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ATP analog-sensitive Pat1 protein kinase for synchronous fission yeast meiosis at physiological temperature

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To study meiosis, synchronous cultures are often indispensable, especially for physical analyses of DNA and proteins. A temperature-sensitive allele of the Pat1 protein kinase (*pat1-114*) has been widely used to induce synchronous meiosis in the fission yeast *Schizosaccharomyces pombe*, but *pat1-114*-induced meiosis differs from wild-type meiosis, and some of these abnormalities might be due to higher temperature needed to inactivate the Pat1 kinase. Here, we report an ATP analog-sensitive allele of Pat1 [Pat1(L95A), designated *pat1-as2*] that can be used to generate synchronous meiotic cultures at physiological temperature. In *pat1-as2* meiosis, chromosomes segregate with higher fidelity and spore viability is higher than in *pat1-114* meiosis, although recombination is lower by a factor of 2–3 in these mutants than in starvation-induced *pat1*$^+$ meiosis. Addition of the *mat-Pc* gene improved chromosome segregation and spore viability to nearly the level of starvation-induced meiosis. We conclude that *pat1-as2 mat-Pc* cells offer synchronous meiosis with most tested properties similar to those of wild-type meiosis.

Introduction

Sexual reproduction depends on formation of haploid gametes from a diploid precursor cell in a process called meiosis. The fission yeast *Schizosaccharomyces pombe* has been an excellent model system for studying meiosis at the molecular level. One of the advantages of using fission yeast is that highly synchronous meiosis can be induced by inactivation of a temperature-sensitive allele of the Pat1 (Ran1) protein kinase (*pat1-114*). Pat1 kinase is a negative regulator of meiosis, and its major target is Mei2, an RNA-binding protein crucial for meiotic entry.[1-5] Highly synchronous meiotic cultures can be obtained by arresting *pat1-114* cells in G_1 by nitrogen starvation and subsequent inactivation of Pat1 by shifting cells to non-permissive temperature (34°C).[6,7] When meiosis is induced in cells by Pat1 inactivation, those cells synchronously undergo premeiotic S phase followed by two rounds of chromosome segregation (meiosis I and meiosis II, respectively) and form spores. This allows study of meiotic events in an entire population, which is particularly important for biochemical and cytological assays.

Although *pat1-114*-induced meiosis is in many aspects similar to wild-type (*pat1*$^+$) meiosis, there are several important differences. The frequency of recombination is reduced; nuclear positioning of centromeres is aberrant; chromosomes frequently missegregate during meiosis I, and spore viability is significantly reduced in *pat1-114*-induced meiosis.[6,8,9] At least some of these defects are due to the absence of mating pheromone signaling, because the chromosome segregation defect can be partially suppressed by ectopic expression of genes from both *mat* loci, which activates the mating pheromone-signaling pathway.[8,10] However, a major disadvantage of using the *pat1-114*-induced meiosis is that it requires elevated temperature (34°C) to inactivate the Pat1. Detrimental effects of elevated temperature on meiosis have been described in numerous studies in references 6 and 11–14. Therefore, it is desirable that a physiological temperature (e.g., 25°C) be used for studying meiosis.

Recent advances in chemical genetics now allow construction of conditional ATP analog-sensitive protein kinase alleles that can be inactivated by adding an inhibitor without the need of elevated temperature.[15,16,17] Here, we construct analog-sensitive alleles of the Pat1 kinase (*pat1-as*), and show that *pat1-as2* [Pat1(L95A)] can be used to generate meiotic cultures that progress through meiosis with a high degree of synchrony at physiological temperature. Importantly, we show that using *pat1-as2* improves the fidelity of chromosome segregation and spore viability to levels near those of fully wild-type meiosis while maintaining high synchrony.

Results

***pat1-as2* can be used to generate synchronous meiotic cultures at physiological temperature.** To inactivate Pat1 conditionally,

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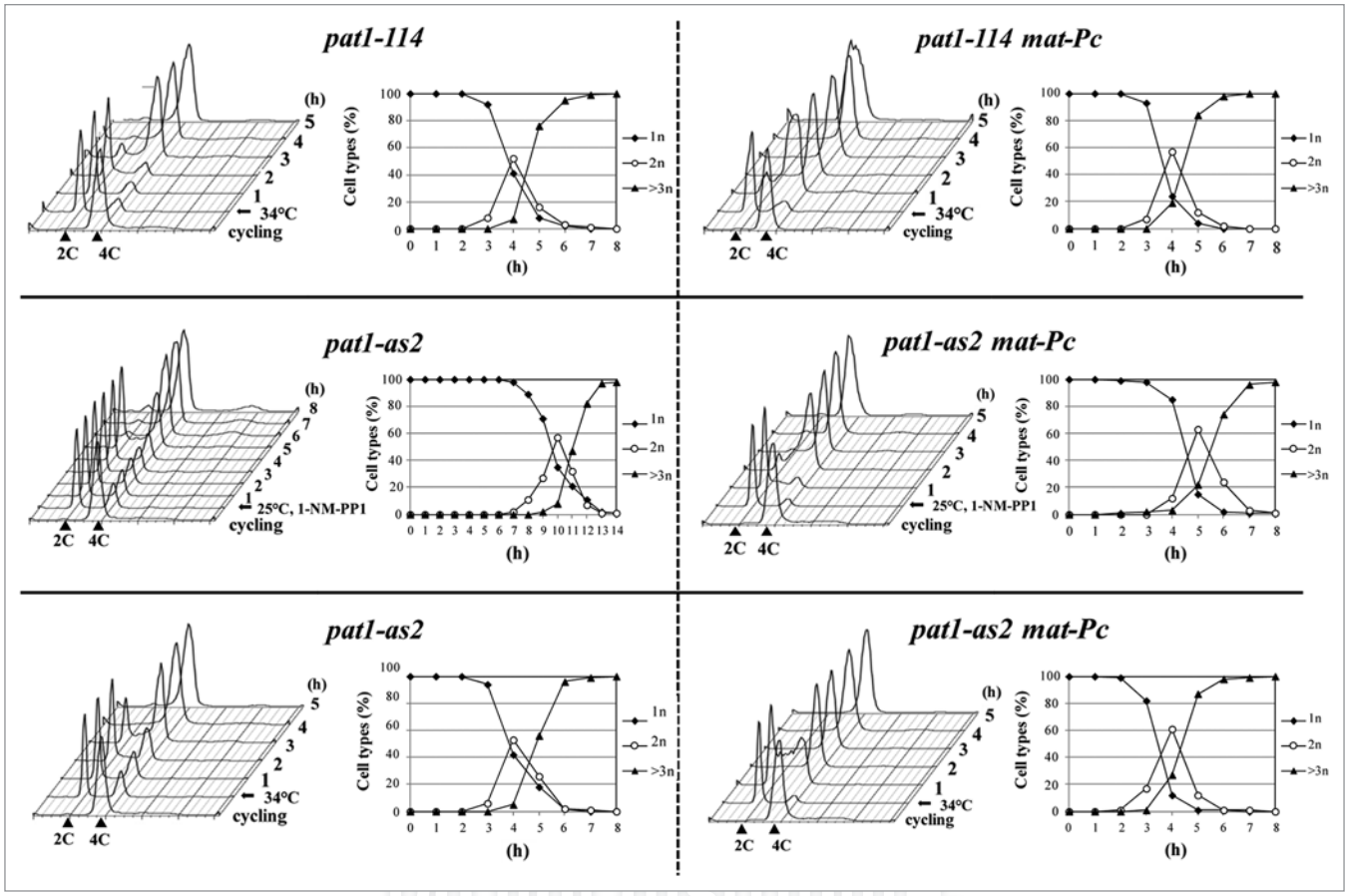
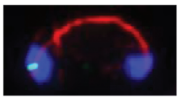
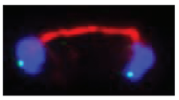


