Dynamic Ubiquitination of the Mitogen-activated Protein Kinase Kinase (MAPKK) Ste7 Determines Mitogen-activated Protein Kinase (MAPK) Specificity**

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Background: Ubiquitination is a post-translational modification that regulates protein behavior.

Results: Pheromone stimulation induces dynamic ubiquitination of the MAPKK Ste7; disruption of this modification leads to altered MAPK signal specificity.

Conclusion: Dynamic ubiquitination is required to maintain the strength and fidelity of the pheromone response.

Significance: This study identifies a novel regulatory mechanism in MAPK cascades, a signaling module that is central to human physiology and disease.

Ubiquitination is a post-translational modification that tags proteins for proteasomal degradation. In addition, there is a growing appreciation that ubiquitination can influence protein activity and localization. Ste7 is a prototype MAPKK in yeast that participates in both the pheromone signaling and nutrient deprivation/invasive growth pathways. We have shown previously that Ste7 is ubiquitinated upon pheromone stimulation. Here, we show that the Skp1/Cullin/F-box ubiquitin ligase SCFCdc4 and the ubiquitin protease Ubp3 regulate Ste7 ubiquitination and signal specificity. Using purified components, we demonstrate that SCFCdc4 ubiquititates Ste7 directly. Using gene deletion mutants, we show that SCFCdc4 and Ubp3 have opposing effects on Ste7 ubiquitination. Although SCFCdc4 is necessary for proper activation of the pheromone MAPK Fus3, Ubp3 is needed to limit activation of the invasive growth MAPK Kss1. Finally, we show that Fus3 phosphorylates Ubp3 directly and that phosphorylation of Ubp3 is necessary to limit Kss1 activation. These results reveal a feedback loop wherein one MAPK limits the ubiquitination of an upstream MAPKK and thereby prevents spurious activation of a second competing MAPK.

MAPK cascades function as signal transduction modules, linking diverse extracellular signals to cell fate decisions. These pathways are initiated by virtually every mitogenic stimulus (1), and they evoke fundamental cellular events, including gene transcription, cell cycle progression, apoptosis, differentiation, and motility (2–5). The core of the MAPK signaling module consists of three protein kinases that are sequentially activated by a phosphorylation cascade as follows: a MAP2 kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK). The MAPKs then phosphorylate effector proteins and transcription factors to alter cellular behavior. So far, 14 MAPKKks, 7 MAPKKs, and 12 MAPKs have been identified in mammalian cells (6). Because these signaling modules are used to mediate such diverse signaling inputs, many will utilize the same signaling proteins to elicit very different outputs. One of the central questions of biology is how cells are able to produce the correct cellular response when so many of the components are shared among different pathways (7).

The yeast mating pathway employs a canonical MAPK cascade. The mating response is mediated by the binding of pheromone to a G-protein-coupled receptor (Ste2/3) that activates a heterotrimeric G-protein, consisting of a Gα subunit (Gpa1) and a Gβγ dimer (Ste4/Ste18). The Gβγ dimer propagates the mating signal through activation of effector proteins, including a protein kinase (Ste20), a kinase scaffolding protein (Ste5), and the Cdc42 GDP-GTP exchange factor (Cdc24). These effectors go on to activate a MAPK cascade composed of a MAPK kinase kinase (Ste11), which phosphorylates and activates a dual specificity MAPK kinase (Ste7), which in turn phosphorylates and activates two related MAPKs (Fus3 and Kss1) (8–10). Major downstream targets of the MAPKs include a transcription factor, Ste12, and a component of the cell cycle regulatory machinery, Far1 (11). Phosphorylation of these proteins leads to new gene transcription, G1 arrest and, eventually, mating competency.

In addition to the pheromone response, MAPK cascades in yeast also mediate responses to environmental stress and nutritional status (12, 13). These pathways utilize two or more signaling proteins that are also involved in the pheromone response pathway. For instance, the pheromone and invasive growth/nutrient deprivation pathways share the MAPKK Ste11 and the MAPKk Ste7 (14, 15). Despite the high degree of similarity between these pathways, yeast have evolved multiple mechanisms to maintain proper activity and specificity within the MAPK cascades (7). Certain modifications such as phosphorylation, which drives the activity of MAPK cascades, are well established as regulators of cellular signaling intensity.

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The abbreviations used are: MAP, mitogen-activated protein; DUB, deubiquitinating protease.

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Other modifications, such as sumoylation, glycosylation, acylation, lipidation, and ubiquitination are also emerging as regulators of cell signaling (16, 17).

