Cyclin-Dependent Kinase 8 Regulates Mitotic Commitment in Fission Yeast

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Temporal changes in transcription programs are coupled to control of cell growth and division. We here report that Mediator, a conserved coregulator of eukaryotic transcription, is part of a regulatory pathway that controls mitotic entry in fission yeast. The Mediator subunit cyclin-dependent kinase 8 (Cdk8) phosphorylates the forkhead 2 (Fkh2) protein in a periodic manner that coincides with gene activation during mitosis. Phosphorylation prevents degradation of the Fkh2 transcription factor by the proteasome, thus ensuring cell cycle-dependent variations in Fkh2 levels. Interestingly, Cdk8-dependent phosphorylation of Fkh2 controls mitotic entry, and mitotic entry is delayed by inactivation of the Cdk8 kinase activity or mutations replacing the phosphorylated serine residues of Fkh2. In addition, mutations in Fkh2, which mimic protein phosphorylation, lead to premature mitotic entry. Therefore, Fkh2 regulates not only the onset of mitotic transcription but also the correct timing of mitotic entry via effects on the Wee1 kinase. Our findings thus establish a new pathway linking the Mediator complex to control of mitotic transcription and regulation of mitotic entry in fission yeast.

Signaling pathways can control the activation of gene expression programs and thereby regulate cell fate determination. In embryonic stem cells, certain gene expression programs allow the cells to self-renew whereas other programs trigger differentiation into specific cell types as a response to developmental signaling (58). Elucidation of how temporal changes in transcription programs are coupled to control of cell growth and division is therefore of fundamental importance for our understanding of developmental processes.

Global gene transcription analysis in yeasts and higher eukaryotes has revealed that a significant proportion of the genome is transcribed in a periodic manner during cell cycle progression (5, 15, 34, 49, 55). Correct periodic regulation is believed to play a critical role in normal cell proliferation, and the genes are often deregulated in different forms of cancer (6). Depending on the organism, the number of periodically expressed genes ranges from ~400 to more than 1,000 (5, 6, 56). These include genes with well-established roles in cell cycle progression, such as those encoding cyclins, transcription factors and protein kinases.

A cluster named *CLB2* in budding yeast (35 genes) or cluster 1 in fission yeast (87 genes) is periodically expressed and activated at mitosis and repressed in G₁ of the next cell cycle (4, 5, 34, 56). In budding yeast, transcription of the *CLB2* cluster is controlled by the forkhead proteins Fkh1 and Fkh2, which cooperate with Mcm1 (a MADS box protein) and the Ndd1 coactivator (27, 28). In fission yeast, forkhead proteins Sep1 and Fkh2 and the MADS box protein Mbx1 regulate mitotic transcription (12, 13, 49, 53). Deletion of the *sep1* gene results in reduced transcription, whereas overexpression of *sep1* induces expression of the same genes. In contrast, deletion of *fkh2* causes elevated levels of gene transcription, suggesting a role for this transcription factor in negative regulation of gene transcription (49). Furthermore, the periodic binding of Sep1 to cluster 1 promoters coincides with gene activation, whereas Fkh2 is bound to those genes when they are repressed, supporting the idea that Sep1 promotes gene expression and Fkh2 represses it (43).

Our understanding of how regulation of *CLB2* or cluster 1

genes is coordinated with mitotic progression has increased in recent years, revealing the importance of phosphorylation of specific transcription factors by Cdk1 and the Polo kinase and dephosphorylation by the CDC14 phosphatase. In *Saccharomyces cerevisiae*, the Cdc28 (Cdk1) kinase phosphorylates both Ndd1 and Fkh2 and these phosphorylation events stimulate direct interactions between Ndd1 and the FHA domain of Fkh2 (18, 45, 46). The Polo kinase Cdc5 is also temporally recruited to *CLB2* gene cluster promoters and phosphorylates Ndd1, which helps to establish a positive feedback loop for *CLB2* cluster activation (17). Similarly, in *Schizosaccharomyces pombe*, the Polo kinase Plo1 phosphorylates the Mbx1 MADS box protein to positively control gene expression in a feedback loop (43). *In vitro*, Mbx1 is also phosphorylated by Cdk1 and dephosphorylated by the Cdc14-like phosphatase Clp1, with Clp1 apparently having a repressive role in controlling gene expression (42). Similar to Mbx1, the Fkh2 protein also becomes phosphorylated during mitosis, but the responsible kinase and the function of this modification have not been described.

Albeit Cdk1 kinase is the master regulator of cell cycle progression in eukaryotic cells and the protein is an important regulator of mitotic transcription, recent studies revealed that other factors are also required to establish global periodic transcription patterns in budding yeast (41). A “transcription network oscillator” has been proposed to operate and couple periodic transcription to the Cdk oscillator (51), even if the molecular basis for this new oscillator remains to be established.

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The Mediator complex is a coregulator of eukaryotic transcription and functions as a bridge between gene-specific transcription regulators and the polymerase II (Pol II) machinery at the promoter (16). Phylogenetic studies have suggested that the Mediator complex is present in most eukaryotic organisms (10, 11). Cyclin-dependent kinase 8 (Cdk8) is a conserved Mediator component found in most eukaryotes. In multicellular organisms, Cdk8 has been associated with signaling pathways related to cell differentiation and neuronal development (29, 31, 47). Early studies identified Cdk8 as a negative regulator of global transcription, but later investigations have modified this conclusion somewhat, since Cdk8 is also required for stimulation of specific genes (24). Cdk8 has been shown to phosphorylate specific transcription factors to regulate their activity (2, 14, 22, 39). Recently, Cdk8 has also been connected to cancer development (21, 26), but a specific role in cell cycle progression has not been demonstrated.

We here demonstrate that Fkh2 and Cdk8 are important regulators of cell cycle progression in fission yeast. We found that Cdk8 controls the levels and activity of Fkh2, which in turn regulates Cdk1 activity via the Wee1 kinase. Our studies reveal a novel pathway whereby cells may couple mitotic gene activation to the control of mitotic entry and suggest that Mediator may be an integrative hub for both transcription regulation and cell cycle progression (33).

