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RESEARCH ARTICLE

Dma1-dependent degradation of SIN proteins during meiosis in *Schizosaccharomyces pombe*

Andrea Krapp and Viesturs Simanis*

ABSTRACT

The *Schizosaccharomyces pombe* septation initiation network (SIN) is required for cytokinesis during vegetative growth and for spore formation during meiosis. Regulation of the SIN during mitosis has been studied extensively, but less is known about its meiotic regulation. Here, we show that several aspects of SIN regulation differ between mitosis and meiosis. First, the presence of GTP-bound Spg1p is not the main determinant of the timing of Cdc7p and Sid1p association with the spindle pole body (SPB) during meiosis. Second, the localisation dependencies of SIN proteins differ from those in mitotic cells, suggesting a modified functional organisation of the SIN during meiosis. Third, there is stage-specific degradation of SIN components in meiosis; Byr4p is degraded after meiosis I, whereas the degradation of Cdc7p, Cdc11p and Sid4p occurs after the second meiotic division and depends upon the ubiquitin ligase Dma1p. Finally, Dma1p-dependent degradation is not restricted to the SIN, as we show that Dma1p is needed for the degradation of Mcp6p (also known as Hrs1p) during meiosis I. Taken together, these data suggest that stage-specific targeted proteolysis plays an important role in regulating meiotic progression.

KEY WORDS: Meiosis, *S. pombe*, Degradation, Dma1, Septation initiation network

INTRODUCTION

Meiosis is a variant of the cell cycle, which gives rise to haploid gametes. In *Schizosaccharomyces pombe*, the product of meiosis is an ascus containing four spores. When starved, cells of opposite mating types undergo a pheromone-dependent arrest of their cell cycles in G1, followed by cytoplasmic and nuclear fusion. Premeiotic DNA synthesis and homologous recombination are followed by the two meiotic divisions. Finally, spores are formed by the deposition of membranes and cell wall around the four nuclei (reviewed by Shimoda, 2004; Shimoda and Nakamura, 2004; Yamamoto, 2004). The mechanisms involved have been revealed by genetic and cytological analysis. The spindle pole body (SPB) is remodelled during meiosis II and changes its appearance from a dot to a crescent shape, as visualised by indirect immunofluorescence (Hagan and Yanagida, 1995; Yoo et al., 1973). This requires Spo2p, Spo13p and Spo15p (Ikemoto et al., 2000; Nakamura et al., 2008; Nakase et al., 2008). Vesicles are recruited to the modified SPB and fuse to give rise to the forespore membrane (FSM), which expands outwards from the SPB and encapsulates the nucleus to form the prespore. Spore-wall material is then deposited between the membranes to form the mature spore (Shigehisa et al., 2010; Tanaka and Hirata, 1982; Yoo et al., 1973). Leading edge proteins (LEPs), such as Meu14p (Okuzaki et al., 2003), function together with a meiotic actin ring (Petersen et al., 1998; Yan and Balasubramanian, 2012) and the meiotic septin complex (Onishi et al., 2010) to orient FSM extension and closure – FSM growth depends on SNARE-vesicle-fusion complexes (Kashiwazaki et al., 2011; Maeda et al., 2009; Nakamura-Kubo et al., 2011; Nakamura et al., 2005; Nakamura et al., 2001).

The SIN and its regulators

During vegetative growth, *S. pombe* cells divide by medial fission. A contractile ring (CAR) is assembled at the centre of the cell during mitosis; at the end of anaphase, contraction of the CAR guides the synthesis of the septum that bisects the cell. A signalling cascade 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 (reviewed by Goyal et al., 2011; Krapp and Simanis, 2008). The SIN is also essential during meiosis (Krapp et al., 2006; Ohtaka et al., 2008; Pérez-Hidalgo et al., 2008; Yan et al., 2008) – SIN mutants complete both meiotic divisions, but fail to form spores.

SIN signalling is modulated by the nucleotide status of the GTPase Spg1p (Schmidt et al., 1997; Sohrmann et al., 1998), which is regulated by the balance of spontaneous nucleotide exchange, 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). This keeps Spg1p in the inactive GDP-bound form during interphase. During mitosis, GTP-bound Spg1p interacts with the protein kinase Cdc7p (Fankhauser and Simanis, 1994; Mehta and Gould, 2006). Signal transmission requires the activity of two additional kinases, Sid1p and Sid2p, each of which has a regulatory subunit – Cdc14p and Mob1p, respectively (Fankhauser and Simanis, 1993; Guertin et al., 2000; Guertin and McCollum, 2001; Hou et al., 2000; Salimova et al., 2000; Sparks et al., 1999). The kinase Plo1p acts upstream of the 'core' SIN kinases (Tanaka et al., 2001).

SIN signalling originates from the SPB in the context of 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). The localisation dependencies between SIN proteins in vegetative cells indicate that all the core components of the SIN require Sid4p and Cdc11p for association with the SPB (Feoktistova et al., 2012; Krapp et al., 2001; Morrell et al., 2004; Tomlin et al., 2002). Cdc16p and Byr4p are interdependent for SPB localisation (Cerutti and Simanis, 1999), and the association of Cdc7p with the SPB

École Polytechnique Fédérale de Lausanne, School of Life Sciences, Institut Suisse de Recherche Expérimentale sur le Cancer UPSIM, SV2.1830, Station 19, CH-1015 Lausanne, Switzerland.

*Author for correspondence (viesturs.simanis@epfl.ch)

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requires Spg1p, but not vice versa (Sohrmann et al., 1998). Cdc14p and Sid1p are interdependent for localisation (Guertin et al., 2000), as are Sid2p and Mob1p (Hou et al., 2000; Salimova et al., 2000). Hierarchically, the localisation of Sid1p and Cdc14p depends upon Cdc7p (Guertin et al., 2000), but not vice-versa. The localisation of Sid2p and Mob1p to the SPB depends only on the scaffold proteins and Cdc7p, but localisation to the CAR requires Cdc7p and Sid1p function (Guertin et al., 2000; Sparks et al., 1999). Sid1p and Cdc7p still localise to the SPB during mitosis in a *sid2* mutant (Guertin et al., 2000; Sparks et al., 1999), but they no longer localise asymmetrically to the new SPB (nSPB) during anaphase B (Feoktistova et al., 2012). Assuming that the localisation of SIN proteins reflects their activity, it has been proposed that the SIN kinases act in a linear sequence – Cdc7p, Sid1p, Sid2p – with a feedback from Sid2p to Cdc11p to promote SIN protein asymmetry (Feoktistova et al., 2012; Guertin et al., 2000; Sparks et al., 1999).

Cdc11p, Sid4p, Spg1p, and Sid2p–Mob1p are associated with the SPB at all stages of meiosis (Krapp et al., 2006). Cdc16p associates with the SPB during oscillatory movement of the nucleus (termed 'horsetail nuclear movement') and with both SPBs during meiosis I, whereas Cdc7p and Sid1p–Cdc14p are absent during these stages. During meiosis II, Cdc16p disappears from the SPBs, to be replaced by Cdc7p and Sid1p–Cdc14p. A Sid2p-related protein kinase called Mug27 (also known as Ppk35 or Slk1) is expressed specifically during meiosis (Ohtaka et al., 2008; Pérez-Hidalgo et al., 2008; Yan et al., 2008). Mug27p localises to the SPB during nuclear division and associates with the FSM during spore formation before being degraded at the end of meiosis II. It does not require the SIN scaffold proteins for association with the SPB (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 cooperate to promote the recruitment of components of the secretory apparatus to allow FSM expansion. Previous studies have shown that the association of all the SIN proteins with the SPB during meiosis depends upon the scaffold proteins Sid4p and Cdc11p (Krapp et al., 2006; Ohtaka et al., 2008; Yan et al., 2008). However, the hierarchy of localisation of the other SIN proteins in meiosis has not been investigated in detail.

The SIN regulator *dma1*

Dma1p, which is related to the mammalian CHFR/RNF8 family of ubiquitin ligases, is an inhibitor of the SIN – strong overexpression of Dma1p inhibits septum formation, producing multinucleated cells (Murone and Simanis, 1996). It regulates the association of Plo1p with the SPB (Guertin et al., 2002) by ubiquitylation of Sid4p (Johnson and Gould, 2011). Dma1p also plays a role in meiosis; after mating between *dma1-D* cells, the two meiotic divisions are completed normally, but the majority of asci have fewer than four spores, which are larger than wild-type spores. Analysis of spore formation reveals defects in FSM extension (Krapp et al., 2010; Li et al., 2010), and the degradation of mug27p is delayed, suggesting that this is regulated by Dma1p (Krapp et al., 2010).

In this paper, we have studied how the SIN is regulated during meiosis. Based upon localisation dependencies, our data indicate that the regulatory hierarchy of the SIN differs significantly between meiosis and mitosis. Furthermore, in contrast to the mitotic cycle, the Spg1p GTPase cycle is not the key factor in promoting the association of SIN proteins with the SPB. Finally, our results reveal stage-specific proteolysis of SIN components during meiosis. Dma1p is required for the degradation of some, but not all, of the SIN proteins, as well as other unrelated targets. Taken together, our data indicate that the regulatory relationships between SIN proteins differ between vegetative growth and meiosis.