Ubiquitination is a tightly regulated process by which the small 76-amino acid protein ubiquitin is attached to lysine residues on target proteins. Attachment of ubiquitin is mediated by a three-step process involving an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase (18). Substrate specificity is controlled by E3, which is frequently recruited via the post-translational modification (phosphorylation) of target proteins. The Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase is a multiprotein complex that utilizes an assortment of F-box proteins to identify phosphorylated substrates (19). Although each component of the SCF is necessary for ubiquitin ligase function, the F-box protein binds directly to the substrate and therefore defines substrate specificity of the SCF complex. Previous work has shown that Cdc34 and the SCF complex regulate mating-associated cell cycle arrest (20–22). We recently demonstrated that SCF complexes are required for proper MAPK activation in Saccharomyces cerevisiae and are likely to target multiple components within the pathway (23). One of these targets, the G-protein a subunit Gpa1 (23), is directly ubiquitinated by SCF in complex with the F-box protein Cdc4. Other components that appear to be degraded in an SCF-dependent manner include Ste4 (23), Ste20 (23), Ste7 (23), and Ste5 (23, 24).

Like many other post-translational modifications, ubiquitination is reversible. Deubiquitinating proteases (DUBs) cleave ubiquitin-ubiquitin and ubiquitin-protein bonds, mediate recruitment of substrates to the proteasome, and remove polyubiquitin chains from substrates to facilitate proteosomal entry. Thus, DUBs have the potential to regulate any ubiquitin-mediated process. There are 17 DUBs in yeast and 95 DUBs in humans, suggesting a high degree of substrate specificity. Additionally, DUB activity is regulated by post-translational modifications, including phosphorylation. New roles in cellular signaling pathways are emerging as new DUB substrates and functionalities are identified (25).

Here, we investigate dynamic ubiquitination of the MAPKK Ste7. Ste7 expression is tightly regulated, and stochastic modeling of the pheromone pathway suggests that very small changes in Ste7 abundance can have a significant impact on pathway activation (26, 27). Additionally, Ste7 is the direct activator of two related MAPKs, Fus3 and Kss1. Although both MAPKs are activated during pheromone signaling, Fus3 is primarily responsible for the mating response (28–30). Kss1 alone is activated during invasive growth (31). We previously found that Ste7 undergoes both SCF-dependent ubiquitination and Ubp3-dependent deubiquitination, indicating that ubiquitination of Ste7 plays a critical role in pheromone pathway activation (32, 33). Given that Ste7 acts upstream of both Fus3 and Kss1, we hypothesized that dynamic ubiquitination of Ste7 contributes to MAPK signaling specificity.

Herein, we demonstrate that Ste7 is recognized by the F-box protein Cdc4 and directly ubiquitinated by the SCF$^{Cdc4}$ ubiquitin ligase. Disruption of SCF$^{Cdc4}$ attenuates the activation of Fus3, but not Kss1. In contrast, disruption of Ubp3 enhances the activation of Kss1, but not Fus3. Finally, we show that Fus3 phosphorylates Ubp3 and that phosphorylation is needed for Ubp3 to limit Kss1 activation in vivo. Taken together, these data demonstrate that dynamic ubiquitination of Ste7 modulates the strength and specificity of MAPK activation and that the cycle of ubiquitination/deubiquitination is controlled by both upstream stimulation and MAPK-mediated feedback loops.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Standard procedures for the growth, maintenance, and transformation of yeast and bacteria and for the manipulation of DNA were used throughout. Yeast strains used in this study were BY4741 (MATa leu2Δ met15Δ his3Δ1 ura3Δ), BY4741-derived strains lacking STE7 (ste7Δ:KANMX4, resistance to geneticin), UBP3 (ubp3Δ:HPMX4, resistance to hygromycin B), KSS1 (kss1Δ::NATMX4, resistance to nourseothricin), TEC1 (tec1Δ::KANMX4), and R1158 (MATa URA3::CMV-tTA leu2Δ met15Δ his3Δ1 ura3Δ) purchased as the yeast Tet-promoter Hughes Collection (yTHC, Open Bio-systems). Deletion strains were validated by PCR amplification of genomic DNA. The Tet-Off strains were originally created from the background strain BY4741 by a one-step integration of the tetracycline-controlled transcriptional activator, under the control of the CMV promoter, at the URA3 locus. A plasmid carrying a kanR-tetO7-TATA cassette was then integrated into the genome replacing the endogenous promoter.

The yeast shuttle plasmids used were pRS315 (CEN, ampR, and LEU2) and pRS316 (CEN, ampR, and URA3). Plasmid pRS423-FUS1-lacZ was described previously (34). pRS315-ADH1-STE7–8HIS and pRS315-ADH1-STE7-FLAG were constructed by PCR amplification and subcloning of the STE7 ORF from pNC766 (35) into the SacI/SacI sites of pRS315-ADH-8HIS or the XmaI site of the yeast shuttle vector pRS315-ADH1-FLAG (36), respectively. pRS315-ADH1-UBP3-FLAG, pRS315-ADH1-UBP3C469A-FLAG, and pRS315-ADH1-UBP3—S695A-FLAG were constructed by amplifying UBP3, ubp3C469A, or ubp3—S695A genes from PEN298, PEN301, and PCS71 (generously supplied by Dr. Eulalia de Nadal, Universitat Pompeu Fabra, Barcelona, Spain (37)), respectively, and subcloned into the XmaI site of pRS315-ADH1-FLAG.

