The role of *Schizosaccharomyces pombe dma1* in spore formation during meiosis

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Summary
Meiosis is a specialised form of the cell cycle that gives rise to haploid gametes. In *Schizosaccharomyces pombe*, the products of meiosis are four spores, which are formed by encapsulation of the four meiosis II nuclei within the cytoplasm of the zygote produced by fusion of the mating cells. The *S. pombe* spindle pole body is remodelled during meiosis II and membrane vesicles are then recruited there to form the forespore membrane, which encapsulates the haploid nucleus to form a prespore. Spore wall material is then deposited, giving rise to the mature spore. The septation initiation network is required to coordinate cytokinesis and mitosis in the vegetative cycle and for spore formation in the meiotic cycle. We have investigated the role of the SIN regulator dma1p in meiosis; we find that although both meiotic divisions occur in the absence of dma1p, asci frequently contain fewer than four spores, which are larger than in wild-type meiosis. Our data indicate that dma1p acts in parallel to the leading-edge proteins and septins to assure proper formation for the forespore membrane. Dma1p also contributes to the temporal regulation of the abundance of the meiosis-specific SIN component mug27p.

Key words: Meiosis, Spore formation, Dma1, Fission yeast

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Introduction
Meiosis is a form of the cell cycle that gives rise to haploid gametes after recombination and reassortment of the genome. In *Schizosaccharomyces pombe*, the products of meiosis are four spores that are highly resistant to environmental insults. Under starvation conditions, cells of opposite mating types undergo a pheromone-dependent arrest of their cell cycles in G1, followed by cytoplasmic and nuclear fusion. Cells then commit to the meiotic cell cycle and undergo premeiotic DNA synthesis, recombination, two meiotic divisions and then form four haploid spores (for reviews, see Shimoda, 2004; Shimoda and Nakamura, 2004; Yamamoto, 2004). Filming of spore formation in living cells (Nakamura et al., 2008), coupled with genetic and cytological analysis, has given rise to a model for spore formation. During meiosis II, the spindle pole body (SPB) changes its appearance from a dot to a crescent shape (Hagan and Yanagida, 1995). This requires the products of the *spo2*, *spo13* and *spo15* genes (Ikemoto et al., 2000; Nakase et al., 2008). Membrane vesicles are recruited to the modified SPB and fuse to give rise to the forespore membrane (FSM), which encapsulates the haploid nucleus to form the prespore. Components such as meu14p, found at the leading edge of the spore, are involved in its closure (Okuzaki et al., 2003). The leading edge proteins (LEPs) function in parallel with the meiotic septin complex to orient FSM extension (Onishi et al., 2010). Spore wall material is then deposited between the membranes to form the spore (Tanaka and Hirata, 1982; Yoo et al., 1973).

The SIN and its regulators
During vegetative growth, cells of the fission yeast *S. pombe* divide by medial fission. A contractile ring (CAR) is assembled at the centre of the cell during mitosis; at the end of anaphase, CAR contraction guides synthesis of the septum that bisects the cell. A group of protein kinases called the 'septation initiation network' (SIN) is essential for cytokinesis. Loss of SIN signalling produces multinucleate cells, whereas constant activation of the SIN results in multiseptated cells (Krapp and Simanis, 2008). The SIN also collaborates with the anillin-related protein mid1p to promote CAR assembly early in mitosis (Hachet and Simanis, 2008; Huang et al., 2008). The SIN and mid1p are both regulated by plo1p (Bahler et al., 1998a; Tanaka et al., 2001), which might provide global coordination of cytokinesis and mitosis (for a review, see Roberts-Galbraith and Gould, 2008). The SIN also plays an essential role during the meiotic cell cycle (Krapp et al., 2006; Yan et al., 2008); SIN mutants complete apparently normal meiotic nuclear divisions but fail to form spores, suggesting a common function for the SIN in coordinating septation and nuclear division in the mitotic cycle, and spore formation with nuclear division in the meiotic cycle.

SIN signalling originates from the spindle pole body (SPB) and is modulated by the nucleotide status of the GTPase spg1p (Schmidt et al., 1997; Sohrmann et al., 1998). This is determined by the balance of spontaneous nucleotide exchange, a putative GEF, etd1p (Garcia-Cortes and McCollum, 2009), and a GTPase-activating protein (GAP), cdc16p (Fankhauser et al., 1993; Minet et al., 1979), with which spg1p interacts through a scaffold, byr4p (Furge et al., 1999; Furge et al., 1998; Song et al., 1996). Signal transmission requires the activity of three protein kinases, each of which has a regulatory subunit: cdc7p-spg1p (Fankhauser and Simanis, 1994; Mehta and Gould, 2006), sid1p-cdc14p (Fankhauser and Simanis, 1993; Guertin et al., 2000; Guertin and McCollum, 2001) and sid2p-mob1p (Hou et al., 2000; Salimova et al., 2000; Sparks et al., 1999). These proteins associate with the SPB via a tripartite scaffold comprising ppc89p, sid4p and cdc11p (Chang and Gould, 2000; Krapp et al., 2001; Morrell et al., 2004; Rosenberg et al., 2006; Tomlin et al., 2002).

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During meiosis, the SIN scaffold proteins, spg1p and sid2p-mob1p are located at the SPB during the horsetail stage, meiosis I and meiosis II (Krapp et al., 2006). Byr4p and cdc16p are on the SPB during the horsetail stage and on both SPBs during meiosis I, whereas cdc7p and sid1p-cdc14p are absent. During meiosis II, byr4p and cdc16p disappear from the SPBs, to be replaced by sid1p-cdc14p and cdc7p. Furthermore, although these proteins all show some asymmetric behaviour during the mitotic cycle (for a review, see Lattmann et al., 2009), they are observed on all the SPBs during the meiotic cycle. This is consistent with the fact that each meiosis II nucleus will be engulfed by a spore that develops from the SPB.

A sid2p-related kinase called *mug27* (also known as *ppk35* or *slk1*; hereafter called *mug27*) is expressed during meiosis (Ohtaka et al., 2008; Perez-Hidalgo et al., 2008; Yan et al., 2008). A *sid2-250 mug27-D* double mutant makes no spores at all, indicating that the two kinases cooperate. The *mug27-D*-null mutant makes smaller spores and the FSM fails to engulf the nucleus, which results in decreased spore viability. Sid2p and mug27p are both observed at the SPB; in addition, mug27p is also present at the SPB and then associates with the FSM during spore formation. Mug27p does not require the SIN scaffold proteins for SPB-association (Ohtaka et al., 2008). Increased expression of the syntaxin psy1p rescues the sporulation defect of *mug27-D sid2-250* (Yan et al., 2008), suggesting that mug27p and sid2p facilitate recruitment of components of the secretory apparatus to allow FSM expansion.

The SIN inhibitor *dma1*

Dma1p, which is related to the mammalian CHFR or RNF8 family of ubiquitin ligases, is an inhibitor of the SIN (Murone and Simanis, 1996), which might function by regulating plo1p at the SPB (Guertin et al., 2002). Dma1p has two functional domains: an N-terminal forkhead-associated (FHA)-domain, which promotes phosphorylation-dependent interaction with other proteins and is required for proper localisation of dma1p (Guertin et al., 2002), and a C-terminal RING-finger, which is a motif found in ubiquitin-protein ligases. Proteins related to dma1p, such as CHFR, have been shown to function as protein-ubiquitin ligases in vitro (e.g. see Bothos et al., 2003; Kang et al., 2004); both domains are essential for its mitotic function in preventing septum formation during mitosis. Strong overexpression of *dma1* inhibits septum formation, producing multinucleate cells. Increased expression of *dma1* does not significantly affect the steady-state level of SIN proteins such as sid2p and mob1p during the mitotic cycle (Guertin et al., 2002) (A.K. and V.S., unpublished data). Genome-wide analysis of meiotic gene expression (Mata et al., 2002) indicates that *dma1* expression increases strongly as cells enter meiosis II.

