Many cell signaling pathways are regulated by phosphorylation, ubiquitination, and degradation of constituent proteins. As with phosphorylation, protein ubiquitination can be reversed, through the action of ubiquitin-specific processing proteases (UBPs). Here we have analyzed 15 UBP disruption mutants in the yeast *Saccharomyces cerevisiae* and identified one (ubp3Δ) that acts specifically in the pheromone response pathway. Upon pheromone stimulation, ubp3Δ mutants accumulate unconjugated polyubiquitin chains as well as polyubiquitinated forms of the mitogen-activated protein kinase kinase Ste7. The ubp3Δ mutants exhibit a potentiated response to pheromone, as measured by *in vivo* MAP kinase activity, transcriptional induction, and cell cycle arrest. Signaling is likewise enhanced upon direct activation of Ste4 (G protein β subunit) and Ste11 (Ste7 kinase) but not the downstream transcription factor Ste12. These findings reveal a mechanism by which pheromone-triggered ubiquitination of Ste7 can modulate the pheromone response *in vivo*.

A broad array of cell signaling molecules act on cell surface receptors and their associated G proteins. In humans, such receptors can detect hormones, neurotransmitters, odors, taste, and light. In the yeast *Saccharomyces cerevisiae*, G protein-coupled receptors (Ste2, Ste3) respond to pheromones that initiate haploid cell fusion, or mating (1). Upon receptor stimulation, the G protein β subunit (Gpa1) binds to GTP and dissociates from the G protein βγ subunits (Ste4/Ste18). The Gβγ dimer can then propagate 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 mitogen-activated protein (MAP)1 kinase cascade composed of Ste11 (MAPK kinase kinase), which phosphorylates and activates a dual specificity kinase Ste7 (MAPK kinase), which in turn phosphorylates and activates Fus3 (MAPK). Known downstream targets of Fus3 in the mating pathway include Ste12, a transcription factor, and Far1, a component of the cell cycle machinery. Phosphorylation of Ste12 and Far1 leads to enhanced transcription of pheromone-inducible genes and arrest of the cell division cycle in the late G1 phase (1).

A general property of signaling cascades is the ability to abrogate their responsiveness over time (2). In yeast, pheromone signaling is attenuated by degradation of the peptide ligand, hydrolysis of GTP by Gα, and dephosphorylation of target proteins (1). Further desensitization is achieved through feedback regulatory mechanisms, including phosphorylation, internalization, and degradation of cell surface receptors (3–5). Another desensitization mechanism involves the expression, phosphorylation, and stabilization of the GTPase-accelerating protein Sst2 (6). These processes allow resumption of cell division after pheromone arrest, whether or not mating is successful.

There is emerging evidence that pheromone signaling is regulated through ubiquitination and degradation of constituent proteins (3–5, 7). Ubiquitination requires ubiquitin-conjugating enzymes, which catalyze the formation of an isopeptide bond between the C-terminal carboxyl group of ubiquitin and Lys side chains of the target protein (8). Conjugated ubiquitin is itself ubiquitinated, resulting in the formation of polyubiquitin chains. These chains are then recognized by the 26 S proteasome, which degrades the substrate protein (9). In the case of some cell surface receptors (e.g., Ste2 and Ste3) ubiquitination can function as a signal for ligand-induced endocytosis. This situation is unusual, however, in that the receptors are largely monoubiquitinated instead of polyubiquitinated and are delivered to the vacuole instead of the proteasome (3–5).

Protein ubiquitination is reversible (10). Following proteolysis of the substrate, ubiquitin is removed from the resulting protein fragments and reused. Moreover, some proteins appear to undergo ubiquitination without being degraded or targeted to the proteasome. For instance, the IκBα protein kinase is regulated through a cycle of stimulus-dependent ubiquitination and deubiquitination (11). The immunoglobulin E receptor is ubiquitinated upon antigen binding but rapidly deubiquitinated upon antigen disengagement (12). In yeast, the ubiquitinated form of the transcription factor Met4 can associate with target promoters but fails to form functional transcription complexes, indicating that ubiquitination can directly regulate Met4 activity (13).