RESULTS

Requirement for SIN proteins during spore formation

Heat-sensitive and null mutants have implicated the SIN and its regulators in spore formation (Krapp et al., 2006; Ohtaka et al., 2008; Pérez-Hidalgo et al., 2008; Yan et al., 2008). We extended this analysis to other components of the SIN. To induce meiosis, we used a mutation in *pat1*, which inhibits meiosis during vegetative growth (Iino and Yamamoto, 1985; Nurse, 1985). Colonies were exposed to iodine vapour, which stains them dark brown if spore-wall material is present. Double mutants of *pat1-114* with *sid2-250*, *mob1-R4* or *cdc7-24* were able to form spores, whereas double mutants of *pat1-114* with *cdc14-118*, *sid1-C14*, *spg1-B8*, *cdc11-136* or *sid4-SA1* were not (supplementary material Fig. S1A). The failure of *cdc14-118* to form spores is consistent with the requirement for its partner Sid1p in spore formation (Krapp et al., 2006), and the results for *spg1*, *cdc11* and *sid4* confirm those of earlier studies (Krapp et al., 2006; Yan et al., 2008). The ability of *sid2-250* to form spores has been documented and explained previously (see Introduction), but it was surprising to find that *mob1-R4* and *cdc7-24* formed spores, because these mutant proteins are inactive in vegetative cells at 32℃ (Goyal and Simanis, 2012). This not due to a partial suppression of these alleles by *pat1-114*, because a similar result was observed for diploids of *cdc7-24*, *cdc7-A20* and *mob1-R4* in a $pat1^+$ background (data not shown). This suggests either that Cdc7p and Mob1p are not required for meiosis or that their residual activity at 32℃ is sufficient during meiosis. Because meiosis is impaired above 33℃ (Crandall et al., 1977), we reduced SIN activity by replacing the promoters of SIN genes with the *rad21* promoter (*rad21P*), which is downregulated during meiosis (Kitajima et al., 2004). We were able to express *spg1*, *cdc7*, *sid1*, *sid2* and *mob1* from *rad21P* at levels sufficient to permit growth during the mitotic cycle. Cells carrying the *rad21P-sid2* allele formed spores after the expression of the gene was turned off (supplementary material Fig. S1B, left panel) but the *rad21P-sid2 mug27-D* mutant did not (supplementary material Fig. S1B, right panel), consistent with previous studies. Meiosis-specific depletion of Spg1p, Cdc7p, Sid1p and Mob1p resulted in the formation of asci containing four nuclei but no spores (supplementary material Fig. S1C), indicating the essential role of these SIN proteins in meiosis. These data indicate either that the meiotic roles of Cdc7-24p and Mob1-R4p are fulfilled by the residual activity in these hypomorphic alleles or that they are impaired only in their mitotic function.

Generation of the active configuration of the SIN at the onset of the second meiotic division is inhibited by Byr4p, but not by Cdc16p: a GAP-independent role for Byr4p?

Examination of Cdc7p–GFP in cells expressing *atb2-mCherry* (atb2–CHY) revealed that, in meiosis II cells with short spindles, Cdc7p was undetectable or very faint (Fig. 1A). Combining Cdc7p–GFP with the kinetochore marker *cnp1-mCherry* showed that Cdc7p–GFP appears at the SPB before the kinetochores have completed their movement to the microtubule ends at the SPB (Fig. 1B). Thus, Cdc7p–GFP associates with the SPB after spindle formation. Next, we investigated the localisation of

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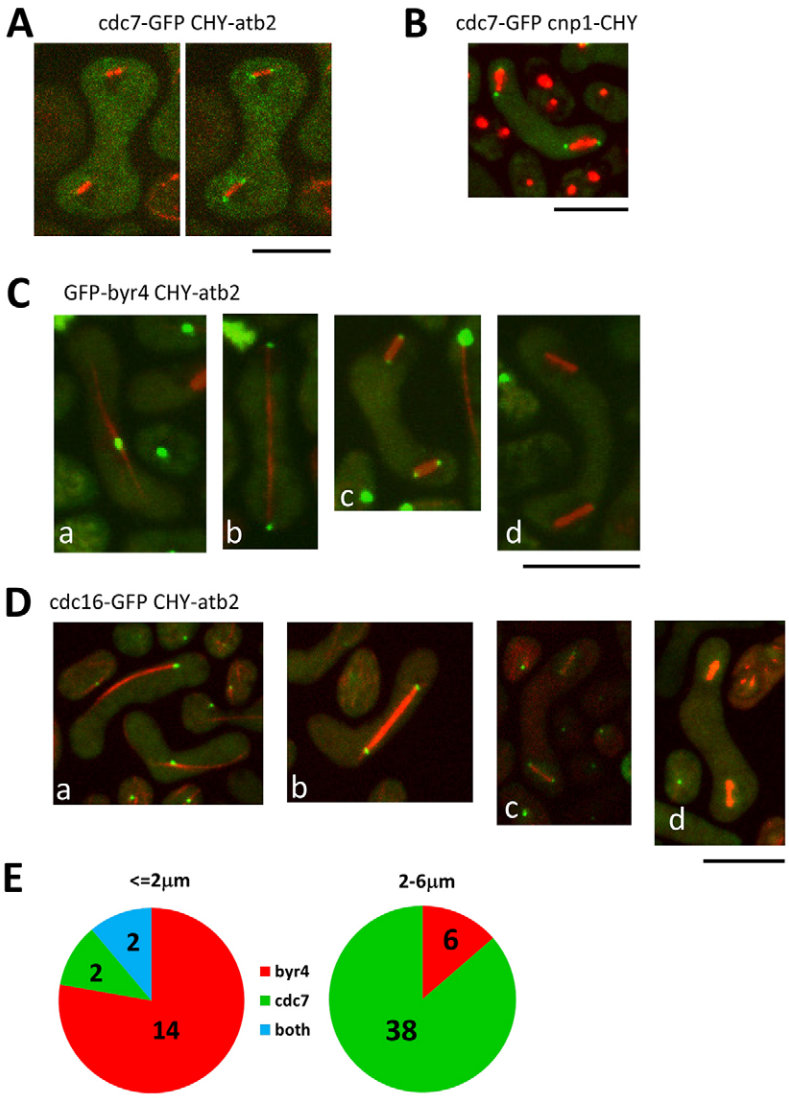

Fig. 1. Meiotic localisation of SIN proteins. The indicated strains were mated and imaged as described in the Materials and Methods. (A) Two images of meiosis II cells are shown, taken 1 minute apart. Cdc7p–GFP (green) is detectable only in the right panel. (B) A cell in meiosis II. Cdc7p–GFP is present at the SPBs, despite the fact that anaphase A has not been completed. (C) The localisation of GFP–Byr4p (green) at various stages of meiosis; (a) horsetail stage, (b) meiosis I, (c) early meiosis II and (d) late meiosis II. GFP–Byr4p is present in the early, but not late, meiosis II cell. (D) The localisation of Cdc16p–GFP at various stages of meiosis; (a) horsetail stage, (b) meiosis I, (c) early meiosis II and (d) late meiosis II. Cdc16p–GFP is present in the early, but not late, meiosis II cell. (E) Quantification of the association of Byr4p and Cdc7p with SPBs in meiosis II. Meiosis II spindles in cells expressing GFP–Byr4p and Cdc7p–CHY were analysed. The data obtained are presented as pie-charts; the numbers within each segment are the number of spindles observed. The spindle-length category is indicated above the chart. The null hypothesis, that the two proteins localise to the SPB in meiosis II independently, irrespective of spindle length, predicts a ratio of 1:1:2 green:red:red+green SPBs. A chi-squared test indicates that the results obtained for both spindle-length categories are significantly different from this ($P<0.001$). Scale bars: 10 μm.

GFP–Byr4p during meiosis. Previous studies (Krapp et al., 2006) did not examine this, owing to the lack of a suitable GFP-tagged *byr4* allele. We found that GFP–Byr4p was associated with the SPB at the horsetail stage, meiosis I and early, but not late, meiosis II (Fig. 1C). A similar pattern of localisation was found for Cdc16p–GFP (Fig. 1D), consistent with our previous analysis (Krapp et al., 2006).

Because the timing of the disappearance of Cdc16p and Byr4p from the SPBs is similar to that of the appearance of Cdc7p and Sid1p at the SPBs (see above), we attempted to investigate the colocalisation of Cdc7p and Byr4p. Unfortunately, rapid photobleaching of Cdc7p–CHY precluded simultaneous detection of the proteins in a timecourse. We therefore analysed images of meiosis II cells and scored the number of spindles that had Cdc7p–CHY, Byr4p–GFP or both associated with the SPBs. We observed that the majority of short spindles (≤2 μm) had GFP–Byr4p associated with the SPB, whereas examination of longer spindles (2–6 μm in length), revealed that the majority displayed a Cdc7p–CHY signal at the SPBs. A minority of SPBs showed both Byr4p and Cdc7p (Fig. 1E). These data are therefore consistent with the idea that Cdc7p–CHY replaces Byr4p–GFP in meiosis II, and that their colocalisation at the SPB is transient, at best.

