Mcp4, a Meiotic Coiled-Coil Protein, Plays a Role in F-Actin Positioning during Schizosaccharomyces pombe Meiosis

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Some meiosis-specific proteins of Schizosaccharomyces pombe harbor coiled-coil motifs and play essential roles in meiotic progression. Here we describe Mcp4, a novel meiosis-specific protein whose expression is abruptly induced at the horsetail phase and which remains expressed until sporulation is finished. Fluorescence microscopic analysis revealed that Mcp4 alters its subcellular localization during meiosis in a manner that partially resembles the movement of F-actin during meiosis. Mcp4 and F-actin never colocalize; rather, they are located in a side-by-side manner. When forespore membrane formation begins at metaphase II, the Mcp4 signals assemble at the lagging face of the dividing nuclei. At this stage, they are sandwiched between F-actin and the nucleus. Mcp4, in turn, appears to sandwich F-actin with Meu14. In mc4pΔ cells at anaphase II, the F-actin, which is normally dumbbell-shaped, adopts an abnormal balloon shape. Spores of mc4pΔ cells were sensitive to NaCl, although their shape and viability were normal. Taken together, we conclude that Mcp4 plays a role in the accurate positioning of F-actin during S. pombe meiosis.

The generation of heritable haploid gametes from diploid parental cells requires meiosis. When fission yeast (Schizosaccharomyces pombe) cells are starved by nitrogen deprivation, two haploid cells with opposite mating types conjugate, after which their nuclei fuse together and then undergo meiosis. This begins with a single round of DNA replication, after which the nucleus begins to oscillate between the cell poles and quickly acquires an elongated shape called the horsetail. At this stage, homologous chromosome pairing and genetic recombination occur. The cells then undergo two rounds of continuous cell division, called meiosis I and meiosis II, after which they start to form the double-layer forespore membrane (FSM) (29). Subsequently, during sporulation, the FSM grows into spore walls. Thus, one cell produces one ascus containing four spores. The spores then await appropriate environmental conditions before starting vegetative growth.

The FSM encapsulates the haploid nuclei and serves as the scaffold for spore wall materials. The formation of FSMs starts on the cytoplasmic side of the spindle pole body (SPB) and goes on to wrap each divided nucleus along with cellular organelles by fusing with membranous vesicles derived from the endoplasmic reticulum (ER) via the Golgi apparatus (15). The spore walls are formed by sorting the cell wall components to the luminal space between the outer and inner layers of the FSMs. After the spore wall is successfully organized, mature spores are released from the ascus by autolysis of the asc cell wall. The movement of the nascent FSM has been determined by studying the behavior of the green fluorescent protein (GFP)-tagged FSM protein Psy1 under a microscope (15). GFP-Psy1 localizes to the plasma membrane before meiosis I and then suddenly disappears before reappearing at the cytoplasmic face of the SPB at metaphase II. Subsequently, it surrounds each of the nuclei. By the time the FSM is closed, GFP-Psy1 surrounds each haploid nucleus. S. pombe spores, which resume vegetative growth when appropriate nutrients are supplied, are more resistant to organic solvents and freezing than are mitotic cells (28). This process, called germination, might correspond to the transition from the quiescent G0 phase to active proliferation in higher eukaryotes. Germinated spores grow out initially by cell expansion, followed by unidirectional cell extension. Cortical actin patches are randomly distributed in the early stage of outgrowth and then localize to one side of spores before the formation of projections (4).

F-actin plays an essential role in the life of S. pombe (19), and its subcellular movement during meiosis has been analyzed in detail (7, 20). Briefly, after meiosis is induced by cell fusion and the cells enter the horsetail phase, F-actin appears as randomly scattered dots. These dots remain scattered during meiosis I, but when the cells proceed to prometaphase or metaphase of meiosis II, they accumulate around the two nuclei. Subsequently, during anaphase II, when the two nuclei both divide in two, F-actin is detected at the extending rim of the cup-shaped FSM. This region of the FSM has been designated the “leading edge” of the FSM (18), where Meu14 and F-actin are partly colocalized (7). By early anaphase II, F-actin is also detected on the opposite side of the nucleus, in the vicinity of the SPB. Finally, in the spores of the mature ascus, F-actin again adopts a scattered localization. During sporulation in budding yeast, i.e., Saccharomyces cerevisiae, actin is essential for maturation of the spore wall but not for the assembly, shaping, and closure of the prosopore membrane, which corresponds approximately to the fission yeast FSM (31).

We previously reported that the meiosis-specific S. pombe proteins Meu13 (14) and Meu14 (18) harbor coiled-coil motifs.
Meu13 plays a pivotal role in homologous pairing and meiotic recombination at meiosis I as well as in the meiotic recombination checkpoint (27). Meu14 localizes to the leading edge of the FSM and is essential for accurate FSM formation. Another protein known to regulate spore formation in S. pombe, namely, Spol15 (6), also contains coiled-coil regions. The coiled-coil motif is important for protein-protein interactions (13). These observations suggested to us that other novel meiosis-specific proteins that play key roles in meiosis by interacting with each other could be identified by screening for coiled-coil proteins whose expression is specifically induced during S. pombe meiosis. Indeed, our comprehensive screening yielded a number of novel meiotic coiled-coil protein (Mcp)-encoding genes (21). We have subsequently shown that one of these, Mcp7, associates with Meu13 and is required for meiotic recombination in S. pombe (21). In addition, Mcp6/Hrs1 localizes at the SPB and is needed for establishing the proper astral microtubule positioning that maintains the horsetail movement of the nucleus (22, 30). Mcp5, the homolog of the budding yeast dynein anchor Num1, localizes to the cell cortex and functions as a dynein anchor that facilitates horsetail movement (23, 32). Here we describe the role that Mcp4 plays in S. pombe meiosis. Our studies show that it regulates the proper positioning of F-actin during FSM formation.