Whether or not the protein substrate is degraded, disassembly of polyubiquitin requires ubiquitin-C-terminal hydrolases and ubiquitin-specific processing proteases (UBPs; also called isopeptidases and deubiquitinating enzymes) that cleave the amide bond between ubiquitin and the substrate protein (10, 14, 15). Of the 17 deubiquitinating enzymes in yeast, 16 are members of the UBP class. The UBP family is extremely divergent, in yeast ranging from 54 to 146 kDa; however, all members contain a signature sequence that includes conserved Cys and His residues needed for catalytic activity (16, 17).
The cellular function of most UBP s is unknown (14). Systematic disruption of the 16 UBP genes in yeast revealed only minimal phenotypic abnormalities, and none proved essential (18). Where a ubp phenotype has been identified, there appears to be little functional overlap among family members (18-23). Perhaps the best characterized member is Doa4 (Ubpd) (24). Doa4 is associated with the proteasome, where it removes ubiquitin from substrate intermediates during the course of proteolysis (19). Cells lacking DOA4 accumulate small polyubiquitinated peptide fragments, and the consequent depletion of free ubiquitin leads to stabilization of other substrates (19, 21, 25, 26). Another well characterized family member is Ubp14 (mammalian IsoT is similar), which appears to act downstream of Doa4 to disassemble free (unanchored) polyubiquitin chains (23).

The large number of UBP s suggests that they may function in specific signaling or developmental pathways. Here, we demonstrate functional regulation of the G protein and MAP kinase signaling cascade by Ubpd in yeast. These experiments reveal a novel mechanism of feedback regulation through pheromone-dependent ubiquitination of the MAP kinase Ste7.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Standard methods for the growth, maintenance, and transformation of yeast and bacteria and for the manipulation of DNA were used throughout (27). The yeast S. cerevisiae strains used in this study are BY4741 (MATa leu2 met15ura3), BY4741-derived mutants lacking STE4, STE7, UBP1, UBP2, UBP3, DOA4 (UBP4, SSV7, NP2, ASI7), UBP5, UBP6, UBP7, UBP9, UBP11, UBP12, UBP13, UBP14, UBP15, and UBP16 (LFP12) (Research Genetics, Huntsville, AL), MHY753 (MATa his3-d200 leu2-3,112 lys2-801 trp1-163 ade2-101), MHY754 (MHY753, cim3-1), and MHY755 (MHY753, cim-1) (28).

Expression plasmids used in this study that have been described previously are pRS316-GAL-STE4 (29) and YCP50-STE11-4 (30) (from George Sprague, University of Oregon). Overexpression of STE12 was achieved by PCR amplification and subcloning into the pYES2.1V5-His-TOPO (2 μM, URA3, GAL1 promoter, CYC1 terminator) (Invitrogen, Carlsbad, CA). PCR primer was 5'-CCA GAA TGA AAG TCC AAA -3'.

Growth, Transcription, Phosphorylation, Degradation, and Ubiquitination Bioassays—The pheromone-dependent growth inhibition (halo) and reporter-transcription assays were conducted as described previously (31). Phosphorylation of Ste4 and Ste7 and ubiquitination of Ste7 were monitored by immunoblotting of whole cell extracts. To monitor the loss of Ste7 over time, midlog cell cultures were treated with 2.5 μM α-factor for 30 min, followed by cycloheximide (10 μg/ml in 0.1% ethanol, final concentrations) for up to 90 min. Growth was stopped by the addition of 10 mM NaN₃ and transfer to an ice bath. Cells were washed and resuspended directly in boiling SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.0005% bromphenol blue) for 10 min, subjected to glass bead homogenization, and clarified by microcentrifugation. Following SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose, the membrane was probed with antibodies to Ste4 at 1:2,000 (from Duane Jenness, University of Massachusetts), Pgk1 at 1:7,500 (from Jeremy Thorner, University of California, Berkeley), ubiquitin at 1:100 (Sigma), or Ste7 at 1:200 (yN-18) (Santa Cruz Biotechnology). Specificity of detection was established using ste4Δ and ste7Δ cell extracts as negative controls.