Cells were grown in selective medium using 2% (w/v) dextrose (d/c) for use in experiments (23). The yTHC strains carrying a kanR-tetO7-TATA cassette was then integrated into the genome replacing the endogenous promoter. The yeast shuttle plasmids used were pRS315 (CEN, ampR, and LEU2) and pRS316 (CEN, ampR, and URA3). Plasmid pRS423-FUS1-lacZ was described previously (34). pRS315-ADH1-STE7–8HIS and pRS315-ADH1-STE7-FLAG were constructed by PCR amplification and subcloning of the STE7 ORF from pNC766 (35) into the SacI/SacI sites of pRS315-ADH-8HIS or the XmaI site of the yeast shuttle vector pRS315-ADH1-FLAG (36), respectively. pRS315-ADH1-UBP3-FLAG, pRS315-ADH1-UBP3C469A-FLAG, and pRS315-ADH1-UBP3—S695A-FLAG were constructed by amplifying UBP3, ubp3C469A, or ubp3—S695A genes from PEN298, PEN301, and PCS71 (generously supplied by Dr. Eulalia de Nadal, Universitat Pompeu Fabra, Barcelona, Spain (37)), respectively, and subcloned into the XmaI site of pRS315-ADH1-FLAG.

Cells were grown in selective medium using 2% (w/v) dextrose. The yeast yTHC strains were grown in selective medium to $A_{600}$ nm~0.8, re-inoculated at 1:80 in medium containing doxycycline at a final concentration 10 $\mu$g/ml, and grown to $A_{600}$ nm~0.8 for use in experiments (23). The yTHC strains were validated by comparing colony growth in the presence or absence of doxycyclin.

Cell Extracts and Immunoblotting—Protein extracts were produced by glass bead lysis in trichloroacetic acid as described previously (38). Protein extracts were resolved by SDS-PAGE, transferred onto nitrocellulose, and subjected to immunoblotting. The following antibodies were used as indicated: phospho44/42 (9101L, Cell Signaling Technology) at 1:1000 and glucose-6-phosphate dehydrogenase (A9521, Sigma) at 1:50,000. Immunoabsorbent species were visualized by chemiluminescent detection (PerkinElmer Life Sciences) of horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology or Bio-Rad). Protein concentration was determined by detergent-compatible protein assay (500-0112, Bio-Rad). Band intensity
was quantified through scanning densitometry using ImageJ (National Institutes of Health). Phospho-Fus3 and phospho-Kss1 were normalized to the glucose-6-phosphate dehydrogenase loading control.

**Protein Turnover Assay**—To measure Ste7 protein stability, TetO2 WT, TetO2 CDC4, and TetO2 MET30 strains were treated with 10 μg/ml doxycycline for 18 h, grown to A_600nm ~0.6–0.8, stimulated with 3 μM α factor for 1 h, and treated with the protein synthesis inhibitor cycloheximide for up to 90 min as described previously (39). Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with Ste7 antibodies at 1:1000 (Santa Cruz Biotechnology).

**Transcription Reporter Assay**—Strains were transformed with the pRS423-FUS1-lacZ reporter plasmid, grown in selective SCD-His medium overnight, and then diluted to A_600nm = 0.2 in fresh medium and grown to A_600nm = 0.8. Cultures were diluted to equalize density, and 90 μl were mixed with 10 μl of α factor at 10× the indicated concentration. After 90 min at 30°C, β-galactosidase activity was measured as described previously (40).

**Preparation and Purification of Recombinant Proteins**—BY4741 yeast cells were transformed with pRS315-ADH1-STE7-FLAG, pRS315-ADH1-UBP3-FLAG, or pRS315-UBP3S695A-FLAG and grown to A_600nm ~1.0 before harvesting by centrifugation at 3000 × g. The cell pellet was frozen in liquid nitrogen and lysed by grinding cells blast-frozen in a 1:0.7 ratio of Lysis Buffer (50 mM Tris-HCl, pH 7.5, 400 mM KCl, 0.1% Triton X-100, 0.2 mM dithiothreitol) supplemented with 10 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 protease inhibitor tablet per 50 ml of buffer (11873580001, Roche Applied Science). The cell lysate was thawed on ice and centrifuged at 15,000 × g for 30 min at 4°C. The supernatant was incubated with EZView anti-FLAG M2 beads (Sigma) for 2 h rotating at 4°C. Beads were harvested by centrifugation at 500 × g and washed three times with 100× bead bed volume of Ubiquitination Buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.2 mM dithiothreitol, 2 mM MgCl2, 20 μM GDP) containing 5% glycerol, 0.1 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml chymostatin. Protein concentration of each sample was determined by the bicinchoninic acid protein assay (Pierce). Protein aliquots were added directly to ubiquitination reactions (23).

