The Fission Yeast Mitotic Regulator *win1*⁺ Encodes an MAP Kinase Kinase Kinase That Phosphorylates and Activates Wis1 MAP Kinase Kinase in Response to High Osmolarity

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The *Schizosaccharomyces pombe win1-1* mutant has a defect in the G2-M transition of the cell cycle. Although the defect is suppressed by *wis1*⁺ and *wis4*⁺, which are components of a stress-activated MAP kinase pathway that links stress response and cell cycle control, the molecular identity of Win1 has not been known. We show here that *win1*⁺ encodes a polypeptide of 1436 residues with an apparent molecular size of 180 kDa and demonstrate that Win1 is a MAP kinase kinase kinase that phosphorylates and activates Wis1. Despite extensive similarities between Win1 and Wis4, the two MAP kinase kinase kinases have distinct functions. Wis4 is able to compensate for loss of Win1 only under unstressed conditions to maintain basal Wis1 activity, but it fails to suppress the osmo-signaling defect conferred by *win1* mutations. The *win1-1* mutation is a spontaneous duplication of 16 nucleotides, which leads to a frameshift and production of a truncated protein lacking the kinase domain. We discuss the cell cycle phenotype of the *win1-1 cdc25-22 wee1-50* mutant and its suppression by *wis* genes.

INTRODUCTION

Investigations on the fission yeast *Schizosaccharomyces pombe* have been central in unraveling the mechanisms that regulate cell cycle transitions, in particular entry into mitosis from G2. Genetical analysis led to the identification of Cdc2, and of its regulators Cdc25 and Wee1 as key players in G2-M regulation. Further investigations have led to the identification of several other genes whose products play a rate-limiting role in the transition into mitosis (MacNeill and Fantes, 1995).

The *S. pombe win1-1* mutant was isolated as a mutation that reverses the effect of *wee1-50* in suppressing the temperature-sensitive cell cycle arrest of *cdc25-22* (Ogden and Fantes, 1986). Among five *wis* genes isolated as multicopy suppressors of the *cdc* phenotype of the triple mutant *win1-1 cdc25-22 wee1-50* (Warbrick and Fantes, 1992), *wis1*⁺ and *wis4*⁺ encode a MAP kinase kinase (MAPKK) and a MAP kinase kinase kinase (MAPKKK), respectively (Warbrick and Fantes, 1991; Samejima *et al.*, 1997). Identification of the *win1*⁺ gene is crucial for understanding how these genes affect the cell cycle phenotype of *win1-1 cdc25-22 wee1-50*. Several attempts to clone the *win1*⁺ gene have been made, but its molecular identity remains unknown (Warbrick and Fantes, 1992).

In addition to their cell cycle effects, Wis1 and Wis4 are components of a stress-responsive MAP kinase signaling pathway. Similar pathways exist in a range of eukaryotic cell types (Brewster *et al.*, 1993; Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994; Millar *et al.*,

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1995; Shiozaki and Russell, 1995). In *S. pombe*, the Wis1 MAPKK is activated in response to osmotic and other types of stress, resulting in increased phosphorylation of the MAP kinase homologue Spc1 (= Sty1 = Phh1) (Millar *et al.*, 1995; Shiozaki and Russell, 1995; Kato *et al.*, 1996). *wis1*⁺ and *spc1*⁺ are essential for survival under conditions of extreme heat, osmolarity, oxidation, or poor nutrition. Wis4 (= Wik1 = Wak1) is a MAPKKK that phosphorylates and activates Wis1; however, the phenotype of *wis4Δ* mutants is not as severe as those of *wis1* deletion strains (Samejima *et al.*, 1997; Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). Activation of Wis1 is observed in *wis4* deletion strains after exposure to high osmolarity, although activation of Wis1 under these conditions requires phosphorylation of Wis1 at the conserved Ser⁴⁶⁹ and Thr⁴⁷³ residues (Samejima *et al.*, 1997). These observations suggest that Wis1 is activated by other MAPKKK(s) in response to osmotic stress, and that Wis4 is therefore one of several redundant MAPKKKs that function upstream of Wis1. Genetic evidence suggests that Win1 is also an activator of Wis1, which functions in parallel with Wis4 and plays a major role in osmostress signaling (Samejima *et al.*, 1997).

Here we identify an MAPKKK structurally homologous to Wis4 and present evidence that it is encoded by *win1*⁺. We show that Win1 activates Wis1 in vivo and in vitro and is a major transducer of osmostress signaling; cells lacking Win1 function show a reduced basal level of Wis1 activity, and their ability to activate Wis1 in response to osmotic stress is severely compromised. *win1-1* is a frameshift mutation, and the mutant gene product is predicted to lack the kinase domain.

MATERIALS AND METHODS

Strains and Media

Standard techniques for handling *S. pombe* are described elsewhere (Moreno *et al.*, 1991; Alfa *et al.*, 1993). *S. pombe* cells were grown in YE (yeast extract medium) or EMM2 (minimal medium) (Alfa *et al.*, 1993). All of the strains used for phosphotyrosine assay carried the tagged *spc1* allele derived from KS1376 (Shiozaki and Russell, 1995).

PCR Primers

For expression of the catalytic domain of the Win1 protein kinase in *S. pombe*, the following sequences were used to design PCR primers to clone the last 400 aa of the gene, which was expressed from the pREP1 *nmt1* promoter: 5'-GTCGACGTAGTATGGACCAACAA-3' and 5'-AGATCTAATGGTGATGGTGATGGTGGCGGCCGCCAA-GTTCCAACGGAGCACCAT-3'.

Physical Mapping of *win1*⁺

One plasmid that contained the *tps19*⁺ gene was isolated by complementation of the temperature-sensitive (ts) growth phenotype of the *tps19* mutant. A 5-kb *Bam*HI fragment from the plasmid was used to screen a P1 phage library (Hoeheisel *et al.*, 1993) and cosmid libraries (Hoeheisel *et al.*, 1993; Mizukami *et al.*, 1993) by hybridization. For selection of *S. pombe* transformants carrying P1 phage and cosmids the *ura4*⁺ gene and *ars1* sequences were first introduced by

a method that exploits a bacterial transposon (Morgan *et al.*, 1996). The *ura4*⁺-*ars1*-tagged phage or cosmids were used to transform the *win1-1 cdc25-22 wee1-50* mutant to score their ability to suppress the ts phenotype.

