Activation of the pheromone-responsive MAP kinase drives haploid cells to undergo ectopic meiosis with normal telomere clustering and sister chromatid segregation in fission yeast

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Summary

Meiosis is a process of importance for sexually reproducing eukaryotic organisms. In the fission yeast *Schizosaccharomyces pombe*, meiosis normally proceeds in a diploid zygote which is produced by conjugation of haploid cells of opposite mating types. We demonstrate that activation of the pheromone-responsive MAPK, Spk1, by the ectopic expression of a constitutively active form of Byr1 (MAPKK for Spk1) induced the cells to undergo meiosis while in the haploid state. Moreover, the induction of meiosis required Mei2 (a key positive regulator of meiosis), but did not require Mei3; Mei3 is normally required to inactivate the Pat1 kinase (a negative regulator of Mei2) thereby allowing Mei2 to drive meiosis. Therefore, expression of a constitutively active form of Byr1 activates Mei2 without the need of Mei3. In cells induced to undergo meiosis by activating the Spk1 MAPK signaling pathway, telomeres clustered at the spindle pole body (SPB) and centromeres detached normally from the SPB during meiotic prophase, and the cells showed the correct segregation of sister chromatids during meiotic divisions. In contrast, in meiosis induced by inactivation of Pat1, sister chromatids segregate precociously during the first meiotic division. Thus, these results suggest that activation of Spk1 drives meiosis in *S. pombe*.

Key words: Telomere, Centromere, Sister chromatid, Meiosis, MAP kinase

Introduction

Meiosis, a process of general importance for sexually reproducing eukaryotic organisms, generates inheritable haploid gametes from a diploid cell. This process of halving the chromosome number is achieved by two consecutive rounds of nuclear division following one round of DNA replication. During the first meiotic division, homologous chromosomes segregate to the opposite spindle poles while sister chromatids move together to the same spindle pole (reductional segregation). During the second meiotic division, sister chromatids segregate to the opposite poles (equational segregation). Reductional segregation in the first meiotic division is a unique feature of meiosis. Another unique feature of meiotic chromosomes, observed in a wide range of organisms, is the formation of the bouquet arrangement, in which chromosomes are bundled at the telomere, resulting in a configuration with a bouquet-like appearance (Bass et al., 1997; Chikashige et al., 1994; Scherthan et al., 1996; Trelles-Sticken et al., 1999). The widespread appearance of telomere clustering in meiotic prophase suggests a special role for the telomere in meiosis.

The fission yeast *Schizosaccharomyces pombe* has provided a particularly striking example of telomere clustering, and has demonstrated a role for telomeres in meiosis (reviewed by de Lange, 1998; Hiraoka, 1998; Yamamoto and Hiraoka, 2001). In this organism, upon induction of meiosis, the nucleus oscillates back and forth between the cell poles with telomeres remaining clustered at the leading edge of the moving nucleus throughout meiotic prophase, which can last for several hours (Chikashige et al., 1994). It has been demonstrated that telomere clustering and the subsequent nuclear movements are important for recombination of homologous chromosomes and the normal progression of meiosis in *S. pombe* (Chikashige and Hiraoka, 2001; Cooper et al., 1998; Kanoh and Ishikawa, 2001; Miki et al., 2002; Nimmo et al., 1998; Shimanuki et al., 1997; Yamamoto et al., 1999; Ding et al., 2004).

In addition, *S. pombe* provides a useful experimental system to study regulatory mechanisms of chromosome behavior during meiosis. In this organism, genetic regulation of the progression of meiosis has been extensively studied (reviewed by Davey, 1998; Yamamoto et al., 1997); induction of meiosis can be easily controlled by manipulation of the nitrogen sources in the culture medium; the rapid process of meiosis and the ease of live cell observation allow examination

of chromosome behavior microscopically continuously throughout meiosis; and the small number of chromosomes (three chromosomes in a haploid genome) simplifies the analysis of chromosome behavior. We have made use of these features to examined regulatory mechanisms of chromosome behavior during meiosis.

Normally, when cultured in a rich medium, *S. pombe* grow as haploid cells. Upon nitrogen starvation, cells stop mitotic growth and produce and secrete mating pheromone, and haploid cells with the opposite mating types, h^+ and h^-, conjugate with each other to produce a diploid zygote. The mating pheromone signal is transduced through the pheromone-responsive MAP kinase (MAPK) cascade. The MAPK cascade is a cellular signal transduction pathway in which an extracellular signal is transduced to the nucleus through sequential activation of kinases by their phosphorylation: MAP kinase kinase kinase (MAPKKK) phosphorylates MAP kinase kinase (MAPKK), which in turn phosphorylates MAPK and phosphorylated MAPK subsequently activates transcription of target genes (reviewed by Cobb and Goldsmith, 1995; Cowan and Storey, 2003; Nishida and Gotoh, 1993). In *S. pombe*, *byr2*, *byr1*, and *spk1* genes encode MAPKKK, MAPKK, and MAPK, respectively, which constitute the MAPK cascade that transduces the mating pheromone signal (Gotoh et al., 1993; Nadin-Davis and Nasim, 1988; Nadin-Davis and Nasim, 1990; Neiman et al., 1993; Toda et al., 1991; Wang et al., 1991).

After cell conjugation, co-operation of h^+ and h^- mating type-specific gene products in the diploid zygote induces zygotic expression of the *mei3* gene (McLeod et al., 1987; Willer et al., 1995). Expression of *mei3* inactivates Pat1 kinase (a negative regulator of Mei2), and inactivation of Pat1 leads to activation of Mei2 which drives meiosis (McLeod and Beach, 1988; Watanabe et al., 1997). In contrast, in haploid cells, which do not normally undergo meiosis, active Pat1 kinase phosphorylates Mei2 and represses its activity (Watanabe et al., 1997): Pat1 kinase phosphorylation of Mei2 affects its localization and turnover (Sato et al., 2002; Kitamura et al., 2001). Thus, normally only h^+/h^- diploid cells, bearing mating-type genes of both mating types, can enter meiosis. However, meiosis can be induced ectopically in haploid cells under conditions that mimic the situation in a diploid zygote: (1) ectopic expression of both mating-type genes (Kelly et al., 1988; Thon and Klar, 1992; Willer et al., 1995), (2) expression of *mei3* (McLeod et al., 1987), (3) inactivation of Pat1 (Iino and Yamamoto, 1985a; Nurse, 1985) and (4) expression of a constitutively active form of Mei2 (Watanabe et al., 1997).

We demonstrate that activation of the pheromone-responsive Spk1 MAPK can induce *S. pombe* haploid cells to undergo ectopic meiosis without zygotic expression of the *mei3* gene, which is normally required for the progression of meiosis. In cells induced to undergo meiosis by activating Spk1 signaling telomeres clustered at the spindle pole body (SPB) and centromeres separated from the SPB normally. Furthermore, sister chromatids exhibited the correct segregation behavior during the meiotic divisions. Thus, activation of Spk1 induces meiosis with normal chromosome behavior.

Materials and Methods

S. pombe strains and culture media

S. pombe strains used are listed in Table 1. Diploid strains homozygous for the *mat* gene locus (h^-/h^-) were constructed by cell fusion (Alfa et al., 1993). Growth media and basic genetic techniques for *S. pombe* have been described previously (Alfa et al., 1993; Moreno et al., 1991). The complete medium YES (YE medium containing 150 mg/l adenine, 200 mg/l leucine, 75 mg/l histidine, 75 mg/l uracil, 75 mg/l lysine) was used for routine culture of *S. pombe* strains. The minimal medium EMM2 containing nutritional supplements when necessary was used as a rich medium for mitotic growth. EMM2 was supplemented with 2 μM thiamine to repress the *nmt1* promoter, and depleted of thiamine to induce the *nmt1* promoter. EMM2-N (EMM2 lacking nitrogen sources) was used for induction of meiosis in wild-type and *pat1-114* cells, and EMM2 depleted of thiamine was used for induction of meiosis by expression of $byr1^{DD}$.

