Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast

Kazuhiro Shiozaki and Paul Russell¹

Departments of Molecular Biology and Cell Biology, The Scripps Research Institute, La Jolla, California 92037 USA

The stress-activated Wis1–Spc1 protein kinase cascade links mitotic control with environmental signals in *Schizosaccharomyces pombe*. Fission yeast *spc1*[−] mutants are delayed in G₂ during normal growth and undergo G₂ arrest when exposed to osmotic or oxidative stress. Here we report that Spc1 also has an important role in regulating sexual development in *S. pombe*. This discovery arose from the observation that Spc1 is activated in response to nitrogen limitation, a key signal that promotes conjugation in fission yeast. Mutant *spc1*[−] cells are defective at arresting in G₁ during nitrogen starvation and exhibit a poor mating ability. These deficiencies correlate with a failure to induce transcription of *ste11*⁺, a gene that encodes a transcription factor responsible for expression of various meiotic genes. Two genes, *atf1*⁺ and *atf21*⁺, were cloned as multicopy suppressors of the *spc1*[−] mating defect. Atf1 and Atf21 are bZIP transcription factors that are most closely related to human ATF-2/CRE-BP1. Spc1 is required for stress-induced phosphorylation of Atf1. Atf1 is required for induction of meiotic genes and stress-response genes, such as *gpd1*⁺ and *pyp2*⁺, that are transcriptionally regulated by Spc1. *atf1*[−] and *spc1*[−] mutants are sensitive to osmotic stress and impaired for sexual development, showing that fission yeast uses a common pathway to respond to cytotoxic stress and nitrogen starvation. However, unlike *spc1*[−] mutants, *atf1*[−] cells have no mitotic cell-cycle defect, indicating that the stress response pathway bifurcates at Spc1 to regulate independently meiosis and mitosis.

[Key Words: ATF-2; meiosis; *Schizosaccharomyces pombe*; stress-activated MAP kinase]

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Studies of *Schizosaccharomyces pombe* have played an instrumental role in uncovering how eukaryotic cells regulate the initiation of mitosis. Cdc2, the cyclin-dependent kinase that is the focus of this control, is maintained in an inhibited state during S and G₂ attributable to phosphorylation of tyrosine-15 carried out by Wee1 and Mik1 kinases (Dunphy 1994). Cdc2 is activated by Cdc25 tyrosine phosphatase to bring about the onset of mitosis. A major aim of recent studies has been to discover how the mitotic control is linked to checkpoint mechanisms that monitor genome replication and integrity, but recently it has become apparent that fission yeast also links the mitotic control with changes in the extracellular environment that affect cell physiology. A key element of this signal transduction pathway is Spc1, a mitogen-activated protein (MAP) kinase homolog that is also known as Sty1 (Millar et al. 1995; Shiozaki and Russell 1995). At optimal growth conditions Spc1 has a

basal activity that is important for determining the timing of mitosis, as shown by the fact that *spc1*[−] cells are ~20% elongated at division compared with wild type. A *spc1*[−] mutation causes G₂ arrest in cells that have a partial loss of Cdc25 activity, confirming that Spc1 activity affects the mitotic control (Millar et al. 1995; Shiozaki and Russell 1995).

Spc1 becomes highly activated in response to various forms of cytotoxic stress, including high osmolarity, oxidative stress, and high temperature (Millar et al. 1995; Shiozaki and Russell 1995; Degols et al. 1996). Failure to activate Spc1 during stress leads to G₂ arrest and cell death. Spc1 is most closely related to a conserved family of stress-activated protein kinases that includes budding yeast Hog1p (Brewster et al. 1993), *Xenopus* Mpk2 (Rouse et al. 1994), murine p38 (Han et al. 1994), and human CSBP1 (Lee et al. 1994). These kinases have a Thr-Gly-Tyr sequence in the region of activating phosphorylations, whereas other members of the MAP kinase family have Thr-Glu-Tyr or Thr-Pro-Tyr at corresponding positions. The MAPK kinase homolog Wis1 (Warbrick and Fantes 1991) activates Spc1 by phosphorylating

¹Corresponding author.

the threonine and tyrosine residues of the Thr-Gly-Tyr motif (Shiozaki and Russell 1995). Inactivation of Spc1 is carried out by tyrosine-specific phosphatases Pyp1 and Pyp2 (Millar et al. 1995; Shiozaki and Russell 1995; Degols et al. 1996), unlike other MAP kinase phosphatases that dephosphorylate both the threonine and tyrosine residues (Alessi et al. 1993; Sun et al. 1993; Doi et al. 1994).

Recent studies have shown that Spc1 is required for the stress-stimulated transcription of several stress response genes, including those encoding glycerol-3-phosphate dehydrogenase (*gpd1*⁺) and trehalose-6-phosphate synthase (*tps1*⁺) (Degols et al. 1996). Activation of Spc1 also leads to expression of the *pyp2*⁺ gene encoding one of tyrosine phosphatases that regulates Spc1 negatively, indicating the existence of negative feedback loop to attenuate Spc1 activity (Millar et al. 1995; Degols et al. 1996). Various forms of stress induce expression of these genes in a *spc1*⁺-dependent manner. Budding yeast Hog1p also regulates stress-inducible genes such as *CTT1*, *GPD1*, and *HSP12*, although Hog1p is reported to be specifically responsive to osmotic stress (Albetyn et al. 1994; Schüller et al. 1994; Varela et al. 1995).

In addition to cytotoxic stress, Spc1 is activated when cells are grown in a synthetic minimal medium (Shiozaki and Russell 1995), suggesting that the Spc1 pathway might also be involved in sensing the nutritional environment. In this paper we extend this observation by showing that Spc1 is activated in response to limitation of a specific nutrient, namely, nitrogen. This finding is important because nitrogen limitation induces a program of sexual development in fission yeast. This program involves secretion of mating pheromones and expression of mating pheromone receptors, followed by G₁ arrest and conjugation between cells of opposite mating types (Egel 1989). Nitrogen starvation also stimulates the meiotic pathway, thus newly formed zygotes normally proceed directly to meiosis, with the end product being four spores enclosed in an ascus. Our findings suggest that activation of the Wis1–Spc1 kinase cascade is important for sexual development, because *spc1*[−] mutants are severely defective at arresting in G₁ and inducing mating genes upon nitrogen starvation. Two multi-copy suppressors of the *spc1*[−] sterile phenotype have been isolated; *atf1*⁺ and *atf21*⁺ encode basic zipper (bZIP) transcription factors that are related most closely to human activating transcription factor 2 (ATF-2)/cAMP response element (CRE)–BP1 binding protein. *atf1*[−] and *spc1*[−] mutants share various phenotypes, including failure to respond to nitrogen starvation, osmo-sensitive growth, and a defect in expression of stress-response genes. Atf1 undergoes Spc1-dependent phosphorylation during stress. These results strongly suggest that Atf1 is a downstream target of Spc1. However, unlike *spc1*[−] cells, *atf1*[−] cells do not show a G₂ delay phenotype, indicating that Spc1 phosphorylates another substrate to regulate mitosis. We propose that the Wis1–Spc1 signal transduction pathway links mitotic and meiotic controls with changes in the extracellular environment that affect cell physiology.

