

Regulation of *wee1*⁺ expression during meiosis in fission yeast

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Author contributions: YM-T and HM designed research; YM-T and HO performed research; YM-T, HO, HA, and HM analyzed data; and HM wrote the paper.

Keywords: Cdk1, *wee1*, *Mei4*, meiosis 1, transcription, pseudogene

Abbreviations: Cdk1, cyclin-dependent kinase 1; MI, meiosis 1; Tyr15, tyrosine-15.

In eukaryotes, the cyclin-dependent kinase Cdk1p (Cdc2p) plays a central role in entry into and progression through nuclear division during mitosis and meiosis. Cdk1p is activated during meiotic nuclear divisions by dephosphorylation of its tyrosine-15 residue. The phosphorylation status of this residue is largely determined by the *Wee1p* kinase and the *Cdc25p* phosphatase. In fission yeast, the forkhead-type transcription factor *Mei4p* is essential for entry into the first meiotic nuclear division. We recently identified *cdc25*⁺ as an essential target of *Mei4p* in the control of entry into meiosis I. Here, we show that *wee1*⁺ is another important target of *Mei4p* in the control of entry into meiosis I. *Mei4p* bound to the upstream region of *wee1*⁺ in vivo and in vitro and inhibited expression of *wee1*⁺, whereas *Mei4p* positively regulated expression of the adjacent pseudogene. Overexpression of *Mei4p* inhibited expression of *wee1*⁺ and induced that of the pseudogene. Conversely, deletion of *Mei4p* did not decrease expression of *wee1*⁺ but inhibited that of the pseudogene. In addition, deletion of *Mei4p*-binding regions delayed repression of *wee1*⁺ expression as well as induction of expression of the pseudogene. These results suggest that repression of *wee1*⁺ expression is primarily owing to *Mei4p*-mediated transcriptional interference.

Introduction

In meiosis, 2 consecutive rounds of chromosome segregation follow a single S phase, producing functional haploid gametes. A high level of homologous recombination occurs between S phase and the first meiotic nuclear division (meiosis I (MI)).^{1,2} The order of meiosis is regulated by the activities of the cyclin-dependent kinase 1 Cdk1p (also known as Cdc2p)³ and the DNA replication and damage checkpoint proteins.⁴ Based on the temporal expression of meiosis-specific genes (early, middle, and late), a transcriptional cascade of various meiotic genes regulates sporulation.^{5,6} In fission yeast, transcription of the middle genes is activated by the meiosis-specific transcription factor *Mei4p*.⁶ *Mei4p* is a member of the group of forkhead transcription factors that bind to the consensus sequence GTAAAYA, also known as FLEX.^{7,8} In fission yeast, there are 4 forkhead transcription factors, namely, *Fkh2p*, *Fhl1p*, *Sep1p*, and *Mei4p*.⁹ These proteins are involved

in the cell cycle, mating, septation, and periodic gene expression.^{10–13} Among them, *Mei4p* is specifically required during meiosis. *Mei4p* is essential for recombination, the first meiotic division, and sporulation.^{8,14} Among the potential *Mei4p* target genes, *spo6*⁺ is required for sporulation⁸ and *mde2*⁺ is required for recombination.^{7,15}

The activity of Cdc2p is not only regulated by the availability of cyclin but by the phosphorylation status of its tyrosine-15 (Tyr¹⁵) residue.^{3,16} During the mitotic cell cycle, the tyrosine kinase *Wee1p* phosphorylates Cdc2p on Tyr¹⁵ and thereby inhibits Cdc2p activity, whereas the tyrosine phosphatase *Cdc25p* dephosphorylates this residue and thereby activates Cdc2p.^{3,16} Similar to its regulation during the mitotic cycle, Cdc2p kinase activity gradually increases from meiotic ‘Start’ and peaks around meiotic nuclear division.^{4,17,18} Consistent with its kinase activity, the Tyr¹⁵ residue of Cdc2p is phosphorylated after meiotic ‘Start’ and decreases during meiotic nuclear division.^{4,17–19} This tyrosine phosphorylation is a rate-limiting step

