Mal3, the *Schizosaccharomyces pombe* homolog of EB1, is required for karyogamy and for promoting oscillatory nuclear movement during meiosis

Silvia Polakova[1], Zsigmond Benko[1], Lijuan Zhang[1], and Juraj Gregan[1,2,*]

[1]Max F. Perutz Laboratories; Department of Chromosome Biology; University of Vienna; Vienna, Austria;
[2]Department of Genetics; Comenius University; Bratislava, Slovak Republic

Two successive rounds of chromosome segregation following a single round of DNA replication enable the production of haploid gametes during meiosis. In the fission yeast *Schizosaccharomyces pombe*, karyogamy is the process where the nuclei from 2 haploid cells fuse to create a diploid nucleus, which then undergoes meiosis to produce 4 haploid spores. By screening a collection of *S. pombe* deletion strains, we found that the deletion of 2 genes, *mal3* and *mto1*, leads to the production of asci containing up to 8 spores. Here, we show that Mal3, the fission yeast member of the EB1 family of conserved microtubule plus-end tracking proteins, is required for karyogamy, oscillatory nuclear movement, and proper segregation of chromosomes during meiosis. In the absence of Mal3, meiosis frequently initiates before the completion of karyogamy, thus producing up to 8 nuclei in a single ascus. Our results provide new evidence that fission yeast can initiate meiosis prior to completing karyogamy.

Introduction

In sexually reproducing organisms, meiosis is the process by which haploid gametes are produced from diploid precursor cells.[1] The fission yeast *Schizosaccharomyces pombe* is a useful model organism for the study of meiosis, as it is amenable to both genetic and cell biological techniques and can be induced to undergo highly synchronous meiosis.[2-7] Moreover, a near-complete, genome-wide deletion collection for fission yeast has been created.[8-11] Upon nitrogen starvation, 2 haploid *S. pombe* cells of opposite mating type, designated *mat1-P* (*h*$^+$) and *mat1-M* (*h*$^-$), mate, and the two haploid nuclei fuse (karyogamy).[12] This process is followed by a single round of DNA replication and 2 successive rounds of chromosome segregation, called meiosis I and meiosis II. Whereas the second meiotic division is similar to mitosis in that sister centromeres segregate to opposite poles, the first meiotic division ensures segregation of homologous centromeres.[1,13] Two meiotic divisions produce 4 haploid nuclei that are encapsulated, and 4 haploid spores are formed in a single ascus.[14]

In this study, we screened a genome-wide *S. pombe* deletion library for mutants defective in chromosome segregation during meiosis. We found that deletion of 2 genes, *mal3* and *mto1*, leads to the production of asci containing up to 8 spores.

In *mal3Δ* mutant zygotes, meiosis frequently initiates before the completion of karyogamy, thus producing up to 8 nuclei in a single ascus. In addition, Mal3 is required for oscillatory nuclear movement and proper segregation of homologous centromeres during meiosis I.

Results

mal3Δ and *mto1Δ* mutant cells produce asci with abnormal spore numbers

To identify novel proteins required for proper chromosome segregation during meiosis, we screened a genome-wide *S. pombe* deletion library (the results of this screen will be published in a separate manuscript). Whereas wild-type cells produce asci containing 4 spores, we found that the deletion of 2 genes, *mal3* and *mto1*, frequently led to the production of asci containing up to 8 spores (**Fig. 1A and B**). However, a fraction of *mal3Δ* and *mto1Δ* asci contained 4 normal spores that were indistinguishable from wild-type spores, suggesting that this phenotype is only partially penetrant. Spore viability, as determined by random spore analysis, was reduced in both *mal3Δ* and *mto1Δ* strains (**Fig. 1B**). A similar phenotype has been reported in recent independent studies.[15,16] Thus, Mal3 and Mto1 are important for the formation of normal 4-spored asci.