Results

Spc1 is activated in response to nitrogen starvation

To examine the activation state of Spc1 after nitrogen starvation, we used a haploid *S. pombe* strain in which the single chromosomal copy of *spc1*⁺ was tagged with a sequence encoding two copies of the HA epitope and six consecutive histidine residues (Shiozaki and Russell 1995). This allowed us to purify Spc1 using Ni²⁺-NTA beads and detect it using anti-HA antibodies. Cells growing exponentially in standard EMM2 minimal medium were collected and resuspended in EMM2 lacking nitrogen. Activation of Spc1 was assessed by monitoring the level of activating tyrosine phosphorylation using anti-phosphotyrosine antibodies. Spc1 tyrosine phosphorylation increased within 0.5 hr of nitrogen depletion and peaked at ~1 hr, then decreased to the initial level by 2.5 hr (Fig. 1A). This result indicates that Spc1 undergoes transient activation upon nitrogen limitation.

*Activation of Spc1 upon nitrogen starvation is required for G₁ cell-cycle arrest and induction of *ste11*⁺ expression*

In cultures of homothallic wild-type cells, nitrogen starvation initiates a program of sexual development in which cells arrest in G₁, mate with partners of the op-

Figure 1. (A) Spc1 undergoes activating tyrosine phosphorylation upon nitrogen starvation. Strain KS1376, in which the genomic copy of *spc1*⁺ encodes an epitope-tagged form of Spc1 that has two copies of the HA epitope and six consecutive histidine residues at the carboxyl terminus, was grown to mid-log phase at 30°C in EMM₂ medium and then cells were collected and resuspended into the same medium lacking a nitrogen source (NH₄Cl). Spc1 was purified using Ni-NTA-agarose beads and analyzed by immunoblotting with anti-phosphotyrosine (anti-pTyr) and anti-HA epitope antibodies. (B) FACS analysis of DNA content in wild-type (wt; PR109) and Δ*spc1* (KS1366) strains after nitrogen starvation. Within 6 hr of nitrogen deprivation a majority of wild-type cells arrested in G₁ with a 1C DNA content, whereas only very few of the Δ*spc1* cells had a 1C DNA content. (C) Δ*spc1* cells are defective in *ste11*⁺ expression. *ste11*⁺ mRNA in wild-type (PR109) and Δ*spc1* (KS1366) strains was monitored at 2 hr intervals after nitrogen starvation by Northern blotting (upper). Ethidium bromide staining of ribosomal RNA shown below indicates nearly equal loading of RNA in each lane.

posite mating type, and then undergo meiosis. A key event in this program is the induction of *ste11*⁺ expression (Sugimoto et al. 1991). Ste11 is a transcription factor containing a high mobility group (HMG) motif that is required for transcription of *mei2*⁺, a gene encoding an essential factor for the initiation of meiosis (Watanabe and Yamamoto 1994). Interestingly, G₁ arrest and induction of *ste11*⁺ expression are not dependent on the presence of cells of opposite mating types, thus events that occur purely in response to nitrogen limitation can be distinguished from those that are dependent on a response to mating pheromones. The role of Spc1 in these processes was evaluated by examining the behavior of a heterothallic *h*⁻ Δ *spc1* strain as it was starved of nitrogen. FACS analysis of DNA content revealed that Δ *spc1* cells failed to arrest in G₁ after nitrogen starvation, whereas a large population of G₁ cells with a 1C DNA content was detected with wild-type cells (Fig. 1B). Expression of *ste11*⁺ mRNA was examined by Northern blotting. Under the experimental conditions used here, accumulation of *ste11*⁺ mRNA was observed 2 hr after withdrawal of nitrogen from the growth medium and returned to the initial level by 4 hr (Fig. 1C). On the other hand, in Δ *spc1* cells the amount of *ste11*⁺ mRNA was very low before and during nitrogen starvation (Fig. 1C), indicating that Spc1 is important for expression of *ste11*⁺. Defects in G₁ arrest and *ste11*⁺ expression after nitrogen starvation were also observed with a Δ *wis1* strain (data not shown), suggesting that activation of the Wis1–Spc1 pathway is important for cellular response to nitrogen limitation.

Isolation of *spc1*⁻ multicopy suppressors

Our studies suggested that Spc1 has a key early role in transmitting the nitrogen starvation signal that triggers the initiation of the sexual development. This proposal was supported by the observation that the efficiency of mating and sporulation was very low in a homothallic *h*⁹⁰ *spc1*–M13 strain (Fig. 2A). With the aim of identifying downstream elements of the Wis1–Spc1 signal transduction pathway, we screened a *S. pombe* genomic DNA library and a cDNA expression library for plasmid suppressors of the *spc1*⁻ mating defect. Strain KS1440 (*h*⁹⁰ *leu1 ura4 spc*–M13) was transformed with library DNA, and plasmids were recovered from sporulating colonies identified by iodine staining (see Materials and Methods). In addition to clones containing *spc1*⁺, plasmids named p2B from the cDNA library and p4U from the genomic library were found to rescue the sterility of the *spc1*⁻ strain (Fig. 2A). In comparison to vector controls, the p2B transformants had an enhanced ability to undergo G₁-arrest after nitrogen starvation (Fig. 2B) and grew much better in high-osmolarity medium (Fig. 2C). Plasmid p4U exhibited a similar ability to rescue the G₁-arrest and osmosensitive defects of *spc1*–M13 cells (data not shown). Plasmid p2B contained a 1.2-kb insert and sequence analysis indicated that the insert cDNA lacked the 5' end (Fig. 3A). A full-length genomic clone was isolated from the genomic library by hybridization

Figure 2. Multicopy suppressors of *spc1*⁻. (A) Patches of *h*⁹⁰ *spc1*–M13 (KS1440) cells transformed with the negative control pREP3 (vector), *pspc1*⁺, which carries *spc1*⁺ cDNA, or cDNA clone p2B were grown at 25°C for 4 days on an EMM₂ agar plate supplemented with 1 μ M thiamine, and then streaked out for photographs. Plasmid *pspc1*⁺ and p2B transformants colonies contained a large number of asci with four spores, indicating rescue of the *spc1*⁻ mating defect, whereas the frequency of asci in the vector control transformants was <1% in this experiment. (B) cDNA p2B rescues partially the G₁ arrest defect of *spc1*⁻ cells after nitrogen starvation. *spc1*–M13 strain KS1147 was transformed by pREP3 vector alone or p2B, and grown in EMM₂ medium supplemented with 1 μ M thiamine. Cells were washed and reinoculated in the same medium without NH₄Cl and their DNA content was examined by flow cytometry analysis after 6 hr at 30°C. (C) Suppression of high-osmolarity sensitive phenotype of *spc1*⁻ strain by cDNA p2B. Strain KS1147 transformed by pREP3 vector, *pspc1*⁺, or p2B was streaked onto EMM₂ medium with thiamine containing 1 M KCl and incubated for 4 days at 32°C. Clone p4U from genomic library also rescued the *spc1*⁻ phenotypes in the same assay (data not shown).