For some experiments, ubiquitinated Ste7 was enriched by immunoprecipitation prior to immunoblotting. Cells were grown to midlog phase and either treated with 2.5 μM α-factor or with water for 1 h. Approximately 100 ml of cells at A₆₀₀ = 1 were harvested and lysed at 4°C in 600 μl of lysis buffer (25 mM Tris-HCl, pH 7.4, 200 mM NaCl, 15 mM EGTA, 15 mM MgCl₂, 0.1% Triton X-100, 100 mM glycine, 1 mM NaN₃, 1 mM dithiothreitol, 10 mM N-ethylmaleimide, 5 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, and 1 μg/ml leupeptin) with the use of acid-washed glass beads and 30-s pulses of vortexing, repeated six times. Samples were centrifuged for 10 min at 6,500 × g, and the resulting supernatant was removed and diluted to a final volume of 1 ml with wash buffer (the same as lysis buffer except without glycerol). Lysates were incubated with 40 μl of yN-18 goat anti-Ste7 antibodies (Santa Cruz Biotechnology) for 90 min on ice. After clarification with 10-min high speed microcentrifugation at 4°C, protein-antibody complexes were precipitated for 1 h at 4°C with 40 μl of 50% slurry of protein G-Sepharose (Amersham Biosciences, Inc.) equilibrated in wash buffer. Immunoprecipitates were collected by centrifugation at 2,000 × g for 30 s, and pellets were washed with wash buffer before final resuspension in 50 μl of 2× SDS-PAGE sample buffer.

RESULTS

It was well established that the pheromone receptors are regulated by ubiquitination. Upon pheromone binding, Ste2 and Ste3 are rapidly phosphorylated, ubiquitinated, endocytosed, and degraded in the vacuole (3-5). Our goal here was to determine if postreceptor signaling events are similarly regulated by ubiquitination. Our initial approach was to examine the ubiquitination and signaling properties of strains lacking each of the known deubiquitinating enzymes. By removing UBP activity from cells, we anticipated that short-lived changes in protein ubiquitination might be detected. This is analogous to using specific phosphatase inhibitors to preserve transient increases in protein phosphorylation.

We initially investigated whether there are any global changes in ubiquitination following stimulation with pheromone. Extracts were prepared from wild type cells and from 15 different ubp deletion mutants, either untreated or treated for 1 h with 2.5 μM α-factor. Whole cell extracts were subjected to SDS-PAGE and immunoblotting, and probed with anti-ubiquitin antibodies. All of the ubp mutants accumulated polyubiquitin chains of varying length and appeared depleted of free ubiquitin and smaller ubiquitin chains (four or fewer) as previously described for doa4 and ubp14 (18, 21, 23). Following pheromone stimulation, the ubp3Δ mutant accumulated additional polyubiquitin chains. There was no effect of pheromone in wild type cells or in any of the other ubp mutants tested (Fig. 1 and data not shown).

