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Role of *SGS1* and *SLX4* in maintaining rDNA structure in *Saccharomyces cerevisiae*

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Abstract

To address the role of *SGS1* in controlling genome stability we previously identified several “*slx*” mutants that require *SGS1* for viability. Here we report the isolation and characterization of temperature-sensitive (ts) *SGS1* alleles in cells lacking *SLX4*. At the non-permissive temperature (37°C) *sgs1-ts slx4* cells progress through S-phase and arrest growth as large-budded cells with at least a 2C DNA content. Analysis of the integrity of the replicated DNA by pulsed-field gel electrophoresis revealed that ChrXII was uniquely altered as it was unable to enter the gel. This defect was specific to the tandem rDNA repeats on ChrXII and occurred as cells progressed through S-phase at 37°C. Reciprocal-shift experiments revealed that viability and ChrXII migration can be restored by allowing Sgs1 to act between G2/M and the subsequent G1 phase. These results suggest that Sgs1 and Slx4 are not required for bulk DNA synthesis but that they play redundant roles in maintaining rDNA structure during DNA replication.

Keywords

SGS1; DNA helicase; DNA replication; rDNA; synthetic-lethal

Introduction

The *SGS1* gene of *Saccharomyces cerevisiae* encodes a member of the RecQ family of DNA helicases. This family includes RECQL, BLM, WRN, RECQL4 and RECQ5 in humans, along with Rqh1 in *S. pombe* and Dmblm and RecQ5 in *D. melanogaster* (Puranam and Blackshear 1994; Ellis et al. 1995; Yu et al. 1996; Murray et al. 1997; Stewart et al. 1997; Kitao et al. 1998; Kusano et al. 1999; Sekelsky et al. 1999). These proteins are characterized by a central DNA helicase domain with homology to *E. coli* RecQ and a large N-terminal domain that is functionally important in yeast (Duno et al. 2000; Mullen et al. 2000). The N-termini of Sgs1 and BLM interact with DNA topoisomerase III (Bennett et al. 2000; Wu et al. 2000; Fricke et al. 2001) while the N-terminus of WRN is unique in that it contains an exonuclease domain (Mushegian et al. 1997; Huang et al. 1998). Like RecQ, the BLM, WRN, and Sgs1 helicases display 3' to 5' DNA unwinding activity in vitro (Umezu et al. 1990; Lu et al. 1996; Gray et al. 1997; Karow et al. 1997; Bennett et al. 1998).

Mutations in the RecQ family genes are often associated with genomic instability and sensitivity to DNA damaging agents (German 1963; Hoehn et al. 1975; Gebhart et al. 1988; Gangloff et al. 1994; Watt et al. 1996; Murray et al. 1997; Stewart et al. 1997). In humans three distinct genetic diseases, Bloom's syndrome, Werner's syndrome and a subset of Rothmund-

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Thomson syndrome, are caused by mutations in *BLM* (Ellis et al. 1995), *WRN* (Yu et al. 1996), and *RECQL4* (Kitao et al. 1998), respectively. In yeast, mutations in *SGS1* result in an increase in chromosome missegregation, increased rates of recombination at the rDNA and elsewhere, impaired sporulation and an increased sensitivity to both DNA damaging agents and the DNA synthesis inhibitor hydroxyurea (Gangloff et al. 1994; Watt et al. 1995; Yamagata et al. 1998; Mullen et al. 2000). *SGS1* mutants also age prematurely possibly due to an increase in extrachromosomal rDNA circles (ERC's) (Sinclair and Guarente 1997).

The prototypical member of this family, the *E. coli* RecQ protein, is required for homologous recombination via the RecF pathway and to inhibit illegitimate recombination (Nakayama et al. 1985; Hanada et al. 1997). Some members of the RecF pathway are required to resume replication following UV-induced DNA damage and RecQ is required to process nascent strands at stalled replication forks (Courcelle et al. 1997; Courcelle and Hanawalt 1999). These data suggest that at least some eukaryotic RecQ proteins might be involved in processing stalled replication forks.

In order to identify the primary target of Sgs1 in yeast we previously isolated a set of mutants that require *SGS1* for viability. Growth of these six “*slx*” mutants requires both the N-terminal and DNA helicase domains of Sgs1, as well as Top3 (Mullen et al. 2001). Most of the *SLX* genes encode novel proteins that are conserved in other species, and in yeast they associate into three distinct complexes: Slx1-Slx4, Slx5-Slx8, and Mms4-Mus81. Mms4-Mus81 is a heterodimeric structure-specific endonuclease that cleaves 3' branches from duplex DNA in vitro (Kaliraman et al. 2001). The Slx1 and Slx4 proteins may also function as a complex since their mutant phenotypes are identical, they co-immunoprecipitate from cell extracts and they interact in a two-hybrid screen (Uetz et al. 2000; Mullen et al. 2001). However, unlike other *SLX* genes, mutations in *SLX1* and *SLX4* have no obvious effect on growth rate, DNA damage sensitivity or sporulation efficiency. In this report we describe the isolation and characterization of temperature-sensitive (*ts*) alleles of *SGS1* in the *slx4Δ* background. We show that *SGS1* and *SLX4* have overlapping roles in the control of rDNA structure during DNA replication. Eliminating both activities simultaneously does not affect bulk DNA synthesis at the rDNA or elsewhere. However, upon entering S-phase the rDNA loses its ability to migrate in pulsed-field gel electrophoresis (PFGE). This defect has previously been shown to correlate with incompletely replicated chromosomes (Hennessy et al. 1991; Desany et al. 1998). Normal chromosome behavior can be restored if Sgs1 is provided prior to mitosis. Based on the role of RecQ in bacteria, we propose that structural defects in the rDNA of *sgs1-ts slx4Δ* cells arise from the failure to restart stalled replication forks.

Materials and Methods

Strains, media and plasmid construction

The yeast strains used in this study are listed in Table 1. Strain construction, growth, and transformation followed standard methods (Rose et al. 1990). The *sgs1-11::loxP* deletion removes all but 15 codons at each end of the *SGS1* open reading frame (Mullen et al. 2000). All plasmids were made in pRS vectors (Sikorski and Hieter 1989). Plasmids pNTS1 (*sgs1-34*), pQTS1 (*sgs1-35*), & pQTS2 (*sgs1-t36*) were isolated after PCR mutagenesis of *SGS1* in pRS415 and screening for temperature-sensitive growth, as described below. The *sgs1-ts* alleles were subcloned as *XhoI/SacI* fragments from pNTS1, pQTS1 and pQTS2 into the corresponding sites of the integrating vectors pRS405 (to create pVK16, 17, and 18, respectively) and pRS404 (to create pVK20, 21, and 22, respectively).

Isolation of *sgs1* temperature-sensitive (*ts*) alleles

sgs1-ts alleles of *SGS1* were isolated following polymerase chain reaction (PCR) mutagenesis and screening for conditional growth in strain NJY1338 which requires plasmid-borne *SGS1* or *SLX4* for viability. Two domains of Sgs1 were individually targeted for mutagenesis by amplifying the appropriate regions of *SGS1* under mutagenic conditions. The PCR product was then used to repair a plasmid containing the corresponding deletion of *SGS1* by *in vivo* recombination (Muhlrad et al. 1992). Plasmid pSM100 (Mullen et al. 2000) contains a modified *SGS1* gene in the *LEU2/CEN* vector pRS415 (Sikorski and Hieter 1989). A unique *NdeI* site was engineered at the natural translation start site of *SGS1* such that the gene consists of a 153 bp *XhoI-NdeI* promoter fragment, a 1810 bp *NdeI-HindIII* fragment encoding the N-terminus and a 2616 bp *HindIII-SacI* fragment encoding the C-terminus of Sgs1. Using pSM100 as template, the N-terminal domain of Sgs1 (aa 1-652) was targeted for mutagenesis using primer 552 (5'-GGTAACGCCAGGGTTTTCCCA-3') within pRS415 and anti-sense primer 553 (TTACCTTGCAAAGTTGCATTT) at position 2061 of *SGS1* (ATG = position 1). The NTP-binding domain (aa 653-996) of *SGS1* was targeted using sense primer 554 (TTGGAAGATGATCTGGAAAAG) at position 1560, and anti-sense primer 555 (TTACCTTGCAAAGTTGCATTT) at position 3214. Gapped versions of pSM100 were prepared by digestion with either *NdeI* and *HindIII* ($\Delta 1 - 1810$ bp for mutagenesis of the N-terminal domain) or *HindIII* and *EagI* ($\Delta 1810 - 2963$ bp for mutagenesis of the NTP-domain). Mutagenic PCR (35 cycles) was performed with 0.2 u μ l Taq DNA polymerase (Promega), 100 μ M primers, 0.25 mM dNTPs, and 0.25mM to 0.5mM Mn⁺⁺ in place of Mg⁺⁺. Gapped plasmids and the PCR products were purified by agarose gel electrophoresis, mixed and used to transform strain NJY1338 (Table 1). Leu⁺ transformants were selected at 25°C, replica-plated to media containing 5-fluoroorotic acid (5-FOA) at 25°C and then replica-plated to yeast extract/peptone/dextrose (YPD) plates (Rose et al. 1990) to screen for viability at 17°C, 25°C and 37°C. For each domain, 4000 transformants were screened. Three mutants were isolated that were inviable at 37°C. One mutant (*sgs1-34*) resulted from mutagenesis of the N-terminus and two mutants (*sgs1-35*, *sgs1-36*) resulted from mutagenesis of the NTP-domain. The rescued plasmids had a wild-type restriction map, indicating that the gaps were repaired correctly.

