Differential regulation of *FUS3* MAP kinase by tyrosine-specific phosphatases *PTP2/PTP3* and dual-specificity phosphatase *MSG5* in *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae* mating pheromone response is mediated by activation of a MAP kinase (Fus3p and Kss1p) signaling pathway. Pheromone stimulation causes cell cycle arrest. Therefore, inactivation of the Fus3p and Kss1p MAP kinases is required during recovery phase for the resumption of cell growth. We have isolated a novel protein tyrosine phosphatase gene, *PTP3*, as a negative regulator of this pathway. Ptp3p directly dephosphorylates and inactivates Fus3p MAP kinase in vitro. Multicopy *PTP3* represses pheromone-induced transcription and promotes recovery. In contrast, disruption of *PTP3* in combination with its homolog *PTP2* results in constitutive tyrosine phosphorylation, enhanced kinase activity of Fus3p MAP kinase on stimulation, and delayed recovery from the cell cycle arrest. Both tyrosine phosphorylation and kinase activity of Fus3p are further increased by disruption of *PTP3* and *PTP2* in combination with *MSG5*, which encodes a dual-specificity phosphatase. Cells deleted for all three of the phosphatases (*ptp2Δptp3Δmsg5Δ*) are hypersensitive to pheromone and exhibit a severe defect in recovery from pheromone-induced growth arrest. Our data indicate that Ptp3p is the major phosphatase responsible for tyrosine dephosphorylation of Fus3p to maintain a low basal activity; it also has important roles, along with Msg5p, in inactivation of Fus3p following pheromone stimulation. These data present the first evidence for a coordinated regulation of MAP kinase function through differential actions of protein tyrosine phosphatases and a dual-specificity phosphatase.

[Key Words: FUS3 MAP kinase; protein tyrosine phosphatase; pheromone response pathway; PTP2; PTP3; MSG5]

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Mitogen-activated protein kinase (MAPK) cascades are used widely in eukaryotic signal transduction (for review, see Blumer and Johnson 1994; Marshall 1994). The typical cascade consists of three distinct kinases. In response to a variety of extracellular stimuli, MAP kinase kinase kinase (MAPKKK/MEKK) activates MAP kinase kinase (MAPKK/MEK) via phosphorylation of serine and threonine residues on MEK (Alessi et al. 1994; Zheng and Guan 1994). MEK in turn activates MAPK through phosphorylation of the conserved threonine/tyrosine motif (TxY) on MAPK (for review, see Cobb and Goldsmith 1995). These three kinases (MEKK, MEK, and MAPK) compose an evolutionarily conserved eukaryotic signaling module that responds to various extracellular stimuli

and elicits a wide range of cellular responses. Examples include mitogenic signaling in mammalian cells, vulval development in *Caenorhabditis elegans* (Eisenmann and Kim 1994), and eye development in *Drosophila* (Dickson and Hafen 1994). In the budding yeast *Saccharomyces cerevisiae*, at least six MAPK pathways are known to regulate a variety of important physiological events, including mating pheromone response, cell wall integrity, osmosensing, pseudohyphal growth, invasive growth, and spore formation (for review, see Herskowitz 1995).

The *S. cerevisiae* mating pheromone response pathway is a well-characterized MAPK signal transduction pathway (for review, see Sprague and Thorner 1992; Herskowitz 1995). Haploid cells of one of the two mating types (**a** or **α**) secrete mating pheromone (**a**-factor or **α**-factor), which binds to its receptors on the surface of cells of the other mating type (**α** or **a**). The mating pheromone receptors are G protein-coupled receptors which,

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on binding of their corresponding ligands, promote dissociation of G_{α} from $G_{\beta\gamma}$. The unleashed $G_{\beta\gamma}$ complex propagates signals via Ste5p and Ste20p, leading to activation of a MAPK cascade that includes Ste11p (MEKK), Ste7p (MEK) (Errede et al. 1993; Zhou et al. 1993), and the Fus3p and Kss1p MAPK that have overlapping functions in mating pheromone response (Elion et al. 1991). Activation of this pathway results in morphological changes, transcriptional activation of pheromone-inducible genes, and G_1 -specific cell cycle arrest, which all prepare haploid cells for upcoming mating events that give rise to diploid cells. Two physiological targets of the Fus3p MAPK have been identified—Ste12p, a transcription factor that activates pheromone-inducible gene expression (Dolan et al. 1989; Errede and Ammerer 1989; Song et al. 1991; Elion et al. 1993); and Far1p, which mediates G_1 cell cycle arrest by inhibiting cyclin-dependent kinase Cdc28–Cln activity (Chang and Herskowitz 1990; Elion et al. 1993; Peter et al. 1993; Peter and Herskowitz 1994).

MAPK activation is a transient event that must be regulated tightly, even in the continuous presence of stimuli. Failure to inactivate MAPK leads to oncogenic transformation in higher eukaryotes (Cowley et al. 1994; Mansour et al. 1994). Constitutive activation of the osmosensing MAPK Hog1p is lethal in *S. cerevisiae* (Maeda et al. 1994). One way to regulate MAPK activity is through controlling the level of phosphorylation by protein phosphatases (for review, see Hunter 1995; Tonks and Neel 1996). Protein phosphatases represent a diverse family including protein serine/threonine phosphatases (PPases) and protein tyrosine phosphatases (PTPases), classified according to their substrate specificity. PTPases are often subcategorized into three groups: receptor PTPases, intracellular PTPases, and dual-specificity PTPases (for review, see Denu et al. 1996). The dual-specificity PTPases are unique in the ability to dephosphorylate not only phosphoserine/phosphothreonine but also phosphotyrosine residues. In mammalian cells, dual-specificity PTPases appear to have an important role in dephosphorylation of MAPKs. However, other phosphatases yet to be identified are also involved in MAPK regulation (for review, see Keyse 1995).

The *S. cerevisiae* mating pheromone pathway is a good representative of MAPK pathway, with well-characterized components of the signaling cascade following activation by mating pheromone. However, little is known about the other equally important side of the pathway—mechanisms of its negative regulation, including repressed pathway activity in the absence of mating pheromone, and recovery from pheromone-induced cell cycle arrest. These negative regulations are essential for normal cell growth and function. Mutations that lead to constitutive activation of the pheromone response pathway result in severe growth defects in *S. cerevisiae* (Miyajima et al. 1987; Stevenson et al. 1992; Brill et al. 1994). As a key molecule that connects upstream signal to downstream effectors, the Fus3p MAPK is a likely target for negative regulation. A dual-specificity phosphatase Msg5p has been shown to down-regulate the

pheromone-activated MAPK pathway by inactivating the Fus3p MAPK via dephosphorylation (Doi et al. 1994). However, cells in which *MSG5* has been deleted are still able to recover from pheromone treatment, suggesting that other gene products may also be involved in down-regulation of this pathway (Doi et al. 1994).

To isolate negative regulators of the pheromone response pathway and investigate the possible involvement of PTPase in regulation of Fus3p MAPK, we performed a genetic screen for dosage suppressors of *STE11-4*. *STE11-4* encodes a constitutively active form of the Ste11p kinase (MEKK), which leads to activation of the pheromone response pathway and results in G_1 arrest even in the absence of pheromone (Stevenson et al. 1992). In this paper, we describe the isolation of a novel phosphotyrosine-specific phosphatase gene, *PTP3*, that suppresses the *STE11-4* phenotypes when present on a multicopy plasmid. We demonstrate that Ptp3p negatively regulates the yeast mating pheromone response. It has critical roles in dephosphorylation, thereby inactivating both the basal and pheromone-activated Fus3p MAPK, whereas Msg5p functions to inactivate Fus3p following pheromone stimulation. These data provide the first evidence for a model of coregulation of MAPK function by a tyrosine phosphatase and a dual-specificity phosphatase, which exert distinct regulations through the same mechanism—dephosphorylation.

Results

Isolation of PTP3 as a multicopy suppressor of STE11-4

The MEK kinase (MEKK) of the *S. cerevisiae* mating pheromone response pathway is encoded by the *STE11* gene. *STE11-4*, a dominant allele of *STE11*, leads to constitutive activation of the pheromone response pathway—high level expression of pheromone responsive genes and cell cycle arrest in G_1 in the absence of pheromone (Stevenson et al. 1992). To isolate negative regulators of the MAPK cascade, we screened a yeast genomic library for multicopy suppressors of the *STE11-4* phenotypes. The yeast strain that we used (SY1866) has a chromosomal *STE11-4* allele, a chromosomal deletion of the *HIS3* marker gene, a pheromone-inducible *FUS1* gene promoter-driven *HIS3* gene integrated at the *FUS1* locus (*FUS1-HIS3*) (Stevenson et al. 1992), and pUZ4 plasmid, a *lacZ* reporter plasmid under the control of pheromone-responsive elements (*PRE-lacZ*) (Cairns et al. 1992). SY1866 was transformed with a multicopy genomic library (Nikawa et al. 1987). Transformants were replica-plated onto agar plates lacking histidine, and colonies that failed to grow on plates lacking histidine were selected. These were putative suppressors of *STE11-4* phenotype.

