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The meiosis-specific Cdc20 family-member Ama1 promotes binding of the Ssp2 activator to the Smk1 MAP kinase

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ABSTRACT Smk1 is a meiosis-specific MAP kinase (MAPK) in budding yeast that is required for spore formation. It is localized to prospore membranes (PSMs), the structures that engulf haploid cells during meiosis II (MII). Similar to canonically activated MAPKs, Smk1 is controlled by phosphorylation of its activation-loop threonine (T) and tyrosine (Y). However, activation loop phosphorylation occurs via a noncanonical two-step mechanism in which 1) the cyclin-dependent kinase activating kinase Cak1 phosphorylates residue T207 in Smk1, and 2) Smk1 autophosphorylates Y209 as MII draws to a close. Autophosphorylation of Y209 and cyclin-dependent kinase activating kinase Cak1 phosphorylates T207 during MI, and 2) Smk1's activation loop phosphorylation occurs via a noncanonical two-step mechanism in which 1) the cyclin-dependent kinase activating kinase Cak1 phosphorylates residue T207 in Smk1, and 2) Smk1 autophosphorylates Y209 as MII draws to a close. Autophosphorylation of Y209 and catalytic activity for substrates require Ssp2, a meiosis-specific protein that is translationally repressed until anaphase of MII. Ama1 is a meiosis-specific targeting subunit of the anaphase-promoting complex/cyclosome that regulates multiple steps in meiotic development, including exit from MII. Here, we show that Ama1 activates autophosphorylation of Smk1 on Y209 by promoting formation of the Ssp2/Smk1 complex at PSMs. These findings link meiotic exit to Smk1 activation and spore wall assembly.

INTRODUCTION Meiosis is a specialized form of cell division in which a diploid cell undergoes one round of DNA replication followed by two rounds of chromosome segregation. While meiosis leads to the formation of egg and sperm cells in vertebrates, it leads to the formation of spores in yeast. In many ascomycetes, including Saccharomyces cerevisiae, meiosis is connected to spore formation by prospore membranes (PSMs) (Neiman, 1998). These double-membraned structures engulf the products of meiosis II (MII) as they are being formed and pinch off to generate four haploid cells within the mother cell. Spore wall assembly takes place within and around PSMs, giving rise to multilayered spore walls that protect the haploids from environmental insults (Neiman, 2005, 2011).

Smk1 is a meiosis-specific MAP kinase (MAPK) that is specifically required for spore morphogenesis (Krisak et al., 1994). The SMK1 promoter is induced by the middle-meiotic transcription factor, Ndt80, as cells exit meiotic prophase (G2) and enter meiosis I (MI) (Pierce et al., 1998; Ahmed et al., 2009; Shin et al., 2010). The Smk1 protein is activated in a noncanonical pathway that is coordinated with the subsequent stages in meiosis. First, the cyclin-dependent kinase (CDK)-activating kinase Cak1 phosphorylates residue T207 in Smk1’s activation loop (Wagner et al., 1997; Whinston et al., 2013). Phosphorylation of T207 is insufficient to activate the kinase, and Smk1 remains catalytically inactive until bound by the Ssp2 activator protein later in the pathway (Tio et al., 2017). Similar to SMK1, SSP2 is controlled by an NDT80-inducible promoter (Chu et al., 1998). However, in contrast to SMK1, SSP2 mRNA is translationally repressed until MII, when the meiosis-specific CDK-like kinase Ima2 triggers its translation (Brar et al., 2012; Berchowitz et al., 2013; Tio et al., 2015). Ssp2 localizes to PSMs, where it binds Smk1 and activates the kinase (Tio et al., 2015, 2017). Smk1 then autophosphorylates Y209, further increasing catalytic activity. Thus, this pathway delivers an activated MAPK to the subcellular location where it is needed (the PSM) at a defined stage in meiosis (exit from MII).
Ssp2 consists of an N-terminal targeting domain (TD) that localizes Ssp2 to the PSM and a kinase-activating domain (KAD). The KAD contains two motifs that have been annotated as RNA-recognition motifs (RRMs) based on sequence similarity. However, an interaction between Ssp2 and RNA has not been reported. Although Ssp2 lacking the TD (Ssp2-ΔTD) is delocalized, it can activate Smk1 autophosphorylation and promote spore wall assembly (Tio et al., 2015). While the translational induction of Ssp2 couples Smk1 activation to anaphase II, other mechanisms may also temporally connect Smk1 activation to completion of the meiotic program.