MATERIALS AND METHODS

Yeast genetic methods. Standard fission yeast media and methods were used (36). Strains used in this study are listed in Table 1. Primer sequences and PCR conditions are available on request. Cell size measurements were done at 25°C, except for the *wee1-50* mutants, which were measured after incubating cells at 36°C for 6 h. For overexpression of *fkh2*, we transformed wild-type (wt) and *wee1-50* cells with the pREP3X-Fkh2 plasmid (12) or an empty control plasmid (pREP3X). The indicated transformants were propagated on selective media under repressive conditions (in the presence of 5 µg of thiamine/ml) and then incubated under inductive conditions overnight at 25°C or 36°C to analyze *wee1-50* cell phenotypes. Gene targeting was performed according to published protocols (54), and mutagenesis of *fkh2* DNA was done using a Lightning Multi site-directed mutagenesis kit (Stratagene). Sequence modifications were always confirmed by sequencing.

For construction of strains expressing mutated versions of Fkh2 with a C-terminal 3× hemagglutinin (HA) epitope tag, we used the pFA6a-3HA-natMX6 plasmid (54). The coding region (excluding the translation stop codon) and 1,000 bp of the upstream region of wild-type *fkh2* were cloned between the PvuII and PacI sites. The 300-bp region immediately downstream of the *fkh2* translation stop was cloned into the SacI-EcoRV sites to create the pFA6a-HA-fkh2WT plasmid. The upstream PvuII-PacI fragment was then replaced by DNA fragments mutated *in vitro* to create mutant versions of the pFA6a-HA-fkh2WT plasmid. Strains expressing Fkh2 with a C-terminal FLAG tag were created by replacing the 3× HA tag with a 1× FLAG epitope in the PacI-AscI sites of the pFA6a-HA-fkh2WT plasmid.

Gene targeting was done as described previously (54), using the nourseothricin (NAT) resistance marker. Colonies appearing on plates were picked, and replacement was confirmed by PCR. When applicable, sequence modifications were confirmed by sequencing of the PCR products. Protein tagging was confirmed by Western blotting, and strains were backcrossed to wild type. For Fkh2 overproduction in *Escherichia coli*, full-length *fkh2* cDNA was cloned into the pTRC plasmid (Invitrogen) between the NcoI and SalI sites such that it contained a C-terminally placed 6×His epitope tag in frame.

For identification of the PEST motifs on Fkh2, we used the epestfind

TABLE 1 Strains used in this study

Strain	Genotype	Source or reference
CG1	<i>h⁻ L972</i>	M. Sipiczki (University of Debrecen)
CG10	<i>h⁻ med13⁺::TAP-kanMX6 ade6-216</i>	19
CG15	<i>h⁺ cdc25-22</i>	M. Sipiczki (University of Debrecen)
CG27	<i>h⁺ med7⁺::TAP-kanMX6 med13::kanMX4</i>	19
CG39	<i>h⁺ cdk8-D158A</i>	19
CG45	<i>h⁻ cdc25-22 med7⁺::myc-kanMX6</i>	This study
CG94	<i>h⁺ cdk8-D158A cdc25-22 med7⁺::myc-kanMX6</i>	This study
CG117	<i>h⁺ fkh2-3HA::kanMX6 cdc25-22</i>	12
CG119	<i>h⁻ cdk8-D158A cdc25-22 fkh2-3HA::kanMX6</i>	This study
CG134	<i>h⁺ fkh2::1FLAG-nat-MX6 cdc25-22</i>	This study
CG152	<i>h⁺ cdc25-22 cdk8-D158A</i>	This study
CG120	<i>h⁺ fkh2::kanR cdc25-22 med7⁺::myc-kanMX6</i>	This study
CG175	<i>h⁺ cdk8-D158A fkh2::kanMX4 cdc25-22</i>	This study
CG186	<i>h⁻ fkh2-3HA::kanR mts3-1 ura4-D18 leu1-32 ade6-M210</i>	C. Gordon (MRC, Edinburgh, United Kingdom)
CG207	<i>h⁻ cdc25-22 fkh2-S322A S375A-3HA::nat-MX6</i>	This study
CG206	<i>h⁻ fkh2-S322A S375A-3HA::nat-MX6</i>	This study
CG209	<i>h⁻ fkh2-3HA::kanR</i>	12
CG271	<i>h⁺ fkh2-S322E S375E-3HA::nat-MX6 ade6-Mx</i>	This study
CG283	<i>h⁻ fkh2-S322E S375E-3HA::nat-MX6 cdc25-22</i>	This study
CG284	<i>h⁺ fkh2-S322E S375E-3HA::nat-MX6 wee1-50</i>	This study
CG211	<i>h⁺ wee1-50 cdk8-D158A</i>	This study
CG215	<i>h⁻ wee1-50 fkh2-S322A S375A-3HA::nat-MX6</i>	This study
CG181	<i>MATa URA3::lexA-lacZ met- his3 ade2 trp1 leu2 gal4D gal80D</i>	43
CG-336	<i>h⁺ cdc25-22 cdk8-3HA::natMX6</i>	This study
CG-236	<i>h⁻ nda3-KM311 fkh2-3HA::kanR</i>	This study
CG-340	<i>h⁻ nda3-KM311 fkh2-322A 375A-3HA::nat-MX6</i>	This study
CG-341	<i>h⁺ nda3-KM311 fkh2-S322E S375E-3HA::nat-MX6</i>	This study
CG-344	<i>h⁻ mts3-1 fkh2-322A S375A-HA::natMX6</i>	This study
CG-349	<i>h⁻ cdk8-D158A fkh2-S332A 375A-3HA::nat-MX6</i>	This study
CG-350	<i>h⁺ fkh2-PESTmut-HA::nat-MX6 ura4-D18 leu1-32</i>	This study

algorithm, which is available at <http://emboss.bioinformatics.nl/cgi-bin/emboss/pepfind>.

Protein methods and purifications. To produce whole-cell protein extracts, 3×10^8 to 5×10^8 cells were collected from nonsynchronous or synchronized cell cultures. After harvest, the cells were suspended in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.05% NP-40, 2 mM sodium orthovanadate, 40 mM β-glycerophosphate, and protease inhibitors (phenylmethylsulfonyl fluoride [PMSF, 100 mM], pepstatin [200 µM], leupeptin [60 µM], and benzamidine and [200 mM]). After bead beating was performed, lysates were cleared by centrifugation (13,000 rpm), resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted to membranes, and probed with the appropriate antibodies. Signals were detected using an ECL kit (GE Healthcare).