screening using the cDNA insert of p2B as a probe (p5A; Fig. 3A). Genomic clone p5A contained a 1698-bp open reading frame (ORF) encoding a protein of 566-amino acid residues, which was named *atf1*⁺ because of its homology to human ATF-2 (see below). Very recently *atf1*⁺ was discovered during the process of sequencing chromosome II of *S. pombe* (Takeda et al. 1995). Like p2B, a multi-copy plasmid pREP1–*atf1*⁺ (Fig. 3A), which expresses full-length *atf1*⁺ under the control of thiamine-repressible *nmt1* promoter (Maundrell 1993), also complemented the mating defect and high-osmolarity sensitivity of *spc1*⁻ mutants in the presence of thiamine (data not shown), indicating that leaky expression of *atf1*⁺ from the repressed *nmt1* promoter was sufficient to rescue the *spc1*⁻ phenotypes. Sequence analysis of the 3.9-kb insert of p4U identified two ORFs (Fig. 3B). A subclone of p4U, p4U-1, containing the *pce1*⁺ ORF encod-

Figure 3. Restriction maps of *atf1*⁺ and *atf21*⁺. (A) Clone p2B isolated from *S. pombe* cDNA library lacks the 5' end of the *atf1*⁺ open reading frame and expresses the carboxy-terminal half of the protein under the *nmt1* promoter (*nmt1p*). The *atf1*⁺ genomic clone p5A was isolated from a *S. pombe* genomic library by hybridization screening using a 1.2-kb insert from p2B. Full-length *atf1*⁺ expressed from the *nmt1* promoter (*pREP1-atf1*⁺) also rescues *spc1*⁻ mating and high osmolarity defects. The plasmid construct used for *atf1*⁺ disruption with the *ura4*⁺ marker is also shown. Restriction enzyme sites: (Ec) EcoRI; (Sc) SacI; (Sp) SphI; (Xb) XbaI. (B) Clone p4U isolated from *S. pombe* genomic library was found to contain two open reading frames *atf21*⁺ and *pce1*⁺ that encode mRNA capping enzyme (Shuman et al. 1994). Subclone p4U-1 containing only *pce1*⁺ failed to rescue *spc1*⁻, whereas *atf21*⁺ expressed from the *nmt1* promoter (*pREP1-atf21*⁺) complemented *spc1*⁻. Disruption of *atf21*⁺ was performed with the plasmid construct shown above. Restriction enzyme sites: (Hd) HindIII; (Ns) NsiI; (RV) EcoRV.

ing a mRNA capping enzyme (Shuman et al. 1994), failed to rescue *spc1-M13* (Fig. 3B). Instead, *spc1-M13* was rescued by expression of the second p4U ORF from the *nmt1* promoter in medium containing thiamine (Fig. 3B). The gene encoding the 355-amino acid protein from this ORF was named *atf21*⁺.

atf1⁺ and *atf21*⁺ encode transcription factors related to human ATF-2

The *atf1*⁺ and *atf21*⁺ genes encode 65-kDa and 41-kDa proteins, respectively. The deduced primary sequences of Atf1 and Atf21 contain a carboxy-terminal bZIP structure that consists of a DNA-binding domain composed of basic residues followed by a leucine zipper structure (Fig. 4A). A data-base search revealed that Atf1 and Atf21 are most homologous to the human ATF-2/CRE-BP1 transcription factor (Hai et al. 1989; Maekawa et al. 1989). *S. pombe* Atf1 and Atf21 show 50% and 55% identity to ATF-2 in the bZIP domains, respectively. Weak similarity is also seen in the amino-terminal domains of human ATF-2 and Atf1 (Fig. 4A), whereas, unlike ATF-2, neither of Atf1 nor Atf21 have a zinc finger motif (Fig. 4B). Another cAMP response element binding protein (CREB)/ATF-related protein in fission yeast, Pcr1, was reported recently (Watanabe and Yamamoto 1996). Pcr1 is a rela-

Figure 4. (A) Comparison of predicted amino acid sequences of Atf1, human ATF-2/CRE-BP1 (Hs ATF-2) (Maekawa et al. 1989) and Atf21. Residues conserved among all three proteins are boxed and shaded residues are conserved between two of the proteins. MAPK phosphorylation sites in ATF-2 (Thr-69 and Thr-71) (Gupta et al. 1995; Livingstone et al. 1995; van Dam et al. 1995) are indicated by filled circles. Residues important for formation of the "leucine zipper" structure are marked by asterisks. GenBank accession nos.: *S. pombe atf1*⁺, U38237; *atf21*⁺, U51566; human ATF-2/CRE-BP1, X15875. Alignments were performed using GeneWorks software (IntelliGenetics). (B) Schematic representation of the structures of human (Hs) ATF-2 and *S. pombe* transcription factors carrying CREB/ATF-like bZIP domain, Atf1, Atf21, and Pcr1. Human ATF-2 carries a zinc finger motif in its amino-terminal domain.

tively small protein of 171-amino acid residues with a bZIP domain located near the amino terminus (Fig. 4B).

atf1⁺ is required for the cellular response to nitrogen starvation and sexual development

Gene disruption experiments were performed to reveal the cellular functions of Atf1 and Atf21 (see Fig. 3A,B). FACSscan analyses of cells revealed that $\Delta atf1$ cells were highly defective in undergoing G₁ arrest upon nitrogen starvation (Fig. 5A). On the other hand, $\Delta atf21$ cells had

Figure 5. (A) FACS analysis of DNA content of wild-type (wt; PR109), $\Delta spc1$ (KS1366), $\Delta atf1$ (KS1497), and $\Delta atf21$ (KS1557) strains after nitrogen starvation. After 8 hr, only a small population of $\Delta atf1$ cells were arrested in G₁ with a 1C DNA content in comparison with wild-type and $\Delta atf21$ cells. (B) $\Delta atf1$ cells are sensitive to high osmolarity. Wild-type (wt; PR109), $\Delta spc1$ (KS1366), $\Delta atf1$ (KS1497), $\Delta atf21$ (KS1557) and $\Delta atf1 \Delta atf21$ (KS1565) cells were streaked onto YES agar plates supplemented with or without 1 M KCl, and incubated for 4 days at 32°C. $\Delta atf21$ mutation showed no apparent effect on the salt sensitivity in both wild-type and $\Delta atf1$ backgrounds. (C) Wild-type (PR109) and $\Delta atf1$ (KS1497) cells were grown at 30°C to mid-log phase in YES medium and then switched to YES + 0.6 M KCl at time 0. Samples were taken at 10-min intervals and processed for Northern blot analysis. Induction of *gpd1*⁺ and *pyp2*⁺ mRNA is abolished in the $\Delta atf1$ cells. The *leu1*⁺ probe serves as a control.

