(residues 305–492, 216–330, or 317–479) *in vitro* (Figure 4A; Supplementary Figure 9). Cdc2p precipitated from cell extracts with anti-hemagglutinin epitope (HA) antibody or Suc1p-coated beads phosphorylated each of the GST–Fkh2p fusion proteins but not GST alone. We found that mutation to alanine of the consensus phosphorylation sites for Cdc2p in each of the Fkh2p fragments (T314 in Fkh2p (216–330), S462 in Fkh2p (317–479), or T314, S462, and S481 in Fkh2p (305–492)) reduced the extent of phosphorylation by Cdc2p. These results thus suggested that Cdc2p phosphorylates at least T314 and S462 residues of Fkh2p *in vitro*. In addition, recombinant human Cdc2p complex, but not the kinase inactive complex, phosphorylated Fkh2p, suggesting that Cdc2p directly phosphorylates Fkh2p (Supplementary Figure 9).

To test whether Fkh2p is phosphorylated on T314 or S462 *in vivo*, we prepared antibodies to Fkh2p peptides containing phosphorylated (p) T314 or pS462. The antibodies (anti-pT314, anti-pS462) specifically recognized the respective Fkh2p peptides containing pT314 or pS462 but not the corresponding nonphosphorylated peptides (Figure 4B). They also recognized wild-type Fkh2p but not the Fkh2p(T314A,S462A) mutant expressed in fission yeast cells (Figure 4C). Fkh2p exhibited multiple forms because of phosphorylation (Buck *et al*, 2004; Bulmer *et al*, 2004). Similarly, multiple bands appeared in Fkh2p(T314A,S462A) mutant (Figure 4C; Supplementary Figure 8), suggesting that multiple bands come from phosphorylation other than these sites.

We, therefore, next examined whether Cdc2p is required for phosphorylation of Fkh2p on T314 or S462 *in vivo*. We first examined a temperature-sensitive *cdc2* mutant. Inactivation of *cdc2*$^{+}$ by a temperature shift resulted in a decrease in the level of Fkh2p phosphorylation on each of these two residues (Figure 4D). We then examined a strain in which the B-type cyclin gene *cig2*$^{+}$ is deleted and found that the level of Fkh2p phosphorylation on T314 and S462 was also decreased (Figure 4D). In addition, shut off of expression of the B-type cyclin gene *cdc13*$^{+}$ induced a slight decrease in the level of Fkh2p phosphorylation on each of these two residues (Figure 4E). To test whether Fkh2p is phosphorylated depending on the cell-cycle stage, cells were transiently arrested in G_1 by the inactivation of *cdc10*$^{+}$ to induce cyclin degradation and released to the cell cycle (Figure 4F and G). In G_1, Fkh2p was found to be dephosphorylated on T314 and S462. On release from G_1, S462 was phosphorylated earlier than T314, although both of these residues were eventually phosphorylated. This may be due to the facts that the major cyclin responsible for phosphorylating these residues may be different and that the expression of the cyclin may vary during the cell cycle. On the basis of these results, we concluded that Cdc2p and the B-type cyclins Cig2p and Cdc13p are required for phosphorylation of Fkh2p on T314 and S462 *in vivo*.

