Regulation of meiotic progression by the meiosis-specific checkpoint kinase Mek1 in fission yeast

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Summary

During the eukaryotic cell cycle, accurate transmission of genetic information to progeny is ensured by the operation of cell cycle checkpoints. Checkpoints are regulatory mechanisms that block cell cycle progression when key cellular processes are defective or chromosomes are damaged. During meiosis, genetic recombination between homologous chromosomes is essential for proper chromosome segregation at the first meiotic division. In response to incomplete recombination, the pachytene checkpoint (also known as the meiotic recombination checkpoint) arrests or delays meiotic cell cycle progression, thus preventing the formation of defective gametes. Here, we describe a role for a meiosis-specific kinase, Mek1, in the meiotic recombination checkpoint in fission yeast. Mek1 belongs to the Cds1/Rad53/Chk2 family of kinases containing forkhead-associated domains, which participate in a number of checkpoint responses from yeast to mammals. We show that defects in meiotic recombination generated by the lack of the fission yeast Meu13 protein lead to a delay in entry into meiosis I owing to inhibitory phosphorylation of the cyclin-dependent kinase Cdc2 on tyrosine 15. Mutation of *mek1*$^+$ alleviates this chekpoint-induced delay, resulting in the formation of largely inviable meiotic products. Experiments involving ectopic overexpression of the *mek1*$^+$ gene indicate that Mek1 inhibits the Cdc25 phosphatase, which is responsible for dephosphorylation of Cdc2 on tyrosine 15. Furthermore, the meiotic recombination checkpoint is impaired in a *cdc25* phosphorylation site mutant. Thus, we provide the first evidence of a connection between an effector kinase of the meiotic recombination checkpoint and a crucial cell cycle regulator and present a model for the operation of this meiotic checkpoint in fission yeast.

Key words: Meiosis, Checkpoint, Mek1, Meiotic recombination, Cell cycle, Fission yeast

Introduction

Eukaryotic cell division consists of a highly regulated sequence of events that must occur in the appropriate order. Checkpoints are control mechanisms that prevent initiation of late events until earlier events have been successfully completed, thus ensuring faithful transmission of genetic information to the progeny (Hartwell and Weinert, 1989). In response to defective cellular processes and/or alterations of genome integrity, these surveillance mechanisms arrest or delay cell cycle progression. Checkpoints pathways are composed of sensors that detect the cellular defect or DNA lesions, generating a signal that is transmitted through a transduction pathway usually formed by protein kinases. Ultimately, checkpoint effectors act on cellular targets, triggering various responses, including cell cycle arrest or delay and DNA repair. In mammals, defects in checkpoint responses cause genomic instability, leading to tumor development (reviewed by Weinert, 1998; Lowndes and Murguia, 2000; Melo and Toczyski, 2002).

Meiosis is a specialized type of cell division that generates haploid gametes from diploid parental cells because a single round of DNA replication is followed by two consecutive nuclear divisions. During meiotic prophase, a complex series of interactions between homologous chromosomes (or homologs) occur. First, chromosomes search for and associate with the homologous partners (pairing). In most (but not all) organisms, these associations are stabilized by synapsis, which is the formation of an elaborate proteinaceous structure (the synaptonemal complex; SC) that holds homologs close together along their entire length. Concomitantly, DNA recombination between homologous chromosomes takes place. In addition to the exchange of genetic information, the result of these interactions is the formation of physical connections between homologs, called chiasmata, which promote correct chromosome segregation during the first meiotic division (reviewed by Roeder, 1997; Smith and Nicolas, 1998; Zickler and Kleckner, 1999; Lee and Amon, 2001).

Meiotic cells possess a surveillance mechanism referred to as the ‘pachytene checkpoint’ or the ‘meiotic recombination checkpoint’ that monitors these critical meiosis-specific events. Meiotic recombination is initiated by DNA double-strand breaks (DSBs), which are repaired using nonsister chromatids as templates. In response to defects in recombination that lead to accumulation of unrepaired DSBs and/or other recombination intermediates, the pachytene checkpoint triggers meiotic cell cycle arrest or delay to prevent meiotic chromosome missegregation (Roeder and Bailis, 2000).

A number of studies in the budding yeast *Saccharomyces cerevisiae* have identified several components of the pachytene checkpoint (Roeder and Bailis, 2000). DNA damage checkpoint proteins that respond to DSBs in vegetative cells

also monitor these lesions during meiosis; however, there are differences between the mitotic DNA damage checkpoint and the meiotic recombination checkpoint. First, some DNA damage checkpoint proteins (e.g., Chk1, and Rad9) are not required for the pachytene checkpoint (Lydall et al., 1996) (P.A.S.-S. and G. S. Roeder, unpublished). Second, it has been proposed that meiotic DSBs are monitored in a meiosis-specific chromosomal context and are not recognized as 'general' damage (Xu et al., 1997). Third, some crucial pachytene checkpoint proteins, such as the nucleolar silencing factor Pch2 or the Mek1 kinase, are produced only during meiosis (Rockmill and Roeder, 1991; San-Segundo and Roeder, 1999). Fourth, the DNA damage checkpoint and the pachytene checkpoint act on different targets of the cell cycle machinery to block cell cycle progression (Leu and Roeder, 1999).

The pachytene checkpoint has been extensively studied only in *S. cerevisiae*, but its operation in worms, flies and mammals has been also reported (Edelmann et al., 1996; Pittman et al., 1998; Yoshida et al., 1998; Ghabrial and Schupbach, 1999; Gartner et al., 2000; MacQueen and Villeneuve, 2001; Abdu et al., 2002). In fact, most (if not all) yeast pachytene checkpoint proteins have homologs in other organisms (Roeder and Bailis, 2000). However, although the fission yeast *Schizosaccharomyces pombe* is a model organism widely used in checkpoint studies during the mitotic cell cycle (Murakami and Nurse, 2000), little is known about surveillance mechanisms of meiosis-specific processes, in particular meiotic recombination.

Here we show that the meiotic recombination checkpoint does indeed operate in *S. pombe*, and we describe a role for a meiosis-specific kinase, Mek1, in this control mechanism. Mek1 contains a forkhead-associated (FHA) domain. FHA motifs are usually implicated in protein-protein interactions regulated by phosphorylation (Durocher et al., 1999). We present evidence indicating that fission yeast Mek1 prevents entry into the first meiotic division (MI) until recombination is completed. The Mek1-dependent negative regulation of MI entry is achieved by maintaining phosphorylation of Cdc2 at Tyr15, at least in part, through inhibition of the Cdc25 phosphatase.

Materials and Methods

Strains and plasmids

Fission yeast strains used in this work are listed in Table 1. To delete the *mek1*$^+$ and *meu13*$^+$ genes, a PCR-based strategy was used (Bahler et al., 1998). The whole ORF was replaced for the *kanMX6* or the *ura4*$^+$ marker. The same approach was used for tagging *mek1*$^+$ with three copies of the HA epitope immediately before the stop codon at its genomic locus. Strains expressing *mek1*$^+$ tagged with GFP integrated in the genome under control of the thiamine-repressible *nmt1* promoter were constructed by transformation with plasmid pLV1 (see below) cut with *Nru*I to target the construct to the *leu1-32* locus. Strain S1299 carrying the *cdc25-9A* allele at the genomic locus was constructed as follows. An *Nde*I-*Sma*I fragment containing the *cdc25-9A* gene was obtained from plasmid pGEX2TN(HpaI)-Cdc25(9A) (Zeng and Piwnica-Worms, 1999) and used to transform a *cdc25-22* temperature-sensitive strain (PN35). Transformants were initially selected on YES plates at 36°C; then, several colonies were patched and replica-plated to YES-phloxin plates containing 0.05% and 0.005% methylmethane sulphonate (MMS). Transformants showing a defective response to MMS were selected and the presence of the nine mutated phosphorylation sites in *cdc25-9A* was verified by DNA sequencing. Growth conditions and strain manipulations were described previously (Moreno et al., 1991). Diploid strains homozygous for the mating type (h^-/h^-) were generated by protoplast fusion (Sipiczki and Ferenczy, 1977). Synchronous meiosis in h^-/h^- *pat1-114/pat1-114* thermosensitive diploid strains was carried out essentially as described previously (Blanco et al., 2001).

To clone the *mek1*$^+$ gene, the *mek1*$^+$ cDNA was amplified by PCR using cDNA obtained from a 3-hour *pat1* meiotic culture (see Fig. 1) with primers mek1-N 5′-TTTTCTCGAG*CATATG*GACTTTTTAT-CACATGCCATG-3′ (*Xho*I site, underlined; *Nde*I site, italicized) and mek1-C 5′-TTTTCCCGGG*CTA*GCGGCCGCCTAGCCGGGAAT-GTTTAAGAGG-3′ (*Sma*I and *Not*I sites, underlined; an added stop codon, italicized). The PCR product was digested with *Xho*I-*Sma*I and cloned into the same sites of the pREP3X vector (Forsburg, 1993), producing plasmid pSS123, which contains *mek1*$^+$ cDNA under the *nmt1(3×)* promoter. The *Nde*I-*Sma*I fragment from pSS123 containing *mek1*$^+$ was cloned into the same sites of pREP41-EGFP-N (Craven et al., 1998), generating plasmid pSS124, which expresses *mek1*$^+$, N-terminally tagged with GFP, from the *nmt1(41×)* promoter. Plasmid pLV1 was constructed by cloning a *Pst*I-*Eco*RI fragment from pSS124 containing *nmt1-GFP-mek1*$^+$ into the same sites of the integrative vector pJK148 (Keeney and Boeke, 1994).