**In Vitro Kinase Assays**—Four μg of GST-Fus3 was preincubated in 40 μl of Kinase Buffer (25 mM HEPES, pH 7.5, 10 mM MgCl2, 150 mM NaCl, 25 mM β-glycerophosphate, 0.3 mM ATP) for 1 h at 30°C. Next, 10 μl of eluted protein from FLAG purification of yeast cells expressing either Ubp3-FLAG or Ubp3S695A-FLAG (or, as a reaction control, yeast expressing untagged Ubp3) was added along with 6 μCi of [γ-32P]ATP (PerkinElmer Life Sciences) and incubated for 3 h at 30°C. The reaction was terminated by the addition of 6× SDS-PAGE sample buffer. Samples were boiled for 5 min and resolved by SDS-PAGE and stained with Coomassie Brilliant Blue (Bio-Rad). 32P incorporation was detected by autoradiography (41).

**In Vitro Ubiquitination Assay**—Ubiquitination reactions were prepared with FLAG/SCF complexes containing FLAG-Skp1/Cdc53/Myc-Rbx1/Cdc4 or Met30 purified from insect cells as described previously (21, 23). Nine μl of purified and eluted SCF complex (~2 pmol) was combined with 13.5 pmol of^{15}N-HisCdc4, 1.0 pmol of^{15}N-HisUba1, 120 pmol of ubiquitin (Boston Biochem), 1.5 pmol of Ste7-FLAG and supplemented with 1 mM ATP, 5 mM MgCl2, and 20 μM GDP in a volume of 15 μl. Reactions were allowed to proceed at 30°C and stopped after 90 min with the addition of boiling SDS-PAGE sample buffer, followed by SDS-PAGE, transfer onto nitrocellulose, and immunoblotted with Ste7 antibodies at 1:1000 (Santa Cruz Biotechnology). Membranes were stripped and reprobed with Cdc4 and Met30 antibodies at 1:1000 and Cdc53 antibodies at 1:1000 (provided by Mark Goebl, University of Indiana Medical School).

**Denaturing Purifications**—Strains transformed with pRS315-ADH1-STE7-8HIS were grown to A_600nm ~1.0; TCA was added to a final concentration of 1%, and cells were harvested by centrifugation at 3000 × g. Supernatant was removed, and cell pellets were snap-frozen in liquid nitrogen and then stored overnight at ~80°C. Frozen cell pellets were thawed on ice in Denaturing Lysis Buffer (6 mg guanidine HCl, 0.1 M NaH2PO4, 20 mM Tris, pH 8.0, 0.1% Antifoam 204 (Sigma)). Cells were lysed by vortexing with glass beads for 10 min at 4°C. Lysates were rocked at 4°C for 60 min to solubilize membrane proteins and centrifuged once at 6000 × g for 1 min and again for 30 min to remove insoluble matter. Protein concentration of each sample was determined by A_280 nm and samples were diluted to equivalent concentrations. Cell lysates were loaded onto TALON cobalt metal affinity resin that had been equilibrated with lysis buffer for 1 h (Clontech) and incubated for 2 h at 4°C with gentle rocking. The resin was washed 10× with Urea Wash Buffer (100 mM Tris, pH 7.0, 500 mM NaCl, 20 mM imidazole, 6 M urea). His_8-tagged proteins were eluted by washing beads twice with imidazole buffer (100 mM Tris, pH 7.0, 500 mM NaCl, 200 mM imidazole). Protein was precipitated from pooled elutions by the addition of ice-cold pure acetone and overnight incubation at ~20°C, followed by centrifugation at 6000 × g for 30 min. Remaining acetone was evaporated, and the precipitated protein was mixed with 1× SDS-PAGE sample buffer,
followed by SDS-PAGE, transfer onto nitrocellulose, and immunoblotting with Ste7 antibodies.