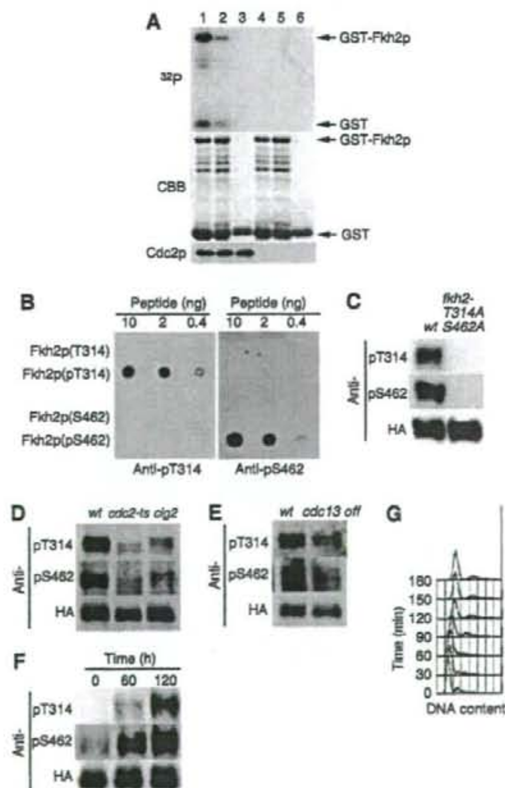

Figure 4 Phosphorylation of Fkh2p on T314 and S462 by Cdc2p *in vitro* and *in vivo*. (**A**) Kinase assays were performed with Cdc2p precipitates prepared from protein extracts of exponentially growing cells expressing hemagglutinin epitope (HA)-tagged forms of Cdc2p (HM6118; lanes 1–3) or not expressing HA (HM6; lanes 4–6) with anti-HA antibody. Substrates (lanes 1–3, respectively) included GST–Fkh2p(305–492), GST–Fkh2p(305–492) containing T314A, S462A, and S481A mutations, or GST alone. Reaction mixtures were separated by SDS-polyacrylamide gel electrophoresis, and proteins were detected by staining with Coomassie brilliant blue (CBB) and autoradiography (^{32}P). Arrows indicate GST and the GST–Fkh2p fusion proteins. The Cdc2p input into each reaction mixture was also examined separately by Western blotting. (**B**) Various amounts (10, 2, or 0.4 ng) of Fkh2p peptides containing phosphorylated or nonphosphorylated T314 or S462 were spotted onto a nitrocellulose membrane and subjected to immunodetection with affinity-purified antibodies (anti-pT314 and anti-pS462) generated in response to the corresponding phosphorylated peptides. (**C**) Cells expressing HA-tagged forms of wild-type Fkh2p (HM5145) or the Fkh2p(T314A,S462A) mutant (HM5722) were grown to mid-log phase at 30°C. Cell lysates were then subjected to immunoprecipitation with antibodies to HA, and the resulting precipitates were subjected to immunoblot analysis with anti-pT314, anti-pS462, and anti-HA, as indicated. (**D**) Cells expressing HA-tagged Fkh2p were either grown to mid-log phase in EMM2 at 24°C and then incubated at 36.5°C for 7 h (*wt*, HM5145; *cdc2-ts*, HM5444) or grown as in (C) (*cig2*, HM5530). Cell lysates were subjected to immunoprecipitation and immunoblot analysis as in (C). (**E**) Cells expressing HA-tagged Fkh2p (*wt*, HM5146; *cdc13 off*, HM5554) were grown to mid-log phase in EMM2 at 30°C, after which thiamine was added to the culture medium to switch off *cdc13*$^{+}$ expression and the cells were incubated for an additional 5 h. Cell lysates were subjected to immunoprecipitation and immunoblot analysis as in (C). (**F**) Cells expressing HA-tagged Fkh2p (HM6107) were synchronized in G_1 by transient temperature arrest and samples taken every 1 h upon release to the permissive temperature. Cell lysates were subjected to immunoprecipitation and immunoblot analysis as in (C). (**G**) DNA content of the cells in (F) was determined by flow cytometric analysis.

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To examine whether phosphorylation of Fkh2p on T314 affects its ability to bind to the upstream region of *ste11*$^+$ containing the FLEX1 and FLEXL1 sites, we performed ChIP analysis with cells expressing phosphomimetic mutants of Fkh2p (Figure 5). The mating efficiency and the induction of *ste11*$^+$ were low in GFP-tagged Fkh2p(S462E) cell-like control cells (Supplementary Figure 2; see Figure 3B and C). At 2 h after nitrogen withdrawal, the amount of Fkh2p(T314E) associated with this genomic region failed to increase compared with that of the wild-type protein. These results thus suggested that the poor mating efficiency of, as well as the impaired induction of *ste11*$^+$ mRNA in, *fkh2-T314E* mutant cells is due to the reduced ability of the Fkh2p(T314E) mutant protein to bind to the upstream region of *ste11*$^+$. Given that we showed that Fkh2p binds directly to the FLEX1 element upstream of *ste11*$^+$ *in vitro*, we next examined the binding activity of the Fkh2p(T314E) mutant by EMSA analysis with a FLEX1 probe (Supplementary Figure 10). The binding activity of Fkh2p(T314E) was only slightly reduced compared with that of the wild-type protein. This fact suggests that an additional mechanism may operate to regulate the binding of Fkh2p by Cdc2p phosphorylation *in vivo*. Together, these findings suggest that, during the mitotic cycle, Cdc2p phosphorylates Fkh2p on T314, leading to the failure of its binding to the upstream region of *ste11*$^+$ at least *in vivo*.

