The Basic Leucine Zipper Domain Transcription Factor Atf1 Directly Controls Cdc13 Expression and Regulates Mitotic Entry Independently of Wee1 and Cdc25 in \textit{Schizosaccharomyces pombe}

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Progression into mitosis is a major point of regulation in the \textit{Schizosaccharomyces pombe} cell cycle, and its proper control is essential for maintenance of genomic stability. Investigation of the G2/M progression event in \textit{S. pombe} has revealed the existence of a complex regulatory process that is responsible for making the decision to enter mitosis. Newer aspects of this regulation are still being revealed. In this paper, we report the discovery of a novel mode of regulation of G2/M progression in \textit{S. pombe}. We show that the mitogen-activated protein kinase (MAPK)-regulated transcription factor Atf1 is a regulator of Cdc13 (mitotic cyclin) transcription and is therefore a prominent player in the regulation of mitosis in \textit{S. pombe}. We have used genetic approaches to study the effect of overexpression or deletion of Atf1 on the cell length and G2/M progression of \textit{S. pombe} cells. Our results clearly show that Atf1 overexpression accelerates mitosis, leading to an accumulation of cells with shorter lengths. The previously known major regulators of entry into mitosis are the Cdc25 phosphatase and the Wee1 kinase, which modulate cyclin-dependent kinase (CDK) activity. The significantly striking aspect of our discovery is that Atf1-mediated G2/M progression is independent of both Cdc25 and Wee1. We have shown that Atf1 binds to the Cdc13 promoter, leading to activation of Cdc13 expression. This leads to enhanced nuclear localization of CDK Cdc2, thereby promoting the G2/M transition.

The mammalian basic leucine zipper domain (bZIP) family transcription factor ATF2 is known to be associated with multiple cellular processes, including stress responses, DNA damage responses, and cell cycle regulation. \textit{Schizosaccharomyces pombe} has a well-characterized ATF2 homolog (Atf1) with functions similar to those of the human ATF2 protein (1–4). It is important for heterochromatin formation and meiotic recombination. Atf1 has also been shown to influence some very important events during \textit{S. pombe} cell division. In \textit{S. pombe}, Atf1 was first isolated as the suppressor of the \textit{asp1} phenotype (1). Asp1 is the major mitogen-activated protein kinase (MAPK) in \textit{S. pombe} and is the homolog of mammalian p38MAPK. Atf1 has also been implicated in many important stages of cell cycle control in \textit{S. pombe}. Atf1 is known to be associated with activation of the spindle orientation checkpoint (5) that controls the metaphase-to-anaphase transition and activation of the anaphase-promoting complex (APC) leading to mitotic exit. It has a synthetic lethal interaction with Cut1 (6). Atf1 is also necessary for accumulation of cells in G1 after nitrogen starvation (1). It has been shown to be important for degradation of the mitotic cyclin Cdc13 by activating the APC/cyclosome (APC/C) ubiquitin ligase (7).

The major point of regulation of the \textit{S. pombe} cell cycle is the transition from G2 phase into mitosis. This transition is dependent on the activity of the cyclin-dependent kinase (CDK) Cdc2. The known important regulators of Cdc2 activity in \textit{S. pombe} are the Wee1 kinase and the Cdc25 dual-specificity phosphatase (8–10). The former inhibits Cdc2 activity by phosphorylating it at Y15, while the latter activates Cdc2 by removing this inhibitory phosphorylation. The regulation of Cdc2 activity, however, is influenced by a host of cellular factors, especially by the MAPK pathway. Spc1 and Cdc25 have been shown to have a synthetic lethal interaction (11). There is evidence for the MAPK pathway being involved in Cdc25 regulation, spindle orientation checkpoint activation, and chromosome segregation (6, 12–14). Clearly, multiple layers of cross talk exist between the MAPK pathway and the factors controlling cell division in \textit{S. pombe}. However, a comprehensive picture of these interconnections is still elusive. We were particularly interested in a systematic investigation of the multiple modes of cell cycle regulation by the MAPK pathway in \textit{S. pombe}. The importance of Atf1 in this regard has not been investigated in detail. ATF2 (the human homolog of Atf1) is known to exhibit both tumor suppressor and oncogenic properties (15–17). ATF2 has also been shown to be regulated by ATM in response to DNA damage (18). The human homologs of both Atf1 and Spc1 (ATF2 and p38, respectively) have emerged as prominent target molecules for cancer therapy. Modulation of ATF2 activity, in fact, holds great promise as a therapeutic strategy against cancer. However, the existing information regarding Atf1 in \textit{S. pombe} (or ATF2 in mammalian systems) is far from comprehensive. Detailed investigation of the role of Atf1 in regulating the \textit{S. pombe} cell cycle shall benefit the development of therapeutic strategies. Therefore, we chose Atf1 for our studies. The aim of the study was to screen for newer modes of regulation of the cell cycle by Atf1. In this report, we present data that suggest novel roles for Atf1 in regulating and promoting the G2/M transition. The striking and unexpected feature of this mode of regulation, as we clearly show, is...
that it is independent of both Cdc25 and Wee1. We show that Atf1 can regulate the expression of Cdc13 and can thus indirectly target the activity of S. pombe cyclin-dependent kinase. These results are distinct from the previously characterized functions of Atf1.

