**A knockout screen for protein kinases required for the proper meiotic segregation of chromosomes in the fission yeast Schizosaccharomyces pombe**

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Reversible protein phosphorylation has been established as the major regulatory mechanism in the cell.1,2 Genome-wide surveys of protein kinases and phosphatases have been instrumental in characterizing novel proteins involved in various processes, including mitosis and meiosis.3-6 The fission yeast *Schizosaccharomyces pombe* is an excellent model organism for studying eukaryotic biology. There are more than one hundred predicted protein kinases encoded by the *S. pombe* genome, and some of them are known to play key roles in meiotic chromosome segregation.7-14 However, a systematic approach to analyze the role of *S. pombe* protein kinases in chromosome segregation during meiosis has not been conducted. While studies of *S. pombe* protein kinases that are essential for cell growth require the use of mutant strains carrying conditional alleles,15 non-essential protein kinases can be analyzed using knockout alleles.16 In our current study, we systematically analyze the role of non-essential *S. pombe* protein kinases in meiotic chromosome segregation. We focus on two protein kinases that show meiotic defects, namely Mph1 kinase, a member of the Mps1 family of spindle assembly checkpoint kinases and Spo4 kinase, the fission yeast ortholog of Dbf4-dependent Cdc7 kinase.

The reduction of chromosome number during meiosis is achieved by two successive rounds of chromosome segregation after just single round of DNA replication. To identify novel proteins required for the proper segregation of chromosomes during meiosis, we analyzed the consequences of deleting *Schizosaccharomyces pombe* genes predicted to encode protein kinases that are not essential for cell viability. We show that Mph1, a member of the Mps1 family of spindle assembly checkpoint kinases, is required to prevent meiosis I homolog non-disjunction. We also provide evidence for a novel function of Spo4, the fission yeast ortholog of Dbf4-dependent Cdc7 kinase, in regulating the length of anaphase II spindles. In the absence of Spo4, abnormally elongated anaphase II spindles frequently overlap and thus destroy the linear order of nuclei in the ascus. Our observation that the *spo4Δ* mutant phenotype can be partially suppressed by inhibiting Cdc2-as suggests that dysregulation of the activity of this cyclin-dependent kinase may cause abnormal elongation of anaphase II spindles in *spo4Δ* mutant cells.

**Introduction**

Reversible protein phosphorylation has been established as the major regulatory mechanism in the cell.1,2 Genome-wide surveys of protein kinases and phosphatases have been instrumental in characterizing novel proteins involved in various processes, including mitosis and meiosis.3-6 The fission yeast *Schizosaccharomyces pombe* is an excellent model organism for studying eukaryotic biology. There are more than one hundred predicted protein kinases encoded by the *S. pombe* genome, and some of them are known to play key roles in meiotic chromosome segregation.7-14 However, a systematic approach to analyze the role of *S. pombe* protein kinases in chromosome segregation during meiosis has not been conducted. While studies of *S. pombe* protein kinases that are essential for cell growth require the use of mutant strains carrying conditional alleles,15 non-essential protein kinases can be analyzed using knockout alleles.16 In our current study, we systematically analyze the role of non-essential *S. pombe* protein kinases in meiotic chromosome segregation. We focus on two protein kinases that show meiotic defects, namely Mph1 kinase, a member of the Mps1 family of spindle assembly checkpoint kinases and Spo4 kinase, the fission yeast ortholog of Dbf4-dependent Cdc7 kinase.