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Submitted: 05/22/2014; Accepted: 06/16/2014

<http://dx.doi.org/10.4161/15384101.2014.946807>

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for entry into meiotic division, given that such entry is promoted by forced dephosphorylation of this residue.¹⁷

We have previously shown that *cdc25⁺* is an essential target of Mei4p for progression through meiotic nuclear division.²⁰ We also proposed that Mei4p is a rate-limiting factor for entry into MI and coordinates cell cycle progression with other meiotic events such as sporulation and double-strand break formation.²⁰ Here, we show that Mei4p also regulates *wee1⁺* expression, but this negatively affects entry into MI.

Results and Discussion

wee1⁺ is a target gene of Mei4p

We previously reported that Mei4p controls phosphorylation of Cdc2p on Tyr¹⁵.²⁰ This suggests that *wee1⁺* is also regulated by Mei4p, in addition to *cdc25⁺*. We examined the expression of *wee1⁺* mRNA during meiosis by Northern blot analysis (Fig. 1A). To induce meiosis synchronously, we used a temperature-sensitive *pat1* strain of *Schizosaccharomyces pombe*.²¹ Cells were synchronized in G₁ phase by nitrogen deprivation and then shifted to the restrictive temperature to induce meiosis. The amount of *wee1⁺* mRNA decreased around the onset of MI (5 h) in wild-type (WT) cells. By contrast, the level of *wee1⁺* mRNA was almost constant in *mei4⁺*-deleted (*mei4*) cells. Furthermore, the level of *wee1⁺* mRNA was decreased earlier (4 h) in *mei4⁺*-overproducing (*mei4⁺*-OP) cells. These results suggest that Mei4p negatively regulates *wee1⁺* mRNA.

We next studied the amount of Wee1p protein during meiosis (Fig. 1B). Like the pattern of its mRNA expression, the level of Wee1p protein decreased around the onset of MI (5 h) in WT

cells and then slightly increased at later time points. However, the amount of Wee1p was almost constant in *mei4* cells. Additionally, the level of Wee1p was decreased earlier in *mei4⁺*-OP cells. These data suggest that the Wee1p protein level is negatively controlled by Mei4p, similar to its mRNA expression. The correlation between the expression of *wee1⁺* at the mRNA and protein levels suggested that regulation of the abundance of *wee1⁺* mRNA is important for the physiological function of this gene.

Regulation of the pseudogene adjacent to *wee1⁺* by Mei4p

To address the mechanism by which Mei4p negatively regulates *wee1⁺* mRNA, we measured the abundance of the gene (SPCC18B5.02c) that is located adjacent to *wee1⁺* (Fig. 1A). SPCC18B5.02c is a cinnamoyl-CoA reductase gene (Sanger Center database, <http://www.sanger.ac.uk/>), although it has frame-shift mutations and is considered to be a pseudogene. The amount of SPCC18B5.02c mRNA increased around MI (5 h) in WT cells, but was not increased in *mei4* cells (Fig. 1A). The induction of this mRNA occurred earlier in *mei4⁺*-OP cells (Fig. 1A). These data suggest that Mei4p positively regulates this pseudogene.

Mei4p binds to FLEX elements adjacent to *wee1⁺*

A 27-bp oligonucleotide harboring a FLEX heptameric core (GTAAAYA) is required for binding to Mei4p in vitro.⁸ We searched for core FLEX sequences in the genomic regions adjacent to *wee1⁺*. Five FLEX sequences (FLEX1, FLEX2, FLEX3, FLEX4, and FLEX5) were found within a 3-kb region upstream of the start codon of *wee1⁺* (Fig. 2A).

