# Cytoplasmic microtubular system implicated in de novo formation of a Rabl-like orientation of chromosomes in fission yeast

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## SUMMARY

**Chromosomes are not packed randomly in the nucleus. The Rabl orientation is an example of the non-random arrangement of chromosomes, centromeres are grouped in a limited area near the nuclear periphery and telomeres are located apart from centromeres. This orientation is established during mitosis and maintained through subsequent interphase in a range of species. We report that a Rabl-like configuration can be formed de novo without a preceding mitosis during the transition from the sexual phase to the vegetative phase of the life cycle in fission yeast. In this process, each of the dispersed centromeres is often associated with a novel Sad1-containing body that is contacting a cytoplasmic microtubule laterally (Sad1 is a component of the spindle pole body (SPB)). The Sad1-containing body was colocalized with other known SPB components, Kms1 and Spo15 but not with Cut12, indicating that it represents a novel SPB-related complex. The existence of the triplex structure (centromere-microtubule-Sad1 body) suggests that the clustering of centromeres is controlled by a cytoplasmic microtubular system. Accordingly, when microtubules are destabilized, clustering is markedly reduced.**

Key words: Centromere, Telomere, Fission yeast, Spindle pole body

## INTRODUCTION

A number of observations reveal that chromosomes are not randomly packed in the nucleus. The spatial distribution of chromosomes within a nucleus is dynamic. Chromosomal location is regulated according to the cell cycle, cell type, or environmental change (Manuelidis, 1990; Cremer et al., 1993; Vourc'h et al., 1993; Dernburg et al., 1995; Janevski et al., 1995; Therman, 1995). Specific local interactions between chromosomes and between chromosomes and nuclear matrices are thought to contribute to the formation of global chromosome arrangements (Marshall et al., 1997). Centromeres and telomeres probably represent important chromosomal domains that are required for regulating chromosome arrangement. Two representative examples, the Rabl orientation and the bouquet arrangements (Comings, 1980; Dernburg et al., 1995; Franklin and Cande, 1999), support this notion. Both share a common feature in that chromosomes are arranged in a polarized manner; centromeres and telomeres are more or less grouped near the nuclear membrane and they are located apart from each other. The bouquet arrangement is formed during the early prophase of meiosis and implicated in efficient homologous pairing (Loidl, 1990; Dernburg et al., 1995; Scherthan, 1997; de Lange, 1998; Niwa et al., 2000). The functional significance of the Rabl orientation is not known.

Alterations in chromosome distribution and conformation are not independent of the preceding condition. There are some cases, however, where such dependency is more apparent. For example, the fact that each chromosome occupies a confined space, a territory, or compartment in an interphase nucleus probably reflects the process of decondensation of condensed chromosomes produced in the previous mitosis. Likewise, the successful condensation of chromosomes might be dependent on such territorialization. The Rabl orientation is an example of a more intimate relation to the previous event, because this orientation is established during mitotic anaphase and maintained through subsequent interphase with decondensed chromosomes. In *Drosophila* larvae, the Rabl orientation persists early in the G1 phase but, in the late G1 phase, it resolves into a non-Rabl configuration, apparently through a process in which the role of heterochromatin is dominant (Csink and Henikoff, 1998). This suggests that the lack of observations of the Rabl orientation in many species, including human tissue cells, is attributable to the short duration of the Rabl configuration after mitosis.

We are interested in the mechanisms involved in the formation of the Rabl configuration, using the fission yeast *Schizosaccharomyces pombe*. In vegetatively proliferating fission yeast cells, centromeres are grouped in a tight cluster, which is formed near the nuclear periphery beneath the spindle pole body (SPB; the centrosome equivalent structure in yeast), and telomeres are positioned apart from the SPB. The clustering of centromeres is disrupted only during the early phase of mitosis and re-established in anaphase (Funabiki et al., 1993). Thus, the Rabl orientation is maintained stably throughout

interphase in this yeast. It is not known if the Rabl configuration is a prerequisite for successful mitosis in this yeast.

Depending on the presence of opposite mating types of cells and nutritional starvation, haploid fission yeast cells are arrested in the G1 phase and conjugate to form zygotes containing a diploid nucleus, and then immediately proceed into meiosis/sporulation (Yamamoto et al., 1997). Before the formation of the diploid nucleus in the zygote, there is a dynamic rearrangement of chromosomes in which telomeres and centromeres switch their positions in the nucleus. This switching occurs in a two-step process (Chikashige et al., 1997). First, before conjugation under the influence of the sex pheromone, telomeres gather at the SPB where centromeres are also clustered. In the next step, as a conjugation-related process, centromeres are released from the SPB to form the ‘bouquet’ arrangement. Thus, the bouquet-like arrangement is formed in fission yeast before entry into meiotic prophase. The bouquet configuration is also formed directly from diploid cells without intervening conjugation, although it is not known whether this is a two-step process. The mechanisms underlying telomere clustering remain to be elucidated. When zygotes are placed in rich media before commitment to meiosis, they enter into the vegetative cell cycle of diploid cells. At some stage during this return-to-growth process (RTG), chromosomes must conform to the Rabl orientation. The present study investigated whether this reformation of the Rabl orientation is dependent on the first mitosis in RTG and, if not, the mechanism involved in the chromosomal rearrangement.

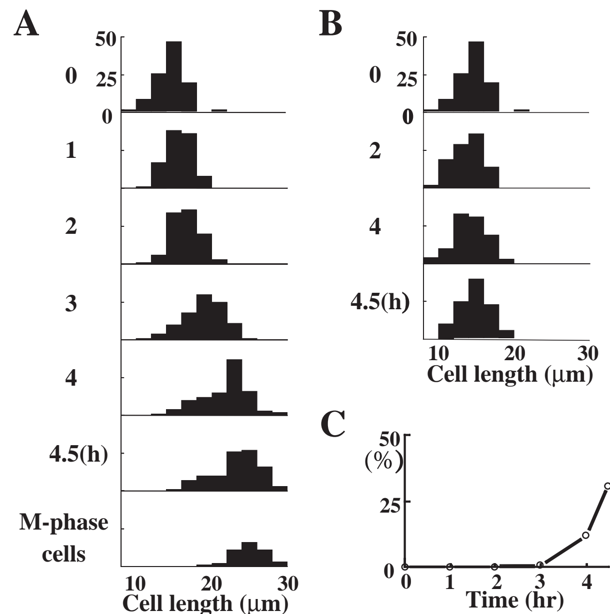



**Fig. 1.** Increase of cell length during RTG. Number of cells with indicated length after the transfer to the rich medium (A) and those kept in the conjugation medium (B). Percentages of cells containing a spindle are shown in (C).