Previous studies have shown that Ama1, a meiosis-specific targeting subunit of the anaphase-promoting complex/cyclosome (APC/C) E3 ubiquitin ligase, positively regulates Smk1 (McDonald et al., 2005). APC/C^Ama1^ is required for multiple steps in meiotic development, and the ama1∆ meiotic phenotype is complex (Cooper et al., 2000; Oelschlaegel et al., 2005; Penkner et al., 2005; Torres and Borchers, 2007; Diamond et al., 2009; Tan et al., 2011, 2013; Okaz et al., 2012). For example, Ama1 targets both Ndd1, a transcriptional regulator that controls progression of the mitotic cell cycle, and Cib4, a mitosis-specific M-phase cyclin, for ubiquitin-dependent degradation during meiotic prophase. Destruction of these mitotic targets is essential for the recombination checkpoint to block meiosis in the presence of persistent recombination intermediates and thus for high-fidelity chromosome segregation (Okaz et al., 2012). Further, Ama1 targets the APC/C targeting subunit Cdc20 for destruction (Tan et al., 2011). As cells complete the meiotic divisions, APC/C^Ama1^ targets another component of the PSM named Ssp1 for destruction in what has been proposed to be a regulatory step in PSM closure (cellularization of the haploids within the mother cell) (Maier et al., 2007; Diamond et al., 2009; Paulissen et al., 2016). In addition, ama1∆ cells disassemble meiotic spindles inefficiently, suggesting that Ama1 promotes exit from MI (Okaz et al., 2012). Although strain-specific differences in the terminal phenotype have been reported, in the SK1 (hypersporulating) strain background, ama1∆ cells block the meiotic program with four nuclear masses surrounded by PSMs. However, these PSMs fail to pinch off and spore wall assembly is undetectable (Diamond et al., 2009).

Although Ama1 has been implicated as an activator of Smk1, the mechanism of activation has not been determined (McDonald et al., 2005). Here, we show that Smk1 and Ssp2 are produced in a timely manner and localize to the PSM in ama1∆ cells. However, Smk1 and Ssp2 do not form a complex in ama1∆ cells, and the Y209 phosphorylated fraction of Smk1 is reduced. In the ama1∆ background, Smk1 and Ssp2 are hyperstabilized, and they accumulate and persist at the PSM. Taken together, these data demonstrate that Smk1 and Ssp2 are poised at the PSM, but their interaction and Smk1 activation require Ama1.

**RESULTS**

**APC/C^Ama1^ is required for Smk1 autophosphorylation**

For determination of whether Ama1 influences the autophosphorylation of Smk1 on Y209, Smk1 tagged on its C-terminus with polyhistidine and a hemagglutinin (HA) epitope (Smk1-HH) was purified under denaturing conditions from postmeiotic ama1∆ and wild-type cells. The relative amounts of phosphorylated Y209 in these samples were analyzed using a phosophospecific antiserum. The fraction of Smk1 phosphorylated on Y209 was substantially decreased in the ama1∆ strain (by 89 and 91% in two separate experiments) (Figure 1A). Ama1 contains conserved APC/C interaction segments termed CB and IR motifs. While ama1-CB∆ and ama1-IR∆ are partially able to support spore wall formation, the double ama1-CB∆,IR∆ mutant is phenotypically indistinguishable from the ama1∆ mutant (Tan et al., 2011). For testing whether Ama1 regulates Smk1 autophosphorylation in an APC/C-dependent manner, Smk1-HH purified from ama1∆ cells containing ama1-CB∆, ama1-IR∆, or ama1-CB∆,IR∆ plasmids was analyzed using the pY209 antiserum.