To detect Fkh2 ubiquitylation, 800 ml of cells from synchronized cultures were collected at the indicated time points and chilled quickly to 4 to 6°C by direct contact with ice. After harvest, the cells were suspended in

lysis buffer supplemented with 20 μ M Lactocystin and 2 mM *N*-ethylmaleimide to inhibit the proteasome and deubiquitinase activities, respectively. Lysates were cleared by centrifugation, and 300 μ l was used for immunoprecipitation of Fkh2 by the use of M2-FLAG agarose (Sigma). Agarose beads were washed with lysis buffer, boiled, and resolved using SDS-PAGE. Proteins were detected by immunoblotting, using anti-FLAG (Sigma) and antiubiquitin (abcam catalog no. ab90376) antibodies. Other antibodies used in this study were anti-Cdk1 Y15 (abcam catalog no. ab47594) (1:1,000), anti-Cdk1 (abcam catalog no. ab5467) (1:1,000), antiactin (abcam catalog no. ab8224) (1:1,000), anti-myc (Sigma catalog no. M4439) (1:1,000), and anti-HA (F-7; Santa Cruz catalog no. sc-7392) (1:500).

For quantification of the relative amounts of different Fkh2 isoforms, the intensities of bands were measured (see Fig. 4A) and the intensities of phosphorylated bands (slower-migrating band) were divided by the intensities of nonphosphorylated bands (faster-migrating band) for each time point. The relative intensities of the two Fkh2 bands were determined (see Fig. 4B).

Recombinant Fkh2 purification and kinase assays. wt and mutant versions of Fkh2 were produced by expression in pLYS BL21 *E. coli* cells. Protein expression was induced in 5-liter cultures (optical density [OD], $\sim$ 0.6) by addition of 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 3 h at 37°C. Cells were lysed in buffer Q containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 2 mM imidazol, 1% Triton, 10% glycerol, and a 1 $\times$ protease inhibitor cocktail. The lysates were cleared by centrifugation and incubated with nickel-agarose (Sigma catalog no. P6611) (1 ml) for 1 h. The beads were washed with buffer Q containing 20 mM imidazol (10 column volumes). Fkh2 was then eluted with buffer Q containing 300 mM imidazol. The eluate was further purified on a heparin column, using a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 10% glycerol, and a 1 $\times$ protease inhibitor cocktail. The bound Fkh2 protein was eluted with a high-salt buffer (800 mM NaCl) and resolved by SDS-PAGE to verify purity. This method has typically resulted in 300 to 800 ng of Fkh2/ μ l with a purity of $\sim$ 80%. The purified Fkh2 protein was used as a substrate for kinase assays with the Cdk8/Mediator, according to a previously published protocol (19). As a positive control, we used a recombinant fragment of the Pol II C-terminal domain (CTD).

Yeast two-hybrid analysis. We followed previously published protocols (43) to analyze protein-protein interactions by the use of the yeast two-hybrid system. We cloned *cdk8* into the bait plasmid pBTM116 and tested two hybrid interactions with *plp1*, *fkh2*, *sep1*, and *mbx1* cloned into the prey plasmid pACT2. Two hybrid interactions were identified using an X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) overlay assay and quantified with a β -galactosidase assay according to a previously published method (43).

ChIP. To detect Mediator binding, wild-type and *cdk8-D158A* cells containing a myc tag on the Mediator core component Med7 or wild-type cells bearing an HA tag on the Cdk8 kinase were synchronized in *cdc25-22* block-release experiments. The same experimental setting was used to detect Fkh2 binding in synchronized wild-type and *cdk8-D158A fkh2-HA* cells. We collected 10^9 cells every 15 min (or 20 min when doing Cdk8-HA chromatin immunoprecipitation [ChIP] experiments or 10 min in the experiments detecting Fkh2 binding) and processed the cells as described previously (52). At the 0-min and 30-min points (or the 40-min time point when doing the Cdk8-HA ChIP experiments), we also collected cells from the untagged *cdc25-22* control strain, using the same experimental conditions.

To detect wild-type and phosphomutant Fkh2-HA binding in asynchronous and mitotically blocked *nda3-KM311* cells, half of the mid-log-stage cultures were processed at 30°C for ChIP and other half of the cultures were incubated for 6 h at 18°C and then processed for ChIP as described previously. Antibodies (anti-myc or anti-HA) were applied at a 1:100 dilution in a 100- μ l volume of chromatin. A 2- μ l volume of ChIP DNA was used for real-time PCRs, and the experi-

ments were done three times with 2 immunoprecipitation repeats in each experiment.

RNA methods, microarray analysis, and mRNA expression measurements. Total RNA was isolated as described previously (57). cDNA was synthesized from 1 μ g of total RNA by the use of a Roche 11483188001 kit, the reaction mixture was diluted 10-fold (200 μ l final volume), and 2 μ l was used for real-time PCR amplification.

For microarray analysis, two biological repeats of exponentially growing wt, *fkh2 Δ* , and *fkh2-S322A S375A* mutant cells were harvested and total RNA was isolated as described above. The quality of the RNA was assessed using an Agilent BIOanalyser. Labeling and microarray hybridization to Affymetrix Yeast 2.0 arrays were carried out using the service of the Bioinformatics and Expression analysis core facility of Karolinska Institutet, Sweden. Analysis of the microarray data was done using Array Star 4 analysis software. The raw data files were submitted to the GEO repository (see below).

Mass spectrometry. The Fkh2 protein was digested with trypsin, dried, reconstituted in 0.1% formic acid, and analyzed on an LTQ-Fourier transform-ion cyclotron resonance (LTQ-FT-ICR) mass spectrometer (MS) (Thermo Fisher Scientific) interfaced with an in-house-constructed nano-liquid chromatography (nano-LC) system. The peptides were separated on a reverse-phase column (200 by 0.050 mm) packed in-house with 3- μ m-diameter Reprosil-Pur C₁₈-AQ particles. Peptides were eluted with an acetonitrile gradient and 0.1% formic acid. LTQ-FT-ICR MS settings were as follows: spray voltage, 1.4 kV; 1 microscan for MS1 scans at 100,000 resolutions (*m/z* 400); full MS mass range, *m/z* 400 to 2,000; data-dependent mode with one MS1; FT-MS scan precursor ions followed by CID (collision-induced dissociation) MS2 scans of the five most abundant doubly, triply, or quadruply protonated ions in each FT-MS scan. Tandem MS (MS/MS) was obtained by CID with collision energy of 25%.

