Gene amplification in the ribosomal RNA genes (rDNA) is associated with selective sister chromatid segregation

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Contents

Abstract

Irrespective of micro organisms or metazoans, cellular components sometimes unevenly inherited to the progenies during cell division. Currently, this phenomenon, which is known as an asymmetric cell division, is shown to be intimately connected with development and cellular homeostasis maintenance. In the asymmetric cell division subcellular constituents including transcripts, proteins, and organelle such as endoplasmic reticulum and centriole segregate unevenly. Moreover, chromosomal DNAs are also inherited unevenly, though the phenomenon remains to be confirmed. In this study, I constructed an assay system that enables us to detect the non-random sister chromatids segregation. Using the system I analyzed if the event is taken place in the budding yeast *Saccharomyces cerevisiae*. As a result, I succeeded in obtaining evidence that two sisters are selected and non-randomly segregated.

As a model system to analyze the non-random chromosome segregation, I employed the rDNA repeats that reside on the chromosome XII in *S. cerevisiae*. In the region, the copy number of rDNA repeats frequently varies and it was expected that distinct sister chromatids arise during DNA replication. For this reason, I assumed the non-random sister chromatid segregation can be observed in the rDNA. I continuously separated the progenies of cell division (the daughter and mother cells) by centrifugal elutriation to trace the fate of sister chromatids that are bearing the rDNA repeats. Consequently, when compared the rDNA copy number in the sorted cells, the daughter and mother cell lineages clearly differed. The daughter lineage constituently inherited a sister chromatid that harbors increased copy number of rDNA, while in the mother lineage the

number did not change. Therefore, it seemed like that the Chr. XII was differentiated during cell division and non-randomly segregated to the progenies. Remarkably, the pattern of sister chromatids inheritance showed the lineage specificity. This indicated that *S. cerevisiae* was recognizing the two sister chromatids.

To explore the *cis* acting mechanisms underlying the non-random sister chromatid segregation, I analyzed the effect of centromere. In *S. cerevisiae*, centromeric sequences that associate with the kinetochore proteins have directionality. And also, some of the mitotic apparatuses involved in chromosome segregation are polarized during cell division. I speculated that there are some relationship between the polarity of centromere and mitotic apparatus, and the non-random sister chromatid segregation. To analyze the relationship, I exchanged the *CEN12* (Chr. XII centromere) sequences with other centromeric fragments that harbors opposite directionality and analyzed the phenotypes in the strain. Although the pattern of sister chromatid segregation was analyzed in the *CEN12*-modified strains, the directionality of centromere seemed not to be important.

As the other case, I also analyzed the effect of the directionality of rDNA repeat. On the tandemly aligned rDNA repeats, several biological processes, including transcription and replication, are performed in unidirectional way. I investigated whether this directionality is involved in the asymmetric chromosome segregation. For this purpose, the rDNA repeat was reconstructed in inverted direction using a strain that lost the rDNA repeat completely. Unexpectedly, the newly introduced rDNA repeats had lost the competency for increasing their

copy number on Chr. XII. Therefore, the effect of the rDNA directionality could not be estimated.

Apart from the *cis* elements, then I wondered if *trans* factors associate with the regulation of the sister chromatid segregation. In the mutants that affect stability (*sir2*Δ), nuclear localization (*heh1*Δ), and segregation (*bud6*Δ) of the rDNA, the fate of sister chromatid segregation was analyzed. In the *sir2*Δ and *heh1*Δ, the segregation pattern of Chr. XII was equivalent to that of WT. In the *bud6*Δ, it was not able to detect the rDNA copy number change by our analysis. From these result, I speculated that nuclear positioning and chromatin structures of the rDNA had little to do with the pattern of sister chromatid segregation.

Finally, I concerned about the possibility that the non-random chromosome segregation specifically occurred in the Chr. XII. To investigate this possibility, I performed BrdU pulse-chase analysis to trace the segregation pattern of whole chromosomes. In this analysis, I could not observe the apparently biased DNA strand retention in 16 chromosomes including the Chr. XII at least when recombination in the rDNA was repressed.

In conclusion, I obtained the first evidence of the non-random chromosome segregation in *S. cerevisiae*. Thus *S. cerevisiae* seems to maintains a system which distinguish the two sister chromatids during cytokinesis. Further study will reveal how such a chromosome segregation was taken place

Introduction

From prokaryote to multicellular eukaryote, organisms maintain their lives with continuous cell division that increases their cellular population. In many species, cell division is not used just as a self-copying program, but functions in diversifying offspring. When cells divide, they give rise to two offspring that are not identical in nature and/or fate. In case of multicellular eukaryotes, such an asymmetry in progenies is observed in the stem cell renewal/differentiation and developmental processes, and necessary to produce various functional tissues from a single fertilized egg. Even in unicellular organisms, such as bacteria and yeast, the asymmetric cell division plays important functions for their survival. For example, bacterium *Bacillus subtilis* divides asymmetrically to form spore upon nutrient starvation and endure in harsh environment (Errington, 1996; McBride *et al.*, 2005). In other situation, unfavorable phenomenon (especially cellular ageing) biasedly appear in one of the progenies to keep the other in juvenile and healthy (Ackermann *et al.*, 2003; Erjavec *et al.*, 2008; Woldringh *et al.*, 1995). Thus, asymmetric cell division commonly acts as a fundamental mechanism that supports physiological activities beyond species.

The mechanisms underlying asymmetric cell division have been studied for a long time. To date, the mechanisms are categorized into roughly two, extrinsic and intrinsic mechanisms (Moore & Lemischka, 2006). When asymmetric cell division is achieved by the extrinsic mechanism, microenvironment (e.g. stem cell niche), in which progenies are exposed after cytokinesis, plays an important role (Mitsiadis *et al.*, 2007). That is to say, cell division itself

produces symmetric two progenies, however, they are differently diversified according to the signals from surroundings. In germline stem cells of *Drosophila melanogaster* and *Caenorhabditis elegans*, the balance of stem cell renewal/differentiation is adjusted in this manner (Byrd & Kimble, 2009; Drummond-Barbosa, 2008). On the other hand, the intrinsic mechanism is guided by biased partitioning of cellular components and the progenies are differentiated during cytokinesis. The budding yeast *Saccharomyces cerevisiae* is a representative model that routinely performs this type of cell division. This yeast produces morphologically and cytologically different progenies, the mother and daughter cells. According to remarkably uneven molecular partitioning, the youthfulness is guaranteed only in the daughter-lineage (Aguilaniu *et al.*, 2003; Erjavec & Nystrom, 2007; Sinclair & Guarente, 1997). Such an event takes place in the other organisms even if their cells were symmetrically divided in appearance, and confers different features to the each progenies by segregating the functional products such as proteins and mRNAs unequally (Dong *et al.*, 2009; Shen *et al.*, 2002). Furthermore, it is also demonstrated that chromosomal DNAs are unequally partitioned (Armakolas & Klar, 2006; Rosenberger & Kessel, 1968). This suggests replicated two sister chromatids are recognized and distinguished in a cell.

Although DNA replication is believed to give rise to equivalent sister chromatids, some studies raised a possibility that it is not true. As suggested in the immortal strand hypothesis, which assumed the selective DNA strands segregation, the individual parental DNA strands are proven to be segregated non-randomly during cytokinesis. In several stem cell lines, the DNA

pulse-chase analysis using thymidine analogue, such as 5-bromo-2-deoxyuridine (BrdU), was performed to trace the ‘old’ parental DNA strand (Conboy *et al.*, 2007; Karpowicz *et al.*, 2009; Karpowicz *et al.*, 2005). These analyses succeeded in showing that the ‘old’ DNA strands preferentially retained in stem cells, while differentiated cells obtain ‘new’ strands. Likewise this result, similar tendency was observed in another assay. In this case, using the inductive recombination and its products as a hallmark, non-random sister chromatids segregation was provided in mouse cells (Armakolas & Klar, 2006). Such a non-random DNA strand segregation is thought to avoid accumulating mutations in specific cells, and save the integrity of their genome (Rando, 2007). Alternatively, it is also considered that sequestration of specific chromosomes might trigger the cellular differentiation, according to the epigenetic differences on DNA (Tajbakhsh & Gonzalez, 2009). However, due to the lack of experimental information that supports these ideas, the significances and mechanisms of the phenomena remain unknown.

Although it had been attempted to elucidate the mechanisms underlying the asymmetric DNA segregation, the limited analytical systems made it difficult to progress the analysis. What is worse, the artificial DNA modification sometimes gives rise to unexpected problems. Currently, the thymidine analogues are principally used to track the fate of chromosome segregation. However, little is known of the side effects of their incorporation into the DNA. It is reported that they modulate various biological functions (Fujii *et al.*, 2002; Ross *et al.*, 2008). For instance, exposure to the BrdU was shown to alter the growth and differentiation pattern in the mouse neural cells (Bannigan, 1985; Nagao *et al.*, 1998). Moreover, BrdU induces the

senescent like phenotypes in the *S. cerevisiae* and mammalian cells (Fujii *et al.*, 2002; Michishita *et al.*, 1999). Thus, thymidine analogues may alter the nature of both chromosomes and cells. Therefore, it cannot be denied that some of the results obtained from the analysis may be artifact. To avoid such problems, it is desirable to use native biological processes for analyzing asymmetric chromosome segregation, though, it is quite troublesome to distinguish the native sister chromatids since the sisters usually looks equivalent in appearance. Except for a study of mating type locus in fission yeast *Schizosaccharomyces pombe* (Klar, 2007), the natural asymmetric sister chromatid segregation is not analyzed.

In this paper, I focused on spontaneously highly recombinogenic ribosomal RNA genes (rDNA) in *S. cerevisiae*, and discussed about its potential asymmetric segregation. In wild type *S. cerevisiae*, the rDNA region consists of ~150 copies of 9.1-kb unit that aligns in tandem on the chromosome XII (Fig. 1). This region is known as one of the most fragile chromosomal part in *S. cerevisiae*, and the number of rDNA copies frequently varies according to the homologous recombination between the copies (Kobayashi *et al.*, 1998; Kobayashi *et al.*, 2004). However, when rDNA copies were lost, the maintenance mechanisms assure recovery of the copy number. This mechanism amplifies rDNA copies in S-phase at the rate of ~1 copy/cell division and the process is found to be inducible (Kobayashi *et al.*, 1998). And the most noticeable point of this process is that unequal sister chromatid recombination proposed to gives rise to distinct sister chromatids. That is to say, the rDNA copy amplifies on the only one of two sister chromatids, and the other sister remains the original copy number. Therefore, I hypothesized that if I use the

number of rDNA repeat as criteria, it may be possible to trace the fate of sister chromatids in ‘natural’ conditions.

I investigated the pattern of the rDNA-bearing sister chromatid segregation both in the daughter- and mother-lineages, and compared the number of rDNA copies between them. As a result, I detected a lineage-specific bias of sister chromatid segregation. Sister chromatid that was segregated toward daughter cell preferentially harbored the amplified rDNA copies, while that of mother cells harbored unchanged copies. Therefore, this indicated that rDNA-bearing chromosome segregated asymmetrically at least when rDNA amplification was induced. Here, I present evidences of the asymmetric sister chromatid segregation and some approaches to investigate the mechanisms.

Material and Methods

Yeast strains, plasmids, and PCR primers used in this study

Yeast strains, plasmid DNA, and PCR primers used in this study are listed in Table 1, 2, and 3, respectively. Plasmids were maintained in *Escherichia coli* DH5α strain, except for pWJ1513 (Sure2). To control the *FOB1* expression under the *GAL7* promoter, cells were pre-cultured in medium containing 2% (w/v) raffinose as a sole carbon source until the induction point. Induction of *FOB1* was triggered by adding galactose solution to the culture to be 2% (w/v). To synchronize the cells cycle in G1 phase by α-factor (Zymo Research Corporation), cells were washed twice in sterilized distilled water and then cultured in medium containing 2 μg/ml of α-factor for 3 h.

Yeast medium

Medium used for yeast culture is listed in Table 4. Medium was prepared as described in (Dan Burke, 2000) with some modification. If necessary, G418 (Sigma), Hygromycin B (Nacalai), and 5-Fluoroorotic acid (5-FOA; Wako) were added to the medium with the concentration shown in Table 4.

DNA preparation and manipulation

Chromosomal DNA from yeast and Plasmid DNA from *E. coli* were prepared using PI-50α and 100 automatic DNA isolation system (KURABO) followed by manufacturer's

instruction, respectively. If necessary, yeast chromosomal DNA was prepared according to the methods in (Dan Burke, 2000).

Standard DNA manipulation procedures were followed by the methods described in (Sambrook, 2001).

Yeast genetic transformation

Yeast genetic transformation was performed with Frozen-EZ Yeast Transformation II Kit (Zymo Research Corporation) according to manufacturer's instruction. Yeast cells were cultured in appropriate liquid medium until mid-log phase (O.D.600 of ~1.0) and collected by centrifugation at 4000 rpm. Cells were washed with 0.5 ~ 1.0 ml of EZ solution 1 and repelleted. After supernatant was discarded, ~1x 10^7 cells were suspended into 50 ul of EZ solution 2 for one transformation reaction. 5 μl of DNA solution (~200 ng/μl) was mixed with the cell suspension and 500 μl of EZ solution 3 was added to it. The mixture was incubated in 30^{o}C for at least 45 min with vigorously mixing every 15 min. Cell mixture was pelleted by centrifugation at 4000 rpm for 3 min, and spread onto an appropriate plate medium. If drug resistance marker was used for selection, cells were cultured in non-selective liquid medium for at least 3 h before spreading.

Plasmid construction

i) Integrative Galactose inducible *FOB1* plasmid

The integrative galactose-inducible *FOB1* Plasmid, pRS304-GAL*FOB1*, was constructed as follow. ~ 3-kb fragment that contains galactose-inducible *FOB1* cassette was excised from YCp*GALFOB1* by *Bam*HI / *Sal*I digestion and sub-cloned into these sites of pRS304. For transformation pRS304-GAL*FOB1* was digested with *EcoR*V, and integration was checked by primer pair of RS2-TRP1 N.

ii) Plasmids used for centromere modification

Plasmids used for modification of chromosome XII centromere (*CEN12*) were constructed as follow. Primer CEN12-AF and CEN12-AR were used to amplify the 650-bp DNA sequences flanking to the left end of *CEN12* (L-segment). This PCR product was digested with *Hind*III / *Pst*I and sub-cloned into these sites of pUC18 to give rise to pCEN12-L. Next, primer CEN12-BF and CEN12-BR was used to amplify the 720-bp DNA sequences flanking to the right end of *CEN12* (R-segment). This PCR product was digested with *Kpn*I / *Xba*I and sub-cloned into these sites of pCEN12-L to give rise to pCEN12-LR. The plasmid pCEN12-LR harbors complete *CEN12* context beside it lacks centromere core sequences. ~120-bp centromere sequence was sub-cloned into *Pst*I / *Xba*I site of this plasmid to yield pCEN12-Rev and pCEN12-CEN5. In case of pCEN12-Rev, *CEN12* sequence was amplified by primer pair CEN12-1 (PstI) / CEN12-2 (XbaI) and sub-cloned into the *Pst*I-*Xba*I site of pCEN12 followed

by digestion with *Pst*I / *Xba*I. And for pCEN12-CEN5, primer pair CEN5-F2 (PstI) / CEN5-R2 (XbaI) was used to amplify CEN5 sequence and PCR product was introduced into *Pst*I / *Xba*I site of pCEN12-LR followed by digestion with *Pst*I / *Xba*I.

The plasmid used for inversing ~6-kb region surrounding the *CEN12* was constructed as follow. Primer CEN12-XLF and CEN12-XLR were used to amplify the 800-bp DNA sequences flanking to the N-terminal region of *DNM1* gene on Chr. XII. This PCR product was digested with *Sma*I / *BamH*I and sub-cloned into these sites of pUC18 to give rise to pCEN12-XL. Next, primer CEN12-XRF and CEN12-XRR was used to amplify the 670-bp DNA sequences adjacent to the C-terminal end of *NOC3* gene. This PCR product was digested with *Sph*I / *Sal*I and sub-cloned into these sites of pCEN12-XL to give rise to pCEN12-XLR. The ~6-kb DNA region between *DNM1* and *NOC3* gene was amplified by primer CEN12-XLA and CEN12-XRB, then sub-cloned into *Sal*I / *BamH*I site of pCEN12-XLR, followed by digestion with those enzymes. This plasmid, pCEN12-Rev2, was used to invert the direction of ~6-kb region including *CEN12*.

iii) Plasmid used for inversion of rDNA direction

Plasmid prDNA-Rev that was used for inversion of rDNA repeat on the chromosome XII was constructed according to the method in (Oakes *et al.*, 2006) with some modification. Primers 127-*Sal*I-*EcoR*I / 128-*Sma*I-*Xho*I were used to amplify the 801-bp DNA segment (the L sequence), which is located 330 bp from the centromere-proximal end ("left boundary") of the native rDNA repeats, and the product was digested with *EcoR*I and *Sma*I. Primers

129-(*Spe*I-*BamH*I) / 130-*Sal*I were used to amplify the 791-bp DNA segment (the R sequence), which is located in each of four 3652-bp *ASP3* repeats adjacent to the telomere-proximal end ("right boundary") of the native rDNA repeats and the product was digested with *Sal*I and *BamH*I. The ~800-bp *EcoR*I-*Sma*I fragment containing the L sequence was inserted into the *EcoR*I and *sma*I sites of pUC18. Similarly, the ~800-bp *Sal*I-*BamH*I fragment containing the R sequence was inserted between the *Sal*I and *BamH*I sites of the same plasmid. The resultant plasmid was then digested with *Spe*I and *Xho*I, and the ~11 kb *Spe*I-*Sal*I fragment [the core fragment containing *Arabidopsis thaliana* genome sequence (N), hygromycin-resistant-rDNA and *HIS3*] obtained from pNOY3286 (Oakes *et al.*, 2006) was inserted between these two sites to give rise prDNA-Rev. To integrate the plasmid into chromosomal *RDN1* locus, prDNA-Rev was used after digestion with *Sal*I.

iv) GFP-*lacI* and *lacO* Plasmid used for visualizing rDNA units

Plasmid pTM-$lacO_{50}$ that was used for rDNA labeling with *lacO* was constructed as follow. Repeats of *lacO* in the pAFS52 were shortened from 256x to 50x by *EcoR*I partial digestion and re-ligation to give rise pAFS52-$lacO_{50}$. Primer TM3 and TM4 was used to amplify IGS1 region in the rDNA unit and sub-cloned into *Kpn*I / XhoI site of pAFS52-$lacO_{50}$ to give pTM1. And Primer TM-Hind and TM-Sph was used to amplify IGS2 rDNA fragment and sub-cloned into *Hind*III / SphI site of pTM1 to give rise to pTM2. *URA3* ORF was amplified from pJJ242 by primer set TM7 / TM8 and introduced into SphI-*Sal*I site of pTM2 to obtain

pTM-*lacO*$_{50}$. To integrate into rDNA region, pTM-*lacO*$_{50}$ was used after digestion with *Kpn*I and *Hind*III. Similarly, pTM-*tetO*$_{50}$ used for rDNA labeling with *tetO* was constructed as follow. Repeats of *tetO* array in the pWJ1513 were shortened from 224x to 50x and gave rise pWJ1513-*tetO*$_{50.}$ ~ 1.5-Kb *tetO* array was excised from pWJ1513-*tetO*$_{50}$ by *Bam*HI / *Bgl*II digestion and sub-cloned into *Bam*HI site of pUC18 to give pUC18-*tetO*$_{50}$. ~1.5-kb IGS2 side of rDNA fragment with *URA3* ORF was amplified from pTM-*lacO*$_{50}$ by primer set TM-Hind / TM8 and sub-cloned into *Hind*III / *Sal*I site of pUC18-*tetO*$_{50}$ to give pIGS1-*tetO*$_{50}$. Primer set TM3-SacI / TM4-EcoRI was used for amplify ~500-bp IGS1 rDNA fragment and sub-cloned into *Sac*I / *Eco*RI site of pIGS1-*tetO*$_{50}$ to give rise to pTM-*tetO*$_{50}$. To integrate into the chromosomal rDNA, pTM-*tetO*$_{50}$ was used followed by digestion with *Hind*III / EcoRI. The correct integration of these fragments was analyzed by primer set TM8 / rDNAa. Insertion was confirmed by the detection of ~2.2-kb PCR fragment.

CEN12 modification

CEN12 modification was performed as follow. Using pRS306 as a template, *URA3* fragment was amplified with primer set pRSCEN12 F / pRSCEN12 R and introduced into the yeast cells. The transformants were selected on SC without uracil plate and precise integration into the left side of *CEN12* (~400-bp distant from CEN sequence) was confirmed by primer set CEN12-AF / CEN12-AR. The strain TAK201 CEN12L::*URA3* was obtained in this way. Next, by using pCEN12-Rev or pCEN12-CEN5 as a template, PCR fragment for *CEN12* substitution

was amplified with the primer set CEN12-AF / CEN12-BR. In case of pCEN12Rev2, ~8-kb fragment produced by *Sma*I / *Sph*I digestion was used for transformation. These fragments were introduced into the CEN12L::*URA3* strain to substitute the chromosomal *CEN12* sequence. Before select the transformants, the transformation mixture was spread onto non-selective YPD (or YPGal) plate and pre-cultured for 2 days. And then the cells were replica-plated onto the SC+5-FOA plate to select the transformants (without pre-culture process, no transformants were obtained). The precise substitution of the *CEN12* was confirmed by sequencing the *CEN12* context. When examine the sequence around *CEN12*, the primer set CEN12-S1/ CEN12-BR was used for obtaining template DNA. And the template was read with the primer either CEN12-S1 or CEN12-S2. The strain CEN12::CEN12-Rev and CEN12::CEN5 was obtained by this way.

DNA sequencing

DNA sequencing was carried out using BigDye® Terminator v3.1 Cycle Sequencing Kits (applied biosystems) and 3130*xl* Genetic Analyzer (applied biosystems) according to manufacturer's instruction.