## MATERIALS AND METHODS

### Strains and media

Strains used in this study: $h^{90}$ *mei1-B102 leu1-32* (BG990), $h^{90}$ *mei1-B102* (BG991), $h^{90}$ *mei1-B102 cdc25-22* (BG992), $h^{90}$ *mei1-B102 cut12-M33GFP::ura4*$^+$ (BG993). The strain carrying the *cut12-M33GFP::ura4*$^+$ was kindly provided by Iain Hagan (Bridge et al., 1998). Plasmid pTN225 carrying GFP-tagged *spo15*$^+$ (Ikemoto et al., 2000) was provided by Chikashi Shimoda, and was used to integrate this fused gene into BG990 to create $h^{90}$ *mei1-B102 spo15-GFP::LEU2* (BG994). Other plasmids used were pDQ105, which carried α-tubulin-GFP gene (obtained from Y. Hiraoka) and pGK157, carrying *kms1-GFP* (M. Shimanuki, unpublished). The Media used were YE (Moreno et al., 1991) (3% glucose, 0.5% yeast extract (Difco)), MEB-Gal (Miyata et al., 1997) (2% malt extract (OXOID), 1% galactose), and MSM (Miyata et al., 1997) (1% galactose, 0.4% mannose, 0.15% $KH_2PO_4$, vitamins as in EMM (Moreno et al., 1991)). Phloxin B plate was described previously (Moreno et al., 1991).

### Culture conditions

All incubations were at 25 or 26°C, unless otherwise indicated. Cell conjugation was induced as described previously (Miyata et al., 1997). Cells were cultured in MEB-Gal to a cell concentration of $1\times10^7$/ml. Cells were washed once with 0.1% glucose and resuspended in MSM at a concentration of $6\times10^7$/ml and incubated for approximately 6 hours until the appropriate number of zygotes was produced. The MSM culture was diluted in YE at $5\times10^6$/ml (time 0 of the RTG). When the *cdc25* mutant was used, the YE culture was transferred to 35°C, 2 hours after the nutritional shift. 100 μl of thiabendazole (Sigma) solution (30 mg/ml in dimethyl sulfoxide (DMSO); Nacalai, Kyoto) was added to 30 ml of the YE culture at the time of the temperature shift. For controls, the same amount of DMSO was added.

To monitor diploid formation of the zygotes, individual zygotes were separated on a YE plate and incubated at 26°C for 4 days (for the *cdc25* mutant 22°C for 6 days). Ploidy of each colony was tested using a flowcytometric method (FACScan) as well as the phloxin B plating method (Moreno et al., 1991).

### Live observation of spindle elongation

BG990 was transformed with pDQ105 (α-tubulin-GFP) and induced for conjugation in MSM medium. Cells were transferred to YE supplemented with 100 μM of thiamine and incubated for 4 hours at 26°C. Cell suspension in the same medium was put on a coverslip-bottomed petri dish and observed with a fluorescence microscope. Images of GFP-labelled spindles were taken every 30 seconds at 25-26°C, with occasional manual focusing. To see spindle elongation in vegetatively proliferating cells, diploid cells were established from the same strain and subjected to live analysis under the same condition. The length of a spindle was measured when both of the spindle ends were in focus or near in focus.

### Indirect immunostaining and fluorescent in situ hybridization

The procedures used in this study were a slight modification of those described previously (Funabiki et al., 1993; Chikashige et al., 1997). Glutaraldehyde and formaldehyde (final concentration of 0.2% and 3%, respectively) were added to a cell culture containing $1\text{-}5\times10^8$ cells, followed by incubation for 1 hour at the same temperature of the culture. Fixed cells were incubated twice in 1 ml of PEM (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$), containing 100 mM glycine to quench unreacted aldehyde groups. Cells were digested with Zymolyase-100T (Seikagaku) and Lysing Enzymes from *Trichoderma harzianum* (Sigma) at 36°C for approximately 20 minutes. Digested cells were incubated in PEM containing 1% Triton X-100 for a few minutes at room temperature and then treated with RNase A. For indirect immunostaining, rabbit anti-Sad1 antibody (Okazaki et al., 2000) and anti-α-tubulin monoclonal antibody, TAT1 (Woods et al., 1989) were used as primary antibodies, and Oregon green (Molecular Probes, Eugene, OR) or Cy3- (Jackson Laboratory,

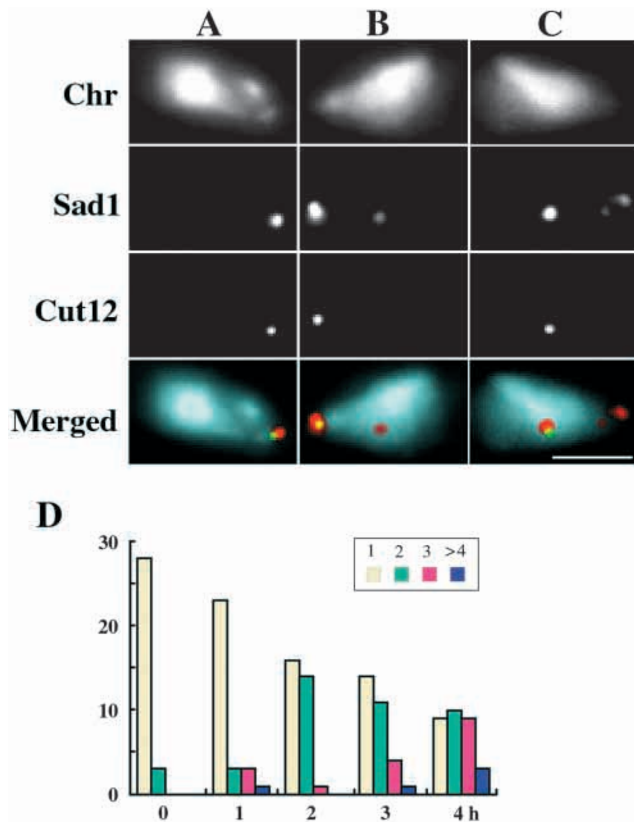



**Fig. 2.** Emergence of the Sad1-containing bodies in the zygotes of strain BG993. (A-C) Chr, DAPI; Sad1, anti-Sad1 antibody; Cut12, GFP-tagged Cut12; Merged: cyan, DAPI; red, Sad1; green, Cut12; overlapped region of Sad1 and Cut12, yellow. Bar, 2.5 µm. (A) 0 hours, (B,C) 4 hours after transferring to the rich medium. (D) Number of cells containing indicated number of the Sad1-bodies per nucleus at the indicated time after transfer.

West Grove, PA) conjugated anti-mouse IgG and Cy3- or Cy5-conjugated anti-rabbit IgG were used as secondary antibodies. When desired, FISH was performed for the immunostained cells, using Cy3- or Cy5-labeled DNA probes produced from cosmid 212 (for subtelomeric regions of chromosomes I and II; Funabiki et al., 1993, cosmid 1228 (for the centromere-proximal region of chromosome II; Mizukami et al., 1993), and pRS140 plasmid (for the centromeric repeats; Funabiki et al., 1993). Chromosomes were stained with 4′,6-diamidino-2-phenylindole (DAPI).

### Microscopy

The Delta Vision System used in the present study was previously described (Shimanuki et al., 1997). For the observation of each nucleus, optical sectioning was performed at intervals of 0.2 µm, computational removal of out-of-focus signals was performed with these images using a deconvolution function integrated in the system. Images presented in the figures were obtained by projecting relevant sections of the images on a single plane.