Mal3 and Mto1 are required for karyogamy

Mal3 is the fission yeast member of the EB1 family of conserved microtubule plus-end tracking proteins.[17] Mto1 is a large coiled-coil protein similar to centrosomin and is required for nucleation of cytoplasmic microtubules.[18,19] Because the absence of the EB1 homolog in budding yeast leads to a defect in karyogamy,[20] we speculated that Mal3 and Mto1 might also be required for karyogamy in fission yeast. Moreover, it has been suggested that in zygotes lacking both the kinesin-like protein Klp2 and the dynein heavy chain Dhc1 meiosis proceeded in the absence of karyogamy, resulting in asci with more than 4 spores.[21] This phenomenon, called "twin meiosis", has been previously observed in tetraploid *S. pombe* zygotes resulting from the mating of 2 diploid cells.[22] Indeed, microscopy of early *mal3Δ* and *mto1Δ* mutant zygotes revealed that zygotes containing only a single nucleus occurred less frequently than in wild-type (data not shown). Live-cell imaging showed that nuclei from 2 haploid wild-type cells fused to create a single nucleus, which then underwent meiosis and produced 4, presumably haploid, nuclei. On the other hand, we observed a karyogamy defect in *tht1Δ* mutant zygotes, as previously reported[23] (**Fig. 2A and B**). Importantly, we observed that *mal3Δ* and *mto1Δ* mutant zygotes frequently entered meiosis before the completion of karyogamy. In these zygotes, the 2 nuclei each underwent 2 successive meiotic divisions, thus producing up to 8 nuclei in a single ascus (**Fig. 2A–C**). Analysis of *mal3Δ* zygotes, wherein one nucleus was labeled with Hht1-mRFP and the other with Hht1-CFP, also confirmed that the nuclei underwent meiotic divisions prior to completing karyogamy (**Fig. 2D**). We conclude that *mal3Δ* and *mto1Δ* mutant zygotes frequently enter meiosis before the completion of karyogamy and produce up to 8 nuclei in a single ascus.

The fission yeast *S. pombe* normally proliferates in a haploid state, but diploid strains can also be obtained. Karyogamy is required to produce a diploid nucleus, which then undergoes meiosis when two haploid cells mate (i.e., zygotic meiosis). However, karyogamy is dispensable for azygotic meiosis in which a diploid cell undergoes meiosis without mating. We reasoned that if the supernumerary spores in *mal3Δ* and *mto1Δ* mutant asci were due to defective karyogamy, azygotic meiosis should restore the normal number of spores in the asci. We therefore constructed *mal3Δ/mal3Δ* and wild-type *mal3+/mal3+* diploid strains and plated them on sporulation medium. Both the wild-type and *mal3Δ/mal3Δ* strains produced azygotic asci containing four spores (**Fig. 3A**). We have not observed any azygotic asci containing more than four spores in the *mal3Δ/mal3Δ* strain (**Fig. 3A** and data not shown). However, spore viability was reduced in the *mal3Δ/mal3Δ* strain compared with wild-type (**Fig. 3B**). This result suggests that in addition to a karyogamy defect, *mal3Δ* mutant cells have other defects that reduce spore viability. It is unlikely that the reduced spore viability in the diploid *mal3Δ/mal3Δ* strain was due to the presence of zygotic asci. We found that approximately 99% of the asci were azygotic, as judged by ascus morphology (data not shown). We therefore conclude that azygotic meiosis restores the normal number of spores but does not fully restore spore viability in *mal3Δ* mutant asci.

Mal3 is required for oscillatory nuclear movement and proper segregation of homologous centromeres during meiosis I

The reduced spore viability in azygotic *mal3Δ* mutant asci suggested that in addition to karyogamy, Mal3 has some other role in meiosis. To identify this additional function of Mal3, we analyzed meiotic chromosome segregation in *mal3Δ* mutant cells.