**RESULTS**

**Ste7 Is Ubiquitinated by SCFCdc4 and Deubiquitinated by Ubp3**—Ste7 lies at the center of a prototypical MAPK cascade; Ste7 is phosphorylated and activated by Ste11, and it in turn phosphorylates and activates the MAPKs Fus3 and Kss1. We have shown previously that Ste7 is ubiquitinated and that this modification occurs in a stimulus-, Ste11-, and SCF-dependent manner; however, the F-box protein that recruits Ste7 to the SCF complex was not identified (33). One member of the F-box family, Cdc4, was shown to be necessary for proper pheromone signaling and to mediate ubiquitination of the G-protein signaling subunit, Gpa1 (23). To determine whether Cdc4 acts on Ste7, we performed immunoprecipitations using C-terminal FLAG-tagged Cdc4 and probed for co-purifying Ste7. As shown in Fig. 1A, Ste7 was indeed detected in association with Cdc4-FLAG, but not FLAG alone, indicating that Ste7 and Cdc4 interact. We then investigated whether SCFCdc4 ubiquitinates Ste7 directly. Ste7 was purified from yeast (to preserve post-translational modifications) and combined with SCFCdc4 isolated from Sf9 cells, E2 ubiquitin-conjugating enzyme Cdc34, E1 ubiquitin-activating enzyme Uba1, ubiquitin (Ub), and yeast-purified Ste7, as indicated. Immunoblots were probed with antibodies against Ste7, Cdc4, or Met30 as indicated. C. Ste7 is stabilized in the absence of Cdc4. Ste7 expression was measured in wild type cells (TetoO, WT), cells deficient in Cdc4 (TetoO, Cdc4), or cells deficient in Met30 (TetoO, Met30) at time 0 and up to 90 min after treatment with the protein synthesis inhibitor cycloheximide. Equal numbers of cells were collected at the indicated times, and protein was extracted. The protein extracts were resolved by SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with antibodies targeting Ste7. D, loss of Cdc4 blocks Ste7 ubiquitination, whereas loss of Ubp3 leads to accumulation of ubiquitinated Ste7. TetO7-CDC4 cells or TetO7-CDC4 cells lacking STE7 or UBP3 were treated with 3 μM α-factor and 10 μg/ml doxycycline (DOX), as indicated. His-tagged Ste7 was purified under denaturing conditions and detected by immunoblotting with antibodies directed against Ste7. Data are representative of at least three independent experiments.
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FIGURE 2. Loss of Cdc4 and Ubp3 has opposing effects on pheromone signaling. Cdc4 and Ubp3 have opposing effects on pheromone-stimulated transcription (23, 32). To compare these directly, pheromone-stimulated transcription was measured in TetO7 WT or TetO7 CDC4 cells treated for 18 h with 10 μg/ml doxycycline (A) and wild type or Ubp3-deficient (ubp3Δ) cells transformed with a pheromone-specific reporter, FUS1-LacZ (B). All results are representative of three independent biological replicates. Error bars are representative of the average ± S.E.

pressible promoter upstream of either CDC4 (TetO7 CDC4) or a noncoding region (TetO7 WT) as a control. As expected, knockdown expression of Cdc4 slowed the degradation of Ste7 (Fig. 1C). We then deleted the de-ubiquitinating protease gene UBP3 so as to preserve the modified form of the substrate (32). His-tagged Ste7 was purified in the presence of 8 M guanidinium, to ensure the removal of nonspecific interacting proteins. These purified samples were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies directed against Ste7 (Fig. 1D). Ste7 purified from unstimulated wild type cells was detected as a single band. Stimulation with α factor produced a higher molecular weight form of Ste7, a consequence of feedback phosphorylation by the MAPK Fus3 (35). This phosphorylated form of Ste7 was also detected in cells lacking Ubp3 (ubp3Δ), indicating that the protease acts to suppress basal MAPK activation. Finally, multiple higher molecular weight forms of Ste7, representing the polyubiquitinated form of the protein, were detected in cells stimulated with 3 μM α factor. In the absence of Ubp3, these higher molecular weight bands were strongly enriched. In the absence of Cdc4, these bands disappeared. Thus, with respect to Ste7 ubiquitination, Ubp3 and SCF-Cdc4 act in opposition to one another.

Cdc4 Promotes Activation of Fus3 and Ubp3 Limits Activation of Kss1—We have shown previously that Cdc4 and Ubp3 have opposing effects on pheromone-stimulated transcription (23, 32). Whereas loss of Cdc4 (TetO7 CDC4) significantly reduced the transcriptional response, disruption of Ubp3 (ubp3Δ) enhanced the response (Fig. 2). In addition, loss of Cdc4 diminished pheromone-dependent activation of Fus3 but not of Kss1 (23). To quantify the effects of Cdc4 and to establish whether Ubp3 affects MAPK activity, we used an antibody that specifically recognizes the dually phosphorylated forms of Fus3 (pp-Fus3) and Kss1 (pp-Kss1) (Figs. 3A and 4A). When Cdc4 expression was repressed (TetO7 CDC4), Fus3 activation was reduced by almost 80% compared with wild type cells (TetO7 WT). In contrast Kss1 activation was unaffected (compare Fig. 3, B and C). Because Fus3 (but not Kss1) expression is induced by α factor treatment (42), we immunoblotted the same samples to measure Fus3 abundance. As anticipated, Fus3 levels were slightly reduced in the TetO7 CDC4 strain (Fig. 3A); however, the modest reduction in total Fus3 could not account for the large reduction in phosphorylated Fus3 (Fig. 3D). These data suggest that ubiquitination of Ste7 is necessary for the proper activation of Fus3 but not Kss1.

We then considered the reciprocal case where Ste7 is strongly ubiquitinated, as occurs in cells that lack Ubp3. In this situation, the activity of Kss1 increased 6-fold (Fig. 4, A and C). Moreover, the slight increases in the total amount of Kss1 could not account for the increased level of activation (Fig. 4, B and E). Conversely, Fus3 activation was unaffected relative to total Fus3 (Fig. 4, A, B, D, and F). These data indicate that sustained ubiquitination leads to hyper-activation of Kss1 but not Fus3. The opposing effects of Ubp3 and Cdc4 on signaling cannot be due to the opposing effects on Ste7 degradation, because loss of either Ubp3 or Cdc4 leads to stabilized expression of Ste7 (Fig. 4A, B, D, and E). Thus, we infer that nonubiquitinated Ste7 (as occurs in the TetO7 CDC4 strain) is a poor activator of Fus3, whereas hyper-ubiquitinated Ste7 (as occurs in the ubp3Δ mutant) strongly activates Kss1. This pattern of increased and decreased MAPK activation mirrors the changes in transcription reporter activity (Fig. 2) (29, 31, 43).

