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Autophosphorylation of the Smk1 MAPK is spatially and temporally regulated by Ssp2 during meiotic development in yeast

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ABSTRACT **Smk1 is a meiosis-specific MAPK that controls spore wall morphogenesis in *Saccharomyces cerevisiae*. Although Smk1 is activated by phosphorylation of the threonine (T) and tyrosine (Y) in its activation loop, it is not phosphorylated by a dual-specificity MAPK kinase. Instead, the T is phosphorylated by the cyclin-dependent kinase (CDK)–activating kinase, Cak1. The Y is autophosphorylated in an intramolecular reaction that requires a meiosis-specific protein named Ssp2. The meiosis-specific CDK-like kinase, Ime2, was previously shown to positively regulate Smk1. Here we show that Ime2 activity is required to induce the translation of *SSP2* mRNA at anaphase II. Ssp2 protein is then localized to the prospore membrane, the structure where spore wall assembly takes place. Next the carboxy-terminal portion of Ssp2 forms a complex with Smk1 and stimulates the autophosphorylation of its activation-loop Y residue. These findings link Ime2 to Smk1 activation through Ssp2 and define a developmentally regulated mechanism for activating MAPK at specific locations in the cell.**

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INTRODUCTION

Mitogen-activated protein kinases (MAPKs) are evolutionarily conserved signal transduction enzymes that are activated in response to diverse extracellular inputs ranging from stress signals, to mitogens, to developmental cues (Chen and Thorner, 2007). MAPKs are activated by dual-specificity MAPK kinases (MAP2Ks) that phosphorylate a conserved threonine (T) and tyrosine (Y) in the activation loop of the MAPK. MAP2Ks are activated by upstream kinases (MAP3Ks) that couple to receptors through a variety of mechanisms (canonical MAPK signaling). MAPKs are also activated by noncanonical mechanisms (Coulombe and Meloche, 2007). These alternative mechanisms include phosphorylation of MAPKs by kinases outside of the MAP2K family and autophosphorylation.

Smk1 is a meiosis-specific MAPK in the yeast *Saccharomyces cerevisiae* that controls the postmeiotic program of spore morphogenesis (Krisak *et al.*, 1994). Sporulation in yeast is a well-suited system to study protein kinase signaling in the context of a developmental program. Similar to differentiation programs in higher eukaryotes, sporulation in yeast is controlled by a transcriptional cascade (Chu *et al.*, 1998). The cascade induces three temporally distinct sets of genes. Early genes are controlled by Ime1; these genes are expressed as meiotic S phase, homologue pairing, and recombination take place (van Werven and Amon, 2011). Middle genes are controlled by the Ndt80 transcription factor; these genes are expressed as meiosis I and II (MI/MII) and spore morphogenesis take place (Winter, 2012). Late genes are expressed during spore maturation. Although many meiotically induced mRNAs are translated soon after they are transcribed, numerous sporulation-specific mRNAs are regulated at the translational level (Brar *et al.*, 2012). This provides an additional layer of control that temporally diversifies the accumulation of meiosis-specific gene products.

Genotype/phenotype studies have shown that Smk1 controls multiple steps in assembly of the spore wall that require different thresholds of Smk1 activity (Wagner *et al.*, 1999). These properties of the Smk1 pathway are different from the characterized properties of canonical MAPK pathways, which generate switch-like (on/off) signaling outputs (Ferrell, 1996; Ferrell and Machleder, 1998). Subsequent studies showed that Smk1 is activated by a noncanonical two-step mechanism that produces a low-activity form of the enzyme early in the program and a high-activity form later

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Abbreviations used: GFP, green fluorescent protein; GST, glutathione *S*-transferase; KAD, kinase-activating domain; MI, meiosis I; MII, meiosis II; MAPK, mitogen-activated protein kinase; PSM, prospore membrane.

(Whinston *et al.*, 2013). In the first step, Cak1, the CDK-activating kinase, phosphorylates Smk1 on its activating T residue (T207; Wagner *et al.*, 1997; Schaber *et al.*, 2002). In the second step, which occurs later in the program, a meiosis-specific protein named Ssp2 activates the intramolecular (*cis*) autophosphorylation of Smk1 on Y209. Although the *SSP2* and *SMK1* transcriptional promoters are both activated by Ndt80, Ssp2 is translated later than Smk1. The differential timing of Smk1 and Ssp2 translation therefore plays a role in producing two activity states of Smk1 as different steps in meiosis are taking place.

Ime2 is a meiosis-specific, CDK-like kinase that has been hypothesized to coregulate meiosis with cell cycle–regulatory CDK, Cdc28 (Benjamin *et al.*, 2003; Honigberg, 2004; Holt *et al.*, 2007; Shin *et al.*, 2010). Ime2 regulates multiple steps in sporulation, including the induction of early genes, premeiotic S phase, entry into and progression through the meiotic division, and completion of meiosis. Ime2 has been shown to promote the phosphorylation of Smk1 (McDonald *et al.*, 2009). Whether Ime2 controls the phosphorylation of T207 and/or Y209 was not determined in this earlier study.

A key step in sporulation involves formation of the prospore membrane (PSM; Neiman, 2011). This double membraneous structure is nucleated at the spindle pole body (centrosome) in the early stages of MII. The PSM grows around the four haploid cells as MII is taking place and pinches off in a cytokinetic process that occurs soon after anaphase II has been completed. Multiple spore wall layers are deposited in and around the PSM to generate four haploid spores.

In this study, we show that Ssp2 is translated at anaphase of MII and that the translation of *SSP2* mRNA requires the catalytic activity of Ime2. The newly translated Ssp2 is localized to the PSM by its amino-terminal region. The carboxy-terminal domain of Ssp2 forms a complex with Smk1 at the PSM and activates the intramolecular autophosphorylation of Smk1 on its activation-loop Y residue. Ssp2 therefore triggers Smk1 activation at the site where Smk1 coordinates spore wall assembly on completion of meiosis. These findings suggest a new mechanism to deliver activated MAPKs to specific cellular locations during developmental programs.

RESULTS

Ime2 activates Smk1 through Ssp2

Ime2 has been shown to promote the activation of Smk1 (McDonald *et al.*, 2009). To determine whether Ime2 induces the phosphorylation of Smk1 on T207, Y209, or both activation-loop residues and to define more precisely the interval in meiosis when Ime2 regulates Smk1, we tested the consequences of inhibiting an analogue-sensitive form of Ime2 (Ime2-as1) on Smk1 phosphorylation using a yeast strain in which *NDT80* is controlled by an estrogen-inducible promoter (referred to as the *NDT80-block/release* background hereafter; Benjamin *et al.*, 2003; Carlile and Amon, 2008). *NDT80-block/release* cells transferred to sporulation medium stall at pachytene due to an *NDT80* deficiency. Addition of estrogen induces *NDT80*, which triggers exit from pachytene and relatively synchronous progression through meiosis and spore formation. *SMK1* and *SSP2* are both controlled by middle promoters that are activated by Ndt80. We treated *NDT80-block/release ime2-as1* cells with estrogen and added the Ime2-as1–specific inhibitor Bn-PP1 at various times thereafter (Figure 1A). Cells were harvested at hourly intervals, and the phosphorylation of Smk1 was assayed by electrophoresis through Phos-tag acrylamide gels and immunoblot analyses (Figure 1B). As previously shown, the first pool of Smk1 that is produced (detectable as MI is being carried out) migrates as a doublet (Whinston *et al.*, 2013). The faster- migrating member of the doublet is unphosphorylated, and the slower form is phosphorylated on T207 (by Cak1). Later in the program, as MII is being completed, a third, slower-migrating form of Smk1 accumulates. This form of Smk1 is phosphorylated on T207 and Y209. The doubly phosphorylated form of Smk1 appears at a later stage than the monophosphorylated form because Ssp2 is produced later than Smk1. Addition of Bn-PP1 at 2 h, when 60% of the cells had completed MI, almost completely eliminated the production of Ssp2 and the doubly phosphorylated form of Smk1. Addition of Bn-PPI at 3 h (when 55% of cells had completed MII) reduced the production of Ssp2 (to ~20% of the level seen in wild-type cells). The relative amount of the doubly phosphorylated form of Smk1 was substantially reduced in these cells, but a low amount was still present (~10% of the wild-type level; Figure 1B, top right). The addition of Bn-PP1 did not influence the relative amount of Smk1 that is singly phosphorylated. These data suggest that Ime2 triggers Ssp2 production and thus Smk1 autophosphorylation during MII.