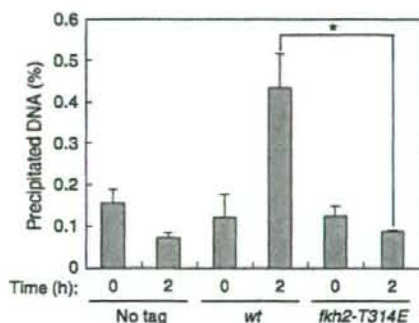

Figure 5 Phosphorylation of Fkh2p on T314 reduces its binding to the FLEX1 sequence upstream of *ste11*$^+$. No tagged cells (No tag, HM6) and cells expressing GFP-tagged wild-type (*wt*, HM5719) or T314E (*fkh2-T314E*, HM5912) mutant form of Fkh2p were treated and subjected to ChIP analysis with antibodies to GFP and the primer set A as in Figure 2D. Data are means ± s.e. of values from three independent experiments. $^*P<0.013$ (Student's *t*-test).

If Cdc2p phosphorylates Fkh2p, which in turn reduces mating efficiency, then Fkh2p functions downstream of Cdc2p. To confirm this notion, we performed a mating assay with several strains harboring a *cig2* deletion (Figure 6A and B). The mating efficiency of *cig2 fkh2*, *cig2 fkh2 fhl1*, *cig2 fkh2 mei4*, *cig2 fkh2 fhl1 mei4*, *cig2 fkh2-T314E*, or *cig2 fkh2-S462E* cells was reduced compared with that of the *cig2* single mutant, although the difference of the mating efficiency among *cig2 fkh2*, *cig2 fkh2-T314E*, *cig2 fkh2-S462E*, and *cig2* cells was not due to a timing in induction of cell-cycle arrest in G_1 phase (Supplementary Figures 4 and 7), suggesting that Fkh2p function overlaps with that of Fhl1p and Mei4p and that Fkh2p acts downstream of Cig2p. We also found that the induction of *ste11*$^+$ mRNA occurred earlier in *cig2*-deleted cells than in *wt* cells (Figure 6C), which likely explains the enhanced mating phenotype of the former cells. However, we cannot exclude the possibility that this phenotype of *cig2*-deleted cells is caused by earlier arrest in G_1 phase in response to nitrogen deprivation (Obara-Ishihara and Okayama, 1994; Supplementary Figures 4 and 7). The induction of *ste11*$^+$ mRNA of *cig2 fkh2-T314E* or *cig2 fkh2-S462E* cells was reduced compared with those of *cig2*-deleted cells (Figure 6D). These results suggested that Fkh2p(314E)