**Results**

A screen for protein kinases required for the proper segregation of chromosomes during meiosis. To identify novel proteins required for the proper segregation of chromosomes during meiosis, we analyzed the consequences of deleting *Schizosaccharomyces pombe* genes predicted to encode protein kinases that are not essential for cell viability. According to the PomBase database, there are 96 non-essential *S. pombe* genes predicted to encode protein kinases.16 In this study, we aimed to analyze knockout alleles from at least two independent sources for a majority of the studied kinases. Therefore, we analyzed kinase knockout alleles created by Bimbo et al.6 or purchased from the Bioneer collection.17,18 In addition, we made 38 knockout alleles according to our protocol described in Gregan et al.19 (Table S1). We failed to obtain knockout alleles of *ppk18* and *ppk19*. We confirmed that *byr1Δ*, *byr2Δ*, *spk1Δ*, *sp1Δ*, *sp2Δ* and *sty1Δ* mutant cells are sterile, which prevented us from analyzing meiotic chromosome segregation in these mutants.20-24 As previously described,25,26 we found that *gad8Δ* mutant cells are also defective in mating. However, we were able to find enough ascii to score meiotic chromosome segregation in *gad8Δ* mutant cells.
Mph1 is required for the proper segregation of homologs during meiosis I. Our screening revealed two mutants (bub1Δ and mphp1Δ) that showed a strong meiotic missegregation phenotype. We focused on mphp1Δ because meiotic chromosome segregation in the bub1Δ mutant has been previously described.11,36 Mph1 (the fission yeast MPS1 homolog) is an evolutionarily conserved protein kinase required for the spindle assembly checkpoint (SAC).37-42 Analysis of cen2-GFP dots in the mature asci of strains carrying homozygous cen2-GFP indicated homolog non-disjunction at meiosis I in mphp1Δ cells (Fig. 1A). To analyze chromosome segregation directly in anaphase I cells, we fixed and stained cells with antibodies against tubulin and GFP. We observed lagging chromosomes (5% of anaphase I cells) and homolog non-disjunction in mphp1Δ cells (Fig. 1B). Analysis of cells in which only one copy of chromosome II was marked by cen2-GFP (heterozygous cen2-GFP) suggested that there were no major defects in the segregation of sister chromatids during meiosis I and meiosis II in mphp1Δ cells (data not shown). We thus conclude that Mph1 is required for efficient homolog disjunction during meiosis I.

Spo4/Spo6 and Spo5 are required to prevent the abnormal extension of anaphase II spindles. The Ddb4-dependent Cdc7 kinase is essential for DNA replication in most eukaryotes.43,44 The fission yeast S. pombe possesses two complexes homologous to Cdc7-Ddb4. While the Hsk1/Dfp1 complex is required for DNA replication during mitosis and meiosis, the Spo4/Spo6 complex is meiosis-specific and dispensable for DNA replication, but it is required for progression of the second meiotic division.33,34 A recent report showing the role of S. cerevisiae Cdc7 kinase in setting up mono-orientation of sister kinetochores during the first meiotic division prompted us to carefully analyze chromosome segregation in spo4Δ and spo6Δ mutants.45

We asked if S. pombe Spo4 kinase and its regulatory subunit Spo6 are required for segregation of sister centromeres during meiosis. Consistent with previous reports,33,34 we observed that most of the spo4Δ and spo6Δ meiotic cells arrested at the binaucleate stage, probably due to fragmentation of meiosis II spindles. However, a small number of cells underwent both meiotic divisions. We scored the segregation of sister centromeres in a strain with only one copy of chromosome I marked with GFP (lys1-GFP).27 S. pombe produces linear asci in which the order of spores reflects the descent of nuclei from the two meiotic divisions.46 This allows detection of the missegregation of sister centromeres by scoring lys1-GFP in mature asci. In about 40% of spo4Δ and spo6Δ asci with four nuclei, lys1-GFP dots occupied both halves of the ascus, which is indicative of missegregation of sister centromeres during meiosis I (equational meiosis I) (Fig. 2A). Segregation of sister centromeres to opposite poles during meiosis I could be caused by the precarious loss of sister-chromatid cohesion. However, we found no evidence of a cohesion defect by monitoring cut3-GFP dots in spo4Δ cells arrested in late prophase I by a mer1Δ mutation (Fig. S1). To investigate more directly the behavior of sister centromeres, we analyzed the segregation of lys1-GFP in fixed cells stained with antibodies against tubulin and GFP. Surprisingly, we could not detect any missegregation in spo4Δ or spo6Δ cells when we analyzed...
spo5Δ mutant elongation of anaphase II spindles. Interestingly, we observed decided to test if other sporulation-deficient mutants show abnor-
mal elongation of anaphase II. We therefore affect the spindle length and timing of anaphase II. We therefore
will be interesting to analyze other sporulation-deficient mutants
to gain more insight into the possible link between sporulation
and the mechanisms governing anaphase II.