Figure 1. Progression of diploid *pat1-114/pat1-114* (JG12209), *pat1-as2/pat1-as2* (JG15620), *pat1-114/pat1-114 mat-Pc* (JG16328) and *pat1-as2/pat1-as2 mat-Pc* (JG16113) cells into meiosis. Cells were cultured to mid log phase in YES-Ade medium, transferred to EMM2-NH_4Cl medium for 16 h at 25°C (*pat1-114* and *pat1-as2*) or for 7 h at 25°C (*pat1-114 mat-Pc* and *pat1-as2 mat-Pc*) to synchronize cells in G_1, then shifted into EMM2 medium and incubated at 34°C or kept at 25°C with addition of 25 μM 1-NM-PP1 to inactivate the Pat1 kinase. Progression of meiosis was monitored by flow cytometry (Cytox Green staining) and by counting the nuclei (DAPI staining) of samples that were collected at the indicated time points after temperature shift or addition of 1-NM-PP1.

we applied a chemical-genetic strategy for sensitizing protein kinases to small-molecule inhibitors.[15,16] We mutated a single codon, that for leucine 95 in the ATP-binding pocket of Pat1, termed the "gate-keeper" residue, to a small residue (glycine or alanine). While Pat1(L95G) mutant (*pat1-as1*) was not fully functional (unpublished data), Pat1(L95A) (*pat1-as2*) appeared to be functional as judged by the nearly wild-type growth of cells on YES medium at 25°C.[17] We also noticed that cells expressing Pat1(L95A) were temperature-sensitive and sporulated at 34°C in the absence of an inhibitor. Importantly, Pat1(L95A) cells grew normally at 25°C on YES medium in the absence of an inhibitor, but very poorly in the presence of the ATP-analog 1-NM-PP1.[17] Thus, we conclude that at 25°C, Pat1(L95A) is functional and confers sensitivity to 1-NM-PP1. We refer to Pat1(L95A) as Pat1-as (Pat1-analog sensitive).

We next analyzed whether the *pat1-as2* allele can be used to generate meiotic cultures that progress through meiosis with a high degree of synchrony. We arrested diploid *pat1-as2/pat1-as2* cells in G_1 by nitrogen starvation and subsequently inactivated the Pat1-as kinase by adding 1-NM-PP1 at 25°C. Analysis of nuclear divisions together with FACS analysis revealed that these cells underwent premeiotic S phase followed by two rounds of chromosome segregation in a very synchronous manner (**Fig. 1**). The level of synchrony in *pat1-as2*-induced meiosis was similar to that in *pat1-114*-induced meiosis, although the onset of S phase and meiotic divisions at 25°C were delayed by about 2–3 h in *pat1-as2*-induced meiosis (**Fig. 1**). A high level of synchrony was also observed in diploid *pat1-as2/pat1-as2* cells, where the mating-pheromone signaling pathway was activated by ectopically expressing *mat-Pc* locus (**Fig. 1**). In this case, S phase was delayed by only about 1 h. We conclude that *pat1-as2* can be used as a tool to generate synchronous meiotic cultures at physiological temperature 25°C.

***pat1-as2* improves fidelity of chromosome segregation and spore viability.** *pat1-114*-induced meiosis differs from normal meiosis in several aspects, such as frequency of recombination, nuclear positioning of centromeres, segregation of sister centromeres during meiosis I, and spore viability.[6,8,9] While the aberrant nuclear positioning of centromeres results from Pat1 inactivation, not the high temperature,[9] some of the other defects might be due to the detrimental effects of elevated temperature, which is used to inactivate the Pat1-114 mutant kinase. To test this, we compared

Strains	Genotype	Induction condition	Anaphase I: reductional [%]	Anaphase I: equational [%]
				
