A Novel Role of Dma1 in Regulating Forespore Membrane Assembly and Sporulation in Fission Yeast

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In fission yeast Schizosaccharomyces pombe, a diploid mother cell differentiates into an ascus containing four haploid ascospores following meiotic nuclear divisions, through a process called sporulation. Several meiosis-specific proteins of fission yeast have been identified to play essential roles in meiotic progression and sporulation. We report here an unexpected function of mitotic spindle checkpoint protein Dma1 in proper spore formation. Consistent with its function in sporulation, expression of dma1+ is up-regulated during meiosis I and II. We showed that Dma1 localizes to the SPB during meiosis and the maintenance of this localization at meiosis II depends on septation initiation network (SIN) scaffold proteins Sid4 and Cdc11. Cells lacking Dma1 display defects associated with sporulation but not nuclear division, leading frequently to formation of ascii with fewer spores. Our genetic analyses support the notion that Dma1 functions in parallel with the meiosis-specific Sid2-related protein kinase Slk1/Mug27 and the SIN signaling during sporulation, possibly through regulating proper forespore membrane assembly. Our studies therefore revealed a novel function of Dma1 in regulating sporulation in fission yeast.

INTRODUCTION

Sporulation in the model organism, the fission yeast Schizosaccharomyces pombe, is a unique biological process where the plasma membrane of daughter cells is assembled de novo within the mother cell cytoplasm. In recent years, intensive studies in fission yeast have led to a better understanding of the events in sporulation, though our knowledge of the molecular mechanisms that regulate meiosis and sporulation is still limited comparing with the mitotic cell cycle (Shimoda, 2004; Ohtaka et al., 2007b). During sporulation, a double-layered membrane called the forespore membrane (FSM) is formed dynamically, and this process is initiated during the second meiotic division. Synthesis of the forespore membrane must be properly coordinated with the second meiotic nuclear division both temporally and spatially, which is essential for accurate distribution of the genome into four haploid spores. A key structure that links these two events is the spindle pole bodies (SPBs), which are the functional equivalent to the centrosomes of animal cells. Thus, the SPB in yeast functions not only to nucleate and organize microtubules, but also to act as a signaling center and a platform that coordinates meiotic cell cycle events.

During meiosis II, at the metaphase-to-anaphase transition, the SPBs are structurally modified as revealed by a transient change in shape from a dot into a crescent (Hagan and Yanagida, 1995). This change results in the formation of a complex multilayered structure called “meiotic plaque,” which is essential for spore formation (Tanaka and Hirata, 1982; Hirata and Shimoda, 1994; Ikemoto et al., 2000). The inner side of the plaque forms the meiotic spindle, whereas its outer side serves as a platform for assembly of the forespore membrane (Tanaka and Hirata, 1982; Hirata and Shimoda, 1994). After SPB maturation and structural modification, membranous vesicles are targeted to the meiotic outer plaque and promote FSM growth and expansion by vesicle fusion. FSM eventually encapsulates each of the four haploid nuclei and then spore walls are synthesized by the accumulation of wall materials—lipids and polysaccharides—between the inner and outer membranes of the forespore (reviewed in Shimoda, 2004).

Quite a number of genes required for sporulation in fission yeast have been identified and functionally analyzed. The constitutive SPB protein Spo15 is located on the meiotic plaque and plays a critical role in SPB modification and sequential recruitment of a few other sporulation-specific SPB proteins (such as Spo13 and Spo2) to assist SPB maturation (Ikemoto et al., 2000; Nakase et al., 2008). When SPB modification is blocked by mutation of Spo15, sporulation is totally abolished (Ikemoto et al., 2000; Nakase et al., 2008). After SPB maturation, the coiled-coil–containing protein Spo3 and the syntaxin-related protein Psy1 participate in SPB targeting of secretory vesicles (Nakamura et al., 2001). FSM outgrowth is initiated at each of the modified SPBs and subsequently proceeds toward the midspindle region with Meu14 as the major component of a ring structure at the leading edge (Okuzaki et al., 2003; Shimoda, 2004). After completion of spore membrane assembly, the spore wall is assembled by the combined action of enzymes, such as Ags1/Mok1, Bgs2 and Chs1 (Hochstenbach et al., 1998; Arel-
At the end of the vegetative cell cycle in fission yeast, a signaling cascade termed the septation initiation network (SIN) is required to regulate the onset of cytokinesis and septum formation (reviewed in McCollum and Gould, 2001; Guerin et al., 2002a; Krapp and Simanis, 2008). The SIN pathway includes a small GTPase (Spg1), three protein kinases (Cdc7, Sid1, and Sid2) and two scaffold proteins (Cdc11 and Sid4). All the members of the SIN localize to the SPBs and are thought to coordinate cytokinesis with completion of chromosome segregation (reviewed in McCollum and Gould, 2001; Guerin et al., 2002a; Krapp and Simanis, 2008). Very recent studies have shown that the SIN components also localize to the SPBs during meiosis and play an important role in FAS assembly (Krapp et al., 2006). The defects in FAS assembly have been correlated with improper localization of the t-SNARE Psy1 in some SIN mutants, however the detailed mechanism linking the SIN and FAS assembly remains unclear (Krapp et al., 2006). It has been recently shown that a meiosis-specific Sid2-like kinase Slk1/Mug27 is expressed specifically during meiosis and localizes to the SPBs during meiosis I and II in an SIN-dependent manner (Ohtaka et al., 2008; Perez-Hidalgo et al., 2008; Yan et al., 2008). Examination on the phenotypes during sporulation in slk1Δ cells suggested that Slk1/Mug27, together with Sid2, plays a role in forespore membrane assembly by facilitating recruitment of components of the secretory apparatus, such as Psy1, to allow FAS expansion (Ohtaka et al., 2008; Perez-Hidalgo et al., 2008; Yan et al., 2008). These studies thereby provided a novel link between the SIN and vesicle trafficking during cytokinesis.