DNA damage, recombination and aging assays

To prepare media, methylmethanesulfonate (MMS, final concentration 0.012%) was added to YPD agar just before pouring and the plates were used within 3 days. Cells were scraped from freshly growing plates, resuspended in water and the OD₆₀₀ was determined. Cells were transferred to a well of a 96-well microtiter plate at OD = 3.0 and serially diluted 1:10 to adjacent wells. A replica-plater was then used to transfer approximately 5 μ l from each well to YPD plates containing MMS or no drug. For recombination assays NJY540 cells carrying the integrated versions of the *sgs1-ts* alleles were maintained on minimal (SD) plates (Rose et al. 1990) lacking adenine, uracil and leucine. Cells were resuspended in water and about 200 cells were spread onto YPD plates containing 20 μ g/ml adenine and incubated at 25°C or 37°C. After 3 days, 7 colonies from each plate were individually picked, resuspended in 150 μ l water, diluted and a volume containing approximately 400 cells was spread onto a YPD plate and an SD plate containing 5-FOA. After 3 days at 25°C, colonies were counted and the percentage of viable cells that were resistant to 5-FOA (uracil⁻) was determined. The experiment was repeated three times. Old yeast cells were isolated as described (Smeal et al. 1996).

Microfluorimetry, CHEF gel and density transfer analyses

Yeast strains were grown in liquid YPD broth at 25°C to OD₆₀₀ = 0.15 and treated with α -factor at 5 μ g/ml for 2 hours to arrest the cells in the G1 phase of the cell cycle. Following

arrest, the cells were removed by filtration, resuspended in water, filtered, and washed a second time with YPD broth. The cells were then sonicated, resuspended in YPD broth, split in half, and incubated with shaking at either 25°C or 37°C. Aliquots of cells were removed at the indicated times, washed with phosphate-buffered saline (PBS) (Harlow and Lane 1988) and fixed in 70% ethanol for 1 hr. The cells were again washed in PBS, resuspended in PBS containing 0.1 mg/ml RNaseA and incubated at 37°C for 2 hrs. Proteinase K was added to 0.04 mg/ml and the cells were incubated for 1 hr at 55°C. The cells were then washed with PBS, sonicated and incubated with propidium iodide at 100 µg/ml for 1 hr. The cells were diluted 1:4 and sonicated prior to microfluorimetric analysis on a Coulter-Epics fluorescence-activated cell sorter (FACS). At least 150,000 events were recorded per sample.

CHEF gel analysis was carried out essentially as described by the manufacturer (Bio-Rad, Hercules, CA). Briefly, the strains were grown in liquid YPD medium at 25°C to OD₆₀₀ = 0.2 and synchronized with α -factor as above. The culture was released at 37°C and cells collected after 3 or 6 hours. The cells were washed and resuspended in cold 50 mM EDTA. Cells (6×10^8) were then treated as described and loaded into a 1.0% agarose gel (Pulse Field Certified Agarose, BioRad) in 0.5 X TBE and subjected to electrophoresis at 6 V/cm at an included angle of 120° with constant recirculation of the buffer at 12°C. Electrophoresis of chromosomal DNA was performed for 24 hours with an initial switch time of 1 min and final switch time of 2 min. Following electrophoresis the gels were stained with ethidium bromide (0.5 µg/ml), photographed and processed for Southern blotting as described (Sambrook et al. 1989).

The density-transfer assay was performed essentially as described (McCarroll and Fangman 1988). NJY1229 (*sgs1-34 slx4Δ*) cells were grown at 25°C for seven generations in heavy minimal medium containing 0.1% ¹³C glucose and 0.01% ¹⁵N ammonium sulfate as carbon and nitrogen sources, respectively. The cells were then arrested in G1 with α -factor (5 µg/ml for 3 hours). The cells were then washed, split and released into light rich medium (YPD) under one of the three following conditions: at 25°C with no drug; at 37°C with α -factor (15 µg/ml); or at 37°C with nocodazole (20 µg/ml). Samples (32×10^6 cells) were taken for analysis after the G1 arrest and 3 hrs after release treatment. The DNA was digested with *Bgl*III and separated on CsCl gradients (McCarroll and Fangman 1988). The gradients were fractionated, the refractive index of every other fraction was determined and every fraction was dot-blotted and probed with a ³²P-labeled rDNA fragment consisting of the 2.7 Kb *Hind*III fragment from the 5' end of the 35S transcript fused to the 0.6 kb *Eco*RI fragment at the 3' end of the 35S transcript.. A Molecular Dynamics PhosphorImager was used to develop the image which was quantitated using IP LabGel software. The positions of unreplicated DNA, corresponding to a refractive index of 1.4050 (heavy-heavy DNA), and fully replicated DNA, corresponding to a refractive index of 1.4040 (heavy-light DNA), were determined.

Results

Generation of temperature-sensitive alleles of *SGS1*

To isolate point mutants of *SGS1* that result in conditional loss-of-function, we took advantage of the fact that a yeast strain lacking the non-essential gene *SLX4* (YLR135W) is dependent on *SGS1* for viability (Mullen et al. 2001). We assumed that mutations in either the N-terminal domain or the DNA helicase domain of Sgs1 could be lethal since Sgs1 proteins lacking small portions of the N-terminus or DNA helicase activity were lethal in the *slx4Δ* background (Mullen et al. 2000). The strain NJY1338 (*slx4Δ sgs1Δ pJM6401/SLX4/URA3/ADE3*), is unable to grow on media containing 5-FOA since selection against the *URA3* plasmid is lethal in this background. Mutagenized *SGS1* genes were introduced into NJY1338 on *LEU2*-based plasmids, swapped for the *SLX4* plasmid by growth on 5-FOA at 25°C, and the resulting colonies screened for loss of viability on YPD plates at 37°C. Three independent plasmids were isolated that conferred ts growth. All three *sgs1-ts* alleles were recessive, as an *slx4Δ*

SGS1 strain containing any of the *sgs1-ts* plasmids was able to grow at 37°C (data not shown). The *sgs1-ts* alleles were sequenced and each found to contain a single missense mutation. The *sgs1-34* mutation (Q31P) mapped to the N-terminus while *sgs1-35* and *sgs1-36* (T980I and E987K, respectively) mapped to the more highly-conserved NTP-binding domain. Thus, missense mutations mapping to either the N-terminus or the DNA helicase domain of Sgs1 are capable of generating *ts* growth in *slx4Δ* cells.

In order to identify allelic differences, each *sgs1-ts* allele was integrated as the only copy of *SGS1* into otherwise wild type (wt) and *slx4Δ* backgrounds (Table 1). A liquid culture of each *sgs1-ts slx4Δ* strain was shifted from 25°C to 37°C and at various times cells were removed and plated to measure colony formation at 25°C (Fig. 1A). Double mutant strains displayed an initial increase in viable cell number after which they rapidly lost viability. The *sgs1-34 slx4Δ* strain exhibited the greatest sensitivity with less than a 2-fold increase in cell number and reduction to 10% viability in 16 hrs. To test whether the thermosensitivity of the *sgs1-ts* alleles was independent of *slx4Δ*, we assayed *sgs1* phenotypes in the *SLX4* background. The recombination phenotype was determined in strain NJY540, which contains the *URA3* marker within the rDNA cluster (Keil and McWilliams 1993). The rate of *URA3* marker loss was determined following growth on non-selective media at 25°C and 37°C. Strain NJY540 (*sgs1Δ*) carrying vector alone lost the *URA3* marker at 12 times the frequency of wt regardless of temperature (Fig. 1B). This elevation in recombination frequency is similar to that observed previously for the *sgs1Δ* null strain (Gangloff et al. 1994; Watt et al. 1996; Mullen et al. 2000). In contrast, the *sgs1-ts* alleles conferred near wt levels of recombination on NJY540 at the permissive temperature and 4- to 10-fold higher levels at 37°C. Thus, with respect to recombination, the *sgs1-ts* alleles are thermosensitive.