Reconstruction of the rDNA repeat on the Chr. XII

The introduction of the inverted-rDNA unit into the Chr. XII was carried out according to the strategies of (Oakes *et al.*, 2006). The NOY984 (*rdnΔΔ* strain, *fob1Δ*) was crossed with NOY408-1b *fob1Δ*::hphMX to give rise to diploid yeast strain dTM1. Plasmid prDNA-Rev was

digested with *Sal*I and the ~13 kb fragment containing the core fragment and the two (L and R) flanking sequences was transformed into the diploid by selecting His^+ transformants. Correct integration of a single inverted-rDNA unit on the chromosome XII derived from NOY984 was established by confirming the presence of the two new connections (N with chromosome XII DNA on the left side of L, and *HIS3* with chromosome XII DNA on the right side of R) by PCR using primer pairs 137 / 174 and 138 / 161. Sporulation and tetrad dissection on SPO medium was carried out in the presence of helper plasmid pNOY130. One of the segregants, rDNA-Rev1, was shown to contain chromosome XII with a single rDNA copy at the original *RDN1* locus. To integrate the second copy for subsequent expansion, the linear ~13 kb fragment obtained after digestion of pNOY3293 with *Spe*I and *Sal*I was then transformed into rDNA-Rev1 and His^+ Leu^+ transformants were selected. One of the transformants rDNA-Rev2, was shown to have the second rDNA copy by PCR using primer pairs 182 / 185

DNA Pulse labeling using 5-bromo-2'-deoxyuridine (BrdU)

BrdU incorporation analysis was performed using TM4 (MAT**a** *bar1Δ* strains that harbor both thymidine kinase and transporter) (Lengronne *et al.*, 2001; Viggiani & Aparicio, 2006). To synchronize cells in G1 phase before BrdU incorporation, they (3×10^6 cells/ml) were incubated in medium containing 40 ng /ml of α-factor for 2.5 h. The cells were pelleted and washed twice in sterilized distilled water, then released into a medium containing 400 μg/ml BrdU (Sigma) to label DNA.

Detection of BrdU-incorporated DNA

Detection of BrdU-incorporated DNA was carried out according to the procedure of (Lengronne *et al.*, 2001) with some modification. After chromosomal DNA was run with CHEF electrophoresis, the DNA was transferred to the Hybond N+ nylon membrane according to the procedures of usual Southern blotting. After DNA cross-linking treatment, the membrane was equilibrated with Buffer I (see Table 5) for 5 min and next in Blocking buffer for 45 min. Immunodetection of BrdU was performed in Buffer I+T (Buffer I containing 0.3% Tween-20) using an Anti-BrdU antibody (1:1000; Sigma), and a secondary Anti-mouse IgG coupled to horseradish peroxidase (1:10000; GE). ECL Western Blotting Detection kit (GE) or Immobilon^{TM} Western (Millipore) were used for chemiluminescent reaction according to manufacturer's instruction. Signal detection was carried out by scanning the membrane with a LumiImager F1 (BOHLINGER MANNHEIM).

DNA labeling with radioactive dCTP

Radio labeled DNA probes for Southern hybridization was obtained as follow. To label DNA fragment with [α-^{32}P] dCTP, Rediprime II DNA Labeling System (GE) or High prime (Roche diagnostic) were used according to manufacturer's instruction. Before the labeling process, template DNA was boiled for 5 min and immediately chilled on ice. About 100 ng of the template DNA was mixed with reaction mixture and 5 μl of [α-^{32}P] dCTP, then incubated for at least 15 min at 37 ^{o}C. After the reaction was finished, labeled DNA was purified by using NICK

Columns (GE) and used for hybridization.

Southern blotting and hybridization

i) Southern blotting

DNA transfer from agarose gel to nylon membrane was carried out as described in (Sambrook, 2001). After electrophoresis, the DNA was depurinated in 0.25 N HCl, denaturated in Denaturation buffer (see Table 5), and neutralized in Neutralization buffer (see Table 5) for 30 minutes, respectively. Next, the DNA was transferred to Hybond N+ Nylon membrane (GE) in 20x SSC by capillary transfer for at least 12 h. Using Stratalinker (Stratagene), DNA was cross-linked to the membrane before the hybridization with 120 mJ of UV (254 nm) irradiation.

ii) Hybridization

The membrane was pre-hybridized in 40 ml Hybridization buffer (Table 5) at 65 ^{o}C for 5 minutes, followed by hybridization in 40 ml of Hybridization buffer containing heat-denatured probe at 65 ^{o}C for overnight in a roller bottle. The membrane was washed in 2 x SSC, 2% SDS for 30 min at 65 ^{o}C and in 0.2 x SSC, 0.2% SDS for 30 min at 65 ^{o}C. Then, the membrane was briefly rinsed with 0.2 x SSC, 0.2% SDS at room temperature. The membrane was exposed to the Imaging plate (Fujifilm) for 1~3 days and signals were detected by BAS-2500 (Fujifilm).

Conditions for Contour-clamped homogenous electric field (CHEF) electrophoresis

Samples for CHEF electrophoresis were prepared as described in (Kobayashi *et al.*, 2001) using ~ 1.0X 10^7 cells per one plug. After DNA in the plugs were digested with restriction enzymes, the sample plugs were washed twice in Tris-EDTA (TE) buffer for 30 min, and then twice in 1X restriction enzyme buffer for 15 min before reaction. Reaction was carried out for at least 12 hr in a 200 ul of 1X reaction buffer with 150 units of enzymes.

Electrophoresis was carried out in a 1.0% agarose gel with 0.5X Tris-borate-EDTA (TBE) buffer, using CHEF-MAPPER (Bio-Rad). The conditions were summarized in Table 6. When perform an electrophoresis, condition was determined according to the experimental purpose. For the experiment in Fig. 8, 9-2 and 11A, samples were run with a pulse time 60 to 120 s and 6.0 V/cm for 40 h at 14 ^{o}C. For the experiment in Fig. 3a), 13B, samples were run with a pulse time 22 to 266 s and 6.0 V/cm for 40 h min at 14 ^{o}C. And for the experiment in Fig. 3b), 4, 6, 7b, 11B, and 12B~D, samples were run with a pulse time 0.22 to 12.91 s and 6.0 V/cm for 15 h and 16 min at 14 ^{o}C.

Separation of mother and daughter cell lineage

Separation and isolation of mother and daughter cells were carried out based on the procedures of (Ganley *et al.*, 2009). A 2L culture of wild type cells was grown to late log phase (O.D.600 = ~3), harvested by centrifugation, washed in PBS buffer, and resuspended in 20 ml of PBS with 2 mg/ml BSA. The cells were sonicated, filtered with a 60 μm filter (Millipore), and

loaded onto an elutriator (Beckman JE5.0 Elutriation System). Elutriation was performed at 2,300 rpm by adjusting the flow rate and cells were separated into 8 fractions. The first two fractions contained the smallest cells were used as the daughter cell fraction and the last two were used as the mother fraction. The number of bud scar was also counted in each fraction to verify the ratio of daughter cells. When separate the daughter and mother cells lineage, elutriation was repeated as follow.

i)Separation of daughter cell lineage

The daughter cell fraction obtained by elutriation was pelleted and re-innoculated into a fresh 1L YPGal culture, then divided just once with checking both O.D.600 and microscopic observation. The cells were harvested and conducted to second elutriation procedure according to the method described above. The first two fractions with the youngest cells were collected, pelleted and re-innoculated into 500 ml YPGal culture. The cells were divided just once and used for third elutriation. These processes were continued until it became unable to obtain sufficient daughter cells for elutriation.

ii)Separation of mother cell lineage

Separation of mother cell lineage was carried out basically same as that of daughter cell lineage. The mother cell fractions obtained by elutriation were pelleted and re-innoculated into a fresh 1L YPGal culture, then divided just once. The cells were harvested and conducted to

second elutriation procedure according to the method described above. The two fractions with the oldest cells were collected, pelleted, and re-innoculated into 500 ml YPGal culture. The cells were divided just once and used for third elutriation. These processes were repeated until it became unable to obtain sufficient mother cells for elutriation.

Microscopy

For epifluorescence microscopy (especially in *ade2* mutant strains), cells were pre-cultured in SC+Adenine (SCA) medium to reduce auto-fluorescence. Before microscopic observation, cells were washed twice in SCA. Next, cells were immobilized on the surface of glass bottom dish (MATSUNAMI) with 0.05% (w/v) concanavalin A. The filters used to visualize GFP (excitation 480 nm; emission 535 nm), 4′,6-diamidino-2-phenylindole (DAPI; excitation 365 nm, emission 450 nm) were from Nikon. Images were acquired by using a cooled CCD camera (Coolsnap HQ-2 from Rooper, Tucson, AZ) mounted on an ECLIPSE Ti microscope (Nikon) with a Plan-Apochromat 100x, 1.4 numerical aperture (NA) objective lens. Images were acquired in the NIS-Elements software (Nikon). Counting bud scars using calcofluor staining was performed as previously described (Lesur & Campbell, 2004).

Results

1. Asymmetric sister chromatids arise after rDNA amplification

i) A model of rDNA copy amplification by unequal sister chromatid recombination.

In the budding yeast *S. cerevisiae*, rDNA copy number is maintained by a dynamic process involving DNA recombination (Kobayashi *et al.*, 1998). Gene amplification that expands rDNA copies plays a crucial role in the process, and currently, a model of its molecular mechanism is proposed as described in Fig. 2. The key of this mechanism is a unidirectional replication fork block, which occurs at the RFB site depends on Fob1 protein (Fig. 2 i)). A DNA replication fork that is moving against the direction of 35S rRNA transcription is arrested at the RFB, and induced DNA double strand break (DSB). This broken DNA is repaired by homologous recombination with sister chromatid. If unequal sister chromatid is used as a template, the number of rDNA copies is altered (Fig. 2 ii), iii)).

According to (Burkhalter & Sogo, 2004), the DSB formation at the RFB is specifically induced on the leading strand of replication fork. Therefore, it is assumed that this strand specificity assures the induction of rDNA amplification on one of two sister chromatids. That is to say, one of two sister chromatids that is synthesized via leading-strand replication is capable to expand its rDNA copies, while on the other sister chromatid, the rDNA copy number doesn't change (Fig. 2). According to this model, it is expected that two distinct sister chromatids appear as a result of rDNA amplification. I tried to detect such an asymmetric sister chromatids and examined the inheritance pattern of them.

ii) rDNA amplification causes distinct sister chromatids that contain different rDNA copy number

To ascertain whether asymmetric sister chromatids are synthesized, the products of rDNA amplification were compared between the daughter and mother cells. Subsequently, the segregation pattern was monitored for several generations. If asymmetric sister chromatids are synthesized, they will be distinguishable from the difference of rDNA copy number between the daughter and mother cells.

To simplify the experimental system, I employed the 2-rDNA copy strain (*fob1Δ*) that harbors only two rDNA repeats on the chromosome XII (Fig. 3). The galactose-inducible *FOB1* gene (GAL-*FOB1*) was introduced into the strain to trigger the rDNA amplification (Fig. 4). As the rDNA amplification requires Fob1, the expressional control of *FOB1* gene by galactose makes it possible to induce the rDNA amplification at specific timing. For the aim of separating the daughter and mother cells, the centrifugal elutriation was employed. The elutriation enables us to separate the intact living cells by size. In the asymmetric cell division, the daughter cells are always much smaller than the mother cells. Therefore, I can separate the daughter and mother cells by this method. As described in Fig. 5B, daughter and mother cells were efficiently fractionated into different fractions. Using this inducible rDNA amplification system, the fate of amplified rDNA copies was examined both in the daughter and mother cells.

The 2-rDNA copy strain was pre-cultured in raffinose medium and transferred to galactose medium to induce Fob1 protein. Cells were incubated in galactose until amplified rDNA appeared (in average for 24~36 h, Fig. 4), and then separated into the daughter and mother

cells by elutriation. Using the fractions (F1, 2 and F6, 7 in Fig. 5B were used as the daughter and mother cells, respectively), chromosomal DNA was purified, digested with *BamH*I, and followed by CHEF electrophoresis. The rDNA repeats were detected by Southern hybridization, and the number of rDNA copies was estimated from the size of the fragments (Fig. 6). In the bulk culture (before elutriation), the majority of rDNA copy was still 2 copies, however, weak amplified bands corresponding to 5 copies were detected (Fig. 6). When the rDNA copy number was compared after elutriation, the amplified products (5 copies) were enriched only in the daughter fractions (Fig. 6). This indicates that the asymmetric sister chromatids had synthesized and amplified one was segregated to the daughter. Further investigation was performed to confirm the result. Using the daughter fractions that harbor 5-copy rDNA as ‘founder cells’, the rDNA amplification and the fate of sister chromatids segregation was monitored for next several rounds of cell cycle.

The ‘Founder cells’ divided once and were separated into the daughter and mother cells. In this round, not only 5-copy, but also 8- and 9-copy rDNA bands were observed in the daughter fractions (Fig. 7B D1). The ratio of 2-copy rDNA was slightly reduced in these fractions (Fig. 7B D1, C). In contrast, in the mother fractions, majority of cells still have 2-copy rDNA (Fig. 7B M1). Since lineage specific inheritance of the rDNA amplification was observed, the offspring of these fractions were classified into the daughter- and mother-lineage, and the number of rDNA copies was detected (Fig. 7B). As a result, in the daughter-lineage, rDNA amplification was consistently observed for 3 generations (Fig. 7B, D1-3). On the other hand, in the mother-lineage,

the majority of rDNA remained 2 copies (Fig. 7B, M1-3). This was also confirmed by the size of undigested Chr. XII (Fig. 8B). By rDNA amplification, the size of Chr. XII gradually increased in the daughter-lineage as generation has gone by, while no detectable size alteration was observed in the mother-lineage (Fig. 8B).

These date suggest that rDNA amplification gives rise to asymmetric sister chromatids, and the segregation pattern of those sisters have a lineage preference. In other words, sister chromatid that was used as a template for rDNA amplification is retained in the mother, on the other hand, the other sister that have altered rDNA copies goes to the daughter cell.

2. Effect of centromeric modification on rDNA amplification and sister chromatid segregation

Next, it was examined whether factors for chromosome segregation, especially centromere, affect the asymmetric sister chromatid segregation and rDNA amplification. In the budding yeast, at least in meiosis, sister chromatids are differentially marked with kinetochore proteins at the centromeric region (Thorpe *et al.*, 2009). In addition, several proteins are specifically loaded onto the daughter spindle pole body (equivalent to the centrosome in the metazoan) during cytokinesis to ensure the proper spindle orientation and asymmetric cell division (Pereira *et al.*, 2001; Vallen *et al.*, 1992). Furthermore, in *S. cerevisiae*, the centromere maintains directionality in its sequences (Lechner & Ortiz, 1996). Thus, polarized mitotic apparatuses pull sister chromatids toward opposite poles during the yeast cell division. Therefore,

it is possible that such apparatuses affect the pattern of sister chromatid segregation.

I considered that modification of centromere (and its directionality on optional chromosome) might alter the dynamics, according to unusual kinetochore-centromere association. To ask this possibility, I modified the *CEN12* (Chr. XII centromere) and monitored the sister chromatid segregation in the strain. In *S. cerevisiae*, the centromere is defined as the ~125-bp consensus sequence that is subdivided into three core domains (CDE-I, II, and III), which associate with Cse4 (CENP-A homolog) and kinetochore proteins (Hegemann & Fleig, 1993; McAinsh *et al.*, 2003). I modified *CEN12* in two different ways (Fig. 9A-C). One is inversion of *CEN12* directionality (CEN12::CEN12-Rev, Fig. 9C), and the other is replacement of *CEN12* with *CEN5* (centromere of Chr. V) (CEN12::CEN5, Fig. 9C). In both cases, flanking sequences that adjacent to *CEN12* remain intact (only ~125-bp CEN region was replaced). These modifications were confirmed by sequencing (Fig. 9B). Growth of the *CEN12*::*CEN5* strain seemed normal, while *CEN12*::*CEN12*-Rev is a little sick (date not shown). Compared to WT, the stability of Chr. XII seemed to be normal in both strains (Fig. 9-2). rDNA amplification was also normally induced (Fig. 9-2).

The pattern of sister chromatid segregation was analyzed in these strains as same as the experiments in Fig. 7. In *CEN12*::*CEN12*-Rev strain, when daughter and mother cells were separated after rDNA amplification, the Chr. XII with amplified rDNA copies was segregated to the daughter cells, likewise in WT (Fig. 9-2B). From the result, it was suggested that centromere (at least its direction) was not important for non-random sister chromatids segregation in the

budding yeast.

3. Directionality of the rDNA units and sister chromatid segregation

In *S. cerevisiae,* rDNA units tandemly align on Chr. XII and several biological processes (e.g. rRNA transcription, replication fork block at RFB, and so on) are carried out in unidirectional way (Fig. 1, 2). To test whether the pattern of sister chromatid segregation is affected by such a directionality, the alignment of the rDNA cluster was turned to opposite direction on Chr. XII (Fig. 10). For this purpose, I constructed a strain based on NOY984 (rdn*ΔΔ* strain that lost rDNA from Chr. XII completely) (Oakes *et al.*, 2006). An inverted rDNA fragment (prDNA-Rev) was prepared and inserted into the *RDN1* locus where rDNA repeats existed before (Fig. 10A). In addition, since rDNA amplification requires at least two rDNA copies for unequal sister chromatid recombination (Kobayashi *et al.*, 2001), the second rDNA fragment (pNOY3293) was inserted into the same site (Fig. 10B). Integration of two rDNA fragments into the target chromosomal region was confirmed by Southern hybridization after CHEF electrophoresis and PCR analysis. Transformants that maintained inverted two rDNA copies were selected and used for subsequent analysis.

To investigate whether inverted-rDNA is capable to amplify the copies on the Chr. XII, *FOB1* gene was re-introduced into the transformants and cultured for ~300 generations. However, no rDNA amplification event was observed in the strains (Fig. 11A). From this result, I speculated that amplified rDNA copies are not inherited to the daughter-lineage, because the

pattern of sister chromatid segregation is inversed in this strain. To examine this idea, I performed centrifugal elutriation to separate the daughter- and mother-lineage and the number of rDNA copies was monitored in both lineages. However, as shown in Fig. 11B, amplified rDNA was not observed in neither of them. When pNOY3292 (a plasmid that was used to re-construct rDNA cluster in original direction) was used for same assay, rDNA repeats were normally amplified (Oakes *et al.*, 2006). Therefore, it was likely that rDNA amplification in opposite direction was inhibited in the budding yeast.

4. Patterns of sister chromatid segregation in *sir2*, *heh1*, and *bud6* mutants

To explore *trans* factors for the asymmetric sister chromatid segregation, I next investigated three mutant strains; *sir2Δ*, *heh1Δ*, and *bud6Δ*. These nonessential genes are involved in maintenance of rDNA and/or asymmetric partitioning of aging factors in different pathways (Kobayashi *et al.*, 2004; Mekhail *et al.*, 2008; Shcheprova *et al.*, 2008). I considered that the phenotypes may have something to do with the sister chromatid segregation and its pattern. Therefore, I examined the possibility by tracing the fate of rDNA amplification and its products in the daughter- and mother-lineage in cells of these mutants.

As the first step of investigation, I checked the phenotypes of *sir2Δ* mutant. The *SIR2* gene encodes a NAD^+-dependent histone deacetylase that regulates chromatin silencing, rDNA recombination, genome stability, and aging (Brachmann *et al.*, 1995; Kaeberlein *et al.*, 1999; Kobayashi *et al.*, 2004). In the absence of Sir2, the stability of rDNA region (Chr. XII) is reduced

due to increased recombination frequency in rDNA (Gottlieb & Esposito, 1989). In addition, asymmetric partitioning of damaged proteins is ruined (Erjavec & Nystrom, 2007). To examine whether these phenotypes are related to the sister chromatid segregation, I deleted *SIR2* in the 2-rDNA copy strain and rDNA amplification was induced (Fig. 3). The number of rDNA copies was monitored for three generations both in the daughter- and mother-lineage by Southern hybridization. As a result, amplified rDNA was observed in the daughter-lineage rather than in mother in the *sir2Δ* mutant, likewise in WT strain (Fig. 7B, 12B).

Similar result was also obtained in the inner nuclear membrane protein mutant, *heh1Δ*. In nucleus, the rDNA region localizes in the nucleolus and its positioning requires association between nuclear membrane and rDNA through protein-protein interaction (Mekhail *et al.*, 2008). Heh1 is a component of the protein network that mediates this rDNA positioning, and depletion of this protein leads to dissociation of rDNA from the nuclear membrane. This positioning defect is shown to destabilize the rDNA region. I examined the pattern of sister chromatid segregation in the *heh1Δ* strain by the same method that was carried out in *sir2Δ* mutant. As shown in Fig. 12C, although the rDNA amplification was weakly induced, amplified rDNA preferentially segregated to the daughter-lineage but not to the mother side. That is to say, the pattern was identical to that of WT.

Next, I investigated the *bud6Δ* mutant. *BUD6* gene encodes an actin-related protein that forms diffusion barrier at the bud neck. This sequesters the extra-chromosomal rDNA circle (ERC), a pop-out molecule from the rDNA by recombination, in the mother cell (Shcheprova *et*

al., 2008). Besides, the gene is involved in other multiple process including chromosome maintenance, and determination of cellular polarity (Huisman *et al.*, 2004; Shcheprova *et al.*, 2008). In this mutant, I performed centrifugal elutriation as the other mutants. However, due to the defect in cytokinesis I could not trace the both progenies more than two generations. And during the period, no rDNA amplification was observed both in daughter- and mother-lineage (Fig. 12D).

From the results, it seemed like that the pattern of sister chromatid segregation was not affected by silencing and localization of rDNA because Sir2 and Heh1proteins were not important for non-random chromosome segregation.