Genetic Mapping of *win1-1*

The *win1-1* locus was mapped on chromosome I by mitotic haploidization of the diploid strain *win1-1*⁺/*ura1-131*⁺/*lys1-171*⁺/*leu1-32*⁺/*ade6-704*⁺/*mat2-102*/h- (Alfa *et al.*, 1993). Preliminary mapping of *win1-1* within chromosome I was carried out by random spore analysis in *swi5* background (Schmidt *et al.*, 1987), followed by fine mapping by tetrad analysis with markers on the short arm of chromosome I. The relative positions of *win1-1*, *tps19*, and *rad1-1* were determined by three-point cross-analysis carried out on random spore colonies.

Purification of Win1 Kinases

Win1 proteins tagged with 6× histidine were expressed from the pREP1 *nmt1* promoter for 12 h at 30°C in the *wis1Δ* strain. *S. pombe* cells were disrupted by glass beads in lysis buffer (50 mM Tris-HCl, pH 8.0, 0.4 M NaCl, 10% glycerol, 10 mM 2-mercaptoethanol, 1 mM PMSF). The cleared lysate was incubated with Ni-NTA-Sepharose beads (Qiagen, Chatsworth, CA) in the presence of 0.1% NP-40 and 20 mM imidazole. The tagged proteins were eluted by the same buffer containing 250 mM imidazole and dialyzed against storage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20% glycerol).

Coupled Kinase Assay

Recombinant GST-Wis1(K349R) (0.16 μg) purified from *Escherichia coli* was incubated in buffer containing 25 μM ATP, with Win1ΔN or Win1ΔN(K1149R) purified from *S. pombe* equivalent to 2 × 10⁷ cells. After 10 min incubation at 30°C, 1.3 μg of catalytically inactive MAPK substrate was added in the presence of [γ-³²P]ATP (0.2 μCi/μl). The mixture was incubated for an additional 10 min at 30°C, and the reactions were stopped by addition of 3× SDS loading buffer. The MAPK substrate GST-Spc1 was purified from an *S. pombe wis1Δ* strain, essentially according to the method of Shiozaki and Russell (1997). The method for purifying GST-Wis1 from *E. coli* is described by Samejima *et al.* (1997).

Phosphotyrosine Assay

S. pombe cells were collected by filtration. Cells were washed once with STOP buffer (50 mM NaF, 100 mM NaCl, 10 mM EDTA, 1 mM NaN₃) before being frozen in liquid nitrogen. Spc1 protein was isolated on Ni-NTA beads (Qiagen) in denaturing buffer (6 M guanidine HCl, 0.1 M Na phosphate, 0.1 M Tris-HCl, pH 8.0) as described by Shiozaki and Russell (1995). Spc1 and phosphotyrosine were detected by Western blotting by ECL (Amersham, Buckinghamshire, UK). The primary antibodies used to detect the hemagglutinin (HA) epitope and phosphotyrosine were 12CA5 (Boehringer Mannheim, Indianapolis, IN) and 4G10 (Upstate Biotechnology, Lake Placid, NY), respectively.

RESULTS

Identification of a Candidate Gene for *win1*⁺

Because attempts to clone the *win1*⁺ gene by complementation were unsuccessful (Warbrick and Fantes, 1992), we decided to clone the *win1*⁺ gene by positional cloning. *win1-1* was mapped on chromosome I, 5 cM from the *tps19* locus in the direction of *rad1*. As a preliminary step to position the *win1*⁺ gene on the physical map of the *S. pombe* genome, the *tps19*⁺ gene was cloned and was used as a probe to screen ordered

cosmid libraries and a P1 phage library (Hoheisel *et al.*, 1993; Mizukami *et al.*, 1993). 3F9 is one of the positive clones in the P1 phage library, and subsequent analysis revealed that it contains the *tps19⁺* gene at the very end of the insert, which extends in the direction of the *rad1* locus (Figure 1A). There is a single region within 3F9 not represented by cosmids from either library. 3F9 suppressed the ts phenotype of the *win1-1 cdc25-22 wee1-50* triple mutant, whereas none of the cosmids tested did (Figure 1A), suggesting that the gene responsible for the suppression, or a part of it, lay in the "gap" region. The DNA of the gap region was recovered by PCR amplification, and its DNA sequence was determined. A single ORF spans the gap, suggesting that it is the *win1⁺* gene. The ORF encodes a polypeptide of 1436 amino acid residues with a protein kinase domain at positions 1127–1410 (Figure 1B). The kinase domain of the Win1 protein is most similar to *S. pombe* Wis4 and *Saccharomyces cerevisiae* SSK2 and SSK22; within the region, 163 of 291 aa residues (56%) are identical to Wis4. The homology extends beyond the kinase domain throughout the protein, apart from the first ~180 residues, where we find no homology to any protein in current databases.

Detection of Full-Length Win1 Protein

With the aim of identifying the full-size *win1⁺* gene product, we constructed a strain in which the chromosomal *win1⁺* gene was modified such that the Win1 protein was tagged with the HA epitope at the carboxyl terminus (Figure 2A). We confirmed by Southern analysis that the construct had integrated at the intended locus. A polypeptide of 180 kDa was detected by the anti-HA antibody (12CA5) in the cell extract of the tagged strain (Figure 2B), whereas no signal was detected in the cell extract of a parental *win1Δ* or untagged wild-type strain.

Win1 Phosphorylates and Activates Wis1 In Vitro

The amino acid sequence similarity of Win1 to MAPKKKs, in particular to Wis4, suggests that Win1 is a protein kinase that phosphorylates Wis1. To test this, we expressed an amino-terminal truncated Win1 protein, Win1ΔN, containing the catalytic domain, in *S. pombe*. We affinity purified the protein and tested whether it could phosphorylate recombinant GST-Wis1(K349R) in vitro (Figure 3A). This substrate purified from bacteria does not autophosphorylate because of a mutation in the conserved lysine residue essential for ATP binding (Figure 3A, lane 5). The GST-Wis1(K349R) was phosphorylated when incubated with the wild-type Win1ΔN. Incubation with the catalytically defective mutant protein Win1ΔN(K1149R), which has the conserved lysine residue in the ATP binding domain mutated to arginine, had a much reduced effect.