## RESULTS

The homothallic *S. pombe* strain that carryies the *mei1-B102* mutation, when induced into the sexual life cycle by nitrogen starvation, makes zygotes that contain a fused nucleus that is blocked before premeiotic DNA synthesis and thus unable to proceed to meiosis (Egel and Egel-Mitani, 1974; Yamamoto et al., 1997). The zygotes, however, can return to the vegetative cell cycle of a diploid cell when appropriate nutrition is re-fed. After the nutritional shift, the zygotes elongated until they became approximately 23 µm in length and then entered into the first mitosis in RTG (Fig. 1A,B). The rate of cell elongation was low up to 2 hours after the nutritional shift, but it became high thereafter as shown in Fig. 1A. First spindles appeared approximately 3 hours after the shift and the proportion of cells with a spindle became more than 30% at 4.5 hours (Fig. 1C). Septation followed the spindle formation about 40 minutes later. In the present study, we investigated the behaviour of chromosomes during RTG using fluorescence in situ hybridization (FISH) and immunostaining.

### Multiple Sad1-containing bodies emerge before the first mitosis

The Sad1 protein is a constitutive SPB component (Hagan and Yanagida, 1995). In vegetatively proliferating cells, the anti-Sad1 antibody produces a single signal in the nucleus except for during the period of nuclear division (Funabiki et al., 1993). In conjugation with the fission yeast, the SPB at the leading edge of each of the approaching nuclei serves to bring two haploid nuclei close (Hirata and Tanaka, 1982; Chikashige et al., 1994; Hagan and Yanagida, 1995). Soon after the nuclear fusion, two SPBs join to form a united structure (Hirata and Tanaka, 1982) that is stained with the anti-Sad1 antibody, usually as an ellipsoid or sometimes as a closely juxtaposed

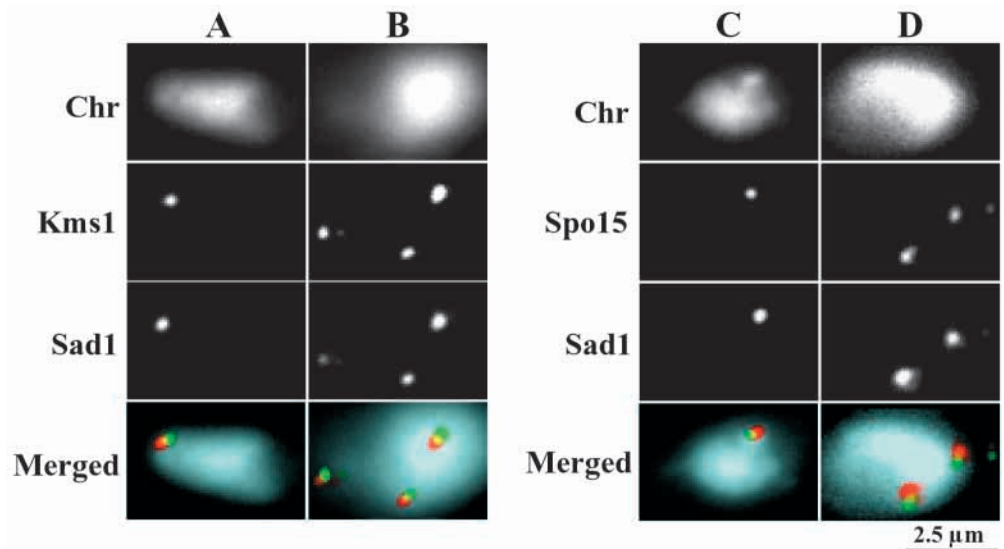



**Fig. 3.** Presence of Kms1 and Spo15 in the Sad1-containing bodies. (A,C) 0 hours, (B,D) 4 hours after the nutritional transfer. In merged images: chromosomes, cyan; Sad1, red; Kms1 and Spo15, green.

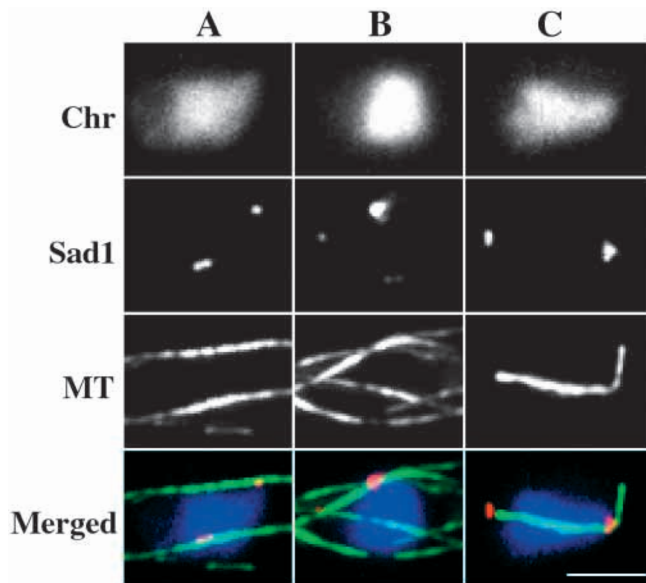



**Fig. 4.** Association of the Sad1-bodies with cytoplasmic microtubules. Wild-type zygotes (BG991) were stained with DAPI (Chr), anti-Sad1 antibody (Sad1) and TAT1 (MT, microtubule). Merged: blue, DAPI; green, MT; and red, Sad1. (A,B) 3 hours, (C) 4 hours after the transfer. A spindle formed during RTG is shown in C. Bar, 2.5 μm.

doublet (Tange et al., 1998; the present study). The anti-Sad1 antibody consistently stained only one site on the fused nucleus before the nutritional shift (Fig. 2A). The number of signals, however, markedly increased approximately 2 hours after the zygotes were transferred to a rich medium. At 4 hours, approximately 70% of the cells that were devoid of a spindle contained multiple Sad1 signals (Fig. 2B,C,D). These multiple signals usually consisted of one intense signal and other fainter signals. These images were clearly different from those of mitotic cells, occasionally observed at this stage, where two Sad1 signals situated at the ends of the spindle are observed with nearly identical intensity (see, for example, Fig. 4C). Another SPB component, Cut12 tagged with GFP (Bridge et al., 1998) was not present at multiple sites but always colocalized with one of the Sad1 stains, usually with the most intensely stained (Fig. 2B,C). The GFP-tagged Cut12 was found as expected to be associated with two SPBs at the ends of a spindle (data not shown), as well as with the SPB before RTG (Fig. 2A). These observations indicate that the multiple Sad1-containing bodies were not the products of ordinal SPB duplication, suggesting that they consisted of a presumed canonical SPB and other Sad1-containing complexes (Sad1-bodies). Using western blot analysis, an increase in Sad1 protein during the RTG process could not be determined, owing to the poor synchrony. In addition, we found no Sad1 protein with altered electrophoretic mobility during this period.