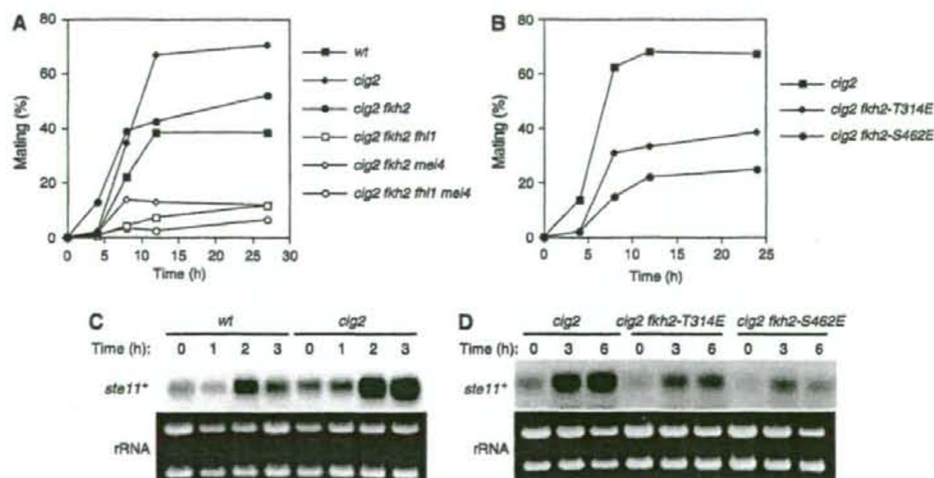

Figure 6 The mating efficiency of *cig2* cells is reduced by mutation of forkhead transcription factors. (**A**) *wt* (HM6), *cig2* (HM5555), *cig2 fkh2* (HM5701), *cig2 fkh2 fhl1* (HM5702), *cig2 fkh2 mei4* (HM5703), or *cig2 fkh2 fhl1 mei4* (HM5704) cells were assayed for mating efficiency as in Figure 1. (**B**) *cig2* (HM5530), *cig2 fkh2-T314E* (HM5924), or *cig2 fkh2-S462E* (HM5925) cells were assayed for mating efficiency as in Figure 1. (**C, D**) Total RNA was extracted from cells treated as in (A) or (B) and was subjected to northern blot analysis of *ste11*$^+$ mRNA.

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or Fkh2p(S462E) acts downstream of Cig2p in both mating and induction of *ste11*$^{+}$ mRNA but not in induction of cell-cycle arrest.

Discussion

Initiation of sexual development in fission yeast requires the temporal coordination of the induction of many genes with cell-cycle progression, but the molecular events that underlie this coordination are not well understood. We have now provided evidence that Fkh2p is phosphorylated by Cdc2p and that this phosphorylation inhibits sexual development by preventing the induction of *ste11*$^{+}$. Although many other processes likely also contribute to control of mating, our data establish a direct connection between initiation of mating and a key regulator of cell-cycle progression.

Our study has revealed the following. (1) The forkhead transcription factors Fkh2p, Fhl1p, and Mei4p have overlapping functions and are required for induction of *ste11*$^{+}$ mRNA and efficient mating. Among these factors, Fkh2p plays a major role in mating. (2) Fkh2p binds to the FLEX1 sequence present upstream of *ste11*$^{+}$, when the kinase activity of Cdc2p is low, with FLEX1 serving as a *cis*-acting element for Fkh2p. (3) Cdc2p phosphorylates Fkh2p on T314 and S462 both *in vivo* and *in vitro*, and phosphorylation of these residues results in inhibition of both *ste11*$^{+}$ induction and mating; phosphorylation on T314 also inhibits the binding of Fkh2p to the FLEX1 sequence *in vivo*.

On the basis of these results, we propose a model for the roles of Cdc2p and Fkh2p in the control of mating. During the mitotic cycle, Cdc2p is active and phosphorylates Fkh2p on T314 and S462. Phosphorylation of T314 may inhibit the binding of Fkh2p to the FLEX1 site upstream of *ste11*$^{+}$ *in vivo*. The mechanism by which phosphorylation of Fkh2p on S462 inhibits the induction of *ste11*$^{+}$ mRNA is unknown, but it is possible that phosphorylation of this residue results in the recruitment of a repressor protein that blocks *ste11*$^{+}$ transcription. In support of this notion, human FoxN3, which is homologous to Fkh2p, binds to a histone deacetylase complex (Scott and Plon, 2003). Nutrient exhaustion in fission yeast triggers the degradation of B-type cyclins and the consequent inactivation of Cdc2p. The absence of the kinase activity of Cdc2p allows the dephosphorylation of Fkh2p on T314 and S462 and the consequent activation of this transcription factor. Fkh2p thus binds to the FLEX1 site upstream of *ste11*$^{+}$ and induces its transcription, thereby triggering sexual development.