Otherwise wt *sgs1-ts* strains were next tested for sensitivity to methylmethanesulfonate (MMS). Ten-fold serial dilutions of cells were spotted onto broth plates in the absence or presence of MMS, and the level of growth was observed after 3 days at permissive and restrictive temperatures. As previously observed, the *sgs1* deletion strain carrying vector alone grew very poorly in the presence of MMS, while the strain carrying wild type *SGS1* grew well at both permissive and restrictive temperatures (Fig. 1C). At 25°C, *sgs1-ts* strains grew at nearly the wild type rate in the absence or presence of mutagen. In contrast, at 37°C, the growth of all three *sgs1-ts* mutants was specifically inhibited in the presence of MMS (Fig. 1C), and the level of growth approximated that of the *sgs1Δ* null strain. As in Fig. 1A, the *sgs1-34* allele showed the most severe effect at 37°C. In fact, *sgs1-34* grew worse than the null strain on YPD at 37°C (Fig. 1C, bottom right panel). We have previously shown that a strain lacking the first 158 amino acids of Sgs1 (*sgs1-ΔN158*) displays “hypermorphic” phenotypes relative to the null. For example, the *sgs1-ΔN158* strain is more sensitive to MMS than the null strain and grows more slowly than the null strain (Mullen et al. 2000). Since the Q31P mutation falls within the first 158 aa, we presume that *sgs1-34* mimics the hypermorphic phenotype of the *sgs1-ΔN158* allele, but only at the restrictive temperature. Finally, immunoblotting of epitope-tagged Sgs1 proteins revealed nearly equal levels of protein in all three mutant and wt strains grown at 25 or 37°C (data not shown). Based on these assays we conclude that the activity of the three *sgs1-ts* alleles is greatly reduced at the restrictive temperature.

***sgs1-ts* alleles affect separate *SGS1* functional domains**

Sgs1 is composed of two functional domains: the N-terminus (amino acids (aa) 1 - 652) and the DNA helicase domain (aa 645 - 1447). These two domains function in *trans* to complement *sgs1Δ* MMS sensitivity and *sgs1Δ slx4Δ* synthetic lethality (Mullen et al. 2000). We used intragenic complementation to test whether one or both domains were defective in the *sgs1-ts* alleles. Strain RLY2 (*sgs1-34 slx4Δ*) was transformed with a series of *SGS1* truncation alleles at 25°C and tested for viability by streaking onto YPD plates and observing growth at 37°C.

As expected, cells transformed with *SGS1* were viable at 37°C while those transformed with vector were not (Table 2). The full-length helicase-defective allele, *sgs1-hd*, as well as an allele that expresses only the N-terminus (aa 1 - 652), also complemented the lethality of the *sgs1-34 slx4Δ* strain at 37°C. In contrast, *SGS1* alleles encoding N-terminal deletions failed to complement this strain. We conclude that *sgs1-34* is defective only in the N-terminal function and retains DNA helicase activity at 37°C. The *sgs1-35* and *sgs1-36* alleles could not be complemented at the non-permissive temperature by *sgs1-hd* or by C-terminal truncations. Thus, complementation of these alleles requires an intact helicase domain. Consistent with this result, *SGS1* alleles encoding N-terminal deletions of up to 158 aa showed full complementation of *sgs1-35* and *sgs1-36* (Table 2). Taken together, we conclude that the NTP-domain mutations have impaired DNA helicase activity but retain N-terminal function at 37°C.

We tested whether the *sgs1-ts* alleles displayed growth defects in other *slxΔ* backgrounds at 37°C. As expected from the physical interaction between Slx1 and Slx4, all three *sgs1-ts* alleles that were isolated in the *slx4Δ* mutant background were lethal in the *slx1Δ* background at the non-permissive temperature (Table 3). The growth of *sgs1-ts mms4Δ* and *sgs1-ts mus81Δ* double mutants was impaired, although not eliminated at 37°C. Finally, the *sgs1-34 slx5Δ* double mutant was inviable at 37°C, while *sgs1-35 slx5Δ* and *sgs1-36 slx5Δ* strains displayed a growth defect at 37°C. As above, we conclude that the thermosensitivity of the *sgs1-ts* alleles is independent of the *slxΔ* background.

SGS1 and SLX4 are not required for bulk DNA synthesis

To address the roles of Sgs1 and Slx4 in cell growth, we assayed cell-cycle progression in *sgs1-ts slx4Δ* cells by flow cytometry. Exponentially growing strains were first arrested with α -factor at the permissive temperature and then released from G1 arrest at 37°C. The *slx4Δ* strain began DNA synthesis between 0.5 and 1 hour after release, and achieved a 2C DNA content by 2 hours (Fig. 2A). At 3 hours, cells containing 1C DNA content appeared while at later time points there is a loss of synchrony in the culture. Nearly identical profiles were obtained with wt cells at 37°C or single and double mutant combinations at 25°C (data not shown). When the *slx4Δ sgs1-34* mutant was released at 37°C, DNA synthesis also began between 0.5 and 1 hour, and 2C DNA content was observed after 3 hours (Fig. 2A). Thus, bulk DNA synthesis was not significantly affected in the double mutant at 37°C. However, unlike single mutants this strain did not produce a 1C peak indicative of G1 cells in the next cell cycle. Instead, the G2 peak broadened and a peak corresponding to 3C to 4C appeared 4 to 6 hours after release. The *sgs1-35* and *-36* strains behaved similarly: DNA synthesis was completed by 2 hours, they failed to produce G1 cells, and they accumulated greater than 2C DNA content at late times. We note that the increase in DNA content observed 4–6 hours at 37°C appears to coincide with the loss of viability in exponential cultures (Fig. 1A).

To confirm the cell cycle results, we examined the morphology of these strains growing at 37°C. At time zero all strains were predominantly unbudded, as expected for cells in the G1 phase of the cell cycle (Fig. 2B). After 4 hours at 37°C, *slx4Δ* cells had lost synchrony and were growing with nearly equal proportions of unbudded, small-budded and large-budded cells. In contrast, double mutant cultures were mostly comprised of large- or multiply-budded cells after 4–6 hours at 37°C. DAPI staining of these cells revealed that most large-budded cells contained a single undivided nucleus at the neck of the mother cell (data not shown). This phenotype is similar to that observed when *SGS1* expression was repressed in the *mms4Δ* background (Mullen et al. 2001) and suggests that most cells arrest growth in the late S or G2 phase of the cell cycle. As in the assays described above, the N-terminal domain mutant, *sgs1-34*, displayed a more severe aberrant budding phenotype than the helicase domain mutants. After 6 hours at 37°C, approximately 30% of these cells were multiply budded compared to 10% or fewer of the helicase domain mutants (Fig. 2B). Taken together, we conclude that DNA synthesis is not

significantly inhibited in the absence of *SGS1* and *SLX4*. However, the majority of these cells arrest growth at late S/G2 and undergo additional DNA synthesis after 4–6 hours. The broad 3C - 4C peak may result from incomplete DNA synthesis in cells that are losing viability.

Altered stability of Chromosome XII in *sgs1-ts slx4Δ* mutants

The terminal phenotype of the double mutants suggested a defect late in DNA replication. To identify defects in chromosomal replication, we used PFGE to search for temperature-dependent changes in chromosome structure. Clamped Homogenous Electric Field (CHEF) gels have previously been used to characterize chromosomal abnormalities associated with replicating yeast DNA (Hennessy et al. 1991; Christman et al. 1993; Desany et al. 1998; Shimada et al. 1999). When cells are arrested before or after DNA replication chromosomes migrate normally in this system, however when cells are arrested during DNA replication, for example by treatment with hydroxyurea (HU), chromosomes are unable to enter the gel (Hennessy et al. 1991; Desany et al. 1998). These incompletely replicated chromosomes fail to enter the gel due to DNA replication forks or bubbles that block its electrophoretic migration (Hennessy et al. 1991; Desany et al. 1998; Shimada et al. 1999).

CHEF gels resolve the 16 individual yeast chromosomes into a ladder of 13 bands following ethidium bromide staining. Under the conditions used here Chromosome XII (ChrXII), which contains the 1 Mb rDNA cluster, migrates as the second largest chromosome. This was confirmed by Southern blotting with an rDNA probe which detected ChrXII as a doublet in all strains (Fig. 3, lower panel). Cells were synchronized with α -factor at 25°C, released at 37°C and harvested after 3 and 6 hours for CHEF gel analysis. Under permissive conditions, the pattern of chromosomal bands obtained from wt and mutant strains was identical (Figs. 3, 6A and data not shown). In contrast, when double mutant cells were shifted to 37°C for 3 or 6 hours, there was a slight decrease in the intensity of most bands and a striking loss of ChrXII from the profile (Fig. 3). Southern blotting with an rDNA probe confirmed the identity of the missing band as ChrXII and indicated that it was not co-migrating with other chromosomal bands (Fig. 3, lower panel). This phenotype is specific to the double mutant at restrictive temperature as ChrXII migration was normal in *sgs1Δ*, *slx4Δ* and *sgs1-ts* single mutants at both temperatures (Figs. 3 and 6A). In addition, the phenotype was independent of synchrony since it was also observed in exponentially growing cells upon shift to 37°C (data not shown). We conclude that ChrXII isolated from *sgs1-ts slx4Δ* strains at 37°C is retained in the well of the gel similar to chromosomes from HU-treated cells.