5. Analysis of segregation pattern of the whole chromosomes by BrdU Pulse-chase

To explore the detailed mechanisms of asymmetric sister chromatids segregation, next I examined whether this phenomenon depends on the rDNA (or its amplification). In the previous section, I focused on the segregation patterns of Chr. XII, and found that they were non-randomly segregated at least upon rDNA amplification (fig. 7B). However, the fate of sister chromatids of other chromosomes remains to be revealed. If the rDNA (or its amplification) is intimately concerned with the fate of sisters, it is conceivable that asymmetric chromosome segregation is exclusively observed in the Chr. XII. In contrast, if other factors (e.g. centromeric function) are much important, similar phenotype can be also observed in other chromosomes. To

examine these possibilities, segregation patterns of whole chromosomes were traced by DNA pulse-chase analysis using BrdU.

I used the *fob1Δ* strain to eliminate rDNA amplification and recombination in the Chr. XII. To adjust quality of cells as previous section, experiment was started with pure daughter cells that were obtained by centrifugal elutriation. After the cells were synchronized in G1 phase by α-factor, they were released into S-phase and the chromosomes were labeled with BrdU. As described in Fig. 13A, this treatment hypothetically gives rise to heteroduplex chromosomes that one of two DNA strands are labeled with BrdU. After this labeling process, the daughter- and mother-lineage of cells were separated likewise in the previous section, and BrdU signal was monitored using anti-BrdU antibody. If the asymmetrical sister chromatids segregation was programmed in a chromosome, it can be distinguished from the differential staining pattern between the daughter and mother cell, by the biased distribution of BrdU-labeled DNAs (Fig. 13A).

As shown in Fig. 13B, the segregation pattern of BrdU incorporated chromosomes was analyzed both in the daughter- and mother-lineage. After the chromosomal DNAs were separated by CHEF electrophoresis, the BrdU-incorporated DNAs were visualized with anti-BrdU antibodies (Fig. 13B). The intensity of the signals that appeared around the position A-G in Fig. 13B was measured and compared among the fractions (Fig. 13C). The results indicated that there was less correlation between the signal intensity of EtBr stained DNAs and BrdU-immunodetection. Although the signals of EtBr stained DNAs were almost equivalent

among the fractions, the BrdU-incorporated DNAs varied (Fig. 13B, C). This may suggest that the asymmetric DNA segregation occurred in the several chromosomes, beside the Chr. XII. Alternatively, as the signal intensity was magnified during the BrdU-chase process, it was also possible that BrdU incorporation was carried out even after cells were released into BrdU free medium.

6. Dynamics of the rDNA region in strains with Chr. XII modification

To monitor the dynamics of the rDNA region in terms of the non-random sister chromatid segregation, I employed the *lacO* / *lacI*-GFP system to visualize individual rDNA unit (Fig. 14). The *lacO* / *lacI*-GFP system enables us to observe optional chromosomal region in living organisms by utilizing the affinity between bacterial *lacO* array and *lacI* protein (Straight *et al.*, 1998; Straight *et al.*, 1996). I planned to label the all rDNA units with *lacO* array to visualize the region in a living yeast cell (Fig. 14).

To manage rDNA visualization using *lacO* / *lacI* system, I used a genetic trick in the 2-rDNA copy strain. 50x *lacO* array was inserted into one of two rDNA units and rDNA amplification was induced to duplicate this *lacO*-modification into newly synthesized copies. Before the amplification, the position of inserted *lacO* array was observed as a single dot in the transformant (Fig. 14B). After induction of amplification, as the copy number increases, the shape and intensity of fluorescence signals were gradually changed (Fig. 14C). To confirm whether this change of signal change was according to the amplification of *lacO*-inserted rDNA

units, the maintenance of *lacO* array was confirmed by Southern hybridization following by digestion with restriction enzymes (Fig. 15A). Chromosomal DNA was digestion with two different enzymes, *Bgl*II and *Sal*I, to know the ratio and interval of *lacO*-inserted rDNA units. When rDNA region was digested with *Bgl*II, both sides of *lacO*-integration site were cleaved to give rise to 4.6- and ~7.0-kb fragment in WT and *lacO*-inserted strain, respectively. By comparing the intensity of these signals, the ratio of *lacO*-inserted unit can be speculated. On the other hand, as *Sal*I recognition site resides only in the *lacO* array, but not in the rDNA unit, digestion with this enzyme enables us to measure the interval of *lacO*-inserted units. If all of the rDNA units were inserted with *lacO* array, *Sal*I digestion produces ~11.5-kb fragment. However, if there are any 'blank' rDNA units that are not labeled with *lacO*, > 22-kb fragment is detected. As shown in Fig. 15A, restriction enzyme digestion indicates that all of the rDNA units are completely inserted with *lacO* array without any 'blank' unit (Fig. 15A). The *lacO*-inserted rDNA units were finally amplified to the WT level (~150 copies) and its nuclear localization was observed. As a result, localization was quite normal and co-localized with several nucleolar proteins (Fig. 15B). Therefore, I concluded that this labeling technique can be used for rDNA visualization in living yeast cell. The system enables us to observe the copy number dependent rDNA structure, dynamics, and so on in the living cell. However, unfortunately, according to the low frequency of rDNA amplification events, I couldn't get visual information to solve the mechanism of the non-random chromosome segregation.

Discussion

The budding yeast *S. cerevisiae* is a well-studied model organism that divides asymmetrically. To date, it has been extensively explored how this asymmetry is created in this yeast and several mechanisms were revealed. For instance, regarding to the proteins and mRNAs partitioning, actin and myosin related factors are elucidated to be involved in (Erjavec & Nystrom, 2007; Muller *et al.*, 2007). As for the pathways for determining the cellular polarity, association of karyogramy proteins was identified (Liakopoulos *et al.*, 2003). However, with respect to the chromosomal DNAs, there was almost no evidence that suggested the non-random segregation of sister chromatids. Although several studies reported about this issue in the yeast, most of them claimed random chromosome segregation. For instance, (Neff & Burke, 1991) showed a negative evidence that denied the non-random segregation of sister chromatids in *S. cerevisiae*. They performed DNA pulse-chase analysis to observe the dynamics of whole chromosomes as I did in the result section, and they concluded sister chromatids segregation seemed to be random. Similarly, (Chua & Jinks-Robertson, 1991) used a recombinant sister chromatid as a tool to provide the random chromosome segregation of Chr.V in diploid *S. cerevisiae* cells. However, here in this study, I revealed that the sister chromatids of Chr. XII were asymmetrically segregated at least upon rDNA amplification (Fig. 6, 7). There was a pedigree preference in sister chromatid segregation and the daughter-lineage cells always received amplified rDNA (Fig. 7, 8). Therefore, the result suggests that the amplification occurs before cytokinesis and then the cell non-randomly partitioned sister chromatids. I believe this

result provides the first affirmative evidence of unequal chromosome inheritance in the budding yeast besides the result of (Ganley *et al.*, 2009). They showed that the rDNA in the mother cell was more unstable than that in the daughter. These observations raised several questions including 'Is the rDNA required for asymmetric segregation?' and 'how does *S. cerevisiae* recognize sister chromatids during cytokinesis?'. Considering the differences from other studies, I speculate that the rDNA directed non-random sister chromosome segregation can take place on the Chr. XII. In this part, I discuss about mechanisms and biological significance of non-random sister chromatid segregation.

1. Is asymmetric sister chromatid segregation associated with rDNA?

Although the principal methodology that is utilized for the analysis of asymmetric chromosome segregation is common in many organisms, the results of those studies vary even if the same organism is used for investigation. In case of mouse, the evidence of asymmetric chromosome inheritance was found in the embryonic stem cells, neural stem cells, endoderm cells, and so on (Armakolas & Klar, 2006; Karpowicz *et al.*, 2005). However, in contrast, in the embryonic neural progenitors, hematopoietic and hair follicle stem cells, the chromosomes were randomly segregated to their offspring (Fei & Huttner, 2009; Kiel *et al.*, 2007; Sotiropoulou *et al.*, 2008; Waghmare *et al.*, 2008). In the germ line stem cells of *D. melanogaster*, biased inheritance of the ancestral DNA strands was selectively occurred in some fractions of the chromosomes, but not all of them (Karpowicz *et al.*, 2009). These results provoke a possibility

that non-random chromosome segregation can take place under the limited conditions and cell types. In other words, the chromosomes that have potential for non-random segregation may be restricted. Therefore, I wondered if Chr. XII (or rDNA) especially triggers non-random chromosome segregation in case of the budding yeast. I am interested in the relationship between rDNA and non-random sister chromatids segregation, and whether rDNA itself has a potential to control the pattern of chromosome segregation. As the structure of rDNA is conserved in the most organisms, the role as a determinant of non-random DNA segregation can be maintained in the other organisms.

i) Is the rDNA a determinant of asymmetric chromosome segregation?

The yeast *S. cerevisiae* maintains 16 chromosomes in the haploid nucleus, although the rDNA resides on one of them. In this study, I focused on the Chr. XII that has the rDNA, and found that its sister chromatids segregated in non-random way (Fig. 6, 7). Given that rDNA region is so unique chromosomal part both in structurally and biologically, the phenomena observed in this study may be derived from its special characteristic. If this is the case, it is conceivable that the Chr. XII is randomly inherited to the progenies in the absence of rDNA repeats. Conversely, if the rDNA repeat was transferred into the other chromosome, the fate of the chromosome will be reprogrammed to non-random segregation. To examine this hypothesis, I constructed a strain that harbors rDNA repeats on the Chr. V that is suggested to be segregated randomly (Chua & Jinks-Robertson, 1991). If the non-random segregation is observed in the

strain, it is supported that rDNA has a potential to partition chromosomes non-randomly. Alternatively, to confirm the hypothesis, it is also required to monitor the fate of sister chromatid segregation in the absence of rDNA and its amplification. Unfortunately, in present, I lack the system to evaluate the non-random segregation in the rDNA-lost Chr. XII and the other 15 chromosomes. As an approach to examine the issue, I performed DNA pulse-chase analysis for monitoring the segregation patterns of whole chromosomes by CHEF electrophoresis (Fig. 13). However, I found that the analysis carried out in this study seemed to be unreliable. I tried to monitor the BrdU signal dilution for several rounds of cell cycle, however, the signal was intensified rather than diluted through cell division (Fig. 13B). It was likely that the BrdU or its derivatives was stored in the cellular nucleotides pool, and incorporated again in the S-phase of next cell cycle. Several system improvements are required to obtain better results and reveal the details of chromosome segregation pattern.

ii) Does rDNA amplification trigger asymmetric chromosome segregation?

Although the state of both chromosomes and cells that undergo asymmetric DNA segregation remains unclear in most model systems, there are several reports that featured about the triggers to guide the event. The non-random DNA segregation that was preferentially observed after recombination in *D. melanogaster* is one of the representative examples of them (Pimpinelli & Ripoll, 1986). In the study, it was suggested that sister chromatids crossover destines the asymmetric chromosome segregation during mitosis. Although the molecular

mechanisms were not elucidated, the recombination seemed to be a key to trigger the event. Alternatively, the replication fork termination was raised as the other cue from the study of *S. pombe (Klar, 2007).* The fission yeast *S. pombe* switches mating cell type depend on the circumstances. During its regulational process, specific DNA strand at the *mat1* locus was 'differentiated' to produce the asymmetric sister chromatids. The inheritance of these sister chromatids is non-random and assure the only one of four cells to switch the mating type. Currently, it is understood that the 'differentiation' process is thought to be controlled by unidirectional DNA replication at the *mat1* locus and its polar termination in the replication termination site (RTS1) (Klar, 2007).

Given that rDNA amplification in *S. cerevisiae* is accompanied with both recombination and replication fork block at the RFB, it is possible that these events leave epigenetic hallmarks on one of the sister chromatids to 'differentiate' it. This may trigger asymmetric chromosome segregation likewise in *D. melanogaster* and *S. pombe*. If this is the case, rDNA amplification is necessary for the asymmetric sister chromatid segregation. Considering the random segregation of the recombined Chr. V sisters (Chua & Jinks-Robertson, 1991), site specific replication termination rather than recombination would be important. Otherwise, the properties of the rDNA recombination are distinct from others, as it does not require Rad51 that is necessary for conventional recombination process. In this study, I could trace the segregation pattern of sister chromatid that contains 'recombined (amplified) rDNA'. Therefore, the pattern of 'recombination free' sister was not detected. It remained unclear whether asymmetric

chromosome segregation takes place depending on the rDNA amplification. To ascertain the issue, DNA pulse-chase analysis with BrdU labeling was considered to be efficient. However, my approach was not sufficient to show a clear result about the issue (Fig. 13). I have been attempting to improve the resolution of the pulse-chase experiment, and I expect that improvement of the system would provide several evidences on the connection between asymmetric chromosome segregation and rDNA amplification.

Currently, the replication termination site (RTS) was commonly observed in the functional chromosomal region including centromere. If there is a connection between the rDNA amplification and asymmetric chromosome segregation, it will provide a universal role of RTS in the non-random DNA partitioning.

iii) The rDNA copy may associate with asymmetric chromosome segregation.

According to technical difficulty for detection of rDNA amplification, I carried out most of the analysis based on the 2-rDNA copy strain (Fig. 3). This copy number is extremely low compared with WT level (~150 copies) and may affect the property of chromosome segregation. In addition, asymmetric helper plasmid partitioning is the other anxiety. The helper plasmid that supports the rRNA synthesis is necessary for growth of the 2-rDNA copy strain. However, likewise the extra chromosomal rDNA circle (ERC), the plasmids tended to be anchored in the mother cell, and the daughter cell seemed to be deprived from efficient rRNA synthesis (date not shown). Given that the yeast cells can lose large portion of rDNA copies from chromosome by

the pop-out recombination, such a situation in nature is possibly mimicked in the 2-rDNA copy strain. This raises a possibility that the asymmetric chromosome segregation can be equipped as a defensive mechanism that rescues the daughter cell from such a crisis of rRNA starvation. If this is the case, asymmetric segregation is possibly observed only when chromosomal rDNA copies are reduced. The other interpretation is that non-random segregation is also used for quality control of rDNA copies. That is to say, it has a role in preventing excess rDNA increase/decrease in the daughter-lineage. In young cells of WT budding yeast, the rDNA copy number is maintained ~150 copies, while once the cells get older, the number becomes larger (Dang *et al.,* 2009). In such a case, the pattern of non-random chromosome segregation may adjust the inheritance of favorable chromosome to the daughter. Although further analysis is required to solve this question, I expect the phenomenon can be a clue to understand the significance of non-random chromosome segregation.

iv) Association of *trans* factors for non-random chromosome segregation

I expected the *trans* factors that affect the rDNA stability may have a role in sister chromatids segregation. To examine the possibility, I monitored 3 mutants. As absence of Sir2 and Heh1 did not alter the mode of sister chromatids segregation, at least the chromatin modification and nuclear localization of rDNA were independent of chromosome segregation (Fig. 12B). With respect to the *bud6*Δ mutant, I could not observe the rDNA amplification, however, the mutant may have a potential to influence the pattern of sister chromatid segregation.

Because Bud6 protein not only interferes with episomal rDNA dynamics, but also interacts with dynein, a molecular motor that is necessary for asymmetric chromosome segregation in mouse (Armakolas & Klar, 2007; Shcheprova *et al.*, 2008). The episomal rDNA molecules (ERC) are positively sequestered in mother cell, according to diffusion barrier (Bud6) and nuclear membrane (pore) proteins (Shcheprova *et al.*, 2008). Likewise this observation, I found the helper plasmid was also subjected to the system, despite it uses centromeric functions for their segregation. These suggest that the rDNA on a plasmid is tightly anchored to the mother's side in a Bud6 dependent manner. It is a fascinating idea if chromosomal rDNA is also captured by this system, and only one of two sister chromatids was tethered to the mother cell. This idea raises a possibility that Bud6 protein affects the pattern of chromosome segregation. To date, several studies suggest that the yeast rDNA locus interacts with actin-network and dynactin, a regulator of dynein, that include Bud6 interactant (Schoner *et al.*, 2008). Recent study showed that part of polarized DNA movement in mouse eggs is carried out in a dynein dependent, but centromere/kinetochore independent manner (Deng *et al.*, 2009). This raises a possibility that the rDNA units are pulled toward the daughter cell independent of conventional mitotic apparatus (Fig. 16A). Therefore the dynamics of chromosomal rDNA may be regulated by Bud6 and other actin-related proteins and their association with rDNA. Further studies in this field will provide important evidences that contribute to reveal the *trans* factors for non-random sister chromosome segregation.

2. Possibility of rDNA independent non-random sister chromatid segregation.

As shown in Fig. 13, besides the Chr. XII, non-random sister chromatid segregation may be occurred in other several chromosomes. This suggests that conventional non-random sister chromatid segregation can take place independent of rDNA. As (Klar, 2007) indicated in their studies, the epigenetic modification on the non-rDNA chromosomal region can lead to non-random sister chromatid segregation. In this chapter, I discuss about the possibility of rDNA independent non-random sister chromatid segregation.

One of the major mechanisms that hypothesized to manage non-random DNA segregation is the dialog between DNA and mitotic apparatus (Tajbakhsh & Gonzalez, 2009). Mitotic apparatus including centromere, kinetochore, and centrosome are key machineries for DNA segregation, and they are shown to be polarized through the protein interactions in several organisms (Liakopoulos *et al.*, 2003; Louie *et al.*, 2004; Yamashita *et al.*, 2003). For instance, the karyogramy protein Kar9 is the typical factor that confers the polarity to the centrosome (Liakopoulos *et al.*, 2003). Recent study in the *S. cerevisiae* provided kinetochore protein Mtw1 can select and tag one of four kinetochore during meiosis or subsequent germination process (Thorpe *et al.*, 2009). Such machineries are thought to destine non-random sister chromatid segregation through interactions with centromere and polarized proteins. In this study, I explored whether *CEN12* is involved in such a chromosome polarization. If *CEN12* has specialized functions to adjust non-random sister chromatid segregation, it is conceivable that the state of Chr. XII is somehow affected by its modifications. In the analysis, although I could not confirm

the patterns of Chr. XII segregation in those strains, at least the rDNA amplification and chromosome stability were seemed not to be affected (Fig. 9, 9-2). As *CEN12* was exchangeable to *CEN5* without remarkable side effect, *CEN12* itself is not likely to encode sequence specific functions for Chr. XII maintenance. In consequence, in the *CEN12*::CEN12-Rev strain, slight growth defect was observed. In this strain, the directionality (alignment of CDE sequences) of centromere was inverted as same as *CEN12*::*CEN5* (Fig.9C). Because the *CEN12*::*CEN5* modification did not affect to the growth, the defect in *CEN12*::CEN12-Rev strain can be caused by the inversion of Watson-Crick strand of *CEN12* rather than directionality of centromere (Fig. 9C). As the *CEN12*::*CEN12*-Rev modification did not alter the pattern of non-random sister chromatid segregation, the connection between chromosome segregation and the growth defect is quite unclear. As one of possibilities, the phenomenon may reflect strand specific recognition of sister chromatids.

Apart from the centromeric sequences, the other region on the Chr. XII may have a role in non-random sister chromatid segregation. (Kim *et al.*, 2006) split the several sites of the Chr. XII to remove some of the regions, such as right or left arm, to explore the biological significance of this specific chromosomal context. This experiment showed an idea that there are *cis* elements to maintain the rDNA. Rather than centromeric sequences, such elements may be involved in the asymmetric segregation of the Chr. XII.

3. Models for the mechanism of asymmetric sister chromatid segregation

Although it is still unclear how the sister chromatids are partitioned in the yeast cell, some possible ideas are raised in this study. Those ideas are summarized as a model shown in Fig. 16, and I am currently working on the issues. In addition to the traditional kinetochore dependent non-random chromosome segregation model (Fig. 16B), I speculated the rDNA directed chromosome segregation (Fig. 16A) takes place in the yeast. I expect that the trial contributes to understand the universal mechanism for non-random chromosome segregation both in the yeast and other organisms (see the legend for detail).

4. The connection between directionality of rDNA repeats and amplification

In this study, I constructed 2-rDNA copy strain with inverted rDNA repeats (Fig. 10), and tried to amplify the repeats on the Chr. XII. However, I found that the rDNA repeats couldn't amplify in the strain (Fig. 11A). Although the rDNA inversion alters the context of neighboring sequence, considering the rDNA repeats on the Chr. V that amplified to WT level (>100 copies) (Oakes *et al.*, 2006), junction of rDNA repeats with those sequences is not involved in. In addition, separation of the daughter and mother cells indicated that the rDNA amplification doesn't occurr in both cell lineages. Therefore, it is likely that the rDNA amplification is inhibited in inverted-rDNA repeats. As for the problems, two interpretations can be possible. One is inhibition of Fob1 dependent unequal sister chromatid recombination, according to the reduced activity of replication fork block and/or recombination. I speculated that in inverted rDNA strain, Fob1 protein fails to interact with RFB site according to the altered properties of

chromatin. Alternatively, if Fob1 protein is able to access to the RFB, subsequent DSB repair process may be restricted to the equal sister chromatid recombination. To ascertain this point, now I am checking the replication fork arrest at the RFB by 2-dimensional gel electrophoresis analysis (Kobayashi *et al.*, 2001). It may suggest which process bothers the rDNA amplification. The other possibility is that rDNA amplification is lethal in a cell with inverted-rDNA repeats according to the problems such as unsuccessful DSB repair following replication fork block and toxicity of amplified rDNA repeats. To examine this possibility, I am going to attempt the other methodology to invert the rDNA repeats by using site-specific recombination system in the budding yeast (Araki *et al.*, 1992). The system probably enables me to invert the rDNA repeats even in a cell with WT level (~150) rDNA copies, and the effect of maintaining inverted-rDNA repeats can be examined.

The relationship between the directionality of rDNA repeats and rDNA amplification is unexpected, but curious finding. As this issue suggests that the direction of repeat sequence has a connection with their properties and functions, the same phenomena may commonly take place among the unidirectional repeat regions in the yeast and the other organisms.

