Monopolar spindle attachment of sister chromatids is ensured by two distinct mechanisms at the first meiotic division in fission yeast

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At meiosis I, sister chromatids attach to the same spindle pole (i.e. monopolar attachment). Mechanisms establishing monopolar attachment remain largely unknown. In the fission yeast *Schizosaccharomyces pombe*, monopolar attachment is established in haploid cells, indicating that homologous chromosomes are dispensable for its establishment. This monopolar attachment requires both mating pheromone signaling and inactivation of Pat1 kinase (a key negative regulator of meiosis). It also requires the meiotic cohesin factor Rec8 but not the recombination factor Rec12. In contrast, in diploid cells, monopolar attachment is established by Pat1 inactivation alone, and does not require mating pheromone signaling. Furthermore, monopolar attachment requires Rec12 in addition to Rec8. These results indicate that monopolar attachment of sister chromatids can be established by two distinct mechanisms in *S.pombe*, one that is pheromone dependent and recombination independent, and a second that is pheromone independent and recombination dependent. We propose that co-operation of these two mechanisms generates the high fidelity of monopolar attachment.

Keywords: centromere/homologous chromosome/
meiosis/recombination/sister chromatid

Introduction

Meiosis is a special type of cell division through which eukaryotic diploid cells produce haploid gametes containing their genetic determinants. In meiosis, two rounds of chromosome segregation follow a single round of DNA replication, thus halving the number of chromosomes per daughter cell. The first round of chromosome segregation (meiosis I) is unique in that sister chromatids move together to the same spindle pole while homologous chromosomes move apart from each other to the opposite poles. How chromosomes are properly segregated at meiosis I is one of the major questions of meiosis. Moreover, understanding its mechanism is clinically important, because chromosome missegregation at meiosis I in human oogenesis is a major cause of fetal miscarriage and trisomy disease (Hassold *et al.*, 1996).

Chromosome segregation depends on poleward forces generated by the mechanical attachments of chromosomes to the spindle. At meiosis I, sister chromatids attach to the

same spindle pole while homologous chromosomes attach to the opposite spindle pole via the spindle microtubules. These chromosomal attachments to the spindle poles result in meiosis I-specific chromosome segregation. Spindle attachment of chromosomes depends on meiosis-specific chromosome organization (Östergren, 1951; Nicklas, 1977; Paliulis and Nicklas, 2000). During meiosis, homologous chromosomes become associated with each other and undergo crossover recombination. This leads to the formation of chiasmata, which maintain homolog association until the onset of anaphase I. The homologous chromosomes that are linked by chiasmata have kinetochores arranged in such a way that the kinetochores of sister chromatids face in the same direction (i.e. mono-orientation), while those of homologous chromosomes face in opposite directions (i.e. bi-orientation). This chromosome organization, with such kinetochore orientation, generates a tendency for each chromosome to attach to the correct spindle pole. Furthermore, with this particular chromosome organization, proper spindle attachment of each chromosome then generates tension at the kinetochores that subsequently stabilizes spindle attachment of the kinetochores (Nicklas, 1997).

It is generally thought that monopolar attachment of sister chromatids requires sister centromere cohesion (see review by Bickel and Orr-Weaver, 1996). This idea is supported by sister chromatid missegregation in several mutants of different organisms that are defective in sister chromatid cohesion (Klein *et al.*, 1999; van Heemst *et al.*, 1999; Mercier *et al.*, 2001; Bickel *et al.*, 2002). In particular, those of the budding yeast *Saccharomyces cerevisiae* have been extensively studied. In this organism, sister chromatid cohesion in both mitosis and meiosis depends on cohesin, a protein complex that is conserved from yeast to vertebrates (reviewed by van Heemst and Heyting, 2000; Nasmyth, 2001). Cohesin is localized on paired sister chromatids and dissociates from them, as their association resolves. In meiosis, their association resolves at chromatid arms in anaphase I and then at the centromeres in anaphase II (Buonomo *et al.*, 2000), and accordingly, meiotic cohesin dissociates first from the arms and then from the centromeres (Klein *et al.*, 1999). In cells lacking a meiotic cohesin subunit Rec8, sister chromatids precociously separate and are frequently missegregated at anaphase I (Klein *et al.*, 1999), demonstrating the essential role of sister centromere cohesion in monopolar attachment of sister chromatids.

In addition, the meiosis-specific centromere structure is probably required for monopolar attachment. Centromere proteins that are required for monopolar attachment of sister chromatids, but not for centromere cohesion, have been identified. In the fission yeast *Schizosaccharomyces pombe*, Rec8 is dispensable for sister centromere cohesion at the onset of anaphase I, albeit essential for sister

Fig. 1. Haploid meiosis induced by the *mat* genes. (A) Changes in nuclear morphology (a) and DNA content (b) of haploid cells containing transcriptionally active mating type genes of the P and M types (strain AY1931) after nitrogen starvation. (B) Microtubule and nuclear morphology (a) and behavior of the single spindle (b) in haploid meiotic cells (strain CRL4221). Microtubules were visualized by GFP-tagged α -tubulin (see Materials and methods): (a) microtubules (green) and chromosomal DNA (red). (b) photos were taken every 2.9 min. Numbers indicate time in minutes. (C) Chromosomal morphology and visualized chromosomal loci at the first (a–f) or second (g and h) division: (a) and (d): chromosomal DNA stained with DAPI; (b) and (e) the visualized chromosomal loci; (c) and (f)–(h) merged images of chromosomal DNA (red) and visualized chromosomal locus (green). Arrows indicate three individual chromosomes. (D) Frequencies of co-segregated sister loci. P and M are the mating type genes at the *mat1* locus or at the ectopic locus. N, number of cells examined. Strains used for analyses were as follows: 1, AY1391; 2, AY1931; 3, AY2082; 4, AY2072.

chromatid cohesion at arms (Molnar *et al.*, 1995), and required for monopolar attachment of sister chromatids, as evidenced by equational segregation of sister chromatids

at anaphase I in cells lacking Rec8 (Watanabe and Nurse, 1999). In *S.cerevisiae*, a meiosis-specific centromere protein Mam1 (also called monopolin), appears to be specifically required for monopolar attachment of sister chromatids (Toth *et al.*, 2000). These proteins likely function to form the meiosis-specific centromere structure that generates sister kinetochore mono-orientation.

It is known that homolog association is required for bipolar attachment of homologous chromosomes, and thereby their proper segregation (for a review, see Hawley, 1988). Homolog association, however, is probably also important for monopolar attachment of sister chromatids. In many organisms, when a pair of sister chromatids are not associated with their homologous partners, the sister chromatids often attach to the opposite poles to subsequently be segregated away from each other (e.g. Maguire, 1987; Hunt *et al.*, 1995; Rebollo and Arana, 1995) or instead to be broken by poleward forces (Darlington, 1939). Homolog association, therefore, does not simply play a role in the bipolar attachment of homologous chromosomes.

How monopolar attachment of sister chromatids is established remains largely unknown. This is partly because monopolar attachment relies on the complex chromosomal organization also involving homologous chromosomes. To further understand the mechanism of monopolar attachment, it is important to assess the contribution of homologous chromosomes to monopolar attachment. Fission yeast is a good model organism for studying this issue, because it has only three chromosomes, and because their meiotic behavior can be easily followed (Chikashige *et al.*, 1994). Here, we examine meiotic sister chromatid behavior in various types of fission yeast cells to assess the contribution of homologous chromosomes. Through this study, we show that the monopolar attachment of sister chromatids is established by two distinct mechanisms: one is independent of homologous chromosomes while the other is dependent on the homologous chromosomes. We provide evidence that the co-operation of these two mechanisms is required for the high fidelity of monopolar attachment.