Microarray data accession number. The raw data files for the mutant as well as wt cells constructed in this work were submitted to the GEO repository (accession number GSE31642).

RESULTS

Inactivation of the Cdk8 kinase activity delays mitotic entry.

Cdk8 is a conserved component of the Mediator complex, but a direct role in cell cycle progression had not been demonstrated. We wanted to investigate if Cdk8 could affect cell cycle progression in fission yeast, and to this end we performed *cdc25-22* block-release experiments. At the restrictive temperature, the *cdc25-22* mutation arrests the cells in G₂, allowing synchronized entry into mitosis when the cells are shifted back to the permissive temperature. We used this approach to synchronize wild-type and *cdk8-D158A* cells in which a single amino acid substitution had inactivated the Cdk8 kinase activity without affecting protein stability (19). Interestingly, Cdk8 was required for normal cell cycle progression, since cytokinesis and septum formation were delayed by $\sim$ 30 min in *cdk8-D158A* cells compared to wild-type cells (Fig. 1A). We did not observe phenotypes related to defective actomyosin ring formation or impaired synthesis of the division septum in the *cdk8-D158A* mutant cells (data not shown), consistent with earlier observations made during large-scale analysis of a fission yeast protein kinase deletion library (8). However, the *cdk8-D158A* mutation seemed to directly affect mitotic entry, since the mitotic index, which measures the fraction of cells in mitosis at any given time point, peaked with a $\sim$ 30-min delay in these mutant cells. The method of synchronization can sometimes affect the analysis, and we therefore repeated the experiment, but this time we synchronized cells by the use of hydroxyurea, which depletes dNTP levels and blocks cells in S phase. We used fluorescence-activated cell sorter (FACS) analysis of DNA content as an

FIG 1 Cdk8 regulates mitotic entry. (A) The mitotic and septation indices of synchronized wild-type and *cdk8-D158A* mutant cells. (B) Measurement of Cdk1-Y15 phosphorylation in synchronously dividing wild-type and *cdk8-D158A* cells.

indication of cell cycle progression and confirmed that the *cdk8-D158A* mutation caused a similar delay in cytokinesis and septum formation when hydroxyurea was used for cell cycle synchronization (data not shown). We concluded that the timing of septation and the mitotic indices most likely reflected an altered mitotic entry in synchronously dividing *cdk8-D158A* cells.

To further explore effects on mitotic entry, we monitored the phosphorylation status of Tyr15 on Cdk1 (the product of the *cdc2* gene in fission yeast). Dephosphorylation of this amino acid is a critical step during entry into mitosis (40). Importantly, we noted a ~30-min delay in Tyr15 dephosphorylation in synchronized *cdk8-D158A* cells (Fig. 1B), supporting the idea that delayed mitotic entry was the cause of the observed phenotypes. In addition, the *cdc25-22 cdk8-D158A* mutant cells divided at an increased cell size (Table 2), which indicates delayed mitotic commitment in fission yeast (37, 44). In summary, we conclude that the Cdk8 kinase is required for the correct timing of mitotic entry in *S. pombe*.

Cdk8/Mediator binds to mitotic promoters in a periodic manner. The Cdk8 kinase is an integral part of the Mediator complex (9), and the observed requirement for the *cdk8* kinase activity for normal mitotic entry could therefore be connected to a function of the Cdk8/Mediator complex during mitotic transcription. Although a direct role for the Mediator complex in periodic transcription has not been reported, earlier microarray studies performed with nonsynchronized cells have revealed alterations of mitotic transcript levels in Mediator mutant strains (30, 35). In fission yeast, expression of a specific subset of genes (cluster 1) is initiated as cells pass through mitosis (49). We therefore investigated if Mediator was recruited to promoters of mitotic genes. We used synchronized cell cultures and monitored binding of the core Mediator subunit Med7-myc to the promoter regions of mitotic genes (*fkh2*, *plp1*, *ace2*, *slp1*, and *rum1*). We found that Med7-myc was bound in a periodic manner to these promoters, with the peak of binding coinciding with the peak of transcription at M phase (Fig. 2A and B and data not shown). The Cdk8 module is not

always associated with the Mediator complex. Therefore, we also monitored binding of Cdk8-HA to mitotic promoters (*ace2* and *slp1*) in synchronized cells. The results showed a periodic binding of Cdk8, similar to that observed with Med7-myc, suggesting that both the core Mediator and the Cdk8 module are recruited to these promoters during mitosis (Fig. 2C and data not shown). In agreement with the observed delay in mitotic progression (Fig. 1), both *ace2* transcription and Mediator recruitment occurred later in the *cdk8-D158A* mutant background (Fig. 2B and D).

Cdk8 interacts with and regulates mitotic phosphorylation of transcription factor Fkh2. Since Cdk8/Mediator binding to mitotic promoters coincided with gene activation, we hypothesized that Cdk8 could directly interact with and phosphorylate regulatory factors associated with mitotic promoters in fission yeast. We therefore performed a two-hybrid screen to test possible interactions between the Cdk8 kinase and proteins implicated in mitotic gene transcription, Sep1, Mbx1, Fkh2, and Plo1 (4, 43). Previous studies had demonstrated that Plo1 interacts with Mbx1 (43), so we used this protein pair as a positive control. We found a specific interaction between Cdk8 and Fkh2, indicating that this transcription factor is a direct target of Cdk8 (Fig. 3A). No interactions were observed between Cdk8 and the other promoter-associated factors. To follow up our finding, we investigated genetic interactions between *fkh2* and *cdk8*. Both analysis of cell cycle progression after synchronization and measurements of cell size in cultures of asynchronously dividing cells demonstrated that deletion of *fkh2* could suppress the mitotic entry delay observed in *cdk8-D158A* cells (Fig. 3B and Table 2), which indicated that the Cdk8 effect on mitotic entry required Fkh2.