To address whether the Spg1p–GAP complex affects the localisation of Cdc7p and Sid1p, we examined the effects of loss of Cdc16p and Byr4p. Both *byr4* and *cdc16* are essential in vegetative cells (Fankhauser et al., 1993; Minet et al., 1979; Song et al., 1996). Analysis of the localisation of Cdc7p–GFP or GFP–Sid1p in vegetatively growing *mob1-R4 byr4-D* or *mob1-R4 cdc16-D* cells revealed a strong signal associated with all SPBs at all stages of the mitotic cycle (supplementary material Fig. S2A), consistent with previous studies in the *cdc16-116* mutant (Guertin et al., 2000; Sohrmann et al., 1998). This is consistent with the idea that, in the absence of GAP activity, Spg1p is predominantly in the GTP-bound form and the SIN signals constitutively (Sohrmann et al., 1998).

To date, we have been unable to generate *rad21P*-alleles of either *cdc16* or *byr4*. We therefore worked in the *mob1-R4* background, which rescues both *cdc16-D* and *byr4-D* (Fournier et al., 2001). The *mob1-R4* mutation did not significantly affect the behaviour of SIN proteins during meiosis in a $byr4^{+}$ or $cdc16^{+}$ background (supplementary material Fig. S1D). Furthermore, the localisation of GFP–Byr4p, Cdc7p–GFP and GFP–Sid1p in meiotic *rad21P-mob1* cells was normal, despite the failure to form spores (supplementary material Fig. S1E), indicating that Mob1p is not required for the localisation of these proteins during meiosis.

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Imaging of meiotic *mob1-R4 byr4-D* cells revealed that Cdc7p–GFP was already present at the SPB during the horsetail stage and the first meiotic division, as well as during meiosis II (Fig. 2Aa–c). However, GFP–Sid1p associated with the SPB only during meiosis II in *mob1-R4 byr4-D* (Fig. 2Ba–c), as in wild-type cells. Thus, the loss of Byr4p promotes the premature recruitment of Cdc7p–GFP, but not GFP–Sid1p, to the SPB. By contrast, during meiosis in *mob1-R4 cdc16-D* cells, a robust Cdc7p–GFP signal was only detected in meiosis II (Fig. 2Af). No signal was detectable at the horsetail stage (Fig. 2Ad), and only a very faint signal was detected in late meiosis I (Fig. 2Ae). Similarly, GFP–Sid1p was only detectable at the SPB in meiosis II (Fig. 2Bd–f).

These data indicate that loss of the Byr4p and Cdc16p have different effects upon SIN protein localisation during meiosis, and suggest that Byr4p, but not the GAP activity of Cdc16p, plays an important role in controlling the timing of Cdc7p and Sid1p recruitment to the SPB in meiosis. Furthermore, because the ablation of either Byr4p or Cdc16p prevented its partner from localising to the SPB at any stage of meiosis (Fig. 2C; data not shown) it is likely that Byr4p exercises its influence upon Cdc7p localisation in the cytoplasm, rather than at the SPB.

The unexpected difference between *byr4-D* and *cdc16-D* described above prompted us to examine the localisation of

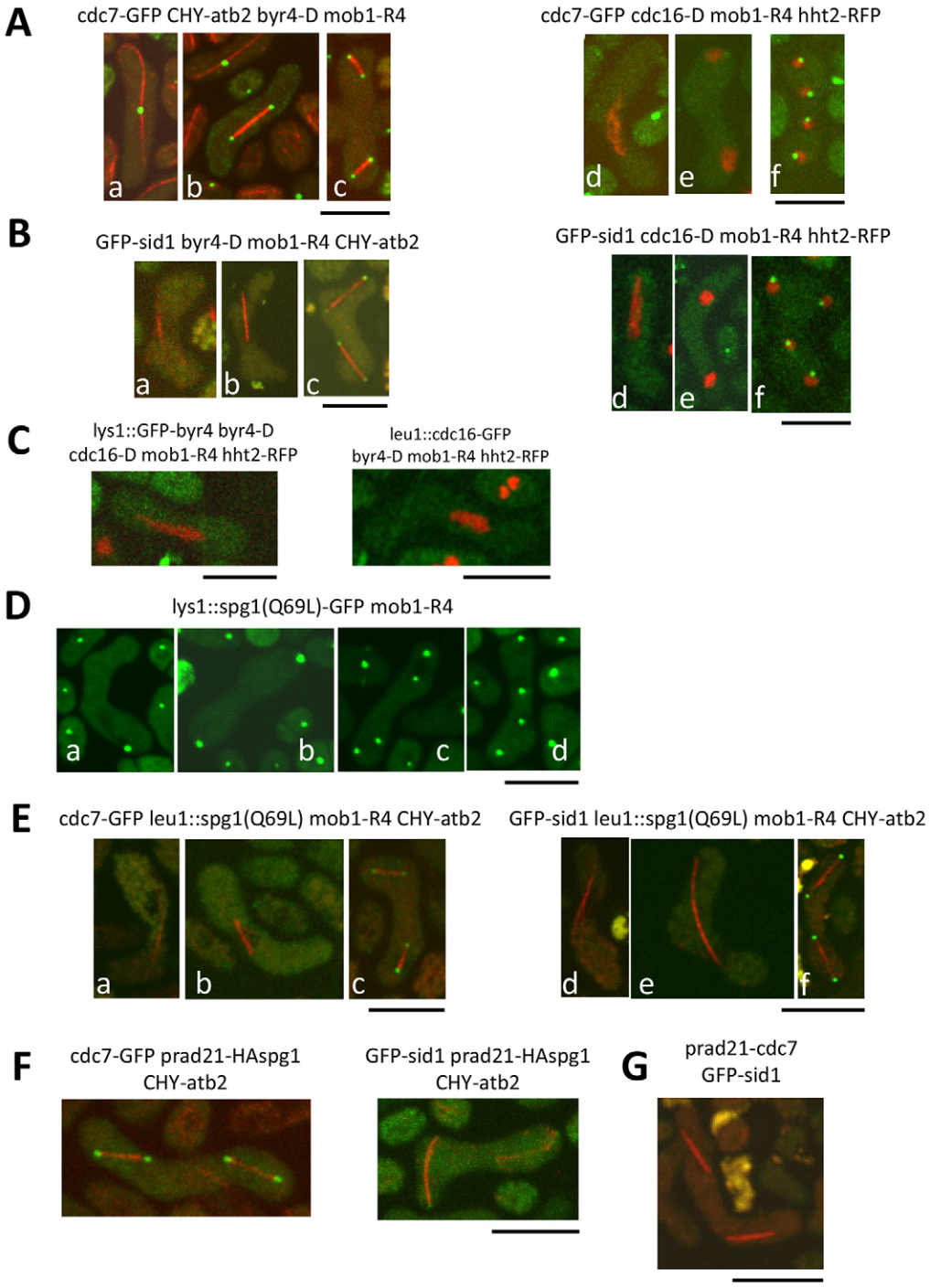

Fig. 2. Meiotic localisation of SIN proteins in SIN signalling mutants. The indicated mutants, expressing GFP-tagged SIN proteins were imaged during meiosis. (A) (a,d) Horsetail stage, (b,e) meiosis I and (c,f) meiosis II. Cdc7p–GFP (green) is present on the SPB at all stages of meiosis in *byr4-D mob1-R4* but not in *cdc16-D mob1-R4*. (B) (a,d) Horsetail stage, (b,e) meiosis I and (c,f) meiosis II. GFP–Sid1p (green) is present on the SPBs only in meiosis II. (C) Horsetail stage cells are shown; note the absence of Cdc16p–GFP (green, right panel) and GFP–Byr4p (green, left panel) if the other subunit of the GAP is absent. (D) (a) Horsetail stage, (b) meiosis I and (c,d) meiosis II. Spg1p(Q69L)–GFP (green) is present on the SPB at all stages of meiosis. (E) (a,d) Horsetail stage, (b,e) meiosis I and (c,f) meiosis II. Cdc7p–GFP and GFP–Sid1p are only associated with the SPBs in meiosis II. (F) Meiosis II cells are shown. Cdc7p–GFP is present on the SPBs and GFP–Sid1p is absent from the SPBs. (G) The panel shows a meiosis II cell. GFP–Sid1p is absent from the SPBs. Scale bars: 10 μm.

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SIN proteins during meiosis in a *spg1* mutant that could not hydrolyse GTP. We integrated *spg1-Q69L* (Schmidt et al., 1997) at the *leu1* locus, as described previously (Fankhauser and Simanis, 1994). *Leu1::spg1-Q69L* is a dominant allele (Schmidt et al., 1997), which can be propagated in mutant backgrounds that rescue the loss of GAP function, such as *mob1-R4* (Fournier et al., 2001). During vegetative growth, both GFP–Sid1p and Cdc7p–GFP localised to the SPB at all stages of the cell cycle in this mutant (supplementary material Fig. S2B), consistent with constitutive Spg1p signalling. Spg1(Q69L)p–GFP associated with the SPB throughout meiosis (Fig. 2D). However, despite the presence of the activated dominant Spg1(Q69L)p–GFP at the SPB, Cdc7p–GFP and GFP–Sid1p associated with the SPB only during meiosis II (Fig. 2E), and spores formed normally (supplementary material Fig. S2D). This suggests that the presence of Spg1p–GTP at the SPB is not the main factor promoting the recruitment of Cdc7p and Sid1p to the SPB during meiosis.