5.Biological significance of asymmetric chromosome segregation in *S. cerevisiae*

To date, several theories about the significance of asymmetric chromosome segregation are proposed. Most of them were linked with the cell differentiation, including regulation of cell robustness, cellular aging, cell fate choice, and so on. Given that the non-random chromosome

segregation was observed from bacteria to metazoan, the conserved function is likely to associate with aging because this process commonly takes place in all organisms.

The ageing related DNA disorders are found in many organisms and one of the examples is accumulation of damage and mutations. Because DNA replication leaves some errors in every round of cell cycle, the DNA strands accumulate them owing to semi-conservative replication. In parallel, as the fidelity of DNA repair and replication machineries are gradually diminished as cells get older, chromosomes become prone to be injured in senescent cells (Sinclair & Oberdoerffer, 2009). According to the immortal strand hypothesis, even in such a condition, parental old DNA strands are retained in the stem cell and protected from those disasters (Rando, 2007). If followed by this manner, when a chromosome is preferentially inherited to the specific pedigree, the integrity of one of two ‘differentiated’ sister chromatids can be preserved. Recent study in our laboratory suggested that ‘damaged’ rDNA (Chr. XII) was preferentially sequestered to the mother cell in *S. cerevisiae* (Ganley *et al.*, 2009). This is possibly related to the mother cell specific accumulation of dysfunctional proteins (Erjavec & Nystrom, 2007) and also inheritance of intact Chr. XII to the daughter cell. Therefore, it is conceivable that the asymmetric sister chromatid segregation functions to release the one of the sisters into the safe environment. In other words, the process can contribute to rejuvenation of the chromosome XII at least in rDNA region. As the asymmetric sister chromatid segregation was accompanied with the rDNA amplification, the recombinational repair in the rDNA may assure the genome integrity. The segregation process may be a mechanism to preserve functional

DNA (in case of rDNA, proper copy number).

In addition to the hypothesis described above, there may be another meanings in the asymmetric sister chromatid segregation. In the population, rDNA amplification was occurred less than 5% of cells in the daughter-lineage (Fig. 7), this means that only the limited pedigree can inherit adaptive functions such as a growth advantage. It is an attractive theme to reveal what makes the difference between the ‘selected’ cells and the others, and I am going to screen how and why such a variation is formed in future.

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Table 1. Yeast strains used in this study

Name	Background	Genotype	Reference
NOY408-1b	W303	*MAT a leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 (150 rDNA copies)*	
TAK201	W303	*MAT a leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 fob1 Δ::HIS3 (2 rDNA copies) pNOY353*	
NOY202	W303	*MAT a leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 fob1 Δ::HIS3 (2 rDNA copies) pRDN-hyg1*	
NOY408-1b Hho1-yEGFP Sik1-mCherry	W303	Same as NOY408-1b except for C-terminus of *HHO1* and *SIK1* ORF was tagged with yEGFP and mCHerry, respectively	This study
Strain used for asymmetric sister chromatid segregation analysis			
NOY202 pRS304-*GALFOB1*	W303	Same as NOY202 except for pRS304-*GALFOB1* was integrated into the *TRP1* locus	This study
Strains used for CEN12 modification analysis			
TAK201CEN12L::*URA3*	W303	Same as TAK201 except for *URA3* ORF was inserted into the left side of chromosome XII centromere (*CEN12*)	This study
CEN12::CEN12-REV	W303	Same as TAK201 except the direction of chromosome XII centromere (*CEN12*) is inverted	This study
CEN12::CEN5	W303	Same as TAK201 except for chromosome XII centromere (*CEN12*) is replaced with chromosome V centromere (*CEN5*)	This study
Strains used for rDNA-inversion analysis			
NOY408-1b fob1D::hphMX	W303	Same as NOY408-1b except for *FOB1* ORF was replaced with hygromycin resistant gene hphMX	This study
NOY984	W303	*MAT α leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 fob1 Δ::HIS3 rdn ΔΔ::HISG (0 rDNA copy) pNOY190*	*Oakes et al., 2006*
dTM1	W303	Dipliod strain between NOY408-1b *fob1 Δ*::hphMX and NOY984	This study
rDNA-rev1	W303	*MAT a leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 fob1 Δ::hphMX rdn ΔΔ::HISG::prDNA-Rev (1 inverted rDNA copy) pNOY190*	This study
rDNA-rev2	W303	*MAT a leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 fob1 Δ::hphMX rdn ΔΔ::HISG::prDNA-Rev::pNOY3293 (2 inverted rDNA copies) pNOY190*	This study
Strains used for analysis of siter chromatid segregation in mutants			
NOY202 pRS304-GALFOB1*sir2*Δ::KAN	W303	*MAT a leu2-3,112 trp1-1::TRP1-GALFOB1 can1-100 ura3-1 ade2-1 his3-11,15 fob1 Δ::HIS3 (2 rDNA copies) pRDN-hyg1*	This study
NOY202 pRS304-GALFOB1 *heh1 Δ*::KAN	W303	*MAT a leu2-3,112 trp1-1::TRP1-GALFOB1 can1-100 ura3-1 ade2-1 his3-11,15 fob1 Δ::HIS3 (2 rDNA copies) pRDN-hyg1*	This study
NOY202 pRS304-GALFOB1*bud6 Δ*::KAN	W303	*MAT a leu2-3,112 trp1-1::TRP1-GALFOB1 can1-100 ura3-1 ade2-1 his3-11,15 fob1 Δ::HIS3 (2 rDNA copies) pRDN-hyg1*	This study
Strains used for BrdU incorporation analysis			
TM3	W303	*MAT a leu2-3,112 trp1-1::TRP1-GALFOB1 can1-100 ura3-1 ade2-1 his3-11,15::p303-BrdU-Inc-HIS3 fob1 Δ::LEU2 AUR1::pAUR101-GPDTK bar1 Δ::hphMX (~30 rDNA copies)*	This study
Strains used for rDNA-labeling with lacO			
TAK201 *RDN1*::lacO	W303	*MAT a leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1::GFP-*lacI-*ADE2 his3-11,15 fob1 Δ::HIS3 RDN1::50x* lacO-URA3 *(2 rDNA copies) pNOY353*	This study
TM1	W303	*MAT a leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1::GFP-*lacI-*ADE2 his3-11,15 FOB1 RDN1::50x* lacO-URA3 *(150-copy rDNA are completely labeled with lacO)*	This study
TM1 Sik1-mCherry	W303	Same as TM1 except for C-terminus of *SIK1* ORF was tagged with mcherry	This study
TM1 Net1-mCherry	W303	Same as TM1 except for C-terminus of *NET1* ORF was tagged with mcherry	This study

Table 2. Plasmids used in this study

Name	Description	Fragment	Marker	Reference
pUC18				
pRS304			*TRP1*	
pRS304-GALFOB1		EcoRV	*TRP1*	This study
pJJ242			*URA3*	
YCpGalFOB1			*TRP1*	
Plasmids used for CEN12 modification				
pCEN12-L	Plasmid containing the DNA sequences flanking to the left end of CEN12		-	This study
pCEN12-LR	Plasmid containing the complete CEN12 context beside it lacks centromere core sequences		-	This study
pCEN12-Rev	Plasmid use for inversion of the CEN12 sequence	PCR (CEN12-AF-CEN12-BF	-	This study
pCEN12-CEN5	Plasmid used for replacing the CEN12 with CEN5	PCR (CEN12-AF-CEN12-BF	-	This study
pCEN12-XL	Plasmid containing the DNA sequences flanking to the N-terminus of *DNM1* gene		-	
pCEN12-XLR	DNA sequences that adjacents to the C-terminus end of *NOC3* gene was inserted into the SalI-SphI site of pCEN12-XL		-	
pCEN12-Rev2	Plasmid use for inversion of the CEN12 sequence	SmaI/SphI	-	
			-	
Plasmids used for rDNA inversion				
pNOY3286	Plasmid containing the core rDNA fragment consists of N, hygromycin-resistant-rDNA and *HIS3*		*HIS3*	Oakes et al. 2006
prDNA-Rev	Plasmid used for integration of inverted-rDNA unit into the RDN1 locus	SalI	*HIS3*	This study
pNOY3293	Plasmid used for integration of second rDNA unit into the RDN1 locus	SalI/SpeI	*LEU2*	Oakes et al. 2006
Plasmids used for rDNA labeling				
pASFS52	Plasmid containing the 256x lacO array		*TRP1*	Straight et al. 1996
pAFS52-lacO50	Plasmid containing the 50x lacO array		*TRP1*	This study
pTM1	IGS1 fragment was inserted into the KpnI-XhoI site of pAFS52-lacO50		*TRP1*	This study
pTM2	IGS2 fragment was inserted into the HindIII-SphI site of pTM1		*TRP1*	This study
pTM-lacO50	Plasmid used for labeling the rDNA unit with lacO	KpnI/HindIII	*TRP1*, *URA3*	This study
pWJ1513	Plasmid containing the 224x tetO array		*URA3*	Torres-Rosell *et al.*,
pWJ1513-tetO50	Plasmid containing the 50x tetO array		*URA3*	This study
pUC18-tetO50	BglII-BamHI tetO50 fragment was inserted into BamHI site of pUC18		-	This study
pIGS1-tetO50	IGS2 fragment and URA3 ORF was inserted into HindIII-SalI site of pUC18-tetO50		*URA3*	This study
pTM-tetO50	Plasmid used for labeling the rDNA unit with tetO	SacI/HindIII	*URA3*	This study
pAFS144	Plasmid containing GFP-lacI fragment	NheI	*HIS3*	Straight et al. 1998
pML22	Plasmid containing tetR-mRFP fragment		*LEU2*	Torres-Rosell *et al.*,
pAFS144-yEGFP	Plasmid containing yEGFP-lacI fragment	BglII	*ADE2*	This study
pKT128	Plasmid containing yEGFP fragment		*KAN*	Purchased from Eurc
Plasmids used for BrdU pulse-chase analysis				
pAUR101-GPD-TK	Plasmid containing thymidine kinase for BrdU incorporation		Aureobasidin^{r}	Katou *et al.*, 2003
p303BrdU-Inc-HIS3	Plasmid containing thymidine kinase and transporter for BrdU incorporation	NheI	*HIS3*	Viggiani & Aparicio

Table 3. PCR primers used in this study

Name	Sequence	Comment
Primers used for confirming pRS304-*GALFOB1* integreration		
RS2	5'-CTTGATTAGGGTGATGGTTCACG-3'	
TRP1 N	5'-TTCACAGGTAGTTCTGGTCC-3'	
Primers used for *CEN12* modification		
CEN12 AF HindIII	5'-CCCCAAGCTTGACAAAAATCAAAGAGGGAG-3'	
CEN12 AR PstI	5'-AAAACTGCAGTTAATATATCCTGTTTTTTTTTCATCATTC-3'	
CEN12 BF XbaI	5'-CTAGTCTAGAACATTTTATTAGCTATTTTTCAAGCA-3'	
CEN12 BR KpnI	5'-ACGGGGTACCTGAAATCACTCGGAGTTTAT-3'	
CEN12 -1 PstI	5'-AAAACTGCAGTTTTATTGTTCGGATAACAA-3'	
CEN12 -2 XbaI	5'-CTAGTCTAGAATCACGTGTAATAAATATTA-3'	
CEN5 F PstI	5'-AAAACTGCAGATCACGTGCTTTTTAAAAAA-3'	
CEN5 R XbaI	5'-CTAGTCTAGATTTTTCTTTTCGGAAATCTA-3'	
CEN5 F2 PstI	5'-AAAACTGCAGTTCTATGAAACATCAAATTAATCACGTGCT-3'	
CEN5 R2 PstI	5'-CTAGTCTAGACTTGAAACCTTTTTTTCTTTTCGGAAATC-3'	
CEN12 S1	5'-AGTGTCGATTAAATCAAAATGTAAAAATGG-3'	
CEN12 S2	5'-CATGGTTTGTAGACAACCAAACTGGTGTAT-3'	
CEN12 XLF	5'-ATCCCCCGGGGACCTGCATCCCAGTACCTCTTCCCAGATT-3'	
CEN12 XLR	5'-ACGCGGATCCGTACTAACAATAAATACAAACATGCATTTT-3'	
CEN12 XLA	5'-ACGCGTCGACCTTGATCGCCACCTTTCTAGGTAATGATAG-3'	
CEN12 XLB	5'-ACGCGGATCCATAAACACTGACCTATAATCACGCCCGCAA-3'	
CEN12 XRF	5'-ACGCGGATCCCCAATCATTTTTGCCGTGTCGAGTAGAATT-3'	
CEN12 XRR	5'-ACGCGTCGACCTAACGATAATCGTGGCTCTTTATATACTT-3'	
pRS CEN12 R	5'-AAGGGTGGGATCAGATCAAAAGGGCGGTCGCAGTGTCAGTACCTCATAAGTGTTAATATATCATTTAAGTCTGTGCGGTATTTCACACCG-3'	
pRS CEN12 F	5'-AAAATAACCACAAAACTTAAAACGAACGTTATTTTGTTCAATTGCTTAATTTGTGAAGATATTATCTACTAGATTGTACTGAGAGTGCAC-3'	
Primers used for construction of prDNA-Rev and rDNA inversion		
127 SalI-EcoRI	5'-CCCGAATTCGTCGACTCTATACTTCTGAGCAGCAATTAAGG-3'	
128 SmaI-XhoI	5'-CCCCCCGGGCTCGAGGTATTACGTGATATACAGTGACAGCC-3'	
129 SpeI-BamHI	5'-GGGGGATCCACTAGTTGAGCATATTTGAGATCTGACTTGCC-3'	
130 SalI	5'-GGGGTCGACGCTCCAACTCAGTTCTATCTCAGCTCC-3'	
182	5'-CTCACACTTGTACTCCATGACTAAACCCCC-3'	Oakes et al., 2006
185	5'-CATGGTTGGCGAAGTAATGGTTTGGCGAAG-3'	Oakes et al., 2006
137	5'-CCGCTGAGACCTTTCCATTGGGTCAGGTCG-3'	Oakes et al., 2006
138	5'-GTACGCCATTGGATCTGGCTACCTAAACCC-3'	Oakes et al., 2006
174	5'-TGGTACCAACGATGTTCCCTCCACCAAAGG-3'	Oakes et al., 2006
161	5'-GCAAACCACAAGTGGACCAAGGTTCCCC-3'	Oakes et al., 2006
Primers used for construction of pTM-lacO and pTM-tetO		
TM3 [a)]	5'- ATCTCGAGTCCCATAACTAACCTACCAT -3'	
TM4 [a)]	5'- ACGGTACCAGTAAATGGCAGTTTCTAGG-3'	
TM-Hind	5'-CCCCAAGCTTACCTACCGACCAACTTTCAT-3'	
TM-Sph	5'-CACATGCATGCTTTAGCATAGGAAGCCAAGA-3'	
TM7	5'- CACATGCATGCAGCTTTTCAATTCATCT-3'	
TM8	5'- ACGCGTCGACATCATTACGACCGAGATTCC-3'	
TM3-SacI	5'- ATGCGAGCTCTCCCATAACTAACCTACCAT -3'	
TM4-EcoRI	5'- ACCGGAATTCAGTAAATGGCAGTTTCTAGG-3'	
rDNA a	5'-TTACTACTATCCTCCCTTCA-3'	

Primers used for preparing a probe for chromsomome XII detection

dRYLLD58W	5'-TTATACCTCCAAGCAAGGTTACGG-3'
dFYLLD58W	5'-GAATGTTGAACTCTGCGGTTATCC-3'

a) Primer set TM3 / TM4 was also used for preparing a probe for *IGS1*

Table 4. Media used for yeast culture

	Constituents	Final concentration
YPD [b) e)]	D-glucose	20 g/L
	Bacto yeast extract	10 g/L
	Bacto pepton	20 g/L
	Bacto agar[a)]	20 g/L
SC [b) e) f)]	D-glucose	20 g/L
	Bacto-yeast nitorogen base (without	6.7 g/L
	Drop out mix [c)]	0.2 g/L
	Supplyments [d)]	
	Bacto agar[a)]	20 g/L
SPO	D-glucose	0.5 g/L
	Bacto yeast extract	1 g/L
	potassium acetate	10 g/L
	Bacto agar[a)]	20 g/L

c) Drop out mix

Constituent	Final concentration (mg /ml)
Adenine sulfate	20
L-Arginine	20
L-Methionine	20
L-Tyrosine	20
L-Isoleucine	20
L-Lysine	20
L-phenylalanine	20
L-Glutamic acid	20
L-Asparatic acid	20
L-Valine	20
L-Threonine	20
L-Serine	20

a)For plate medium

b) For YPGal and SGal medium, 2% D-glucose was replaced with 2% D-galactose

d) Depends on the puopose, L-Histidine, L-Leucine, L-Tryptophan, and Uracil was supplied

e) To culture or select drug resistance strains, antibiotics were added with the concentration shown below

G418	500 ug/ml	Sigma
Hygromycin B	300 ug/ml	Nacalai

f) For the counter selection of ura3- cells, 5-Fluoroorotic Acid (5-FOA) was added with the concentration shown below

5-FOA	0.1% (w/v)	WAKO

Bacto-pepton, Bacto-yeast extract, Bacto-agar, and Bacto-Yeast nitrogen base (without amino acids) were purchased from (BD). Sugars, amino acids, and other supplements used for auxotrophic complementation were all purchased from WAKO.

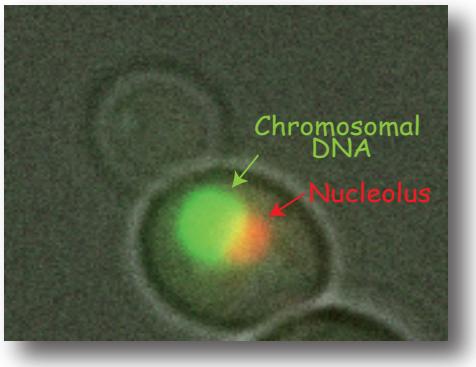
Table 5. Buffers used for Southern hybridization and BrdU immuno detection

Name	Constituents		Final Conc.
Buffers used for Southern hybridization			
Denaturation buffer		g/ 3L	
	NaOH	60	0.5 M
	NaCl	262.9	3M
Neutralization buffer		g/ 3L	
	Tris-HCl (pH = 7.0)	181.7	0.5 M
	NaCl	526.5	1.5 M
Hybridization buffer		g/ 100 ml	
	BSA	1 g	50 mg/ml
	0.5 M EDTA (pH = 8.0)	0.2 ml	1 mM
	20% SDS	35 ml	7%
	1M Na2HPO4 (pH = 7.2)	40 ml	0.4 M
	SDW	24.8 ml	
Buffers used for BrdU immunodetection			
Buffer I		g/ 1 L	
	Tris-HCl (pH = 7.5)	12.1	0.1 M
	NaCl	8.77	0.15M
Blocking buffer	1% Blocking reagent (Roche) in Buffer I		
Buffer I+T	0.3% Tween20 in Buffer I		

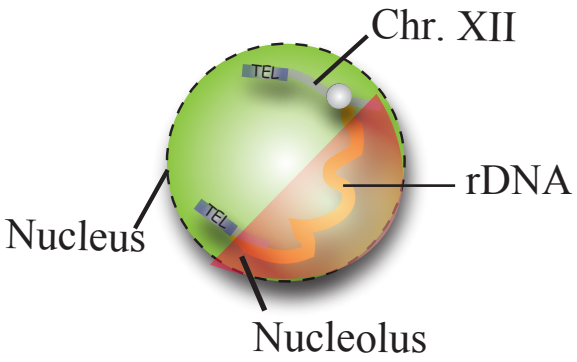
Table 6. Conditions for CHEF electrophoresis

Size resolution	Pulse time (second)		Gradient (V/cm)	Included angle	Temperature	Run time
	Int. Sw	Fin. Sw				
1.5 ~ 3.5 Mb	300.0	900.0	6.0	120^{o}	$14^{o}C$	68 h
5 ~150 Kb	22.0	265.9	6.0	120^{o}	$14^{o}C$	15 h 16 min
5 Kb ~ 2.5Mb	0.2	12.9	6.0	120^{o}	$14^{o}C$	15 h 16 min
0.6 ~ 1.6 Mb	60.0	120.0	6.0	120^{o}	$14^{o}C$	40 h

A.



B.



C.