Table 1. *S. pombe* strains used in this study

Strain	Genotype
AY193-3A	*h⁻ leu1-32 his7⁺::lacI-GFP lys1⁺::lacOp*
CRL239	*h⁻ leu1-32*
CRL544	*h⁻ leu1-32 ura4-D18 mei2::ura4⁺*
CRLc92	*h⁻ ura4-D18 spk1::ura4⁺ pat1-114 lys1⁺::CFP-sad1⁺ pro1⁺::taz1⁺-GFP*
TG7	*h⁻ leu1-32 ura4-D18 spk1::ura4⁺ lys1⁺::spk1⁺-GFP*
TG30	*h⁻ leu1-32 ura4-D18 mei3::ura4⁺*
TG43	*h⁻ leu1-32 ura4-D18 spk1::ura4⁺ lys1⁺::spk1^{AF}-GFP*
TG44	*h⁻ leu1-32 ura4-D18 spk1::ura4⁺ lys1⁺::spk1^{AY}-GFP*
TG45	*h⁻ leu1-32 ura4-D18 spk1::ura4⁺ lys1⁺::spk1^{TF}-GFP*
TG143	*h⁻/h⁻ leu1-32/leu1-32 ura4-D18/ura4⁺ ade6⁺/ade6-210 lys1⁺::lacOp/lys1⁺ his7⁺::lacI-GFP/his7⁺*
TG157	*h⁺/h⁻ leu1-32/leu1-32 ura4-D18/ura4⁺ ade6⁺/ade6-210 lys1⁺::lacOp/lys1⁺ his7⁺::lacI-GFP/his7⁺*
TG202	*h⁻ ura4-D18 mei2::ura4⁺ pat1-114 lys1⁺::CFP-sad1⁺ pro1⁺::taz1⁺-GFP*
TG208	*h⁻ leu1-32 ura4-D18 sxa2::ura4⁺ lys1⁺::taz1⁺-GFP sad1⁺::sad1⁺-Dsred::LEU2*
TG263	*h⁻ leu1-32 ura4-D18 sxa2::ura4⁺ mei2::ura4⁺ lys1⁺::taz1⁺-GFP sad1⁺::sad1⁺-Dsred::LEU2*
TG265	*h⁻ leu1-32 ura4-D18 sxa2::ura4⁺ spk1::ura4⁺ lys1⁺::taz1⁺-GFP sad1⁺::sad1⁺-Dsred::LEU2*
TG292	*h⁻ pat1-114 lys1⁺::CFP-sad1⁺ pro1⁺::taz1⁺-GFP*
TG309	*h⁻ leu1-32 lys1⁺::CFP-sad1⁺ pro1⁺::taz1⁺-GFP*
TG335	*h⁻ leu1-32 ura4-D18 spk1::ura4⁺ lys1⁺::CFP-sad1⁺ pro1⁺::taz1⁺-GFP*
TP106-3C	*h⁻ leu1-32 ura4-D18 spk1::ura4⁺*

References of genotypes are as follows: *his7⁺::lacI-GFP* and *lys1⁺::lacOp* (Nabeshima et al., 1998); *mei2::ura4⁺* (Watanabe et al., 1988); *spk1::ura4⁺* (Toda et al., 1991); *pat1-114* (Iino and Yamamoto, 1985a); *sxa2::ura4⁺* (Imai and Yamamoto, 1992); *mei3::ura4⁺* (Y. Imai and M. Yamamoto, personal communication); *lys1⁺::CFP-sad1⁺*, *pro1⁺::taz1⁺-GFP*, *lys1⁺::spk1⁺-GFP*, *lys1⁺::spk1^{AF}-GFP*, *lys1⁺::spk1^{AY}-GFP* and *lys1⁺::spk1^{TF}-GFP* (this study); *lys1⁺::taz1⁺-GFP* and *sad1⁺::sad1⁺-Dsred::LEU2* (Chikashige and Hiraoka, 2001).

Plasmid construction

Oligonucleotide primers used for PCR are listed in Table 2. The plasmids used for overexpression of *byr1*$^+$ were constructed as follows. A DNA fragment of the entire *byr1*$^+$ open reading frame (ORF) was amplified by PCR using pFO2-byr1$^+$, which contains the *byr1* promoter and ORF, as the template and the forward primer BYR1-1F and the reverse primer BYR1-1R. This DNA fragment was digested with *Sal*I and cloned in the *Sal*I site of pREP1 and 81 (Maundrell, 1993). The resulting constructs were designated pREP1-byr1$^+$ and pREP81-byr1$^+$, respectively. Similarly, the plasmid used for overexpression of *byr1*^{DD} was constructed using pFO2-byr1^{DD}, a derivative of pBS-byr1^{DD} (Ozoe et al., 2002), as the template. The resulting constructs were designated pREP1-byr1^{DD} and pREP81-byr1^{DD}.

The plasmids for integration of GFP fusion of *spk1*$^+$, *spk1*^{AF}, *spk1*^{AY} and *spk1*^{TF} genes were constructed as follows. A DNA fragment containing the *GFP* ORF followed by the *nmt1* terminator was cloned in the *Bam*HI and *Sac*I sites of pYC36, an integration plasmid with the partial *lys1* gene. The resulting construct was designated pTY1. The DNA fragment containing the *spk1* promoter (from –998 to –1) and ORF was amplified by PCR using genomic DNA as the template and the forward primer *spk1-Sal*I and the reverse primer *spk1-Bam*HI. This DNA fragment was digested with *Sal*I and *Bam*HI and cloned into the *Sal*I and *Bam*HI sites of pTY1. The resulting construct was designated pTY1-spk1$^+$. The QuickChange site-directed mutagenesis kit (Stratagene) was used for site-directed mutagenesis. Mutagenic PCR was carried out with pBS-spk1$^+$, which contains the *spk1*$^+$ ORF in pBluescript (Stratagene), as the template and the forward primer SPK1AF-F and the reverse primer SPK1AF-R for the *spk1*^{AF} mutant; the forward primer SPK1AY-F and the reverse primer SPK1AY-R for the *spk1*^{AY} mutant; and the forward primer SPK1TF-F and the reverse primer SPK1TF-R for the *spk1*^{TF} mutant. The *Xba*I-*Pst*I fragment of pTY1-spk1$^+$ was replaced with the *spk1* mutant fragments. The resulting constructs were designated pTY1-spk1^{AF}, pTY1-spk1^{AY} and pTY1-spk1^{TF}. These plasmids were integrated into the chromosome at the *lys1-131* gene locus in *spk1Δ* cells. Integration was confirmed by PCR. The *spk1*$^+$*-GFP* fusion gene complements *spk1Δ* sterility, indicating that the *spk1*$^+$*-GFP* fusion gene is functional.

The plasmid used for integrating the *taz1*$^+$*-GFP* fusion gene at the *pro1* gene locus was constructed from the plasmid that was used for integrating the *taz1*$^+$*-GFP* fusion gene at the *lys1* gene locus (Chikashige and Hiraoka, 2001) by replacing the partial *lys1* gene with the partial *pro1* gene. This plasmid was integrated into the chromosome at the *pro1-1* gene locus. Integration was confirmed by PCR. The *taz1*$^+$*-GFP* fusion gene is functional, as described previously (Chikashige and Hiraoka, 2001).

The plasmid for integration of the *CFP-sad1*$^+$ fusion gene was constructed by replacing serine, tyrosine, and glycine at residues 64-66 of GFP with leucine, threonine and tryptophan, respectively (the DNA sequence is CTTACTTGG). A DNA fragment containing the *sad1* promoter (from –614 to –1) followed by the first seven amino acids of the coding sequence of *CFP* was amplified by PCR using genomic DNA as the template and the forward primer sad1P-up and the reverse primer ATG-GFsac. This DNA fragment was digested with *Bam*HI and *Sac*I and cloned into the *Bam*HI and *Sac*I sites of pYC36 lacking the *Xho*I and *Sal*I sites. A DNA fragment lacking the first five amino acids of the coding sequence of *CFP* was amplified by PCR using the forward primer sac-GFP and the reverse primer GFP-XS. This DNA fragment was digested with *Bam*HI and *Xho*I and cloned into the *Bam*HI and *Xho*I sites of pCST3, which contains the *nmt1* terminator. The *Sac*I fragment containing the partial *CFP* ORF followed by the *nmt1* terminator was cloned into the *Sac*I site of pYC36 containing the *sad1* promoter followed by the partial *CFP* ORF described above. Finally the *Bam*HI fragment containing the *sad1*$^+$ ORF amplified by PCR was cloned into the *Bgl*II site of this plasmid. The resulting plasmid was integrated into the chromosome at the *lys1-131* gene locus. Integration was confirmed by PCR. The *CFP-sad1*$^+$ fusion gene complements *sad1Δ* lethality, indicating that the *CFP-sad1*$^+$ fusion gene is functional.

Culture conditions

For induction of normal meiosis, wild-type *h*$^+$/*h*$^-$ diploid cells were grown in liquid YES at 33°C to mid-log phase; after two washes in EMM2-N, cells were resuspended in liquid EMM2-N and incubated at 26°C.

For induction of meiosis by expression of *byr1*^{DD}, cells with a pREP-byr1^{DD} plasmid were grown in liquid EMM2 with 2 μM thiamine at 33°C to mid-log phase; after two washes in EMM2 without thiamine, cells were resuspended in liquid EMM2 without thiamine and incubated at 26 or 30°C.