We then tested whether this phosphorylation by Win1 led to activation of Wis1 kinase activity. GST-Spc1 was expressed in an *S. pombe wis1Δ* strain, and the purified protein was used as a substrate to assay Wis1 kinase activity in vitro. GST-Wis1 protein produced in bacteria shows very low kinase activity (Figure 3B, lane 1). Wis1 kinase activity was greatly enhanced after preincubation with Win1 protein in the presence of ATP (Figure 3B, lane 2). The kinase-defective mutant Win1ΔN (K1149R) had a much reduced effect (Figure 3B, lane 3): a likely reason for the difference in Figure 3B between lanes 1 and 3 is a contribution from a minor population of phosphorylated Wis1 molecules (see Figure 3A, lane 2). GST-Wis1(K349R) did not phosphorylate GST-Spc1 under any conditions (Figure 3B, lanes 4 and 5). These observations show that Win1 is able to activate Wis1 in vitro and excludes the possibility that Win1 directly phosphorylates GST-Spc1. These data thus show that phosphorylation by Win1 is sufficient to activate the Wis1 kinase.

Conserved Wis1 Phosphorylation Sites Are Required for Activation by Win1 In Vivo

We asked whether Win1 activates Wis1 in vivo by examining the effect of overexpression of the *win1⁺* gene in *S. pombe*. In many cases, the amino termini of MAPKKK homologues are not essential for their kinase activity, and indeed, the truncated proteins are often more active than their full-length counterparts in vivo (Cairns *et al.*, 1992; Lee and Levin, 1992; Stevenson *et al.*, 1992; Maeda *et al.*, 1995; Samejima *et al.*, 1997; Shiozaki *et al.*, 1997). The carboxyl-terminal 400 residues of Win1, Win1ΔN, which contains the entire catalytic domain, was expressed from the *nmt1* promoter, so that its transcription was regulatable by thiamine (Maundrell, 1990). In vivo Wis1 kinase activity was assayed by the level of phosphotyrosine on Spc1 (Shiozaki and Russell, 1995). Overexpression of *win1ΔN* in wild-type cells resulted in more phosphotyrosine on Spc1, whereas the kinase-defective mutant allele of *win1ΔN* had no such effect (Figure 4A). We detected no phosphotyrosine in *wis1Δ* cells under these conditions, consistent with several reports that *wis1⁺* is essential for tyrosine phosphorylation of Spc1 (Millar *et al.*, 1995; Shiozaki *et al.*, 1995).

MAPKKs have conserved serine/threonine and threonine residues between kinase subdomains VII and VIII, and phosphorylation of these residues plays a central role in the regulation of kinase activity (Alessi *et al.*, 1994; Zheng and Guan 1994). Wis1 has such residues at positions 469 and 473, and they are essential for response to osmotic stress (Samejima *et al.*, 1997). *wis1-4* is an allele that has both Ser⁴⁶⁹ and Thr⁴⁷³ mutated to nonphosphorylatable glutamate residues. Cells carrying this allele are insensitive to overexpression of *win1ΔN*. The phosphotyrosine level on

Spc1 remained at the same level in the *wis1-4* mutant, irrespective of the expression level of *win1* Δ N (Figure 4), suggesting that phosphorylation at Ser⁴⁶⁹ and Thr⁴⁷³ of Wis1 is essential for the action of Win1.

Hyperactivation of Wis1 by phosphorylation is toxic to wild-type cells and inhibits colony formation (Samejima *et al.*, 1997), as is overexpression of the *wis1*⁺ gene (Shiozaki and Russell, 1995). Consistent with the phosphotyrosine data shown above, overexpression of *win1* Δ N was toxic in wild-type cells, and colony formation was inhibited (Figure 4). In contrast, overexpression of mutant *win1* Δ N(K1149R) protein had no such effect, showing that increased kinase activity was responsible for the toxicity. The toxic phenotype was suppressed in *wis1* Δ and *wis1-4* strains, suggesting that Win1 function requires phosphorylation of Wis1 at Ser⁴⁶⁹ and Thr⁴⁷³ (Figure 4). Taken together, these results suggest that Win1 phosphorylates these residues to activate Wis1.

win1-1 Mutation Site Lies in the New MAPKKK Coding Region

We asked whether the DNA from a *win1-1* strain has a mutation in the ORF encoding the new MAPKKK. The ORF region was amplified by PCR from genomic DNA isolated from a *win1-1* strain, and the PCR product was used as a template for DNA sequencing. An insertion of 16 nucleotides was found compared with the wild-type sequence. The wild-type sequence has a pair of repetitive sequences (TATCCTTCA) at positions 1347...1355 and 1364...1372 (Figure 5). In *win1-1*, the heptamer sequence, GTCGTTC, which is flanked by the TATCCTTCA repeat, is duplicated with an extra copy of TATCCTTCA in between (Figure 5). This duplication of 16 nucleotides results in a frameshift that replaces the carboxyl 1002 residues including the kinase domain with 23 irrelevant amino acids (Figure 5). The mutant protein thus has 456 residues in total, with the amino-terminal portion of the original 433 residues. The presence of such a mutation in *win1-1* strain strongly suggests that the ORF is the *win1*⁺ gene. Moreover, the disruption of this ORF produced phenotypes very similar, if not identical, to *win1-1* (see below).

Disruption of the MAPKKK Gene Is Sufficient to Give *win1-1* Phenotype

The chromosomal region that encodes the last 278 residues (1159–1436) of the *win1*⁺ gene was replaced by the *S. pombe* *ura4*⁺ gene, removing most of the protein kinase catalytic domain from the genome. The resulting strain is viable, and in a cross between the deletion strain and *win1-1*, all of the 33 tetrads dissected were parental ditype, confirming very close linkage of the deletion mutation to *win1-1*. The chromosomal deletion is hereafter referred to as *win1* Δ .

Figure 1. (A) An approximate restriction map of 3F9 and selected cosmids from ordered cosmid libraries (Hoheisel *et al.*, 1993; Mizukami *et al.*, 1993). The positions of *Bam*HI sites, particularly those a long distance apart, may not be accurate. (a) Activity of suppression of the *ts* phenotype of the *win1-1 cdc25-22 wee1-50* mutant. (B facing page) Deduced amino acid sequence of the Win1 MAPKKK. The nucleotide sequence is available from the EMBL, GenBank, and DNA Database of Japan databases under the accession number AJ223190. The amino acid sequences of the products of *S. pombe* *wis4*⁺ and *S. cerevisiae* SSK2 and SSK22 genes are aligned with the Win1 sequence. Residues conserved among three or four proteins are indicated in white letters on black, and residues conserved between any pair of proteins are indicated in black letters on gray. The first 83 residues of SSK2 are not shown here.

