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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 SCF^{Cdc4} and the ubiquitin protease Ubp3 regulate Ste7 ubiquitination and signal specificity. Using purified components, we demonstrate that SCF^{Cdc4} ubiquitinates Ste7 directly. Using gene deletion mutants, we show that SCF^{Cdc4} and Ubp3 have opposing effects on Ste7 ubiquitination. Although SCF^{Cdc4} 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 MAP[2] kinase kinase 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 the 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 $G\alpha$ subunit (Gpa1) and a $G\beta\gamma$ dimer (Ste4/Ste18). The $G\beta\gamma$ 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, G_1 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 MAPKKK 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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[2] The abbreviations used are: MAP, mitogen-activated protein; DUB, deubiquitinating protease.

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 α 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 proteasomal 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 (MAT**a** *leu2*Δ *met15*Δ *his3-1 ura3*Δ), BY4741-derived strains lacking *STE7* (*ste7*::KANMX4, resistance to geneticin), *UBP3* (*ubp3*::HPHMX4, resistance to hygromycin B), *KSS1* (*kss1*::NATMX4, resistance to nourseothricin), *TEC1* (*tec1*::KANMX4), and R1158 (MAT**a** *URA3*::CMV-tTA *leu2*Δ *met15*Δ *his3*-1 *ura3*Δ) purchased as the yeast Tet-promoter Hughes Collection (yTHC, Open Biosystems). 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, amp^R, and *LEU2*) and pRS316 (CEN, amp^R, 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 SalI/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-$UBP3^{C469A}$-FLAG, and pRS315-ADH1-$UBP3^{S695A}$-FLAG were constructed by amplifying *UBP3*, $ubp3^{C469A}$, 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 μg/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: phospho-p44/42 (9101L, Cell Signaling Technology) at 1:1000 and glucose-6-phosphate dehydrogenase (A9521, Sigma) at 1:50,000. Immunoreactive 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, $TetO_7$ WT, $TetO_7$ CDC4, and $TetO_7$ MET30 strains were treated with 10 μg/ml doxycycline for 18 h, grown to $A_{600\,nm}$ ~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 $A_{600\,nm}$ = 0.2 in fresh medium and grown to $A_{600\,nm}$ = 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-UBP3^{S695A}-FLAG and grown to $A_{600\,nm}$ ~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 $MgCl_2$, 20 μM GDP) supplemented with 5% glycerol, followed by elution with 2× bead bed volume of the Ubiquitination Buffer containing 0.25 mg/ml 3× FLAG peptide (Sigma). Protein aliquots were frozen and stored at −80 °C (23).

Yeast ^{His}Cdc34 E2 was purified from *Escherichia coli*, yeast ^{His}Uba1 E1 was purified from yeast, and yeast SCF E3 complexes were purified from insect cells infected with baculoviruses expressing yeast FLAG-Skp1, Cdc53, Myc-Rbx1, and HA-Cdc4 or HA-Met30 for 40 h as described previously (21). Cells were disrupted in NETN buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 0.5% Nonidet P-40, 0.2 mM dithiothreitol, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride) supplemented with one protease inhibitor tablet per 50 ml and cleared by centrifugation at 15,000 × *g* for 30 min at 4 °C. Typically, 3 ml of NETN buffer was used per 0.5×10^8 cells. For immunoprecipitation, 300 μl of cell lysate was incubated with 10 μl of EZView anti-FLAG M2 beads with rotating for 1 h at 4 °C. Beads were washed three times with 500 μl of NETN buffer for 5 min each with rocking and three quick washes with 500 μl of Ubiquitination Buffer. Bound protein was eluted from the beads with 2× 10 μl of Ubiquitination Buffer supplemented with 0.25 mg/ml 3× FLAG peptide for 10 min each. Eluted protein was added directly to ubiquitination reactions (23). Purification of GST-Fus3 constructs was performed as described previously (41).

In Vitro Kinase Assays—Four μg of GST-Fus3 were preincubated in 40 μl of Kinase Buffer (25 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 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 Ubp3^{S695A}-FLAG (or, as a reaction control, yeast expressing untagged Ubp3) was added along with 6 μCi of [γ-^{32}P]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). ^{32}P 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 ^{His}Cdc34, 1.0 pmol of ^{His}Uba1, 120 pmol of ubiquitin (Boston Biochem), 1.5 pmol of Ste7-FLAG and supplemented with 1 mM ATP, 5 mM $MgCl_2$, 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 immunoblotting 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 $A_{600\,nm}$ ~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 M guanidine HCl, 0.1 M NaH_2PO_4, 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,

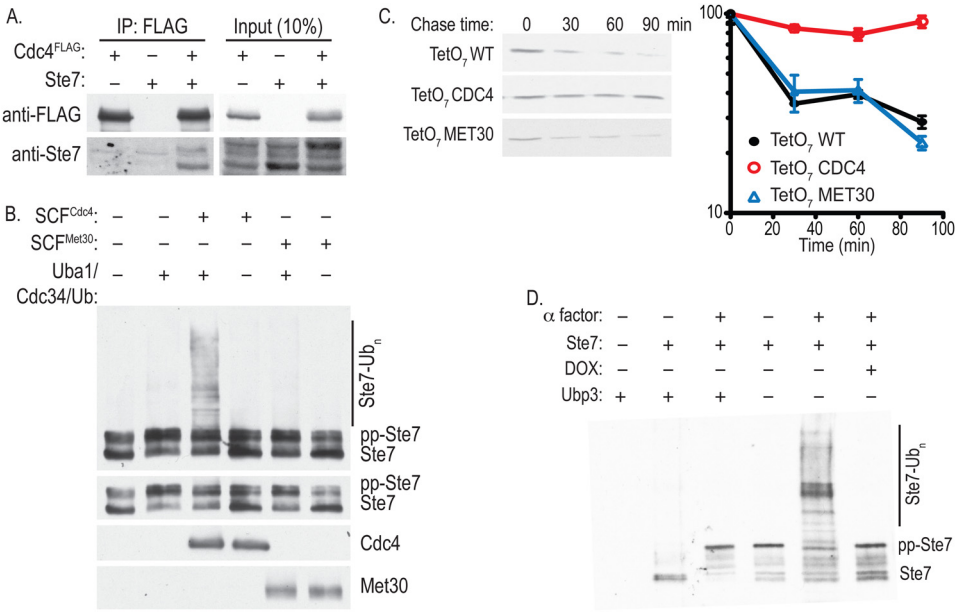

FIGURE 1. **Ste7 is ubiquitinated by SCF^{Cdc4} and deubiquitinated by Ubp3.** *A,* Ste7 binds the F-box protein Cdc4. BY4741 cells were transformed with plasmids expressing Ste7 and FLAG-tagged Cdc4. Cdc4-FLAG was immunoprecipitated (*IP*), separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies directed against FLAG or Ste7, as indicated. *B,* SCF^{Cdc4} is sufficient to ubiquitinate Ste7. *In vitro* ubiquitination assays were performed with either SCF^{Cdc4} or SCF^{Met30} purified 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 ($TetO_7$ WT), cells deficient in Cdc4 ($TetO_7$ CDC4), or cells deficient in Met30 ($TetO_7$ 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. $TetO_7$-CDC4 cells or $TetO_7$-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.

followed by SDS-PAGE, transfer onto nitrocellulose, and immunoblotting with Ste7 antibodies.

