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Meiotic actin rings are essential for proper sporulation in fission yeast

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Summary

Sporulation is a unique form of cytokinesis that occurs following meiosis II in many yeasts, during which four daughter cells (spores) are generated within a single mother cell. Here we characterize the role of F-actin in the process of sporulation in the fission yeast Schizosaccharomyces pombe. As shown previously, we find that F-actin assembles into four ring structures per ascus, referred to as the meiotic actin ring (MeiAR). The actin nucleators Arp2/3 and formin For3 assemble into ring structures that overlap with Meu14, a protein known to assemble into the so-called leading edge, a ring structure that is known to guide forespore membrane assembly. Interestingly, F-actin makes rings that occupy a larger region behind the leading edge ring. Time-lapse microscopy showed that the MeiAR assembles near the spindle pole bodies and undergoes an expansion in diameter during the early stages of meiosis II, followed by closure in later stages of meiosis II. MeiAR closure completes the process of forespore membrane assembly. Loss of the MeiAR leads to excessive assembly of forespore membranes with a deformed appearance. The rate of closure of the MeiAR is dictated by the function of the septation initiation network (SIN). We conclude that the MeiAR ensures proper targeting of the membrane biogenesis machinery to the leading edge, thereby ensuring the formation of spherical spores.

Key words: Fission yeast, Meiotic actin ring, SIN, Cytokinesis

Introduction

In many organisms, cytokinesis is achieved through the use of an actomyosin ring containing F-actin, type II myosin and several other proteins (Balasubramanian et al., 2004; Fujiwara and Pollard, 1976; Fujiwara et al., 1978; Glotzer, 2001; Glotzer, 2005; Schroeder, 1968; Schroeder, 1973). The contraction of actomyosin rings is presumed to guide membrane assembly during cytokinesis (Dobbelaere and Barral, 2004). In recent years, the fission yeast Schizosaccharomyces pombe has been used as a model organism for the study of cytokinesis, because of the ease with which genetic and cell biological analysis are applied in this organism (Gould and Simanis, 1997; McCollum and Gould, 2001; Wu and Pollard, 2005; Wu et al., 2006). During mitosis, fission yeast cells divide through the use of an actomyosin-based contractile ring (Balasubramanian et al., 1992; Balasubramanian et al., 1994; Balasubramanian et al., 1998; Chang et al., 1997; Eng et al., 1998; Fankhauser et al., 1995; Le Goff et al., 2000; Marks et al., 1986; McCollum et al., 1995; Naqvi et al., 2000; Wong et al., 2000; Wu et al., 2003). The actomyosin ring is assembled at the onset of mitosis and constricts concomitant with the centripetal addition of new membranes and the division septum at the end of mitosis (Balasubramanian et al., 2004; Wu and Pollard, 2005; Wu et al., 2006).

In fission yeast, contraction of the actomyosin ring, as well as the formation of new membrane and the division septum, are regulated by a signaling cascade termed the septation initiation network (SIN) (McCollum and Gould, 2001; Simanis, 2003); the functional equivalent in budding yeast is called the mitotic exit network (MEN) (Balasubramanian et al., 2004; Bardin and Amon, 2001). The SIN is a GTPase-regulated protein kinase cascade consisting of three protein kinases (Cdc7, Sid1 and Sid2) and one small GTPase, Spg1 (Fankhauser and Simanis, 1994; Guertin et al., 2000; Hou et al., 2000; Schmidt et al., 1997; Sparks et al., 1999). Mob1 and Sid2 form a complex and are downstream of the SIN pathway (Balasubramanian et al., 2004; Hou et al., 2000). Cdc16 and Byr4 function as part of a two-component GTPase-activating protein (GAP) that inhibits Spg1 (Furge et al., 1998; Furge et al., 1999; Li et al., 2000). All of the SIN components seem to be anchored to the spindle pole body (SPB) through the SIN scaffold complex containing Sid4 and Cdc11 (Krapp et al., 2001; Li et al., 2000; Tomlin et al., 2002).