Genetic procedures

Spore viability was assayed by tetrad dissection. The frequency of meiotic intergenic recombination was determined by random spore analysis. Crosses were performed on MEA plates and, after 2 days, spores were isolated, grown on YES plates and replica-plated to minimal medium. The number of recombinant spores was counted and normalized to the total number of viable spores.

Northern and western blotting

RNA preparation and northern blot analysis were performed as described previously (Blanco et al., 2001) using a *mek1*$^+$ PCR fragment amplified with oligomers mek1-N and mek1-C as a probe. Total protein extracts were prepared as described elsewhere (Blanco et al., 2000). For western blot analysis, ~60 μg of total extracts were run on 12% SDS-PAGE gels, transferred to nitrocellulose and probed with the following antibodies: mouse monoclonal anti-HA (12CA5; 0.15 mg/ml), rabbit polyclonal anti-Cdc2 (C2; 1:200 dilution), rabbit polyclonal anti-phospho-Cdc2(Tyr15) (Cell Signaling Technology; 1:1000 dilution) and mouse monoclonal anti-tubulin (TAT1; 1:1000 dilution). Goat anti-rabbit or goat anti-mouse antibodies conjugated to horseradish peroxidase (Amersham) were used as secondary antibodies (1:3500 and 1:2000 dilution, respectively). Immunoblots were developed using the Luminol Reagent (Santa Cruz Biotechnology) or the SuperSignal kit (Pierce).

Flow cytometry

Flow cytometric analysis was performed on a Becton-Dickinson FACSscan using propidium iodide staining of cells (Sazer and Sherwood, 1990).

Microscopy

For the analysis of meiotic progression, cells were fixed in 70% ethanol and processed for DAPI staining of nuclei as described previously (Moreno et al., 1991). To study the subcellular localization of Mek1-HA, immunofluorescence analysis was performed essentially as described previously (Santos and Snyder, 1997), except that PEMBAL buffer (100 mM PIPES pH 6.9, 1 mM EGTA, 1 mM MgSO4, 1% BSA, 0.1% azide, 0.1 M L-lysine HCl)

Table 1. Yeast strains

Strain	*Genotype*
PN22	*h⁻ leu1-32*
PN35	*h⁺ leu1-32 cdc25-22*
S145	*h⁻ leu1-32 wee1-50*
S176	*h⁺ leu1-32 ade6-M210 cdc2-3w*
S778	*h⁺ leu1-32 ade6-M216*
S781	*h⁻ leu1-32 ade6-M210*
S898	*h⁺ leu1-32 ura4-259 cdc25::ura4⁺ cdc2-3w*
S964	*h⁻/h⁻ pat1-114/pat1-114 ade6-M216/ade6-M210 leu1-32/leu1-32*
S1285	*h⁺ his5-303*
S1291	*h⁺ ade6-M216 leu1-32 mek1::kanMX6*
S1292	*h⁻ ade6-M210 leu1-32 mek1::kanMX6*
S1293	*h⁻/h⁻ pat1-114/pat1-114 ade6-M216/ade6-M210 leu1-32/leu1-32 mek1::kanMX6 /mek1::kanMX6*
S1294	*h⁻/h⁻ pat1-114/pat1-114 ade6-M216/ade6-M210 leu1-32/leu1-32 mek1-3HA-kanMX6/mek1-3HA-kanMX6*
S1295	*h⁻/h⁻ pat1-114/pat1-114 ade6-M216/ade6-M210 leu1-32/leu1-32 ura4-d18/ura4-d18 meu13::ura4⁺/meu13::ura4⁺*
S1296	*h⁻/h⁻ pat1-114/pat1-114 ade6-M216/ade6-M210 leu1-32/leu1-32 ura4-d18/ura4-d18 meu13::ura4⁺/meu13::ura4⁺ mek1::kanMX6/mek1::kanMX6*
S1297	*h⁻ leu1-32 ade6-M210 nmt1(41X)-mek1-GFP::leu1⁺*
S1298	*h⁻/h⁻ pat1-114/pat1-114 ade6-M216/ade6-M210 nmt1(41X)-mek1-GFP::leu1⁺/leu1-32*
S1299	*h⁺ leu1-32 cdc25-9A*
S1300	*h⁻ leu1-32 mek1::kanMX6*
S1301	*h⁺ his5-303 mek1::kanMX6*
S1302	*h⁺ leu1-32 ade6-M210 nmt1(41X)-mek1-GFP::leu1⁺ cdc25-9A*
S1306	*h⁻/h⁻ pat1-114/pat1-114 ade6-M216/ade6-M210 leu1-32/leu1-32 meu13::kanMX6⁺/meu13::kanMX6⁺*
S1307	*h⁻/h⁻ pat1-114/pat1-114 ade6-M216/ade6-M210 leu1-32/leu1-32 meu13::kanMX6⁺/meu13::kanMX6 cdc25-9A/cdc25-9A*
S1308	*h⁻/h⁻ pat1-114/pat1-114 ade6-M216/ade6-M210 leu1-32/leu1-32 cdc25-9A/cdc25-9A*

was used instead of PBS. Mouse monoclonal anti-HA antibody (HA.11, Covance) was used at 1:150 dilution. Goat anti-mouse antibody conjugated to CY3 (Jackson ImmunoResearch Labs) was used as the secondary antibody (1:200 dilution). Cells were visualized using a Zeiss Axioplan2 fluorescence microscope equipped with a Hamamatsu CCD camera.

Results

S. pombe Mek1: a meiosis-specific member of the FHA family of checkpoint kinases

Bioinformatic analysis of the fission yeast genome (http://www.sanger.ac.uk/Projects/S_pombe/) revealed that *S. pombe* possesses a putative open reading frame (SPAC14C4.03), which encodes a 445 amino-acid protein with a high degree of similarity to the *S. cerevisiae* meiosis-specific kinase Mek1 (Fig. 1A; 34% identitity, 54% similarity, BLAST e-value: 4×10^{-58}). In addition to the kinase domain (amino acids 160-421), *S. pombe* Mek1 contains a FHA domain (amino acids 62-116), which is characteristic of a conserved family of kinases involved in different checkpoint responses in eukaryotic cells (Fig. 1B) (Durocher et al., 1999).

The *S. pombe mek1⁺* gene was cloned by polymerase chain reaction (PCR) using specific primers and genomic DNA or cDNA as the template (see Materials and Methods; Fig. 1C). No PCR product corresponding to *mek1⁺* was amplified when cDNA obtained from vegetative cells was used (Fig. 1C). However, a PCR fragment of the expected size was obtained when cDNA from *S. pombe* cells at the early stages of meiosis was used (Fig. 1C), indicating that the *mek1⁺* gene is only expressed in meiotic cells. The difference in size of the *mek1⁺* fragment amplified from genomic DNA or from cDNA is consistent with the presence of the two predicted introns in the gene.

The *S. pombe* Mek1 protein is produced during meiotic prophase and localizes to the nucleus

Expression of the *mek1⁺* gene was monitored by northern blot analysis during a *pat1*-driven synchronous meiosis (Fig. 2A-C). Consistent with the results shown above, no expression of *mek1⁺* is detected in vegetatively growing cells; its expression is induced at the same time as the onset of premeiotic S phase and reaches the maximum level during the period corresponding to meiotic prophase; then, *mek1⁺* mRNA levels decrease as cells enter into the first meiotic division (Fig. 2A-C). The production of the Mek1 protein was analyzed by western blot using anti-HA antibodies in a meiotic time course of *pat1-114* diploid cells expressing a functional version of *mek1⁺* tagged with three copies of the HA epitope. The kinetics of Mek1-HA production is similar to the one described above for *mek1⁺* mRNA (Fig. 2D).

To determine the subcellular location of Mek1, immunofluorescence analysis of *pat1-114 mek1-HA* diploid cells was carried out using anti-HA antibodies. The Mek1 protein localizes to the nucleus of meiotic cells during the horse-tail movement period (Fig. 2E). No staining is detected in control cells lacking the HA epitope (data not shown).

Spore viability and meiotic recombination are reduced in the *mek1* mutant

To study Mek1 function during meiosis in fission yeast, the *mek1⁺* gene was deleted. The *mek1* mutant completes meiotic divisions and sporulation, generating morphologically normal four-spore asci. However, tetrad dissection revealed that spore viability is reduced in the *mek1* mutant compared to wildtype (64% versus 89%, respectively). Although the overall decrease in spore viability of *mek1* is not dramatic, the fraction of tetrads containing four viable spores is significantly reduced compared to wildtype (~16% versus ~78%; Fig. 3A). No excess of tetrads

containing 4, 2 and 0 viable spores is observed, suggesting that spore death in the *mek1* mutant is not due to chromosome nondisjunction during meiosis I. Intergenic recombination was examined in the *leu1-his5* interval on chromosome II. The *mek1* mutant displays a ~2.4-fold reduction in meiotic recombination in this region (Fig. 3B).