We examined whether Mei4p can bind to the identified FLEX sequences adjacent to *wee1⁺* by performing a chromatin immunoprecipitation (ChIP) assay. ChIP analysis showed that Mei4p associated with the FLEX1, FLEX2, FLEX3/4, and FLEX5 sites of *wee1⁺*, as well as with the FLEX-D site of *spo6⁺*, which was used as a positive control (Fig. 2B). This binding was greatly reduced at the 0 h time point, when the level of Mei4p is low.²⁰ Furthermore, the association of Mei4p with a genomic region located ~15 kb or 30 kb downstream of the start codon of *wee1⁺* was minimal. These results showed that Mei4p binds to the FLEX sites adjacent to *wee1⁺* in vivo when the abundance of *wee1⁺* mRNA is low.

To examine whether Mei4p can bind directly to the FLEX sites adjacent to *wee1⁺* in vitro, we performed an electrophoretic mobility shift assay (EMSA) with a fusion protein composed of glutathione S-transferase (GST) and the forkhead domain of Mei4p

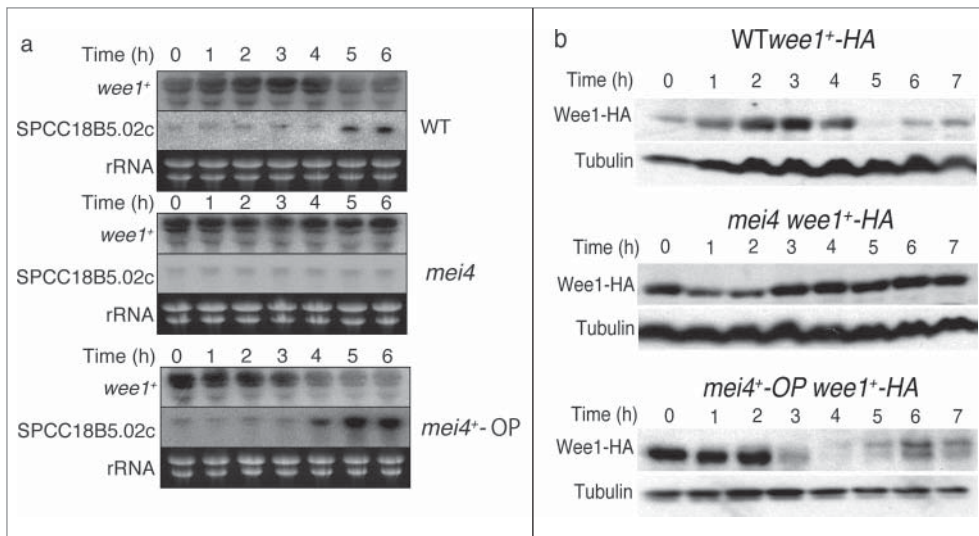
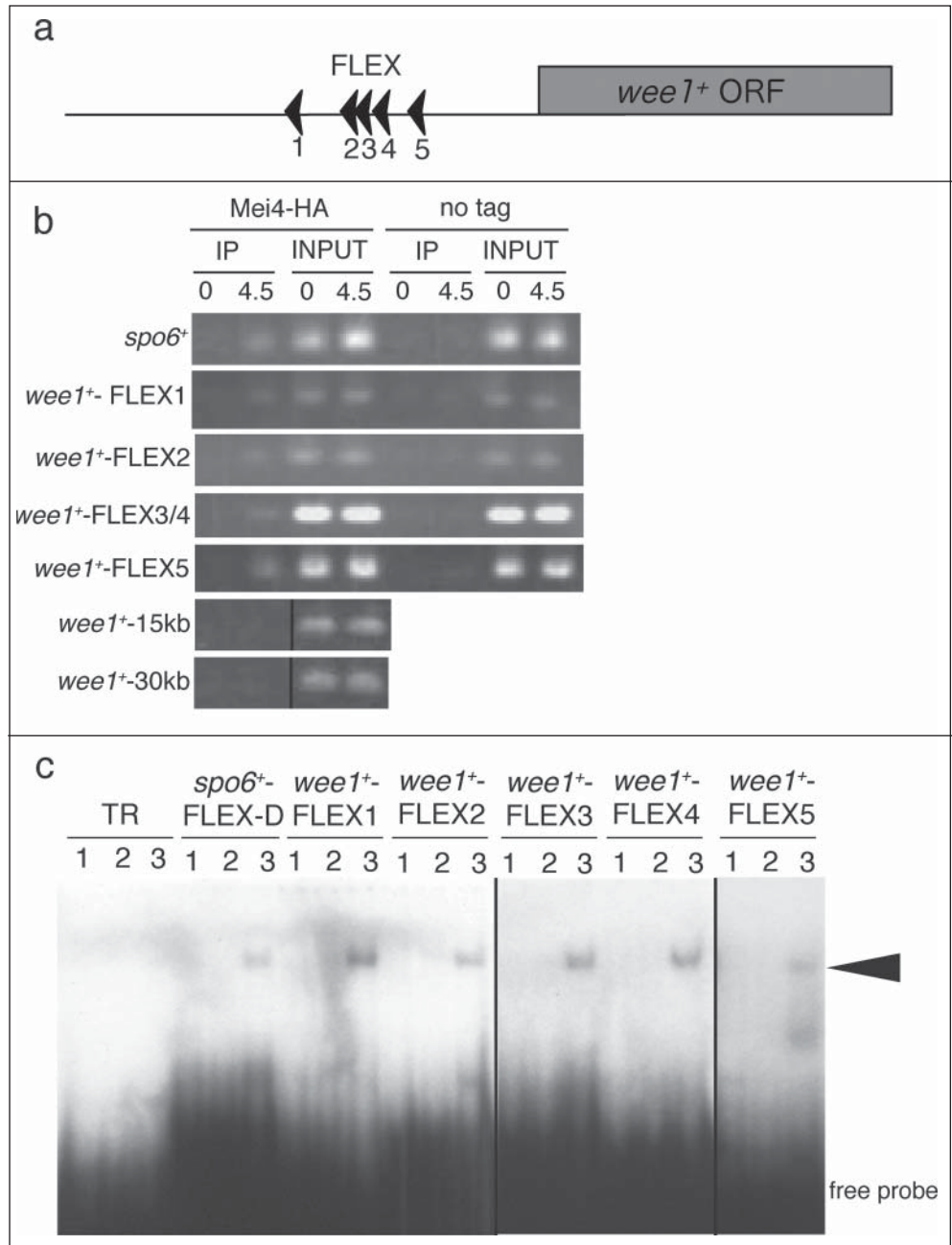