### The Sad1-body contains other SPB components

It is known that Kms1 and Spo15 are constitutively associated with the SPB, although the loss of these factors causes recognisable phenotypes only during the sexual phase (Shimanuki et al., 1997; Niwa et al., 2000; Ikemoto et al., 2000). GFP-tagged forms of these proteins were used to examine whether they were associated with the Sad1-bodies. Fig. 3 shows that both of the factors were associated with all of the Sad1-stains. There were some cases, however, in which some of the Spo15-GFP signals were not associated with them, but the reason why is not known. Nevertheless, the result indicated that novel SPB-related complexes that contain some SPB components but that are devoid of Cut12 were produced in the process of RTG.

### The Sad1-bodies were associated with microtubules

Cells were stained with an anti-α-tubulin antibody (TAT1) together with the anti-Sad1 antibody after the nutritional shift. Each of the Sad1-bodies as well as the SPB often contacted a cytoplasmic microtubule laterally (Fig. 4A,B). Occasionally,

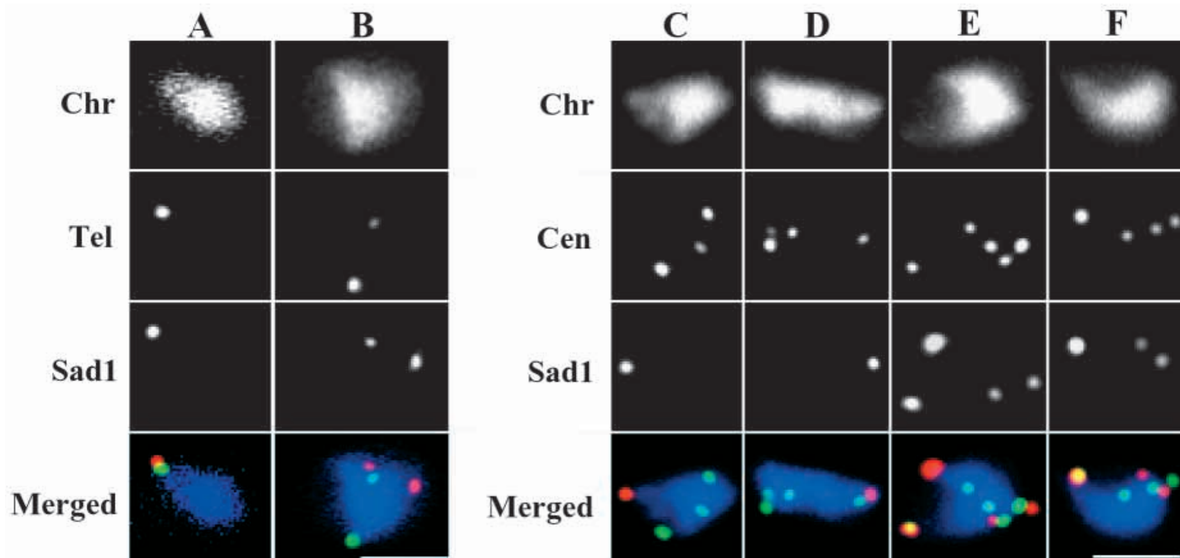
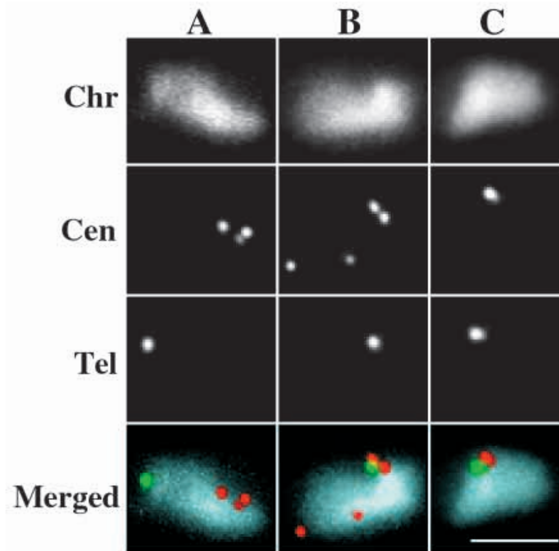



**Fig. 5.** Positioning of telomeres and centromeres during RTG in wild-type zygotes of BG991. Chr, DAPI; Tel, FISH with cos212; Cen, FISH with pRS140; Sad1, anti-Sad1 antibody; Merged: blue, DAPI; green, cos212 (in A,B) or pRS140 (in C-F); red, Sad1. (A) 0 hours, (B) 4 hours (C,D) 0 hours, (E,F) 3 hours after transfer. Bars, 2.5 μm.



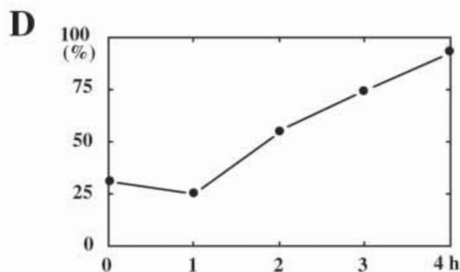
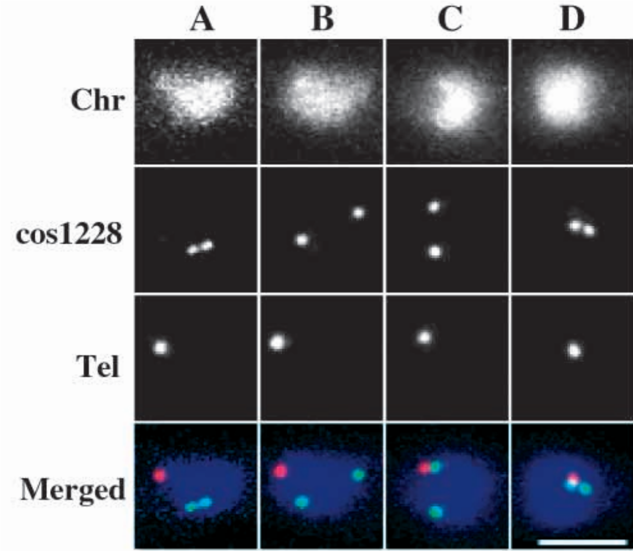
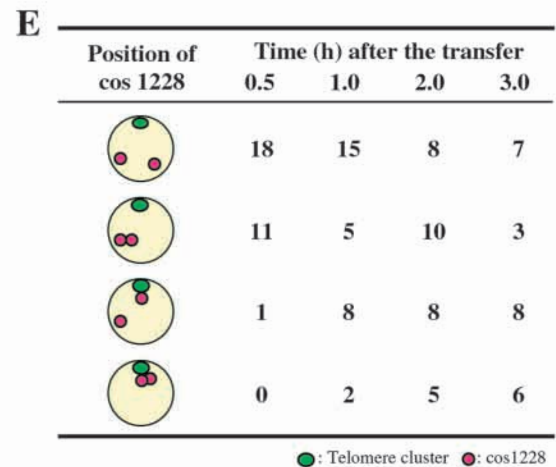




**Fig. 6.** Accumulation of centromeres near the telomere cluster. Wild-type zygotes (BG991) were prepared only for FISH. (A-C) Chr, DAPI; Cen, Cy3-labeled pRS104; Tel, Cy5-labeled cos212; Merged: cyan, DAPI; red, Cen; and green, Tel. (A) 0 hours, (B,C) 4 hours after transfer. Bar, 2.5 µm. (D) The frequency of the telomere clusters that were associated with at least one centromere.