Aberrant migration of chromosome XII requires multiple tandem repeats of rDNA

A possible cause of the altered mobility of ChrXII is that the entire chromosome has a unique structure, in which case all portions of the chromosome would be retained in the well. Alternatively, it might be due to the 100–200 tandem repeats of rDNA present on ChrXII, or to a feature unique to each rDNA repeat unit. To test these possibilities, we digested chromosome preparations from *sgs1-34 slx4Δ* mutants with restriction enzymes prior to CHEF analysis and Southern blotting. *NotI* digests ChrXII into six large fragments but does not cut within the rDNA array. Southern blotting with probes specific for either rDNA or the flanking DNA would determine whether the migration defect was due to the rDNA cluster or the structure of ChrXII itself. We used the *TOP3* gene as a probe for flanking DNA and observed a band migrating at the expected size of 0.25 Mb, regardless of the growth temperature (Fig. 4A, left panel). In contrast, the *NotI* fragment containing the rDNA failed to migrate into the gel when isolated from cells grown at 37°C (Fig. 4A, right panel). When chromosomes were isolated at 25°C, *NotI* digestion resulted in a homogeneous rDNA band (approximately 1 Mb) which migrated near the full-length ChrXII bands. Thus, the aberrant migration of ChrXII is specific to the rDNA at the non-permissive temperature.

To test whether this aberrant migration was specific to each rDNA repeat or the cluster of repeats, chromosome preparations from *sgs1-34 slx4Δ* cells were digested with *SacI*, which cuts once in each of the rDNA repeats. Digestion products were then analyzed as above except the duration of electrophoresis was reduced to prevent the unit length rDNA fragments (9.1 kb) from migrating off the gel. We observed that *SacI* digestion produced single rDNA fragments that entered the CHEF gel regardless of the growth temperature (Fig. 4B). Thus, the failure of ChrXII to enter the CHEF gel is a characteristic of the rDNA array, not of the repeat unit itself.

We suspected that the anomalous behavior of rDNA from *sgs1-34 slx4Δ* strains at the non-permissive temperature might be related to the excision of extra chromosomal rDNA circles (ERCs) in aging cells (Sinclair and Guarente 1997). To examine the effect of *slx* mutations on the premature excision of ERCs, we measured ERC levels by Southern blotting DNA isolated from young and old (7 - 8 generations) cells with wild-type, *sgs1Δ* and *slx4Δ* mutant backgrounds (Mullen et al. 2001). As previously reported, there was no evidence of ERCs in any of the young cells; the rDNA was limited to a linear DNA fragment of 23 Kb that arises from the shearing of genomic DNA (Fig. 5). After 7 generations all of the strains produced ERCs, but their formation in *sgs1Δ* or *slx4Δ* mutants was not quantitatively different from wild type. We conclude that ERC formation does not occur prematurely in *slx4Δ* or *sgs1Δ* mutants and is therefore unlikely to be involved in the altered structure of the rDNA in the *sgs1-ts slx4Δ* double mutants.

Altered mobility of Chromosome XII requires cell-cycle progression

The altered structure of ChrXII might be due to defects in transcription, recombination, or DNA replication. If it were due to defects in transcription or recombination, it would be expected to be independent of cell-cycle position while a defect in DNA replication should require passage through S-phase. To distinguish between these possibilities, we synchronized the growth of *sgs1-ts slx4* cells by arresting them in G1 with α -factor at 25°C. Cells were then released at 37°C in the presence of additional α -factor to hold them in G1, or in the presence of nocodazole to allow them to progress to G2/M. After three hours the cells were harvested for CHEF analysis. As shown in Figure 6A, ChrXII entered the gel when the cells were held in G1 at 37°C, but failed to enter the gel when the cells were allowed to progress through the cell cycle. The simplest explanation for these results is that the rDNA undergoes a structural change during the S-phase of the cell cycle.

Since the FACS analysis presented in Fig. 2A may have failed to detect defects in rDNA replication, we assayed this directly using a density-shift protocol. The *sgs1-34 slx4Δ* strain was grown in heavy isotopes at 25°C and arrested in G1 with α -factor. The cells were then released from the α -factor block into light medium at 25°C, or at 37°C in the presence of either additional α -factor or nocodazole. After three hours DNA was isolated, fractionated by equilibrium density sedimentation and the fractions were blotted with an rDNA specific probe. As shown in Fig. 6B, DNA isolated prior to release sedimented with a peak at fraction 11 while DNA from cells released at 25°C peaked at fraction 15. These fractions correspond to HH and HL densities, respectively, as judged by refractive index. When the cells were released at 37°C in the presence of additional α -factor its DNA remained HH, while that from cells released at 37°C in the presence of nocodazole peaked at fraction 15 corresponding to HL DNA. CHEF gel analysis of these samples confirmed that ChrXII failed to enter the gel upon release into nocodazole at 37°C (data not shown). We conclude that rDNA synthesis in this strain is not significantly inhibited at 37°C.

Given that rDNA replication was essentially complete in the absence of these activities, we asked whether Sgs1 activity could be provided following S-phase to correct the defect in ChrXII structure. As above, double mutant cells were arrested in G1 with α -factor and released at 37°

C in the presence of nocodazole. Three hours later cells were released from the nocodazole block at 25°C in the presence or absence of α -factor. Samples were taken at each condition for CHEF analysis. As before, ChrXII migration was severely inhibited when cells were allowed to progress through S-phase to the nocodazole block at 37°C (Fig. 7). However, when cells were released from the nocodazole block into YPD at the permissive temperature, ChrXII migration returned to normal (Fig. 7) and cell proliferation continued (data not shown). Moreover, a subsequent S phase is not required to correct ChrXII structure as it migrated normally when cells were returned to the permissive condition in the presence of α -factor. We conclude that Sgs1 is not required for DNA replication and that its function can be provided following bulk DNA synthesis to correct defects in rDNA structure.

Discussion

In order to gain further insight into the function of Sgs1 in yeast, we used *SLX4* mutants to isolate conditional alleles of *SGS1* and search for defects that occur upon Sgs1 inactivation. *SLX4* is one of six non-essential genes previously shown to be required for viability in the absence of *SGS1* (Mullen et al. 2001). Two other genes isolated in this screen, *MMS4* and *MUS81*, encode a heterodimeric structure-specific endonuclease (Kaliraman et al. 2001). Interestingly, Slx4 functions in a complex with Slx1 (Mullen et al. 2001) which was recently shown to be homologous to a conserved class of UvrC-Intron-type (URI) endonucleases (Aravind and Koonin 2001). Thus, *SLX4* and *SLX1* appear to encode a heterodimeric endonuclease that functions in a conserved DNA repair or recombination pathway, like Mms4-Mus81.

We isolated *SGS1* mutations that map to either the DNA helicase domain (*sgs1-35* and *sgs1-36*) or the N-terminus (*sgs1-34*). Although these two classes of mutations exhibited phenotypes consistent with previous structure-function analysis (Mullen et al. 2000), they were equally defective in controlling rDNA structure in the *slx4 Δ* background. This supports the idea that Sgs1 requires the Top3-interacting domain, not just DNA helicase activity, for wt function. When *sgs1-ts slx4 Δ* cells were shifted to the non-permissive temperature they replicated their DNA and arrested in late S/G2. These results strongly support the idea that *SLX4* and *SGS1* are not required for bulk DNA synthesis. The fact that all three *SGS1* alleles behaved similarly suggests that these alleles do not exhibit a delay or “leakiness” in their inactivation at 37°C. Moreover, the viability of these cells was dramatically reduced if they were held at 37°C for longer than 5 hours and FACS analysis revealed that additional of DNA synthesis takes place at this time. We suggest that the first round of replication in *slx4 Δ sgs1-ts* cells at 37°C is defective and that a checkpoint transiently arrests the cell cycle in late S/G2. In the absence of repair, adaptation ensues and the cells enter an aberrant, and lethal, cell cycle. The increase in multiply-budded cells and 4C levels of DNA content indicate that cells begin this cell cycle without undergoing a normal mitosis.