**FIGURE 1:** APC/C^Ama1^ promotes Smk1 autophosphorylation on Y209. (A) SMK1-HH strains containing the indicated mutations were harvested after most cells had completed MI (9 h postinduction; greater than 75% of cells had completed MI in all cases). Smk1-HH was purified under denaturing conditions and quantitated. Comparable amounts of Smk1-HH were then analyzed using a phosho-specific antiserum (pY209) and an HA antibody (Smk1). Quantitation of the pY209:Smk1 band ratios are shown below each lane with the values of the AMA1 strains (first and third lanes from the left) set at 1.0 to facilitate comparison with the ama1∆ strains (second and fourth lanes from left). (B) ama1∆ SMK1-HH cells containing the indicated AMA1 plasmids were collected at 9 h postinduction, Smk1-HH was purified, and pY209 levels were assayed as in A. (C) SMK1-HA cells that are otherwise wild type (WT), ama1∆, or ama1∆ cib4∆ ndd1 were transferred to sporulation medium and collected at the indicated time points (h). Total protein was analyzed by phostag-acrylamide gel electrophoresis and immunoblot analyses using an HA antiserum. Unphosphorylated (0P), monophosphorylated (1P), or doubly phosphorylated (2P) forms of Smk1 are indicated. The fraction of Smk1 that is doubly phosphorylated at the 8-h time point in wild-type, ama1∆, and ama1∆ cib4∆ ndd1 cells in this experiment is 47, 3, and 8%, respectively.
While plasmid-encoded *AMA1* is not as effective as *AMA1* expressed from its natural chromosomal location in promoting Smk1 autophosphorylation, *AMA1* plasmids did increase the fraction of autophosphorylated Smk1 in *ama1*Δ cells. Both the *ama1*-CBΔ and the *ama1*-IRΔ plasmids also increased the fraction of Smk1 that was autophosphorylated on Y209, while the *ama1*-CBΔ/IRΔ mutant plasmid had little or no effect (Figure 1B). These findings indicate that the role of Ama1 in activating Smk1 autophosphorylation is dependent on binding the APC/C.

Ama1 exerts its influence on the Smk1 signaling pathway after the late stage of meiotic G2 when Ndt80 is induced (Whinston *et al.*, 2013). To investigate the interplay between Ssp2 and Ama1 in controlling Smk1 stability, we monitored Smk1-HA in *ama1*Δ cells expressing detectable Smk1-GFP and Ssp2-GFP within a given experiment were identical, and intensities of the fluorescence signals in the two strains as MII was being completed were indistinguishable. Importantly, as MII was being completed, Smk1-GFP and Ssp2-GFP fluorescence was tightly localized to PSMs in more than 95% of the fluorescent wild-type and *ama1*Δ cells. Taken together, these data indicate that Ama1 exerts its influence on the Smk1 signaling pathway after Ssp2 and Smk1 are localized at the PSM.

**AMA1 promotes formation of the Smk1-Ssp2 complex at the PSM**

To investigate whether Ama1 is required for formation of the Ssp2/Smk1 complex, we used glutathione S-transferase (GST)-tagged forms of Ssp2. Ssp2-ΔTD-GST is more soluble than full-length Ssp2-GST, and this facilitates analyses of the Ssp2/Smk1 interaction. Ssp2-ΔTD-GST was purified from *ama1*Δ and wild-type cells expressing *SMK1-HH*. The amount of Smk1-HH that copurified with Ssp2-ΔTD-GST in postmeiotic *ama1*Δ cells was 7 ± 3% of that seen in wild type (n = 3) (Figure 2). These data indicate that Ama1 controls the Smk1 pathway by promoting the interaction of Ssp2 and Smk1.

Smk1 localizes to PSMs during anaphase II (Tio *et al.*, 2015). Although Ssp2 is found almost exclusively at PSMs and may play a role in retaining Smk1 at PSMs, Smk1 localizes to PSMs in the absence of Ssp2. We transformed wild-type and *ama1*Δ strains containing the HTB2-mCherry histone marker with multicopy plasmids containing Ssp2-green fluorescent protein (SSP2-GFP) or *SMK1-GFP* and monitored cells that were completing meiosis by fluorescence microscopy (Figure 3). Although Smk1-GFP and Ssp2-GFP fluorescence was observed in a fraction of these cells that varied from experiment to experiment (a consequence of plasmid loss), the fractions of wild-type and *ama1*Δ cells with detectable Smk1-GFP and Ssp2-GFP within a given experiment were identical, and intensities of the fluorescence signals in the two strains as MII was being completed were indistinguishable. Importantly, as MII was being completed, Smk1-GFP and Ssp2-GFP fluorescence was tightly localized to PSMs in more than 95% of the fluorescent wild-type and *ama1*Δ cells. Taken together, these data indicate that Ama1 exerts its influence on the Smk1 signaling pathway after Ssp2 and Smk1 are localized at the PSM.