JG15458 x JG12226	*pat1*$^{+}$ *(P/M)*	25°C	98.7 ± 0.6	1.3 ± 0.6
JG16022	*pat1-as2 (M/M)*	25°C, 1-NM-PP1	44.3 ± 3.4	55.7 ± 3.4
JG16022	*pat1-as2 (M/M)*	34°C	29.9 ± 4.0	70.1 ± 4.0
JG16022	*pat1-as2 (M/M)*	25°C, 1-NM-PP1, P-factor	89.6 ± 3.9	10.4 ± 3.9
JG16022	*pat1-as2 (M/M)*	34°C, P-factor	85.4 ± 2.3	14.6 ± 2.3
JG16113	*pat1-as2 (M/M, mat-Pc)*	25°C, 1-NM-PP1	95.7 ± 1.1	4.3 ± 1.1
JG16113	*pat1-as2 (M/M, mat-Pc)*	34°C	93.2 ± 1.4	6.8 ± 1.4

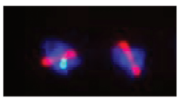
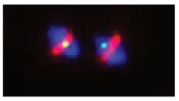
Strains	Genotype	Induction condition	Metaphase II	
				
JG15458 x JG12226	*pat1*$^{+}$ *(P/M)*	25°C	98.1 ± 0.4	1.9 ± 0.4
JG16022	*pat1-as2 (M/M)*	25°C, 1-NM-PP1	48.8 ± 1.8	51.2 ± 1.8
JG16022	*pat1-as2 (M/M)*	34°C	31.6 ± 3.1	68.4 ± 3.1
JG16022	*pat1-as2 (M/M)*	25°C, 1-NM-PP1, P-factor	91.7 ± 2.9	8.3 ± 2.9
JG16022	*pat1-as2 (M/M)*	34°C, P-factor	86.8 ± 4.2	13.2 ± 4.2
JG16113	*pat1-as2 (M/M, mat-Pc)*	25°C, 1-NM-PP1	97.3 ± 0.6	2.7 ± 0.6
JG16113	*pat1-as2 (M/M, mat-Pc)*	34°C	94.1 ± 1.2	5.9 ± 1.2

Figure 2. Analysis of segregation of sister centromeres during meiosis I. Wild-type *pat1*$^{+}$ (JG12226) cells carrying *cen2*-GFP (*cen2*-proximal loci were visualized by the LacI-GFP fusion protein bound to *lac* operator array inserted about 5 kb from *cen2*)[8] were crossed to cells lacking *cen2*-GFP (JG15458) and sporulated in EMM2-NH_4Cl medium. Diploid *pat1-as2/pat1-as2* (JG16022) and *pat1-as2/pat1-as2 mat-Pc* (JG16113) cells carrying heterozygous *cen2*-GFP were cultured to mid log phase in YES-Ade medium, then transferred to EMM2-NH_4Cl medium for 16 h at 25°C to synchronize cells in G_1, then shifted into EMM2 medium and released into meiosis by adding 25 μM 1-NM-PP1 inhibitor at 25°C or by shifting the cells to 34°C. Mating pheromone-signaling was triggered either by addition of synthetic P-factor (70 μg/ml) or expression of the *mat-Pc* as indicated. Cells in anaphase I or metaphase II were fixed, stained with Hoechst 33342 and antibodies against tubulin and GFP and examined under a fluorescence microscope. Segregation of chromosome II labeled with *cen2*-GFP was scored in 600 anaphase I cells and in 200 metaphase II cells.

recombination, chromosome segregation and spore viability in wild-type cells and in cells where meiosis was induced by inactivation of Pat1 either by higher temperature (*pat1-114*, 34°C and *pat1-as2*, 34°C) or by adding inhibitor (*pat1-as2*, 25°C).

While in wild-type cells, sister centromeres segregate to the same pole in anaphase I, we observed that in cells where meiosis was induced by inactivation of Pat1-as at 34°C (note that *pat1-as2* is temperature-sensitive), sister centromeres segregated to the same pole in only 30% of anaphase I cells but 45% when meiosis was induced by inhibiting Pat1-as at 25°C (**Fig. 2**). We confirmed, as previously reported in reference 8, that triggering mating pheromone-signaling either by ectopically expressing *mat-Pc* or by adding P-factor increased the fidelity of segregation of sister centromeres during anaphase I. In cells induced into meiosis by inactivation of Pat1 by higher temperature (34°C), sister centromeres segregated to the same pole in 85% of cells treated with P-factor and in 93% of cells containing *mat-Pc*. The fidelity of chromosome segregation was further improved in cells where the mating pheromone-signaling pathway was activated and meiosis was induced by inhibiting Pat1-as at 25°C (**Fig. 2**). Proper segregation was seen in 90% of *pat1-as2* cells treated with P-factor and 96% of *pat1-as2 mat-Pc* cells. Thus, the fidelity of chromosome segregation in synchronously induced *pat1-as2* cells is nearly as high as that in wild-type cells.

Next, we determined spore viability by both tetrad and random spore analyses. Cells induced into meiosis by inactivation of Pat1 by higher temperature (34°C) produced spores with low viability (about 30%). Notably, spore viability was higher (about 57%) when cells were induced into meiosis by inactivation of Pat1-as at 25°C (**Table 1**). Triggering mating pheromone-signaling, either by addition of P-factor or addition of the *mat-Pc,* increased spore viability to about 73% and 80%, respectively, in cells induced into meiosis by inactivation of Pat1 by higher temperature (34°C). Spore viability was further improved in cells

Table 1. Spore viability of *S. pombe* strains

Strain	Alleles		P-factor addition	Mutation	Temperature	1-NM-PP1	Viability (%)	
	mat1	Ectopic	-				TA[a]	RSA[b]
JG11315	*P/M*	-	-	none	25°C	-	91.5	92.5
JG12209	*M/M*	-	-	*pat1-114*	34°C	-	31.5	29.2
JG16022	*M/M*	-	-	*pat1-as2*	25°C	+	56.0	55.8
JG16022	*M/M*	-	-	*pat1-as2*	34°C	-	30.5	32.1
JG15912	*P/P*	-	-	*pat1-as2*	25°C	+	58.5	57.4
JG15912	*P/P*	-	-	*pat1-as2*	34°C	-	31.5	27.8
JG16022	*M/M*	-	+	*pat1-as2*	25°C	+	79.5	81.3
JG16022	*M/M*	-	+	*pat1-as2*	34°C	-	72.0	73.6
JG16113	*M/M*	*mat-Pc*	-	*pat1-as2*	25°C	+	87.5	85.4
JG16113	*M/M*	*mat-Pc*	-	*pat1-as2*	34°C	-	81.0	77.8

[a]TA - spore viability examined by tetrad analysis (tetrads examined ≥ 75). [b]RSA - spore viability examined by random spore analysis (spores examined ≥ 6,000). Data are the means of three independent experiments. SEM was < 10% of the mean.