Co-immunoprecipitation Assay—Transformed yeast strains were grown to $A_{600\,nm}$ ~1.0, harvested by centrifugation at 3000 × *g*, and resuspended in FLAG buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 25 mM β-glycerophosphate, 0.1% Triton X-100, protease inhibitor mixture pellets (Roche Applied Science, 1 pellet/25 ml of buffer)). Cells were lysed by vortexing with glass beads for 10 min at 4 °C. Lysates were rocked at 4 °C for 30 min to solubilize membrane proteins and centrifuged once at 6000 × *g* for 1 min and again for 30 min to remove insoluble matter. Protein content of the supernatant was determined by DC Protein Assay (Bio-Rad) and equalized. For FLAG immunoprecipitations, 20 μl of M2 FLAG affinity resin (Sigma) equilibrated with FLAG Lysis Buffer was added to each sample. After 2 h of gentle rocking at 4 °C, the resin was collected by centrifugation at 500 × *g* for 1 min. Resin was washed four times with 1.5 ml of FLAG Lysis Buffer, and bound proteins were eluted twice with 2.5 mg/ml 3× FLAG peptide (Sigma).

Invasive Growth Assay—Five μl of an overnight saturated culture of the indicated strains from BY4741 background were spotted onto a yeast extract peptone dextrose (YPD, complete medium) agar plate and grown at 30 °C for 3 days. Total growth of strains was imaged, and then the plate was gently washed under a stream of water to remove noninvasive cells from the surface of the agar. The plates were allowed to dry briefly before re-imaging to document invasive growth.

RESULTS

Ste7 Is Ubiquitinated by SCF^{Cdc4} 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 α 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. 1*A*, Ste7 was indeed detected in association with Cdc4-FLAG, but not FLAG alone, indicating that Ste7 and Cdc4 interact. We then investigated whether SCF^{Cdc4} ubiquitinates Ste7 directly. Ste7 was purified from yeast (to preserve post-translational modifications) and combined with SCF^{Cdc4} isolated from *Sf9* cells, E2 (Cdc34) purified from *E. coli*, as well as purified E1 (Uba1), ubiquitin, and ATP. In addition to Cdc4, we tested a closely related F-box protein, Met30, as a control. As shown in Fig. 1*B*, Ste7 was ubiquitinated by SCF^{Cdc4}, but not SCF^{Met30}. These data reveal that Cdc4 binds to Ste7, and the SCF^{Cdc4} complex is sufficient to ubiquitinate the protein.

We next examined whether Cdc4 is necessary, as well as sufficient, for the ubiquitination of Ste7. Because Cdc4 is an essential gene, we used a yeast strain containing a tetracycline-re-

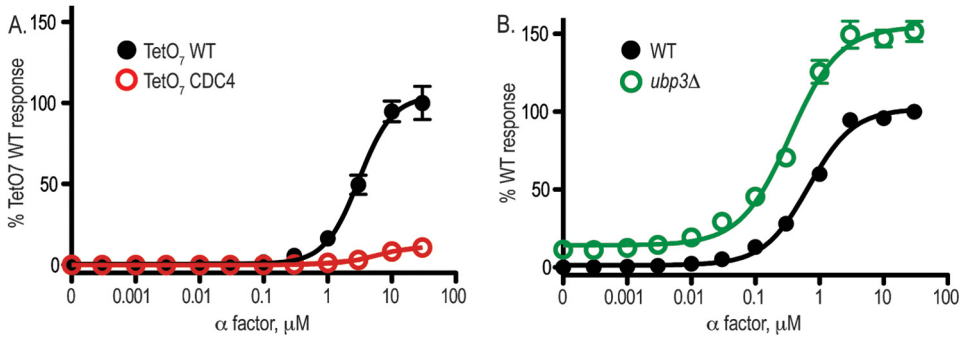

FIGURE 2. **Loss of Cdc4 and Ubp3 has opposing effects on pheromone signaling.** Cdc4 and Ubp3 have opposing effects on pheromone-stimulated transcription (23, 32). To compare these directly, pheromone-stimulated transcription was measured in $TetO_7$ WT or $TetO_7$ CDC4 cells treated for 18 h with 10 μg/ml doxycycline (*A*) and wild type or Ubp3-deficient (*ubp3*Δ) 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* ($TetO_7$ CDC4) or a noncoding region ($TetO_7$ WT) as a control. As expected, knockdown expression of Cdc4 slowed the degradation of Ste7 (Fig. 1*C*). 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. 1*D*). 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 (*ubp3*Δ), 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 ($TetO_7$ CDC4) significantly reduced the transcriptional response, disruption of Ubp3 (*ubp3*Δ) 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. 3*A* and 4*A*). When Cdc4 expression was repressed ($TetO_7$ CDC4), Fus3 activation was reduced by almost 80% compared with wild type cells ($TetO_7$ 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 $TetO_7$ CDC4 strain (Fig. 3*A*); however, the modest reduction in total Fus3 could not account for the large reduction in phosphorylated Fus3 (Fig. 3*D*). 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. 1*C*) (32). Thus, we infer that nonubiquitinated Ste7 (as occurs in the $TetO_7$ CDC4 strain) is a poor activator of Fus3, whereas hyper-ubiquitinated Ste7 (as occurs in the *ubp3*Δ 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 *ubp3*Δ 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