**TABLE 1. Strains used in this study**

| Strain | Genotype | Source |
|--------|----------|--------|
| CD16-1 | h<sup>+</sup> h<sup>-</sup> ade6-M210/ade6-M216 cyh1<sup>+/+</sup>;lys5-391 | C. Shimoda |
| CD16-5 | h<sup>-</sup> ade6-M210/ade6-M216 cyh1<sup>+/+</sup>;lys5-391 | C. Shimoda |
| TP4-5A | h<sup>-</sup> ade6-M210 leu1-32 ura4-D18 | C. Shimoda |
| TP4-1D | h<sup>-</sup> ade6-M216 his2 leu1-32 ura4-D18 | C. Shimoda |
| AO24 | h<sup>-</sup> ade6-M216 leu1-32 ura4-D18 mcpc4<sup>-</sup> | This study |
| AO28 | h<sup>-</sup> ade6-M216 his2 leu1-32 ura4-D18 mcpc4<sup>-</sup>/ur4<sup>-</sup> | This study |
| AO89 | h<sup>-</sup> ade6-M216/ade6-M216 ura4-D18 ura4-D18 leu1-32/leu1-32 pat1-114/pat1-114 mcpc<sup>+</sup>/mcpc<sup>-</sup> | This study |
| AO64 | h<sup>+</sup> ade6-M210 leu1-32 ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 | This study |
| AO00 | h<sup>+</sup> leu1-32 prRTG41-mcpc<sup>+</sup> | This study |
| YN68 | h<sup>+</sup> leu1: gfp-psyl<sup>-</sup>; leu1<sup>-</sup> | C. Shimoda |
| AO185 | h<sup>+</sup> ade6-M210 leu1-32 ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 meu14<sup>-</sup>;ura4<sup>-</sup> | This study |
| AO187 | h<sup>+</sup> ade6-M210 leu1-32 ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 meu14<sup>-</sup>;ura4<sup>-</sup> | This study |
| AO192 | h<sup>+</sup> ade6-M210 leu1-32 ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 meu14<sup>-</sup>;ura4<sup>-</sup> | This study |
| AO189 | h<sup>+</sup> leu1-32 ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 prRTG41-mcpc<sup>+</sup> | This study |
| AO190 | h<sup>+</sup> leu1-32 ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 prRTG41-mcpc<sup>+</sup>ΔC | This study |
| AO125 | h<sup>+</sup> leu1: gfp-psyl<sup>-</sup> ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 | This study |
| AO181 | h<sup>+</sup> leu1-32 prRTG41-mcpc4ΔC | This study |
| FY13596<sup>+</sup> | h<sup>+</sup> cmt1<sup>-</sup> gfp::kan<sup>-</sup> ade6-M210 leu1-32 | YGRC/NBRP |
| AO168 | h<sup>+</sup> ade6-M210 leu1-32 prRep1<sup>-</sup>-1<sup>-</sup>c<sup>-</sup> ura4-D18 prRTG2-mcpc<sup>4</sup>ΔC | This study |
| AO165 | h<sup>+</sup> ade6-M216 leu1-32 his2 ura4-D18 his3 cdc12 mcpc4<sup>-</sup>/[^3]ura4-D18 | This study |
| AO162 | h<sup>+</sup> ade6-M210 ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 | This study |
| AO194 | h<sup>+</sup> ade6-M210 leu1-32 ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 prRep1<sup>-</sup>-gfp-psyl<sup>-</sup> | This study |
| AO196 | h<sup>+</sup> ade6-M210 leu1-32 ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 spn6<sup>-</sup> gfp::kan<sup>-</sup> | This study |
| AO200 | h<sup>+</sup> ade6-M210 ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 cut15<sup>-</sup> gfp::ura4<sup>-</sup>/URA2 | This study |
| ST272 | h<sup>+</sup> ade6-M210 his2 leu1-32 ura4-D18 his3 cdc12 | Our stock |
| NP16-6B | h<sup>+</sup> ade6-M216 ura4-D18 | Our stock |
| AO54 | h<sup>+</sup> ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 | This study |
| AO122 | h<sup>+</sup> his2 leu1-32 ura4-D18 his3 cdc12 mcpc4<sup>-</sup>/[^3]ura4-D18 | This study |
| MS105-1B | h<sup>+</sup> ade6-M26 ura4-D18 | Our stock |
| MS111w1 | h<sup>+</sup> ade6-M26 ura4-D18 leu1-32 his2 | Our stock |
| T81-1 | h<sup>+</sup> ura4<sup>-</sup> | Our stock |
| NP32-2A | h<sup>+</sup> leu1-32 his2 ura4-D18 | Our stock |
| AO127 | h<sup>+</sup> ade6-M26 ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 | This study |
| AO128 | h<sup>+</sup> his2 ade6-M26 ura4-D18 mcpc4<sup>-</sup>/[^3]ura4-D18 | This study |
| IZ670 | h<sup>+</sup> ade6-M210/ade6-M216 ura4-D18 ura4<sup>-</sup>/pat1-114/pat1-114 | M. Yamamoto |
| AO115 | h<sup>+</sup> ade6-M210/ade6-M216 ura4-D18 ura4<sup>-</sup>/pat1-114/pat1-114 mcpc4<sup>-</sup>/[^3]ura4-D18 | This study |
| AO356 | h<sup>+</sup> ade6-M210/ade6-M216 ura4-D18 ura4<sup>-</sup>/pat1-114/pat1-114 mcpc4<sup>-</sup>/[^3]ura4-D18 | This study |

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**MATERIALS AND METHODS**

**Yeast strains, media, and molecular biology.** The S. pombe strains used in this study are listed in Table 1. The complete media yeast extract-peptone-dextrose (YPD) and yeast extract (YE), synthetic Edinburgh minimal medium 2 (EMM2), and the sporulation media malt extract (ME) and EMM2-nitrogen (EMM2-N) were used. Induction of synchronous meiosis was assessed as described previously (27). We used the high-copy-number plasmid prRTG41 driven by its nmt41 promoter for overproduction experiments (18).