no defect in undergoing G₁ arrest. Homothallic $\Delta atf1$ strains had a low mating frequency, whereas $\Delta atf21$ cells appeared to be normal in mating and sporulation (data not shown). $\Delta atf1$ and $\Delta spc1$ mutants were also defective in meiosis; 31% of diploid cells homozygous for $\Delta atf1$ and 20% of $\Delta spc1$ diploids performed meiosis in sporulation medium, compared to a value of 78% with wild-type diploid cells. In addition, the $\Delta atf1$ and $\Delta spc1$ mutations suppressed meiosis induced by the temperature-sensitive *pat1-114* mutation. *pat1*⁺, also known as *ran1*⁺, encodes a protein kinase (McLeod and Beach 1986) that regulates negatively the initiation of meiosis (Iino and Yamamoto 1985; Nurse 1985). Growth of *pat1-114* mutants at the restrictive temperature results in the initiation of meiosis, regardless of cell ploidy or the presence of nitrogen in the growth medium. It was found that $\Delta atf1 pat1-114$ and *spc1-M13 pat1-114* double mutants formed colonies of mitotically dividing cells at 32°C, whereas *pat1-114* cells did not form colonies be-

cause they underwent lethal haploid meiosis (data not shown). The $\Delta atf1$ conjugation defects are consistent with those recently described by Takeda and colleagues (1995). Our findings suggest that upon nitrogen starvation *Spc1* and *Atf1* function in the same pathway to promote mating and meiosis by bringing about G₁ cell cycle arrest and inducing *ste11*⁺ transcription.

atf1⁻ cells are defective in the response to osmotic stress

The findings described above, indicating that *Atf1* and *Spc1* have important roles in mediating the cellular response to nitrogen starvation, prompted an examination of whether *Atf1* might also have a role in regulating the cellular response to osmotic stress. We found that $\Delta atf1$ cells grew very poorly on medium supplemented with 1 M KCl (Fig. 5B), although the growth defect of $\Delta atf1$ cells was less severe than that of $\Delta spc1$ cells. Importantly, the osmosensitive growth caused by $\Delta spc1$ was not enhanced in $\Delta spc1 \Delta atf1$ double mutants (data not shown), which implies that *Atf1* and *Spc1* function in a linear pathway. The $\Delta atf21$ mutation did not affect the high osmolarity sensitivity of wild-type and $\Delta atf1$ cells (Fig. 5B), indicating that *Atf21* does not have a major role in osmoregulation.

These findings suggested that *Atf1* may be involved in promoting transcription of stress response genes that are regulated by the *Wis1-Spc1* signal transduction pathway, such as *gpd1*⁺, *tps1*⁺, and *pyp2*⁺ (Miller et al. 1995; Degols et al. 1996). Indeed, osmotic stress-induced expression of *gpd1*⁺, which encodes glycerol-3-phosphate dehydrogenase (Pidoux et al. 1990), was abolished in $\Delta atf1$ cells (Fig. 5C). Expression of *pyp2*⁺ was similarly defective in $\Delta atf1$ cells (Fig. 5C), indicating that *Atf1* is a part of the negative feedback loop in which *Spc1*-stimulated induction of *pyp2*⁺ expression attenuates *Spc1* activity (Miller et al. 1995; Degols et al. 1996).

Atf1 regulation by *Spc1*

Our genetic findings indicated that *Atf1* probably functions downstream of *Spc1* in the regulation of sexual differentiation and stress response; therefore, we examined the biochemical relationship between *Atf1* and *Spc1* kinase. To facilitate analysis of *Atf1* protein, the chromosomal *atf1*⁺ gene was tagged with a sequence encoding the hemagglutinin antigen (HA) epitope and six consecutive histidine residues (see Materials and Methods). The resultant strain was indistinguishable from wild type, indicating that *Atf1* protein with the carboxy-terminal tag was fully functional (data not shown). *Atf1* protein purified from unstressed wild-type cells migrated as a somewhat diffuse band with an apparent molecular mass of ~85 kD in SDS-PAGE (Fig. 6A, lane 1). When cells were stressed in a medium supplemented with 0.6 M KCl for 10 min, *Atf1* migrated with a reduced mobility of ~90 kD (Fig. 6A, lane 2). In contrast, *Atf1* protein purified from a $\Delta spc1$ strain migrated as a less diffuse ~80 kD protein species both before and during osmotic stress (Fig. 6A, lanes 3,4). To determine whether the *Atf1*

Figure 6. (A) Spc1-dependent phosphorylation of Atf1 in vivo. Wild-type (KS1479, lanes 1,2,5–7) and Δ spc1 (KS1572, lanes 3,4) strains carrying a chromosomal copy of epitope tagged *atf1*⁺ were grown in YES medium at 30°C, and aliquots were harvested before (lanes 1,3) and after osmostress in YES + 0.6 M KCl for 10 min (lanes 2,4–7). Atf1 protein tagged with HA epitopes and six His residues was purified on Ni-NTA beads and analyzed by SDS-PAGE followed by immunoblotting with anti-HA antibodies. For lanes 5–7, samples were incubated with buffer alone (lane 5), alkaline phosphatase (CIP, lane 6), or alkaline phosphatase and phosphatase inhibitors (CIP + inh, lane 7) before electrophoresis. (B) *atf1*⁺ mRNA accumulates with osmostress in a *spc1*⁺-dependent manner. Wild-type (PR109; solid bar) and *spc1*-M13 (KS1147; open bar) strains were treated with 0.6 M KCl as in Fig. 5C, and *atf1*⁺ mRNA was examined by Northern blotting. Data were quantified and calibrated using a control blot with *leu1*⁺ probe as a standard. Even before osmostress (at time 0), the level of *atf1*⁺ mRNA detected in *spc1*⁻ cells was lower than wild type.

mobility shifts were caused by phosphorylation, Atf1 protein isolated from osmostressed wild-type cells was incubated with calf intestine alkaline phosphatase (CIP). After phosphatase treatment, Atf1 protein migrated as a less diffuse protein species with faster mobility (Fig. 6A, lane 6), whereas no change was seen if phosphatase inhibitors were included in the incubation (Fig. 6A, lane 7). These data show that Atf1 is phosphorylated in vivo in response to osmostress in a *spc1*⁺-dependent manner.

As shown in Figure 6A, the amount of Atf1 protein recovered from a wild-type strain was two- to threefold higher than in a Δ spc1 strain. Therefore, we examined whether *atf1*⁺ mRNA expression was regulated by Spc1. Northern blotting analysis demonstrated that in unstressed conditions the level of *atf1*⁺ mRNA was reduced ~50% in *spc1*-M13 cells as compared with wild type (Fig. 6B, time 0). A transient increase in the amount of *atf1*⁺ mRNA was observed when wild-type cells were

stressed by 0.6 M KCl (Fig. 6B), whereas no increase in the amount of *atf1*⁺ mRNA was observed in *spc1*-M13 mutant cells. This finding indicates that expression of *atf1*⁺ mRNA is also regulated by Spc1, possibly through the regulation of Atf1 itself.

There is considerable evidence from studies of mammalian cells indicating that the stress-activated Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) phosphorylate ATF-2 directly (Gupta et al. 1995; Livingstone et al. 1995; van Dam et al. 1995). This conclusion is supported by the detection of stable in vitro binding between JNK1 and ATF-2. Interestingly, this binding is mediated by a region of ATF-2 that is separate from the sites of phosphorylation. We carried out experiments to evaluate whether the Spc1-dependent phosphorylation of Atf1, as established by the data shown in Figure 6A, might involve direct phosphorylation of Atf1 by Spc1 kinase. As a first step in this analysis, we asked whether Atf1 coprecipitated with a protein kinase. These studies were facilitated by expression of glutathione *s*-transferase (GST)-Atf1 fusion protein in *S. pombe*, which rescued both *spc1*⁻ and *atf1*⁻ osmosensitive phenotypes (data not shown). GST-Atf1 was purified by glutathione (GSH)-Sepharose affinity precipitation and then incubated with Mg²⁺ and [γ -³²P]ATP (Fig. 7A). GST-Atf1 became highly phosphorylated in this assay, indicating that GST-Atf1 coprecipitated with a protein kinase that phosphorylates GST-Atf1 efficiently. Importantly, this phosphorylation was abolished when GST-Atf1 was expressed and recovered from Δ spc1 cells (Fig. 7A).