Lee and coworkers have reported the isolation of a conditional-lethal allele of *SGS1* in the presence of the *srs2 Δ* mutation (Lee et al. 1999). *SRS2* encodes a 3' - 5' DNA helicase (Rong and Klein 1993) and, in contrast to our results, these authors conclude that at least one of these two DNA helicases is required for DNA replication fork movement in yeast. We used *slx4* to screen for *sgs1-ts* alleles because, like others, we observe only a synthetic-sickness, not synthetic-lethality in the *sgs1 Δ srs2 Δ* double mutant (Gangloff et al. 2000; Klein 2001; Mullen et al. 2001). Moreover, the sickness of the *sgs1 Δ srs2 Δ* double mutant is dependent on homologous recombination and not a failure to replicate DNA (Gangloff et al. 2000; Klein 2001; McVey et al. 2001). Taken together with the present study, we find no evidence for Sgs1 as a primary replicative DNA helicase. Instead, the function of Sgs1 in DNA replication appears to be required late in DNA replication and may be locus specific.

One molecular defect in *slx4Δ sgs1-ts* cells at the non-permissive temperature is the loss of structural integrity of the rDNA as assayed by its inability to enter a CHEF gel. Previous work with hydroxyurea and the *cdc46* replication mutant suggested that the inability of chromosomal DNA to enter a CHEF gel is due to abnormal structures that result from incomplete replication (Hennessy et al. 1991; Desany et al. 1998). Indeed, we find that this unusual behavior is dependent on *slx4Δ sgs1-ts* cells progressing through S phase. In contrast to HU-treatment and *cdc46* inactivation, in which no chromosomal DNA enters the gel, most of the DNA from *slx4Δ sgs1-ts* cells at 37°C behaves normally with the exception of the rDNA. Given the specific defect in these cells, we conclude that Slx4 and Sgs1 play an important role late in rDNA replication. Such a result is consistent with the presence of Sgs1 in the nucleolus and its role in maintaining nucleolar structure (Sinclair et al. 1997; Frei and Gasser 2000). Interestingly, defects in DNA topoisomerase I, which is enriched in the nucleolus, also lead to an inability of rDNA to enter a CHEF gel (Muller et al. 1985; Christman et al. 1993). In this case however, when *top1Δ* cells are grown to saturation, rDNA regains the ability to enter the gel (Christman et al. 1993). Presumably another protein that can relieve torsional stress (e.g., Top2) replaces Top1, albeit with reduced efficiency, thereby completing rDNA replication. It is known that the rDNA has unique topological problems since simultaneously reducing both Top1 and Top2 result in excision of rDNA circles and inhibition of rDNA transcription (Brill et al. 1987; Kim and Wang 1989). Just as Top1 and Top2 have overlapping functions in relieving topological stress at the rDNA, we propose that Sgs1-Top3 and Slx1-4 share responsibility for dealing with another unique aspect of rDNA replication.

In contrast to idea that Sgs1 is directly involved in promoting DNA replication, reciprocal-shift experiments demonstrate that Sgs1 can fulfill its role after most, if not all, DNA has been replicated. It is formally possible that the ability of Sgs1 to act following G2/M is unique to this system; it could be dependent on the absence of Slx4 or the rDNA substrate. We think this is unlikely and suggest that in wild type cells Sgs1 performs its function during and/or after DNA replication. This execution point is consistent with models that propose a role for RecQ proteins in the resolution of stalled replication forks. This idea was originally suggested by the fact that RecQ DNA helicase functions at stalled replication forks in *E. coli* and that Rqh1 is required for restarting replication in HU-arrested *S. pombe* (Stewart et al. 1997; Courcelle and Hanawalt 1999). We suggest that Sgs1-Top3 and Slx1-4 function throughout the genome and that the apparent specificity for rDNA reflects the fact that rDNA replication involves numerous stalled replication forks. The replication fork barrier, which prevents forks from progressing into the path of the 35S transcription unit, stalls one fork at every rDNA origin or approximately every 5th rDNA repeat (Walmsley et al. 1984; Brewer and Fangman 1988). In the absence of Sgs1 and Slx4, most rDNA repeats should be replicated completely by the fork moving in the direction of rDNA transcription, however one in five repeats could be defective if the replication block is not properly processed. This may explain why individual rDNA repeats appear to migrate normally in CHEF gels, but the entire rDNA cluster does not. We suggest that each replicon of approximately 5 rDNA repeats contains one partially replicated repeat that inhibits its movement in CHEF gels. While we cannot rule out the possibility that Slx1-4 functions exclusively at the rDNA, we favor the idea that it functions globally, like Cdc46 and Top1, and that its mutant phenotypes are exacerbated at the rDNA.

Early models of replication repair proposed that stalled forks might regress to form Holliday junctions (Higgins et al. 1976). Support for this idea is provided by the fact that the Holliday junction binding proteins RuvAB are required for processing arrested replication forks in *E. coli* (Seigneur et al. 1998). Interestingly, molecular studies have shown that Holliday junctions form in the rDNA of yeast specifically during S-phase (Zou and Rothstein 1997). The fact that another RecQ family member, the BLM helicase, is capable of branch migrating a Holliday junction suggested that BLM might reverse the formation of Holliday junctions at stalled replication forks (Karow et al. 2000). Sgs1 might play a similar role at the rDNA of yeast where

stalled forks are expected to form at every origin. Given this model, the Slx1-4 complex might be required in the absence Sgs1 to inhibit the formation of Holliday junctions or to resolve them with its putative nuclease activity. An alternative model is that the Slx1-4 complex processes subsequent recombination intermediates that occur in the absence of Sgs1. These models predict that the Slx1-4 complex should be enriched in the nucleolus and interact with DNA in a structure-specific manner. These predictions are currently being tested.