We then examined whether UBp3 can modulate the pheromone response pathway. For these experiments, we compared signaling in wild type and each of the ubpΔ mutants using three standard bioassays. First, we measured pheromone-dependent growth arrest (31). In this assay, cells are spread onto solid medium and exposed to α-factor pheromone spotted onto sterile filter disks. The resulting zone of growth inhibition provides an indication of initial pheromone sensitivity (halo size) and long term desensitization (halo turbidity). Of the 15
ubp mutants tested, only ubp3Δ exhibited a larger than normal zone of growth inhibition, indicating a ~5-fold enhancement in pheromone sensitivity (Fig. 2A and data not shown). Second, we measured short term pheromone signaling using a reporter transcription assay, consisting of the pheromone-inducible FUS1 promoter fused to the lacZ (β-galactosidase) reporter (31). Compared with wild type cells, the ubp3Δ mutant exhibited a 1.7-fold increase in β-galactosidase activity at the maximum effective dose and an elevated basal activity (i.e. no pheromone added) but no change in the EC
50 (Fig. 2B). No difference was observed for the doa4Δ or ubp14Δ mutants. Finally, we measured pheromone-dependent MAP kinase activity using two endogenous substrates, the G protein β subunit (Ste4) and the MAP kinase kinase (Ste7). Feedback phosphorylation by the MAP kinases Fus3 (or Kss1, which has partially overlapping function) significantly reduces the electrophoretic mobility of each substrate and is conveniently monitored by immunoblotting with anti-Ste7 and anti-Ste4 antibodies (32, 33). In either case, the ubp3Δ mutation resulted in a substantial increase in basal and pheromone-stimulated phosphorylation (Fig. 2C). The extent of basal and pheromone-dependent phosphorylation was greater for Ste7 than Ste4. No difference was observed for either the doa4Δ or ubp14Δ mutants.

The data presented in Fig. 2 reveal that MAP kinase activity is elevated in UBP3-deficient cells. This is likely to be responsible for the increase in transcriptional activity and growth arrest exhibited in these mutants. The same immunoblots also revealed a very high molecular weight form of Ste7 in ubp3Δ cells treated with pheromone, a phenomenon commonly observed with proteins that have undergone polyubiquitination. Accumulation of the presumed ubiquitinated form of Ste7 is dose-dependent (Fig. 3A). Ubiquitinated Ste7 is barely visible in the wild type cells even at the highest concentrations of α-factor sufficient to trigger growth arrest. Thus, accumulation of ubiquitinated Ste7 is dependent on pheromone and is not simply a result of cell division arrest.

To confirm that Ste7 is ubiquitinated, the protein was immunoprecipitated with anti-Ste7 antibodies and analyzed by immunoblotting with anti-ubiquitin antibodies. This enrichment scheme again yielded a high molecular weight band recognized by the ubiquitin antibodies (Fig. 3B). To confirm these results, we monitored expression of Ste7 in cim3–1 and cim5–1 mutant strains, which are deficient in 26 S proteasome activity (28). Upon pheromone stimulation, expression of ubiquitinated and nonubiquitinated Ste7 increased in both mutants and in particular the cim3-1 strain (Fig. 3C). Taken together, these results demonstrate that pheromone stimulation promotes the ubiquitination of Ste7.

Ubiquitination typically leads to protein degradation. However, the ubp3Δ mutant accumulates polyubiquitinated forms of Ste7, suggesting that Ste7 degradation is slowed in these cells. To test this possibility, we monitored Ste7 degradation in ubp3Δ mutant and wild type cells treated with pheromone. Cells in midlog phase were treated with cycloheximide to block new protein synthesis and analyzed by immunoblotting with Ste7 antibodies. In both strains, the overall level of the native (nonubiquitinated) Ste7 dropped rapidly when translation was blocked, but the ubp3Δ mutation extended the half-life of the

![Image](https://example.com/image.png)
Ubiquitination and Regulation of Ste7

Many biological processes are regulated through the modification of existing proteins. Modification by ubiquitination typically leads to destruction of the substrate protein. With regard to cell signaling, most work has focused on ubiquitination of tyrosine kinase receptors, transcription factors, and components of the cell cycle machinery. More recently, attention has turned to ubiquitination of G protein-coupled receptors and their downstream targets (7, 41-43). There has also been growing interest in mechanisms of protein deubiquitination (10). Our results indicate that ubiquitination of Ste7 is stimulated by pheromone and that deubiquitination of Ste7 specifically requires the Ubp3 enzyme. In the absence of Ubp3 activity, Ste7 expression is elevated, and this leads to elevated MAP kinase activity, transcription, and growth arrest.