Interestingly, during meiosis, a different type of cytokinesis regulates the formation of gametes (spores) in S. pombe and several other yeasts and fungi (Neiman, 2005; Shimoda, 2004). An extensive body of genetic and genomic approaches has led to the identification of the SPO (sporulation defective) and MEU (meiotic expression upregulated) genes, products of which have been shown to be important for forespore membrane assembly and proper sporulation (Hirata and Shimoda, 1992; Yamamoto, 1996). Characterization of the MEU gene products has revealed an important role for the leading edge proteins (LEPs), such as Meu14, which assembles into a pair of rings per mitotic spindle and guides forespore membrane assembly (Okuzaki et al., 2003). In addition, analysis of many of the SPO and MEU gene products has led to the identification of elements of the membrane trafficking machinery, such as v-SNAREs and t-SNAREs that specifically function during forespore membrane assembly.
Recent studies have shown that, as in mitosis, sporulation is dependent on the SIN. SIN proteins localize to SPBs during meiosis and the pathway is activated during the second meiotic division. In some SIN mutant cells, the forespore membrane (FSM) assembly is defective, resulting in unencapsulated nuclei (Krapp et al., 2006; Ohtaka et al., 2008; Perez–Hidalgo et al., 2008; Yan et al., 2008). Although SIN has been analyzed, it is not fully understood how F-actin participates in this process and whether SIN regulates actin cytoskeletal function during meiosis.

In this study, we have characterized the dynamics and function of the actin cytoskeleton during meiosis and sporulation. We characterize the meiotic actin ring (MeiAR), which contains F-actin and its nucleators, and appears to participate in proper assembly of the forespore membrane. We also report that the rate of closure of the meiotic actin ring is strongly influenced by the SIN.

Results
F-actin assembles into ring structures during sporulation

Previous studies have shown that F-actin assembles into ring-like structures during meiosis II in fission yeast (Itadani et al., 2006; Ohtaka et al., 2007; Petersen et al., 1998). Consistent with this, we found that F-actin assembles in rings in meiosis II (Fig. 1A). Staining of fixed wild-type cells with FITC–phalloidin and DAPI revealed a striking array of F-actin arrangements. In cells undergoing horsetail movement (Fig. 1A, cell 1) of chromosomes, F-actin was detected in patches throughout the forming ascus. Similarly, F-actin was detected in patches in cells that had completed meiosis I (Fig. 1A, cells 2 and 3). Around the time of initiation of chromosome segregation in meiosis II (Fig. 1A, cells 4 and 5), F-actin started to accumulate near the nuclei. In cells in which chromosome segregation had initiated during meiosis II (Fig. 1A, cells 6 and 7), F-actin organization was apparent as four ring structures, which we term meiotic actin rings (MeiARs). Following full segregation of chromosomes (Fig. 1A, cells 8–12) the diameter of the F-actin rings decreased. In cells that had completed sporulation (Fig. 1A, cell 13), F-actin was detected in randomly organized patches.

Because previous studies had not investigated the dynamics of F-actin during sporulation, we used green fluorescent protein (GFP) fused to the calponin homology domain (CHD) of Rng2 to detect the dynamics of F-actin ring assembly (Karagiannis et al., 2005). The GFP–CHD fusion, which binds to all F-actin components, has been used previously to study the dynamics of F-actin in the mitotic cell cycle (Karagiannis et al., 2005). We also performed live-cell imaging to analyze the spatial relationship between F-actin and Meu14 (a marker of the leading edge) during sporulation. Before meiosis II, GFP–CHD patches were randomly distributed in the cytoplasm (Fig. 1B, 20 minutes, −3 minutes). At metaphase II, the number of GFP–CHD patches in the cytoplasm gradually decreased, whereas bright GFP–CHD signals started to accumulate at each of the four SPBs (Fig. 1B, Fig. 1C, visualized with the nuclear envelope marker Cut11–mCherry). Subsequently, the four bright GFP–CHD dots each formed a donut-like structure, indicative of the initiation of assembly of the FSM (Fig. 1B). During this stage, the size of the GFP–CHD rings began to expand to the maximum diameter (Fig. 1B) (0–20.5 minutes) and GFP–CHD overlapped with the leading edge ring protein marker Meu14–mCherry (Fig. 1C, 0–20 minutes, bottom panel). However, after anaphase II (Fig. 1C), the GFP–CHD rings started to shrink in diameter until they disappeared completely, which indicates closure of the FSMs (Fig. 1B, 20.5–58.5 minutes and 1C, 24–66 minutes). During this shrinking stage, the fluorescence of the ring-like structure of GFP–CHD was rather broad and followed the leading edge Meu14 rings (Fig. 1C, right panel). Finally, the GFP–CHD diffused into each newly formed spore (Fig. 1B, 61.5 minutes and 1C, 72 minutes). The results are shown schematically (Fig. 1D). These studies established that F-actin assembled into rings that follow the leading edge during FSM assembly.