In this study, we have examined the role of dma1p in meiosis. Mating between cells lacking dma1p produces a majority of asci with less than four spores, despite the fact that the two meiotic divisions are completed normally. Analysis of spore formation indicates that the spores are larger than those of wild type, and reveal problems in FSM formation. Our data indicate that the timing of the degradation of the sid2p-family protein kinase mug27p might be regulated by dma1p.

Results

Analysis of *dma1* expression and dma1p during meiosis

Because dma1p is an important regulator of the SIN in the mitotic cycle (see Introduction), we examined whether dma1p plays any role in meiosis. Northern blotting of RNA extracted from diploid cells undergoing a *pat1-114*-induced meiosis revealed a marked increase in the steady-state level of *dma1* mRNA when cells were undergoing the transition from meiosis I to meiosis II (Fig. 1A), consistent with the data from a genome-wide meiotic expression analysis (Mata et al., 2002). Protein extracts prepared under denaturing conditions revealed that dma1p-GFP levels increased significantly in parallel with RNA levels and remained elevated through to the end of meiosis II (Fig. 1B).

Localisation of dma1p-GFP in mating cells revealed a broad signal at the point of fusion between the two cells (Fig. 1C, lower panel). No signal was observed associated with any discrete structure during horsetail movement or meiosis I, although western blotting indicated that dma1p-GFP is present (Fig. 1B). In meiosis II, dma1p-GFP localised to the SPB and the FSM (Fig. 1C, upper panel). It was then observed as an intense dot between the separating nuclei, overlying the spindle midzone in anaphase II (Fig. 1D). At the end of meiosis II, the dma1p-GFP signal was observed as a dot, which colocalised with two different SPB markers, spg1p and cut12p (Fig. 1E). Formation of the crescent-shaped meiotic spindle pole plaque, and hence FSM formation, requires spo15p (Ikemoto et al., 2000); in a *spo15-D*-null mutant, dma1p-GFP was still observed as a dot at the meiosis II SPBs and also between the separating nuclei, although the signal was less intense than in wild-type cells (Fig. 1F). This indicates that SPB-association of dma1p-GFP in meiosis II is independent of SPB remodelling. In a *spo3-D*-null mutant in which FSM expansion is compromised (Nakamura et al., 2008), dma1p-GFP was observed at the SPB and on the FSM (Fig. 1F).

Meiosis in the absence of *dma1* function produces asci with less than four spores

Previous studies have shown that dma1p regulates the SIN during the mitotic cell cycle (Guertin et al., 2002; Murone and Simanis, 1996). Because the SIN is essential for spore formation, we examined whether *dma1*::*ura4*$^{+}$ cells (*dma1-D*) were affected during meiosis. A *dma1-D* h^{90} strain was allowed to mate and undergo meiosis; examination of asci revealed that only 40% of them contained four spores, whereas the remainder contained three (35%), two (17%), one (8%) or no (1%) spores (Fig. 2A,C; figures for a typical experiment are given). Staining of asci revealed that 97% of asci that contained at least one spore also contained four condensed nuclei, consistent with completion of the two meiotic divisions (Fig. 2B). The control mating of h^{90} *dma1*$^{+}$ cells gave rise to >99% four-spored asci. Similar results were obtained when a h^{+}/h^{-} *dma1-D*/*dma1-D* diploid was starved and sporulated (4 spores, 48%; 3 spores, 38%; 2 spores, 5%; 1 spore, 1.5%; no spores, 7%). Measurement of the diameter of the spores indicated that those produced following a *dma1-D* meiosis had a diameter of 3.45 μm ± 0.47 μm (n=292) compared with wild-type spores, which had a diameter of 3.0 μm ± 0.38 μm (n=280; P<0.001).

Dma1p comprises two functional domains (see Introduction); h^{90} RF and FHA point mutants of *dma1* were allowed to mate and undergo meiosis. Analysis of asci revealed that inactivation of either domain produced a result similar to the *dma1-D* mutant (Fig. 2C), indicating that both the RF and FHA domains of dma1p are essential for its meiotic role in spore formation. The increased number of asci with no spores suggests that the presence of a mutant dma1p might be more deleterious than its absence.

The *dma1-D* meiotic phenotype is reminiscent of that of the null mutant of the APC/C regulator *mfr1*, where asci frequently contain fewer than four spores (Blanco et al., 2001). This prompted us to

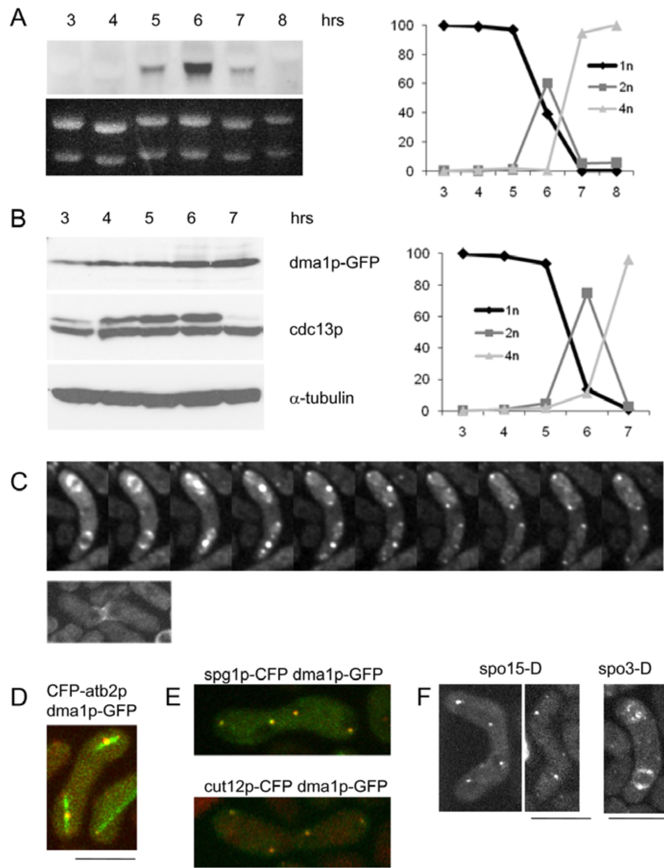

Fig. 1. Analysis of *dma1* expression and localisation during meiosis. (**A**) A culture of *pat1-114* diploid cells was induced to enter meiosis; samples were withdrawn at the indicated times after shift and RNA was extracted. A northern blot was probed as described in the Materials and Methods. The upper panel shows the *dma1* signal, the lower panel shows the Ethidium-Bromide-stained gel prior to transfer. The graph shows progression through meiosis in the experiment; 1n, 2n and 4n indicate one, two and four nuclei, respectively. (**B**) A culture of diploid *pat1-114 dma1-GFP* cells was induced to enter meiosis; samples were withdrawn and protein was extracted at the indicated time (hours) after shift. A western blot was probed with antiserum to detect GFP, cdc13p or α-tubulin. The kinetics of meiotic progression are shown in the panel on the right. 1n, 2n and 4n indicate one, two and four nuclei, respectively. (**C**) *dma1-GFP* h^+ and h^- cells were mated; cells were mounted for imaging as described in the Materials and Methods. The upper panels show a cell pictured at 4-minute intervals during meiosis II. The lower panel shows a pair of cells fusing during conjugation. (**D**) A cell expressing *CFP-atb2* and *dma1-GFP* was photographed during meiotic anaphase II. Note the presence of a bright GFP signal between the dividing nuclei, overlying the spindle midzone. (**E**) Cells expressing *dma1-GFP* and either *spg1-CFP* or *cut12-CFP* were photographed at the end of meiosis II. The image presented is a merge of the CFP (red) and GFP (green) channels; note that the SPB signal is yellow. (**F**) *dma1-GFP spo15-D* and *dma1-GFP spo3-D* cells were examined during meiosis II. Scale bars: 10 μm.