In addition to phosphorylating nuclear transcription factors, MAPKs phosphorylate a number of upstream components and pathway regulators, including Ste7 and Ubp3 (see below). Such feedback phosphorylation events often lead to diminished signaling over time. Thus, we considered whether Ubp3 contributes to MAPK desensitization. Wild type and ubp3Δ cells were treated with α factor for 30 min and then washed with pheromone-free media to allow the cells to recover. Activation of Fus3 and Kss1 was monitored by immunoblotting prior to stimulation, 30 min after stimulation, and at several times after the removal of pheromone (Fig. 5). Once again, Kss1 was hyper-activated in the absence of Ubp3. Moreover, the activity of both Fus3 and Kss1 remained high even 30 min after removal of the stimulus, whereas in wild type cells the MAPK activity returned to base line within 15 min after α factor removal (Fig. 5, A–C). The effect is particularly striking for Kss1 activation. However, the initial levels of Kss1 activity, and to a lesser extent Fus3 activity, are higher in the mutant, and these differences in baseline activity could account for the observed differences in system recovery. Regardless, these data reveal that Ubp3 is required to dampen the pheromone signaling pathway.

Sustained Kss1 activation leads to invasive growth, a differentiation program wherein haploid yeast adopt a filamentous
morphology that enhances their ability to invade the substratum and adhere to solid media. Deletion of UBP3 leads to sustained Kss1 activation. To determine how Ubp3 affects invasive growth, cells were plated on solid media for 3 days. The plates were then washed with water to detach cells that had not invaded the agar. In agreement with previous reports, wild type yeast (BY4741) did not invade solid media (44). In contrast, cells lacking Ubp3 were capable of invasion (Fig. 5D). The invasive capability of the ubp3Δ mutant was dependent on the integrity of Ste7 as well as of Tec1, a transcription factor that is activated by Kss1 and required for invasive growth. Deletion of Kss1 did not alter the ability of ubp3Δ cells to invade solid agar, in agreement with previous observations (44). In particular, deletion of Kss1 is thought to promote invasive growth by relieving Dig1/Dig2-mediated inhibition of Ste12 and Tec1, which mediate transcription of genes containing filamentous response elements (43–45).

Fus3 Phosphorylates and Activates Ubp3—The enzymatic activity of ubiquitin proteases was reported to be inhibited by phosphorylation (46). Sole et al. (37) demonstrated that the stress-responsive MAPK Hog1 phosphorylates Ubp3 at Ser-695 to modulate transcriptional responses to high salt conditions; in the absence of Ubp3 phosphorylation, osmoreponsive gene transcription is reduced (37). Prior to that, Ptacek et al. (47) used a kinome-wide screen to show that Ubp3 is phosphorylated by the MAPK Fus3. To validate the results of the earlier kinome screen, we performed in vitro kinase reactions using Fus3 purified from E. coli and Ubp3 from S. cerevisiae. Purified Fus3 becomes autophosphorylated on Tyr-182, resulting in partial activation of the kinase (42, 48). As shown in Fig. 6A, Fus3 phosphorylated Ubp3; furthermore, phosphate incorporation was largely abrogated in the case of a phosphorylation site mutant, Ubp3S695A (37).

We then investigated the functional consequences of Ubp3 phosphorylation. To this end, we compared MAPK activation in a ubp3Δ mutant strain containing plasmid-borne UBP3, the phosphorylation site mutant ubp3S695A, or a catalytically inactive mutant ubp3C469A (Fig. 6, B and C). Whereas UBP3 restored normal MAPK activity, the phosphorylation site and functionally inactive mutants did not (Fig. 6, B and C). Finally, we considered whether Ubp3 phosphorylation contributes to desensitization. The ubp3Δ cells expressing UBP3, ubp3S695A, or ubp3C469A were treated with α factor for 30 min and then washed as described above (Fig. 5). Activation of Fus3 and Kss1 was monitored by immunoblotting prior to stimulation, 30 min after stimulation, and at several times after the removal of pheromone. As shown in Fig. 7, A–C, loss of Ubp3 phosphorylation resulted in sustained MAPK activation, similar to that seen in Ubp3-deficient cells (Fig. 7, D–F). We conclude that Fus3 phosphorylates Ubp3 and that phosphorylation is required for full activity of Ubp3 in vivo.
**DISCUSSION**

Ste7 is a MAPKK that participates in both the pheromone and the invasive growth pathways. During the pheromone response, Ste7 activates Fus3 and Kss1; during invasive growth, Ste7 activates only Kss1. Moreover, Ste7 expression is tightly regulated, and its low level of expression makes it a limiting factor in MAPK signaling cascades (49). Thus, post-translational modification or degradation of Ste7 is likely to have a significant impact on the strength and specificity of pheromone signaling. Here, we demonstrate that Ste7 is ubiquitinated by SCFCdc4 and deubiquitinated by Ubp3. Loss of SCFCdc4 leads to diminished activation of Fus3. Loss of Ubp3, or loss of Ubp3 phosphorylation, causes spurious activation of Kss1 and the invasive growth response. These findings establish that proper control of Ste7 ubiquitination is required for proper signal specificity (Fig. 8).