An SPB-localized protein, Dma1, has been identified as a negative regulator of mitotic exit and cytokinesis in late mitosis (Murone and Simanis, 1996; Guerin et al., 2002b). In mitotic cells, Dma1 localizes at the SPBs through interaction with Sid4, which is a scaffold protein required for recruiting SIN components to the SPB (Chang and Gould, 2000; Guerin et al., 2002a; Guerin et al., 2002b). So far, nothing is known about the function of Dma1 in meiosis. Whether Dma1 negatively regulates SIN components in meiosis as it does in mitotic exit and cytokinesis remains an open question. According to the S. pombe genome-wide transcriptome analysis, expression of dma1+ is clearly up-regulated during meiosis (Mata et al., 2002), suggesting that Dma1 may be involved in regulation of meiotic events. Here we report that indeed Dma1 is also localized to the SPB during meiosis and plays a pivotal role in FAS formation and sporulation. Our results suggest that Dma1 fulfills its function during sporulation possibly through cooperating with the SIN and meiosis-specific kinase Slk1/Mug27.

MATERIALS AND METHODS

Yeast Strains, Media, and Culture Conditions

Schizosaccharomyces pombe strains used in this study are listed in Table 1. Yeast strains were constructed by either random spore method or by tetrad analysis. Yeast cells were grown on YES medium or minimal media with appropriate supplements (Moreno et al., 1991). Solid malt extract (ME) medium or synthetic Edinburgh minimal medium without nitrogen (EMM-N) was used for mating and sporulation. Homothallic yeast strains were induced to enter meiosis and sporulation on ME for 24 h at 30°C or for 14 h at 37°C or for 14 h at 25°C, 28°C, 30°C, or 35°C and processed for staining (DAPI or Hoechst) or imaging experiments as the case may be. The induction of synchronous meiosis and mitotic time course experiments using pat1-114 mutants were performed as described previously (Loidl and Lorenz, 2009). Growth temperatures were 25°C (permissive) for temperature-sensitive strains, and 30°C for all other strains.

Construction of Gene Replacement Strains Carrying dma1Δ511A or dma1Δ252F

The fragments containing dma1Δ511A (344-804 bp) or dma1Δ252F (1-572 bp) flanked by ~500 bp dna1+ 5' UTR and ~500 bp dma1 3' UTR regions were amplified by fusion PCR's using primers containing Plsu and Sall sites. Primers used were: dma1-5'UTR/500'-Sall-F: 5'-GGGCTTGCAATGCTGAGAATTCTGC-3'; dma1-ARP/500'-Sall-R: 5'-GACCGATGCCATCGGATATCCATC-3'; dns34-UTR-3'F: 5'-CCTCCGGGAATAAACAGTTATTTTCTTCACTCA-3'; dma1-3'UTR-F: 5'-CCTCCGGGAATAAACAGTTATTTTCTTCACTCA-3'; dma1-5'UTR-5'R: 5'-GACCGATGCCATCGGATATCCATC-3'; dma1-3'UTR-3'R: 5'-GACCGATGCCATCGGATATCCATC-3';

RNA and Protein Methods

Total RNA was isolated from cells collected at each time points of the meiotic time course by using the TRIzol Reagent (Roche, Indianapolis, IN) and following manufacturer’s instructions. RT was performed with PrimeScript-RT and following manufacturer’s instructions. RT was performed with PrimeScript-RT reagent Kit (Takara Bio) at 37°C for 15 min followed by treatment at 85°C for 5 s. Quantitative real-time PCR was performed on a Rotor-Gene 3000A instrument using SYBR Premix Ex Taq (Takara). Relative expression levels were calculated by the ΔCT method normalized to act1+ RNA levels. The melting curve in each individual measurement was monitored to guard against nonspecific amplification. Primer pairs to detect dma1+ and act1+ transcripts were as follows: dma1-F:5'-GTCGCCATCGGCAAGG-3' and dma1-R-781: 5'-GCCGG-3' and dma1-R-781: 5'-GCCGG-3' and dma1-5'R-781: 5'-GTCGCCATCGGCAAGG-3' (amplifying a 254bp fragment); act1-F: 5'-CGATGCATACGCACTCA-3' and act1-R: 5'-CGATGCATACGCACTCA-3' (amplifying a 200bp fragment). Total protein extracts were made using the 8M urea extraction protocol (Masai et al., 1995). Protein extracts were run on 8–10% SDS-PAGE gels and transferred to nitrocellulose membranes (GE, Munich, Germany). For detection, mouse monoclonal anti-GFP (Roche or Santa Cruz Biotechnology, Santa Cruz, CA) or anti-TAT1 antibodies and following manufacturer’s instructions. RT was performed with PrimeScript-RT reagent Kit (Takara Bio) at 37°C for 15 min followed by treatment at 85°C for 5 s. Quantitative real-time PCR was performed on a Rotor-Gene 3000A instrument using SYBR Premix Ex Taq (Takara). Relative RNA levels were calculated from ΔCT values and normalized to act1+ RNA levels. The melting curve in each individual measurement was monitored to guard against nonspecific amplification. Primer pairs to detect dma1+ and act1+ transcripts were as follows: dma1-F:5'-GTCGCCATCGGCAAGG-3' and dma1-R-781: 5'-GCCGG-3' and dma1-5'R-781: 5'-GTCGCCATCGGCAAGG-3' (amplifying a 254bp fragment); act1-F: 5'-CGATGCATACGCACTCA-3' and act1-R: 5'-CGATGCATACGCACTCA-3' (amplifying a 200bp fragment).

For immunoblot experiments, whole-cell lysates were prepared in NP-40 buffer (6 mM Na₂HPO₄, 4 mM NaH₂PO₄, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na₃VO₄) and lysates were subjected to immunoprecipitation with anti-GFP (366), Molecular Probes, Eugene, OR) antibodies and Western blot analyses with anti-GFP or anti-Myc (9E10, Roche) antibodies as were previously described (Guerin et al., 2002b).

Spore Viability

Dissected spores’ viability was determined as described previously (Ohtaka et al., 2007a; Ohtaka et al., 2008). Briefly, 0° haploid wild-type or dma1Δ strains were grown on YE plates at 30°C. Cells were mated and sporulated on ME plates at 30°C for 3–4 d. The spores from 4-spore asci were separated on YE agar plates using a micromanipulator (Schnell Biotec, Germany). The plates were incubated at 30°C for 4–5 d, after which spore viability was calculated.