Mek1 regulates meiotic cell cycle progression in fission yeast

Using *pat1* strains to induce synchronous meiosis, kinetics of meiotic progression were examined in the *mek1* mutant in comparison with the wildtype (Fig. 4A). In the *mek1* mutant, the first meiotic division occurs reproducibly ~30 minutes faster than in the otherwise isogenic wildtype (Fig. 4A). Since *mek1*$^+$ expression is induced at the time of premeiotic S phase (although its peak of expression is reached at prophase; Fig. 2), DNA replication was carefully monitored by FACS during synchronous meiosis in *mek1* and wild-type cells at 15 minute time points (Fig. 4B). This analysis revealed that premeiotic DNA replication takes place with the same kinetics in both wild-type and *mek1* cells. Therefore, since entry into meiosis I occurs earlier in *mek1*, this implies that meiotic prophase is shorter in the absence of Mek1.

To further characterize this observation, the effect of expressing high levels of Mek1 was also studied. A green fluorescent protein (GFP)-tagged version of *mek1*$^+$ was placed under control of the thiamine-regulated *nmt1* promoter and integrated at the *leu1* locus (see Materials and Methods). To induce overexpression of *mek1*$^+$, thiamine was removed 14 hours prior to transferring the cells to medium lacking nitrogen (Fig. 4C). The production of Mek1-GFP was followed by microscopic examination of the cells throughout the experiment. A control culture, in which thiamine was always

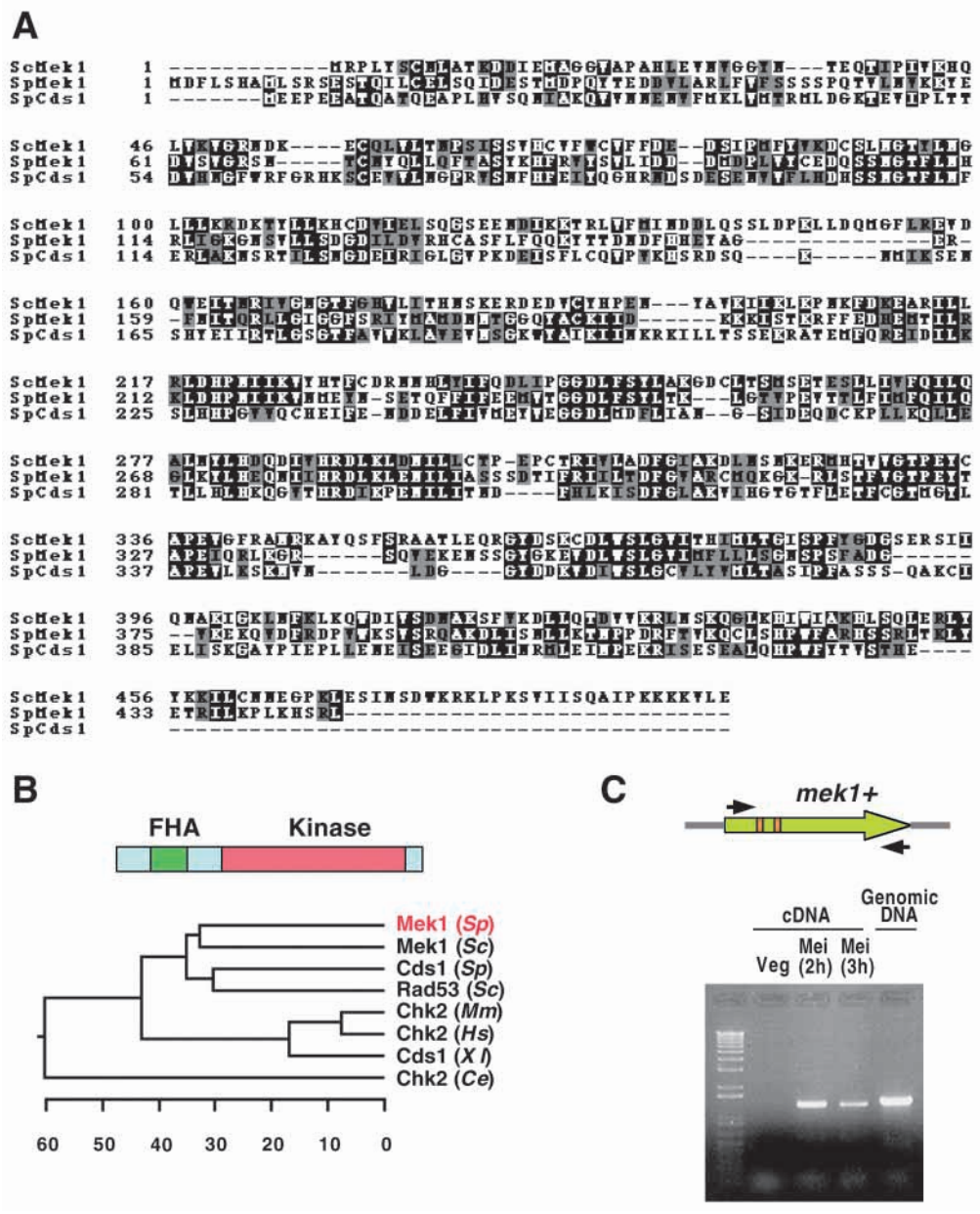


Fig. 1. Fission yeast Mek1 belongs to the family of Cds1/Rad53/Chk2 checkpoint kinases and is expressed only in meiotic cells. (A) Alignment of the protein sequences of *S. cerevisiae* Mek1 (ScMek1), *S. pombe* Mek1 (SpMek1) and *S. pombe* Cds1 (SpCds1). Identities are highlighted in black and conservative substitutions in gray. Numbers at the left represent amino-acid positions. Alignment and shading were performed with ClustalW 1.8 and Boxshade 3.21, respectively, at the BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/). (B) Phylogenetic tree of the family of FHA checkpoint kinases. The clustal method with PAM250 residue table was used. Sp, *S. pombe*; Sc, *S. cerevisiae*; Mm, *M. musculus*; Hs, *H. sapiens*; Xl, *X. laevis*; Ce, *C. elegans*. A schematic representation of the functional motifs in this protein family (with the exception of Rad53, which contains two FHA domains) is shown. (C) PCR amplification of *S. pombe mek1*$^+$ using primers mek1-N and mek1-C (small arrows; see Materials and Methods). The following DNA templates were used: cDNA from vegetative *S. pombe* cells (Veg), cDNA from meiotic cells (S964) at 2 hours and 3 hours in meiosis (Mei 2h and Mei 3h, respectively) and *S. pombe* genomic DNA.

present and therefore *mek1*$^+$ expression was repressed, was also examined. FACS analysis revealed that both cultures were blocked in G1 to the same extent and underwent premeiotic S phase with similar kinetics (Fig. 4D); however, *nmt1*-driven expression of *mek1*$^+$ resulted in a significant delay (~1 hour) of the first meiotic division (Fig. 4E). Interestingly, the Mek1-GFP signal disappeared as cells entered meiosis I, and binucleate cells containing GFP signal were rarely observed (data not show). Thus, these results suggest that the Mek1 kinase negatively regulates entry into meiosis I.

Ectopic overexpression of *mek1*$^+$ in vegetative cells causes cell cycle arrest by inhibiting Cdc25 function

In order to understand how Mek1 regulates cell cycle progression, high levels of the protein were produced in vegetative cells using the *nmt1* promoter. Interestingly, ectopic overproduction of Mek1 results in inhibition of growth (Fig. 5A). Microscopic examination revealed that Mek1-overproducing cells are highly elongated and contain a single undivided nucleus (Fig. 5B), a phenotype that resembles the G2/M arrest induced by activation of the DNA integrity checkpoints or by overproduction of the Cds1 or Chk1 checkpoint kinases (Furnari et al., 1997; Boddy et al., 1998).