To confirm that Ime2 controls the phosphorylation of Smk1 on Y209, we assayed Smk1 tagged with polyhistidine and hemagglutinin (HA; Smk1-HH) with a phosphospecific antiserum. *smk1-HH ime2-as1* homozygotes were treated with Bn-PP1 at various times. Cells were harvested later, when Smk1 and Ssp2 levels were high in untreated cells and Y209 was maximally autophosphorylated (8 h postinduction). Smk1-HH was then purified and assayed using a phosphospecific antiserum specific for phosphorylated Y209 (Figure 1C). Bn-PP1 eliminated detectable pY209 immunoreactivity when it was added during prophase or MI (4 and 5 h, respectively), partially inhibited pY209 when added as cells were carrying out MII (6 h), and barely inhibited pY209 when most cells had completed MII (7 h). These experiments confirm that Ime2 catalytic activity is required for Smk1 to autophosphorylate Y209.

In contrast to Smk1, Ssp2 protein accumulates with a substantial delay compared with its mRNA (Whinston *et al.*, 2013). Ssp2 mRNA was also previously identified in genome-wide ribosome-profiling experiments as a member of a set of mRNAs that are translationally repressed until MII (Brar *et al.*, 2012). We monitored the level of *SSP2* mRNA in synchronous *NDT80-block/release* cells treated with Bn-PPI and found that Ime2 inhibition did not influence *SSP2* mRNA levels (Figure 1D). These data suggest that Ime2 is required to induce the translation of *SSP2* mRNA.

The inhibition of *ime2-as1* can block cells at the end stages of MII before spore wall formation, before nuclear segregation, before meiotic S -phase, and even before entry into the program, depending on when the inhibitor is added (Benjamin *et al.*, 2003; McDonald *et al.*, 2009; Berchowitz *et al.*, 2013). Consistent with previous studies, the addition of Bn-PP1 to *NDT80-block/release ime2-as* cells at 3 h (when ~50% of the cells had completed anaphase II) blocked most of the cells with elongated MII spindles before spore formation (Berchowitz *et al.*, 2013). Chromosome segregation and spindle disassembly occur in *ssp2Δ* and *smk1Δ* backgrounds (Krisak *et al.*, 1994; Sarkar *et al.*, 2002). Thus, in addition to triggering Smk1 autophosphorylation via Ssp2 at anaphase II, Ime2 likely triggers additional processes at this stage that are required for successful completion of the program.

Ssp2 and Smk1 colocalize to the prospore membrane

We next investigated the pattern of Smk1 and Ssp2 staining in cells at various stages of meiosis. Smk1 and Ssp2 were undetectable before MI (Figure 2A, top). Smk1 was first detected throughout the cell, as MI was taking place (Figure 2A, middle). During MII, Smk1 staining increased, and this was accompanied by its localization to the area of the cell where PSMs are located (the zone surrounding