Table 2. Meiotic recombination in *pat1* strains

Strain[a]	Mutation	*ade6-M26 x 52*[b]	*lys3-ura1*[c]	*ade6-arg1*
GP1943	none	3900, 5600 20000*	33/280 = 12% 14 cM	ND[d] 38% = 68 cM[e]
GP7382	*pat1-as2*	1300, 1600 7600*	69/876 = 8% 9 cM	146/743 = 20% 27 cM
GP1973	*pat1-114*	2000 5000, 5200*	290/420 = 7% 8 cM	ND[d]

[a]Recombination data are from sporulated diploid cultures: two meiotically induced cultures for *pat1*$^+$ at 25°C, three for *pat1-as2* at 25°C with 1-NM-PP1, and three for *pat1-114* at 34°C. [b]Measured as Ade$^+$ colonies per 10^6 total colonies. *Indicates meiotic inductions done concurrently with higher Ade$^+$ frequency in all strains; other experiments were done on a different day. [c]Data are the number of recombinants/number of spore colonies tested. Genetic distance in cM was calculated using Haldane's equation. [d]ND, not determined. [e]From Davis et al. (2008)[18]. Recombination data are from sporulated diploid cultures: two meiotically induced cultures for *pat1*$^+$, three for *pat1-as2*, and three for *pat1-114*. The reduction is based on the mean frequencies from all colonies scored.

where the mating pheromone-signaling pathway was activated and meiosis was induced by inhibiting Pat1-as at 25°C: spore viability was increased from about 57% to about 80% or 86% by addition of P-factor or the *mat-Pc* gene (**Table 1**).

In summary, precocious segregation of sister centromeres and low spore viability in cells induced into meiosis by inactivation of Pat1 by higher temperature (34°C) is partly due to high temperature (34°C), and using *pat1-as2* to induce meiosis at 25°C improves fidelity of segregation of sister centromeres as well as spore viability. The fidelity of chromosome segregation and spore viability are almost at the wild-type level when induction of meiosis by inhibiting Pat1-as at 25°C is combined with the activation of mating pheromone-signaling pathway.

Meiotic recombination is slightly reduced in the *pat1-as2* strain. The frequency of recombination in *pat1-as2* strains in the presence of inhibitor was measured both for gene conversion (intragenic recombination) and for crossovers (intergenic recombination); the results were compared with those in *pat1*$^+$ and *pat1-114* strains (**Table 2**). The recombinant frequency was reduced about 3-fold for intragenic *ade6* recombination and about 2-fold in the intergenic *ade6-arg1* and *lys3-ura1* intervals in *pat1-as2* strains compared with those in *pat1*$^+$ strains. This reduction of recombination was also seen in the *pat1-114* strain, suggesting that this deficiency is a result of the loss of the active Pat1 kinase, regardless of the higher temperature.

Discussion

Development of the *pat1-114* as a tool to induce synchronous meiotic cultures has been crucial for progress in the field of *S. pombe* meiosis. However, a major disadvantage of using *pat1-114* is the higher temperature (34°C) needed to inactivate the Pat1-114 mutant kinase. Here, we show that this disadvantage can be overcome by using the *pat1-as2* allele, which allows induction of synchronous meiosis at physiological temperature (25°C). Importantly, *pat1-as2*-induced meiosis eliminates some, but not all, abnormalities observed in *pat1-114*-induced meiosis. Ectopic expression of genes from both *mat* loci, which activates mating pheromone-signaling,[8] eliminates further abnormalities, such as improving the fidelity of chromosome segregation and spore viability of *pat1-as2*-induced meiosis (**Table 1 and Fig. 2**). Induction of *pat1-as2 mat-Pc* with inhibitor at 25°C results in chromosome segregation at MI and spore viability only slightly different from those of wild type. In addition, cells arrest in G_1 and upon induction replicate their DNA more quickly than do *pat1-114* cells (**Figs. 1 and 3**). Although recombination in *pat1-as2* at 25°C is reduced by a factor of 2–3 compared with that in *pat1*$^+$ (**Table 2**), the addition of mating-pheromone or *mat-Pc* may increase recombination to the wild-type level. Thus, *pat1-as2 mat-Pc* cells offer the most synchronous meiosis with characteristics close to that of wild-type meiosis.

Another advantage of using *pat1-as2* is that it can be combined with temperature-sensitive alleles of other essential proteins. For example, meiosis can be induced by inhibiting *pat1-as2* at 25°C, and at the desired time, a temperature-sensitive form of a different protein can be inactivated by shifting the culture to non-permissive temperature (e.g., 34°C). This will, of course, introduce