**Gene disruption of mcpc4**. We disrupted the mcpc4<sup>-</sup> gene by replacing it with the ura4<sup>-</sup> cassette according to a previously described method (22). Briefly, we made two pBSK-GD-Mcp4 primers consisting of 100 bp from either end of the mcpc4<sup>-</sup> gene; each primer was also flanked with 20 bp of the ura4<sup>-</sup> containing pBluescript II KS<sup>-</sup> vector sequence, as follows: forward primer, 5'<sup>-</sup>TCAAGG CAGGCTTAAAAAGATGATATATAAACTTCAAGAAGAACGGA GTTAAATATTATTTAAATCTGATCCTGGGATTTAGATCTTCTATA ATGGCAAGAATGCGATAAAG-3<sup>+</sup>; and reverse primer, 5'<sup>-</sup>ATGCTTGAA CAGGACCTTTAAAACATTTTATCCCAAATATAGGATGACCTCA AATCGGAGAATTTGGGAAAAATTTAATTAGAAATACAAATATA Δ GTCGACGCGTATGCGAGAAG-3<sup>+</sup>. The underlined sequences belong to the pBluescript II KS<sup>-</sup> vector. The primers generated a 2.0-kb PCR product.
containing the urad4 cassette. This product was introduced into the haploid strains TP4A and TP4-1D, and the Ura+ transformants were screened by PCR analysis to identify the mcp4-urad4 strain. All of the mutants that were isolated were backcrossed three times with the wild-type strains.

**Construction of Mcp4-3HA-expressing strain.** To prepare the Mcp4-3HA construct, we performed PCR and obtained a DNA fragment carrying the open reading frame (ORF) and 3′ downstream region of the mcp4+ gene. The following primers were used to obtain the Mcp4 ORF: mcp4 ORF-F (5′-GGAGCCCTGTGAAGACAAATG-3′) and mcp4 ORF-R (5′-GCCGCCGCG CGTTGCGTTCATTTACGAGAG-3′). The underlined sequences denote the artificially introduced restriction enzyme sites for NdeI and NotI, respectively. The following primers were used to obtain the 3′ downstream region: mcp4 3UTR-F (5′-GCCGCCGCGTTTGTATTTCATTAATTTAC-3′) and mcp4 3UTR-R (5′-GCACTACTTACGACCTCTAGTTTGAAT-3′). The underlined sequences denote the artificially introduced restriction enzyme sites for Smal and SacI, respectively. The 3′ downstream region was inserted into the 3HA-containing pREP vector via Smal-SacI, cut out by NotI-EcoRI, and then inserted into the pT7 BlueT vector containing the Mcp4 ORF. The construct was then cut out by EcoRI-SpeI and inserted into the urad4-1 containing pBlueScript II KS(+) vector, which was subsequently digested with PstI and introduced into the haploid strain TP4-1D. The Ura+ transformants were then screened by PCR.

**Fluorescence microscopic observation and immunofluorescence.** Fluorescence microscopic observations were performed as described previously (22). Cells were cultured in 10 ml EMM2 with supplements until they reached mid-log phase at 28°C. The cells were collected by centrifugation, washed three times with 1 ml EMM2-N, and induced to enter meiosis by incubation in EMM2-N at 28°C for 10 h. For immunofluorescence experiments, meiotic cells were fixed after the procedure, using glutaraldehyde. For glutaraldehyde fixation, 10 ml of this was added to a 1/6 volume of prewarmed 30% electron microscope (EM)-grade formaldehyde and fixed for 1 h at 28°C. The cells were then washed three times in one culture volume of 0.1 M Na-PIPES, pH 6.8, 1 mM croscopy (EM)-grade formaldehyde and fixed for 1 ha t 28°C. The cells were then cultures were added directly to a 1/6 volume of prewarmed 30% electron microscopy (EM)-grade formaldehyde and fixed for 1 h at 28°C. The cells were then washed three times in one culture volume of 0.1 M Na-PIPES, pH 6.8, 1 mM croscopy (EM)-grade formaldehyde and fixed for 1 h at 28°C. The cells were then observed using a fluorescence microscope (BX51; Olympus) with a charge-coupled device (CCD) camera System. Images were acquired using Photoshop 7.0 (Adobe). The Golgi complex/endosome morphology of fission yeast cells was visualized under a fluorescence microscope. Mcp4-3HA expression did not impair meiotic progression and spore formation, and no more than 1 spore per 300 cells was observed. In other words, the Mcp4-3HA protein tagged with three copies of the HA epitope was visualized with the anti-HA antibody (Boehringer Mannheim, Germany). Subsequently, we added an Alexa 488-conjugated goat anti-rabbit antibody (Invitrogen) for TAT1, an Alexa 488-conjugated goat anti-rabbit antibody (Invitrogen) for the anti-Sad1 antibody, and an Alexa 594-conjugated goat anti-rat immunoglobulin G (Molecular Probes Inc.) for the anti-HA antibody. The samples were then stained with 0.2 mg/ml Hoechst 33342 in phosphate-buffered saline (PBS; 150 mM NaCl, 40 mM HEPES, 500 mM K2HPO4, and 10 mM KH2PO4) for 1 min and mounted with anti-fade mounting medium containing p-phenylenediamine. Fluorescence images of these cells were observed using a fluorescence microscope (BX51; Olympus) with a charge-coupled device (CCD) camera System. Images were acquired using Photoshop 7.0 (Adobe). The Golgi complex/endosome and vacuoles of fission yeast wild-type and mutant cells were labeled with FM4-64 (Molecular Probes Inc.) (see below).