We next attempted to understand why spo4Δ and spo6Δ mutant
cells fail to maintain the proper length of anaphase II spindles.
Although molecular mechanisms that regulate the length of ana-
phase II spindles are poorly characterized, tight regulation of
cyclin-dependent protein kinase (CDK) activity is known to be
essential for progression through several stages of the cell cycle,
including anaphase.14,49 Cells expressing non-degradable cyclin B
have prolonged anaphase B, and chromosomes segregate much
further than in wild-type cells.50 We therefore speculated that
dysregulation of CDK activity may cause abnormal elongation of
anaphase II spindles in spo4Δ and spo6Δ mutant cells. To test this
possibility, we introduced a conditional analog-sensitive allele of
cdc2-as (Fig. 3)),51 which encodes the fission yeast CDK,
into spo4Δ cells. Whereas approximately 60% of spo4Δ asci with
four nuclei contained cen2-GFP dots in both halves of the ascus,
we observed a reduction to just 31% in cells where Cdc2-as was
inhibited by adding inhibitor (Fig. 5). We observed a partial sup-
pression of the spo4Δ mutant phenotype by cdc2-as, even in the
absence of inhibitor, suggesting that Cdc2-as may not be fully
functional. Thus, we conclude that inhibition of Cdc2-as by add-
ing ATP-analog 1-NM-PP1 partially suppresses the mutant phe-
notype of spo4Δ cells.

Taken together, we conclude that Spo4 and Spo6 are dispens-
able for proper segregation of chromosomes during meiosis I, but
Spo4 kinase activity is required to prevent the abnormal elon-
gation of spindles during meiosis II. Our observation that the
spo4Δ mutant phenotype can be partially suppressed by inhibiting
Cdc2-as suggests that dysregulating the activity of this
cyclin-dependent kinase may cause the abnormal elongation of
anaphase II spindles in spo4Δ mutant cells.

**Figure 2.** Segregation of sister centromeres in spo4Δ and spo6Δ meiotic cells. (A) The wild-type strain h−-lys1-GFP (wt) (JG11338) and h−-lys1-GFP strains carrying knockout alleles of spo4 (spo4Δ) (JG14885) or spo6 (spo6Δ) (JG14888) were crossed to h+ strains of the same genotype but lacking
lys1-GFP (JG11339, JG14872 and JG14879, respectively). Similarly, strains carrying a knockout allele of spo4 transformed with a plasmid carrying either a wild-type allele of spo4 (spo4Δ spo4+) (JG14911) or a “kinase-dead” allele of spo4 (spo4Δ spo4K95A) (JG14913) were crossed to h+ strains of the same
genotype but lacking lys1-GFP (JG14903 and JG14907, respectively). Cells were sporulated and stained with Hoechst. Segregation of chromosome I was scored in at least 100 asci. (B) The strains described in (A) were fixed and stained with antibodies against tubulin and GFP. DNA was visualized by Hoechst staining. Cells were examined under a fluorescence microscope and segregation of chromosome I, marked by lys1-GFP, was scored in 100
anaphase I or anaphase II cells.

lys1-GFP in anaphase I and anaphase II cells (Fig. 2B). In rare
cases, sister lys1-GFP sequences segregated to opposite halves. We
attribute this to recombination taking place between a centro-
mere and the lys1 locus. Interestingly, we observed abnormally
elongated anaphase II spindles in both spo4Δ and spo6Δ mutant
cells. These elongated spindles overlapped and thereby indicated
that corresponding nuclei that separated during meiosis II were
no longer adjacent (Fig. 3). Live cell imaging showed that the
abnormal elongation of spindles in the spo4Δ mutant cells pushed
sister nuclei apart and thus destroyed the linear order of nuclei
in the ascus such that the two spores at one end of the ascus
contained non-sister nuclei (Fig. 4). This abnormal expansion
of meiosis II spindles is likely due to the absence of Spo4 kinase
activity, because only wild-type spo4, not the “kinase-dead”
spo4K95A allele, rescued this phenotype (Figs. 2A and 3). We also
observed that anaphase II was longer in spo4Δ mutant cells
(21.5 min +/- 5.1) as compared with wild-type cells (9.3 min +/- 
2.3), suggesting that Spo4 is required for the timely completion
of anaphase II.