Results

Homologous chromosomes are not required for monopolar spindle attachment of sister chromatids at meiosis I

Monopolar attachment of sister chromatids is established at meiosis I when the sister chromatids are associated with their homologous chromosomes. To elucidate whether homologous chromosomes are required for monopolar attachment of sister chromatids in *S.pombe*, we induced meiosis in haploid cells in which a homologous set of chromosomes is absent. In *S.pombe*, meiosis is normally induced in a diploid cell that is formed by the fusion of haploid cells of the P and M mating types, and under nitrogen-starved conditions, the diploid cell undergoes meiosis to form four spores. The mating type is determined by mating type-specific genes at the *mat1* locus, and induction of meiosis requires co-expression of those genes of both mating types. If both mating type genes are co-expressed in a haploid cell, the cell enters meiosis in the haploid state (i.e. haploid meiosis; Thon and Klar, 1992).

Table I. Spore viability of various fission yeast strains

Ploidy	Strain	Allele ^a		Mutations ^b	Spore viability (%) ^c
		<i>mat1</i>	Ectopic		
Haploid	AY1391	<i>P</i>	<i>M</i>	None	2.3
	AY2072	<i>P</i>	<i>M</i>	None	2.0
	AY2082	<i>M</i>	<i>P</i>	None	5.9
	CRL245	<i>M</i>	None	<i>pat1</i>	4.6
Diploid	AY1731	<i>P/M</i>	None	None	74.5 ^d
	AY167d	<i>M/M</i>	None	<i>pat1</i>	37.1 ^d
	AY210d	<i>M/M</i>	None	<i>pat1 rec8</i>	18.5
	AY2001	<i>M/M</i>	None	<i>pat1 rec12</i>	10.6
	AY2463d	<i>M/M</i>	<i>mat-Pc</i>	<i>pat1</i>	69.0

^aAllele: mating type alleles at the *mat1* locus and the ectopic locus where the *mat* gene(s) is integrated.

^bMutations: a mutation(s) in the strains. None, no mutations.

^cSpore viability examined by random spore analysis.

^dAverage of two independent experiments.

Table II. Sister locus behavior at meiosis II in various cell types

Strain	Cell type (%) ^a					<i>N</i>
	1	2	3	4	Others	
AY1931	55.7	18.2	3.4	21.6	1.1	88
AY191-6C	51.3	40.0	3.8	5.0	0	80
AY1731	98.8	0	0	0	1.2	80
AY167d	78.2	1.8	14.5	5.5	0	55

^aCell type: cells were classified into five classes according to the number of nuclei (open circles below) and *lys1*-GFP signals (dots in open circles below).

N, number of cells examined.

We created haploid cells that co-express both mating type genes by introducing the opposite mating type genes at an ectopic chromosomal locus (see Materials and methods). These cells underwent meiosis in the haploid state under nitrogen-starved conditions (Figure 1A). After nitrogen starvation, most of the cells in the G₂ phase underwent one cell division to enter G₁. This was shown by a transient increase in the number of cells containing two DNA masses (Figure 1A, a, 3 h) and a shift of a DNA peak from 2C to 1C (Figure 1A, b, 3 and 4 h). A fraction of these cells underwent DNA replication, demonstrated by an increase in the 2C DNA peak (Figure 1A, b, 6–8 h), and subsequently, two nuclear divisions generated three or four DNA masses [Figure 1A, a, 8–11 h; note, however, that the population of cells that underwent two divisions varied among the different strains used and individual experiments (40–70%)]. As seen in meiosis of wild-type diploid cells, radial microtubules were formed from the edge of the elongated nucleus before nuclear divisions (Figure 1B, a, left), and one and two spindles, respectively, were formed at the first and second divisions (Figure 1B, a, middle and right). The cells eventually formed one to four spores whose viability was less than one-tenth that of wild-type diploid cells (Table I, AY1391, AY2082, AY2072 and AY1731).

Sister chromatid behavior was examined by visualizing several chromosomal loci on three different chromosomes using fluorescence *in situ* hybridization or the *lac* repressor/operator recognition system, together with chromosomal DNA staining (see Materials and methods). Specifically, we examined two rDNA loci located near both telomeres of chromosome III, the *lys1*⁺ locus on chromosome I, the *ade8*⁺ locus on chromosome II and the *ade6*⁺ locus on chromosome III. All these loci gave similar results; i.e. three individual chromosomes were clearly discernible during anaphase I, one of which contained the signal (Figure 1C, a–c, arrows). Furthermore, in the majority of cells, two DNA masses formed via meiosis I appeared to be unequal in size and the signal of the locus was observed in only one of these DNA masses (Figure 1C, a–f, and D). These observations indicate that, in most cases, sister chromatids moved to the same pole without separating from each other at meiosis I. After meiosis II, sister chromatids mostly separated from each other, as in diploid meiosis (Table II, AY1731), because the signal was observed mostly in two of the three or four DNA masses generated [73.9% (= 55.7% + 18.2%); Table II, AY1931; Figure 1C, g and h]. Sister chromatid separation at anaphase II indicates that sister centromere cohesion persists until anaphase II, as in normal diploid meiosis. From these observations, we conclude that homologous chromosomes are not required for the monopolar attachment of sister chromatids at meiosis I.

Monopolar spindle attachment of sister chromatids is not established in *pat1*-induced haploid meiosis

The mating type gene products lead to the inactivation of Pat1 (Ran1) kinase, which is a key negative regulator of meiosis in *S.pombe* (reviewed by Yamamoto *et al.*, 1997). Inactivation of Pat1 kinase alone is sufficient to induce meiosis, and haploid cells bearing a *pat1* temperature-sensitive (ts) allele undergo haploid meiosis at the restrictive temperature (Iino and Yamamoto, 1985a). We next examined sister chromatid behavior in *pat1*-induced haploid meiosis to determine whether Pat1 inactivation alone is sufficient to establish monopolar attachment of sister chromatids.

Fig. 2. Haploid meiosis induced by Pat1 inactivation. (A) Changes in nuclear morphology (a) and DNA content (b) of *pat1* haploid cells (strain AY191-6C) after a temperature shift. (B) Microtubule and nuclear morphology (a) and behavior of the single spindle (b) in *pat1* haploid cells (strain CRL246 or CRL298): (a) microtubules (green) and chromosomal DNA (red); (b) photos were taken every 2 min. Numbers indicate time in minutes. (C) Chromosomal morphology and the visualized chromosomal loci at the first (a–f) or second (g and h) division; (a) and (d) chromosomal DNA stained with DAPI; (b) and (e) visualized chromosomal loci; (c) and (f)–(h) merged images of chromosomal DNA (red) and visualized chromosomal locus (green). (D) Frequencies of co-segregated various sister loci. Strains used for analyses were as follows: 1, CRL245; 2, AY191-6C.