In principle, the G2/M arrest triggered by *mek1*$^+$ ectopic overexpression may be caused by inhibition of the Cdc25 phosphatase or activation of the Wee1 or Mik1 kinases. To identify which cell cycle regulator(s) is the target of Mek1, the protein was overproduced in mutants defective in either Cdc25 or Wee1 function. Since *cdc25*$^+$ is an essential gene, to analyze whether Cdc25 is involved in the Mek1-dependent arrest, a *cdc2-3w* strain, which bypasses the requirement for Cdc25 was used (Russell and Nurse, 1986). Like in the wild-type cells, overexpression of *mek1*$^+$ in *cdc2-3w* results in cell elongation,

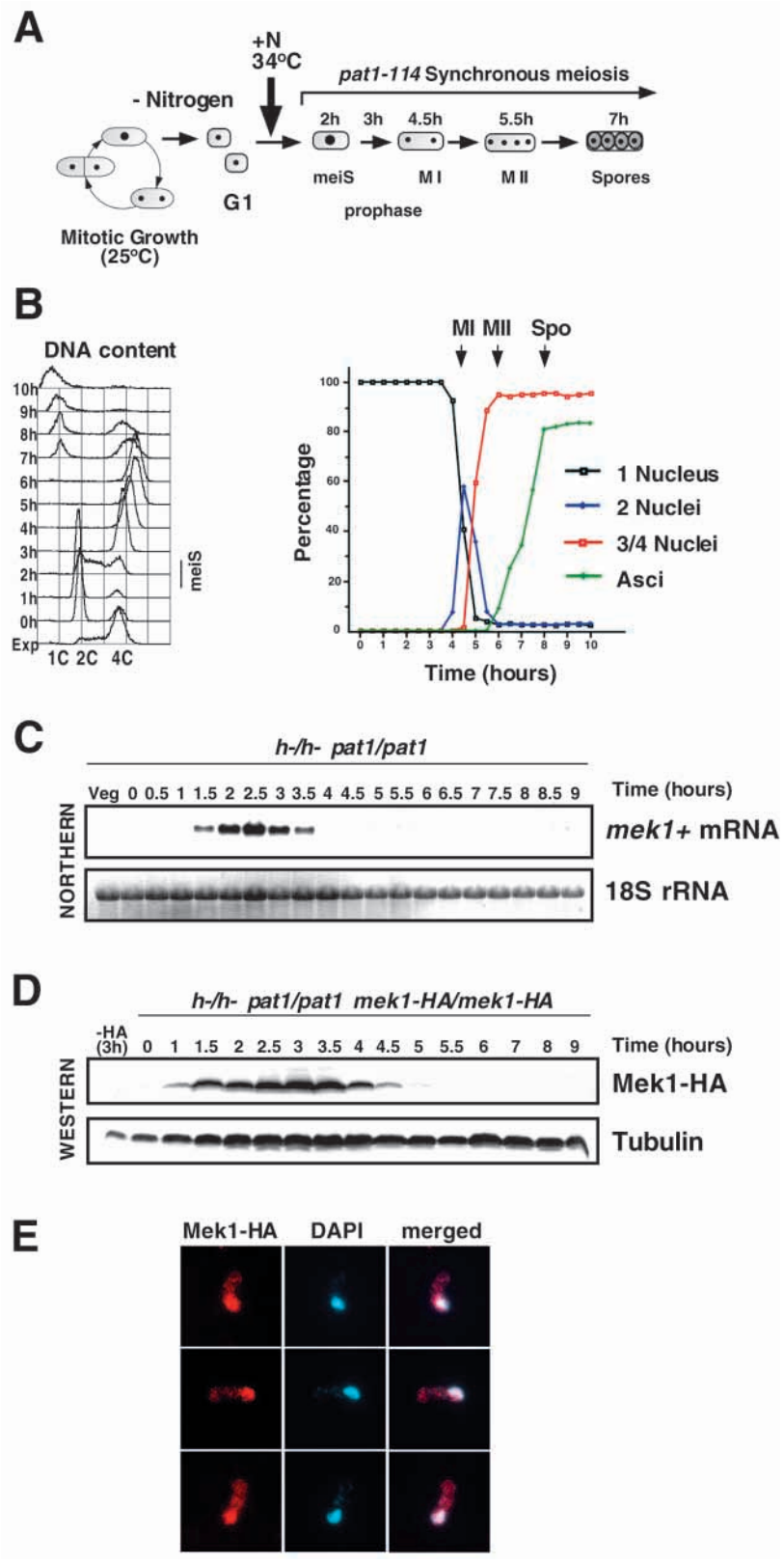
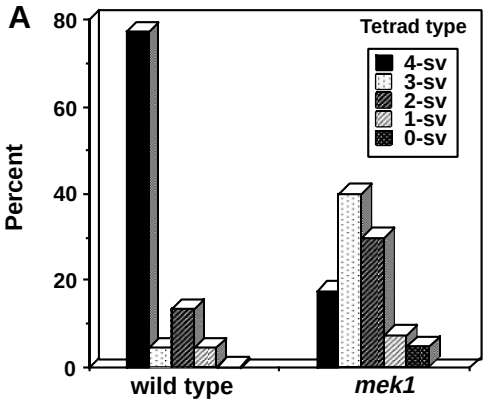


Fig. 2. Mek1 is a meiosis-specific nuclear protein. (A) Schematic representation of *pat1*-driven synchronous meiosis. Vegetatively growing cells are blocked in G1 by nitrogen starvation during ~14 hours and then induced to enter meiosis synchronously by inactivating the Pat1 kinase at 34°C (time 0 in B, C and D). The approximate timing of the major meiotic landmarks, as determined in (B), is indicated. (B) Synchronous meiosis of strain S964. Left panel, DNA content measured by FACS analysis. The period in which premeiotic DNA replication takes place is indicated (meiS). Note that the 1C peak that appears after 7 hours corresponds to free spores that are released from asci owing to sonication during the preparation of cells for FACS. Right panel, meiotic progression was followed by DAPI staining of nuclei and sporulation by microscopic observation of asci. The peaks of meiosis I (MI), meiosis II (MII) and spore formation (Spo) are indicated. (C) Northern blot analysis of *mek1*$^+$ expression during the synchronous meiosis of strain S964 shown in B. 18S rRNA levels are shown as a loading control. (D) Western blot analysis of Mek1-HA production during a synchronous meiosis in strain S1294. Tubulin is presented as a loading control. (E) Immunofluorescence analysis of cells from strain S1294 (*mek1-HA*), after 3 hours of induction of meiosis, stained with DAPI (blue) and anti-HA antibodies (red). The merged image is presented in the right column. Three representative cells are shown.

but this phenotype is largely suppressed in a *cdc2-3w cdc25Δ* strain (Fig. 5C). By contrast, *wee1-50* cells overexpressing *mek1*$^+$ at the restrictive temperature still manifest the elongation phenotype (Fig. 5C). These observations suggest that Cdc25, but not Wee1, is a target of Mek1.

Our results are consistent with the possibility that *S. pombe* Mek1 may be the meiosis-specific counterpart of the FHA family Cds1 checkpoint kinase (Fig. 1). When DNA integrity checkpoints are activated, Cds1 and Chk1 phosphorylate and inhibit Cdc25; several residues of Cdc25 phosphorylated by Cds1 have been identified (Zeng et al., 1998; Furnari et al., 1999; Zeng and Piwnica-Worms, 1999). In order to investigate whether the Mek1-induced G2/M arrest in vegetative cells is also mediated by phosphorylation of Cdc25, the effect of Mek1 overproduction was examined in the *cdc25-9A* mutant, which contains nine Cds1 phosphorylation sites changed to alanine and is impaired in the checkpoint response to DNA damage and replication blocks (Zeng and Piwnica-Worms, 1999). Importantly, the cell cycle arrest phenotype caused by Mek1 overproduction is significantly less severe in *cdc25-9A* cells compared to wildtype (Fig. 5D). Thus, our results suggest that Mek1 phosphorylates Cdc25 on at least some of the same residues as Cds1, leading to inactivation of Cdc25 function and resulting in cell cycle arrest. Nevertheless, since *nmt1-mek1*$^+$ *cdc2-3w cdc25Δ* and *nmt1-mek1*$^+$ *cdc25-9A* cells, in the absence of thiamine, still show a partial arrest (Fig. 5; data not shown), targets of Mek1 other than Cdc25 may exist.



Cross	Recombinant spores (Leu+ His+)	Total spores	Frequency of recombination (%)	Fold decrease
wild type	206	1633	12.6	1
mek1	101	1934	5.2	2.4

Fig. 3. The *mek1* mutant is defective in spore viability and meiotic recombination. (A) Distribution of tetrad types. The percentages of tetrads with 4, 3, 2, 1 and 0 viable spores (4-sv, 3-sv, 2-sv, 1-sv and 0-sv, respectively) are represented. Tetrads were dissected from crosses between wild-type (S778×S781) and *mek1* strains (S1291×S1292) after 2 days on MEA plates. (B) The frequency of intergenic meiotic recombination was measured on the *leu1-his5* interval on chromosome II by random spore analysis of crosses between wildtype (PN22 x S1285) and *mek1* (S1300×S1301) strains.

A Mek1-dependent meiotic recombination checkpoint also operates in fission yeast

The results reported above revealed that the consequences of high levels of Mek1 in vegetative cells to a certain extent mimic the DNA integrity checkpoint responses. However, *mek1*$^+$ is normally expressed only during meiotic prophase; therefore, we investigated whether Mek1 carries out a checkpoint function during meiosis in fission yeast. The alterations observed in meiotic progression when *mek1*$^+$ is deleted or when *mek1*$^+$ is overexpressed (Fig. 4) are also consistent with such a regulatory role for Mek1.