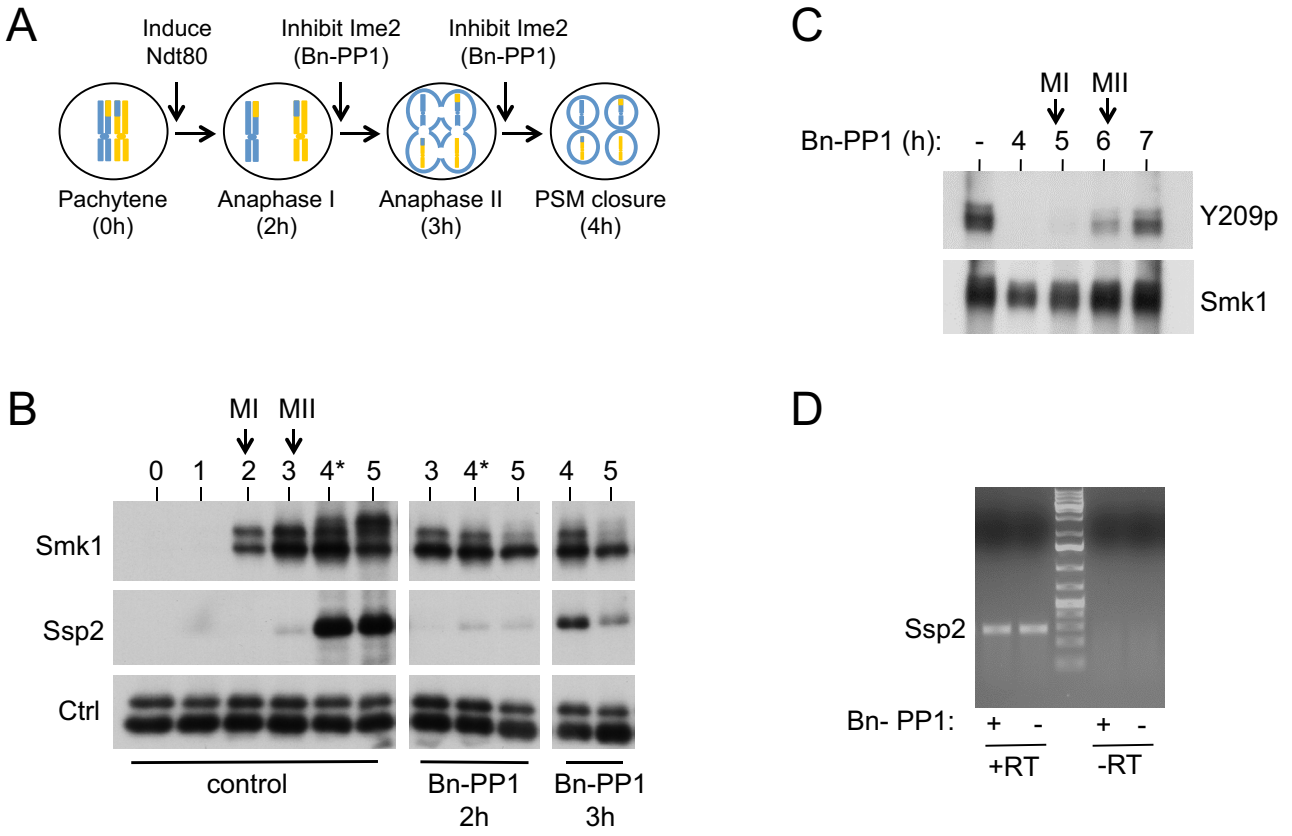

FIGURE 1: Ime2 promotes Smk1 autophosphorylation via Ssp2. (A) Steps in meiosis/spore formation and the *NDT80-block/release ime2-as* experimental strategy. Spore wall morphogenesis is an elongated process that is initiated between 3 and 4 h when PSM closure occurs. (B) *NDT80-block/release ime2-as* cells were induced to enter meiosis, and Bn-PP1 was added 2 h later (as anaphase I is taking place) or 3 h later (as anaphase II is taking place). Cell extracts were collected at the indicated times after estradiol addition (h) and analyzed by electrophoresis in gels containing Phos-tag acrylamide, followed by immunoblot analyses with the indicated antibodies. An antibody against PSTAIRE that detects Cdc28 (lower band) was used as a control for protein loading (Ctrl). The timing of MI and MII (midpoint of maximum) is indicated by downward arrows. (C) *ime2-as SMK1-HH* cells were transferred to sporulation medium, and Bn-PP1 was added at the indicated times (h). At 4 h, >80% of the cells have completed S phase, and most cells are in the later stages of meiotic prophase. MI and MII timing is indicated as in A. All cells were collected 8 h after transfer to sporulation medium, when untreated cells are assembling spore walls, Ssp2 and Smk1 levels are high, and the fraction of Smk1 that is phosphorylated on Y209 is near maximal. Cells were lysed, Smk1 was purified, and the phosphorylation of Y209 was monitored by electrophoreses (in the absence of Phos-tag) and immunoblot analyses using the phosphospecific antiserum (Y209p) and an HA antibody to control for Smk1 levels (Smk1). (D) *NDT80-block/release ime2-as1* cells were treated with 1-Bn-PP1 at 2 h after *NDT80* induction as indicated. The cells were collected 4 h after *NDT80* induction (they correspond to the samples in B labeled with an asterisk). RNA was prepared and analyzed for SSP2 mRNA by reverse transcription PCR in reactions containing (+) and lacking (–) reverse transcriptase (RT) to control for contaminating DNA.

the incipient spores, where spore walls will be assembled; Figure 2A, bottom). Ssp2 was undetectable until anaphase II. Ssp2 was almost always localized at or near the PSM, similar to Smk1 (Figure 2A, bottom).

To define more precisely the stage of MII when Ssp2 accumulates, we assayed its staining in cells that had been counterstained for tubulin and DNA. Ssp2 was undetectable in all cells before anaphase II and was detectable in almost all cells that were in anaphase II or later (representative images are shown in Figure 2B and quantified in Figure 2C). The vast majority of Ssp2 showed a PSM-like staining pattern. In the rare instances in which Ssp2 staining was more diffuse (see Figure 2B, second row from the bottom, for an example), cells were trapped as DNA masses were segregating. The Ssp2 that appears outside of the PSM as anaphase II is occurring may therefore represent newly translated Ssp2 that is in transit to the PSM. We also analyzed Smk1 staining in these experiments. In contrast to Ssp2, Smk1 was detected in almost all cells that had completed anaphase of MI. The fraction of cells that were Smk1 positive remained high throughout meiosis (Figure 2C). These data show that Smk1 accumulates gradually starting early in meiosis, Ssp2 accumulates in a more switch-like manner at anaphase II, and both of these proteins accumulate at or near the PSM thereafter.

An alternative approach to visualize Smk1 and Ssp2 is to tag them with green fluorescent protein (GFP). Whereas single-copy *SMK1-GFP* and *SSP2-GFP* are functional, the levels of both of these proteins are too low to permit visualization using standard methods (Li *et al.*, 2007). However, we were able to detect Smk1-GFP and Ssp2-GFP using multicopy plasmids. In these experiments,

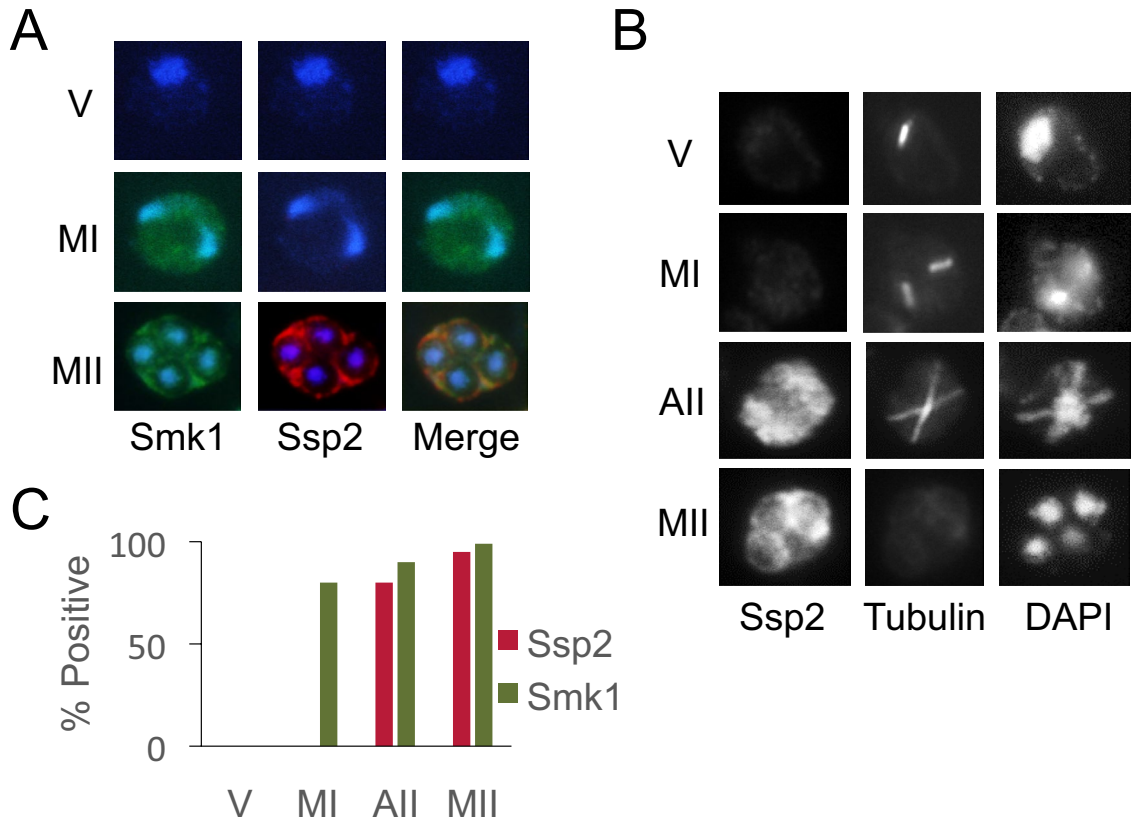

FIGURE 2: Smk1 and Ssp2 localization during meiosis. (A) *Smk1-HA SSP2-Myc* diploids were fixed and immunostained with anti-HA (Smk1, green) and anti-Myc (Ssp2, red) and also with DAPI (DNA, blue). (B) Immunofluorescence of Ssp2 and tubulin, counterstained with DAPI as indicated. (C) Quantitation of immunofluorescence signals for Ssp2 and Smk1 determined by counting 100 cells analyzed as in B.