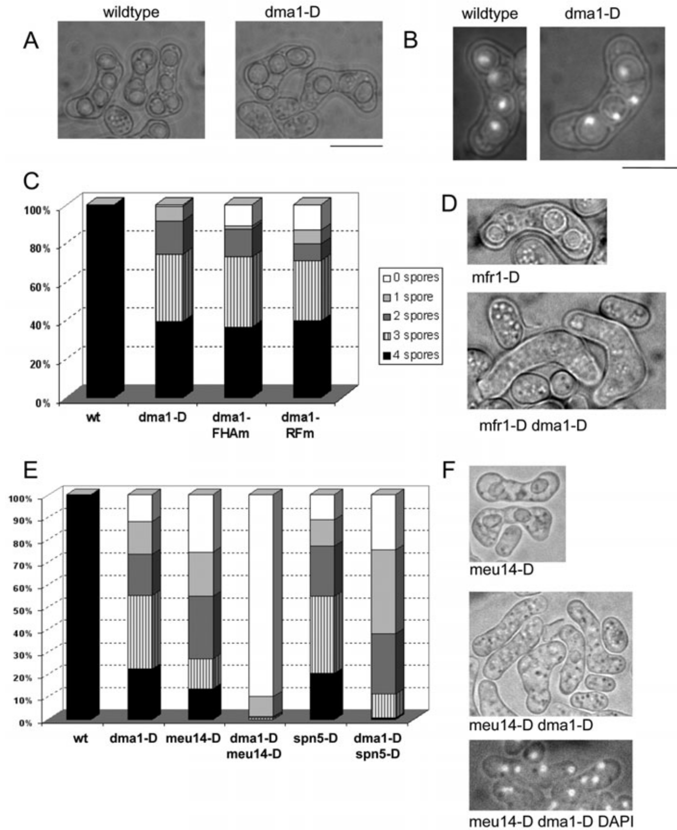

Fig. 2. Meiosis in the absence of dma1p frequently gives rise to asci with fewer than four spores. (**A**) The indicated h^{90} strains were mated as described in the Materials and Methods. After three days at 25°C, asci were suspended in EMM-N and photographed. Scale bar: 10 μm. (**B**) Cells from the same mating as A were fixed, stained with DAPI and photographed with combined fluorescence and transmission illumination. (**C**) Asci from the indicated h^{90} matings were scored according to the number of spores in the asci produced. Dma1-RFm and Dma1-FHAm indicate mutants in the RING-finger and Forkhead-associated domains, respectively. The number of spores in the asci was determined after 60 hours at 29°C. At least 100 asci were scored for each strain. (**D**) *mfr1-D* and *mfr1-D dma1-D* h^{90} cells were allowed to self-mate. After three days, asci were suspended in EMM-N and photographed. Note the absence of spores in the double-null mutant. (**E**) Matings between h^+ and h^- strains of the indicated genotypes were performed. In the case of *meu14-D*, an h^{90} strain was used. The number of spores in the asci was determined after 60 hours at 29°C. At least 100 asci were scored for each strain. (**F**) Samples of the indicated matings analysed in E were photographed. Note that although spores are almost completely absent in the *dma1-D meu14-D* mutant, DAPI staining reveals the presence of four nuclei in the asci.

examine the meiotic phenotype of *mfr1-D dma1-D* cells; we found a synergistic effect in the double-null mutant in that few or no spores were formed after meiosis (Fig. 2D), indicating that they do not function in a single linear pathway.

Microdissection of three- and four-spored asci from a *dma1-D* h^{90} mating revealed that the overall spore viability was 89% and 94%, respectively; the wild-type control gave 99% viable spores (Table 1A). One spore gave rise to a microcolony of approximately 20 cells; this phenotype might be produced by a spore inheriting an unbalanced number of chromosomes (Niwa and Yanagida, 1985). All the viable progeny examined appeared haploid (irrespective of the number of spores in the ascus), as judged by the size of cells and colony colour on medium containing Phloxin B. To examine this further, three- and four-spored asci from the cross *dma1*::*ura4*$^+$ *ade6-M210 leu1-32* h^- × *dma1*::*ura4*$^+$ *ade6-M216 leu1-32* h^+ were dissected and replica-plated onto media without adenine and containing limiting adenine (Moreno et al., 1991) to reveal the colour difference between the two *ade6* alleles. These two *ade6* alleles complement in trans and are frequently

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Table 1

A. Spore viability from *dma1-D* meiosis		Number of viable spores				
	Total	Four	Three	Two	One	None
dma1-D four-spored asci	63	49 (78%)	12 (19%)	2 (3%)	0	0
dma1-D three-spored asci	91	NA	72 (79%)	12 (13%)	3 (3%)	4 (4%)
Wild type	52	50 (96%)	2 (4%)	0	0	0

NA, not applicable. Spores from asci containing either three or four visible spores were separated by microdissection and colonies were allowed to form at 29°C. Colonies were counted after 3 or 4 days, and the fate of 'missing' spores was determined by microscopy. Only asci in which spores were liberated in 24 hours or less were counted in this analysis. For the wild-type control, 52 four-spored asci were dissected from an *ade6-M210 leu1-32* h^- × *ade6-M216 leu1-32* h^+ cross.

B. Delayed release of spores from *dma1-D* h^{90} mating	Number of asci	Spores released in 24 hours	Spores released in 72 hours
dma1-D four-spored asci	59	38 (64%)	51 (86%)
dma1-D three-spored asci	72	40 (56%)	56 (78%)
Wild type	70	69 (99%)	69 (99%)

Asci were placed on YE (yeast extract) plates at 25°C and examined at intervals thereafter for the liberation and germination of spores from the ascus.

used to select and maintain diploids. If diploid progeny are generated in a *dma1-D* meiosis, then some cells should inherit both alleles and be adenine prototrophs. After dissection of 37 four-spored asci, we observed that 32 gave rise to four colonies and five gave rise to only three colonies (overall spore viability of 96%). In 32 three-spored asci, 25 gave rise to three colonies, whereas five produced only two; in both instances, the remaining spores failed to germinate (overall viability 93%). After replica plating, all the progeny were adenine auxotrophs and growth on limiting adenine medium produced dark red (*ade6-M210*) to light red (*ade6-M216*) colony colours, consistent with 2:2 segregation of the *ade6* alleles in the four-spored asci and either one or two of each kind in the three-spored asci where three colonies arose. This is consistent with all the progeny being haploid. In addition, we noted that significant numbers of asci from *dma1-D* homozygous meiosis failed to release spores in the first 24 hours after placement on yeast extract (YE) plates (Table 1B). The reason for the delayed germination of *dma1-D* spores is unclear, but might reflect alterations in the composition of the spore wall or alterations in the expression of *agn2*, which is required for the release of spores from the ascus (Dekker et al., 2007).

To examine chromosome segregation, we used the lacI-GFP–lys1::lacO detection system (Nabeshima et al., 1998) to examine the segregation of the chromosome I centromere in *dma1-D* h^{90} mating. In crosses where both parent cells carried the lacI-GFP–lys1::lacO detection system, we observed in *dma1-D* cells that 90.9% (n=88) of asci had four detectable cen1 signals after meiosis II; a wild-type control showed only 98.3% (n=120) of nuclei with a cenI signal. Because >90% of spores are viable in four-spored asci, the absence of strong cen1 signal at the end of meiosis II in might not reflect loss or mis-segregation of chromosomes because *S. pombe* cells are intolerant of aneuploidy (Niwa and Yanagida, 1985).