In several organisms, including budding yeast, *C. elegans* and mouse, defects at intermediate steps in the meiotic recombination process trigger the so-called 'pachytene checkpoint' or 'meiotic recombination checkpoint', which blocks meiotic cell cycle progression, thus preventing the formation of defective gametes. To study whether a similar response also occurs in fission yeast, meiotic progression in the *S. pombe meu13* mutant, which is defective in chromosome pairing and meiotic recombination (Nabeshima et al., 2001), was carefully examined using *pat1*-driven synchronous meiosis. Meu13 is the homolog of the *S. cerevisiae* Hop2 protein; the *hop2* mutant triggers the pachytene checkpoint in budding yeast (Leu et al., 1998). The *meu13* mutant completes meiosis and sporulation, as described previously (Nabeshima et al., 2001), but displays a ~30 minute delay in entering meiosis I compared to wildtype (Fig. 6A). For example, at the 4 hour time point, only ~15% of *meu13* cells had undergone meiosis I, compared with ~50% in the wild-type strain (see arrows in Fig. 6A; a representative time course is presented, but the *meu13* delay has been observed in three independent experiments). Introduction of a *rec12* mutation, which abolishes initiation of meiotic recombination suppresses this delay (data not shown), suggesting that it is due to the presence of recombination intermediates that trigger the meiotic checkpoint. Interestingly, mutation of *mek1*$^+$ also alleviates the meiotic delay of *meu13* (Fig. 6A); the *meu13 mek1* double mutant proceeds to the first meiotic division with similar kinetics to that of the *mek1* single mutant (i.e., even faster than wildtype; Fig. 4). Moreover, whereas spore viability in *meu13* only shows a slight reduction in comparison with wildtype (62% versus 77%, respectively), it is significantly reduced in the *meu13 mek1* double mutant (40%). Thus, during meiosis in fission yeast, Mek1 participates in a surveillance mechanism that delays cell cycle progression in response to defective recombination, which is important to promote viability of the meiotic progeny.

The above results (Fig. 5) suggest that Cdc25 is a target of Mek1. Since Cdc25 dephosphorylates Tyr15 of Cdc2 promoting G2/M transition, the status of Tyr15 phosphorylation was examined during synchronized meiosis of wild-type, *mek1*, *meu13* and *meu13 mek1* strains (Fig. 6B). In agreement with the checkpoint-dependent meiotic delay of the

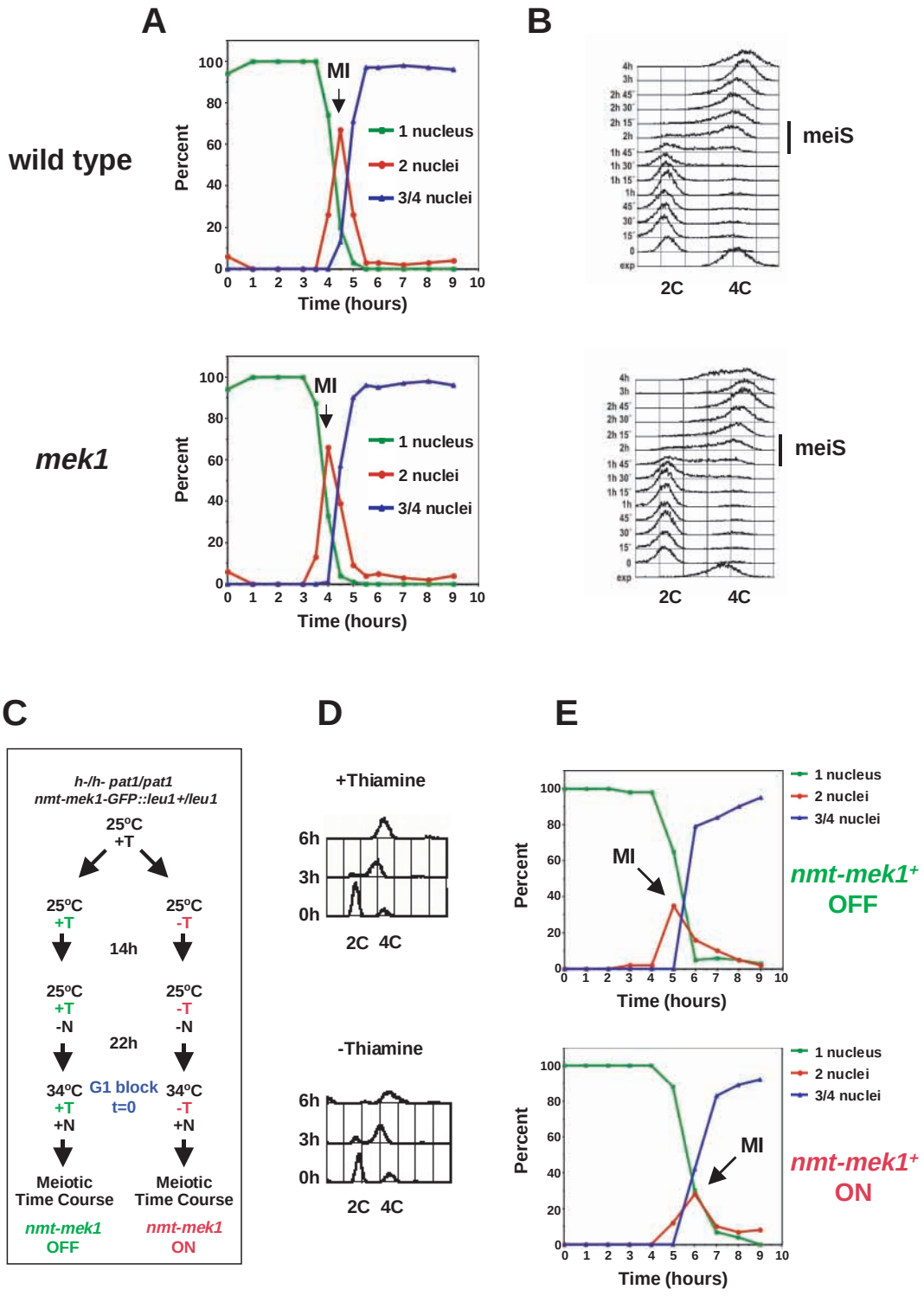


Fig. 4. Mek1 regulates entry into meiosis I. (A) The *mek1* mutant enters meiosis I faster than wildtype. Kinetics of meiotic progression of wildtype (S964; upper panel) and *mek1* (S1293; lower panel) was followed by DAPI staining of nuclei. Note that the peak of meiosis I (MI) occurs ~30 minutes earlier in the *mek1* mutant. A representative time course is presented, but the experiment was repeated at least three times, the same results being obtained. (B) FACS analysis of the meiotic time courses shown in A. Note that the timing of premeiotic S phase (meiS) is the same in both the wildtype (upper panel) and *mek1* mutant (lower panel). (C,D,E) High levels of Mek1 delay entry into meiosis I. (C) Schematic representation of the experimental procedure used for overexpression of *mek1*$^+$ from the thiamine-repressible *nmt1* promoter during meiosis in strain S1298. T, thiamine; N, nitrogen. (D) FACS analysis of meiotic time courses of S1298 in the presence (upper panel) or in the absence (lower panel) of thiamine. (E) Kinetics of meiotic progression monitored by DAPI nuclear staining during meiotic time courses of strain S1298 in the presence of thiamine (*nmt-mek1*$^+$ OFF) or in the absence of thiamine (*nmt-mek1*$^+$ ON). Note that the peak of meiosis I (MI) takes place ~1 hour later when Mek1 is overproduced.

meu13 mutant, phosphorylation of Tyr15 persists longer than in wildtype (compare the 4 hour time point in Fig. 6B). By contrast, in both *mek1* and *meu13 mek1* strains, dephosphorylation of the Cdc2 tyrosine 15 occurs earlier, consistent with higher levels of Cdc25 phosphatase activity in the absence of Mek1 and correlating with a faster meiotic progression in these strains.

The *cdc25-9A* mutant is defective in the meiotic recombination checkpoint

Our overexpression studies in vegetative cells suggest that the Mek1-dependent regulation of Cdc2 tyrosine 15 phosphorylation is exerted through inhibition of Cdc25. To directly demonstrate that Cdc25 is required for the fission yeast meiotic recombination checkpoint, meiotic progression was examined in strains carrying the phosphorylation-deficient *cdc25-9A* allele (Fig. 7). Like deletion of *mek1*$^+$, the *cdc25-9A* mutant alleviates the meiotic delay of *meu13*; for example, at the 4 hour time point, ~20% of the *meu13* cells had undergone meiosis I, compared with ~60% in the *cdc25-9A meu13* double mutant (see arrows in Fig. 7).