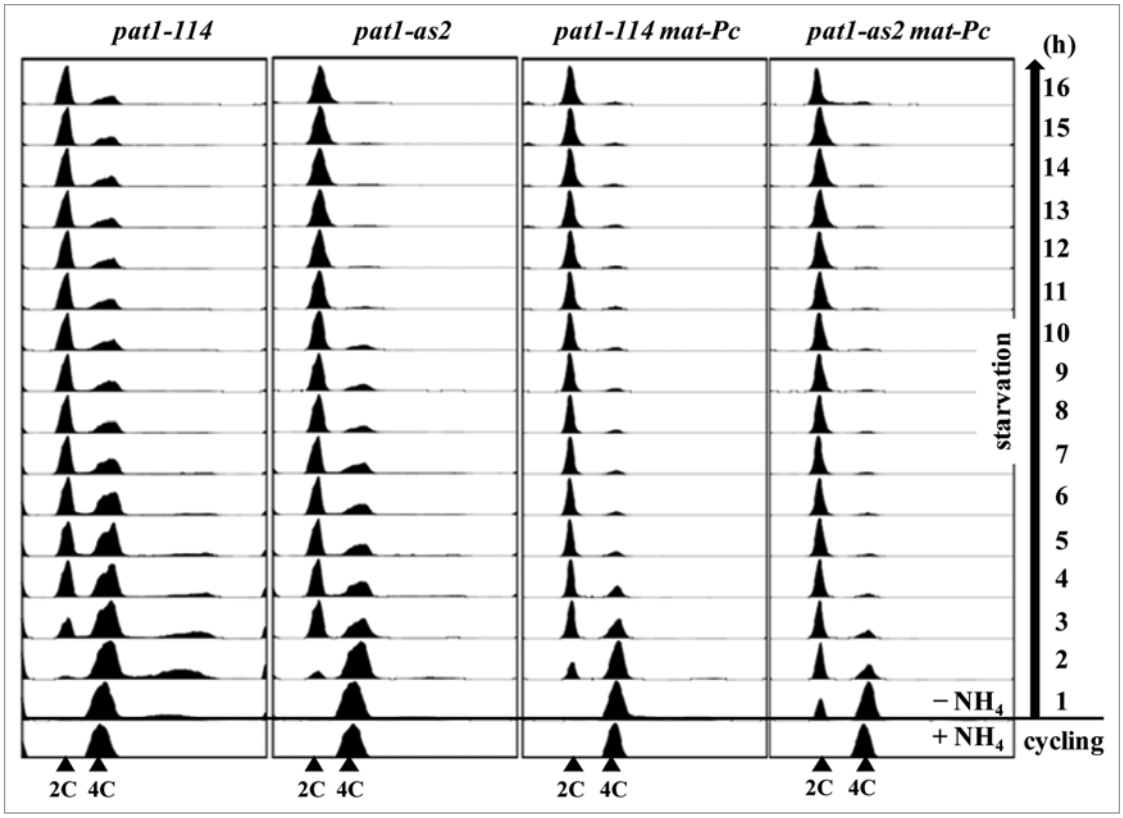


Figure 3. *mat-Pc* shortens the time for nitrogen starvation-induced G_1 arrest and DNA replication. Diploid *pat1-114/pat1-114* (JG12209), *pat1-as2/pat1-as2* (JG15620), *pat1-114/pat1-114 mat-Pc* (JG16328) and *pat1-as2/pat1-as2 mat-Pc* (JG16113) cells were cultured to mid-log phase in YES-Ade medium and transferred to EMM2-NH_4Cl medium. Cells were harvested at the indicated times and analyzed by flow cytometry (Cytox Green staining).

all the negative effects of higher temperature. Nevertheless, some of the meiotic processes occur normally at 34°C; therefore, this may still be a useful experimental approach. In addition to being useful for generating synchronous meiotic cultures, *pat1-as2* provides a valuable tool for elucidating Pat1 function itself.

We anticipate that the *pat1-as2* allele will be useful for additional studies of meiosis in which synchrony is important. For example, determination of the timing of events, such as the localization of proteins to chromosomes by microscopy, is simplest when cells are all at the same stage. Biochemical analyses, such as assaying protein abundance or modification and determining the frequency and structure of DNA intermediates of recombination, are most powerful with synchronous cultures, which can give maximal signals. In addition, novel DNA species, such as single-end invasion intermediates[19] not yet reported in *S. pombe*, may be detectable at the lower temperature because of a possible longer life-time during meiosis that progresses more slowly.

Materials and Methods

***S. pombe* strains and general methods.** The strains used and sources of alleles are listed in **Table 3**. Standard molecular and genetic procedures and media for growth have been described by Forsburg et al.[20] and Moreno et al.[21] Transformation of *S. pombe* with plasmids for deletion and integration was performed using a lithium acetate method as previously described by Gregan et al.[22-26]

Deletion of the *pat1* gene. DNA flanking the *pat1* gene (SPBC19C2.05) was PCR-amplified from genomic DNA using primers 5'-AAA ATC TAG Acgc aag cgt tga ttg tcg at-3' and 5'-AAA ACT CGA Ggt ccc aat tga tgg cga aaa-3' for the upstream region and primers 5'-AAA ATC TAG Att cgt att cca aaa gct tag ttt gc-3' and 5'-AAA AAG ATC Ttc gct acc gca cgt tgt ttt-3' for the downstream region (upper case letters indicate sequences used for the cloning, and lower case letters indicate *S. pombe* sequences). The products were digested with XbaI, ligated to each other and cloned into a pCloneNat1 vector (EF101285),[22,27] carrying drug resistance markers for *E. coli* (ampicillin) and *S. pombe* (nourseothricin) using XhoI and BglII enzymes. The resulting pCloneNat1-Δ*pat1* plasmid (p132) was amplified in *E. coli*, linearized by cutting with XbaI and used to delete one copy of the *pat1* gene in diploid JG11315 strain. The deletion of the *pat1* gene, which is essential for viability[1,5] was confirmed by colony PCR and by tetrad analysis.

Creation of *pat1* analog-sensitive (as) mutants and testing their sensitivity to ATP analogs. The *pat1* gene together with its promoter and terminator regions was PCR-amplified from genomic DNA using primers 5'-ATA TCT CGA Gcg att gtg ttt cct tct cat cc-3' and 5'-ATA TGG ATC Cgg tga tac aat atg act gca tgc-3'. The sequence was cloned into a pCloneHyg1