**Ama1 and Ssp2 regulate Smk1 stability**

Previous work suggested that Ssp2 not only activates Smk1 but that it also has a stabilizing influence on Smk1 as cells complete meiosis (Whinston *et al.*, 2013). To investigate the interplay between Ssp2 and Ama1 in controlling Smk1 stability, we monitored Smk1-HA in *ama1*Δ and *ssp2Δ* cells at various times after transfer to sporulation medium (Figure 4).

Smk1 accumulation in wild-type cells is biphasic. The first phase starts around 5 h postinduction, when many cells are in pachytene, the late stage of meiotic G2 when *Ndt80* is induced (Whinston *et al.*, 2013). Smk1 accumulation continues during the 5- to 6.5-h interval as most cells are carrying out MII. This first phase of Smk1 accumulation will be referred to as “meiotic.” During the 6.5- to 8-h interval, as cells complete MII and Ssp2 is translated, the amount of Smk1-HA further increases. This second phase of Smk1 accumulation will be referred to as “postmeiotic.” Previous studies have shown that Ssp2 translation and Smk1 autophosphorylation occur during the postmeiotic phase of Smk1 accumulation (Whinston *et al.*, 2013; Tio *et al.*, 2015), which is undetectable in *ssp2Δ* cells. The postmeiotic Smk1 accumulation was also muted in the *ama1*Δ mutant. One
remnants. Taken together, these findings suggest that, while Ama1 stabilizes Smk1 by promoting association with Ssp2 as MII is being completed, it is also required for the developmentally regulated elimination of Smk1 after key steps in spore wall formation have been completed. Smk1 could be persistent in the ama1Δ mutant at late time points (24 h postinduction), because ama1Δ blocks the program before the point when Smk1 degradation is induced and/or because Ama1 plays a direct role in eliminating Smk1 once key steps in the program have been completed.

**Ssp2 is persistently phosphorylated when Smk1 is inactive**

It has previously been shown that the Ssp2 that is translated during anaphase II is rapidly phosphorylated and that Smk1 autophosphorylation and Ssp2 dephosphorylation occur around the same time (Whinston *et al*., 2013). These observations led to the suggestion that the dephosphorylation of Ssp2 plays a role in triggering Smk1 activation. We analyzed the phosphorylation of Ssp2-MYC by phostag-acrylamide electrophoresis in wild-type and ama1Δ cells at various times postinduction (Figure 5). When Ssp2-Myc is first produced (6.5 h), two major forms are present in both wild-type and ama1Δ cells. The electrophoretic resolution of the major slower-migrating form (labeled Ssp2-P in Figure 5) from the faster-migrating form of Ssp2 requires the inclusion of phostag-acrylamide in the electrophoretic gel, indicating that Ssp2-P is phosphorylated. While the relative amount of Ssp2-P decreases substantially as wild-type cells progress through meiosis, the decrease in the Ssp2-P:Ssp2 ratio is not observed in ama1Δ cells. These findings indicate that Ssp2 is inefficiently dephosphorylated in ama1Δ cells.

Cdc20 family members are known to promote the dephosphorylation of cell cycle regulatory proteins that have been phosphorylated by CDKs (Pesin and Orr-Weaver, 2008). The persistent phosphorylation of Ssp2 in ama1Δ cells might prevent Ssp2 and Smk1 from forming a stable complex or it could be a consequence of a failed Ssp2:Smk1 interaction. Previous studies have demonstrated that a catalytically inactive form of Smk1 (Smk1-K69R) binds to Ssp2-GST indistinguishably from wild-type Smk1 (Tio *et al*., 2017). We next tested Ssp2-MYC phosphorylation in smk1Δ and smk1Δ-K69R backgrounds containing and lacking AMA1. Ssp2 is persistently hyperphosphorylated in all of the mutants tested (Figure 5B). These observations suggest that activated Smk1 is required to promote the dephosphorylation of Ssp2 and that the dephosphorylation of Ssp2 is a consequence of Smk1 activation and not a regulatory step that controls formation of the Ssp2/Smk1 complex. We also assayed Ssp2-ATD-Myc in wild-type and ama1Δ cells using phostag-acrylamide electrophoresis and found no evidence that this truncated protein was differentially phosphorylated at different stages of meiosis (Figure 5C). These observations are consistent with Ama1 controlling Ssp2/Smk1 complex formation through a mechanism that is independent of Ssp2 dephosphorylation.