Figure 1. Expression of *wee1⁺* and SPCC18B5.02c during meiosis and the effects of *mei4⁺* deletion or overexpression. Cells of the indicated genotypes (wild-type (WT), HM1307; *mei4*, HM2163; *mei4⁺*-OP, HM4582; WT *wee1⁺*-HA, HM4732; *wee1⁺*-HA, *mei4*, HM4833; and *mei4⁺*-OP *wee1⁺*-HA, HM4735) were subjected to synchronous induction of meiosis as described in the Materials and Methods. Samples were collected at the indicated time points thereafter and subjected to Northern blot analysis of *wee1⁺* and SPCC18B5.02c mRNA (rRNA (rRNA) was stained with ethidium bromide as the loading control) (A) or Western blot analysis with anti-HA and anti- α -tubulin (loading control) antibodies (B).

Figure 2. Mei4p binds to the FLEX sequences upstream of *wee1*⁺ both in vivo and in vitro. (A) Schematic representation of the positions of the FLEX sequences associated with *wee1*⁺. The arrowheads indicate the direction of the FLEX sequence from 5' to 3'. ORF, open reading frame; (B) A chromatin immunoprecipitation assay of FLEX sites associated with *wee1*⁺ was performed with antibodies against HA or control IgG at the indicated times after induction of meiosis in cells expressing (Mei4-HA) or not expressing (no tag) *mei4*⁺-HA. *wee1*⁺-15kb and *wee1*⁺-30kb were about 15 kb and 30 kb downstream, respectively, of the start codon of *wee1*⁺ and were used as negative controls. The FLEX-D site of *spo6* was used as a positive control. The images of the immunoprecipitates of *wee1*⁺-15kb and *wee1*⁺-30kb are different from those of the inputs, but the experimental conditions were the same; (C) Electrophoretic mobility shift assay analysis of *wee1*⁺ FLEX sites with a recombinant glutathione S-transferase (GST)-Mei4p fusion protein. Buffer only (lane 1), GST (lane 2), or purified recombinant GST-Mei4p (71–182) (lane 3) were incubated with the indicated labeled oligonucleotide probe. The TR probe is unrelated to the FLEX sequence and was used as a negative control. The arrowhead indicates shifted bands. The image of *wee1*⁺-FLEX3 and *wee1*⁺-FLEX4 is different from the rest of the image, but the experimental conditions were the same.