**FM4-64 and rhodamine-phalloidin staining.** To visualize the fission yeast vacuole, the cells were labeled with the lipophilic dye FM4-64 according to the method described by Kitaj et al. (9), with some modifications. Briefly, the cells were grown to the exponential phase in EMM-N at 28°C, after which the two cell populations were harvested by centrifugation at 4°C and resuspended in ice-cold EMM or EMM-N. FM4-64 was added to a final concentration of 80 μM, and the cells were incubated at room temperature for 15 min. Thereafter, the cells were harvested by centrifugation at 700 × g for 3 min at 4°C, washed with resuspension in ice-cold fresh EMM or EMM-N to remove free FM4-64, and incubated at room temperature. The cells were then harvested after 5 min to visualize the Golgi complex/endosomes or after 10 min to visualize the vacuoles, washed with ice-cold PBS, and immediately examined under a fluorescence microscope.

To visualize F-actin, rhodamine-phalloidin staining was performed by using the method of Sawin and Nurse (26), with some modifications. Briefly, growing cultures were added directly to a 1/6 volume of prewarmed 30% electron microscopy (EM)-grade formaldehyde and fixed for 1 h at 28°C. The cells were then washed three times in one culture volume of 0.1 M Na-PIPES, pH 6.8, 1 mM EGTA, and 1 mM MgCl2 (PEM), extracted for 30 min with PEM-1% Triton X-100, and washed three additional times with PEM. Three hundred units of rhodamine-phalloidin (Molecular Probes) was resuspended in 1.5 ml methanol, divided into 15-μl aliquots, evaporated in a Speed-Vac machine, and stored at −20°C. For staining, one aliquot was resuspended in 50 μl PEM, and 7 μl of this was added to no more than 1 μl of fixed, extracted cell pellet. For mounting, 0.5 μl of stained cells was spotted onto a glass slide, followed by 2.5 μl of PEM mounting medium containing 1 mg/ml P-phenylenediamine as an antifade agent.

**Sensitivity to NaCl.** Homothallic haploid cells were grown on yeast extract-peptone-dextrose plates at 33°C, and the cells were mated and sporulated on ME plates at 28°C for 4 days. At the end of the culture, the ascal walls were spontaneously dissolved, and single spores were liberated. The spores were separated on YE plus histidine (YEHI) agar plates, with or without 0.1 M or 0.17 M NaCl, by use of a micromanipulator (Singer Instruments, Somerset, United Kingdom). The plates were incubated at 30°C for 5 days, after which NaCl sensitivity was calculated.

**RESULTS**

**mcp4+** is a meiosis-specific gene that generates a coiled-coil protein. **mcp4+** is one of the seven genes encoding meiosis-specific coiled-coil proteins that we isolated by our screening analysis, as reported previously (21). Mcp4 consists of 355 amino acids and harbors a putative coiled-coil motif close to its C terminus (Fig. 1A). Homology searches using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) failed to identify orthologues in other organisms, which indicates that Mcp4 is specific to S. pombe.

We first investigated the meiotic expression pattern of mcp4+ by Northern blot analysis, using RNAs obtained from CD16-1 (h+/h−) and CD16-5 (h+/h+) cells harvested at various times after commencement of nitrogen starvation. CD16-1 cells undergo meiosis upon nitrogen starvation, unlike CD16-5 cells. This analysis revealed that mcp4+ displays meiosis-specific transcription that starts just before the nuclear division in meiosis I (Fig. 1B). This result confirms a previous report showing that mcp4+ transcription is elevated several fold during the meiosis I nuclear division (11).

To accurately express the mcp4+ protein during meiosis, we constructed the mcp4+-3ha strain, which expresses the Mcp4 protein tagged with three copies of the HA epitope at its C-terminal end. To obtain synchronized meiotic progression, we replaced the mcp4+ gene of the pat1-114 strain with the mcp4+-3ha fusion gene. The pat1-114 mcp4+-3ha diploid cells were then induced to enter synchronized meiosis by a temperature shift, and their lysates were subjected to Western blot analysis using the anti-HA antibody as the probe. We first confirmed that the meiotic progression and spore morphology of pat1-114 mcp4+-3ha and pat1-114 diploid cells were similar (data not shown). Western blot analysis showed that the Mcp4-3HA protein migrated at the expected size and was expressed only during meiosis, from 2.5 to 8 h after the temperature shift (Fig. 1C). In other words, the Mcp4-3HA protein was first observed at the horsetail phase and remained detectable until sporulation occurred. This timing of Mcp4 expression is considered to be more accurate than the result obtained by Northern blotting using the CD16-1 strain (Fig. 1B) because synchronized meiosis is accurate when the pat1-114 strain is used. This means that Mcp4 appears only 30 min after Meu13 becomes detectable. Meu13 is another meiosis-specific coiled-coil protein that regulates the meiotic recombination checkpoint (27) and plays a pivotal role in homologous pairing and meiotic recombination at meiosis I (14).

**Mcp4-3HA is detected as cytoplasmic dots during meiosis.** To examine the subcellular localization of the Mcp4 protein during meiosis, we utilized the homothallic haploid AO64 strain (h90 mcp4-3ha), which can be visualized under a fluorescence microscope. Mcp4-3HA expression did not impair meiotic progression.