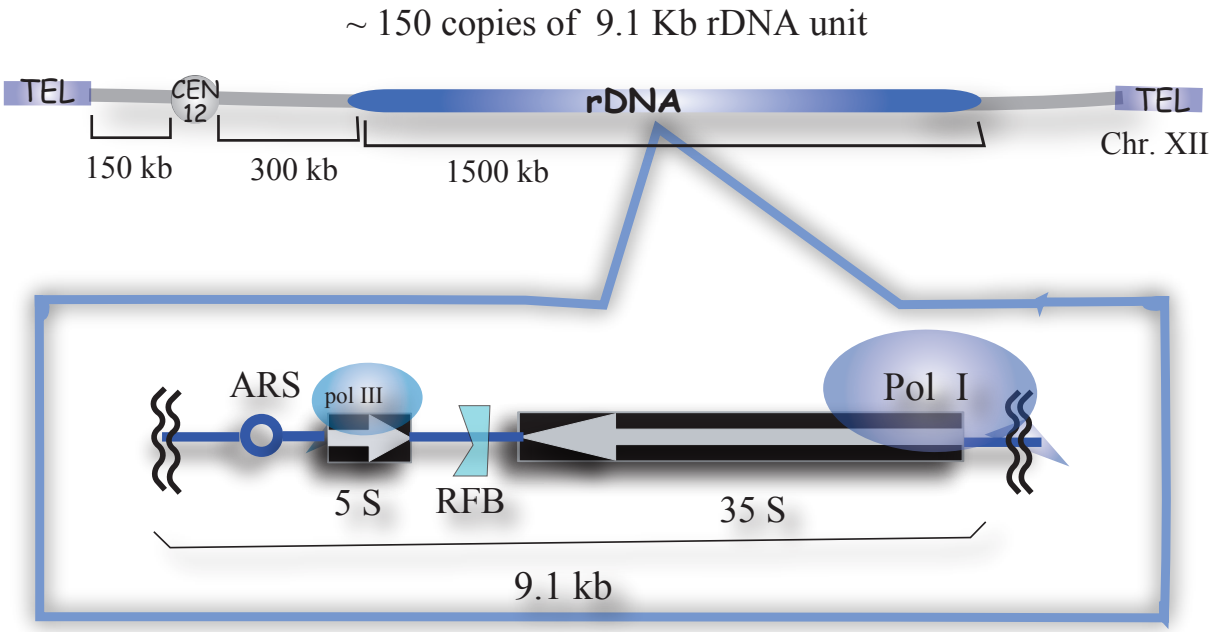


Fig. 1. The rDNA in the yeast *Saccharomyces cerevisiae*

Fig. 1. The rDNA in the yeast *Saccharomyces cerevisiae*

A. Fluorescent image of *S. cerevisiae* nucleus was shown. The chromosomal DNA and nucleolus are visualized with Hho1-yEGFP (histone H1) and Sik1-mCherry (nucleolar marker), respectively. **B.** The schematic diagram of the Chr. XII positioning in the nucleus. Apart from the other chromosomal loci, the rDNA region of the Chr. XII is specifically compounded by the crescent shaped nucleolus. **C.** The structure of the Chr. XII. The rDNA occupies an approximately 1.5 Mb region consisting of 100-200 tandem copies of a 9.1 kb repeat on the right arm of chromosome XII. Each repeat contains the genes for 5S and 35S rRNAs, as well as replication fork block sequence (RFB) and replication origin (ARS). The 35S and 5S rRNA are transcribed separately by RNA polymerase I and III, respectively.

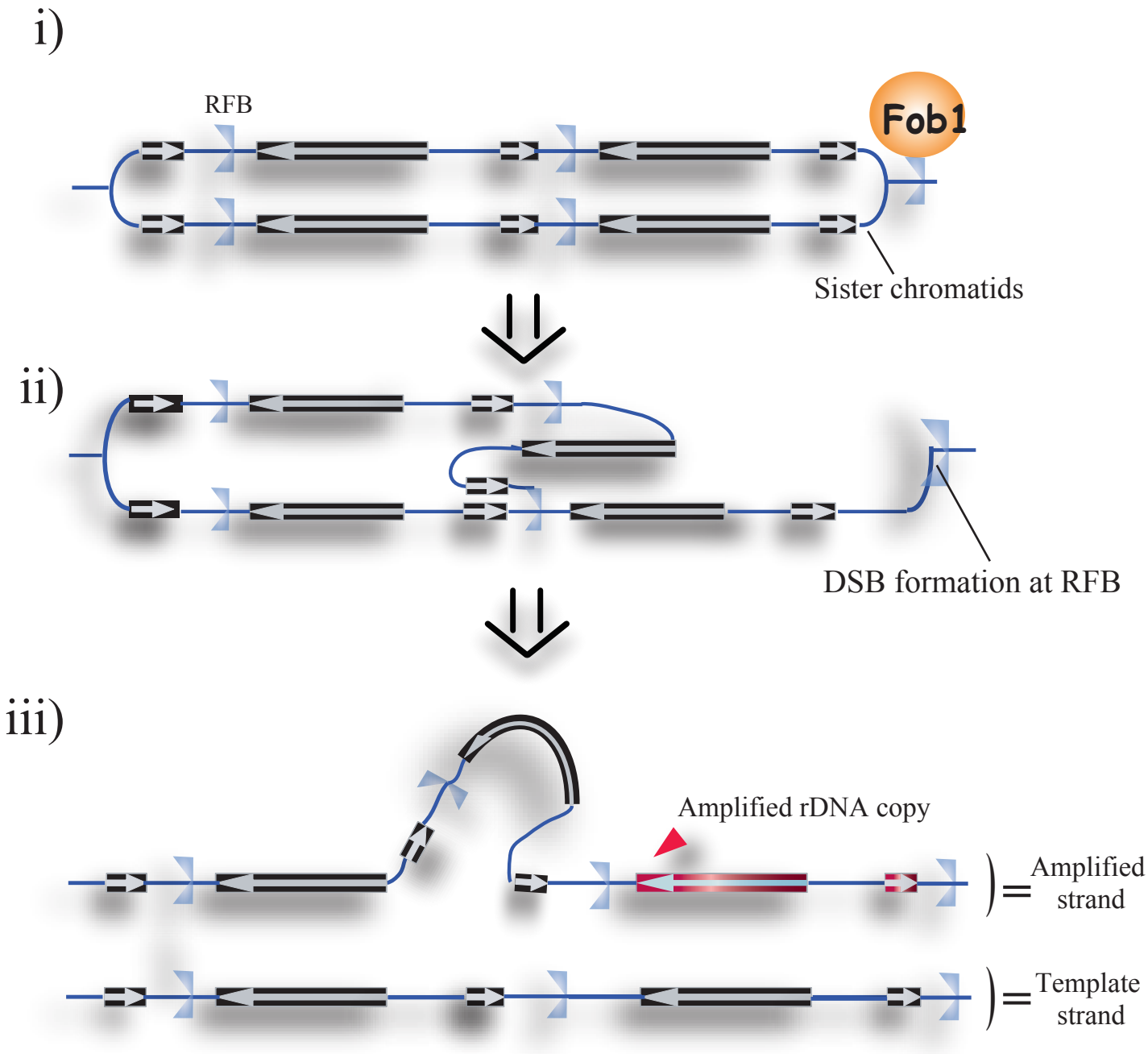


Fig. 2. A Model for rDNA amplification in *S. cerevisiae*

Fig. 2. A model for rDNA amplification in *S. cerevisiae*

The proposed model for the rDNA copy number expansion in *S. cerevisiae* was illustrated. **i)** In the S-phase, the leading strand replication that moves opposite to the 35S rRNA transcription is arrested at the RFB region in Fob1 dependent manner. This replication fork block event causes DSB nearby the RFB and stimulates recombinational repair. **ii)** The DSB is repaired by homologous recombination using the other sister chromatid as a template. A strand invasion takes place and new replication fork is formed. **iii)** If the strand invasion occurs at a site in an upstream repeat, gain of new rDNA repeat (amplified rDNA) is triggered in the one of two sister chromatids. Consequently, two distinct sister chromatids arise and they are distinguishable by their rDNA copy number.

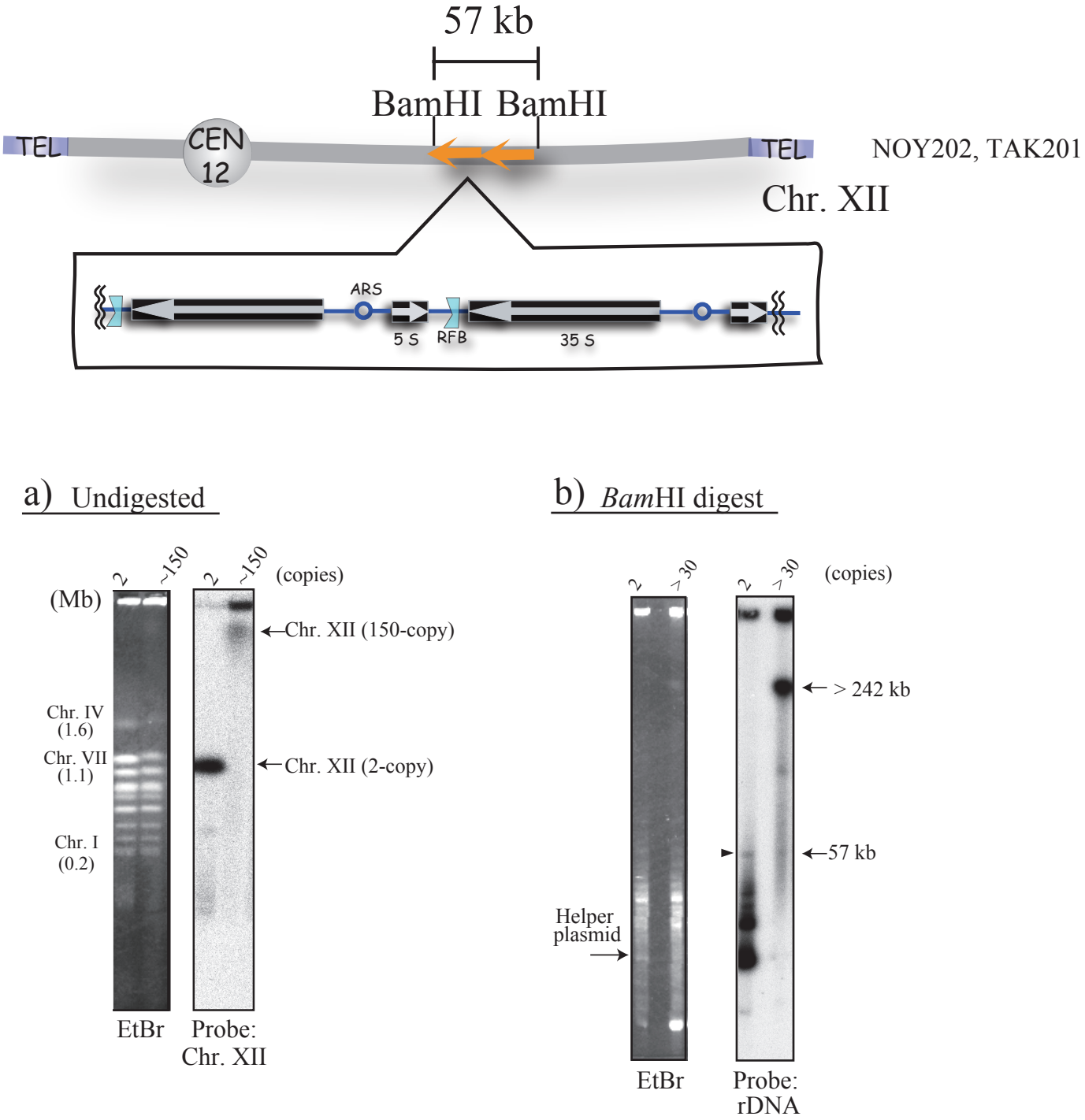


Fig. 3. 2-rDNA copy strain that was used for the analysis of rDNA amplification

Fig. 3. 2-rDNA copy strain that was used for the analysis of rDNA amplification

The rDNA region in the 2-rDNA copy strain (NOY202 and TAK201) was shown. As illustrated in the upper part, the rDNA repeats in the strain was shorten from ~150 to 2 copies, as confirmed by CHEF electrophoresis and subsequent Southern hybridization. **a)** The size of the Chr. XII was compared between WT (~150 copies) and NOY202 (2 copies). As indicated with arrows, the Chr. XII was detected around ~1 Mb position in 2-rDNA copy strain and > 2 Mb position in WT. For Southern hybridization, Chr. XII probe was used. **b)** The size of the rDNA region in 2-rDNA copy strain that was digested with *Bam*HI. *Bam*HI recognition sites exist not in the rDNA unit, but in both side of rDNA region. Therefore, the Chr. XII fragment that was digested with *Bam*HI reflects the size of rDNA region and copy numbers. Before rDNA amplification was induced, *Bam*HI digestion causes the 57-Kb fragment that including 2-rDNA repeat. Upon the rDNA amplification, the position of the fragment moved to the upper side. For Southern hybridization, probe for the IGS1 region was used to detect the rDNA.

A.

B.

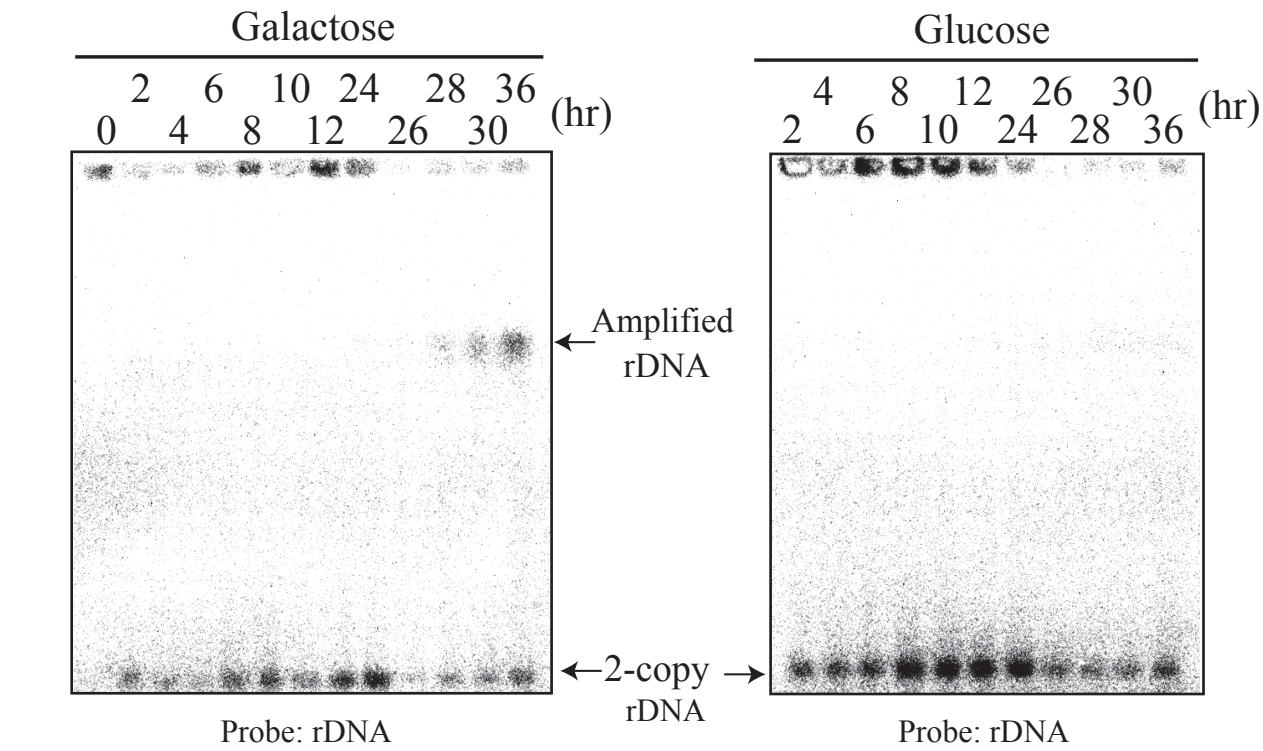


Fig.4. Induction of rDNA amplification by galactose inducible *FOB1* gene

Fig. 4. Induction of rDNA amplification by galactose inducible *FOB1* gene

Galactose inducible Fob1 expression system was introduced into NOY202 and the pattern of rDNA amplification was observed. **A.** The cassette containing a *FOB1* ORF that was fused to *Gal7* promoter and terminator (Gal-*FOB1*). This construct expresses Fob1 protein when galactose exists in the medium as an available carbon source (left). However, in the presence of glucose, its expression is not induced (right). **B.** Detection of amplified rDNA. The *Gal-FOB1* construct was integrated into the *TRP1* locus of NOY202, and Fob1 expression was induced in the strain. The cells were pre-cultured in raffinose medium and separated into two. The half of them was transferred to galactose medium and the rest was innoculated into glucose medium. Both of the cells were cultured simultaneously and chromosomal DNA was purified at the time point indicated on the top of the gel images. The Chromosomal DNA was digested with *Bam*HI and detected by Southern hybridization followed by CHEF electrophoresis. For Southern hybridization, probe for the IGS1 region was used to detect the rDNA. As indicated with arrow, the amplified rDNA appeared when cells were cultured in galactose medium for 26~36 h. And when cells were cultured in glucose medium, such products were not detected.

A.

Pomp flow rate (ml/min)	25	35	50	60	75	85	95	105
FRACTION NO.	F1	F2	F3	F4	F5	F6	F7	F8

B.

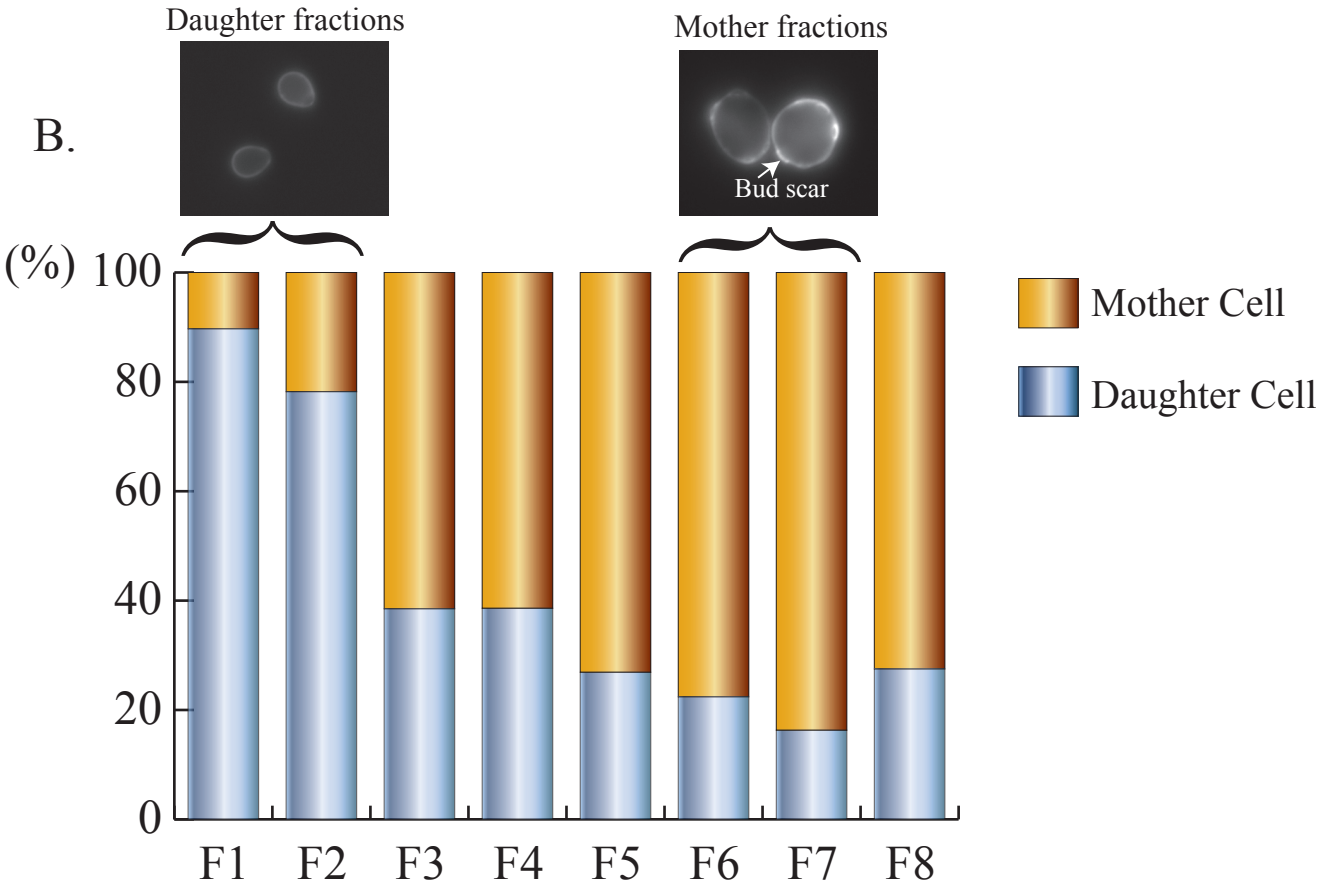


Fig. 5. Separation of the daughter and mother cells by centrifugal elutriation

Fig. 5. Separation of the daughter and mother cells by centrifugal elutriation

A. Parameters of pomp flow rate for the centrifugal elutriation were shown. The bulk yeast cell culture was classified into 8 fractions according to the differential flow rate. **B.** The typical result of the centrifugal elutriation was depicted. Ratio of the daughter and mother cells in the each fraction were estimated by calcofluor staining of bud scar. The bud scar is formed on the cellular surface when a yeast mother cell gives rise to daughter, and remained until the cell dies. Therefore, the bud scar is a hallmark of mother cell. As the ratio of cells with bud scar (mother cell) and without it (daughter cells) suggested, the first 2 fractions (F1 and F2) were highly enriched in daughter cells, while the later 2 fractions (F6 and F7) contains a lot of mother cells. Based on this result, fractions F1 and F2 were treated as daughter fractions, and F6 and F7 were as mother fractions in this study.