To examine the consequences of eliminating Spg1p function upon SIN protein localisation, we analysed a *rad21P-spg1* self-mating. In contrast to its localisation in mitotic cycle, during which Spg1p is required to load Cdc7p to the SPB (Sohrmann et al., 1998), we observed that Cdc7p–GFP was recruited to the poles in meiosis II (Fig. 2F), whereas GFP–Sid1p was barely detectable (Fig. 2F). These data indicate that one of the essential roles of Spg1p in meiosis is to promote the association of Sid1p, but not Cdc7p, with the SPB in meiosis II. However, we found that the association of GFP–Sid1p with the SPB in meiosis II was abolished in the absence of Cdc7p (Fig. 2G), indicating that Cdc7p is still required to load Sid1p to the SPB. Furthermore, the localisation of Cdc16p–GFP at the SPB during meiosis I and the horsetail stage was unaffected by the loss of Cdc7p (supplementary material Fig. S2F). In the mitotic cycle, Byr4p association with the SPB requires the presence of either Cdc7p or Byr4p (Krapp et al., 2008). Spg1p–GFP in *rad21P-cdc7* cells was found to associate with the SPB during the horsetail stage, meiosis I and both early and late meiosis II (supplementary material Fig. S2E). Because neither Byr4p nor Cdc16p are associated with the SPB in late meiosis II (see above), we conclude that the association of Spg1p–GFP with the SPB in meiosis II is independent of both Cdc7p and Byr4p. Similarly, analysis of *mob1-R4 cdc16-D* meiosis revealed that the localisation of Spg1p–GFP was not affected by the loss of *cdc16* (supplementary material Fig. S2C) during the horsetail stage or the first meiotic division, despite the absence of Cdc7p (Fig. 2A; supplementary material Fig. S2C). A model consistent with these data is presented in the discussion.

Byr4p is degraded after the completion of meiosis I

Because Byr4p and Cdc16p no longer associate with the SPB as cells progress through meiosis II, we analysed their steady-state levels during meiosis. The levels of Cdc16p showed a gradual decline as cells progressed through meiosis II (supplementary material Fig. S3J). By contrast, the steady-state level of Byr4p decreased after meiosis I (Fig. 3A). This decrease was dependent on the proteasome, because Byr4p was still detected at late stages in *mts2-1* cells (supplementary material Fig. S3A). Byr4p levels still decreased in the *sid4-SA1* mutant, in which the association of all SIN proteins (except Ppc89p) with the SPB is lost (Krapp et al., 2001; Rosenberg et al., 2006; Tomlin et al., 2002), indicating that stable association with the SPB is not required for the degradation of Byr4p, which most likely occurs in the cytoplasm (Fig. 3B). Finally, neither Dma1p nor the meiosis-II-specific anaphase promoting complex (APC/C) cofactor Mfr1p was required for the degradation of Byr4p (supplementary material Fig. S3E,F).

To determine which stages of meiosis needed to be completed for Byr4p to be degraded, we examined the steady-state level of the protein in various mutants. In *ddb1-D* cells, meiosis arrests at the horsetail stage owing to a failure to complete premeiotic S phase (Holmberg et al., 2005), whereas *mei4-D* cells fail to complete meiosis I (Horie et al., 1998); we found that the steady-state level of Byr4p remained elevated in both mutants (Fig. 3C; supplementary material Fig. S3B). The essential meiotic function of Ddb1p can be bypassed by deleting one of its targets, *spd1*; in the *ddb1-D spd1-D* mutant, Byr4p was once again degraded (supplementary material Fig. S3C), indicating that Ddb1p–Cul4p is not involved. The *mes1-D* mutant arrests after meiosis I with an activated APC/C (Izawa et al., 2005); the steady-state level of Byr4p decreased and GFP–Byr4p was not detected in binucleate cells that had disassembled the meiosis I spindle (Fig. 3D). Similar results were observed in a *spo6-D* strain (supplementary material Fig. S2G; Fig. S3D), which is delayed at the meiosis I–II transition (Nakamura et al., 2000). Taken together, these data indicate that Byr4p degradation requires completion of the first meiotic division.

Cdc7p, Cdc11p and Sid4p are degraded after the completion of meiosis II

To investigate the mechanisms involved in turning off the SIN at the end of meiosis, we analysed the steady-state levels of SIN-activator proteins after the end of meiosis II. The steady-state levels of Spg1p and Cdc14p (supplementary material Fig. S3G,H) did not change significantly during meiosis. Our previous study (Krapp et al., 2010) showed that, in contrast to Mug27p, the steady-state levels of Sid2p and Mob1p do not change significantly during meiosis. The Sid2p–GFP fusion protein clearly differs from the wild-type protein, and its localisation to the SPB during interphase is an artefact of the GFP tag (Grallert et al., 2012; Salimova et al., 2000). We therefore used the Sid2p antiserum described previously (Grallert et al., 2012) to examine Sid2p during meiosis. Western blotting revealed that Sid2p is present throughout meiosis and that its level declines after the end of meiosis II (supplementary material Fig. S3K). Indirect immunofluorescence (data not shown) revealed that Sid2p localised at the SPBs in meiosis I and II, consistent with the analysis of the GFP-tagged allele (Krapp et al., 2006). The steady-state level of GFP–Sid1p increased during meiosis I, and then declined to a level similar to that seen in early meiosis as cells progressed through meiosis II (supplementary material Fig. S3I). This reflects the changes in *sid1* mRNA level during meiosis (Mata et al., 2002). In contrast to this, the steady-state levels of Cdc7p, Cdc11p and Sid4p all declined significantly after the completion of meiosis II (Fig. 4A–C); however, this decline was abolished in *mts2-1*, indicating that it is due to proteolysis (supplementary material Fig. S4A–C). Moreover, we also observed slower-migrating forms of Cdc7p–GFP, Cdc11p–HA and Sid4p–GFP, starting from the meiosis I–II transition; treatment of the Cdc11p-HA immunoprecipitate with alkaline phosphatase converted much of the protein to a faster-migrating form, indicating that this slower migration is due, at least in part, to phosphorylation (supplementary material Fig. S4D). The degradation of Cdc7p and Cdc11p depends upon the completion of meiosis II, because they are not degraded in *spo6-D* cells

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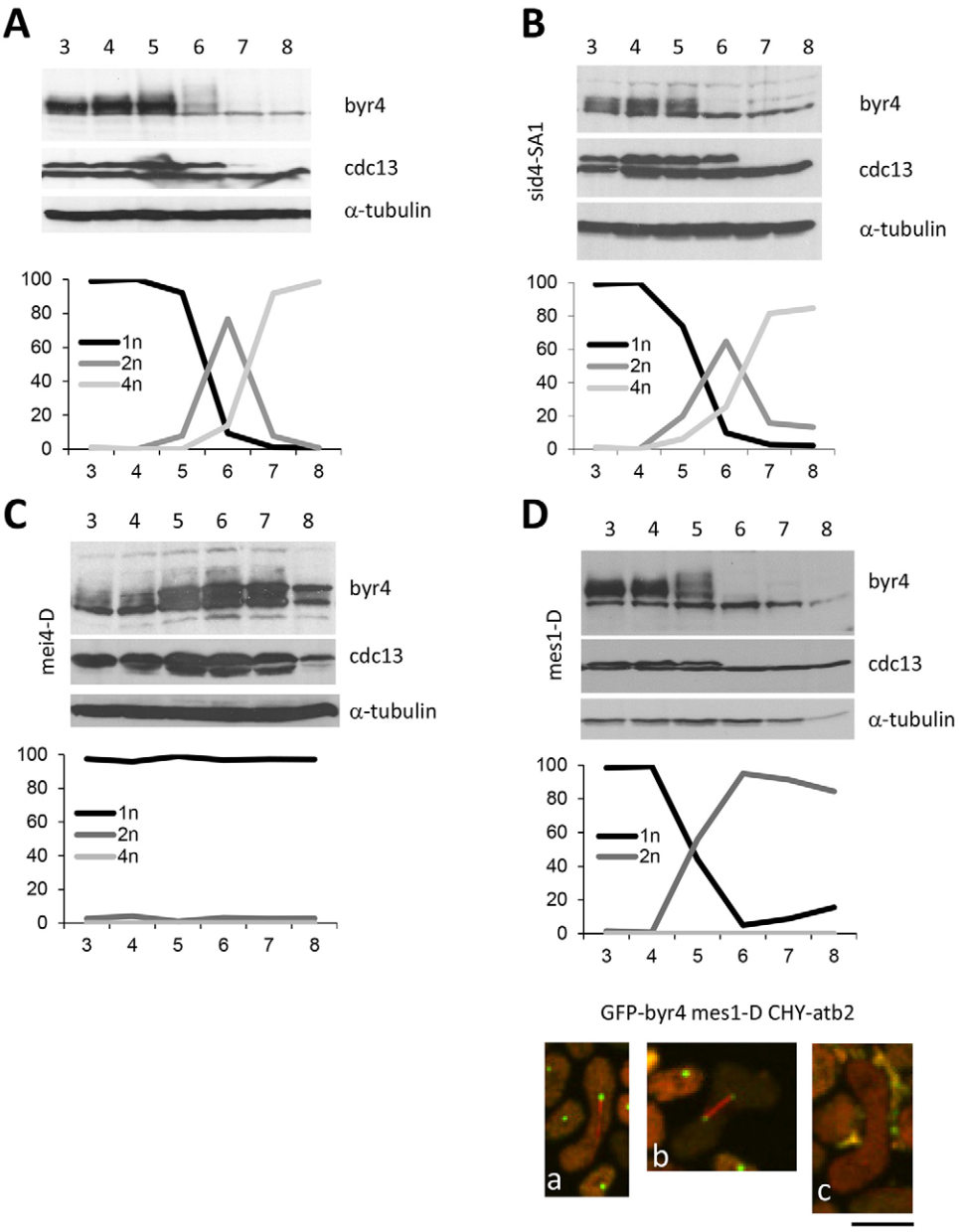