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and spore formation (data not shown), which indicates that the tagged Mcp4 protein is fully functional. Cells carrying mcp4/H11001 were induced to enter meiosis and were fixed before Hoechst 33342, anti-HA antibody, and anti-Sad1 antibody staining (3), which identified the DNA, Mcp4-3HA, and the SPB, respectively. The stage of meiosis of the cells was determined by the number of nuclei and the morphology of the SPB. Typical images taken at each meiotic stage are shown in Fig. 2A.

The fluorescence signal from Mcp4-3HA was not detected in vegetative growing cells, which further confirmed that Mcp4 expression is meiosis specific (Fig. 2A, top panels). During meiosis (Fig. 2A, remaining panels), the Mcp4-3HA signals first appeared faintly in the cytoplasm at the horsetail phase. At metaphase I, these signals accumulated in the cytoplasm as randomly scattered dots. At anaphase I, the signals became concentrated into many bright dots located around each of the two nuclei. At metaphase II, which is when the SPB duplicates and the two nuclei move to prepare for meiosis II, the Mcp4 dots remained around the nuclei. At early anaphase II, some dots were separate from the DNA (chromatin), as the chro-
mesome shapes became deformed prior to nuclear division. This suggests that Mcp4 encircles the nuclear membrane but not the chromatin during anaphase I and metaphase II. At late anaphase II, the Mcp4 dots appeared to form a ring close to the perinucleus (see white arrowheads); the SPB is located on the opposite side of the nucleus. Thus, the Mcp4 ring and the SPB effectively sandwich the nucleus at late anaphase II. The Mcp4 ring is probably at the leading edge of the FSM. Upon
FIG. 3. Mcp4-GFP localizes side by side with F-actin during meiosis. (A) Microscopic analysis of Mcp4 and F-actin localization during meiosis. The homothallic haploid strain AO192 (h<sup>90</sup> mcp4<sup>-3ha</sup> crn1<sup>-gfp</sup>), which expresses Crn1-GFP, was induced to enter meiosis, after which the cells were chemically fixed and stained with Hoechst 33342 and anti-HA antibody to detect DNA (blue) and Mcp4-3HA (red), respectively. Crn1-GFP, which marks the F-actin complex, was visualized directly through its GFP-derived fluorescence. The merged images are shown schematically in the rightmost panels. (B) Enlarged view (left) and its schematic depiction (right) of the merged image at late anaphase II indicated by the white arrowhead in panel A. The localization of Meu14 that was revealed in Fig. 2C and D is also shown. (C) Microscopic analysis of the meiotic localization patterns of F-actin and ectopically expressed Mcp4-GFP. The homothallic haploid strain AO00, which bears the mcp4<sup>-gfp</sup> plasmid and expresses Mcp4-GFP under the control of the nmt<sup>41</sup> promoter, was cultured in EMM containing 1 μg/ml thiamine with supplements and then transferred to EMM without thiamine to induce the expression of Mcp4-GFP. Subsequently, 10 h after the first medium replacement, the cells were transferred to fresh EMM-N without thiamine to induce meiosis. Cells at various stages of meiosis were chemically fixed and stained with Hoechst 33342 and 4 μM rhodamine-phalloidin to visualize the DNA (blue) and F-actin (red), respectively. Mcp4-GFP is shown in green. The merged images are shown in the rightmost panels. (D) Mcp4-GFP localization is independent of F-actin polymerization. mcp<sup>Δ</sup> cells were transformed with
sporulation, the Mcp4 dots again loosely surrounded the nucleus. We confirmed that these cytoplasmic dots were not derived from the antibody background (see http://www.biken.osaka-u.ac.jp/lab/molgenet/Supplementary_data_EC.pdf). To confirm whether Mcp4 localizes at the leading edge of FSM, we examined the subcellular localization of Mcp4 with an FSM marker, GFP-Psy1. Indeed, Mcp4-3HA signals were detected at the leading edge of GFP-Psy1 signals, or the FSM (Fig. 2E).

To confirm this subcellular distribution of Mcp4 during meiosis, we immunostained tubulin in meiotic AO64 cells with the TAT1 antibody as well as visualizing Mcp4-3HA. This revealed that the Mcp4 dots accumulated at the junctions between the chromatin and tubulin at late anaphase II (Fig. 2B) (see http://www.biken.osaka-u.ac.jp/lab/molgenet/Supplementary_data_EC.pdf). We also compared the subcellular localization of Mcp4 with that of Meu14, which also forms a ring at the leading edge of the FSM (18). We found that Mcp4 is sandwiched between the nuclear chromatin and Meu14-GFP at late anaphase II (Fig. 2C). Notably, the late anaphase II image in Fig. 2C revealed a space between Mcp4 and Meu14. When the F-actin in cells carrying Meu14-GFP was stained with rhodamine-phalloidin in late anaphase II, it was found that this space was occupied by F-actin (Fig. 2D).

The detection of Mcp4 in the vicinity of the nucleus at metaphase II suggested that Mcp4 may localize in association with the nuclear membrane. Thus, we examined if Mcp4-3HA colocalizes with Cut15-GFP, which is known to exist in the nuclear membrane (12). We found that the two proteins displayed distinct localizations (see http://www.biken.osaka-u.ac.jp/lab/molgenet/Supplementary_data_EC.pdf), which indicates that the association of Mcp4 with the nuclear membrane, if any, is very weak. We also examined if Mcp4-3HA colocalizes with a septin homolog, Snp6-GFP, because the localization of budding yeast septin, an essential factor for sporulation, resembles that of Mcp4 (10). However, their localizations were different at both metaphase I and metaphase II (see http://www.biken.osaka-u.ac.jp/lab/molgenet/Supplementary_data_EC.pdf).