Among ~5000 transformants, 25 His⁻ colonies were isolated. Only three of them displayed plasmid-dependent suppression of the G_1 arrest phenotype (Fig. 1A,B). When reintroduced into SY1866 cells, these plasmids also resulted in decreased β -galactosidase activity from

Figure 1. Suppression of the constitutively active *STE11-4* mutant by multicopy *PTP3*. (A) Suppression of *FUS1*–*HIS3* expression by *PTP3* in SY1866 (*STE11-4 FUS1::HIS3*). SY1866 cells harboring a control vector or YEp13M4–*PTP3* (two independent transformants) were streaked onto synthetic complete (SC) agar plates lacking the indicated nutrients. (L) Leucine; (W) tryptophan; (H) histidine. L and W were for selection of *PTP3* and PRE–*lacZ* plasmids, respectively. AT (3-aminotriazole, 30 mM) was added to enhance the difference in *HIS3* expression. (B) Suppression of *G₁* cell cycle arrest of *STE11-4* cells (SY1866) by multicopy *PTP3*. Cell morphology of *STE11-4* cells carrying a control vector (left) or YEp13M4–*PTP3* (right) were examined by Normaski microscopy. Multicopy *PTP3* suppressed *G₁* arrest and decreased the percentage of cells with shmoo caused by the *STE11-4* allele. (C) Suppression of PRE–*LacZ* expression by multicopy *PTP2* or *PTP3*, but not *PTP1*. β -Galactosidase expression from PRE–*LacZ* (pUZ4) in the absence of α -factor was measured by β -galactosidase activity in *STE11-4* cells (SY1866) harboring a control vector YEp13 (column 1), YEp13–*PTP1* (column 2), YEp13–*PTP2* (column 3), YEp13M4–*PTP3* (column 4), or YEp13M4–*ptp3*^{C814G}, encoding a catalytically deficient mutant Ptp3p (column 5). Activity was calculated as $[\text{OD}_{420} \times 1000] / [600 \times \text{time}(\text{min}) \times \text{volume of culture used in the assay}(\text{ml})]$. The control activity (column 1) was 7.5 units and normalized to 100%. Each value is the mean activity measured from three independent transformants. (D) Predicted amino acids sequence alignment of *S. cerevisiae* Ptp3p, Ptp1p (Guan et al. 1991), and Ptp2p (Guan et al. 1992). The PILEUP program of Wisconsin Genetics Computation Group was used. Conserved residues are highlighted. The catalytically essential and conserved cysteine in all PTPases is indicated by an asterisk.

the PRE–*lacZ* reporter gene (data not shown; see also Fig. 1C). All three plasmids gave identical restriction digestion patterns, and one of them was characterized in this study.

Deletion analysis identified a 5.5-kb segment of the insert to be responsible for suppression of the *STE11-4* phenotypes (data not shown). Sequencing of the entire region revealed an open reading frame (ORF) encoding a polypeptide with 975 amino acid residues, which shares significant sequence similarity to the two known yeast protein tyrosine phosphatases, Ptp1p (Guan et al. 1991)

and Ptp2p (Guan et al. 1992; James et al. 1992; Ota and Varshavsky 1992). Therefore, the suppressor gene was named *PTP3*. The predicted Ptp3p amino acid sequence shows highest similarity to Ptp2p in both the carboxy-terminal PTPase catalytic domain and the amino-terminal domain (Fig. 1D).

Multicopy PTP2 or PTP3 results in decreased transcription from pheromone-inducible genes

Pheromone-inducible gene transcription was measured

with a reporter plasmid carrying a PRE-*lacZ* fusion gene (pUZ4). SY1866 cells (*STE11-4*) with this plasmid exhibited high β -galactosidase activity in the absence of α -factor (Fig. 1C, column 1). Transformation of *PTP3* gene carried on a multicopy plasmid resulted in ~80% reduction of the β -galactosidase activity in SY1866 (Fig. 1C, column 4). To test whether the phosphatase activity of Ptp3p is required for its suppression of *STE11-4*, the codon for the conserved active site cysteine residue (Cys814) in *PTP3*, which is absolutely required for catalysis in all PTPases (Denu et al. 1996), was mutated to encode glycine (C814G). Multicopy *ptp3*^{C814G} was much less effective in down-regulating the β -galactosidase expression, suggesting that the catalytic activity of Ptp3p is required for the suppression (Fig. 1C, column 5). The slight reduction of β -galactosidase activity in cells carrying *ptp3*^{C814G} could be attributable to sequestration of Fus3p by the mutant Ptp3p, through formation of a non-dissociating complex between the mutant phosphatase and its substrate Fus3p. Although similar observations have also been made with other inactive phosphatase mutants (Sun et al. 1993; Hannig et al. 1994), further evidence is needed for this hypothesis. The similarity between *PTP2* and *PTP3* suggests functional overlap between two PTPases. Consistent with this hypothesis, expression of multicopy *PTP2* also reduced the β -galacto-

sidase activity from the PRE-*lacZ* fusion gene in *STE11-4* cells (Fig. 1C, column 3), whereas *PTP1* expression had no effect (Fig. 1C, column 2). The inhibitory effect of multicopy *PTP2* or *PTP3* on the pheromone-responsive elements suggests that *PTP2* and *PTP3* regulate the pheromone response pathway negatively.

The role of *PTP2* and *PTP3* in cellular recovery after pheromone stimulation

To further test the role of *PTP* genes in pheromone response, isogenic yeast strains with single, double, and triple disruptions of *PTP1*, *PTP2*, and *PTP3* were generated. Single and combined disruptions had no defect on growth and displayed normal mating efficiency (data not shown), suggesting that *PTP1*, *PTP2*, and *PTP3* are not required for the initial response to pheromone. The pheromone-induced G₁ arrest and recovery were then examined by halo assays (Sprague 1991). All disruptants had a similar sensitivity to pheromone as judged by halo sizes (Fig. 2A, *ptp1* Δ cells were not shown). However, microscopic examination of the halo zones showed that *ptp2* Δ *ptp3* Δ cells had a slight defect in recovery (Fig. 2B). Wild-type cells were able to form microcolonies within the halos (Fig. 2B, upper left). In contrast, no microcolo-

Figure 2. Involvement of *PTP2* and *PTP3* in recovery from pheromone stimulated arrest. All assays were performed on *MATa* cells with the indicated genotypes. (A) The α -factor halo assay. Approximately 10^5 cells of each of the following strains, wild-type T (Y568), *ptp2* Δ (Y572), *ptp3* Δ (Y567), or *ptp2* Δ *ptp3* Δ (Y578), were mixed with melted soft top agar and plated on YPD agar plates. The amount of α -factor spotted on the filter disks are as follows. (Center) 0 μ g; (from top left, clockwise) 1, 2, 10, and 20 μ g. Photos were taken after 2-day incubation at 30°C. (B) Deletions of *PTP2* and *PTP3* affect microcolony formation in halos. The halo assay was performed as described in A. Microcolonies formed around the discs containing 20 μ g α -factor were visualized under microscope. (C) Deletions of *PTP2* and *PTP3* affect the recovery of cells from pheromone-induced arrest. Approximately 10^6 wild-type or *ptp2* Δ *ptp3* Δ cells from logarithmic-phase cultures in YPD medium were treated with α -factor for 3 hr and the arrested cells were resuspended in fresh medium without α -factor. Relative colony formation unit (CFU), which is the ratio of cell number at different time points versus cell number at the 0 time point. The data are representative of three independent experiments. (D) Effect of multicopy *PTP3* on the recovery of wild-type cells. Cells carrying control vector YEpl3, YEpl3-*PTP2*, YEpl3M4-*PTP3*, or YEpl3M4-*ptp3*^{C814G} were plated on SC-Leu soft agar and halo assays were performed with the indicated amounts of α -factor. Photos were taken after 2-day incubation at 30°C. Experiments were repeated using independent transformants.

nies were observed in the halos formed on the *ptp2Δ ptp3Δ* double disruption cells (Fig. 2B, lower right). This defect was observed in *ptp2Δptp3Δ* double disruption cells and *ptp1Δ ptp2Δptp3Δ* triple disruption cells only, suggesting functional redundancy between *PTP2* and *PTP3*.