Haploid cells of the *pat1* ts mutant were arrested in G₁ phase under nitrogen-starved conditions and then induced into meiosis by a shift to the restrictive temperature

(Figure 2A). As observed for *mat* gene-induced haploid meiosis, the cells underwent DNA replication (Figure 2A, b, 2–4 h) and two meiotic divisions to form three or four DNA masses (Figure 2A, a, 5–9 h), two of which mostly contained the signal of the visualized loci [91.3% (= 51.3% + 40.0%); Table II, AY191-6C; Figure 2C, g and h], and eventually formed spores with low viability (Table I, CRL245). In addition, radial microtubules were formed before nuclear divisions, and one and two spindles, respectively, were formed during the first and second divisions (Figure 2B, a). Dynamic behavior of the spindle at the first division was also similar to that observed in *mat* gene-induced haploid meiosis (Figures 1B, b, and 2B, b). Chromosomal behavior at meiosis I, however, was strikingly different. The two DNA masses formed through this division appeared to be equal in size (Figure 2C, a and d), and both contained the signal of the loci in most cells (Figure 2C, b, c, e and f, and D). This indicates that sister chromatids attached to the opposite poles and separated from each other at meiosis I. From these observations, we conclude that Pat1 inactivation alone is not sufficient to establish monopolar attachment of sister chromatids, as well as to maintain sister centromere cohesion at anaphase I. Thus, the mating type gene products play an essential role in monopolar attachment of sister chromatids in addition to Pat1 inactivation.

Mating pheromone signaling is required for monopolar spindle attachment of sister chromatids in *pat1*-induced haploid meiosis

Schizosaccharomyces pombe has two forms of each mating type gene (*i*-type and *c*-type). *mat-Pc* and *mat-Mc* are essential for both cell conjugation and meiosis and the others, *mat-Pi* and *mat-Mi* are solely essential for meiosis (Kelly *et al.*, 1988). If only one form of the gene is required for monopolar attachment, *pat1* haploid cells expressing such genes of the P and M mating types are expected to establish monopolar attachment. To test this idea, we constructed *pat1* haploid cells that express both *mat-Pi* and *mat-Mi*, or both *mat-Pc* and *mat-Mc* (see Materials and methods) and induced meiosis in these cells. The haploid *pat1* cells expressing both *mat-Pi* and *mat-Mi* underwent meiosis after a temperature shift, like those expressing solely *mat-Pi* or *mat-Mi* (Figure 3A). They underwent meiosis of a similar timing (Figure 3A, a and b) and failed to establish monopolar attachment (Figure 3A, c, and D, 1, 3 and 5). In contrast, the cells containing both *mat-Pc* and *mat-Mc* underwent meiosis differently. They started meiosis ~2 h earlier (Figure 3B, a and b) and the majority established monopolar attachment of sister chromatids (Figure 3B, c, and D, 2, 4 and 6). Three individual chromosomes were also visible during anaphase I, indicating that sister chromatid cohesion was maintained during anaphase I (data not shown). From these results, we conclude that *mat-Pc* and *mat-Mc* are required for monopolar attachment of sister chromatids as well as maintenance of sister chromatid cohesion in *pat1*-induced haploid meiosis, but *mat-Pi* and *mat-Mi* are not.

One of the essential tasks of *c*-type *mat* genes is induction of the mating pheromone response (reviewed by Yamamoto *et al.*, 1997). We next examined whether mating pheromone signaling is required for monopolar attachment of sister chromatids. We triggered mating

pheromone signaling in *h⁻ pat1* haploid cells by treating them with the mating pheromone, P factor, and then induced meiosis. In this analysis, the *sxa2⁺* gene, which encodes a P factor-specific protease, was disrupted in the cells to enhance their sensitivity to P factor (Imai and Yamamoto, 1994). Haploid *pat1* cells bearing *sxa2* mutation underwent meiosis at the restrictive temperature like *sxa2⁺ pat1* haploid cells and showed sister chromatids separation at meiosis I (Figure 3C, a–c, and D, #7). On the

other hand, the cells treated with P factor showed cell elongation (Figure 3C, d), indicating that they are responding to the mating pheromone. In these cells, sister chromatids mostly moved together toward the same pole (Figure 3C, d–f, and D, #8). From these results, we conclude that mating pheromone signaling, in addition to Pat1 inactivation, is required to establish monopolar attachment of sister chromatids.

Monopolar attachment of sister chromatids in haploid meiosis is dependent on meiotic cohesin but not on recombination

Meiotic cohesin Rec8 has been shown to be required for monopolar attachment of sister chromatids in diploid meiosis (Watanabe and Nurse, 1999). We next examined the Rec8 function in haploid meiosis. Haploid cells bearing the *rec8⁺* deletion allele (Parisi *et al.*, 1999) were induced to meiosis by the *mat* genes. These cells underwent the meiotic events with a timing similar to that of *rec8⁺* cells. In these cells, sister chromatids mostly separated from each other at meiosis I (Figure 4A, a, and B, #1). Thus, monopolar attachment of sister chromatids is

Fig. 4. Roles of Rec8 and Rec12 at meiosis I in haploid cells. (A) Sister locus behavior in cells lacking Rec8 (a, strain AY2081) or Rec12 (b, strain AY2071). Red and green indicate chromosomal DNA and GFP-visualized locus, respectively. (B) Frequencies of co-segregated sister loci. Strains used for analyses were as follows: 1, AY2081; 2, AY2071.

Fig. 3. Effect of *i*-type or *c*-type *mat* genes or mating pheromone signaling on *pat1*-induced haploid meiosis. Meiosis induced in haploid *pat1* cells containing transcriptionally active *i*-type (A) or *c*-type (B) *mat* genes of both types (strain CRL2411 or CRL2412). Changes in nuclear morphology (a) and DNA content (b) after a temperature shift. rDNA locus (green) and chromosomal DNA (red) after the first division (c). (C) Sister locus behavior at meiosis I in haploid *pat1* cells in the presence (d–f) or absence (a–c) of mating pheromone response: (a and d) chromosomal DNA stained by DAPI; (b and e) the GFP-visualized *lys1⁺* locus; (c and f) merged images of chromosomal DNA (red) and visualized chromosomal locus (green). (D) Frequencies of co-segregated sister loci: none, no ectopic *mat* gene; *M(i+c)*, *mat-Mi* and *mat-Mc*; *P(i+c)*, *mat-Pi* and *mat-Pc*. Strains used for analyses were as follows: 1, CRL2411; 2, CRL2412; 3, AY1396; 4, AY1395; 5, AY1902; 6, AY1901; 7 and 8, AY195-1C.

Fig. 5. Rec8 behavior in haploid meiosis. (A) Localization of GFP-tagged Rec8 in haploid cells undergoing meiosis. Left and right panels show *mat* gene-induced meiosis (strain AY2211) and *pat1*-induced meiosis (strain AY221-8A), respectively: (a) before nitrogen starvation (left panels) or shifting temperature (right panels); (b) meiotic prophase; (c) and (d) meiosis I; (e) after meiosis II. (B) Colocalization of Rec8-GFP (green) with Mis12-HA (red) in mitotically growing cells (strain AY311-19C). Chromosomal DNA is shown in white. (C) Live observation of Rec8-GFP at meiosis I in haploid *pat1* cells (strain AY221-8A). Numbers indicate time in minutes. Red and green indicate chromosomal DNA and Rec8-GFP, respectively. (D) Chromosome spread of haploid meiotic cells: (a) *mat* gene-induced meiosis (strain AY2211); (b) *pat1*-induced meiosis (strain AY221-8A). (E) Changes in Rec8-GFP expression and localization during haploid meiosis: (a–c) *mat* gene-induced meiosis (strain AY2211); (d–f) *pat1*-induced meiosis (strain AY309-9C). Numbers indicate times after nitrogen starvation or a temperature shift. (a and d) DNA contents of the cells analyzed by FACS; (b and e) transcripts of Rec8-GFP (upper rows) detected by northern blotting. Cam1 was used as an internal control (lower rows); (c and f), Rec8-GFP localization (upper panels) and chromosomal DNA morphology (lower panels).

dependent on Rec8 in haploid meiosis, as in diploid meiosis.