These findings indicate that Spc1 or another kinase whose activity is dependent on Spc1 copurifies with and phosphorylates GST-Atf1. To distinguish between these possibilities, we first asked whether Spc1 was present in the GST-Atf1 samples prepared by GSH-Sepharose affinity precipitation. GST-Atf1 and unfused GST were expressed in a strain in which endogenous Spc1 was tagged with the HA epitope. GST-Atf1 and GST were purified by GSH-Sepharose precipitation. Spc1 was detected in the GST-Atf1 sample but not with GST alone (Fig. 7B). Control experiments, in which GST-Atf1 was coexpressed with HA epitope-tagged forms of Wee1 kinase, Nim1 kinase, and Cig2 B-type cyclin, confirmed that the detection of HA-tagged Spc1 in GST-Atf1 sample was not attributable to a nonspecific interaction between the HA epitope tag and GST-Atf1 (data not shown).

These results supported the conclusion that Spc1 may be directly responsible for the phosphorylation of GST-Atf1 in the GSH-Sepharose precipitates. This possibility was explored further by comparing the two-dimensional tryptic phosphopeptide map of GST-Atf1 phosphorylated by its coprecipitating kinase with the map generated by direct phosphorylation of GST-Atf1 with purified Spc1 kinase. The GST-Atf1 isolated from Δ spc1 cells was treated with irreversible protein kinase inhibitor *p*-fluorosulfonyl-benzoyl 5'-adenosine (FSBA) (Zoller and Taylor 1979) to inactivate coprecipitating kinases, and then incubated with Mg²⁺ and [γ -³²P]ATP together

Figure 7. (A) Spc1-dependent phosphorylation of Atf1 by its coprecipitating protein kinase. GST-Atf1 proteins were purified from *spc1*⁺ (wt; KS1376) and Δspc1 (KS1366) strains that had been grown in EMM₂ + 0.6 M KCl for 10 min. GST-Atf1 proteins were incubated in the presence of Mg^{2+} and [γ - ^{32}P]ATP and subjected to SDS-PAGE. Coomassie blue (CB) staining of purified GST-Atf1 shows that approximately equal levels of GST-Atf1 were recovered from the two strains. An autoradiogram of the gel (^{32}P) shows that in vitro phosphorylation of GST-Atf1 is dependent on Spc1. Note that the phosphorylated GST-Atf1 appears as a somewhat more diffuse band by CB staining, indicating that phosphorylation affects the electrophoretic mobility of GST-Atf1. (B) Physical interaction of Atf1 and Spc1. GST and GST-Atf1 fusion proteins expressed in KS1376 strains were precipitated using GSH-Sepharose beads. After extensive washes, proteins bound to the beads were analyzed by immunoblotting with anti-GST (upper) and anti-HA (lower) antibodies. Spc1HA protein was detected in the GST-Atf1 sample and not detected in the GST control sample. (C) Tryptic phosphopeptide maps of GST-Atf1 proteins phosphorylated by a coprecipitating "Atf1 kinase" (top), purified GST-Spc1 (middle), and a mixture of the same amount of both samples (bottom). ^{32}P -labeled GST-Atf1 proteins were subjected to trypsin digestion and spotted to origins shown by circles. Peptides were developed by electrophoresis at pH 1.9 (left, anode; right, cathode) in the first dimension, which is followed by ascending chromatography in the second dimension. Because Spc1 does not phosphorylate GST in vitro (data not shown), all of the phosphopeptides in the middle panel are derived from Atf1. Note that the map of the top panel is more expanded than the others because of faster ascension of the chromatography buffer.

with GST-Spc1 purified from *S. pombe*. This reaction led to extensive phosphorylation of GST-Atf1, and the two-dimensional tryptic phosphopeptide pattern of GST-Atf1 phosphorylated by purified Spc1 kinase was very similar to the pattern generated by analysis of GST-Atf1 that had been phosphorylated by coprecipitating "Atf1 kinase" (Fig. 7C). A mixing experiment confirmed that the four major phosphopeptides (labeled 1–4 in Fig. 7C) in the two samples migrated with identical properties. These data provide strong evidence indicating that

Spc1 is the protein kinase that coprecipitates with and phosphorylates GST-Atf1.

Atf1 is not involved in G₂-M cell cycle control

Our findings indicate that Atf1 is a downstream target of Spc1 that regulates G₁ arrest, sexual development, and the osmotic stress response. However, unlike *spc1*[−] mutants that show a G₂ cell-cycle delay, Δatf1 cells divided at a wild-type cell length in both rich YES and minimal EMM₂ media (data not shown). This observation was confirmed by the following findings. The cell lengths at which $\Delta\text{atf1 cdc25-22}$ double mutants and *cdc25-22* single mutants divided were nearly identical at 25°C, a permissive temperature of *cdc25-22* mutation, which is in contrast to *cdc* arrest phenotype exhibited by *spc1-M13 cdc25-22* double mutant grown at 25°C (Fig. 8A). Furthermore, the *cdc* phenotype of a *spc1-M13 cdc25-22 wee1-50* triple mutant at 35°C (Shiozaki and Russell 1995) was not rescued by overexpression of *atf1*⁺ (data not shown). These observations strongly suggest that the mitotic induction activity of Spc1 is transmitted by an Atf1-independent pathway.

Bifurcation of the Spc1 pathway was also implied by comparing the effects of Wis1 overproduction in Δspc1 and Δatf1 cells. Previous studies showed that Δspc1 cells

Figure 8. (A) The Δatf1 mutation does not cause a G₂ cell-cycle delay. A *cdc25-22* mutant strain (GL84) was crossed with *spc1-M13* (KS1310) or Δatf1 (KS1497) strains and segregants of *cdc25-22*, *spc1-M13 cdc25-22*, and $\Delta\text{atf1 cdc25-22}$ were grown at 25°C. *cdc25-22* and $\Delta\text{atf1 cdc25-22}$ double mutant cells grew well at this temperature with a moderate elongated cell morphology, whereas *spc1-M13 cdc25-22* cells showed a *cdc* arrest phenotype. Bar, 10 μm . (B) Phenotype of wild-type (wt; PR109), Δspc1 (KS1366), and Δatf1 (KS1497) strains upon Wis1 overexpression. Strains transformed with either pREP1 vector or pREP1-*wis1*⁺ were streaked on an EMM₂ plate without thiamine to induce the expression of Wis1 from the *nmt1* promoter. The plate was photographed after 3 days at 30°C. Wis1 overproduction severely impaired growth in wild type, had a somewhat less severe effect in Δatf1 cells, and had very little effect on Δspc1 cells.

are almost completely refractive to the lethal cell lysis phenotype caused by Wis1 overproduction (Shiozaki and Russell 1995). In contrast, Wis1 overproduction severely decreased the size of $\Delta atf1$ colonies, although $\Delta atf1$ colonies were larger than those of wild-type cells overproducing Wis1 (Fig. 8B). Microscopic examination revealed that Wis1 overproduction severely deformed cell shape in $\Delta atf1$ cells but did not cause extensive lysis. The $\Delta atf21$ mutation had no effect on the toxicity of Wis1 overproduction (data not shown). This result shows that the toxic effect of Spc1 hyperactivation is not solely dependent on Atf1 or Atf21, nor presumably on genes, such as $gpd1^+$ that are regulated by Atf1.