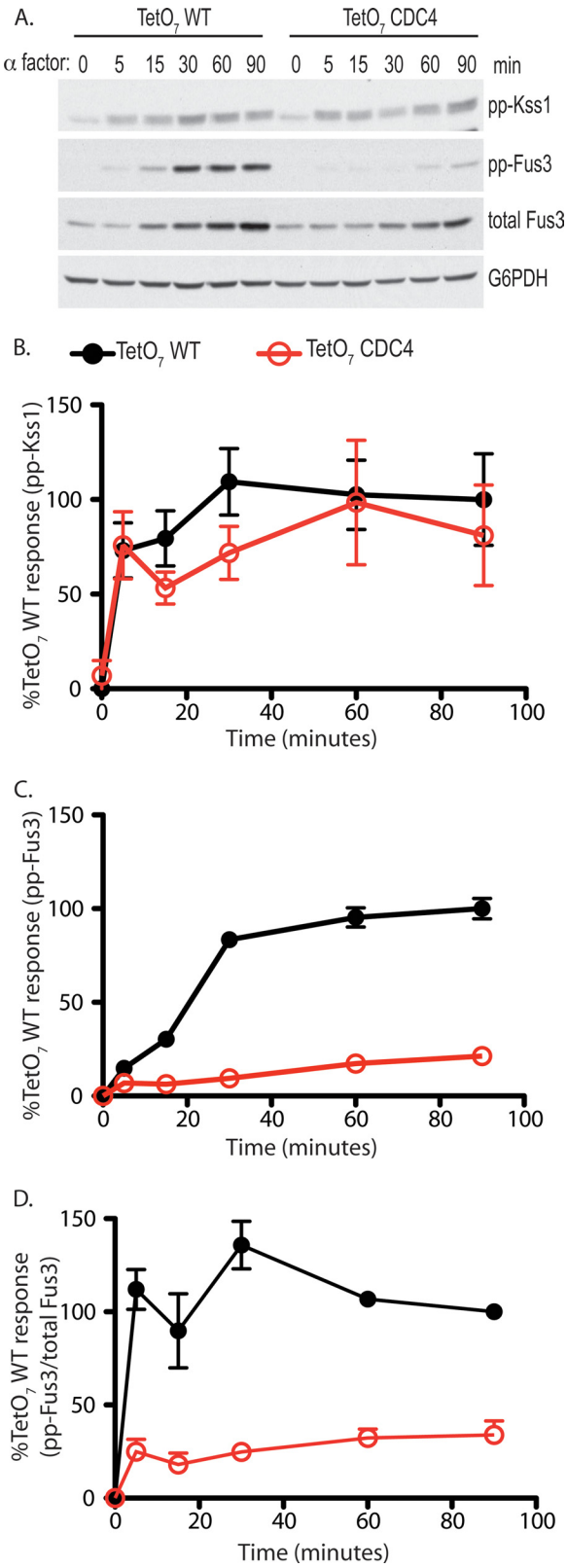

FIGURE 3. **Loss of Cdc4 specifically decreases Fus3 activation.** MAP kinase phosphorylation was measured in whole cell lysates from $TetO_7$-WT or $TetO_7$-CDC4 cells, treated for the indicated times with 10 μg/ml doxycycline, and then stimulated with 3 μM α factor (pheromone) for the indicated times (*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 signal in wild type cells after 90 min of α factor stimulation, normalized to the loading control (*B* and *C*) or to total Fus3 (*D*). Results are an average of three independent biological replicates. *Error bars* are representative of the average ± S.E. *G6PDH*, glucose-6-phosphate dehydrogenase.

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. 5*D*). The invasive capability of the *ubp3*Δ 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 *ubp3*Δ 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, osmoresponsive 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. 6*A*, Fus3 phosphorylated Ubp3; furthermore, phosphate incorporation was largely abrogated in the case of a phosphorylation site mutant, $Ubp3^{S695A}$ (37).

We then investigated the functional consequences of Ubp3 phosphorylation. To this end, we compared MAPK activation in a *ubp3*Δ mutant strain containing plasmid-borne *UBP3*, the phosphorylation-site mutant $ubp3^{S695A}$, or a catalytically inactive mutant $ubp3^{C469A}$ (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 *ubp3*Δ cells expressing *UBP3*, $ubp3^{S695A}$, or $ubp3^{C469A}$ 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*.

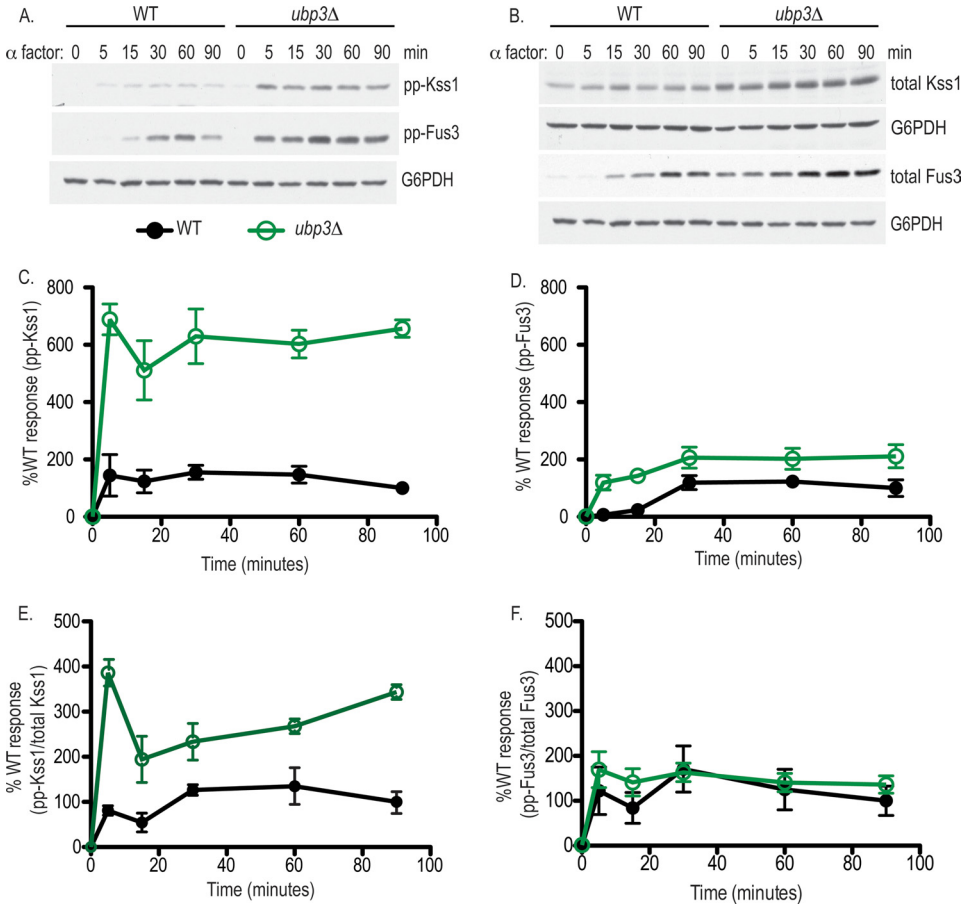

FIGURE 4. **Loss of Ubp3 specifically enhances Kss1 activation.** MAP kinase phosphorylation was measured in whole cell lysates from wild type or Ubp3-deficient (*ubp3*Δ) cells stimulated with 3 μM α factor (pheromone) for the indicated times. Whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies directed against phosphorylated Fus3 and Kss1 (*A*), total Fus3 or Kss1 (*B*), and GAPDH (loading control). Please note that total Fus3 was probed on the same immunoblot as phosphorylated Fus3 and Kss1 and therefore uses the same loading control. Band intensities are expressed as a percentage of maximum signal in wild type cells after 90 min of α factor stimulation, normalized to the loading control (*C* and *D*) or to total Fus3 or Kss1 (*E* and *F*). Total Kss1 samples were run in parallel on a separate gel. Results are an average of three independent biological replicates. *Error bars* are representative of the average ± S.E. *G6PDH*, glucose-6-phosphate dehydrogenase.