Interestingly, Fkh2 is phosphorylated during mitotic progression, but the responsible kinase has not been identified (12). Our observations supported a model in which Fkh2 was directly involved in Cdk8-dependent promotion of mitotic entry and raised the possibility that Fkh2 could be a target for the Cdk8 kinase activity. To investigate this possibility, we synchronized cells by the use of the *cdc25-22* mutation and monitored Fkh2 during cell cycle progression in wild-type and *cdk8-D158A* cells. As previ-

TABLE 2 Cell size at division of the indicated strains^a

Strain description	Cell length (μm) (mean ± SD; n > 50)
wt	13.9 ± 0.8
<i>cdc25-22</i>	21.2 ± 1.66
<i>wee1-50</i>	6.9 ± 0.8
<i>cdk8-D158A</i>	14.5 ± 0.6
<i>cdk8-D158A cdc25-22</i>	24.98 ± 3.85
<i>cdk8-D158A wee1-50</i>	6.9 ± 0.7
<i>cdc25-22 fkh2Δ</i>	20.6 ± 3.2
<i>cdc25-22 cdk8-D158A</i>	24.98 ± 3.85
<i>cdc25-22 cdk8-D158A fkh2Δ</i>	19.77 ± 3.38
<i>fkh2-S322A S375A-HA</i>	20.0 ± 0.82
<i>fkh2-S322A S375A-HA cdc25-22</i>	29.8 ± 4.45
<i>fkh2-S322A S375A-HA wee1-50</i>	6.8 ± 0.7
<i>fkh2-S322E S375E</i>	11.17 ± 0.92
<i>fkh2-S322E S375E cdc25-22</i>	18.85 ± 1.32
<i>fkh2-S322E S375E wee1-50</i>	7.09 ± 1.13
<i>fkh2-S322A S375A-HA cdk8-D158A</i>	23.74 ± 2.0
<i>fkh2-PESTmut</i>	12.04 ± 0.74

^a Cell sizes of strains containing the *wee1-50* allele were measured after incubation at 36°C.

FIG 2 Cdk8/Mediator binds to mitotic promoters in wt and *cdk8-D158A* mutant cells. Data represent the results of ChIP analysis performed using synchronously dividing cells. Samples were collected at the indicated time points. Asterisks indicate time points corresponding to an untagged control strain. (A) Med7-myc binding to the mitotic *slp1* promoter in wt cells. (B) Med7-myc binding to the mitotic *ace2* promoter in wt and *cdk8-D158A* cells. (C) Cdk8-HA binding to the *ace2* promoter in wt cells. Septation indices are included to show that the HA tag does not affect cell cycle progression. (D) Real-time PCR measurement of *ace2* transcription. Peaks of mRNA levels in both wt and *cdk8-D158A* cells coincided with the peak of Mediator complex binding. All measurements were repeated three times, and the mean values are shown, with error bars representing standard deviations.

ously demonstrated, Fkh2 migrates as a doublet in SDS-PAGE, with the slower-migrating band corresponding to a phosphorylated isoform (12). In wild-type cells, the levels of the phosphorylated protein changed during cell cycle progression, with a peak in mitosis. In the *cdk8-D158A* mutant strain, we still observed a phosphorylated band, but the intensity was much lower and there were no periodic variations or a peak during mitosis (Fig. 4A and B). Interestingly, the peak of Fkh2 phosphorylation in wild-type cells coincided with transcription of mitotic gene *ace2*, suggesting that Cdk8 may phosphorylate Fkh2 at the promoter (Fig. 4C). We also noted a variation in the total Fkh2 protein levels during the cell cycle (Fig. 4A). In wild-type cells, Fkh2 levels peaked at M phase and decreased thereafter. This increase in Fkh2 protein levels was not apparent in *cdk8-D158A* cells, and the overall Fkh2 protein levels were lower in the mutant background (Fig. 4A).

Cdk8-dependent phosphorylation of Fkh2 on S322 and S375 *in vitro* and *in vivo*. We next set out to identify the amino acids in Fkh2 phosphorylated by Cdk8. Purified Cdk8/Mediator complex could efficiently phosphorylate recombinant Fkh2 *in vitro*. In Mediator complexes lacking Med13, the levels of Cdk8 are severely depleted (50) and, using this mutant form of Mediator, we observed much lower levels of Fkh2 phosphorylation, supporting the idea that Cdk8 and no other contaminating kinase was responsible for the phosphorylation observed *in vitro* (Fig. 4D). To identify the specific amino acid residues phosphorylated, we performed mass spectrometry analysis of recombinant Fkh2 phosphorylated

in vitro by Cdk8. We identified three phosphorylated amino acid residues, serines S14, S322, and S375 (data not shown), which were absent from nonphosphorylated control Fkh2. Interestingly, two of these sites, S322 and S375, are also phosphorylated *in vivo*, as demonstrated by a recent phosphoproteome analysis (7), suggesting that these sites could be specific targets of Cdk8 phosphorylation *in vitro*. Indeed, replacement of either S322 or S375 by alanine caused a strong reduction in Cdk8-dependent phosphorylation *in vitro* (Fig. 4E and F).

The *in vitro* specificity of a kinase may be different from that observed *in vivo*. To obtain further evidence for the functional relevance of the observed phosphorylation events, we replaced the serines with alanine residues (S322A and S375A) by gene targeting of the native *fkh2* locus (Fig. 5A). We also engineered a yeast strain carrying S322E and S375E mutations. Glutamic acid resembles the structure of a phosphorylated serine, and the S322E and S375E mutations therefore had the potential to mimic phosphorylated Fkh2. We monitored Fkh2 by immunoblot analysis of the wt and *fkh2* mutant strains, but since nonsynchronized wild-type cells reside mostly in G₂, it was difficult to distinguish the mitotically phosphorylated form of Fkh2 from the nonphosphorylated protein (data not shown). To enhance resolution, we arrested cells in early mitosis by using the cold-sensitive *nda3-KM311* mutant. Under these conditions, we were readily able to detect a band representing slower mobility in wt cells, corresponding to the phosphorylated form of Fkh2 (Fig. 5B). The pattern was similar to