Because Rec8 is required for proper recombination (DeVeaux and Smith, 1994), we next examined whether the recombination process is required for monopolar attachment of sister chromatids in haploid meiosis. We eliminated the recombination process by disrupting the *rec12*⁺ gene, which encodes a protein homologous to

budding yeast Spo11p and is required for the initiation of the recombination process (Keeney *et al.*, 1997; Cervantes *et al.*, 2000). In the cells lacking Rec12, sister chromatids moved predominantly to the same pole (Figure 4A, b, and B, #2), indicating that monopolar attachment is Rec12 independent. Thus, monopolar attachment of sister chromatids in haploid meiosis is not dependent on the recombination process.

In diploid meiosis, Rec8 transcription increases before premeiotic DNA replication, and Rec8p localized on almost the entire chromosomes until anaphase I (Parisi *et al.*, 1999; Watanabe and Nurse, 1999). It then disappears from the chromosome arms during anaphase I, but it remains at the centromeres until the onset of anaphase II. Bipolar attachment of sister chromatids seen in *pat1* haploid cells may be caused by the loss of Rec8 from chromosomes. Alternatively, bipolar attachment may be caused by delayed expression of Rec8 after DNA replication, which brings about equational sister chromatid segregation at meiosis I in diploid cells (Watanabe *et al.*, 2001). To test these possibilities, we examined localization and transcription of Rec8 in haploid meiosis using GFP-tagged Rec8 (Rec8-GFP) placed under its own promoter, which functions similar to wild-type Rec8 (Watanabe and Nurse, 1999). We found that localization of Rec8-GFP and its expression in *pat1*-induced meiosis were largely similar to those seen in *mat* gene-induced meiosis. Rec8-GFP was transcribed at a low level and localized at centromeres in mitotically growing cells, as shown by its colocalization with a centromere protein Mis12 (Goshima *et al.*, 1999) (Figure 5A, a, B, and E, b and c, 0 h). Both in *mat* gene-induced meiosis and in *pat1*-induced meiosis, Rec8 transcription gradually increased (Figure 5E, b, and e) and Rec8-GFP accumulated in the nucleus before DNA replication (Figure 5A, b, and E, c and f). In *mat* gene-induced meiosis, nuclear accumulation of Rec8 became evident at 5 h after nitrogen starvation (Figure 5E, c) and DNA replication occurred between 6 and 7 h (Figure 5E, a). In *pat1*-induced meiosis, Rec8 nuclear accumulation became evident at 3 h after a temperature shift (Figure 5E, f) and DNA replication occurred between 3 and 4 h (Figure 5E, d). Rec8-GFP was retained on the chromosomes after chromosome spread (Figure 5D), indicating that nuclear-accumulated Rec8 is tightly associated with chromosomes in either of the meioses, as seen in diploid meiosis (Watanabe and Nurse, 1999). Furthermore, Rec8 disappeared from the chromosome arms during anaphase I but remained localized at the centromeres, as shown by persistence of its localization at the leading edges of the chromosomes at anaphase I (Figure 5A, c and d, and C). Rec8 eventually disappeared at anaphase II (Figure 5A, e). Therefore, bipolar attachment of sister chromatids in *pat1*-induced haploid meiosis is not due to a loss of Rec8 from the chromosomes or delayed expression of Rec8 after DNA replication.

Recombination is required for the high fidelity of monopolar attachment of sister chromatids at meiosis I in diploid cells

As described above, the majority of the cells established monopolar attachment of sister chromatids in *mat* gene-induced haploid meiosis. However, a fraction of them showed sister chromatid separation (10–22%; Table III,

Table III. Sister locus separation at meiosis I in various cell types

Strains	<i>mat1</i> allele	Mutations or ectopic <i>mat</i> gene	Sister locus separation (%) ^a	Locus examined	N
Haploid meiosis					
AY1931	<i>P</i>	<i>M</i>	22.2	<i>lys1</i> ⁺	55
Zygotic diploid meiosis ^b					
AY167-1D × CRL173	<i>P/M</i>	None	4.3	<i>lys1</i> ⁺	140
AY260-2A × AY278-7D	<i>P/M</i>	None	0	<i>cen2</i>	120
MK9 × MK10	<i>P/M</i>	<i>rec7</i>	9.0	<i>lys1</i> ⁺	100
MK67 × MK95	<i>P/M</i>	<i>rec10</i>	3.0	<i>cen2</i>	100
AY199-1C × AY248-2B	<i>P/M</i>	<i>rec12</i>	12.0	<i>lys1</i> ⁺	150
AY199-2A × AY261-28A	<i>P/M</i>	<i>rec12</i>	4.2	<i>cen2</i>	190
AY258-2B × AY258-3B	<i>P/M</i>	<i>mes1</i>	2.7	<i>lys1</i> ⁺	110
AY259-11C × AY259-1B	<i>P/M</i>	<i>rec12 mes1</i>	6.7	<i>lys1</i> ⁺	120
Azygotic diploid meiosis ^c					
AY167d	<i>M/M</i>	<i>pat1</i>	38.2	<i>lys1</i> ⁺	113
AY2463d	<i>M/M</i>	<i>pat1 mat-Pc</i>	5.0	<i>lys1</i> ⁺	80

^aSister locus separation: percentage of cells that contain two *lys1*⁺ or *cen2* sister loci partitioned in two DNA masses at meiosis I.

^bZygotic diploid meiosis: meiosis induced in a diploid zygote that was formed by conjugation of two haploid cells.

^cAzygotic diploid meiosis: meiosis induced in a diploid cell without cell conjugation.

N, number of cells examined.

AY1931), which rarely occurs in normal diploid meiosis. These observations imply that homologous chromosomes are not absolutely required for monopolar attachment, but are required for its high fidelity. To examine if recombination between homologous chromosomes is required for the high fidelity of monopolar attachment, we examined sister chromatid behavior at meiosis I in diploid *rec7*, *rec10* and *rec12* mutant cells, which are defective in recombination (Lin and Smith, 1994, 1995; Molnar *et al.*, 2001). Sister chromatid behavior was monitored by visualizing the centromere-linked locus of chromosome I (*lys1*⁺; ~30 kb from the centromere) or that of chromosome II (*cen2*; ~5 kb from the centromere) on one of the homologous chromosomes. In wild-type diploid cells, sister loci were predominantly observed in only one of the two DNA masses (Table III, AY167-1D × CRL173 and AY260-2A × AY278-7D). Separation of the *lys1*⁺ locus observed in a small fraction of the cells probably resulted from recombination between the centromere and the *lys1*⁺ locus. On the other hand, in all the recombination-deficient diploid cells, separation of the sister loci was observed at a significant level (Table III, MK9 × MK10, MK67 × MK95, AY199-1C × AY248-2B and AY199-2A × AY261-28A). This separation was not caused by meiosis II, because the separation was not abolished by introduction of a *mes1* mutation that blocks the cell cycle before meiosis II (Kishida *et al.*, 1994) (Table III, AY258-2B × AY258-3B and AY259-11C × AY259-1B). From these results, we conclude that the high fidelity of monopolar attachment depends on recombination between homologous chromosomes.