**An Ssp2 mutant that fails to bind Smk1 is persistently phosphorylated**

It has previously been shown that deletions that eliminate residues at Ssp2's carboxy terminus eliminate the ability of Ssp2 to form a complex with Smk1 (Tio *et al*., 2015). During the course of our studies, we identified several missense mutations in the C-terminal coding region of Ssp2 that cause a sporulation-defective phenotype (unpublished data). One of these defective alleles contains F to A changes at residues 307 and 327 of the protein (referred to later as ssp2-F/A). These substitutions change aromatic amino acids in Ssp2, one of which (F307) was previously shown to be required for the interaction of Ssp2 with Smk1. The ssp2-F/A cell line lacks cell wall integrity and fails to complete meiosis. This phenotype is not rescued by either the wild-type or the catalytically inactive Smk1-K69R, suggesting that Smk1 kinase activity is not required for this interaction. It would be of interest to compare the phosphorylation profile of ssp2-F/A with that of wild-type and Smk1-K69R, as well as with that of the catalytically inactive Smk1-A241E (unpublished results).

**Ssp2 localizes to the PSM in ama1Δ cells**

Wild-type and ama1Δ cells containing the nuclear HTB2-mCherry marker (strains LH902 and TY110 respectively) transformed with multicopy plasmids containing either SMK1-GFP (top) or SSP2-GFP (bottom) (see Table 2) were transferred to sporulation media and viewed by fluorescence microscopy as they completed meiosis.
Ssp2 is correlated with uncomplexed Ssp2 in meiotic cells, dephosphorylation of Ssp2 is a downstream consequence of Smk1 activation and not a mechanism used by Ama1 to regulate formation of the Ssp2/Smk1 complex.

**DISCUSSION**

Metaphase II in *S. cerevisiae* is followed in rapid succession by anaphase II, exit from MII, PSM encapsulation of the haploid, and spore wall assembly. The APC/C E3 ubiquitin ligase functions as a major driver of these events through the use of the Cdc20, Cdh1, and Ama1 targeting subunits (Cooper and Strich, 2011). The meiosis-specific targeting subunit Ama1 plays a particularly prominent role in controlling exit from MII, PSM closure, and spore wall assembly. In this study we have shown that Ama1 triggers activation of the Smk1 MAPK by regulating the interaction of Ssp2 and Smk1 at the PSM.

**SMK1 and SSP2** are both transcriptionally induced by Ndt80 as cells exit meiotic G2 and enter MI (Ahmed et al., 2009; Shin et al., 2010). While SMK1 mRNA is translated shortly after transcription, SSP2 is a member of a subset of genes whose mRNAs are repressed until the meiosis-specific Ime2 CDK-like kinase triggers their translation at MII by phosphorylating the Rim4 translational repressor (Brar et al., 2012; Berchowitz et al., 2013, 2015; Tio et al., 2015). This mechanism provides the first-level temporal control that prevents Smk1 activation until the MII spindle is present and PSM outgrowth is occurring. It has previously been shown that Smk1 and Ssp2 localize to the growing PSM independently (Tio et al., 2015). In the present study, we show that ama1Δ decreases the activation state of Smk1. Although both Smk1 and Ssp2 localize to the PSM, the interaction between Smk1 and Ssp2 is substantially reduced in the ama1Δ background. These findings suggest the Smk1 is poised for activation at the PSM as MII is taking place and that an Ama1-dependent step promotes formation of the Ssp2/Smk1 complex, thereby triggering activation of the kinase. Ama1 also promotes exit from MII (disassembly of the anaphase II spindle), and it is required for PSM closure (Diamond et al., 2009; Okaz et al., 2012). These observations suggest that Ama1 couples these events to Smk1 activation, thereby delaying spore wall assembly until meiosis has been completed and cellularization of the four haploid cells has taken place within the mother cell.