(amino acids 71–182) as described previously,²⁰ together with radioactive oligonucleotides containing FLEX1, FLEX2, FLEX3, FLEX4, or FLEX5 of *wee1*⁺ as probes. A band shift with each of the 5 *wee1*⁺ FLEX probes was observed only in the presence of the fusion protein, not in its absence or in the presence of GST alone (Fig. 2C). These were similar to the results obtained with a *spo6*⁺ FLEX-D probe, which was used as a positive control.⁸ A shifted band was not detected with the GST-Mei4p fusion protein and a negative control (TR) oligonucleotide (Fig. 2C). These data suggested that Mei4p binds directly in vitro to all 5 FLEX elements located close to *wee1*⁺.

Role of the FLEX sites in the control of *wee1*⁺ mRNA expression

We next examined whether the FLEX sequences associated with *wee1*⁺ function as transcriptional cis elements. We deleted

the chromosomal region containing FLEX1, FLEX2, FLEX3, and FLEX4 of *wee1*⁺ (Fig. 3A). In the resulting cells, *wee1*⁺ mRNA was downregulated 1 h later than in WT cells following the induction of meiosis, and induction of the adjacent pseudogene was also delayed (Fig. 3B). This result shows that 4 of the FLEX elements located close to *wee1*⁺ are required for efficient down-regulation of *wee1*⁺ mRNA and up-regulation of the adjacent pseudogene mRNA. This suggests that these sites serve as cis-acting elements for Mei4p.

Negative regulation of *wee1*⁺ expression by Mei4p

This study of the role of Mei4p in the control of meiotic nuclear division in fission yeast revealed that: (1) the major target of Mei4p in this context is *wee1*⁺, in addition to *cdc25*⁺; (2)

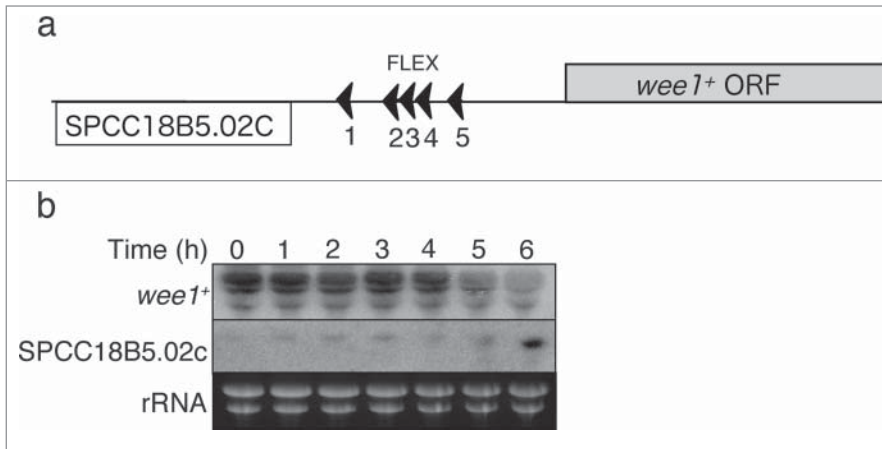


Figure 3. Effects of deletion of the FLEX sites near to *wee1*⁺ on *wee1*⁺ gene expression. (A) Schematic representation of the *wee1*⁺ and SPCC18B5.02c loci according to the Sanger Center database. ORF, open reading frame; (B) The region of the *wee1*⁺ gene containing FLEX1, FLEX2, FLEX3, and FLEX4 was deleted (HM5678). Meiosis was induced in the resulting cells as described in Fig. 1. Samples were collected at the indicated time points after meiosis induction and subjected to Northern blot analysis of *wee1*⁺ and SPCC18B5.02c mRNA. Ribosomal RNA (rRNA) was stained with ethidium bromide as the loading control.