Actin nucleators and actin interactors localize to the MeiAR

Assembly of actin filaments from their monomeric subunits requires the actin nucleators Arp2–Arp3 (Arp2/3) and formins (Baum and Kunda, 2005; Campellone and Welch, 2010; Chesarone and Goode, 2009). The Arp2/3 complex is known to nucleate and organize actin into a dense network of short, branched actin filaments (Goley and Welch, 2006). By contrast, formins are required for unbranched actin cable formation during interphase and mitosis (Chang et al., 1997; Evangelista et al., 2003; Feierbach and Chang, 2001; Pryune et al., 2002). The mYFP–Arp2/3 complex (Wu and Pollard, 2005) colocalized with Meu14–mCherry at the leading edge throughout the entire process of FSM assembly (Fig. 2A). For3–GFP also colocalized and constricted in concert with the Meu14–mCherry rings (Fig. 2A). The type I myosin Myo1 is known to recruit the Arp2/3 complex, and is involved in actin polymerization during mitosis (Itadani et al., 2006; Lee et al., 2000). Consistently, Myo1 was also detected at the leading edge (Fig. 2B). We noticed that a significant fraction of F-actin in the MeiAR trailed the leading edge (Fig. 2A–C). These experiments suggested that F-actin polymerized at the leading edge potentially through the action of the nucleators such as Arp2/3 complex and the formin For3.

We then tested whether other known actin cytoskeletal regulators and cytokinetic proteins localized to the MeiAR. Of the proteins tested, we found that the fimbrin Fim1 (Nakano et al., 2001; Wu et al., 2001) and Sla2 (an actin-patch protein related to budding yeast End4) (Castagnetti et al., 2005; Ge et al., 2005; Gourlay et al., 2003; Holtzman et al., 1993) localized to the MeiAR (Fig. 2D). Interestingly, the myosin regulatory light chain Rlc1 was also detected at the MeiAR (Fig. 2E), although other actomyosin ring components, such as Rng2–IQGAP, the F-BAR protein Cdc15, the UCS protein Rng3 and the formin Cdc12 were not readily detected at the MeiAR.

For3 and Arp2/3 are important for MeiAR assembly and function

We then set out to understand the role of the several of the components of the MeiAR in forespore membrane assembly and sporulation. To this end, we first tested the role of For3 in sporulation. Whereas wild-type cells assembled spherical spores of roughly equal size (Fig. 3A), for3Δ cells generated spores with an abnormal morphology and size (Fig. 3A). These effects were more obvious when GFP–Psy1 was used as a marker for spore membranes (Fig. 3A, bottom panels). In this analysis we found
that whereas wild-type spores had smooth membranes underlying the spherical spores, $for3\Delta$ spores were of abnormal morphology and had small protrusions of membranous material (Fig. 3A,B). Time-lapse microscopy showed that the protrusions of membranous material arose around the end of forespore membrane assembly (Fig. 3C).