FIG 3 Cdk8 physically and functionally interacts with transcription factor Fkh2. (A) Two-hybrid interaction between Cdk8 and Fkh2. β -Galactosidase (β -Gal) assays were performed in triplicate, and error bars represent standard deviations. The table displays the combinations of different plasmids used in two-hybrid analysis. As a positive control, we used Plo1 and Mbx1, which have previously been described as interacting (43). (B) The septation indices of the indicated mutants were followed in synchronized cell cultures. The delay of septation in the *cdk8-D158A* mutant was suppressed by the deletion of *fkh2*.

that obtained in the *cdc25-22* synchronization experiment shown in Fig. 4A (time points 10 and 20 min). Furthermore, in both the *fkh2-S322A S375A* (*fkh2-S2A*) and *fkh2-S322E S375E* (*fkh2-S2E*) mutant strains, the phosphorylated Fkh2 protein band was lost, demonstrating that S322 and S375 are bona fide phosphorylation sites *in vivo* (Fig. 5B). We also observed that the levels of Fkh2 were significantly reduced in the *fkh2-S2A* mutant strain, similar to what had been observed in the *cdk8-D158A* mutant. In contrast, the protein levels did not change significantly in the *fkh2-S2E* mutant.

Cdk8 regulates Fkh2 protein levels by preventing proteasome-dependent degradation. Sequence analysis using the epest-find algorithm revealed two putative PEST sequence motifs spanning the region in which Cdk8 phosphorylated Fkh2 (Fig. 5A), which we named PEST1 (score, 7.95) and PEST2 (score, 13.25). PEST sequences are often identified in rapidly degraded proteins and may thus represent signals for proteolytic degradation (48). Association of these sequence motifs with the Cdk8 phosphorylation sites in Fkh2 and the observed effects on protein levels in the *fkh2-S2A* mutant cells raised the possibility that Cdk8-dependent phosphorylation could control Fkh2 protein levels via the proteasome-dependent degradation pathway. In support of this notion, we found that Fkh2 was ubiquitinated and that the levels of this modification were increased in cells arrested before entry into the M phase (Fig. 5C). Therefore, the levels of Fkh2 ubiquitination were the highest when the Fkh2 protein levels were the lowest.

To investigate if the proteasome could affect Fkh2 protein levels, we used the *mts3-1* strain that harbored a temperature-sensitive mutation in a subunit of the 26S proteasome (25) and monitored Fkh2 protein level changes in both wild-type and *fkh2-S2A*

mutant cells. At the permissive temperature, the level of the Fkh2-S2A mutant protein was much lower than that of the wt Fkh2. After a shift to the restrictive temperature (36°C), we could observe an increase in the level of wt Fkh2. Interestingly, we could observe an even more dramatic increase in the nonphosphorylatable Fkh2-S2A mutant protein levels (Fig. 5D).

One of the 2 identified PEST domains (PEST2) included S375, which we had found to be phosphorylated by Cdk8. To investigate the functional importance of PEST2, we deleted a stretch of 8 amino acids, including S375 (Fig. 5A and E; Fkh2-PESTmut) by gene targeting at the native locus. This deletion stabilized Fkh2, and the protein levels were increased compared to those seen with the wild-type cells (Fig. 5E). Our findings thus suggest that the PEST2 domain is involved in the regulated degradation of Fkh2 and that the mitotic phosphorylation of Fkh2 by Cdk8 may inhibit this process.

Fkh2 phosphorylation controls the timing of mitotic entry via Wee1. We next investigated if changes in Fkh2 phosphorylation could influence the timing of mitotic entry. Interestingly, the *fkh2-S2A* cells were ~50% larger than the wt control cells at division, indicating delayed entry into mitosis (Fig. 6A and Table 2). We also found that the Cdk1-Tyr15 phosphorylation levels were higher in *fkh2-S2A* cells, further supporting the idea that the non-phosphorylatable Fkh2 mutant directly affected mitotic entry (Fig. 6B). We also analyzed the *fkh2-S2E* mutant strain. In this mutant, we observed cells dividing at reduced size (Fig. 6A and Table 2), suggesting premature entry into mitosis. Therefore, the *fkh2-S2A* and *fkh2-S2E* mutants had opposite effects on mitotic progression. For comparison, we also analyzed *fkh2-PESTmut* cells and found that these divided at a smaller size, similar to that of the *fkh2-S2E* mutant. Therefore, mutations disrupting the PEST domain or mimicking Cdk8 phosphorylation had similar effects on mitotic progression. By manipulating the phosphorylation status of Fkh2, we could either delay or advance cells through the cell cycle. The *fkh2-S2A* and *fkh2-S2E* mutations did not cause any other apparent morphological changes (Fig. 6A, data not shown).

In fission yeast, the Wee1 kinase and the Cdc25 phosphatase directly regulate Cdk1-Tyr15 phosphorylation and entry into mitosis. We wanted to genetically determine which of these two factors was influenced by Cdk8-dependent phosphorylation of Fkh2. We observed that the *fkh2-S2A* mutant caused a synthetic phenotype when combined with *cdc25-22*. The double mutant entered mitosis at what was already a much larger cell size at the permissive temperature (Table 2). Neither *fkh2-S2A* nor *cdk8-D158A* created synthetic phenotypes with *wee1-50*, which indicated that the Cdk8-Fkh2 pathway was signaling through Wee1 to control mitotic commitment. In agreement with this notion, the *fkh2-S2E* mutant suppressed the mitotic delay phenotype of *cdc25-22* at the permissive temperature but did not cause a synthetic phenotype when combined with the *wee1-50* mutation (Table 2).

Others have demonstrated that overexpression of *fkh2* causes lethality and delayed entry into mitosis (12). Interestingly, we found that inactivation of *wee1* suppressed the mitotic delay phenotype that was associated with *fkh2* overproduction, supporting the idea that Fkh2 controls mitotic entry via Wee1 (Fig. 7). Furthermore, overexpression of the nonphosphorylatable Fkh2-S2A protein could suppress the effect on the timing of mitotic entry, even if the mutations of serine to alanine failed to suppress the lethality caused by *fkh2* overproduction (data not shown).