**MATERIALS AND METHODS**

**Fission yeast strains, media, and growth conditions.** *S. pombe* strains used in this study are listed in Table 1. Cells were grown as described by Moreno et al. (19). All cells were grown at 30°C in yeast extract with supplements (YES) medium unless indicated otherwise. For overexpression experiments, cells were grown overnight in Eagle’s modified medium (EMM)-Leu supplemented with 20 μM thiamine, harvested, washed, and resuspended in EMM-Leu, and incubated for another 24 h at 30°C.

**Microscopy.** *S. pombe* cells were grown as indicated and fixed with 70% ethanol after harvesting. They were rehydrated and examined using an Olympus BX51 fluorescence microscope at a magnification of 40× unless mentioned otherwise. Fission yeast nuclei were stained with 2 μg/ml DAPI (4’-6-diamidino-2-phenylindole). Bright-field images were taken using unstained cells. All images were taken and processed with the use of identical parameters. Cell length analysis was done using ImageJ software (20).

**Viability assays.** Cells were first grown to saturation and then normalized by measurement of absorbance at 595 nm. Ten-fold serial dilutions were then made, and 5 μl was spotted onto the indicated plates. Plates were then incubated at the indicated temperatures for 4 days before being photographed.

**S. pombe transformations.** One milliliter of an overnight *S. pombe* culture in YES was harvested and then resuspended in 0.5 ml PEGLLET (10 mM Tris [pH 8], 1 mM EDTA, 0.1 M lithium acetate, 40% polyethylene glycol [PEG]). Five micro liters of denatured salmon sperm DNA (10 mg/ml) was added to it. One microgram of the purified plasmid DNA was then added to this mixture and allowed to stand overnight at room temperature, after which the cells were resuspended in 150 μl YES and spread onto appropriate selection plates.

**Cloning of Atf1bZIPΔ.** The region from bp 1 to 1410 of the Atf1 gene was amplified using the following primers and cloned between the Ndel and Smal sites of the pREP41 vector: forward, ATTACATAGTGGCCG TCTCCCGT; reverse, ATATCGGGGTATTTTGGAAAT.

**RNA isolation and real-time PCR.** Total cellular yeast RNA was isolated from the samples after lysing the cells using RNA extraction buffer (1 M Tris [pH 8], 0.5 M EDTA [pH 8], 4 M NaCl), phenol-chloroform-isooamyl alcohol (25:24:1), and glass beads. It was followed by phenol-chloroform extraction and DNAseI (Thermo Scientific) treatment. About 1 μl of the isolated RNA was converted to cDNA using reverse transcriptase (Moloney murine leukemia virus [M-MuLV] reverse transcriptase; Thermo Scientific). Real-time PCR was performed in an Applied Biosystems Real Time Fast 7500 instrument using SYBR green reagent (Applied Biosystems). Melt curve analysis was done to confirm the absence of primer dimers and nonspecific amplification products. Primers used for real-time PCR are as follows: Cdc13, forward, GGATGACTACCCCGTGC TTAAAAC, and reverse, TGGAAAGACACAGTGGCTTTCTT; 18S rRNA, forward, TGTCATGGAACCTGGCAATGCTC, and reverse, GCAAGCCATGGATGCTCC.