Ssp2 is hyperphosphorylated shortly after it is translated (during MII), and it accumulates in a dephosphorylated form around the time that Smk1 autophosphorylation takes place. On the basis of these findings, we previously hypothesized that the dephosphorylation of Ssp2 is required for Smk1 activation (Whinston et al., 2013).

**Acids in the C-terminal RRM-like motif of Ssp2 that align with residues in well-studied RRMs that interact with cognate RNAs** (Daubner et al., 2013). Phos-tag acrylamide electrophoretic analyses revealed that the F/A substitutions prevent the postmeiotic accumulation of autophosphorylated Smk1, similar to ssp2Δ, and that Ssp2-F/A is persistently phosphorylated and comigrates with Ssp2-P (Figure 6A). While wild-type Ssp2-ΔTD-GST formed a stable complex with Smk1, Ssp2-ΔTD-F/A-GST did not (Figure 6B). Nevertheless, the F/A form of Ssp2 fused to GFP localized to PSMs (Figure 6C). Thus, the behavior of Ssp2-F/A in otherwise wild-type cells resembles that of wild-type Ssp2 in the ama1Δ mutant. Collectively, these data suggest that, although hyperphosphorylated
In this study, we show that, although Ssp2 remains hyperphosphorylated in ama1Δ cells, it also remains hyperphosphorylated in smk1K69R cells in which Ssp2 is complexed to Smk1 (Tio et al., 2015, 2017). Further, we have shown that a mutant form of Ssp2 that cannot bind Smk1 is persistently hyperphosphorylated. Moreover, Ama1 controls the binding of Ssp2-ΔTD, which does not appear to be differentially phosphorylated during meiosis. Smk1 is phosphorylated exclusively on its activation loop, and unphosphorylated, singly phosphorylated, and doubly phosphorylated Smk1 can form a complex with Ssp2 (Tio et al., 2017). Thus, although targeting sub-units of the APC/C are well known to control cell cycle events by influencing the dephosphorylation of regulatory proteins, these data indicate that Ama1 does not trigger Ssp2/Smk1 binding by promoting the dephosphorylation of either of these proteins. One possibility is that an inhibitory protein in the PSM that is targeted for regulated protein destruction as sporulation draws to a close.

Our data suggest that Smk1 and Ssp2 are destroyed by an AMA1-dependent pathway after spore wall assembly has occurred, leading to an absence of Smk1 and a reduced amount of Ssp2 in mature spores. While Smk1 may be recruited to the APC/C by Ama1, it is also likely that the block in meiotic progression observed in ama1Δ cells prevents cells from reaching the point in meiotic development at which Smk1 degradation can occur. While most canonical MAPK signaling is opposed by phosphatases that remove phosphate from the activation loop, Smk1 appears to remain fully phosphorylated until it is degraded. These findings suggest that Smk1 signaling is terminated by regulated protein destruction as sporulation draws to a close.

**FIGURE 5:** Ssp2 is persistently phosphorylated in ama1Δ cells. (A) SSP2-MYC wild-type and ama1Δ cells were incubated in sporulation medium for the indicated times (h), and total protein was analyzed by phostag-acrylamide gel electrophoresis and immunoblot analyses using a Myc antibody (Ssp2) or an antibody that recognizes Cdc28 as indicated. (B) SSP2-MYC cells harboring the indicated mutations were incubated in sporulation medium for 9.5 h and analyzed by phostag-acrylamide gel electrophoresis and immunoblot analysis using a Myc antibody. Greater than 80% of cells had completed MII in all cases. (C) SSP2-MYC and SSP2-ΔTD-MYC cells with and without AMA1 were collected at the indicated times (h) and analyzed by phostag-acrylamide gel electrophoresis and immunoblot analyses using a Myc antibody.