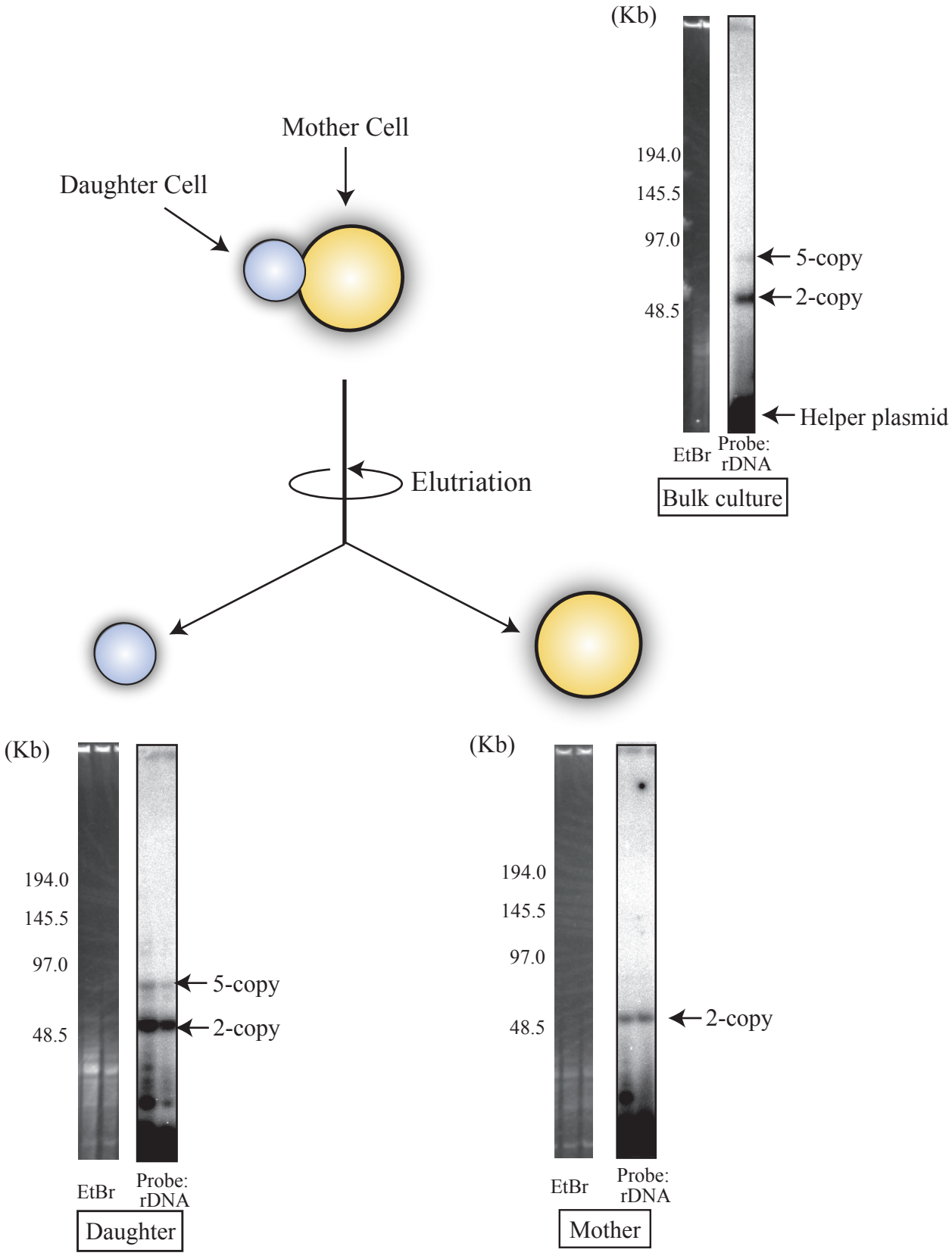


Fig. 6. rDNA amplification gives rise to asymmetric sister chromatids

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The cells of 2-rDNA copy strain (NOY202 pRS304-*GalFOB1*) were separated by centrifugal elutriation after rDNA amplification was induced. The number of rDNA copies was monitored by *Bam*HI digestion. Before the bulk culture was separated into the daughter and mother cells, two distinct rDNA signals were detected by Southern hybridization with IGS1 probe (top). One of the signals is derived from original 2-copy rDNA and the other is the amplified 5-copy rDNA from the length of *Bam*HI digested fragment. The separated daughter and mother fractions were differentially inherited these chromosomes as shown in bottom. The daughter cell fractions inherited both (2- and 5-copy) rDNA (bottom left), while the mother fractions received only the 2-copy (bottom right).

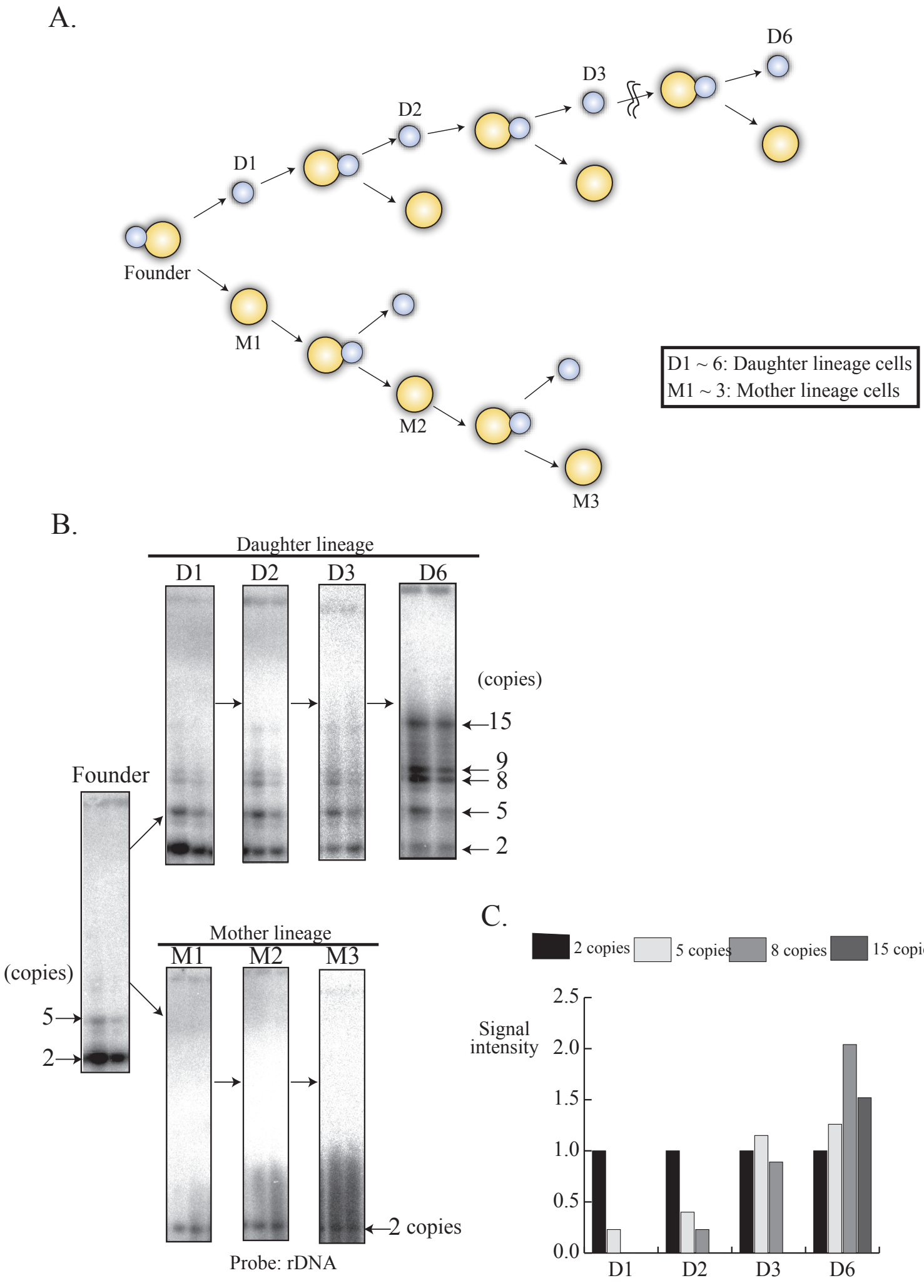


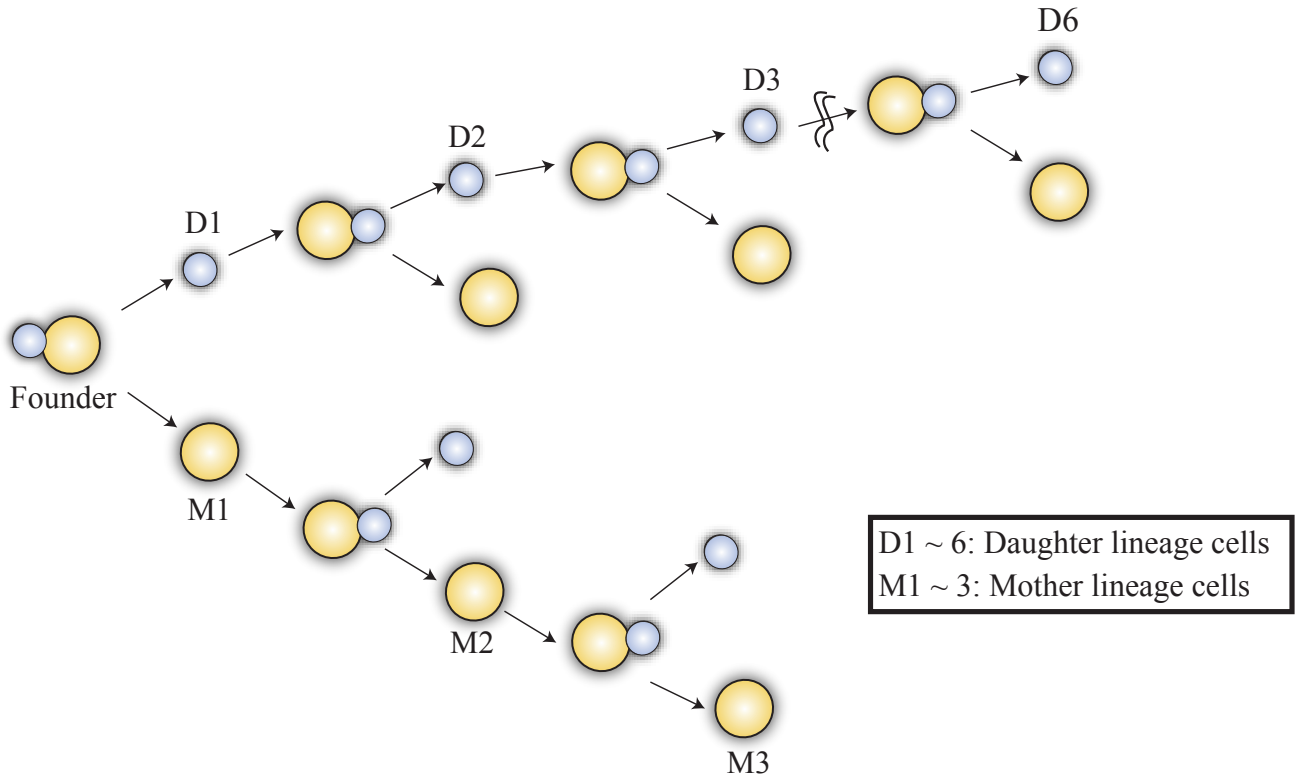
Fig. 7. The inheritance of the Chr. XII sister chromatids is non-random.

Fig. 7. The inheritance of the Chr. XII sister chromatids is non-random.

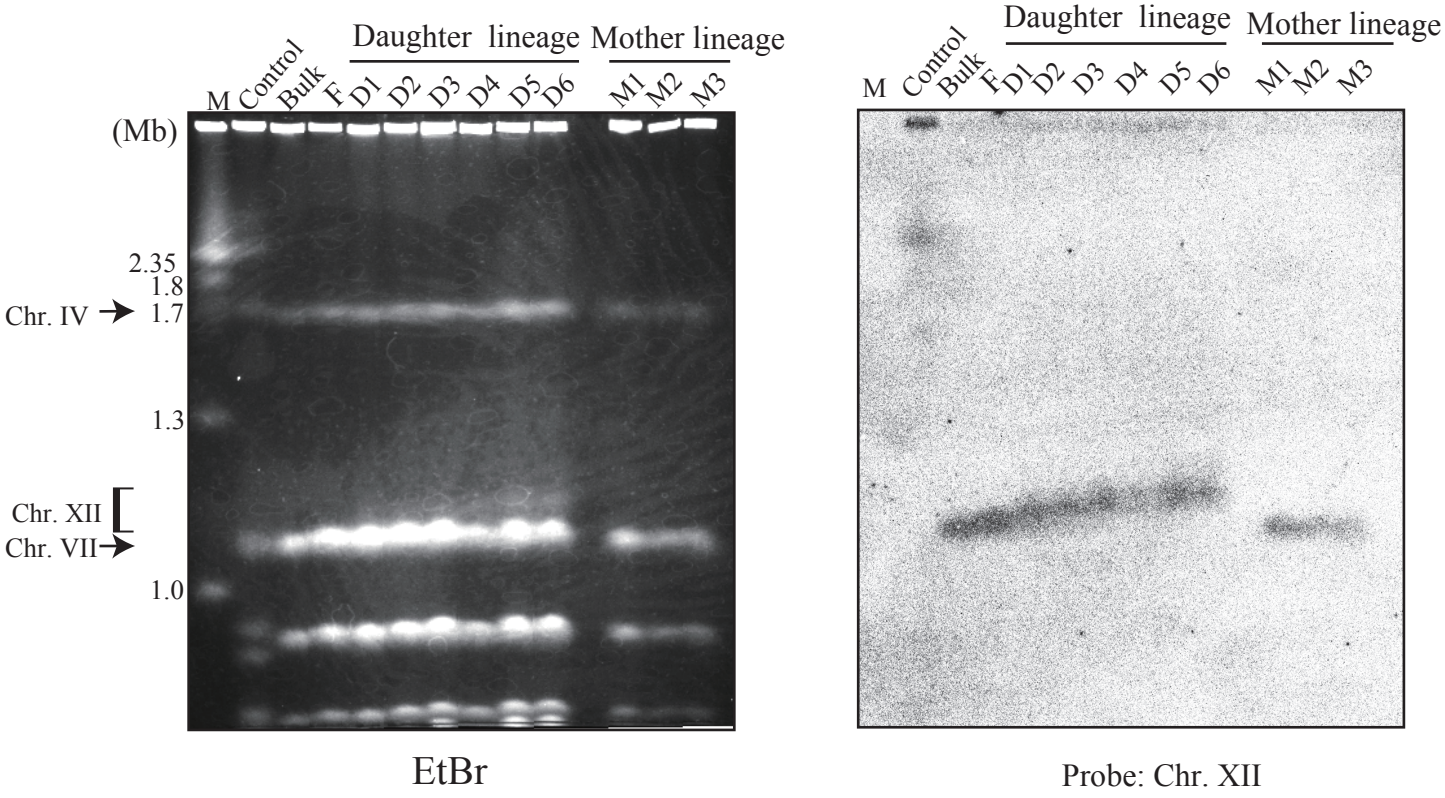
A. Schematic diagram of the separation of the daughter- and mother-lineage cells by the sequential centrifugal elutriation. The two lineages were separated after the founder cells gave rise to daughter cells. **(The daughter-lineage)** The daughter of the founder cells (D1) was obtained by elutriation and divided once. Then, the second elutriation was performed to obtain the daughter of the D1 cells (D2). The D2 cells divided once and the culture was separated by third elutriation to obtain the daughter of the D2 cells (D3). Likewise this way, elutriation was repeated to collect daughter cells until obtain the great-grand-child of the D3 cells (D6). **(The mother-lineage)** The founder cells were re-collected by elutriation after they gave rise to the daughter cells. These cells were defined as M1. The M1 cells divided once and separated into daughter and mother cells. The mother (once divided M1 cells) cells was defined as M2. The same process was repeated once more to obtain the once divided M2 cells (M3). The D1~6 cells consist of the youngest daughter cell line originated from the founder cells, while the M1~3 cells are the founder cells that underwent several cell divisions. According to the technical difficulty, I could not obtain M4~6 cells in the mother-lineage. **B.** The rDNA copy number was monitored in the daughter- (D1~6) and mother-lineage (M1~3) cells. The chromosomal DNAs were digested with *Bam*HI and rDNA copy number was estimated by Southern hybridization with IGS1 probe. In the daughter-lineage, in addition to the 2 and 5 copies of rDNA repeats, signals of 8, 9, and 15 copies were newly detected. On the other hand, in the Mother-lineage, the rDNA copies were remained in 2-copy in the majority of cells. **Raf**, and **Gal** indicate the bulk yeast culture grown in

raffinose and galactose medium, respectively. **C.** Relative signal intensity among the 2- to 15-copy rDNA was compared in the daughter-lineage. The signal intensity was normalized in each fraction by using the 2-copy rDNA signal as a standard.

A.



B.



M: *Hansenula wingei* chromosomal DNA

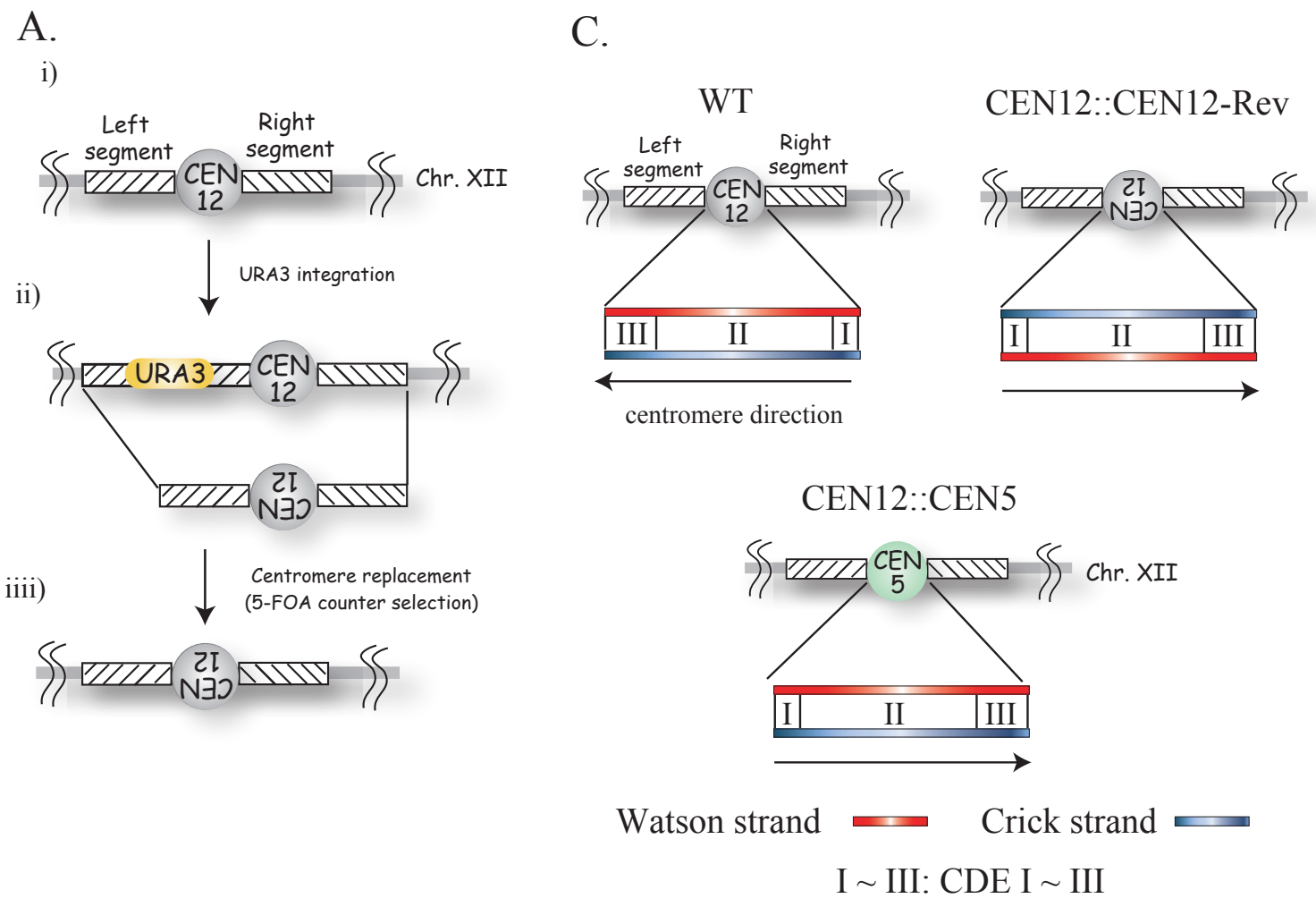
Control: *S. cerevisiae chromosomal* DNA (150-copy rDNA)

F: Founder cell

Fig. 8. The inheritance of the Chr. XII sister chromatids is non-random.

Fig. 8. The inheritance of the Chr. XII sister chromatids is non-random.

A. Schematic diagram of the separation of the Daughter- and Mother-lineage cells by the sequential centrifugal elutriation. **B.** Size of the Chr. XII in the daughter- and mother-lineage was compared. The native chromosomal DNA (undigested chromosome) of D1~6 and M1~3 cells were run with CHEF electrophoresis. The Chr. XII was detected by Southern hybridization with Chr. XII probe. **M:** *Hansenula wingei* chromosomal DNA, **C:** control *S. cerevisiae* chromosomal DNA (150 copies of rDNA units), **Bulk:** bulk yeast culture before separating the founder cell, **F:** founder cell.