Several phenotypes conferred by the *win1* Δ mutation were examined and compared with the *win1-1* mutant. Dividing *win1* Δ cells are longer than the wild type during growth on EMM (our unpublished observations), suggesting a mitotic delay as in *win1-1* (Ogden and Fantes, 1986). The double mutant, *cdc25-22 wee1-50* and the *win1-1* single mutant can grow and make colonies at 36°C. However, the combination of the three mutations makes a cell unable to grow at this temperature, and the cell shows a *cdc* phenotype (Ogden and Fantes, 1986). The *win1* Δ mutation had a similar effect, and extremely elongated cells were observed with the triple mutant *win1* Δ *cdc25-22 wee1-50* (our unpublished observations).

Consistent with the demonstration that the Win1 MAPKKK is an activator of Wis1 (Figures 3 and 4), the phosphotyrosine content of Spc1 in *win1* Δ was lower than in the wild-type strain (our unpublished observations), as in *win1-1* (Samejima *et al.*, 1997). Moreover, the toxicity of overexpression of *wis1*⁺ was alleviated in *win1* Δ strain just as in *win1-1* and *wis4* Δ strains (our unpublished observations). The Wis1 kinase is activated when the wild-type cell is exposed to high osmolarity, and its substrate Spc1 is rapidly tyrosine phosphorylated (Millar *et al.*, 1995; Shiozaki and Russell, 1995). On the contrary, the phosphotyrosine level remained low in both *win1* Δ and *win1-1* strains (our unpublished observations; Samejima *et al.*, 1997). This suggests that Win1 is required to activate Wis1 in response to high osmolarity.