**Preparation of denatured cell extracts and immunoblotting.** Cell pellets were resuspended in 20% trichloroacetic acid (TCA) and vortexed at maximum speed for five 1-min pulses after adding glass beads. The solution was then transferred to a fresh microcentrifuge tube (to remove the glass beads) and then centrifuged at 13,000 rpm for 15 mins. All steps were done at 4°C, and samples were kept in ice. The pellet was then washed thrice with 70% ethanol, air-dried, and resuspended in 5× SDS-PAGE loading buffer. The samples were boiled at 100°C for 5 min before loading onto 12% SDS-polyacrylamide gels. After transferring onto polyvinylidene difluoride (PVDF) membranes, immunoblotting was done using anti-Cdc13 antibodies (ab10873; Abcam) at 1:1,000 dilutions. Immunoblots were developed using Lumiglo reagent (Cell Signaling number 7003).

**ChIP analysis.** Chromatin immunoprecipitation (ChIP) assays for testing the binding of Atf1 were done as described before (21) with minor modifications. Briefly, 200 ml of exponentially growing cells at an optical density (OD) of 0.6 was treated with 3% formaldehyde for cross-linking and incubated at 25°C for 30 min, followed by treatment with 125 mM glycine for 10 min at 25°C. The cells were then harvested, 400 μl of lysis buffer (50 mM HEPES–NaOH [pH 7.5], 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 50 mM sodium fluoride, 0.1 M sodium vanadate, 1% SDS, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride [PMSF], protease inhibitor cocktail) was added along with glass beads, and the cells were lysed by vortexing at 4°C. The lysates were then collected and sonicated at an amplitude of 50% for 15 min with 30-s on-off cycles to get fragments of 500 to 1,000 bp. The lysates were centrifuged at 15,000 rpm for 5 min, and the supernatant was carefully collected. Around 20 μl of the lysate was stored separately as the input control (whole-cell extracts before immunoprecipitation [WCE]). To the remaining lysate, anti-hemagglutinin (HA) antibody (1:100) was added, and the lysates were nuted for 4 h. Thirty microliters of 50% slurry of protein A Sepharose was then added. The samples were then nuted for another 6 h at 4°C. The beads were washed successively with lysis buffer, lysis buffer containing 0.5 M NaCl, wash buffer (10 mM Tris [pH 8], 250 mM LiCl, 0.5% sodium deoxycholate, 1 mM EDTA), and finally with Tris-EDTA (TE), pH 8.0. After the last wash, the beads were resuspended in STE (10 mM Tris [pH 8], 1 mM EDTA, 150 mM NaCl) and incubated at 65°C for 10 min. Beads were allowed to settle, and the supernatants were collected. The process was repeated twice. These supernatants and the STE collected previously were all incubated at 65°C for another 6 h. Samples were then cooled to room temperature and treated with proteinase K (final concentration, 0.5 mg/ml) at 37°C for 2 h. Extraction with phenol-chloroform-isooamyl alcohol (25:24:1) was done, and nucleic acids were precipitated using 1/10 volume of 3 M NaOAc, pH 5.5, 0.5 μl glycogen (20 mg/ml), and 2.25 volumes of ethanol. This was followed by RNase treatment and reprecipitation.

**Table 1** Strains and plasmids used in this study

| Strain or plasmid no. | Relevant genotype or description | Source |
|-----------------------|---------------------------------|--------|
| GS001                 | h-leu1–32 ura4–D18              | Paul Russell |
| GS027                 | h-atf1::ura4+ leu1–32          | Kazuhiro Shiozaki |
| GS017                 | h-leu1::lacZ50                 | Yeast Genetic Resource Centre |
| GS048                 | h-leu1–32 ura4–D18 cdc25–22    | Paul Russell |
| GS117                 | h-leu1–32 ura4–D18 atf1::ura4   | Paul Russell |
| GS185                 | h-cdc2GFP::nat leu1–32 ura4–D18 ade6–M210 | Francisco J. Navarro |
| GS224                 | h-leu1–32 ura4–D18 atf1Ata4Hisura4+ | Paul Russell |
| GS041                 | h-leu1 cdc2-3w                  | Yeast Genetic Resource Centre |
| GS046                 | h-leu1 cdc2-1w                  | Yeast Genetic Resource Centre |
| GS044                 | h-leu1 cdc2-33                  | Yeast Genetic Resource Centre |
| GS192                 | h-cdc13–117 leu1–32 ura4–D18 ade6–M210 | Kathy Gould |
| pGS017                | pREP41                          | Yeast Genetic Resource Centre |
| pGS018                | pREP41+Atf1                     | Elizabeth A. Veal |
| pGS045                | pREP41+Atf1bZIPΔ                | This study |
| pGS042                | pREP1+Cdc13                     | Kathy Gould |