In synchronous *pat1–114*-induced meiosis, *mal3Δ* mutant cells underwent both meiotic divisions with kinetics similar to those of the wild type (**Fig. S1**). Analysis of *cen2*-GFP dots in 4-spore asci of a strain in which both copies of chromosome II contained *cen2* sequences marked with GFP (*cen2*-GFP) indicated meiosis I homolog nondisjunction and missegregation of sister centromeres in *mal3Δ* mutant cells (**Fig. 4A**). We observed a similar missegregation in azygotic *mal3Δ/mal3Δ* asci (data not shown). To investigate chromosome segregation directly in anaphase I cells, we fixed zygotic asci and stained them with antibodies against GFP and tubulin. In wild-type cells, homologous centromeres faithfully segregated to opposite poles during anaphase I. Next, we analyzed *mal3Δ* zygotes that underwent successful karyogamy, as indicated by a single anaphase I spindle. In 6% of *mal3Δ* anaphase I cells, we observed that homologous centromeres segregated to the same pole (**Fig. 4B**). Missegregation of homologous chromosomes during meiosis I could be due to several reasons including a failure to produce
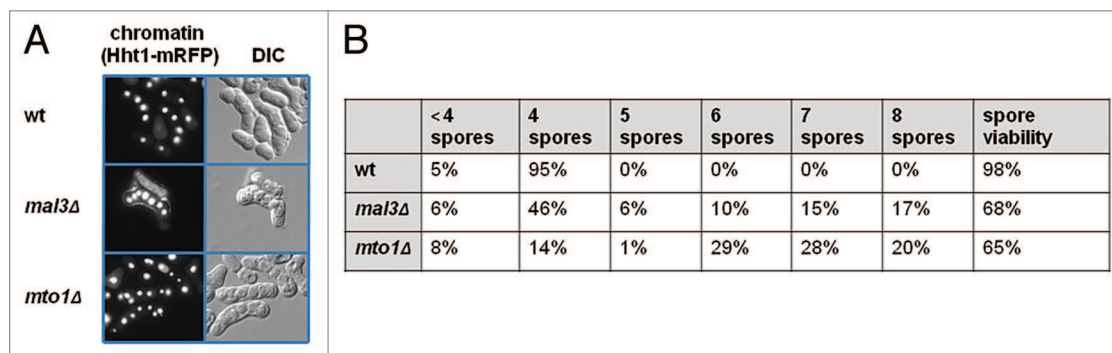

	<4 spores	4 spores	5 spores	6 spores	7 spores	8 spores	spore viability
wt	5%	95%	0%	0%	0%	0%	98%
mal3Δ	6%	46%	6%	10%	15%	17%	68%
mto1Δ	8%	14%	1%	29%	28%	20%	65%

Figure 1. Mal3 and Mto1 are important for the formation of normal 4-spored asci. (**A**) Spore morphology was analyzed in a wild-type *h90* strain (wt) (JG16917) and in *h90* strains carrying the knockout allele of either *mal3* (*mal3Δ*) (JG16923) or *mto1* (*mto1Δ*) (JG17037). Cells expressing Hht1-mRFP were used to allow visualization of chromatin. Cells were sporulated on EMM2-NH₄Cl plates and examined by fluorescence microscopy. (**B**) A wild-type *h90* strain (wt) (JG12618) and *h90* strains carrying the knockout allele of either *mal3* (*mal3Δ*) (JG17118) or *mto1* (*mto1Δ*) (JG17043) were sporulated on EMM2-NH₄Cl plates and the number of spores was determined in at least 100 asci. In addition, spore viability was determined by random spore analysis. Data shown are the means of 3 independent experiments.