Table 3. *S. pombe* strains used in this study

Strain	Genotype[a]
JG11315	*h⁻/h⁺ leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216*
JG15101	*h⁻/h⁺ leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216 pat1::Nat^R/pat1⁺*
JG12209	*h⁻/h⁻ ade6-M210/ade6-M216 pat1-114/pat1-114*
JG12226	*h⁻ leu1-32 lys1-131 ura4-D18 cen2(D107)::Kan^R ura4⁺-lacO his7⁺::lacI-GFP*
JG15404	*h⁻ leu1-32 ade6-M216 ura4-D18 pat1::Nat^R pat1-as(L95G)::Hyg^R*
JG15458	*h⁺*
JG15466	*h⁻ ade6-M216 pat1::Nat^R pat1-as(L95A)::Hyg^R*
JG15912	*h⁺/h⁺ ade6-M210/ade6-M216 pat1::Nat^R/pat1::Nat^R pat1-as(L95A)::Hyg^R/pat1-as(L95A)::Hyg^R cen2(D107)::Kan^R-ura4⁺-lacO his7⁺::lacI-GFP*
JG15978	*h⁻ ade6-M210 pat1::Nat^R pat1-as(L95A)::Hyg^R cen2(D107)::Kan^R-ura4⁺-lacO his7⁺::lacI-GFP*
JG16022	*h⁻/h⁻ ade6-M210/ade6-M216 pat1::Nat^R/pat1::Nat^R pat1-as(L95A)::Hyg^R/pat1-as(L95A)::Hyg^R cen2(D107)::Kan^R-ura4⁺-lacO his7⁺::lacI-GFP*
JG16113	*h⁻/h⁻ ade6-M210/ade6-M216 pat1::Nat^R/pat1::Nat^R pat1-as(L95A)::Hyg^R/pat1-as(L95A)::Hyg^R cen2(D107)::Kan^R-ura4⁺-lacO his7⁺::lacI-GFP lys1::BleoMX-mat-Pc*
JG16328	*h⁻/h⁻ lys1/lys1 ade6-M210/ade6-M216 pat1-114/pat1-114 lys1::BleoMX-mat-Pc*
GP1943	*h⁻/h⁺ ade6-M26/ade6-52 +/lys3-37 ura1-171/+ +/pro1-1*
GP1973	*h⁻/h⁻ ade6-M26/ade6-52 +/lys3-37 ura1-171/+ +/pro1-1 pat1-114*
GP7382	*h⁻/h⁻ ade6-M26/ade6-52 pat1::Nat^R/pat1::Nat^R pat1-as(L95A)::Hyg^R/pat1-as(L95A)::Hyg^R arg1-114/+ +/lys3-37 +/ura1-171*

[a]Alleles other than commonly used autoxtrophies are described in the following references: *pat1::Nat^R*,[17] *pat1-as(L95G)::Hyg^R*,[17] *pat1-114*,[4] *cen2(D107)::Kan^R-ura4⁺-lacO his7⁺::lacI-GFP*[8].

vector (EF101286),[22] carrying drug resistance markers for *E. coli* (ampicillin) and *S. pombe* (hygromycin B) using XhoI and BamHI restriction sites, resulting in pCloneHyg1-*pat1* plasmid (p133). Site-directed mutagenesis (QuikChangeII *Site Directed Mutagenesis Kit*, Agilent Technologies, Inc.,) of this plasmid was used to change leucine 95 of Pat1, predicted to be the "gatekeeper" residue by a protein kinase sequence database (sequoia.ucsf.edu/ksd/sequences/family178/family178.html), to glycine or alanine. Oligonucleotides used for mutagenesis were as follows: 5'-aag acg cca ttt atg tcg tt*g gc*c agt att gtc cga atg g-3' (sense) and 5'-cca ttc gga caa tac tg*g cc*a acg aca taa atg gcg tct t-3' (anti-sense) for the Leu95Gly mutant and 5'-aag acg cca ttt atg tcg tt*g ccc* agt att gtc cga atg g-3' (sense) and 5'-cca ttc gga caa tac tg*g gc*a acg aca taa atg gcg tct t-3' (anti-sense) for the Leu95Ala mutant (mutant codons are italicized). The mutations were confirmed by sequencing. The resulting plasmids pCloneHyg1-*pat1-as(L95G)* (p134) and pCloneHyg1-*pat1-as(L95A)* (p135) were linearized with Psp5II and transformed into diploid Δ*pat1/pat1⁺* strain JG15101. The transformants were sporulated on EMM2-NH_4Cl plates (3.0 g/l potassium hydrogen phthalate, 2.2 g/l Na_2HPO_4, 1.0% (w/v) glucose, 2.0% agar, supplemented with amino acids, salts, vitamins and minerals) at 25°C for 36 h, and the haploids carrying mutant *pat1-as*(*L95G*) (further referred to as *pat1-as1*) or *pat1-as(L95A)* (further referred to as *pat1-as2)* alleles were selected based on resistance to Nourseothricin and Hygromycin. Correct integration of mutant alleles was confirmed by colony PCR. The *pat1-as* alleles were further PCR-amplified from genomic DNA and sequenced to finally prove the presence of the expected *pat1-as* alleles.

Sensitivity of *pat1-as* mutants to ATP analogs was tested as described by Cipak et al.[17] Briefly, serial dilutions of exponentially growing *pat1⁺* (JG15458) and *pat1-as* mutants (JG15404, JG15466) were plated on YES plates (5.0 g/l yeast extract, 3.0% glucose, 0.1 g/l L-leucine, 0.1 g/l L-lysine hydrochloride, 0.1 g/l L-histidine, 0.1 g/l uracil, 0.15 g/l adenine sulfate, 2.0% agar) supplemented with 25 μM ATP analogs, namely 1-NM-PP1 (4-amino-1-*tert*-butyl-3-(1'-naphtylmethyl)pyrazolo[3,4-d]pyrimidine) or 2-NA-PP1 (4-amino-1-*tert*-butyl-3-(2'-naphthyl)pyrazolo[3,4-d]pyrimidine). The plates were incubated at 25°C for 3 d and examined for visible colonies.