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 SCF^{Cdc4} and deubiquitinated by Ubp3. Loss of SCF^{Cdc4} 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 MEKKα 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,

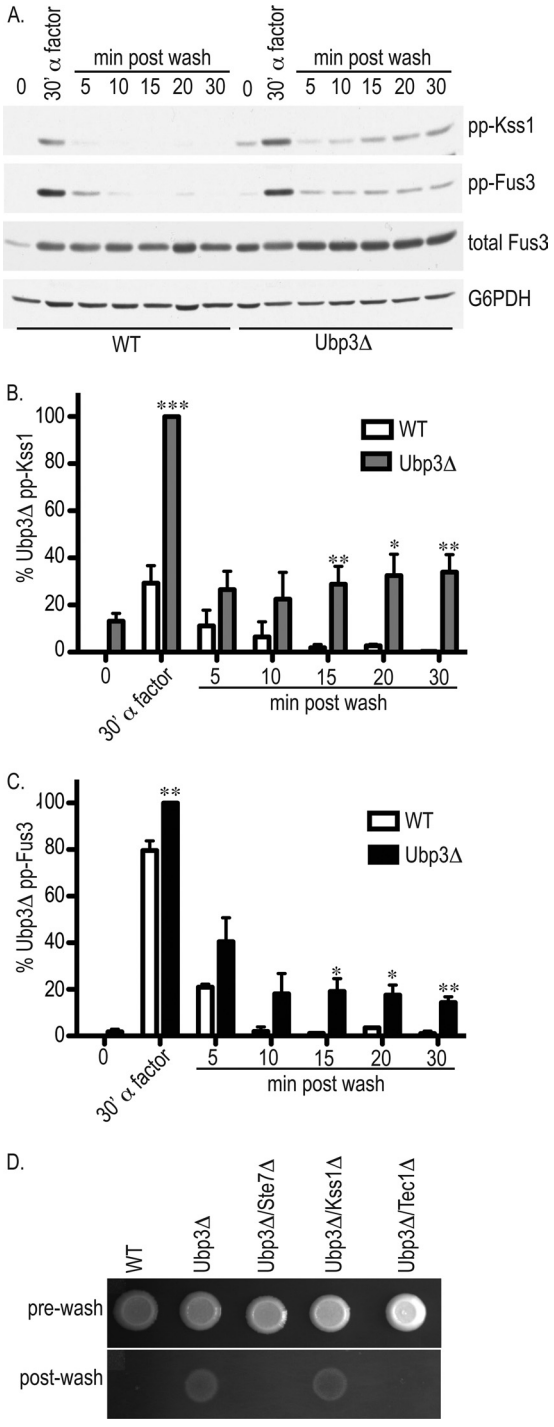

FIGURE 5. **Loss of Ubp3 leads to sustained pheromone signaling and misactivation of the invasive growth response.** Wild type (*WT*) and Ubp3-deficient (*ubp3*Δ) cells were stimulated with 3 μM α factor (pheromone) for 30 min, washed, and placed in fresh pheromone-free media for an additional 30 min. 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 *ubp3*Δ cells after 30 min of α factor stimulation. *D,* indicated strains were spotted on YPD plates and grown at 30 °C for 3 days. Plates were imaged, washed under running water to remove nonadherent yeast, and then imaged again. 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.

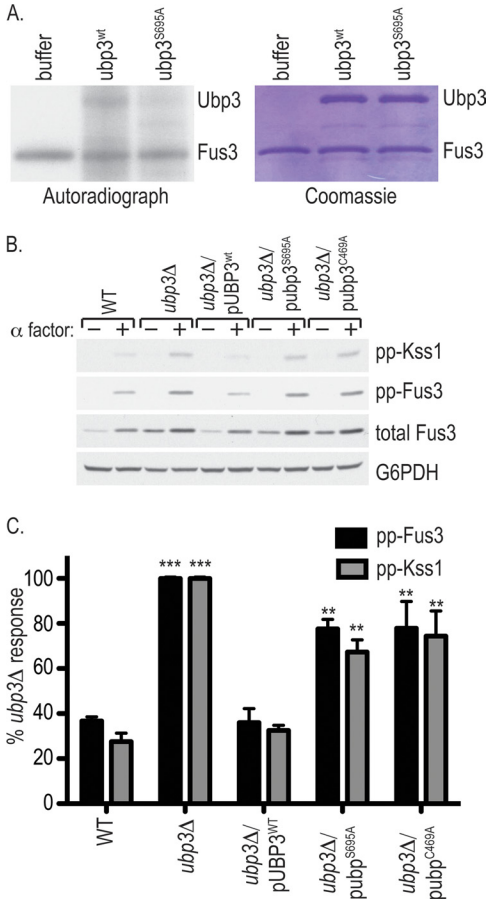

FIGURE 6. **Fus3 phosphorylates Ubp3 to limit pheromone-stimulated MAP kinase activation.** *A, in vitro* kinase assays were performed using Fus3 purified from *E. coli*, FLAG-tagged Ubp3, or the phospho-site mutant Ubp3^{S695A} purified from yeast and [γ-^{32}P]ATP for 3 h at 30 °C. The reactions were 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. ^{32}P-autophosphorylated protein was not detected in a parallel experiment using catalytically inactive Fus3^{K42R} (data not shown). *B,* wild type (*WT*) and *ubp3*Δ cells transformed with an empty vector (WT and *ubp3*Δ lanes) or with plasmids expressing Ubp3, the phospho-site mutant Ubp3^{S695A}, or catalytically inactive Ubp3^{C469A}. 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 *ubp3*Δ 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.