chiasmata, which link homologous chromosomes. Microtubule-dependent movement of chromosomes during meiotic prophase (known as horsetail movement) is known to be required for normal levels of meiotic recombination and chiasma formation.[24-26] Live-cell imaging of *mal3Δ* cells showed that horsetail movement was impaired in all 30 zygotes examined (**Fig. 2** and data not shown). We observed a similar defect in *mto1Δ* mutant cells, where all 10 examined zygotes showed impaired horsetail movement (**Fig. 2**

and data not shown). We also observed a reduced number of horsetail nuclei in fixed cells (**Table 1**). It is unlikely that the observed defect in horsetail movement is due to the absence of karyogamy, because we also observed impaired horsetail movement in *mal3Δ* and *mto1Δ* mutant zygotes that underwent successful karyogamy (data not shown). Moreover, analysis of *tht1Δ* mutant zygotes provides additional evidence that horsetail movement can occur in the absence of karyogamy (**Fig. 2B**).[23]
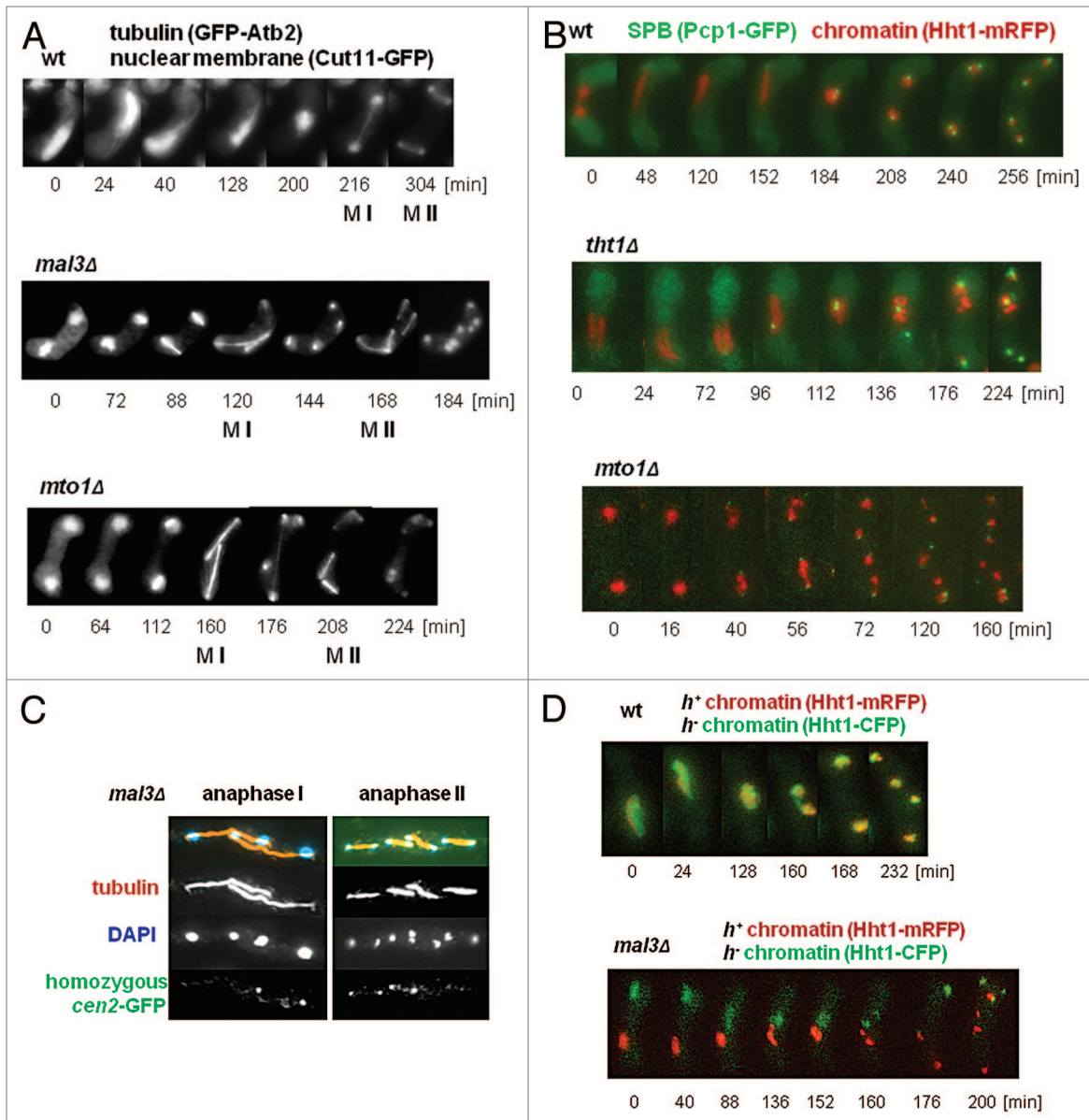