E

| Position of cos 1228 | Time (h) after the transfer | | | |
|---|---|---|---|---|
| | 0.5 | 1.0 | 2.0 | 3.0 |
| | 18 | 15 | 8 | 7 |
| | 11 | 5 | 10 | 3 |
| | 1 | 8 | 8 | 8 |
| | 0 | 2 | 5 | 6 |

●: Telomere cluster ●: cos1228

**Fig. 7.** Accumulation of a centromere-proximal sequence near the telomere cluster. Zygotes of BG991 were prepared only for FISH. (A-D) Chr, DAPI; cos1228, Cy3-labelled cos1228; Tel, Cy5-labelled cos212; Merged: blue, DAPI; green, cos1228; red, cos212. Bar, 2.5 µm. Images were taken 0.5 hours (A,B) and 3 hours (C,D) after transfer. (E) Number of cells with indicated type of relative positioning of centromeres against the telomere cluster.

two or three Sad1-bodies were attached to the same microtubule.

## Chromosome dynamics in the returning process

The chromosome behaviour was examined using FISH. A cosmid DNA (cos212) was used as a telomere probe that recognized telomeres of chromosomes I and II, and pRS140 DNA was used as a probe for all of the centromeres (Funabiki et al., 1993). First, we verified that the telomeres made a single cluster near the SPB in the zygotes produced in the nitrogen-depleted medium (Fig. 5A). Telomeres remained in a single cluster, probably near the same SPB, until 3 hours after the shift. At 4 hours, 65% of the zygotes still contained a single cluster, but the remaining cells contained 2 to 3 telomere signals (Fig. 5B). Centromeres, by contrast, were observed as multiple (usually 3 to 4) foci at the time of the nutritional shift (Fig. 5C,D). Only a small population of the centromeres was located near the SPB (see Fig. 5D). After transfer to the rich medium, as the Sad1-bodies emerged and increased in number, the majority of the centromeres were associated with either the Sad1-bodies or the SPB (Fig. 5E,F).

Consistent with the above result, there was a clear tendency for centromeres to associate with the telomere cluster, as revealed by simultaneous FISH for telomeres and centromeres (Fig. 6). Within 4 hours, the telomere cluster was associated with at least one centromere in almost all of the cells (Fig. 6D). Cells in which all of the centromeres were located close to the telomere cluster were occasionally observed (Fig. 6C). A similar experiment was performed with a unique probe, cos1228 DNA, which has been mapped close to the centromere on chromosome II (Mizukami et al., 1993). As shown in Fig. 7, the position of this centromeric probe shifted closer to the telomere cluster as the incubation time in the rich medium increased. Also, each of the homologous centromeres behaved independently from each other with regard to the association with the telomere cluster. These results suggested that centromeres and telomeres could make a single cluster, perhaps transiently, before the first mitosis.

To confirm that centromeres could make a cluster near the SPB before the first mitosis, we examined chromosome behaviour in the *cdc25-22* temperature-sensitive mutant. At the

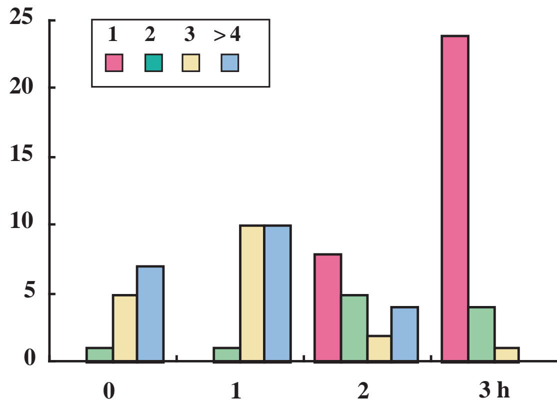
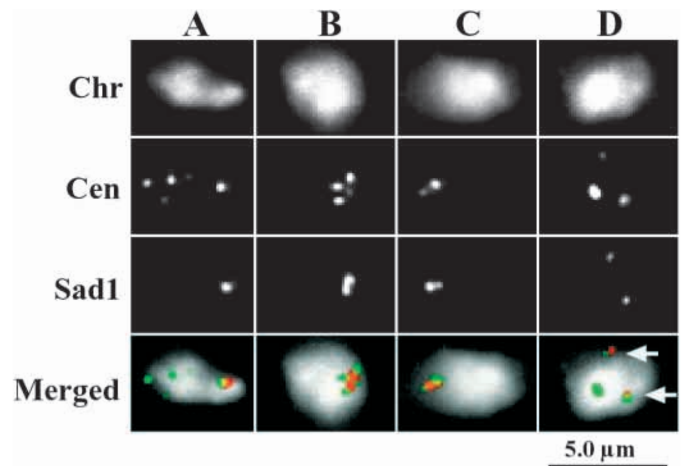



**Fig. 8.** Clustering of centromeres in the *cdc25* mutant zygotes during RTG. Number of cells with indicated number of centromere signals are plotted against the time of incubation. When centromere signals were in contact, they were regarded as one signal. The single centromere cluster was always observed at the SPB. 0 hours is the time point when the incubation temperature was shifted to 35°C.




| | E | | | | F | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 hr | 3 hrs after the shift | | | 0 hr | 3 hr | 4 hr | |
| | YE | YE | DMSO | TBZ | YE | YE | YE | TBZ |
| Clustered | 0 | 16 | 14 | 0 | 0 | 23 | 28 | 26 |
| Scattered | 30 | 13 | 16 | 30 | 30* | 7 | 1 | 4 |

● : SPB ● : Centromere

**Fig. 9.** Effect of a microtubule-destabilizing agent on the centromere clustering. Zygotes of the *cdc25* mutant (BG992) were transferred into the rich medium and 2 hours later the incubation temperature was raised to 35°C (time 0). Cells were stained with DAPI (Chr), Cy3-labelled pRS140 (Cen) and anti-Sad1 antibody (Sad1). Merged images: white, DAPI; green, centromeres; red, Sad1. (A) 0 hours, (B-D) 3 hours after transfer. YE (B) or DMSO (C) was added as a control. (D) Cells were treated with 100 µg/ml of thiabendazole (TBZ). (E) Number of cells with clustered or scattered centromeres. Scattered centromeres are present in at least two sites. (F) In a separate experiment, TBZ was added 3 hours after the temperature shift and centromere clustering was observed 1 hour later. (E,F) Red, presumed SPB; green, centromeres. *This number includes two nuclei with clustered centromeres but detached from the SPB.