Smk1-GFP was detected in a diffuse pattern in cells undergoing MI. It subsequently associated with the PSM before anaphase II (Figure 3A). Ssp2-GFP was undetectable until anaphase II, at which time it was exclusively present at the PSM. It was suggested that PSM formation is abnormal in *ssp2Δ* cells (Li *et al.*, 2007). However, PSMs appear to surround nuclei normally and pinch off to form single cells in both *ssp2Δ* and *smk1Δ* backgrounds, as judged by the pattern of Spo20$^{1-51}$-RFP fluorescence, a well-characterized marker for the PSM (Neiman, 1998; Figure 3B). These findings suggest that *SMK1* and *SSP2* are not required for the major steps of PSM outgrowth and cytokinesis. We also observed that Ssp2-GFP localizes to the PSM in an *smk1Δ* background and Smk1-GFP localizes to the PSM in an *ssp2Δ* background, suggesting that Ssp2 and Smk1 can localize to the PSM independently (unpublished data).

To investigate further the colocalization of Smk1 and Ssp2, we tested the interaction of Smk1-HA and Ssp2-Myc using a proximity ligation assay (PLA), which relies on pairs of DNA-tagged antibodies that permit a PCR fragment to be generated when two antigens are in close proximity (the proximity limit is ~40 nm; Fredriksson *et al.*, 2002). An antigen-specific PLA signal for Smk1 and Ssp2 was detected, but only in cells that had completed anaphase II (Figure 3C). These data confirm that Smk1 and Ssp2 are close to one another and coexist only after anaphase II has been completed.

Ssp2 and Smk1 form a complex

To investigate whether Ssp2 and Smk1 exist in a physical complex, we purified functional Smk1-glutathione *S*-transferase (GST) from postmeiotic *SSP2-Myc* cells and purified functional Ssp2-GST from postmeiotic *SMK1-HA* cells. Ssp2-Myc protein was significantly enriched in the Smk1-GST preparations, and Smk1-HA protein was significantly enriched in the Ssp2-GST preparations, whereas neither Smk1-HA nor Ssp2-Myc was enriched when the purification protocol was carried out with extracts lacking a GST fusion (Figure 4). These data indicate that Smk1 and Ssp2 exist in a physical complex.

The amino-terminal half of the 371-residue Ssp2 protein is predicted to be substantially more disordered than the carboxy-terminal half. We generated a series of amino-terminal deletions starting with full-length *SSP2-GST* to interrogate the function of the disordered region. The deletion of 26, 63, and even 137 residues did not noticeably affect the ability of *SSP2-GST* to promote spore formation, as assayed by phase contrast microscopy (Figure 5A). Since carrying out these experiments, we have found that the amino-terminal 161 residues (almost all of the disorded portion) of Ssp2 can also be deleted without affecting its ability to promote spore formation. In contrast, deletion of 170 (or 190) residues caused a Spo$^-$ phenotype similar to *ssp2Δ*. We also deleted the carboxy-terminal 20 residues; this also caused an *ssp2Δ*-like Spo$^-$ phenotype. The deletion mutants were also assayed using a fluorescence assay that detects incorporation of dityrosine into the outer layer of the spore wall (Figure 5B). These data demonstrate that the 26, 63, and 137 amino-terminal residues of Ssp2 are not essential for this late step in spore wall formation. Taken together, these data show that the carboxy-terminal segment of Ssp2 (residues 162–371) is essential, whereas the amino-terminal segment is not, for Ssp2 function.

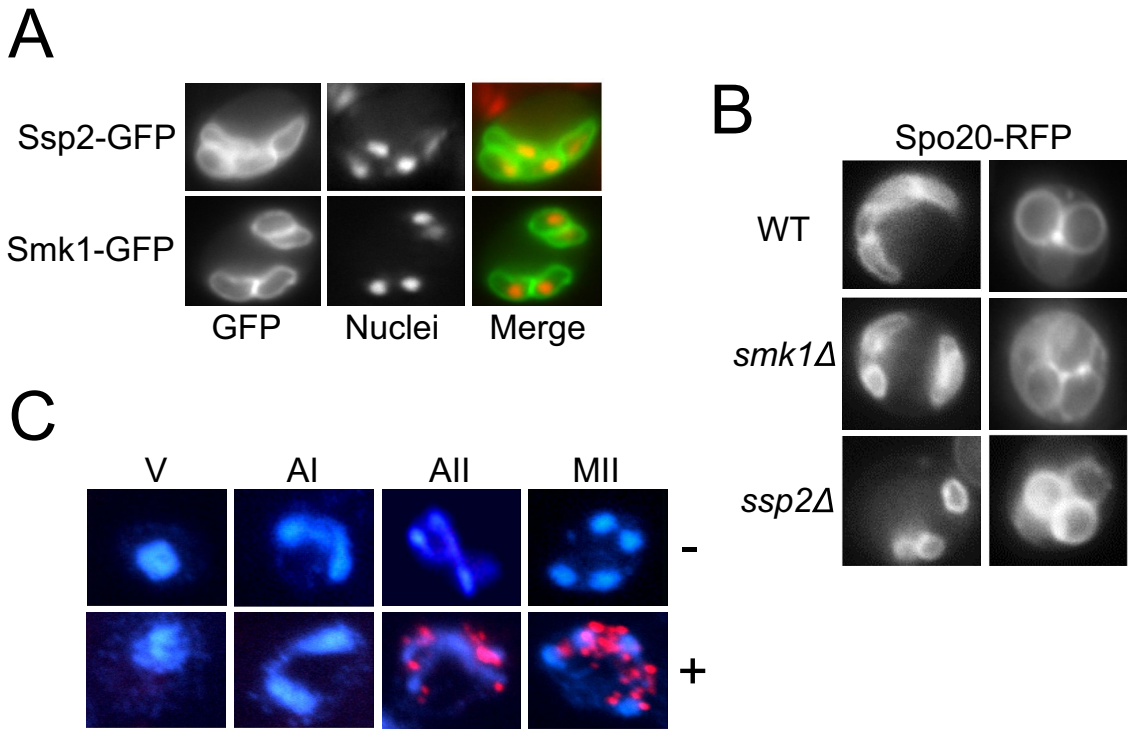

FIGURE 3: Ssp2-GFP and Smk1-GFP localize to the PSM. (A) *HTB2-mCherry* diploids harboring multicopy Smk1-GFP or Ssp2-GFP plasmids were sporulated, and living cells were visualized using fluorescence microscopy. (B) Live-cell imaging of Spo20-RFP in sporulating wild-type, *smk1Δ*, or *ssp2Δ* cells soon after anaphase II (left) and later in the program (right). (C). PLA for Smk1-HA and Ssp2-Myc. *SMK1-HA* cells containing *SSP2-Myc* (+) or untagged *SSP2* (–) were fixed and incubated with corresponding primary antibodies conjugated to DNA, and PCR was performed. The PCR products were detected using fluorescently labeled proximity probes and visualized by fluorescence microscopy (red signal).

We next tested whether the set of truncated Ssp2-GST proteins physically interact with Smk1-HH. Ssp2-GST proteins were purified, and these preparations were tested using antibodies specific for Ssp2-GST and Smk1-HH (Figure 5C). The Smk1-HH was also purified from a fraction of these cultures using a nickel-affinity resin; this material was tested with the pY209 antiserum. In all cases, the *SSP2-GST* deletion mutants that supported sporulation interacted with Smk1 and activated Y209 autophosphorylation. In contrast, all of the *SSP2-GST* proteins that were Spo$^-$ failed to interact with Smk1 and failed to activate Smk1 autophosphorylation. These data show that residues 137–371 of Ssp2 are sufficient for Smk1/Ssp2 complex formation and Smk1 activation. More recently, we found that residues 162–371 of Ssp2 are also sufficient to form a complex with Smk1 and to activate Y209 autophosphorylation. We therefore refer to residues 162–371 as the kinase-activating domain (KAD) hereafter. A notable feature of the GST-interaction data shown in Figure 5C is that the amount of Ssp2-GST in the purified samples increased as amino acids were deleted from the amino-terminal end of the protein. This is not a consequence of differential expression or stability of the various deletion constructs, since comparable amounts of these proteins are present in total extracts. Instead, this is a consequence of differential recovery of the Ssp2 protein.

Ssp2 is targeted to the PSM via its amino-terminal region

We replaced the GST moiety in a subset of the *SSP2-GST* deletants with GFP and visualized the proteins in living cells as they progressed through sporulation. All of the amino-terminal deletions tested altered the tight PSM fluorescence pattern of Ssp2-GFP. A

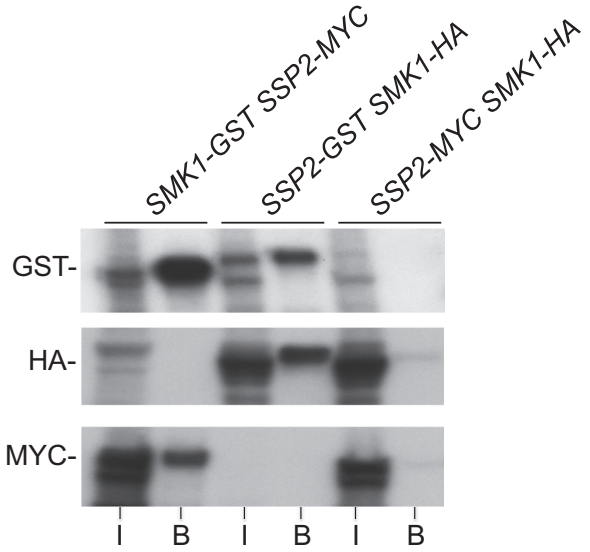

FIGURE 4: Smk1 and Ssp2 exist in a complex. Cells of the indicated genotype were transferred to sporulation media, harvested at 8 h, when Smk1 and Ssp2 are both present at nearly maximal levels, and extracts prepared. Subsequently Smk1-GST or Ssp2-GST was purified using glutathione–agarose beads. Bound protein was eluted with reduced glutathione and analyzed with GST, HA, or MYC antibodies as indicated. The *SMK1-HA SSP2-MYC* strain, lacking a GST-fusion protein, was processed in parallel as a negative control. I, input; B, bound protein.

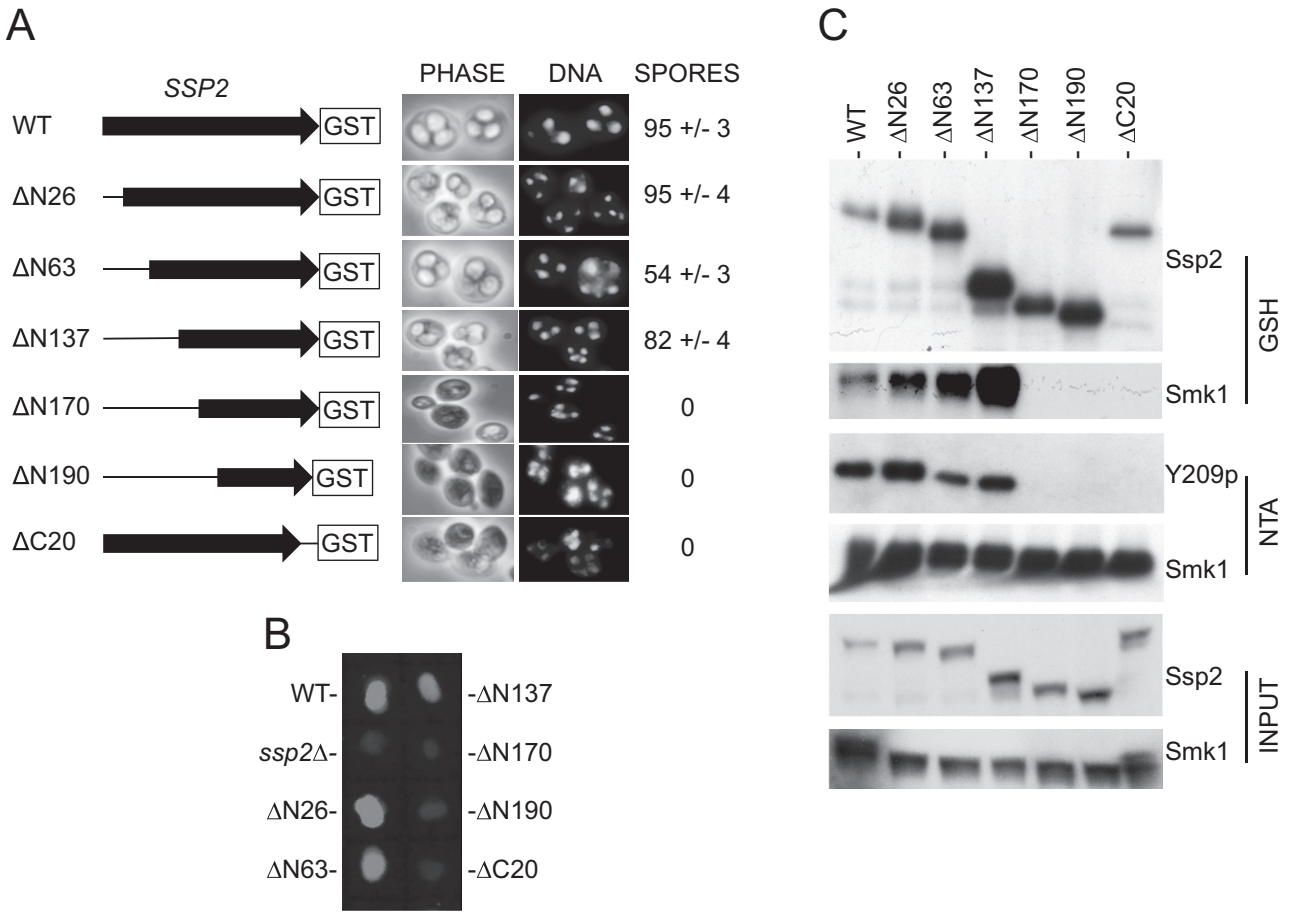

FIGURE 5: The carboxy-terminal region of Ssp2 is sufficient to form a complex with Smk1, activate Smk1 autophosphorylation, and promote spore formation. (A) The deletions (lines) in the *SSP2* open reading frame (solid arrows) fused to GST (boxes) were integrated at the *SSP2* genomic locus, and the resulting strains were crossed by an *ssp2Δ SMK1-HH* haploid. The diploids were transferred to sporulation medium, cells were collected at 24 h postinduction, stained with DAPI, and examined by phase contrast (PHASE) and fluorescence (DNA) microscopy. The percentage of meiosis-positive cells that formed spore walls (SPORES) represents the average of three isolates (100 cells scored/isolate ± SD). (B) The indicated mutants were assayed for spore formation using a fluorescence assay that detects incorporation of dityrosine into insoluble material. (C) The *SSP2-GST* deletion strains diagrammed in A were lysed at 8 h postinduction, and Ssp2-GST was purified using glutathione beads (GSH) and analyzed by immunoblot analyses with an antiserum against GST (Ssp2) or HA (Smk1). In parallel, cells were lysed in 6 M guanidine, Smk1-HH was purified with Ni–nitrilotriacetic acid beads (NTA), and bound proteins were analyzed by immunoblot analyses with a Y209p phosphospecific antiserum (Y209p) or HA as a control for total Smk1 levels (Smk1). A fraction of the extract (Input) was analyzed to control for total Ssp2 and Smk1.

fraction of the Ssp2-GFP protein lacking amino-terminal residues 1–137 (which is functional) was present at the PSM, but a significant fraction was also dispersed throughout the cell. The construct lacking amino-terminal residues 1–170 (which is nonfunctional) was dispersed in the cell but differed from the construct lacking residues 1–137 by being mostly absent from the PSM. The construct lacking the 20 carboxy-terminal residues (which is nonfunctional) localized to the PSM similar to the full-length construct (compare to the full-length pattern in Figure 3A).