Figure 2. Mal3 and Mto1 are required for karyogamy. (**A**) A wild-type *h⁹⁰* strain (wt) (JG16918) and *h⁹⁰* strains carrying the knockout allele of either *mal3* (*mal3Δ*) (JG16922) or *mto1* (*mto1Δ*) (JG17040) were plated on EMM2-NH₄Cl plates and analyzed by live-cell imaging. Tubulin and the nuclear membrane were visualized using GFP-Atb2 and Cut11-GFP, respectively. Numbers below the images represent elapsed time in minutes. Meiosis I (MI) and meiosis II (MII) are indicated. (**B**) A wild-type *h⁹⁰* strain (wt) (JG16917) and *h⁹⁰* strains carrying the knockout allele of either *tht1* (*tht1Δ*) (JG17039) or *mto1* (*mto1Δ*) (JG17037) were plated on EMM2-NH₄Cl plates and analyzed by live-cell imaging. The spindle pole body (SPB) and chromatin were visualized using Pcp1-GFP and Hht1-mRFP, respectively. Numbers below the images represent elapsed time, in minutes. (**C**) *h⁹⁰* cells carrying *cen2*-GFP and the knockout allele of *mal3* (*mal3Δ*) (JG17118) were sporulated on EMM2-NH₄Cl plates, fixed and immunostained for tubulin and GFP. DNA was visualized by DAPI staining. (**D**) The wild-type *h⁻* strain expressing Hht1-CFP (JG17004) was crossed to the *h⁺* strain expressing Hht1-mRFP (JG17000) (wt). An *h⁻* strain expressing Hht1-CFP and carrying the knockout allele of *mal3* (JG16998) was crossed to an *h⁺* strain expressing Hht1-mRFP and carrying the knockout allele of *mal3* (JG16997) (*mal3Δ*). Cells were plated on EMM2-NH₄Cl plates and analyzed by live-cell imaging. Numbers below the images represent elapsed time, in minutes.

We also observed *mal3Δ* and *mto1Δ* mutant zygotes in which 2 spindles overlapped and thus destroyed the linear order of nuclei in the ascus (**Fig. 2A and C**). A similar phenomenon has been previously observed in *spo4Δ* and *spo6Δ* mutant zygotes where abnormally elongated anaphase II spindles overlapped.[27] However, reductional segregation of homozygous *cen2*-GFP suggested that the overlapping spindles in *mal3Δ* zygotes were anaphase I spindles (**Fig. 2C** and data not shown).

Taken together, we show that Mal3 is required for oscillatory nuclear movement during meiotic prophase and also for proper segregation of homologous centromeres during meiosis I.

Discussion

Systematic genetic screens using genome-wide gene deletion collections provide a powerful tool for identifying key regulators

Table 1. The frequency of horsetail nuclei is reduced in *mal3Δ* and *mto1Δ* mutant zygotes

	Horsetail shape of the nucleus	Regular shape of the nucleus
wt (JG12618) - 8.5 h	76%	24%
mal3Δ (JG17118) - 8.5 h	14%	86%
mto1Δ (JG17043) - 8.5 h	30%	70%
wt (JG12618) - 9.5 h	62%	38%
mal3Δ (JG17118) - 9.5 h	20%	80%
mto1Δ (JG17043) - 9.5 h	40%	60%