A recent study revealed that the fission yeast meiotic septins and the proteins at the leading edge of the FSM (leading-edge proteins, LEPs) function in parallel pathways to orient growth of the FSM. Double mutants between the meiotic septins and a LEP, *meu14-D*, revealed an additive effect upon spore formation (Onishi et al., 2010). To examine the genetic relationship of *dma1* to the LEPs and meiotic septins, we examined the effects of constructing double mutants between them. We found that a *dma1-D* produced a synergistic effect with both the meiotic septin mutant *spn5-D* and the LEP mutant *meu14-D* (Fig. 2E). In the *spn5-D dma1-D* double mutant, less than 1% of asci contained four spores, whereas almost 20% contained none. The effect observed in the *dma1-D meu14-D* double mutant was even more pronounced, with 90% of asci containing no visible spores (Fig. 2E,F). DAPI staining of asci revealed four nuclei in the *dma1-D meu14-D* asci, suggesting that the meiotic divisions had been completed (Fig. 2F). The observation that *dma1-D* displayed additive effects with both the *spn5-D* and *meu14-D* suggests that dma1p does not act in a single linear pathway with either of these two protein complexes and is consistent with dma1p having multiple roles in meiosis.

Taken together, the data presented above lead us to conclude that meiosis in a *dma1-D* background produces spores that are larger than those of wild type and of reduced viability. A majority of asci contain fewer than four spores, although the two meiotic divisions appear to be completed normally. Analysis of *dma1* point mutants indicates that both of its functional domains are required during meiosis. Thus, whereas dma1p is not essential for meiosis or spore formation, it is important for the formation of normal, four-spored asci.

Forespore development is abnormal in *dma1-D* cells

The observation that the size and number of spores produced following meiosis in the absence of dma1p are aberrant suggests a role for dma1p in regulating synthesis of the FSM. Therefore, we analysed formation of the FSM using a GFP-tagged syntaxin psy1p, which is an FSM-resident protein (Nakamura et al., 2008). As shown in Fig. 3A,B, in wild-type meiosis, the FSM expands from the plaque-like SPB to engulf the four nuclei, forming the prespore (Nakamura et al., 2008). By contrast, in *dma1-D* meiosis, although FSM expansion began normally, FSM development became aberrant on one of the nuclei, producing bleb-like structures that eventually coalesced to give small multi-lobed structures (Fig. 3A); these usually did not contain any DNA (Fig. 3C). Similar data were obtained using spo3p-GFP to follow FSM formation (data not shown).

We also examined the localisation of meu14p, which is localised at the leading edge of the FSM (Fig. 4A,B) and might also affect meiotic SPB function (Okuzaki et al., 2003). We observed extra meu14p-GFP rings forming during FSM expansion (Fig. 4A,C), consistent with the data obtained using psy1p-GFP and spo3p-GFP as markers.

FSM development requires spo15p-dependent remodelling of the SPB in meiosis II (Ikemoto et al., 2000). Examination of

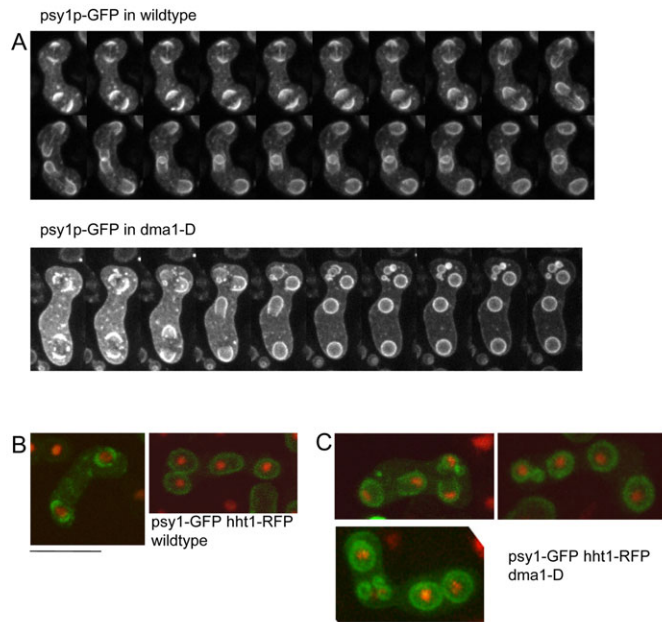

Fig. 3. Forespore membrane formation is aberrant in *dma1-D* meiosis. (**A**) Matings between h^- and h^+ wild-type or h^- and h^+ *dma1-D* cells expressing psy1p-GFP were filmed during forespore membrane (FSM) development in meiosis II. The time interval between frames is 2 minutes in the wild-type and 4 minutes in the *dma1-D* cells. (**B**,**C**) Matings between h^- and h^+ wild-type or h^- and h^+ *dma1-D* cells expressing spo3p-GFP (green) and hht1-RFP (red) were photographed. Scale bars: 10 µm.

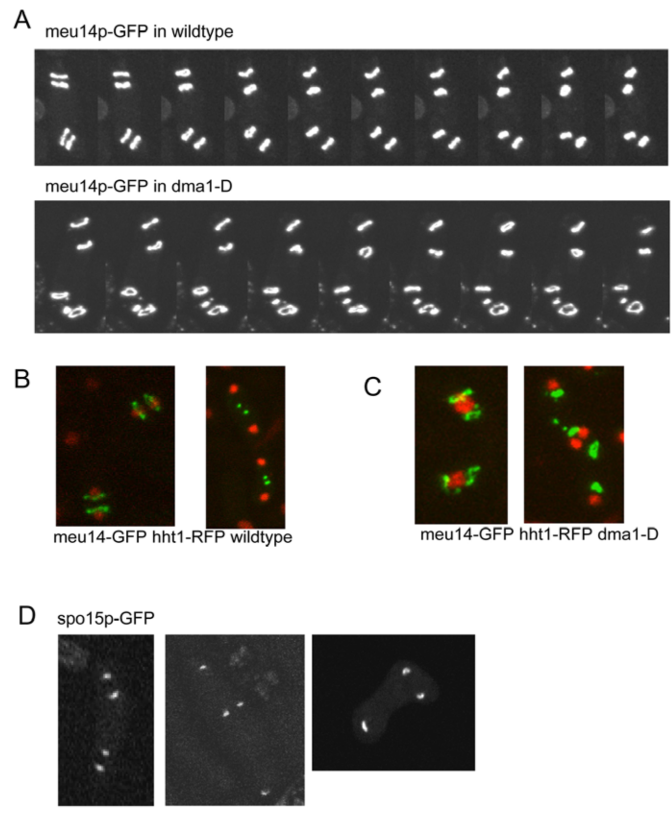

Fig. 4. The distribution of meu14p is aberrant in *dma1-D* meiosis. (**A**) Matings between h^- and h^+ wild-type or h^- and h^+ *dma1-D* cells expressing meu14p-GFP were filmed during FSM development in meiosis II. The time interval between frames is 2 minutes. (**B**,**C**) Matings between h^- and h^+ wild-type or h^- and h^+ *dma1-D* cells expressing meu14p-GFP (green) and hht1-RFP (red) were photographed. (**D**) Meiotic *dma1-D* cells expressing spo15-GFP were examined. Meiosis II cells are shown in each case. Note that in the left-most panel the two other nuclei overlap. Scale bars: 10 µm.

spo15p-GFP revealed the presence of elongated and crescent-shaped SPB structures in meiotic cells (Fig. 4D); whether the crescent structure of the meiotic SPB is normal in *dma1-D* will require further analysis by electron microscopy. However, the observation that FSM deposition, which absolutely requires SPB remodelling and spo15p function (Ikemoto et al., 2000), occurs in *dma1-D* cells suggests that the SPB is modified at the onset of meiosis II. Taken together, these data indicate that FSM development during meiosis II is aberrant in *dma1-D* cells, which leads to the formation of asci containing fewer than four spores.