Ssp2-GFP constructs containing only the amino-terminal 138 or 101 residues (ΔC233 and ΔC270 constructs, respectively) were localized to the PSM (Figure 6). In contrast, an Ssp2-GFP construct containing only the amino-terminal 50 residues (ΔC320) was localized to the interior of the developing spores. This pattern is similar to the pattern of fluorescence observed when the entire Ssp2 open reading frame had been deleted (i.e., when only GFP is expressed from the *SSP2* promoter). These experiments suggest that features in the 50- to 101-residue segment of Ssp2 are necessary for PSM localization. These experiments also demonstrate that amino-terminal residues 1–101 of Ssp2 are sufficient to localize (GFP) to the PSM. We refer to residues 1–101 as the targeting domain (TD) hereafter (Figure 7).

DISCUSSION

Development and differentiation require that signal transduction is coordinated with morphogenetic processes in time and space. In this study, we showed that a MAPK (Smk1) that is essential for a morphogenetic program (spore wall morphogenesis) is activated by a spatiotemporally regulated activator of *cis*-autophosphorylation (Ssp2) at the sites where spore walls are assembled (the PSM). Below, we discuss mechanisms controlling the timing/stage of autophosphorylation, and then we discuss mechanisms controlling its location in the cell. We propose a generalizable model for controlling activation of MAPKs and MAPK-like enzymes that may be relevant to

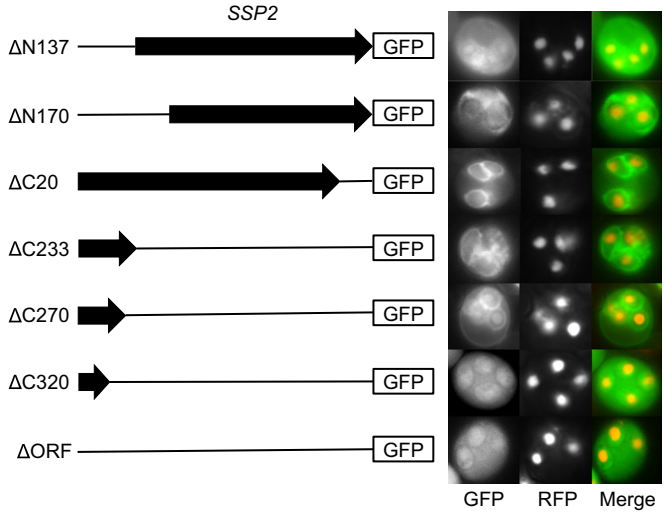

FIGURE 6: The amino-terminal region of Ssp2 is sufficient to target Ssp2 to the PSM. Multicopy plasmids containing the deletions (indicated by the lines) in the Ssp2 open reading frame (solid arrows) fused to GFP (boxes) as indicated were transformed into an *HTB2-mCherry* diploid. Transformants were transferred to solid sporulation medium and cells examined at various times thereafter by fluorescence microscopy. The Δ233, Δ270, and Δ320 alleles produce 138-, 101-, and 51-residue fragments of Ssp2, respectively. The ΔORF allele (bottom) lacks the entire *SSP2* open reading frame.

delivering activated kinase to morphogenetic intermediates that exist at discrete stages during cellular differentiation programs.

Temporal control of MAPK activation

Smk1 protein starts to accumulate during MI, soon after the *SMK1* gene is transcribed. Ssp2 protein accumulates later. Because Ssp2 is required for Smk1 autophosphorylation, this generates an interval during which Smk1 is present in a monophosphorylated (low-activity) state and an interval during which Smk1 is present in a dually phosphorylated (high-activity) state. In this study, we found that although *SSP2* mRNA is present in cells that are undergoing MI, Ssp2 protein is not detected until anaphase II. Genome-wide ribosome-profiling analyses of meiotic cells previously identified multiple sets of middle mRNAs that are translationally induced at distinct meiotic stages (Brar *et al.*, 2012). In that study, *SSP2* mRNA was found to be translationally induced as MII is being completed. The data presented here are consistent with the ribosome-profiling data. Another example of a middle-meiotic (Ndt80-inducible) mRNA that is translationally regulated is *CLB3*, which is translationally repressed until the initiation of MII (Carlile and Amon, 2008). Berchowitz *et al.* (2013) demonstrated that an RNA-binding protein, Rim4, translationally represses *CLB3* mRNA until MII and that Ime2 down-regulates Rim4 to permit Clb3 translation. Our data are consistent with the possibility that Ime2 also triggers Ssp2 translation by derepressing Rim4. However, if this is the case, there must be additional mechanisms that modulate the differential timing of Ssp2 and Clb3 translation. PSM outgrowth/encapsulation, disassembly of the spindle, and activation of Smk1 all appear to take place during a short interval as Ssp2 translation is being derepressed. Further studies are required to elucidate how Ime2 controls the translation of *SSP2* mRNA and how the Ime2-dependent induction of different mRNAs in meiosis is temporally diversified.

Spatial control of MAPK activation

Spore wall assembly occurs within and around the two PSM bilayers that pinch off after exit from MII (Neiman, 2011). During spore wall assembly, the outer PSM bilayer is discarded, and the inner bilayer becomes the plasma membrane of the mature spores. The PSM therefore exists transiently, yet it is essential for this morphogenetic program. In this study, we showed that Smk1 localizes to the PSM as it is surrounding the haploid cells and that Ssp2 localizes to the PSM in a switch-like manner at anaphase II. We also showed that the amino-terminal portion of Ssp2 (the TD) is necessary for localizing Ssp2 to the PSM and that the TD is also sufficient to target a heterologous protein (GFP) to the PSM. The TD of Ssp2 is predicted to be disordered and is rich in basic amino acids. It is possible that the TD directly interacts with negatively charged phospholipids in the PSM. If this is the case, the TD may interact with specific phospholipids that are enriched in the PSM, since TD-GFP is not localized to other membranes in the cell. Further studies are required to determine whether the TD directly interacts with phospholipids and, if so, how PSM targeting specificity is achieved.