Fig. 3. Analysis of the steady-state level of Byr4p in meiosis. Cells expressing the indicated mutant protein in a *pat1-114* background were induced to undergo meiosis by incubation at 32°C. The number above each lane indicates the number of hours after the induction of meiosis. The graph indicates mononucleated (1n), binucleated (2n) and tetranucleated (4n) cells. *x*-axis, time (hours); *y*-axis, percentage. Western blots were probed with antibody against Byr4p. The mitotic cyclin Cdc13p and α-tubulin served as controls. In the Cdc13 blot, the upper band is Cdc13p and the lower band is non-specific. In panel D, the micrographs show a horsetail-stage cell (a), a meiosis I cell with a spindle (b) and a binucleate cell without a spindle (c) that has completed meiosis I, but not formed a detectable meiosis II spindle. Note the absence of GFP–Byr4p (green) in c. Scale bars: 10 μm.

(supplementary material Fig. S4E; data not shown). To test whether their degradation requires their association with the SPB, we examined the level of Cdc11p in *sid4-SA1* cells and that of Cdc7p in *cdc11-136* cells, mutant backgrounds that compromise the SPB association of these proteins – the levels of both proteins decreased (Fig. 4D,E). Furthermore, the slower-migrating forms of Cdc11p were not observed in *sid4-SA1* cells, indicating that, as in the mitotic cycle (Krapp et al., 2003), phosphorylation of Cdc11p requires its association with the SPB. Taken together, these data demonstrate that SPB association is not essential to trigger the degradation of Cdc7p and Cdc11p at the end of meiosis II.

Spore formation is not required for the removal of SIN proteins from the SPB in meiosis II

To examine at which stage SIN proteins no longer associated with the SPB, we investigated the localisation of Cdc7p in late meiotic cells using the markers Meu14p–GFP and Psy1p–GFP to assess completion of the FSM. While the FSM was still being formed, we observed that Cdc7p was associated with the SPB (Fig. 5A, left panel, left cell; Fig. 6D); once the leading-edge complex was no longer visible and the FSM was completed, Cdc7p was no longer associated with the SPB (Fig. 5A, right panel, left cell; Fig. 6D).

Because the removal of Cdc7p from the SPB correlated with FSM closure, we tested whether this is necessary for Cdc7p destruction. We examined the level and localisation of Cdc7p–GFP in *spo15-D*, which cannot initiate FSM formation, and *meu14-D*, where FSM extension and closure is impaired. Previous studies showed that Cdc7p can associate with the SPB in a *spo15-D* or *meu14-D* mutant in early meiosis II (Krapp et al., 2006). However, after the completion of meiosis II, Cdc7p–GFP no longer associated with the SPB in either *spo15-D* or *meu14-D* (Fig. 5B), and it was degraded at the end of meiosis II (Fig. 5C,D), indicating that neither the removal of Cdc7p from the SPB nor its degradation require spore formation.

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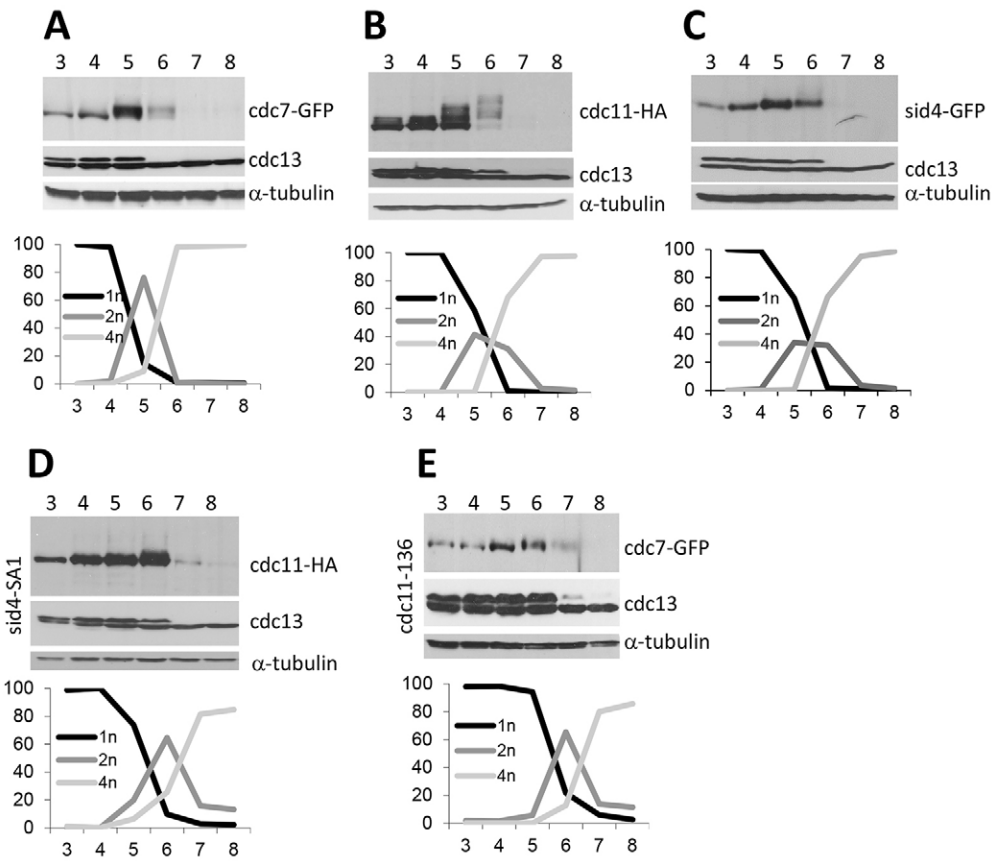

Fig. 4. Analysis of the steady-state levels of SIN proteins during meiosis. Cells bearing the indicated mutant or tagged allele in a *pat1-114* background were induced to undergo meiosis by incubation at 32°C. The western blots were probed with an antibody to detect the tag. Graphs and western blots are labelled as described for Fig. 3.

The degradation of SIN proteins in late meiosis depends upon Dma1p

Previously, we showed that Dma1p is required for the timely elimination of Mug27p (Krapp et al., 2010). Therefore, we tested whether Dma1p is also required for the elimination of Cdc7p, Cdc11p and Sid4p after the completion of meiosis II. We observed that all three proteins were not degraded at late times in meiosis in *dma1-D* (Fig. 6A–C). Consistent with this, comparison of the SPB-associated Cdc7p–CHY signal in cells expressing Psy1p–GFP that had completed FSM closure showed a faint signal in *dma1*$^{+}$ cells, but a bright signal in *dma1-D* cells (Fig. 6D). Moreover, Cdc7p–GFP, Cdc11p–GFP and Sid4p–GFP all remained associated with the completed spores in a *dma1-D* meiosis (Fig. 6E,F). The slower-migrating forms of all three proteins accumulated in *dma1-D*, indicating that their modification does not require Dma1p.

The SPB protein Mcp6p (also known as Hrs1p) is downregulated by phosphorylation and proteolysis to permit formation of the meiosis I spindle. Failure to degrade Mcp6p leads to sporulation defects (Funaya et al., 2012) similar to those observed in *dma1-D* cells (Krapp et al., 2010). Therefore, we examined whether Dma1p is required for the elimination of Mcp6p. In *dma1*$^{+}$ cells, Mcp6p–GFP was degraded at the onset of meiosis I (Fig. 7A,D), confirming previous studies (Funaya et al., 2012). By contrast, in a *dma1-D* mutant, Mcp6p remained associated with the SPBs throughout mitosis (Fig. 7B,E). Similar results were obtained in the *dma1(I194A)* mutant, in which the mutant Dma1p cannot bind to its E2 enzyme (Johnson and Gould, 2011) (Fig. 7C). These data indicate that the degradation of Mcp6p is dependent upon Dma1p, and suggest that Dma1p might have multiple targets throughout meiosis.

DISCUSSION

The regulation of SIN signalling has been studied extensively, and a basic picture of how it works in vegetative cells has emerged. It is unclear whether these SIN regulation ‘rules’ also apply during meiosis. In this paper, we have begun to examine how the SIN is regulated in meiosis, and we have found significant differences between the mitotic and meiotic regulation of the SIN.