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

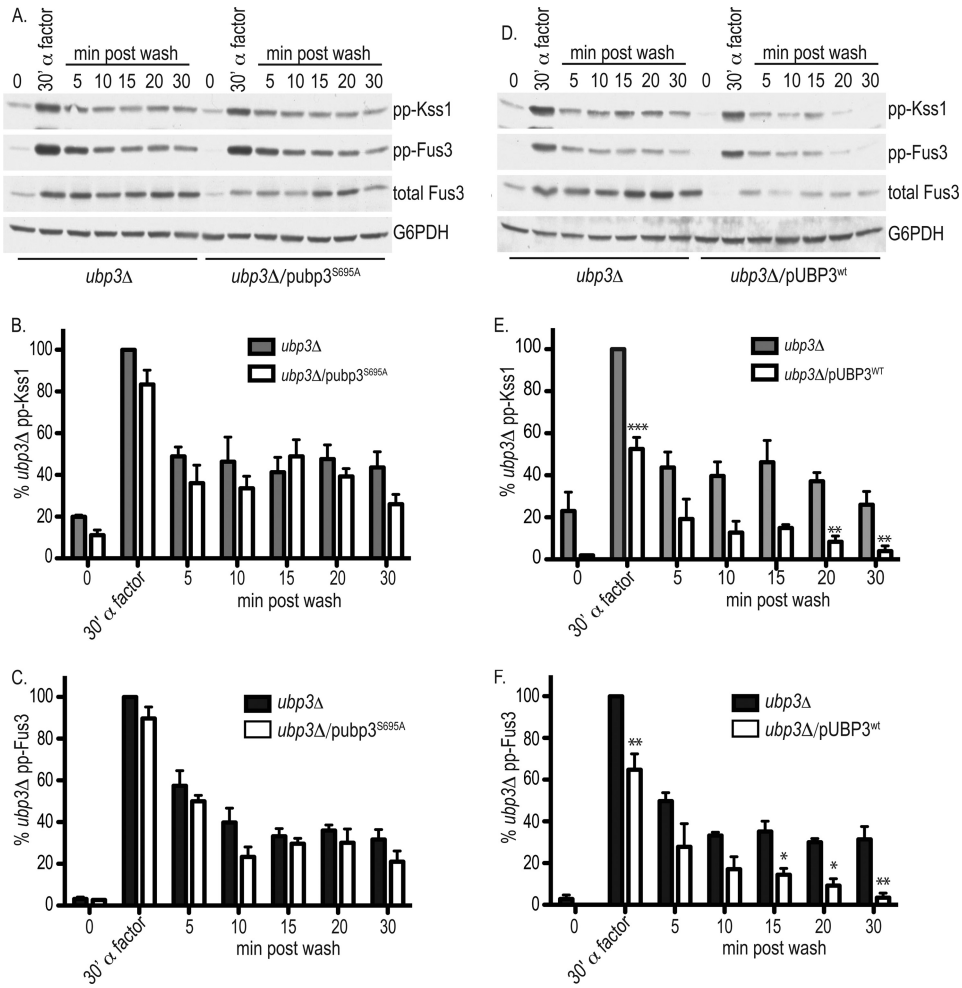

FIGURE 7. **Cells expressing Ubp3^{S695A} exhibit sustained MAP kinase activation.** Ubp3-deficient (*ubp3*Δ) cells transformed with an empty vector or with plasmids expressing the phospho-site mutant Ubp3^{S695A} (*A–C*) or Ubp3^{wt} (*D–F*) were stimulated with 3 μM α factor (pheromone) for 30 min, 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 Kss1 (*B* and *E*) and phosphorylated Fus3 (*C* and *F*) in *ubp3*Δ 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.

complex, SCF^{Cdc4}, directly ubiquitinates 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 (Fbxw6), 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 SCF^{Fbw7} (or SCF^{Cdc4}) 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*Δ mutant exhibits a substantial increase in feedback-phosphorylated Ste7, comparable with that seen in response to pheromone stimulation (Fig. 1*D*). 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. 4*B*). 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-

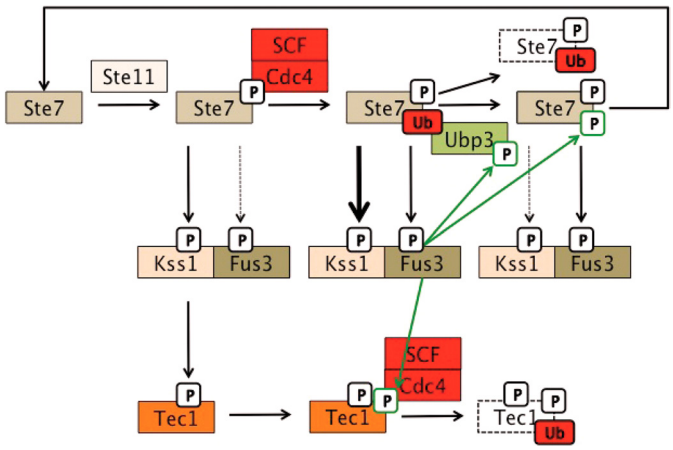

FIGURE 8. **Speculative model of the role of Ste7 ubiquitination (*Ub*) in the yeast pheromone pathway.** Upon pheromone stimulation, the G-protein activates a kinase cascade that includes Ste20 (not shown), Ste11, Ste7, and the MAPKs Fus3 and Kss1. The MAPKs phosphorylate transcription factors in the nucleus, including Ste12 (promotes mating, not shown) and Tec1 (promotes invasive growth). Ste7 is directly ubiquitinated by SCF/Cdc4 (this work). When Ste11 or SCF is not active, cellular pools of Ste7 are stabilized (32). When Ubp3 is not active, there is an accumulation of polyubiquitinated Ste7 (32). Also indicated are three mechanisms that promote pheromone pathway specificity (*green lines*). First, Fus3 phosphorylates Tec1, which is then desumoylated, ubiquitinated, and degraded (76–78, 82, 95). Loss of Tec1 prevents transcription of genes necessary for invasive growth. However, Kss1 activity is still elevated in cells that lack functional Fus3 (30, 42, 96, 97), pointing to additional mechanisms of cross-pathway inhibition upstream of the MAPKs. A second mechanism of cross-pathway inhibition requires Fus3 phosphorylation of Ste7 (35). When Ste7 is phosphorylated, Kss1 is less active (26). A third mechanism depends on Fus3 phosphorylation of Ubp3. When Ubp3 is phosphorylated, Kss1 is again less active (this work). Thus, feedback phosphorylation of either Ste7 or Ubp3 preserves Fus3 signaling and limits inappropriate activation of a competing MAPK, Kss1.

talk. Here, we show that the F-box protein Cdc4 promotes the activation of Fus3 but not Kss1 and acts in part by ubiquitination of a shared upstream kinase Ste7. At least four other proteins have been found to preferentially activate Fus3 but not Kss1. The scaffold protein Ste5 is well known to bind to Fus3 (but not Kss1) and is specifically required for Fus3 activation. More recently, the phosphatidylinositol 4-kinase Stt4 was shown to promote activation of Fus3 and to do so by recruiting Ste5 to the plasma membrane (84–86). Another phosphatidylinositol 4-kinase Pik1 likewise activates Fus3 while simultaneously inhibiting Kss1 (87). Finally, the phosphatidylinositol 3-kinase Vps34 promotes activation of Fus3 but not Kss1 (88). With the exception of Ste5, however, there is little evidence that these regulators are subject to dynamic regulation by pheromone stimulation (86). In contrast, ubiquitination of Ste7 is strictly dependent on pheromone stimulation and subsequent phosphorylation by Ste11 (33).

Prior genetic epistasis analysis had indicated that Ubp3 acts late in the pathway whereas Cdc4 acts early. Although overexpression of *STE4* or the constitutively active *STE11-4* results in MAPK activation in the absence of pheromone, knockdown expression of Cdc4 failed to dampen signaling in these cases (23). In contrast, deletion of Ubp3 still potentiated the response (32). Thus, although Ubp3 acts primarily between the MAPKKK and the MAPK, the functional effects of Cdc4 are primarily at the level of the G-protein. We propose that Cdc4 regulates overall signal output at the level of the G-protein, but it also regulates signal specificity at the level of the MAPKK.

