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

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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 *spo4*Δ 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 *spo4*Δ 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.[6] 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 *byr1*Δ, *byr2*Δ, *spk1*Δ, *ssp1*Δ, *ssp2*Δ and *sty1*Δ 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 *gad8*Δ mutant cells are also defective in mating. However, we were able to find enough asci to score meiotic chromosome segregation in *gad8*Δ mutant cells.

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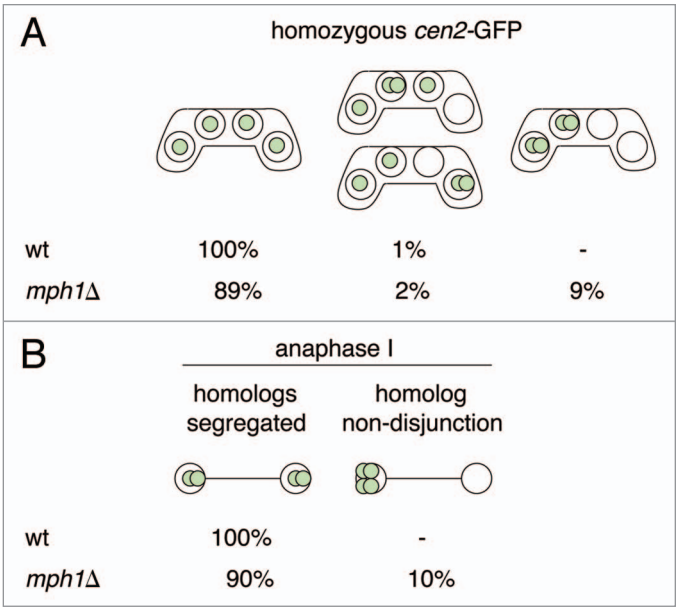


Figure 1. Mph1 is required for the proper segregation of recombined homologous chromosomes during meiosis I. (**A**) The meiotic segregation of chromosome II was scored in a wild-type *h90 cen2*-GFP strain (JG12618) and an *h90 cen2*-GFP strain carrying the knockout allele of *mph1* (*mph1*Δ) (JG15607). Cells were stained with Hoechst and examined under the fluorescence microscope. Chromosome segregation was scored in at least 100 asci. (**B**) The strains described in (**A**) were fixed and immunostained for tubulin and GFP. DNA was visualized by Hoechst staining. A total of 100 anaphase I cells were examined under a fluorescence microscope, and the segregation of chromosome II, marked by *cen2*-GFP, was scored.

To analyze chromosome segregation, we introduced knockout alleles into a haploid homothallic h^{90} strain where chromosome I or chromosome II was marked with GFP (*lys1*-GFP[27] or *cen2*-GFP[28]). These strains generate cells of both mating types and undergo mating and meiosis on sporulation medium. We sporulated mutant cells, stained nuclei with Hoechst dye and scored segregation of GFP dots in asci. In selected mutant strains, we also stained fixed cells with Hoechst dye and antibodies against tubulin and GFP in order to investigate chromosome segregation directly in anaphase I and anaphase II cells. Of the 88 mutants analyzed, 81 had no apparent meiotic phenotype. Two mutants (*bub1*Δ and *mph1*Δ) showed strong defects in chromosome segregation during meiosis, and five mutants (*hhp2*Δ, *ppk24*Δ, *mug27*Δ, *spo4*Δ and *atg1*Δ) showed various meiotic defects, such as a weak missegregation phenotype, lagging chromosomes or asci with more than four DNA masses (data not shown). Meiotic defects in *hhp2*Δ, *spo4*Δ and *mug27*Δ mutant cells have previously been described.[10,29-34] Atg1 is an evolutionarily conserved protein kinase that is required for autophagy, and a defect in sporulation has been described in *Saccharomyces cerevisiae atg1*Δ mutant cells.[35] In fission yeast *atg1*Δ mutant cells we observed that spore viability, as determined by random spore analysis, was strongly reduced (8% spore viability) compared with wild-type cells (86% spore viability). The role of Ppk24 in meiosis is not known and will be interesting to analyze in the future.