In the simplest terms, ubiquitination mediates the destruction of proteins. Accordingly, regulation of proteins by ubiquitination generally occurs at a relatively slow time scale, comparable with the regulation of protein expression by gene induction. In contrast, regulation by protein phosphorylation occurs more rapidly. Since the initial discovery of the ubiquitin proteasome system, however, the role of ubiquitination has expanded to include rapid processes such as protein trafficking, protein-protein interactions, and dynamic regulation of the signal transduction machinery (50–52). Indeed there is growing evidence that ubiquitination can in some cases modulate enzyme activity. This realization helps us to reconcile how protein ubiquitination might contribute to short term regulation of protein kinase activity.

Not surprisingly, disturbances in the balance of ubiquitination/deubiquitination have important consequences for a variety of signaling systems. An E2 ubiquitin-conjugating enzyme, UbcB, and a DUB, UbpB, mediate the spatial and temporal regulation of the MAPKKK MEKK to control the developmental timing and patterning of pre-stalk and pre-spore cells in the slime mold *Dictyostelium discoideum* (53). In *Drosophila melanogaster*, mutations in the E3 ubiquitin ligase Highwire or the DUB fat facets disrupt synaptic development (54–56). The pathogen *Yersinia* disrupts MAPK signaling by secreting YopJ, a putative DUB that acts at the level of the MAPKK (57). Similarly, Yang *et al.* (58) showed that cycles of ubiquitination and deubiquitination regulate growth factor-mediated activation of AKT. CYLD, a DUB that regulates signaling by NF-κB, RhoA,
and JNK (59–63), suppressed growth factor-stimulated ubiquitination and activation of AKT and removed ubiquitin from AKT under serum-starved conditions (58). These studies provide some striking examples of how dynamic ubiquitination can influence MAPK activity.

It has long been known that MAPKs are hyperactivated in cancer. In recent years, the SCF family of ubiquitin ligases has emerged as an important regulator of the cell cycle, and aberrant SCF function is now linked to cancer progression (64–70). Although ubiquitination has been shown to influence MAPK signaling events, this is the first study to directly demonstrate SCF-mediated ubiquitination of a component of a MAP kinase cascade. Previously, we reported that the same ubiquitin ligase...

**FIGURE 5.** Loss of Ubp3 leads to sustained pheromone signaling and misactivation of the invasive growth response. Wild type (WT) and Ubp3-deficient (ubp3Δ) cells were stimulated with 3 μM α factor (pheromone) for 30 min, washed, and placed in fresh pheromone-free media for an additional 30 min. Samples were taken at the indicated time points. A, whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies directed against phosphorylated Fus3 and Kss1, total Fus3, and GAPDH (loading control). Band intensities are expressed as a percentage of maximum phosphorylated Fus3 (B) or phosphorylated Kss1 (C) in ubp3Δ cells after 30 min of α factor stimulation. All results are representative of three independent biological replicates. Error bars are representative of the average ± S.E. (one-way analysis of variance, ***p < 0.0005; ***, p < 0.005; *, p < 0.05). G6PDH, glucose-6-phosphate dehydrogenase.

**FIGURE 6.** Fus3 phosphorylates Ubp3 to limit pheromone-stimulated MAP kinase activation. A, in vitro kinase assays were performed using Fus3 purified from E. coli, FLAG-tagged Ubp3, or the phospho-site mutant Ubp3Ser695A purified from yeast and [γ-32P]ATP for 3 h at 30°C. The reactions were stopped by the addition of boiling sample buffer, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue. The stained gel was then dried, and phosphorylation was detected by autoradiography. 32P-autophosphorylated protein was not detected in a parallel experiment using catalytically inactive Fus3K42R (data not shown). B, wild type (WT) and ubp3Δ cells transformed with an empty vector (WT and ubp3Δ lanes) or with plasmids expressing Ubp3, the phospho-site mutant Ubp3Ser695A, or catalytically inactive Ubp3Cys469A. MAP kinase phosphorylation was measured in cells stimulated with 3 μM α factor for 15 min. Whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies directed against phosphorylated Fus3 and Kss1, total Fus3, and GAPDH (loading control). C, band intensities are expressed as a percentage of maximum phosphorylated Fus3 and phosphorylated Kss1 in ubp3Δ cells after 30 min of α factor stimulation. All results are representative of three independent biological replicates. Error bars are representative of the average ± S.E. (one-way analysis of variance, ***p < 0.0005; ***, p < 0.005). G6PDH, glucose-6-phosphate dehydrogenase.
complex, SCFCdc4, directly ubiquitinas the heterotrimeric G-protein α subunit, Gpa1 (23). Gpa1 is in turn needed to regulate MAPK signaling in yeast. These results reveal a specialized function for Cdc4 in pheromone regulation.