B

win1	MENILDPSPVNSHILENGSRSSINPILDSRLDRKTFEKAHRRSLTLLSSFTSSMLELPNNKEENHRRPSVARSSSDRSKASAKEDLFSEAFRMAEQPP	100
wis4	MLEHTFYPAEDRFEPILLEHSEPVNFVKENAKSYVR	37
SSK22	MMDLNQQQKAAGGRVLAPHHTISSKLVK	31
SSK2	QLFNRLPGIPQGGFFHQNAISGSSSSSARSSRRPSNIGLPLKPNPQOSLPKLSTQVPVPHKKVEASKTESEI IKKPAVPVNSNPDLLTPTPLVISPGLAS	183
win1	AEALTISTPVDPINIDELDRAYAVSPSDTSNLLHPTSSSSSIPIPIKNAGHSNLDHPIRPSLQSSISSNRI-----IKSPGKEDDMMHGRSSISSPMI	194
wis4	QGFAHQSLMDNLVDSTESTKRSNFVSHIPLTPSHSGQSEKLMSRTSHSPYISPTMSYTNHSPANLTRNSSFNHQHYSTPLRSPPSMRGRGIDVNS	122
SSK22	RLSSHSS-----HKLSRSDLKALGSETISD-----GPSQLTFKDRVVFNESLYLKKLKKTLADDDYTRGKTLNRYE	99
SSK2	LNTTNTSIMSTPQNITNTQTSKNGHIPTRSQPNGSTSSSTLQDIVTTNNSQSRVSGHHGGSTSLRTYKKQVVLNEQLYLRMRNRANDDYTRGIVASSNFE	283
win1	DVEHNSITAVPSKTKNLPEKPKRSHKLRNSITFAKMKIILKRKSQRLRLSSSLKCFDEPYDNDPSLSIRRDSSTYYFSNVNETYDEEDSDLDSETSTVN	294
wis4	HYPHI-----SRPRTSSDSQKMYTRAPVDVYYYIQENPYFNNIDQDSISDKSLPSTNQSLHSEEDTESDNDFSSEIHPEFD	198
SSK22	EDDGD-----DEIIRLSNGDRIDEDLHSGVKFFSTTPYCKRMRS-----	137
SSK2	DDEENFSNGKEDDLELMDLLKVEGEDK-DNDFNFGYNFITSSTKNNENVMSLNLYLKGKLDWLRDNNNDQPCETEDEEWSHILGSED-----	372
win1	WQSVNLNPLSLSDDDMAVPPKNKERFEWQYMLTSLVIGDIVRSEKLRRLKRIASSREGNSDYSENLAWEIWNLTTHRSVDSYRENLKHLRTGMVDVLAH	394
wis4	IDVFFYKVNILYDESLOQPEKKRERLEWHSMSSVLKGDVMQTEKRRRLTEPDGHSQTY-IS-EVQLGLQAWHCHGLNADQAEVRKISREGVEPVEREV	296
SSK22	-----DSDELAWNIEATERFKWQSMRLARVLKGDIVKGEKRTANQVKKPLNKE-LSDEHMLKAWLNKRTMQEMEQSLTYLSDSSDSVEBEI	214
SSK2	-----LLSKLEQNPVMNRFPEWQYMLSKVLKGDIVRNEKRTANQVKGKPGFNTQ-FSDIMIELKAWNNGRTVEDQNKSLRIFSDSTDSVEQEI	460
win1	MNHWDDES--NELTPIVAVDNL--QRLDKYERLYSRSSILQEHSLYASESFQHKDVLITAYSNVTHALEIQVNIIRSVVGNEMDITKNTTN--SINNV	489
wis4	IDFQIQDE--ETTTPLPEQVTEILERVEQCKOFYISSREBENVPLSASKFEYKLNALISWSNVMSIQVETVLVLRQWGNDFDLTMRTP--QFNVD	391
SSK22	MKQTPQKGLSLDALAEALQDMMNRVHVSVSYWENLKKYKDKPITNTAFTARTIVMNSWLNFKTNLTLRQQLDDMNKRFSPISSSDNC--QEDFD	323
SSK2	MASKLEDNM-SADAAETIK-SIVDYYRVLNLMNIRKHAETIKTKTEARNRRLTILNSMLKFKNFDTNIAIYKKIVGNKELESTTEVDNTTVNL	558
win1	SQISNGPVEFRFYRETGLIRAREORI---MTNMNVSLSVNCNTIVTVADDLSYGLPLIADDYMRLLSFPRLIKFEFLNRLSACENITSISLPTISL	585
wis4	LDDFKIALSVAVRIKREYIKTASPSGWSLPTNVIEDYDNLVLDSEKFEYKRLTLRLSSGNKL--YKKEIFLENENAFINEHIYWGNGDIHMAQGS	487
SSK22	GVPOWNCMKMLIAEOLMKKEKNIBSFQKKIFVPLSPWMLKLLHFIIVYRETLTKMKIKYPYERLSRLAFFPYLHKVILTRLSYARKLNKPTMMIDCM	408
SSK2	DPAVFATNCKRFAEQIMKEKIDELIFQKKIFFPLAPVILKARFFLLKYQKIMWELNLSYLDQDEFLMLFPMRLVQDILILRLSYARKIIONPTLMMIDCM	658
win1	LDDLNTMKVAVHIIQOHTVLKIPFRDSDKFDENOSNLNVLVASEKFEYNLHRRVNRNGCALL--HFKEITELEGMBDFLAVCPHEHGFQIMSKSL	683
wis4	LDDFKIALSVAVRIKREYIKTASPSGWSLPTNVIEDYDNLVLDSEKFEYKRLTLRLSSGNKL--YKKEIFLENENAFINEHIYWGNGDIHMAQGS	585
SSK22	IDDFNAFTRLSVQLRYTLTKYCSNLPF---DDDFPTPEFNVIEAIRKLEFLNLLIDSSKQ---NRAPELILKYMDHLKNTGHYLNGAETVYINBEL	502
SSK2	MDFFSTYIKLAVQMKPTVASVCNDWFF---KKKIPPEFDHTVVEGLKFFYSIIEELIYSGKNSFKTSKEPELLIKKYMEMFRNVYVDDAGELIAABET	755
win1	SLVGEILTNINRLKDLQOGEDTDDSLIT-SFYKVLDCVRIRPKMSTRIKHAHLESCENYIKENSISLQIRLEESNMVLTITAS-TEHBCAV	766
wis4	YLSNLSLLNVRHVESHNGERTERTASLT-NMYSTLLKNTQIRPKRLLRSESTNSRENASDPVISEGHLPDVRNRTSTGHFLAYTAN-LERDGVFV	684
SSK22	KLFLRLVFKLOFYLLKQCFEPTFANASEAKRLSSIFENLGAEMKRLKIRNSNLLVKARONSAYVCIN--HNAQLVKKLDAHYPLVMSGNTPESSGVVM	600
SSK2	KLFLRLVHRLHMLLQOCTEPEKLENEAAEKMLVQITEILGSMKREKLNRTNLTKARONFVRKYIE--DHNVLKQLKEGHILITVQGYTEQNGTYL	853
win1	IYP-----<-----GHLVDSNLLREVLISM	800
wis4	IAD-----<-----HTLSENEALKALFS	717
SSK22	FAAPPELLGCDNDITLILRLNKSIGCDLVPKLIGNNENVYDITTKETDLNLLVSKGDESKGIPYRVVANSSSLLDRHAHQSKKNFSTDFQHLDEKN	715
SSK2	IGSPPELLGCKDDITLITKNSDIGCDLVPKLEINNSLTIYNALDDNWSNSSLGSDISNDGTPFY---YIKNDLTQPSYNGNRVNRNEDFENSRSSTE	949
win1	TFNKGDNNFESVPPYAVVLADSSICNGHVTDLIDIEV---SISIAP---NCVREVTLATANQSVIEEYFISVYDVTSLVDSAKANSKINKQMT	892
wis4	KDINLEFIQONCSYVLIICPGHETVAKRIEKVVDVDF---SVLLKT---NVRVITIASNKRHELOAKSVQFOSGLVLAVFCRSSITRVYKFEI	809
SSK22	KFLFLVLEVALSSGLAVLYPGEVVDGCVYKPGNNLFPASNEMLKGIKNPNTLVKARONSAYVCIN--HNAQLVKKLDAHYPLVMSGNTPESSGVVM	814
SSK2	EEFYBLETRLNSGLVVLITQEBLLIESEMNLSDNRTIKPEGLNKVYI---PNSIDLMCGSSYALVYQDRFQCGSSVSF-LEKKSSETVYKNLQ	1046
win1	KIKRNSLKLALSLLD---VIQITRTYHEMNCQNLHYSESYALIEFAORLMRLSILDASSIGLIRKRIOLALISAVGFIYEDCSEPTDRNFFRWYVTALE	988
wis4	RLSNLCMRISSTVVD---CVSAVREACSVNCHDIYHYVFSAAAEFORILFLSFDPSYWTCLKRKITSIAVENSISFCDECPDLMORKFFRWGVALF	905
SSK22	KINKAYYKLYTVLNNYKGLGFSFMKQCPG---NELNLSFPMGRDFORSELKYNAFSSKRYVLIIFLVKLGMMALKPLVSECDPTDORFTRWCVLMD	911
SSK2	RINKAYFRCTYSVLEKNTKIVITTEKVSVP---NDLNNHFLGGRDFGLNRLIRIVVANNEKRSITILLMLRISLSCMLKFLABECDPTDORFTRWCVLMD	1143
win1	FAMIMTYSNLLMDKKSEELKEKVGKCVALLLHFDVMGTKHAGRSMDQAGDIPARLVNNSDRSRLSDNELASFVKEEVMEHRIIELESNRDRLYK	1088
wis4	FLMLTRONNILLIDAMLEKIREKVGKSAFLLTHFDVVGAKSKVAQKLRSETEVSSSPRLTSFGDVEEALSQIQLOKETMIRIDELEIBERNNTLE	1005
SSK22	FAMOMTSYNNILALNVKQOELKERVSVCSLLISHFDVMGARAT-----BAENGMOQARLNI--DDEENIDDEEATLEINSRLRLEAKTLEKTMKR	1002
SSK2	FAMHVSQNNILALDECOBSSEKRISECMSSLLISHFTIIGARST-----EVEKINQOARSNL--DDEVDVDDMMQVNSEFRVQSIMLEBERKRN	1234
win1	SQILGRVLDDITKENRILKELASSKSNITTRWQGGILGSGSPGVYRAVNLTGDLMAVKEVALKPRISRPMIKRIKCEMLVLELFDHPNVVSYGVE	1105
wis4	RLAIGHVLDDSVFRNRDFTKLASSFSNITTRWQGGHVFYRSGMFGDYYTGVNMTGDLAVKEIKLOSRIFRSTVQDHNEMITVLERLNHPNVVYGYVE	1102
SSK22	PROMKRVLDATDQCKNYLLSLASSLNVSMRWQKRSFICGGTFCGVYSAINLENGSILAVKEIKIHTTTIMKKIFPLIKBEMTVLEMNHPNVVYGYVE	1334
SSK2	PHOTERVLDSDCKNYVLSLASSLNVSMRWQKRNFIQGTTEGRVYSAVDLNGSILAVKEIKIHTTTIMKKIFPLIKBEMSVLELNLNHPNVVYGYVE	1334
win1	VHREKVNIFMELCQSSLEFEFTRYGRIEDELMOVYVQLLEGLAYITHSCVSHQDVKPENILLDHNGIMRFDPGSAKMSGASAKIFEQLTQOESEEF	1288
wis4	VHREKVNIFMELCQSSLEADLLAHGRIEDENLVYVQLLEGLAYITHSHILHRDHKFNILLDHRCMIKYSDFCGALYVSPPTDPEVRYEDIOPE---	1202
SSK22	VHREKVNIFMELCQSSLASLLDHGRIEDENLVYVQLLEGLAYIHSQSVVHRDHKFNILLDNGHLYKYVDGRTARTVVGSRRTVNRNAVQDFGVE	1202
SSK2	VHREKVNIFMELCQSSLAALLDHGRIEDENLVYVQLLEGLAYIHSQSVVHRDHKFNILLDNGHLYKYVDGRTARTVVGSRRTVNRNAVQDFGVE	1434
win1	EKDSEFLQHLQDNR-----GYSLGTFTYMAPEITLGNP-SERVGAMQVSLGCVLEVMTGSPFPRLLDNHFSIMYHIAAHNPETIADQLSPLCQNF	1382
wis4	QLHLAGTPMYMAPEITLGTG-KGDFGAMQVSLGCVLEVMTGSPFSEMDNEWAIMYHVAAMHTSIPQNEKITSSEARDF	1280
SSK22	TKS-----LNMEMTPMYMAPEITHSGSAVKGLGADVWALGCVLEVMTGSPFSEMDNEWAIMYHVAAGRIPQLENREEMTAAGRAF	1286
SSK2	HEDVTHVSDSKAVKNNENALLDMGTPMYMAPESITGSTITKGLGADVWALGCVLEVMTGSPFSEMDNEWAIMYHVAAGHTIQFETKREVESAGMKF	1534
win1	LKRCFVSDPNORAPAELEMDPMVYPLRAGTEFDLMNSSSVESAPSTNGAPLEL•	1436
wis4	IEQCFERDEQRRAVELLTHPMITDERKKTIITMPATIKKTLSTHTEEKTAQLLAGRHVDSKAETDSLAASYKEESALPVASNVGLRQPNELRID	1380
SSK22	LERCLVQDPTMRRAVELLIDPMIQTREIAPGNSEKQDVPILSS•	1314
SSK2	LERCLIQNSRRASAVELLEMDPMVQIREIAPGDDSSSTDEERE•	1579
wis4	SINLPPAIVTPDTINYSD•	1399