In summary, our results indicate that the meiotic recombination checkpoint in fission yeast inhibits entry into

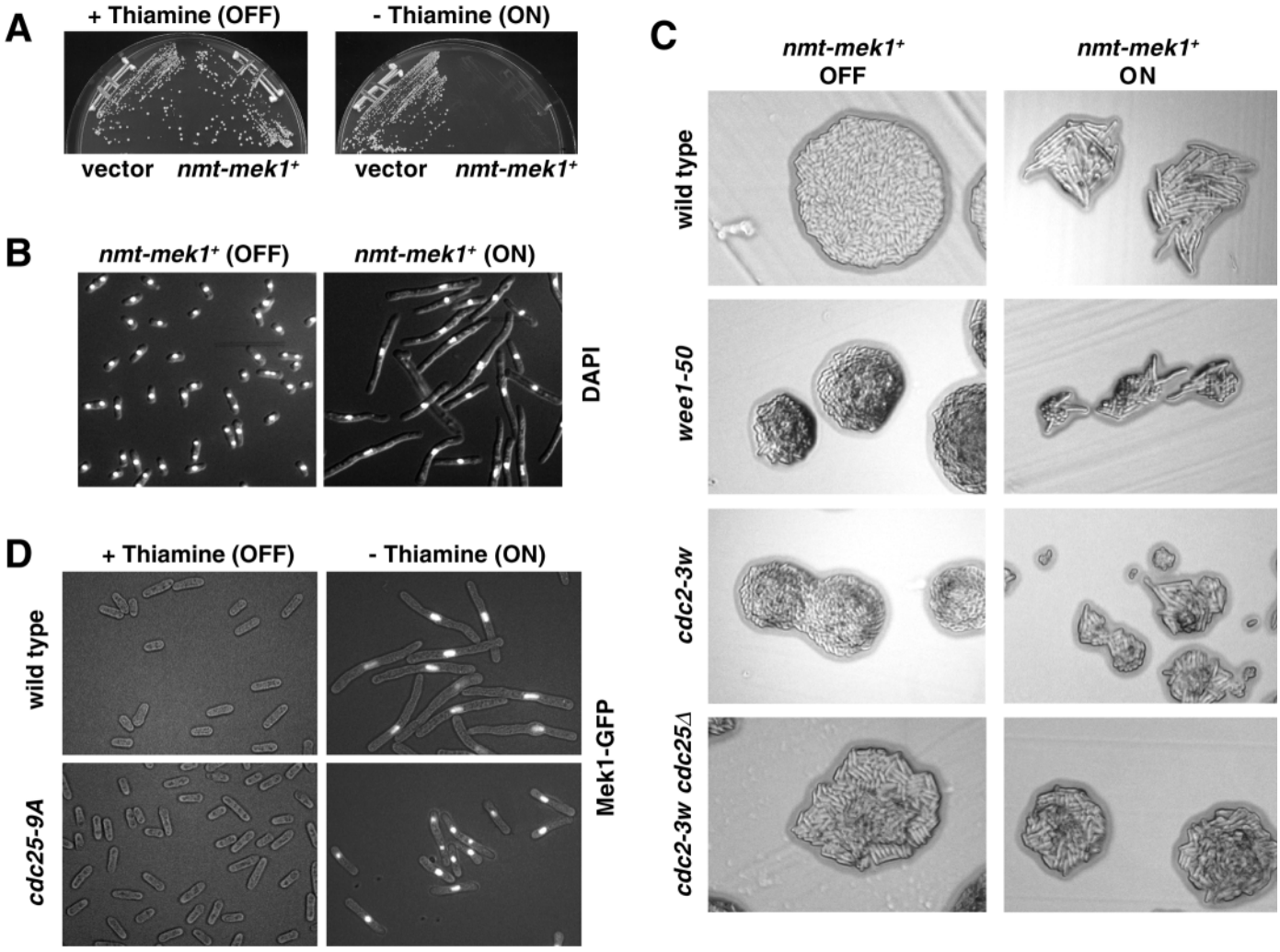


Fig. 5. Ectopic overexpression of *mek1*$^+$ in vegetative cells causes Cdc25-dependent G2/M cell cycle arrest. (A) Wild-type cells (PN22) transformed with the pREP3X vector or with plasmid pSS123 (*nmt-mek1*$^+$) were streaked out on plates containing thiamine (*nmt1* promoter OFF) or lacking thiamine (*nmt1* promoter ON) and incubated for 3 days at 30°C. (B) Cells from strain S1297, which contain an integrated *nmt1-mek1-GFP* construct, were incubated in *nmt1*-repressing conditions (OFF) or *nmt1*-inducing conditions (ON) for 24 hours. Nuclei were stained with DAPI. Backlight reveals cell bodies. (C) Wild-type (PN22), *wee1-50* (S145), *cdc2-3w* (S176) and *cdc2-3w cdc25Δ* (S898) cells transformed with pSS124 were grown on plates containing or lacking thiamine (*nmt-mek1*$^+$ OFF and *nmt-mek1*$^+$ ON, respectively). For wildtype, *cdc2-3w* and *cdc2-3w cdc25Δ*, plates were incubated at 30°C, whereas *wee1-50* cells were incubated at 36°C to inactivate Wee1 function. Microcolonies were photographed after ~30 hours. Note that *cdc25Δ* cells do not elongate in response to *mek1*$^+$ overexpression. (D) Strains S1297 (wildtype) and S1302 (*cdc25-9A*), which contain *nmt1-mek1-GFP* integrated in the genome were incubated in the absence (OFF) or in the presence (ON) of thiamine for 22 hours. Cells were visualized at the fluorescence microscope using a GFP filter. Backlight reveals cell bodies. Note that in the absence of thiamine Mek1-GFP accumulates in the nucleus of both wildtype and *cdc25-9A*, but *cdc25-9A* cells elongate considerably less than wildtype; in addition, *cdc25-9A* binucleate dividing cells are observed frequently.

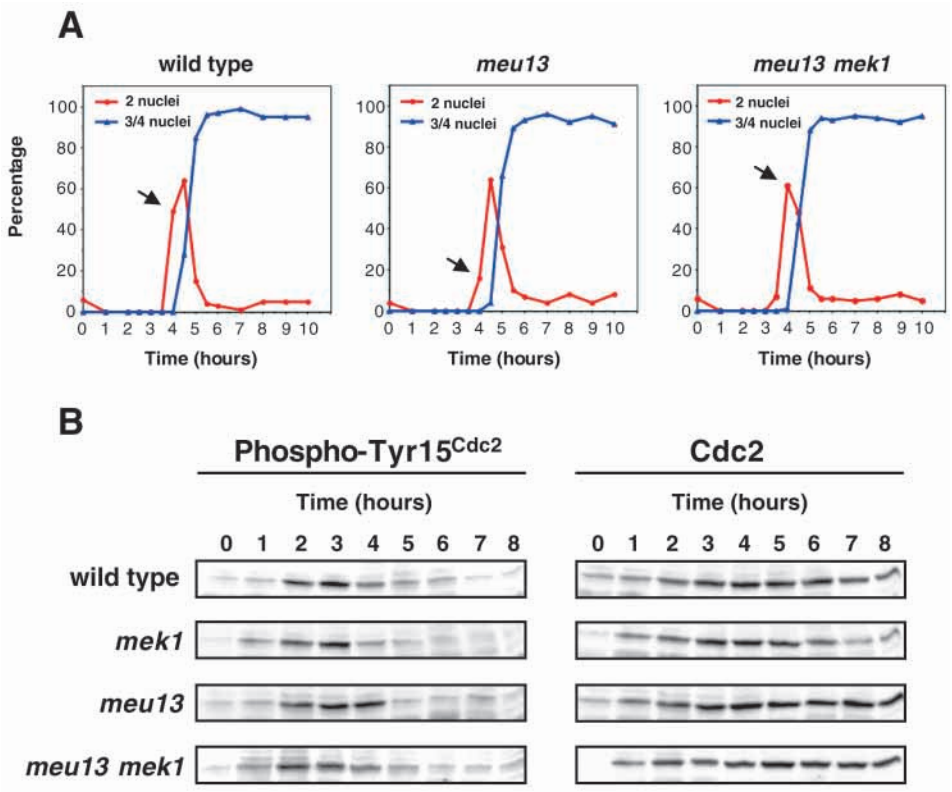


Fig. 6. The Mek1-dependent meiotic recombination checkpoint regulates phosphorylation of Cdc2 on Tyr15. (A) Meiotic time courses of wild-type (S964), *meu13* (S1295) and *meu13 mek1* (S1296) strains. The percentage of cells that have undergone the first meiotic division (2 nuclei) or both meiotic divisions (3/4 nuclei) is shown. Note that the *meu13* mutant shows a delay (~30 minutes) in entering meiosis I that is alleviated by the *mek1* mutation. Arrows pointing to the percentage of binucleate cells at the 4 hour time point are shown to highlight the *meu13* delay. (B) Western blot analysis of the meiotic time courses shown in A, also including a *mek1* strain (S1293; see Fig. 4A), using anti-phospho-Cdc2(Tyr15) and anti-Cdc2 antibodies. Note that phosphorylation of Cdc2 on Tyr15 persists longer when the meiotic recombination checkpoint is triggered (i.e., in the *meu13* mutant) but not when the checkpoint is inactivated by mutation of *mek1*$^+$.

meiosis I by Mek1-dependent inhibitory phosphorylation of Cdc25, which contributes, at least in part, to maintaining Cdc2 phosphorylated on tyrosine 15 (Fig. 8).