Nutrient exhaustion and Cdk inactivation

We have shown that phosphorylation of Fkh2p by Cdc2p results in efficient inhibition of mating, indicating that inactivation of Cdc2p is required for efficient mating. Consistent with this notion, inhibition of cyclin degradation, downregulation of a Cdk inhibitor, or overproduction of Cig2p inhibits mating (Obara-Ishihara and Okayama, 1994; Yamaguchi *et al*, 1997; Kitamura *et al*, 1998; Stern and Nurse, 1998). It has been known that *cig2* cells show enhanced mating, probably because of the upregulation of Ste11p at both mRNA (this study) and protein levels (Kjaerulff *et al*, 2007) in addition to the enhanced G_1 arrest (Obara-Ishihara and Okayama, 1994). There are therefore two distinct mechanisms by which mating is controlled by Cdc2p. First, exhaustion of nutrients, especially that of nitrogen, induces G_1 arrest, which requires inactivation of Cdc2p mediated by cyclin degradation or upregulation of a Cdk inhibitor (Yamaguchi *et al*, 1997; Kitamura *et al*, 1998; Stern and Nurse, 1998). Second, during the mitotic cycle, when nutrients are available, Cdc2p phosphorylates Fkh2p and thereby inhibits both induction of *ste11*$^{+}$ mRNA and mating. Fkh2p is not required for the G_1 arrest induced by nitrogen deprivation, suggesting that it is specifically required for *ste11*$^{+}$ induction. In other words, Cdc2p may actively inhibit mating by phosphorylating Fkh2p. We propose that these two controls ensure that mating occurs only in G_1 phase when the activity of Cdc2p is low.

It has been recently shown that Cdc2p directly phosphorylates Ste11p, which inhibits its DNA binding activity (Kjaerulff *et al*, 2007). Therefore, Cdc2p inhibits sexual differentiation through Ste11p at both the mRNA (this study) and post-translational levels (Kjaerulff *et al*, 2007). It is possible that Fkh2p-dependent mechanism is less important than the more direct Cdc2 phosphorylated mechanism. However, the switch between mitosis and meiosis is vitally important to fission yeast, and it is likely that this organism having two levels of control in regulating Ste11p expression to achieve this goal. These two controls of Ste11p by Cdc2p may reinforce to repress differentiation outside G_1.

ste11^{+} as a target gene of Fkh2p in mating

We have shown that *ste11*$^{+}$ is a critical target of Fkh2p in the control of mating. In addition to Rst2p and Ste11p (Kunitomo *et al*, 2000; Higuchi *et al*, 2002), Fkh2p is thus required for the induction of *ste11*$^{+}$ mRNA and mediates its effect by binding to the upstream region of the gene. Nutrient limitation therefore triggers *ste11*$^{+}$ expression by at least two separate signaling pathways: it reduces PKA activity, thereby activates Rst2p, leading to the production of *ste11*$^{+}$ mRNA, and it inactivates Cdc2p, thereby activates Fkh2p, again resulting in the induction of *ste11*$^{+}$ mRNA. The latter mechanism also contributes to the coordination of cell-cycle progression and sexual development.

Regulation of Fkh2p by phosphorylation

The sequence similarity among forkhead proteins is largely limited to the DNA-binding domain. We have now detected substantial similarity of the region surrounding the T314 phosphorylation site in Fkh2p of fission yeast to FoxN3 forkhead proteins of various species. In budding yeast, Fkh2p is phosphorylated predominantly on residues in its C-terminal region by Cdk1p *in vitro* (Ubersax *et al*, 2003; Pic-Taylor *et al*, 2004). Phosphorylation of at least some of these residues (S683, T697 and S771) is important for recruitment of Ndd1p (Pic-Taylor *et al*, 2004). These Cdk1p phosphorylation sites of Fkh2p in budding yeast do not appear to be conserved in fission yeast. In mammalian cells, FoxM1 forkhead proteins are transcriptional regulators important for cell-cycle progression similar to fission yeast Mei4p (Costa *et al*, 2003; Laoukili *et al*, 2005; Murakami-Tonami *et al*, 2007). FoxM1B undergoes extensive phosphorylation by several kinases, with Cdks phosphorylating T596 in the activation domain of the mouse protein, a process that is essential for the recruitment of coactivator proteins and transcriptional activity (Major *et al*, 2004). In addition, the transcriptional activity of FoxM1C is regulated by Cdk-mediated phosphor-