Figure 1B.

Figure 2. Detection of the full-length *win1*⁺ gene product. (A) Strategy for constructing a strain carrying tagged Win1 (ED1423) by homologous recombination. The *win1*⁺ gene in the haploid wild-type strain was disrupted by the *S. pombe ura4*⁺ gene to generate a *win1*Δ strain (ED1370). Then the *ura4*⁺ marker of the deletion strain was replaced by a sequence encoding the tagged carboxyl-terminal domain of Win1, using 5-fluoro-orotic acid selection (Grimm *et al.*, 1998). The shaded box indicates the kinase domain. (B) Western blot of *S. pombe* cell extract from ED1423. Anti-HA antibody (12CA5) was used as the primary antibody.

Win1 and Wis4 Have Distinct Biological Roles in Stress Response

Previous reports have demonstrated that Wis4 phosphorylates and activates Wis1. However, the question of whether Wis4 transmits an osmotic stress signal to Wis1 is controversial, and the physiological function of Wis4 is not established (Samejima *et al.*, 1997; Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). We tested whether introduction of multiple copies of the *wis4*⁺ gene could suppress the phenotypes of *win1-1* observed when the strain is challenged by high osmolarity.

Wild-type cells undergo morphological changes on a high-osmolarity medium, just as when the *wis1*⁺ gene is overexpressed from a strong promoter or when *wis1* is activated by ectopic overexpression of a truncated MAPKKK gene (Shiozaki and Russell, 1995; Samejima *et al.*, 1997). Wild-type cells are short and swollen on medium containing 1.2 M KCl, whereas *win1-1* cells are more elongated than on normal medium (Samejima *et al.*, 1997; Figure 6A). The *win1-1* strain carrying a *wis4*⁺ plasmid appears more similar to *win1-1* than to the wild type on high-osmolarity medium, in that it retains the same width as on EMM and cell length is greater than on the control medium (Figure 6A). *win1-1* cells carrying multiple copies of *wis4*⁺ are shorter than the control *win1-1* cells in both conditions (with or without KCl). Thus a single copy of the *win1*⁺ gene (in wild-type cells) allows morphological change in response to high osmotic conditions. However, in the absence of *win1*⁺ function, even the presence of multiple copies of *wis4*⁺ does not restore this ability.

Figure 3. Win1 phosphorylates and activates Wis1 in vitro. (A) Win1 phosphorylates Wis1. In vitro kinase activity of the catalytic domain of Win1 purified from *S. pombe* was tested with recombinant GST-Wis1(K349R) as a substrate. (B) Win1 activates Wis1. Wis1 proteins were preincubated with Win1 proteins in the presence of unlabeled ATP. Then, Wis1 kinase activity was tested by assaying incorporation of radioactive phosphate into GST-Spc1 after adding γ -labeled ATP.

Another effect of the *win1-1* mutation relevant to osmotic stress response is a change in the phosphotyrosine content of Spc1. In wild-type cells, Spc1 is rapidly tyrosine phosphorylated after exposure to 0.6 M KCl, whereas this response is lost in *win1-1* cells. Although the presence of multiple copies of *wis4*⁺ in *win1-1* cells raised the basal level of tyrosine phosphorylation, we observed no significant increase in response to KCl treatment (Figure 6B). This observation argues against a major role of *wis4* in response to high osmolarity.