For induction of meiosis by inactivation of Pat1 kinase, cells of the temperature-sensitive *pat1-114* mutant were grown in liquid YES at 26°C (permissive temperature) to mid-log phase; after two washes in EMM2-N, cells were resuspended in liquid EMM2-N and incubated for 16 hours at 26°C. After resuspending in liquid EMM2-N, cells were incubated at 34°C (restrictive temperature). At the restrictive temperature the Pat1 kinase is inactivated in *pat1-114* mutant cells (Iino and Yamamoto, 1985a; Nurse, 1985).

P-factor treatment was carried out as follows: *h*$^-$ *sxa2Δ* cells were grown in liquid YES at 33°C to mid-log phase; after two washes in EMM2-N, cells were resuspended in liquid EMM2-N with 0.5 μg/ml synthetic P-factor (Imai and Yamamoto, 1994) and incubated at 30°C.

Table 2. Oligonucleotide primers used in this study

Name	Sequence
BYR1-1F	5′-CTGTCGACATGTTTAAACGACGTCG-3′
BYR1-1R	5′-GAGTCGACTTACGAGGATCTAAAG-3′
spk1-*Sal*I	5′-TTGTCGACTTGAGAGTTTCAACGGATTTATCT-3′
spk1-*Bam*HI	5′-TCGGATCCTCTGAAATTTACTTCACGAAATAT-3′
SPK1AF-F	5′-GGAAATCCTGGCTTTATGGCTGAGTTTGTCGCAACTCGTTGG-3′
SPK1AF-R	5′-CCAACGAGTTGCGACAAACTCAGCCATAAAGCCAGGATTTCC-3′
SPK1AY-F	5′-GGAAATCCTGGCTTTATGGCTGAGTATGTCGCAACTCG-3′
SPK1AY-R	5′-CGAGTTGCGACATACTCAGCCATAAAGCCAGGATTTCC-3′
SPK1TF-F	5′-CCTGGCTTTATGACTGAGTTTGTCGCAACTCGTTGG-3′
SPK1TF-R	5′-CCAACGAGTTGCGACAAACTCAGTCATAAAGCCAGG-3′
sad1P-up	5′-AACACGCGAATTGCTGGATTTGGATCCTATCAGCTTCAAGTACTTA-3′
ATG-GFsac	5′-GAA**GAGCTC**TTCTCCTTTACTCATTGATGTATAAAAGGCTATTTGA-3′
sac-GFP	5′-GCGGGATCC**GAGCTC**TTCACTGGAGTTGTCCCAATTCTTG-3′
GFP-XS	5′-AGCCCGGGCTCGAGTTTGTATAGTTCATCCATGCCATGTG-3′

The underlining indicates the codons mutated to make *spk1* mutants; bold indicates the artificial *Sac*I site, which does not change CFP amino acid sequences.

Immunofluorescence staining and FISH

Cells were multiply stained by immunofluorescence followed by FISH as described previously (Chikashige et al., 1997; Nabeshima et al., 2001). Cells were fixed in EMM2 with 3.7% formaldehyde and 0.1% glutaraldehyde for 15 minutes at 26°C. The SPB was stained with an anti-Sad1 antibody (1:1,000 dilution) (Hagan and Yanagida, 1995) and detected with Alexa green-conjugated anti-rabbit antibodies (1:100 dilution) (Molecular Probes). Cells were then re-fixed in PEM with 3.7% formaldehyde and 0.1% glutaraldehyde for 5 minutes at 26°C. FISH was carried out as described previously (Yamamoto et al., 1999) except that chromosomal DNA was denatured for 5 minutes at 75°C and hybridization was carried out for 10 hours at 40°C. The following fluorescently labeled DNA probes were used. Cosmid cos212, which hybridizes to both ends of chromosomes I and II (Funabiki et al., 1993) was used as a telomere probe. Plasmid pRS140, which hybridizes to centromeric repeats of all three chromosomes (Chikashige et al., 1989), was used as a centromere probe. Cos212 and pRS140 were labeled with Cy5-dUTP (Amersham) and Texas Red-dUTP (Molecular Probes), respectively, using an oligonucleotide tailing kit (Boehringer-Mannheim).

Fluorescence microscopy

Microscope images were obtained using a DeltaVision microscope system (Applied Precision, Inc., Seattle, WA) as described by Haraguchi et al. (Haraguchi et al., 1999). Optical section images were collected at focus intervals of 0.4 μm. In the presented figures, relevant sections of the images are projected.

Flow cytometry

Samples for flow cytometry were prepared following a protocol from the Forsburg lab (available on the Internet at http://pingu.salk.edu/flow/protocols/ycc.html). Briefly, cells were fixed in 70% ethanol for 30 minutes at 4°C. Fixed cells were incubated in 50 mM sodium citrate with 0.1 mg/ml RNase A for 2 hours at 37°C. After RNase treatment, cells were stained with 1 μM SYTOX green (Molecular Probes) and sonicated for a few seconds. The DNA content was determined using an Epics XL flow cytometer (Beckman Coulter).

Results

Byr1^{DD}, a constitutively active MAPKK for Spk1 MAPK, induces ectopic meiosis

Because telomeres cluster at the SPB in response to the mating pheromone in *S. pombe* (Chikashige et al., 1997), we wished to examine whether the pheromone-responsive *byr2*/*byr1*/*spk1* MAPK cascade is directly involved in telomere clustering. To this end, we attempted to activate Spk1 MAPK without mating pheromone signaling by using a constitutively active form of Byr1, the MAPKK for Spk1. This was accomplished by constructing a mutant Byr1 mimicking its phosphorylated state by replacing both serine (S), at residue 214, and threonine (T), at residue 218, with aspartic acid (D) (Ozoe et al., 2002) (Fig. 1A). The mutated *byr1* (designated *byr1^{DD}*) was introduced into *h*$^{-}$ haploid cells on a plasmid and expressed under the control of the thiamine-repressible *nmt1* promoter. The wild-type *byr1* (*byr1*$^{+}$) plasmid and the plasmid vector alone were