To test whether the observed effects were related to MeiAR assembly and function defects in $for3\Delta$ cells, we imaged the
Fig. 2. Localization of actin nucleators and interactors to the leading edge during FSM formation in *S. pombe*. (A) Time-lapse images showing the colocalization of For3–GFP (green) and Meu14–mCherry (red) (top panel, MBY6237), mYFP–Arp3 (green) and Meu14–mCherry (red) (middle panel, MBY6228) and mYFP–Arp2 (green) and Meu14–mCherry (red) (bottom panel, MBY6206) in cells undergoing FSM formation. Enlarged images (right panels) show that mYFP–Arp2, mYFP–Arp3 and For3–GFP colocalized with Meu14–mCherry at the leading edge of FSM at late meiosis II, respectively. (B) Time-lapse images showing the colocalization of Myo1–GFP (green) with Meu14–mCherry (red) at the leading edge in cells (MBY6048) undergoing FSM assembly. Enlarged image (right panel) shows that Myo1–GFP colocalized with Meu14–mCherry at the leading edge of FSM at late meiosis II. (C) Time-lapse images showing the colocalization of GFP–CHD (green) with Arp3–mCherry (red) at the leading edge of FSM at late meiosis II. (D) Time-lapse images showing the colocalization of Fim1–GFP (green) with Meu14–mCherry (red) (top panel, MBY7589), Sla2–GFP (green) and Meu14–mCherry (red) (bottom panel, MBY7591) at the leading edge in cells undergoing FSM assembly. Enlarged images (right panel) show that Fim1–GFP, Sla2–GFP colocalized with Meu14–mCherry at the leading edge of FSM at late meiosis II. Images were taken using an inverted confocal microscope except that For3–GFP image in A (bottom panel), Fim1–GFP and Sla2–GFP images in D were taken using a spinning-disk confocal microscope. (E) Rlc1–3GFP colocalizes with Meu14–mCherry to the leading edge in the meiotic cells (MBY7609). Arrows show the meiotic cells. Images were taken using an Olympus microscope. Scale bars: 10 μm.
behaviour of F-actin in wild-type and for3Δ cells undergoing meiosis and sporulation. As described earlier (Fig. 1B) GFP–CHD assembled into MeiARs that underwent normal assembly and constriction (Fig. 3D). F-actin cables were also detected in wild-type cells (Fig. 3D, arrows). By contrast, MeiARs were significantly smaller in diameter in for3Δ cells (Fig. 3D,E) and

Fig. 3. For3 is required for proper sporulation. (A) Abnormally shaped spores and small protrusions of membranous material were observed in for3Δ spores. Wild-type (MBY3664) and for3Δ cells (MBY7583) expressing GFP–Psy1 were induced to undergo meiosis at 31°C for 2 days. Arrows show the protrusions of GFP–Psy1 in asci. (B) Quantification of GFP–Psy1 morphology in wild-type (MBY3664) and for3Δ asci (MBY7583). Cells were induced to undergo meiosis on YPD plates at 31°C for 24 hours. n≥500 for each genotype. (C) Time-lapse microscopy of GFP–Psy1 in for3Δ (MBY7583) shows that the protrusions of membranous material arise around the end of FSM assembly. Images were taken at 31°C. The GFP–Psy1 formed cup-shaped and round FSMs (0–18 minutes) but failed to terminate the assembly of FSMs (24–96 minutes), resulting in the protrusion of membranous material around the end of the FSM assembly (102 minutes). Arrows show the protrusions of GFP–Psy1. Images were taken using a spinning-disk confocal microscope. (D) Time-lapse images showing the dynamics of GFP–CHD in wild-type (MBY5998) and for3Δ cells (MBY6030) undergoing meiosis and sporulation. Arrows show F-actin cables detected during FSM formation in the wild-type cell. By contrast, actin cables were undetectable in for3Δ cells. Images were taken using a spinning-disk confocal microscope. (E) Comparison of the maximal diameter of GFP–CHD rings between wild-type (MBY5998) and for3Δ (MBY6030) cells. The maximum diameter of GFP–CHD rings was measured by MetaMorph software. n≥60 for each genotype. Means and s.d. are represented. (F) Graph shows the duration of the two phases of GFP–CHD assembly and disassembly depicted in the upper panel of F in wild-type (MBY5998) and for3Δ (MBY6030) cells. The MeiAR formation process was divided into two phases as follows: Phase I, from the initiation of MeiAR formation to the time at which the diameter of the MeiAR is maximal; Phase II, the time at which the large MeiAR begins to reduce in size until MeiAR forms a dot (≤1 µm). The depictions indicate the dynamics of GFP–CHD rings (green) and Cut11–mCherry (black) during FSM formation. n≥30 for each genotype. Means and s.d. are represented. Scale bars: 10 µm.
actin cables were undetectable (Fig. 3D). In addition, the time taken for the assembly and disassembly of MeiARs was significantly increased in for3Δ cells (Fig. 3F, assembly is indicated as Phase I and disassembly is indicated as Phase II). These experiments established that For3 function was important, although not essential, for aspects of MeiAR assembly, function and proper sporulation.