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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REFERENCES

1. Pelech, S. L., and Sanghera, J. S. (1992) Mitogen-activated protein kinases: versatile transducers for cell signaling. *Trends Biochem. Sci.* **17,** 233–238
2. Davis, R. J. (1993) The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.* **268,** 14553–14556
3. Crews, C. M., and Erikson, R. L. (1993) Extracellular signals and reversible protein phosphorylation: what to Mek of it all. *Cell* **74,** 215–217
4. Errede, B., and Levin, D. E. (1993) A conserved kinase cascade for MAP kinase activation in yeast. *Curr. Opin. Cell Biol.* **5,** 254–260
5. Roux, P. P., and Blenis, J. (2004) ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol. Mol. Biol. Rev.* **68,** 320–344
6. Cargnello, M., and Roux, P. P. (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol. Mol. Biol. Rev.* **75,** 50–83
7. Chen, R. E., and Thorner, J. (2007) Function and regulation in MAPK signaling pathways: lessons learned from the yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1773,** 1311–1340
8. Bardwell, L. (2005) A walk-through of the yeast mating pheromone response pathway. *Peptides* **26,** 339–350
9. Bardwell, L., Cook, J. G., Inouye, C. J., and Thorner, J. (1994) Signal propagation and regulation in the mating pheromone response pathway of the yeast *Saccharomyces cerevisiae*. *Dev. Biol.* **166,** 363–379

10. Marsh, L., Neiman, A. M., and Herskowitz, I. (1991) Signal transduction during pheromone response in yeast. *Annu. Rev. Cell Biol.* **7,** 699–728
11. Elion, E. A., Satterberg, B., and Kranz, J. E. (1993) FUS3 phosphorylates multiple components of the mating signal transduction cascade: evidence for STE12 and FAR1. *Mol. Biol. Cell* **4,** 495–510
12. Posas, F., Takekawa, M., and Saito, H. (1998) Signal transduction by MAP kinase cascades in budding yeast. *Curr. Opin. Microbiol.* **1,** 175–182
13. Levin, D. E., and Errede, B. (1995) The proliferation of MAP kinase signaling pathways in yeast. *Curr. Opin. Cell Biol.* **7,** 197–202
14. Gagiano, M., Bauer, F. F., and Pretorius, I. S. (2002) The sensing of nutritional status and the relationship to filamentous growth in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* **2,** 433–470
15. Palecek, S. P., Parikh, A. S., and Kron, S. J. (2002) Sensing, signalling, and integrating physical processes during *Saccharomyces cerevisiae* invasive and filamentous growth. *Microbiology* **148,** 893–907
16. Molina, M., Cid, V. J., and Martín, H. (2010) Fine regulation of *Saccharomyces cerevisiae* MAPK pathways by post-translational modifications. *Yeast* **27,** 503–511
17. Whelan, J. T., Hollis, S. E., Cha, D. S., Asch, A. S., and Lee, M. H. (2012) Post-transcriptional regulation of the Ras-ERK/MAPK signaling pathway. *J. Cell. Physiol.* **227,** 1235–1241
18. Hershko, A., and Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem.* **67,** 425–479
19. Cardozo, T., and Pagano, M. (2004) The SCF ubiquitin ligase: insights into a molecular machine. *Nat. Rev. Mol. Cell Biol.* **5,** 739–751
20. Henchoz, S., Chi, Y., Catarin, B., Herskowitz, I., Deshaies, R. J., and Peter, M. (1997) Phosphorylation- and ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor Far1p in budding yeast. *Genes Dev.* **11,** 3046–3060
21. Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J., and Harper, J. W. (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* **91,** 209–219
22. Feldman, R. M., Correll, C. C., Kaplan, K. B., and Deshaies, R. J. (1997) A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* **91,** 221–230
23. Cappell, S. D., Baker, R., Skowyra, D., and Dohlman, H. G. (2010) Systematic analysis of essential genes reveals important regulators of G protein signaling. *Mol. Cell* **38,** 746–757
24. Garrenton, L. S., Braunwarth, A., Irniger, S., Hurt, E., Künzler, M., and Thorner, J. (2009) Nucleus-specific and cell cycle-regulated degradation of mitogen-activated protein kinase scaffold protein Ste5 contributes to the control of signaling competence. *Mol. Cell. Biol.* **29,** 582–601
25. Reyes-Turcu, F. E., Ventii, K. H., and Wilkinson, K. D. (2009) Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. *Annu. Rev. Biochem.* **78,** 363–397
26. Hao, N., Yildirim, N., Nagiec, M. J., Parnell, S. C., Errede, B., Dohlman, H. G., and Elston, T. C. (2012) Combined computational and experimental analysis reveals mitogen-activated protein kinase-mediated feedback phosphorylation as a mechanism for signaling specificity. *Mol. Biol. Cell* **23,** 3899–3910
27. Hao, N., Behar, M., Elston, T. C., and Dohlman, H. G. (2007) Systems biology analysis of G protein and MAP kinase signaling in yeast. *Oncogene* **26,** 3254–3266
28. Breitkreutz, A., Boucher, L., and Tyers, M. (2001) MAPK specificity in the yeast pheromone response independent of transcriptional activation. *Curr. Biol.* **11,** 1266–1271
29. Farley, F. W., Satterberg, B., Goldsmith, E. J., and Elion, E. A. (1999) Relative dependence of different outputs of the *Saccharomyces cerevisiae* pheromone response pathway on the MAP kinase Fus3p. *Genetics* **151,** 1425–1444