restrictive temperature, this mutation blocks entry into mitosis (Millar and Russell, 1992). Zygotes produced at the permissive temperature (25°C) were transferred to the rich medium and shifted to the restrictive temperature (35°C) 2 hours later. Control cells were maintained in the nitrogen-depleted medium. As shown in Fig. 8, approximately 80% of the cells contained centromeres clustered at the SPB within 3 hours of the temperature shift. In the control cells, the SPB was associated with at least one centromere in 33% (7/21) of the cells at the time of the temperature shift, and in 55% (12/22) of the cells at 3 hours after the shift. Thus, there was a slight increase even in the absence of nutrition, although it was not statistically significant. It should be noted, however, that centromeres did not produce a single cluster near the SPB in any of the control cells. These results indicated that centromeres relocated to the SPB in the cells arrested before entry into mitosis. In a few cases, telomeres remained near the SPB, but in the majority of the cases some of the telomeres were already detached from the SPB, leaving the centromere cluster there and resulting in the formation of a Rabl-like configuration. Therefore, the Rabl-like orientation can be established without prior mitosis. The results also suggested that positional switching of telomeres and centromeres occurred during the RTG via a two-step process. To verify that the temperature shift caused the arrest of the G2/M transition, we shifted the temperature back to 25°C. As much as 30% of the total cell population contained a spindle approximately 30 minutes after the temperature shift, indicating a certain level of synchronous transition to M-phase.

### Inhibition of microtubule formation interfered with centromere clustering

Because the *cdc25* mutant was useful for observing the centromere clustering during the RTG, we tested whether thiabendazole, an inhibitor of microtubule formation, affected the centromere clustering. The results summarized in Fig. 9 indicate that the drug markedly interfered with the centromere clustering. We verified that cytoplasmic microtubules became less stable following treatment (data not shown). There were multiple Sad1 signals in the TBZ-treated cells although their intensity was generally reduced, whereas essentially all of the nuclei in control cells contained only a single Sad1 signal, which is consistent with centromere clustering (Fig. 9). It should be noted that the scattered centromeres in the TBZ-treated cells appeared to be still associated with the Sad1-bodies (see Fig. 9D). These results strongly suggest that cytoplasmic microtubules are involved in the centromere clustering and that Sad1-bodies mediate the clustering, probably by connecting centromere(s) to a microtubule. It might also be suggested that the connection between the Sad1-body and centromere is not dependent on cytoplasmic microtubules.

We then examined whether the effect of the drug was against the gathering of centromeres or the maintenance of the centromere clustering. To this end, we added TBZ to cells in which centromeres were already clustered in the *cdc25* mutant at the restrictive temperature. One hour after the drug

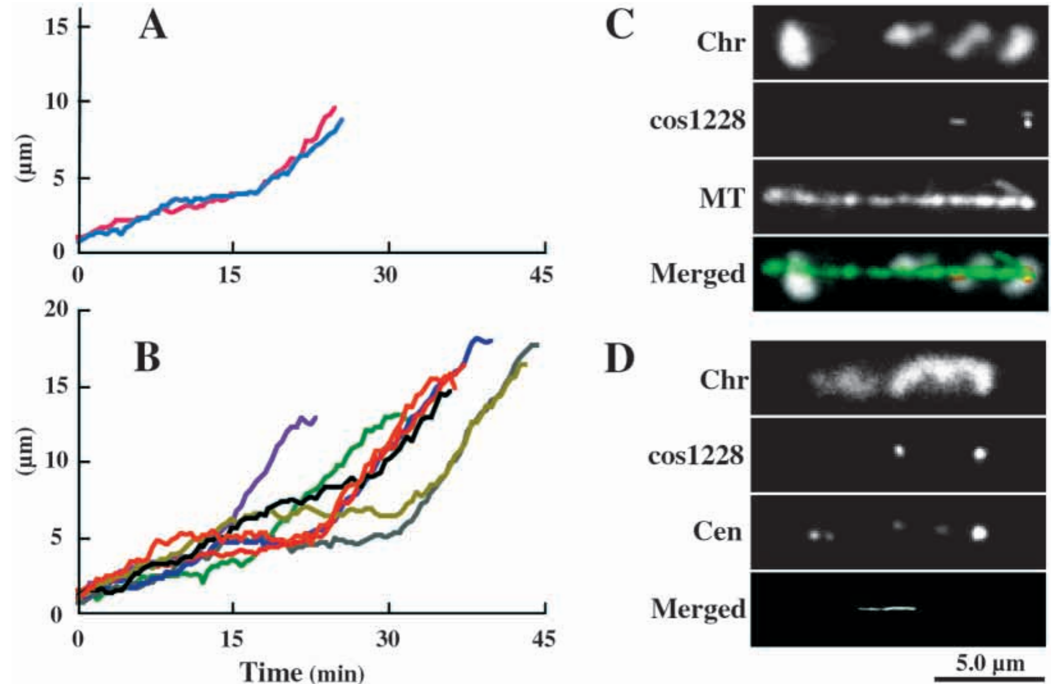



**Fig. 10.** Spindle dynamics and chromosome segregation in the first mitosis during RTG. Live observation of spindle elongation in RTG (B). (A) Spindles in ordinary mitoses of diploid cells were measured. (C,D) Typical images of early-to-late anaphase of the first mitosis. Chr, DAPI; cos1228, Cy3-labelled cos1228; MT, TAT1/Cy5; Cen, Cy5-labelled pRS140; Merged: white, DAPI; red, cos1228; green, TAT1 (C) and pRS140 (D).

addition, there was no significant reduction in the centromere clustering, suggesting that the microtubules were not required for the maintenance of the centromere cluster beneath the SPB (Fig. 9F). Interestingly, however, while centromeres remained clustered after the drug treatment, a few Sad1-containing bodies (without associated centromere) appeared in more than half of nuclei (data not shown). Similar experiments were carried out with vegetatively growing haploid *cdc25* mutant cells that were arrested in G2 phase. As anticipated, centromere clustering at the SPB was not affected by the treatment with 100 μg/ml of TBZ (data not shown).

### Unordinary features of the first mitosis

As noted above, when cells reached a certain length in the RTG, a short spindle was formed and then it elongated to drive chromosome segregation. The first mitosis showed several unusual features compared with ordinary mitoses in vegetatively proliferating diploid cells. The spindle dynamics in live cells with GFP-tagged microtubules was observed. In ordinary diploid mitoses, the elongation of a spindle proceeded in the three-phase mode (Nabeshima et al., 1998), the second phase, corresponding to the metaphase when the elongation paused, occurred when the spindle was approximately 3.5 μm and lasted for about 8 minutes (Fig. 10A). Spindle elongation in the first mitosis also showed three phases, however, the spindle length of the second phase varied from 3.5 to 8 μm and its duration also varied from 3 to 25 minutes (Fig. 10B). Perhaps related with the longer metaphase spindle, the first mitosis was missing the U-shape stage (Toda et al., 1981), which would be seen in early anaphase in an ordinal mitosis. Furthermore, we found that even after the formation of a spindle, telomere cluster could be maintained in about 40% of the cells that contained a spindle less than 4 μm in length, although, in these cells, the cluster was often released from the SPB. This indicated that the telomere cluster could be sustained without the associated SPB. When the spindle became more than 4 μm long, however, all of the telomere clusters were disrupted into multiple subclusters.