Having established that For3 was important for MeiAR function, we investigated whether the Arp2/3 complex was important for MeiAR function and/or sporulation. To address this question we tested the ability of arp3-c1 cells to sporulate at the restrictive temperature of 19°C (McCollum et al., 1996). Although arp3-c1 cells were capable of performing meiosis I and II, as evidenced from the presence of tetranucleated asci, spores were not detected in these asci (Fig. 4A). Unlike in wild-type cells (Fig. 1A), F-actin was not observed in MeiARs, establishing that Arp2/3 complex was also important for MeiAR assembly and/or its maintenance. Collectively, these experiments establish that actin is polymerized at the leading edge of the MeiAR and that the nucleators and F-actin at the MeiAR participate in proper sporulation.

Because the regulatory light chain for myosin II, Rlc1, was detected at the MeiAR, we addressed whether myosin II might power constriction of the MeiAR. Rlc1 is known to interact with two myosin II heavy chains, Myo2 and Myp2 (Le Goff et al., 2000; Moteigi et al., 2004; Naqvi et al., 2000). Meiocytes of the temperature sensitive myo2-E1 mutant and the myp2Δ null mutant were able to assemble spores of normal appearance (Fig. 4B,C,D). Owing to the synthetic lethality of the myo2-E1 mpy2Δ double mutants, we have been unable to investigate potential sporulation defects in these double mutants. Our studies therefore established that Myo2 and Myp2 were individually dispensable for sporulation.

**F-actin is required for coordination of FSM assembly with progression of the leading edge**

To further investigate the role of F-actin in sporulation, we treated cells with Latrunculin A (Lat A) to prevent actin polymerization (Aysscoug et al., 1997) and carried out time-lapse analysis during sporulation. We compared the morphologies of the FSM and the nucleus in DMSO and Lat A treated cells by live examination of the dynamics of GFP–Psy1, Meu14–mCherry and mCherry–Atb2. Psy1 is a fission yeast homolog of mammalian syntaxin 1A, which is a t–SNARE (soluble NSF attachment protein receptor) protein involved in membrane assembly (Maeda et al., 2009; Nakamura et al., 2001; Nakamura et al., 2008; Shimoda, 2004). Psy1 disappears from the plasma membrane upon exit from meiosis I and reappears in the nascent FSMs (Maeda et al., 2009; Nakamura et al., 2001; Nakamura et al., 2008). Thus, monitoring the movement of GFP–Psy1 allowed us to visualize the process of FSM formation. Observation of the mCherry–Atb2 allowed us to monitor the progression through the meiotic cell cycle.

First, we established that Lat A treatment led to a complete dispersal of actin nucleators For3 and Arp2/3 from the leading edge as well as the loss of all detectable F-actin and the MeiAR (supplementary material Fig. S1). Time-lapse movies showed that, in comparison to DMSO-treated cells, the localization of GFP–Psy1 was significantly different in cells treated with Lat A. DMSO-treated cells developed FSMs marked by Psy1 and formed four nucleated forepores (Fig. 5A). In cells undergoing meiosis II, treatment with Lat A led to excessive proliferation of FSMs whose geometry was that of abnormal spheres in which several bubble-like outgrowths were noticed (Fig. 5B). Interestingly, these bubble-like structures were visualized just at the point of the closure of FSM (Fig. 5B, arrows). The bubble structures extended to form long tubes and stopped growing at around the time spore walls would have been deposited. Normal meiotic spindles were detected in Lat-A-treated cells, ruling out the possibility that improper FSM growth was a consequence of abnormal mitosis (Fig. 5B). To understand the detailed morphology of the bubble structures, we examined them by electron microscopy (EM). EM observation in wild-type cells revealed four normal spores that encapsulate nuclei, rough endoplasmic reticulum, Golgi complexes and mature spore walls.
By contrast, in Lat-A-treated cells, many of the bubbled spores containing haploid nuclei retained a canal-like passage of cytoplasm between the mother and daughter spores (Fig. 5D, ii) and displayed ruffled, seemingly fragile spore walls (Fig. 5D, iii). Moreover, the fragile spore walls did not stain with Anillin Blue, implying defects in spore wall formation and/or maturation (data not shown). All these experiments established that a key role of MeiAR during meiosis II is to coordinate the FSM assembly with the progression of the leading edge.