The first divergence lies in the importance of the nucleotide status of Spg1p and its GAP proteins in regulating the recruitment of SIN effector proteins, such as Cdc7p and Sid1p, to the SPB. Spg1(Q69L) is a dominant signalling-active allele that promotes the association of Cdc7p and Sid1p with the SPB at all stages of the cell cycle and induces septum formation when expressed in vegetative cells. By contrast, the expression of this allele during meiosis does not result in the recruitment of Cdc7p or Sid1p to the SPB outside of meiosis II. This is also true if the GAP component Cdc16p is absent. However, in contrast to this, loss of the GAP scaffold protein Byr4p results in premature recruitment of Cdc7p, but not Sid1p, to the SPB, as early as the horsetail stage. This indicates firstly, that the presence of Spg1p–GTP is insufficient per se to promote the association of Cdc7p with the SPB and, secondly, that Byr4p, but not Cdc16p, is required to restrain the association of Cdc7p with the SPB in early meiosis. The difference between the phenotypes of *cdc16-D* and *byr4-D* suggests that this role of Byr4p is independent of its scaffolding function in the Spg1p GAP. Furthermore, because Cdc16p and Byr4p remain interdependent for SPB association during meiosis, it is likely that Byr4p enacts this role in the cytoplasm. Future studies will investigate this novel role of Byr4p.

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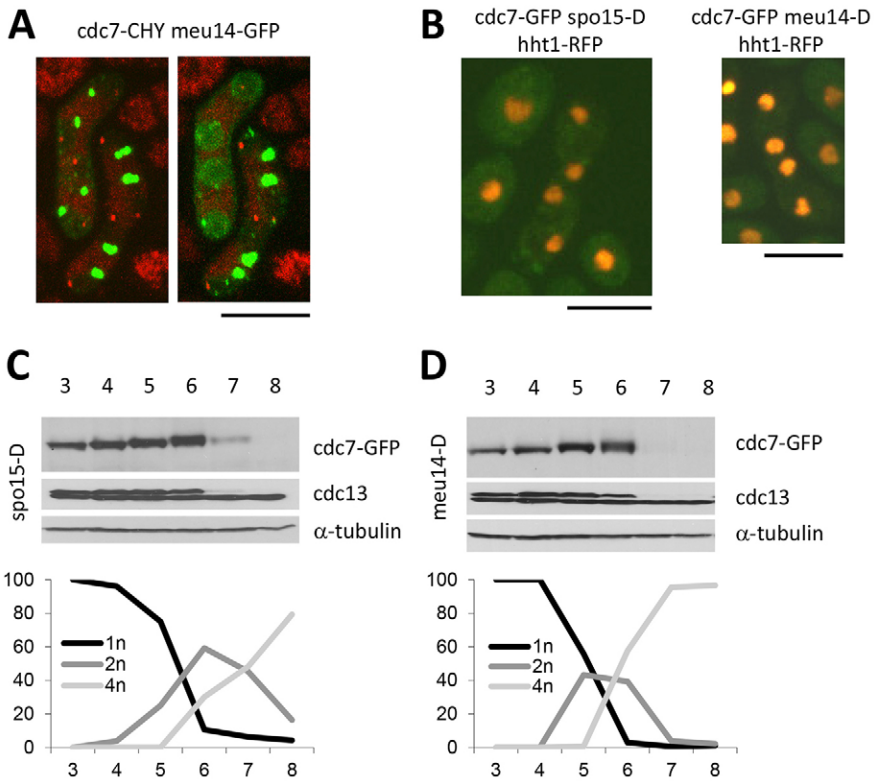

Fig. 5. Analysis of Cdc7p at the end of meiosis. (A) The indicated strain was imaged during late meiosis. The two frames were taken 5 minutes apart. While the FSM is incomplete (left panel, left cell), Cdc7p–CHY (red) is present on the SPBs, and it is absent after the leading-edge complex has dissociated, indicating closure of the FSM. (B) Tetranucleated cells of the indicated strains were imaged. Cdc7p–GFP is absent from the SPBs in these cells. Scale bars: 10 μm. (C,D) The indicated mutants, expressing Cdc7p–GFP in a *pat1-114* background, were induced to undergo meiosis by incubation at 32°C. Western blots were probed with an antibody to detect the GFP tag. Graphs and western blots are labelled as described for Fig. 3.

An earlier study showed that Byr4p recruits Fin1p (the *S. pombe* orthologue of nimA) to the SPB in mitosis, and that Fin1p also regulates signalling flux through the SIN (Grallert et al., 2004). The steady-state level of *fin1* mRNA increases at the meiosis I–II transition (Mata et al., 2002); it is not known whether this is reflected at the protein level. Given these findings, it will be of interest to determine whether Fin1p contributes to the regulation of Byr4p during meiosis.

The second difference is the hierarchy of SIN protein association with the SPB. The association of Spg1p–GFP with the SPB is independent of both Cdc7p and the GAP, in contrast to the situation in mitotic cells (Krapp et al., 2008). Because Spg1p can interact directly with the SIN scaffold Cdc11p in vegetative cells (Morrell et al., 2004), it is possible that Cdc11p also provides a direct anchor in meiosis. During vegetative growth, Spg1p is required for the localisation, but not the activity, of Cdc7p (Mehta and Gould, 2006; Schmidt et al., 1997). However, in meiosis, Cdc7p association with the SPB in meiosis II is independent of Spg1p. Strikingly, during meiosis, it is Sid1p, rather than Cdc7p, which depends upon Spg1p for its association with the SPB, suggesting that an important role of Spg1p during meiosis is to promote the SPB association of Sid1p. The requirement for Cdc7p in the correct localisation of Sid1p could be explained if active Cdc7p creates a phosphorylation-dependent binding site for Sid1p or Spg1p at the SPB. It is also possible that Spg1 is required to activate, but not direct the localisation of Cdc7p during meiosis (the opposite of the situation in vegetative growth). The active Cdc7p would then promote the stable association of Sid1p with the SPB (see Fig. 7F). These ideas are consistent with the observation that, in the absence of Byr4p, Cdc7p localises to the SPB before Sid1p. However, it is also possible that some other event (for example, Dma1p-dependent modification of a protein) is required for Sid1p recruitment in the absence of Byr4p.

The mechanism for turning the SIN off differs between meiosis and mitosis. In the mitotic cycle, ‘resetting’ of the SIN to the interphase configuration correlates with contractile ring closure and does not involve degradation of Cdc7p or scaffold proteins (García-Cortés and McCollum, 2009). In contrast to the situation in vegetative growth, we have found that several SIN proteins are degraded during meiosis. The GAP scaffold Byr4p is degraded after meiosis I, independently of Dma1p. The scaffolds Cdc11p and Sid4p, as well as Cdc7p, are degraded at the end of meiosis II, and this requires Dma1p, summarised in Fig. 7G. Moreover, although the Cdc7p signal decreases at the time of FSM closure, this is not obligatory either for its removal from the SPB or its degradation.

The only SIN component that is clearly regulated by proteolysis in vegetative cells is Byr4p, which is degraded by the proteasome when not complexed to Spg1p. This is thought to be a mechanism to regulate the amount of Spg1p GAP during vegetative growth (Krapp et al., 2008). Overexpression of *nuc2*, which encodes a component of the APC/C, blocks cytokinesis (Kumada et al., 1995). It is involved in turning the SIN off after completion of cytokinesis, by blocking the formation of the Spg1p–Cdc7p complex (Chew and Balasubramanian, 2008). Ubiquitylation also plays a modulatory, rather than a destructive role in regulating the SIN, because Sid4p ubiquitylation by Dma1p in mitotically arrested cells prevents the association of Plo1p with the SPB (Johnson et al., 2013; Johnson et al., 2012; Johnson and Gould, 2011). This Dma1p-dependent ubiquitylation does not cause Sid4p degradation, but probably modifies its scaffolding activity. How Dma1p modifies the activity of Sid4p in vegetative cells, while triggering its destruction in meiosis II is unclear. The outcome might depend upon the combination of post-translational modifications of Sid4p, which could target Dma1p to different sites on the protein. Alternatively, ubiquitylation by Dma1p might be