DISCUSSION

We have identified a second MAPKKK homologue of the Wis4 class in *S. pombe*. Several lines of evidence argue that it is encoded by the *win1*⁺ gene, previously only identified by the single *win1-1* mutation. The tight genetic linkage between *win1-1* and a marker integrated at the new MAPKKK gene (*win1Δ*) suggests that their chromosomal locations are very close. The genetic distance of 5 cM between *win1-1* and a nearby marker, *tps19*, agrees well with the physical distance of 30–40 kb from *tps19*⁺ to the MAPKKK gene. It is the only gene on 3F9 that is not represented on any of the overlapping sets of cosmids, and the fact that 3F9, but no cosmids, suppresses the ts phenotype of the *win1-1 cdc25-22 wee1-50* triple mutant suggests strongly that the MAPKKK gene is *win1*. Further supporting evidence is the discovery of a frameshift mutation within the MAPKKK ORF in the *win1-1* strain, which would cause a truncation eliminating the carboxyl-terminal kinase domain. Consistent with this, the phenotypes of *win1-1* and *win1Δ* are almost identical. Furthermore, all the phenotypes of *win1-1* can be very well explained by Win1 being an MAPKKK that activates Wis1. Further work will be needed for a full characterization of Win1: in particular the isolation of a full-length clone, which has so far eluded us, for reasons discussed below.

In a previous report, we showed that Wis1 can be activated by phosphorylation at its conserved activation residues, presumably because of the action of one or more MAPKKKs, one of which is Wis4. We also reported that Wis4 could not account for the response to osmotic stress but showed that the response was almost abolished by a mutation in *win1*, whose molecular identity was not then known. We now show that Win1 is an MAPKKK homologous to Wis4. This explains very well that although phosphorylation of Wis1 is essential for activation of Wis1 in response to high osmolarity, *wis4*⁺ is not required, and that Win1 activates Wis1 in parallel to Wis4. *S. pombe* Mcs4, the presumed receptor of a bacterial-like two-component phospho-relay system, has been reported to act upstream of the Wis4–Wis1–Spc1 pathway (Cottarel, 1997; Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). By analogy with the HOG pathway in *S. cerevisiae* where a similar two-component system is implicated in detection of high osmolarity and in the activation of two MAPKKKs (Maeda *et al.*, 1994), it might be expected that Wis4 plays a role in transmitting osmotic stress signals to the Wis1–Spc1 pathway. However, there is so far no evidence for *wis4*⁺ playing a major role in osmotic stress signaling in wild-type cells. In addition, the data we present here show that *wis4*⁺ cannot suppress the *win1-1* phenotypic defects that are manifest under conditions of high osmolarity. A model that

Figure 4. Win1 activates Wis1 in vivo. (A) Wis1 kinase activity in vivo was assayed by phosphotyrosine blot of Spc1 protein isolated from the strains indicated. The HA blot shows the relative amounts of HA-tagged Spc1. The truncated *win1*⁺ gene was expressed from pREP1 *nmt1* promoter by growth of cells in the absence of thiamine for 12 h. (B) The effect of overexpression of active or inactive Win1 catalytic domains was tested in various genetic backgrounds. Each strain was streaked on an EMM plate lacking thiamine, and the *win1* gene was overexpressed from the pREP1 *nmt1* promoter. The details of the strains given here are ED1259, wild type; ED1313, *wis1-4*; and ED1254, *wis1Δ*; and plasmids: pWN43, *Win1ΔN*; and pWN46, *Win1ΔN(K1149R)*.

summarizes these and other observations is shown in Figure 7.

The strategies for detection of high osmolarity and transduction of these signals vary in different organisms. In *Saccharomyces cerevisiae*, a pair of MAPKKKs, Ssk2p and Ssk22p, are required to transduce the signal from the bacterial-like two-component system, which uses phosphate transfer from histidine to aspartate, to the Pbs2 MAPKK homologue (Parkinson and Kofoid, 1992; Maeda *et al.*, 1994). Pbs2 can alternatively be activated by the Ste11 MAPKKK in an unknown way that does not involve the SLN1–YPD1–SSK1 two-component system (Posas and Saito, 1997), indicating that a two-component phospho-relay system is not the only way to detect and transduce high-osmolarity signals. In mammalian cells, multimerization of receptor tyrosine kinases such as the epidermal growth factor, interleukin 1, and tumor necrosis factor receptors in response to high osmotic stress leads to activation of a c-jun NH₂-terminal kinase (JNK) via MEKK-1, but no evidence for the presence of a bacterial-like two-component system has been reported (Rosette and Karin, 1996). A better understanding of the Win1 MAPKKK is expected to pave the way to understanding the mechanism of detection and transduction of high-osmolarity signals in *S. pombe*.

wild type

1335 TATGAACGTTTATATCCTTCACGTCGTTCTATCCTTCAGGAACATTCATTATATGCT
422 Y E R L Y P S R R S I L Q E H S L Y A

TCAGAATCTTTTCAGCACAAAGTTGGATGTCTTGACGGCTTATTCTAATGTCACATCATGCGCTA
S E S F Q H K L D V L T A Y S N V T H A L

win1-1

1335 TATGAACGTTTATATCCTTCACGTCGTTCTATCCTTCACGTCGTTCTATCCTTCAGG
422 Y E R L Y P S R R S I L H V V L S F R

AACATTTCATTATATGCTTCAGAATCTTTTCAGCACAAAGTTGGATGTCTTGACGGCTTATTCTA
N I H Y M L Q N L F S T S W M S

Figure 5. Mutation site of *win1-1*. The nucleotide sequences of part of the ORF of the MAPKKK in wild-type and *win1-1* strains are shown with deduced amino acid sequences. The repetitive sequences are shown in bold. The mutant protein sequence generated by the frameshift is underlined.