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ylation (Luscher-Firzlaff *et al*, 2006; Wierstra and Alves, 2006). Furthermore, Cdk2 phosphorylates FoxO1 and thereby reduces its transcriptional activity (Huang *et al*, 2006). Regulation of forkhead proteins by Cdk-mediated phosphorylation thus appears to be evolutionarily conserved in many eukaryotes, although the number and location of phosphorylation sites appear to vary among species.

Materials and methods

Yeast strains, media, and genetic methods

All media and standard methods were as described previously (Moreno *et al*, 1991). The procedures for gene disruption and N-terminal or C-terminal tagging of proteins were also as described previously (Bahler *et al*, 1998). The *S. pombe* strains used in this study are listed in Supplementary Table 1.

Primers and probes

Oligonucleotide primers and probes used in this study are listed in Supplementary Table 2.

Mating assay

After exponential growth in YE4S medium, cells were cultured in EMM2 medium for 12–16 h at 30°C to a density of 1×10^7–2×10^7 cells/ml, washed several times with EMM2 medium without a nitrogen source, resuspended at a density of 2×10^7 cells/ml, and shook gently. After the incubation for the indicated times at 30°C, samples of the cell suspension were collected and the number of zygotes was counted with the use of a light microscope. The percent mating frequencies were calculated by dividing the number of zygotes (one zygote counted as two cells) by the number of total cells.

Construction of fkh2 mutants

To construct a mutant lacking the 7-bp core sequence of FLEX1, we performed the polymerase chain reaction (PCR) with genomic DNA and the primers 1088 and 1089. The amplified DNA fragment was introduced by transformation into a strain in which the FLEX1 region was replaced with *ura4*$^+$. Colonies resistant to 5-fluoroorotic acid (5-FOA) were selected, and the 7-bp deletion of FLEX1 was verified by sequencing.

To construct strains with mutations of the putative Cdc2p phosphorylation sites of Fkh2p, we performed site-directed mutagenesis with the *fkh2*$^+$ coding region amplified by PCR with the primers 1167 and 1172. The primers used for mutagenesis were as follows: 1167, 1168, 1169, 1170, 1171, and 1172 for *fkh2-T314A S462A S481A*; 1167, 1270, 1271, 1272, 1273, and 1172 for *fkh2-T314E S462E S481E*; 1167, 1270, 1271, and 1172 for *fkh2-T314E*; 1167, 1272, 1373, and 1172 for *fkh2-S462E*; and 1167, 1038, 1273, and 1172 for *fkh2-S481E*. The plasmids were then used as templates for PCR with primers 1167 and 1172, and the resulting DNA fragments were introduced into *fkh2::ura4*$^+$ cells (HM5657) by transformation. Colonies resistant to 5-FOA were selected, and the mutations were confirmed by PCR and sequencing.

Protein extraction, immunoprecipitation, and immunoblot analysis

Protein extracts were prepared and immunoblot analysis was performed as described previously (Shimada *et al*, 2005). Immunoprecipitation was also performed as previously described (Shimada *et al*, 1999) with the exception described below. Mouse monoclonal antibody to HA (1:1000) was obtained from Roche. Polyclonal antibodies specific for phosphorylated forms of Fkh2p were generated in rabbits with the keyhole limpet hemocyanin-conjugated peptides AKTRKpTPRKRS (residues 309–319) for phospho-T314 and GSYDTIpSPYRN (residues 456–466) for phospho-S462 as antigens. Immunoblot analysis was performed with affinity-purified anti-pT314 (1:100 dilution) or anti-pS462 (1:50 dilution) after immunoprecipitation of HA-tagged Fkh2p with anti-HA antibody. Immune complexes were detected with horseradish peroxidase-conjugated goat antibodies to mouse or rabbit immunoglobulin G (both at 1:1000 dilution, Amersham) and ECL reagents (Amersham).