spo4Δ and spo6Δ mutant cells are sporulation-defective, and
we speculated that processes involved in spore formation might
affect the spindle length and timing of anaphase II. We therefore
decided to test if other sporulation-deficient mutants show abnor-
mal elongation of anaphase II spindles. Interestingly, we observed
abnormally elongated anaphase II spindles in spo5Δ mutant cells
(Fig. S2). Spo5 is a putative RNA-binding protein required for
spore formation, but its molecular function is not known.47,48 It
will be interesting to analyze other sporulation-deficient mutants
to gain more insight into the possible link between sporulation
and the mechanisms governing anaphase II.
their normal partitioning to opposite spindle poles and leads to the occurrence of homolog non-disjunction events in meiosis I. Although the stringency of the SAC may be reduced during meiosis I, cells lacking a functional spindle assembly checkpoint (SAC) still require for the proper segregation of recombinated homologous chromosomes during meiosis I (Mph1) and to maintain the proper length of anaphase II spindles (Spo4, Spo5 and Spo6). Mph1 is a member of the Mps1 family of protein kinases that are not essential for cell viability. This analysis uncovered new proteins required for meiotic recombination (Rec24, Rec25, Rec27, Mde2 and Dill). Our current study is focused on a systematic analysis of S. pombe genes predicted to encode protein kinases that are not essential for cell viability. This analysis uncovered new proteins required for the proper segregation of recombinated homologous chromosomes during meiosis I (Mph1) and to maintain the proper length of anaphase II spindles (Spo4, Spo5 and Spo6).

Mph1 is a member of the Mps1 family of protein kinases required for the spindle assembly checkpoint (SAC). Although the stringency of the SAC may be reduced during meiosis I, cells lacking a functional spindle assembly checkpoint enter anaphase I precociously, which does not allow sufficient time for recombinated homologous chromosomes to complete their normal partitioning to opposite spindle poles and leads to the occurrence of homolog non-disjunction events in meiosis I. Although homolog non-disjunction during meiosis I can be caused by various defects, such as a failure to undergo meiotic recombination or defective sister-chromatid cohesion along chromosome arms, it is likely that the homolog non-disjunction phenotype observed in mph1Δ cells is due to a precocious entry into anaphase I.

Discussion

The strategy of knocking out selected groups of genes has proven to be an efficient way to identify key regulators of meiotic chromosome segregation. In fission yeast, such a strategy led to the identification of the protector of centromeric cohesion (Sgo1) and new proteins required for meiotic recombination (Rec24, Rec25, Rec27, Mde2 and Dill). Our current study is focused on a systematic analysis of S. pombe genes predicted to encode protein kinases that are not essential for cell viability. This analysis uncovered new proteins required for the proper segregation of recombinated homologous chromosomes during meiosis I (Mph1) and to maintain the proper length of anaphase II spindles (Spo4, Spo5 and Spo6).

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Dbf4-dependent Cdc7 kinase is essential for eukaryotic DNA replication during both mitosis and meiosis. In addition to its role in origin firing, Dbf4 is also required after S phase to ensure mono-orientation of sister kinetochores during the first meiotic division in the budding yeast S. cerevisiae. Our observation that Spo4, the fission yeast ortholog of Dbf4-dependent Cdc7 kinase, is not required for mono-orientation of sister kinetochores during meiosis I is not surprising, given that the Pcs1/Mde4 complex, the fission yeast counterpart of the budding yeast monopole subcomplex Csm1/Lrs4, is also dispensable for the mono-orientation process. However, there are two orthologs of the Cdc7 kinase in the fission yeast S. pombe (Spo4 and Hsk1) and a possible involvement of the Hsk1 kinase in the mono-orientation of sister kinetochores during meiosis I remains to be tested. Unexpectedly, we discovered that Spo4 kinase activity is required to maintain the proper length of anaphase II spindles. The control of spindle length is critical for both mitosis and meiosis. However, the molecular mechanisms and proteins involved are poorly characterized. Spindle length depends on the coordinated actions of motor proteins and factors that control tubulin polymerization and depolymerization, such as MCAK, Klp2 and γ-tubulin.