Microscopy

RFP or GFP-fusion proteins were observed in cells after fixation with cold methanol or in live cells. For DAPI (4', 6-diamidino-2-phenylindole, Sigma, St. Louis, MO) staining of cells fixed with cold methanol, washed in PBS and resuspended in PBS plus 1 μg/ml DAPI. Nucleus staining of live cells was performed with Hoechst 33342 (Sigma) at 1–2 μg/ml in EMM or PBS. Photomicrographs were obtained using a Nikon 80i fluorescence micro-
Table 1. Yeast strains used in this study

| Name | Genotype | Source |
|------|----------|--------|
| JY1  | h− ade6-210 leu1-32 ura4-D18 | Lab stock |
| JY2  | h+ ade6-210 leu1-32 ura4-D18 | Lab stock |
| JY78 | h− dma1::ura4+ ade6-21x leu1-32 ura4-D18 | Lab stock |
| JY92 | h+ dma1::ura4+ ade6-21x leu1-32 ura4-D18 | Lab stock |
| JY79 | h+ spo3-GFP+ >>LEU2 leu1-32 | This study |
| JY760| h+ spo3-GFP+ >>LEU2 dma1::kan6 ade6-210 leu1-32 ura4-D18 | YGRC |
| JY810| h+ pat1-114/j14 pat1-114 dma1::GFP::kan6 ade6-210/ad6-216 leu1-32/leu1-32 ura4-D18 | This study |
| JY812| h+ pat1-114/j14 pat1-114 dma1::ura4+ dma1::ura4+ ade6-210/ad6-216 leu1-32/leu1-32 ura4-D18 | This study |
| JY904| h+ spo15-GFP::leu1+ sad1-GFP+ >>kan6 ade6-21x leu1-32 ura4-D18 | This study |
| JY903| h+ spo15-GFP::leu1+ sad1-GFP+ >>kan6 dma1::ura4+ ade6-21x leu1-32 ura4-D18 | This study |
| JY909| h+ sid4-GFP::kan6 ade6-21x leu1-32 ura4-D18 | This study |
| JY887| h+ sid4-GFP::kan6 dma1::ura4+ ade6-21x leu1-32 ura4-D18 | This study |
| JY911| h+ cut12-EGFP::ura4+ ade6-21x leu1-32 ura4-D18 | This study |
| JY888| h+ cut12-EGFP::ura4+ dma1::kan6 ade6-21x leu1-32 ura4-D18 | This study |
| JY1200| h+ skl1/mug27::ura4+ ade6-210 leu1-32 ura4-D18 | M. Balasubramanian |
| JY1286| h+ skl1/mug27::ura4+ dma1::kan6 ade6-210 leu1-32 ura4-D18 | M. Balasubramanian |
| JY1202| h+ skl1/mug27::GFP::ura4+ ade6-210 leu1-32 ura4-D18 | M. Balasubramanian |
| JY1206| h+ pat1-114/j14 pat1-114/mug27::GFP::ura4+ ade6-210 leu1-32 ura4-D18 | YGRC |
| JY1347| h+ pat1-114/j14 skl1/mug27::[skl1/mug27::9myc-3’UTR-LEU2] dma1::GFP::kan6 ade6-210 leu1-32 ura4-D18 | This study |
| JY1293| h+ pat1-114/j14 skl1/mug27::[skl1/mug27::9myc-3’UTR-LEU2] dma1::ura4+ ade6-210 leu1-32 ura4-D18 | This study |
| JY1558| h+ plo1-24C ade6-21x leu1-32 ura4-D18 | This study |
| JY1537| h+ dma1::ura4+ plo1-24C ade6-21x leu1-32 ura4-D18 | This study |
| JY1252| h+ sid4-A1 ade6-21x leu1-32 ura4-D18 | This study |
| JY1232| h+ sid4-A1 dma1::ura4+ ade6-21x leu1-32 ura4-D18 | This study |
| JY1233| h+ sid4-GFP::kan6 cdc11-123 ade6-21x leu1-32 ura4-D18 | This study |
| JY1234| h+ sid4-GFP::kan6 cdc11-123 dma1::ura4+ ade6-21x leu1-32 ura4-D18 | This study |
| JY1596| h+ spo1-106 ade6-21x leu1-32 ura4-D18 | This study |
| JY1358| h+ dma1::ura4+ spo1-106 ade6-21x leu1-32 ura4-D18 | This study |
| JY1598| h+ cdc7-24 ade6-21x leu1-32 ura4-D18 | This study |
| JY1599| h+ dma1::ura4+ cdc7-24 ade6-21x leu1-32 ura4-D18 | This study |
| JY1358| h+ cdc7-24 ade6-21x leu1-32 ura4-D18 | This study |
| JY1234| h+ sid2-250 ade6-21x leu1-32 ura4-D18 | This study |
| JY1299| h+ sid2-250 dma1::ura4+ ade6-21x leu1-32 ura4-D18 | This study |
| JY1598| h+ mob1-1 ade6-21x leu1-32 ura4-D18 | This study |
| JY1599| h+ mob1-1 dma1::ura4+ ade6-21x leu1-32 ura4-D18 | This study |
| JY1229| h+ dma1::GFP::kan6 spo15::ura4+ ade6-21x leu1-32 ura4-D18 | This study |
| JY1258| h+ dma1::GFP::kan6 spo15::ura4+ ade6-216 leu1-32 ura4-D18 | This study |
| JY1260| h+ dma1::GFP::kan6 sid4-A1 ade6-21x leu1-32 ura4-D18 | This study |
| JY1262| h+ dma1::GFP::kan6 cdc11-123 ade6-210 leu1-32 ura4-D18 | This study |
| JY1276| h+ dma1::GFP::kan6 sad1-RFP::kan6 ade6-21x leu1-32 ura4-D18 | This study |
| JY1248| h+ leu1+ >>psylp1pGFP-psy1pGFP-ath2−::kan6 dma1::ura4+ ade6-21x leu1-32 ura4-D18 | This study |
| JY1278| h+ leu1+ >>psylp1pGFP-psy1pGFP-ath2−::kan6 dma1::ura4+ ade6-216 leu1-32 ura4-D18 | This study |
| JY1244| h+ sid2-GFP::ura4+ dma1::kan6 ade6-210 leu1-32 ura4-D18 | This study |
| JY1280| h+ cdc11-GFP::kan6 ade6-21x leu1-32 ura4-D18 | This study |
| JY1246| h+ cdc11-GFP::kan6 ade6-21x leu1-32 ura4-D18 | This study |
| JY1203| h+ pat1-114/j14 skl1/mug27::[skl1/mug27::GFP-3’UTR-LEU2] ade6-216 leu1-32 ura4-D18 | YGRC |
| JY1356| h+ pat1-114/j14 skl1/mug27::[skl1/mug27::GFP-3’UTR-LEU2] ade6-21x leu1-32 ura4-D18 | This study |
| JY1294| h+ pat1-114/j14 skl1/mug27::[skl1/mug27::GFP-3’UTR-LEU2] dma1::ura4+ ade6-216 leu1-32 ura4-D18 | This study |
| JY1295| h+ pat1-114/j14 skl1/mug27::[skl1/mug27::GFP-3’UTR-LEU2] dma1::ura4+ ade6-216 leu1-32 ura4-D18 | This study |
| JY1296| h+ leu1+ >>psylp1pGFP-psy1pGFP-dma1::APA6 ade6-216 ura4-D18 | This study |
| JY1298| h+ leu1+ >>psylp1pGFP-psy1pGFP-dma1::APA6 ade6-216 ura4-D18 | This study |
| JY1329| h+ pat1-114 dma1::GFP::kan6 ade6-216 | This study |
| JY1339| h+ pat1-114 dma1::ura4+ ade6-216 | This study |
| JY111| h+ cen2>>lacO-kan6::ura4+ his7+>>GFP-lacI-NLS leu1-32 lys1 ura4-D18 ade6-M216 | Y. Watanabe |
| JY470| h+ dma1::ura4+ cen2>>lacO-kan6::ura4+ his7+>>GFP-lacI-NLS ura4-D18 leu1-32 ade6-21x lys1 | This study |
| JY1477| h+ nmt1-GFP-skl1::leu1+ skl1-GFP::ura4+ ade6-210 leu1-32 ura4-D18 | This study |
| JY1563| h+ nmt1-GFP-skl1::leu1+ skl1-GFP::ura4+ ade6-210 leu1-32 ura4-D18 | This study |
scope coupled to a cooled CCD camera (Hamamatsu, ORCA-ER) and image processing and analysis was carried out using Element software (Nikon) and Adobe Photoshop. For time-lapse microscopy, homothallic wild-type or dma1Δ cells carrying GFP-Psy1 and GFP-Abf2 were first induced to enter meiosis on solid ME plates for 10–12 h. Live cells were then spotted on a glass slide containing 2% agar pad with EMM-N at room temperature (~25°C). Images were acquired every 3–10 min (with a 400–600 ms exposure time).