Mei4p down-regulates *wee1*⁺ mRNA, whereas it up-regulates the adjacent pseudogene mRNA; and (3) FLEX sequences are located close to *wee1*⁺ and function as cis-regulatory elements for Mei4p.

We propose the following model (Fig. 4). Before MI, *wee1*⁺ is transcribed by an unknown transcription factor, whereas the adjacent pseudogene is not expressed. When Mei4p is produced, Mei4p binds to the FLEX regions close to *wee1*⁺ and displaces the unknown transcription factor at the onset of MI. The affinity of Mei4p for the FLEX regions is likely stronger than that of the unknown transcription factor. Thereafter, the level of *wee1*⁺ mRNA decreases, as well as the level of Wee1p protein. Conversely, the level of the pseudogene mRNA increases. Due to the downregulation of Wee1p, phosphorylation of the Tyr¹⁵ residue

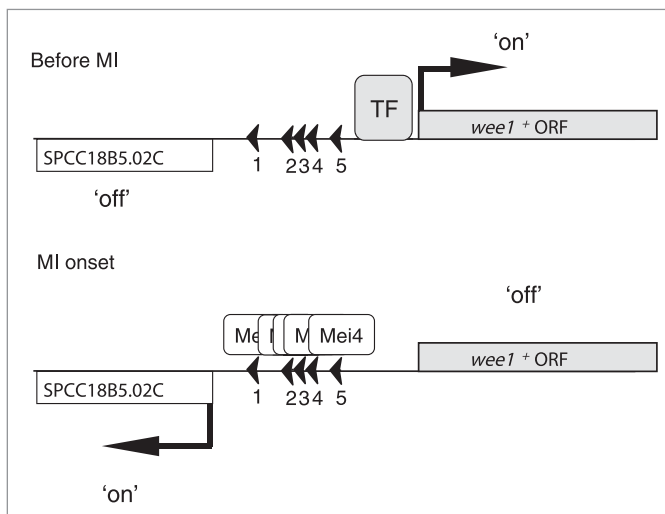


Figure 4. Model of *wee1*⁺ regulation during meiosis. See the text for details. MI, meiosis I; ORF, open reading frame; TF, transcription factor.

of Cdc2p decreases, Cdc2p is activated, and MI begins. In this way, Mei4p negatively regulates *wee1*⁺, whereas it positively regulates transcription of the adjacent pseudogene. In the process of transcriptional interference, a transcriptional event affects another transcriptional process.²² Therefore, inhibition of *wee1*⁺ expression is likely primarily owing to Mei4p-mediated transcriptional interference.

Positive and negative regulation of genes by the same transcription factor has been described previously. In budding yeast, Fkh1p and Fkh2p both activate and inhibit transcription.²³ The serine-dependent factor Cha4p also serves as both an activator and repressor of transcription simultaneously.²⁴ In mammals, the tumor suppressor p53 up-regulates the transcription of genes involved in several essential processes including cell cycle control and apoptosis.²⁵

It also acts as a transcriptional repressor by competing with more efficient activators for DNA binding or by blocking the functions of DNA-bound activators.^{26–28} Similar to p53, it is possible that Mei4p negatively regulates its target genes by competing with an activator for binding to overlapping sites in the gene promoter. The promoter context, such as the relative positions of the Mei4p-binding sites and other factors, may influence whether Mei4p activates or represses transcription.