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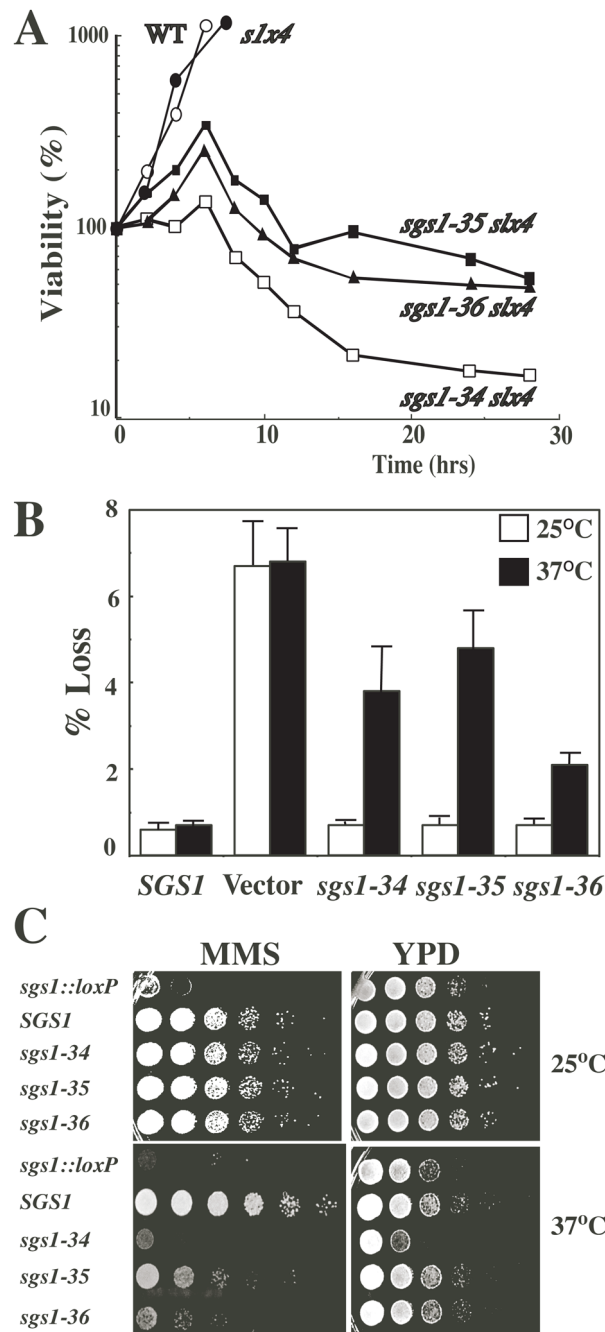
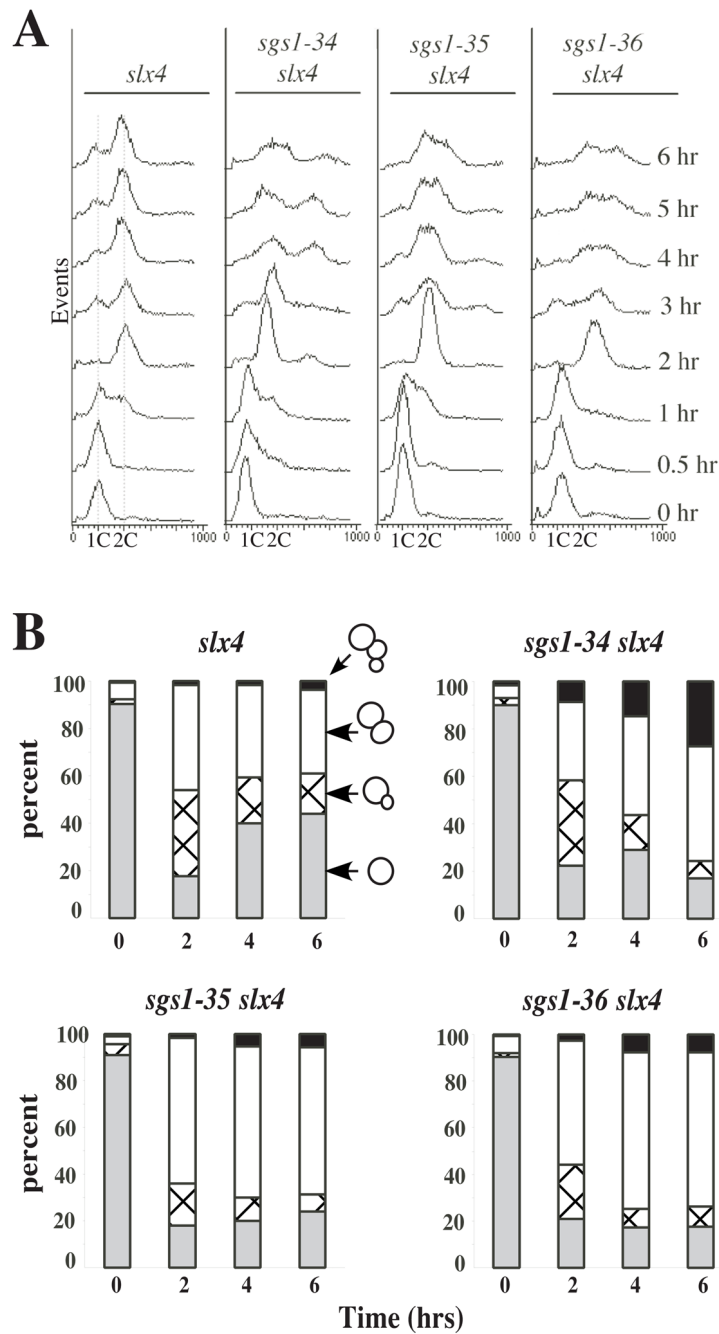
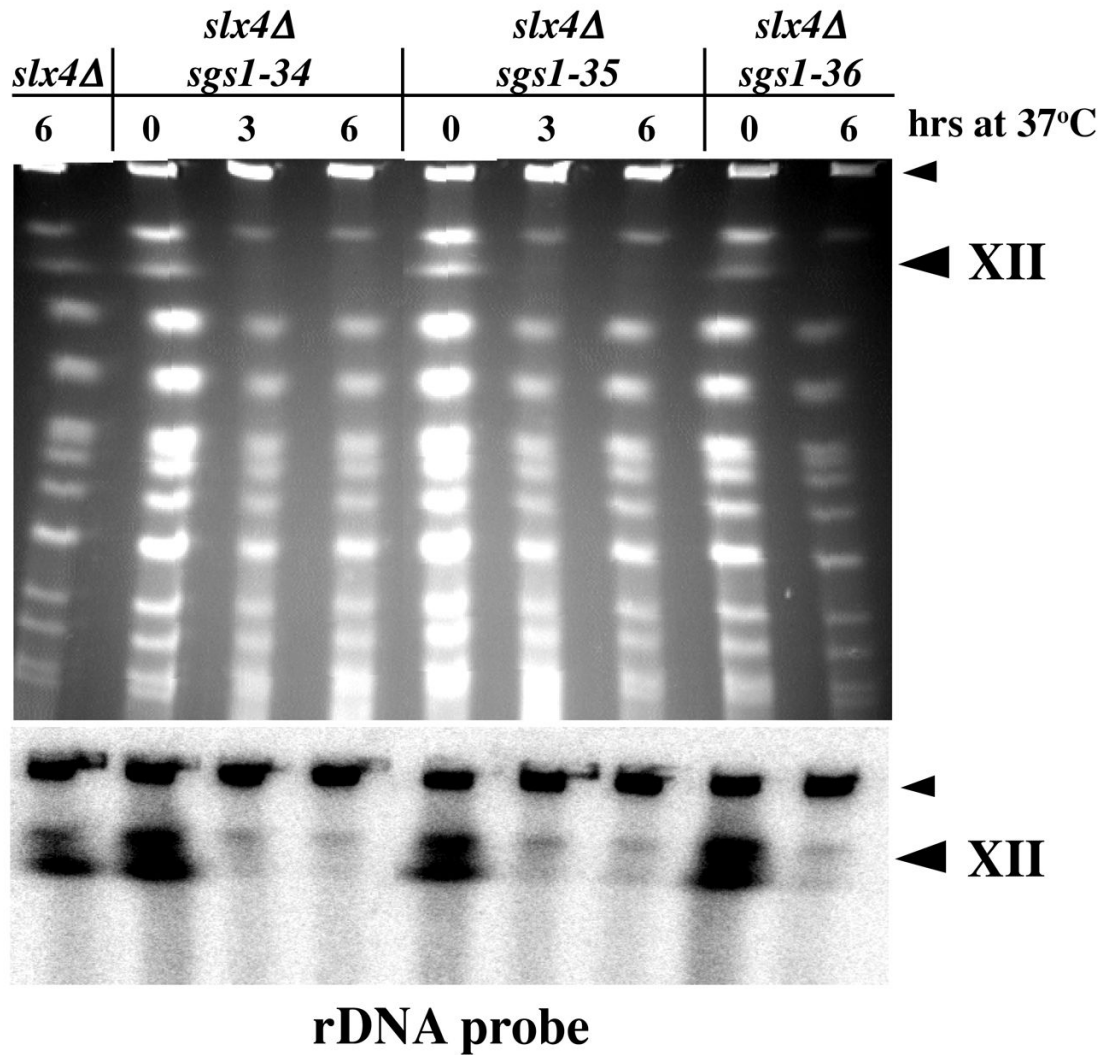


Fig. 1. Conditional *SGS1* activity. **A** Strains of the indicated genotype were grown to exponential phase in YPD broth at 25°C, transferred to 37°C for the indicated times, and the percentage of viable cells determined. **B** Recombination rate was determined by stably transforming the indicated *SGS1* allele, or vector alone, into strain NJY540 (*sgs1::loxP*) which contains the *URA3* gene integrated at *RDNI*. The number of uracil auxotrophs was determined following 3 days growth in the absence of selection at the indicated temperature. The percentage of cells undergoing a marker loss event was determined and is presented as the mean \pm SD from seven independent colonies. **C** *SGS1*-dependent hypersensitivity to DNA damaging agents was determined by resuspending strains of the indicated genotype and spotting them in 10-fold

serial dilutions on YPD plates containing MMS or no drug. The plates were photographed following three days at the indicated temperature.

**Fig. 2.**

Cell cycle arrest of *sgs1-ts slx4Δ* cells. The indicated strains were grown to exponential phase at 25°C and synchronized by arresting growth with α -factor. After release into YPD broth at 37°C, aliquots were removed at the indicated times and fixed in ethanol. **A** One portion of each aliquot was stained with propidium iodide and analyzed by flow cytometry. **B** Another portion was microscopically examined for budding morphology: gray, single cells; crosshatch, small budded cells; white, large budded cells; black, aberrant or multiply-budded cells. Data is presented as a percentage of at least 100 cells.

**Fig. 3.**

Altered electrophoretic mobility of ChrXII in *sgs1-ts slx4Δ* strains. The indicated strains were grown to exponential phase at 25°C, arrested with α -factor and released at 37°C for the indicated times. Cells were collected and prepared for CHEF gel electrophoresis in agarose plugs. The gel was stained with ethidium bromide (top) and Southern blotted using an rDNA fragment as probe (bottom). The bottom half of the blot had no signal and is not presented. The large arrowhead indicates the position of ChrXII and the small arrowhead indicates the position of the wells.