Smk1 must phosphorylate substrates located at or near the PSM to coordinate spore wall morphogenesis (Huang *et al.*, 2005). It is therefore somewhat surprising that sporulation takes place normally in a mutant lacking the TD (*ssp2-ΔN137*). However, Ssp2-Δ137-GFP is found both at the PSM and throughout developing spores (Figure 6). It is possible that delocalized Ssp2-Δ137-GFP previously formed a complex with Smk1 at the PSM, where it activated Smk1 for autophosphorylation and phosphorylation of target proteins. If so, this delocalized pool of protein may have previously carried out its function. In prior studies, we demonstrated that it is possible to cripple *SMK1* transcription by ~80–90% before sporulation phenotypes are observed (Pierce *et al.*, 1998). These findings may also be relevant to the *ssp2-Δ137* phenotype, since they imply that the *SMK1* pathway is robust enough to withstand perturbations that reduce the fraction of Smk1 that is properly localized. Although Smk1 and Ssp2 exist in a complex, and the complex may play a role in retaining Smk1 at the PSM once the complex has formed, Ssp2 is not required to recruit Smk1 to the PSM. The mechanism that recruits Smk1 to the PSM remains to be determined but is likely to be essential for Ssp2-dependent autophosphorylation of Y209.

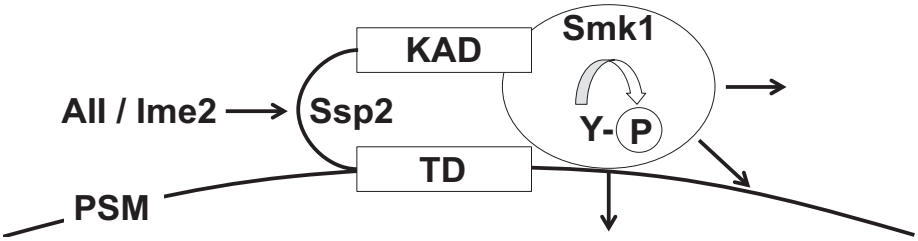

FIGURE 7: Model for the spatiotemporal regulation of Smk1 autophosphorylation by Ssp2. The transcriptional cascade of meiosis and Ime2-dependent translational regulatory mechanisms generate a pulse of Ssp2 protein at anaphase of MII (AII). The amino-terminal targeting region of Ssp2 (TD) targets the kinase-activating domain (KAD) to the PSM, which is enveloping haploid cells at this time. The interaction of the TD-localized KAD with Smk1 at the PSM activates the *cis*-autophosphorylation of the Smk1 activation-loop Y residue, thereby activating the Smk1 MAPK specifically at the PSM.

Strain	Genotype	Source
ALY62	*MATa/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 smk1::LEU2/smk1::LEU2*	McDonald *et al.* (2009)
AXS12	*MATa/MATα ura3/ura3 leu2::hisG/leu2::hisG lys2/lys2 trp1::hisG/trp1::hisG his3/HIS3 ho::LYS2/ho::LYS2 SMK1-3HA-HIS3/SMK1-3HA-HIS3 SSP2-13MYC-KANMX6/SSP2-13MYC-KANMX6 ura3::PGPD1-GAL4(848).ER::URA3/ura3::PGPD1-GAL4(848).ER::URA3 pGAL1-NDT80::TRP1/pGAL1-NDT80::TRP1 IME2(M146G)-13MYC::TRP1/IME2(M146G)-13MYC::TRP1*	This study
EWY122	*MATa/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 his4-N and/or his4-G trp1::hisG/TRP1 SMK1-3HA::KANMX6/SMK1-3HA::KANMX6 ssp2Δ/ssp2Δ*	Shin *et al.* (2010)
GOY29	*MATa/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 SMK1-3HA::HIS3/SMK1-3HA::HIS3 SSP2-13MYC::KANMX6/SSP2-13MYC::KANMX6*	This study
JTY52	*MATa/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 SMK1-3HA::HIS3/SMK1-3HA::HIS3 SSP2-GST::TRP1/SSP2-GST::TRP1*	This study
JTY59	*MATa/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 SMK1-GST::KANMX6/SMK1-GST::KANMX6 SSP2-MYC::KANMX6/SSP2-MYC::KANMX6*	This study
JTY68	*MATa/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 SMK1-3HA::HIS3/SMK1-8HIS-HA::LEU2 SSP2-ΔC20-GST::TRP1/ssp2-KANMX6::URA3*	This study
JTY70	*MATa/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 SMK1/SMK1-8HIS-HA::LEU2 SSP2-ΔN26-GST::TRP1/ssp2-KANMX6::URA3*	This study
JTY71	*MATa/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 SMK1/SMK1-8HIS-HA::LEU2 SSP2-ΔN63-GST::TRP1/ssp2-KANMX6::URA3*	This study
JTY72	*MATa/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 SMK1/SMK1-8HIS-HA::LEU2 SSP2-ΔN137-GST::TRP1/ssp2-KANMX6::URA3*	This study
JTY73	*MATa/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 SMK1/SMK1-8HIS-HA::LEU2 SSP2-ΔN170-GST::TRP1/ssp2-KANMX6::URA3*	This study
JTY74	*MATa/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 SMK1/SMK1-8HIS-HA::LEU2 SSP2-ΔN190-GST::TRP1/ssp2-KANMX6::URA3*	This study
JTY93	*MATa/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 SMK1-8HIS-HA::LEU2/SMK1-8HIS-HA::LEU2 IME2(M146G)-13MYC::TRP1/IME2(M146G)-13MYC::TRP1*	This study
LH902	*MATa/MATα ho::hisG/ho::hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA HTB2-mCherry-TRP1$^{C.g.}$/HTB2-mCherry-TRP1$^{C.g.}$*	Parodi *et al.* (2012)

TABLE 1: Yeast strains.

Spatiotemporal activation of MAPK during morphogenetic programs

Taken together, the data in this study suggest a model in which Ssp2 triggers activation of the Smk1 MAPK at a specific stage of meiosis (anaphase II) at a specific location (the PSM; Figure 7). In this model, timing of protein kinase activity is specified by a transcriptional cascade that is temporally diversified by translational regulatory mechanisms, whereas location is specified by a kinase-activating domain that is physically linked to a targeting domain. Related mechanisms may regulate other MAPKs. For example, although the mating pheromone–responsive Fus3 MAPK in yeast is activated by a canonical MAPK pathway, the Ste5 scaffold, which recruits the kinases in this pathway, also contains a motif that can activate the *cis*-autophosphorylation of Fus3 on its activating Y residue (Bhattacharyya *et al.*, 2006). Multiple mammalian protein kinases in the CMGC group of kinases (cyclin-dependent kinase, MAP kinase, glycogen synthase kinase, CDK-like kinase) have been shown to autophosphorylate activation-loop Y residues in *cis*. The best-characterized examples include the Erk7/8, DYRK, and GSK3ß protein kinases, which autophosphorylate their activating Y residues as these proteins are being produced (Abe *et al.*, 2001; Lochhead *et al.*, 2005, 2006; Klevernic *et al.*, 2006; Kinstrie *et al.*, 2010). The findings in this study are consistent with Smk1 autophosphorylation being coupled to the production of new kinase. The Ssp2/Smk1 pathway may be viewed as a starting point for understanding how the production of protein kinases and *cis*-autophosphorylation are spatially controlled during developmental programs.