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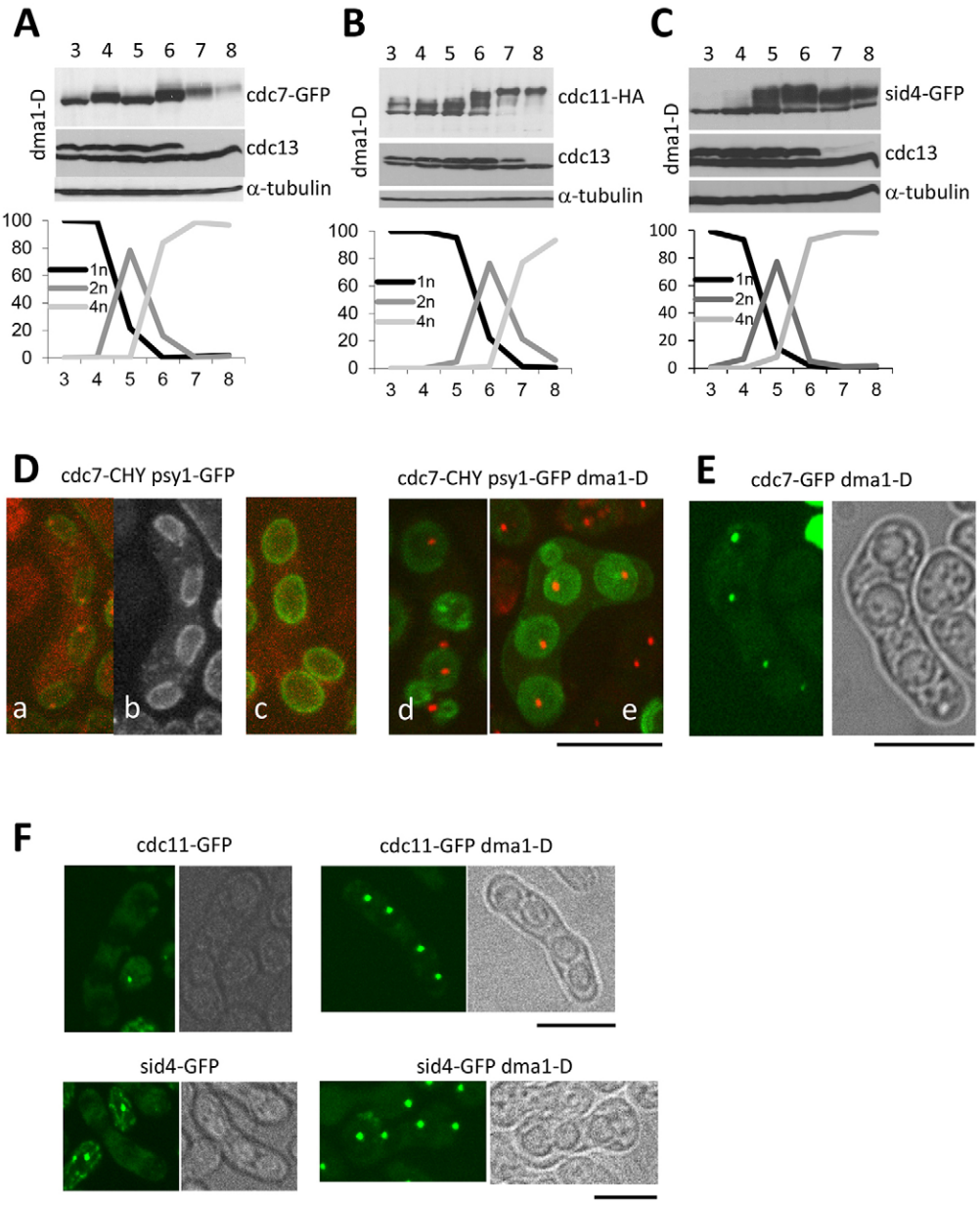

Fig. 6. Analysis of the effects of *dma1-D* on the degradation of SIN proteins in meiosis. (A–C) *dma1-D* cells expressing the indicated tagged SIN protein (right-hand side of top panel) in a *pat1-114* background, were induced to undergo meiosis by incubation at 32°C. Western blots were probed with an antibody to detect the tag. The number above each lane indicates the number of hours after the induction of meiosis. Graphs and western blots are labelled as described for Fig. 3. (D) Mating cells expressing the indicated tagged alleles in a *dma1*$^{+}$ (a–c) or *dma1-D* background (d,e) were examined. Note that the Cdc7p–CHY image (a) is faint in the *dma1*$^{+}$ compared with that in the *dma1-D* background. The asci shown (c,d,e) have all completed the FSM, as judged by the unbroken Psy1–GFP signal (green). To see the incomplete FSM in *dma1*$^{+}$, the green channel (Psy1p–GFP) for panel a is presented adjacent to the merged image (b). In panels d and e, note the presence of aberrant FSM formation as described previously (Krapp et al., 2010). (E,F) Homozygous matings of strains expressing the indicated tagged allele in a *dma1-D* or *dma1*$^{+}$ background were imaged when asci had formed. For each image pair, the left panel is the GFP channel and the right panel is the brightfield image. In the *dma1-D* background, the GFP-tagged proteins remain associated with the SPB, whereas the equivalent signals are not detected in the *dma1*$^{+}$ background. Scale bars: 10 μm.

the trigger for additional meiosis-specific modifications that result in degradation. Understanding the mechanisms governing stage-specific SIN protein degradation will be the subject of future studies.

Dma1p is also required for the elimination of Mcp6p prior to meiosis I. This raises the possibility that Dma1p has targets throughout meiosis, and that its activity extends beyond SIN regulation. Dma1p does not associate stably with the SPB during the horsetail stage or meiosis I (Krapp et al., 2010), which suggests that its activity towards Mcp6p is mediated in the cytoplasm. A proteome-wide search for other Dma1p-dependent degradation events in meiosis will be the subject of future studies.

The budding yeast counterpart of the SIN is the mitotic exit network (MEN). Although the regulatory wiring of the MEN and SIN differ slightly in vegetative cells, previous studies have shown that the regulation of the MEN also differs significantly between meiosis and mitosis. During vegetative growth, MEN signalling originates from the SPB and involves a cascade of protein kinases, the activities of which are modulated by a GTPase (reviewed by Simanis, 2003; Weiss, 2012). By contrast, during meiosis, MEN signalling does not require either SPB association or the scaffold protein used in vegetative growth. Furthermore, signalling is regulated by controlling the association of one of the kinases with its regulatory subunit (Attner and Amon, 2012). Thus, in both yeasts, this signalling pathway undergoes significant adaptations for its role in meiosis.

A definitive answer to the question of why a subset of SIN proteins should be degraded during meiosis awaits mapping and mutagenesis of ubiquitylation sites on the targeted proteins. Nonetheless, the data we have obtained raise some interesting ideas regarding the possible role of protein degradation in regulating the SIN during meiosis.

Elimination of Cdc7p and the scaffold proteins Cdc11p and Sid4p might appear to be ‘overkill’ if the degradation serves only to inactivate the SIN. It is possible that the relevant Dma1p target is Sid4p and that, in late meiosis, Cdc11p and Cdc7p are unstable

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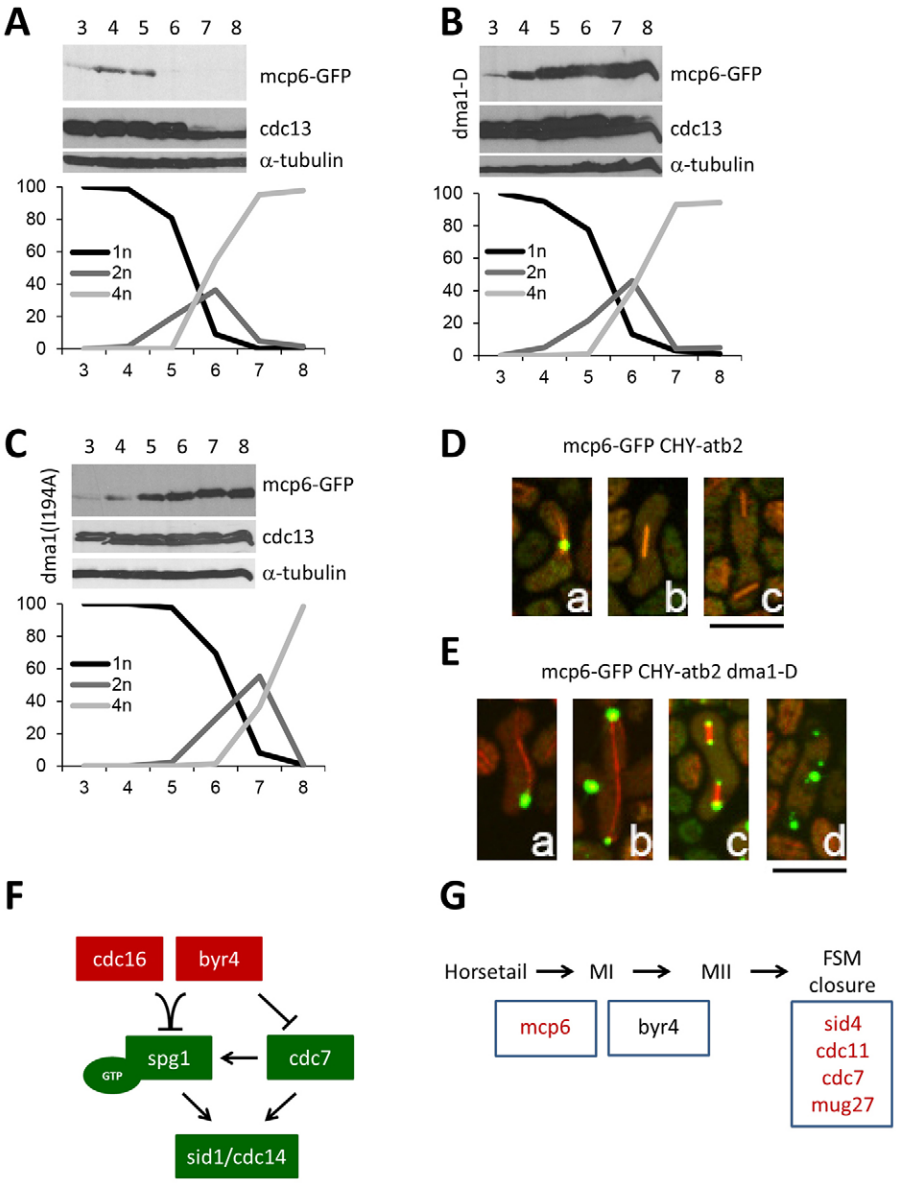