Ubp3 is one of three UBPs originally isolated based on their ability to cleave a ubiquitin-β-galactosidase fusion test protein (47). A ubp3Δ mutant has a slight growth defect but no detectable change in total cellular ubiquitin-specific processing protease activity. Ubp3 was later shown to copurify with Sir4 (48), a factor necessary for transcriptional silencing (49). Deletion of UBP3 results in a marked increase in silencing at telomeres and the HML mating type locus, suggesting that Ubp3 is an inhibitor of silencing (48). The mechanism of Ubp3 function in

...
Ubiquitination and Regulation of Ste7

**A**

![Graph](image)

**Fig. 4.** The ubp3Δ mutant potentiates transcriptional induction upon activation by STE4 and STE11 but not STE12. Cells were transformed with a plasmid containing the FUS1 promoter-lacZ reporter and a plasmid that overexpresses either wild type STE4 (A), constitutively active STE11-4 (B), or wild type STE12 (C). Cells were treated with the indicated concentrations of α-factor, and β-galactosidase activity was determined as described above. Absolute values at 0 and 100 μM α-factor are as follows: 8,874 ± 209 and 10,345 ± 502 (wild type strain, STE4 plasmid), 14,539 ± 666 and 22,405 ± 1,343 (ubp3Δ, STE4), 13,859 ± 311 and 38,611 ± 380 (wild type, STE11-4), 24,111 ± 339 and 64,680 ± 727 (ubp3Δ, STE11-4), 23,135 ± 1,241 and 25,353 ± 1,093 (ubp14Δ, STE11-4), 14,539 ± 727 (ubp3Δ, 1,241 and 24,999 ± 2,641 (wild type, STE12), and 7,893 ± 414 and 25,353 ± 1,093 (ubp14Δ, STE12). Data shown are typical of three independent experiments performed in triplicate. Error bars, S.E.

**B**

![Graph](image)

**C**

![Graph](image)

**Fig. 5.** Fus3 expression is elevated in the ubp3Δ mutant. Whole cell extracts were prepared from wild type, ubp3Δ, ubp4Δ/doa4Δ, and ubp14Δ mutant strains, either untreated (−) or treated with 2.5 μM α-factor (+) for 1 h. Samples were resolved by 7.5% SDS-PAGE and immunoblotting and probed using anti-Fus3 polyclonal antiserum.

perhaps acting on just a subset of ubiquitinated substrates. Individual UBPs could have specific roles in the reversal of regulatory ubiquitination (11), editing of inappropriately ubiquitinated proteins (12), and regeneration of active ubiquitin from adducts with small molecular mass (13). A possible model for this context is not known. Although HML silencing is needed to preserve mating type and mating efficiency, the ubp3Δ mutant does not confer a sterile phenotype (48). Indeed, our findings indicate that ubp3Δ actually enhances pheromone-dependent transcription and growth arrest and does so through action upstream of the transcription factor Ste12.

Perhaps the best characterized UBP is Doa4. Doa4 is physically associated with the proteasome (19, 25) and appears necessary for deubiquitination of polyubiquitin-substrate intermediates prior to their degradation. Doa4 might also function to rescue inappropriately ubiquitinated proteins from degradation. Mutants of doa4 have reduced cellular levels of free ubiquitin but elevated levels of ubiquitinated peptides, evidently the remnants of proteins degraded by the proteasome (21). Even when ubiquitin is restored to normal or strongly elevated levels, however, degradation of a test substrate (ubiquitin-β-galactosidase fusion) is diminished. These findings indicate that the doa4Δ mutant is also defective for a postubiquitination step in the ubiquitin-proteasome pathway (19).

Another well characterized UBP is Ubp14, the yeast counterpart of mammalian isopeptidase T (22, 23) and Drosophila Ubpa (22). This enzyme acts primarily on unanchored ubiquitin chains generated as intermediates in substrate degradation (15). A ubp14Δ mutant exhibits a defect in ubiquitin-dependent proteolysis and an accumulation of unanchored polyubiquitin chains (23); these chains are thought to inhibit proteolysis by competing with polyubiquitinated substrates on the 26 S proteasome (50, 51).