**TABLE 1** Strains and plasmids used in this study
RESULTS AND DISCUSSION

Atf1 is important for mitotic entry in *S. pombe*. While screening for a regulatory relationship between Atf1 and the major cell cycle regulatory molecules in *S. pombe*, we found that deletion of Atf1 exaggerates the temperature sensitivity of the *cdc25-22* ts mutant (Fig. 1A). However, the overexpression of Atf1 was not able to rescue the temperature sensitivity of the *cdc25-22* ts mutant. In order to investigate the physiological relevance of these findings, we looked at the phenotypes of these cells at both the permissive (25°C) and restrictive (37°C) temperatures. As expected, the *cdc25-22* cells transformed with the empty vector were arrested at the G2/M boundary and became elongated at 37°C. Surprisingly, the cells overexpressing Atf1 did not show any phenotypic evidence of a G2 arrest and had similar cell lengths at both 25°C and 37°C (Fig. 1B). The cells transformed with the empty vector had average cell lengths of 15.7 μm and 33.3 μm at 25°C and 37°C, respectively, while the ones overexpressing Atf1 had average cell lengths of 15.6 μm and 15.5 μm at 25°C and 37°C, respectively (Fig. 1C).

The *cdc25-22Δatf1* cells, however, had an average cell length that was slightly higher than that of the *cdc25-22* cells at 37°C (Fig. 1B). The length of *cdc25-22Δatf1* cells changed from 16.1 μm at the permissive temperature to 38.7 μm at the nonpermissive temperature. The change for the *cdc25-22* cells was from 16.5 μm to 34.8 μm. We then took exponentially growing cultures of both *cdc25-22* and *cdc25-22Δatf1* cells at 25°C and shifted them to 37°C for 4 h before returning them back to the permissive temperature of 25°C. We followed the growth of both these mutants and found that the *cdc25-22Δatf1* cells exhibited a significant delay in resuming cell division after being released to the permissive temperature (Fig. 2A), as indicated by their lower growth rate. These results show that Atf1 is important for resuming mitotic entry in these cells. We argued that if deletion of Atf1 slows down the entry into mitosis, the opposite, i.e., overexpression of Atf1, should accelerate entry into mitosis. We therefore overexpressed Atf1 for 24 h in *cdc25-22* cells growing at 25°C. We then shifted the cultures of *cdc25-22* transformed with either the empty vector (φ) or pREP41-Atf1 from 25°C to 37°C for 4 h. During these 4 h, the *cdc25-22* cells were arrested at the G2/M boundary and did not exhibit any significant increase in cell number. However, the cells overexpressing Atf1 continued to divide and exhibited similar growth rates at both 25°C and 37°C (Fig. 2B).

All these results clearly show that Atf1 can promote mitotic entry.
entry in *S. pombe* and can do so independently of Cdc25. This indicates the presence of a parallel Atf1-dependent pathway for regulating G2/M transition in *S. pombe*.

Our results showed that even though Atf1 overexpression rescued the *cdc25-22* cells from a G2 arrest, it failed to rescue the temperature sensitivity of these mutants. This indicated that the mitotic acceleration achieved through enhanced Atf1 levels could not give any survival advantage to the cells. It therefore seemed possible that these cells were entering into mitosis aberrantly. Indeed, we found that the *cdc25-22* cells overexpressing Atf1 had abnormal nuclear morphology at 37°C (Fig. 2C). These cells show abnormal septation and unequal chromosome segregation. This clearly shows that although Atf1 can promote mitotic entry, it does not have the ability to communicate with the cellular machinery responsible for ensuring proper timing of mitosis. The resulting aberrant mitotic entry therefore leads to mitotic catastrophe, and thus the cells do not gain any survival advantage in spite of being rescued from the G2 block.