Recovery was also assayed by monitoring the increase in colony-forming units (CFU) after cells were released from pheromone arrest (Moser et al. 1996). The rate of relative CFU increase for *ptp2Δptp3Δ* cells was reproducibly lower than that of the wild-type cells (Fig. 2C). This slower increase of CFU was not attributable to a slower growth rate of *ptp2Δptp3Δ* cells because the same growth rate was observed for wild-type or *ptp2Δptp3Δ* cells without pheromone pretreatment (data not shown).

The effect of *PTP2* or *PTP3* overexpression on the recovery process of wild-type cells was also examined by the halo assay. Wild-type cells (Y568) carrying the vector alone, multicopy *PTP2*, *PTP3*, or *ptp3^{C814G}* gene formed similarly sized halos. However, the halos formed on lawns of cells carrying multicopy *PTP3* filled in more rapidly than those on lawns of wild-type cells carrying the control vector or multicopy *PTP2* (Fig. 2D). Furthermore, the ability of *PTP3* to promote recovery required the tyrosine phosphatase activity. The rapid filling in of the halos was not observed on lawns of cells containing multicopy *ptp3^{C814G}*, which encodes the catalytically inactive Ptp3p (Fig. 2D). These results demonstrate a role of *PTP3* in the recovery phase of the pheromone response without exhibiting a measurable effect in the initial response.

Ptp2p and Ptp3p down-regulate the Fus3p MAPK activity in vivo

The fact that *PTP3* was isolated as a multicopy suppressor of *STE11-4* leads to the hypothesis that it functions downstream of *STE11* in the pheromone response pathway. In addition, Ptp3p is a phosphotyrosine-specific phosphatase as shown in Figure 4A, below. We have also shown that the negative regulation of *PTP3* on pheromone-inducible gene expression (*PRE-lacZ*) requires its tyrosine phosphatase activity (Fig. 1C). These observations suggest that Ptp3p may directly dephosphorylate tyrosine-phosphorylated components downstream of *STE11-4*. The *FUS3* and *KSS1* MAPKs (Elion et al. 1990) are the only downstream components known to require tyrosine phosphorylation for activation in the pheromone response pathway (Gartner et al. 1992; Ma et al. 1995).

To test whether *PTP3* regulates *FUS3*, the Fus3p MAPK activity was determined in wild-type *ptp2Δ*, *ptp3Δ*, or *ptp2Δptp3Δ* cells during pheromone stimulation. Fus3p was tagged with a influenza virus HA epitope at the amino terminus. The tagged *FUS3* is fully functional, as confirmed by complementation of the sterile phenotype of *fus3Δkss1Δ* cells by *HA-FUS3* (data not shown). The HA-Fus3p protein was immunoprecipitated from whole-cell extracts and the Fus3p MAPK activity was assayed using myelin basic protein (MBP) as an in

vitro substrate, in the presence of [γ -³²P]ATP. In wild-type cells, pheromone treatment resulted in activation of Fus3p kinase activity (Fig. 3A, top, lanes 3,4). The kinase activity detected was specific to HA-Fus3p, because little activity was detected from extracts of cells expressing either untagged Fus3p or an HA-tagged Fus3p mutant Y182F (Fig. 3A, top, lanes 1,2,5,6). *ptp2Δ* had no effect on HA-Fus3p kinase activity (Fig. 3A, top, lanes 7,8). In contrast, disruption of *PTP3* resulted in a twofold increase of pheromone-induced Fus3p activity (Fig. 3A, top, lane 10). Double disruption of *PTP2* and *PTP3* gave rise to a threefold increase of Fus3p kinase activity (Fig. 3A, top, lanes 11,12). When the pheromone-induced tyrosine phosphorylation of Fus3p was examined by anti-phosphotyrosine (anti-Pi-Tyr) immunoblots, similar levels of induced tyrosine phosphorylation were observed in wild-type and *ptp2Δ* cells (Fig. 3A lanes 1–8, middle); whereas a dramatic increase in basal tyrosine phosphorylation (in the absence of pheromone) of Fus3p was observed in *ptp3Δ* cells (Fig. 3A, middle, lanes 9,10). Basal and pheromone-induced tyrosine phosphorylation levels were further increased in *ptp2Δptp3Δ* cells (Fig. 3A, middle, lanes 11,12). Fus3p, like all MAPK, requires phosphorylation at a particular tyrosine residue in the conserved TxY motif (Tyr 182) for activation and signaling (Gartner et al. 1992). When Tyr182 was replaced by a phenylalanine (Y182F), no kinase activity of Fus3p was observed in either wild-type or *ptp2Δptp3Δ* cells (Fig. 3A, top, lanes 5,6,13,14). Furthermore, the induced tyrosine phosphorylation of Fus3p in wild-type, and both basal and induced tyrosine phosphorylation of Fus3p in *ptp2Δptp3Δ* cells were eliminated by this mutation (Fig. 3A, middle). Anti-HA immunoblots were performed to ensure that equal amounts of HA-Fus3p were used in all samples in the above experiments (Fig. 3A, bottom).

The effect of *ptp2Δptp3Δ* disruption on Fus3p activity was further analyzed by comparing the time course of kinase activation during pheromone treatment of wild-type and *ptp2Δptp3Δ* cells (Fig. 3B, top). When wild-type cells were stimulated with α -factor, HA-Fus3p kinase activity increased within 15 min, peaked at 1 hr, and declined to the prestimulated level after 4 hr of stimulation (Fig. 3B, top left, for quantitation, see C). In contrast, activation of Fus3p kinase activity was much more rapid in *ptp2Δ ptp3Δ* cells (Fig. 3B, top right), reaching a higher maximum activity within 15 min and was sustained for at least 1 hr; furthermore, Fus3p activity declined to a level higher than the pre-stimulated level after 4 hr of stimulation (Fig. 3C). Pheromone-induced tyrosine phosphorylation was also examined in parallel by anti-Pi-Tyr immunoblots. In wild-type cells, the time course of tyrosine phosphorylation was concurrent with the kinase activities. In contrast, the tyrosine phosphorylation of Fus3p was readily detectable even without pheromone stimulation in *ptp2Δptp3Δ* cells. The tyrosine phosphorylation level was increased further by pheromone stimulation, then declined in 4 hr to a detectable level comparable with the prestimulated level (Fig. 3B, middle). These data demonstrate that *PTP3* regulates Fus3p activity by regulating phosphorylation of

type (Y568, lanes 1,2), *ptp2Δ* (Y572, lanes 3,4), *ptp3Δ* (Y567, lanes 5,6) and *ptp2Δptp3Δ* (Y578, lanes 7,8) cells carrying vector (lanes 1,3,5,7) or HA-epitope-tagged CDC28 (pSF19, lanes 2,4,6,8) were harvested at logarithmic-phase. Cdc28p-HA was immunoprecipitated and histone H1 kinase activity was determined (bottom). The activities were normalized to the activity in the wild-type cells carrying the vector (lane 1). Cdc28p-HA protein levels in the immunoprecipitates were determined by anti-HA immunoblots (top).

Fus3p, presumably at Tyr182 in the TxY motif. Interestingly, tyrosine dephosphorylation and deactivation of Fus3p still occurred in *ptp2Δptp3Δ* cells, suggesting the existence of additional deactivation mechanisms (see below).

To distinguish whether the effect of *ptp3Δ* and *ptp2Δptp3Δ* disruptions on Fus3p kinase activity was specific or attributable to a global effect on kinases, the Cdc28p kinase activity was examined in wild-type, *ptp2Δ*, *ptp3Δ*, or *ptp2Δptp3Δ* cells. A functional HA-tagged Cdc28p (Sorger and Murray 1992) was immunoprecipitated from exponentially growing cells and kinase activities were determined. Cdc28p kinase activity was not affected by the disruptions of *PTP2* and *PTP3* (Fig. 3D). This result suggests that disruptions of *PTP2* and *PTP3* have no general effects on protein kinase activities.