In mouse oocytes, the Mos-MAP kinase pathway has been shown to control spindle elongation during meiosis I. Interestingly, the phenotype of spo4Δ cells is similar to that of S. cerevisiae cells depleted for Cdc15. The MEN (mitotic exit network pathway) component Cdc15 is a protein kinase required for the formation of mature spores and for proper spindle disassembly after meiosis II. Finally, it has been shown that cells expressing non-degradable cyclin B have prolonged anaphase B. Indeed, our observation that the anaphase II spindle defect in spo4Δ cells can be partially suppressed by inhibiting the fission yeast cyclin-dependent kinase Cdc2 suggests that the activity of this cyclin-dependent kinase may be dysregulated in spo4Δ mutant cells. In the future, identifying the relevant Spo4 targets will be essential for elucidating the mechanism controlling the length of meiosis II spindles.

In summary, our current study, together with many previous reports (e.g., refs. 29 and 71–73), demonstrates that reversible protein phosphorylation and protein kinases play a major role in ensuring the proper segregation of chromosomes during meiosis. In the long run, greater knowledge of these processes may help us understand the origins of human meiotic aneuploidy, which can lead to miscarriages and genetic disorders such as Down syndrome.

Materials and Methods

Strains and general methods. The genotypes of the yeast strains used in this study are listed in Table S2. Schizosaccharomyces pombe strains were maintained and grown using standard
ligated with pREP41 digested with the same restriction enzymes (XmaI and XhoI), thus creating pREP41-H2A.2-TagBFP. Primers F hom R SacI (AAA CGA GCT CTC AAC TCT CCG TAG AGT AT) and R hom R MluI (AAA AAC GCG TCG AAA TGT CTT ATC TTG CCG CA) were used to amplify region near his7 locus from the plasmid p245, the PCR product was digested with MluI and SacI restriction enzymes and ligated with pREP41-hta2-TagBFP digested with the same restriction enzymes (MluI and SacI digest removed ARS sequence from the pREP41-hta2-TagBFP plasmid). ApaI restriction enzyme was used to linearize the plasmid before transformation into yeast. The plasmid pREP41-hta2-TagBFP-leu2 (p244) was used to create strains JG16499, JG16486, JG16662 and JG16663.

Time-lapse fluorescence microscopy. Cells were grown on Edinburgh Minimal Medium (EMM)-leu plates overnight at 32°C and subsequently plated on PMG-N plates (24 h at 25°C) to induce meiosis. Cells were resuspended in liquid PMG-N and transferred to a glass-bottom microwell dish (MatTek, Ashland) coated with 1 μl of 2 mg/ml lectin BS-1 (Sigma-Aldrich). Fluorescence microscopy of live cells was performed using epi-fluorescence microscope Olympus Cell R system equipped with Olympus MT-20 150W mercury arc burner, Halogen Lamp 100W, Hamamatsu ORCA-ER CCD camera, 60x/1.42 PlanApoN oil immersion objective and standard filter sets: DAPI (excitation 381–392 nm, emission 420–460 nm) and CY3 (excitation BP547–572, emission 5669–623 nm). All the experiments were performed at 25°C. Three-dimensional time-lapse images of cells were taken with seven optical Z-sections, with 1 μm z distance, 3 and 4 min intervals. Image and data analyses were performed in ImageJ.

The length of anaphase II was determined in four spo4Δ and eight wild-type cells using the above described Olympus Cell R system.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental materials may be found here: www.landesbioscience.com/journals/cc/article/23513

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