Mph1 is required for the proper segregation of homologs during meiosis I. Our screening revealed two mutants (*bub1*Δ and *mph1*Δ) that showed a strong meiotic missegregation phenotype. We focused on *mph1*Δ because meiotic chromosome segregation in the *bub1*Δ 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 *mph1*Δ 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 *mph1*Δ 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 *mph1*Δ 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 Dbf4-dependent Cdc7 kinase is essential for DNA replication in most eukaryotes.[43,44] The fission yeast *S. pombe* possesses two complexes homologous to Cdc7-Dbf4. 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 *spo4*Δ and *spo6*Δ 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 *spo4*Δ and *spo6*Δ meiotic cells arrested at the binucleate 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 *spo4*Δ and *spo6*Δ 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 precocious loss of sister-chromatid cohesion. However, we found no evidence of a cohesion defect by monitoring *cut3*-GFP dots in *spo4*Δ cells arrested in late prophase I by a *mei4*Δ 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 *spo4*Δ or *spo6*Δ cells when we analyzed

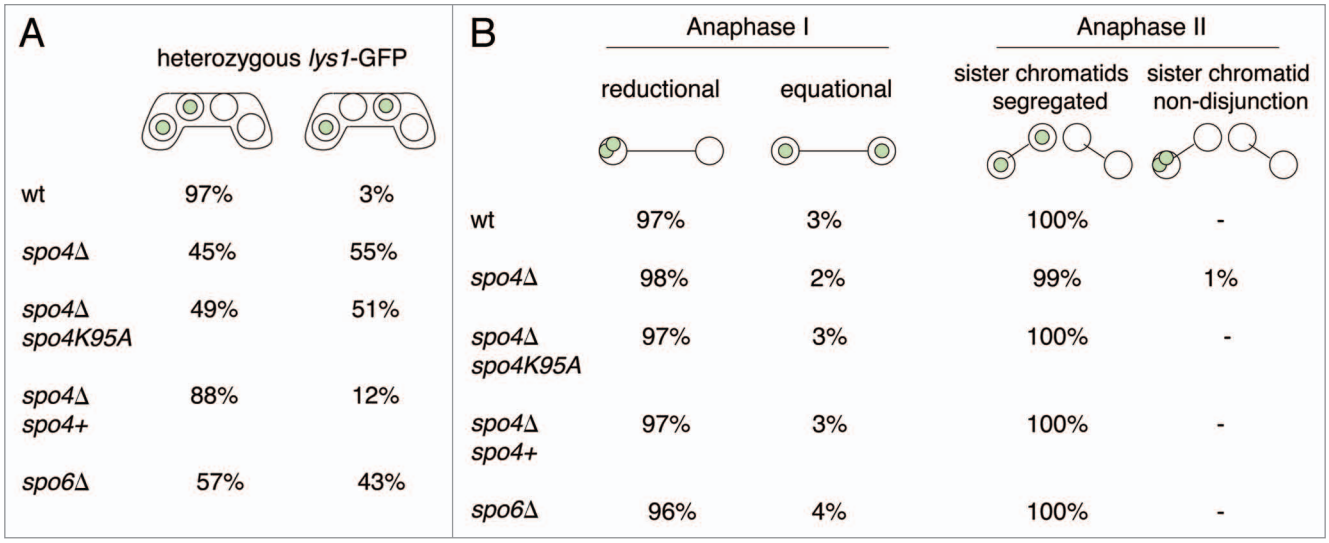


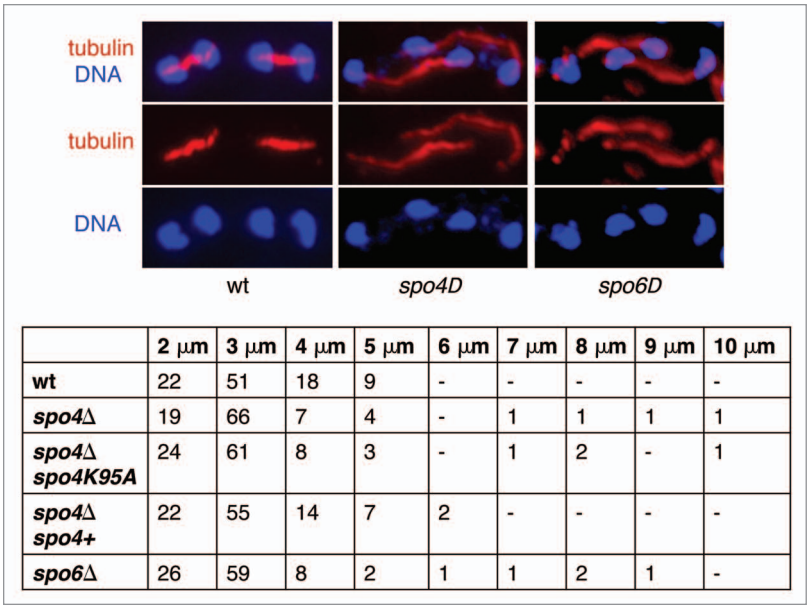
Figure 2. Segregation of sister centromeres in *spo4*Δ and *spo6*Δ meiotic cells. (**A**) The wild-type strain *h*– *lys1*-GFP (wt) (JG11338) and *h*– *lys1*-GFP strains carrying knockout alleles of *spo4* (*spo4*Δ) (JG14885) or *spo6* (*spo6*Δ) (JG14888) were crossed to *h*+ strains of the same genotype 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* (*spo4*Δ *spo4*+) (JG14911) or a "kinase-dead" allele of *spo4* (*spo4*Δ *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 centromere and the *lys1* locus. Interestingly, we observed abnormally elongated anaphase II spindles in both *spo4*Δ and *spo6*Δ 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 *spo4*Δ 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 *spo4*Δ 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.

*spo4*Δ and *spo6*Δ 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 abnormal elongation of anaphase II spindles. Interestingly, we observed abnormally elongated anaphase II spindles in *spo5*Δ 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.

We next attempted to understand why *spo4*Δ and *spo6*Δ mutant cells fail to maintain the proper length of anaphase II spindles. Although molecular mechanisms that regulate the length of anaphase 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 *spo4*Δ and *spo6*Δ mutant cells. To test this possibility, we introduced a conditional analog-sensitive allele of *cdc2* (*cdc2-as*) (**Fig. S3**),[51] which encodes the fission yeast CDK, into *spo4*Δ cells. Whereas approximately 60% of *spo4*Δ 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 suppression of the *spo4*Δ 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 adding ATP-analog 1-NM-PP1 partially suppresses the mutant phenotype of *spo4*Δ cells.

Taken together, we conclude that Spo4 and Spo6 are dispensable for proper segregation of chromosomes during meiosis I, but Spo4 kinase activity is required to prevent the abnormal elongation of spindles during meiosis II. Our observation that the *spo4*Δ 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 *spo4*Δ mutant cells.