The *win1-1* mutation contains an insertion of 16 nucleotides within the amino-terminal domain. The *win1-1* mutant was obtained fortuitously during an

attempt to isolate plasmids that suppress *wee1-50* and is probably a spontaneous mutation (Ogden and Fantes, 1986). It appears that the extra sequence has

Figure 6. *wis4*⁺ does not suppress the *win1-1* defect under high-osmolarity conditions. The *win1-1* strain carrying multiple copies of a genomic fragment containing the *wis4*⁺ gene was compared with the wild-type strain. (A) Cells in a colony were photographed after 3 d on EMM+ 1.2 M KCl or 2 d on EMM (control). (B) The phosphotyrosine content of Spc1 in wild-type or *win1-1* strains determined by anti-phosphotyrosine antibody. The HA blot shows the relative amounts of HA-tagged Spc1. Cells were collected by filtration before or 10 min after exposure to 0.6 M KCl.

Figure 7. Stress response pathway in fission yeast. Win1 MAPKKK phosphorylates Wis1 MAPKK in response to high osmolarity. Heat and oxidative stresses activate the pathway independently of Win1 and Wis4 MAPKKs (Samejima *et al.*, 1997). Spc1 is also known as *Sty1* or *Phh1*. Wis4 is synonymous with *Wak1* and *Wik1*.

arisen by duplication of a tandemly repeated sequence and the short sequence between the repeats. We observe an occasional reversion of *win1-1* phenotype, which might be explained by a similar oblique recombination event reversing the original mutation. The tandem duplication present in the *win1⁺* gene may help explain why it has proved so difficult to obtain full-length clones either by screening of several libraries (Warbrick and Fantes, 1992) or by attempting to subclone the activity from phage 3F9 (Samejima, unpublished observations). Propagation of tandem repeats in *E. coli* can lead to recombination and loss of nucleotide sequence, which in the case of *win1⁺* would cause a frameshift and loss of function. However there may be other factors involved, because both of the ordered cosmid libraries constructed have a gap at the same location, which corresponds to the *win1⁺* gene.

Until the molecular identity of the *win1⁺* gene and nature of the *win1-1* mutation were known, the phenotype of the triple mutant could not be fully interpreted. We have shown that Win1 is an MAPKKK that activates the Wis1 MAPKK, and that *win1-1* is a loss of function mutation. These findings account for the basis of our previous observation that Wis1 is less active in the *win1-1* mutant (Samejima *et al.*, 1997). Reduced Wis1 activity in the *win1-1* mutant is one factor responsible for the cell cycle defect of *wee1-50 cdc25-22 win1-1* at 35°C, because *cdc25-22 wee1-50* is viable at this temperature. It is reduced Wis1 activity rather than the specific loss of Win1 activity that seems to be important, because the combination of *cdc25-22*

wee1-50 with other mutations that reduce the level of Wis1 activity (e.g., *spc1Δ* and *wis4Δ*) all result in similar phenotypes (Shiozaki and Russell, 1995; our unpublished data). Because *wee1-50 win1-1* is viable at 35°C, lethality of the triple mutant also requires the *cdc25-22* defect. By analogy with the synthetic lethality of the *cdc25-22* mutation with *wis1Δ* or *spc1Δ* (Shiozaki and Russell, 1995, 1996), the lethality of *wee1-50 cdc25-22 win1-1* at 35°C is probably a combined result of two defects: one in the Wis1 pathway and the other in Cdc25 activity. This rather simplified view of the phenotype might be useful in understanding the suppression of *wee1-50 cdc25-22 win1-1* by the various *wis* genes. Suppression could occur either by alleviation of the *cdc25-22* mutation or by gain of Wis1 activity to compensate for the partial loss attributable to the *win1-1* mutation. Overexpression of the various *wis* genes might suppress by either mechanism.

The *win1-1* mutant gene encodes a truncated protein lacking a kinase domain, and it is therefore impossible for *wis* genes to restore Win1 kinase activity per se in *win1-1* mutants. Enhanced Wis1 kinase activity, however, can compensate for loss of *win1* function. For instance, overexpression of *wis1⁺* or *wis4⁺* increases the total kinase activity of Wis1 available and substitutes for the missing contribution from *win1⁺* under unstressed conditions. Unlike the case for *wis1⁺* and *wis4⁺*, the underlying mechanism for suppression by other *wis* genes is not evident. The *wis2⁺* gene encodes a cyclophilin of the Cyp-40 class (Weisman *et al.*, 1996). Its predicted peptidyl-prolyl isomerase activity may be required for activation or stabilization of one or more of the kinases in the osmosensing cascade, because some protein kinases (e.g., Wee1 and Src) need a chaperone complex for their function in vivo (Aligue *et al.*, 1994; Gerber *et al.*, 1995; Dey *et al.*, 1996). It is, however, more plausible that *wis2⁺* (and *wis3⁺*) suppress the reduced activity of the *cdc25-22* mutant protein, because they suppress other mutations in a *cdc25-22 wee1-50* background, and neither *wis2⁺* nor *wis3⁺* suppresses the *win1-1* single mutant (Warbrick and Fantes, 1992).

The *win1-1 cdc25-22 wee1-50* mutant provides a rather sensitive way to isolate genes in two apparently distinct mitotic inducing systems, the *wis1* pathway and Cdc25 activation. The effect of a slight enhancement of Cdc25 phosphatase activity in a *cdc25-22* mutant strain might be more pronounced in a *wee1* mutant background, which might increase the sensitivity of cells to a weak suppressor. However, *cdc25-22 wee1-50* cells are able to grow at all temperatures, so that no selection for suppressors of this type is available. The partial reduction of Wis1 activity by *win1-1* in *cdc25-22 wee1-50 win1-1* cells appears to offer suitable conditions for selection of suppressors. Another advantage of screening for regulators of Cdc25 in a *wee1* mutant background over a simple screen with the

cdc25-22 single mutant is that the former approach should avoid isolation of suppressors such as *nim1*⁺, which suppress the *cdc25-22* defect by an indirect mechanism. For these reasons, this genetic screen might contribute to our understanding of the complex regulation of Cdc25 phosphatase activity (Kumagai and Dunphy, 1992; Kovelman and Russell, 1996; Furnari *et al.*, 1997; Peng *et al.*, 1997). It is likely (although not proven) that Wis2 and Wis3 act on Cdc25 or one of its regulators, and further characterization of these proteins should be highly informative. With regard to the *wis1* pathway, a more exhaustive screen for multicopy suppressors of the triple mutant might identify the putative cell cycle effector(s) of Spc1, in addition to the activators of Spc1 such as Wis1 and Wis4 that have been isolated to date.

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