Figure 3. The kinase activities and tyrosine phosphorylation levels of Fus3p are elevated in *ptp3Δ* and *ptp2Δptp3Δ* cells. (A) Enhanced Fus3p kinase activity and tyrosine phosphorylation in *ptp3Δ* single (Y567) and *ptp2Δ ptp3Δ* double-disruption cells (Y578). Logarithmic-phase cells expressing FUS3 (p414ADH-FUS3, lanes 1,2), HA-epitope-tagged FUS3 (p414ADH-HAFUS3, lanes 3,4,7-12) or HA-tagged fus3^{Y182F} (HA-fus3Y182F, lanes 5,6,13,14) were stimulated with 2.5 μM α-factor for 30 min. HA-Fus3p was immunoprecipitated with anti-HA mAb and its kinase activities were determined by in vitro MBP kinase assays (top). The MBP phosphorylation levels were quantitated with a PhosphorImager (Molecular Dynamics) and normalized to the unstimulated wild-type cells (lane 3). In parallel, anti-Pi-Tyr and anti-HA immunoblots were performed on the same immunoprecipitated samples to determine tyrosine phosphorylation levels (middle), and HA-Fus3p or HA-fus3p^{Y182F} protein levels (bottom). *IgG(H) denotes the immunoglobulin heavy chain. (B) Time course of pheromone-induced Fus3p kinase activity in wild-type cells (Y568) or *ptp2Δ ptp3Δ* cells (Y578). Logarithmic cells expressing FUS3 (lane 1,2) and HA-FUS3 (lanes 3-8) were stimulated with 2.5 μM α-factor for the indicated times. Immunoprecipitations, in vitro MBP kinase assays (top), anti-Pi-Tyr (middle), and anti-HA immunoblots (bottom) were performed as described in A. (C) The Fus3p kinase activities from B were quantitated and normalized to the activity in the unstimulated wild-type cells (lane 3 in B). Relative kinase activities were plotted against the time of pheromone stimulation. (▲) Activity in wild-type; (□) Activity in *ptp2Δptp3Δ*. (D) Cdc28p kinase activity is not affected by *ptp2Δ* and *ptp3Δ* disruption. Wild-

Ptp3p dephosphorylates phosphotyrosine residues and inactivates in vitro

The predicted catalytic domain of Ptp3p was purified as a glutathione S-transferase (GST) fusion protein (GST-Ptp3p). The fusion protein dephosphorylated *p*-nitrophenyl phosphate and tyrosine phosphorylated Raytide (data not shown). Furthermore, it dephosphorylated specifically in vitro Tyr-phosphorylated casein by v-Src, but not casein-phosphorylated on Ser/Thr by protein kinase A (Fig. 4A). In addition, the phosphatase activity was inhibited by sodium vanadate, a known PTPase inhibitor (data not shown). These results indicate that Ptp3p possesses phosphotyrosine-specific phosphatase activity.

Direct dephosphorylation of Fus3p by Ptp3p was examined in vitro. The entire coding region of FUS3 was

Figure 4. Ptp3p dephosphorylates and inactivates Fus3p in vitro. (A) Phosphotyrosine-specific phosphatase activity of Ptp3p. Casein was phosphorylated in vitro on tyrosine residues by ν -Src ($\square$), or on serine residues by protein kinase A (PKA) ($\blacklozenge$). Equal amount of Tyr- or Ser-phosphorylated casein was incubated with GST-Ptp3p for 30 min at 37°C and the amount of ^{32}P -phosphate released was determined by liquid scintillation counting. ($\square$) ^{32}P released from Tyr-phosphorylated casein; ($\blacklozenge$) ^{32}P released from Ser-phosphorylated casein. (B) Tyrosine-specific dephosphorylation of Fus3p by Ptp3p in vitro. In vitro ^{32}P -labeled Fus3p by autophosphorylation was incubated for 30 min with buffer (lane 1), the indicated amounts of Ptp3p (lanes 2,3), Ptp3p in the presence of vanadate (lane 4), or GST (lane 5). Half of the samples were subjected to SDS-PAGE and autoradiography (top). The phosphorylation levels of Fus3p was quantitated and normalized to the control (lane 1). The other half of samples were subjected to anti-Pi-Tyr immunoblotting (middle). This same membrane was stripped and reprobed with anti-Fus3p antibody to determine the Fus3p protein levels (bottom). (C) Inactivation of Fus3p kinase activity by Ptp3p. In vitro autophosphorylated Fus3p was treated the same as in B. The Fus3p kinase activity was then determined with MBP kinase assay and normalized to the control (lane 1).

fused to GST and GST-Fus3p was purified from *Escherichia coli*. Autophosphorylated and partially activated Fus3p was obtained by incubating GST-Fus3p with [γ - ^{32}P]ATP (Errede et al. 1993; Doi et al. 1994; and data not shown). When the in vitro-activated GST-Fus3p was

incubated with recombinant GST-Ptp3p, which contains the catalytic domain of Ptp3p, significant dephosphorylation of GST-Fus3p was observed. Dephosphorylation by GST-Ptp3p was inhibited by vanadate, and no dephosphorylation was observed when GST was used as a control (Fig. 4B, top). The incomplete dephosphorylation of GST-Fus3p by GST-Ptp3p is likely attributable to the fact that the autophosphorylated Fus3p also contains phosphoserine/phosphothreonine (Errede et al. 1993; Doi et al. 1994; and data not shown). The tyrosine dephosphorylation of GST-Fus3p by GST-Ptp3p was confirmed further by anti-Pi-Tyr immunoblots (Fig. 4B, middle) and phospho-amino acid analysis (data not shown). Anti-Fus3p immunoblot was also performed to ensure equal amount of GST-Fus3p was used in the above experiments. In vitro MBP kinase assays demonstrated that GST-Fus3p was inactivated by incubation with GST-Ptp3p, and that the inactivation was prevented by vanadate (Fig. 4C). GST had no effect on Fus3p activity (Fig. 4C, lane 5), and GST-Ptp3p does not directly dephosphorylate MBP (data not shown). In addition, GST-Ptp3p treatment also inactivated Fus3p immunoprecipitated from pheromone stimulated cells (data not shown). Together, these data indicate that Ptp3p can directly dephosphorylate Fus3p on phosphotyrosine residues and inactivate its kinase activity.

ptp2 Δ ptp3 Δ msg5 Δ triple disruption cells have increased sensitivity to pheromone and a deficiency in recovery

MSG5, which encodes an inducible dual-specificity PT-Pase, has been shown to negatively regulate Fus3p activity (Doi et al. 1994). Our data with *PTP2* and *PTP3* raised the possibility that these three PTPase genes may cooperate in the regulation of Fus3p. To test this hypothesis, the *MSG5* gene was disrupted either singly or in combination with *PTP2* and *PTP3* disruptions, and pheromone response of the resulting strains was examined. Sensitivity to pheromone of these strains was determined by halo assay. The halos formed on *ptp2 Δ ptp3 Δ msg5 Δ* cells were significantly larger than those of the other strains (Fig. 5A,B) suggesting increased sensitivity of *ptp2 Δ ptp3 Δ msg5 Δ* cells toward pheromone.

The ability of a single cell to recover from a uniform concentration of pheromone was measured by a plate assay (Moser et al. 1996). An equal number of cells (~300–500) were plated on YPD plates containing various concentrations of α -factor and cell viability was assessed by colony formation (Fig. 5C,D). *ptp2 Δ ptp3 Δ* double-disruption cells and *msg5 Δ* single-disruption cells had a modest defect in recovery, whereas *ptp2 Δ ptp3 Δ msg5 Δ* triple disruption cells had a severe defect, compared with wild-type (Fig. 5C). Taken together, the *ptp2 Δ ptp3 Δ msg5 Δ* triple disruption increased sensitivity toward pheromone and resulted in a deficiency in recovery.

Synergistic effects of ptp2 Δ , ptp3 Δ , and msg5 Δ on Fus3p kinase activity

The defect of *ptp2 Δ ptp3 Δ msg5 Δ* cells could be attribut-

Figure 5. *ptp2Δptp3Δmsg5Δ* triple-disruption cells have increased sensitivity to pheromone induced arrest and display a defect in recovery. All assays were performed on MATa cells with the indicated genotypes. (A) Halo assays were performed on wild-type (Y568), *ptp2Δptp3Δ* (Y578), *msg5Δ* (Y170), or *ptp2Δptp3Δmsg5Δ* (Y579) cells, as described in Fig. 2A. The amount of α -factor spotted on the filter discs are as follows. (Center) 0 μ g; (from top left, clockwise) 1, 2, 5, and 10 μ g. Photos were taken after 2-day incubation at 30°C except for the *ptp2Δptp3Δmsg5Δ* cells, which were incubated for 5 days. (B) The relationship between halo sizes and pheromone dosage. The halo sizes from A were measured in triplicate as the distance from the edge of the filter disc to the edge of the halo. Mean values from three independent experiments were plotted against the log of α -factor dosages applied on the filter disks. ($\blacktriangle$ Wild-type; ($\square$) *ptp2Δptp3Δ*; ($\triangle$) *msg5Δ*; ($\bullet$) *ptp2Δptp3Δmsg5Δ*). (C) Plate assay for recovery after exposure to pheromone treatment. Same number of wild-type (Y568), *ptp2Δptp3Δ* (Y578), *msg5Δ* (Y170), or *ptp2Δptp3Δmsg5Δ* (Y173) cells from logarithmic-phase cultures were plated on YPD plates containing different concentrations of α -factor. Photos were taken after the majority of the colonies reached similar sizes. (D) Relative colony formation unit (CFU) of wild-type and *ptp2Δptp3Δmsg5Δ* cells. CFU from C was determined and normalized to the CFU on plates containing no pheromone. Mean values of two independent experiments were plotted against the log of pheromone concentration on the plates. ($\blacktriangle$ Wild-type; ($\bullet$) *ptp2Δptp3Δmsg5Δ*).