Monopolar attachment of sister chromatids is established at meiosis I in diploid *pat1* cells in a *Rec12*-dependent manner

As recombination increases the fidelity of monopolar attachment of sister chromatids, we next examined whether the recombination causes monopolar attachment of sister chromatids. We induced meiosis in *pat1* diploid cells homozygous for the mating type loci (Iino and

Yamamoto, 1985b). During meiosis in these cells, homologous chromosomes do recombine (Iino and Yamamoto, 1985b; Cervantes *et al.*, 2000; A.Yamamoto, unpublished observation), but the mating pheromone response does not occur because of the homozygosity at the *mat1* locus. Because monopolar attachment is not established in the absence of the mating pheromone signaling in *pat1*-induced haploid meiosis, if recombination does not cause monopolar attachment, sister chromatids separate from each other, as in haploid *pat1* cells. Conversely, if recombination causes monopolar attachment, sister chromatids should move to the same pole.

Such diploid *pat1* cells efficiently underwent meiosis after a temperature shift (Figure 6A, a and b). In these cells, sister chromatids frequently attached to the same pole at meiosis I, unlike those of haploid *pat1* cells. The GFP-visualized centromere-linked *lys1*⁺ (Figure 6B, a, and F, #3 and 4; Table III, AY167d) or *ade6*⁺ (~170 kb from the centromere; Figure 6F, #5) locus on one of the homologous chromosomes showed that sister chromatids moved to the same pole in ~60% of the cells. Furthermore, homologous chromosomes mostly separated from each other, as the GFP-visualized *lys1*⁺ loci on both homologous chromosomes showed mostly a 2:2 segregation pattern after meiosis I (Figure 6C). Therefore, monopolar attachment of sister chromatids is established together with bipolar attachment of homologous chromosomes at meiosis I in diploid *pat1* cells.

After meiosis II, unseparated *lys1*⁺ sister loci were observed in 20% of the cells (Figure 6B, b; Table II, AY167d), suggesting that sister chromatids that move to the same pole at meiosis I were randomly segregated in meiosis II (an expected population for a random segregation is ~30%, that is, one half of the population of cells with unseparated sister chromatids at meiosis I). Therefore, sister centromere cohesion is probably not established or maintained at meiosis I in the diploid *pat1* cells. Nonetheless, a significant fraction of the sister chromatids were properly segregated, as shown by their relatively high spore viability (Table I, AY167d).

Fig. 6. Diploid meiosis induced by Pat1 inactivation. (A) Changes in nuclear morphology (a) and DNA content (b) of diploid *pat1* cells (strain AY167d) after a temperature shift. (B) Behavior of the sister loci at the first (a) or second (b) division (strain AY167d). The *lys1*⁺ locus on one of the homologous chromosomes is visualized. (C) Behavior of the chromosomal loci on both homologous chromosomes at meiosis I (strain AY190d). The *lys1*⁺ loci on both homologous chromosomes are visualized. Photos indicate a cell showing 2:2 (a) or 3:1 (b) segregation patterns. Numbers indicate the populations of the cells. (D) Behavior of the sister loci at meiosis I in diploid *pat1* cells lacking Rec8 (strain 210d, a) or Rec12 (strain AY2001, b). (E) Behavior of the sister loci at meiosis I in a diploid *pat1* cell expressing both *mat-Pc* and *mat-Mc* (strain AY2463d). Red and green, respectively, indicate chromosomal DNA and the GFP-visualized chromosomal locus. (F) The populations of co-segregated sister loci. Closed or open bars indicate the populations of co-segregated *lys1*⁺ or *ade6*⁺ sister loci, respectively. The type of meiosis; meiosis induced in diploid wild-type cells (Wt meiosis) or diploid *pat1* cells homozygous for the *mat1* locus (*pat1* meiosis). Other factors: a mutation or an ectopic *mat1* gene. Strains used for analyses were as follows: 1, AY167-1D × CRL173; 2, AY241-11C × CRL173; 3, AY167d; 4, AY2101; 5, AY215d; 6, AY2001; 7, AY210d; 8, AY2463d.

We next examined whether monopolar attachment in the diploid *pat1* cells depends on recombination. Disruption of *rec12*⁺ gene in diploid *pat1* cells significantly reduced the frequency of monopolar attachment of sister chromatids (Figure 6D, a and F, #6), as well as spore

viability (Table I, AY2001). The frequency of monopolar attachment was similarly reduced together with the spore viability by Rec8 depletion, which, like Rec12 depletion, also causes reduced recombination (Figure 6D, b, and F, #7; Table I, AY210d). Thus, monopolar attachment in *pat1*-induced diploid meiosis depends on both Rec12 and Rec8, and is distinct from that seen in *mat* gene-induced haploid meiosis. These results indicate that recombination probably causes monopolar attachment of sister chromatids in diploid *pat1* cells.

Triggering the mating pheromone signaling increases the fidelity of monopolar attachment to a wild-type level in diploid *pat1* cells

Our results suggest that the high fidelity of monopolar attachment of sister chromatids is generated by the co-operation of two distinct mechanisms: one is independent of homologous chromosomes, while the other is dependent on recombination between the homologous chromosomes. To confirm this hypothesis, we induced mating pheromone signaling in diploid *pat1* cells using *c*-type *mat* genes, as the mating pheromone signaling activates the homolog-independent mechanism in *pat1*-induced meiosis. Diploid *pat1* cells expressing both *mat-Pc* and *mat-Mc* underwent the mating pheromone response, as judged by their elongation and the early initiation of meiosis. In these cells, monopolar attachment of sister chromatids was established at a level similar to that of wild-type cells (Figure 6E and F, #8; Table III, AY2463d). Spore viability was also increased to wild-type levels (Table I, AY2463d), indicating that chromosome segregation was almost normal. Thus, induction of the mating pheromone signaling generated faithful monopolar attachment of sister chromatids in diploid *pat1* cells. This result supports the idea that the high fidelity of monopolar attachment is generated by the co-operation of the two mechanisms.

Discussion

In this study, we have shown that monopolar attachment of sister chromatids at meiosis I is established without the aid of homologous chromosomes in *S.pombe*. However, we have shown that recombination between homologous chromosomes significantly increases the fidelity of monopolar attachment, and that the recombination also probably causes monopolar attachment. Our results indicate that monopolar attachment is established by two distinct mechanisms, one that is homolog independent and one homolog dependent, and suggest that the co-operation of these two mechanisms is required for the high fidelity of monopolar attachment. As discussed below, we propose that the homolog-independent mechanism produces mono-orientation of sister kinetochores and the homolog-dependent mechanism further ensures their mono-orientation and/or generates a preference for the monopolar attachment of sister chromatids, resulting in faithful monopolar attachment at meiosis I.

Homolog-independent, monopolar attachment of sister chromatids

Our conclusion that homologous chromosomes are not required for monopolar attachment of sister chromatids comes from the finding that monopolar attachment is

established in haploid meiosis. It has been observed in many organisms that a pair of sister chromatids move to the same pole (that is, show monopolar attachment) even when they are not associated with their homologous chromosomes by chiasmata (e.g. Maguire, 1987; Hunt *et al.*, 1995; Rebollo and Arana, 1995). Thus, homolog-independent, monopolar attachment of sister chromatids is probably common in eukaryotes.

We found that depletion of meiotic cohesin subunit, Rec8, caused equational sister chromatid segregation at meiosis I in haploid cells, as was shown previously for diploid cells (Watanabe and Nurse, 1999). Thus, Rec8 is essential for homolog-independent, monopolar attachment of sister chromatids. Furthermore, Rec8 is apparently required for the persistence of sister centromere cohesion until anaphase II. Given the centromere localization of Rec8, it is likely that Rec8 forms a meiosis-specific centromere structure, which generates sister kinetochore mono-orientation and maintains sister centromere cohesion (Watanabe and Nurse, 1999).