30. Sabbagh, W., Jr., Flatauer, L. J., Bardwell, A. J., and Bardwell, L. (2001) Specificity of MAP kinase signaling in yeast differentiation involves transient *versus* sustained MAPK activation. *Mol. Cell* **8,** 683–691
31. Tedford, K., Kim, S., Sa, D., Stevens, K., and Tyers, M. (1997) Regulation of the mating pheromone and invasive growth responses in yeast by two MAP kinase substrates. *Curr. Biol.* **7,** 228–238
32. Wang, Y., and Dohlman, H. G. (2002) Pheromone-dependent ubiquitination of the mitogen-activated protein kinase kinase Ste7. *J. Biol. Chem.* **277,** 15766–15772
33. Wang, Y., Ge, Q., Houston, D., Thorner, J., Errede, B., and Dohlman, H. G. (2003) Regulation of Ste7 ubiquitination by Ste11 phosphorylation and the Skp1-Cullin-F-box complex. *J. Biol. Chem.* **278,** 22284–22289
34. Hoffman, G. A., Garrison, T. R., and Dohlman, H. G. (2002) Analysis of RGS proteins in *Saccharomyces cerevisiae*. *Methods Enzymol.* **344,** 617–631
35. Maleri, S., Ge, Q., Hackett, E. A., Wang, Y., Dohlman, H. G., and Errede, B. (2004) Persistent activation by constitutive Ste7 promotes Kss1-mediated invasive growth but fails to support Fus3-dependent mating in yeast. *Mol. Cell. Biol.* **24,** 9221–9238
36. Zeller, C. E., Parnell, S. C., and Dohlman, H. G. (2007) The RACK1 ortholog Asc1 functions as a G-protein β subunit coupled to glucose responsiveness in yeast. *J. Biol. Chem.* **282,** 25168–25176
37. Solé, C., Nadal-Ribelles, M., Kraft, C., Peter, M., Posas, F., and de Nadal, E. (2011) Control of Ubp3 ubiquitin protease activity by the Hog1 SAPK modulates transcription upon osmostress. *EMBO J.* **30,** 3274–3284
38. Hao, N., Behar, M., Parnell, S. C., Torres, M. P., Borchers, C. H., Elston, T. C., and Dohlman, H. G. (2007) A systems-biology analysis of feedback inhibition in the Sho1 osmotic-stress-response pathway. *Curr. Biol.* **17,** 659–667
39. Garrison, T. R., Zhang, Y., Pausch, M., Apanovitch, D., Aebersold, R., and Dohlman, H. G. (1999) Feedback phosphorylation of an RGS protein by MAP kinase in yeast. *J. Biol. Chem.* **274,** 36387–36391
40. Chasse, S. A., and Dohlman, H. G. (2004) Identification of yeast pheromone pathway modulators by high-throughput agonist response profiling of a yeast gene knockout strain collection. *Methods Enzymol.* **389,** 399–409
41. Parnell, S. C., Marotti, L. A., Jr., Kiang, L., Torres, M. P., Borchers, C. H., and Dohlman, H. G. (2005) Phosphorylation of the RGS protein Sst2 by the MAP kinase Fus3 and use of Sst2 as a model to analyze determinants of substrate sequence specificity. *Biochemistry* **44,** 8159–8166
42. Gartner, A., Nasmyth, K., and Ammerer, G. (1992) Signal transduction in *Saccharomyces cerevisiae* requires tyrosine and threonine phosphorylation of FUS3 and KSS1. *Genes Dev.* **6,** 1280–1292
43. Chou, S., Lane, S., and Liu, H. (2006) Regulation of mating and filamentation genes by two distinct Ste12 complexes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **26,** 4794–4805
44. Cook, J. G., Bardwell, L., and Thorner, J. (1997) Inhibitory and activating functions for MAPK Kss1 in the *S. cerevisiae* filamentous-growth signalling pathway. *Nature* **390,** 85–88
45. Bardwell, L., Cook, J. G., Zhu-Shimoni, J. X., Voora, D., and Thorner, J. (1998) Differential regulation of transcription: repression by unactivated mitogen-activated protein kinase Kss1 requires the Dig1 and Dig2 proteins. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 15400–15405
46. Kessler, B. M., and Edelmann, M. J. (2011) PTMs in conversation: activity and function of deubiquitinating enzymes regulated via post-translational modifications. *Cell Biochem. Biophys.* **60,** 21–38
47. Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R., McCartney, R. R., Schmidt, M. C., Rachidi, N., Lee, S. J., Mah, A. S., Meng, L., Stark, M. J., Stern, D. F., De Virgilio, C., Tyers, M., Andrews, B., Gerstein, M., Schweitzer, B., Predki, P. F., and Snyder, M. (2005) Global analysis of protein phosphorylation in yeast. *Nature* **438,** 679–684
48. Bhattacharyya, R. P., Reményi, A., Good, M. C., Bashor, C. J., Falick, A. M., and Lim, W. A. (2006) The Ste5 scaffold allosterically modulates signaling output of the yeast mating pathway. *Science* **311,** 822–826
49. Hao, N., Yildirim, N., Wang, Y., Elston, T. C., and Dohlman, H. G. (2003) Regulators of G protein signaling and transient activation of signaling: experimental and computational analysis reveals negative and positive feedback controls on G protein activity. *J. Biol. Chem.* **278,** 46506–46515
50. Clague, M. J., Liu, H., and Urbé, S. (2012) Governance of endocytic trafficking and signaling by reversible ubiquitylation. *Dev. Cell* **23,** 457–467
51. Dores, M. R., and Trejo, J. (2012) Ubiquitination of G protein-coupled receptors: functional implications and drug discovery. *Mol. Pharmacol.* **82,** 563–570
52. Husnjak, K., and Dikic, I. (2012) Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. *Annu. Rev. Biochem.* **81,** 291–322