able to failure to inactivate Fus3p MAPK. To test this hypothesis, Fus3p kinase activity was examined in *ptp2Δptp3Δmsg5Δ* cells by immunoprecipitations and MBP kinase assays, as described previously (Fig. 6A, top). The pheromone-stimulated activity in either *ptp2Δptp3Δ* double- (Fig. 6A, top, lanes 5,6) or *msg5Δ* single-disruption cells (Fig. 6A, top, lanes 7,8) was three-fold higher than the stimulated activity in wild type (Fig. 6A, top, lanes 1–4), although in *ptp2Δptp3Δmsg5Δ* cells, the pheromone-stimulated Fus3p activity increased 15-fold. Tyrosine phosphorylation levels of Fus3p were compared in parallel (Fig. 6A, middle). The *msg5Δ* single disruption did not cause any change of the basal tyrosine phosphorylation, and the pheromone-induced tyrosine phosphorylation level was slightly higher than that of wild-type cells. In contrast, the *ptp2Δptp3Δ* disruption caused an increase in both basal and induced tyrosine phosphorylation levels, and the triple disruption resulted in further enhancement of tyrosine phosphorylation.

Next, the time course of Fus3p kinase activation following pheromone stimulation was investigated (Fig. 6B, for quantitation, see C). Like *ptp2Δptp3Δ* cells (Fig. 3B), *msg5Δ* cells also displayed increased Fus3p kinase activity, whereas *ptp2Δptp3Δmsg5Δ* triple disruption further

enhanced Fus3p activity. Concomitantly, a similar trend was observed in the Fus3p tyrosine phosphorylation levels in these cells. The induced tyrosine phosphorylation was slightly enhanced in *msg5Δ* cells and was of longer duration than in the wild-type cells (Fig. 3B, middle, cf. lanes 2–5 with lanes 6–10). In contrast, triple disruption resulted in higher basal as well as pheromone-stimulated tyrosine phosphorylation levels. The synergistic effects of *ptp2Δptp3Δmsg5Δ* triple disruption on Fus3p kinase activity and tyrosine phosphorylation indicate that the three PTPases—Ptp2p, Ptp3p, and Msg5p—are all involved in the regulation of Fus3p MAPK.

Discussion

Cooperation of Ptp2p, Ptp3p, and Msg5p in regulation of Fus3p MAPK

Activation and inactivation of MAPKs are both necessary for the regulated responses of cells to a variety of extracellular stimuli. In *S. cerevisiae*, mating pheromone stimulation induces specific gene expression and cell cycle arrest at the G₁ stage to prepare cells for subse-

Figure 6. Enhancement of Fus3p kinase activity in *ptp2Δptp3Δmsg5Δ* triple-disruption cells. (A) Fus3p kinase activity and tyrosine phosphorylation in wild-type (Y568), *ptp2Δptp3Δ* double-disruption (Y578), *msg5Δ* (Y170), or *ptp2Δptp3Δmsg5Δ* triple-disruption (Y173) cells. Logarithmic cells with the indicated genotypes carrying *FUS3* (lanes 1,2) or *HA-FUS3* (lanes 3–10) were stimulated with 2.5 μM α-factor for 30 min. Immunoprecipitation with anti-HA mAb, in vitro MBP kinase assays (top), anti-Pi-Tyr (middle) and anti-HA immunoblots (bottom) were performed as described in Fig. 3A. (B) Time course of Fus3p kinase activity and tyrosine phosphorylation in wild-type (Y568), *msg5Δ* (Y170), or *ptp2Δptp3Δmsg5Δ* triple-disruption (Y173) cells. Cells were stimulated with 2.5 μM α-factor for the indicated times. Immunoprecipitations, in vitro MBP kinase assays (top), anti-Pi-Tyr (middle) and anti-HA immunoblots (bottom) were performed as described in Fig. 3A. (C) The kinase activity from Fig. 6B was quantitated and normalized to the activity in unstimulated wild-type T cells carrying HA-Fus3p (top, lane 2, in B). Relative activities were plotted against time of pheromone stimulation. (▲) Activity in wild-type; (△) activity in *msg5Δ*; (●) activity in *ptp2Δptp3Δmsg5Δ*.

quent mating. After prolonged stimulation, cells recover from G₁ arrest, whether or not mating actually occurs. The recovery involves down-regulation of the MAPK cascade. In this study, we characterized a new protein, phosphotyrosine-specific phosphatase Ptp3p, which negatively regulates the Fus3p MAPK cascade. We demonstrated that Ptp3p has an important role in maintaining a low basal activity of Fus3p in the absence of pheromone and in down-regulating Fus3p MAPK during recovery from pheromone stimulation. We also present data suggesting that Ptp2p, a PTPase identified previously, has a partially overlapping function with Ptp3p. Double disruption of *PTP2* and *PTP3* results in a slight defect in recovery. Furthermore, both tyrosine phosphorylation and kinase activity of Fus3p are enhanced in *ptp2Δptp3Δ* cells. Interestingly, a significant decrease in Fus3p activity still occurred in *ptp2Δptp3Δ* cells after 2 hr of pheromone stimulation. Two possible mechanisms may explain this observation. First, it is known that Fus3p can phosphorylate and inhibit Ste7p (Gartner et al. 1992; Errede et al. 1993). Second, *MSG5*, which encodes a dual-specificity phosphatase, is induced by pheromone and can dephosphorylate Fus3p (Doi et al. 1994). Therefore, the faster and stronger activation of Fus3p in *ptp2Δptp3Δ* cells may enhance the induction of *MSG5* and/or the feedback inhibition of Ste7p, which results in a more rapid deactivation of Fus3p. Our genetic data clearly support the model that Fus3p is regulated coordinately by

PTP2, *PTP3*, and *MSG5*. Triple disruption of *PTP2*, *PTP3*, and *MSG5* dramatically increases the sensitivity of cells to pheromone and results in a pronounced defect in recovery. Consistently, the Fus3p activity is enhanced greatly and inactivation is impaired significantly in *ptp2Δptp3Δmsg5Δ* cells.

Although *PTP2*, *PTP3*, and *MSG5* are all involved in dephosphorylation of Fus3p, they have distinct roles under different conditions. Ptp3p is required for the basal Tyr-dephosphorylation of Fus3p because Fus3p is constitutively phosphorylated on the critical tyrosine residue (Tyr182) in *ptp3Δ* and *ptp2Δptp3Δ* cells. Ptp3p is clearly more important than Ptp2p in regulation of Fus3p because Fus3p is not constitutively phosphorylated in *ptp2Δ* cells. Basal tyrosine phosphorylation of Fus3p was not increased in *msg5Δ* cells. However, tyrosine phosphorylation and kinase activity of Fus3p are prolonged in *msg5Δ* cells on pheromone stimulation. It is worth noting that *MSG5* mRNA level is induced by pheromone (Doi et al. 1994), whereas *PTP2* and *PTP3* are not (data not shown). Based on these observations, we propose that *PTP3*, and to a less extent, *PTP2*, have critical roles in dephosphorylating Fus3p and keeping pheromone response pathway at a low basal activity in the absence of pheromone. They also facilitate recovery by inactivating Fus3p following pheromone stimulation. We propose that *Msg5p* primarily participates in the inactivation of Fus3p following pheromone stimulation. Consistent

with this model, Fus3p in *ptp2Δptp3Δ* cells shows a more rapid activation kinetics than wild type because the tyrosine is constitutively phosphorylated. *MSG5* disruption does not alter the activation kinetics. Therefore, Fus3p MAPK is coordinately regulated by phosphotyrosine specific Ptp2p and Ptp3p, and a pheromone-inducible dual-specific *Msg5p* in the pheromone signal transduction pathway, giving the first example of co-regulation of kinase activity by tyrosine-specific phosphatase and dual-specificity phosphatase (Fig. 7).

