 except for *fob1*Δ*::LEU2*
TAK610	Same as 1bAS172 except for *fob1*Δ*::LEU2*
2774-21	*MAT*α *cdc2-2 his7 leu2-3,112 hom3 ura3 can1*
Plasmid	
pNOY102	Multicopy plasmid carrying *GAL7-35S* rDNA 2μ, *URA3* (Nogi et al., 1991)
pNOY117	*CEN6*, *ARSH4*, *TRP1*, *RPA135* (Kobayashi et al., 1998)
pNOY705	A derivative of pRS303 (Sikorski and Hieter, 1989) carrying 9.1 kb rDNA with two *ura3* fragments inserted (Fig. 5A).
YCplac33	*CEN* plasmid vector, *URA3*, *ARS1* (Gietz and Sugino, 1988)
pTAK606	YCplac33 carrying *RAD52*
pTAK607	YCplac33 carrying *SIR2*

structed from strains NOY408-1b. The *SIR2* gene was disrupted by *HIS3* as described above and the FLAG epitope tag sequence was added to the 3′-end of the *MCD1* protein coding region by the gene replacement method with *TRP1* as a selection marker gene. Plasmid pNOY705 used for measurements of recombination frequency within a single rDNA repeat was constructed as a derivative of the *HIS3* integration plasmid pRS303 (Sikorski and Hieter, 1989) and carries a 9.1 kb rDNA repeat containing two *ura3* fragments inserted between the SalI and BamHI sites, as shown in Figure 5A. One of the fragments ("5′-*ura3*") is the 657 bp HindIII-StuI fragment of *URA3* (together with 22 bp derived from the vector) and is integrated at MscI (4486; numbering with respect to the Pol I start site as +1) in the 25S coding region. The other fragment ("3′-*ura3*") is the 693 bp EcoRV-SmaI fragment of *URA3* and is integrated at the NheI site (2054) in the 18S coding region after blunt-ending. These two *ura3* fragments share a 248 bp (EcoRV to StuI) common sequence for homologous recombination to generate Ura^+ recombinants.

Measurements of Recombination Frequency and Rate in rDNA

pNOY705 was cleaved with *PflM*1 and integrated into WT and *sir2*Δ strains (see Figure 5A). Independent *HIS3* transformants were then isolated and analyzed for the frequency of mitotic recombination between the two *ura3* fragments to form Ura^+ recombinants using the method of median described by Lea and Coulson (1949). Briefly, 30–50 cells derived from a single transformant were inoculated into each of nine tubes containing 1.5 ml SC medium. The cultures were grown to saturation, and appropriate dilutions were plated on SC and SC-Ura. The frequency of mitotic recombination was estimated from the median of the distribution of recombinants as described by Lea and Coulson (1949).

For measurements of the rate of marker loss in rDNA, cells carrying *URA3* in rDNA (see legends to Table 1 and Figure 5) were grown in SC-Ura medium to saturation. The frequency of Ura^- cells were determined by spotting or by spreading aliquots of suitable dilutions on SC plates with and without 5-FOA, followed by incubation for colony counts.

Other Methods

Analysis of chromosome XII by CHEF electrophoresis and determination of rDNA repeat numbers by Southern were carried out as described previously (Kobayashi et al., 1998). RFB activity was analyzed by 2D gel electrophoresis as described previously (Brewer and Fangman, 1987). Quantification of the results shown in Figure 3 was done after transfer of gels on membrane followed by a phosphorimager analysis. Chromatin immunoprecipitation assay was done based on the method described by Tanaka et al. (1997), except that Dynabeads protein A was used as described (Kamimura et al., 2001). Two different concentrations of DNA samples were used to obtain concentration-dependent signals after PCR amplification of relevant DNA regions. PCR products were separated by agarose gel electrophoresis, stained with EtBr, photographed and quantified using NIH image software.

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