Mcp4-GFP localizes in the vicinity of F-actin. Since the subcellular localization of Mcp4 partially resembled that of F-actin (20), we examined whether Mcp4 colocalizes with F-actin during meiosis. For this purpose, we crossed the mcpc4+ strain with FY13596 to generate a strain that expresses Mcp4-GFP from the native promoter. However, we could not observe a fluorescence signal due to little expression of the fusion protein. Thus, we next prepared strain AO00, which carries a plasmid expressing Mcp4-GFP from the nmt41 promoter. This allowed us to visualize the fluorescent signal of Mcp4-GFP under a fluorescence microscope. These cells were induced to enter meiosis, after which they were fixed and stained for F-actin. As shown in Fig. 3C, the F-actin signals behaved very similarly to the Cn1-GFP signals shown in Fig. 3A. Briefly, at metaphase I and anaphase I, Mcp4-GFP and F-actin both occurred as nonoverlapping cytoplasmic dots, while at metaphase II, Mcp4-GFP was sandwiched by the nucleus on one side and F-actin on the other (red arrowheads). This sandwiching of Mcp4 became more apparent at anaphase II (white arrowhead). After sporulation, Mcp4-GFP and F-actin again appeared as nonoverlapping dots located loosely around the spore nuclei.

To investigate whether this subcellular localization of Mcp4 is dependent on the proper localization of F-actin, we treated AO00 with latrunculin A, which depolymerizes F-actin (8). Since polymerization of F-actin is required for normal spore formation, the addition of latrunculin A (50 μM) to the medium inhibits meiotic progression and causes abnormal spore formation (20). When Mcp4-GFP was observed in the absence (Fig. 3D, panel i) or presence (Fig. 3D, panel ii) of latrunculin A, Mcp4 rings were observed in the cells without rhodamine-phalloidin signals from polymerized F-actin (Fig. 3D, panel ii). However, localization of Mcp4-GFP was aberrant in the cells during the process of abnormal spore formation, which was caused by the addition of latrunculin A (data not shown). Thus, to minimize the effect of sporulation, we performed a pulse-chase experiment in which we added a high concentration of latrunculin A (20 μM) to the cells (AO192) at metaphase I or metaphase II and then chemically
fixed them after 1 h of incubation; this caused depolymerization of F-actin, but no spore formation occurred at this stage of meiosis. We found that Mcp4-3HA localized similarly in either the absence or presence of latrunculin A, even in the cells without fluorescent signals from Crn1-GFP (see http://www.biken.osaka-u.ac.jp/lab/molgenet/Supplementary_data_EC.pdf). These results indicate that formation of the Mcp4 ring is independent of F-actin polymerization.

Accurate positioning of Mcp4 at anaphase II depends on proper FSM formation. The subcellular localization pattern of Mcp4-3HA and Mcp4-GFP during meiosis suggested that Mcp4 plays a role in the proper positioning of the FSM. We previously reported that Meu14 plays an essential role in the formation of the FSM by localizing at its leading edge (18). Thus, we examined the subcellular localization of Mcp4-GFP in meu14Δ cells. Mcp4-3HA behaved normally at metaphase I (randomly scattered dots in the cytoplasm) as well as at anaphase I and metaphase II (perinuclear accumulation) (Fig. 2A and 4A and B, top panels; data not shown). At anaphase II, however, Mcp4-GFP did not accumulate near the junction.
points of tubulin and the nuclei (Fig. 4B). Instead, as demonstrated by the enlarged views shown in Fig. 4A, panel ii, and B, panel ii (arrows), many of the Mcp4-GFP signals were randomly scattered away from the perinucleus.

We also examined the subcellular localization of F-actin during meiosis II in meu1Δ cells. We found that F-actin polymerization occurred normally in meu1Δ cells, since F-actin signals were detected (Fig. 4C and D). However, the positioning of F-actin during anaphase II was abnormal in these cells, as F-actin occurred as scattered dots in the cytoplasm away from the nucleus (Fig. 4C and D, orange arrows). Thus, Meu14 is required for the proper localization of both Mcp4 and F-actin during anaphase II.

**Mcp4 is required for the proper alignment of F-actin at the FSM.** To examine the meiotic role of Mcp4 in more detail, we constructed a null mutant lacking the 

\[ \textit{mcp4}^{+} \] gene \( (\textit{mcp4Δ}) \). We first investigated whether Mcp4 is required for the development of the FSM by visualizing GFP-Psy1 in \( \textit{mcp4Δ} \) cells. During meiosis II, Psy1 translocates from the plasma membrane to the nascent FSM (15). Thus, monitoring the movement of GFP-Psy1 allows us to visualize the process of FSM formation. Time-lapse observation of GFP-Psy1 in live \( \textit{mcp4Δ} \) cells under a fluorescence microscope revealed a normal subcellular distribution and movement of GFP-Psy1 (data not shown). Moreover, \( \textit{mcp4Δ} \) cells developed FSMs and formed four nucleated spores as efficiently as did wild-type cells (data not shown).

Next, to investigate how F-actin behaves during FSM formation in \( \textit{mcp4Δ} \) cells, we compared the subcellular localizations of F-actin and GFP-Psy1 under a fluorescence microscope. At metaphase II, we found that F-actin behaved normally (Fig. 5A and B, white arrowheads in upper panels). However, at anaphase II, when the FSM was almost closed (as visualized by GFP-Psy1), the F-actin signals formed a balloon shape in \( \textit{mcp4Δ} \) cells rather than a dumbbell shape (Fig. 5A, panel i, and B, panel i, red arrowheads in the bottom panels). The disparate F-actin signals are shown by the enlarged pictures (Fig. 5A, panel ii, and B, panel ii, red arrows and arrowhead) and their schematic renditions (right panels). Bar graphs summarizing the proportion of cells harboring zero to four dumbbell or balloon shapes clearly indicate that almost all \( \textit{mcp4Δ} \) cells have aberrant balloon-shaped F-actin at anaphase II (Fig. 5C). Thus, Mcp4 helps to regulate the positioning of F-actin at the aperture of the closing FSM.