RESULTS

Dma1 Expression Is Significantly Up-Regulated during Meiosis
According to previously published microarray expression data, dma1+ mRNA is expressed during meiotic prophase and peaked between meiosis I and meiosis II (Mata et al., 2002). To more accurately examine the expression of the dma1+ at the mRNA and protein levels during meiosis, we used the haploid dma1−/GFP strain, which expresses Dma1 protein tagged with the GFP epitope at its C-terminal end (Guertin et al., 2002b). To obtain synchronous meiosis, we used the pat1-114 temperature-sensitive strain, which enters meiosis in a highly synchronous manner when it is shifted to the restrictive temperature (Iino and Yamamoto, 1985). The cell lysates from a time course were subjected to total RNA isolation or protein extraction and subsequent quantitative RT-PCR (qRT-PCR) or immunoblot analysis using the anti-GFP antibodies. These analyses revealed that dma1− indeed displayed meiosis-specific up-regulation of transcription, its mRNA level began to increase during prophase and peaked at ~4.5–5 h after the induction of meiosis (Figure 1B). These data are consistent with the microarray expression data (Mata et al., 2002). In parallel, Dma1 protein levels peaked at ~5.5 h and started to decrease at the end of the second meiotic nuclear division (Figure 1C).

Dma1 Is Localized at the Spindle Pole Body during Meiosis and Maintenance of this Localization at Meiosis II Depends on SIN Scaffold Proteins

In mitosis, Dma1 exhibits distinct localization at the SPBs and cell division site from metaphase to anaphase, a pattern similar to some of the SIN components (Guertin et al., 2002b). To gain insight into the cellular role of Dma1 in meiosis, the localization of this protein was analyzed. Dma1-GFP localization was examined in homothallic (h0) cells after induction of meiosis by nitrogen starvation. The SPB marker Sad1 tagged with RFP (RFP-Sad1) was used to monitor cell cycle progression. As shown in Figure 2A, Dma1-GFP was first observed as a dot-like signal at horsetail stage, and during meiosis I and II Dma1-GFP was visible as one, two, or four foci, which were always colocalized with the RFP-Sad1 signals. This colocalization pattern of Dma1 and Sad1 suggested that Dma1 localized to the SPB in meiotic cells similarly to SIN components (Krapp et al., 2006). As sporulation commenced, Dma1-GFP gradually disappeared from the four SPBs, and it showed slight accumulation in the cytoplasm of asci (Figure 2A).

In mitosis, core components of the fission yeast SIN signaling are anchored to the outer plaque of the SPB by Cdc11 and Sid4 (Krapp et al., 2001), and Dma1 is recruited to SPB through its interaction with Sid4 (Guertin et al., 2002b). We wondered whether Dma1’s localization to SPB during meiosis and sporulation also requires SIN proteins. To test this possibility, Dma1-GFP localization was examined in temperature-sensitive homothallic cdc11-123 and sid4-A1 strains after they were induced into meiosis and sporulation at permissive (25°C) and nonpermissive (28°C for sid4-A1 and 35°C for cdc11-123) temperatures. We found that Dma1-GFP localized normally at SPBs from metaphase I to metaphase II in these mutants at nonpermissive temperatures (Figure 2, B and C, and data not shown). At anaphase II, however, many of the Dma1-GFP signals did not accumulate at SPBs (Figure 2, B and C). These observations suggested that retention of Dma1 localization to SPBs during meiosis II and sporulation depends on the SIN scaffold proteins Cdc11 and Sid4, although the initial recruitment is independent of these two proteins. This pattern of localization dependency is reminiscent of the meiosis-specific kinase Slk1/Mug27, whose stable localization at SPB in meiotic anaphase II also requires Cdc11 and Sid4 (Yan et al., 2008). Furthermore, similarly to Slk1/Mug27 (Yan et al., 2008), Dma1 does not require Spo15 and Spo3 for its proper maintenance at SPBs (Fig. S1).