**MATERIALS AND METHODS**

**Yeast strains, culture conditions, and plasmids**

All yeast strains in this study were in the SK1 background (Table 1). Cells were grown in vegetative cultures in YEPD (1% yeast extract, 2% peptone, 2% glucose) supplemented with adenine to 40 µg/ml, or SD (0.67% yeast nitrogen base without amino acids, 2% glucose, and nutrients essential for auxotrophic strains) at 30°C. Sporulation assays were performed by inoculating vegetative cells from YEPD into YEPA (1% yeast extract, 2% peptone, 2% potassium acetate) supplemented with adenine to 40 µg/ml and growing them overnight at 30°C to a density of 10⁷ cells/ml. Cells were pelleted by centrifugation, washed, and resuspended 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) at 4 × 10⁷ cells/ml, and incubated in a roller drum at 30°C. Estradiol-inducible NDT80 cells were sporulated as previously described (Whinston et al., 2013) except that β-estradiol was added to 2 µM at 6 h postinduction. Plasmids used in this study are listed in Table 2. The plasmid pRS314-SSP2 was made by ligating a PCR fragment containing the SSP2 open reading frame with 500 base pairs upstream and 500 base pairs downstream as a KpnI/Spe1 fragment into pRS314. pRS314-SSP2-F307A,F327A was generated from pRS314-SSP2 by site-directed mutagenesis. The SSP2-F307A,F327A-GFP plasmid was generated from pJ82 by site-directed mutagenesis. Mutated genes in plasmids were sequenced in their entirety to assure that only the desired substitutions were present. Wild-type SSP2 was replaced with SSP2-ΔTD-F/A-GST using the strategy that was described for SSP2-ΔTD-GST, except that the source of DNA for the SSP2 PCR was pRS314-SSP2-F307A,F327A instead of wild-type genomic DNA (Tio et al., 2015). The genomic copy of SSP2-ΔTD-MYC in EWY196 and EWY198 was generated by replacing the GST segment of SSP2-ΔTD-GST with MYC using a previously described strategy (Tio et al., 2015).

**Purification of proteins**

Purification of GST-tagged proteins was performed as described in Tio et al. (2015). Briefly, 1 × 10⁹ cells were collected using centrifugation and resuspended in 1 ml of lysis buffer (300 mM NaCl, 5 mM MgCl₂, 25 mM TrisCl, pH 7.4, 0.5% NP-40, and protease inhibitors to the concentrations specified; Schindler and Winter, 2006). The cells were lysed using three 40-s and one 30-s pulse of a Mini-Beadbeater 24 with 1 min cooling on ice in between pulses. A low-speed centrifugation was used to separate the whole-cell extract from the beads, and a 15,000 rpm spin at 4°C for 10 min was used to clear the cell lysate. Cleared lysates were added to 20 µl of glutathione-Sepharose 4B and incubated for 2 h at 4°C with end-over-end rotation. The beads were washed two times with lysis buffer and two times with wash buffer, and the proteins were eluted with 25 mM reduced glutathione in 25 mM Tris HCl (pH 7.4). The eluted proteins were precipitated with trichloroacetic acid (TCA), washed with acetone, and analyzed by gel electrophoresis. Smk1-HH proteins were subjected to a 25% reduction in glutathione in 25 mM Tris HCl (pH 7.4) prior to purification because of their instability. One milliliter of bead slurry was used per gram of total cell lysate.
purified under denaturing conditions as previously described (Corbi et al., 2014). In brief, \(2 \times 10^8\) cells were collected by centrifugation and lysed with NaOH. The proteins were precipitated with TCA, resuspended in denaturing buffer, and purified using nickel beads.

**Immunoblot analyses**

Sporulating cells were lysed with NaOH, and proteins were precipitated with TCA and solubilized with 8 M urea, as previously described (Knop et al., 1999). This protocol maximizes extraction of membrane-associated proteins from TCA precipitates and has been shown to efficiently extract PSM proteins from sporulating cells. After electrophoresis, proteins were transferred to an Immobilon-P membrane and probed with mouse anti-HA.11 (1:10,000) for Smk1 and mouse anti-MYC (1:5000) and mouse anti-GST (1:500) for Ssp2, as previously described (Tio et al., 2015). Smk1-Y209p analyses were performed as previously described (Whinston et al., 2013). Alkaline phosphatase–conjugated goat anti-mouse immunoglobulin G (1:5000) (Promega) was used as a secondary antibody.

Protein samples analyzed using phostag-acrylamide gels were prepared as described above, except that EDTA was omitted from the gel-loading buffer. Samples were electrophoresed through an 8% acrylamide gel containing 50 \(\mu\)M phostag-acrylamide and 100 \(\mu\)M MnCl\(_2\). After electrophoresis, the gel was washed in transfer buffer with 2 mM EDTA for 20 min followed by transfer buffer without EDTA. Proteins were transferred to Immobilon-P membranes and analyzed as described above.