Fig. 7. The SPB protein Mcp6p requires Dma1p for degradation. (A–C) Wild-type, *dma1-D* or *dma1(I194A)* cells, expressing Mcp6p–GFP in a *pat1-114* background were induced to undergo meiosis by incubation at 32˚C. Westerns were probed with an antibody to detect the tag. Graphs and western blots are labelled as described for Fig. 3. (D,E) The indicated strains were imaged as they underwent meiosis; (a) horsetail stage, (b) meiosis I, (c) meiosis II and (d) post-meiosis II. Mcp6p–GFP is absent after the horsetail stage in wild-type cells but not in *dma1-D* cells. Scale bars: 10 μm. (F) Model of the 'dual-key' regulation of the SIN during meiosis. Red boxes indicate inhibitors of SIN signalling, green boxes represent components required for signal transmission. Arrowed lines indicate activation events. T-end lines indicate inhibition. (G) Summary of the timing of the degradation during meiosis of the proteins examined in this study; see text for details. Gene names written in red indicate Dma1p-dependent degradation of the corresponding protein. The E3 enzyme that targets Byr4p is unknown.

if not bound to a scaffold. Alternatively, it might be necessary to eliminate multiple SIN proteins for other reasons. For example, Cdc11p and Sid4p might serve as scaffolds for non-SIN proteins. Two studies are noteworthy in this context: first, the mitotic B-type cyclin Cdc13p also associates with Cdc11p (Morrell et al., 2004); second, in budding yeast, Nud1p, the functional counterpart of Cdc11p, binds to different proteins in meiosis and mitosis and is destroyed at the end of meiosis II (Knop and Strasser, 2000), suggesting that destruction of scaffold proteins in meiosis might be a theme that has been conserved through evolution. Another possibility is that these SIN proteins are eliminated from the spore to prevent untimely assembly of signalling complexes as it germinates. However, there is no indication that significant numbers of *dma1-D* spores undergo inappropriate septation during the germination process (Krapp et al., 2010). Alternatively, the SIN might be silenced to prevent it from inhibiting the morphology network, which is required to establish polar growth (Gupta et al., 2013; Ray et al., 2010), in the germinating apolar spore. It is noteworthy that *dma1-D* spores are slow to germinate and resume polar growth (Krapp et al., 2010). Future studies will attempt to distinguish these possibilities.

Our data are consistent with a dual-key model (Fig. 7F) for the recruitment of SIN proteins to the SPB during meiosis II. We propose that the simultaneous presence of Cdc7p and Spg1p–GTP is required for the association of Sid1p with the SPB in meiosis II. Degradation of Byr4p at the end of meiosis I would permit SPB-association of Cdc7p. It would also generate Spg1p-GTP, allowing Spg1p-dependent Sid1p SPB association. Future studies will examine whether there is a direct interaction between Spg1p and Sid1p in meiosis. This model explains why neither Spg1p(Q69L) nor loss of Cdc16p results in the recruitment of Sid1p and Cdc7p early in meiosis, because the continued presence of Byr4p would inhibit the association of Cdc7p with the SPB. The model therefore posits a dual role for Byr4p in meiosis; it regulates Spg1p, together with Cdc16p, and controls the association of Cdc7p with the SPB independently of its GAP function. The simplest mechanism would be a direct interaction between Cdc7p and Byr4p but, to date, there is no

evidence of which we are aware for direct *in vivo* interaction between the proteins in vegetative cells. Therefore, more complex alternatives, such as Byr4p sequestering an activator of Cdc7p, must also be considered. Elimination of Byr4p after meiosis I would free Cdc7p to associate with the SPB. The meiotic binding partner of Cdc7p at the SPB is unknown, but a direct association with Cdc11p has been demonstrated in vegetative cells (Feoktistova et al., 2012). Our future studies will test these ideas and attempt to elucidate the mechanism(s) governing the SPB association of SIN proteins during meiosis.

In budding yeast, an overall decrease in the amount of protein has been noted as cells progress through meiosis (Betz and Weisner, 1976; Zubenko and Jones, 1981). Analysis of specific SPB-associated proteins has also revealed stage-specific protein degradation throughout meiosis (Knop and Strasser, 2000). Targeted stage-specific proteolysis of cyclins and other proteins is already established as important for meiotic progression in *S. pombe* (Blanco et al., 2001; Funaya et al., 2012; Izawa et al., 2005; Kimata et al., 2008; Okuzaki et al., 2010; Pérez-Hidalgo et al., 2008). Furthermore, studies in *Saccharomyces cerevisiae* using 2D gels have revealed some changes in protein level during meiosis (Grassl et al., 2010; Scaife et al., 2010). We have shown in this study that Dma1p activity is required for the degradation of proteins at different stages of meiosis. Our results suggest that progression through meiosis will be regulated, in part, by stage-specific protein degradation. Punctual degradation might ensure orderly meiotic progression and prevent the inappropriate reinitiation of meiotic events. A case in point is provided by the failure to upregulate the expression of the APC/C regulator *mfr1*, which results in failure to terminate meiosis properly (Aoi et al., 2013). The role of protein degradation in regulating meiotic progression and the ubiquitin ligases involved will be the subject of future studies. In summary, our data reveal that the regulation of SIN activity in meiosis involves a complex interplay of phosphorylation and degradation to bring about the activation and then silencing of the SIN.

MATERIALS AND METHODS

Yeast methods

Standard methods and media were used for the growth of *S. pombe* (Moreno et al., 1991). YE or EMM2 media were used as required; EMM2 without NH_4Cl was used for nitrogen starvation. *S. pombe* strains bearing GFP-, m-Cherry-, HA- or myc-tagged or null alleles of SIN genes have been described previously (see Krapp et al., 2006; Krapp et al., 2010 for references). The *CHY-atb2* allele was a gift from the Toda laboratory (London Research Institute, London, UK). The *mcp6-GFP* strain was received from the Tanaka laboratory (University of Leicester, Leicester, UK). *dma1(I194A)* was a gift from the Gould laboratory (Vanderbilt University Medical Center, Nashville, TN). GFP-tagged Psy1p and Meu14p, and the null alleles of *meu14* and *spo15* were obtained from the Yeast National Bioresearch Project (Japan). *mei4-D* and *mes1-D* were obtained from the Yamamoto laboratory (Kasuza DNA Research Institute, Tokyo, Japan). *mug27-D* and *mfr1-D* were obtained from the Moreno laboratory (Institute of Functional Biology and Genomics, Salamanca, Spain). The *cnp1-CHY* strain was obtained from the Hagan laboratory (Paterson Institute for Cancer Research, Manchester, UK). *ddb1-D* and *spd1-D* were obtained from the Carr laboratory (University of Sussex, Sussex, UK). Expression of the SIN genes under control of the *rad21* promoter was performed by oligonucleotide-mediated tagging, as described previously (Bähler et al., 1998) for *pFA-rad21P-HA*. The vector was as described previously (Kitajima et al., 2004) and was obtained from the Tanaka laboratory. The *GFP-byr4* strain was created by placing the *byr4* promoter upstream of the GFP–Byr4 fusion. This construct was then integrated into either the *leu1* or the *lys1* gene. The GFP-tagged *byr4* gene inserted at either locus rescues a *byr4* deletion. The *spg1(Q69L)* mutant was integrated into the *leu1* or *lys1* gene in a *mob1-R4* background.

Standard techniques (Moreno et al., 1991) were used to create the strains described in the text. To examine the effect on spore formation, as well as to examine the localisation of GFP-tagged proteins during meiosis, strains of opposite mating types expressing chromosomally GFP-tagged alleles were mated on EMM-N plates at 25℃ for 20 hours, resuspended in EMM-N and mounted for imaging. Spore-wall formation was monitored by exposing cells to I_2 vapours.

Synchronous meiosis in *pat1-114* temperature-sensitive mutants was performed as follows. Cells were grown in EMM plus supplements (adenine, leucine, uracil, as required) (100 µg/ml), washed and resuspended in EMM-N plus supplements (10 µg/ml) at a density of $2–3\times10^6$ cells/ml. After 16 hours at 25℃, most cells were arrested in G1 phase and the culture was shifted to 32℃ in the presence of EMM containing 0.5 g/l NH_4Cl and 10 µg/ml supplements to induce meiosis.

Microscopy

DAPI staining was performed on cells that had been fixed with 70% (v/v) ethanol, as described previously (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. The presence of asci with mature spores was determined by using phase-contrast microscopy.

Examination of GFP-tagged proteins in living cells was performed using an inverted Olympus IX 81 microscope equipped with a confocal scanner (CSU-W1; Yokogawa Electric Corporation), a EMCCD ImageEM B/W camera (Hamamatsu) and 60×/1.42 NA plan apo objective. Images were collected using VisiView software (Visitron Systems GmbH). Images were assembled in Adobe Photoshop CS5.

Protein methods

Total protein extracts were made using the trichloroacetic acid (TCA) extraction protocol (Foiani et al., 1994). Protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes (Protran, Whatman). Primary antibodies against GFP (raised in-house), Sid2 (Hagan laboratory), Cdc13 (Moreno laboratory), c-myc (Santa Cruz Biotechnology), HA (Roche), Byr4 and tubulin (TAT-1; Keith Gull, University of Oxford, Oxford, UK) were used as described by the authors. Secondary antibodies conjugated to horseradish peroxidise and enhanced chemiluminescence (ECL) western blotting reagents (Amersham) were used to visualise the bands. Immunoprecipitation and phosphatase treatment of Cdc11p–HA has been described previously (Krapp et al., 2001).

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Competing interests

The authors declare no competing interests.

Author contributions

Experiments conceived by V.S. and A.K., performed by A.K. Paper written by V.S. and A.K.

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Supplementary material

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