Discussion

A meiotic FHA kinase in fission yeast

Here we report the functional characterization of the meiosis-specific *mek1*$^+$ gene product in fission yeast. *S. pombe* Mek1 belongs to the family of protein kinases containing FHA domains. One group of this family, including *S. cerevisiae* Rad53, *S. pombe* Cds1 or mammalian Chk2, are key regulators of the checkpoint responses to DNA damage and/or replication blocks in mitotically dividing cells. Upon checkpoint activation, phosphorylation of different substrates by Rad53/Cds1/Ckh2 brings about different cellular responses, such as cell cycle arrest/delay and DNA repair (Rhind and Russell, 2000). By contrast, other members of the FHA family of kinases, including *S. cerevisiae* Mek1, *C. elegans* Chk2 and the *S. pombe* Mek1 protein described in this paper, function during meiosis. Consistent with their meiotic roles, these genes display a meiosis-specific pattern of expression (this work) (Rockmill and Roeder, 1991; Higashitani et al., 2000; MacQueen and Villeneuve, 2001; Oishi et al., 2001).

The *mek1* mutant of *S. cerevisiae* is proficient in pairing, but displays reduced (although not abolished) meiotic recombination, reduced spore viability, makes only short stretches of SC and is defective in meiotic sister chromatid cohesion (Rockmill and Roeder, 1991; Bailis and Roeder, 1998). By contrast, inactivation of the *chk-2* gene in *C. elegans* results in a strong pairing defect and lack of crossover recombination, but SC formation and chromosome morphogenesis are apparently normal (Higashitani et al., 2000; MacQueen and Villeneuve, 2001; Oishi et al., 2001). Our results indicate that, like the budding yeast homolog, *S. pombe* Mek1 is also required for normal levels of meiotic interhomolog recombination and spore viability. Because fission yeast lacks SC, no role for *S. pombe* Mek1 in SC development can be proposed, but the characteristic horse-tail morphology adopted by the prophase nucleus in *S. pombe* (Chikashige et al., 1994) appears to be normal in the *mek1* mutant (L. P.-H., S.M. and P.A.S.-S., unpublished).

Despite the different meiotic phenotypes resulting from inactivation of these meiotic FHA kinases during an unperturbed meiosis in these model organisms, there is a common role for them; both budding and fission yeast Mek1, as well as Chk2 in worms, are essential components of the meiotic cell cycle control mechanism called pachytene checkpoint or meiotic recombination checkpoint, which arrests or delays meiotic cell cycle progression when recombination is incomplete (this work) (Bailis and Roeder, 2000; MacQueen and Villeneuve, 2001). It is possible that checkpoint function, like that of the mitotic counterparts of this protein family, could be the evolutionarily conserved role for these proteins, and the diverse meiotic phenotypes observed may reflect the peculiarities of meiosis in the different organisms as revealed, for example, by the different relationship between synapsis and initiation of recombination in *S. cerevisiae* and *C. elegans* or the absence of SC in *S. pombe* (Villeneuve and Hillers, 2001).

Meiotic defects in the *S. pombe mek1* mutant

We have shown that the *mek1* mutant in *S. pombe* shows reduced interhomolog meiotic recombination, decreased spore

viability and a shorter meiotic prophase. In principle, the defect in meiotic recombination can be explained by a reduction in the number of initiating events (i.e., DSBs) and/or by increasing the number of DSBs repaired using a sister (instead of a nonsister) chromatid. In *S. cerevisiae*, Mek1 appears to be required both for the generation of wild-type levels of DSBs (Xu et al., 1997) as well as for the proper choice of recombination partner (Thompson and Stahl, 1999).

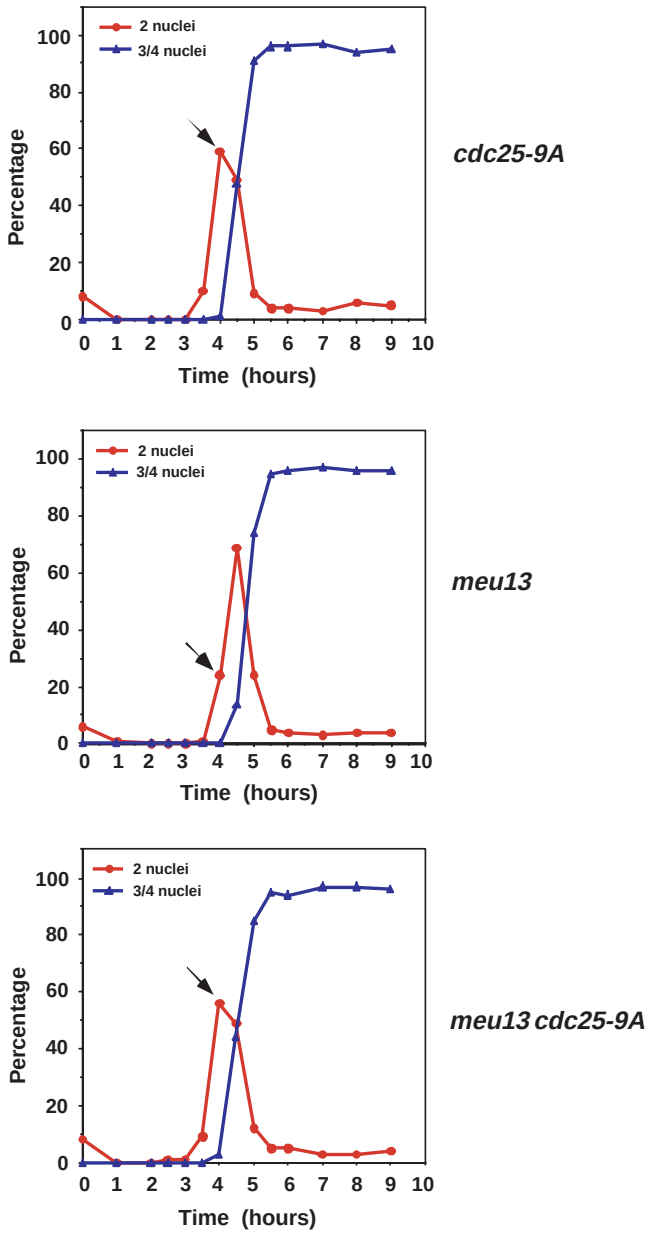


Fig. 7. The meiotic recombination checkpoint is impaired in the *cdc25-9A* phosphorylation site mutant. Meiotic time courses of *cdc25-9A* (S1308; top panel), *meu13* (S1306; middle panel) and *meu13 cdc25-9A* (S1307, lower panel) strains. The percentages of cells that have undergone the first meiotic division (2 nuclei) or both meiotic divisions (3/4 nuclei) are shown. Arrows pointing to the percentage of binucleate cells at the 4 hour time point are presented to highlight the suppression of the *meu13* delay by the *cdc25-9A* mutation.

Alternatively, the accelerated progression through prophase in the fission yeast *mek1* mutant may cause entry into meiosis I with unrepaired/unresolved recombination intermediates in a fraction of cells, resulting in reduced recombination frequency and spore inviability. The observed random pattern of spore death in *mek1* is consistent with this possibility and suggests that nondisjunction of homologs at meiosis I, as a consequence of the failure to recombine, is not the only cause of spore death in *mek1* as this would result in an excess of asci with two or zero viable spores (Molnar et al., 1995).

In contrast to the original checkpoint definition (Hartwell and Weinert, 1989), the direct participation of checkpoint proteins in the monitored cell cycle event appears to be the rule rather than the exception. For example, in addition to the *mek1* phenotypes mentioned above, other pachytene checkpoint mutants in budding yeast, such as *rad24*, *rad17* and *mec1-1*, show decreased crossing over, increased ectopic recombination, increased unequal sister-chromatid exchange, defective chromosome synapsis and reduced spore viability (Lydall and Weinert, 1995; Lydall et al., 1996; Grushcow et al., 1999; Thompson and Stahl, 1999). The same observation applies for DNA damage and replication checkpoint proteins such as Rad53 (another FHA Kinase member; Fig. 1) and Mec1, which perform essential functions during DNA replication in the mitotic cell cycle (Desany et al., 1998).

Mek1-dependent regulation of meiotic cell cycle

We have observed that kinetics of meiotic progression in fission yeast depends on Mek1 dosage. Lack of Mek1 results in a more rapid entry into meiosis I, whereas high levels of Mek1 lead to delayed meiotic progression. Premeiotic S phase is not affected, suggesting that Mek1 function negatively regulates the prophase to meiosis I transition. Supporting this notion, western blot analysis of Mek1 throughout meiotic time courses revealed that the protein rapidly disappears as cells enter the first meiotic division.