Localisation of SIN proteins and SIN regulators in *dma1-D* meiosis

Because the SIN is required for spore formation (Krapp et al., 2006; Yan et al., 2008) and dma1p regulates the SIN in the mitotic cycle (Guertin et al., 2002; Murone and Simanis, 1996), we examined the localisation of SIN proteins in meiosis in the *dma1-D* background. We found that the localisation of all SIN-GFP proteins examined (Fig. 5; data not shown) was qualitatively similar to that previously described in $dma1^+$ cells (Krapp et al., 2006), in that the signals were associated with all SPBs depending on the stage of the meiotic cycle, with sid1p and cdc7p associated with the SPB only in meiosis II, as expected (Fig. 5A). The localisation of both sid2p and mob1p also resembled that seen in a wild-type meiosis (see Fig. 5B,C); both GFP-tagged proteins were observed on all four SPBs in meiosis II. Western blotting showed that the steady-state levels of both mob1p (Fig. 5B) and sid2p (Fig. 5C) did not change significantly during meiosis. However, we observed that sid2p displayed a slower migrating form at 7 hours after induction of meiosis in *dma1-D* cells (Fig. 5C); the wild-type cells did not accumulate detectable amounts of this form of sid2p. The nature of the modification is unknown (see Discussion), but it was observed in three separate experiments (data not shown).

As dma1p might regulate the association of plo1p with the SPB (Guertin et al., 2002), we examined whether the absence of dma1p influenced the meiotic localisation of plo1p-GFP (Bahler et al., 1998a). We found that, in $dma1^+$ meiosis, three spots were observed during the horsetail stage (Fig. 6A, cell 1), which colocalise with the kinetochore marker cnp1p (Fig. 6B). During meiosis I, plo1p-GFP was observed first as a number of dots along the spindle and on the SPB, then on the SPBs and faintly on the elongating spindle (Fig. 6A, cells 2 and 3; Fig. 6B), becoming fainter at the end of anaphase I (Fig. 6A, cell 4). During meiosis II, plo1p-GFP was observed at all four SPBs (Fig. 6A) and faintly on the spindle. The intensity of the SPB signal decreased during meiosis II (Fig. 6A, cells 5–9; n=5), eventually becoming too faint to detect during anaphase; this resembles the mitotic localisation of plo1p (Mulvihill et al., 1999). Thus, in addition to its mitotic association with the SPB and spindle, during meiosis I, plo1p-GFP also associates with kinetochores.

In *dma1-D* meiosis, the localisation was similar up to meiosis II (Fig 7A, cells 1–3); during meiosis II, a signal was observed initially at all four SPBs, then, during anaphase II, the signal

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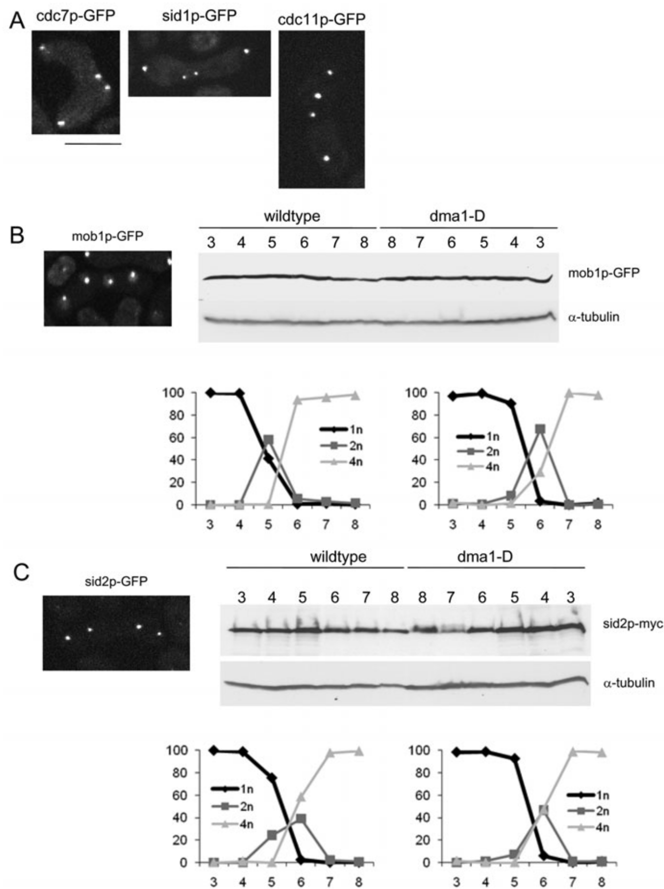

Fig. 5. Localisation of SPB markers and proteins involved in SIN signalling during wild-type and *dma1-D* meiosis. (**A**) Matings between h^+ and h^- *dma1-D* cells expressing the indicated GFP-tagged SIN proteins were examined. Meiosis II cells are shown in each case. Scale bar: 10 μm. (**B**,**C**) *pat1-114* and *pat1-114 dma1-D* cells expressing either mob1p-GFP or sid2p-myc or sid2p-GFP (for imaging) were induced to undergo meiosis. Protein samples were prepared at the indicated times (hours) after shift and western blots were probed with sera against the indicated proteins. The kinetics of meiotic progression are shown below the western blot (wild type on the left, *dma1-D* on the right). The images show a meiosis II cell in each case. B, data for mob1p-GFP; C, data for sid2-GFP and sid2-myc.

became asymmetric. Nine cells were filmed; in seven cells, plo1p-GFP remained associated with two of the four SPBs, one from each spindle (Fig. 7A, cells 4–8), whereas in two cells, plo1p-GFP remained associated with only one of the four SPBs (data not shown). Analysis of the plo1p-GFP by western blotting revealed that the level of plo1p-GFP decreased as cells progressed through meiosis II and that this was preceded by the appearance of a modified, slower-migrating form of the protein at the transition from meiosis I to meiosis II in both *dma1*$^+$ and *dma1-D* meiosis (Fig. 7B). In summary, we conclude that although plo1p-GFP localises symmetrically in meiotic *dma1*$^+$ cells, in the absence of dma1p, the meiosis II SPBs behave asymmetrically with regard to plo1p-GFP localisation.

Dma1p is required for timely elimination of mug27p

Mug27p is the meiosis-specific orthologue of sid2p (see Introduction). Because *mug27-D* cells produce small spores, whereas *dma1-D* cells produce large spores, we tested whether the steady-state levels of mug27p were affected in *dma1-D*. We observed that mug27p levels peaked at the transition from meiosis I to meiosis II and then declined at the time of spore formation (Fig. 8A), consistent with previous studies (Ohtaka et al., 2008; Perez-Hidalgo et al., 2008; Yan et al., 2008). By contrast, in *dma1-D* meiosis, mug27p persisted beyond the onset of meiosis II (Fig. 6A). Significantly, whereas in wild-type mug27p levels decline before cdc13p, in *dma1-D* they remain elevated after cdc13p levels have decreased (Fig. 8A). However, localisation of mug27p-GFP to the SPB was similar in wild-type and *dma1-D* meiosis (Fig. 8B). We conclude that dma1p plays a role in the timely elimination of mug27p. To study the extent to which the effects of the absence of dma1p upon spore formation were due to mug27p, we constructed a *dma1-D mug27-D* double-null mutant; we found that although meiosis produced four nuclei, less than 1% of asci contained any spores (Fig. 8C). Filming of psy1-GFP in this mutant showed strongly aberrant FSM extension, which rapidly collapsed to form small multi-lobed structures (Fig. 8D). This synergy between the null mutants demonstrates that the meiotic effects caused by the absence of dma1p are not mediated solely via mug27p. We attempted to examine the effects of increased *dma1* expression on spore formation and mug27p levels; however, increased expression of *dma1*$^+$ blocks cytokinesis and interferes with mitotic progression (Murone and Simanis, 1996), and we found that expression of *dma1*$^+$ from the full-strength *nmt1* promoter interferes with chromosome segregation during meiosis and, hence, no spores were observed (data not shown). Whether intermediate levels of overexpression of *dma1* affects spore formation will be the subject of future studies.