Regulation of MAPK by phosphotyrosine-specific and dual-specific phosphatases

PTP2 is one of the major phosphatases for the inactivation of the Hog1p MAPK in the high osmolarity response pathway, whereas *PTP3* has a limited role in this pathway (Maeda et al. 1994; Wurgler-Murphy et al. 1997). Interestingly, the relative contribution of *PTP2* and *PTP3* to regulation of Fus3p kinase is reversed, in that *PTP3* is more important than *PTP2* in the Fus3p regulation. It is reasonable to conclude that *PTP2* and *PTP3* have overlapping, as well as distinct physiological functions. Two phosphotyrosine-specific phosphatases, pyp1 and pyp2 in *Schizosaccharomyces pombe*, have been demonstrated to regulate the *spc1/styl* MAPK in osmolarity and cell cycle regulation (Millar et al. 1995; Shiozaki and Russell 1995). pyp1 and pyp2 appear to be the major, if not the sole phosphatases, responsible for *spc1/styl* MAPK inactivation. Unlike the regulation of *spc1/styl* and Hog1p MAPKs, where only phosphotyrosine-specific phosphatases predominate, phosphotyrosine phosphatases (Ptp2p and Ptp3p) as well as an inducible dual-spe-

cific phosphatase (*Msg5p*) are important for the regulation of Fus3p MAPK in *S. cerevisiae*. These three phosphatases make differentiated contributions to the overall regulation of Fus3p MAPK.

Similar mechanisms may be operating in higher eukaryotes. In mammalian cells, a family of inducible dual-specificity phosphatases that are related to *Msg5p* have been implicated in the inactivation of MAPK. However, several observations suggest that additional regulators exist (Sun and Tonks 1994; Keyse 1995). For example, a vanadate-sensitive tyrosine phosphatase has been implicated in ERK1 and ERK2 inactivation (Alessi et al. 1995). Because MAPKs require both Thr and Tyr phosphorylation to be fully active, dephosphorylation of either Thr or Tyr is sufficient for inactivation (for review, see Cobb and Goldsmith 1995). The existence of multiple inactivation mechanisms might be necessary for the complex regulation of MAPKs. Sustained ERK activation correlates with nuclear translocation, whereas during transient ERK activation, the kinase remains in the cytoplasm (Chen et al. 1992). Interestingly, the inducible dual-specificity phosphatases are localized primarily in the nucleus (Keyse 1995). What turns off the active MAPKs in the cytoplasm? Although cytoplasmic PT-Pases responsible for ERK1 and ERK2 inactivation in mammalian cells have not yet been identified, Ptp2p and Ptp3p may be prototypes for this family of MAPK regulators. The yeast pheromone response pathway continues to be an excellent system to explore the complexities of MAPK regulation.

Materials and methods

Strains and yeast genetic methods

S. cerevisiae strains used in this study are listed in Table 1. Standard methods were used for yeast genetic manipulations (Rose et al. 1990; Guthrie and Fink 1991). Gene replacements were performed to disrupt *PTP1*, *PTP2*, and *PTP3* in isogenic strains. *MSG5* was disrupted by PCR-based method (Wach et al. 1994). Southern blots, PCR, and tetrad dissection analysis were performed to verify all gene disruptions.

Genetic screen for multicopy suppressors of STE11-4

To isolate yeast genomic sequences that suppress the effect of the *STE11-4* mutant, the reporter plasmid pUZ4 (Cairns et al. 1992) was transformed into SY1866. SY1866 + pUZ4 was then transformed with 5 μ g multicopy yeast genomic library in YEpl3M4 (2 μ /LEU2; Nikawa et al. 1987) and plated onto SC-Leu-Trp plates. Transformants were replica-plated to SC-Leu-Trp-His + 40 mM 3-amino-1,2,4-triazole plates. His⁻ transformants were identified and inoculated into SC-Leu-Trp liquid medium and assayed for β -galactosidase activity. Library plasmids were recovered from His⁻ transformants and suppression of the *STE11-4* phenotypes was retested by re-transformation. The GenBank accession number for the *PTP3* sequence reported in this paper is AF006304.

β -Galactosidase assays and microscopy

β -Galactosidase activity of cells bearing pUZ4 was assayed as described (Dolan and Fields 1990). The activity was calculated

Figure 7. Model for the regulation of Fus3p MAPK by Ptp3p, Ptp2p, and *Msg5p* in the mating pheromone response pathway. The Fus3p MAPK is regulated by the coordinated actions of tyrosine specific PTPases Ptp3p, Ptp2p and dual-specificity phosphatase *Msg5p*. Ptp3p has more important roles in the regulation of Fus3p Tyr-phosphorylation than Ptp2p does. Ptp3p and Ptp2p regulate tyrosine phosphorylation levels of Fus3p both in the absence and following pheromone stimulation; whereas *Msg5p* is only induced following pheromone stimulation.

Table 1. *S. cerevisiae* strains used in this work

Strains	Genotype	Reference/Source
Y568	<i>MATa trp1 his3 leu2 ura3</i>	Lab stock
Y258	<i>MATa trp1 his3 leu2 ura3 ptp1Δ::URA3</i>	Guan et al. (1991)
Y572	<i>MATa trp1 his3 leu2 ura3 ptp2Δ::LEU2</i>	Guan et al. (1992)
Y567	<i>MATa trp1 his3 leu2 ura3 ptp3Δ::URA3</i>	this study
Y578	<i>MATa trp1 his3 leu2 ura3 ptp2Δ::LEU2 ptp3Δ::URA3</i>	this study
Y680	<i>MATa trp1 his3 leu2 ura3 ptp1Δ::URA3 ptp2Δ::LEU2 ptp3Δ::URA3</i>	this study
Y170	<i>MATa trp1 his3 leu2 ura3 msg5Δ::kan^r</i>	this study
Y173	<i>MATa trp1 his3 leu2 ura3 msg5Δ::kan^r ptp2Δ::LEU2 ptp3Δ::URA3</i>	this study
SY1866	<i>MATa STE11-4 fus1::FUS1::HIS3 leu2 ura3 trp1 his3Δ200::ura3 pep4Δ::ura3 can1</i>	Stevenson et al. (1992)

as $[\text{OD}_{420} \times 1000]/[\text{OD}_{600} \times \text{time}(\text{min}) \times \text{volume of culture used in the assay (ml)}]$. Morphology of cells in logarithmic phase was examined by Normaski microscopy and photographed (Sprague 1991).

Plasmids

Plasmid constructions were performed by standard methods (Sambrook et al. 1989). YEpl3-PTP2 contains a *Bam*HI-*Bam*HI fragment of *PTP2* in YEpl3 (2 μ /LEU2). YEpl3M4-PTP3 (2 μ /LEU2) is a genomic clone of *PTP3* isolated from the screening. YEpl3M4-ptp3^{C814G}, Cys814 to Gly mutant of *PTP3*, was generated by site-directed mutagenesis using Altered Sites kit (Promega) and mutagenic primer 5'-CAACCTGCGGTACCATTGAACCAAA (mutated sequence is underscored). A *Sal*I-*Sal*I fragment of *PTP3* was subcloned into pGEM-3Z (Promega) and a 1.07-kb *Nsi*I-*Nsi*I fragment was substituted by 1.1-kb *Hind*III-*Hind*III fragment of *URA3* to construct pGEM3Z-ptp3Δ::URA3. The *Xba*I-*Sal*I fragment of *PTP3*, which encodes the catalytic domain, was subcloned into pGEX-KG to create pKG-PTP3 (Guan and Dixon 1991). pGEX2T-FUS3 was constructed as described (Errede et al. 1993). To construct p414ADH1-FUS3HA, a 129-bp *Bam*HI-*Bgl*II PCR fragment containing the triple HA epitope was subcloned into pBluescript KS(-) (Stratagene). The *FUS3* ORF was amplified by PCR using the following primers: 5'-GCGGATCCATGCCAAAGAG-AATTG and 5'-GCGGATCCCTAACTAAATATTCGTTCC (*Bam*HI site is underscored) and subcloned into the *Bgl*II site of pSK-HA, resulting in 3XHA tag at the amino terminus. A 1.34-kb *Bam*HI-*Sst*I fragment of *FUS3HA* was subcloned into p414ADH1 (*CEN/TRP1*) (Mumberg et al. 1995). For expression of untagged *FUS3*, the PCR fragment was subcloned into p414ADH1 (*CEN/TRP1*). Both p414ADH-FUS3HA and p414ADH-FUS3 can complement the sterile phenotype of *fus3Δkss1Δ* double-disruption cells. HA-*fus3*^{Y182F} was constructed by PCR site-directed mutagenesis using the following primers: 5'-GTCAGCCGCGGACAATTCAGAGCCACAGGTCAGCAAAGCGGCATGACCGAATTCGTGGCCACACGTTGGTACAG and 5'-GGTCAAAAACAAGCATACGC (mutated sequence is underscored).