Since little is known about the molecular mechanisms controlling the meiotic cell cycle in fission yeast in comparison with the regulation of the mitotic cell cycle, we used ectopic overexpression of *mek1*$^{+}$ in vegetative cells as a tool for identifying potential cell cycle targets of Mek1. We found that high levels of Mek1 in vegetative cells lead to G2/M arrest, the same effect observed when the homologous Cds1 checkpoint kinase is overproduced (Boddy et al., 1998). In *S. pombe*, the G2/M transition depends on the phosphorylation status of Cdc2 on Tyr15 (reviewed by Moser and Russell, 2000). The Wee1 and Mik1 kinases inhibit Cdc2 activity by phosphorylation of Tyr15, whereas the Cdc25 phosphatase activates Cdc2 by removing the phosphate of Tyr15, thus promoting the G2/M transition. Our results suggest that Mek1-induced cell cycle arrest partly results from inhibition of Cdc25 rather than activation of Wee1, because the effect of Mek1 overproduction, as manifested by cell elongation, is diminished in *cdc25Δ* and *cdc25-9A* strains, but is not significantly altered in the absence of Wee1 function. The *cdc25-9A* mutant lacks the relevant Cds1 phosphorylation sites and shows a much weaker cell cycle arrest response to high Mek1 levels. Given the sequence similarity between Mek1 and Cds1 (Fig. 1), these results strongly suggest that Mek1 directly phosphorylates Cdc25, promoting its inhibition. Nevertheless, our results indicate that

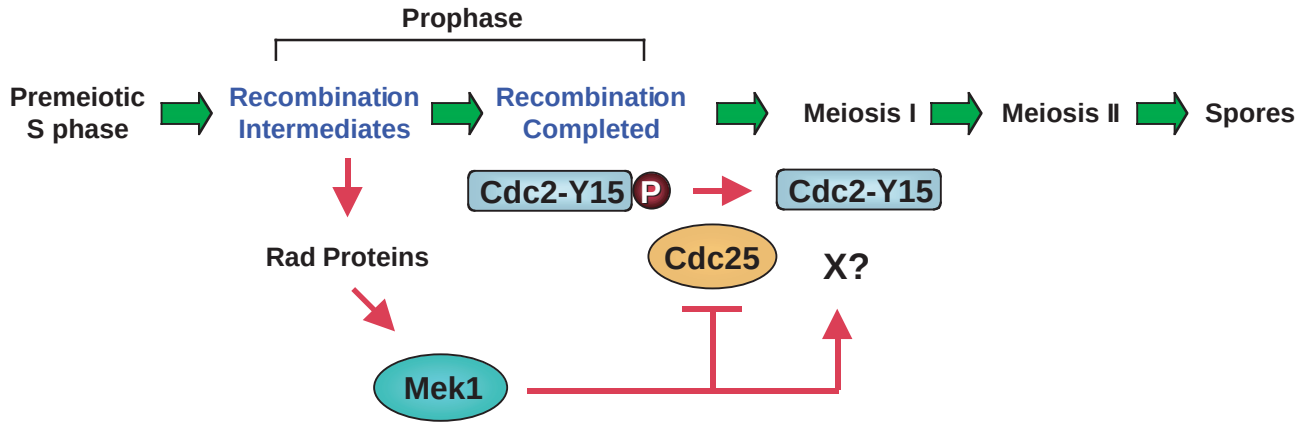


Fig. 8. A model for the meiotic recombination checkpoint pathway in fission yeast. See text for details.

Cdc25 is not the only target of Mek1; the possible involvement of Mik1 in the Mek1-dependent regulation of cell cycle remains to be investigated.

The meiotic recombination checkpoint in *S. pombe*: a role for Mek1

Our results show that defects at intermediate steps in the recombination pathway (induced by a *meu13* mutation) trigger a meiotic cell cycle delay in fission yeast mediated by inhibitory phosphorylation of the cyclin-dependent kinase Cdc2 on Tyr15. Like its budding yeast homolog, *S. pombe* Mek1 is an important component of this meiotic recombination checkpoint; mutation of *mek1*$^{+}$ alleviates the *meu13* delay. Although the checkpoint-induced delay is not very prolonged (~30 minutes), it appears to be important for the viability of the meiotic products. The extent of the meiotic delay in *meu13* roughly correlates with a delay in DSBs repair (Shimada et al., 2002); therefore it is formally possible that *S. pombe* mutants with more profound defects in recombinational repair of DSBs may exhibit stronger meiotic delays. However, although detailed analysis of meiotic progression in such mutants has not been reported, there is no evidence for a robust meiotic block in fission yeast. For example, the *rhp51* mutant is able to complete meiosis and sporulation despite the presence of unrepaired DSBs (Zenvirth and Simchen, 2000; Boddy et al., 2001).

The pachytene checkpoint pathway has been extensively studied in *S. cerevisiae*, and several components have been identified (see introduction) (reviewed by Roeder and Bailis, 2000). It has been proposed that Mek1-dependent phosphorylation of Red1 is required for checkpoint-induced arrest in response to unrepaired recombination intermediates. Once recombination has been completed, dephosphorylation of Red1 by Glc7 allows pachytene exit and entry into meiosis I (Bailis and Roeder, 1998; Bailis and Roeder, 2000). Pachytene arrest is achieved, at least in part, by inhibition of Cdc28/Clb1 activity, both by Swe1-mediated inhibitory phosphorylation of Cdc28 on Tyr19 (the equivalent of Tyr15 in *S. pombe* Cdc2) and by limiting Ndt80-dependent transcription of *CLB1* (Chu and Herskowitz, 1998; Hepworth et al., 1998; Leu and Roeder, 1999; Tung et al., 2000). An additional branch of the checkpoint, which targets the Sum1 transcriptional repressor has been recently reported (Lindgren et al., 2000). When the pachytene checkpoint is activated, the Swe1 kinase accumulates in a hyperphosphorylated (and presumably activated) form (Leu and Roeder, 1999); however, the molecular mechanisms regulating Swe1 stability and phosphorylation are unknown.

As mentioned above, in *S. cerevisiae*, phosphorylation of Red1 (a component of the lateral elements of the SC) by Mek1 is important for transducing the pachytene checkpoint signal. In *S. pombe*, however, there is no SC and, although SC-like structures called linear elements have been described (Bahler et al., 1993), BLAST searches reveal that no obvious *RED1* homolog exist in the fission yeast genome (http://www.sanger.ac.uk/Projects/S_pombe/). Therefore, targets of Mek1 other than Red1 must exist in fission yeast. Our experiments expressing Mek1 in different *S. pombe* mutant backgrounds during vegetative growth suggest that Mek1 phosphorylates Cdc25. Moreover, we have shown that the phosphorylation-deficient *cdc25-9A* mutant is impaired in the meiotic checkpoint response to the presence of recombination intermediates. We thus provide the first evidence of a direct connection between an effector kinase of the meiotic recombination checkpoint (Mek1) and a key component of the cell cycle machinery (Cdc25). Consistent with our findings, an essential role for Cdc25 in promoting meiosis I has been reported (Iino et al., 1995).

As in budding yeast, here we show that the meiotic recombination checkpoint in *S. pombe* regulates the phosphorylation of Cdc2 on Tyr15; however, we propose that the checkpoint-induced meiotic delay would, in part, be mediated by the Mek1-dependent inhibitory phosphorylation of Cdc25 and not by the activation of Wee1. This contrasts with the situation in *S. cerevisiae*, in which Mih1 (the Cdc25 homolog) seems to be dispensable for pachytene checkpoint function (Leu and Roeder, 1999). Thus, although the meiotic checkpoint arrests or delays meiosis by maintaining inhibitory phosphorylation of the cyclin-dependent kinase in both budding and fission yeast, different cell cycle regulators are targeted in each organism. It has been recently described that the Mrc1 protein is required for activation of Rad53 and Cds1 during the replication checkpoint in both budding and fission yeast (Alcasabas et al., 2001; Tanaka and Russell, 2001). Whether activation of Mek1 by the pachytene

checkpoint also requires Mrc1 or other adaptor proteins remains to be tested.

A model for the fission yeast meiotic recombination checkpoint pathway

In agreement with our observations, during the preparation of this paper, the existence of a meiotic recombination checkpoint in *S. pombe* has also been reported (Shimada et al., 2002). These authors show that the mitotic DNA integrity checkpoint Rad proteins also respond to unrepaired DSBs during meiosis in fission yeast, as they do in *S. cerevisiae* (Lydall et al., 1996). It has been recently shown that Mec1 and the Rad24 group of budding yeast checkpoint proteins do indeed localize to the sites of DSBs, acting as sensors of damage (Kondo et al., 2001; Melo et al., 2001; Hong and Roeder, 2002). Combining our results and those of Shimada et al., we propose the following model for the action of the meiotic recombination checkpoint in fission yeast (Shimada et al., 2002) (Fig. 8). The presence of ongoing recombination (presumably unrepaired DSBs) is sensed by the group of Rad checkpoint proteins, generating a signal that results in activation of the Mek1 kinase. Mek1, in turn, phosphorylates Cdc25, and possibly other as yet unknown substrate(s), which contribute to the maintenance of Tyr15 phosphorylation of Cdc2, thus inhibiting meiosis I entry. When recombination has been completed, the inhibitory signal disappears and dephosphorylation of Cdc2 on Tyr15 promotes the first meiotic division.

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