Discussion

Our results indicate that the Wis1–Spc1 pathway regulates at least two different downstream targets (Fig. 9). One branch proceeds by Atf1 to cause G_1 arrest and induce $ste11^+$ expression in response to nitrogen limitation, and to induce expression of genes such as $gpd1^+$ and $pyp2^+$ in response to cytotoxic stress. The second

branch regulates the onset of mitosis. Thus, the Wis1–Spc1 pathway bifurcates to regulate both meiosis and mitosis in response to changes in the extracellular environment.

Identification of a *S. pombe* ATF-2 homolog as a downstream element of the stress-activated Wis1–Spc1 kinase cascade

Two structurally related genes, $atf1^+$ and $atf21^+$, encoding bZIP transcription factors, have been identified as multicopy suppressors of the sterility and osmosensitivity of $spc1^-$ cells. Atf1 and Atf21 are most homologous to human ATF-2/CRE-BP1 (Hai et al. 1989; Maekawa et al. 1989). Evidence presented in this paper strongly indicates that Atf1 is a downstream component of the Wis1–Spc1 pathway. First, $\Delta atf1$ and $\Delta spc1$ mutants exhibit very similar stress and sexual development deficiencies. Both mutants show osmosensitive growth and fail to induce the expression of $gpd1^+$ and $pyp2^+$. $\Delta atf1$ and $\Delta spc1$ cells do not arrest in G_1 and show severe defects in sexual differentiation after nitrogen deprivation. Second, $\Delta spc1$ mutant phenotypes are not enhanced in $\Delta spc1 \Delta atf1$ double mutants. Third, overexpression of $atf1^+$ complements the $spc1^-$ defects. Fourth, Atf1 is phosphorylated upon stress in a $spc1^+$ -dependent manner. Fifth, GST–Atf1 coprecipitates with a kinase that phosphorylates Atf1; this kinase activity is abolished in a $spc1^-$ mutant. Sixth, the tryptic phosphopeptide map derived from GST–Atf1 phosphorylated by its coprecipitating kinase closely matches the pattern derived from GST–Atf1 phosphorylated by purified GST–Spc1. Finally, Spc1 was detected in GST–Atf1 samples purified by GSH–Sepharose precipitation.

Human ATF-2 is phosphorylated on Thr-69 and Thr-71 by several kinases belonging to the extended MAP kinase family (Abdel-Hafiz et al. 1992; Gupta et al. 1995; Livingstone et al. 1995; Raingeaud et al. 1995, 1996; van Dam et al. 1995). Phosphorylation by MAP kinase increases in vitro DNA-binding activity of ATF-2, and alanine substitution of Thr-69 and Thr-71 abolishes the transcriptional activity of Gal4–ATF-2 fusion protein in vivo. ATF-2 activity is stimulated by UV and genotoxic agents, and bacterially produced GST–ATF-2 fusion protein binds to stress-activated kinases JNK/SAP kinase and p38. The identification of Atf1 as a downstream element of the Spc1 signal transduction system further extends a similarity between yeast and mammalian stress-sensing MAP kinase pathways. Although MAP kinase cascades are conserved signal transduction modules, this is the first example that the structural similarity between the mammalian and yeast pathways has been detected to the level of downstream transcription factors. Because stress response is a basic and essential mechanism for cells to survive environmental changes, a high degree of conservation in the cellular mechanism to sense stress is perhaps not surprising.

Genes regulated by Atf1

Atf1 regulates the transcription of the stress response

Figure 9. The Spc1 stress and nutrient sensor pathway. The pathway is stimulated by various forms of environmental changes that cause cellular stress and by nitrogen limitation. The Wis1 MAP kinase homolog activates Spc1 by phosphorylating Thr171 and Tyr173. Elements acting upstream of Wis1 have not yet been identified. Spc1 is inactivated by Pyp1 and Pyp2 tyrosine phosphatases, which dephosphorylate Tyr173. Expression of $pyp1^+$ is constitutive. Spc1 promotes meiosis after nitrogen starvation and other stress responses through the Atf1 transcription factor, which is required for induced expression of $ste11^+$, $gpd1^+$, $tps1^+$ (data not shown) and $pyp2^+$. $atf1^+$ expression is also stimulated by Spc1 activation, possibly through Atf1 itself, which may amplify transmitted signals. Stress-induced transcription of $pyp2^+$ constitutes a negative feedback loop to attenuate the Spc1 activation. Spc1 is expected to have additional targets to promote G_2/M in the mitotic cell cycle, because unlike $\Delta spc1$, $\Delta atf1$ mutation does not cause a G_2 delay or rescue Wis1 overproduction lethality.

gene *gpd1*⁺, the *pyp2*⁺ tyrosine phosphatase gene, and the *ste11*⁺ gene that regulates the commitment to meiosis. It has also been reported that the glucose-repressible gene *fbp1*⁺, which encodes fructose-1,6-bisphosphatase (Vassarotti and Friesen 1985), is also regulated by *atf1*⁺ (Takeda et al. 1995). The *fbp1*⁺ findings are consistent with the observation that Spc1 is activated in response to glucose starvation (G. Degols and P. Russell, unpubl.). In mammalian cells, ATF-2 is implicated in the expression of transforming growth factor- β 2 (Kim et al. 1992), interferon β (Du et al. 1993), E-selectin (De Luca et al. 1994), and *c-jun* (van Dam et al. 1995). More recent studies with ATF-2-deficient mice, which have severe chondrodysplasia and neurological abnormalities, have indicated that ATF-2 is required for stress-induced expression of the E-selectin gene but may not be required for expression of other genes whose promoters bind ATF-2 in vitro, including interferon β and *c-jun* (Reimold et al. 1996). Like *pyp2*⁺, transcription of genes encoding dual specificity phosphatases that are proposed to dephosphorylate MAP kinases are induced by various forms of stress (Keyse and Emslie 1992; Ishibashi et al. 1994; Liu et al. 1995). It will be important to determine whether expression of these stress-induced MAP kinase phosphatase genes are regulated by ATF-2.

Induction of the Ste11 transcription factor is one of the early events in the process of sexual differentiation. Ste11 expression leads to transcription of a number of genes required for conjugation and meiosis, including the mating type genes and *mei2*⁺ (Sugimoto et al. 1991). Expression of *ste11*⁺ is regulated negatively by the cAMP-dependent protein kinase (PKA) pathway (Sugimoto et al. 1991). Nitrogen deprivation from the growth medium lowers the intracellular concentration of cAMP (Mochizuki and Yamamoto 1992), which leads to derepression of *ste11*⁺. We have shown that nitrogen starvation also induces activation of Spc1 kinase and Spc1 is required for *ste11*⁺ transcription. Spores are extremely resistant to all forms of stress, thus it may be significant that *S. pombe* uses a single MAP kinase pathway to regulate induction of stress response genes and to promote initiation of sexual development. Counteracting activities of stress-activated MAP kinase homologs and PKA in transcriptional regulation has also been reported in budding yeast (Schüller et al. 1994), although it is not known which transcription factors are involved.