Discussion

In the present study, we have examined whether the SIN regulator dma1p plays a role in meiosis. We have found that if cells undergo the meiotic cycle without dma1p, more than 60% of asci contain fewer than four spores. However, in the vast majority of cases, the asci contain four nuclei, indicating that the two meiotic divisions have been completed successfully.

Analysis of three-spored asci indicates that the viability of the spores is high and the progeny are haploid. This argues against extensive mis-segregation of chromosomes, as *S. pombe* is intolerant of aneuploidy (Niwa and Yanagida, 1985). The presence of four DAPI staining bodies also argues against encapsulation of more than one nucleus by a growing FSM as being responsible for the absence of one or more spores.

Dma1p localises to the SPB in meiosis II; however, its localisation there does not require this remodelling, as it is still observed at the dot-like SPB in a *spo15* mutant, which does not reorganise the SPB. The nature of the meiotic SPB anchor for dma1p is unknown, although by analogy with the mitotic cycle (Guertin et al., 2002), sid4p might play this role. Dma1p also appears to associate with the expanding FSM early in meiosis II. In mid-anaphase, dma1p-GFP is observed as very bright dot between the two dividing nuclei; the only protein we are aware of that shows a similar localisation is pad1p-GFP, which is a subunit of the proteasome (Wilkinson et al., 1998). To date, we have been unable to find conditions that allow both dma1p and the pad1p to be visualised simultaneously; nonetheless, this raises the possibility that dma1p might regulate the proteasome at some point(s) in meiosis. The role for dma1p at the point of fusion between mating cells is at present unclear. F-actin is present around, but excluded from, the region of cell fusion (Petersen et al., 1998). It is possible

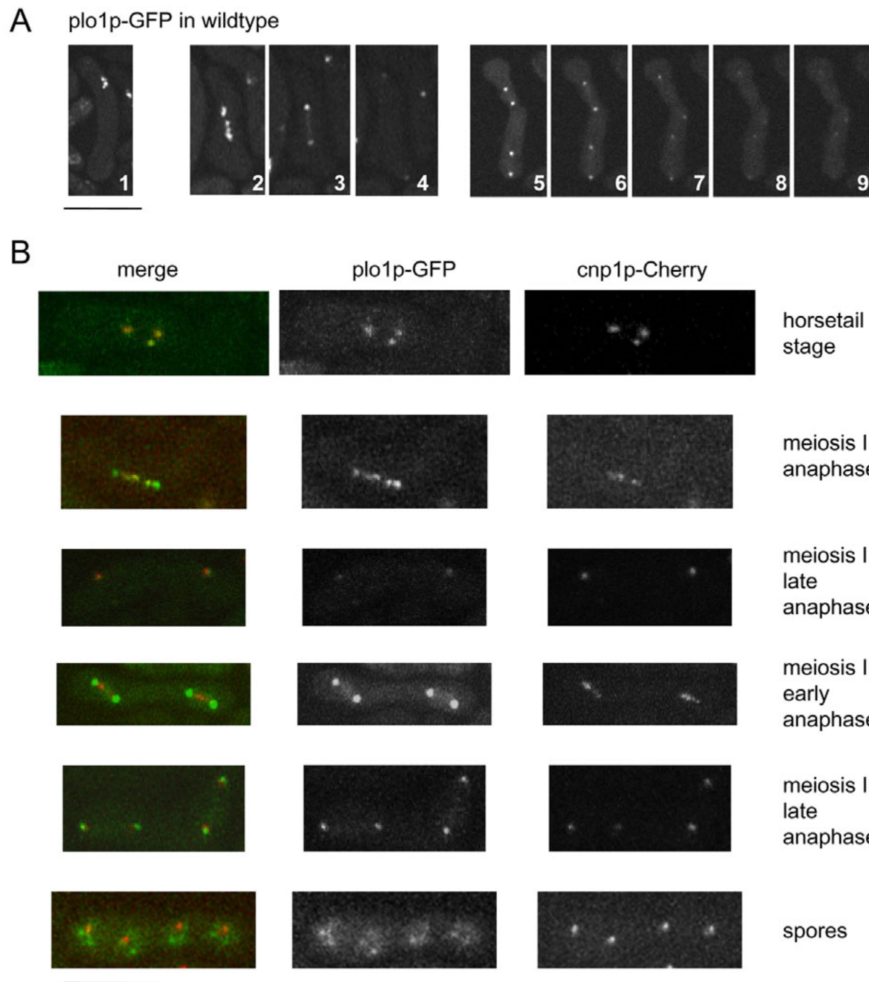

Fig. 6. Localisation of plo1p-GFP in *dma1*$^+$ meiosis. (**A**) *Dma1-D* cells expressing plo1-GFP were mated and filmed at intervals during meiosis; see text for details. Cells 2–4 are images of the same cell taken 15 minutes apart. Cells 5–9 are images of a single cell taken 5 minutes apart. (**B**) The strains *plo1-GFP cnp1-Cherry* and *plo1-GFP cnp1*$^+$ were mated. Cells were mounted 24 hours later and photographed. The panel shows cells at various stages of meiosis, with a merge and the separated red and green channels. Scale bars: 10 µm.

that the presence of dma1p prevents any attempt to construct a CAR or division septum at this site.

The data presented here demonstrate that *dma1-D* meiosis frequently produces asci containing fewer than four spores. Analysis of null mutants for meiosis-specific genes has identified a number of mutants that produce the same phenotype (Martin-Castellanos et al., 2005). The one whose mutant phenotype most strongly resembles that of *dma1-D* is *mfr1-D* (Blanco et al., 2001). This raised the possibility that dma1p might regulate mfr1p and thus control the activity of the meiotic APC/C. However, the strong additive effects of these two null mutants mean that this simple hypothesis is unlikely to be correct and imply that if dma1p does act upon mfr1p (or vice versa) to regulate the APC/C during meiosis then dma1p and/or mfr1p must have additional targets.

This screen also identified mug27p. The absence of mug27p produces smaller spores and it has been proposed that mug27p regulates FSM expansion; we have found that mug27p persists for longer than usual in *dma1-D* meiosis. It is possible that this accounts for the production of larger spores. Preliminary data indicate that increased expression of the *mug27* gene from the *nmt1* promoter produces spores that are approximately 10% larger than those in wild type but most asci contain four spores (data not shown). Because mug27p levels do eventually decrease in *dma1-D*, this task is either performed by a redundant mechanism that does not depend upon dma1p, or the absence of dma1p reduces the efficiency of the elimination mechanism for mug27p. At present, it is not understood how mug27p levels are reduced at the end of meiosis; our data indicate clearly that dma1p contributes to this, although it is unclear whether it does so directly. To date, we have failed to detect any two-hybrid interaction between dma1p and mug27p (data not shown) and analysis of proteins associated with dma1p in meiotic cells by mass spectrometry did not reveal any peptides derived from mug27p (our unpublished data).