FIG 4 Cdk8 phosphorylates Fkh2 *in vitro* and *in vivo*. (A) Periodic phosphorylation of Fkh2 during mitosis in synchronized wt and *cdk8-D158A* mutant cells. Fkh2-HA and actin were revealed by immunoblotting. Please note that the protein extracts were separated on one single SDS-PAGE gel, followed by immunoblotting on one membrane, thus allowing direct comparison of protein levels between wt and *cdk8-D158A* mutant cells. (B) The relative intensities of the phosphorylated and nonphosphorylated Fkh2 bands displayed in panel A. (C) Transcription of the *ace2* mitotic gene in wt and *cdk8-D158A* cells, measured by real-time PCR in the same synchrony experiment as described for panel A. (D) *In vitro* kinase assay performed with purified recombinant Fkh2. Mediator complexes containing Cdk8 (Cdk8/Med) or depleted of Cdk8 (Δ med13/Med) were incubated with recombinant Fkh2 in the presence of [γ - 32 P]ATP. The phosphorylated proteins were separated by SDS-polyacrylamide gel electrophoresis and identified by autoradiography (P32) or Coomassie staining (CBB). (E) *In vitro* kinase assay performed as described for panel B but with recombinant Fkh2 S322A. (F) *In vitro* kinase assay performed as described for panel B but with recombinant Fkh2 S375A.

Even if Fkh2 phosphorylation has a distinct effect on protein stability and mitotic entry, it is apparently not required for expression of genes regulated by the Fkh2 transcription factor. Microarray analysis demonstrated that the transcription of more than 600 genes was changed at least 2-fold in the *fkh2* deletion mutant, whereas the mutant strain carrying a nonphosphorylatable *fkh2*-S322A S375A mutation had only a minor impact on global gene transcription in nonsynchronized cells (Fig. 8). In a similar way, the effects of the *cdk8-D158A* mutation were mild in a nonsynchronized cell culture but delayed the timing of mitotic transcription by 30 min in synchronized cells (see above).

Fkh2 phosphorylation status affects promoter binding. We also investigated if promoter binding by Fkh2 was changed in the *cdk8-D158A* mutant strain. In wt cells, there is a drop in *ace2* promoter occupancy during the M phase, after which Fkh2 occupancy increases (43). In agreement with the lower levels of the Fkh2 protein in *cdk8-D158A* mutant cells, Fkh2 occupancy was decreased at the *ace2* promoter and the reappearance of Fkh2 after mitosis occurred more slowly than in wt cells (Fig. 9 A). We also

monitored whether the *fkh2-S2A* and *fkh2-S2E* mutants affected Fkh2 occupancy. Given the genetic interaction between *cdc25-22* and *fkh2-S2A* (Table 2), we had to synchronize the cells with *nda3-KM311*, which arrested the cells in early mitosis. In wt cells, Fkh2 binding was high before synchronization (mainly G₂) but dropped about 7.6-fold after arrest in early mitosis (Fig. 9 B). In the *fkh2-S2A* mutant strain, binding in the nonsynchronized cells was more than 10-fold lower than that observed in wt cells, and the occupancy was further decreased (5.8-fold) upon mitotic arrest. For the *fkh2-S2E* mutant, we also saw a decrease of Fkh2 binding in nonsynchronized cells, but the effect was less pronounced than that observed in the *fkh2-S2A* strain. Interestingly, arrest in mitosis caused only a less than 1.9-fold decrease in Fkh2 binding in the *fkh2-S2E* mutant cells. In fact, Fkh2 occupancy was even higher in the mitotically arrested *fkh2-S2E* mutant cells compared to the wt control. Therefore, the phosphorylation-mimicking mutations of S322 and S375 reduced the dynamic changes in Fkh2 promoter occupancy seen in wt cells, consistent with the observed effects on overall Fkh2 protein levels.

FIG 5 Cdk8 phosphorylates Fkh2 *in vivo* and controls protein stability via the proteasome. (A) Schematic representation of the Fkh2 protein, displaying the forkhead-associated (FHA) and the forkhead (FKH) domains. The sequence view shows the phosphorylated residues (asterisks) and the PEST sequence motifs that were identified using the epestfind algorithm. The boxed sequence was deleted in the *fkh2-PESTmut* strain. (B) Immunoblot analysis of Fkh2 arrested in early mitosis performed using the cold-sensitive β -tubulin mutant *nda3-KM311* mutation at 18°C. Antibodies against the HA tag were used to analyze levels of wt Fkh2, Fkh2-S2A, and Fkh2-S2E. Actin is visualized as a loading control. (C) Ubiquitylation of Fkh2 during mitosis. Fkh2-FLAG and untagged control (Ctr) cells were synchronized using *cdc25-22* block release, and samples were collected for FLAG immunoprecipitation (Ip) and probed with anti-FLAG and antiubiquitylation antibodies to detect Fkh2 and the ubiquitin modification. (D) Western blot analysis of Fkh2 or Fkh2-S2A in *mts3-1* mutant cells after incubation for 6 h at 30°C or 36°C. (E) Immunoblot analysis of Fkh2 protein levels in nonsynchronized wt and *fkh2-PESTmut* strains. Actin is shown as a loading control.

DISCUSSION

Periodic transcription of mitotic genes and the transition to mitosis must be coordinated events in eukaryotic cells. Exactly how this coordination takes place is not completely understood, but it is at least partially controlled by the classical mitotic kinases Polo and Cdk1 in yeast and by the Polo kinase Plk1 in higher eukaryotes (23, 38). Data presented here demonstrate that the Cdk8 kinase also plays an important role in the regulation of mitotic entry and the onset of mitotic transcription in fission yeast. Unlike Cdk1 or the Polo kinases, Cdk8 does not seem to be required for gene activation *per se*, since the levels of Mediator binding and mitotic gene transcription remain largely unchanged in the Cdk8 kinase mutant. This observation also explains the relative mild effects on mitotic genes observed in earlier microarray studies performed using nonsynchronized *cdk8-D158A* cells (30).

Instead, Cdk8 is needed for the correct timing of mitosis and inactivation of *cdk8* results in a concomitant delay in mitotic entry and transcription. The effects of Cdk8 on mitosis are at least partially mediated via the Fkh2 transcription factor. Fkh2 is a target for Cdk8, which specifically phosphorylates amino acids S322 and S375 both *in vivo* and *in vitro*. A nonphosphorylatable *fkh2* mutant in which the phosphorylated serines have been replaced by alanines mimics the phenotypes observed in the *cdk8-D158A* mutant strain. Furthermore, the periodic phosphorylation of Fkh2, which normally peaks during mitosis, is lost in the kinase-deficient *cdk8-D158A* mutant cells.