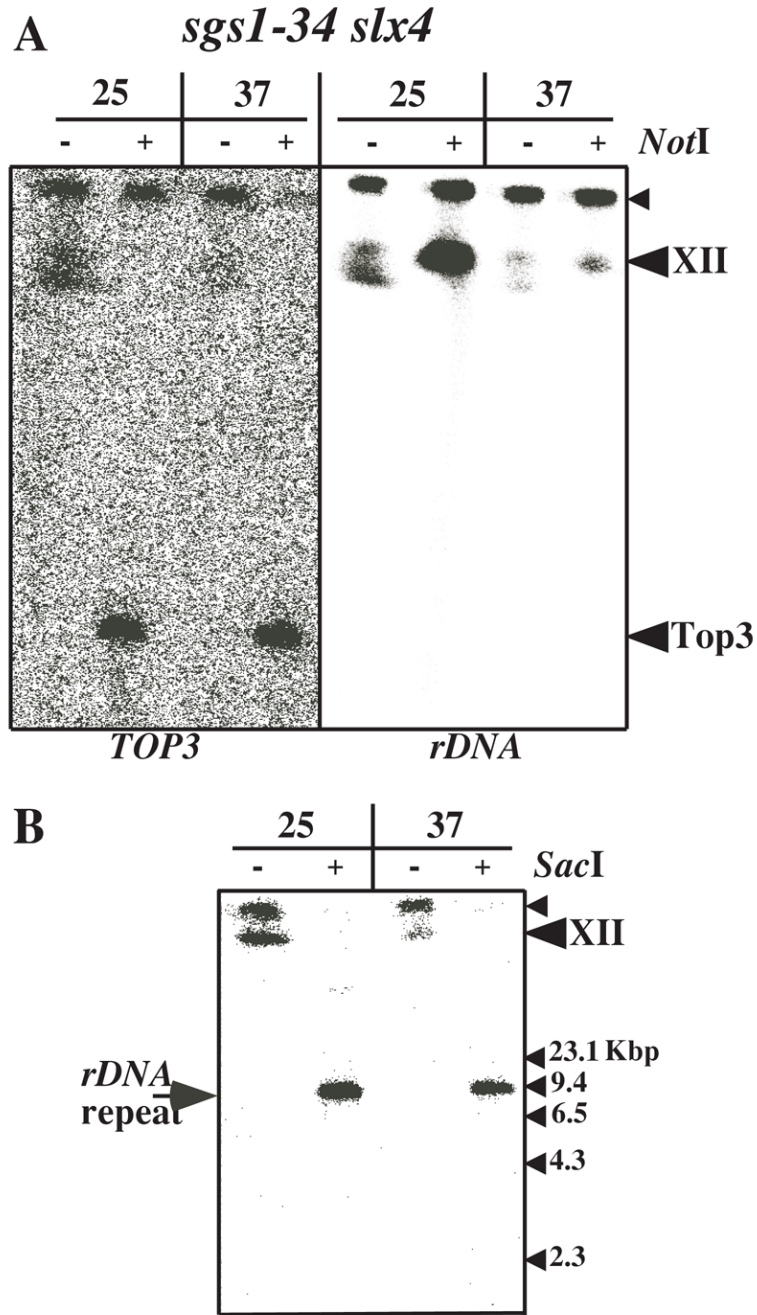


Fig. 4. Multiple rDNA repeat units are required for the altered electrophoretic mobility of ChrXII. The *sgs1-34 slx4* strain was grown to exponential phase at 25°C, arrested with α -factor and released at 37° for 6 hours. Cells were collected and prepared for CHEF gel electrophoresis in agarose plugs. The plugs were digested with *NotI* or *SacI* as indicated. **A** Following electrophoresis the gel was blotted using the complete *TOP3* coding region (left) or an rDNA fragment (right) as probe. **B** The electrophoresis of chromosomal DNA was performed as above except that the electrophoresis was stopped after 7 hours. The gel was then Southern blotted using an rDNA fragment as probe.

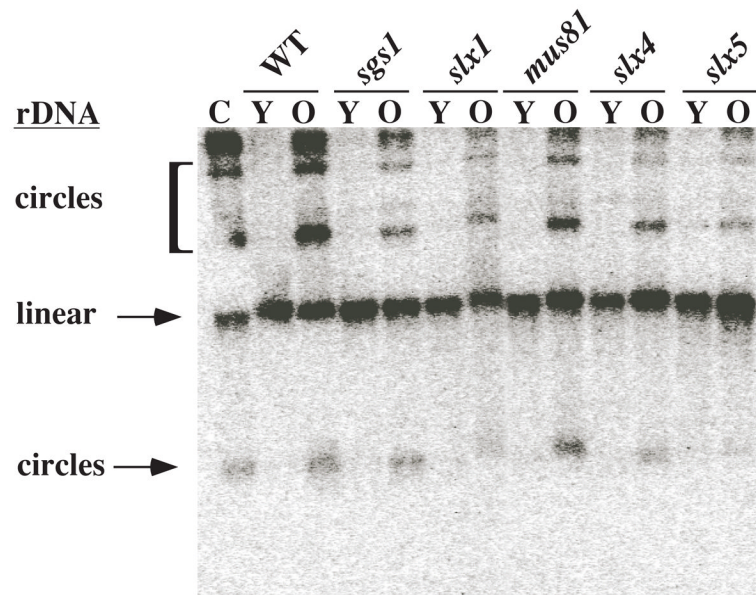


Figure 5.

Extra-chromosomal rDNA circle formation in *sgs1Δ* and *slx1Δ* strains. Total DNA was isolated from sorted seven-generation old cells and unsorted young cells. This DNA was subjected to one-dimensional gel electrophoresis and Southern blotted using an rDNA fragment as probe. The average bud scar counts of sorted cells was determined to be between 7 and 8. A control strain (C; *top1 top2-4*) excises rDNA circles at 25°C and is used as a marker for ERC migration. Y, young cells; O, old cells.

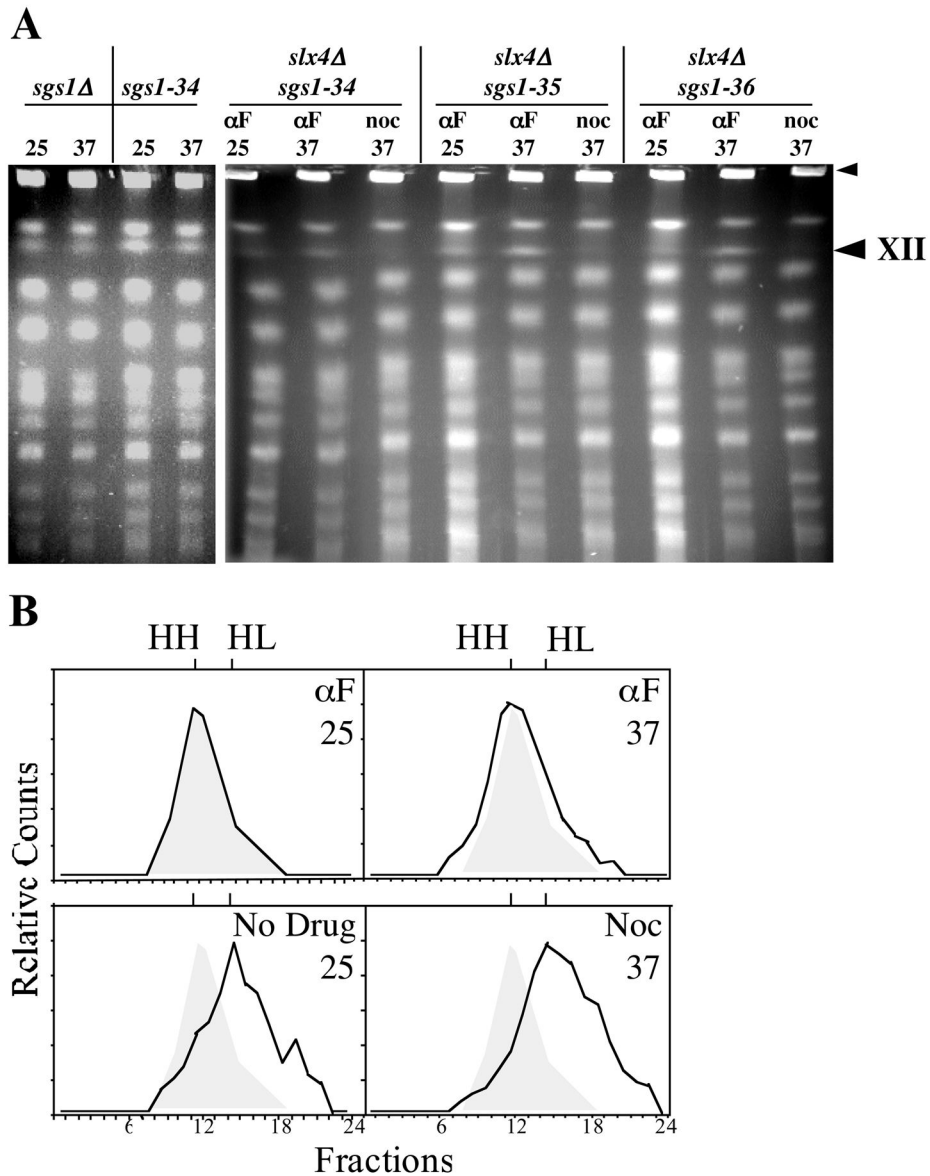


Fig 6. Replication of the rDNA is required for the altered mobility of ChrXII in *sgs1-ts slx4Δ* cells. **A** Cells were synchronized and analyzed as in Fig 3. Left panel: the *sgs1Δ* and *sgs1-34* single mutants were released from α F block and incubated for 6 hours in YPD broth at the indicated temperature. Right panel: the indicated double mutants were released into medium containing α -factor or nocodazole and incubated for 3 hrs at the indicated temperature. Microscopic examination confirmed that 98% of the cells held in α -factor were unbudded and 97% of the cells blocked in nocodazole were large-budded. Cells were harvested and analyzed by CHEF gel electrophoresis. The large arrowhead indicates the position of ChrXII and the small arrowhead indicates the position of the wells. **B** Density shift analysis of rDNA replication. *sgs1-34 slx4Δ* cells were grown in heavy minimal medium and arrested with α -factor at 25°C (α F/25). Cells were then washed, and aliquots released at 25°C into light medium (No Drug/25) or at 37°C into light medium containing α -factor (α F/37) or nocodazole (Noc/37). After the indicated treatments the cells were harvested and the DNA was isolated, fractionated by equilibrium gradient density sedimentation, and analyzed by Southern blotting. The positions

of unreplicated heavy-heavy (HH) and fully replicated heavy-light DNA (HL) are indicated. The initial HH peak is shown for comparison as a shaded peak in the released samples.