**Atf1 targets CDK activity independently of Wee1 in order to regulate G2/M transition in *S. pombe*.** We then tried to investigate the mechanism of Atf1-mediated mitotic acceleration. Since we found its function to be independent of Cdc25, we investigated the other possibility that Atf1 promotes mitotic entry by inhibiting Wee1, the CDK inhibitory kinase. To investigate this possibility, we overexpressed Atf1 in *wee1-50* mutant cells. These cells harbor a temperature-sensitive allele of *wee1*, the permissive and nonpermissive temperatures for the same being 25°C and 37°C, respectively. We found that Atf1 overexpression exaggerates the temperature-sensitive phenotype of *wee1-50* cells (Fig. 3A). Consistent with our hypothesis, this suggests that Wee1 and Atf1 have antagonistic roles. It also suggests that Atf1 does not target CDK activity through Wee1 and that its effect on mitotic entry is independent of the presence of a functional Wee1 as well. To further confirm this, we shifted the growing cultures of *wee1-50* cells over-
expressing either the empty vector (φ) or Atf1 from 25°C to 37°C and monitored their growth rate. As expected, inactivation of Wee1 (at the nonpermissive temperature) resulted in acceleration of growth. However, the cells overexpressing Atf1 exhibited a higher increase in growth rate than the empty vector controls (Fig. 3B). This shows that Atf1 overexpression and Wee1 inactivation have additive effects on the increase in the growth rate of S. pombe cells. Thus, Wee1 is not targeted by Atf1. In other words, Atf1-mediated regulation of mitotic entry is independent of Wee1. Wee1 and Cdc25 are the major regulators of Cdc2 (CDK) activity during mitotic transition. Since we found that Atf1 can function independently of both these regulators, it seemed possible that Atf1 may target the regulation of Cdc2 through a separate, parallel pathway. To test this possibility, we use the cdc2.33 ts mutant cells. These cells carry a temperature-sensitive allele of Cdc2. When incubated at the nonpermissive temperature of 37°C, these cells are arrested at either the G2/M or the G1/S boundary, depending upon which phase of the cell cycle they were in when the temperature was elevated to nonpermissive levels. Since S. pombe cells spend almost 70% of their time in G2, the cdc2.33 mutants show a predominantly elongated phenotype characteristic of a G2/M arrest at the nonpermissive temperature. We argued that if Atf1 does target Cdc2, then there should not be any phenotypic manifestation of Atf1 overexpression in cdc2.33 cells at 37°C, and that is exactly what was observed in our experiments. Exponentially growing cultures of cdc2.33 cells overexpressing either the empty vector (φ) or Atf1 were shifted from 25°C to 37°C for 4 h and observed microscopically. We found that both the cells were arrested predominantly at the G2/M boundary, showing an increase in cell length (Fig. 4A). Thus, a functional Cdc2 protein is absolutely essential for Atf1-mediated acceleration of mitotic entry. This is also supported by our observation that Atf1 had no accelerating effect on the growth rate of cdc2.33 cells at the nonpermissive temperature (Fig. 5D). This growth acceleration, however, was not abolished in cells harboring Wee1 and Cdc25-independent mutant alleles of Cdc2 (cdc2-1w and cdc2-3w, respectively [Fig. 5]). These cells showed changes in growth rate similar to those of the wild-type (wt) cells.

We then checked the phenotype resulting from Atf1 overexpression in cdc13-117 ts mutants. Cdc13 is a B-type cyclin and forms a complex with Cdc2, and this complex formation is essential for activating Cdc2 and promoting mitosis in S. pombe. Hence, at the nonpermissive temperature these mutants are also arrested at G2/M boundary due to the absence of a functional Cdc2-Cdc13 complex. Atf1 overexpression could not rescue these mutants from the G2 arrest, and they exhibited elongated phenotypes irrespective of the presence or absence of Atf1 overexpression (Fig. 4B).

All these results suggest that Atf1 targets the activation of Cdc2 to accelerate mitosis.