We also examined the meiotic progression, spore morphology, and spore viability of \( \textit{mcp4Δ} \) cells and found that all were almost completely normal (see http://www.biken.osaka-u.ac.jp/lab/molgenet/Supplementary_data_EC.pdf). Examination of almost completely normal (see http://www.biken.osaka-u.ac.jp/lab/molgenet/Supplementary_data_EC.pdf). Examination of

The C-terminal domain of Mcp4 harboring the coiled-coil motif participates in proper localization of Mcp4. Next, to investigate the role that the coiled-coil domain of Mcp4 plays in its meiotic function, we examined the subcellular localization of Mcp4 lacking the C-terminal coiled-coil domain \( (\textit{mcp4ΔC}) \) (Fig. 6A). We first examined how ectopically expressed intact and truncated Mcp4 proteins localize in mitotic cells stained with the fluorescent styryl dye FM4-64, which stains the Golgi/endosome membranes after 5 min of uptake and then the vacuole membrane 60 min later (2). In both Mcp4-GFP- and Mcp4ΔC-GFP-expressing cells, we did not find any GFP signals in the Golgi complex/endosome (Fig. 6C, panels i and ii, upper panels). The observation that Mcp4ΔC-GFP and Mcp4-GFP do not localize at stable Golgi/endosome structures was confirmed by the failure of these proteins to colocalize with cyan fluorescent protein (CFP)-labeled Rer1, an ER/Golgi complex shutting protein (17, 24) (Fig. 6B).

However, Mcp4ΔC-GFP (Fig. 6C, panel ii, lower panels) appeared to colocalize with the 60-minute FM4-64 signal in the vacuole, unlike the intact Mcp4 protein (Fig. 6C, panel i, lower panels). This is probably due to the highly hydrophobic nature of Mcp4ΔC-GFP.

Next, we examined the role that the coiled-coil domain of Mcp4 plays in meiosis. Since we could not detect the Mcp4-GFP signal by using the native promoter, as described above, we ectopically expressed the intact Mcp4-GFP or truncated Mcp4ΔC-GFP protein. Comparison of the fluorescence signals from GFP and FM4-64 revealed that Mcp4-GFP dots did not colocalize with WM4-64, which primarily stained the vacuole during meiosis (Fig. 6D, panel i). In contrast, the Mcp4ΔC-GFP signals did not occur as scattered dots, but rather, they aggregated into small rings that did not colocalize with FM4-64 signals but were localized in the vicinity of the vacuole (Fig. 6D, panel ii).

When we compared the localization patterns of the truncated Mcp4 protein and F-actin, we found that while intact Mcp4-GFP was sandwiched between the nucleus and F-actin (Fig. 6E, panel i, white arrowheads), Mcp4ΔC-GFP appeared to have lost its proper sandwiched distribution. Instead, it displayed a large ring-shaped structure (Fig. 6E, panel ii, white arrowheads). Nonetheless, we found that the sporulation of cells ectopically expressing Mcp4ΔC-GFP appeared to be almost normal (data not shown). Taken together, these results indicate that the C-terminal domain of Mcp4 harboring a coiled-coil motif is required for the proper localization of Mcp4.