We also found that Pat1 inactivation alone is insufficient to establish monopolar attachment of sister chromatids in haploid cells. We have shown that, in addition to Pat1 inactivation, mating pheromone signaling is required for establishing monopolar attachment. What is the role of mating pheromone signaling? It is most likely that mating pheromone signaling is required for the proper functioning of Rec8. Rec8 is localized to chromosomes, and it must be expressed before premeiotic DNA replication to execute its functions (Watanabe *et al.*, 2001). We have shown that mating pheromone signaling is not essential for chromosome localization of Rec8 or its expression. However, we cannot exclude the possibility that mating pheromone signaling is required for Rec8 to localize at subregions within the centromere. Rec8 accumulates at both the inner and the outer centromere regions, unlike a mitotic cohesin Rad21, which accumulates predominantly at the outer centromere region (Watanabe *et al.*, 2001). Mating pheromone signaling may be required for Rec8 accumulation at the inner centromere region, which is likely required for its meiosis-specific functions. Alternatively, mating pheromone signaling may induce or activate other factors that co-operate with Rec8 or regulate Rec8 functions at centromeres. Cdc2 kinase, which is a master regulator of the cell cycle, may be one such factor, because Cdc2 appears to accumulate at the centromeres upon induction of mating pheromone signaling (Decottignies *et al.*, 2001). Cdc2 also accumulates in the nucleus upon induction of mating pheromone signaling, and this nuclear accumulation may be a cause of the early initiation of meiosis seen in *pat1* haploid cells with mating pheromone response.

Rec8-mediated, mono-orientation of sister kinetochores is probably generated during the premeiotic G₁/S phase, because Rec8 is required during this phase for monopolar attachment (Watanabe *et al.*, 2001). At this stage of meiosis, striking changes in centromere positioning take place in *S.pombe*, that is, the centromeres are located near the spindle pole body (SPB) during mitotic interphase (Funabiki *et al.*, 1993) but are away from it during the premeiotic stage (Chikashige *et al.*, 1994). Furthermore, the centromere component, Nuf2, disappears from centromeres during this phase (Nabetani *et al.*, 2001).

Disappearance of Nuf2 and the detachment of centromeres from the SPB may allow the centromeres to be reconstructed in a Rec8-dependent manner to generate sister kinetochore mono-orientation. Studies of kinetochore structures in insect cells suggest that a single kinetochore is formed for a pair of sister chromatids at meiosis I, leading to monopolar attachment of the sister chromatids (Goldstein, 1981; Rufas *et al.*, 1989). Similarly, a single kinetochore may be formed in *S.pombe* through centromere reconstruction. Further studies will be required to determine the centromere structure that causes monopolar attachment of sister chromatids at meiosis I.

Homolog-dependent, monopolar attachment of sister chromatids

Although homologous chromosomes are not required for monopolar attachment of sister chromatids, recombination between the homologous chromosomes increases the high fidelity of monopolar attachment, and also causes monopolar attachment. In many organisms, sister chromatids occasionally separate from each other when they are not linked to their homologous chromosomes by chiasmata (e.g. Maguire, 1987; Hunt *et al.*, 1995; Rebollo and Arana, 1995). In addition, in *spo13* diploid cells of *S.cerevisiae*, monopolar attachment of sister chromatids is established in a recombination-dependent manner (Klapholz *et al.*, 1985; Shonn *et al.*, 2002). Thus, recombination probably plays a similar role in monopolar attachment of sister chromatids in other organisms. In *S.cerevisiae*, however, recombination does not seem to be required for the high fidelity of monopolar attachment, because in recombination-deficient *spo11* diploid cells, sister chromatid separation still appears to be very rare, as in wild-type diploid cells (Klein *et al.*, 1999). This difference may result from a specific property of the budding yeast centromere (Tyler-Smith and Florida, 2000).

How does recombination contribute to monopolar attachment of sister chromatids? Considering the fact that bipolar attachment of homologous chromosomes is established together with monopolar attachment of sister chromatids in *pat1*-induced diploid meiosis, we propose two possibilities, which are not mutually exclusive. One possibility is that chiasma (recombination)-mediated homolog association changes chromosome configuration, generating both the mono-orientation of sister kinetochores and bi-orientation of homologous kinetochores. In this model, only chiasmata formed in the vicinity of centromeres may efficiently orient kinetochores in the proper directions. The considerable level of sister chromatid separation in *pat1*-induced diploid meiosis may be explained by an occasional lack of chiasmata in the vicinity of the centromere. An alternative possibility is that chiasma-mediated homolog association generates a preference for monopolar attachment of sister chromatids together with bipolar attachment of homologous chromosomes because such attachments generate tension at kinetochores that stabilizes spindle attachment of kinetochores. In this model, the spindle checkpoint may play a critical role, because it prevents cells from entering anaphase when tension is absent at kinetochores or the kinetochores are not attached to the spindle, and thereby strengthens the preference. However, in our preliminary observation, the frequency of monopolar attachment is not