Thus, Doa4 appears to remove ubiquitin chains from proteins already committed to degradation by the proteasome (25), while Ubp14 acts to disassemble free ubiquitin chains (23). Notably, neither mutant affects the pheromone response. Moreover, the defects that are associated with doa4Δ and ubp14Δ appear not to be rescued by other UBP isoforms, even when present on high copy plasmids. Thus, it seems likely that each UBP shares a common catalytic activity but can have highly specific cell regulatory functions in vivo, perhaps acting on just a subset of ubiquitinated substrates. Individual UBPs could have specific roles in the reversals of regulatory ubiquitination (11), editing of inappropriately ubiquitinated proteins and regeneration of active ubiquitin from adducts with small cellular nucleophiles (such as glutathione) that may be produced by side reactions. Another possibility is that different UBPs recruit only selected substrates to the proteasome (Fig. 6). This model would explain how Ste7 can be ubiquitinated but not degraded in ubp3Δ cells.

A modest increase in Ste7 expression could well account for the enhanced signaling observed in the ubp3Δ mutant. Ste7 appears to be the limiting component of the MAP kinase cascade. Estimates from quantitative immunoblotting studies revealed the number of Ste7 molecules (~2,000/cell) to be considerably lower than either Fus3 or Kas1 (~5,000 each) (52). Thus,
a small change in Ste7 expression could easily account for the increased activity of Fus3/Kss1 observed in vivo. Indeed, it was shown previously that modest overexpression of STE7 (using a CYC1 promoter and a low copy CEN plasmid) yielded a 1.4-fold increase in pheromone-stimulated gene transcription (32). An intriguing question is whether the ubiquitinated Ste7 species retains kinase activity and how this activity might compare with that of the nonubiquitinated species. Ste7 is also required for invasive growth (1), suggesting that Ubp3 might regulate this signaling pathway as well. However, examination of this possibility could be challenging because of the lack of satisfactory quantitative assays of invasive growth similar to those used for the mating pathway.

A more general question is whether UBp control of MAP kinase activity could lead to altered cell differentiation or transformation. Specific deubiquitinating enzymes have been shown to regulate cellular growth in a number of other organisms. In Dicystostelium, UbAp-deficient cells grow and respond normally to starvation growth conditions but fail to continue development to the stage where pulses of cAMP trigger aggregation and fruiting body formation (22). Another isoform, UbPB, was identified in a two-hybrid screen using MEKKa (MAPKKK) as bait (53). Cells deficient in either MEKKa or UbPB develop precociously and exhibit abnormal cell type patterning. Deletion of UBPB alone was shown to diminish MEKKa expression and was stated to increase the abundance of ubiquitinated MEKKa (53). In Drosophila, the UBP fas determines cell growth and cell differentiation during eye development (54, 55). Antagonizing ubiquitination by the neuronal overexpression of fas or yeast UBP2 leads to synaptic overgrowth and defects in neurotransmitter release. This phenotype is very similar to the loss-of-function phenotype of hiw, a putative synaptic E3 ubiquitin ligase (56). In mammals, the protooncogene tre-2 encodes a deubiquitinating enzyme, and the tre-2 oncoprotein exhibits transforming activity in 3T3 fibroblasts (25, 57). Likewise, the deubiquitinating enzyme encoded by unp is tumorigenic in transgenic mice (58, 59). It is not known if any of these transforming mutations alter MAPKK ubiquitination or activity.

In summary, we have demonstrated that Ste7 is regulated by ubiquitination and that Ste7 ubiquitination is regulated by pheromone and Ubp3. Our results imply that other Ubp isoforms will have highly selective cell-regulatory functions and that MAP kinase kinases in other organisms may be similarly regulated by growth factor stimulation.

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Pheromone-dependent Ubiquitination of the Mitogen-activated Protein Kinase Ste7
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