	2 μm	3 μm	4 μm	5 μm	6 μm	7 μm	8 μm	9 μm	10 μm
wt	22	51	18	9	-	-	-	-	-
spo4Δ	19	66	7	4	-	1	1	1	1
spo4Δ spo4K95A	24	61	8	3	-	1	2	-	1
spo4Δ spo4+	22	55	14	7	2	-	-	-	-
spo6Δ	26	59	8	2	1	1	2	1	-

Figure 3. The anaphase II spindles are abnormally expanded in spo4Δ and spo6Δ mutant cells. A wild-type h90 strain and h90 strains carrying either a knockout allele of spo4 (spo4Δ) (JG14875), or a knockout allele of spo6 (spo6Δ) (JG14882) or a knockout allele of spo4 transformed with a plasmid carrying either a wild-type allele of spo4 (spo4Δ spo4+) (JG14906) or a "kinase-dead" allele of spo4 (spo4Δ spo4K95A) (JG14910) were sporulated, fixed and stained with antibodies against tubulin and GFP. DNA was visualized by Hoechst staining. The length of meiosis II spindles was determined in 100 zygotes.

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 Dil1).[52-55] 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 recombined 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).[37-42] Although the stringency of the SAC may be reduced during meiosis I,[56-58] cells lacking a functional spindle assembly checkpoint enter anaphase I precociously, which does not allow sufficient time for recombined homologous chromosomes to complete their normal partitioning to opposite spindle poles[59] and leads to the occurrence of homolog non-disjunction events in meiosis I.[36,59-61] 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.

Dbf4-dependent Cdc7 kinase is essential for eukaryotic DNA replication during both mitosis and meiosis.[44] 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*.[45] 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 monopolin subcomplex Csm1/Lrs4, is also dispensable for the mono-orientation process.[62] 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.[63-68] In mouse oocytes, the Mos-MAP kinase pathway has been shown to control spindle elongation during meiosis I.[69] 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.[70] Finally, it has been shown that cells expressing non-degradable cyclin B have prolonged anaphase B.[50] 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.[74]