Dma1 Is Not Required for Meiotic Progression and Chromosome Segregation, but Is Required for Proper Spore Formation

Because Dma1 expression was significantly up-regulated and it localized at SPBs during meiosis (Figures 1 and 2), we investigated possible function(s) of this protein in meiosis. To analyze meiotic cell cycle progression in the dma1Δ mutant, we induced synchronous meiosis in haploid pat1-114.
strains. As shown in Figure 3A, after temperature shift, dma1Δ/h9004 cells proceeded through meiotic nuclear divisions with kinetics identical to that of the wild-type (as compared with Figure 1A). During the first and second meiotic divisions, chromatin segregation appeared almost normal, as revealed by visualization of cen2-GFP (Kitajima et al., 2003b) (Supplemental Figure S2). However, we noticed that in the absence of dma1Δ/h11001, the initiation of spore formation was delayed for 2 h compared with wild-type cells, and also the efficiency of spore formation was dramatically dropped with only 60% of cells containing spores (Figure 3B). Therefore, we concluded that Dma1 is not essential for progression through the stages of meiosis before spore formation.

In the course of the synchronized meiosis experiment described above, we noticed that dma1Δ cells gave rise to large number of abnormal spores (data not shown). Therefore, we next undertook a more detailed examination of the defects in spore formation in dma1Δ cells. Heterothallic (h+ and h−) or homothallic (h0) wild-type cells placed under nitrogen-limiting conditions underwent mating followed by meiosis and sporulation, leading to the formation of four-spored asci (Figure 4A). In contrast, abnormalities related to sporulation were observed when dma1Δ cells were placed under the same nitrogen-limiting conditions (Figure 4A). Quantitative analyses revealed that a significant fraction (~75–80%) of dma1Δ asci contained one, two, or three, instead of four, spores, indicating incomplete spore formation within these asci (Figure 4B). This was also true for diploid dma1Δ cells when they were induced to undergo meiosis and sporulation (Figure 4, A and B). We found the negative effect of dma1Δ on spore formation was recessive, as we observed almost normal spore formation when a wild-type haploid strain mated with a dma1Δ haploid (Figure 4A).

The spore viability of four-spore asci was analyzed by tetrad dissection and random spore analysis, and we observed normal-looking spores produced by mated dma1Δ cells showed slightly reduced germination efficiency compared with wild-type cells (Figure 4C and data not shown). This indicated although some mated dma1Δ cells managed to form four spores, a portion of the spores could not germinate to form colonies, presumably because of defective FSM and spore wall assembly (see below).
Dma1 contains two recognized domains: an N-terminal FHA domain and a C-terminal RING-H2 (RH2) finger (RF) (Murone and Simanis, 1996; Guertin et al., 2002b). To determine which domain is more specifically required for the role Dma1 plays in spore formation, we generated partial deletion mutants (Dma1\textsuperscript{ΔFHA} or Dma1\textsuperscript{ΔRF}) in which either FHA or RF domain was deleted in the genome (Figure 4D). Examination of spore formation in these mutant cells indicated that absence of either domain led to increase of asci containing fewer than four spores similar to dma1\textDelta cells (Figure 4E). These data showed that both FHA and RF domains are required for the function of Dma1 in regulating sporation.

**Spore Membrane Assembly Is Aberrant in dma1\textDelta Cells, But Meiotic Plaque Assembly Is Normal**

To understand the molecular basis of the spore formation defects in dma1\textDelta cells, we examined the localization of several proteins that participate at distinct steps during sporulation. The formation of the crescent-shaped meiotic outer plaque during SPB modifications depends on the SPB-associated protein Spo15 (Ikemoto et al., 2000). Because Dma1 is already present at the SPB from the early stage of FSM assembly, we decided to test whether the modification of the SPB is impaired in dma1\textDelta cells. We found that the localization of Spo15 and the assembly of the Spo15 crescents were unaffected in dma1\textDelta cells (Supplemental Figure S3). Our observation of the SPB protein Cut12 by using a chromosomally GFP-tagged allele (cut12-GFP) (Bridge et al., 1998) also revealed the presence of the typical crescent-shaped SPBs in dma1\textDelta cells (Supplemental Figure S3). These data indicated that Dma1 is not required for the meiosis-specific modification of the SPB.

It is known that after SPB maturation, the syntaxin-related protein Psy1 participates in the process of FSM development by promoting targeting of membranous vesicles to the meiotic outer plaque (Nakamura et al., 2001; Shimoda, 2004). So we next investigated the localization and behavior of Psy1 in wild-type and dma1\textDelta cells using strains that simultaneously express GFP-Psy1 and GFP-Atb2, which labeled FSM and tubulin, respectively. Similar to what we observed in wild-type cells, GFP-Psy1 in dma1\textDelta cells appeared as two pairs of bright crescent near the SPBs at metaphase II and formed two pairs of cup-like structures facing each other in the asci (Figure 5A). However, we found small-sized GFP-Psy1 aggregates without hollow internal space in many dma1\textDelta asci (Figure 5, A and B). These aggregates were often observed in the same asci as normal-looking sphere-like structures (Figure 5, A and B), suggesting that they could have derived from collapsed or shrunk sphere-like structures. Other defects in GFP-Psy1 localization in dma1\textDelta asci included the following: a small proportion of asci containing only three GFP-Psy1 aggregates or spheres, as well as many asci (almost 40%) containing more than four (up to 8) discernable GFP-Psy1 aggregates or spheres (Figure 5, A–C). To investigate whether the normal-looking GFP-Psy1 spheres could result in normal spore formation, we counted the asci with one, two, three, or four normal-looking GFP-Psy1 spheres. Interestingly, the frequencies of each class corresponded very well to the frequencies of asci containing same number of spores (compare Figure 5D with Figure 4B). This suggested that once the FSM formed normally, the relatively normal spore development was followed.