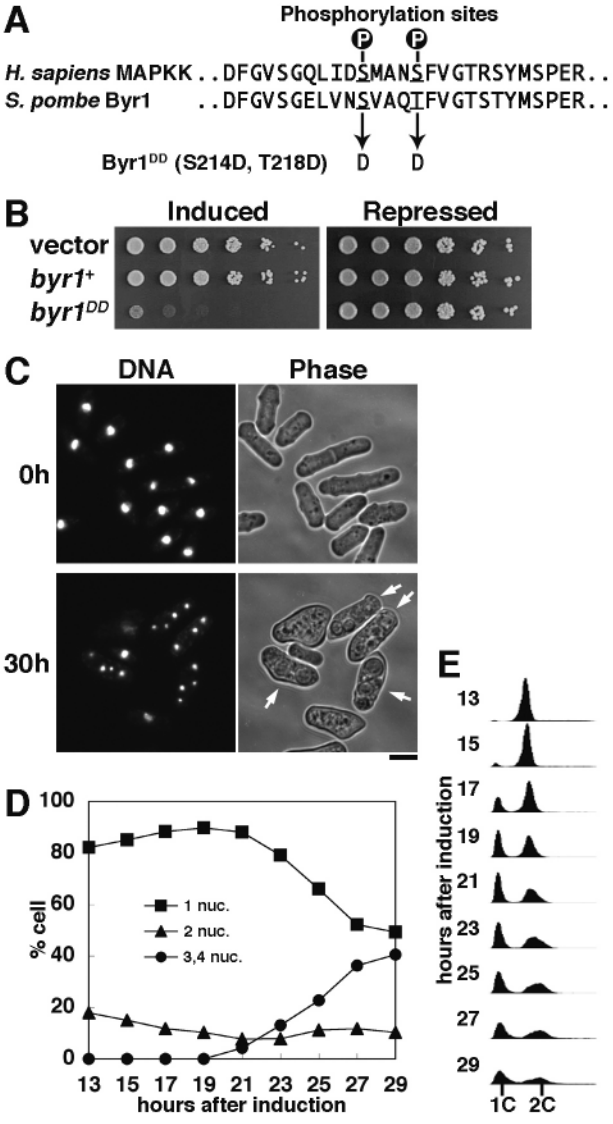


Fig. 1. Progression of meiosis induced by the expression of *byr1^{DD}*. (A) Amino acid sequences of *H. sapiens* MAPKK and *S. pombe* Byr1. MAPKKK phosphorylation sites of *H. sapiens* MAPKK and the corresponding sites of Byr1 are underlined. To make a constitutively active form of Byr1 (Byr1^{DD}), these sites of Byr1 were replaced with aspartic acid (shown as 'D'). (B) Mitotic growth defects in cells expressing *byr1^{DD}*. *h*$^{-}$ wild-type (CRL239) cells were transformed with either pREP81 (vector), pREP81-byr1^{+} (*byr1*$^{+}$) or pREP81-byr1^{DD} (*byr1^{DD}*). Each transformant was cultured to mid-log phase in liquid EMM2 with thiamine. After removing thiamine, fivefold serial dilutions of each transformant were spotted onto EMM2 plates with (right panel, 'Repressed') or without thiamine (left panel, 'Induced'). Plates were photographed after 4 days incubation at 30°C. (C) *h*$^{-}$ wild-type (CRL239) cells carrying pREP81-byr1^{DD} were induced to undergo ectopic meiosis in liquid EMM2 at 30°C, and fixed at 30 hours after induction. Nuclei were stained with DAPI. Bright-field images of the same cells are also shown (Phase). Arrows indicate cells with three or four nuclei, or spores. Scale bar: 5 μm. (D) Progression of meiosis in cells expressing *byr1^{DD}*. *h*$^{-}$ wild-type (CRL239) cells carrying pREP81-byr1^{DD} were induced to undergo ectopic meiosis in liquid EMM2 at 30°C. The number of nuclei per cell was counted every 2 hours from 13 to 29 hours after induction. At least 300 cells were scored for each time point. During analysis, the cell concentration was kept between 1×10^{6} and 2×10^{7} cells/ml by diluting with EMM2. (E) DNA content, measured by flow cytometry. The same cell samples used in D were examined.

used as controls. In h^+/h^- diploid cells, the mating pheromone response is immediately followed by zygotic expression of mating-type genes and entrance into meiosis, making it difficult to separate regulatory mechanisms downstream of the pheromone response from those downstream of the zygotic expression of mating-type genes. In contrast, in haploid cells, the response to mating pheromone does not include zygotic expression of mating-type genes. Thus, we expected that haploid cells expressing *byr1*^{DD} would allow us to examine telomere clustering without inducing meiosis.

Unexpectedly, however, we found that expression of *byr1*^{DD} induced ectopic meiosis in haploid cells (without zygotic expression of mating-type genes). Under conditions that repress the *nmt1* promoter, cells continued normal mitotic cell cycling (Fig. 1B, right panel). Under conditions that induce the *nmt1* promoter, the cells expressed *byr1*^{DD} and stopped mitotic growth, even when cultured in a rich medium (EMM2) (Fig. 1B, left panel). Under the same conditions, control cells receiving the vector or expressing *byr1*$^+$ continued normal mitotic cell cycling (Fig. 1B, left panel). Before induction of *byr1*^{DD}, cells were the regular rod-like shape (Fig. 1C, 0 h). After induction of *byr1*^{DD}, cells became deformed and entered meiosis (Fig. 1C, 30 h). The altered cell shape is similar to that which occurs in response to mating pheromone. These haploid cells underwent two meiotic nuclear divisions to form spores, about 30 hours after induction (Fig. 1D). Flow cytometry showed that cells with a 1C DNA content began to accumulate at 15 hours, and reached a maximum level (about 50%) at 21 hours (Fig. 1E). As a control, cells expressing *byr1*$^+$ did not show any such changes under identical conditions (data not shown). These results indicate that expression of *byr1*^{DD} alone induces ectopic meiosis in the asexual state without nutritional starvation.

Sister chromatids segregate correctly in meiosis induced by expression of *byr1*^{DD}

In order to follow chromosomal events during meiosis induced by expression of *byr1*^{DD}, we examined the behavior of sister chromatids during the meiotic divisions. For this purpose, we marked the *lys1* locus located near the centromere of chromosome I using the lacI-GFP/lacO recognition system (Nabeshima et al., 1998). In the case of diploid cells, one of the homologous chromosomes was marked to enable us to follow the behavior of sister chromatids. First, as a control for normal meiosis, h^+/h^- diploid cells, which bear mating-type genes of both h^+ and h^- mating types, were induced to undergo meiosis upon nitrogen starvation (Fig. 2A-D, left). In the majority of these cells, sister chromatids moved to the same pole at the first meiotic division (Fig. 2A,B) and segregated to opposite poles at the second meiotic division (Fig. 2C,D). We next examined h^-/h^- diploid cells (Fig. 2A-D, middle) and h^- haploid cells (Fig. 2A-D, right), which bear only the h^- mating-type gene and normally do not enter meiosis upon nitrogen starvation. These cells were induced to enter meiosis by expression of *byr1*^{DD}. Our observations showed that in most cases the separation of sister chromatids was relatively normal in both h^-/h^- diploid cells and h^- haploid cells expressing *byr1*^{DD} (Fig. 2A-D). While haploid cells expressing *byr1*^{DD} formed predominantly inviable spores (spore viability of 6.8%), as expected, h^-/h^- diploid cells formed spores with relatively high viability (67.3%), but somewhat lower than that in h^+/h^- diploid cells (82.7%). These results indicate that meiosis induced by expression of *byr1*^{DD} shows the correct segregation pattern of sister chromatids.

Induction of meiosis by expression of *byr1*^{DD} requires Spk1

We next examined whether induction of meiosis by *byr1*^{DD} required the activity of the Spk1 protein. For this purpose, *byr1*^{DD} was expressed in *spk1*deletion (*spk1Δ*) cells. We also constructed putative unphosphorylatable forms of Spk1 by replacing either, or both, threonine (T) at residue 199 and tyrosine (Y) at residue 201 with alanine (A) and phenylalanine

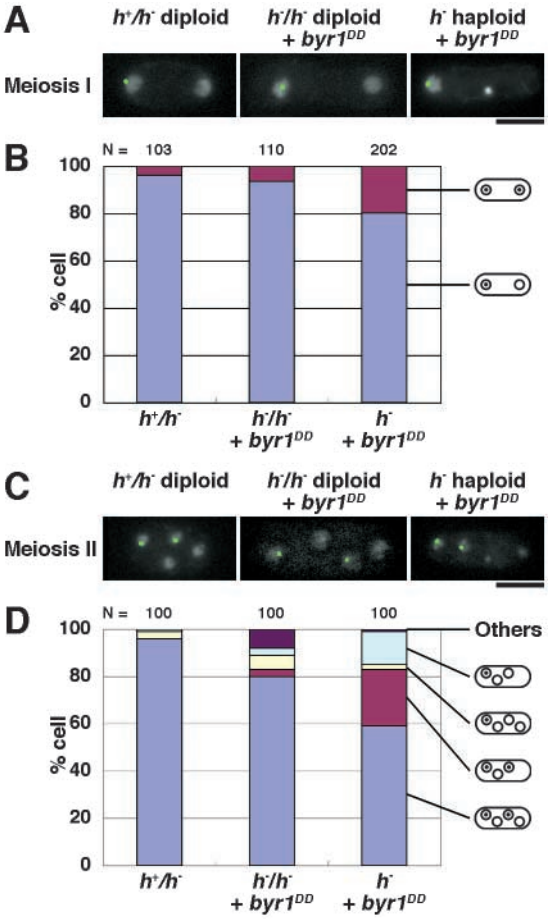
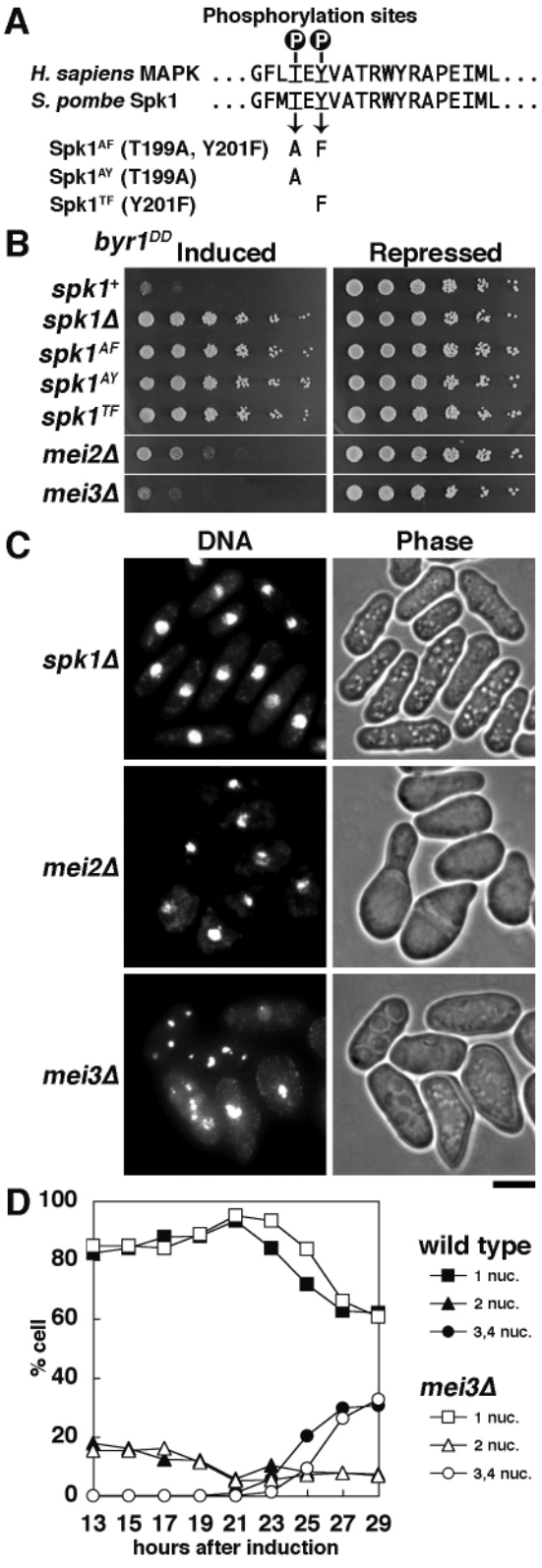