The data presented here indicate that growth of the FSM is aberrant in *dma1-D*. Although FSM expansion begins normally, we observe asynchrony in membrane closure and what appear to be additional FSM initiation events, producing multi-lobed structures. FSM organisation and shape are determined by the cooperative action of LEPs and the meiotic septins (Onishi et al., 2010). Our data reveal significant negative genetic interactions of *dma1-D* both with an LEP-null mutant and a septin-null mutant. This indicates that dma1p does not act in a single linear pathway with either of these two protein complexes in the process of spore formation and is consistent with dma1p having multiple roles in meiosis.

The study of Yan et al. (Yan et al., 2008) demonstrated that sid4p is required for spore formation by using a *sid4* shut-off strain; this frequently gave rise to asci with two, rather than four, spores even though the two meiotic divisions had been completed. Dma1p is known to localise at the SPB via sid4p (Guertin et al., 2002); however, the phenotypes are different, so it is not clear at

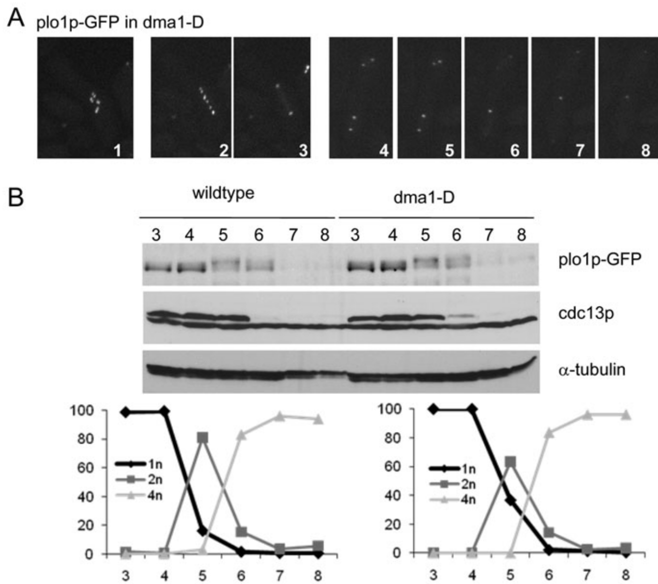

Fig. 7. Protein levels of plo1-GFP during meiosis and localisation of plo1p-GFP in *dma1-D* meiosis. (**A**) Cells expressing plo1-GFP were mated and filmed at intervals during meiosis; see text for details. Cells 2 and 3 are images of the same cell taken 15 minutes apart. Cells 4–8 are images of a single cell taken 5 minutes apart. (**B**) *pat1-114* and *pat1-114 dma1-D* cells expressing plo1-GFP were induced to undergo meiosis. Protein samples were prepared at the indicated times (hours) after shift and western blots were probed with sera against GFP, cdc13p or α-tubulin. The kinetics of meiotic progression are shown below the western blot (wild type on the left, *dma1-D* on the right).

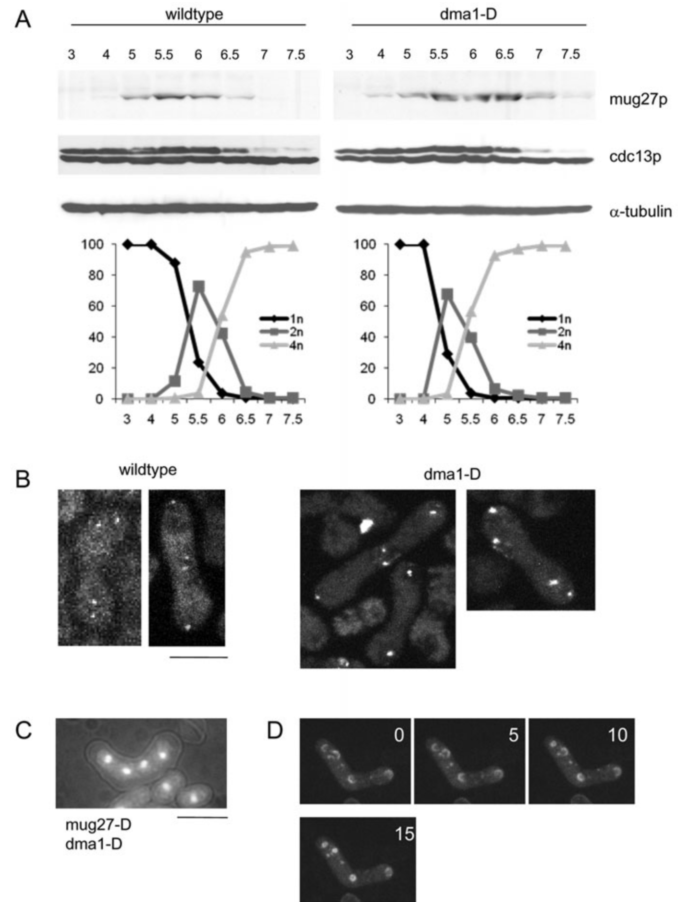

Fig. 8. Dma1p affects the timing of the elimination of mug27p during meiosis. (**A**) *pat1-114* and *pat1-114 dma1-D* cells expressing *mug27-GFP* were induced to undergo meiosis. Protein samples were prepared at the indicated times (hours) after shift and western blots were probed with sera against the indicated proteins. The kinetics of meiotic progression are shown below the western blot (wild type on the left, *dma1-D* on the right). (**B**) Localisation of mug27p-GFP. Wild-type or *dma1-D* h^+ and h^- cells expressing mug27p-GFP were mated and imaged as described in the Materials and Methods. The images show meiosis II cells for each strain. Scale bar: 10 μm. (**C**) *Mug27-D dma1-D* h^+ and h^- strains were mated and asci were examined by staining with DAPI after 3 days at 25°C. Note that although four nuclei are present, there are no spores in the double-mutant ascus. Scale bar: 10 μm. (**D**) *Mug27-D dma1-D* h^+ and h^- strains expressing psy1-GFP were mated and filmed during spore formation. The time indicated represents minutes after the first frame shown.

present whether the effects of the absence of dma1p are mediated via regulation of the SIN. In this context, it is noteworthy that the failure to form four-spored asci in *dma1-D* is not rescued by increasing SIN activity through overexpression of *spg1* (data not shown).

Localisation of SIN proteins and regulators of the SIN show that all the proteins examined segregate as previously described in a wild-type meiosis. Thus, mislocalisation of the 'core' mitotic SIN proteins is unlikely to be the primary cause of the failure to form spores. A notable exception to this is the SIN regulator plo1p. Although the role of plo1p in *S. pombe* meiosis has not been analysed extensively to date, its horsetail–meiosis I localisation at the kinetochores is consistent with the role described for its *Saccharomyces cerevisiae* orthologue cdc5p in assuring kinetochore orientation during meiosis I (Lee and Amon, 2003). The localisation of plo1p during the 'mitotic-like' meiosis II division resembles its localisation in mitosis (Mulvihill et al., 1999). The plo1p-GFP signal is first detected strongly on all four meiosis II SPBs; as anaphase progresses, the signal grows fainter and plo1p-GFP is also detected on the spindle. In meiotic *dma1-D* cells, the behaviour of SPBs in meiosis II differs from that in wild type; although plo1p-GFP initially associates with all four SPBs, it subsequently becomes asymmetric during meiosis II. Although the SIN proteins sid1p, cdc14p, cdc16p, byr4p and cdc7p display such behaviour in the mitotic cycle, they segregate symmetrically during meiosis, even in the absence of dma1p. The majority of meioses examined showed 2:2 segregation of meiosis II SPBs that retained plo1p to those that did not, whereas the most frequent class of aberrant asci had three spores. Thus, there is not an obvious correlation between the aberrant segregation of plo1p-GFP and a failure to form spores.