The Wis1-Spc1-Atf1 pathway is also essential to establish a G₀-like quiescent state with nutritional deprivation. Cells lacking any component of the pathway quickly lose viability after they enter stationary phase (Warbrick and Fantes 1991; Takeda et al. 1995). This *spc1*⁻ defect is complemented by overexpression of *atf1*⁺ (K. Shiozaki and P. Russell, unpubl.). An important goal of future studies will be to identify the Atf1-regulated genes that are required for the asexual response to nutritional starvation.

Other downstream targets of Spc1

Unlike Δ *spc1*, the Δ *atf1* mutation does not cause a G₂

delay or synthetic lethality in a *cdc25-22* background, implying that a distinct branch of the pathway mediates the mitotic induction activity of Spc1. Little is known about this branch of the signal transduction pathway. It is possible that a protein kinase cascade extends downstream of Spc1, as p38 kinase, a mammalian homolog of Spc1, has been implicated as an activator of MAPK-associated protein kinase-2 (Freshney et al. 1994; Rouse et al. 1994). The *cdc* arrest phenotype of a *spc1-M13 cdc25-22 wee1-50* triple mutant at 35°C suggests that Spc1 can function independently of Wee1 and Cdc25 to regulate mitosis (Shiozaki and Russell 1995). Mutational inactivation of Mik1, a second kinase that inhibits Cdc2 (Lundgren et al. 1991), had no effect in a *spc1-M13* strain, indicating that Mik1 is not regulated by Spc1. The substrate of Spc1 in this pathway could be another transcription factor, although Atf21 does not appear to be involved, as Δ *atf21* cells show no cell-cycle defect (data not shown). One might predict that such a transcription factor would be a high-copy suppressor of the *spc1*⁻ *cdc25-22* synthetic lethal interaction, therefore experiments to identify such a gene are currently under way.

Materials and methods

Yeast strains and general techniques

Schizosaccharomyces pombe strains used in this study are listed in Table 1. They are derivatives of 972h⁻ and 975h⁺ (Mitchison 1970). Growth media and basic genetic and biochemical techniques for fission yeast have been described (Alfa et al. 1993). Yeast extract medium YES and synthetic minimal medium EMM₂ were used in growing *S. pombe* cells.

Purification and detection of Spc1HA6H protein

In strain KS1376 the genomic copy of *spc1*⁺ is tagged with a sequence encoding two copies of the HA epitope and six consecutive histidine (Shiozaki and Russell 1995). KS1376 was grown in EMM₂ medium at 30°C to midlog phase. Cells were harvested and washed three times on a filtration apparatus with the same medium but lacking the nitrogen source NH₄Cl (EMM₂-N), then reinoculated in EMM₂-N at 30°C. Aliquots of cells were harvested by filtration and Spc1HA6H protein was purified on Ni²⁺-NTA-agarose beads (Qiagen Inc.) as described (Shiozaki and Russell 1995). Immunoblotting was performed using anti-HA (12CA5) and anti-phosphotyrosine (4G10, Upstate Biotechnology) monoclonal antibodies.

Isolation and disruption of *atf1*⁺ and *atf21*⁺ genes

Strain KS1440 was transformed with a *S. pombe* genomic library in pUR19 (Barbet et al. 1992) and a *S. pombe* cDNA library constructed using the pREP3 vector in which expression of insert cDNA is regulated by the *nmt1* promoter (Maundrell 1993). Transformants were plated onto EMM₂ (3% glucose) agar plates, and then replica-plated onto 1% glucose EMM₂ plates. Cells transformed with the cDNA library were plated on EMM₂ supplemented with 1 μ M thiamine. After incubation at 26°C for 4 days, sporulating colonies were identified by staining with iodine vapor. Three of ~70,000 cDNA library transformants and two of ~50,000 genomic library transformants showed plasmid-dependent sporulation. Recovered plasmids were analyzed by PCR and DNA hybridization. Plasmids p2B from the cDNA

Table 1. *S. pombe* strains used in this study (all strains are leu1-32 ura4-D18)

Strains	Genotype	Source or reference
PR109	<i>h</i> ⁻	Lab stock
PR636	<i>h</i> ⁹⁰	Lab stock
CHP428	<i>h</i> ⁺ <i>his7-366 ade6-M210</i>	C.S. Hoffman (Boston College, Chestnut Hill, MA)
CHP429	<i>h</i> ⁻ <i>his7-366 ade6-M216</i>	C.S. Hoffman
GL84	<i>h</i> ⁺ <i>cdc25-22</i>	Lab stock
LW117	<i>h</i> ⁻ <i>nim1HA6H(ura4⁺)</i>	L. Wu and P. Russell, unpubl.
FY144	<i>h</i> ⁻ <i>pat1-114</i>	S.L. Forsburg (Salk Institute, La Jolla, CA)
JM544	<i>h</i> ⁻ <i>wis1::ura4⁺</i>	Lab stock
KS1172	<i>h</i> ⁻ <i>pat1-114 spc1-M13</i>	This study
KS1310	<i>h</i> ⁻ <i>spc1-M13</i>	Shiozaki and Russell 1995
KS1366	<i>h</i> ⁻ <i>spc1::ura4⁺</i>	Shiozaki and Russell 1995
KS1374	<i>h</i> ⁺ <i>spc1-M13 cdc25-22 wee1-50</i>	Shiozaki and Russell 1995
KS1376	<i>h</i> ⁻ <i>spc1HA6H(ura4⁺)</i>	Shiozaki and Russell 1995
KS1440	<i>h</i> ⁹⁰ <i>spc1-M13</i>	This study
OM1442	<i>h</i> ⁻ <i>HA-cig2⁺</i>	Mondesert et al. 1996
KS1479	<i>h</i> ⁻ <i>atf1HA6H(ura4⁺)</i>	This study
KS1497	<i>h</i> ⁻ <i>atf1::ura4⁺</i>	This study
KS1501	<i>h</i> ⁹⁰ <i>atf1::ura4⁺</i>	This study
KS1523	<i>h</i> ⁻ <i>atf1::ura4⁺ cdc25-22</i>	This study
KS1533	<i>h</i> ⁻ <i>atf1::ura4⁺ spc1::ura4⁺</i>	This study
KS1542	<i>h</i> ⁻ <i>atf1::ura4⁺ pat1-114</i>	This study
KS1557	<i>h</i> ⁻ <i>atf21::ura4⁺</i>	This study
KS1562	<i>h</i> ⁺ <i>his7-366 ade6-M210 atf1::ura4⁺</i>	This study
KS1565	<i>h</i> ⁻ <i>atf1::ura4⁺ atf21::ura4⁺</i>	This study
KS1570	<i>h</i> ⁻ <i>his7-366 ade6-M216 atf1::ura4⁺</i>	This study
KS1572	<i>h</i> ⁻ <i>spc1::ura4⁺ atf1HA6H (ura4⁺)</i>	This study
KS1575	<i>h</i> ⁺ <i>his7-366 ade6-M210 spc1::ura4⁺</i>	This study
KS1578	<i>h</i> ⁻ <i>his7-366 ade6-M216 spc1::ura4⁺</i>	This study
KS1579	<i>h</i> ⁹⁰ <i>atf21::ura4⁺</i>	This study
LW1585	<i>h</i> ⁻ <i>wee1HA</i>	L. Wu and P. Russell, unpubl.