Interestingly, the Fkh2 orthologue in budding yeast, ScFkh2, is present at mitotic promoters all through the cell cycle (28). ScFkh2 is phosphorylated by Cdc28 (Cdk1) in a cell cycle-dependent manner, and the phosphorylation is required to stimulate

FIG 6 Fkh2 influences mitotic entry. (A) Microscopic images of wild-type, *fkh2-S322A S375A*, and *fkh2-S322E S375E* mutant cells. Nuclei and septa were stained. Delayed or advanced mitotic entry caused an increase or decrease of cell size at division in the mutants. Bar, 3.5 μ m. (B) Western analysis of Cdc2-Y15 levels in the indicated strains, asynchronously dividing at 25°C. The relative intensities of Cdc2-Y15 compared to the total Cdc2 protein levels are indicated under the panel.

FIG 7 Overexpression of *fkh2* in wt and *wee1-50* cells. Cells were transformed with a vector containing the *fkh2* gene under the control of the *nmt1* promoter or empty vector as a control. Cells were propagated overnight under induced or noninduced conditions at 25°C or 36°C. Bar, 5 μ m.

Fkh2 interactions with the Ndd1 coactivator, which in turn is required for periodic transcription of mitotic genes (27, 45, 46). In fission yeast, there is no apparent orthologue of Ndd1. In addition, Cdk8, rather than Cdk1, is responsible for Fkh2 phosphorylation during mitosis. Our findings thus suggest that, apart from the ability to bind mitotic promoters, the functions of Fkh2 are different in the two distantly related yeasts, reflecting the plasticity of the control mechanisms, while similar sets of proteins are still

utilized in both organisms. However, it is worth noticing that we were still able to observe lower levels of nonperiodic Fkh2 phosphorylation in the *cdk8-D158A* mutant strain. In contrast, the Fkh2-S2A mutation, which removed the phosphorylatable amino acid residues, completely lost the slower-migrating, phosphorylated protein species (Fig. 5B). This finding could suggest the existence of a second, partially redundant kinase.

Our data demonstrate that Cdk8 prevents proteasome-depen-

FIG 8 Microarray profile of *fkh2* Δ and *fkh2*-S322A S375A (S2A) mutants. (A) Scatter plot image of changes in gene expression. The two oblique lines represent a 2-fold cutoff. (B) Chart showing the number of genes affected in the *fkh2* Δ and *fkh2*-S2A mutants. (C and D) Venn diagrams showing total numbers representing overlap of the genes (C) and overlap of mitotic cluster genes (D) that changed more than 2-fold in the *fkh2* Δ and *fkh2*-S2A mutants.

FIG 9 Fkh2 phosphorylation status affects promoter binding. (A) ChIP analysis of Fkh2 binding to the *ace2* promoter during mitosis in synchronized wt and *cdk8-D158A* mutant cells. Samples were collected at the indicated time points. (B) ChIP analysis of Fkh2, Fkh2-S2A, and Fkh2-S2E binding to the *ace2* promoter. Cells were either nonsynchronized (NS) or arrested in early mitosis (M) by the use of the cold-sensitive β -tubulin mutant *nda3-KM311* mutation at 18°C.

dent Fkh2 degradation and thus regulates protein levels during cell cycle progression. The ability of Cdk8 to control the precise levels of a target protein is not without precedence. In mammalian cells, Cdk8 directly phosphorylates the SMAD and Notch transcription factors. Interestingly, PEST sequence motifs are also found in the linker region of the SMAD transcription factor, where it is targeted by Cdk8 during the canonical BMP and transforming growth factor beta (TGF- β) signaling (2). The phosphorylated SMAD is recognized by a ubiquitin ligase, leading to proteasome-mediated degradation of the phosphorylated protein. In a related way, phosphorylation of Notch also leads to ubiquitin-dependent degradation (22). The ability of Cdk8 to regulate SMAD and Notch protein turnover is important, since the precise levels of these transcription factors are crucial for their effects on cell fate determination (22). Our data also suggest that similar mechanisms controlling transcription programs with cell growth and division may also operate through the PEST2 domain-dependent protein degradation of Fkh2. An important difference between Fkh2 and the mammalian transcription factors SMAD and Notch is that Fkh2 phosphorylation inhibits interactions with proteins mediating proteasome-dependent degradation and thus stabilizes the protein. This finding thus represents a new role for Cdk8 and demonstrates that the kinase may both increase and decrease the turnover of protein targets.

Our genetic data suggest that the Cdk8-Fkh2 pathway signals through the Wee1 kinase to promote mitotic entry. How the control over Fkh2 protein turnover by phosphorylation affects Wee1 activity is currently unknown, but it is unlikely to operate through the control of gene expression of *cdc25* or *wee1*, as the levels of those transcripts remain unchanged in the nonphosphorylatable *fkh2* mutant. The coupling between the mitotic and transcription control machineries may in fact be mediated by physical interactions. In support of this idea, the anillin-like protein Mid1, which is involved in division plane positioning (3), has recently been shown to control the transcription of mitotic genes (1) and to interact *in vivo* with both Fkh2 and Wee1 (1, 37). In future studies, we plan to investigate whether Mid1 is part of a machinery that connects the transcription and mitotic control networks.

Cdk/cyclin activities drive the periodicity of eukaryotic cell cycle events (51). However, recent findings suggest that some cell cycle events, such as centrosome duplication, Cdc14-release oscillator, and periodic transcription in budding yeast, can occur independently of the Cdk/cyclin oscillator (32, 41). There must therefore be mecha-

nisms that couple the Cdk oscillator to the transcription network oscillator in order to ensure coordination of these events and correct cell cycle progression; i.e., mitotic transcription must be coordinated with mitotic entry and vice versa. Our data suggest that the transcription regulator Cdk8/Mediator complex may at least partially control the Cdk oscillator in fission yeast. However, even if this conclusion could explain many of the findings described here, much more experimental work is required to establish a role for the Cdk8/Mediator as a transcriptional network oscillator and to determine the molecular mechanisms of this process.

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