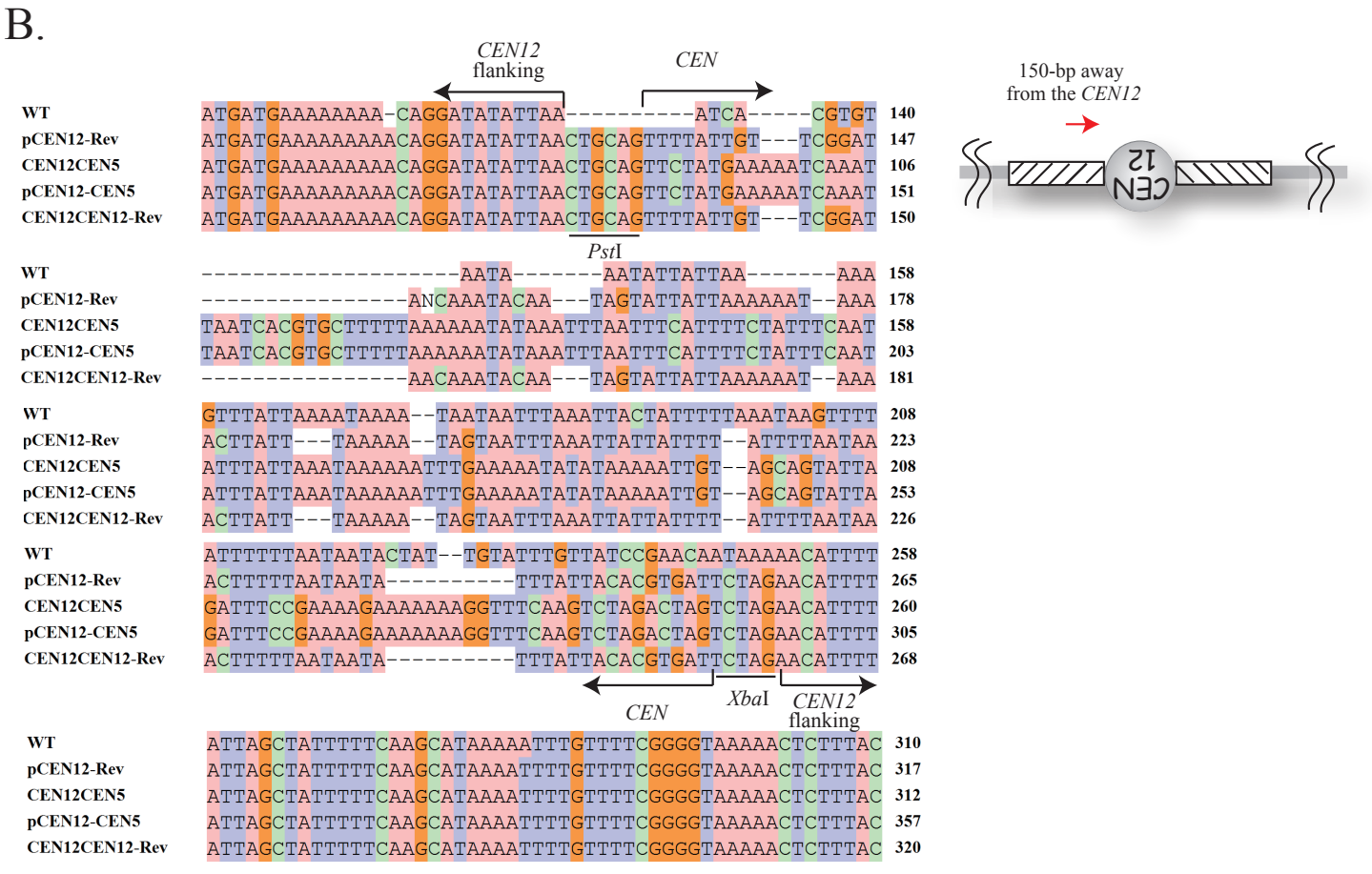


Fig. 9. Modification of chromosome XII centromere (*CEN12*)

Fig. 9. Modification of chromosome XII centromere (*CEN12*)

A. The methodology for *CEN12* modification. Left and right part of *CEN12* adjacent DNA sequences were depicted as L- and R- segment. **i)** The *URA3* ORF was introduced into the middle of the L-segment (~400-bp distant from CEN12). **ii)** Then, the *URA3* ORF was substituted with the PCR fragment that harbors L- and R- segment with modified *CEN* sequences. **iii)** The 5-FOA resistant cells were counter selected to obtain the *CEN12* substituted (*ura*-) cells. **B.** The results of sequence analysis in the *CEN12* modified strains were shown. The *CEN12* context was sequenced in WT, CEN12::CEN12-Rev, CEN12::CEN5, and vectors. The results were aligned to compare the difference between them. **C.** Structural difference of the *CEN12* region in the constructed strains. The direction of the centromere CDE core elements is indicated with arrow, and Watson/Crick strands are distinguished with red and blue bar, respectively. Compared with WT *CEN12* (left-forward *CEN*), CEN12::CEN12-Rev and CEN12::CEN5 strain have right-forward *CEN* element on the Chr. XII. Moreover, in the CEN12::CEN12-Rev strain, the Watson and Crick DNA strand is inverted from the WT.

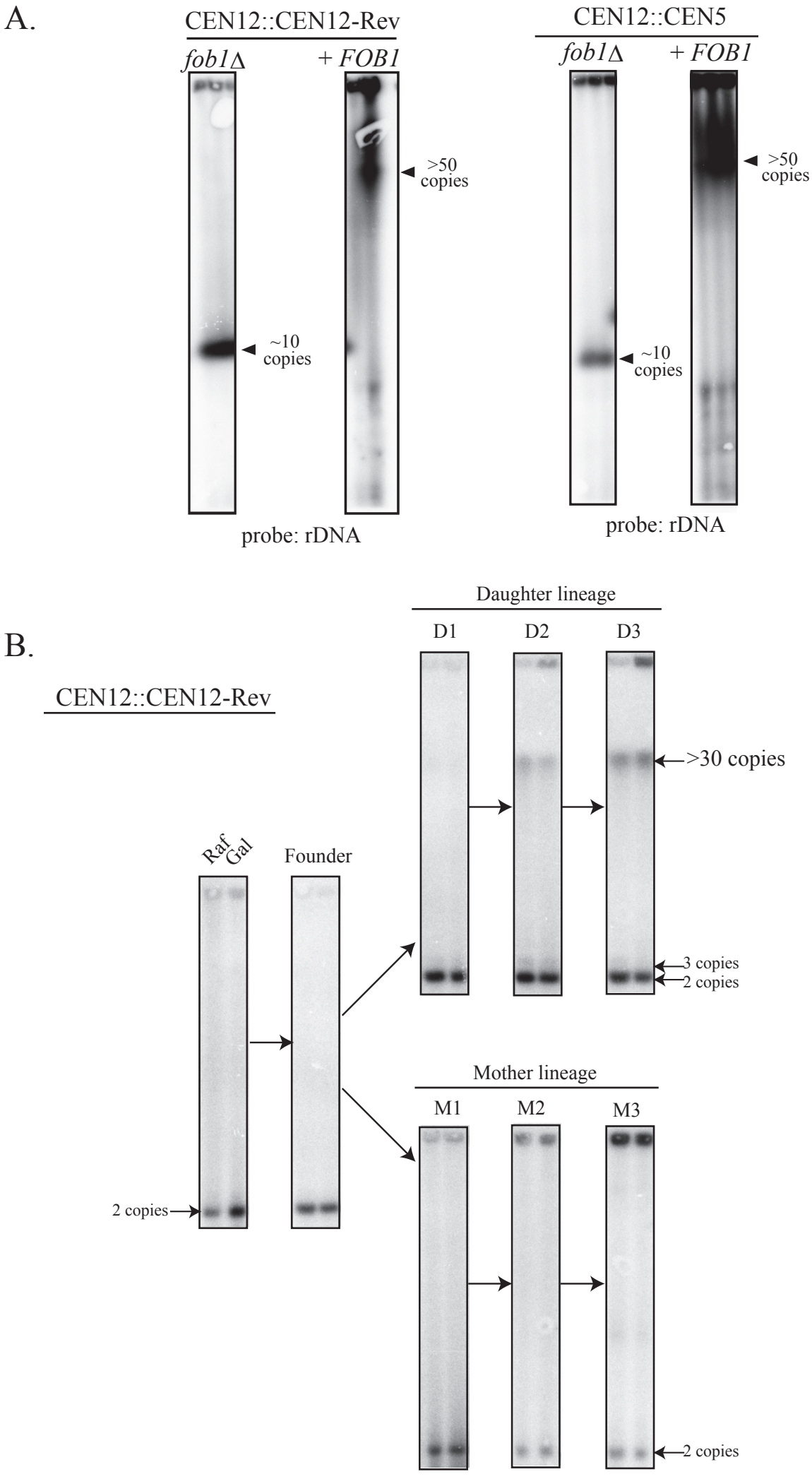


Fig. 9-2. rDNA amplificaton in the *CEN12* modified strains

Fig. 9-2. rDNA amplification in the *CEN12* modified strains

A. The rDNA amplification in the *CEN12* modified strains was investigated. The state of the Chr. XII in *CEN12* modified strains were compared in *fob1Δ* and *+FOB1* condition. **B.** The pattern of sister chromatids segregation in the *CEN12*::*CEN12*-Rev strain. The rDNA copy number was monitored in the daughter- (D1 ~ 3) and mother-lineage (M1 ~ 3) cells in *CEN12*::*CEN12*-Rev. The chromosomal DNA was digested with *Bam*HI and rDNA copy number was estimated by Southern hybridization with IGS1 probe. The estimated rDNA copy number was depicted in the side of gel image.

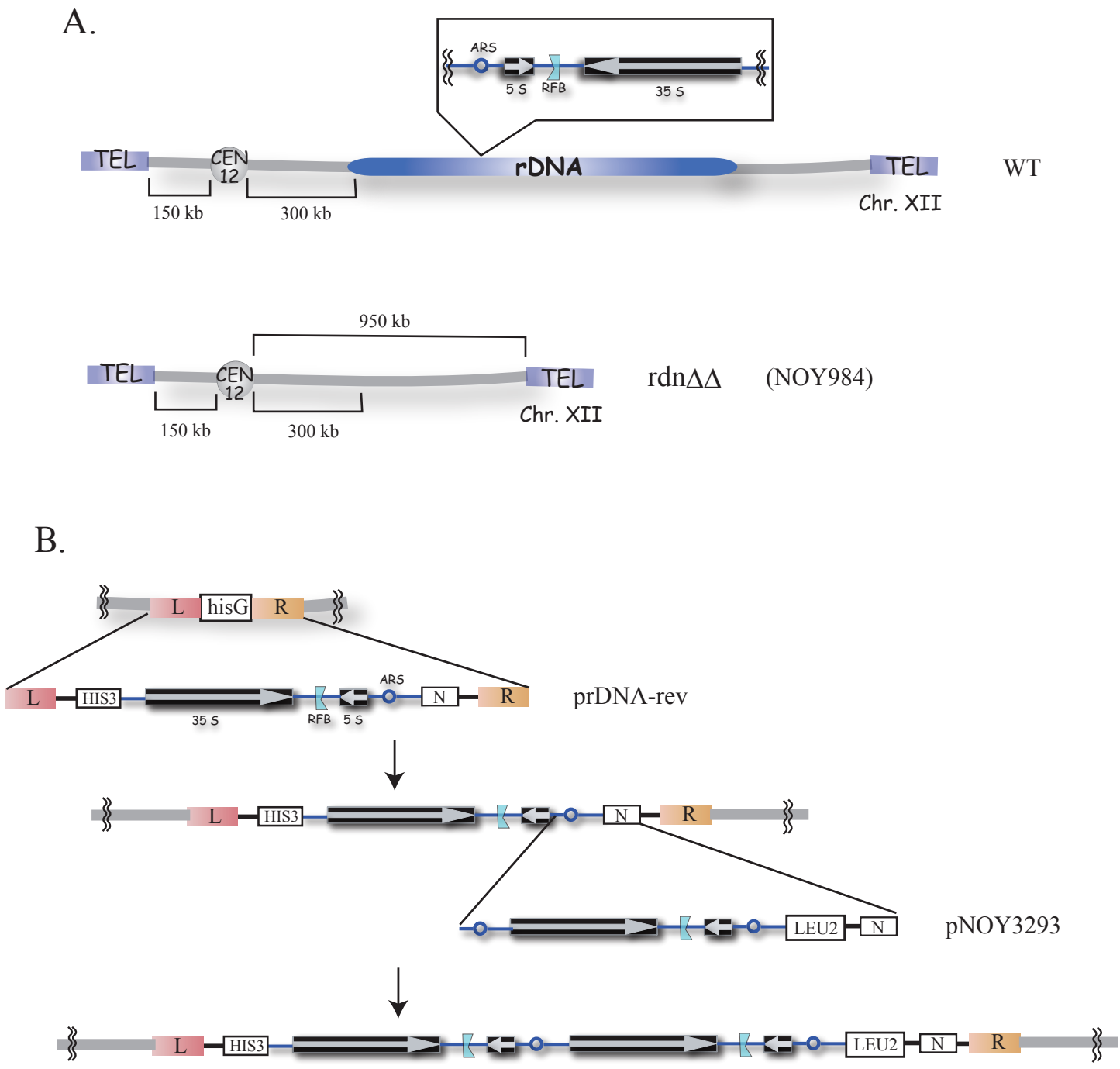


Fig. 10. Construction of reverse-directed rDNA on Chr. XII

Fig. 10. Construction of reverse-directed rDNA on Chr. XII

The rDNA repeats were reconstructed on the Chr. XII to align opposite direction. The strategy for this modification was basically followed by *oakes et al.* (2006). **A.** The structure of the Chr. XII in WT and *rdnΔΔ* (NOY984) strain. In the NOY984, rDNA repeats are completely lost from the Chr. XII. **B.** General strategy used for the integration of rDNA into the *RDN1* locus. Two separate steps and DNA fragments used for integration of rDNA were shown. The sequences flanking the site of integration are shown as L and R. For integration of rDNA at the original *RDN1* site in *rdn*ΔΔ::*hisG* strains, the *Escherichia coli hisG* sequence is between L and R flanking sequences. The rDNA cassette (prDNA-Rev) that consists of an entire 9.1-kb rRNA gene copy flanked by the *HIS3* gene and a non-yeast sequence N from *A. thaliana*, was used after digestion with *Sal*I for first step of rDNA integration. This produces 1-copy inverted rDNA on the Chr. XII. The DNA fragment used for the second step is an ~13-kb fragment obtained after digestion of pNOY3293 with *Spe*I and *Sal*I. PCRs using primer pair 182/185 produce 1,800-bp fragments for correct integration at the second step.

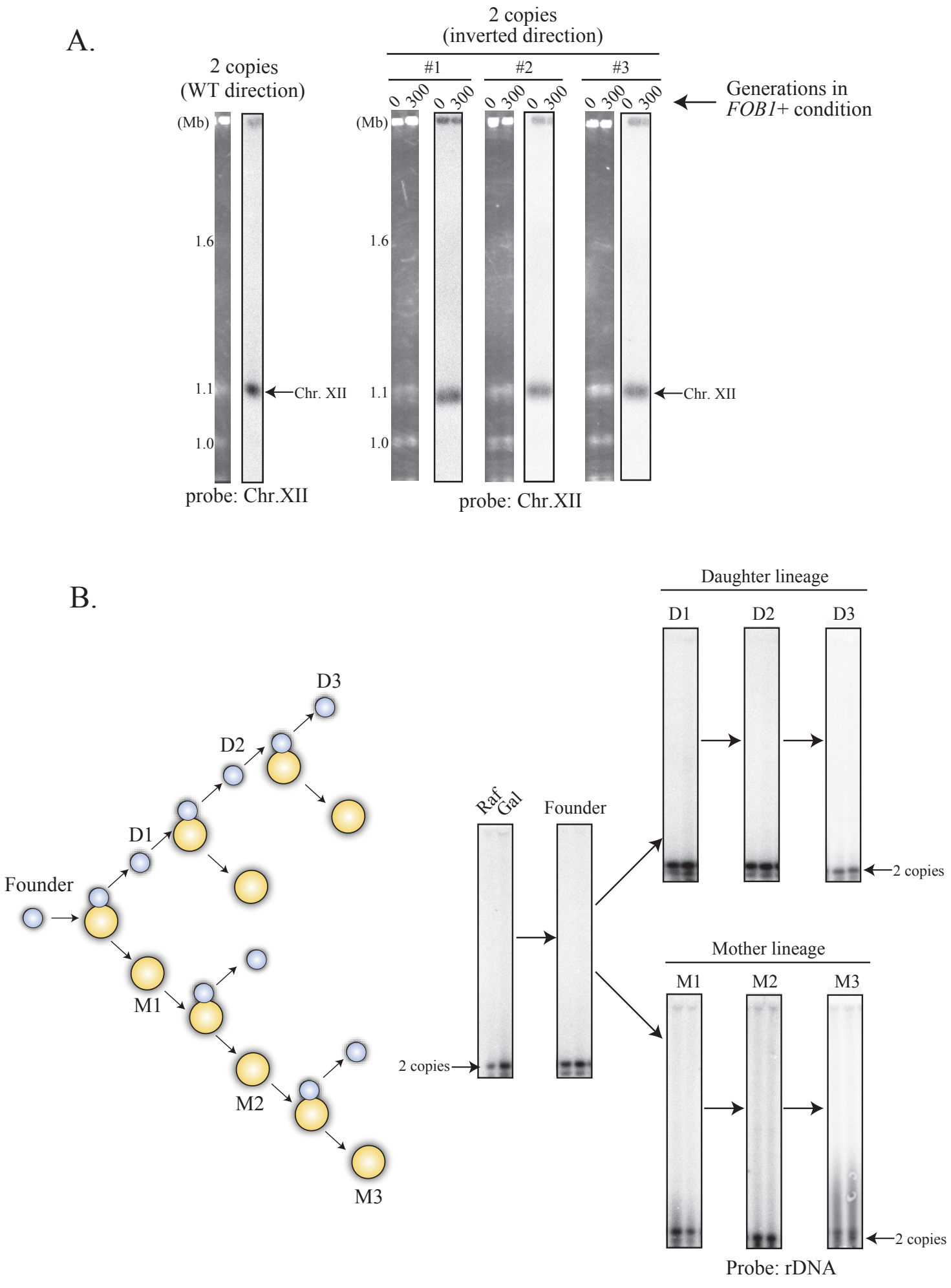


Fig. 11. Inhibition of copy number expansion in the reverse-directed rDNA units

Fig. 11. Inhibition of copy number expansion in the reverse-directed rDNA units.

Fob1 protein expression was induced in the rDNA-rev2 strain, which has two reverse-directed rDNA units. Subsequent rDNA copy number expansion was observed and compared with other 2-rDNA copy strains. **A.** rDNA-rev2 cells were cultured in *FOB1+* condition for ~300 generations, and size of the Chr. XII was detected by Southern hybridization. The analysis was performed in independent 3 transformants. **B.** The rDNA copy number was monitored in the daughter- (D1 ~ 3) and mother-lineage (M1 ~ 3) cells in rDNA-rev2. The chromosomal DNA was digested with *Bam*HI and rDNA copy number was estimated by Southern hybridization with IGS1 probe. The estimated rDNA copy number was depicted in the side of gel image.

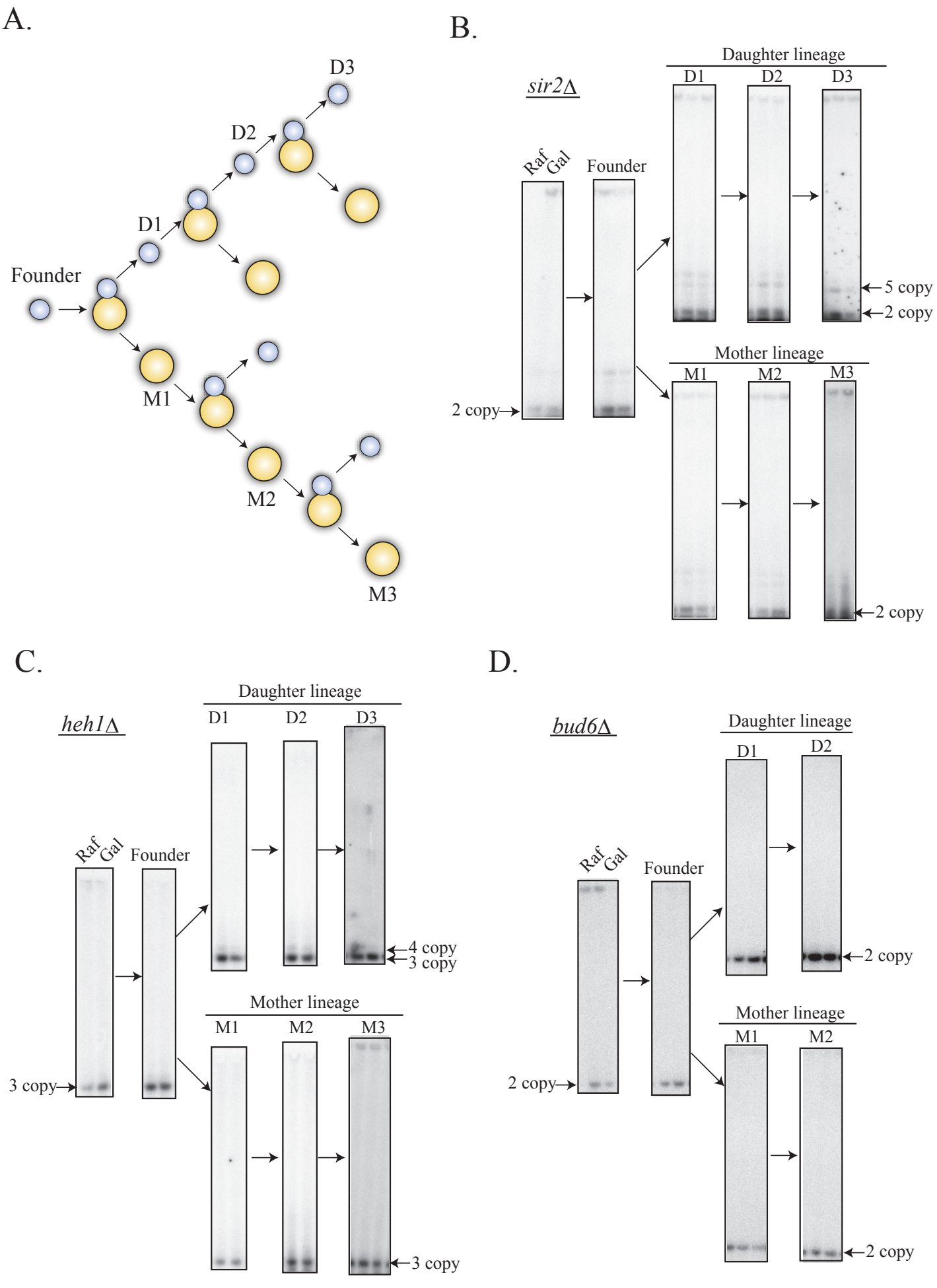


Fig. 12. Sister chromatid segregation in the mutants that destabilize rDNA

Fig. 12. Sister chromatid segregation in the mutants that destabilize rDNA.