The finding that SPBs in the same meiotic cycle behave in a non-equivalent manner in the absence of dma1p strongly suggests that dma1p is involved in regulating behaviour of the SPB during meiosis, and raises the intriguing possibility that, as in the mitotic cycle (Grallert et al., 2004), fission yeast SPBs might have different properties according to their age. In this context, it is noteworthy that, in *Saccharomyces cerevisiae*, the SPB component nud1p (the orthologue of cdc11p in *S. pombe*) helps to distinguish the ages of the four SPBs (Gordon et al., 2006). The transient appearance of a slower migrating form of sid2p in during meiosis II in *dma1-D* cells suggests that dma1p influences the abundance of this form of sid2p during meiosis. Sid2p and mug27p cooperate during spore formation, so it is possible that this is an active form of sid2p that persists for longer than usual. However, many other models are

possible and future experiments will investigate the nature of the modification and its functional significance.

In the mitotic cycle, dma1p is considered to be an inhibitor of the SIN when the cell cycle is perturbed (see Introduction) but, in an unperturbed cell cycle, the main effect is an increased chromosome loss rate (Murone and Simanis, 1996). The absence of dma1p during meiosis results in a failure to form spores from one or more of the SPBs, implying that dma1p might play a more important role in meiosis. Increased expression of *mug27* slightly increases spore size but does not affect spore number (see Results). If one role for dma1p is to regulate mug27p, then, in its absence, persistent mug27p activity might perturb FSM development, leading to formation of bigger spores and aberrant FSM expansion events. By contrast, loss of both dma1p and mug27p strongly inhibits spore formation. Taken together with the genetic interactions of *dma1-D* with *mfr1* and the meiotic septins, our data are consistent with the view that dma1p plays multiple roles during meiosis in addition to regulating the meiotic SIN components such as mug27p.

A common feature of the meiotic localisation of dma1p is its association with sites of membrane remodelling, such as the point of fusion between cells and the expanding FSM. It is also noteworthy that dma1p is found between the two nuclei, where closure of the FSM will occur. In *Saccharomyces cerevisiae*, removal and degradation of ssp1p from the FSM is essential for spore formation (Maier et al., 2007). Because the RING-finger of *dma1* is required for its meiotic activity, it is tempting to speculate that dma1p will promote the degradation and/or inactivation of an equivalent protein. However, BLAST searches have not revealed a clear counterpart of ssp1p in fission yeast. The fact that dma1p and the SIN proteins are localised at the SPB in meiosis suggests that, as is the case in the mitotic cycle, where mitosis and cytokinesis are coordinated from the SPB, the meiotic SPB acts as a signalling centre to coordinate nuclear division and spore formation.

Materials and Methods

Yeast strains, media and culture conditions

Media have been described previously (Moreno et al., 1991); complete yeast extract (YE) medium or EMM2 were used as appropriate. For nitrogen starvation, cells were grown in EMM-N, which is EMM2 lacking NH_4Cl.

Fission yeast strains bearing GFP-tagged alleles of SIN genes used in this study have been described previously (Guertin et al., 2000; Krapp et al., 2001; Salimova et al., 2000; Sohrmann et al., 1998; Sparks et al., 1999). *dma1::ura4+* was constructed by (Murone and Simanis, 1996). Dma1p was tagged with GFP at its C-terminus according to standard techniques (Bahler et al., 1998b). FHA (R64A; H88A) and RF (C210S) mutants of *dma1* were constructed by Justine Shaw (J. Cassidy, Controlling cytokinesis in the fission yeast *Schizosaccharomyces pombe*: the role of dma1 and ubc8, PhD thesis, University of Lausanne, 2005) using standard techniques and integrated into the *dma1* locus, expressed from the *dma1* promoter. A full description of the properties of these mutants is in preparation (J. Shaw, A.K. and V.S., unpublished data). GFP-tagged spo3p, psy1p, spo15p and meu14p have been described previously (Ikemoto et al., 2000; Nakamura et al., 2001; Okuzaki et al., 2003) and were obtained from the Shimoda laboratory or the Yeast National Bioresearch Project, Japan. Plo1p-GFP, pcp1p-GFP and sad1p-CFP have been described previously (Bahler et al., 1998a; Flory et al., 2002; Wu et al., 2003). Mug27p-GFP and *mug27::ura4+* were described in Perez-Hildalgo et al. (Perez-Hidalgo et al., 2008) and *mfr1-D* was described in Blanco et al. (Blanco et al., 2001); all were obtained from the Moreno laboratory (Salamanca, Spain). The cnp1-Cherry strain (Alvarez-Tabares et al., 2007) was obtained from the Hagan laboratory. Standard techniques (Moreno et al., 1991) were used to create the strains described in the text. For examination of GFP-atb2p, the *GFP-atb2* gene was expressed from its own promoter on a multicopy plasmid; the construction is not toxic to cells.

To examine the effect on spore formation and size, as well as to localise GFP-tagged proteins during meiosis, strains of opposite mating types expressing chromosomally GFP-tagged alleles were mated on EMM-N plates at 25°C for 20 hours, resuspended in EMM-N and mounted. Spore size was measured from photographs using Adobe Photoshop.

Diploid strains were obtained by mating on EMM-N plates (Blanco et al., 2001). Synchronous meiosis in *pat1-114/pat1-114* temperature-sensitive mutants was performed as follows. *h-/h-pat1-114/pat1-114* diploid cells were cultured in YE medium at 25°C for 1 day and transferred to EMM plus supplements (100 μg/ml) for another day. These cells were then washed and resuspended in EMM-NH_4Cl plus supplements (10 μg/ml) at a density of $2–3\times10^6$ cells/ml. After 16 hours at 25°C, most cells were arrested in G1 phase and the culture was shifted to 34°C in the presence of EMM containing 0.5 g/l NH_4Cl and 10 μg/ml supplements to induce meiosis.

RNA and protein methods

RNA was extracted using the RNeasy Kit from Qiagen. Five micrograms of total RNA were glyoxylated (Glyoxal Sample Dye, Ambion) and run on a 1.2% agarose gel. RNA was transferred onto a positively charged nylon membrane (Roche) and hybridised with a DIG-labelled probe (DIG Northern Kit, Roche) covering the *dma1* open reading frame.

Total protein extracts were made using the trichloroacetic acid (TCA) extraction protocol (Foiani et al., 1994). Protein extracts were run on SDS-PAGE gels and transferred to nitrocellulose membranes (Protran, Whatman). Primary antibodies against GFP (this laboratory), cdc13 (Moreno laboratory, Salamanca, Spain), c-myc (Santa Cruz Biotechnology) and tubulin (TAT-1; Keith Gull, Oxford, UK) were used. Secondary antibodies conjugated to horseradish peroxidise and ECL western blotting reagents (Amersham) were used to visualise the bands.

Microscopy

DAPI staining (1 μg/ml) was performed on cells that had been fixed with 70% ethanol as described previously (Balasubramanian et al., 1997; Moreno et al., 1991). To estimate the proportion of cells in meiosis I, meiosis II or sporulation, we determined the percentage of cells with one, two or four nuclei after DAPI (1 μg/ml) staining and the percentage of asci with mature spores with phase-contrast microscopy.

Examination of GFP-tagged proteins in living cells was performed using a Zeiss Axiovert 200 microscope equipped with a confocal scanner (CSU10; Yokogawa Electric Corporation), a coolSNAP HQ camera (Photometrics) and 63×1.4 NA plan-apo or 100×1.4 NA plan-apo objective. Images were collected using Metamorph software (Universal Imaging, version 4.5). Images were assembled in Adobe Photoshop 7 or CS and Powerpoint 2003.

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