Loss of SIN affects MeiAR dynamics

During cytokinesis coupled with progression of mitosis in vegetative cells, the SIN is required for actomyosin ring maintenance, new membrane formation and cell wall assembly (Balasubramanian et al., 2004; Krapp et al., 2004; Krapp and Simanis, 2008; McCollum and Gould, 2001). Given that the maintenance and constriction of the actomyosin ring in mitosis depended on SIN, we assessed whether the rate of progression or constriction of the MeiAR depended on the SIN.
To address this question, we made use of a strain in which the gene encoding Sid4 was placed downstream of the promoter of the rad21 gene, which is turned off during meiosis. In previous work, we have shown that such cells assemble four abnormal spores, two of which are larger and the other two smaller than spores in wild-type cells (supplementary material Fig. S2) (Yan et al., 2008). In fixed $P_{rad21-sid4}$ cells, two of the four MeiARs were larger than the other two in later stages of meiosis II (Fig. 6A), consistent with a role for SIN in MeiAR progression and constriction.

Fig. 6. SIN is required for MeiAR constriction. (A) F-actin localization during anaphase II in representative $P_{rad21-sid4}$ cells (MBY3507). F-actin was stained with phalloidin (green) and DNA was stained with DAPI (red) in fixed $P_{rad21-sid4}$ cells. Two types of MeiARs with different diameters are shown: smaller, normal constriction MeiARs (arrows) and broad, slow constriction MeiARs (asterisks). (B) Time-lapse images show the dynamics of GFP–CHD at the leading edge in wild-type cells (top panel, MBY6896) and $P_{rad21-sid4}$ cells (bottom panel, MBY6002). Top panel: each of the four MeiARs present in an ascus constricted with similar kinetics in wild-type cells (arrows), whereas the other two were significantly slower (asterisks). The time, at which GFP–CHD ring (MeiAR) had the maximal diameter is indicated as 0 minute. (C) Graph shows the quantification of the diameters of the GFP–CHD rings as a function of time in wild-type (MBY5998), $P_{rad21-sid4}$ (normal MeiAR constriction, abnormal MeiAR constriction) (MBY6002) and $sid2-250 slk1\Delta$ cells from maximal diameters to the closure of the GFP–CHD rings. The diameter of GFP–CHD rings was measured using MetaMorph software ($n=30$ for each genotype). Means and s.d. are represented. The depictions indicate the dynamics of GFP–CHD rings (green) and Cut11–mCherry (black) during FSM formation in wild-type, $P_{rad21-sid4}$ and $sid2-250 slk1\Delta$ cells. (D) Time-lapse images show the dynamics of Arp3–mCherry and For3–GFP at the leading edge in $P_{rad21-sid4}$ cells. Two types of MeiAR constriction are shown: two of the MeiARs in each genotype cell constricted with kinetics similar to wild-type cells (arrows), whereas the other two were significantly slower (asterisks). Images were taken by a spinning disk confocal except images in A were taken by an inverted confocal microscope. Scale bars, 10 µm.
To understand the dynamics of the MeiAR in P_{rad21-sid4} cells, we carried out time-lapse imaging experiments. In wild-type cells, each of the four MeiARs present in an ascus constricted with similar kinetics such that the closure of the MeiAR, from approximately a diameter of 3.1 μm to a diameter of under 1 μm took roughly 21 minutes (Fig. 6B,C). By contrast, in P_{rad21-sid4} cells, two of the MeiARs constricted with kinetics similar to wild-type cells, whereas the other two were significantly slower. The diameter of the two slower MeiARs only decreased to approximately 1.6 μm in an even longer time frame (Fig. 6C).

In addition to the SIN scaffold protein mutants, we tested whether downstream elements of the SIN were involved in regulating MeiAR dynamics. To this end, actin dynamics was tested in double mutants defective in the two SIN kinases Sid2 and Slk1 that function at the end of the SIN cascade during meiosis (Ohlta et al., 2008; Perez-Hidalgo et al., 2008; Yan et al., 2008). A similar slow-down of MeiAR constriction was also detected in sid2-250 slk1A cells (Fig. 6C; supplementary material Fig. S3). Collectively, the analysis of the MeiAR structure containing F-actin that assembles during sporulation, although the dynamics of this structure have not been studied in detail (Itadani et al., 2006; Petersen et al., 1998). We have confirmed and extended these studies, through the dynamic analysis of additional actin cytoskeletal proteins and the role of the SIN.