**DISCUSSION**

Mcp4 is required for accurately positioning F-actin at anaphase II. In the present study, we characterized the function of the meiosis-specific \( \textit{mcp4}^{+} \) gene, which is highly expressed during the meiotic cell cycle only. We found by Northern blot and Western blot analyses that the expression of the \( \textit{mcp4}^{+} \) gene is required for the proper localization of Mcp4.
FIG. 5. Phenotypes of mcp4Δ cells during meiosis. (A and B) Mep4 is required for proper formation of the F-actin structure at anaphase II. GFP-Psy1-expressing mcp4+ (YN68) (A) and mcp4Δ (AO125) (B) strains were induced to enter meiosis by nitrogen starvation. After 10 h, the cells were chemically fixed and stained with rhodamine-phalloidin to detect F-actin. (i) Typical fluorescence microscope images at metaphase II and anaphase II. The images for F-actin (red), GFP-Psy1 (green), and DNA (blue) are merged in the right panels. (ii) Enlarged views of the images indicated by the arrowheads in panels i and their schematic renditions. F-actin forms a dumbbell shape in mcp4+ cells (red arrows in panel A, part ii) but a balloon shape in mcp4Δ cells (red arrowhead in panel B, part ii). Bars, 10 μm. (C) Quantitative analysis of the cell populations harboring zero to four dumbbell or balloon shapes in the mcp4+ or mcp4Δ strain at anaphase II. The bar graphs were drawn by counting the F-actin shapes in 30 mcp4+ and 31 mcp4Δ cells. (D to F) mcp4Δ spores are more salt sensitive than mcp4+ spores. mcp4+ (YN68) and mcp4Δ (AO125) spores were separated by a manipulator onto YEH agar plates, with or without 0.1 M or 0.17 M NaCl. The plates were incubated at 30°C for 5 days. (D) Sizes of colonies of mcp4+ spores after 2 or 5 days on a YEH plate in the absence (0 mM) or presence (175 mM) of NaCl. (E) Viability of mcp4Δ spores under high NaCl conditions. Spores of mcp4+ and mcp4Δ cells were dissected by a micromanipulator. The data shown are the average values for at least three independent assays (at least 80 spores were dissected per assay). The standard deviations are indicated by the error bars. (F) Quantitative analysis of spore populations that could not make colonies when returned to the YEH plate under high sodium salt conditions. Typical images of the germinated spores for mcp4+ and mcp4Δ strains are shown in the insets. The data shown are the average values for at least three independent assays. At least 8 or 22 spores were dissected per assay for the mcp4+ or mcp4Δ strain, respectively. The standard deviations are indicated by the error bars.
FIG. 6. The C-terminal coiled-coil domain of Mcp4 is required for proper localization of this protein during meiosis. (A) Schematic depiction of Mcp4 and Mcp4ΔC. The coiled-coil motif is indicated by the black box. (B) Comparison of the localization patterns in mitotic cells of ectopically expressed Mcp4ΔC-GFP and CFP-labeled Rer1. Rer1 is an ER/Golgi complex shuttling protein (17, 24). Mitotic AO168 (mcp4/H9004 C-GFP rer1/H11001-cfp) cells were cultured in EMM without thiamine. The merged images are shown in the rightmost panel. (C to E) Comparison of the localization patterns of ectopically expressed Mcp4-GFP (i) and Mcp4ΔC-GFP (ii) in mitotic (C) and meiotic (D and E) pREP41 (mcp4/C-gfp or mcp4ΔC-gfp) cells. (C) The cells were cultured in EMM without thiamine and stained with FM4-64 (red). This allowed us to visualize the Golgi complex/endosome 5 min after uptake and then the vacuole 60 min later. (D and E) The cells were cultured in EMM containing 1 μg/ml thiamine with supplements and then transferred to EMM without thiamine to induce Mcp4-GFP expression. Subsequently, 10 h after the first medium exchange, the cells were transferred to fresh EMM-N without thiamine to induce meiosis. After incubation for 10 h, live cells were observed under a fluorescence microscope in the presence of FM4-64 (red) to visualize the localization of the Golgi complex/endosome and the vacuole (D), or the cells were chemically fixed and stained with rhodamine-phalloidin (red) to detect F-actin (E). The green and blue signals are from Mcp4ΔC-GFP and Hoechst 33342-stained DNA, respectively. The merged images are shown in the rightmost panels. The images denoted by the white arrowheads in panel E are enlarged and depicted schematically in the bottom panels. Bars, 10 μm.
gene is abruptly induced during the premeiotic S phase, after which it is constantly expressed until sporulation is finished (Fig. 1). Fluorescence microscopy of fixed cells expressing Mcp4 tagged with an HA epitope also confirmed this expression profile (Fig. 2). These observations suggest that Mcp4 may play a role in S. pombe meiosis. Indeed, we found that mcp4Δ is required for the production of healthy gametes and that spores of mcp4Δ cells were sensitive to NaCl (Fig. 5D to F). Although a previous study sought to comprehensively identify and characterize the subcellular localization of S. pombe proteins involved in meiosis and mitosis (1, 25), Mcp4 was not mentioned. Thus, the subcellular localization described in this report is novel.

Examination of the Mcp4-3HA (Fig. 2) and Mcp4-GFP (Fig. 3) signals revealed that Mcp4 moves during meiosis from a scattered distribution in the cytoplasm to a pericellular localization at anaphase I. This meiotic redistribution of Mcp4 resembles that of F-actin (20) but differs in a few essential features, as follows. First, Mcp4 assembles around the nucleus earlier than F-actin, which still occurs as randomly scattered dots in the cytoplasm at anaphase I, as determined by Crn1-GFP and rhodamine-phalloidin signals (anaphase I panels in Fig. 3A and C). Second, Mcp4 and F-actin rarely colocalize during meiosis. Instead, they seem to occur side by side, even when both show an apparently random distribution in the cytoplasm. This is clearly revealed when F-actin assembles around the nuclear surface in metaphase II, at which point Mcp4 already shows a perinuclear distribution (metaphase II panels of Fig. 3A and C). Third, when the FSM is initiated, Mcp4 is sandwiched between F-actin and the nucleus (Fig. 3B). Analysis of Meu14 localization (Fig. 2C and D) suggested that F-actin, in turn, is sandwiched between Mcp4 and Meu14 (Fig. 3B). Subcellular localizations of Mcp4 and its related proteins at metaphase II and anaphase II are depicted schematically in Fig. 7.

Since the subcellular localization of Mcp4 after anaphase II partially coincides with that of the FSM, we examined whether Mcp4 is involved in FSM formation by examining the localization of Mcp4 in meu14Δ cells. Meu14 plays an essential role in the FSM by localizing at its border (18). Indeed, the localization of Mcp4 near the junctions of tubulin and the nuclei was largely disturbed in meu14Δ cells (Fig. 4A and B). This strain also showed aberrant Mcp4 localization prior to FSM engulfment of each of the daughter nuclei. Furthermore, meu14Δ cells also showed impaired F-actin positioning (Fig. 4C and D). Nonetheless, spore formation and spore viabilities were almost normal in mcp4Δ cells, indicating that Mcp4 plays a significant role in FSM formation. These observations suggest that the accurate positioning of Mcp4 at anaphase II depends on accurate FSM formation but that Mcp4 is not involved in FSM formation.

Although Mcp4 has a coiled-coil motif, it is unlikely that it interacts biochemically with other meiotic coiled-coil proteins, such as Spo15, Psy1, Sec9, and Meu14 (16, 18), that are essential for proper spore formation, because the localization patterns of these proteins during meiosis differ temporally and/or spatially from the localization pattern of Mcp4. Nonetheless, we found that the coiled-coil domain of Mcp4 is required for its proper localization (Fig. 6). Thus, Mcp4 may act in meiosis by associating with an as yet unknown partner via its coiled-coil domain. The identification of this partner awaits additional studies.

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