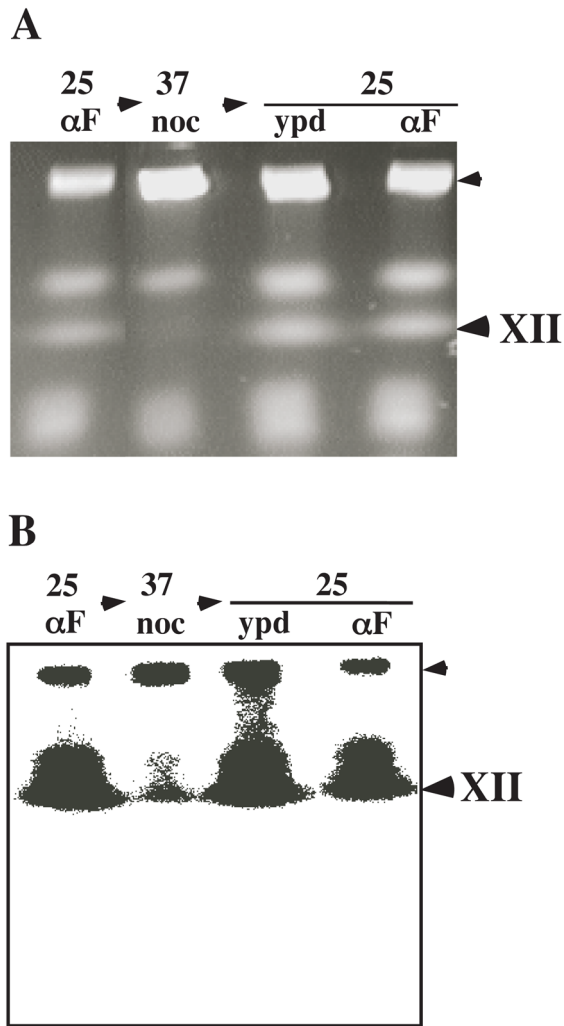


Fig. 7. Reciprocal shift analysis of ChrXII mobility. *sgs1-34 slx4Δ* cells were arrested with α -factor at 25°C (25/ α F) and released at 37°C in the presence of nocodazole (37/noc) for three hours. The cells were then washed and released at 25°C in the absence (25/ypd) or presence (25/ α F) of α -factor for three hours. After the indicated treatments cells were harvested and DNA was analyzed by CHEF gel electrophoresis. **A** Ethidium bromide stain. **B** Southern blot with rDNA probe.

Table 1

List of Strains

Name	Genotype	Reference or Source
CHY125	<i>MATa ade2-1 ade3::hisG ura3-1 his3-12,15 trp1-1 leu2-3,112 can1-100</i>	Mullen et al. (2000)
K1875	<i>MATa ade2-1 ura3-52 his4-260, trp1-H3 leu2-3,112 lys2-ΔBX-CANI-LYS2 can1 rDNA::ADE2 rDNA::URA3</i>	Keil and McWilliams (1993)
W303-1a	<i>MATa ade2-1 ura3-1 his3-1,15 trp1-1 leu2-3,112 can1-100</i>	Thomas and Rothstein (1989)
NY560	<i>MATa ade2-1 ade3::hisG ura3-1 his3-12,15 trp1-1 leu2-3,112 can1-100 sgs1-11::loxP slx4-11::loxP [pJM500::SGS1 URA3 ADE3]</i>	This study
NY1338	NY560 but containing [pJM6401::SLX4 URA3 ADE3] instead of pJM500	This study
VKY1227	<i>MATa ade2-1 ade3::hisG ura3-1 his3-12,15 trp1-1 leu2-3,112 can1-100 sgs1-11::loxP slx4-11::loxP [pJM6401::SLX4 URA3 ADE3]</i>	This study
NY1228	NY560 <i>LEU2::SGS1</i> FOA ^R	This study
NY1229	NY560 <i>LEU2::sgs1-34</i> FOA ^R	This study
NY1230	NY560 <i>LEU2::sgs1-35</i> FOA ^R	This study
NY1231	NY560 <i>LEU2::sgs1-36</i> FOA ^R	This study
NY540	K1875 <i>sgs1-11::loxP</i>	Mullen et al. (2000)
NY1256	NY540 <i>LEU2::SGS1</i>	This study
NY1257	NY540 <i>LEU2::sgs1-34</i>	This study
NY1248	NY540 <i>LEU2::sgs1-35</i>	This study
NY1249	NY540 <i>LEU2::sgs1-36</i>	This study
NY1250	NY540 <i>LEU2::pRS405</i>	This study
NY531	CHY125 <i>sgs1::loxP</i>	Mullen et al. (2000)
NY1251	NY531 <i>TRP1::pRS404</i>	This study
NY1252	NY531 <i>TRP1::SGS1</i>	This study
NY1253	NY531 <i>TRP1::sgs1-34</i>	This study
NY1254	NY531 <i>TRP1::sgs1-35</i>	This study
NY1255	NY531 <i>TRP1::sgs1-36</i>	This study
RLY1	NY560 <i>TRP1::SGS1</i> FOA ^R	This study
RLY2	NY560 <i>TRP1::sgs1-34</i> FOA ^R	This study
RLY3	NY560 <i>TRP1::sgs1-35</i> FOA ^R	This study
RLY4	NY560 <i>TRP1::sgs1-36</i> FOA ^R	This study

Table 2

Intragenic complementation of *sgs1-ts sIx4Δ1*

SGS1 allele	Protein product ²	<i>sgs1-34</i>		<i>sgs1-35</i>		<i>sgs1-36</i>	
		25°C	37°C	25°C	37°C	25°C	37°C
<i>SGS1</i>	1 – 1447	+	+	+	+	+	+
<i>sgs1-hd</i>	1 – 1447	+	+	+	-	ND	ND
<i>sgs1-ΔIC795</i>	1 – 652	+	+	+	-	+	-
<i>sgs1-ΔNI58</i>	159 – 1447	+	-	+	+	+	+
vector (pRS415)	none	+	-	+	-	+	-

¹ Strains RLY2, RLY3, and RLY4 (*sgs1Δ sIx4Δ TRP1::sgs1-ts*) were transformed with the indicated *SGS1* truncation alleles in pRS415 at 25°C and tested for viability by streaking on YPD plates and observing growth after three days at 37°C. +, growth; -, no growth. ND: Not Done.

² Expressed amino acids of Sgs1. The *sgs1-hd* allele expresses Sgs1 with the K706A mutation (Lu *et al.* 1996).

Table 3

Phenotype of *sgs1-ts* alleles in various *SLX* mutant backgrounds¹

<i>SGS1</i> allele	<i>slx1Δ</i>		<i>sgs1Δ</i>		<i>mms4Δ</i>		<i>sgs1Δ</i>		<i>mus81Δ</i>		<i>sgs1Δ</i>		<i>slx4Δ</i>		<i>sgs1Δ</i>		<i>slx5Δ</i>		<i>sgs1Δ</i>		
	25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C	
<i>SGS1</i>	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
<i>sgs1-34</i>	++	-	++	+	++	+	++	+	++	+	++	+	++	-	+	+	+	+	+	-	-
<i>sgs1-35</i>	++	-	++	+	++	+	++	+	++	+	++	+	++	-	++	+	++	++	++	+	+
<i>sgs1-36</i>	++	-	++	+	++	+	++	+	++	+	++	+	++	-	++	+	++	++	++	+	+

¹The indicated double mutant strains containing plasmid pJM500 (*SGS1/URA3*) were transformed with the indicated *sgs1-ts* allele in pRS415 at 25°C and tested for viability by streaking on plates containing 5-FOA and observing growth after three days at 25°C or 37°C.

++, wt growth; + slow growth; -, no growth.