We have shown that the MeiAR consists of among others, F-actin, its nucleators and binding proteins. Previous studies have identified the presence of a ring-like structure containing F-actin that assemblies during sporulation, although the dynamics of this structure have not been studied in detail (Itadani et al., 2006; Petersen et al., 1998). We have confirmed and extended these studies, through the dynamic analysis of additional actin cytoskeletal proteins and the role of the SIN.

We have shown that the MeiAR consists of among others, F-actin, its nucleators For3 and Arp2/3 and actin interactors Myo1, Sla2, Fim1. In addition, previous work has shown that the calmodulin-like protein Cam2 and Myo1 localize to the MeiAR (Itadani et al., 2006; Toya et al., 2001). Interestingly, although For3 and Arp2/3 colocalize with the leading-edge protein Meu14, F-actin itself tracks this structure. The presence of the nucleating proteins at the leading edge suggests that the MeiAR might be composed of de novo generated actin filaments rather than those redistributed from other regions of the cell. The fact that For3 and Arp2/3 localize to the ring suggests that linear actin cables and branched actin filaments are nucleated in the vicinity of the leading edge, and the assembled F-actin tracks the leading edge. Detailed imaging using electron microscopy using myosin head S1 decoration will be necessary to establish the organization of actin filaments in the MeiAR (Kamasaki et al., 2007). Because proteins such as Myo1 and Sla2 localize to the MeiAR, it is likely that the MeiAR is also a site for endocytic events (Ge et al., 2005; Toya et al., 2001). Myo1 has been shown to participate in actin polymerization, through Arp2/3 and it is therefore also possible that Myo1 facilitates MeiAR assembly. The sporulation-defective phenotype of myo1 mutants is consistent with both possible molecular roles of Myo1.

We have shown that loss of function of For3 leads to defects in the assembly, maintenance and constriction of the MeiAR, which in turn generates spores of abnormal morphology. Because for3A cells are also defective in actin cable (non-MeiAR) assembly, it is possible that the loss of actin cables generates the sporulation defective phenotype. We believe this is unlikely because many properties of the MeiAR (such as time taken for assembly and disassembly and the diameter of the ring) are disturbed in for3A cells. Although we have been unable to carry out time-lapse imaging experiments on arp3-c1 cells, as a result of technical limitations, we have shown that loss of Arp2/3 complex function leads to destabilization of the MeiAR and leads to sporulation defects. Collectively, these experiments establish that linear actin cables and branched actin filaments, generated at the MeiAR, are important for proper sporulation.

We have shown that the regulatory light chain of myosin II, Rcl1p, localizes to the MeiAR. Rcl1 is known to bind Myo2 and Myp2, the two proteins related to myosin II heavy chains in fission yeast (Le Goff et al., 2000; Motegi et al., 2000; Naqvi et al., 2000). Cells defective individually for Myo2 and Myp2 function are able to generate normal spores, suggesting that actomyosin contractility, as observed in mitotic cells, might not operate during cytokinesis. However, we are unable to confirm this effect because of the synthetic lethality observed in myo2-E1 myp2A double mutants. It is also possible that Rcl1p might partner other myosins (rather than myosin II) to facilitate sporulation.

What then is the function of the MeiAR? We have shown that the loss of formin For3 leads to abnormally shaped spores, which results from improper forespore membrane assembly. We have also shown that arp3-c1 cells are defective in MeiAR assembly and sporulation. Compelling evidence for the role of F-actin and the MeiAR in sporulation was obtained by analyzing cells treated with the actin polymerization inhibitor Lat A. In Lat-A-treated cells, we noticed that FSM assembly was not arrested by the absence of F-actin. Rather, we found that the integrity of the leading edge ring (visualized by using Meu14) was compromised, leading to excessive proliferation of disorganized forespore membranes containing Psy1–syntaxin in cells devoid of detectable F-actin. Thus the primary function of the MeiAR might be to reinforce the organization and stability of the leading edge ring.