The pattern of amplified rDNA inheritance was monitored in the *sir2*, *heh1*, and *bud6* mutants. **A.** Schematic diagram of the separation of the daughter- and mother-lineage cells by the sequential centrifugal elutriation. **B-D.** The rDNA copy number was monitored in the daughter- (D1~2 or 3) and mother-lineage (M1~2 or 3) cells in *sir2*, *heh1*, and *bud6* mutants. The chromosomal DNAs were digested with *Bam*HI and rDNA copy number was estimated by Southern hybridization with IGS1 probe. The estimated rDNA copy number was depicted in the side of gel image.

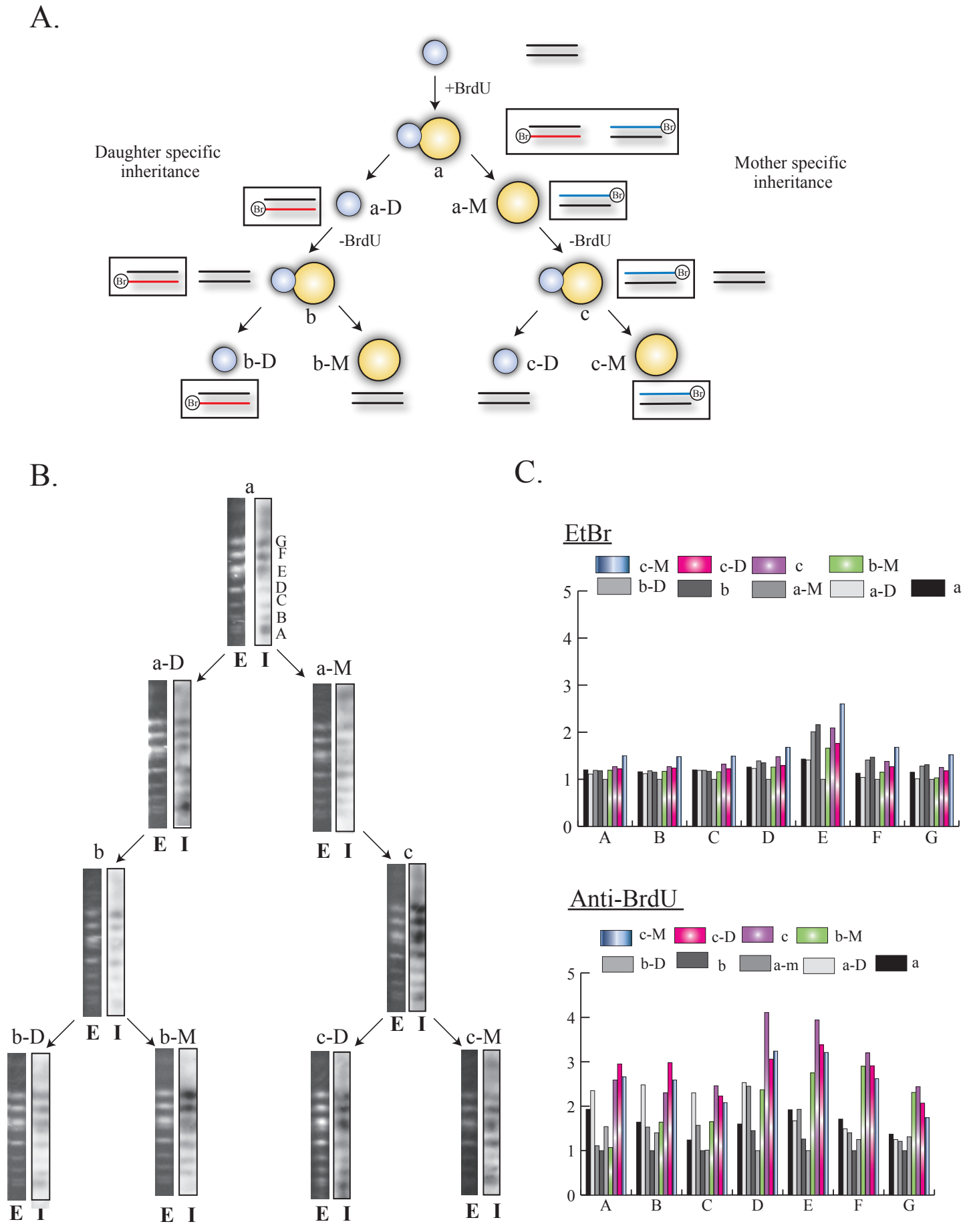


Fig. 13. BrdU Pulse-chase analysis for tracing the segregation pattern of whole chromosomes

Fig. 13. BrdU Pulse-chase analysis for tracing the segregation pattern of whole chromosomes. **A.** A model of hypothetical non-random BrdU retention. The BrdU-labeled DNA strand was depicted in red and blue bar, and the native DNA strand was in black bar. The DNA pulse labeling with BrdU produces the heteroduplex sister chromatids which one of two DNA strand (Watson or Crick strand) was labeled with BrdU (upper part). If the chromosomes are non-randomly inherited to the progenies, the daughter- or mother-lineage specific retention of BrdU-labeled chromosome takes place (middle). In such a case, the retained chromosomes are segregated to next generation with the same manner and BrdU-labeled DNAs are inherited as mirror image between the daughter- and mother-lineage (bottom). **B.** The daughter- and mother-lineage cells were separated by the centrifugal elutriation to collect the cells corresponding to a ~ c-M in Fig. 13A. The chromosomal DNA of the cells was run with CHEF electrophoresis and incorporated BrdU was detected by anti-BrdU antibody. **C.** Comparison of the signal intensity pattern between EtBr stained DNA and BrdU immunodetection. The signal intensity of the EtBr stained DNA and BrdU immunodetection was measured at the position of A~G in Fig. 13B. The results were normalized among the cell fraction using the lowest signal as a standard.

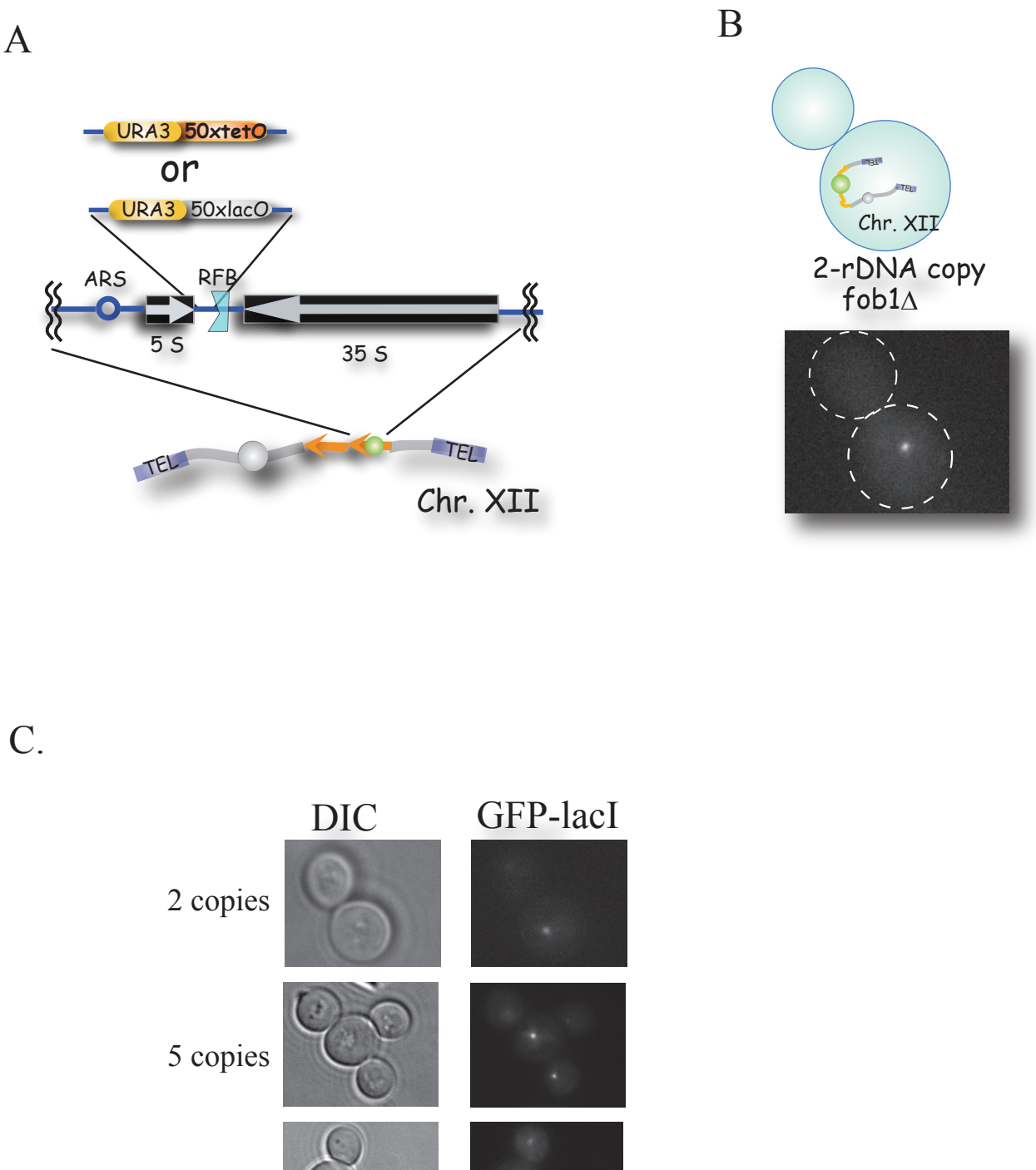
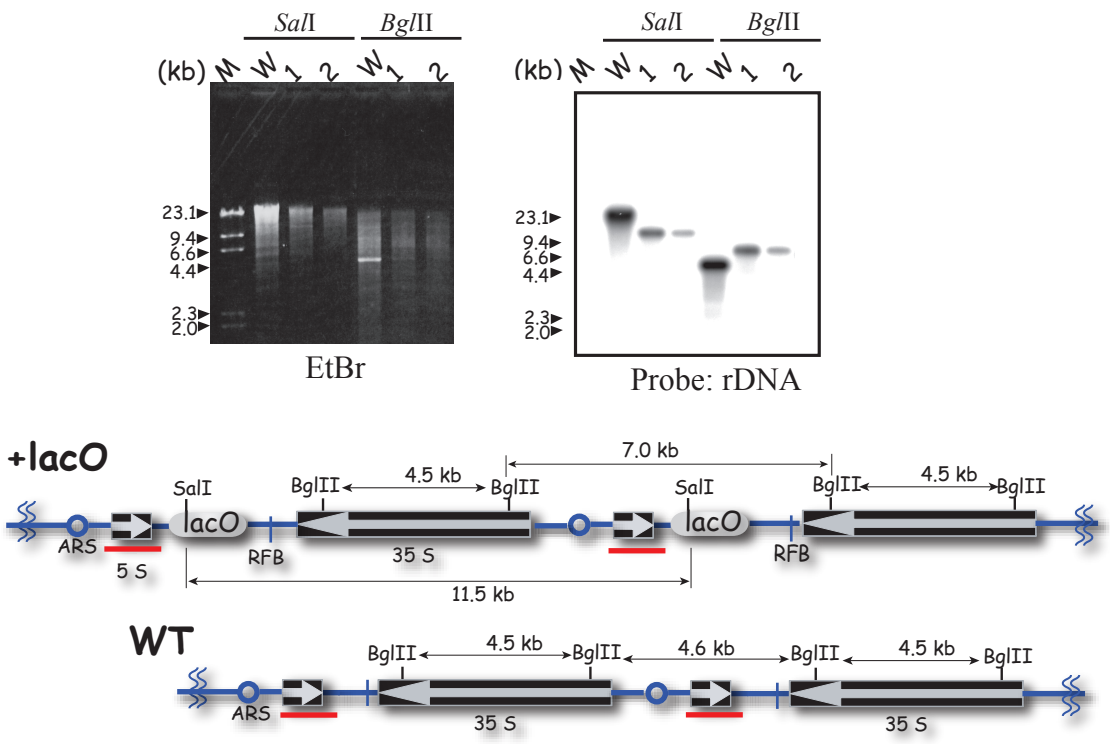


Fig. 14. Develoment of New rDNA labeling technique using *lacO* / *lacI*-GFP system

Fig. 14. Development of new rDNA labeling technique using lacO / lacI-GFP system.

Strategy of the novel bioimaging technique for rDNA visualization was shown. **A.** General strategy used for the insertion of *lacO* array into the IGS1 region. The cassette that consists of 50x *lacO* array flanked by the *URA3* gene and the sequences flanking the site of insertion was used after digestion with *Kpn*I and *Hin*dIII. This construct was introduced into the one of two rDNA copy in TAK201 (2-rDNA copy, *fob1Δ*). **B.** Observation of the localization of inserted lacO array in a yeast cell. The *lacO* array in the rDNA region was visualized with GFP-lacI fusion protein. Single dot appeared in the cell indicating the position of *lacO* array. **C.** Expansion of the *lacO*-labeled rDNA unit and its result. The rDNA amplification was induced in the 2-rDNA copy strain, which one of two rDNA units was labeled with *lacO*. The difference was compared among the cells with 2, 5, 10, and 20-copy rDNA by visualizing the *lacO* array with GFP-*lacI*.

A



B.

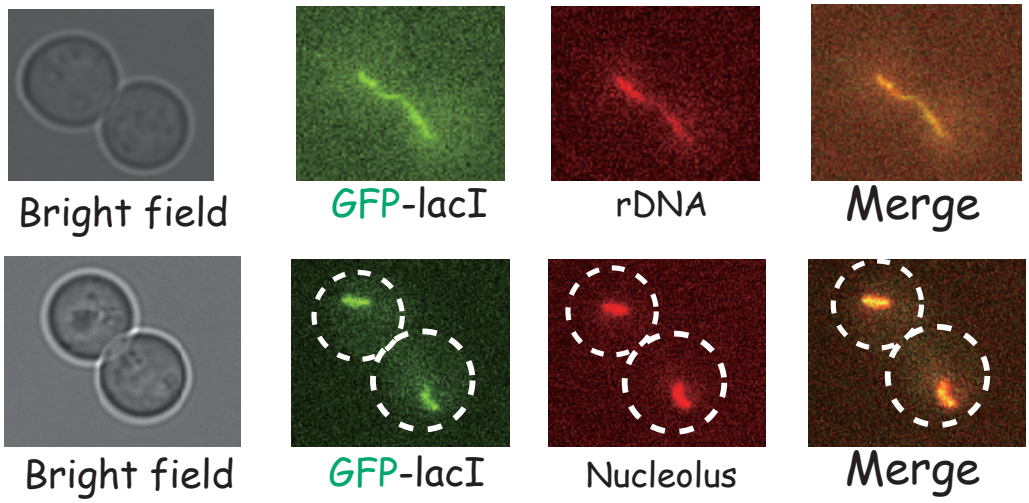


Fig. 15. Observation of the rDNA units that is completely labeled with *lacO*

Fig. 15. Observation of the rDNA units that is completely labeled with *lacO*

The number of rDNA repeats was recovered to ~150-copy after the rDNA was labeled with *lacO* in 2-rDNA copy strain. The state of the rDNA units was examined in that strain (TM1). **A.** Restriction enzyme digestion for confirming the *lacO* integration pattern in TM1. Chromosomal DNAs were purified from WT and TM1 strain and digested with *Bgl*II or *Sal*I. And then, to compare the structure of individual rDNA unit, Southern hybridization with IGS1 probe was performed. As depicted in the restriction map of the rDNA unit (bottom), digestion with the two enzymes produces specific signal in WT and *lacO*-inserted rDNA unit. When the rDNA units were digested with *Bgl*II, both sides of *lacO*-inserted site were cleaved to give rise to 4.6- and ~7.0-kb fragment in WT and *lacO*-inserted unit, respectively. On the other hand, *Sal*I cleaves one site in the *lacO* array, but not in rDNA unit. If the whole rDNA units were completely labeled with *lacO* array, *Sal*I digestion produces ~11.5-kb fragment. If there are any 'blank' rDNA units that are not labeled with *lacO*, > 21-kb fragment is detected. The probe that is used for Southern hybridization was depicted with red bar. **M:** λ / *Hin*dIII size standard, **W:** WT rDNA units, **1,2:** strains with *lacO*-inserted rDNA units. **B.** Co-localization of the GFP-*lacI* signal with yeast nucleolar proteins. Signal of GFP-lacI in the TM1 strain was compared with representative nucleolar proteins. Fluorescent protein (mCherry) was fused to the C-terminus of rDNA marker (*NET1*) and nucleolar marker (*SIK1*) gene, and visualized under the microscope. The merged image indicates the GFP-*lacI* signal colocalize with both of the proteins in nucleus.

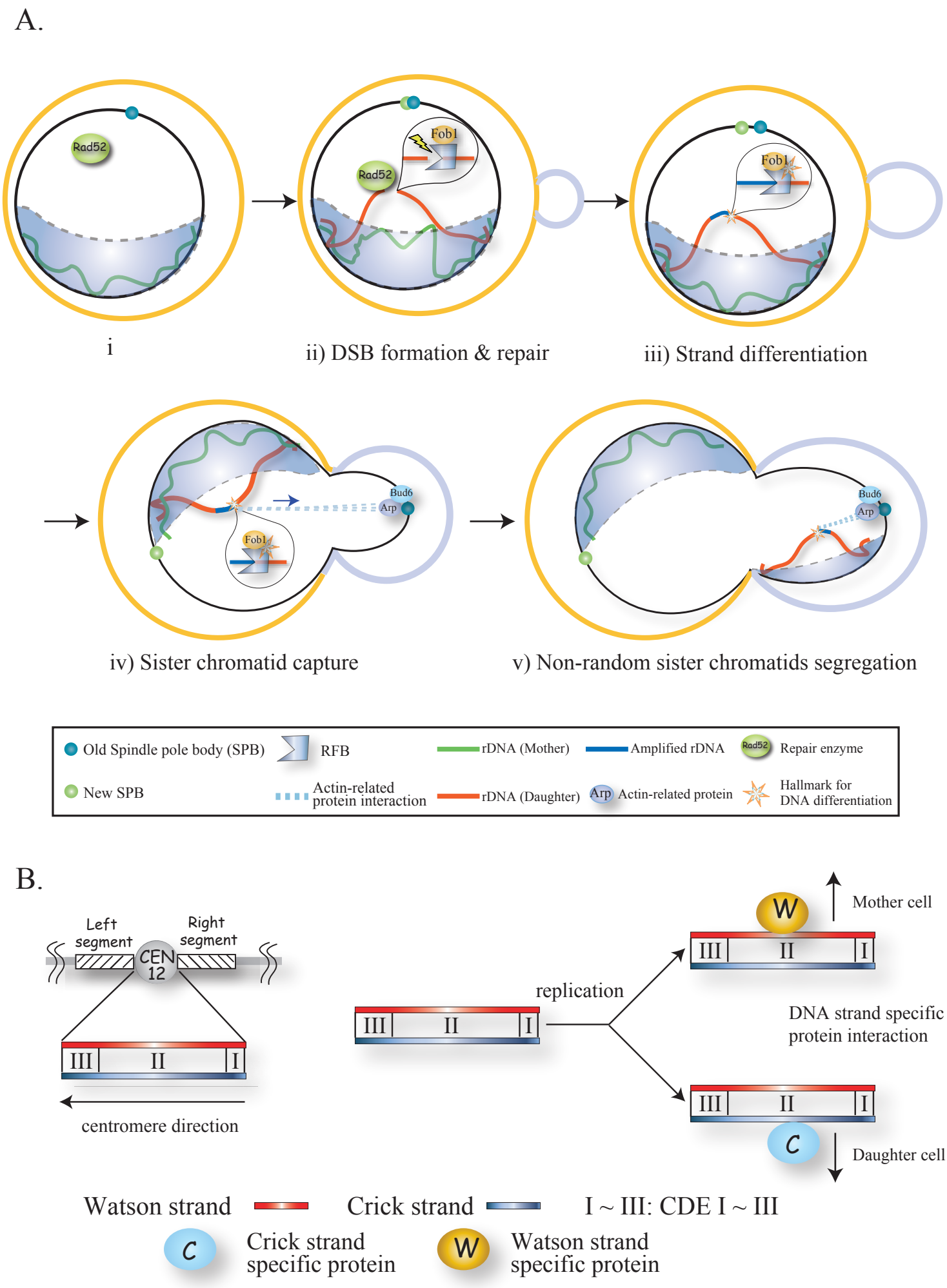


Fig. 16. Models for asymmetric sister chromatids segregation in *S. cerevisiae*

Fig 16. Models for the mechanism of non-random sister chromatid segregation in *S. cerevisiae.*

Ideas for the mechanisms of non-random sister chromatids segregation were summarized as a model. **A.** A model for the rDNA directed asymmetric sister chromatids segregation. **I.** Before a cell enters into S-phase, the rDNA region is compounded by nucleolus and sequestered from repair enzyme (Rad52). **II.** In S-phase, DSB is formed near the RFB sequence in a Fob1 dependent manner, and positioning of the rDNA alters to permit the access of Rad52. **III.** The number of rDNA copies is amplified by unequal sister chromatids recombination. Additionally, the amplified rDNA units are molecularly 'differentiated' by epigenetic modification. **IV.** The 'differentiated' rDNA is captured by the actin- (or dynein-) related proteins that determine the polarity of sister chromatids segregation. Some of the daughter specific proteins including Bud6 pull the sister chromatid with amplified rDNA toward the daughter side through the functions of actin-network (or dynein motor activities). **V.** The sister chromatid with amplified rDNA is preferentially segregated toward the daughter cell by the guidance of the actin-network (or dynein), thus results in non-random sister chromatid segregation. **B.** A model for the centromere dependent asymmetric sister chromatids segregation. As Thorpe et al. (2009) indicated and I suggested, the Watson / Crick DNA strands of *CEN* sequence can be distinguished in the yeast. If the interaction of kinetochore proteins with *CEN* is carried out in Watson (or Crick) strand specific manner, the sister chromatids can be non-randomly segregated.