library and p4U from the genomic library, which did not hybridize to *spc1⁺* probe, were subjected to further analysis. Genomic clone of *atf1⁺* (p5A) was isolated from the genomic library in pUR19 by hybridization screening using a 1.2-kb insert of p2B as a probe. For the *atf1⁺* and *atf21⁺* gene disruption, a diploid strain constructed from CHP428 and CHP429 was transformed with the construct shown in Figure 3 carrying the *ura4⁺* marker gene [Grimm et al. 1988]. Stable Ura⁺ transformants were selected and disruption of one of *atf1⁺* or *atf21⁺* loci in the diploids was confirmed by Southern hybridization. After sporulation, haploid Ura⁺ segregants were back-crossed with a wild-type strain (PR109) and used for further experiments.

Northern blotting

RNA isolation and detection of *gpd1⁺* and *pyp2⁺* mRNA by hybridization have been described [Degols et al. 1996]. The *ste11⁺* coding sequence was amplified by PCR using *S. pombe* genomic DNA as a template with a pair of primers XH-ST11 (5'-CCGCTCGAGTTCTTTAACAGCCGAGCAA-3') and ST11-NT (5'-TTGCGGCCGCCAAATAAATTAGAAATGGGC-3'). This PCR product was cloned into pCRII and used as a probe. The *atf1⁺* cDNA clone p2B was used as a probe to detect *atf1⁺* mRNA. Quantification of the results was performed using PhosphorImager [Molecular Dynamics].

Meiotic assay of diploid strains

Diploid strains were constructed by interrupted mating of wild-type CHP428 and CHP429, the isogenic Δ *atf1* derivatives

KS1562 and KS1570, and the isogenic Δ *spc1* derivatives KS1575 and KS1578. Diploids were maintained by selection for adenine prototrophy generated by complementation between *ade6-M210* and *ade6-M216* alleles. Meiosis was induced by incubating diploid cells on sporulation medium malt extract for 2 days at 30°C. Cells were stained by 4',6-diamidino-2-phenylindole (DAPI) and observed under the fluorescence microscope. Frequency of meiosis/sporulation was calculated according to a previously described equation [Kunitomo et al. 1995].

Expression of Atf1HA6H protein

The ~0.6-kb sequence encoding the carboxyl terminus of Atf1 (residues 363–566) was amplified by PCR with 5' primers NDE-363 (5'-GGAATTCCATATGCCATCTGTTTACGGCGATAC-3', *NdeI* restriction site is underlined) and 2B-NOT (5'-TAGTTTAGCGGCCGCGTACCCTAAATTGATTCTTTGAGC-3', *NotI* restriction site is underlined). After digesting with *NdeI* and *NotI*, this fragment was cloned into pRIP42-HA6H, a derivative of pREP1-HA6H [Wu and Russell 1993] to add a carboxy-terminal tag of two copies of HA epitope and six consecutive histidine residues. The resultant plasmid was digested by *PstI* and *NdeI* and religated to eliminate the *nmt1* promoter sequence, then used for transformation of wild-type *S. pombe* strain (PR109) after being linearized at the *EcoRI* site in *atf1⁺*. Stable Ura⁺ transformants were selected and integration of the plasmid at *atf1⁺* locus was confirmed by Southern hybridization. The Atf1HA6H construct is expressed from *atf1⁺* promoter. Atf1HA6H was purified and detected by the procedures used for Spc1HA6H (see above). Phosphatase treatment of

Atf1HA protein was performed with CIP (Boehringer Mannheim) following a published procedure (Lanker et al. 1996).

Expression of GST-Atf1 fusion protein

The *atf1*⁺ ORF was amplified by PCR with primers NDE-S1 (5'-GGAATTCCATATGTCCTCCCGTCAATACTTCCAC-3', *Nde*I restriction site is underlined) and A1-NOT (5'-TAGTTTAGCGGCCGCTCATCTAGTACCCTAAATTGATTC-3', *Not*I restriction site is underlined) and cloned into the *Nde*I-*Not*I sites of pREP1-KZ, which expresses GST fusion constructs from the *nmt1* promoter. The resultant plasmid pREP1-KZ-*atf1*⁺ and a control plasmid pJL205 (Leatherwood et al. 1996), which expresses unfused GST from the *nmt1* promoter, were used to transform strain KS1376, which expresses Spc1HA6H (Shiozaki and Russell 1995). Plasmids pREP1-KZ-*atf1*⁺ and pJL205 were also transformed into strain LW1585, which expresses Wee1 kinase tagged with three copies of the HA epitope; strain LW117, which expresses Nim1 kinase tagged with two copies of HA and six histidine residues; and strain OM1442, which expresses Cig2 B-type cyclin with a tag of three copies of HA (Mondesert et al. 1996). Expression from the *nmt1* promoter was induced by thiamine depletion. GST and GST-Atf1 proteins were precipitated using glutathione (GSH)-Seph-
 arose following the procedures described previously (Shiozaki and Russell 1995). Proteins bound to the Sepharose beads were analyzed by immunoblotting with anti-GST and anti-HA antibodies.

In vitro phosphorylation of GST-Atf1

GST-Atf1 protein from strains KS1376 [*spc1HA6H(ura4*⁺)] and KS1366 (*Δspc1*) incubated in a medium containing 0.6 M KCl for 10 min was recovered on GSH-Sepharose beads as described above and washed three times in KA buffer [25 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, 0.1 mM EGTA, 0.1 mM Na₃VO₄, 1 mM DTT]. The phosphorylation reaction by the coprecipitated protein kinase was performed in the same buffer containing 50 μM [γ -³²P]ATP for 10 min at 25°C. For in vitro phosphorylation of Atf1 by purified Spc1 kinase, GST-Atf1 was purified by GSH-Sepharose precipitation from strain KS1366 and subjected to three successive treatments by 1 mM FSBA at 30°C for 15 min in KA buffer without dithiothreitol (DTT). The Sepharose beads were then washed three times with KA buffer to eliminate the FSBA. GST-Spc1 protein expressed in wild-type PR109 strain (Shiozaki and Russell 1995) was purified on GSH-Sepharose beads and added to FSBA-treated GST-Atf1. The mixture was incubated at 30°C for 20 min in KA buffer supplemented with 5 mM glutathione and 50 μM [γ -³²P]ATP. Phosphorylation of GST-Atf1 was analyzed by SDS-PAGE and the ³²P-labeled GST-Atf1 bands were excised after autoradiography. Tryptic peptide mapping was performed as described (Boyle et al. 1991) using pH 1.9 buffer for the first dimension electrophoresis and phosphochromatography buffer for the second dimension ascending chromatography.

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Note added in proof

The *atf1*⁺ gene was also recently independently identified as *gad7*⁺ by Kanoh et al. (1996).

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K Shiozaki and P Russell

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