To further examine in detail how the FSM formation defects is generated in live dma1\textDelta cells, we compared the behavior of GFP-Psy1 by time-lapse microscopy in asci from wild-type and dma1\textDelta cells after two metaphase II spindles were fully assembled (as indicated by GFP-Atb2). As previously reported (Nakamura et al., 2008; Yan et al., 2008), GFP-Psy1 in wild-type cells progressively formed crescent-shaped structures besides the SPBs at metaphase II, later expanded as four cup-like structures, and eventually became four round mature structures in the asci (Figure 6A). However, we observed several types of defects in FSM development in dma1\textDelta cells (Figure 6B). These defects roughly fell into three classes: (1) initially FSM formation was normal and appeared as sphere structure, but subsequently it became smaller or collapsed (asterisks in Figure 6B); (2) crescent-shaped structures did not properly develop into cup-like structures (open arrows in Figure 6B); and (3) crescent-shaped structures broke into multiple GFP-Psy1-containing structures which could not develop into round mature FSMs (arrows in Figure 6B).

Collectively, our data suggested that the inability of dma1\textDelta cells to form proper ascospores might result from defective FSM development at different stages, such as failed transition from crescent structure to cup-like structure, abnormal further expansion of the spore membrane, or the failed maintenance of the round structure at the spore periphery.

**Negative Genetic Interactions between spo3-GFP and dma1\textDelta Mutant**

Because we found the defects of forespore membrane development in dma1\textDelta cells, we were interested in knowing the effect of compromised FSM proteins on sporulation in dma1\textDelta. Spo3 is a coiled-coil–containing protein required for...
the assembly of the forespore membrane. While cells deleted for spo3<sup>+</sup> completely fail to form the forespore membrane (Nakamura et al., 2001), cells with GFP tagged Spo3 (Spo3-GFP) present only slightly compromised spore formation (Perez-Hidalgo et al., 2008; Yan et al., 2008). Through analysis of the dma1<sup>Δ</sup> cells carrying spo3-GFP, we found strong negative genetic interaction between dma1<sup>Δ</sup> and spo3-GFP, as we observed severe impairment in sporulation in these cells (Figure 7A). Similar to our observations with GFP-Psy1, we also found collapsed Spo3-GFP signals within cytoplasm in dma1<sup>Δ</sup> cells (Figure 7B), consistent with the idea that Dma1 is involved in maintaining the growth of FSM during sporulation. One major difference between the localization of Spo3-GFP and that of GFP-Psy1 in dma1<sup>Δ</sup> asci is that collapsed Spo3-GFP was frequently found away from nuclei, whereas aggregated GFP-Psy1 usually overlapped with or attached to nuclei (compare Figure 5B and Figure 7B). These results showed that re-
during mitotic exit and cytokinesis, Dma1 may act to antagonize SIN signaling by inhibiting the SPB localization of SIN components including the upstream SIN activator Plo1 kinase (Guerin et al., 2002b). Recent studies have shown that the SIN components localize to the SPBs during meiosis and play an important positive role in forespore membrane assembly during sporulation (Krapp et al., 2006). Because the sporulation defects we observed in \( \text{dma1}\Delta \) cells are similar to those reported for SIN mutants, we wondered whether Dma1 and SIN proteins possibly perform overlapping functions in the regulation of sporulation. We constructed several homothallic double mutants between \( \text{dma1}\Delta \) and temperature-sensitive SIN or SIN activator mutants, including the \( \text{sid4}\Delta-1, \text{cdc11}\Delta-123, \text{spg1}\Delta-106, \text{cdc7}\Delta-24, \text{sid1}\Delta-239, \text{mob1}\Delta-1, \text{sid2}\Delta-250 \) and \( \text{plo1}\Delta-24C \) mutants. Except for \( \text{dma1}\Delta \text{sid4}\Delta-1 \) and \( \text{dma1}\Delta \text{spg1}\Delta-106 \), which could not survive at 30°C and thus the analyses of these mutants were only carried out at 25°C, examination of sporulation in other mutants was performed at both permissive and semipermissive temperatures (25 and 30°C). Our results showed that spore formation defects observed in the \( \text{dma1}\Delta \) single mutant were not significantly enhanced by most SIN mutations (Figure 8, A and B and Supplemental Figure S4). However, we observed that homothallic \( \text{dma1}\Delta \text{spg1}\Delta-106 \) and \( \text{dma1}\Delta \text{mob1}\Delta-1 \) cells were completely unable to sporulate under conditions in which single \( \text{spg1}\Delta-106 \) or \( \text{mob1}\Delta-1 \) mutants were not apparently compromised for sporulation (Figure 8, A and B), suggesting that Dma1 might function in parallel with Spg1 and Mob1 in sporulation. This result is quite similar to the previous observations that the spore formation is severely impaired in \( \text{slk1}\Delta \text{spg1}\Delta-106 \) and \( \text{slk1}\Delta \text{mob1}\Delta-4 \) double mutants at permissive temperature, which allowed the deduction that the SIN and Slk1 function in parallel but not in the same pathway in spore formation (Perez-Hidalgo et al., 2008; Yan et al., 2008).

Recent studies have shown that a \( \text{Sid2}\Delta \)-related meiosis-specific kinase Skl1/Mug27 and SIN proteins perform overlapping functions in the regulation of sporulation in fission yeast (Perez-Hidalgo et al., 2008; Yan et al., 2008). Our studies on Dma1 strongly suggested it also regulates sporulation in an independent pathway from SIN signaling. We wondered whether Dma1 and Slk1 were also functionally overlapping in regulating sporulation. To test this, we constructed a homothallic double mutant \( \text{dma1}\Delta \text{slk1}\Delta \) and analyzed its spore formation efficiency. We found that the double mutant was completely unable to form spores, suggesting that Dma1 and Slk1 regulate sporulation in an independent manner (Figure 8, C and D). Thus, it seems likely there are at least three parallel signaling pathways involved in controlling proper sporulation in fission yeast (see Figure 9).