Fig. 2. Sister chromatid cohesion in cells expressing *byr1*^{DD}. Segregation of sister chromatids in meiosis I (A,B) and in meiosis II (C,D). (A,C) h^+/h^- diploid (TG157) cells (left), h^-/h^- diploid (TG143) cells carrying pREP1-byr1^{DD} (middle), and h^- haploid (AY193-3A) cells carrying pREP81-byr1^{DD} (right) were induced to undergo meiosis and observed by fluorescence microscopy without fixation. The *lys1* locus near the centromere (green) was stained using the lacI-GFP/lacO recognition system. In diploid cells, one of two *lys1* loci was stained. The nucleus (white) was stained with Hoechst 33342. Scale bars: 5 μm. (B,D) Patterns of segregation were classified into two categories in B or five categories in D based on the number of nuclei (open circles) and the number of *lys1*-GFP signals (dots in the circle). Numbers of the cells examined (N) are shown at the top of each graph.

(F), respectively (designated Spk1^{AY}, Spk1^{TF}, and Spk1^{AF}; see Fig. 3A). GFP fusion *spk1*$^{+}$, *spk1*^{AY}, *spk1*^{TF} or *spk1*^{AF} constructs were integrated into the chromosome of *spk1Δ* cells (see Materials and Methods). Upon nitrogen starvation, GFP fluorescence of these constructs was observed predominantly in the nucleus (data not shown). When expression of *byr1*^{DD} was induced in any *spk1* mutants cells, the cells continued mitotic growth with no changes in cell morphology (Fig. 3B,C). Expression of *byr1*^{DD} in *spk1Δ* cells was confirmed by western blot analysis (data not shown). These results indicate that induction of meiosis by *byr1*^{DD} is dependent on Spk1 function, and suggest that its predicted phosphorylation sites are important for its function.



Induction of meiosis by expression of *byr1*^{DD} requires Mei2, but not Mei3

In the normal process of meiosis, Mei2 and Mei3 are essential: expression of *mei3* inactivates Pat1, and inactivation of Pat1 leads to activation of Mei2, which drives meiosis (McLeod and Beach, 1988; Watanabe et al., 1997). Thus, we examined whether induction of meiosis by expression of *byr1*^{DD} requires Mei2 or Mei3. We expressed the *byr1*^{DD} construct in haploid cells disrupted for either *mei2* or *mei3* genes. Expression of *byr1*^{DD} in *mei2Δ* and *mei3Δ* cells was confirmed by western blot analysis (data not shown). In both *mei2Δ* and *mei3Δ*, expression of *byr1*^{DD} caused mitotic growth defects and an altered morphology (Fig. 3B,C). *mei3Δ* cells expressing *byr1*^{DD} entered meiosis and completed sporulation (Fig. 3C); the progression of meiosis was similar to that in wild-type cells (Fig. 3D). Therefore, induction of meiosis by *byr1*^{DD} is independent of Mei3. However, *mei2Δ* cells expressing *byr1*^{DD} did not undergo meiosis or sporulation (Fig. 3C). Thus, Mei2 is required for entry into meiosis. This finding suggests that expression of *byr1*^{DD} leads to activation of Mei2 independently of Mei3 to drive meiosis in haploid cells (see Discussion; Fig. 7).

Fig. 3. Mitotic growth defects in mutant cells expressing *byr1*^{DD}. (A) Amino acid sequences of *H. sapiens* MAPK and *S. pombe* Spk1. MAPKK phosphorylation sites of *H. sapiens* MAPK and the corresponding sites of Spk1 are underlined. To make unphosphorylatable forms of Spk1, these sites in Spk1 were replaced with either or both alanine (A) and phenylalanine (F). These mutated Spk1 constructs are designated Spk1^{AY}, Spk1^{TF}, and Spk1^{AF} as indicated. (B) *h*$^{-}$ *spk1*$^{+}$ (TG7) cells, *h*$^{-}$ *spk1Δ* (TP106-3C) cells, *h*$^{-}$ *spk1*^{AF} (TG43) cells, *h*$^{-}$ *spk1*^{AY} (TG44) cells, *h*$^{-}$ *spk1*^{TF} (TG45) cells, *h*$^{-}$ *mei2Δ* (CRL544) cells, and *h*$^{-}$ *mei3Δ* (TG30) cells were transformed with pREP81-byr1^{DD}. Each transformant was cultured in liquid EMM2 with thiamine. After removing thiamine, fivefold serial dilutions of each transformant were spotted onto EMM2 plates with (right panel, 'Repressed') or without thiamine (left panel, 'Induced'). Plates were photographed after 4 days incubation at 30°C. (C) *h*$^{-}$ *spk1Δ* (TP106-3C) cells, *h*$^{-}$ *mei2Δ* (CRL544) cells, and *h*$^{-}$ *mei3Δ* (TG30) cells carrying pREP81-byr1^{DD} were cultured in liquid EMM2 at 30°C to induce ectopic meiosis, and fixed at 30 hours after induction. The nuclei were stained with DAPI. Bright-field images of the same cells are also shown (Phase). Scale bar: 5 μm. (D) Progression of meiosis in *mei3Δ* cells expressing *byr1*^{DD}. *h*$^{-}$ *mei3Δ* (TG30) cells carrying pREP81-byr1^{DD} were induced to undergo meiosis in liquid EMM2 at 30°C. The number of nuclei per cell was counted every 2 hours from 13 to 29 hours after induction (open symbols). As a control, *h*$^{-}$ wild-type (CRL239) cells carrying pREP81-byr1^{DD} were examined (filled symbols). At least 300 cells were scored for each time point. During analysis, the cell concentration was kept between 1×10^{6} and 2×10^{7} cells/ml by diluting with EMM2.

Telomere clustering induced by expression of *byr1^{DD}* requires Spk1

We then asked whether telomeres are clustered at the SPB in cells induced to undergo meiosis by expression of *byr1^{DD}*. The positions of telomeres and the SPB were determined by GFP-fused Taz1 (Taz1-GFP) and CFP-fused Sad1 (CFP-Sad1), respectively. Taz1 is a telomere-binding protein (Cooper et al., 1997), and Sad1 is a component of the SPB (Hagan and Yanagida, 1995). The expression of *byr1^{DD}* induced a change in cell shape, and in these deformed cells telomeres clustered at the SPB during meiotic prophase (Fig. 4A), as in meiosis in wild-type cells (Chikashige et al., 1994). The number of cells with telomeres clustered at the SPB increased after induction of *byr1^{DD}*, accompanied by an increase in the number of cells with an altered cell shape (Fig. 4C). In *spk1Δ* cells expressing *byr1^{DD}*, telomeres were separated from the SPB (Fig. 4B,C). Thus, we concluded that telomere-SPB clustering is induced by the expression of *byr1^{DD}* through Spk1 activity.

Because centromeres are separated from the SPB during meiotic prophase in normal meiosis (Chikashige et al., 1994; Chikashige et al., 1997), we also examined whether centromeres were separated from the SPB in cells expressing *byr1^{DD}*. In these experiments, the positions of centromeres, telomeres and the SPB were determined by FISH and immunofluorescence staining of fixed cells. In the meiotic prophase nuclei, characterized by an elongated shape, telomeres were clustered at the SPB and centromeres were separated from the SPB in all 30 nuclei examined (Fig. 4D). Collectively, our results show that telomere clustering to the SPB and centromere separation from the SPB take place in meiosis induced by *byr1^{DD}*, as occurs in wild-type cells undergoing meiosis.