Nuclear morphology was scored in at least 100 uninuclear zygotes. The indicated strains were sporulated on EMM2-NH$_4$Cl plates for 8.5 or 9.5 h, stained with DAPI and examined under the fluorescence microscope.
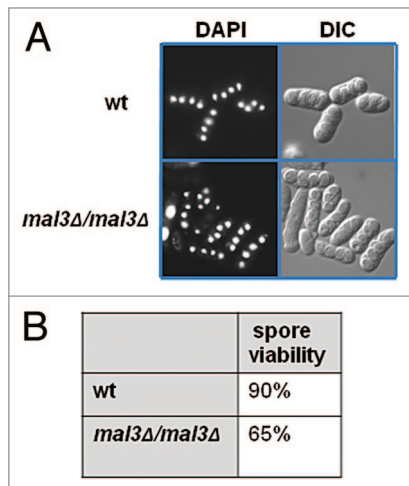

Figure 3. Azygotic meiosis restores normal numbers of spores but does not fully restore spore viability in *mal3Δ* mutant asci. (**A**) Wild-type *mal3+/mal3+* (wt) (JG17122) or *mal3Δ/mal3Δ* (JG17120) diploid cells were sporulated on EMM2-NH$_4$Cl plates, stained with DAPI and examined by fluorescence microscopy. (**B**) Strains as indicated in (**A**) were sporulated on EMM2-NH$_4$Cl plates and spore viability was examined by random spore analysis. Data shown are the means of 3 independent experiments.

of various cellular processes, including chromosome segregation.[28] The fission yeast *S. pombe* is an important model organism sharing many features with higher eukaryotes. The availability of the *S. pombe* deletion-strain collection allowed us to screen for mutants defective in chromosome segregation during meiosis. In our current study, we report that the deletion of 2 genes, *mal3* and *mto1*, leads to the production of asci containing up to 8 spores. We observed that in the absence of Mal3, meiosis frequently initiates before the completion of karyogamy, thus producing up to 8 nuclei in a single ascus. In addition to karyogamy, Mal3 is required for horsetail nuclear movement and proper segregation of homologous centromeres during meiosis.

We speculate that the phenotype observed in *mal3Δ* mutant zygotes is due to a defect in cytoplasmic microtubules, which are known to be required for several processes, including nuclear migration during karyogamy and horsetail nuclear movement.[24,29] Indeed, whereas microtubules are prominent during karyogamy and meiotic prophase in wild-type zygotes, we rarely observed microtubules in *mal3Δ* mutant zygotes prior to meiotic divisions (**Fig. 2A** and data not shown). Moreover, a recent study showed that treatment with anti-microtubule drugs mimics the *mal3Δ* mutant phenotype.[15] Interestingly, we noticed that in some *mal3Δ* zygotes nuclear congression was completed, but fusion of nuclei did not occur (data not shown), raising the possibility that Mal3 is required for both nuclear congression and fusion. Similarly to Mal3, Mto1 is also known to be required for proper microtubule function.[18,19] This raises the possibility that the meiotic phenotypes
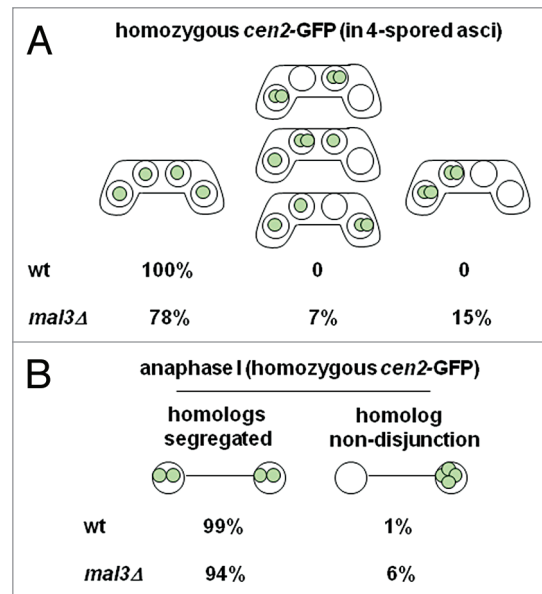