Atf1 overexpression leads to increased Cdc13 expression, which is responsible for mitotic acceleration. We then investigated the mechanisms by which Atf1 may regulate or promote Cdc2 activity in S. pombe cells. We overexpressed Atf1 in wt cells carrying a green fluorescent protein (GFP)-tagged genomic copy of Cdc2. As expected, Atf1 overexpression shortened the average length of the wt cells from 14 μm to 11.9 μm. We looked at the Cdc2 nuclear localization phenotype of these cells and found that a larger population of cells overexpressing Atf1 (42%) exhibited nuclear localization (Fig. 6A) than those transformed with the empty vector (35%). Thus, Atf1 was somehow enhancing the extent of nuclear localization of Cdc2. This probably led to the observed accelerated mitotic entry in these cells. The nuclear import of Cdc2 is regulated mainly by its binding partner Cdc13 (cyclin). The enhanced localization of Cdc2 inside the nucleus is reminiscent of the phenotype seen for cells that can maintain high levels of Cdc13 (22). Hence, it seemed possible that either Atf1 stabilized the Cdc2-Cdc13 complex or it increased the Cdc13 levels in the cell. Both these events could eventually lead to enhanced nuclear localization of Cdc2 and acceleration of mitosis. Hence, we investigated whether Atf1 overexpression could regulate G2/M transition by elevating Cdc13 expression in S. pombe cells. Indeed, we found that wild-type S. pombe cells overexpressing Atf1 showed increased levels of the Cdc13 transcript while Δatf1/cdc25-22 cells that had been synchronized at the G2/M boundary showed decreased Cdc13 expression compared to cdc25-22 cells under similar conditions (Fig. 6B). Real-time PCR analysis showed that Atf1 overexpression led to a 5-fold induction in the level of Cdc13 transcription while its deletion led to a dramatic 50% decrease in Cdc13 expression. We also checked the levels of the Cdc13 protein.
found that the increase in transcript levels was also manifested as increased Cdc13 protein levels (Fig. 6C). It is interesting that Atf1 has earlier been shown to be important for Cdc13 degradation and mitotic exit. This function, however, has been shown to be independent of its activity as a transcription factor, and a truncated version of Atf1 lacking the C-terminal bZIP domain (important for DNA binding and dimerization) has been shown to be fully capable of triggering Cdc13 degradation via activation of the anaphase-promoting complex. We expressed the Atf1bZIPΔ mutant in cdc25-22 cells. The Atf1bZIPΔ mutant protein failed to rescue the cells from a G2 arrest (Fig. 6D). This was not surprising, as we have shown that transcriptional activity of Atf1 is responsible for the observed mitotic acceleration.

In order to validate that the increase in Cdc13 levels was indeed the reason for the phenotypes observed in our initial experiments, we checked the phenotypes of both cdc25-22 and cdc2.33 cells overexpressing Cdc13. These cells exhibited phenotypes indistinguishable from those observed for Atf1 overexpression (Fig. 7A). Moreover, overexpression of Cdc13 also rescued the temperature sensitivity of Δatf1cdc25-22 cells, further proving that a delay in mitotic entry was responsible for their decreased viability (Fig. 7B). Interestingly, overexpression of Cdc13 was found to be toxic for the cdc25-22 mutant even at 25°C. When grown in liquid cultures, the cdc25-22 cells transformed with Cdc13 lose viability after 4 or 5 generations, while the Δatf1cdc25-22 cells continue to grow at 33°C even after repeated passages (our unpublished data). The Δatf1cdc25-22 cells have low levels of Cdc13, and this gives rise to their exaggerated ts phenotype compared with the cdc25-22 mutant alone (Fig. 1A). When this deficiency is corrected by supplying additional Cdc13, the double mutant is rescued from the “enhanced” temperature sensitivity (Fig. 7B). This happens because the synthetically supplied Cdc13 compensates for the inability of these cells to enter into mitosis efficiently. However, the cdc25-22 mutants have no defect in Cdc13 accumulation and a synthetic increase in Cdc13 levels leads to aberrant mitosis and kills these cells. It is also possible that the presence of Atf1 enhances the stability of the Cdc2-Cdc13 complex in addition to elevating the cellular levels of Cdc13. This could also contribute to the inability of cdc25-22 mutants to tolerate increased Cdc13 levels.

The above set of experiments clearly show that Atf1 can regulate mitotic entry by enhancing Cdc13 expression. The increased levels of Cdc13 would lead to an increase in the levels of the active Cdc2-Cdc13 complex, thereby leading to an acceleration of mitotic entry. However, the surprising aspect of CDK regulation that these experiments bring to light is that a simple increase in Cdc2-Cdc13 concentrations can completely bypass the need for removal of the Y15 inhibitory phosphorylation of Cdc2 by Cdc25. We believe that the increase in Cdc2-Cdc13 concentrations stabilizes the interactions between the Cdc2 kinase and its substrates. As a result, the inhibitory effect of Y15 phosphorylation on these kinase-substrate interactions can be overcome. This can then lead to acceleration of mitotic entry even in the absence of Cdc25 activity.