### The first mitosis is not accurate in chromosome segregation

We observed lagging and nondisjoined chromosomes using FISH with a centromere-proximal unique probe cos1228 derived from chromosome II. In approximately 10% of cells containing a late anaphase spindle, there were such aberrantly segregating chromosomes (Fig. 10C,D). In accordance with this observation, a genetic analysis showed that 34% of the first mitoses failed to produce two stable diploid cells. They produced dead cells, haploid or cells with unknown ploidy. By contrast, more than 95% of ordinary diploid mitoses faithfully produced two diploid cells.

## DISCUSSION

The present study demonstrates that a Rabl-like chromosome orientation could be established without a preceding mitosis in the RTG process from the sexual phase of the life cycle in fission yeast. This was shown most clearly in the *cdc25* mutant, which arrested before entry into mitosis. Under the experimental conditions, as many as 80% of cells displayed a clustered centromere near the SPB; approximately 95% of those cells contained telomeres detached from the SPB, producing a typical Rabl-like configuration. Because a substantial portion of the arrested cells entered into mitosis following the temperature shift, it is likely that the Rabl-like chromosome configuration formed in the *cdc25* mutant is capable of subsequent mitosis. In wild-type cells, such configuration was rarely observed during RTG, suggesting that the Rabl-like configuration was only transiently formed before the first mitosis or that mitosis can take place without its formation. These alternative possibilities remain to be tested experimentally (e.g. using living cells). An important question remains regarding whether the centromere clustering or the interaction between centromere(s) and the SPB (Sad1-body) is

a prerequisite condition for the first mitosis to occur. Efforts to address these issues will help to provide clues to understand the unusual features of the first mitosis.

Jin et al. (2000) recently showed that centromeres are clustered as a rosette around the SPB in interphase nuclei of *Saccharomyces cerevisiae*, and this configuration could be formed de novo without the passage through mitotic anaphase (Jin et al., 2000). In the budding yeast, even in interphase nuclei there are intranuclear microtubules that appear to connect the SPB with centromeres. Since such intranuclear microtubules are missing in the fission yeast, mechanisms involved in de novo clustering of centromeres are likely to differ in these yeasts. Nevertheless, the presence of such mechanisms that allow de novo reformation of Rabl-like configuration in two different organisms strongly suggests the importance of organized chromosome arrangement for successful chromosome maintenance and transmission.

The centromere clustering observed in the present study is reminiscent of the de novo formation of telomere clustering during the early phase of meiosis (Dernburg et al., 1995; Scherthan et al., 1996; Bass et al., 1997). This is a key step towards formation of the bouquet arrangement, which is important for the pairing and recombination of homologous chromosomes (Loidl, 1990; Dernburg et al., 1995; Roeder, 1997; Niwa et al., 2000). In mouse spermatogenesis, scattered telomeres move to the periphery of the nucleus and then gather beneath the centrosome to form the telomere cluster (Scherthan et al., 1996). This is similar to the formation of telomere clusters in fission yeast, because in both cases the site of telomere clustering is close to the MTOC. In maize meiosis de novo, telomere clustering has been clearly shown using 3D microscopic observation (Bass et al., 1997). In all of these cases, the mechanism underlying the clustering is not known, but there is circumstantial evidence suggesting that cytoplasmic microtubules are involved (Dernburg et al., 1995; Scherthan, 1997; Dawe, 1998).

An important finding of the present study is that the Sad1 protein is located in multiple sites on the periphery of the nucleus during RTG. In the ordinary mitotic cell cycle, the localization of the Sad1 protein is confined to the SPB (Hagan and Yanagida, 1995). The functional significance of this unusual multiplication of the Sad1 localization, or the formation of the Sad1-bodies, was immediately inferred from the fact that the Sad1-body was often associated with centromere(s) as well as with one of the cytoplasmic microtubules. It is conceivable that de novo centromere clustering is driven by the microtubular system. Consistent with this notion, we demonstrated that thiabendazole, a microtubule destabilizing agent, strongly interferes with centromere clustering in the RTG process. We have also shown that the effect of the drug was against the gathering of the centromere-associated Sad1-bodies but not against the maintenance of the centromere clustering.

The Sad1 protein belongs to the SUN-domain protein family, identified in a wide range of species (Malone et al., 1999; M. Shimanuki, unpublished), which is required for spindle formation in fission yeast and for nuclear migration in the nematode. The Sad1 protein contains a putative membrane-spanning segment and, when mildly overexpressed, it localizes to the nuclear periphery in addition to the SPB (Hagan and Yanagida, 1995). This situation resembles the present result, although it differs in one important aspect. In Sad1 overexpression, none of the subsidiary Sad1 signals were associated with microtubules (Hagan and Yanagida, 1995), whereas Sad1-bodies produced in the RTG process often associated with microtubules. At present, there is only limited information regarding the components of the Sad1-body. Further studies will be needed to elucidate the structure and function of the Sad1-body as well as the SPB. They also will benefit from understanding how chromosomes are rearranged within the nucleus, in response to extranuclear signals that might be conveyed though the SPB.

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## REFERENCES

**Bass, H. W., Marshall, W. F., Sedat, J. W., Agard, D. A. and Cande, Z. W.** (1997). Telomeres cluster de novo before the initiation of synapsis: A three-dimensional spatial analysis of telomere positions before and during meiotic prophase. *J. Cell Biol*. **137**, 5-18.

**Bridge, A. J., Morphew, M., Bartlett, R. and Hagan, I. M.** (1998). The fission yeast SPB component Cut12 links bipolar spindle formation to mitotic control. *Genes Dev*. **12**, 927-942.

**Chikashige, Y., Ding, D. Q., Funabiki, H., Haraguchi, T., Mashiko, S., Yanagida, M. and Hiraoka, Y.** (1994). Telomere-led premeiotic chromosome movement in fission yeast. *Science* **264**, 270-273.

**Chikashige, Y., Ding, D. Q., Imai, Y., Yamamoto, M., Haraguchi, T. and Hiraoka, Y.** (1997). Meiotic nuclear reorganization: Switching the position of centromeres and telomeres in the fission yeast *Schizosaccharomyces pombe*. *EMBO J*. **16**, 193-202.

**Comings, D. E.** (1980). Arrangement of chromatin in the nucleus. *Hum. Genet*. **53**, 131-143.

**Cremer, T., Kurz, A., Zirbel, R., Dietzel, S., Rinke, B., Schrock, E., Speicher, M. R., Mathieu, U., Jauch, A., Emmerich, P. et al.** (1993). Role of chromosome territories in the functional compartmentalization of the cell nucleus. *Cold Spring Harbor Symp. Quant. Biol.* **58**, 777-792.

**Csink, A. K. and Henikoff, S.** (1998). Large-scale chromosomal movements during interphase progression in Drosophila. *J. Cell Biol.* **143**, 13-22.