Figure 4. Mal3 is required for proper segregation of chromosomes during meiosis. (**A**) Meiotic segregation of chromosome II was scored in a wild-type *h90 cen2*-GFP strain (wt) (JG12618) and in an *h90 cen2*-GFP strain carrying the knockout allele of *mal3* (*mal3Δ*) (JG17118). Cells were sporulated on EMM2-NH$_4$Cl plates, stained with DAPI and examined by fluorescence microscopy. Chromosome segregation was scored in at least 100 asci. (**B**) Strains as described in (**A**) were fixed and immunostained for tubulin and GFP. DNA was visualized by DAPI staining. 100 anaphase I cells were examined by fluorescence microscopy, and segregation of chromosome II marked by *cen2*-GFP was scored.

observed in *mto1Δ* and *mal3Δ* zygotes are due to similar defects. However, further experiments are required to understand the role of Mal3 and Mto1 during meiosis.

Our current study contributes to the understanding of how regulators of microtubule function ensure proper segregation of chromosomes during meiosis. Moreover, we believe that in the long run, greater knowledge of the processes that promote proper segregation of chromosomes during meiosis might help us to understand the origins of human meiotic aneuploidy, the leading cause of miscarriages and various genetic disorders.[30]

Materials and Methods

Strains, growth media and general methods

The genotypes of the yeast strains used in this study are listed in **Table 2**. Standard media (YES, EMM) were used to maintain and grow the *S. pombe* strains.[31-33] To induce mating and meiosis, cells were grown in liquid YES to mid-log phase at 32 °C, washed 3 times with water, transferred to EMM2-NH$_4$Cl plates, and incubated at 25 °C.[4] The transformation of *S. pombe* was performed using the lithium acetate method as previously described.[34] The *S. pombe* genes were deleted as described previously.[34] The immunostaining and microscopy used to analyze chromosome segregation in *S. pombe* zygotes were performed as described in Rabitsch et al.[35]

To determine spore viability, an appropriate number of spores (~100 000), obtained by overnight digestion of asci with

β-glucuronidase (Sigma-Aldrich), was plated onto YES plates. Germinating and non-germinating spores were counted under the microscope after 30 h of incubation at 32 °C.[36] It is possible that a small fraction of abnormal spores has not been included in our random spore analysis, leading to an overestimation of spore viability in the *mto1Δ* and *mal3Δ* mutant strains.

Live-cell imaging

Live-cell imaging was performed according to the protocol of Kovacikova et al.,[27] with minor modifications. To induce meiosis, cells were grown in liquid YES to mid-log phase at 32 °C, washed 3 times with water and plated on EMM2-NH$_4$Cl plates. After incubation at 25 °C for 10 h (*h^{90}* strains) or 14 h (*h$^+$ × h$^-$* cross), the cells were resuspended in 1 ml of liquid EMM2-NH$_4$Cl, cells were sonicated (Sonoplus HD2070, Bandelin, 3 cycles, 20 s/cycle, power 40%), and 200 μl of cell suspension was transferred to a glass-bottom microtiter plate (MatTek, Ashland) coated with 2 μl of 2 mg/ml lectin BS-1 (Sigma-Aldrich). After a 5 min incubation, the cell suspension was removed, and 3 ml of liquid EMM2-NH$_4$Cl media was added into the microtiter plate. Live-cell fluorescence microscopy was performed at 25 °C using an Olympus Cell R microscope equipped with an Olympus MT-20 150W mercury arc burner, a 100 W Halogen Lamp, a Hamamatsu ORCA-ER camera, and a 60×/1.42 PlanApoN oil-immersion objective. eGFP, eCFP, and dsRED filters were used for imaging. Time-lapse images of cells were taken at 8 min intervals with seven optical *z*-sections, with a 1-μm *z* distance.