Control of *wee1*⁺ during mitosis versus meiosis

During the mitotic cell cycle, the level of *wee1*⁺ mRNA does not fluctuate markedly, although the Wee1p protein level moderately fluctuates in fission yeast.²⁹ Recently, it has been proposed that Wee1p is localized at the center of the cell and its activity is regulated by the medially-located sensor Cdr2 in actively proliferating cells.^{30,31} In this way, a geometry-sensing mechanism is used to coordinate cell length and division. However, during meiosis, cells do not grow and cell length is relatively constant. Therefore, it would be difficult to use a geometry-sensing mechanism to regulate Wee1p. It seems likely that Mei4p evolved later to transcriptionally regulate *wee1*⁺ mRNA during meiosis.

Simultaneous transcriptional regulation of *wee1*⁺ and *cdc25*⁺

We have shown that Mei4p regulates *wee1*⁺ (this study) and *cdc25*⁺ simultaneously. This simultaneous regulation is related to 2 observations. First, overexpression of *mei4*⁺ induces earlier meiotic nuclear division and Mei4p is a rate-limiting factor for entry into MI.²⁰ Simultaneous regulation of *wee1*⁺ and *cdc25*⁺ by Mei4p decreases *wee1*⁺ mRNA expression and increases *cdc25*⁺ mRNA expression. Consequently, the protein levels of Wee1p and Cdc25p change according to their mRNA levels. Therefore, phosphorylation of the Tyr¹⁵ residue of Cdc2p decreases rapidly and its kinase activity increases quickly. Second, when *mei4*⁺ is disrupted, MI does not occur.^{8,20} Simultaneous

regulation of *wee1*⁺ and *cdc25*⁺ likely contributes to this tight control. We conclude that *Mei4p* is a critical regulator of MI by activating *cdc25*⁺ transcription and inhibiting *wee1*⁺ transcription.

Experimental Procedures

Fission yeast strains

The strains used in the present study were constructed according to standard procedures³² and are listed in Table S1. Synchronous meiosis was induced in liquid culture with temperature-sensitive *pat1* mutants as described previously.²

Primers and probes

The primers and double-stranded oligonucleotides used in the present study are listed in Table S2.

Isolation of RNA and Northern blot analysis

Total RNA was isolated from synchronized *pat1* cells as described previously.³² Aliquots (5–6 μg) of the isolated RNA were subjected to Northern blot analysis. For probes, fragments of open reading frames were amplified from genomic DNA by PCR with the following sets of primers: 477 and 478 for *wee1*⁺ and 1040 and 1041 for *SPCC18B5.02c*.

Immunoblot analysis

Whole cell extracts (20 μg of protein) were prepared by the boiling method¹⁷ and analyzed as described previously.^{17,33}

ChIP

ChIP and quantitative real-time PCR analyses were performed as described previously.²⁰ Aliquots of precipitated DNA as well as DNA fragments isolated from 0.4 mg of whole cell extract were subjected to semi-quantitative PCR analysis with the following primers: *wee1*⁺–FLEX1, 915 and 916; *wee1*⁺–FLEX2, 866

and 867; *wee1*⁺–FLEX3/4, 868 and 869; *wee1*⁺–FLEX5, 913 and 914; *wee1*⁺–15K, 870 and 871; and *wee1*⁺–30K, 872 and 873.

EMSA analysis

EMSA analysis was performed as described previously.²⁰ The double-stranded oligonucleotides used as probes were as follows: TR, 742 and 743; *spo6*⁺–FLEX, 740 and 741; *wee1*⁺–FLEX1, 907 and 908; *wee1*⁺–FLEX2, 909 and 910; *wee1*⁺–FLEX3, 860 and 861; *wee1*⁺–FLEX4, 862 and 863; and *wee1*⁺–FLEX5, 911 and 912.

Deletion of *wee1*⁺ FLEX elements from the chromosome

The chromosomal region containing FLEX1, FLEX2, FLEX3, and FLEX4 of *wee1*⁺ was replaced with a PCR-generated DNA fragment containing the corresponding region interrupted by *kan*^r.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank C Shimoda and P Nurse for providing yeast strains, and A Tochigi for technical help.

Funding

This work was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to H M and Y M-T).

Supplemental Materials

Supplemental data for this article can be accessed on the publisher's website.

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