Table IV. Strain list

Strain	Genotype
AY1391	<i>h⁺ ade6-M210 ura4-D18 lys1⁺::mat-M(i+c)</i>
AY1395	<i>h⁺ ade6-M210 leu1-32 ura4-D18 pat1-114 lys1⁺::mat-Mc</i>
AY1396	<i>h⁺ ade6-M210 leu1-32 ura4-D18 pat1-114 lys1⁺::mat-Mi</i>
AY167-1D	<i>h⁺ ade6-M210 leu1-32 ura4-D18 his7⁺::lacI-GFP lys1⁺::lacOp</i>
AY167d	<i>h⁺/h⁻ leu1-32/leu1⁺ ura4-D18/ura4⁺ his7⁺::lacI-GFP/his7⁺ lys1⁺::lacOp/lys1-131 pat1-114/pat1-114</i>
AY1731	<i>h⁺/h⁻ ade6-M210/ade6-M216 leu1-32/leu1⁺ ura4-D18/ura4⁺ his7⁺::lacI-GFP/his7⁺ lys1⁺::lacOp/lys1-131</i>
AY191-6C	<i>h⁺ pat1-114 his7⁺::lacI-GFP lys1⁺::lacOp</i>
AY195-1C	<i>h⁻ ura4-D18 pat1-114 his7⁺::lacI-GFP lys1⁺::lacOp sxa2::ura4⁺</i>
AY190d	<i>h⁺/h⁻ leu1-32/leu1⁺ ura4-D18/ura4⁺ his7⁺::lacI-GFP/his7⁺ lys1⁺::lacOp/lys1⁺::lacOp</i>
AY1901	<i>h⁺ ura4-D18 pat1-114 his7⁺::lacI-GFP lys1⁺::lacOp cen2(D107)::mat-Mc-URA3</i>
AY1902	<i>h⁺ ura4-D18 pat1-114 his7⁺::lacI-GFP lys1⁺::lacOp cen2(D107)::mat-Mi-URA3</i>
AY1931	<i>h⁺ ura4-D18 his7⁺::lacI-GFP lys1⁺::lacOp cen2(D107)::mat-M(i+c)-URA3</i>
AY199-1C	<i>h⁻ leu1-32 rec12-152::LEU2</i>
AY199-2A	<i>h⁺ leu1-32 rec12-152::LEU2</i>
AY2001	<i>h⁻/h⁺ ade6-L52/ade6⁺ leu1-32/leu1-32 ura4-D18/ura4⁺ pat1-114/pat1-114 lys1-131/lys1⁺::lacOp his7⁺/his7⁺::lacI-GFP rec12-152::LEU2/rec12-152::LEU2</i>
AY2071	<i>h⁺ leu1-32 ura4-D18 rec12-152::LEU2 ade6[::kan^r-ura4⁺-lacOp] his7⁺::lacI-GFP lys1⁺::mat-M(i+c)</i>
AY2072	<i>h⁺ leu1-32 ura4-D18 ade6[::kan^r-ura4⁺-lacOp] his7⁺::lacI-GFP lys1⁺::mat-M(i+c)</i>
AY2081	<i>h⁻ leu1-32 ura4-D18 rec8::ura4⁺ ade8[::kan^r-ura4⁺-lacOp] his7⁺::lacI-GFP lys1⁺::mat-P(i+c)</i>
AY2082	<i>h⁻ leu1-32 ura4-D18 ade8[::kan^r-ura4⁺-lacOp] his7⁺::lacI-GFP lys1⁺::mat-P(i+c)</i>
AY210d	<i>h⁻/h⁺ leu1-32/leu1⁺ ura4-D18/ura4-D18 pat1-114/pat1-114 rec8::ura4⁺/rec8::ura4⁺ his7⁺::lacI-GFP/his7⁺ lys1⁺::lacOp/lys1-131</i>
AY2101	<i>h⁺/h⁺ leu1-32/leu1⁺ ura4⁺/ura4-D18 pat1-114/pat1-114 his7⁺::lacI-GFP/his7⁺ lys1⁺::lacOp/lys1-131</i>
AY215d	<i>h⁺/h⁺ his2⁺/his2-245 leu1-32/leu1⁺ lys1-131/lys1⁺ ura4-D18/ura4⁺ pat1-114/pat1-114 ade6[::kan^r-ura4⁺-lacOp]/ade6⁺ his7⁺::lacI-GFP/his7⁺</i>
AY221-8A	<i>h⁺ ade6-M210 pat1-114 rec8⁺::GFP-kan^r</i>
AY2211	<i>h⁺ ade6-M216 lys1⁺::mat-M(i+c) rec8⁺::GFP-kan^r</i>
AY241-11C	<i>h⁺ lys1-131 ura4-D18 ade6[::kan^r-ura4⁺-lacOp] his7⁺::lacI-GFP</i>
AY248-2B	<i>h⁺ leu1-32 rec12-152::LEU2 his7⁺::lacI-GFP lys1⁺::lacOp</i>
AY2463d	<i>h⁻/h⁺ leu1-32/leu1⁺ ura4⁺/ura4-D18 pat1-114/pat1-114 lys1⁺::mat-Pc/lys1⁺::lacOp his7⁺/his7⁺::lacI-GFP</i>
AY258-2B	<i>h⁺ leu1-32 lys1-131 mes1::LEU2 his7⁺::lacI-GFP</i>
AY258-3B	<i>h⁻ his2-245 leu1-32 ura4-D18 mes1::LEU2 lys1⁺::lacOp</i>
AY259-11C	<i>h⁺ leu1-32 rec12-152::LEU2 mes1::LEU2 lys1⁺::lacOp his7⁺::lacI-GFP</i>
AY259-1B	<i>h⁻ his2-245 leu1-32 lys1-131 rec12-152::LEU2 mes1::LEU2</i>
AY260-2A	<i>h⁺ ade6-M210 leu1-32 lys1-131 ura4-D18 cen2(D107)::kan^r-ura4⁺-lacOp his7⁺::lacI-GFP</i>
AY261-28A	<i>h⁻ leu1-32 lys1-131 ura4-D18 rec12-152::LEU2 cen2(D107)::kan^r-ura4⁺-lacOp his7⁺::lacI-GFP</i>
AY278-7D	<i>h⁻ leu1-32 lys1-131 ura4-D18 his7⁺::lacI-GFP</i>
AY309-9C	<i>h⁺ pat1-114 rec8⁺::GFP-kan^r</i>
AY311-19C	<i>h⁻ leu1-32 ura4-D18 pat1-114 mis12⁺::3HA-LEU2 rec8⁺::GFP-kan^r</i>
CRL173	<i>h⁻ ade6-M216</i>
CRL245	<i>h⁻ pat1-114</i>
CRL246	<i>h⁻ leu1-32 lys1-131 pat1-114</i>
CRL2411	<i>h⁻ pat1-114 lys1⁺::mat-Pi</i>
CRL2412	<i>h⁻ pat1-114 lys1⁺::mat-Pc</i>
CRL298	<i>h⁻ leu1-32 ura4-D18 pat1-114 sxa2::ura4⁺</i>
CRL4221	<i>h⁺ leu1-32 lys1⁺::mat-M(i+c)</i>
MK9	<i>h⁺ leu1-32 ura4-D18 rec7::ura4⁺ lys1⁺::lacOp his7⁺::lacI-GFP</i>
MK10	<i>h⁻ ade6-M216 lys1-131 ura4-D18 rec7::ura4⁺</i>
MK67	<i>h⁺ ade6-M216 leu1-32 rec10-155::LEU2</i>
MK95	<i>h⁻ leu1-32 rec10-155::LEU2 cen2(D107)::kan^r-ura4⁺-lacOp his7⁺::lacI-GFP</i>

significantly altered in *pat1* diploid cells by depleting a spindle checkpoint factor, Mad2 (A.Yamamoto, unpublished observation), suggesting that monopolar attachment seen in *pat1* diploid cells is not dependent on the spindle checkpoint. Further studies will be required to elucidate precisely how recombination contributes to monopolar attachment of sister chromatids.

Conclusions

In this study, we have shown that monopolar attachment of sister chromatids at meiosis I is established by two distinct mechanisms in *S.pombe*. Furthermore, we have shown that each of these mechanisms can be specifically investigated in *mat* gene-induced haploid meiosis or in *pat1*-induced diploid meiosis. It is clear that more players need to be

identified to understand the precise mechanisms of monopolar attachment. These two distinct forms of meiosis will probably make a significant contribution to unveiling the functions of these players and lead to further understanding of the mechanism of chromosome segregation during meiosis.

Materials and methods

Strains and media

The fission yeast strains used in this study are listed in Table IV. Culture media were prepared as described by Moreno *et al.* (1991) except that YE medium containing 75 mg/ml adenine sulfate (YEA) was used for the routine culture of cells. Genetic techniques used in this study are also described by Moreno *et al.* (1991). Diploid cells homozygous for the *mat* loci were constructed by protoplast fusion (Alfa *et al.*, 1993).

Integration of the mating type genes at an ectopic locus of the chromosome

DNA cassettes of P and M types, each of which contained the two mating type-specific genes and their promoters, were amplified by PCRs using genomic DNA of homothallic wild-type cells as a template, and synthetic oligonucleotide primers. Primers used for amplifying the P cassette were CGACCTCGAGCTGTATGCATATGCATATGC (primer A) and CCCCTCGAGCCGAATGAATAATACCTTAA. Primers used for amplifying the M cassette were primer A and CCCCTCGAGCAGCATAT-TTCGATAGAATC (primer B). The amplified DNA fragments were digested by *XhoI* and inserted at the *SalI* site of an integration plasmid, pYC36 or pAY149. pYC36 contains a DNA fragment encoding a portion of *lysI*⁺ gene. pAY149 contains a budding yeast *URA3* gene as a selection marker and a 1.7 kb genomic DNA fragment (D107) that is excised by *HindIII* from the chromosomal region located ~5 kb from the centromere of chromosome II (this fragment contains an N-terminal portion of the *rec6*⁺ gene). The resultant plasmids were integrated at either the *lysI*⁺ or D107 locus.