Table 2. Strains used in this study

Strain	Genotype
JG12618	*h^{90} ade6-M216 leu1-32 lys1-131 ura4-D18 cen2 (D107)::KanR-ura4$^+$-lacO his7$^+$::lacI-GFP*
JG17118	*h^{90} ade6-M216 leu1-32 lys1-131 ura4-D18 cen2 (D107)::KanR-ura4$^+$-lacO his7$^+$::lacI-GFP mal3::natMX4*
JG17043	*h^{90} ade6-M216 leu1-32 lys1-131 ura4-D18 cen2 (D107)::KanR-ura4$^+$-lacO his7$^+$::lacI-GFP mto1::natMX4*
JG16918	*h^{90} leu1-32 ura4-D18 his3-D1 lys1$^+$::Pnmt1-GFP-atb2 hht1-CFP-kanMX6 sid4-mRFP-kanMX6::leu1$^+$ cut11-3xPk-GFP-ura4$^+$*
JG17040	*h^{90} leu1-32 ura4-D18 his3-D1 lys1$^+$::Pnmt1-GFP-atb2 hht1-CFP-kanMX6 sid4-mRFP-kanMX6::leu1$^+$ cut11-3xPk-GFP-ura4$^+$ mto1::natMX4*
JG16922	*h^{90} leu1-32 ura4-D18 his3-D1 lys1$^+$::Pnmt1-GFP-atb2 hht1-CFP-kanMX6 sid4-mRFP-kanMX6::leu1$^+$ cut11-3xPk-GFP-ura4$^+$ mal3::natMX4*
JG16917	*h^{90} ade6-M210 leu1-32 ura4-D18 hht1-mRFP-his3$^+$MX6 pcp1-GFP-kanMX6::leu1$^+$*
JG16923	*h^{90} ade6-M210 leu1-32 ura4-D18 hht1-mRFP-his3$^+$MX6 pcp1-GFP-kanMX6::leu1$^+$ mal3::natMX4*
JG17037	*h^{90} ade6-M210 leu1-32 ura4-D18 hht1-mRFP-his3$^+$MX6 pcp1-GFP-kanMX6::leu1$^+$ mto1::natMX4*
JG17039	*h^{90} ade6-M210 leu1-32 ura4-D18 hht1-mRFP-his3$^+$MX6 pcp1-GFP-kanMX6::leu1$^+$ tht1::natMX4*
JG16998	*h$^-$ leu1-32 ura4-D18 his3-D1 hht1-CFP-kanMX6 mal3::natMX4*
JG16997	*h$^+$ ade6-M210 leu1-32 ura4-D18 hht1-mRFP-his3$^+$MX6 pcp1-GFP-kanMX6::leu1$^+$ mal3::natMX4*
JG17004	*h$^-$ leu1-32 ura4-D18 his3-D1 hht1-CFP-kanMX6*
JG17000	*h$^+$ ade6-M210 leu1-32 ura4-D18 hht1-mRFP-his3$^+$MX6 pcp1-GFP-kanMX6::leu1$^+$*
JG11318	*h$^-$ leu1-32 ura4-D18 ade6-M210*
JG16990	*h$^-$ leu1-32 ura4-D18 ade6-M210 mal3::natMX4*
JG17134	*h$^+$ ade6-M216 ura4-D18 leu1-32*
JG17135	*h$^+$ ade6-M216 ura4-D18 leu1-32 mal3::kanMX6*
JG17120	*h$^-$/h$^+$ leu1-32/ leu1-32 ura4-D18/ ura4-D18 ade6-M210/ ade6-M216 mal3::natMX4/ mal3::natMX4*
JG17122	*h$^-$/h$^+$ leu1-32/ leu1-32 ura4-D18/ ura4-D18 ade6-M210/ ade6-M216*
JG12017	*h$^-$ pat1-114 ade6-M210*
JG17045	*h$^-$ pat1-114 ade6-M210 mal3::natMX4*

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/26815

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