Studies of the contractile actomyosin ring in mitotic cells have shown that the stability of these rings and their constriction are dependent on the SIN (Balasubramanian et al., 2004; Hachet and Simanis, 2008; Mishra et al., 2004; Wu et al., 2006). Curiously, we have found that the rate of migration of the leading edge was significantly reduced in meiotic cells defective in SIN, leading to abnormal FSM assembly and sporulation. These observations reveal that despite the differential nature of cytokinesis in mitotic and meiotic cells, SIN might play an important role in coordinating membrane assembly events with actin cytoskeletal dynamics. Future studies should address whether SIN kinases phosphorylate related substrates in mitosis and meiosis to coordinate membrane assembly with actin dynamics.

Materials and Methods

Yeast strains, media and culture conditions
Fission yeast cells were grown on YES medium or minimal medium with appropriate supplements (Moreno et al., 1991). Yeast strains were constructed by either random spore germination method or by tetrad analysis. Growth temperatures were 24°C (permissive) for temperature-sensitive strains, and 30°C for all other strains. S. pombe cells were sporulated at 30°C, except for temperature-sensitive strains which were sporulated at 24°C, 19°C or 34°C in different experiments. Solid yeast extract peptone dextrose (YPD) medium or EMM5S medium (for nmt1-gfp-chd) were used for inducing meiosis. Strains used in this study are listed in supplementary material Table S1.
F-Actin and DAPI staining  
Fission yeast cells, grown and induced to enter meiosis on solid YPD medium, were fixed with 4% paraformaldehyde at the growth temperature for 15 minutes. Fixed cells were then rinsed three times with phosphate-buffered saline (PBS). To stain DNA and F-actin, fixed cells were permeabilized with 1 × PBS, pH 7.4, solution containing 1% Triton X-100 for 5 minutes, followed by three washes with 1 × PBS at room temperature. F-actin specific 2-photon fluorescence (DAPI) was added to the cell suspension to visualize the nuclei. Alexa-Fluor-488-conjugated phalloidin at a final concentration of 100 ng/ml was used to stain F-actin.

Microscopy and data analysis  
For time-lapse microscopy, cells were induced to undergo meiosis on solid YPD medium or EMMS5 (for strains containing mtd1Δ–gfp–cod1Δ) for 16 hours at 28°C or 24 hours at the permissive temperature for temperature-sensitive strains. Cells were spotted on a glass slide containing a 2% agar pad. For Latrunculin A (Lat A) treatment experiments, cells were treated with 100 μM Lat A or equal volume of DMSO at room temperature for 20 minutes, and placed on EMMS5 + 2% agar pads (containing 100 μM Lat A or DMSO). Time-lapse analysis was conducted at room temperature (22–24°C).

We used a 100 × 1.4 NA objective lens (Nikon, Melville, NY) on a spinning disc confocal microscope (UltraView ERS, PerkinElmer Life And Analytical Sciences, Waltham, MA) with 488 nm and 561 nm lasers and a cooled charge-coupled device camera (ORCA-AG, Hamamatsu, Bridgewater, NJ). Maximum intensity projections of color images were created using MetaMorph software. Images in figures are maximum intensity projections of Z-sections spaced at 0.5 μm. For confocal microscopy (Zeiss LSM10; Thornwood, NY), images were taken with a 100 × 1.4 NA PlanApo objective lens and analyzed with LSM 5 browser software. Z-stack images were taken at 0.5 μm intervals and reconstructed in three dimensions using the projection module. Some microscopic images were captured using Nikon DX71 fluorescence microscope equipped with a Photometrics Cool SNAP ES camera (Tucson, AZ).

Construction of arp3–mCherry, cut11–mCherry and meu14–mCherry  
To generate fusion of arp3, cut11 and meu14 with mCherry at the chromosome locus, DNA fragments containing the C-terminus of arp3, cut11, meu14 were first amplified by PCR and were cloned into pJK210–mCherry (pCDL1207), yielding pJK210–arp3–mCherry (pCDL1524), pJK210–cut11–mCherry (pCDL1526) and pJK210–meu14–mCherry (pCDL1527), respectively. These plasmids were linearized and were transformed into the wild-type strain (MBY1238). Cells were incubated on YPD plates at 28°C for 20 hours. Cells were collected and resuspended in 2% DMSO at room temperature for 20 minutes, and placed on EMMS5 + 2% agar pads containing 100 μM Lat A or DMSO. Time-lapse analysis was conducted at room temperature (22–24°C).

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**Table S2. Primers and plasmids used in this study**

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