MATERIALS AND METHODS

Yeast strains, culture conditions, and plasmids

All yeast strains used in this study were in the SK1 background (Table 1). Vegetative cultures were maintained in YAPD (1% yeast extract, 2% peptone, 2% glucose) or SD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, nutrients essential for auxotrophic strains). For sporulation experiments, cells were grown overnight in YEPA (1% yeast extract, 2% peptone, 2% potassium acetate) to a density of 10^7 cells/ml (mid log). Cells were collected by centrifugation, washed in 2% potassium acetate, resuspended to 4×10^7 cells/ml in sporulation medium (2% potassium acetate, 10 μg/ml adenine, 5 μg/ml histidine, 30 μg/ml leucine, 7.5 μg/ml lysine, 10 μg/ml tryptophan, 5 μg/ml uracil), and placed on a roller drum at 30°C. Synchronous sporulation in the estradiol-inducible

Plasmid	Marker	Source
pJ82	*SSP2*-GFP + 2μ *LEU2*	Sarkar *et al*. (2002)
pJT10	*SMK1*-GFP + 2μ *LEU2*	This study
pJT13	*SSP2ΔC20*-GFP + 2μ *LEU2*	This study
pJT14	*SSP2ΔN137*-GFP + 2μ *LEU2*	This study
pJT20	*SSP2ΔN170*-GFP + 2μ *LEU2*	This study
pJT22	*SSP2Δ*-GFP + 2μ *LEU2*	This study
pJT24	*SSP2ΔC320*-GFP + 2μ *LEU2*	This study
pJT25	*SSP2ΔC270*-GFP + 2μ *LEU2*	This study
pJT26	*SSP2ΔC233*-GFP + 2μ *LEU2*	This study
pRS426-RFP-SPO20$^{51\text{-}91}$	P_{TEF2}-RFP-*SPO20*$^{51\text{-}91}$ + 2μ *LEU2*	Nakanishi *et al*. (2007)

TABLE 2: Plasmids.

NDT80 system was carried out as described (Whinston *et al*., 2013). In brief, sporulation was induced as described. At 6 h postinduction, β-estradiol was added to 2 μM to induce expression of *NDT80*. Ime2-as1 was inhibited by adding Bn-PP1 (a generous gift from Kevin Shokat, University of California, San Francisco, CA) to 10 μm. Plasmids used in this study are listed in Table 2.

Purification of proteins

For purification of GST-tagged Smk1 and Ssp2, 1×10^9 sporulating cells (8 h postinduction) were collected by centrifugation, lysed in 1 ml of lysis buffer (LB; 300 mM NaCl, 5 mM $MgCl_2$, 25 mM Tris-Cl, pH 7.4, 0.5% NP-40, with protease inhibitors at the concentrations as specified; Schindler and Winter, 2006) with four 40-s pulses of a Mini-Beadbeater-24 (BioSpec, Bartlesville, OK) with 1 min incubations on ice between each pulse. Whole-cell extracts were separated from the glass beads and centrifuged at 15,000 rpm for 10 min at 4°C. Lysates were added to 80 μl of glutathione–Sepharose 4B (GE Healthcare, Pittsburgh, PA) that had been washed in LB and incubated for 2 h at 4°C with end-over-end rotation. Beads were washed twice with LB, twice with wash buffer (25 mM Tris-Cl, pH 7.4, 0.5% NP-40), and subsequently eluted with reduced 25 mM glutathione (Acros Organics) in 25 mM Tris-Cl, pH 7.4. Eluted proteins were precipitated with trichloroacetic acid, washed in acetone, and analyzed by gel electrophoresis.

Smk1-HH proteins were purified from meiotic cells under denaturing conditions as previously described (Corbi *et al*., 2014). Briefly, 2×10^8 sporulating cells (8 h after transfer to sporulation medium) were collected by centrifugation. Collected cells were lysed with NaOH, and proteins were precipitated with trichloroacetic acid (TCA). Precipitated proteins were resuspended in denaturing buffer and purified with nickel beads (Chen *et al*., 2005).

Immunofluorescence

We harvested 4×10^7 sporulating cells either 6 or 8 h after transfer to sporulation medium to enrich for MI and MII cells, respectively, and fixed them with 3.7% formaldehyde for 1 h at 30°C. Cells were washed with water three times, resuspended in 1 M sorbitol/1× phosphate-buffered saline (PBS), and digested with 0.5 mg/ml Zymolyase 20-T for 20 min at 23° C. Spheroplasted cells were permeabilized with 1% Triton X-100/1.2 M sorbitol, washed, and placed onto a polylysine-coated slides and incubated with 3% bovine serum albumin for 20 min at room temperature. Subsequently cells were incubated with rabbit polyclonal anti-Myc (Santa Cruz Biotechnology, Dallas, TX), mouse monoclonal anti-HA (HA.11 from Covance), or mouse monoclonal anti-tubulin (a gift from Erica Johnson, Thomas Jefferson University, Philadelphia, PA) as indicated at room temperature for 2.5 h. Slides were subsequently incubated with secondary antibodies (Cy3-labeled goat anti-rabbit or 5-(4,6-dichlorotriazinyl) aminofluorescein–labeled donkey anti-mouse; Jackson ImmunoResearch, West Grove, PA) at room temperature for 1 h. All antibodies were used at 1:200 except for the tubulin antibody, which was used at 1:20. After washing, mounting media (Prolong gold antifade containing 4′,6-diamidino-2-phenylindole [DAPI] from Molecular Probes, Thermo Scientific, Waltham, MA) was added, and cells were visualized using a Leica DM-RXA microscope with oil immersion 60× magnification. For the PLA, 4×10^7 sporulating cells with Smk1-HA and Ssp2-Myc were fixed, permeabilized, and processed as described. Slides were then processed according to the instructions provided with the Duolink PLA Starter Kit (Sigma-Aldrich, St. Louis, MO).

Electrophoresis and immunoblot analyses

For Ime2-as1 experiments, sporulating cells were lysed with NaOH, and proteins were prepared by TCA as described (Schaber *et al*., 2002). Electrophoresis was carried out using Phos-tag acrylamide as described (Whinston *et al*., 2013). Proteins were transferred to an Immobilon-P membrane and probed for HA with a 1:10,000 dilution of HA.11 monoclonal antibody (Berkeley Antibody Company), for Myc with a 1:5000 dilution of 9E10 anti–c-myc monoclonal antibody (Berkeley Antibody Company, Richmond, CA), for the PSTAIRE epitope with a 1:10,000 monoclonal antibody (Sigma-Aldrich), and for GST with a 1:500 dilution of GST monoclonal antibody (Santa Cruz Biotechnology). Smk1-Y209p phospho-specific analyses were carried out as previously described (Whinston *et al*., 2013). Alkaline phosphatase–conjugated goat anti-mouse immunoglobulin G (Promega, Madison, WI) diluted 1:5000 was used to detect immunoreactivity.

Miscellaneous assays and procedures

For phenotypic analyses of cells carrying truncated derivatives of Ssp2, sporulating cells were fixed with ethanol and stained with DAPI as previously described (Krisak *et al*., 1994). Cells were photographed under wet mount using a Nikon Optiphot equipped for epifluorescence. For measuring sporulation efficiency, meiosis-positive cells identified by DAPI fluorescence were scored for the presence of refractile spore compartments by phase contrast microscopy. Cells were scored as sporulation positive if two or more refractile spores were observed in a single meiosis-positive ascus. The fluorescence assay for incorporation of dityrosine into insoluble material was performed as previously described (Wagner *et al*., 1999). For live-cell imaging, 8 μl of sporulating cells carrying GFP/red fluorescent protein plasmids were visualized and photographed using a Leica DM-RXA with oil immersion 60× magnification. For RT-PCR, 1×10^8 cells were lysed with glass beads as described. RNA was extracted using RNeasy Kit (Qiagen, Valencia, CA), and 3 μg of total RNA was reverse transcribed with oligonucleotides specific for the *SMK1* open reading frame. *SSP2* cDNA was quantitated using standard PCR.

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