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

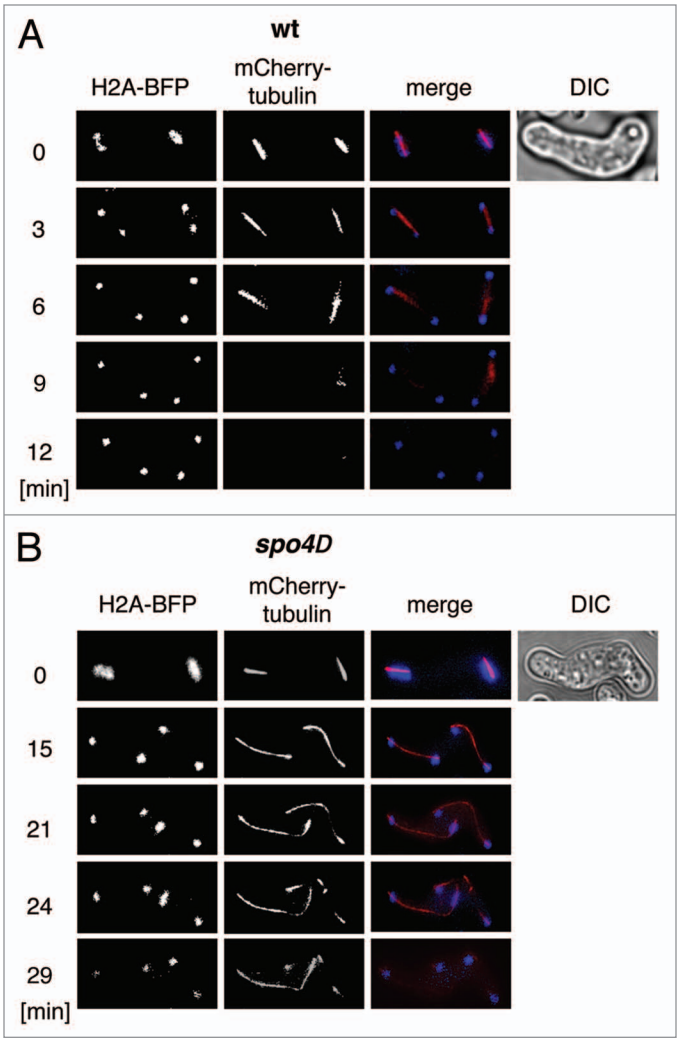


Figure 4. Live-cell analysis of anaphase II spindles in *spo4*Δ cells. The wild-type strain *h*– mCherry-*atb2 hta2*-TagBFP (wt) (JG16499) (**A**) or the h– mCherry-*atb2 hta2*-BFP strain carrying a knockout allele of spo4 (*spo4*Δ) (JG16662) (**B**) was crossed to *h*+ strains of the same genotype (JG16486 and JG16663, respectively). Cells were sporulated and spindle elongation during meiosis II was analyzed by live cell imaging. The numbers indicate time in minutes. Anaphase II spindles were analyzed in four *spo4*Δ and eight wild-type cells.

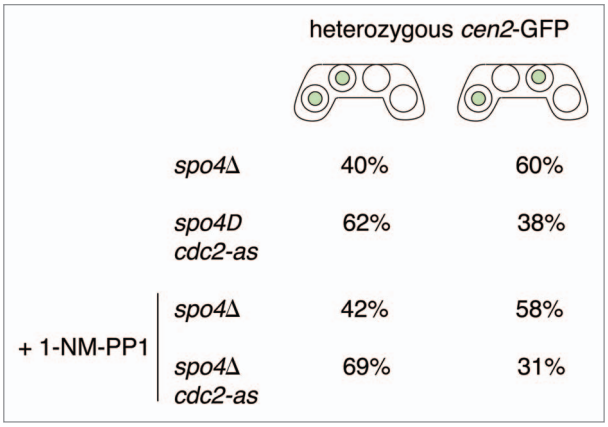


Figure 5. Inactivation of Cdc2-as partially suppresses the mutant phenotype of *spo4*Δ cells. The *h*– *cen2*-GFP strain carrying either a knockout allele of *spo4* (*spo4*Δ) (JG14873) or a knockout allele of *spo4* and an analog-sensitive allele of *cdc2* (*spo4*Δ *cdc2-as*) (JG16848) were crossed to *h*+ strains of the same genotype but lacking *cen2*-GFP (JG14872 and JG16858, respectively) and plated on PMG-N plates. After 24–43 h of incubation at 25°C cells were washed with water and incubated in a liquid PMG-N medium with or without inhibitor (5μM 1-NM-PP1) at 25°C for 4–7 h. Cells were stained with Hoechst to visualize DNA and segregation of *cen2*-GFP was scored in at least 50 asci.

conditions.[15,75,76] The transformation of *S. pombe* was performed using the lithium acetate method as previously described.[19]

To create plasmid pREP41-hta2-TagBFP-leu2 (p244), we used primers F gwTagBFP NheI (CGC GGC TAG CTC TGA ATT GAT TAA AGA GAA TAT GC) and R gwTagBFP XmaI (CGC GCC CGG GTT AGT TCA ATT TGT GTC CTA ACT TAG) to amplify gwTagBFP from Gateway®TagBFP-AS-C entry clone plasmid (Evrogen FP177). The amplified product containing gwTagBFP was digested with *Nhe*I restriction enzyme and ligated with *Nhe*I-digested *hta2*-containing PCR product amplified from the plasmid p245 using primers F H2A.2 XhoI (AAA ACT CGA GAT GTC TGG AGG TAA ATC TGG TGG TA) and R H2A.2 NheI (AAA AGC TAG CAG CAC CAG CAC CGG CTC CGG CA). The ligated product was digested with XmaI and *Xho*I and ligated with pREP41 digested with the same restriction enzymes (XmaI and *Xho*I), 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 *Mlu*I and *Sac*I restriction enzymes and ligated with pREP41-hta2-TagBFP digested with the same restriction enzymes (*Mlu*I and *Sac*I) (*Mlu*I and *Sac*I digest removed ARS sequence from the pREP41-hta2-TagBFP plasmid). *Apa*I 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, 60×/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 *spo4*Δ 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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