Atf1 directly binds to Cdc13 promoter and enhances Cdc13 transcription. Although the timing of Cdc13 expression and degradation during the cell cycle of S. pombe is well known, very little
information is available about the regulators involved in Cdc13 expression. We wanted to determine whether the transcription factor Atf1 is directly responsible for activating Cdc13 transcription or if it does so by influencing the expression of some other transcription factor, which may in turn regulate Cdc13 transcription. For this, we did chromatin immunoprecipitation experiments using a strain carrying a HA-6His-tagged copy of Atf1. The location of the target regions (upstream of Cdc13 transcription

FIG 6 Atf1 overexpression leads to enhanced nuclear localization of Cdc2 through activation of Cdc13 transcription. (A) Images of wild-type S. pombe cells having a GFP-tagged genomic copy of Cdc2 and overexpressing Atf1 were taken. At least 100 cells were counted to determine the extent of nuclear localization of Cdc2. Bar, 10 μm. Representative images of 3 independent experiments are shown. (B) Real-time PCR analysis of Cdc13 expression in wild-type cells overexpressing Atf1 at 30°C and in cdc25-22 and Δatf1cdc25-22 cells (synchronized at the G2/M boundary). 18S rRNA expression was used for normalization. Data represent means of three independent experiments. Statistical analysis was done using the Graph Pad Prism application. *, P < 0.05. (C) Atf1 was overexpressed in wild-type S. pombe cells, and the levels of the Cdc13 protein were detected by immunoblotting. (D) Bright-field images of cdc25-22 cells overexpressing Atf1bZIPΔ after incubation at the indicated temperatures for 4 h. Bar, 10 μm. Representative images of 3 independent experiments are shown.

FIG 7 Increase in Cdc13 expression can rescue the temperature sensitivity of Δatf1cdc25-22 cells. (A) Bright-field images of cdc2.33 and cdc25-22 cells overexpressing Cdc3 after incubation at the indicated temperatures for 4 h. Bar, 10 μm. (B) cdc25-22 and Δatf1cdc25-22 cells transformed with pREP41 (φ) or pREP41+Cdc13 were grown to log phase in the presence of thiamine, and then serial dilutions were spotted onto EMM-Leu plates with or without thiamine. The plates were incubated at the indicated temperatures for 4 days before being photographed. Representative images of 3 independent experiments are shown.

FIG 8 Chromatin immunoprecipitation analysis of Atf1-HA bound to Cdc13 promoter. (A) Location of the regions R1 and R2 on the genomic regions upstream of Cdc13 transcription start site (+1). These regions were tested for association with Atf1 using ChIP assays. (B) Exponentially growing wild-type S. pombe cells (no tag) or cells carrying a HA-6His-tagged genomic copy of Atf1 were harvested and lysed after formaldehyde cross-linking, and chromatin bound to Atf1-HA was obtained after immunoprecipitation with anti-HA antibodies (α-HA-IP). Recovered DNA was analyzed by PCR amplification with primers designed against the regions R1 and R2. WCE, whole-cell extracts before immunoprecipitation; -ve, no-template control for PCR.
start site) is shown schematically in Fig. 8A. Our results clearly show that the Atf1 protein does bind upstream of the Cdc13 promoter and the region R2 was always associated with the protein (Fig. 8B). An internal control for the experiments is represented by the region R1, which was never found to be associated with the Atf1 protein. Thus, Atf1 directly binds upstream of the Cdc13 promoter and activates Cdc13 transcription.

Based on these findings, we propose a model for the regulation of mitotic entry by Atf1, which is summarized in Fig. 9. Increase in Atf1 levels leads to a concomitant increase in Cdc13 levels. This results in the formation of larger amounts of the Cdc2-Cdc13 complex, which then translocates to the nucleus. This increase in the nuclear concentration of the Cdc2-Cdc13 kinase leads to accelerated mitotic entry. This event is completely independent of the activity of Cdc25, and so the cells can progress into mitosis even in the absence of Cdc25 when the Cdc13 levels increase in an Atf1-dependent manner.

Our findings together with those of Ors et al. (7) also bring to light a novel paradigm for coordination of mitotic entry and exit in S. pombe cells, whereby the same protein (Atf1) controls both mitotic entry and exit in S. pombe simply by controlling both the expression and degradation of the mitotic cyclin Cdc13. These contrasting functions are associated with separate domains of the Atf1 protein. The C-terminal bZIP domain is absolutely essential for Cdc13 expression. The N-terminal domain, on the other hand, is responsible for Cdc13 degradation. These findings also indicate that the cell must have a mechanism for the temporal regulation of substrate binding by these domains during both mitotic entry and exit so that the relevant functions predominate at appropriate times during mitosis.

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