53. Chung, C. Y., Reddy, T. B., Zhou, K., and Firtel, R. A. (1998) A novel, putative MEK kinase controls developmental timing and spatial patterning in *Dictyostelium* and is regulated by ubiquitin-mediated protein degradation. *Genes Dev.* **12,** 3564–3578
54. Xiong, X., Wang, X., Ewanek, R., Bhat, P., Diantonio, A., and Collins, C. A. (2010) Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. *J. Cell Biol.* **191,** 211–223
55. Fischer, J. A., and Overstreet, E. (2002) Fat facets does a Highwire act at the synapse. *BioEssays* **24,** 13–16
56. DiAntonio, A., Haghighi, A. P., Portman, S. L., Lee, J. D., Amaranto, A. M., and Goodman, C. S. (2001) Ubiquitination-dependent mechanisms regulate synaptic growth and function. *Nature* **412,** 449–452
57. Yoon, S., Liu, Z., Eyobo, Y., and Orth, K. (2003) *Yersinia* effector YopJ inhibits yeast MAPK signaling pathways by an evolutionarily conserved mechanism. *J. Biol. Chem.* **278,** 2131–2135
58. Yang, W. L., Jin, G., Li, C. F., Jeong, Y. S., Moten, A., Xu, D., Feng, Z., Chen, W., Cai, Z., Darnay, B., Gu, W., and Lin, H. K. (2013) Cycles of ubiquitination and deubiquitination critically regulate growth factor-mediated activation of Akt signaling. *Sci. Signal.* **6,** ra3
59. Harhaj, E. W., and Dixit, V. M. (2011) Deubiquitinases in the regulation of NF-κB signaling. *Cell Res.* **21,** 22–39
60. Sun, S. C. (2010) CYLD: a tumor suppressor deubiquitinase regulating NF-κB activation and diverse biological processes. *Cell Death Differ.* **17,** 25–34
61. Massoumi, R. (2010) Ubiquitin chain cleavage: CYLD at work. *Trends Biochem. Sci.* **35,** 392–399
62. Yang, Y., Sun, L., Tala, Gao, J., Li, D., Zhou, J., and Liu, M. (2013) CYLD regulates RhoA activity by modulating LARG ubiquitination. *PLoS One* **8,** e55833
63. Reiley, W., Zhang, M., and Sun, S. C. (2004) Negative regulation of JNK signaling by the tumor suppressor CYLD. *J. Biol. Chem.* **279,** 55161–55167
64. Satija, Y. K., Bhardwaj, A., and Das, S. (2013) A portrayal of E3 ubiquitin ligases and deubiquitylases in cancer. *Int. J. Cancer*, in press
65. Driscoll, J. J., and Woodle, E. S. (2012) Targeting the ubiquitin+ proteasome system in solid tumors. *Semin. Hematol.* **49,** 277–283
66. Jung, Y. S., Qian, Y., and Chen, X. (2012) Pirh2 RING-finger E3 ubiquitin ligase: its role in tumorigenesis and cancer therapy. *FEBS Lett.* **586,** 1397–1402
67. Mocciaro, A., and Rape, M. (2012) Emerging regulatory mechanisms in ubiquitin-dependent cell cycle control. *J. Cell Sci.* **125,** 255–263
68. Wang, G., Chan, C. H., Gao, Y., and Lin, H. K. (2012) Novel roles of Skp2 E3 ligase in cellular senescence, cancer progression, and metastasis. *Chin. J. Cancer* **31,** 169–177
69. Silverman, J. S., Skaar, J. R., and Pagano, M. (2012) SCF ubiquitin ligases in the maintenance of genome stability. *Trends Biochem. Sci.* **37,** 66–73
70. Inuzuka, H., Fukushima, H., Shaik, S., and Wei, W. (2010) Novel insights into the molecular mechanisms governing Mdm2 ubiquitination and destruction. *Oncotarget* **1,** 685–690
71. Hubbard, E. J., Wu, G., Kitajewski, J., and Greenwald, I. (1997) sel-10, a negative regulator of lin-12 activity in *Caenorhabditis elegans*, encodes a member of the CDC4 family of proteins. *Genes Dev.* **11,** 3182–3193
72. Moberg, K. H., Bell, D. W., Wahrer, D. C., Haber, D. A., and Hariharan, I. K. (2001) Archipelago regulates cyclin E levels in *Drosophila* and is mutated in human cancer cell lines. *Nature* **413,** 311–316
73. Maruyama, S., Hatakeyama, S., Nakayama, K., Ishida, N., and Kawakami, K. (2001) Characterization of a mouse gene (Fbxw6) that encodes a homologue of *Caenorhabditis elegans* SEL-10. *Genomics* **78,** 214–222
74. Koepp, D. M., Schaefer, L. K., Ye, X., Keyomarsi, K., Chu, C., Harper, J. W., and Elledge, S. J. (2001) Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. *Science* **294,** 173–177
75. Welcker, M., and Clurman, B. E. (2008) FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. *Nat. Rev. Cancer* **8,** 83–93
76. Wang, Y., and Dohlman, H. G. (2006) Pheromone-regulated sumoylation of transcription factors that mediate the invasive to mating developmental switch in yeast. *J. Biol. Chem.* **281,** 1964–1969
77. Bao, M. Z., Schwartz, M. A., Cantin, G. T., Yates, J. R., 3rd, and Madhani, H. D. (2004) Pheromone-dependent destruction of the Tec1 transcription factor is required for MAP kinase signaling specificity in yeast. *Cell* **119,** 991–1000
78. Brückner, S., Köhler, T., Braus, G. H., Heise, B., Bolte, M., and Mösch, H. U. (2004) Differential regulation of Tec1 by Fus3 and Kss1 confers signaling specificity in yeast development. *Curr. Genet.* **46,** 331–342
79. Andersson, J., Simpson, D. M., Qi, M., Wang, Y., and Elion, E. A. (2004) Differential input by Ste5 scaffold and Msg5 phosphatase route a MAPK cascade to multiple outcomes. *EMBO J.* **23,** 2564–2576
80. Madhani, H. D., Styles, C. A., and Fink, G. R. (1997) MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation. *Cell* **91,** 673–684
81. Randles, L., and Walters, K. J. (2012) Ubiquitin and its binding domains. *Front. Biosci.* **17,** 2140–2157
82. Chou, S., Huang, L., and Liu, H. (2004) Fus3-regulated Tec1 degradation through SCFCdc4 determines MAPK signaling specificity during mating in yeast. *Cell* **119,** 981–990
83. Bardwell, L. (2006) Mechanisms of MAPK signalling specificity. *Biochem. Soc. Trans.* **34,** 837–841
84. Garrenton, L. S., Young, S. L., and Thorner, J. (2006) Function of the MAPK scaffold protein, Ste5, requires a cryptic PH domain. *Genes Dev.* **20,** 1946–1958
85. Winters, M. J., Lamson, R. E., Nakanishi, H., Neiman, A. M., and Pryciak, P. M. (2005) A membrane binding domain in the ste5 scaffold synergizes with Gβγ binding to control localization and signaling in pheromone response. *Mol. Cell* **20,** 21–32
86. Garrenton, L. S., Stefan, C. J., McMurray, M. A., Emr, S. D., and Thorner, J. (2010) Pheromone-induced anisotropy in yeast plasma membrane phosphatidylinositol-4,5-bisphosphate distribution is required for MAPK signaling. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 11805–11810
87. Cappell, S. D., and Dohlman, H. G. (2011) Selective regulation of MAP kinase signaling by an endomembrane phosphatidylinositol 4-kinase. *J. Biol. Chem.* **286,** 14852–14860
88. Slessareva, J. E., Routt, S. M., Temple, B., Bankaitis, V. A., and Dohlman, H. G. (2006) Activation of the phosphatidylinositol 3-kinase Vps34 by a G protein α subunit at the endosome. *Cell* **126,** 191–203
89. Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., and Bernards, R. (2005) A genomic and functional inventory of deubiquitinating enzymes. *Cell* **123,** 773–786
90. Wing, S. S. (2003) Deubiquitinating enzymes–the importance of driving in reverse along the ubiquitin-proteasome pathway. *Int. J. Biochem. Cell Biol.* **35,** 590–605
91. Wilkinson, K. D., Tashayev, V. L., O'Connor, L. B., Larsen, C. N., Kasperek, E., and Pickart, C. M. (1995) Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T. *Biochemistry* **34,** 14535–14546
92. Komander, D., Clague, M. J., and Urbé, S. (2009) Breaking the chains: structure and function of the deubiquitinases. *Nat. Rev. Mol. Cell Biol.* **10,** 550–563
93. D'Arcy, P., Brnjic, S., Olofsson, M. H., Fryknäs, M., Lindsten, K., De Cesare, M., Perego, P., Sadeghi, B., Hassan, M., Larsson, R., and Linder, S. (2011) Inhibition of proteasome deubiquitinating activity as a new cancer therapy. *Nat. Med.* **17,** 1636–1640
94. Mao, P., and Smerdon, M. J. (2010) Yeast deubiquitinase Ubp3 interacts with the 26 S proteasome to facilitate Rad4 degradation. *J. Biol. Chem.* **285,** 37542–37550
95. Wang, Y., Abu Irqeba, A., Ayalew, M., and Suntay, K. (2009) Sumoylation of transcription factor Tec1 regulates signaling of mitogen-activated protein kinase pathways in yeast. *PLoS One* **4,** e7456
96. Roberts, R. L., and Fink, G. R. (1994) Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. *Genes Dev.* **8,** 2974–2985
97. Madhani, H. D., and Fink, G. R. (1997) Combinatorial control required for the specificity of yeast MAPK signaling. *Science* **275,** 1314–1317