Halo assays and recovery assays

Halo assay was performed as described (Elion et al. 1990) using $\sim 10^5$ cells. Photos were taken after 2 days of incubation at 30°C. Microcolonies formed within halos were visualized with a microscope using 10 $\times$ objective. Recovery from pheromone-induced G₁ arrest was assayed as described (Moser et al. 1996). *MATa* cells were grown to O.D.₆₀₀ ~ 0.2 in YPD and stimulated with 3 μ M α -factor for 3 hr at 30°C. Cells were collected by

filtration, washed with YPD, and resuspended in fresh YPD. The number of viable cells was determined at 1-hr intervals and colony forming units were normalized to values measured immediately following pheromone withdrawal. A plate assay was performed to quantify the ability of a single yeast cell to adapt to a uniform concentration of pheromone and resume growth (Moser et al. 1996). *MATa* cells were grown to 2 to 5 $\times 10^5$ per ml in YPD and aliquots were sonicated and then plated at a 10⁻³ dilution onto freshly prepared 20-ml YPD plates containing various concentrations of α -factor. The number of colonies on YPD plates at each α -factor concentration was determined and normalized to values on plates without α -factor.

Preparation of yeast extracts, immunoprecipitations, and in vitro kinase assays

Yeast extracts for HA-Fus3p immunoprecipitations and MBP kinase assays were prepared as described (Zhou et al. 1993) with modifications. Yeast cells were grown to an O.D.₆₀₀ of 0.7–0.8 and stimulated with 2.5 μ M α -factor. Approximately 50 ml of culture was harvested and cells were broken by vortexing with glass beads in lysis buffer (50 mM Tris-Cl at pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 15 mM *p*-NPP, 15 mM Na₂H₂P₂O₇, 25 mM NaF, 0.25 mM orthovanadate, 1 mM PMSF, 40 μ g/ml of aprotinin, 20 μ g/ml of leupeptin, and 1 mM DTT). Protein concentrations were determined by Bio-Rad protein assay (Bradford assay) with BSA as a standard. Cell extract containing 200 μ g of total proteins was mixed with 2 μ g anti-HA mAb 12CA5 (BAbCO) in a total volume of 200 μ l. After 2 hr incubation at 4°C, samples were incubated with 50 μ l 50% suspension of pre-washed protein A-agarose (Pierce) at 4°C for 1 hr. Immune-complexes were washed three times with 1 ml of lysis buffer. For kinase assays, samples were washed three additional times with 1 ml of kinase buffer (25 mM HEPES at pH 7.2, 15 mM MgCl₂, 5 mM EGTA, 1 mM DTT, 0.1 mM orthovanadate, 15 mM *p*NPP, 1 mM PMSF) and resuspended in 20 μ l of kinase buffer. Reactions were started by addition of a 10 μ l mixture containing 20 μ g MBP (Sigma), 40 μ M ATP, 30 μ Ci [γ -³²P]ATP (7000 Ci/mmol, ICN) in kinase buffer. After 20 min incubation at 30°C with shaking, reactions were terminated by addition of 30 μ l of 2 $\times$ SDS-PAGE sample buffer. MBP phosphorylation was analyzed by 15% SDS-PAGE and autoradiography. Phosphorylation was quantified by phosphorimaging scanning (Molecular Dynamics).

For Cdc28p-HA kinase activity assay, cells harboring the empty vector or pSF19 (Sorgor and Murray 1992) were harvested at logarithmic-phase (O.D.₆₀₀ = 0.7–1.0). Total cell extract was prepared in modified H buffer (Surana et al. 1991) (25 mM MOPS at pH 7.2, 15 mM MgCl₂, 15 mM EDTA, 1 mM DTT, 1 mM Sodium orthovanadate, 1 mM PMSF, 40 μ g/ml of aprotinin, and

20 µg/ml of leupeptin) and immunoprecipitation was performed as described above. H1 kinase assay on immunoprecipitant was performed as described (Surana et al. 1991) and H1 phosphorylation was analyzed by 12.5% SDS-PAGE followed by autoradiograph and phosphorImaging quantitation.

Immunoblotting

Immuno-complexes from extract containing 200 µg of total proteins were subjected to 10% SDS-PAGE followed by electroblotting to PVDF membrane (Millipore). Tyr-phosphorylation of Fus3p was detected with anti-Pi-Tyr mAb 4G10 (UBI) (1:2000 dilution in TBST, 15 mM Tris-Cl at pH 7.6, 150 mM NaCl, and 0.05% Tween-20 plus 5% BSA). HA-Fus3p was detected with anti-HA mAb 12CA5 (BAbCO) (1:2000 dilution in TBST plus 5% nonfat dry milk). Immunoblots were developed using ECL detection kit (Amersham).

In vitro dephosphorylation and inactivation

GST-Ptp3p fusion protein was purified from *E. coli* strain BL21DE3 carrying pKG-PTP3 as described (Guan and Dixon 1991). *p*-Nitrophenyl phosphate (*p*-NPP), ³²P-labeled raytide and casein were used as in vitro phosphatase substrates. Labeling of raytide and casein by protein kinase A and p60^{src} were performed as described (Guan and Dixon 1990).

In vitro dephosphorylation of GST-Fus3p was performed as described (Doi et al. 1994) with modifications. Two-hundred nanograms GST-Fus3p was incubated with 50 µCi [γ-³²P]ATP (7000 Ci/mmol, ICN) in 20 µl of kinase buffer (25 mM MOPS at pH 7.2, 15 mM MgCl₂, 5 mM EGTA, 10 mM DTT, 40 µM ATP and 1 mM MnCl₂) at 30°C. After 90 min, 10 µl of mixture including 100 mM Tris-HCl at pH 7.0, 10 mM DTT, 20 ng, or 50 ng purified GST-Ptp3p was added and followed by 30-min incubation at 30°C. Half of the reaction mixture (15 µl) was withdrawn and subjected to 10% SDS-PAGE and electroblotted to PVDF membrane (Millipore). Phosphorylation levels of GST-Fus3p were visualized by autoradiography. Anti-Pi-Tyr immunoblot was performed on the same membrane. The same membrane was stripped and reprobed with anti-Fus3p polyclonal antibody (gift of S.L. Pelech, University of British Columbia, Vancouver, Canada). The remaining half (15 µl) of reaction mixture was subjected to MBP kinase assay as described above.

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References

- Alessi, D.R., Y. Saito, D.G. Campbell, P. Cohen, G. Sithanandam, U. Rapp, A. Ashworth, C.J. Marshall, and S. Cowley. 1994. Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. *EMBO J.* **13**: 1610-1619.
- Alessi, D.R., N. Gomez, G. Moorhead, T. Lewis, S.M. Keyse, and P. Cohen. 1995. Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. *Curr. Biol.* **5**: 283-295.
- Blumer, K.J. and G.L. Johnson. 1994. Diversity in function and regulation of MAP kinase pathways. *Trends Biochem. Sci.* **19**: 236-240.
- Brill, J.A., E.A. Elion, and G.R. Fink. 1994. A role for autophosphorylation revealed by activated alleles of FUS3, the yeast MAP kinase homolog. *Mol. Biol. Cell.* **5**: 297-312.
- Cairns, R.K., S.W. Ramer, and R. Kornberg. 1992. Order of action of components in the yeast pheromone response pathway revealed with a dominant allele of the STE11 kinase and the multiple phosphorylation of the STE7 kinase. *Genes & Dev.* **6**: 1305-1318.
- Chang, F. and I. Herskowitz. 1990. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G1 cyclin, CLN2. *Cell* **63**: 999-1011.
- Chen, R.H., C. Sarnecki, and J. Blenis. 1992. Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol. Cell. Biol.* **12**: 915-927.
- Cobb, M.H. and E.J. Goldsmith. 1995. How MAP kinases are regulated. *J. Biol. Chem.* **270**: 14843-14846.
- Cowley, S., H. Paterson, P. Kemp, and C.J. Marshall. 1994. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* **77**: 841-852.
- Denu, J.M., J.A. Stuckey, M. Saper, and J.E. Dixon. 1996. Form and function in protein dephosphorylation. *Cell* **87**: 361-364.
- Dickson, B. and E. Hafen. 1994. Genetics of signal transduction in invertebrates. *Curr. Opin. Genet. Dev.* **4**: 64-70.
- Doi, K., A. Gartner, G. Ammerer, B. Errede, H. Shinkawa, K. Sugimoto, and K. Matsumoto. 1994. MSG5, a novel protein phosphatase promotes adaptation to pheromone response in *S. cerevisiae*. *EMBO J.* **13**: 61-70.
- Dolan, J.W. and S. Fields. 1990. Overproduction of the yeast STE12 protein leads to constitutive transcriptional induction. *Genes & Dev.* **4**: 492-502.
- Dolan, J.W., C. Kirkman, and S. Fields. 1989. The yeast STE12 protein binds to the DNA sequence mediating pheromone induction. *Proc. Natl. Acad. Sci.* **86**: 5703-5707.
- Eisenmann, D.M. and S.K. Kim. 1994. Signal transduction and cell fate specification during *Caenorhabditis elegans* vulval development. *Curr. Opin. Genet. Dev.* **4**: 508-516.
- Elion, E.A., P.L. Grisafi, and G.R. Fink. 1990. FUS3 encodes a cdc2+/CDC28-related kinase required for the transition from mitosis into conjugation. *Cell* **60**: 649-664.
- Elion, E.A., J.A. Brill, and G.R. Fink. 1991. Functional redundancy in the yeast cell cycle: FUS3 and KSS1 have both overlapping and unique functions. *Cold Spring Harbor Symp. Quant. Biol.* **56**: 41-49.
- Elion, E.A., B. Satterberg, and J.E. Kranz. 1993. FUS3 phosphorylates multiple components of the mating signal transduction cascade: Evidence for STE12 and FAR1. *Mol. Biol. Cell.* **4**: 495-510.
- Errede, B. and G. Ammerer. 1989. STE12, a protein involved in cell-type-specific transcription and signal transduction in yeast, is part of protein-DNA complexes. *Genes & Dev.* **3**: 1349-1361.
- Errede, B., A. Gartner, Z. Zhou, K. Nasmyth, and G. Ammerer. 1993. MAP kinase-related FUS3 from *S. cerevisiae* is activated by STE7 in vitro. *Nature* **362**: 261-264.
- Gartner, A., K. Nasmyth, and G. Ammerer. 1992. Signal trans-