Quite unexpectedly, we noticed that Slk1-GFP signals in \( \text{dma1}\Delta \) cells seemed brighter than those observed in wild-type cells (Fig. S5A). This observation was supported by our quantification of intensities of Slk1-GFP signals in both wild-type and \( \text{dma1}\Delta \) cells (Supplemental Figure S5B). Furthermore, we found that deletion of \( \text{dma1}\Delta \) caused elevated Slk1 protein level and delayed decay of Slk1 during meiosis II and sporulation when cells were synchronously induced into meiosis (Supplemental Figure S5C). These data suggested that brighter Slk1-GFP signals in \( \text{dma1}\Delta \) cells were most likely derived from its higher protein level. Interestingly, we also found that the complexes immunoprecipitated by anti-GFP antibodies (recognizing Dma1-GFP) contained Skl1 (Supplemental Figure S5D), indicating that these two proteins are at least present in the same complex even if not directly interacting during certain period of meiosis. However, we failed to observe any aberrant sporulation or disrupted spore formation upon overexpression of Slk1 (Supplemental Figure S5E). Also, elevated level of Skl1 did not enhance or rescue sporulation defects in \( \text{dma1}\Delta \) cells.

**Figure 5.** Spore membrane assembly is aberrant in \( \text{dma1}\Delta \) cells. (A) Localization of GFP-Psy1 in wild-type and \( \text{dma1}\Delta \) cells. Wild-type and \( \text{dma1}\Delta \) cells simultaneously expressing both GFP-Psy1 and GFP-Atb2 were induced to undergo meiosis on ME plate at 30°C. Meiotic cells were visualized by fluorescence and DIC microscopes. Examples of cells at meiosis I, II, and sporulation are shown. Abnormal FSMs are observed frequently in \( \text{dma1}\Delta \) ascis. (B) Abnormal number and morphology of GFP-Psy1 and failed encapsulation of nuclei in \( \text{dma1}\Delta \) cells. \( \text{dma1}\Delta \) cells expressing only GFP-Psy1 were induced to undergo meiosis on ME plate at 30°C. Live meiotic cells were visualized by fluorescence and DIC microscopes. Examples of cells at meiosis I, II, and sporulation are shown. Abnormal FSMs are observed frequently in \( \text{dma1}\Delta \) ascis. (C) Abnormal number and morphology of GFP-Psy1 and failed encapsulation of nuclei in \( \text{dma1}\Delta \) cells. \( \text{dma1}\Delta \) cells expressing only GFP-Psy1 were induced to undergo meiosis on ME plate at 30°C. Live meiotic cells were visualized by fluorescence and DIC microscopes. Examples of cells at meiosis I, II, and sporulation are shown. Abnormal FSMs are observed frequently in \( \text{dma1}\Delta \) ascis. (D) Abnormal number and morphology of GFP-Psy1 and failed encapsulation of nuclei in \( \text{dma1}\Delta \) cells. \( \text{dma1}\Delta \) cells expressing only GFP-Psy1 were induced to undergo meiosis on ME plate at 30°C. Live meiotic cells were visualized by fluorescence and DIC microscopes. Examples of cells at meiosis I, II, and sporulation are shown. Abnormal FSMs are observed frequently in \( \text{dma1}\Delta \) ascis.

\( \text{Dma1 Functions in Parallel with SIN Signaling and Meiosis-Specific Kinase Slk1/Mug27 during Forespore Membrane Assembly} \)

During mitotic exit and cytokinesis, Dma1 may act to antagonize SIN signaling by inhibiting the SPB localization of SIN
Therefore, although an aberrant amount of Slk1 is retained as a consequence of dma1/H11001 deletion, simply overexpressing Slk1 alone does not mimic the dma1/H9004 defect in spore formation, suggesting that Dma1 may regulate levels of multiple proteins in addition to Slk1 during meiosis and sporulation.

DISCUSSION

It has been previously reported that Dma1, which resides at SPB after mitotic metaphase, functions as a negative regulator of mitotic exit and cytokinesis in late mitosis (Murone and Simanis, 1996; Guertin et al., 2002b). In the present study, we analyzed functions of dma1+ in meiosis and sporulation. We found dma1+ was transcriptionally induced during meiotic prophase and accumulated at meiosis I and II, during which time it localized to the SPB. Although cells lacking dma1+ displayed minor chromosome segregation defects during meiosis, the majority of dma1Δ cells underwent meiosis I and II without obvious defects, instead these cells could not produce ascis with the normal number of spores. These observations suggest that the inability of dma1Δ mutants to sporulate properly is unrelated to defects in progression through meiosis I and II and fidelity of nuclear division. Rather, our careful analyses of spore formation by following the localization and behavior of t-SNARE (syntaxin) Psy1 in dma1Δ cells revealed that the defect was due to inability to properly develop or maintain the FSM, and a similar observation was independently reported in a recent study (Krapp et al., 2010). These data suggested Dma1 is required for the proper formation of the FSM and/or assembly of the spore wall. This conclusion is further supported by our results in which dma1Δ cells carryingSpo3-GFP were completely unable to sporulate under conditions in which Spo3-GFP alone was only negligibly compromised for sporulation. We believe that the localization and function of untagged Spo3 protein is also defective when dma1+ gene is deleted, because both Spo3 and Psy1 are involved in FSM assembly during sporulation, and previous studies have shown that Spo3 and Psy1 are functionally coupled and they affect each other’s localization at FSM (Nakamura et al., 2001; Maeda et al., 2009). In a recent study, Simanis and colleagues also examined the localization of Meu14 which is localized at the leading edge of the FSM and found extra Meu14-GFP rings forming during FSM expansion in dma1Δ mutants. This observation suggests the proper closure of FSM is also disregulated in the absence of dma1+. Recent studies have shown that SIN components are actively involved in spore formation (Krapp et al., 2006). The phenotype of dma1Δ in sporulation has strong similarity to what has been observed in SIN mutants, including distorted localization of Psy1, and this has been correlated with the
cause for defective FSM extension and assembly in SIN mutants (Krapp et al., 2006). The disrupted localization of Psy1 in SIN and dma1Δ mutants is consistent with the assumption that the SIN signaling and Dma1 might fulfill their functions, at least in part, by controlling membrane and/or protein trafficking to the FSM during sporulation (Krapp et al., 2006 and this study). Therefore, our studies established a functional connection between Dma1 and the SIN signaling in regulating sporulation in fission yeast.