To activate mating pheromone signaling, we constructed diploid *h⁻/h⁻* strains, in which the *h⁺*-specific mating pheromone P-factor was artificially expressed from the *mat-Pc* gene.[8] To create diploid *h⁻/h⁻* cells expressing *mat-Pc* gene, pCloneBleoMX-*mat-Pc* plasmid (p165) was used. To create the pCloneBleoMX-*mat-Pc* plasmid, *lys1-mat-Pc* was PCR-amplified from pYC36 vector[8] using primers 5'-ATA TTT AAT TAA ttt ttt gaa cgc taa act ttc taa g-3' and 5'-CCC CCT CGA Gaa atg att cta tcg tat cc-3'. The amplified fragment containing *lys1-mat-Pc* was digested with PacI and XhoI enzymes and cloned into pCloneBle1 vector (GQ354685). The resulting pCloneBleoMX-*mat-Pc* plasmid (p165) was linearized with PpuMI and integrated into the *lys1⁺* locus of *pat1-114* and *pat1-as2* diploid strains.

Induction of meiosis, monitoring of progression of meiosis and analysis of chromosome segregation. Diploid *pat1-114/pat1-114* and *pat1-as2/pat1-as2* strains were grown in YES-Ade liquid medium to an OD_{600} = 0.5 at 25°C. The cells were collected by centrifugation, resuspended in EMM2-NH_4Cl medium (3.0 g/l potassium hydrogen phthalate, 2.2 g/l Na_2HPO_4, 1.0% (w/v) glucose, supplemented with salts, vitamins and minerals) and incubated at 25°C for 16 h [*pat1-114* (JG12209) and *pat1-as2* (JG16022)] or for 7 h [*pat1-114 mat-Pc* (JG16328) and *pat1-as2*

mat-Pc (JG16113)] to arrest cells in G_1 (**Fig. 3**). The cells were resuspended in fresh EMM2 medium [3.0 g/l potassium hydrogen phthalate, 2.2 g/l Na_2HPO_4, 5.0 g NH_4Cl, 1.0% (w/v) glucose, supplemented with salts, vitamins and minerals] and induced into meiosis by shifting to 34°C or by adding 25 μM 1-NM-PP1 at 25°C. To induce the mating pheromone response in *pat1-as2* cells (JG16022), synthetic P-factor (Peptide 2.0 Inc.) was added to the EMM2 medium at a concentration of 70 μg/ml. To monitor the progression of meiosis, 0.5 ml of culture was fixed in 70% ethanol. Nuclear divisions were monitored by counting the number of nuclei after DAPI staining. Pre-meiotic S phase was monitored by flow cytometry. To follow the segregation of sister centromeres, the cells in various stages of meiosis (anaphase I and metaphase II) were collected, fixed in 2.0% paraformaldehyde and treated with 0.5 mg/ml Zymolase T-100 (cat# 8062H, MP Biomedicals, Inc.) to digest the cell walls. Primary antibodies TAT1 mouse monoclonal anti-tubulin (1:200, kindly provided by Dr. Gull) and rabbit polyclonal anti-GFP (1:800, cat# A11122, Molecular Probes) were used, followed by secondary antibodies Alexa Fluor 568 goat anti-mouse IgG (H + L), (1:500, cat# A11031, Molecular Probes) and Alexa Fluor 488 goat anti-rabbit IgG (H + L), (1:500, cat# A11034, Molecular Probes). DNA was visualized by staining with Hoechst 33342 (cat# H-3570, Molecular Probes).