**Dawe, R. K.** (1998). Meiotic chromosome organization and segregation in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **49**, 371-395.

**Dernburg, A. F., Sedat, J. W., Cande, Z. W. and Bass, H. W.** (1995). Cytology of telomeres. In *Telomeres* (ed. E. H. Blackburn and C. W. Greider), pp. 295-338. New York: Cold Spring Harbor Laboratory Press.

**de Lange, T.** (1998). Ending up with the right partner. *Nature* **392**, 753-754.

**Egel, R. and Egel-Mitani, M.** (1974). Pre-meiotic DNA synthesis in the fission yeast. *Exp. Cell Res.* **88**, 127-134.

**Franklin, A. E. and Cande, W. Z.** (1999). Nuclear organization and chromosome segregation. *Plant Cell* **11**, 523-534.

**Funabiki, H., Hagan, I., Uzawa, S. and Yanagida, M.** (1993). Cell cycle-dependent positioning and clustering of centromeres and telomeres in fission yeast. *J. Cell Biol*. **121**, 961-976.

**Hagan, I. and Yanagida, M.** (1995). The product of the spindle formation gene *sad1*$^+$ associates with the fission yeast spindle pole body and is essential for viability. *J. Cell Biol*. **129**, 1033-1047.

**Hirata, A. and Tanaka, K.** (1982). Nuclear behavior during conjugation and meiosis in the fission yeast Schizosaccharomyces pombe. *J. Gen. Appl. Microbiol.* **28**, 263-274.

**Ikemoto, S., Nakamura, T., Kubo, M. and Shimoda, C.** (2000). *S. pombe* sporulation-specific coiled-coil protein Spo15p is localized to the spindle pole body and essential for its modification. *J. Cell Sci.* **113**, 545-554.

**Janevski, J., Park, P C. and De Boni, U.** (1995). Organization of centromeric domains in hepatocyte nuclei: rearrangement associated with de novo activation of the vitellogenin gene family in Xenopus laevis. *Exp. Cell Res*. **217**, 227-239.

**Jin, Q. W., Fuchs, J. and Loidl, J.** (2000). Centromere clustering is a major

determinant of yeast interphase nuclear organization. *J. Cell Sci*. **113**, 1903-1912.

**Loidl, J.** (1990). The initiation of meiotic chromosome pairing: the cytological view. *Genome* **33**, 759-778.

**Malone, C. J., Fixsen, W. D., Horvitz, H. R. and Han, M.** (1999). UNC-84 localizes to the nuclear envelope and is required for nuclear migration and anchoring during C. elegans development. *Development* **126**, 3171-3181.

**Manuelidis, L.** (1990). A view of interphase chromosomes. *Science* **250**, 1533-1540.

**Marshall, W. F., Fung, J. C. and Sedat, J. W.** (1997). Deconstructing the nucleus: global architecture from local interactions. *Curr. Opi. Genet. Dev.* **7**, 259-263.

**Millar, J. B. and Russell, P.** (1992). The cdc25 M-phase inducer: an unconventional protein phosphatase. *Cell* **68**, 407-410.

**Miyata, M., Doi, H., Miyata, H. and Johnson, I. M.** (1997). Sexual co-flocculation by heterothallic cells of the fission yeast *Schizosaccharomyces pombe* modulated by medium constituents. *Antonie van Leeuwenhoek* **71**, 207-215.

**Mizukami, T., Chang, W. I., Garkavtsev, I., Kaplan, N., Lombardi, D., Matsumoto, T., Niwa, O., Kounosu, A., Yanagida, M., Marr, T. G. et al. (**1993). A 13 kb resolution cosmid map of the 14 Mb fission yeast genome by nonrandom sequence-tagged site mapping. *Cell* **73**, 121-132.

**Moreno, S., Klar, A. and Nurse, P.** (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* **194**, 795-823.

**Niwa, O., Shimanuki, M. and Miki, F.** (2000). Telomere-led bouquet formation facilitates homologous chromosome pairing and restricts ectopic interaction in fission yeast meiosis. *EMBO J*. **19**, 3831-3840.

**Okazaki, K., Okayama, H. and Niwa, O.** (2000). The polyubiquitin gene is essential for meiosis in fission yeast. *Exp. Cell Res*. **254**, 143-152.

**Roeder, G. S.** (1997). Meiotic chromosomes: it takes two to tango. *Genes Dev*. **11**, 2600-2621.

**Scherthan, H.** (1997). Chromosome behaviour in earliest meiotic prophase. In *Chromosomes Today.* Vol. 12 (ed. N. Hengiques-Gil, J. S. Parker and M. J. Puertas), pp. 217-248. London: Chapman and Hall.

**Scherthan, H., Weich, S., Schwegler, H., Heyting, C., Harle, M. and Cremer, T.** (1996). Centromere and telomere movements during early meiotic prophase of mouse and man are associated with the onset of chromosome pairing. *J. Cell Biol.* **134**, 1109-1125.

**Shimanuki, M., Miki, F., Ding, D. Q., Chikashige, Y., Hiraoka, Y., Horio, T. and Niwa, O.** (1997). A novel fission yeast gene, *kms1*$^{+}$, is required for the formation of meitoic prophase-specific nuclear architecture. *Mol. Gen. Genet.* **254**, 238-249.

**Tange, Y., Horio, T., Shimanuki, M., Ding, D. Q., Hiraoka, Y. and Niwa, O.** (1998). A novel fission yeast gene, *tht1*$^{+}$, is required for the fusion of nuclear envelopes during karyogamy. *J. Cell Biol.* **140**, 247-258.

**Toda, T., Yamamoto, M. and Yanagida, M.** (1981). Sequential alterations in the nuclear chromatin region during mitosis of the fission yeast Schizosaccharomyces pombe: video fluorescence microscopy of synchronously growing wild-type and cold-sensitive cdc mutants by using a DNA-binding fluorescent probe*.* *J. Cell Sci*. **52**, 271-287.

**Therman, E.** (1995). Chromosome behavior in cell differentiation: a field ripe for exploration? *Genetics* **141**, 799-804.

**Vourc'h, C., Taruscio, D., Boyle, A. N. and Ward, D. C.** (1993). Cell cycle-dependent distribution of telomeres, centromeres, and chromosome-specific subsatellite domains in the interphase nucleus of mouse lymphocytes. *Exp.Cell Res*. **205**, 142-151.

**Woods, A., Sherwin, T., Sasse, R., MacRae, T. H., Baines, A. J. and Gull, K.** (1989). Definition of individual components within the cytoskeleton of *Trypanosome brucei* by a library of monoclonal antibodies. *J. Cell Sci*. **93**, 491-500.

**Yamamoto, M., Imai, Y. and Watanabe, Y.** (1997). Mating and sporulation in Schizosaccharomyces pombe. In *The molecular and Cellular Biology of the Yeast Saccharomyces* (ed. J. R. Pringle, J. R. Broach and E. W. Jones), pp. 1037-1106. New York: Cold Spring Harbor Laboratory Press.