In this study, our detailed characterization on sporulation defects in dma1Δ mutant cells supports our conclusion that Dma1 positively regulates sporulation, similarly to the SIN and the meiosis-specific kinase Slk1/Mug27 (Krapp et al., 2006; Ohtaka et al., 2008; Perez-Hidalgo et al., 2008; Yan et al., 2008). This is surprising and rather different from the case during mitosis where Dma1 plays an antagonizing role on SIN signaling. Our genetic analyses in double mutants between dma1Δ and SIN mutations or slk1Δ suggested that...
Dma1, SIN, and Slk1 might work in parallel in regulating sporulation, and these pathways probably converge at regulating membrane and/or protein trafficking required for proper FSM development (Figure 9). However, the precise mechanisms linking Dma1, SIN, Slk1, and forespore membrane assembly is not fully understood, and it is not clear whether Dma1, SIN, and Slk1 carry out a common function during sporulation, or act through distinct mechanisms. Our observation that overexpression of dma1Δ in wild-type background during meiosis led to defective spore formation (data not shown) suggests that elevated level of Dma1 is detrimental to proper spore development. It also tells us that it is possible that the levels or activities of one or more proteins must be under precise control by Dma1 during meiosis, too much or too little of these proteins may cause defective sporulation. Consistent with our assumption that Dma1 may regulate several target proteins and have multiple roles in meiosis, Simanis and colleagues have recently shown that dma1Δ also displayed additive sporulation defects with septin mutant spn5Δ and leading edge protein mutant meu14Δ (Krapp et al., 2010).

One interesting, but puzzling, observation in our study was that dma1Δ only showed negative genetic interactions with a few SIN mutations (such as spg1-106 and mob1-1) but not with other hypomorphic alleles tested. This is quite similar to observations in a previous study, in which slk1Δ only showed synthetic phenotypes with the same set of SIN mutants but not others (Yan et al., 2008). One obvious difference between dma1Δ and slk1Δ is that slk1Δ also completely lost ability to sporulate when function of Sid2 (the most downstream SIN component) was compromised (Perez-Hidalgo et al., 2008; Yan et al., 2008) while dma1Δ did not (our data in this study). So, if the genetic interaction between slk1Δ and upstream SIN mutant spg1-106 could be interpreted as a direct consequence of reduced function of the downstream Sid2, this explanation could not be applied to dma1Δ. It is possible that Dma1 and Slk1 have distinct patterns of overlapping function with certain SIN proteins.

In mitosis, the recruitment of Dma1 to SPB is dependent on SIN scaffold protein Sid4 but not Cdc11 (Guertin et al., 2002b). During meiosis, although the initial recruitment of Dma1 to SPBs is not dependent on SIN proteins, its subsequent stable maintenance at SPBs at anaphase II clearly requires both Cdc11 and Sid4. Interestingly, our genetic analyses suggested that SBP-localized Dma1 has different functions during mitosis and meiosis. During mitosis Dma1 antagonizes the SIN, but during meiosis, Dma1 and the SIN appear to perform similar functions. It is currently unclear how Dma1 switches from its inhibitory role on SBP-localized SIN components during mitotic exit and cytokinesis to its positive effect on sporulation. Several possibilities may exist, for example, Dma1 may switch its binding or functional partners in mitosis and meiosis, or Dma1 is differently regulated during mitosis and meiosis. Search for mitosis- and meiosis-specific binding partners of Dma1 might be able to help us to clarify this issue more thoroughly.

The SPB plays two different roles during meiosis, one is for microtubule assembly and the other for construction of the FSM (reviewed in Shimoda, 2004). The latter function is accompanied by meiotic SBP modification. Although many SBP proteins develop a characteristic crescent shape during the late stages of meiosis as the outer plaque is remodeled to promote spore formation, the SBP-localized SIN proteins and Dma1 do not show this shape (Krapp et al., 2006 and this study). Moreover, neither SBP modification nor initiation of the FSM formation is apparently impaired when SIN signaling is compromised or when dma1Δ is absent (Krapp et al., 2006; Krapp et al., 2010; and this study). These observations suggest that both Dma1 and SIN proteins do not necessarily associate with the modified SBP in order to fulfill their functions in FSM development.

Similarly to Dma1, the meiosis-specific kinase Slk1/Mug27 has been shown to be positively required for sporulation in fission yeast (Ohtaka et al., 2008; Perez-Hidalgo et al., 2008; Yan et al., 2008; and this study). However, one puzzling observation in our study is that Slk1 is somehow negatively regulated by Dma1 and a similar result has been reported recently by others (Krapp et al., 2010). As compared with wild-type cells, we could detect stronger signals of Slk1-GFP at SBPs and elevated overall protein level in dma1Δ cells. Our result showing the coexistence of both Dma1 and Slk1 in a immunoprecipitated complex during meiosis suggests it is probably related to their functional connection. However, forced overexpression of Slk1 did not cause any obvious sporulation defects as observed in dma1Δ cells, so the elevated Slk1 level does not appear to be the direct cause of the sporulation defect in dma1Δ cells, and Dma1 may act through multiple partners in addition to Slk1. Alternatively, it is possible that in certain circumstances, Dma1 may negatively fine tune the signals from Slk1 for the process of FSM formation. Dma1 has long been considered to be a potential E3 ubiquitin ligase since its discovery (Murone and Simanis, 1996; Guertin et al., 2002b), as it contains a RING-H2 finger domain which is present in some confirmed E3 ubiquitin ligases. Whether the RING finger domain in Dma1 carries E3 ubiquitin ligase activity and whether this activity is directly required for its function in sporulation regulation are interesting questions for future studies to answer. Our observation that RING finger-deleted Dma1 cannot faithfully support sporulation is consistent with this possibility. If Dma1 indeed played roles in negatively regulating some proteins’ level in sporulation through its potential ubiquitin E3 ligase activity, then the identification of candidate molecules will be an important direction for future studies.
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