Assay for meiotic recombination. Cultures were grown to saturation in EMM2*[28] at 25°C, diluted to OD_{600} = 0.1 in EMM2*, and grown overnight at 25°C. When the OD_{600} reached 0.3–0.4, the cells were collected by centrifugation, washed once with water, suspended in EMM2*-NH_4Cl, and incubated at 25°C for 18 h. NH_4Cl was added to 0.5%; 20 μM 1-NM-PP1 was added to *pat1-as2* cultures, or the temperature was raised to 34°C for *pat1-114* cultures to initiate meiosis. At 24 h, the cells from 10 ml of culture were washed three times with water, suspended in 0.5 ml of water with 5 μl of glusulase, and incubated for 6 h at 25°C. 0.5 ml of 60% ethanol was added, and the mixture incubated at RT for 15 min. The spores were washed three times with water and suspended in 1 ml of water. The spores were plated and recombination frequencies determined as described in reference 29.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Nurse P. Mutants of the fission yeast *Schizosaccharomyces pombe* which alter the shift between cell proliferation and sporulation. Mol Gen Genet 1985; 198:497-502; http://dx.doi.org/10.1007/BF00332946.
2. Watanabe Y, Yamamoto MS. S. pombe *mei2*+ encodes an RNA-binding protein essential for premeiotic DNA synthesis and meiosis I, which cooperates with a novel RNA species meiRNA. Cell 1994; 78:487-98; PMID:7520368; http://dx.doi.org/10.1016/0092-8674(94)90426-X.
3. Beach D, Rodgers L, Gould J. *ran1*+ controls the transition from mitotic division to meiosis in fission yeast. Curr Genet 1985; 10:297-311; PMID:3870979; http://dx.doi.org/10.1007/BF00365626.
4. Iino Y, Yamamoto M. Mutants of *Schizosaccharomyces pombe* which sporulate in the haploid state. Mol Gen Genet 1985; 198:416-21; http://dx.doi.org/10.1007/BF00332932.
5. Iino Y, Yamamoto M. Negative control for the initiation of meiosis in *Schizosaccharomyces pombe*. Proc Natl Acad Sci U S A 1985; 82:2447-51; PMID:16593556; http://dx.doi.org/10.1073/pnas.82.8.2447.
6. Bähler J, Schuchert P, Grimm C, Kohli J. Synchronized meiosis and recombination in fission yeast: observations with *pat1-114* diploid cells. Curr Genet 1991; 19:445-51; PMID:1878997; http://dx.doi.org/10.1007/BF00312735.
7. Kakui Y, Sato M, Tanaka K, Yamamoto M. A novel fission yeast *mei4* mutant that allows efficient synchronization of telomere dispersal and the first meiotic division. Yeast 2011; 28:467-79; PMID:21449049; http://dx.doi.org/10.1002/yea.1851.
8. Yamamoto A, Hiraoka Y. Monopolar spindle attachment of sister chromatids is ensured by two distinct mechanisms at the first meiotic division in fission yeast. EMBO J 2003; 22:2284-96; PMID:12727894; http://dx.doi.org/10.1093/emboj/cdg222.
9. Chikashige Y, Kurokawa R, Haraguchi T, Hiraoka Y. Meiosis induced by inactivation of Pat1 kinase proceeds with aberrant nuclear positioning of centromeres in the fission yeast *Schizosaccharomyces pombe*. Genes Cells 2004; 9:671-84; PMID:15298676; http://dx.doi.org/10.1111/j.1356-9597.2004.00760.x.
10. Asakawa H, Hayashi A, Haraguchi T, Hiraoka Y. Dissociation of the Nuf2-Ndc80 complex releases centromeres from the spindle-pole body during meiotic prophase in fission yeast. Mol Biol Cell 2005; 16:2325-38; PMID:15728720; http://dx.doi.org/10.1091/mbc.E04-11-0996.
11. Lu BC. Dark dependence of meiosis at elevated temperatures in the basidiomycete *Coprinus lagopus*. J Bacteriol 1972; 111:833-4; PMID:5053883.
12. Malik CP. Effect of variations in temperature on meiosis in Gagea reticulata schultes. Nature 1960; 187:805-6; PMID:14420307; http://dx.doi.org/10.1038/187805b0.
13. Francis KE, Lam SY, Harrison BD, Bey AL, Berchowitz LE, Copenhaver GP. Pollen tetrad-based visual assay for meiotic recombination in Arabidopsis. Proc Natl Acad Sci U S A 2007; 104:3913-8; PMID:17360452; http://dx.doi.org/10.1073/pnas.0608936104.
14. Loidl J. Effects of elevated temperature on meiotic chromosome synapsis in *Allium ursinum*. Chromosoma 1989; 97:449-58; http://dx.doi.org/10.1007/BF00295029.
15. Gregan J, Zhang C, Rumpf C, Cipak L, Li Z, Uluocak P, et al. Construction of conditional analog-sensitive kinase alleles in the fission yeast *Schizosaccharomyces pombe*. Nat Protoc 2007; 2:2996-3000; PMID:18007635; http://dx.doi.org/10.1038/nprot.2007.447.
16. Bishop AC, Ubersax JA, Petsch DT, Matheos DP, Gray NS, Blethrow J, et al. A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature 2000; 407:395-401; PMID:11014197; http://dx.doi.org/10.1038/35030148.
17. Cipak L, Zhang C, Kovacikova I, Rumpf C, Miadokova E, Shokat KM, et al. Generation of a set of conditional analog-sensitive alleles of essential protein kinases in the fission yeast *Schizosaccharomyces pombe*. Cell Cycle 2011; 10:3527-32; PMID:22030861; http://dx.doi.org/10.4161/cc.10.20.17792.
18. Davis L, Rozalén AE, Moreno S, Smith GR, Martín-Castellanos C. Rec25 and Rec27, novel linear-element components, link cohesin to meiotic DNA breakage and recombination. Curr Biol 2008; 18:849-54; PMID:18514516; http://dx.doi.org/10.1016/j.cub.2008.05.025.
19. Hunter N, Kleckner N. The single-end invasion: an asymmetric intermediate at the double-strand break to double-holliday junction transition of meiotic recombination. Cell 2001; 106:59-70; PMID:11461702; http://dx.doi.org/10.1016/S0092-8674(01)00430-5.
20. Sabatinos SA, Forsburg SL. Molecular genetics of *Schizosaccharomyces pombe*. Methods Enzymol 2010; 470:759-95; PMID:20946835; http://dx.doi.org/10.1016/S0076-6879(10)70032-X.
21. Moreno S, Klar A, Nurse P. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol 1991; 194:795-823; PMID:2005825; http://dx.doi.org/10.1016/0076-6879(91)94059-L.
22. Gregan J, Rabitsch PK, Rumpf C, Novatchkova M, Schleiffer A, Nasmyth K. High-throughput knockout screen in fission yeast. Nat Protoc 2006; 1:2457-64; PMID:17406492; http://dx.doi.org/10.1038/nprot.2006.385.
23. Gregan J, Rabitsch PK, Sakem B, Csutak O, Latypov V, Lehmann E, et al. Novel genes required for meiotic chromosome segregation are identified by a high-throughput knockout screen in fission yeast. Curr Biol 2005; 15:1663-9; PMID:16169489; http://dx.doi.org/10.1016/j.cub.2005.07.059.

24. Rumpf C, Cipak L, Dudas A, Benko Z, Pozgajova M, Riedel CG, et al. Casein kinase 1 is required for efficient removal of Rec8 during meiosis I. Cell Cycle 2010; 9:2657-62; PMID:20581463; http://dx.doi.org/10.4161/cc.9.13.12146.
25. Rumpf C, Cipak L, Schleiffer A, Pidoux A, Mechtler K, Toli-Nørrelykke IM, et al. Laser microsurgery provides evidence for merotelic kinetochore attachments in fission yeast cells lacking Pcs1 or Clr4. Cell Cycle 2010; 9:3997-4004; PMID:20935472; http://dx.doi.org/10.4161/cc.9.19.13233.
26. Rumpf C, Cipak L, Novatchkova M, Li Z, Polakova S, Dudas A, et al. High-throughput knockout screen in *Schizosaccharomyces pombe* identifies a novel gene required for efficient homolog disjunction during meiosis I. Cell Cycle 2010; 9:1802-8; PMID:20404563; http://dx.doi.org/10.4161/cc.9.9.11526.
27. Spirek M, Benko Z, Carnecka M, Rumpf C, Cipak L, Batova M, et al. S. pombe genome deletion project: an update. Cell Cycle 2010; 9:2399-402; PMID:20519959; http://dx.doi.org/10.4161/cc.9.12.11914.
28. Hyppa RW, Smith GR. Using *Schizosaccharomyces pombe* meiosis to analyze DNA recombination intermediates. Methods Mol Biol 2009; 557:235-52; PMID:19799186.
29. Smith GR. Genetic analysis of meiotic recombination in *Schizosaccharomyces pombe*. Methods Mol Biol 2009; 557:65-76; PMID:19799177.