Since its discovery in S. cerevisiae, Cdc4 orthologs have been identified in a number of species, including Caenorhabditis elegans (SEL-10), D. melanogaster (Archipelago), mouse (Fbw6), and humans (Fbw7) (71–74). In humans, Fbw7 mediates the degradation of the oncogenes cyclin E, MYC, JUN, and Notch (75), and Fbw7 mutations are associated with many forms of cancer. Collectively, these studies suggest that SCFFbw7 (or SCFCdc4) is likely to regulate multiple components of pro-oncogenic MAPK cascades. More broadly, these studies indicate that dynamic ubiquitination is an evolutionarily conserved regulatory mechanism in cell fate decisions.

Among the best understood of cell fate decisions are those mediated by Fus3 (mating) and Kss1 (invasion) (Fig. 8). Previous studies have shown that Fus3 prevents Kss1 from activating invasive growth genes (26, 30, 31, 76–81) and acts by phosphorylating and triggering the degradation of the invasive growth-specific transcription factor Tec1 (77, 82). In addition, it had been suggested that Fus3 limits the strength and duration of Kss1 signaling by phosphorylating an upstream substrate (30, 83). Recent studies revealed a feedback loop wherein Fus3 phosphorylates Ste7 and thereby limits the activation of Kss1 but not Fus3 itself (26). Here, we describe an additional feedback loop that is centered on the ubiquitin protease Ubp3. In the absence of Ubp3 or of Ubp3 phosphorylation, there is an accumulation of ubiquitinated Ste7, which specifically enhances the activation of Kss1 and leads to spurious activation of the invasive growth response. Notably, the ubp3 mutant exhibits a substantial increase in feedback-phosphorylated Ste7, comparable with that seen in response to pheromone stimulation (Fig. 1D). Thus, Ubp3 is likely to regulate signal specificity at least in part through the feedback phosphorylation mechanism. Ubp3 deletion does not appreciably alter Kss1 abundance (Fig. 4B). However, other targets of Ubp3, including targets downstream of the MAPK, cannot be excluded.

Signal transduction systems will often share core signaling components yet maintain specificity and avoid pathway cross-

FIGURE 7. Cells expressing Ubp3S695A exhibit sustained MAP kinase activation. Ubp3-deficient (ubp3Δ) cells transformed with an empty vector or with plasmids expressing the phospho-site mutant Ubp3S695A (A–C) or Ubp3wt (D–F) were stimulated with 3 μM α factor (pheromone) for 30 min, washed, and placed in fresh pheromone-free media for an additional 30 min. Samples were taken at the indicated times. Whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies directed against phosphorylated Fus3 and Kss1, total Fus3, and GAPDH (loading control). Band intensities are expressed as a percentage of maximum phosphorylated Fus3 and Kss1, total Fus3, and GAPDH (loading control). All results are representative of three independent biological replicates. Error bars are representative of the average ± S.E. (one-way analysis of variance, ***, p < 0.0005; **, p < 0.005; *, p < 0.05). G6PDH, glucose-6-phosphate dehydrogenase.
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Left unresolved is how Ubp3 limits Kss1 signaling and activation of the invasive growth pathway. Ubp3 is one of a diverse family of deubiquitinating proteases that mediates the breakdown and removal of ubiquitin in various contexts. There are 16 DUBs in yeast and over 80 in humans (89). DUBs can be classified into three functional categories. First, DUBs can trim ubiquitin chains to achieve a specific length/arrangement (90); second, they can recycle ubiquitin chains to maintain the necessary cellular levels of free ubiquitin (91); third, they remove ubiquitin chains from substrates (92). This last function is perhaps the most important in terms of signaling as it can directly affect protein turnover, activation, and localization. Finally, although DUBs have long been thought to reverse ubiquitination and thereby slow protein degradation, they are now known to actively recruit ubiquitinated substrates to the proteasome (93, 94). Thus, the loss of Ubp3 likely prevents proteasomal recruitment of ubiquitinated Ste7, accounting for its accumulation in the cell.

In summary, the yeast pheromone response is regulated by multiple feedback loops, many of which center on the addition and removal of post-translational modifications. Exposure to pheromone activates components of the MAP kinase cascade, but this is followed by feedback signals, including phosphorylation and ubiquitination of Ste7, and Ste7 regulators including Ubp3. Together, these feedback events help maintain signaling specificity. More broadly, our findings point to the MAPKK as a central target of feedback regulation and the control of cell signaling specificity.

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