Band intensities were quantitated using ImageJ software. For comparison of Ssp2-\(\Delta\)TD-GST/Smk1-HH binding between wild-type and ama1\(\Delta\) cells, the ratios of the Smk1-HH:Ssp2-\(\Delta\)TD-GST signals from samples that bound glutathione-Sepharose 4B beads were divided by the ratios from the corresponding input samples. All immunoblot experiments presented in this study were repeated at least three times, except for the experiments presented in Figure 1, A and C, which were repeated twice.

**FIGURE 6:** A binding-defective Ssp2 mutant is persistently phosphorylated and localized to the PSM. (A) Ssp2-MYC, ssp2\(\Delta\), and ssp2-F/A-MYC cells were collected at the indicated times after transfer to sporulation medium, and total protein was analyzed by phostag-acrylamide gel electrophoresis and immunoblot analyses using a Myc antibody (Ssp2), an HA antibody (Smk1), or an antibody that recognizes Cdc28 as indicated. (B) SMK1-HH/SMK1 strains of the indicated Ssp2 genotype (strains TPY11-13) were collected at 9 h postinduction (>80% MII cells in all cases), protein was purified using glutathione resin and assayed with antibodies against HA (Smk1) or GST as indicated. Smk1 autophosphorylation in the same samples was assayed by purifying Smk1-HH using Ni-NTA beads followed by immunoblot analyses with pY209 antiserum and an HA antibody. (C) WT cells containing the HTB2-mCherry marker (strain LH902) transformed with multicopy plasmids containing either Ssp2-GFP or ssp2-F/A-GFP as indicated were transferred to sporulation media and viewed by fluorescence microscopy as they completed meiosis.

**FIGURE 7:** Model for the role of Ama1 in the Smk1 pathway. Smk1 and Ssp2 are localized to the PSM during MII (left). At this stage, Smk1 has been phosphorylated on its activation-loop T by Cak1 as shown, but this is insufficient to activate the kinase. Ssp2 is also phosphorylated on at least one residue at this stage, but the identity of the protein kinase(s) is unknown. Upon exit from MII, Ama1 complexed to the APC/C promotes binding of Ssp2 to Smk1, triggering the autophosphorylation of Smk1’s activation loop Y residue. Although Ssp2 is dephosphorylated once the Ssp2/Smk1 complex has formed, Ssp2 dephosphorylation is a consequence of complex formation and does not appear to regulate Ssp2/Smk1 binding. Because complex formation does not appear to be regulated by reversible phosphorylation, we posit that Ama1 targets a repressor (I), shown to interact with Ssp2 for simplicity, for ubiquitin-dependent destruction, thus coupling exit from MII to Smk1 activation.
lation efficiency was analyzed by phase-contrast microscopy, with

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All strains are in the SK1 background. The ssp2Δ allele in strain JTY4 is deleted for codons 42–242 of the SSP2 open reading frame and lacks a marker as described (Sarkar et al., 2002).

**TABLE 1: Yeast strains.**

**Microscopy**

For assessment of meiotic progression, sporulating cells were fixed with ethanol, stained with 4′,6-diamino-2-phenyindole, and photographed under wet mount using a Nikon Optiphot equipped for epifluorescence as previously described (Krisak et al. 1994). Sporulation efficiency was analyzed by phase-contrast microscopy, with cells containing two or more spores per ascus scored as positive. For live-cell imaging, it was necessary to use multicopy plasmids to detect the Smk1-GFP and Ssp2-GFP signals. Cells containing an integrated nuclear HTB2-mCherry marker (Table 1) carrying 2µ-based SMK1-GFP or SSP2-GFP plasmids (Table 2) were inoculated from selective medium into YEPα for overnight growth and transferred to
sporulation medium as previously described (Tio et al., 2015). A Leica DM-RXA with oil immersion was used to image 8 μl of sporulating cells, using a 60x lens, at the indicated times. Completion of MI was monitored using the HTB2-mCherry marker and further assayed for GFP fluorescence. The fraction of postmeiotic cells that showed GFP fluorescence varied between trials from 8% to 20% due to plasmid loss. All Smk1-GFP and Ssp2-GFP experiments described in this study were repeated independently at least three times with a minimum of 100 fluorescent cells examined.

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REFERENCES