- duction in *Saccharomyces cerevisiae* requires tyrosine and threonine phosphorylation of FUS3 and KSS1. *Genes & Dev.* **6**: 1280–1292.
- Guan, K.L. and J.E. Dixon. 1990. Protein tyrosine phosphatase activity of an essential virulence determinant in *Yersinia*. *Science* **249**: 553–556.
- . 1991. Eukaryotic proteins expressed in *Escherichia coli*: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192**: 262–267.
- Guan, K.L., R.J. Deschenes, H. Qiu, and J.E. Dixon. 1991. Cloning and expression of a yeast protein tyrosine phosphatase. *J. Biol. Chem.* **266**: 12964–12970.
- Guan, K., R.J. Deschenes, and J.E. Dixon. 1992. Isolation and characterization of a second protein tyrosine phosphatase gene, PTP2, from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **267**: 10024–10030.
- Guthrie, C. and R.G. Fink. 1991. *Guide to yeast genetics and molecular biology*. Academic Press, San Diego, CA.
- Hannig, G., S. Otilie, and R.L. Erikson. 1994. Negative regulation of mitosis in fission yeast by catalytically inactive pyp1 and pyp2 mutants. *Proc. Natl. Acad. Sci.* **91**: 10084–10088.
- Herskowitz, I. 1995. MAP kinase pathways in yeast: For mating and more. *Cell* **80**: 187–197.
- Hunter, T. 1995. Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. *Cell* **80**: 225–236.
- James, P., B.D. Hall, S. Whelen, and E.A. Craig. 1992. Multiple protein tyrosine phosphatase-encoding genes in the yeast *Saccharomyces cerevisiae*. *Gene* **122**: 101–110.
- Keyse, S.M. 1995. An emerging family of dual specificity MAP kinase phosphatases. *Biochim. Biophys. Acta* **1265**: 152–160.
- Ma, D., J.G. Cook, and J. Thorner. 1995. Phosphorylation and localization of Kss1, a MAP kinase of the *Saccharomyces cerevisiae* pheromone response pathway. *Mol. Biol. Cell* **6**: 889–909.
- Maeda, T., S.M. Wurgler-Murphy, and H. Saito. 1994. A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **369**: 242–245.
- Mansour, S.J., W.T. Matten, A.S. Hermann, J.M. Candia, S. Rong, K. Fukasawa, G.F. Vande Woude, and N.G. Ahn. 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* **265**: 966–970.
- Marshall, C.J. 1994. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr. Opin. Genet. Dev.* **4**: 82–89.
- Millar, J.B., V. Buck, and M.G. Wilkinson. 1995. Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast. *Genes & Dev.* **9**: 2117–2130.
- Miyajima, I., M. Nakafuku, N. Nakayama, C. Brenner, A. Miyajima, K. Kaibuchi, K. Arai, Y. Kaziro, and K. Matsumoto. 1987. GPA1, a haploid-specific essential gene, encodes a yeast homolog of mammalian G protein which may be involved in mating factor signal transduction. *Cell* **50**: 1011–1019.
- Moser, M.J., J.R. Geiser, and T.N. Davis. 1996. Ca²⁺-calmodulin promotes survival of pheromone-induced growth arrest by activation of calcineurin and Ca²⁺-calmodulin-dependent protein kinase. *Mol. Cell. Biol.* **16**: 4824–4831.
- Mumberg, D., R. Muller, and M. Funk. 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156**: 119–122.
- Nikawa, J., P. Sass, and M. Wigler. 1987. Cloning and characterization of the low-affinity cyclic AMP phosphodiesterase gene of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7**: 3629–3636.
- Ota, I.M. and A. Varshavsky. 1992. A gene encoding a putative tyrosine phosphatase suppresses lethality of an N-end rule-dependent mutant. *Proc. Natl. Acad. Sci.* **89**: 2355–2359.
- Peter, M. and I. Herskowitz. 1994. Direct inhibition of the yeast cyclin-dependent kinase Cdc28-Cln by Far1. *Science* **265**: 1228–1231.
- Peter, M., A. Gartner, J. Horecka, G. Ammerer, and I. Herskowitz. 1993. FAR1 links the signal transduction pathway to the cell cycle machinery in yeast. *Cell* **73**: 747–760.
- Rose, M.D., F. Winston, and P. Hieter. 1990. *Methods in yeast genetics: A laboratory course manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Shiozaki, K. and P. Russell. 1995. Cell cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. *Nature* **378**: 739–743.
- Song, D., J.W. Dolan, Y.L. Yuan, and S. Fields. 1991. Pheromone-dependent phosphorylation of the yeast STE12 protein correlates with transcriptional activation. *Genes & Dev.* **5**: 741–750.
- Sorger, P.K. and A.W. Murray. 1992. S-phase feedback control in budding yeast independent of tyrosine phosphorylation of p34cdc28. *Nature* **355**: 365–368.
- Sprague, G., Jr. 1991. Assay of yeast mating reaction. *Methods Enzymol.* **194**: 77–93.
- Sprague, G.F., Jr. and J.W. Thorner. 1992. Pheromone response and signal transduction during the mating process of *Saccharomyces cerevisiae*. In *The molecular and cellular biology of the yeast Saccharomyces*. (ed. E.W. Jones, J.R. Pringle, and J.R. Broach), pp. 657–744. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Stevenson, B.J., N. Rhodes, B. Errede, and G. Sprague Jr. 1992. Constitutive mutants of the protein kinase STE11 activate the yeast pheromone response pathway in the absence of the G protein. *Genes & Dev.* **6**: 1293–1304.
- Sun, H. and N.K. Tonks. 1994. The coordinated action of protein tyrosine phosphatases and kinases in cell signaling. *Trends Biochem. Sci.* **19**: 480–485.
- Sun, H., C.H. Charles, L.F. Lau, and N.K. Tonks. 1993. MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. *Cell* **75**: 487–493.
- Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A.B. Fletcher, and K. Nasmyth. 1991. The role of CDC28 and cyclins during mitosis in the budding yeast *S. cerevisiae*. *Cell* **65**: 145–161.
- Tonks, N.K. and B.G. Neel. 1996. From form to function: Signaling by protein tyrosine phosphatases. *Cell* **87**: 365–368.
- Wach, A., A. Brachat, R. Pohlmann, and P. Philippsen. 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**: 1793–1808.
- Wurgler-Murphy, S.M., T. Maeda, E.A. Witten, and H. Saito. 1997. Regulation of the *Saccharomyces cerevisiae* HOG1 mitogen-activated protein kinase by the PTP2 and PTP3 protein tyrosine phosphatases. *Mol. Cell. Biol.* **17**: 1289–1297.
- Zheng, C.F. and K.L. Guan. 1994. Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. *EMBO J.* **13**: 1123–1131.
- Zhou, Z., A. Gartner, R. Cade, G. Ammerer, and B. Errede. 1993. Pheromone-induced signal transduction in *Saccharomyces cerevisiae* requires the sequential function of three protein kinases. *Mol. Cell. Biol.* **13**: 2069–2080.

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