Mei2 is involved in telomere clustering induced by mating pheromone

Because our results suggested that expression of *byr1^{DD}* induces activation of Mei2 in haploid cells, we examined whether telomere clustering observed in cells expressing *byr1^{DD}* depends on Mei2. In *h$^-$ mei2Δ* haploid cells expressing *byr1^{DD}* 37 hours after induction, 70% of the cells had changed shape, and telomeres were clustered in 46% of the deformed cells (data not shown). In contrast, in haploid cells expressing wild-type *mei2*, telomeres were clustered in almost all the deformed cells (Fig. 4C). This result showed that telomeres undergo clustering, but to a limited extent in the absence of Mei2, suggesting that Mei2 is involved in telomere clustering.

To confirm this result, we further examined telomere clustering in haploid cells treated with mating pheromone. In these experiments, we used *h$^-$ sxa2Δ* cells that are hypersensitive to mating pheromone P-factor (Imai and Yamamoto, 1992); the positions of telomeres and the SPB were determined by Taz1-GFP and DsRed-fused Sad1 (Sad1-DsRed), respectively. These cells exhibit telomere clustering without entering meiosis (Chikashige et al., 1997). When *h$^-$ sxa2Δ* cells were treated with synthetic P-factor, these cells changed shape, which is an indication of the cellular response to P-factor. In those cells with altered shape, clustering of telomeres at the SPB was observed (Fig. 5A), as reported previously (Chikashige et al., 1997). Time-course observations after P-factor treatment indicated that the number of cells with

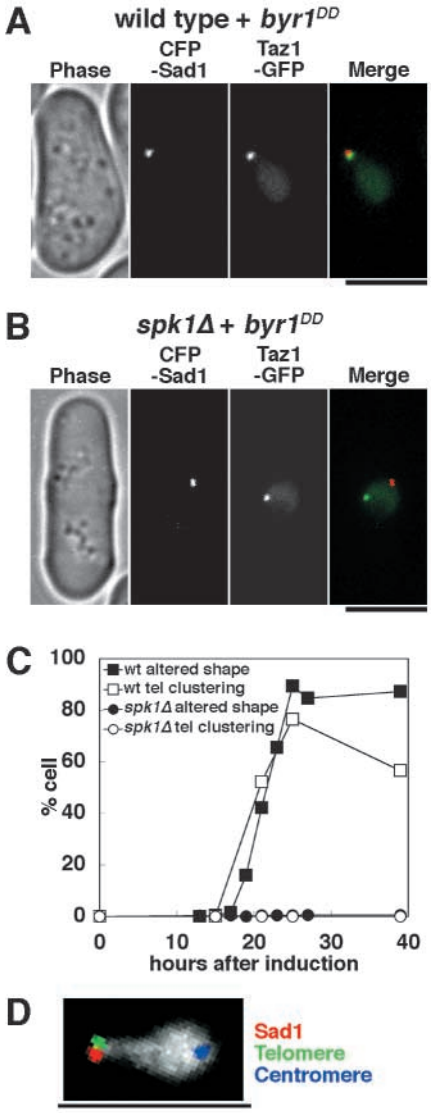


Fig. 4. Nuclear localization of telomeres in cells expressing *byr1^{DD}*. *h$^-$* wild-type (TG309) cells (A) and *h$^-$ spk1Δ* (TG335) cells (B) carrying pREP81-byr1^{DD} were cultured in liquid EMM2 for 27 hour at 30°C to induce ectopic meiosis, and observed by fluorescence microscopy without fixation. The SPB (red) was stained with CFP-Sad1, and telomeres (green) were stained with Taz1-GFP. Bright-field images of the same cells are also shown (Phase). Scale bars: 5 μm. (C) Time course of cell shape alteration ('altered shape') and telomere clustering ('tel clustering') after induction of *byr1^{DD}* in *h$^-$* wild-type (TG309) cells and *h$^-$ spk1Δ* (TG335) cells carrying pREP81-byr1^{DD}. To calculate the percentage of cells with altered shape, at least 130 cells were scored for each time point. To calculate the percentage of cells with telomere clustering, at least 50 cells were scored for each time point. (D) *h$^-$* wild-type (CRL239) cells carrying pREP81-byr1^{DD} were induced to undergo ectopic meiosis in liquid EMM2 at 30°C, and fixed at 20 hours after induction. The SPB (red) was stained with anti-Sad1 antibodies; telomeres (green) and centromeres (blue) were stained by FISH using cos212 and pRS140 as DNA probes, respectively. The nucleus (white) was stained with DAPI. Scale bar: 5 μm.

telomere-SPB clustering increased with time and was accompanied by an increase in the number of cells with an altered cell shape (Fig. 5B). However, *h$^-$ sxa2Δ spk1Δ* cells

showed neither a change in shape nor telomere clustering (Fig. 5F,G). This result confirmed that telomere clustering induced by mating pheromone is dependent on Spk1. We then examined the nuclear position of telomeres in *mei2Δ* cells treated with mating pheromone. Pheromone-treated *h⁻ sxa2Δ mei2Δ* cells showed an altered morphology to a similar extent as *h⁻ sxa2Δ* cells, but had less telomere clustering. As shown in Fig. 5E, in about one third of the deformed cells, telomeres were clustered near the SPB (an example shown in Fig. 5C) while in the rest of the cells telomeres were separated from the SPB (an example shown in Fig. 5D). At 48 hours after mating pheromone treatment, the percentage of cells that formed a telomere-SPB cluster was less than at 24 hours, whereas the total number of cells and the percentage of cells with altered shape were not changed (data not shown), suggesting that the formation of telomere clustering is not delayed, but instead an activity involved in telomere clustering is reduced in *h⁻ sxa2Δ mei2Δ* cells. These results indicate that mating pheromone can induce telomere clustering partially, but not fully, in the absence of Mei2. Thus, telomere clustering may be regulated by Mei2-dependent and -independent pathways downstream of Spk1 (see Discussion; Fig. 7). However, telomeres were regularly clustered in *h⁻ sxa2Δ mei3Δ* cells (data not shown), indicating that telomere clustering does not require Mei3.

Telomere clustering induced by Pat1 inactivation does not require Spk1

Because telomere clustering to the SPB partly required Mei2, we asked whether telomere clustering is induced by inactivation of Pat1 kinase in haploid cells. We examined the position of telomeres in haploid cells induced to undergo meiosis by Pat1 inactivation. In these experiments, we used the temperature-sensitive *pat1-114* mutant, in which Pat1 is inactivated at the restrictive temperature (Iino and Yamamoto, 1985a; Nurse, 1985). Cells of the *h⁻ pat1-114* mutant were arrested in G1 by nitrogen starvation and then shifted to the restrictive temperature to induce meiosis. These cells underwent meiotic nuclear divisions synchronously (Fig. 6A). In these cells, telomeres were separated from the SPB before temperature shift, and were clustered near the SPB at 4-6 hours after temperature shift (Fig. 6B,C). Thus, Pat1 inactivation also causes telomere clustering in haploid cells. As the role for Pat1 inactivation is to activate Mei2, it is expected that telomere clustering induced by Pat1 inactivation requires Mei2. In fact, *pat1-114 mei2Δ* cells did not progress to meiosis, as expected (Beach et al., 1985; Iino and Yamamoto, 1985b) (Fig. 6D), and did not show telomere clustering (Fig. 6E,F). Next we examined whether telomere clustering induced by Pat1 inactivation requires Spk1. In *pat1-114 spk1Δ* cells, the progression of meiosis was similar to that in *pat1-114* cells (Fig. 6G), and the percentage of cells that formed telomere clusters was not significantly reduced (Fig. 6H,I). These results indicate that