Isolation of RNA and northern blot analysis

Total RNA was extracted and northern blot analysis was performed as described previously (Shimada *et al*, 2005). The probe used for northern analysis was a ^{32}P-labeled 1.3-kb *Pvu*II fragment of *ste11*$^+$.

Production of recombinant Fkh2p proteins

To express GST-tagged versions of Fkh2p in *Escherichia coli*, we amplified DNA fragments encoding Fkh2p (amino acids 305–492 containing the *wt* sequence or T314A, S462A, and S481A mutations; amino acids 216–330 containing the *wt* sequence or T314A or T314E mutations; amino acids 317–479 containing the *wt* sequence or the S462A mutation) by PCR using the constructs mentioned above as templates. The primers used here were as follows: 1367 and 1368 for amino acids 305–492; 1002 and 1003 for amino acids 216–330; 1411 and 1412 for amino acids 317–479. The PCR products were digested and cloned them into the corresponding sites of pGEX5X-1 (Pharmacia Biotech). The expression and purification of the GST fusion proteins were performed as described previously (Smith and Johnson, 1988).

EMSA analysis

Double-stranded oligonucleotide probes used are as follows: the primers 1141 and 1142 (FLEX1), 1179 and 1180 (FLEXL1), 1181 and 1182 (FLEXL2), 1183 and 1184 (FLEXL3), or 1143 and 1144 (TR). Purified GST–Fkh2p(216–330) (0.2 μg) was incubated for 30 min at room temperature with 20 μg of ^{32}P-labeled probe in a total volume of 5 μl containing 50 mM Tris–HCl (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$, 15% glycerol, 1 mM dithiothreitol, and poly(dI-dC) (0.1 mg/ml). In some instances, samples were incubated for 15 min at room temperature either with a 100-fold molar excess of nonradioactive competitor oligonucleotide or with 0.9 μg of antibodies to GST (Santa Cruz Biotechnology) before addition of the probe. DNA–protein complexes were resolved by nondenaturing electrophoresis on a 5% polyacrylamide gel, which was then dried and subjected to autoradiography.

ChIP analysis

ChIP was performed as described previously (Saitoh *et al*, 1997) with some modifications. Immunoprecipitation was performed with anti-GFP conjugated to magnetic beads (Dynal). Primer set A comprised 1325 and 1326, and primer set B consisted of 1327 and 1328. For detection of the *dis3*$^+$ region as a negative control, the primers 1329 and 1330 were used. For detection of the *cdc15*$^+$ upstream region, the primers 1569 and 1570 were used. PCR amplification was performed with SYBR Green PCR Master Mix containing immunoprecipitated DNA (or total DNA) and a mixture of the three sets of primers. The amounts of the DNA were determined by the $\Delta\Delta^{CT}$ method with the use of an ABI prism 7700 instrument and the primers described above.

Preparation of recombinant Cdc2/CyclinB1 complex

Baculoviruses expressing Myc- and His × 6-tagged human Cdc2 wild type or kinase dead (K33M) and CyclinB1 were generated by cotransfection of pVL1392-Cdc2 or pVL1392-CyclinB1 with the linearized baculovirus DNA (BaculoGold; BD biosciences) into Sf9 cells. After amplification of the virus, Sf9 were infected with both Cdc2 wild type and CyclinB1 viruses or Cdc2 kinase dead and CyclinB1 viruses for 72 h. Cell lysates were prepared and 0.1 mg of protein immunoprecipitated with anti-Myc antibody (Santa Cruz) was used for kinase assay.

Kinase assay

Protein extracts were prepared with glass beads in lysis buffer as described previously (Shimada *et al*, 1999). The kinase assay was performed as described previously (Murakami and Nurse, 1999).

Statistics

Experiments with at least three replicates were carried out and statistical analyses were performed by Student's *t*-test. Values of $P < 0.05$ are considered significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Acknowledgements

We thank P Nurse and K Kitamura for yeast strains and K Okazaki for plasmids and helpful discussion. We also thank H Kojima and Y Katsuno for technical assistance. This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to HM).

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