The *c*- or *i*-type *mat* gene was integrated into chromosomes as follows. A DNA fragment containing *mat-Pc*, *mat-Mi* or *mat-Mc* was amplified by PCR using the cloned P or M cassette as a template and synthetic oligonucleotides as primers (for *mat-Pc*, primer A and CCCCTCGA-GAATGATTCTATCGTATCC; for *mat-Mi*, primer A and CCCCTCGAGATCTAAAAAGTCGTAGGAAA; for *mat-Mc*, CCCCTCG-AGATCACATAGGATTGAATTG and primer B). The amplified fragments were digested by *XhoI* and inserted at the *SalI* site of an integration plasmid, pYC36 or pAY149. A *BamHI*-*XhoI* fragment of the amplified P cassette containing *mat-Pi* was inserted between the *BamHI* and *SalI* sites of pYC36 or pAY149 and integrated at the *lysI*⁺ or D107 locus. Each cloned fragment contained the promoter that was required for expression of each gene, as shown by induction of meiosis in *h*⁻ cells containing both *mat-Pi* and *mat-Pc* plasmids or *h*⁺ cells containing both *mat-Mi* and *mat-Mc* plasmids under nitrogen-starved conditions.

Fluorescence visualization of chromosome loci

The rDNA locus on chromosome III was visualized by fluorescence *in situ* hybridization as described previously (Yamamoto *et al.*, 1999). The *lysI*⁺, *ade6*⁺, *ade8*⁺ and *cen2*-proximal loci were visualized by the *lacI*/*lacO* recognition system (Robinett *et al.*, 1996; Straight *et al.*, 1996; Nabeshima *et al.*, 1998). Tandem repeats of the *lacO* sequence integrated at the *lysI*⁺ locus and GFP-tagged *lacI* were derived from strain MKY7A-4 (Nabeshima *et al.*, 1998). The *lacO* tandem repeats were integrated at the *ade6*⁺, *ade8*⁺ or *cen2*-proximal locus by two-step integration: first, a DNA fragment containing a partial *ura4* gene was integrated at the locus, and secondly, the *lacO* tandem repeats were integrated at the locus using the *ura4*⁺ sequence as a target. A DNA fragment containing the partial *ura4*⁺ gene was amplified by PCR, using two DNA primers: CGC-GGATCCGAATTGTTGGCTTTGATGGAAG and CGCGGATCCTT-AGTCGCTACATAAAATTTTACC, with a genomic DNA fragment containing *ura4*⁺ gene as a template. The amplified DNA fragment was digested by *BamHI* and inserted at the *BglII* site of a plasmid, pFA6a-kanMX6 (Wach *et al.*, 1997), giving pCT33-6. To integrate a partial *ura4* gene at the *ade8*⁺ locus, a DNA fragment containing both the partial *ura4* and *kan*^r genes was amplified by PCR using two DNA primers: CAAAATAATAAATCTGATGGTCTTCTCAAGGTGGAGCATTGTG-TAGAGACTACTTAGTGTGGTTCCTCACTGTCCATTCCACCAC-AACAGGTAGCGGATCCCGGGTTAATTAA and TCTTCTTG-ACTACTCGGCCAGCTTGATGAGACAAGACAGCAATCTGCCG-TGACACTCATGACGACTAGCACCGTGCTTAGTCATAGCCGA-ATTGAGCTCGTTTAAA, with pCT33-6 as a template. The amplified DNA fragment was integrated at the *ade8*⁺ locus of wild-type cells (*h*⁹⁰ *leu1 lysI ura4*), as described previously (Bähler *et al.*, 1998). To integrate the partial *ura4* gene at the *ade6*⁺ locus, a PCR-amplified *BglII*-*EcoRI* DNA fragment of pCT33-6 was inserted between *BamHI* and *EcoRI* sites in an open reading frame of the *ade6*⁺ gene of a genomic DNA fragment. This DNA fragment was then integrated at the *ade6*⁺ locus. To integrate the partial *ura4* gene near the centromere of chromosome II, a *BamHI*-*SpeI* fragment of pCT33-6 was inserted between *BglII* and *SpeI* sites of the D107 DNA fragment. This fragment was then integrated at the D107 locus. Integration of *lacO* repeats at the *ura4* target sequence was carried out as follows. A DNA fragment containing the *ura4*⁺ gene was inserted at the *NaeI* site of pBluescript plasmid (Stratagene, La Jolla, CA), giving pCT30. Then, a *SalI*-*XhoI* fragment of pSV2-dhfr8.32 (Robinett *et al.*, 1996) containing *lacO* tandem repeats was inserted at the *XhoI* site of pCT30, giving pCT31. pCT31 was linearized by *SmaI* and introduced into the cells containing the target *ura4* gene. The integrants were

selected by *ura*⁺ phenotype. At all steps, integration was confirmed by colony PCR and Southern hybridization analyses.

Induction of meiosis and monitoring the progression of meiosis

Haploid cells containing opposite mating type genes at the ectopic locus were grown in YEA liquid medium to a density of $2-5 \times 10^7$ cells/ml at 33°C. Cells were suspended in an equal volume of EMM2 medium lacking a source of nitrogen (EMM2-N), and induced into meiosis by further incubation at 26°C. *pat1* haploid cells or *pat1* diploid cells homozygous for the *mat1* allele were grown in YEA liquid medium to a density of $2-5 \times 10^6$ cells/ml at 26°C. They were suspended in EMM2-N medium and incubated at 26°C for 14–16 h. They were then resuspended in fresh EMM2-N medium and induced into meiosis by further incubation at 34°C. To induce the mating pheromone response in *h*⁻ *pat1* haploid cells, P factor was added to the EMM2-N medium at a concentration of 0.5 µg/ml during the 26°C incubation. To monitor the progression of meiosis, a portion of the cell culture was taken at regular intervals and chromosomal DNA morphology or DNA content of the cells was analyzed. The DNA content of the cells was analyzed using a protocol from the Forsburg laboratory (available on the Internet at <http://pingu.salk.edu/flow/protocols/yc.html>).

Analysis of microtubule morphology and spindle behavior

Microtubules were visualized by GFP-tagged α 2-tubulin, and their morphology and behavior were examined in living cells, as described (Yamamoto *et al.*, 2001).

Analysis of Rec8 localization

Rec8-GFP was observed in living cells as described previously for GFP-tagged dynein heavy chain (Yamamoto *et al.*, 1999). Chromosome spread was carried out, as described (Bähler *et al.*, 1993), and the Rec8-GFP on the spread chromosomes was visualized by rabbit polyclonal anti-GFP antibody (1:1000 dilution; a gift from Dr J.R.McIntosh, Colorado University). To visualize HA-tagged Mis12 (Goshima *et al.*, 1999) together with the Rec8-GFP, cells were fixed with 3.7% formaldehyde in the culture medium for 20 min at 25°C. The Mis12-HA and Rec8-GFP were stained as described (Hagan and Hyams, 1988), using 16B12 mouse anti-HA antibody (1:10000 dilution; Babco, Richmond, CA) and the rabbit anti-GFP antibody.

Northern blot analysis of Rec8 transcript

Rec8-GFP and Cam1 transcripts were detected using PCR-amplified genomic DNA fragments as probes. The DNA fragment for Rec8 detection was amplified using two DNA primers: GCAGTTACTA-CTCGCAGAAGA and TGGCTAAATGCCTGGGCCACT, and that for Cam1 detection was amplified using primers, ACTACCCGTAACCTTACAGAT and TTGGAAGAAATGACACGAGAG. Hybridization signals were detected using ECL Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences Corp.).

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