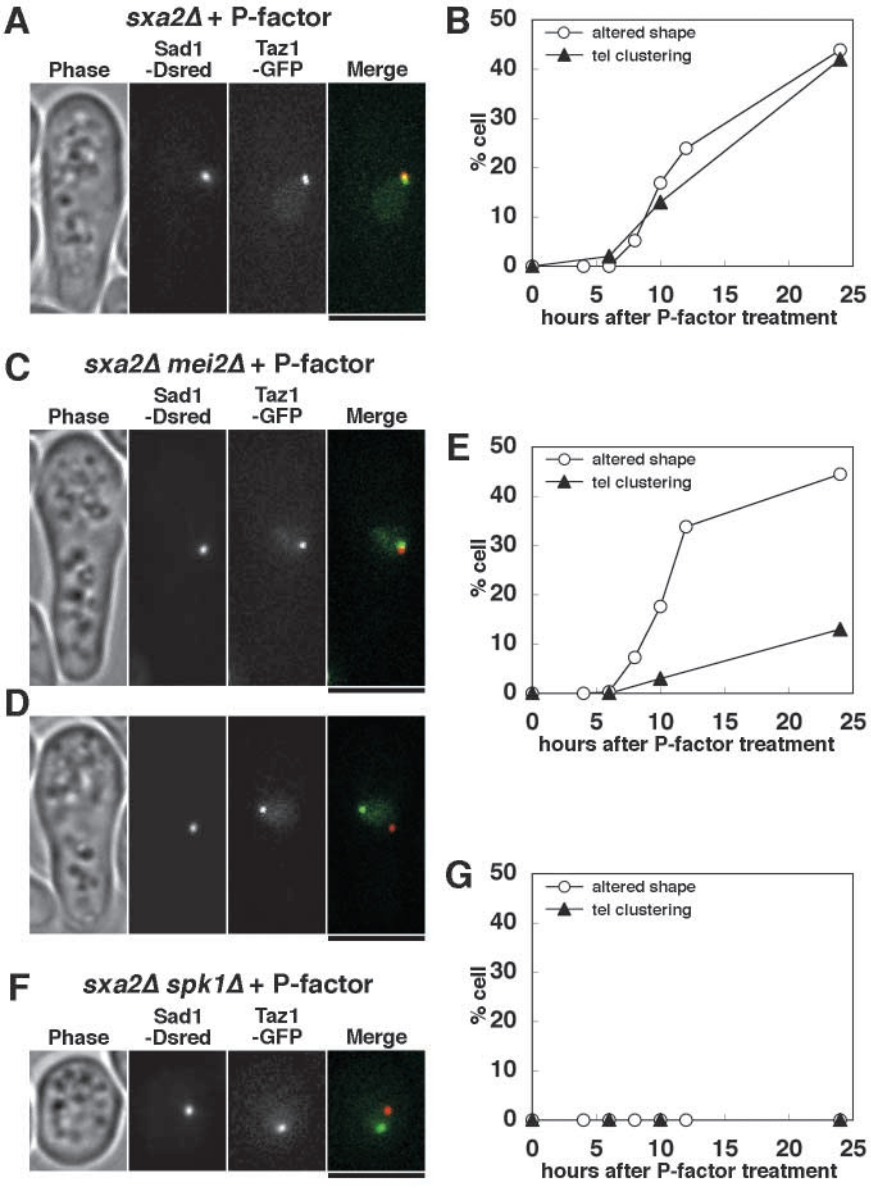


Fig. 5. Nuclear localization of telomeres in cells treated with mating pheromone. *h⁻ sxa2Δ* (TG208) cells (A,B), *h⁻ sxa2Δ mei2Δ* (TG263) cells (C-E), and *h⁻ sxa2Δ spk1Δ* (TG265) cells (F,G) were treated with the synthetic P-factor, and observed by fluorescence microscopy without fixation. (A,C,D,F) The SPB (red) and telomeres (green) were stained with Sad1-DsRed and Taz1-GFP, respectively. Bright-field images of the same cells are also shown (Phase). Scale bars: 5 μm. (B,E,G) Time course of cell shape alteration ('altered shape') and telomere clustering ('tel clustering') after P-factor treatment. To calculate the percentage of cells with altered shape, 200 cells (B,G) or 130 cells (E) were scored for each time point. To calculate the percentage of cells with telomere clustering, at least 50 cells were scored for each time point (B,E,G).

inactivation of Pat1, through activation of Mei2, can fully induce telomere clustering in the absence of Spk1, suggesting that Pat1 is downstream Spk1 (see Discussion; Fig. 7).

Discussion

Activation of MAPK drives meiosis in asexual cells

We have demonstrated that activation of pheromone-responsive MAPK is sufficient for the induction of meiosis in *S. pombe*. Expression of *byr1*^{DD}, a constitutively active form of Byr1 MAPKK, induced haploid cells in a rich medium to undergo meiosis and generate spores, although, most of these spores were inviable. When diploid cells were induced to undergo meiosis by expression of *byr1*^{DD}, regular cohesion of sister chromatids at the first meiotic division and their segregation at the second meiotic division were achieved, and meiosis was completed with viable spores. Telomere clustering to the SPB and centromere separation from the SPB during meiotic prophase were also achieved in *byr1*^{DD}-induced meiosis, as is the case during naturally occurring meiosis. Thus, activation of pheromone-responsive MAPK can drive cells to undergo a phenotypically normal meiosis.

Regulation of meiosis induction

When *S. pombe* diploid cells enter meiosis physiologically, Mei3 inactivates Pat1, and inactivation of Pat1 leads to activation of Mei2. Thus, both Mei2 and Mei3 are required for induction of meiosis. However, it is known that Mei2 and Pat1 are both involved in conjugation of haploid cells as the frequency of conjugation is reduced, but not eliminated, in a *mei2* mutant (Egel, 1973) and conjugation is ectopically induced at a semipermissive temperature in *pat1-114* (Beach et al., 1985). Because Mei3 is expressed only in diploid cells, this Mei2 function in haploid cells must be regulated by a Mei3-independent pathway. From these observations, it has been proposed that Mei2 activity is regulated by stepwise inactivation of Pat1. Mei3-independent partial inactivation of Pat1 takes place in response to mating pheromone before cell conjugation. Later, after cell conjugation, Mei3-dependent full

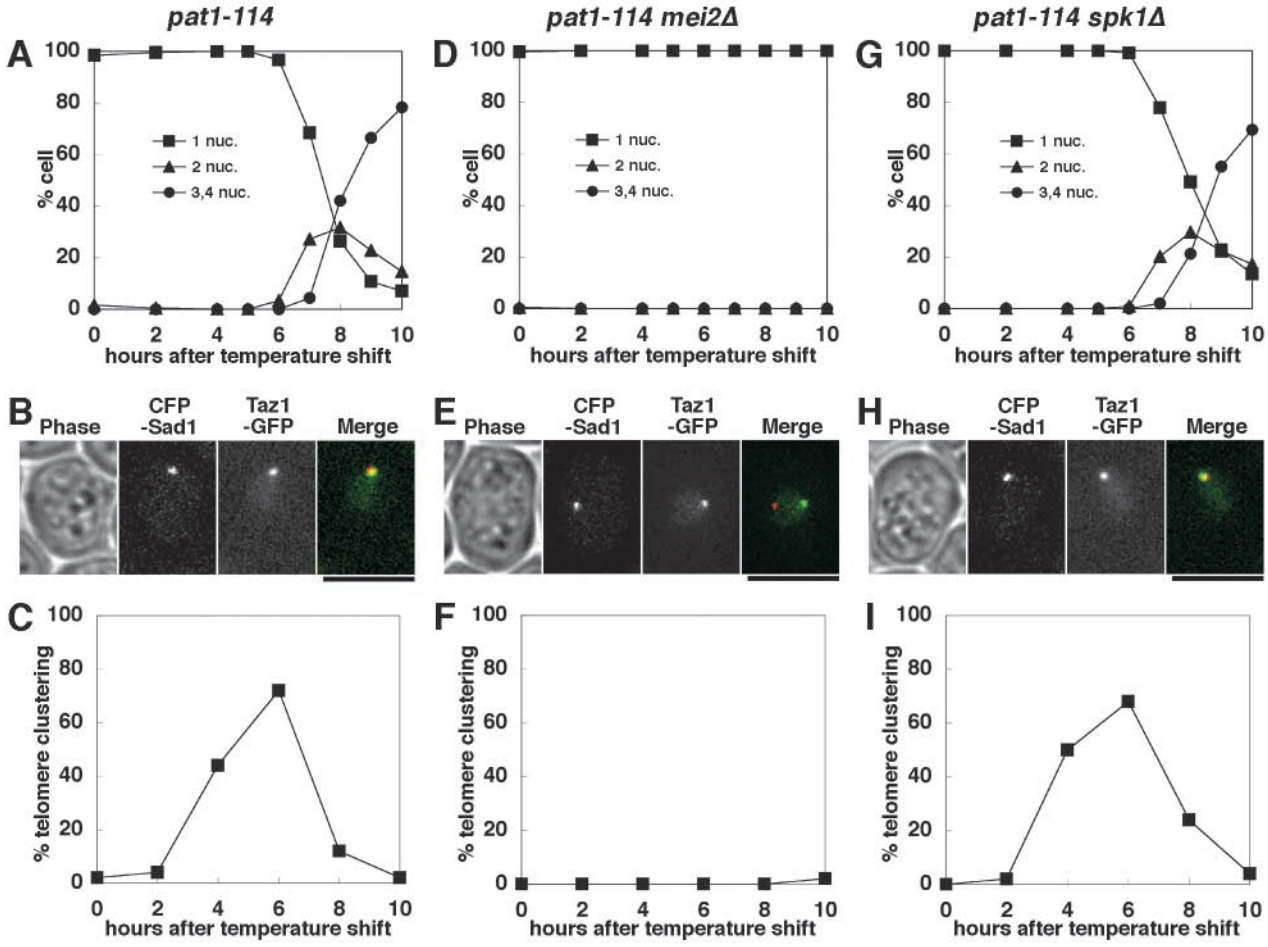


Fig. 6. Nuclear localization of telomeres in *pat1-114* mutant cells. *h*$^{-}$ *pat1-114* (TG292) cells (A-C), *h*$^{-}$ *pat1-114 mei2Δ* (TG202) cells (D-F), and *h*$^{-}$ *pat1-114 spk1Δ* (CRLc92) cells (G-I) were shifted to the restrictive temperature to induce ectopic meiosis, and observed by fluorescence microscopy without fixation. (A,D,G) Progression of meiosis after shifting to the restrictive temperature. The number of nuclei per cell was counted for up to 10 hours after induction of meiosis. At least 250 cells were scored for each time point. (B,E,H) The SPB (red) was stained with CFP-Sad1, and telomeres (green) were stained with Taz1-GFP. Bright-field images of the same cells are also shown (Phase). Scale bars: 5 μm. (C,F,I) Time course of telomere clustering after shifting to the restrictive temperature. To calculate the percentage of cells with telomere clustering, at least 50 cells were scored for each time point.

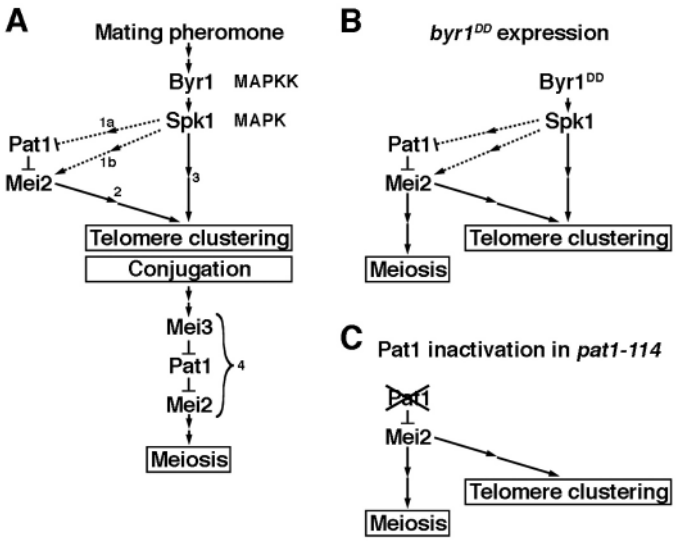


Fig. 7. A model for genetic regulation of meiosis and telomere clustering. Regulatory pathways in the normal process of meiosis (A), in meiosis induced by *byr1*^{DD} expression (B), and in meiosis induced by Pat1 inactivation (C). Activation and inhibition are indicated by arrows and crossing bars, respectively. Direct biochemical interaction has been proved for the inhibition of Pat1 by Mei3, the inhibition of Mei2 by Pat1, and the activation of Spk1 by Byr1 (indicated by a single arrow or bar). Other regulatory pathways indicated by two successive arrows or bars are conceptual, and could be indirect.

inactivation of Pat1 further activates Mei2 to induce meiosis (Nielsen and Egel, 1990).

We found that the induction of meiosis by *byr1*^{DD} required Mei2, but not Mei3. We propose the following model to explain our observations. In haploid cells, mating pheromone signaling modulates Mei2 activity at an intermediate level to promote conjugation without inducing meiosis (Fig. 7A, pathway 2). This is achieved by Mei3-independent inactivation of the Pat1 kinase (Fig. 7A; pathway 1a), or Pat1-independent activation of Mei2 (Fig. 7A; pathway 1b), or both, taking place upon Byr1 MAPKK signaling. Partial activation of Mei2 also induces telomere clustering (Fig. 7A, pathway 2). Normally, full activation of Mei2 mediated by Mei3-dependent inactivation of Pat1 kinase is required to induce meiosis (Fig. 7A, pathway 4). Overexpression of *byr1*^{DD}, however, may activate Mei2 sufficiently to induce not only telomere clustering, but also ectopic meiosis even in the absence of Mei3 (Fig. 7B). Pat1 inactivation in *pat1-114* also fully activates Mei2 to induce ectopic meiosis in haploid cells (Fig. 7C).

Similar Mei3-independent, Mei2-dependent induction of meiosis is also observed when a truncation of Sla1 (an *S. pombe* homolog of the human La protein) is expressed in *S. pombe* (Tanabe et al., 2003). In addition, truncated Sla1 interacts with Pat1, and *sla1* deletion partially suppresses the temperature sensitivity of *pat1-114* cells. Thus, Sla1 is a possible candidate for a Mei3-independent inhibitor of Pat1 in haploid cells. Ste7, another candidate Pat1 inhibitor, is required for conjugation. Ste7 is expressed during mating but degraded after conjugation when meiosis is initiated (Matsuyama et al., 2000). Because *pat1-114 ste7Δ* cells enter meiosis, but do not mate, at a semipermissive temperature, it is suggested that Ste7 inactivates Pat1 to promote conjugation but simultaneously represses meiosis by modulating Mei2 activity to an intermediate level. This inference is further supported by the finding that Ste7 interacts with both Pat1 and Mei2 in the two-hybrid assay. However, neither Sla1 nor Ste7 are major initiating factors of meiosis induced by *byr1*^{DD} because expression of *byr1*^{DD} was found to induce meiosis in haploid cells disrupted for *sla1* or *ste7* (K. Tanabe and M.K., unpublished results; T.G.Y. and Y.H., unpublished results).

Regulation of telomere clustering to the SPB

Our results indicate that telomere clustering induced by expression of *byr1*^{DD} or by treatment with mating pheromone fully depends on Spk1, and partially depends on Mei2 (Fig. 7B), suggesting that Mei2-dependent (Fig. 7A, pathway 2) and Mei2-independent pathways (Fig. 7A, pathway 3) are involved in Spk1-mediated induction of telomere clustering. In contrast, telomere clustering induced by inactivation of Pat1 fully depends on Mei2, but not on Spk1 (Fig. 7C), suggesting that Pat1 is downstream Spk1. The Mei2-dependent pathway for telomere clustering may be driven by partial inactivation of Pat1 (Fig. 7A; pathway 1a), or partial activation of Mei2 (Fig. 7A; pathway 1b); similarly to that proposed for conjugation. An alternative explanation for the Mei2-independent pathway is that formation and maintenance of telomere clustering are regulated by separate pathways: a telomere cluster at the SPB could be induced by the Spk1 pathway but Mei2 could be required to maintain a stable cluster of telomeres. Although telomere clustering is regulated downstream of Spk1, direct targets of Spk1 kinase have not been identified in *S. pombe*.

Several protein components have been identified that are involved in telomere-SPB clustering, and various mutants are known that lack clustering: for example, telomere-binding protein mutants *taz1* (Cooper et al., 1998; Nimmo et al., 1998) and *rap1* (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001) and the mutants *kms1* (Shimanuki et al., 1997) and *dot* (Jin et al., 2002) that both impair the structural integrity of the SPB. Telomere clustering is also defective to some extent in cytoplasmic dynein mutant (*dhc1Δ* or *dlc1Δ*) cells (Miki et al., 2002; Yamamoto et al., 1999); these motor proteins may mediate the telomere tethering movement. In addition, the *dhc1Δ* and *dlc1Δ* mutants exhibit a delay in nuclear fusion (Miki et al., 2002; Yamamoto et al., 1999), and a similar delay is observed in *mei2Δ* cells (Shimoda et al., 1985). Thus, microtubule motor activities may be activated downstream Mei2.

Regulation of centromere-SPB separation and sister chromatid segregation

When cells are induced to undergo meiosis by expression of *byr1*^{DD}, centromeres are separated from the SPB, and sister chromatids segregate properly during meiotic divisions. In contrast, in meiosis induced by Pat1 inactivation, centromeres are partially associated with the SPB (Chikashige et al., 2004), and sister chromatids segregate precociously during the first meiotic division (Yamamoto and Hiraoka, 2003). These results suggest that Pat1 inactivation is not sufficient for the correct behavior of meiotic chromosomes, and that a Pat1-independent regulatory pathway under the control of MAPK signaling is

involved in centromere-SPB separation, and also in sister chromatid cohesion at the first meiotic division (Fig. 7A, pathway 3). Although the biological significance of centromere-SPB separation remains unknown, this process may be important for formation of meiosis-specific sister kinetochore structures. We speculate that meiosis-specific sister kinetochore structures that are crucial for cohesion of sister chromatids during meiosis are not formed as a result of the inactivation of Pat1 kinase alone, but are formed in cooperation with an as yet-unknown pathway(s) under the regulation of MAPK signaling.

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